# Chapter One

## INTRODUCTION

Foodborne diseases constitute a budding problem triggering significant burden of disability and mortality in most countries. Recent assessments indicate that the annual global burden of foodborne disease is >600 million cases of illness, with almost 420,000 deaths (WHO, 2015). Africa and South East Asia have the highest occurrence of foodborne diseases and the highest death rates among all ages (Havelaar, 2016). A tainted food, regardless of the level of hazards, poses health threats to consumers and economic burdens on communities and nations (Mensah *et al.*, 2012).

Food poisoning and diarrhoea caused by foods contaminated by *Citrobacter* have been reported (Doulgeraki *et al*., 2011). Currently, the incidence of shigellosis worldwide is highest among children less than five years of age (Taneja and Mewara, 2016). *Escherichia coli* is one of the major foodborne pathogens of foods of animal origin with wide variability of virulence (Kobayashi *et al.,* 2002; Johnson *et al.,* 2005). *Aeromonas* have been connected with several foodborne outbreaks and are increasingly being isolated from patients with traveler's diarrhoea (Von Graevenitz, 2007). The true burden of illnesses caused by *Bacillus cereus* is unknown probably because they commonly occur as irregular cases, rather than in major outbreaks (Logan *et al*., 2011).

Foodborne infection is endemic in Nigeria with more than one million cases per annum according to the Integrated Disease Surveillance and Response (IDSR), and most cases are not reported (FMoH, 2014). It has been estimated that more than 200,000 deaths occur per annum from foodborne pathogens caused by contaminated foods through improper processing, preservation and service in Nigeria (WHO, 2009a).

Regrettably, all food groups add to the burden of foodborne diseases. Meat, eggs and seafoods cause the highest burden (Havelaar, 2016). Most people are inadvertently exposed to microbial hazards from several sources (Okafor *et al.,* 2017; Amini *et al.,* 2012), which can cause diseases that go unreported, because their symptoms are mistaken for other illnesses such as malaria (Ajayi and Salaudeen, 2014). Mild gastroenteritis has been reported to be common among people that consume snails regularly (Serrano *et al*., 2004).

*Achatina achatina* is a terrestrial gastropod of the family "Achatinidae" which has been listed among edible snails (European Commission, 2004). In Africa, the edible giant snails belong to two genera: *Achatina* and *Archachatina.* The most common species in West Africa is *Achatina achatina* (Hodasi, 1984). Snail meat (also called Congo meat) is high in protein, low in fat and a source of iron, magnesium, calcium, and zinc. The nutritive value of snail meat is comparable to that of domestic livestock (Ademolu *et al.,* 2004; Malik *et al.,* 2011; Babalola and Akinsoyinu, 2009). By the content of histidine, glutamic acid, aspartic acid and threonine, snail meat is ahead of chicken, beef and fish (Sando *et al.,* 2013). It is an ideal meat for diabetics and those with vascular diseases such as heart attack, cardiac arrest, hypertension and stroke (Fumilayo, 2008). In 2010 and 2011, world snail market recorded a turnover of 10 billion Euros per year with consumption of about 400,000 tons of snails, of which only 50,000 tons were produced in snail farms (Toader, 2012). There is a growing interest in heliciculture, i.e. snail rearing for meat and sale, in Nigeria (Omole, 2001).

The identification of potential hazards and determining which hazards need to be controlled is known as hazard analysis (Reij and van Schothorst, 2000). In this approach, the presence of a potentially harmful agent at a detectable level in food is used as a basis

for legislation and/or risk management action (Barlow *et al.,* 2015). In hazard identification, an association between the foodborne pathogen and food is created (Lammerding and Paoli, 1997). It aims at prevention or reduction of the presence of pathogenic microorganisms. Such control commonly requires a food chain approach, because end-product control is not effective (Barlow *et al.*, 2015).

Snail meat is more prone to microbial contamination than meat from other animals, because snails are in continuous contact with soil and debris thereby exposed to various microorganisms. There is very limited research on the bacterial assessment of snail meat along the supply chain from market to table/fork which makes it difficult to evaluate the level of hazards associated with it. The literature on snails is rather replete with research focused on the microbiological quality assessment and preservation methods (Legaspi and Jovellanos, 1990; Tetty *et al.,* 1997; Serrano *et al.*, 2004; Ezeama, 2004; Kirkan *et al.*, 2006; Ezeama *et al.,* 2007; Okonkwo and Anyaene, 2009; Antwi, 2009; Adegoke *et al.,* 2010; Omenewa *et al.,* 2011; Adagbada *et al*., 2011; Ebenso *et al.,* 2012; Emelue *et al.,* 2013; Nyoagbe *et al*., 2016).

In Nigeria, the Federal government revised the national policy on food safety, and even mapped out its implementation strategy to, among other intents, establish an effective early warning system that has the capacity to detect, trace and prevent outbreaks of food borne illnesses before transmission (FMoH, 2014). The federal government desires to achieve comprehensive, effective collaboration and coordination of food safety practices from farm to table nationwide by adopting the integrated food management system approach. The National Food Safety Management Committee (NFSMC) is the entity established to implement the food safety policy.

One of the goals targeted towards the implementation of this policy is to minimize the incidence of risks associated with physical, chemical, and biological hazards in foods and water. One of the steps proposed by NFSMC is to conduct a workshop to identify high risk foods (FMoH, 2014). To achieve its aim, such a workshop will require data on various hazards in foods in Nigeria. Such data is not available for many foods such as snail meat.

**Statement of the Problem**

Edible land snails contain bacteria that are suspected to pose public health threats (Adegoke *et al.,* 2010; Omenewa *et al.,* 2011; Ebenso *et al.,* 2012). Also, regular consumers of snails are often plagued by mild gastroenteritis (Serrano *et al*., 2004). The report on *Aeromonas* food poisoning that involved only a 64-year old man that consumed already prepared snails (*Achatina* spp.) in northern Nigeria (Agbonlahor *et al*., 1982) and other studies gauging the pathogenicity of *Staphylococcus aureus* isolated from edible snails in southern Nigeria (Efuntoye *et al*., 2011), have provided some information on possible bacterial hazards in edible snails.

However, information on molecular studies of presumptive pathogens in snails is not available and it is not known if common methods of processing and cooking improve the bacteriological quality of snails for consumption.

Hence, it has become prudent to assess the bacterial hazards in edible land snails in order to contribute data that will promote safer snail meat along the value chain.

**Significance of the Study**

This study will provide information on important bacterial hazards associated with edible land snails in South East, Nigeria and the efficacy of different processing methods and cooking in eliminating these pathogens from edible snails. Such information, if made available to handlers and consumers, will improve the levels of hygiene and adequacy of preparing edible land snails for consumption at home. It will also provide data useful for developing legislations that will promote the sale of safer edible snails thereby reduce the burden of foodborne diseases in the population.

**Aim**

The aim of this study is to assess bacterial hazards associated with edible land snails (*Achatina achatina*) from selected markets in South East, Nigeria.

**Specific Objectives**

1. To determine the aerobic plate count of edible land snails (*Achatina achatina*) obtained from three major markets in South East, Nigeria.
2. To determine the prevalence and load of coliforms in the edible snails for sale in the markets.
3. To identify six presumptive pathogenic bacteria from edible snails for sale in the markets using phenotypic tests.
4. To determine the prevalence and load of six presumptive pathogenic bacteria in the edible snails for sale in the markets.
5. To determine the prevalence of virulence potentials by phenotypic tests of presumptive pathogenic isolates in the edible snails for sale in the markets.

6. To determine the prevalence of antibiotic resistance of presumptive pathogenic isolates in the edible snails for sale in the markets.
7. To identify selected bacterial pathogens in the edible snails for sale in the markets using 16S rRNA gene sequencing technique.
8. To detect toxin genes in selected pathogenic isolates in the edible snails for sale in the markets using Polymerase Chain Reaction (PCR).
9. To determine the processing method that will reduce the bacterial load of snail meat during culinary preparation.

# Chapter Two

# LITERATURE REVIEW

## 2.1 Edible Snails

Snails are the largest group of mollusks. They are classified under "Gastropoda" because they move with waves of muscular contraction on their foot (Antwi, 2012). Snails have two body parts which include: Head-foot, and Visceral mass. The visceral mass is protected by a calcerous shell secreted by mantle cavity. The organs of digestion, excretion and reproduction are found within the visceral mass. The shell is composed of several layers that are made of precipitated organic calcium carbonate (Toader-Williams and Golubkina, 2009). Snails require calcium to produce the shell and a whitish fragile calcified material known as epiphragm (Toader, 2012).

According to European Commission (2004), snails for human consumption include the following species: *Helix pomatia, H. aspersa, H. lucorum,* and species of the family "Achatinidae":

(a) *Helix pomatia* measures about 45mm across the shell. It is also called then "Roman snail", "Apple snail", "Lunar", "La vignaiola", the German "Weinbergschnecke", the French "Escargot de Bourgogne" or "Burgundy snail" or "Gros blanc". It is a native over a large part of Europe. It lives in wooded mountains and valleys up to 2,000 meters (6,000 feet) altitude and in vineyards and gardens. The Romans may have introduced it into Britain. Immigrants introduced it into United States in Michigan and Wisconsin. Many consumers prefer *H. pomatia* to *H. aspersa* for its flavor and larger size, as the "Escargot par excellence".

(b) *H. aspersa* is also known as the French "Petit gris", "Small grey snail", the "Escargot chagrine", or "La zigrinata". The shell of a mature adult has 4-5 whorls and measures 30 – 45mm across. It is native to the shores of Mediterranean and up

the coast of Spain and Frnace. It is found on many British Isles, where the Romans introduced it in the first century A.D. In the early 1800s, the French brought it into California where it has become a serious pest. These snails are now common through out the United States of America. It was introduced into several Eastern and Gulf States even before 1850, and later introduced into other countries such as South Africa, New Zealand, Mexico, and Argentina. *H. aspersa* has a life span of 2–5 years. This species is more adaptable to different climates and conditions than many snails, and is found in woods, fields, sand dunes, and gardens. This adaptability has increased the range of *H. aspersa*, and also makes its rearing easier and less risky.

(c) *H. lucorum* or Turkish snail is a large, edible air-breathing land snail or escargot, a terrestrial pulmonate gastropod mollusk in the family "Helicidae". It originates from the Black sea region, adjacent Asia Minor, today's western and central Turkey. Now, it is also found on the central Balkan Peninsula (southern Romania, Bulgaria, Thrace, as far as Albania) and Italy west of the Apennine. The species does not occur in Germany, but has been introduced in Austria, south of Vienna; it has also been introduced in parts of southern France and on the Iberian Peninsula. With a shell diameter of between 30 and 60 mm, the Turkish snail is usually larger than the Roman snail (*H. pomatia*). It is only active at night and after heavy rains. The shell form of Turkish snail is similar to that of Roman snail: globular with a depressed spire and largely rounded red-brown strips around the whorls. The shell walls are thick and the surface is irregularly striped; the aperture looks oblique and has a thickened rim of a reddish or brownish colour. It is not cultivated but is collected from nature. In delicatessen shops in central Europe, cultivated Roman snails (*H. pomatia*) are sometimes sold in the more colourful shells of *H. lucorum*.

(d) Species of the family "Achatinidae" (New Latin, from Greek "agate") is a family of medium to large sized tropical land snails, terrestrial pulmonate gastropod mollusks from Africa. The family includes 13 genera. Well known species include: *Achatina achatina* the giant African snail, and *Achatina fulica* the giant East African snail. These snails are among the largest terrestrial snails and may reach 20-30cm. The shell of an agate is larger than it is wide. It is conical with an extended body whorl and a blunt apex. Presently, giant African land snails are found almost anywhere the natural environment is right. They are mainly herbivores feeding on fruits and vegetables, but have been known to feed on dead animals to provide protein. In many places, they are serious agricultural pests that cause considerable crop damages. Also due to their large size, their slime and faecal material create a nuisance as does the odour that occurs when something like poisoned bait causes large numbers to die. The United States of America has made considerable efforts to eradicate *Achatina* (EC, 2004).

## 2.2 Snailery in Nigeria

The habitat of edible land snails spans from the dense tropical high forest in southern Nigeria to the fringing riparian forests of the derived Guinea Savanna (Odaibo, 1997; Fagbuaro *et al.,* 2006). In Nigeria, edible land snails aestivate (i.e. bury themselves in the soil or hide beneath stones in order to avoid direct solar radiation) from November to March each year, and re-surface during the rainy season (Fagbuaro *et al.,* 2006).

Furthermore, edible land snails are collected from the wild/nearby bushes, or purchased from the open market, or from snail farms. The population of edible land snails in the wild has declined considerably because of human activities such as deforestation, pesticide use, slash and burn agriculture, bush fires and premature harvesting of snails. Persistence of these factors will culminate in the extinction of edible land snails in Nigeria.

In addition, seasonality of supply of edible land snails from the wild limits their use for meat on a continuous basis. All these justify rearing of edible land snails in farms just as done for poultry, sheep and goats. Two main systems of snail farming are practiced: indoor and out-door systems. A modification of the out-door system, in which the snails are confined out-doors in enclosures and fed both synthetic and natural diets, fits into the Nigerian farming system (NAERLS, 1995).

The Federal Ministry of Agriculture and Rural Development (FMARD), is a ministry of the federal government of Nigeria that regulates agricultural research, agricultural and natural resources, forestry and veterinary research all over the country. The ministry relies on the membership list of Snail Farmers Association of Nigeria (SFAN) which is a subset of All Farmers Association of Nigeria (AFAN) on the event of workshops, sensitization campaigns and provision of loans for these farmers. The ministry has recognized the growing interest in the rearing and marketing of snails, and has trained

several women and youths on snailery. Nodu *et al.* (2003) found consumption level of snail meat among people of Bori (a southern city in Nigeria) to be as high as 70%.

The snail meat industry, though not well established, is projecting the snail meat as a healthy alternative to the conventional meat and as an economically vibrant mini-livestock. Also, there seems to be a considerable international trade and demand for snail meat (Emslie, 1982). Data on the extent to which the snail market contributes to Nigeria's economy is lacking.

The Nigeria Agricultural Quarantine Service (NAQ) has the mandate of ensuring that all plants, animals and aquatic produce/products leaving the shores of Nigeria meet international standards (FMoH, 2014). However, there is no legislation that lays down the specific requirements that ensure safe snail meat from farm to table. The food supply chain in Nigeria faces a number of challenges such as diversities in culture, lifestyles, agricultural practices, mode of food production, handling, storage, preparation, transportation and eating habits (FMoH, 2014).

Ebenso *et al.* (2012) noted the critical need for producers, retailers, processors and consumers to take responsibility to prevent contamination, cross contamination, mishandling as well as proper holding, storage and cooking of snail meat to eradicate foodborne pathogenic bacteria. This can only be possible if the existing National Policy on Food Safety and Implementation Strategy (NPFSIS) is strengthened at each enterprise level at the federal, state and LGAs platforms (FMoH, 2014).

## 2.3 Bacterial Pathogens in Snails

Unlike meat from vertebrates, snail meat is more prone to microbial contamination which begins with the feeding habit of snails. Because formulated feeds for snails are not available in the market, it has become common practice for snail rearers to use vegetables, plant leaves and kitchen wastes to feed snails (Chah and Inegbedion, 2013). It has been reported that gastropods find mammalian faeces (manure) an attractive food source (Speiser, 2001). In addition, the regular ingestion of bacteria from the soil (Walker *et al*., 1999), worsen their potential to be pathogen laden. Ebenso *et al*. (2012) found pathogens (including coliforms) in snail meat sampled from various markets in Niger Delta, Nigeria to be far above recommended microbiological limits.

Microbial contamination of snail meat inadvertently continues during collection of edible land snails from market women who get their snails directly from the forest. This is because snails' movement is characterized by continuous contact of their body with soil and debris thereby exposing snails to various microorganisms.

The flowchart of traditional processing of fresh water snail is shown in Figure 1.

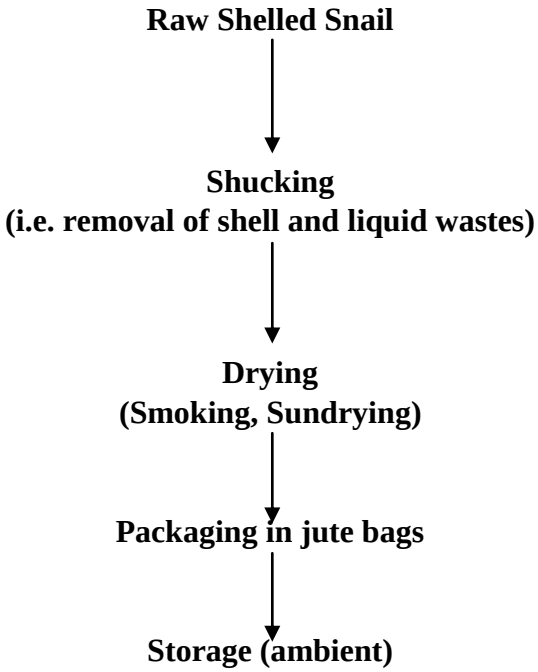



**Figure 1: Flowchart of traditional processing of fresh water snail (Ezeama *et al.*, 2007)**

Each step of processing snail meat has the potential of influencing the microbiological quality. Tettey *et al*. (1997) conducted a preliminary survey of the processing procedure, raw materials used, handling, packaging, and storage of smoked-dry snail meat. The outcome indicated lack of hygienically controlled processing procedure, the use of poor quality snail meat, poor handling and storage procedures for the unpackaged smoked-dry product. Total aerobic count/g of smoked-dry meat ranged from $1.88 \times 10^5$ to $2.77 \times 10^{18}$ cfu/g. Food borne illnesses due to consumption of snail meat may occur when the mollusks that contain pathogenic microorganisms are consumed raw or improperly cooked.

Akinboade *et al*. (1980) and Akpavie *et al*. (2000) showed that various bacteria that are potential pathogens inhabit different organs and tissues including lungs, hemocyanin, liver, kidney, crop, and stomach of clinically healthy edible land snails. Total viable count (log CFU/g) ranging from 6.61 to 8.29 and coliform count ranging from 5.61 to 8.50 in *Achatina* spp. have been reported in Ghana (Nyoagbe *et al*., 2016). Total viable counts in *Cornu aspersum* and *Helix lucorum* varieties of wild snails sampled in Greece were determined to range from 6.0 to 8.0 log CFU/g and microbial counts enumerated in snail meat of *C. aspersum* decreased following the application of processing steps (Parlapani *et al*., 2014). Adegoke *et al*. (2010) analysed different varieties of market snails (*Achatina fulica*, *Limcolaria sp*., and *Helix pomatia*) in Uyo, Akwa Ibom state and found that total bacterial counts ranged from 8.0 to 8.1 log CFU/g, coliform count ranged from 7.1 to 7.3 log CFU/g, *Salmonella/Shigella* count ranged from 7.5 to 7.8 log CFU/g. These studies show there are differences in total bacterial counts among varieties of snails and sources of samples, but the reasons for the differences were not explained.

Andrews *et al*. (1975) isolated *Salmonella* in 84 of 270 Moroccan snails (*Helix aspersa*) with the use of both the pre-enrichment and direct selective enrichment procedures. Serotyping of some *Salmonella* isolates in snails (*Achatina achatina*) revealed the presence of *S. manhattan, S. ndolo, S. reading, S. uppsala*, and *S. typhimurium* (Obi and Nzeako, 1980). In Ghana, Nyoagbe *et al*. (2016) determined *Salmonella* counts (log CFU/g) in market snails (*Achatina*) ranged from 2.91 to 7.39 on Xylose Lactose Desoxycholate (XLD) agar. In Greece, *Salmonella* was detected in wild snails (*C. aspersum* and *H. lucorum*) using selective enrichment procedures (Parlapani *et al*., 2014).

In India, a study on the bacterial diversity of the gastrointestinal tract of *Achatina fulica* using culture-independent and culture-dependent methods reported that an apparent feature of bacterial communities in these snails' gastrointestinal tract was the abundance of members of the genus *Citrobacter* (Pawar *et al*., 2012). Silva *et al*. (2013) found *Citrobacter freundii* in 30% of wild fresh water snails (*Biomphalaria glabrata*) in Brazil. *Citrobacter* has been reported to be present in *Helix pomatia* at Romania (Cîrlan *et al*., 2010).

Obi and Nzeako (1980) isolated *Shigella sonnei* from *Achatina achatina* in Eastern part of Nigeria. Nyoagbe *et al*. (2016) found staphylococcal count (log CFU/g) in market snails (*Achatina*) to range from 2.66 to 7.68 on Baird-Parker agar enriched with egg-yolk emulsion. Efuntoye *et al*. (2011) demonstrated that some *Staph. aureus* isolates in snails (*Archachatina marginata*, *Achatina achatina* and *Achatina fulica*) obtained from south west Nigeria were enterotoxigenic.

Obi and Nzeako (1980) analysed *Aeromonas hydrophila* isolates recovered from *Achatina achatina* in Eastern part of Nigeria and found 58% were enterotoxigenic, and their

toxin was shown to be heat labile. Agbonlahor *et al*. (1982) reported the first case of *A. hydrophila* food poisoning in a 60-year old man after a meal of edible land snails. The snails had been collected from a swampy forest in a village near Oshogbo in Western Nigeria. The *A. hydrophila* isolate was resistant to cephalotin, ampicillin, penicillin, and sensitive to gentamicin, tetracycline, chlorampbenicol, nalidixic acid and nitrofurantoin. Silva *et al*. (2013) found *A. sobria* in 20% of wild fresh water snails (*Biomphalaria glabrata*) in Brazil. Generally, all these reports (except Pawar *et al*., 2012) were not based on molecular studies.

**2.4 Enterotoxins in Food**

Enterotoxins are short, extracellular proteins that are water soluble. They are most commonly described as very stable and are resistant to heat as well as degrading enzymes (Le Loir *et al*., 2003). The enterotoxins, which are classified as superantigens, display common characteristics such as pyrogenicity, immune suppression, and a mitogenic effect on T cells (Le Loir *et al*., 2003).

Bacteria that produce enterotoxins by different mechanisms include: *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Clostridium perfringes,* and *Vibrio cholerae* (Popoff, 2011). The main causes of food borne illness are bacteria (66%), chemicals (26%), virus (4%) and parasites (4%). The two most common types of food borne illness are intoxication and infection. Intoxication occurs when toxins produced by the pathogens cause food poisoning, while infection is characterized by the ingestion of food containing pathogens (CDC, 2011; Addis and Sisay, 2015).

The genes encoding the different enterotoxins are carried and disseminated by different mobile genetic elements such as plasmids and phages (Omoe *et al*., 2003; Schelin *et al*., 2011). Convenience foods offer a suitable growth environment for toxin-producing bacteria such as *S. aureus* which is able to grow and express virulence in a wide variety of foods such as milk products, mixed foods, meat and meat products, egg and egg products, cakes and ice cream (Adams and Moss, 2008). Staphylococcal food poisoning is often associated with growth in protein-rich foods such as meat and dairy products (Le Loir *et al*., 2003).

*S. aureus* can be killed through heat treatment of the food, but the enterotoxins are very heat resistant (Le Loir *et al*., 2003). As little as 20ng-100ng of Staphylococcal enterotoxin (SE) can cause food poisoning (Asao *et al*., 2003). The majority of reported staphylococcal food poisoning outbreaks are associated with the classical enterotoxins: SEA – SEE, with SEA being the most common cause of staphylococcal food poisoning (Kerouanton *et al*., 2007).

Generally, growth of *S. aureus* is necessary for enterotoxin production. Some of the factors that affect *S. aureus* growth and enterotoxin production include: Temperature, pH, water activity, NaCl, oxygen, redox potential, and presence of *Lactococcus lactis* (Adams and Moss, 2008; Schelin *et al*., 2011). Staphylococcal enterotoxins (SEs) are the toxins that induce emesis, while the enterotoxin-like proteins (SE*ls*) either lack emetic activity or have not yet been tested for this (Lina *et al*., 2004). Twenty one SEs or SE*l*s have been identified and designated SEA – SE*l*V (Schelin *et al*., 2011). These toxins (SE and SE*l*) are globular single-chain proteins with molecular weights ranging from 22 – 29 KDa (Thomas *et al*.,

2007). SEA is resistant to proteolytic enzymes, thus retaining its activity in the digestive tract after digestion (Bergdoll, 1989).

SEA and SED were found to decrease in boiled ham after a period of accumulation (Marta *et al*., 2011). Schelin *et al*. (2011) acknowledges that some studies have reported the disappearance of SEA in broth, minced food, raw and pasteurized milk. The apparent decrease in enterotoxin levels could simply be an analytical artifact, such as loss of serological recognition using immuno-based methods such as ELISA, which is a technique commonly used to detect enterotoxins. It has also been proposed that proteases produced by lactic acid bacteria cause the decrease in SEA levels, or that SEA becomes cell-associated and is, therefore, not detected (Hallis *et al*., 1991).

Diagnosis of staphylococcal food poisoning is generally confirmed either by the recovery of at least $10^5$ *S. aureus*/g from food or by detection of SEs in food (Hennekinne *et al*., 2012). Three types of methods are used to detect bacterial toxins in foods: (a) immunological methods, (b) biossays, (c) molecular methods. Immunological methods are the most commonly employed.

**2.5 Indicator Organisms and Pathogens**

The intestines of warm blooded animals contain large numbers of non-pathogenic bacterial species, which are known as indicators of faecal pollution and also contain pathogens such as pathogenic strains of *Escherichia coli*, parasites such as protozoans and viruses (Murray *et al*., 2007). Indicator groups of bacteria have the advantage of being enumerated inexpensively and easily for quantifying the performance of a production process,

when particular pathogens or spoilage organisms might be difficult to detect (Jordan *et al*., 2006).

According to Kornacki (2011) the term indicator organism is used to describe:

(a) A (theoretical) non-pathogenic bacteria whose presence/absence indicates the possible presence or absence of a pathogen

(b) A measure of the hygienic or sanitary conditions of surfaces in a food processing environment

(c) An organism or test that reflects the microbiological quality of a product

(d) An organism whose presence or level indicates the potential for future spoilage

(e) As a "surrogate" organism, for example those organisms that model the behaviour of pathogens under certain conditions. Surrogate organisms are harmless microbes with correlated survival and growth parameters to specific pathogens. They are frequently used to validate the efficacy of critical limits for critical control points, can be naturally occurring or artificially added and should be non-pathogenic (Kornacki, 2010; Ma *et al*., 2007).

Moreover, some indicators are not specific organisms but assays for groups of organisms, such as coliforms, enterobacteriaceae, and aerobic plate count. These various uses cause confusion and disagreement among food safety professionals when the term "indicator" is used.

The criteria for a suitable "indicator" organism (Medema *et al*., 2003) include that:

(a) It should be absent in unpolluted water and present when the source of pathogenic microorganisms of concern is present

(b) It should not multiply in the environment

(c) It should be present in greater numbers than the pathogenic microorganisms

(d) It should respond to natural environmental conditions and water treatment processes in a manner similar to the pathogen of concern

(e) It should be easy to isolate, identify and enumerate

(f) It should be inexpensive to test thereby permitting numerous samples to be taken

(g) It should not be a pathogenic microorganism (to minimize the health risk to analysts)

The four indicator groups include: aerobic plate count (APC), total enterobacteriaceae, total coliforms, *E. coli* biotype I (Jordan *et al.,* 2006). APC is used to quantify the density of aerobic bacteria by unit area of perhaps carcass surfaces, when a general measure of process hygiene is required during meat production. The other three indicator groups are available for assessing the amount of contamination on meat arising from gut contents which includes both that originating directly from the alimentary tract and that arising indirectly through the integument or processing environment. Coliforms are the most frequently studied indicators due to their inclusion into drinking water regulations (Wu *et al.,* 2011).

The occurrence of a specific pathogen is relatively infrequent with less than a few percent of the population infected at any given time except during outbreaks or in areas where it is endemic. An infected individual excretes a specific pathogen in the faeces for a short period of time ranging from few days to weeks. Therefore, the occurrence of such pathogen in the environment depends on whether a particular pathogen is circulating within the community at the time of sampling (Payment and Locas, 2011). The occurrence of indicators and pathogens in water will be a function of occurrence in the population, dilution of sewage, morphology of the microorganisms, water treatments, and environmental and biotic factors

(Hurst *et al.,* 2007). Bacterial indicators can predict the probable presence of pathogens in water, but they cannot predict precisely the level of occurrence.

Variations in pathogen prevalence in population, dilution, retention, and die-off result in conditions where relationships/correlations between any pathogen and any indicator are random, site-specific, or time-specific (Payment and Locas, 2011). Many studies have conflicting results with regards to quantitative relationships between indicators and pathogens. Several studies have not found correlations among indicators and pathogens (Noble and Fuhrman, 2001). However, several investigators have observed relationships between the presence of traditional indicators and illnesses. *E. coli* in well water was found to be significantly associated with gastrointestinal illness in family members (Raina *et al.,* 1999). Wu *et al.* (2011) concluded that indicator organisms are possibly correlated with pathogens if sufficient data are available.

There is lack of quantitative evidence describing how the measurements collected are related to both the occurrence of bacterial hazards in red meat or to the amount of faecal contamination. Jordan *et al.* (2006) conducted analysis to provide quantitative description of the degree of association between concentrations of different indicator organisms in red meat and found that the correlation between coliforms and enterobacteriaceae is consistently higher than the correlation between *E. coli* biotype I and enterobacteriaceae (except for sheep carcass) and also consistently higher than correlation between *E. coli* biotype I and coliforms.

## 2.6 Hazard Analysis in Food Safety Assessment

Hazard analysis is one of the approaches used to ensure food safety. In this approach, the presence of a potentially harmful agent at a detectable level in food is used as a basis for legislation and/or risk management action (Barlow *et al.,* 2015). It aims at prevention or reduction of the presence of pathogenic microorganisms. Such control commonly requires a food chain approach, because end-product control is not effective. The ineffectiveness of end-product control stems from the following: low prevalence of contaminated products, low concentrations of microorganisms, and the potential of microorganisms to grow.

Hazard analysis can be applied throughout the food chain from farm, through processing, packaging, retailing to purchase by the consumer, and are often effective. However, a zero risk approach is normally not feasible for microbiological agents as many hazards occur "naturally" at primary production (example in the soil and faeces of production animals). Microbiological criteria define the acceptability of a product, a batch of food stuffs, or a process, based on the absence, presence or number of microorganisms, and/or on the quantity of their toxins/metabolites, per unit(s) of mass, volume, area or batch (CAC, 1997). They can be used as tools to assess the safety and quality of foods, but cannot guarantee safety.

Hazard-based approach is often useful and efficient, but it can be too stringent when the actual impact of control on the human health risk is not known. Barlow *et al.* (2015) summarized the situations in which hazard-based approaches are appropriate:

(a) When exposure conditions cannot be predicted or estimated with any confidence, but there is an immediate need for rapid communication of information on potential hazards.

(b) When no threshold for the adverse effect can be identified.

(c) When exposure is avoidable.

(d) When use in food is not permitted in law.

Currently, there is no food safety management system that ensures safe snail meat from farm to table. This is because there is scarcity of data needed to design such system and, as a result, consumers have been put at risk in Nigeria.

**2.7 Microbiological Risk Assessment (MRA)**

The major element in assuring that sound science is used to establish standards, guidelines and other recommendations for food safety is risk assessment (Gkogka *et al.,* 2013). The risk of illness from consuming food that contains microbiological hazards depends on the different types of hazards, food matrices and susceptibility of consumers (Magnusson *et al.,* 2012). Microbiological risk assessment is a structured, systematic approach to integrate and evaluate information from diverse sources concerning the origin and fate of pathogens along the food chain and to determine the magnitude of public health risks (Lammerding, 2006). The Sanitary and Phytosanitary measures Agreement specifies that decisions on whether a food can be considered safe and fit for international trade has to be based on science and particularly on risk assessment. The World Trade Organisation relies on the Codex Alimentarius to specify how risk assessment should be performed (Reij and Schothorst, 2000). According to Codex Alimentarius Commission (1999), the general principles of MRA include:

(a.) Principle 1: MRA should be soundly based upon science.

(b.) Principle 2: There should be functional separation between risk assessment and risk management.

(c.) Principle 3: MRA should be conducted according to a structured approach that includes: Hazard identification, Exposure assessment, Hazard characterization and Risk characterization.

(d.) Principle 4: MRA should clearly state the purpose of the exercise, including the form of risk estimate that will be the output.

(e.) Principle 5: The conduct of MRA should be transparent.

(f.) Principle 6: Constraint (such as cost, resources or time) that impact on MRA should be identified and possible consequences described.

(g.) Principle 7: Description of uncertainty and source of the uncertainty during the risk.

(h.) Principle 8: Data and data collection systems should be of sufficient quality and precision that uncertainty in the risk estimate can be determined and minimized.

(i.) Principle 9: MRA should explicitly consider the dynamics of microbiological growth, survival and death in foods and the complexity of the interaction between human and agent following consumption as well as the potential for further spread.

(j.) Principle 10: Wherever possible, risk estimates should be re-assessed overtime by comparison with independent human illness data.

(k.) Principle 11: MRA may need re-evaluation as new relevant information becomes available.

Therefore, risk assessment is concerned with the scientific evaluation of known or potential health effects resulting from human exposure to food borne hazards and consists of

four steps described below (WHO, 2009b; CAC, 1999; Makita *et al.,* 2012; Schelin *et al.,* 2011; Rajkovic, 2014).

**(i) Hazard Identification:**

It is predominantly a qualitative process. Its purpose, for microbial agents, is to identify the microorganisms or their toxins of concern with food. Hazards can be identified from relevant data (information) sources, such as scientific literature, databases of food industry, government agencies, relevant international organizations and solicitation of opinions from experts. Areas from which data can be generated are outlined below (CAC, 1999; Tuominen *et al.,* 2001):

(a) Clinical studies

(b) Epidemiological studies and surveillance

(c) Laboratory animal studies

(d) Investigations of the characteristics of microorganisms

(e) Interaction between microorganisms and their environment through the food chain: from primary production up to (and including) consumption

(f) Studies on analogous microorganisms and situations

**(ii) Exposure Assessment:**

This entails an assessment of the extent of actual or anticipated human exposure. Exposure assessments might be based on the potential extent of food contamination by a particular agent or its toxins, and on dietary information. It should specify the unit of food that is of interest i.e., the portion size in most/all cases of acute illness. Microbial pathogen levels can be dynamic and substantially increase with abused conditions. Hence, the exposure

assessment should describe the pathway from production to consumption. Certain factors which must be considered in exposure assessment include the following (CAC, 1999):

(a.) The frequency of contamination of foods by the pathogenic agent and its level in those foods overtime

(b.) Patterns of consumption

(c.) The role of the food handler as a source of contamination

(d.) The amount of hand contact with the product

(e.) Potential impact of abusive environmental time/temperature

Scenarios can be constructed to predict the range of possible exposures. Such scenarios might reflect effects of processing, such as hygienic design, cleaning and disinfection, as well as the time/temperature and other conditions of the food history, food handling and consumption patterns, regulatory controls and surveillance systems. It estimates the level, within various levels of uncertainty, of microbiological pathogens or microbiological toxins, and the likelihood of their occurrence in foods at the time of consumption. Qualitatively, foods can be categorized according to the likelihood of its contamination at its source. Predictive Microbiology can be a useful tool in exposure assessment.

**(iii) Hazard Characterization:**

Qualitative or quantitative description of the severity and duration of adverse effects that may result from the ingestion of a microorganism or its toxin in food are provided in this step. A dose-response assessment should be performed if the data are obtainable. Several important factors that relate both to the microorganism and the human host need to be considered in hazard characterization (CAC, 1999):

**(a.)In relation to the microorganism:** Replication capability of microorganisms,virulence and infectivity of microorganisms depending on interaction with host and environment, Transfer of genetic materials between microorganisms, Secondary and tertiary transmission of microorganisms, Substantial delay of onset of clinical symptoms, Continued excretion of microorganisms by carrier individuals and continued risk of spread, Low doses of some microorganisms can cause severe effect, Food attributes may alter microbial pathogenicity.

**(b.) In relation to the host:** Genetic factors, Increased susceptibility due to breakdown of physiological barriers, Individual host susceptibility features such as age, pregnancy, nutrition, health and medication status, immune status, concurrent infections and previous exposure history, Population characteristics such as access to and use of medical care, persistence of the organism in the population and population immunity

In the absence of a known dose-response relationship, risk assessment tools such as expert elicitations could be used to consider various factors (example infectivity) necessary to describe hazard characterizations. Moreover, experts may be able to devise ranking systems for characterizing severity and/or duration of disease.

**(iv) Risk Characterization:**

This is defined as an estimation of the probability of occurrence and severity of known or potential adverse health effects in a population based on hazard identification, hazard characterization and exposure assessment. It is in this step that the results of risk assessment are presented. These results are provided in the form of risk estimates and risk descriptions that project answers to the questions risk managers pose to risk assessors (WHO, 2009c). Risk characterization depends on available data and expert judgements. The degree of

confidence in the final estimation of risk will depend on the variability, uncertainty and assumptions identified in all previous steps (CAC, 1999).

Because sources of food contaminants are as numerous as the contaminants, the roles of food groups and bacterial food borne pathogens in food borne infections are yet to be established in most developing countries. Also, there is a disorganised sampling and quality control of foodstuffs because of inadequate coordination between surveillance, food laboratories and food inspection services. Furthermore, the emphasis is on sampling for enforcement purposes and often there is no systematic monitoring for food contaminants as well as no surveillance systems capable of identifying common agents of foodborne diseases (Nguyen *et al*., 2014; Mensah and Julien, 2011). The risk is further aggravated by the increase in food outlets so much that at least a meal is believed to be consumed away from home daily (FMoH, 2014).

**2.8 Antibiotic Resistance in Bacteria**

Antimicrobials consist of substances that inhibit or have killing effect on microorganisms in a clinical setting or for reducing bacterial loads in materials and surfaces and include antibiotics and chemical biocides. Chemical biocides are used for disinfection in the food processing environment (Mathur and Singh, 2005). However, antibiotics are used in food animals to treat clinical diseases, to prevent and control common disease events, and to enhance animal growth (Mc Ewen and Fedorka-Cray, 2002).

The presence of antimicrobial-resistant bacteria in food products is another important public health issue because of the potential for the transfer of antimicrobial-resistant foodborne pathogens to human populations. Moreover, antimicrobial-resistant bacteria may

represent a reservoir of resistance genes transferable to pathogenic or commensal bacteria of the human digestive tract (Capita and Alonso-Calleja, 2013). The emergence of drug resistance has been observed following the introduction of each new class of antibiotics and the threat is compounded by a slow drug development pipeline (Spellberg *et al*., 2004; Norrby *et al*., 2005).

WHO states that more than 25,000 people die each year in the European Union due to infections caused by antibiotic-resistant bacteria (Lawley, 2013). Due to normal genetic variations in bacterial populations, individual organisms may carry mutations that render antibiotics ineffective, transferring a survival advantage to the mutated strain (Landers *et al*., 2012). Also, advantageous mutations can be transferred through plasmid exchange within the bacterial colony when antibiotics is present, resulting in increase of the resistance trait (Courvalin, 2008).

Generally, antimicrobial resistance is the capacity of an organism to resist the growth inhibitory or killing effect of an antimicrobial beyond the normal susceptibility of the organism (Mathur and Singh, 2005). Bacteria can be resistant to antibiotics by using several mechanisms: enzymatic degradation of antibiotics, antibiotic target modification, alteration of the cell wall permeability, and use of alternative pathways to escape the activity (Verraes *et al*., 2013). Prudent use of antibiotics with the intent of preserving their effectiveness for serious infections have been advocated (Belongia *et al*., 2005).

Food may act as a vector for the transfer of antimicrobial resistant bacteria and antimicrobial resistance genes to humans (Verraes *et al*., 2013). There is significant potential threat to human health arising from inappropriate use of antibiotics in food animals. This is because resistant pathogens propagated in these animals are bound to enter the food chain and

could be disseminated in food products (Cui *et al*., 2005; Garofalo *et al*., 2007). Commensal bacteria in livestock may serve as reservoirs for resistance genes that could potentially be transferred to pathogenic organisms in humans (Landers *et al*., 2012). Bergogne-Berezin (1997) listed the routes of contamination of foods with antimicrobial resistant organisms which included:

(a.) Faecal contamination during slaughter of animal

(b.) Faecally contaminated water used for irrigation

(c.) The environment

(d.) Handling by the butcher and consumer

A recent study estimated 1.5% as the probability of exposure to $10^3$ cfu of cephalosporin-resistant *E. coli* through consumption of a meal containing chicken meat (Depoorter *et al*., 2012). Tetracycline resistance is prevalent in lactic acid bacteria associated with raw meat (Gevers *et al*., 2003).

It has been demonstrated that sub-lethal food preservation stresses such as heat stress, acid and salt stress can significantly alter phenotypic antimicrobial resistance in food-related pathogens such as *E. coli*, *Salmonella* Typhimurium and *Staphylococcus aureus.* While sub-lethal high temperature decreased antimicrobial resistance, increased salt or reduced pH conditions increased the phenotypic antimicrobial resistance. It was observed that some of the pathogens continue to express higher levels of phenotypic antimicrobial resistance after removal of stress (Mc Mahon *et al*., 2007).

Bacteria present in food products can survive after the application of a food processing or preservation technique. It is possible that their growth is inhibited resulting in stressed or sub-lethally damaged bacterial cells. Food processing and/or preservation techniques can kill or inactivate bacteria. Such dead bacterial cells can stay intact or be lysed due to cell wall damage. As a consequence, the bacterial DNA, including the eventual present antimicrobial resistance genes, are released into the environment. As soon as the DNA has been released, antimicrobial resistance genes may, theoretically, be transferred to other bacteria by transformation. However, most food processing methods reduce bacterial load (Verreas *et al*., 2013; Rajkovic *et al*., 2010; Wesche *et al*., 2009).

There is indirect evidence of the food borne transfer of Extended Spectrum β-Lactamase (ESBL) genes of poultry to humans. Ninety-four percent of the tested chicken meat isolates contained ESBL genes (Leverstein-van Hall *et al*., 2011). ESBL are hydrolytic enzymes that mediate resistance to extended-spectrum cephalosporins. The detection of ESBL-producing bacterium means that the bacterium possesses the therapeutic resistance to all extended-spectrum cephalosporins, indeed to all cephalosporins, aztreonam and penicillins (Nteimam, 2005).

The notably virulent strain of *E. coli* O104:H4, which caused the major fatal outbreak of infection in Germany in 2011, was resistant to a number of antibiotics, including ampicillin, trimethoprim, cephalosporins and tetracycline. It was also found to possess a plasmid-borne gene for ESBL production. *E. coli* strains with the ESBL gene are often resistant to a wide range of important therapeutic antibiotics, and infections are notoriously difficult to treat. The presence of ESBL in food borne pathogens is an emerging concern (Lawley, 2013).

Agbonlahor *et al*. (1982) showed that *Aeromonas hydrophila* isolate was resistant to cephalotin, ampicillin, penicillin, and sensitive to gentamicin, tetracycline, chlorampbenicol, nalidixic acid and nitrofurantoin. Recently, it has been shown that ofloxacin was the most effective antibiotic against bacterial isolates (*E. coli, Enterobacter, Klebsiella pneumonia, Pseudomonas vulgaris, P. aeruginosa, Salmonella, Shigella*) recovered from snails (*Achatina achatina*), while Ceftriaxone and Augmentin were the least effective on the isolates (Onifade and Aiyenuro, 2018). *Staph. aureus* isolates from snails (*Archachatina marginata*, *Achatina achatina* and *Achatina fulica*) have been reported to show resistance to augmentin, cloxacillin, cefuroxime and amoxycillin-clavulanic acid and other antibiotics tested (Efuntoye *et al*., 2011).

Scientific studies have come to the conclusion that soil is a major reservoir of antibiotic resistance genes (Martinez, 2008; Finley *et al*., 2013; Fitzpatrick and Walsh, 2016).

**2.9 National Policy on Food Safety and its Implementation Strategy**

The National Policy on Food Safety and its Implementation Strategy (NPFSIS) is expected to provide the framework for identification of National Food Safety objectives and formulation of suitable laws, regulations and guidelines for relevant sectors of the food supply chain needed to improve public health and trade (FMoH, 2014). It is also intended to establish an effective early warning system that has the capacity to detect, trace and prevent outbreaks of food borne illnesses before they spread. The desire and determination of government in the NPFSIS is to achieve comprehensive, effective collaboration and coordination of food safety practices from farm-to-table nationwide by adopting the Integrated Food Management System approach. To this end, the NPFSIS proposed the establishment of National Food Safety

Management Committee (NFSMC) as the entity that will coordinate the National Food Safety System, while the Inter Ministerial Committee on Food Safety (IMCFS) shall be established as the entity that oversees the NFSMC. The composition of the IMCFS shall be the Honourable Ministers in charge of:

(a) Health,

(b) Industry, trade and investment,

(c) Agriculture and rural development,

(d) Science and technology,

(e) Environment.

The committee shall be free to co-opt any other member from among stakeholders as deemed necessary. On the other hand, the NFSMC shall be established by the IMCFS to implement the NPFSIS and shall report to the IMCFS. The functions of the NFSMC shall be:

(i) Advise the IMCFS on matters related to food safety

(ii) Coordinate all programmes related to food safety

(iii) Carry out strategic planning, monitor performance and periodically evaluate progress of NPFSIS

(iv) Facilitate the design, and coordinate training programmes for all stakeholders along the food supply chain

(v) Coordinate **risk assessment** and management

(vi) Initiate and coordinate the drafting of the proposed food safety bill

(vii) Facilitate the development and/or updating of standards, regulations, guidelines, code of practice, manuals, SOPs, etc for public and private sectors

(viii) Inform the public and private sectors about current and emerging food safety issues

(ix) Coordinate programmes and seek financial and technical assistance from donor agencies and development partners (FMoH, 2014).

The memberships of the NFSMC shall include one representative of the following among others:

(a) Federal Ministry of Health (FMoH)

(b) Federal Ministry of Industry, trade and investment (FMITI)

(c) Federal Ministry of Agriculture and rural development (FMARD)

(d) Federal Ministry of Science and technology

(e) Federal Ministry of Environment

(f) Federal Ministry of Finance

(g) Federal Ministry of Justice

(h) Federal Departments of Agriculture

(i) Nigeria Agricultural Quarantine Services (NAQS)

(j) National Agency for Food and Drug Administration and Control (NAFDAC)

(k) Standard Organisation of Nigeria (SON)

(l) Institute of Public Analysts of Nigeria

(m) Mycotoxicology Society of Nigeria

(n) Nigerian Institute of Food Science and Technology

and other stakeholders to be invited when needed (FMoH, 2014).

The NPFSIS further states that the existing relationship between the International Committees and the national regulatory agencies will continue to subsist. These committees will continue with their mandates, preparation of national delegations and production of country position on CAC, OIE, IPPC and SPS matters. Moreover, the existing multi-sectorial

approach to food safety where responsibilities for food safety control are shared by relevant MDAs, States and LGAs will still continue to perform their relevant statutory mandates under this policy. However, the statutory legislation/functions of the concerned MDAs will be re-examined to reduce to the barest minimum overlaps and duplications of functions (FMoH, 2014).

For the successful implementation of this policy, **four** goals have been identified.

*Goal I*: To modernize the Nigerian food safety regulatory framework in line with international best practices.

***Goal II*: To minimize the incidence of risks associated with physical, chemical and biological hazards in foods and water**.

*Goal III*: To strengthen institutional capacity for food safety.

*Goal IV*: To improve information and communication systems for food safety.

These goals are further defined by their objectives, strategies and activities. Goal II is the most elaborate and has 4 objectives and 8 strategies (FMoH, 2014).

The objectives, strategies and the responsible bodies are presented (Table 1).

**Table 1: The Objectives, Strategies and Responsible bodies for Goal II**

| Objectives | Strategies | Responsible bodies |
|---|---|---|
| Identify high risk food and water borne disease sources | Conduct a workshop to identify top high risk foods in Nigeria | NFSMC |
| Establish a mechanism to prevent food and water borne hazards | Promote the adoption of appropriate protocols for the use and handling of food additives, processing aids, agrochemicals, feed and veterinary drugs in-line with international best practices | NFSMC, FMARD, NAFDAC, SON, NESREA, NAQS |
| | Establish and implement food safety management control systems such as GAP, GHP, GMP, GEP, GSP, HACCP & ISO 22000, CAC guidelines by the respective regulatory bodies | NFSMC, SON |
| | Ensure that all imported and exported food products are in compliance with World Trade Orrganisation, Sanitary and Phytosanitary measures agreements and other international legislations | FMITI, NFSMC, SON |
| | Strengthen the national food defence system to avert food borne illnesses and hazards | NFSMC |
| Enhance food borne illnesses surveillance and response | Strengthen sentinel sites at each of the states and the FCT with up to date facilities to investigate food borne illnesses, identify causative agents and trace outbreaks to their respective sources | FMoH, NFSMC |
| | Strengthen the National Food Risk Assessment Coordinating Centre | NABDA |
| | ➢ ***Conduct a detailed study of the identified high risk foods to monitor chemical and microbial contaminants and generate useful data*** **(Activity 25)** | NAFDAC |
| Improve inspections, compliance and enforcement systems | Strengthen the capacity of regulatory inspectors, auditors and compliance systems | NFSMC |

(FMoH, 2014)

# Chapter Three

# MATERIALS AND METHODS

## 3.1 Collection of Snail Samples

Three states in the South East of Nigeria were selected for this study which were Enugu, Ebonyi and Anambra States. A central market serving as the largest platform for sale of live edible snails in each state was selected for this study. The markets were Ogbete main market at Enugu State, Abakiliki meat market at Ebonyi State and Nkwo Igboukwu market at Anambra State. A total of 300 live edible snails (*Achatina achatina*), made up of 100 samples from each market, were collected from three states in the south east zone of Nigeria from May, 2016 – October, 2016 and April, 2017 – July, 2017. Edible snails (*A. achatina*) were identified by the Zoology Department of Nnamdi Azikiwe University, Awka. They were identified according to their shape, size, markings, colour, spire angle, sculpture and aperture form (Igbinosa *et al*., 2016; Raut and Barker, 2002). Edible snails displayed for sale in the market (Figure 2) were aseptically collected, with sterile hand gloves, in plastic containers sterilized with 70% alcohol and dried with commercially available sterile paper towel. Samples were immediately transported to the laboratory for analysis.

## 3.2 Sample Preparation

Shells of the snails were surface sterilized with 70% ethanol before being aseptically shucked with a sterile iron rod to extract the meat. Lab blender was sterilized with 70% ethanol. Fifty gram of sample was homogenized in 450 ml of Ringers solution using the lab blender for 2 mins at medium speed. The homogenate was used for serial dilution (1:10). Aliquot (0.1 ml) of appropriately diluted sample was used for determination of bacterial counts.

**Figure 2: Edible land snails (*Achatina achatina*).**

### 3.3 Determination of Total Aerobic Plate Count

Plate count agar medium was used. It was prepared according to manufacturer's instructions and maintained at $45^0$C. Aliquot (0.1 ml) of appropriate dilution ($10^{-6}$-$10^{-8}$) of the homogenate was pipetted into sterile petri dish and the molten agar media was poured into the petri dish. The plate was swirled to mix the homogenate with the agar media. It was done in triplicates for each sample. Plates were incubated aerobically at $37^0$C for 24 hours after which colonies were counted and recorded.

### 3.4 Determination of Coliform Count

MacConkey agar medium was used which was prepared according to manufacturer's instructions. Aliquot (0.1 ml) of appropriate dilution was plated out on the agar medium. It was done in triplicates for each sample. Plates were incubated aerobically at $37^0$C for 24 hours after which pink colonies were counted and recorded.

### 3.5 Determination of the Prevalence of Selected Presumptive Pathogens

Salmonella-Shigella agar, Eosin Methylene Blue agar (EMB), Mannitol Salt agar (MSA), Thiosulfate Citrate Bile salt Sucrose agar (TCBS) and Brain Heart Infusion agar (BHI) were used and were prepared according to manufacturer's instructions.

Aliquot (0.1 ml) of appropriate dilution was directly plated out on appropriate agar media, except Brain Heart Infusion agar, specific for each pathogen and incubated aerobically at $37^0$C for 24 hours. It was done in triplicates for each sample. Typical colonies were counted and recorded after 24 hours.

Appropriate dilutions were heated in a water bath at $80^0$C for 10 mins before being plated on Brain Heart Infusion agar. It was done in triplicates for each sample. Plates were incubated aerobically at $37^0$C for 24 hours after which typical colonies of *Bacillus cereus* were counted and recorded.

### 3.6 Isolation and Identification of Selected Bacterial Pathogens

The procedure for identification of the six selected pathogens were based on the United Kingdom Standards for Microbiology Investigations as published by Public Health England (2014 and 2015).

#### 3.6.1 Isolation and Identification of *Salmonella*

Aliquot (5 ml) of the homogenate was enriched in Selenite F broth (45 ml) for 24 hours, after which a loopful was streaked on Salmonella-Shigella agar and aerobically incubated at $37^0$C for 24 hours. Presumptive colonies (white colonies with black centres) were subcultured in Tryptose Soy agar and solates were subjected to further tests such as Gram staining, catalase test, motility test, oxidase test, indole test, urease test and triple sugar iron test.

#### 3.6.2 Isolation and Identification of *Shigella*

Presumptive colonies (white colonies without black centres) on Salmonella-shigella agar were subcultured in Tryptose Soy agar and isolates were subjected to further tests such as Gram staining, catalase test, motility test, oxidase test, urease test and glucose fermentation test.

### 3.6.3 Isolation and Identification of *Escherichia coli*

Aliquot (5 ml) of the homogenate was enriched in lactose broth (20 ml) for 18 hours, after which a loopful was streaked on EMB agar and aerobically incubated at $37^0$C for 24 hours. Presumptive colonies (blue-black colonies with green metallic sheen and dark centres) on EMB agar were streaked on sorbitol MacConkey agar and subcultured in Tryptose Soy agar and isolates were subjected to further tests such as Gram staining, catalase test, indole test, urease test and citrate test.

### 3.6.4 Isolation and Identification of *Staphylococcus aureus*

Aliquot (5 ml) of the homogenate was enriched in Nutrient broth containing 3% NaCl (20 ml) for 24 hours, after which a loopful was streaked on Mannitol Salt agar and aerobically incubated at $37^0$C for 24 hours. Presumptive colonies (yellow colonies) were subcultured in Tryptose Soy agar and isolates were subjected to further tests such as Gram staining, catalase test and coagulase test.

### 3.6.5 Isolation and Identification of *Vibrio*

Aliquot (5 ml) of the homogenate was enriched in Nutrient broth containing 3% NaCl (20 ml) for 24 hours, after which a loopful was streaked on Thiosulfate Citrate Bile salt Sucrose (TCBS) agar and aerobically incubated at $37^0$C for 24 hours. Presumptive colonies (yellow colonies) on TCBS agar were subcultured in Tryptose Soy agar and isolates were subjected to further tests such as Gram staining, oxidase test and motility test.

### 3.6.6 Isolation and Identification of *Bacillus cereus*

Aliquot (5 ml) of the homogenate was heated in a water bath at $80^0$C for 10 mins and enriched in BHI broth (20 ml) for 24 hours, after which a loopful was streaked on BHI agar and aerobically incubated at $37^0$C for 24 hours. Presumptive colonies (raised grey colonies) on BHI agar were subcultured in Tryptose Soy agar and isolates were subjected to further tests such as Gram staining, spore staining and motility test.

## 3.7 Biochemical Tests for Identification of Isolates

### 3.7.1 Catalase test

A drop of 3% hydrogen peroxide was placed on a clean grease-free glass slide. A loopful of 24 hour-culture of the isolate was emulsified on the slide. Immediate appearance of bubbles was a positive reaction.

### 3.7.2 Oxidase test

A drop of freshly prepared 1% solution of oxidase reagent was placed on a piece of filter paper. With a wireloop, a colony of the isolate was collected and rubbed on the area of the filter paper impregnated with oxidase reagent. Change of colour to purple blue in few seconds was a positive result.

### 3.7.3 Motility test

The Sulphide Indole Motility (SIM) medium was used for this test. The medium was prepared according to the manufacturer's instruction. The tubes containing the semi-solid medium were stab-inoculated with 24- hour culture of the isolates and incubated at $37^0$C for 36 hours. Presence of bacterial growth diffusing away from the line of inoculation was positive result.

### 3.7.4 Glucose fermentation test

Twenty-four hour cultures of the isolates were inoculated in tubes each containing 9ml of peptone water and 1ml of 1% glucose solution. Phenol red was used as indicator and Durham's tube was also inserted. Tubes were incubated at $37^0$C for 48 hours. Colour change from red to pink was positive and presence of gas bubbles was indicated in the Durham's tube.

### 3.7.5 Indole test

Twenty-four hour cultures of the isolates were inoculated in tubes each containing 4ml of peptone water and incubated at $37^0$C for 96 hours. To view results, freshly prepared Kovac's reagent (0.5 ml) was added to the tubes. Presence of red colour within seconds in the alcohol (top) layer was positive result.

### 3.7.6 Urease test

Urea agar medium was used for this test. Twenty-four hour cultures of the isolates were inoculated on urea agar slant and incubated at $37^0$C. The results were viewed after 4 hours and 24 hours of incubation. Presence of purple-pink colour was positive result.

### 3.7.7 Triple Sugar Iron test

Triple sugar iron (TSI) agar was used for this test. Twenty-four hour cultures of the isolates were inoculated on TSI agar slant and incubated at $37^0$C for 24 hours. Results were reported as slant/butt/gas production/hydrogen sulphide production where applicable.

### 3.7.8 Citrate Utilization test

Simmon's citrate agar was used for this test. Twenty-four hour cultures of the isolates were inoculated on the agar slant and incubated at $37^0$C for 24 hours. Presence of blue colour was a positive result.

### 3.7.9 Coagulase test

Twenty-four hour broth cultures of the isolates (0.8 ml) were inoculated into (0.2 ml) plasma in tubes and gently mixed before incubation for 3 hours. Presence of clotting in the tube indicated a positive result.

### 3.7.10 Spore staining

A smear of the twenty-four hour broth culture was made on a clean slide and steam-fixed on a beaker of boiling water until the underside of the slide was covered with drops of condensed water. The smear was then flooded with 5% aqueous malachite green and warmed for about 1 min before rinsing with cold water. Safranin (0.5%) was added for 30 secs and rinsed. The slide was blotted dry before examining under the microscope. Spores appeared green while vegetative cells appeared red.

## 3.8 Determination of Virulence Potentials of Presumptive Pathogenic Isolates

### 3.8.1 Haemolysis test

Blood agar supplemented with 5% human blood was used. Isolates were streaked on the blood agar and incubated at $35^0$C for 24 hours. Greenish or clear zones of haemolysis around colonies indicated alpha and beta haemolysis respectively.

### 3.8.2 Protease test

Nutrient agar supplemented with 1% casein was used. Isolates were streaked on the medium and incubated at $37^0$C for 24 hours. Zone of clearing around colonies was a positive result.

### 3.8.3 Starch hydrolysis test

Nutrient agar supplemented with 1% starch was used. Isolates (0.1ml) were spot inoculated on the medium and incubated at $37^0$C for 24 hours. Before examining plates, plates were flooded with iodine solution. Zone of clearing around colonies was a positive result.

### 3.8.4 Gelatinase test

Nutrient agar supplemented with 1.2% gelatin was used. Isolates were streaked on the medium and incubated at $37^0$C for 24 hours. Before examining plates, plates were flooded with acid mercuric chloride solution. Zone of clearing around colonies was a positive result.

### 3.8.5 Lecithinase test

Nutrient agar supplemented with 10% egg yolk emulsion was used. Isolates were spot inoculated on the medium and incubated at $37^0$C for 3-7 days. Zone of precipitation/opacity around the colonies was positive result.

### 3.8.6 Biofilm formation assay

Brain heart infusion (BHI) agar supplemented with 5g of sucrose and 0.8g of Congo red dye per 100ml of BHI agar was used. Isolates were streaked on the medium and incubated at $37^0$C for 24 hours. Black colonies with dry crystalline consistency indicated positive result.

## 3.9 Determination of Antibiotic Resistance in Presumptive Pathogenic Isolates

Kirby-Bauer disc diffusion method was used. The methodology in the CLSI M02-A12 Supplement (CLSI, 2015) was followed in this study. The isolates were emulsified in saline to a 0.5 McFarland opacity standard and inoculated on Mueller Hinton agar using swab stick. After 30 mins, discs of antibiotics (Ampicillin, Amoxycillin/Clavulanic acid, Ceftazidime, Cefotaxime, Cephalexin, Ciprofloxacin, Gentamicin, Nalidixic acid, Pefloxacin, Septrin, Streptomycin, Ofloxacin, Cloxacillin, Amoxycillin, Chloramphenicol, Erythromycin, Levofloxacin, Norfloxacin and Rifampicin) were placed on the cultured plates. Plates were incubated aerobically at $37^0$C for 24 hours. Zones of inhibition were measured with a ruler and recorded. The results of the antibiotic sensitivity test of the isolates were interpreted according to the M100 supplement (CLSI, 2017).

## 3.10 Identification of Selected Isolates Using 16S rRNA Gene Sequencing

Ten bacterial isolates found to possess some virulence potentials were subjected to identification by 16S rRNA gene sequencing.

### 3.10.1 Extraction of DNA

DNA was extracted from the bacterial isolates using ZR Fungal/Bacterial DNA miniprep™ kit (Zymo Research, USA) in accordance with the manufacturer's instructions.

Briefly, 50-100 mg (wet weight) bacterial cells were washed and resuspended in 200 µl of sterile phosphate buffered saline (PBS) in a ZR BashingBead™ Lysis Tube. The cells were then processed in a cell disruptor at maximum speed for 5 mins. A lysis solution (750 µl) was added to help lyse cells during the mechanical lysis step. The ZR BashingBead™ Lysis Tube containing the cells were centrifuged at 10,000 x g for 1 min. The supernatant (400 µl) of the lysed solution was filtered into the Collection Tube using a Zymo-Spin™ IV Spin Filter and centrifuged at 7,000 x g for 1 min. Fungal/Bacterial DNA Binding Buffer (1,200 µl) was added to the filtrate in the Collection Tube. About 800 µl of the mixture from the last step was transferred to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuged at 10,000 x g for 1 min. The flow through from the Collection Tube was discarded and the last step was repeated. DNA pre-wash buffer (200 µl) was added to the Zymo-Spin™ IIC Column and centrifuged at 10,000 x g for 1 min. After which it was washed with 500 µl Fungal/Bacterial DNA wash buffer. Then, the column was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl of DNA Elution Buffer was added directly to the column matrix, and then centrifuged at 10,000 x g for 30 seconds to elute the DNA.

### 3.10.2 PCR Amplification

Amplification of 16S rRNA gene was carried out in a thermocycler (Applied Biosystems, USA) using the following Primers: 27F: AGAGTTTGATCMTGGCTCAG and 1525R: AAGGAGGTGWTCCARCCGCA. The PCR program was carried out in 10 µl reaction volumes containing 2 µl DNA template (10 ng/µl), 1 µl 10xPCR buffer, 1 µl $Mgcl_2$ (25mM), 0.5 µl of each primer (5 pMol/µl), 0.1 µl Taq DNA polymerase (5 U/µl), 0.8 µl dNTPs (2.5 mM/µl), 1 µl DMSO and 3.1 µl of sterile water. The PCR amplification has initial

DNA denaturation at $94^0$C for 5 mins, followed by 36 cycles of denaturation at $94^0$C for 30 secs, annealing at $56^0$C for 30 secs and extension at $72^0$C for 45 secs which was followed by a final extension at $72^0$C for 7 mins. The amplicon (5 µl) was analysed by electrophoresis (Bio-Rad) in 1.5% agarose gel (Invitrogen™) at 100 volts for 45 mins followed by staining with 1% solution of ethidium bromide (50 µl/l) and destaining with Tris-acetate-EDTA buffer for 10 mins. A 50-bp DNA ladder (NEB) was used as a molecular marker. Gels were visualized by UV transillumination and recorded with Polaroid 667 instant film (Lane, 1991).

### 3.10.3 Sequencing and Analysis

Purified DNA mixture (4 µl) was added to 4 µl of Big-Dye Terminator Reaction Mix (Applied Biosystems, USA), followed by 1.6 µl of primer and 0.4 µl of deionized water. The sequencing reaction was then purified using ethanol/EDTA precipitation method. Sequences of the fragments were determined using 3130xl genetic analyzer (Applied Biosystems) at International Institute of Tropical Agriculture, Ibadan.

The sequences were edited to remove the PCR primer binding sites and manually corrected using Finch TV version 1.4.0. The sequences of the isolates were automatically compared, using the BLAST, against reference sequences of bacteria available in databank with accession date: 7th July 2018 (www.ncbi.nlm.nih.gov/). The identity of each isolate was determined based on the highest % sequence similarity to its relatives in the databank. The 16S rRNA sequence data for the bacterial isolates sequenced in this study were deposited in the Genbank and accession numbers were obtained.

### 3.11 Detection of Specific Toxin Genes by PCR in Selected Isolates Recovered From Snails

Some isolates (*Staphylococcus* and *Bacillus*) that were found to demonstrate some virulence potentials, based on phenotypic tests, were selected for detection of toxin genes by polymerase chain reaction (PCR) method. DNA was extracted from the selected bacterial isolates using ZR Fungal/Bacterial DNA miniprep$^{TM}$ kit (Zymo Research, USA) in accordance with the manufacturer's instructions as discussed earlier (Section 3.10.1).

Touchdown PCR was done according to Don *et al*. (1991) to detect the presence of five toxin genes (*hbla, hblc, nhea, nheb* and *cytk*) in selected *Bacillus* isolates and two toxin genes (*sea* and *exhc*) in selected *Staphylococcus* isolates. The primer sequences used in this study for the amplification of the toxin genes are listed in Table 2. The primers were synthesized by Integrated DNA Technologies (IDT), Belgium.

**Table 2: Primers used in this study for the detection of toxin genes in selected bacterial isolates recovered from snails**

| Target gene | Primer sequence (5′-3′) | Amplicon size (Base pairs) |
|---|---|---|
| *Sea* | F-TAA GGA GGT GGT GCC TAT GG<br>R-CAT CGA AAC CAG CCA AAG TT | 180 |
| *Exhc* | F-GAATAAATATTATGGAGTCTCTCCTGATC<br>R-CCATAGTATTTCAATCCAAAATCAGTAC | 525 |
| *Hbla* | F-CAAGGTGCAGATGTTGATGC<br>R-GAACGCCCGAATATTGAG | 352 |
| *Hblc* | F-AATGGTCATCGGAACTCTAT<br>R-CTCGCTGTTCTGCTGTTAAT | 731 |
| *Nhea* | F-TACGCTAAGGAGGGGCA<br>R-GTTTTTATTGCTTCATCGGCT | 500 |
| *Nheb* | F-CTATCAGCACTTATGGCAG<br>R-ACTCCTAGCGGTGTTCC | 770 |
| *Cytk* | F-ACAGATATCGG(GT)CAAAATGC<br>R-TCCAACCCAGTT(AT)(GC)CAGTTC | 809 |

(Chen *et al*., 2007; Nazari *et al*., 2014; Hansen and Hendriksen, 2001; Owusu-Kwarteng *et al*., 2017)

For PCR amplification, the reaction mixture (25 µl) contained 3.0 µl of the appropriate DNA template (10 ng/µl), 1 µl of primer F (5 pMol/µl), 1 µl of primer R (5 pMol/µl), 1.0 µl Dimethyl sulphoxide, 2.0 µl of Deoxynucleoside triphosphates (2.5 Mmol/l), 2.5 µl of 10× PCR buffer, 1.0 µl of $MgCl_2$ (25 mM/l), 0.1 µl of *Taq* DNA polymerase (5 U/µl), and 13.4 µl of distilled water in each reaction tube. The reaction was performed in a thermal cycler (GeneAMP 9700, Applied Biosystems, USA) with initial denaturation at 94°C for 5 min followed by 9 cycles of denaturation at 94°C for 15 sec, primer annealing at 55°C for 20 sec and extension at 72°C for 30 sec, followed by 35 cycles of denaturation at 94°C for 15 sec, primer annealing at 45°C for 20 sec and extension at 72°C for 30 sec, followed by a final extension at 72°C for 7 min, after which a hold temperature at 10°C was maintained. The presence of amplicons was determined by electrophoresis of the reaction products (10 µl) in a 1.5% agarose gel at 100V for 1.5 hr with 1X SB buffer (22.5 g of anhydrous boric acid/litre and 4g of NaOH/litre) and a 50-bp DNA ladder (NEB) as a molecular marker. Gels were stained with ethidium bromide solution (5 µl of 10 mg/ml) and documentation was done using the Enduro gel system.

### 3.12 Determination of the Processing Method that Will Reduce the Bacterial Load of Snail Meat During Culinary Preparation.

High loads of bacteria, which included pathogens, were found in samples of *A. achatina* studied. This study was carried out to determine the efficacy of the different methods used in processing and cooking snails in eliminating these bacteria in order to ensure safety of snail meat to consumers.

### 3.12.1 Collection of samples for processing and cooking

Hundred samples of live snails (*Achatina achatina*) were collected from Ogbete main market, Enugu. The weight of each of the sample before shucking ranged from 120g – 195g. Each sample was collected into a sterile plastic container with lid and transported to the laboratory for processing, cooking and bacteriological analysis.

### 3.12.2 Processing and cooking of samples

The samples were randomly divided into five batches representing different processing methods. All utensils (bowls, spoons and knives) were washed with hot water and detergent. Utensils were also sterilized with 70% ethanol and dried with commercially available sterile paper towel. Water for processing and cooking was autoclaved at $121^0$C for 15 mins before use. For each batch of samples, the shells were surface sterilized with 70% ethanol and subjected aseptically to stages involved in processing and cooking which are shucking, evisceration, desliming, washing, parboiling and cooking (Figure 3). Five different desliming agents (potassium alum, wood ash, citrus lime, garri and cassava-retting water) were used for each processing method. During processing, the average contact time with the desliming agent was 5 mins. However, the desliming stage was concluded when the samples had lost the slimy texture.

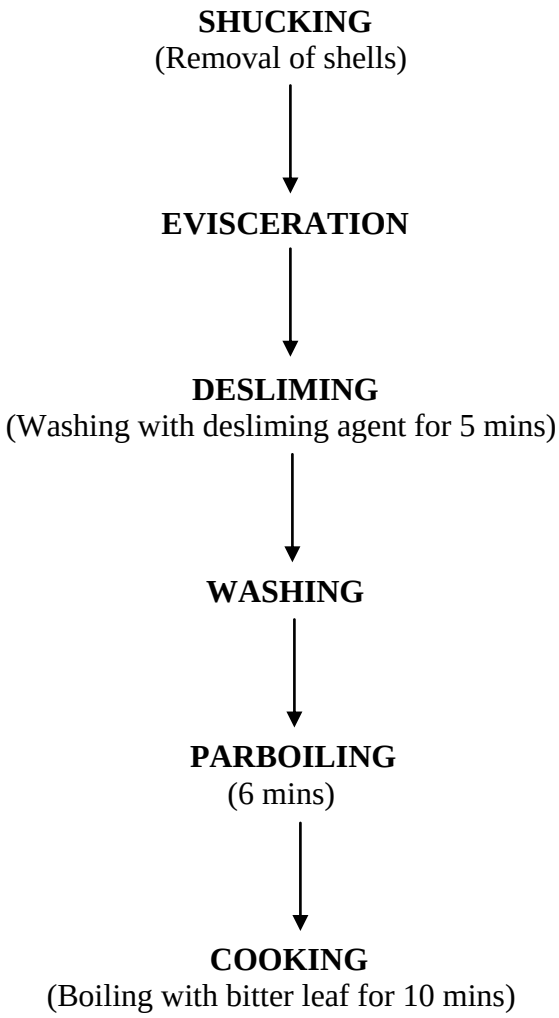



**Figure 3: Stages of processing and cooking of edible snail.**

### 3.12.3 Bacteriological Analysis

For each batch, samples (50g) were collected at each stage of processing and cooking for bacteriological analysis. Each sample was homogenized in 450ml of Ringers solution using a lab blender for 2 mins at medium speed. The homogenate was used for serial dilution (1:10). Aliquot (0.1ml) of appropriate dilution was plated on seven different media for enumeration of six selected presumptive pathogens namely: *Salmonella, Shigella, Staphylococcus aureus, Escherichia coli, Vibrio* and *Bacillus cereus*. The media were: Plate count agar, MacConkey agar, Salmonella-shigella agar, EMB agar, Mannitol salt agar, TCBS agar and BHI agar.

Total aerobic plate count, coliform count and viable counts for each selected pathogen were determined using the methods discussed earlier (sections 3.3 - 3.5). Typical colonies were counted and recorded.

### 3.13 Data Analysis

Descriptive statistics such as means and frequencies were used to present some of the findings. All data on plate counts were converted to logarithmic value. Analysis of variance (ANOVA) was performed using statistical web version software package available in *Vassarstat* website (http://vassarstats.net/).

Phylogenetic trees were constructed by the neighbour-joining method using web version software package of Phylogeny.fr (http://www.phylogeny.fr/index.cgi).

# Chapter Four

## RESULTS

### 4.1 Total aerobic plate count in edible land snails

The mean aerobic plate counts of samples ranged from 8.43 Log CFU/g - 9.61 Log CFU/g. Samples from Ogbete market had the highest mean total aerobic plate count (9.32 ± 0.308 Log CFU/g) while the lowest mean count was found in Igboukwu samples (8.74 ± 0.312 Log CFU/g) (Figure 4). ANOVA (Appendix 3) revealed significant differences between total aerobic plate counts of the following samples: Igboukwu and Abakaliki samples ($p < 0.05$), Igboukwu and Ogbete samples ($p < 0.01$), Abakaliki and Ogbete samples ($p < 0.01$).

### 4.2 Coliform count in edible land snails

Samples from Abakaliki market had the highest mean count of coliforms (7.63 ± 0.389 Log CFU/g) followed by Ogbete samples (7.49 ± 0.358 Log CFU/g) and Igboukwu samples (7.41 ± 0.191 Log CFU/g) (Figure 5). ANOVA (Appendix 4) indicated significant differences between coliform counts of the following samples: Igboukwu and Abakaliki samples ($p < 0.01$), Abakaliki and Ogbete samples ($p < 0.05$). There was no significant difference between Igboukwu and Ogbete samples.

### 4.3 Viable counts of presumptive bacterial pathogens in edible land snails

The edible land snails were analysed for viable counts of colonies characteristics of the following pathogens: *Salmonella*, *Shigella*, *E. coli*, *Staphylococcus*, *Vibrio* and *Bacillus* on Salmonella-Shigella agar, Eosin Methylene Blue agar (EMB), Mannitol Salt agar (MSA), Thiosulfate Citrate Bile salt Sucrose agar (TCBS) and Brain Heart Infusion agar (BHI) respectively.

The highest mean presumptive *Salmonella* count was found in Abakaliki samples (7.24 ± 0.210 Log CFU/g) followed by Igboukwu and Ogbete samples with 6.39 ± 0.114 Log CFU/g and 6.37 ± 0.219 Log CFU/g respectively (Figure 6). ANOVA (Appendix 5) showed that there were significant differences between presumptive *Salmonella* counts of the following samples: Igboukwu and Abakaliki samples ($p < 0.01$), Abakaliki and Ogbete samples ($p < 0.01$). There were no significant differences between Igboukwu and Ogbete samples ($p < 0.01$).

Abakaliki samples were found to contain the highest mean presumptive *Shigella* count (4.61 ± 0.354 Log CFU/g) followed by Igboukwu (4.43 ± 0.284 Log CFU/g) and Ogbete samples (4.03 ± 0.571 Log CFU/g) as shown in Figure 7. ANOVA (Appendix 6) revealed significant differences between presumptive *Shigella* counts of the following samples: Igboukwu and Ogbete samples ($p < 0.01$), Igboukwu and Enugu samples ($p < 0.01$), Abakaliki and Ogbete samples ($p < 0.01$).

The highest mean count of presumptive *E. coli* was found in Igboukwu samples (7.14 ± 0.170 Log CFU/g) followed by Abakaliki (6.95 ± 0.179 Log CFU/g) and Ogbete samples (5.65 ± 0.239 Log CFU/g) as shown in Figure 8. ANOVA (Appendix 7) confirmed significant differences between all groups of samples analysed ($p < 0.01$).

The highest mean count of *Staphylococcus* was found in Abakaliki samples (4.74 ± 0.192 Log CFU/g) followed by Ogbete samples (4.66 ± 0.757 Log CFU/g). *Staphylococcus* was not detected in Igboukwu samples (Figure 9). There was no significant difference between Abakaliki and Ogbete samples (Appendix 8).

The highest mean count of presumptive *Vibrio* was found in Ogbete samples (4.80 ± 0.473 Log CFU/g) followed by Abakaliki (3.44 ± 0.197 Log CFU/g) and Igboukwu samples (3.10 ± 0.052 Log CFU/g) (Figure 10). There were significant differences between all groups of samples analysed ($p < 0.01$) (Appendix 9).

Presumptive *Bacillus cereus* counts revealed that Abakaliki samples had the highest mean count of presumptive *Bacillus cereus* (4.50 ± 0.136 Log CFU/g) followed by Ogbete (3.48 ± 0.135 Log CFU/g) and Igboukwu samples (3.25 ± 0.130 Log CFU/g) as shown in Figure 11. There were significant differences between all groups of samples analysed ($p < 0.01$) (Appendix 10).

**4.4 Prevalence of selected presumptive pathogens in edible land snails**

All 300 samples of edible land snails analysed in this study were found to be contaminated with presumptive *Salmonella* species irrespective of the source of the samples (Table 3). Presumptive *Shigella* species were recovered from 60% of all samples analysed, with the highest frequency occurring in Ogbete samples (80%) followed by Abakaliki samples (60%) and Igboukwu samples (40%). Presumptive *E. coli* was detected in 90% of all samples analysed. Presumptive *E. coli* was found in 100%, 90% and 80% of Ogbete, Igboukwu and Abakaliki samples respectively. *Staphylococcus* was recovered from 36.7% of all samples analysed, 30% and 80% of Abakaliki and Ogbete samples respectively. No *Staphylococcus* was found in Igboukwu samples. Presumptive *Vibrio* species was recovered from 76.6% of all samples analysed: 90%, 70% and 70% of Ogbete, Igboukwu and Abakaliki samples were found to be contaminated by presumptive *Vibrio* respectively. Presumptive *B. cereus* was detected in 80% of all samples analysed: 90%, 80% and 70% of Ogbete, Abakaliki and Igboukwu samples respectively (Table 3).

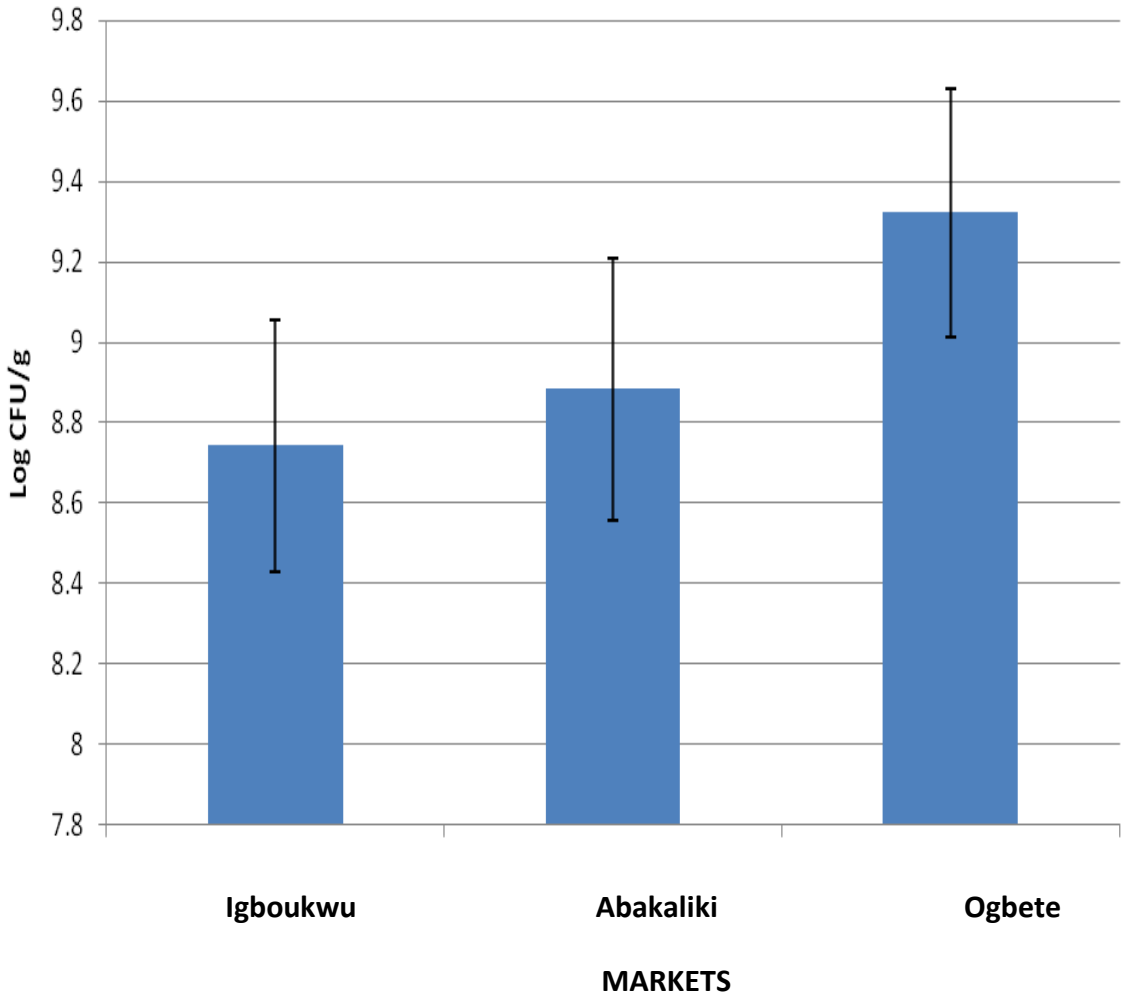



**Figure 4: Mean Aerobic Plate Counts of bacteria in edible land snails from three major markets in South East Nigeria.**

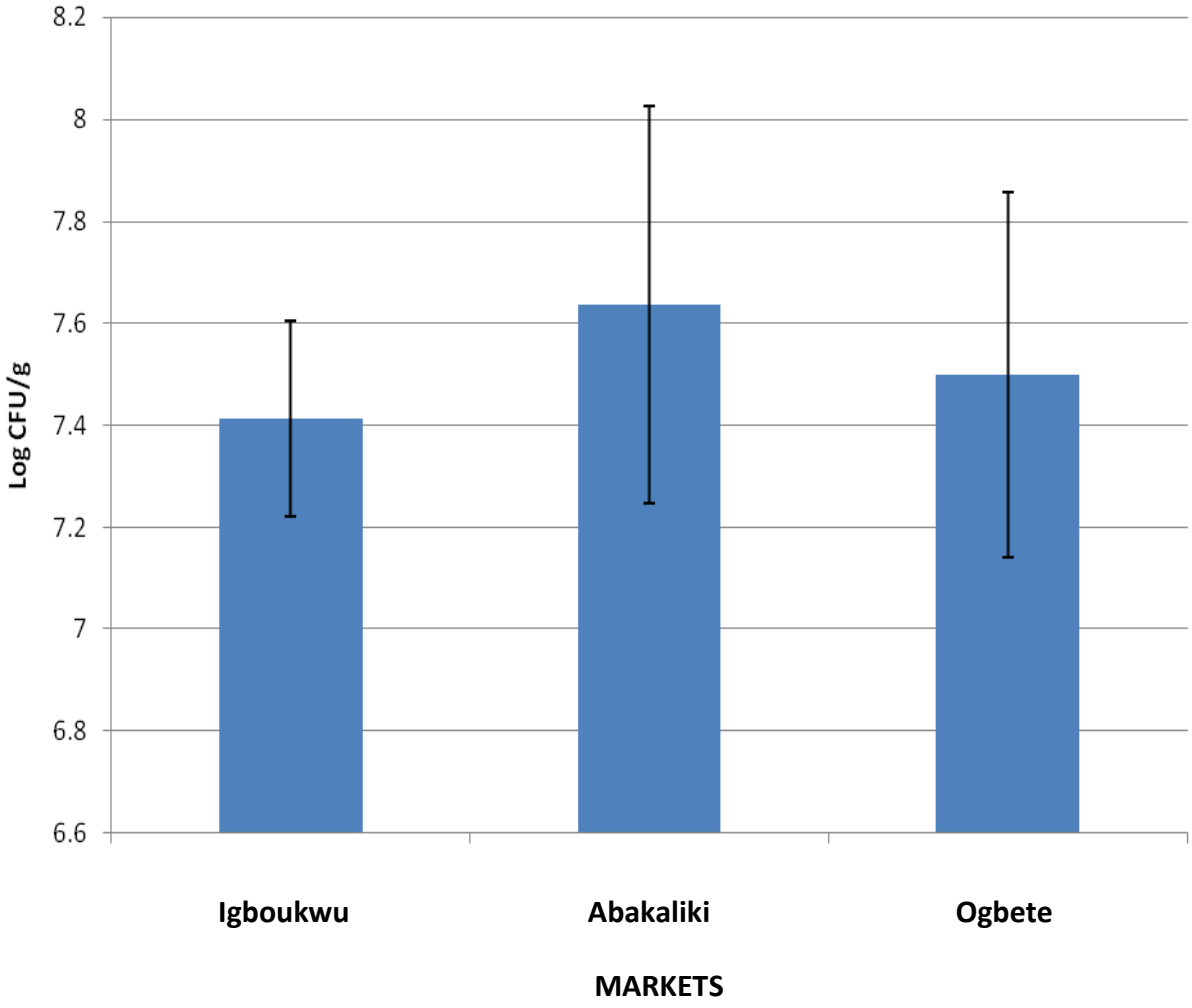



**Figure 5: Mean Counts of Coliforms in edible land snails from three major markets in South East Nigeria.**

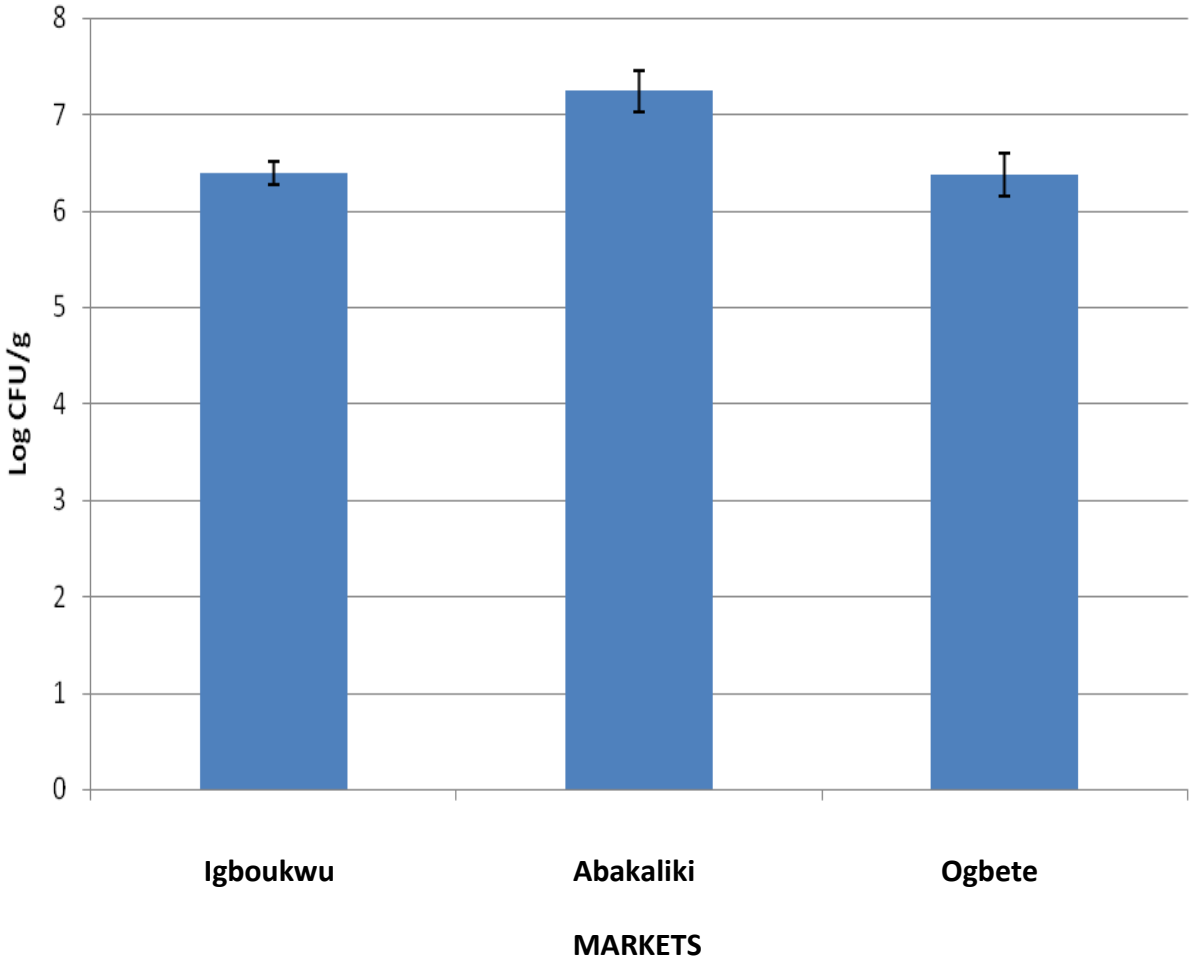



**Figure 6: Mean Counts of presumptive *Salmonella* in edible land snails from three major markets in South East Nigeria**

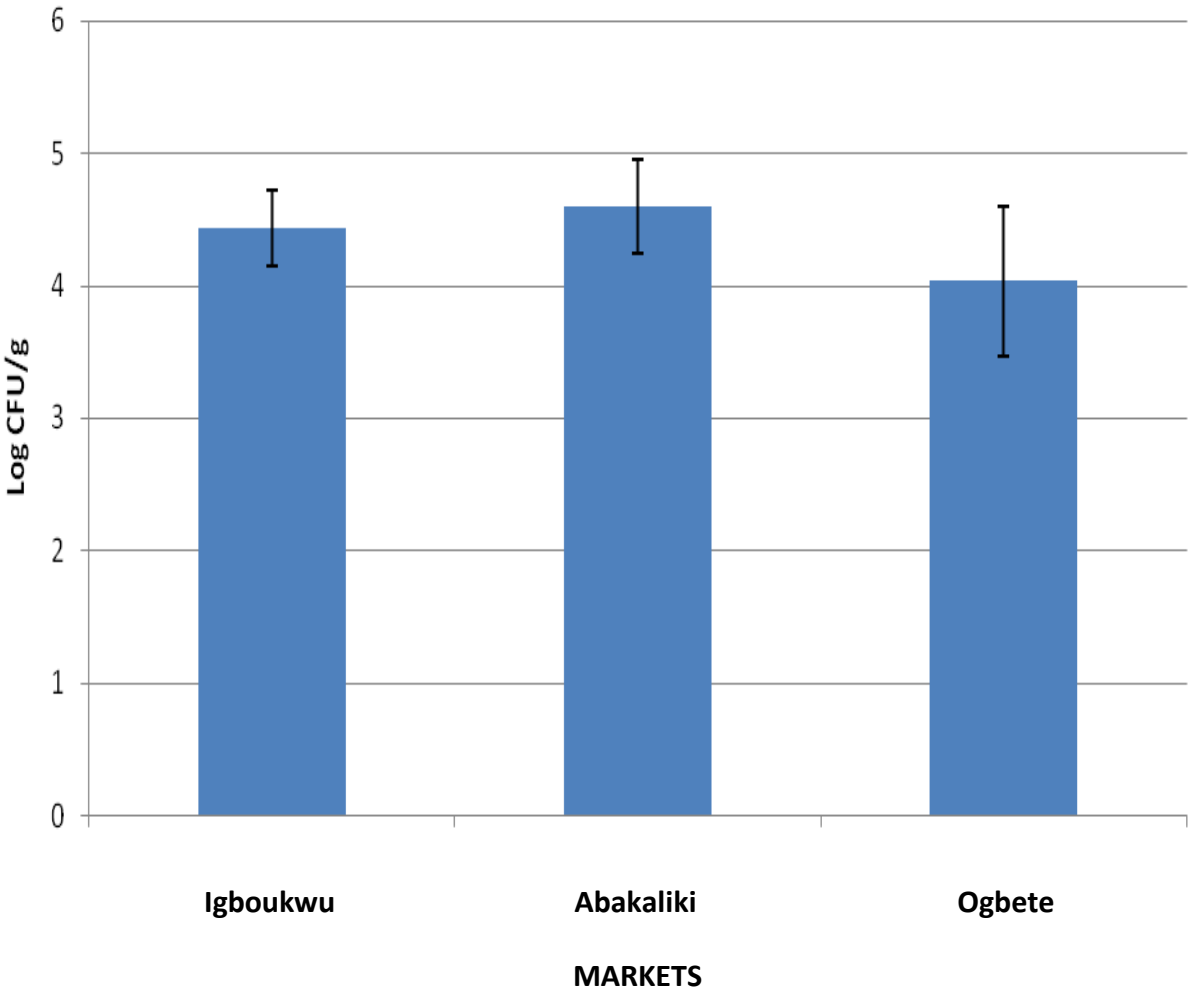



**Figure 7: Mean Counts of presumptive *Shigella* in edible land snails from three major markets in South East Nigeria.**

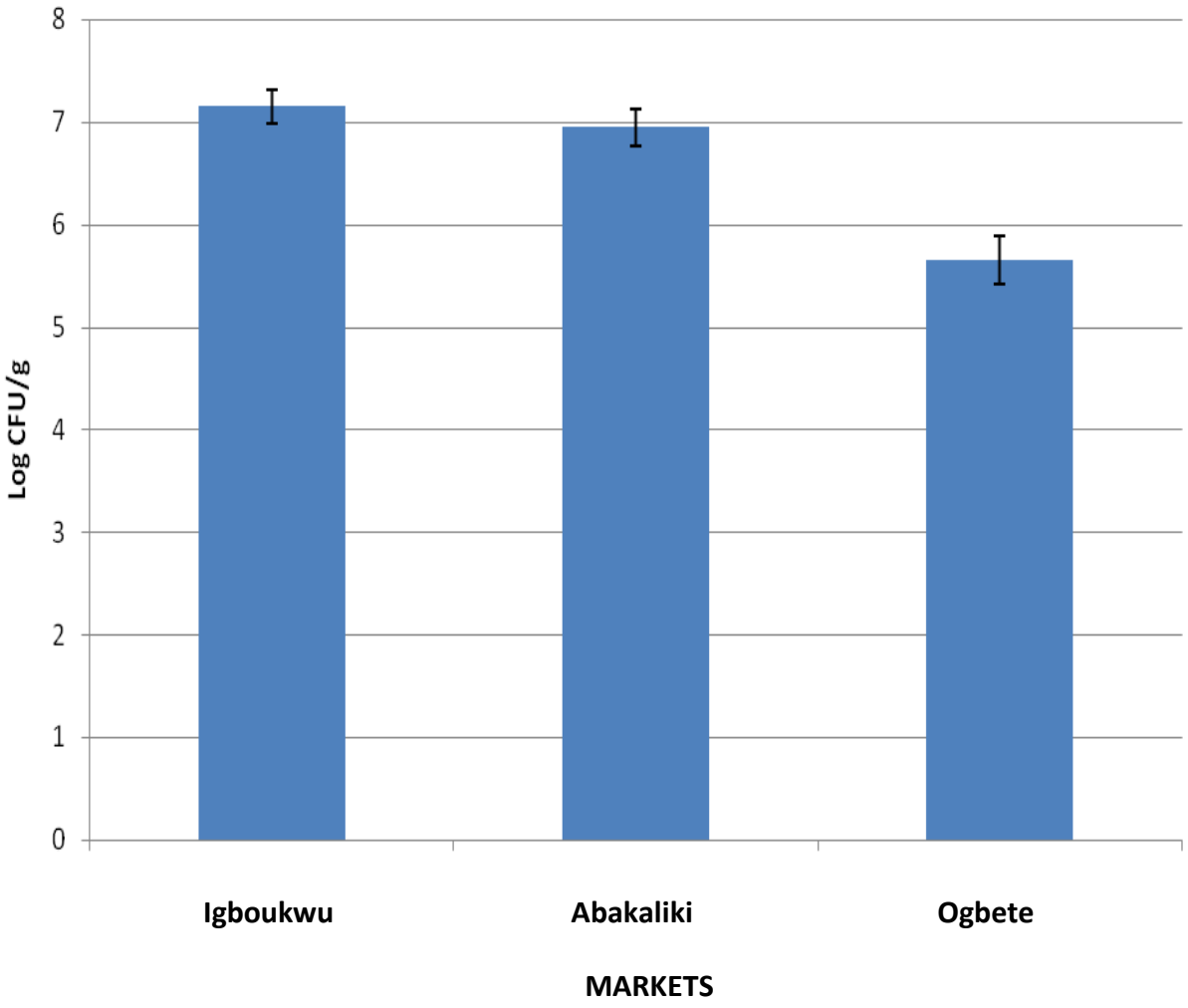



**Figure 8: Mean Counts of presumptive *E. coli* in edible land snails from three major markets in South East Nigeria.**

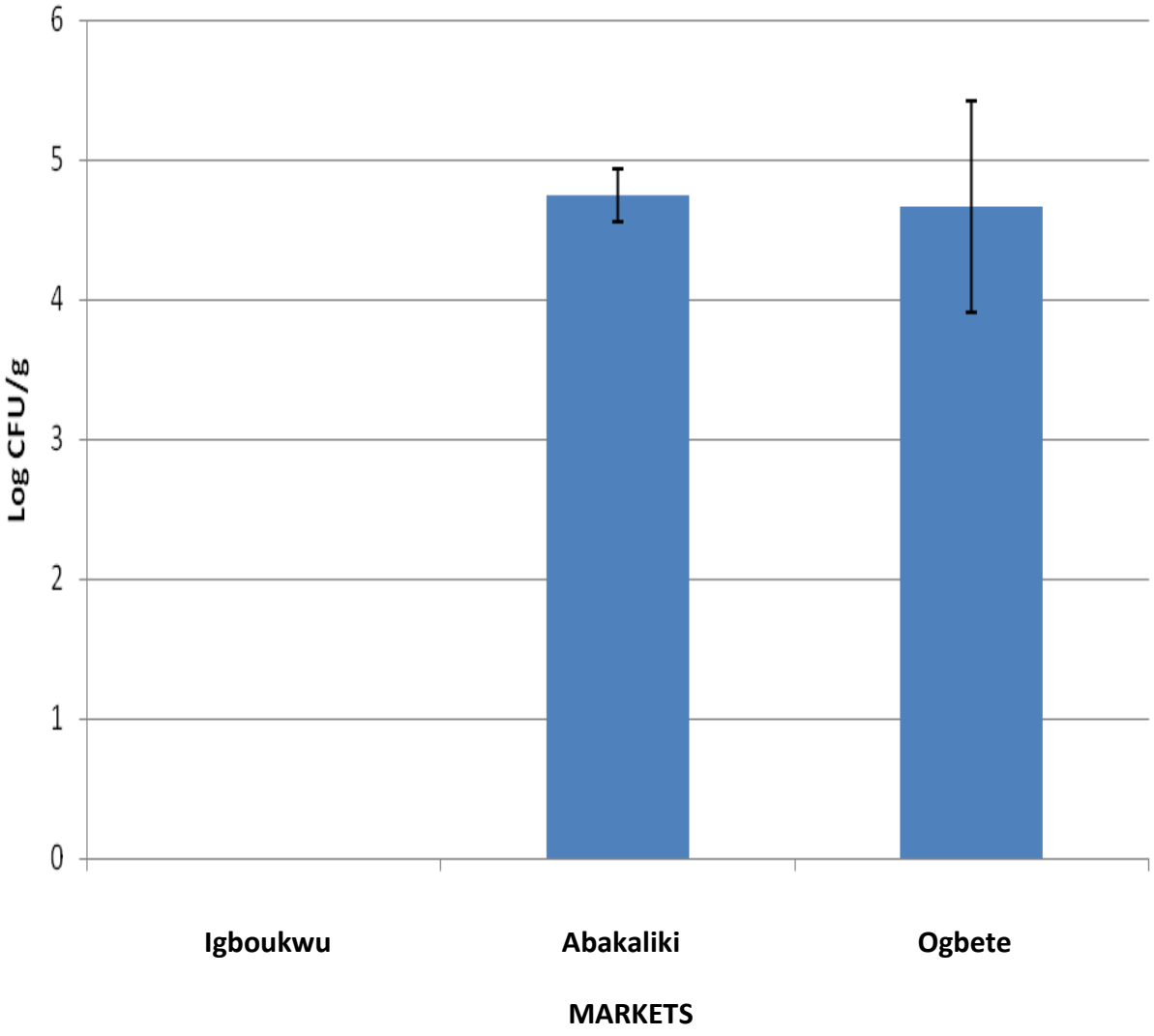



**Figure 9: Mean Counts of *Staphylococcus* in edible land snails from three major markets in South East Nigeria.**

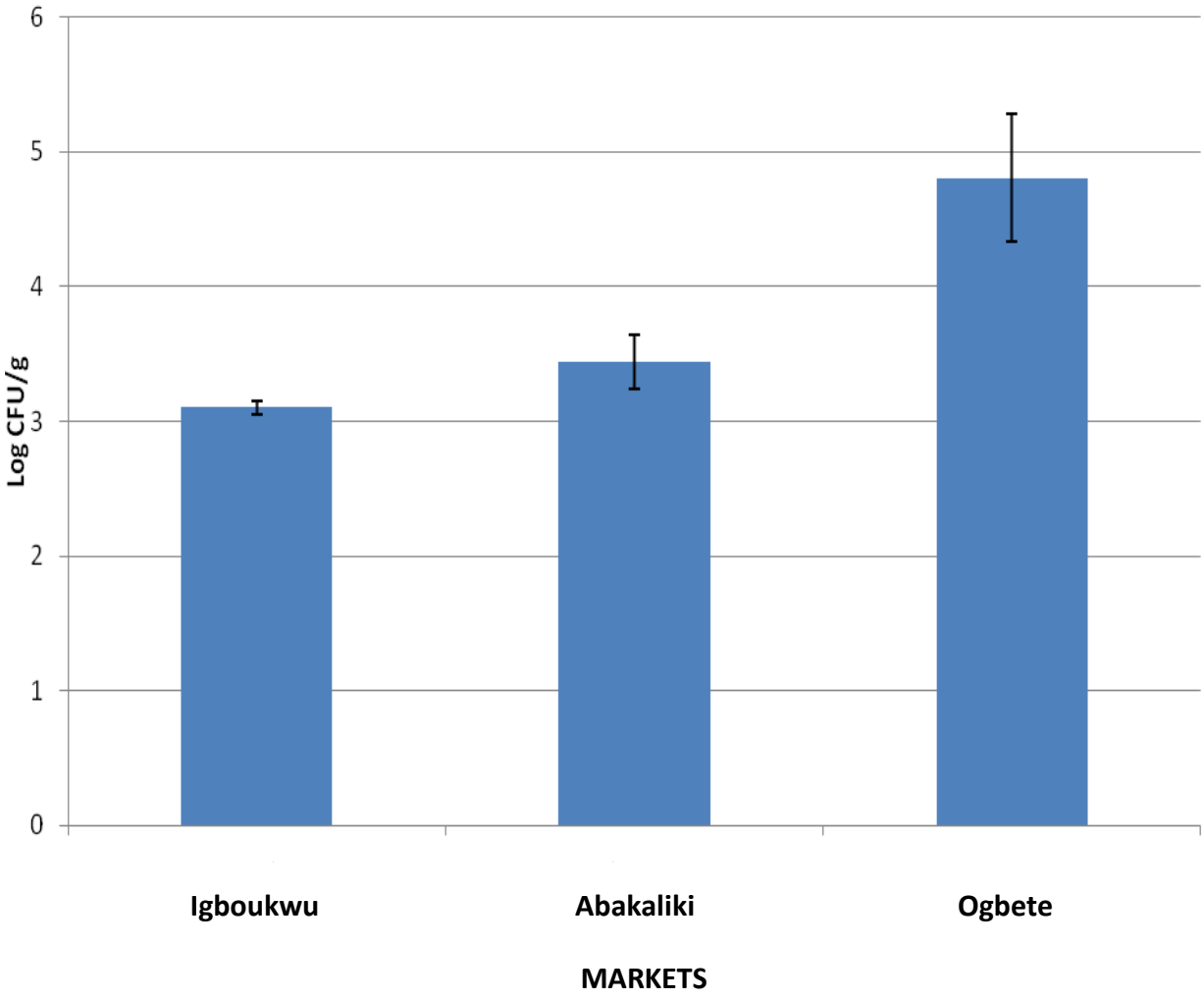



**Figure 10: Mean Counts of presumptive *Vibrio* in edible land snails from three major markets in South East Nigeria.**

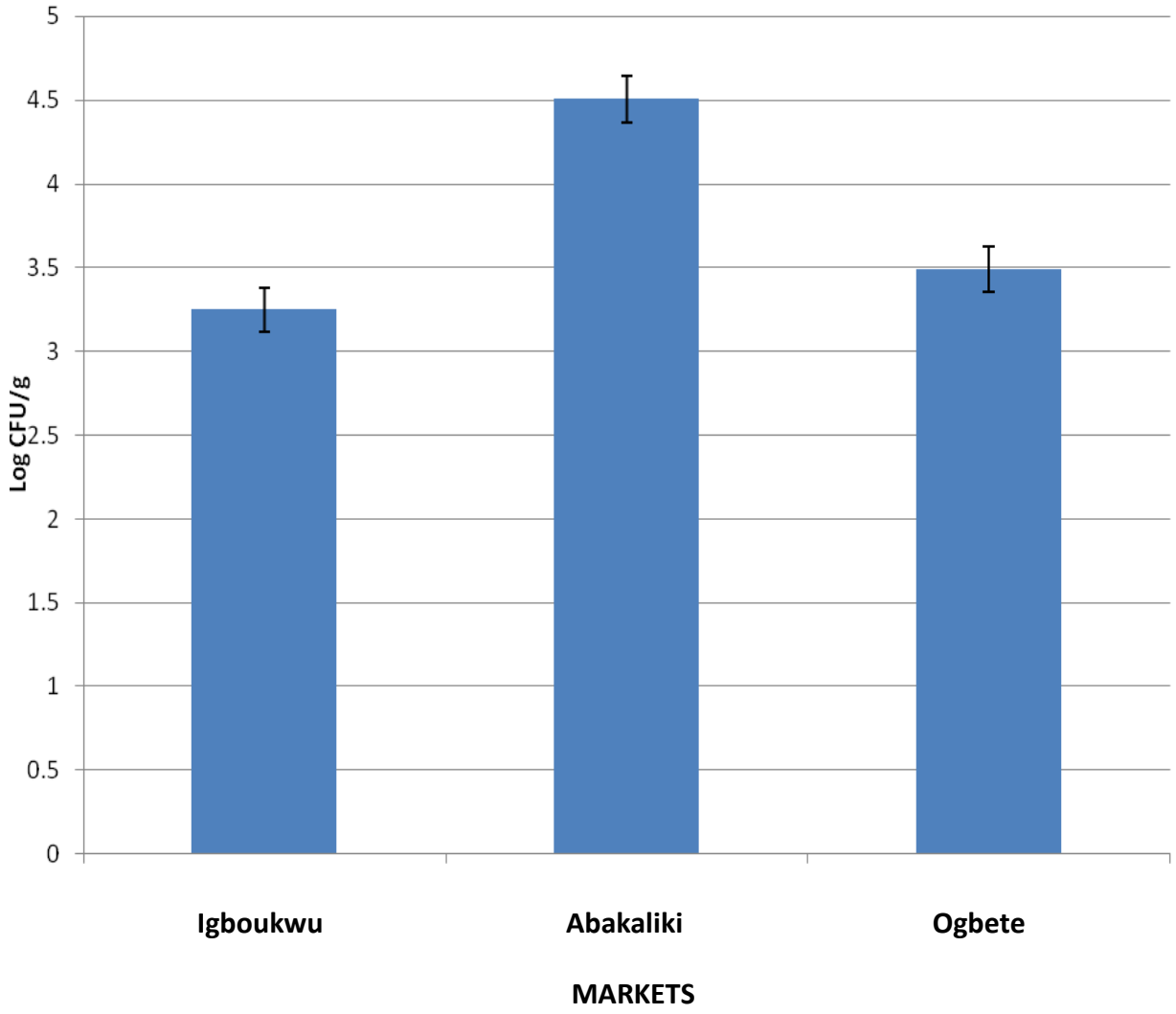



**Figure 11: Mean Counts of presumptive *Bacillus cereus* in edible land snails from three major markets in South East Nigeria.**

**Table 3: Prevalence of presumptive pathogens in edible land snails from three major markets in South East.**

| Presumptive Pathogen | Igboukwu (%) | Abakaliki (%) | Ogbete (%) | Total (%) |
|---|---|---|---|---|
| *Salmonella* | 100 | 100 | 100 | 100 |
| *Shigella* | 40 | 60 | 80 | 60 |
| *E. coli* | 90 | 80 | 100 | 90 |
| *Staphylococcus* | NIL | 30 | 80 | 36.7 |
| *Vibrio* | 70 | 70 | 90 | 76.6 |
| *B. cereus* | 70 | 80 | 90 | 80 |

The prevalence of different levels of bacterial loads in edible snails analysed in this study showed that all samples had total aerobic plate counts $>10^8$ CFU/g. Most samples (86.7%) had coliform counts ranging from $>10^6$ to $10^8$ CFU/g. Presumptive *Salmonella* counts ranged from $>10^6$ to $10^8$ CFU/g in all the samples, presumptive *Shigella* count was $<10^4$ CFU/g in 35% of the samples. Presumptive *E. coli* count was $>10^6$ to $10^8$ CFU/g in 60% of the samples. The presumptive staphylococci count was $>10^4$ to $10^6$ CFU/g in 26.7% of the samples. Presumptive *Vibrio* count was $<10^4$ CFU/g in 43.3%, presumptive *Bacillus cereus* count was $<10^4$ CFU/g in 60% of the samples (Table 4).

### 4.5 Prevalence of virulence potentials among presumptive pathogenic isolates in edible land snails

The results of the prevalence of virulence potentials in pathogens isolated from Igboukwu, Abakaliki and Ogbete samples are presented in Tables 5-10.

One hundred percent and 50% of the presumptive *Salmonella* isolates in Abakaliki and Ogbete samples respectively were found to be haemolytic while none of the Igboukwu isolates were haemolytic. Protease activity was detected in 65.6%, 0.0% and 33.3% of presumptive *Salmonella* isolates recovered from Igboukwu, Abakaliki and Ogbete samples respectively. Also, Biofilm formation test was positive in 65.6%, 70.0% and 33.3% of presumptive *Salmonella* isolates recovered from Igboukwu, Abakaliki and Ogbete samples respectively (Table 5).

Table 6 showed that haemolytic activity was detected in 0.0%, 33.3% and 50% of *Shigella* isolates from Igboukwu, Abakaliki and Ogbete samples respectively. Protease activity was detected only in *Shigella* isolates from Ogbete samples. None of the *Shigella* isolates was found to be potential biofilm producers.

As shown in Table 7, haemolytic activity was detected in only *E. coli* isolates (40%) recovered from Igboukwu samples. Abakaliki (50%) and Ogbete (40%) *E. coli* isolates were found to be proteolytic. Moreover, Igboukwu (50%), Abakaliki (25%) and Ogbete (30%) *E. coli* isolates were found to exhibit potentials for biofilm formation.

Prevalence of virulence potentials in *Staphylococcus* isolates from snails are presented in Table 8. All staphylococci isolates recovered from Abakaliki samples were found to be positive for haemolysis, gelatinase, protease, lecithinase and biofilm formation tests. Also, the staphylococci isolates from Ogbete samples were all found to be positive for all virulence associated tests, except biofilm formation (51.2%).

Prevalence of virulence potentials in presumptive *Vibrio* isolates from snails are presented in Table 9. All presumptive *Vibrio* isolates from Igboukwu samples were found to be positive for all virulence associated tests, except biofilm formation (70%). Also, presumptive *Vibrio* isolates from Abakaliki and Ogbete samples were positive for lecithinase and amylase tests. On the other hand, 50% of Abakaliki presumptive *Vibrio* isolates were positive for haemolysin, while Ogbete isolates were found to be negative. About 30% and 50% of presumptive *Vibrio* isolates from Ogbete samples were positive for biofilm formation and protease tests respectively. Also, 66.6% and 50% of presumptive *Vibrio* isolates from Abakaliki samples were positive for biofilm formation and protease tests respectively.

Prevalence of virulence potentials in presumptive *Bacillus cereus* isolates from market snails are presented in Table 10. All presumptive *Bacillus cereus* isolates recovered from the three states were found to be positive for gelatinase and lecithinase tests. All presumptive *Bacillus cereus* isolates from Igboukwu and Abakaliki samples were positive for haemolysin and 50% of Ogbete isolates were positive. About 51%, 50% and 33.3% of presumptive *Bacillus cereus* isolates from Igboukwu, Abakaliki and Ogbete samples were positive for biofilm formation tests respectively. Generally, > 70% of presumptive *Bacillus cereus* isolates recovered from the three states exhibited proteolytic activity.

**Table 4: Prevalence of different bacterial loads in edible land snails from three major markets in South East Nigeria.**

| **Bacterial Counts** | **$<10^4$ CFU/g [b](%)** | **$>10^4 - 10^6$ CFU/g (%)** | **$>10^6 - 10^8$ CFU/g (%)** | **$>10^8$ CFU/g (%)** | **Total (%) [c]N=300** |
|---|---|---|---|---|---|
| [a]APC | NIL | NIL | NIL | 100 | 100 |
| Coliforms | NIL | NIL | 86.7 | 13.3 | 100 |
| *Salmonella* | NIL | NIL | 100 | NIL | 100 |
| *Shigella* | 35 | 25 | NIL | NIL | 60 |
| *E. coli* | NIL | 30 | 60 | NIL | 90 |
| *Staphylococcus* | 10 | 26.7 | NIL | NIL | 36.7 |
| *Vibrio* | 43.3 | 33.3 | NIL | NIL | 76.6 |
| *Bacillus cereus* | 60 | 20 | NIL | NIL | 80 |

[a]APC: Aerobic Plate Count.
[b]%: Percent of samples with bacterial load.
[c]N: Number of samples analysed.
NIL: Bacterial colonies not present.

**Table 5: Prevalence of virulence potentials in presumptive *Salmonella* species isolated from edible land snails in three major markets in South East Nigeria.**

| Virulence Property | Igboukwu ([a]N=64) [b]No.(%) | Abakaliki (N=50) No.(%) | Ogbete (N=72) No.(%) |
|---|---|---|---|
| **Haemolysin** | NIL | 50 (100) | 36(50) |
| **Protease** | 42(65.6) | NIL | 24(33.3) |
| **Biofilm formation** | 42(65.6) | 35(70.0) | 24(33.3) |

[a]N: Number of isolates tested.
[b]No.(%): Number of positive isolates (Percent of isolates positive).
NIL: None tested positive.

**Table 6: Prevalence of virulence potentials in *Shigella* species isolated from edible land snails in three major markets in South East Nigeria.**

| Virulence Property | Igboukwu ([a]N=25) [b]No.(%) | Abakaliki (N=30) No.(%) | Ogbete (N=38) No.(%) |
|---|---|---|---|
| **Haemolysin** | NIL | 10(33.3) | 19(50) |
| **Protease** | NIL | NIL | 13(34.2) |
| **Biofilm formation** | NIL | NIL | NIL |

[a]N: Number of isolates tested.
[b]No.(%): Number of positive isolates (Percent of isolates positive).
NIL: None tested positive.

**Table 7: Prevalence of virulence potentials in *E. coli* isolated from edible land snails in three major markets in South East Nigeria.**

| Virulence Property | Igboukwu ([a]N=50) [b]No.(%) | Abakaliki (N=44) No.(%) | Ogbete (N=70) No.(%) |
|---|---|---|---|
| **Haemolysin** | 20(40) | NIL | NIL |
| **Protease** | NIL | 22(50) | 28(40) |
| **Lecithinase** | NIL | NIL | NIL |
| **Biofilm formation** | 25(50) | 11(25) | 21(30) |

[a]N: Number of isolates tested.
[b]No.(%): Number of positive isolates (Percent of isolates positive).
NIL: None tested positive.

**Table 8: Prevalence of virulence potentials in *Staphylococcus* isolated from edible land snails in three major markets in South East Nigeria.**

| Virulence Property | *Abakaliki ([a]N=25) [b]No.(%) | Ogbete (N=41) No.(%) |
|---|---|---|
| **Haemolysin** | 25(100) | 41(100) |
| **Gelatinase** | 25(100) | 41(100) |
| **Protease** | 25(100) | 41(100) |
| **Amylase** | NIL | 41(100) |
| **Lecithinase** | 25(100) | 41(100) |
| **Biofilm formation** | 25(100) | 21(51.2) |

* Note: *Staphylococcus* was **not** detected in samples from **Igboukwu** market.
[a]N: Number of isolates tested.
[b]No.(%): Number of positive isolates (Percent of isolates positive).
NIL: None tested positive.

**Table 9: Prevalence of virulence potentials in presumptive *Vibrio* species isolated from edible land snails in three major markets in South East Nigeria.**

| Virulence Property | Igboukwu ([a]N=32) [b]No.%) | Abakaliki (N=40) No.(%) | Ogbete (N=45) No.(%) |
|---|---|---|---|
| **Haemolysin** | 32(100) | 20(50) | NIL |
| **Gelatinase** | 32(100) | 25(62.5) | 45(100) |
| **Protease** | 32(100) | 20(50) | 30(66.6) |
| **Amylase** | 32(100) | 40(100) | 45(100) |
| **Lecithinase** | 32(100) | 40(100) | 45(100) |
| **Biofilm formation** | 24(75) | 20(50) | 15(33.3) |

[a]N: Number of isolates tested.
[b]No.(%): Number of positive isolates (Percent of isolates positive).
NIL: None tested positive.

**Table 10: Prevalence of virulence potentials in *Bacillus cereus* isolated from edible land snails in three major markets in South East Nigeria.**

| Virulence Property | Igboukwu ([a]N=35) [b]No.(%) | Abakaliki (N=32) No.(%) | Ogbete (N=66) No.(%) |
|---|---|---|---|
| **Haemolysin** | 35(100) | 32(100) | 33(50) |
| **Gelatinase** | 35(100) | 32(100) | 66(100) |
| **Protease** | 28(80) | 32(100) | 66(100) |
| **Amylase** | 10(28.5) | 8(25) | 33(50) |
| **Lecithinase** | 35(100) | 32(100) | 66(100) |
| **Biofilm formation** | 18(51.4) | 16(50) | 22(33.3) |

[a]N: Number of isolates tested.
[b]No.(%): Number of positive isolates (Percent of isolates positive).

### 4.6 Prevalence of antibiotic resistance in presumptive pathogenic isolates in edible land snails

The prevalence of antibiotic resistance in selected pathogens isolated from edible land snails are presented in Tables 11-16.

Results showed that among the presumptive *Salmonella* isolates recovered from the three markets, Ogbete isolates were found to be resistant to most antibiotics compared to isolates from Igboukwu and Abakaliki samples. Fifty percent, 35%, 30%, 50%, 30%, 25% and 35% of the presumptive *Salmonella* isolates in Ogbete samples were found to be resistant to ampicillin, amoxycillin/clavulanic acid, ceftazidime, ceftriaxone, gentamicin, nalidixic acid and streptomycin respectively. Most Abakaliki presumptive *Salmonella* isolates (70%) were resistant to ceftriaxone and nalidixic acid, while 80% of Igboukwu isolates were resistant to amoxycillin/clavulanic acid. However, all presumptive *Salmonella* isolates from these states were sensitive to ciprofloxacin, pefloxacin, septrin and ofloxacin (Table 11).

The prevalence of antibiotic resistance in *Shigella* isolates recovered from edible land snails showed that 30%, 65%, 25% and 65% of the Ogbete *Shigella* isolates were resistant to ampicillin, ceftazidime, cephalexin and ceftriaxone respectively. Abakaliki isolates (20%) were resistant to only ceftriaxone, while Igboukwu isolates were sensitive to all antibiotics used in this study (Table 12).

The prevalence of antibiotic resistance in *E. coli* isolates recovered from edible land snails showed that most *E. coli* isolates recovered from Igboukwu (75%) and Abakaliki (60%) samples were resistant to ceftazidime. Some Ogbete isolates (25%, 10% and 30%) were resistant to amoxycillin/clavulanic acid, ceftazidime and ceftriaxone, respectively. Twenty percent, 75%, 50%, and 45% of Igboukwu *E. coli* isolates were resistant to amoxycillin/clavulanic acid, ceftazidime, ceftriaxone, and streptomycin, respectively. Thirty

percent, 40%, 60%, 20% and 30% of Abakaliki *E. coli* isolates were resistant to ampicillin, amoxycillin/clavulanic acid, ceftazidime, ceftriaxone and streptomycin, respectively. All *E. coli* isolates recovered from the three markets were sensitive to ciprofloxacin, gentamicin, pefloxacin, septrin and ofloxacin (Table 13).

The prevalence of antibiotic resistance in *Staphylococcus* isolates recovered from edible land snails showed that Abakaliki (65% and 60%) and Ogbete (40% and 50%) *Staphylococcus* isolates were resistant to chloramphenicol and norfloxacin, respectively. Thirty percent of Abakaliki isolates were found to be resistant to cloxacillin, while Ogbete isolates were sensitive to cloxacillin. All *Staphylococcus* isolates were sensitive to 70% of antibiotics used in this study (Table 14).

The prevalence of antibiotic resistance in presumptive *Vibrio* isolates recovered from edible land snails showed that 40%, 26.6% and 20% of Igboukwu presumptive *Vibrio* isolates were resistant to ceftazidime, ceftriaxone and nalidixic acid, respectively. Also, 25%, 50% and 40% of Abakaliki presumptive *Vibrio* isolates were resistant to ampicillin, ceftazidime and ceftriaxone, respectively. Moreover, 40%, 45% and 35% of Ogbete presumptive *Vibrio* isolates were resistant to ampicillin, amoxycillin/clavulanic acid and ceftriaxone, respectively. All isolates of presumptive *Vibrio* were sensitive to 70% of antibiotics used in this study (Table 15).

The prevalence of antibiotic resistance in presumptive *Bacillus cereus* isolates recovered from edible land snails showed that 10%, 5%, 65% and 60% of Ogbete presumptive *B. cereus* isolates were resistant to cloxacillin, amoxycillin, chloramphenicol and norfloxacin, respectively. Abakaliki presumptive *B. cereus* isolates (50% and 75%) were

resistant to chloramphenicol and norfloxacin, respectively. A cloxacillin-resistant presumptive *B. cereus* isolate was recovered from Igboukwu samples. However, all presumptive *B. cereus* isolates were found to be sensitive to 60% of antibiotics used in this study (Table 16).

Generally, some isolates were found to exhibit multidrug resistance. Antibiotic resistance patterns detected in these isolates are presented in Table 17.

A total of 15 different antibiotic resistance patterns were detected among the multi-resistant isolates. Among the *E. coli* isolates, five types of antibiotic resistance patterns (4 and 1 patterns) were found in Igboukwu and Abakaliki samples, respectively. Among the presumptive *Vibrio* isolates, four types of resistance patterns (2, 1 and 1 patterns) were found in Abakaliki, Igboukwu and Ogbete samples, respectively. Among the presumptive *Salmonella* isolates, three types of resistance patterns (1, 1 and 2 patterns) were found in Igboukwu, Abakaliki and Ogbete samples, respectively. Two resistance patterns were detected in *Shigella*, *Staphylococcus* and *Bacillus* isolates recovered from Ogbete and Abakaliki samples.

Presumptive *Salmonella* isolates from Igboukwu and Abakaliki samples were each found to exhibit multi-resistance to 5 antibiotics. Furthermore, the multi-resistant isolates were found to be most resistant to Ceftazidime > Ceftriaxone > Ampicillin > Nalidixic acid = Streptomycin = Amoxycillin/clavulanic acid = Norfloxacin = Chloramphenicol > Cephalexin > Cloxacillin. Multi-drug resistance was most prevalent in Abakaliki and Igboukwu isolates tested in this study.

**Table 11: Prevalence of antibiotic resistance in presumptive *Salmonella* species isolated from edible land snails in three major markets in South East Nigeria.**

| Antibiotics | Igboukwu ([a]N=20) [b]No.(%) | Abakaliki (N=40) No.(%) | Ogbete (N=40) No.(%) |
|---|---|---|---|
| **Ampicillin** | 4(20) | 14(35) | 20(50) |
| **Amoxy/Clav. Acid** | 16(80) | 14(35) | 14(35) |
| **Ceftazidime** | 7(35) | 12(30) | 12(30) |
| **Ceftriaxone** | 5(25) | 28(70) | 20(50) |
| **Cephalexin** | 4(20) | NIL | NIL |
| **Ciprofloxacin** | NIL | NIL | NIL |
| **Gentamicin** | NIL | NIL | 12(30) |
| **Nalidixic acid** | 4(20) | 28(70) | 10(25) |
| **Pefloxacin** | NIL | NIL | NIL |
| **Septrin** | NIL | NIL | NIL |
| **Streptomycin** | NIL | NIL | 14(35) |
| **Ofloxacin** | NIL | NIL | NIL |

[a]N: Number of isolates tested,
[b]No.(%): Number of resistant isolates (Percent of isolates resistant),
NIL: None.

**Table 12: Prevalence of antibiotic resistance in *Shigella* species isolated from edible land snails in three major markets in South East Nigeria.**

| Antibiotics | Igboukwu ([a]N=20) [b]No.(%) | Abakaliki (N=20) No.(%) | Ogbete (N=20) No.(%) |
|---|---|---|---|
| **Ampicillin** | NIL | NIL | 6(30) |
| **Amoxy/Clav. Acid** | NIL | NIL | NIL |
| **Ceftazidime** | NIL | NIL | 13(65) |
| **Ceftriaxone** | NIL | 4(20) | 13(65) |
| **Cephalexin** | NIL | NIL | 5(25) |
| **Ciprofloxacin** | NIL | NIL | NIL |
| **Gentamicin** | NIL | NIL | NIL |
| **Nalidixic acid** | NIL | NIL | NIL |
| **Pefloxacin** | NIL | NIL | NIL |
| **Septrin** | NIL | NIL | NIL |
| **Streptomycin** | NIL | NIL | NIL |
| **Ofloxacin** | NIL | NIL | NIL |

[a]N: Number of isolates tested,
[b]No.(%): Number of resistant isolates (Percent of isolates resistant),
NIL: None.

**Table 13: Prevalence of antibiotic resistance in *E. coli* isolated from edible land snails in three major markets in South East Nigeria.**

| Antibiotics | Igboukwu ([a]N=20) [b]No.(%) | Abakaliki (N=10) No.(%) | Ogbete (N=20) No.(%) |
|---|---|---|---|
| **Ampicillin** | NIL | 3(30) | NIL |
| **Amoxy/Clav. Acid** | 4(20) | 4(40) | 5(25) |
| **Ceftazidime** | 15(75) | 6(60) | 2(10) |
| **Ceftriaxone** | 10(50) | 2(20) | 6(30) |
| **Cephalexin** | NIL | NIL | NIL |
| **Ciprofloxacin** | NIL | NIL | NIL |
| **Gentamicin** | NIL | NIL | NIL |
| **Nalidixic acid** | NIL | NIL | NIL |
| **Pefloxacin** | NIL | NIL | NIL |
| **Septrin** | NIL | NIL | NIL |
| **Streptomycin** | 9(45) | 3(30) | NIL |
| **Ofloxacin** | NIL | NIL | NIL |

[a]N: Number of isolates tested,
[b]No.(%): Number of resistant isolates (Percent of isolates resistant),
NIL: None.

**Table 14: Prevalence of antibiotic resistance in *Staphylococcus* isolated from edible land snails in three major markets in South East Nigeria.**

| Antibiotics | Abakaliki (N=20) No.(%) | Ogbete (N=30) No.(%) |
|---|---|---|
| **Cloxacillin** | 6(30) | NIL |
| **Amoxycillin** | NIL | NIL |
| **Chloramphenicol** | 13(65) | 12(40) |
| **Ciprofloxacin** | NIL | NIL |
| **Erythromycin** | NIL | NIL |
| **Gentamicin** | NIL | NIL |
| **Levofloxacin** | NIL | NIL |
| **Norfloxacin** | 12(60) | 15(50) |
| **Rifampicin** | NIL | NIL |
| **Streptomycin** | NIL | NIL |

Note: *Staphylococcus* was not detected in any sample from Anambra state.
[a]N: Number of isolates tested,
[b]No.(%): Number of resistant isolates (Percent of isolates resistant),
NIL: None.

**Table 15: Prevalence of antibiotic resistance in presumptive *Vibrio* species isolated from edible land snails in three major markets in South East Nigeria.**

| Antibiotics | Igboukwu ([a]N=30) [b]No.(%) | Abakaliki (N=20) No.(%) | Ogbete (N=20) No.(%) |
|---|---|---|---|
| **Ampicillin** | NIL | 5(25) | 8(40) |
| **Amoxy/Clav. Acid** | NIL | NIL | 9(45) |
| **Ceftazidime** | 12(40) | 10(50) | NIL |
| **Ceftriaxone** | 8(26.6) | 8(40) | 7(35) |
| **Cephalexin** | NIL | NIL | NIL |
| **Ciprofloxacin** | NIL | NIL | NIL |
| **Gentamicin** | NIL | NIL | NIL |
| **Nalidixic acid** | 6(20) | NIL | NIL |
| **Pefloxacin** | NIL | NIL | NIL |
| **Septrin** | NIL | NIL | NIL |
| **Streptomycin** | NIL | NIL | NIL |
| **Ofloxacin** | NIL | NIL | NIL |

[a]N: Number of isolates tested,
[b]No.(%): Number of resistant isolates (Percent of isolates resistant),
NIL: None.

**Table 16: Prevalence of antibiotic resistance in presumptive *B. cereus* isolated from edible land snails in three major markets in South East Nigeria.**

| Antibiotics | Igboukwu ([a]N=20) [b]No.(%) | Abakaliki (N=20) No.(%) | Ogbete (N=40) No.(%) |
|---|---|---|---|
| **Cloxacillin** | 1(5) | NIL | 4(10) |
| **Amoxycillin** | NIL | NIL | 2(5) |
| **Chloramphenicol** | NIL | 10(50) | 26(65) |
| **Ciprofloxacin** | NIL | NIL | NIL |
| **Erythromycin** | NIL | NIL | NIL |
| **Gentamicin** | NIL | NIL | NIL |
| **Levofloxacin** | NIL | NIL | NIL |
| **Norfloxacin** | NIL | 15(75) | 24(60) |
| **Rifampicin** | NIL | NIL | NIL |
| **Streptomycin** | NIL | NIL | NIL |

[a]N: Number of isolates tested,
[b]No.(%): Number of resistant isolates (Percent of isolates resistant),
NIL: None.

**Table 17: Antibiotic resistance patterns in pathogens isolated from edible land snails in three major markets in South East Nigeria.**

| Antibiotic Resistance Pattern | Presumptive Pathogen | Source |
|---|---|---|
| **AMP-CEP-NA-CTX-CAZ** | *Salmonella* | Igboukwu |
| **AMP-NA-AMC-CTX-CAZ** | *Salmonella* | Abakaliki |
| **S-AMC-CTX-CAZ** | *E. coli* | Abakaliki |
| **AMP-CTX-CAZ** | *Vibrio* | Abakaliki |
| **CLX-NOR-CHL** | *Staphylococcus* | Abakaliki |
| **AMC-CTX-CAZ** | *E. coli* | Igboukwu |
| **S-CTX-CAZ** | *E. coli* | Igboukwu |
| **S-CAZ** | *E. coli* | Igboukwu |
| **NA-CAZ** | *Vibrio* | Igboukwu |
| **NA-CAZ** | *E. coli* | Igboukwu |
| **CTX-CAZ** | *Vibrio* | Abakaliki |
| **NOR-CHL** | *B. cereus* | Abakaliki |
| **NOR-CHL** | *Staphylococcus* | Abakaliki |
| **CTX-CAZ** | *Salmonella* | Ogbete |
| **CTX-CAZ** | *Shigella* | Ogbete |
| **S-AMP** | *Salmonella* | Ogbete |
| **AMP-CEP** | *Shigella* | Ogbete |
| **AMP-AMC** | *Vibrio* | Ogbete |
| **NOR-CHL** | *B. cereus* | Ogbete |

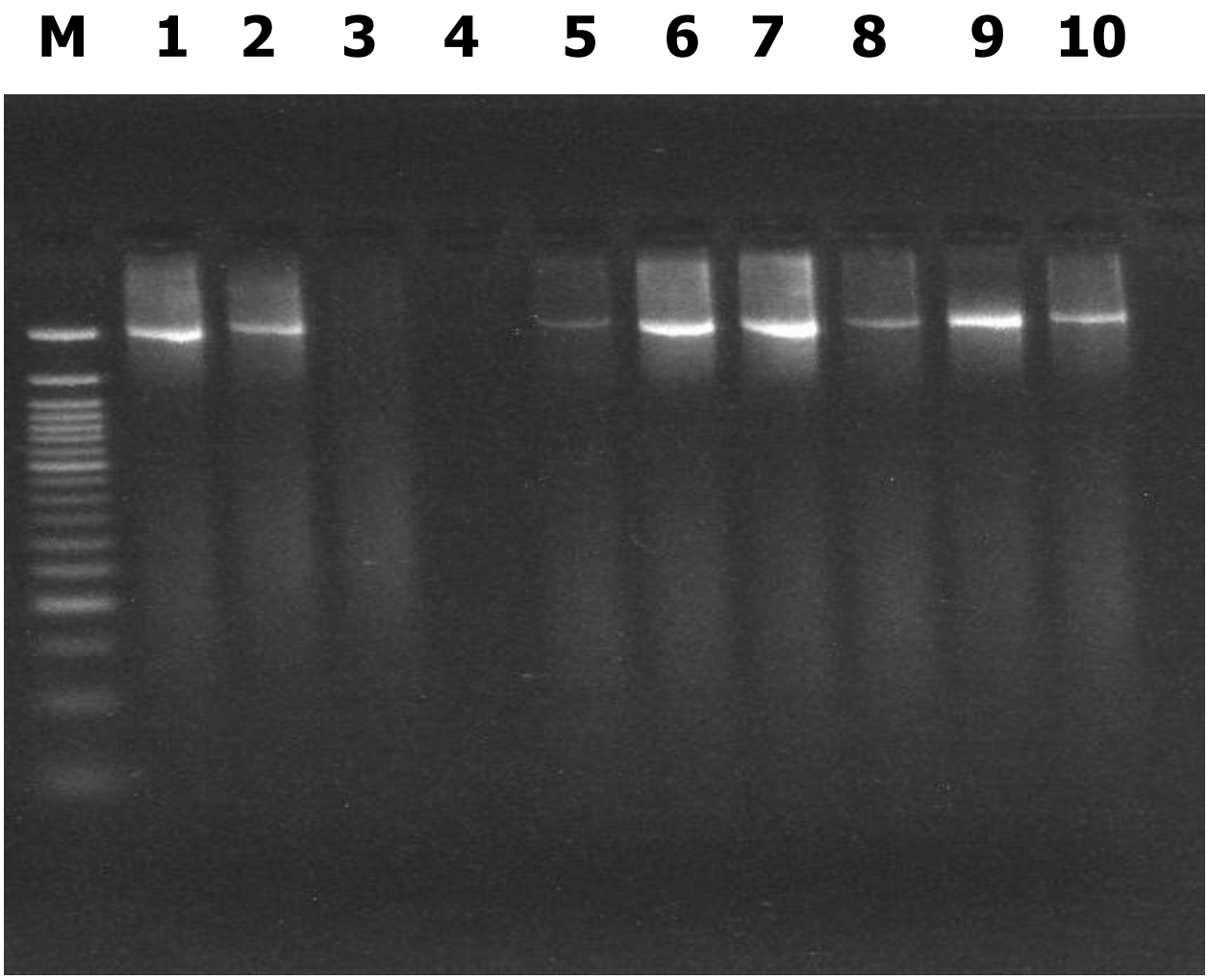
AMP: Ampicillin, CEP: Cephalexin, NA: Nalidixic acid, CTX: Ceftriaxone, CAZ: Ceftazidime, AMC: Amoxy/Clav. acid, S: Streptomycin, CLX: Cloxacillin, NOR: Norfloxacin, CHL: Chloramphenicol

## 4.7 Identification of selected bacterial pathogens in edible land snails using 16S rRNA gene sequencing

PCR amplification of the 16S rRNA genes of the selected isolates yielded good bands after electrophoresis, except for isolate 0196AN that had no band (Lane 4, Figure 12). Analysis of 16S rRNA gene sequence was used for identification of the ten selected pathogens. BLAST of the nucleotide sequences of these isolates on NCBI website revealed species of the following genera as shown in Table 18: *Escherichia, Citrobacter, Staphylococcus, Aeromonas* and *Bacillus*, which in some cases differed from the presumptive identity (Table 19). Phylogenetic trees showing the relationship between 16S rRNA sequences of these isolates and sequences of other similar isolates on GenBank are presented in Figures 13-19.

## 4.8 Detection of toxin genes by PCR in selected presumptive pathogenic isolates in edible land snails.

The results of the PCR analysis to detect some toxin genes in some *Staphylococcus* and *Bacillus* isolates are presented in Tables 20 and 21. Gel images from the PCR analysis are presented in Appendices 11 – 17. Results showed that *Sea* gene was detected only in *Staphylococcus sciuri* (0181EN) (Table 20). However, *exhc* gene was not detected in any of the *Staphylococcus* isolates. The results of PCR analysis to detect toxin genes in *Bacillus* isolates recovered from edible land snails are summarized in Table 21. The *nheb* gene was the only gene detected in all three *Bacillus* isolates screened in this study, while *hblc* gene was not detected in any of the isolates. Only isolate 0186EN was found to possess four (*hbla, nhea, nheb* and *cytk*) out of the five toxin genes used in this study. While isolate 0177EN possessed *hbla* and *nheb*, 0197AN was found to habour *nhea* and *nheb*.




**(M) Ladder (50 bp); (1) 0163EN; (2) 0180EN; (3) 0186EN; (4) 0196AN; (5) 0184EN; (6) 0193AN; (7) 0173EN; (8) 0177EN; (9) 0181EN; (10) 0197AN.**

**Figure 12: Amplicons of 16S rRNA genes of selected isolates targeted for sequencing**

**Table 18: Identity of nine selected pathogens in edible land snails determined by 16S rRNA gene sequencing**

| Isolate Code | Identity of Isolate | Accession Number |
|---|---|---|
| 0163EN | *Escherichia fergusonii* | MK522152 |
| 0173EN | *Citrobacter freundii* | MK526907 |
| 0177EN | *Bacillus cereus* | MK530202 |
| 0180EN | *Staphylococcus arlettae* | MK518344 |
| 0181EN | *Staphylococcus sciuri* | MK518066 |
| 0184EN | *Aeromonas hydrophila* | MK530176 |
| 0186EN | *Bacillus thuringiensis* | MK530172 |
| 0193AN | *Citrobacter freundii* | MK527109 |
| 0197AN | *Bacillus cereus* | MK530171 |

**Table 19: Comparison of the Identity of nine selected pathogens determined by phenotypic and molecular studies**

| Isolate Code | Identity by phenotypic studies | Identity by molecular studies |
|---|---|---|
| 0163EN | *Escherichia coli* | *Escherichia fergusonii* |
| 0173EN | *Salmonella* | *Citrobacter freundii* |
| 0177EN | *Bacillus cereus* | *Bacillus cereus* |
| 0180EN | *Staphylococcus* | *Staphylococcus arlettae* |
| 0181EN | *Staphylococcus* | *Staphylococcus sciuri* |
| 0184EN | *Vibrio* | *Aeromonas hydrophila* |
| 0186EN | *Bacillus cereus* | *Bacillus thuringiensis* |
| 0193AN | *Salmonella* | *Citrobacter freundii* |
| 0197AN | *Bacillus cereus* | *Bacillus cereus* |

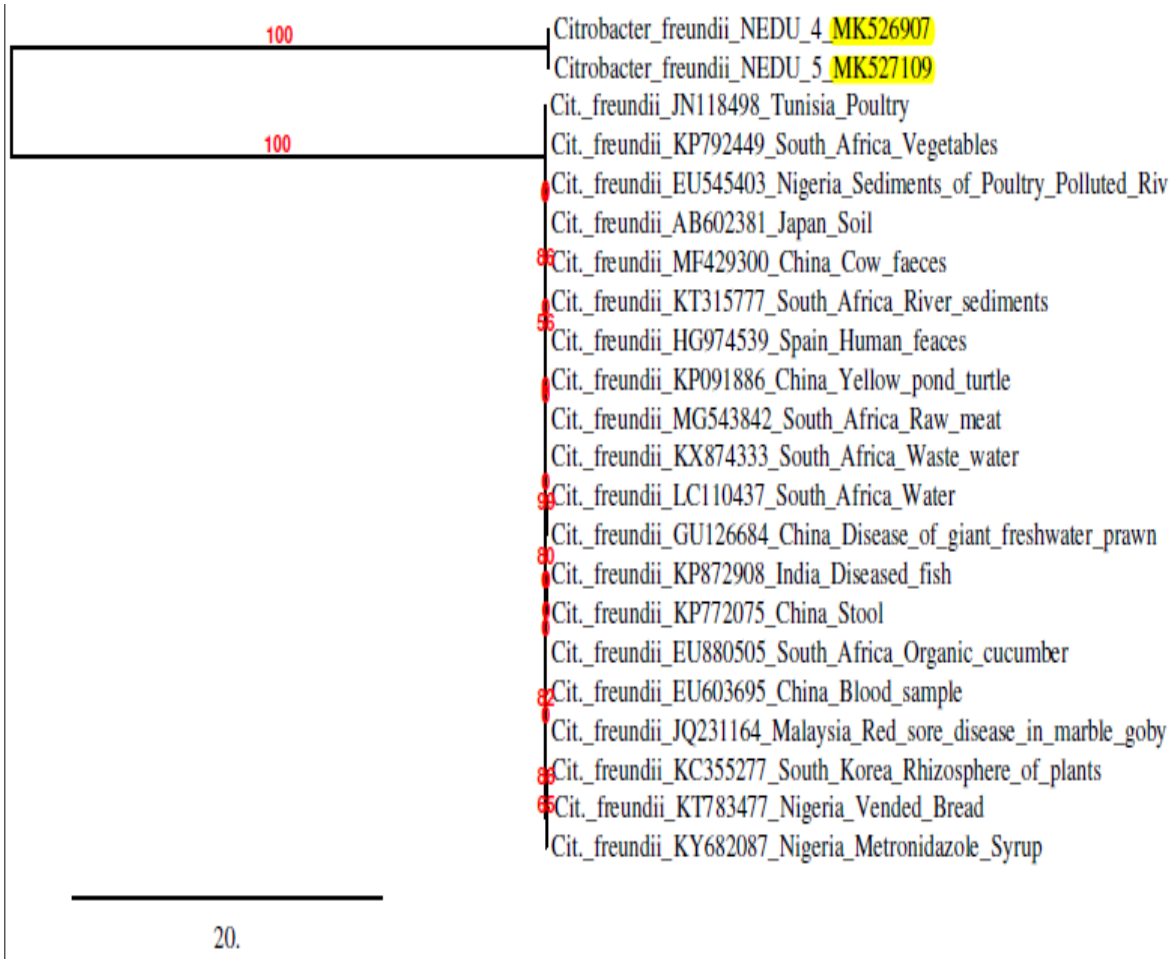



**Figure 13: Phylogenetic tree showing the relationship of *Citrobacter freundii* NEDU 4 and NEDU 5 to other members of the *Citrobacter freundii* based on 16S rRNA gene sequences**

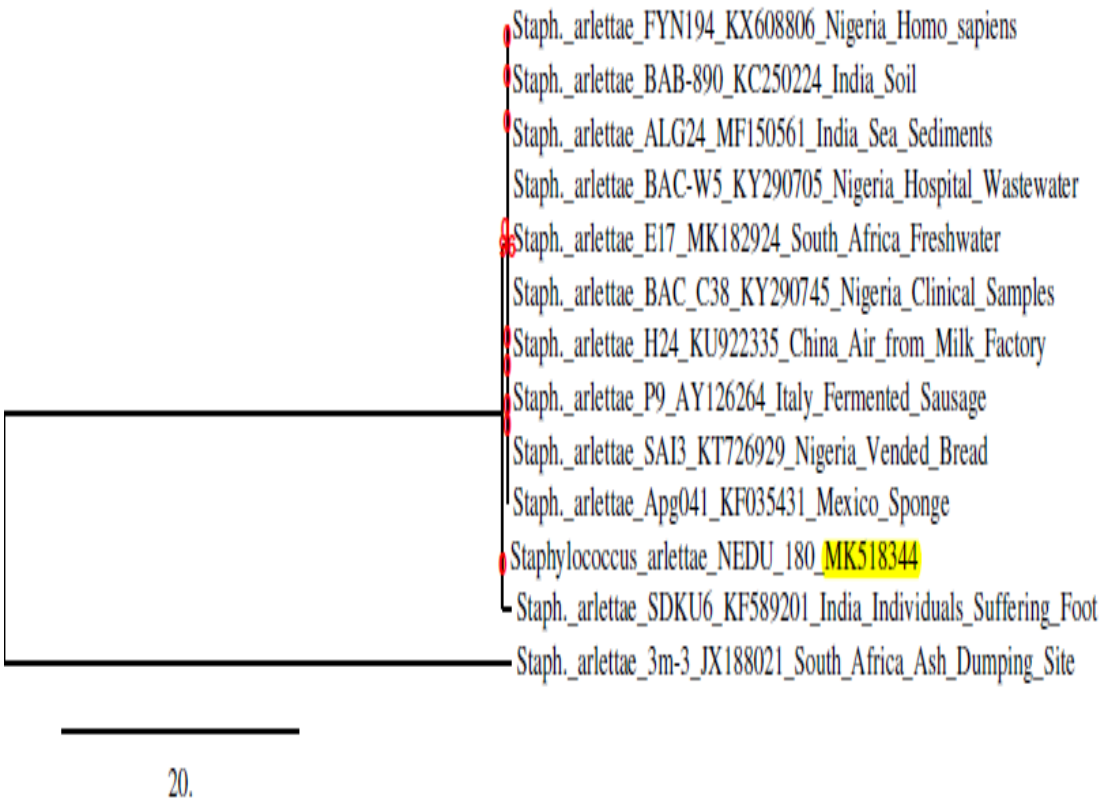



**Figure 14: Phylogenetic tree showing the relationship of *Staphylococcus arlettae* NEDU 180 to other members of the *Staphylococcus arlettae* based on 16S rRNA gene sequences**

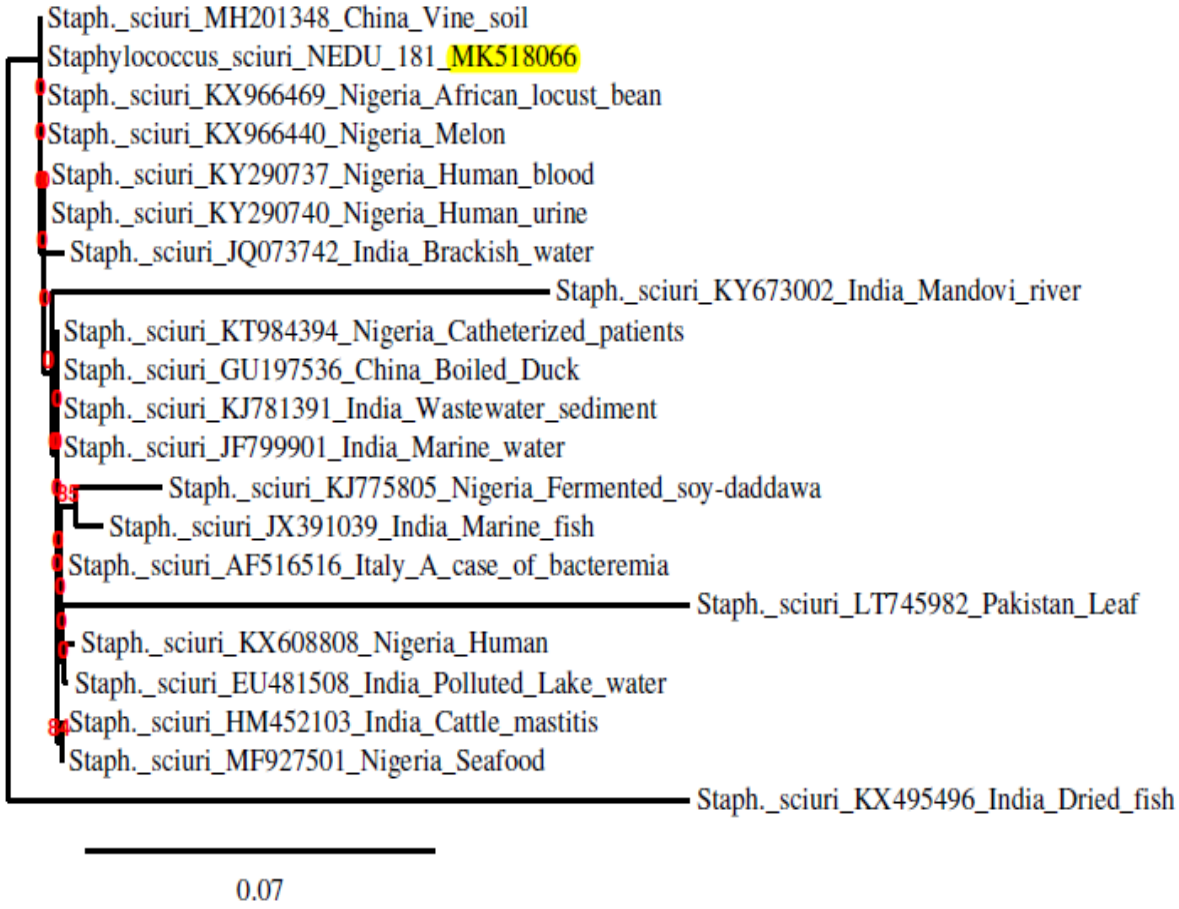



**Figure 15: Phylogenetic tree showing the relationship of *Staphylococcus sciuri* NEDU 181 to other members of the *Staphylococcus sciuri* based on 16S rRNA gene sequences**

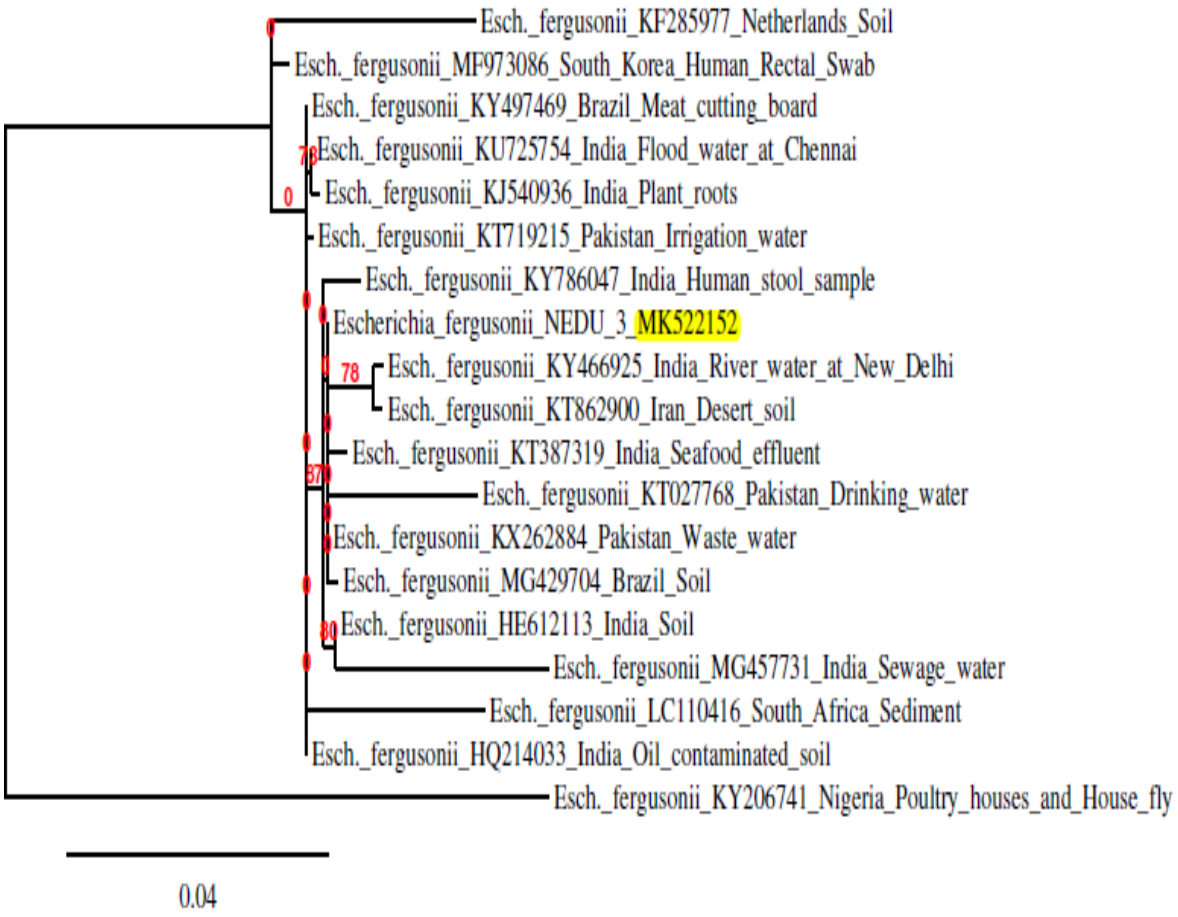



**Figure 16: Phylogenetic tree showing the relationship of *Escherichia fergusonii* isolate NEDU 3 to other members of the *Escherichia fergusonii* based on 16S rRNA gene sequences**

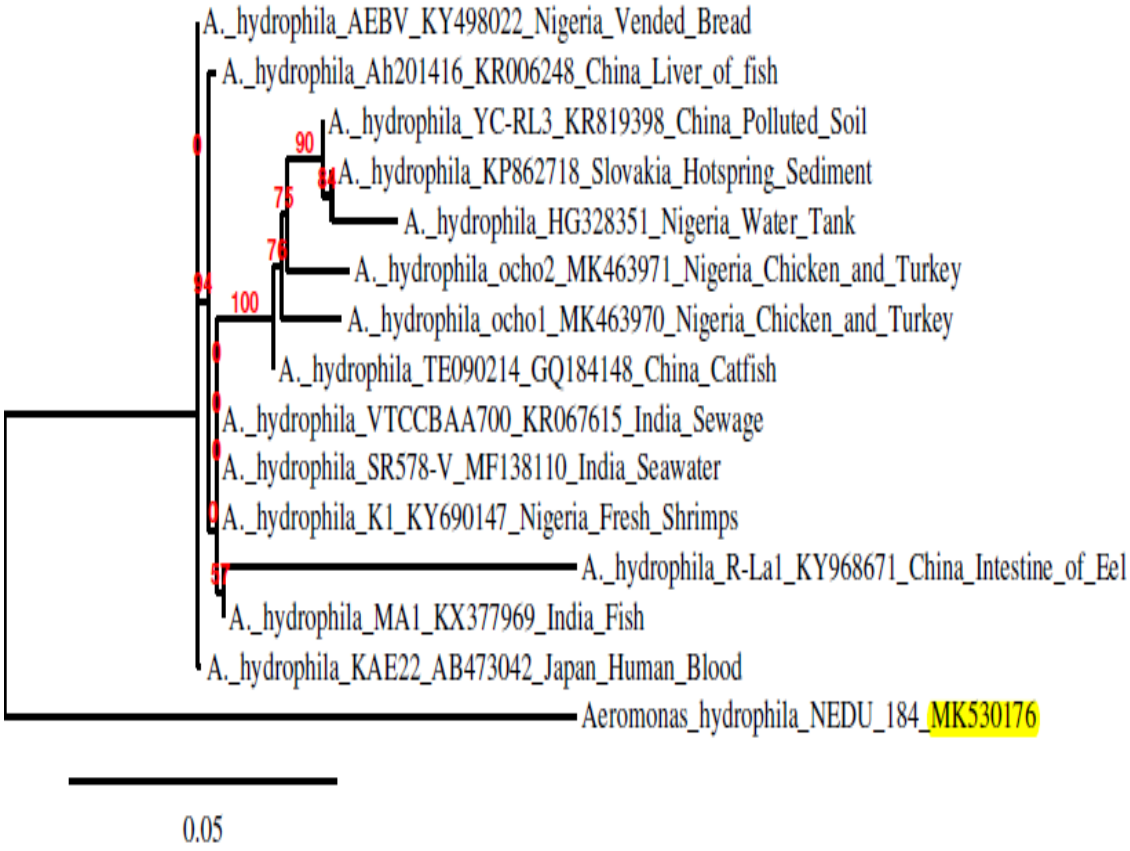



**Figure 17: Phylogenetic tree showing the relationship of *Aeromonas hydrophila* NEDU 184 to other members of the *Aeromonas hydrophila* based on 16S rRNA gene sequences**

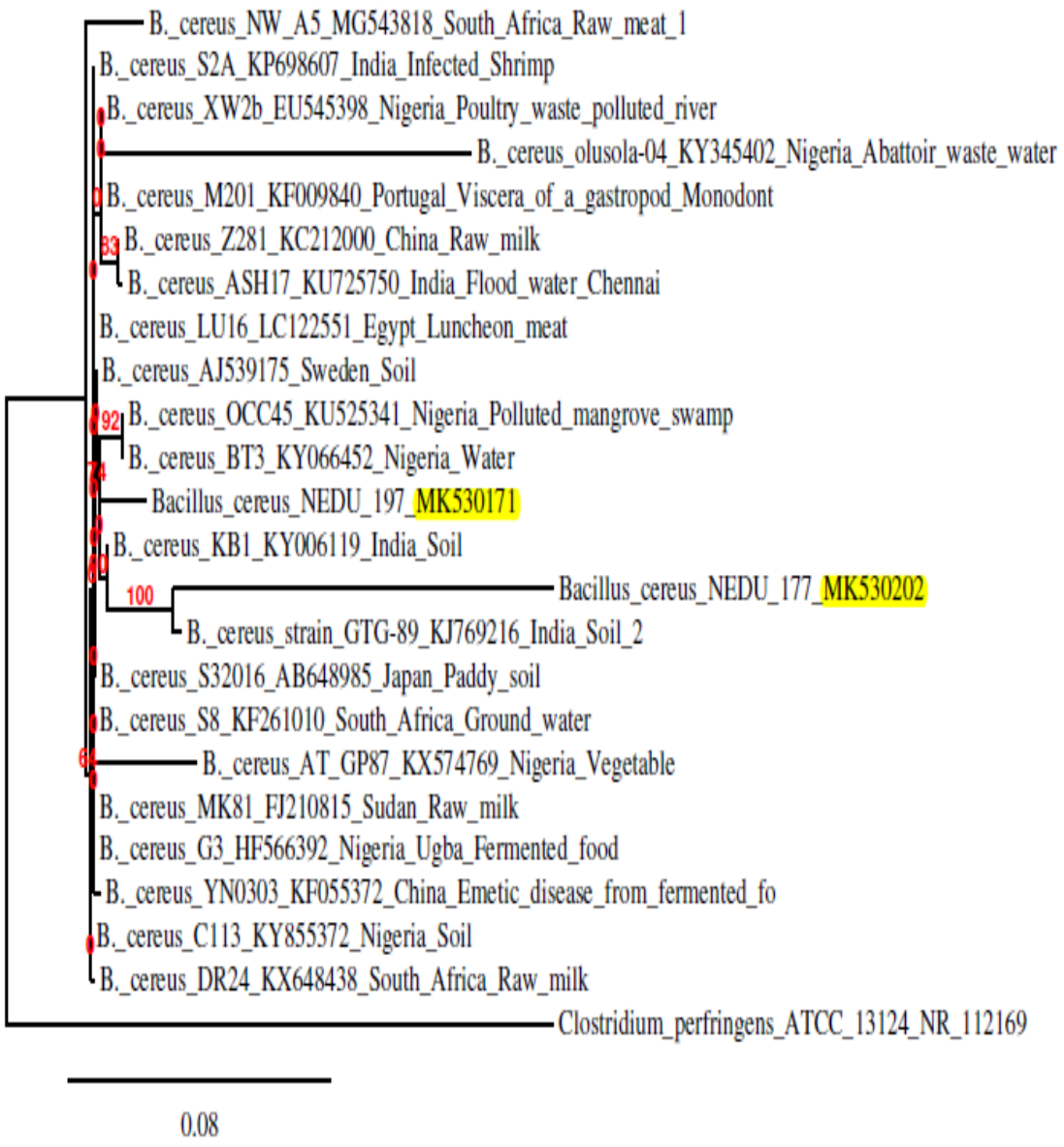



**Figure 18: Phylogenetic tree showing the relationship of *Bacillus cereus* NEDU 177 and NEDU 197 to other members of the *Bacillus cereus* based on 16S rRNA gene sequences**

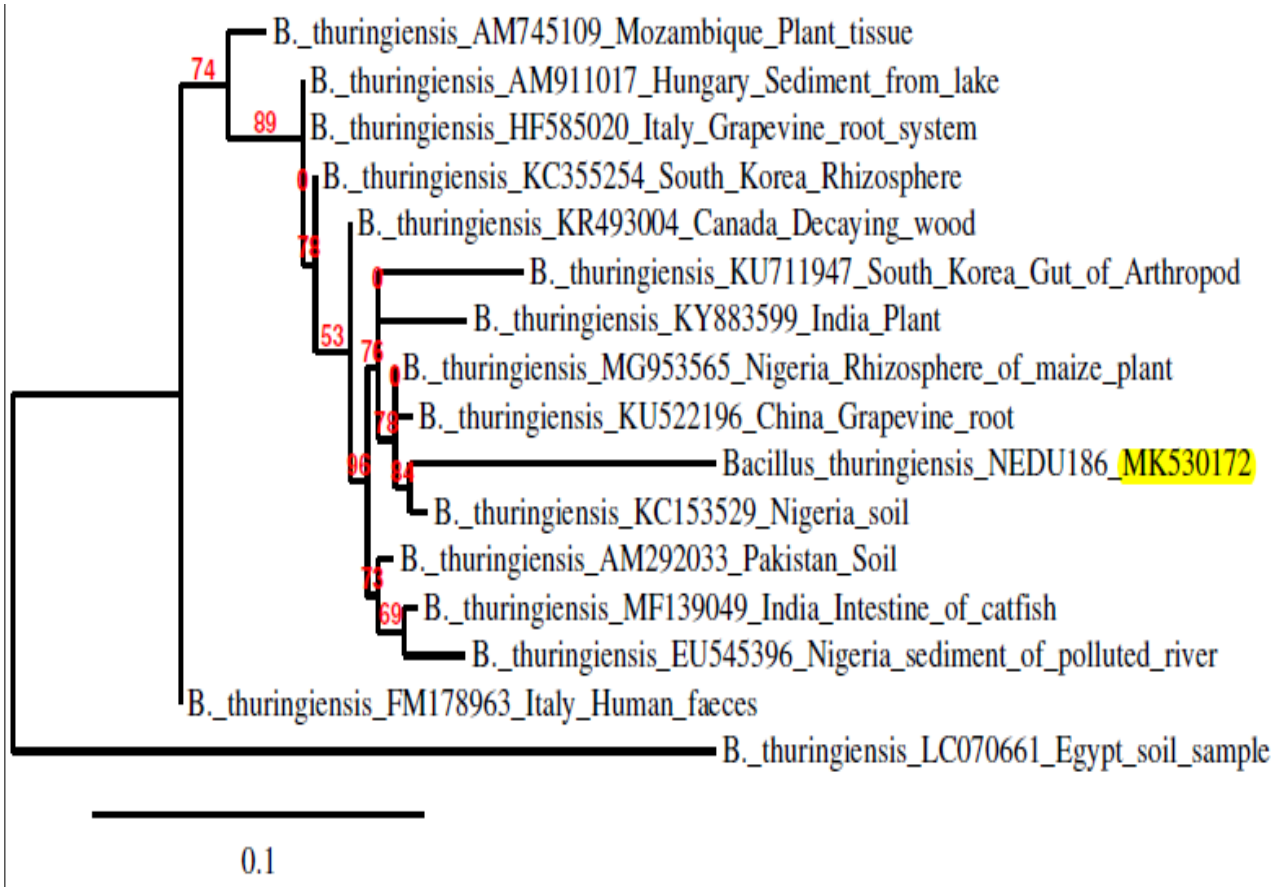



**Figure 19: Phylogenetic tree showing the relationship of *Bacillus thuringiensis* isolate NEDU 186 to other members of the *Bacillus thuringiensis* based on 16S rRNA gene sequences**

**Table 20: Toxin genes detected by PCR in *Staphylococcus* and Bacillus isolates recovered from edible land snails**

| Identity of Isolate (Accession number) | *Sea* | *Exhc* | *Hbla* | *Hblc* | *Nhea* | *Nheb* | *cytk* |
|---|---|---|---|---|---|---|---|
| *Staph. arlettae* (MK518344) | - | - | NA | NA | NA | NA | NA |
| *Staph. Sciuri* (MK518066) | + | - | NA | NA | NA | NA | NA |
| *B. thuringiensis* (MK530172) | NA | NA | + | - | + | + | + |
| *B. cereus* (MK530202) | NA | NA | + | - | - | + | - |
| *B. cereus* (MK530171) | NA | NA | - | - | + | + | - |

**NA: Not Applicable**

### 4.9 Determination of the Processing Method that Will Reduce the Bacterial Load of Snail Meat during Culinary Preparation.

The effect of different processing methods on the aerobic plate counts of bacteria in five batches of snail meat showed that the initial mean counts at the end of shucking ranged from 8.79 Log CFU/g to 9.55 Log CFU/g among batches of samples. However, mean aerobic plate counts of samples were found to decrease along the stages of processing. At the desliming stage, the wood ash-deslimed batch of samples had the least mean bacterial count (5.98 Log CFU/g) followed by potassium alum-deslimed batch (6.92 Log CFU/g), cassava water-deslimed batch (7.04 Log CFU/g), garri-deslimed batch (7.72 Log CFU/g) and lime-deslimed batch (8.16 Log CFU/g). These represent the following reductions for each batch of samples: 2.95 Log CFU/g, 1.3 Log CFU/g, 0.99 Log CFU/g, 0.98 Log CFU/g and 0.93 Log CFU/g for wood ash, alum, lime, cassava water and garri deslimed batches respectively. However, at the end of the washing stage, there were increases in the mean bacterial counts for the batches deslimed with wood ash (6.89 Log CFU/g) and cassava water (7.35 Log CFU/g), but their mean counts were found to be lowest (2.44 Log CFU/g and 2.67 Log CFU/g) at the end of the cooking stage (Figure 20).

The effect of processing methods on mean coliform counts for snail meat is presented in Figure 21. The initial mean coliform counts were above 6.85 Log CFU/g across the five batches of samples at the end of shucking. The mean counts decreased across all batches along the stages of processing. No coliform was recovered at the last stages of processing and cooking.

At the desliming stage, various levels of reductions in mean counts of coliforms were observed across batches which include: 2.78 Log CFU/g, 1.02 Log CFU/g, 0.29 Log CFU/g,

0.22 Log CFU/g and 0.09 Log CFU/g reductions for wood ash, alum, cassava water, lime and garri deslimed batches, respectively. However, there was increase in mean coliform counts of the ash-deslimed batch (5.63 Log CFU/g) at the end of washing stage, while the mean count of the cassava water-deslimed batch (6.73 Log CFU/g) remained almost the same at this stage.

The effect of processing methods on mean counts of *Citrobacter* in snail meat is presented in Figure 22. The initial mean counts across batches of samples at the shucking stage were above 5.47 Log CFU/g. The mean counts decreased across batches along the stages of processing, except for the cassava water-deslimed batch which increased at the washing stage (6.42 Log CFU/g). *Citrobacter* was not recovered in any batch of samples at the end of parboiling and cooking stages. Considering the desliming stage, there were various levels of reductions in mean *Citrobacter* counts across batches of samples which include: 3.85 Log CFU/g, 1.88 Log CFU/g, 0.91 Log CFU/g, 0.57 Log CFU/g and 0.35 Log CFU/g reductions for wood ash, alum, garri, lime and cassava water deslimed batches respectively. However, there were increases in mean counts of presumptive *Citrobacter* for wood ash (3.95 Log CFU/g) and alum (3.49 Log CFU/g) deslimed batches at the end of washing.

The effect of processing methods on mean counts of *Shigella* in snail meat is presented in Figure 23. The initial mean counts across batches of samples at the shucking stage ranged from 6.42 Log CFU/g to 4.43 Log CFU/g. The mean counts decreased across batches along the stages of processing, except for the ash-deslimed batch which increased at the washing stage (4.26 Log CFU/g). *Shigella* was not recovered in any batch of samples at the end of parboiling and cooking stages. At the desliming stage, there were various levels of reductions in mean *Shigella* counts across batches of samples which include: 1.68 Log CFU/g,

1.49 Log CFU/g, 1.49 Log CFU/g, 1.10 Log CFU/g and 0.11 Log CFU/g reductions for lime, wood ash, alum, cassava water and garri deslimed batches respectively.

The effect of processing methods on mean counts of *E. coli* in snail meat is presented in Figure 24. The initial mean counts across batches of samples at the shucking stage ranged from 6.57 Log CFU/g to 7.59 Log CFU/g. The mean counts of *E. coli* decreased across batches along the stages of processing, except for the ash-deslimed batch which increased at the washing stage (4.90 Log CFU/g). *E. coli* was not recovered in any batch of samples at the end of parboiling and cooking stages. At the desliming stage, various levels of reductions in mean *E. coli* counts were observed across batches of samples which include: 3.05 Log CFU/g, 1.19 Log CFU/g, 0.92 Log CFU/g, 0.52 Log CFU/g and 0.39 Log CFU/g reductions for wood ash, alum, cassava water, lime and garri deslimed batches respectively.

The effect of processing methods on mean counts of *Staphylococcus* in snail meat is presented in Figure 25. The initial mean counts across batches of samples at the shucking stage ranged from 5.47 Log CFU/g to 6.38 Log CFU/g. The mean counts of *Staphylococcus* decreased across batches along the stages of processing, except for the ash-deslimed batch which increased at the washing stage (4.41 Log CFU/g). *Staphylococcus* was not recovered in any batch of samples at the end of parboiling and cooking stages.

At the desliming stage, various levels of reductions in mean *Staphylococcus* counts were observed across batches of samples which include: 2.13 Log CFU/g, 1.22 Log CFU/g, 1.19 Log CFU/g, 0.39 Log CFU/g and 0.20 Log CFU/g reductions for wood ash, lime, cassava water, alum and garri deslimed batches respectively.

The effect of processing methods on mean counts of *Aeromonas* in snail meat is presented in Figure 26. The initial mean counts across batches of samples at the shucking stage ranged from 3.75 Log CFU/g to 5.51 Log CFU/g. The mean counts of *Aeromonas* decreased across batches along the stages of processing, except for the wood ash (3.62 Log CFU/g) and garri (4.63 Log CFU/g) deslimed batch which increased at the washing stage. *Aeromonas* was not recovered in any batch of samples at the end of parboiling and cooking stages. At the desliming stage, various levels of reductions in mean *Aeromonas* counts were observed across batches of samples which include: 3.24 Log CFU/g, 1.99 Log CFU/g, 1.06 Log CFU/g, 0.69 Log CFU/g and 0.33 Log CFU/g reductions for lime, wood ash, garri, cassava water and alum deslimed batches respectively.

The effect of processing methods on mean counts of *Bacillus* in snail meat is presented in Figure 27. The initial mean counts across batches of samples at the shucking stage ranged from 4.3 Log CFU/g to 5.54 Log CFU/g. The mean counts of *Bacillus* decreased across batches along the stages of processing. *Bacillus* was recovered in all batches of samples at all stages of processing and cooking. At the desliming stage, various levels of reductions in mean *Bacillus* counts were observed across batches of samples which include: 1.02 Log CFU/g, 1.00 Log CFU/g, 0.44 Log CFU/g, 0.37 Log CFU/g and 0.21 Log CFU/g reductions for lime, wood ash, cassava water, alum, and garri deslimed batches respectively. The final mean counts of *Bacillus* at the end of the cooking stage ranged from 2.53 Log CFU/g to 3.24 Log CFU/g.

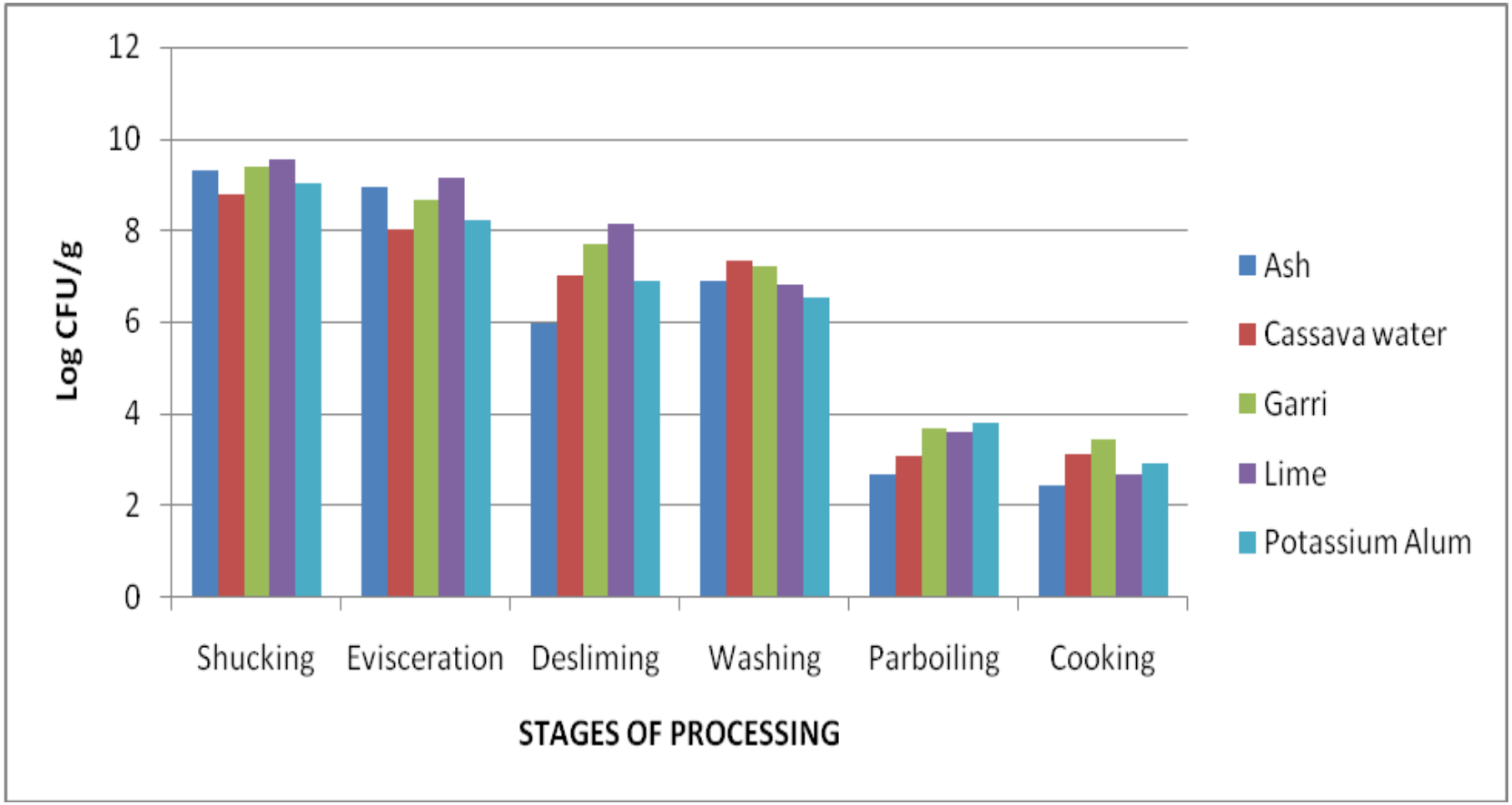



**Fig. 20: Effect of different processing methods on Mean Aerobic Plate counts of bacteria in snail meat.**

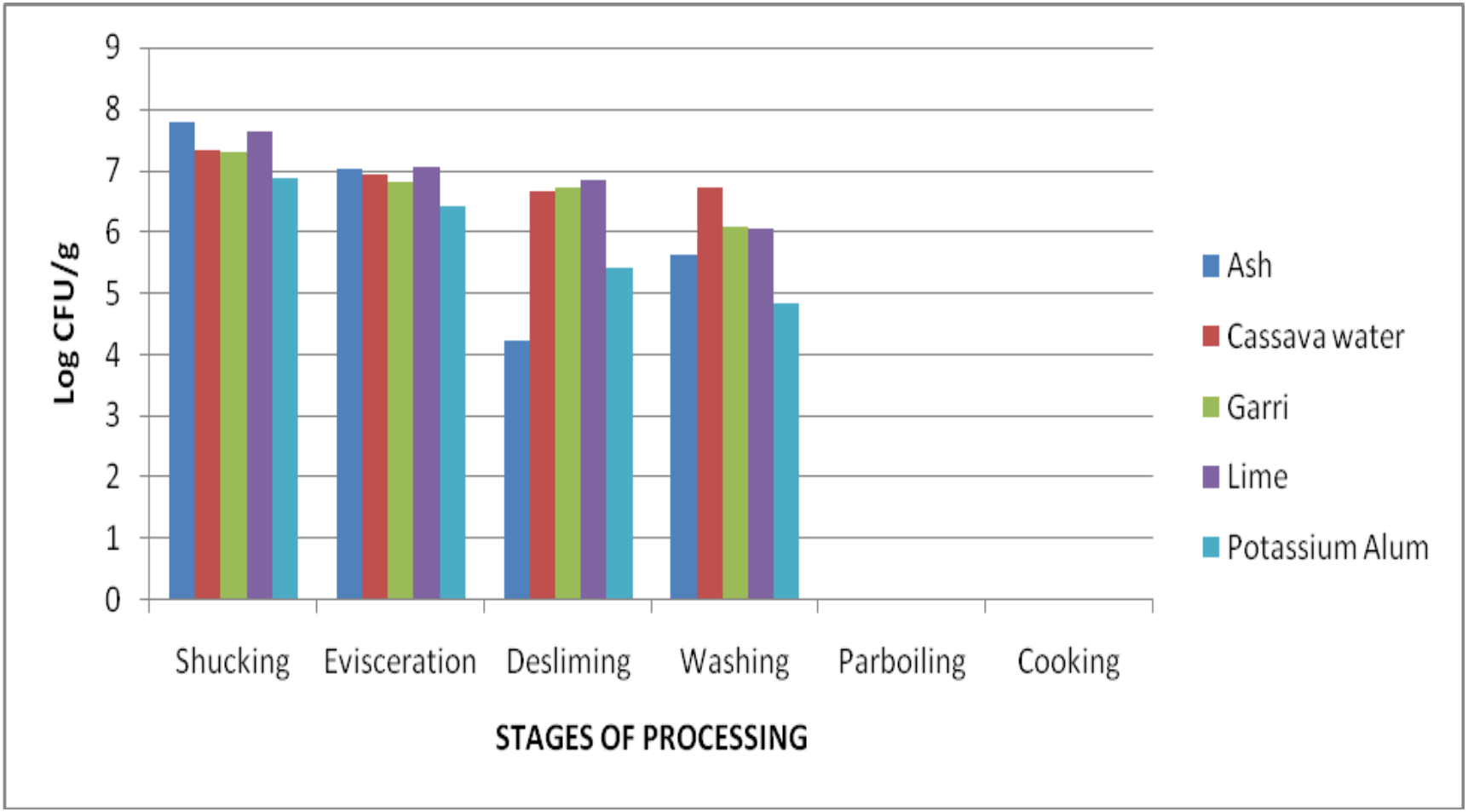



**Fig. 21: Effect of different processing methods on Mean Coliform counts in snail meat.**

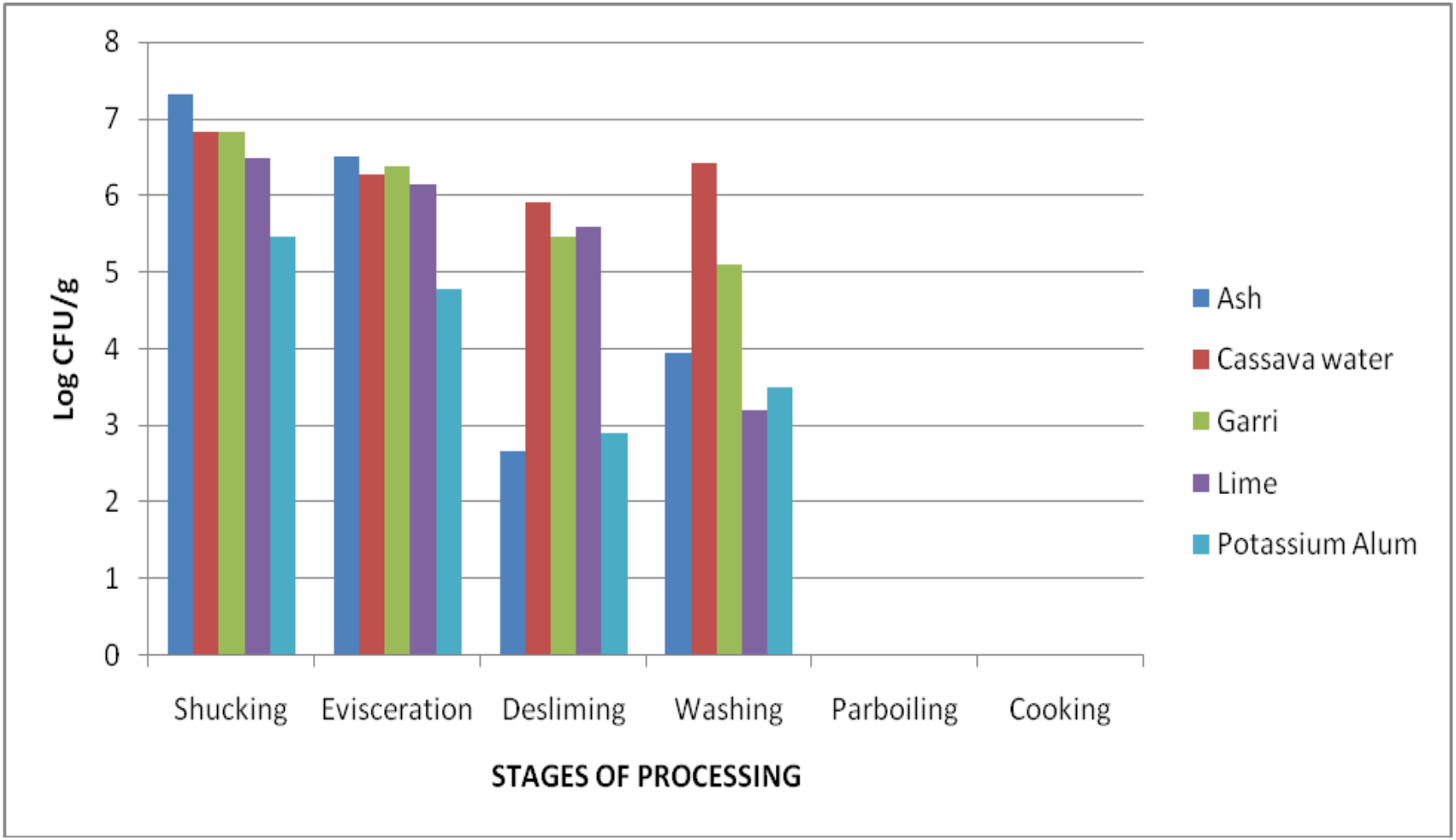



**Fig. 22: Effect of different processing methods on Mean counts of presumptive *Citrobacter* in snail meat.**

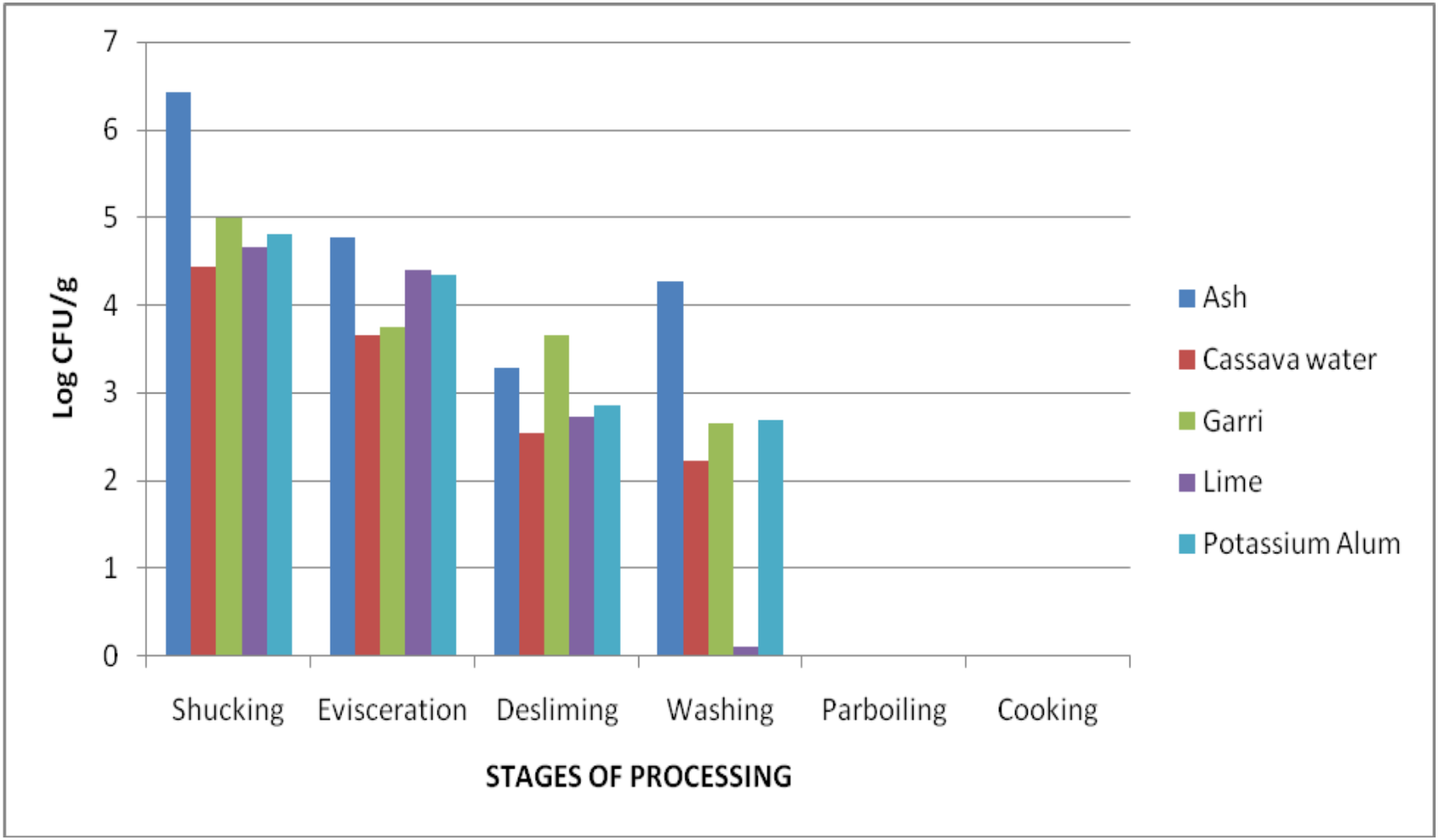



**Fig. 23: Effect of different processing methods on Mean counts of *Shigella* in snail meat.**

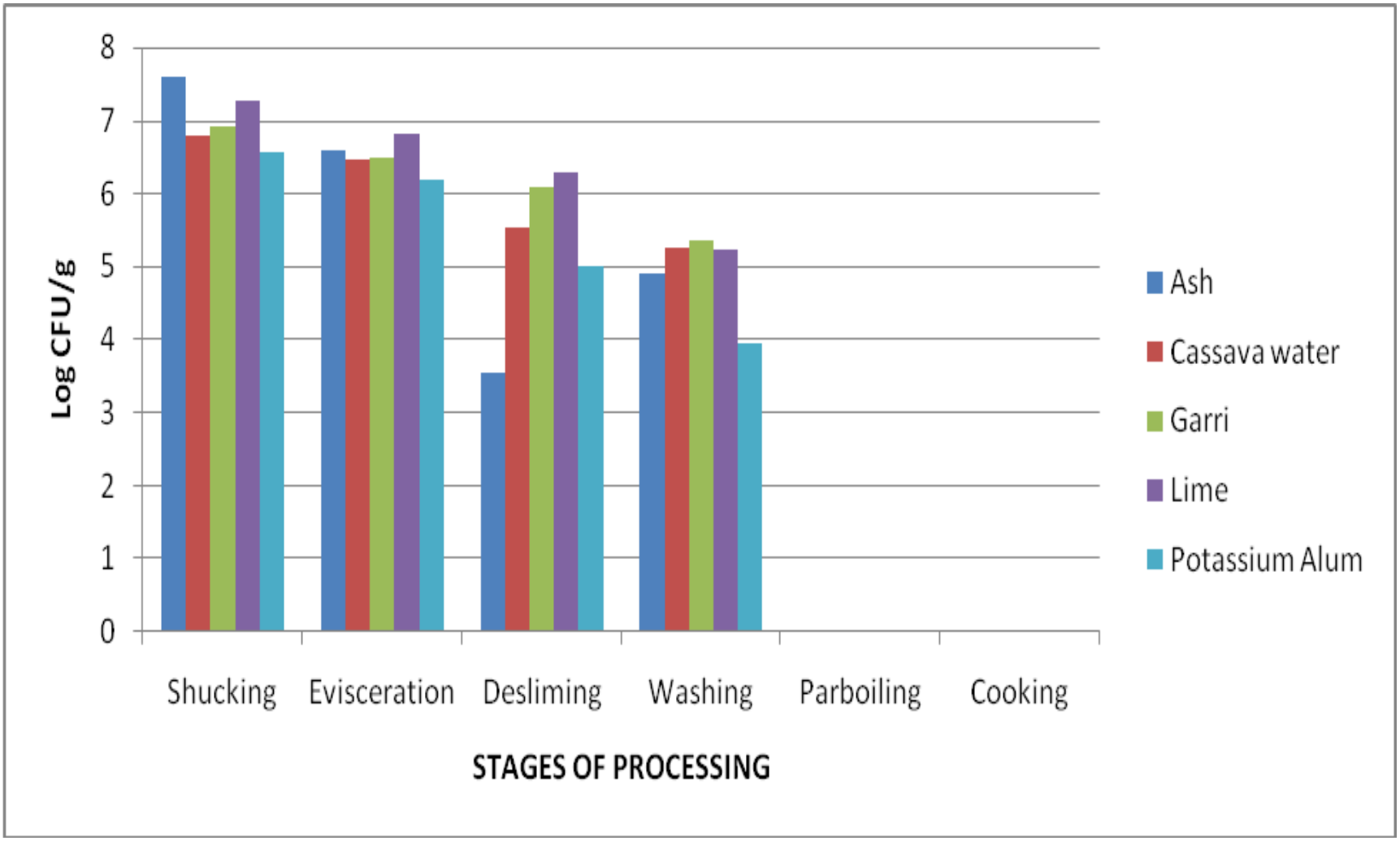



**Fig. 24: Effect of different processing methods on Mean counts of *E. coli* in snail meat**

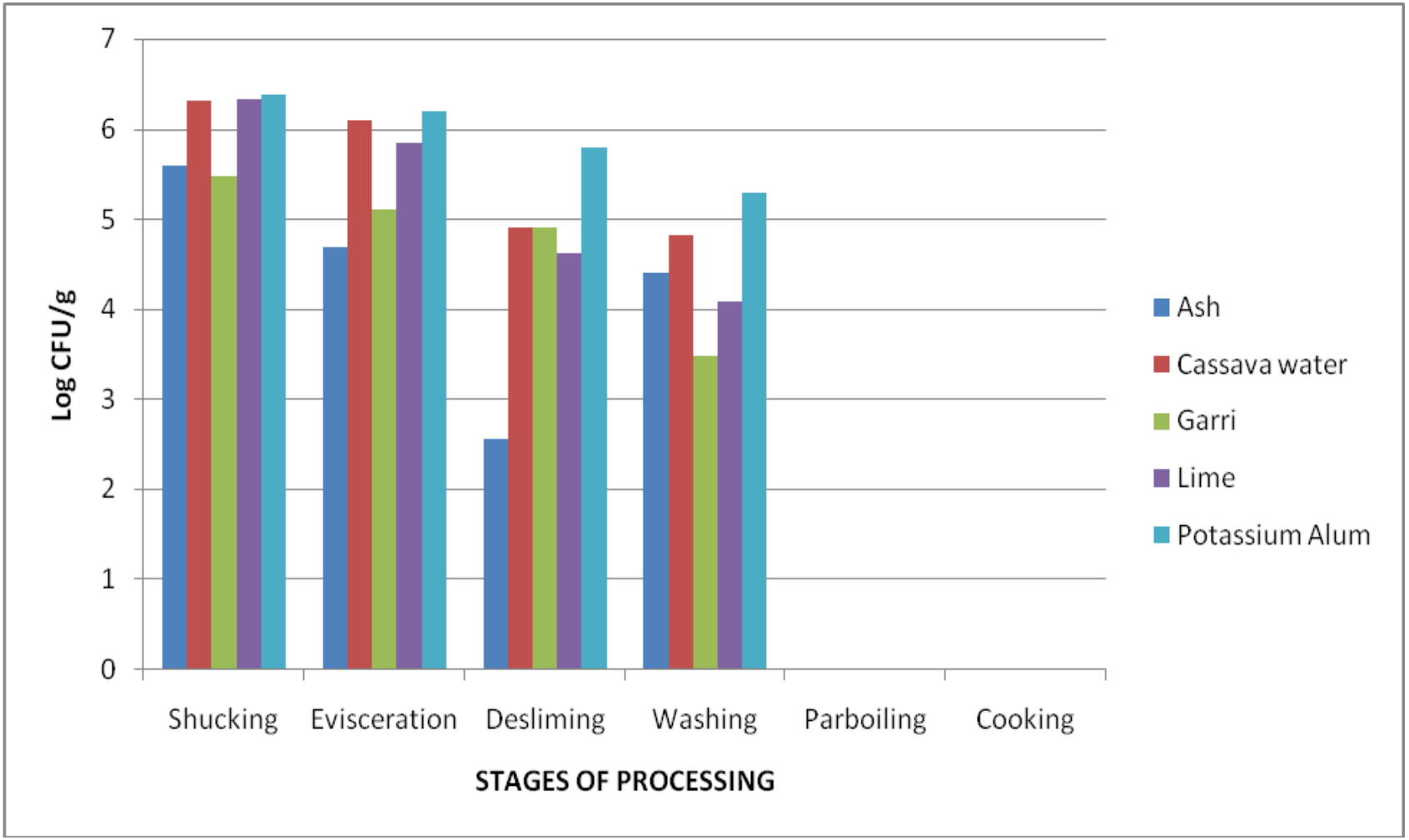



**Fig. 25: Effect of different processing methods on Mean count of *Staphylococcus* in snail meat.**

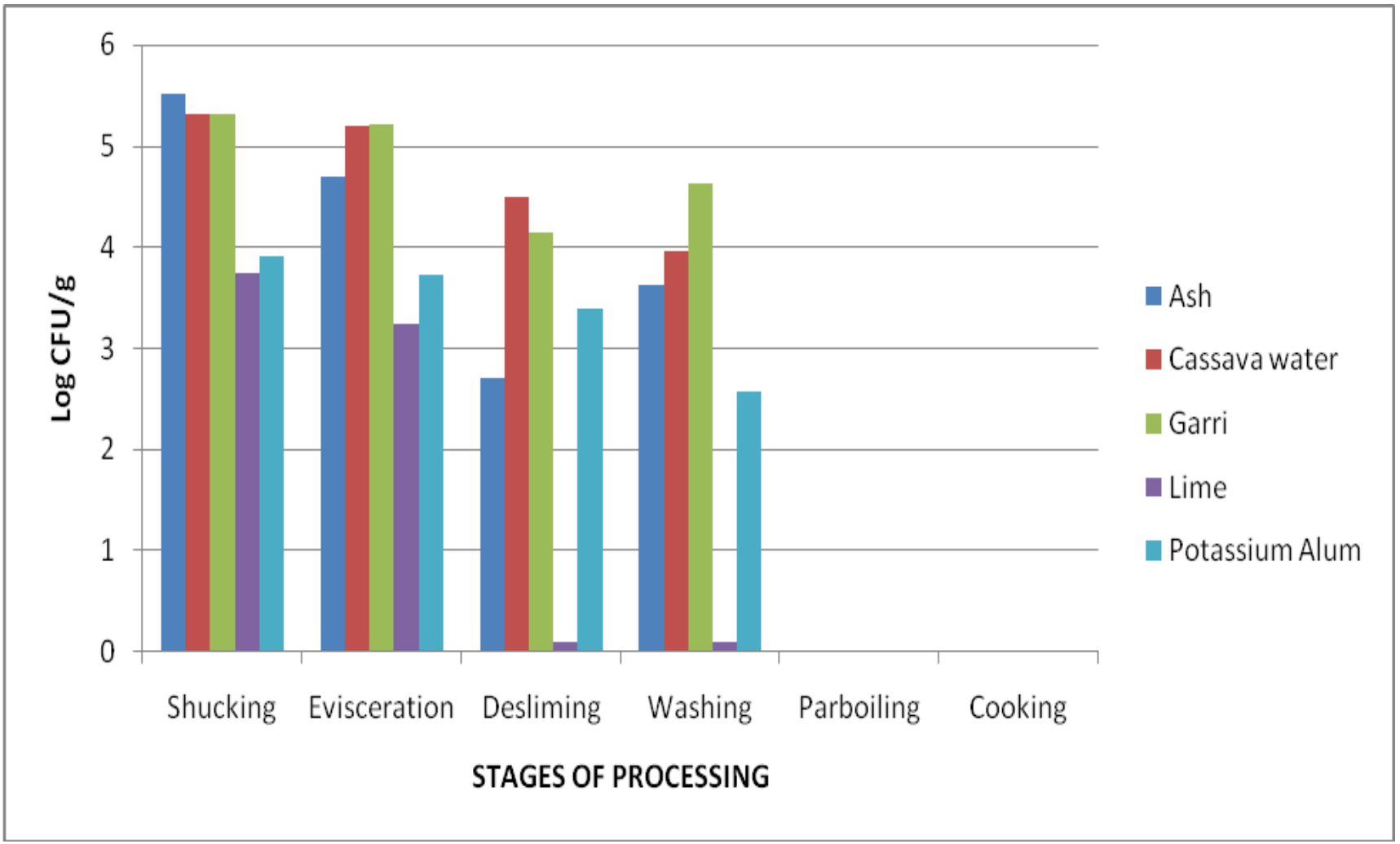



**Fig. 26: Effect of different processing methods on Mean counts of *Aeromonas* in snail meat.**

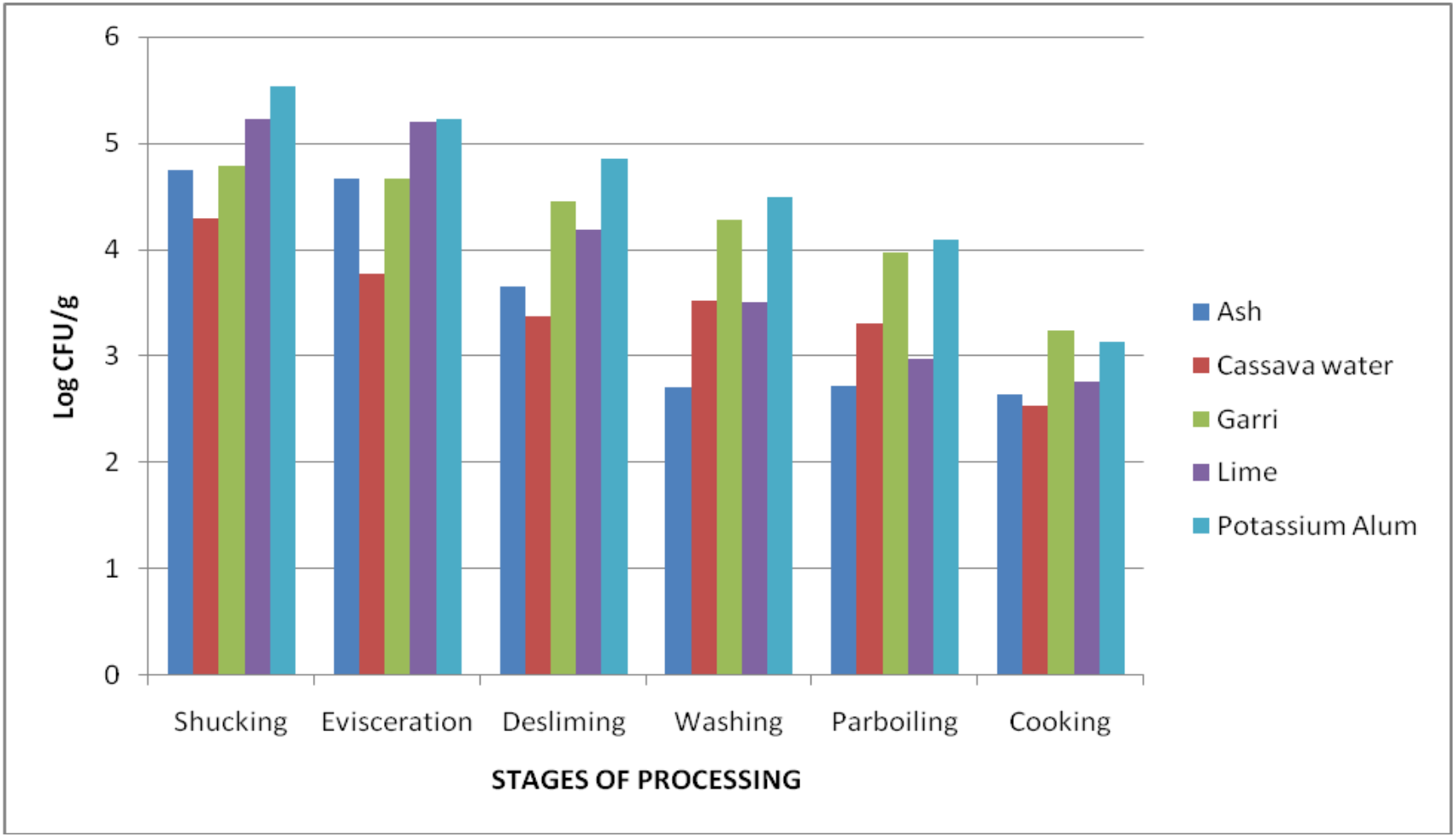



**Fig. 27: Effect of different processing methods on Mean counts of *Bacillus* in snail meat.**

## Chapter five

## DISCUSSION

The findings of this study have demonstrated that edible land snails for sale in three major markets (Igboukwu market in Anambra state, Abakaliki Meat market in Ebonyi state and Ogbete market in Enugu state) contain various levels of high loads of bacterial indicators and pathogens. The mean aerobic plate count of samples analysed in this study ranged from 8.43 to 9.61 Log CFU/g (Figure 4). This appears close to the findings of other related studies: Adegoke *et al*. (2010) reported total aerobic bacterial count in market snails at Akwa Ibom state was 8.0 Log CFU/g. In Ghana, Nyoagbe *et al*. (2016) reported that total viable count ranged from 6.61 to 8.29 Log CFU/g. However, Temelli *et al*. (2006) found the average total aerobic bacterial count in live snails in Turkey to be 6.85 Log CFU/g. Also, mean aerobic counts varied significantly ($p < 0.01$) between the three states from which samples were collected (Figure 4), probably because of the difference in the nature of soil and debris present in the natural habitats of these snails. Aerobic plate count is generally used as a means of assessing the overall microbial quality of raw ingredients (Siragusa *et al*., 1998). According to ICMSF (1986), the acceptable upper limit of total aerobic bacterial load for seafoods is 5.0 Log CFU/g and this limit has been cited in most research articles till date. It is important to note that all snail samples analysed in this study had total aerobic plate counts $>10^8$ CFU/g (Table 4). This implies that 100% of market snails analysed pose microbiological risk to handlers and consumers. However, the use of the aerobic plate count as an indicator for the presence of specific pathogens is generally not accepted (Siragusa *et al*., 1998). Snails that are usually sold in the markets are collected from the forest. Such snails are in high demand among consumers (Nyoagbe *et al*., 2016). Also, they are usually purchased alive in the market

and brought into homes where they are handled and prepared in the raw state in domestic kitchens.

Coliform counts are used for assessing the amount of contamination on meat arising from gut contents which include both those originating directly from the alimentary tract and those arising indirectly through the integument or processing environment. Coliforms are the most frequently studied indicators (Wu *et al.*, 2011). The acceptable upper limit of total coliform is 2.0 Log CFU/g (ICMSF, 1986). In this study, the coliform counts were >2.0 Log CFU/g in all samples analysed (Figure 5 and Table 4). Similar coliform count in snails has been previously reported (Adegoke *et al.*, 2010; Nyoagbe *et al*., 2016), though the prevalence of levels of loads of coliforms in snails is scarce in literature. It is appropriate to note that snails discharge their faeces within their habitat (Ibom *et al*., 2012) and may explain the high loads of coliforms observed in this study.

The presumptive *Salmonella* isolates were found to be strains of *Citrobacter* based on molecular studies (Table 19). This implies that 100% of snail samples, in the present study, were contaminated with *Citrobacter* rather than presumptive *Salmonella* as stated earlier (Table 3). This observation is supported by another study in India on the bacterial diversity of the gastrointestinal tract of *Achatina fulica* using culture-independent and culture-dependent methods. The study also concluded that an apparent feature of bacterial communities in snails' gastrointestinal tract was the abundance of members of the genus *Citrobacter* (Pawar *et al*., 2012). The highest mean *Citrobacter* count was found in Ebonyi samples (7.24 ± 0.210 Log CFU/g) followed by Anambra and Enugu samples with 6.39 ± 0.114 Log CFU/g and 6.37 ± 0.219 Log CFU/g respectively (Figure 6). No significant differences were found between Anambra and Enugu samples ($p < 0.01$). *Citrobacter* is classically considered a

resident commensal of the intestinal tracts of both humans and animals (Guerrant *et al*., 1976). It is also prevalent in soil and water through contamination from the waste materials of animals. A study concluded that healthy pet turtles are a potential carrier of *C. freundii* (Sabrina-Hossain *et al*., 2017). Therefore, turtles, as sea animals, are likely to be the source of *Citrobacter* around swampy environments where most snails are collected.

Ebonyi samples were found to contain the highest mean *Shigella* count (4.61 ± 0.354 Log CFU/g) followed by Anambra (4.43 ± 0.284 Log CFU/g) and Enugu samples (4.03 ± 0.571 Log CFU/g) (Figure 7). Most studies on snails have reported the presence of *Shigella* without indicating its level of concentration (Adagbada *et al*., 2011). Several aquatic bodies have been found to contain *Shigella*, and thus another potential source of infection may be aquatic food which may play a role in transmission of *Shigella* if such food is harvested from sewage-contaminated water (Iwamoto *et al*., 2010). The number of *Shigella* cells required to initiate infection ranges from $10^1$ to $10^4$ cells/person (Dupont *et al*., 1989; Heymann, 2004). Since 60% of snails analysed in the present study exceeded 10 CFU/g (Table 4), this probably implies that such percentage of snails represent health threat to handlers and consumers. When market snails are brought into homes, snail handlers and cooks have the tendency for direct hand-to-mouth exposure to pathogens that may be present in these snails, and cross contamination of the kitchen environment and ready-to-eat foods may always follow. Currently, the incidence of shigellosis worldwide is highest among children less than five years of age (Taneja and Mewara, 2016).

The highest mean count of *E. coli* was found in Anambra samples (7.14 ± 0.170 Log CFU/g), while Enugu samples had the lowest mean count (5.65 ± 0.239 Log CFU/g) as shown in Figure 8. Sixty percent of the market snails in this study had *E. coli* count > 6.0 Log CFU/g

(Table 4) which is within the range of counts noted for resulting in diarrhoeal diseases 6.0 to 9.0 Log CFU/g (Kornacki and Marth, 1982).

The mean Staphylococcal count in samples analysed in this study ranged from 4.66 to 4.74 Log CFU/g (Figure 9). There were no significant differences between Ebonyi and Enugu samples ($p < 0.01$). *Staphylococcus* was not detected in Anambra samples. The only study that quantified the level of *Staphylococcus* in snails reported a range between 2.66 and 7.68 Log CFU/g (Nyoagbe *et al*., 2016). Diagnosis of staphylococcal food poisoning is generally confirmed by the recovery of at least 5.0 Log CFU/g from food (Halpin-Dohnalek and Marth, 1989; Hennekinne *et al*., 2012). It is suggested that since *Staphylococcus* is also present in intestinal tract, raw meat may contain *Staphylococcus* due to contamination with intestinal content during evisceration (Bhalla *et al*., 2007).

Presumptive *Vibrio* isolate was found to be a strain of *Aeromonas* based on molecular studies (Table 19). The mean count of *Aeromonas* was found to range from 3.10 to 4.80 Log CFU/g. There were significant differences between samples collected from different states ($p < 0.01$) (Figure 10). The infectious dose of *Aeromonas* species in foods is not known (Isonhood and Drake, 2012). In another study, mesophilic aeromonads were isolated from 26% of vegetable samples, 70% of meat and poultry samples, and from 72% of fish and shrimps. Numbers of motile aeromonads present in these samples varied from < 2.0 to > 5.0 Log CFU/g (Neyts *et al*., 2000). In the present study, 76.6% of the snail samples contained *Aeromonas*. This is important because snails feed on assortment of plant and animal species including algae (Okafor, 1989). *Aeromonas* species are widely distributed in the aquatic environment (Palumbo, 1996; Neyts *et al*., 2000) and their prevalence in various water and food sources represents a significant public health threat (Wu *et al*., 2007).

The results of this study indicate that *Bacillus* counts for Ebonyi samples had the highest mean count of *Bacillus* (4.50 ± 0.136 Log CFU/g) followed by Enugu (3.48 ± 0.135 Log CFU/g) and Anambra samples (3.25 ± 0.130 Log CFU/g) as shown in Figure 11. Nyoagbe *et al*. (2016) reported similar levels of *Bacillus* in snails ranging from 1.53 to 4.90 Log CFU/g. It is often noted that *Bacillus cereus* levels > 3.0 Log CFU/g have resulted in illness (Harmon *et al*., 1992). In order to promote the consumption of products in domestic and export markets, it is important to ensure such products are free from pathogenic microorganisms (Dhanze *et al*., 2013).

Proteolysis, haemolysis and biofilm formation are the common phenotypic virulence factors found in diarrhoea-associated *Citrobacter freundii* (Chen *et al*., 2002). In this study, the most prevalent virulence factors among the *Citrobacter* isolates were biofilm formation and haemolysin production (Table 5). In another study, which involved *C. freundii* isolates from healthy pet turtles, haemolysin production was not detected among the isolates and it was concluded that lack of haemolysis indicates the inability of the isolates to break down red blood cells and cause hemolytic uremic syndrome in humans (Sabrina-Hossain *et al*., 2017). Biofilm formation has been reported in *Citrobacter* isolates from clinical samples (Pardia *et al*., 1980). In the present study, 100% and 70% of Ebonyi *Citrobacter* isolates were found to be haemolysin and biofilm producers, which implies that these isolates demonstrate a greater potential public health risk compared to isolates from other locations.

Absorption of congo red from an agar medium containing the dye (indirect method of detecting biofilm formation) appears to correlate with virulence in *Shigella* species (Maurelli *et al*., 1984). None of the *Shigella* isolates was found to form biofilm in this study (Table 6).

Haemolytic activity was only detected in 50 *E. coli* isolates (40%) recovered from Anambra samples (Table 7). Bashar *et al.* (2011) reported that among 60 *E. coli* isolates from poultry chicken faeces, 59% were found to demonstrate haemolysis. Also, several studies have investigated haemolytic activity in *E. coli* isolates and their correlation with virulence (Chart *et al.*, 1998; Reingold *et al.*, 1999; Bashar *et al.*, 2011). Moreover, Bisht *et al.* (1977) observed association between haemolysin and necrotoxin production among the *E. coli* isolates from cases of acute gastroenteritis, chronic diarrhoea as well as healthy human population.

All staphylococci isolates recovered from snail samples in this study were found to be positive for haemolysis, gelatinase, protease, lecithinase and biofilm formation tests (Table 8). These are pathogenic factors that have been reported among *Staphylococcus* isolates possessing virulence genes (Bertelloni *et al*., 2015; Schaumburg *et al*., 2011; Coelho *et al*., 2009; Cifrian *et al*., 1996; Akinjogunla *et al*., 2014).

Analysis of pathogenic mechanisms associated with *Aeromonas* has identified various virulence factors including toxins with haemolytic, cytotoxic and protease activities among others (Chopra *et al*., 2000; Singh *et al*., 2009; Cascon *et al*. 2000). From Table 9 of the results of this study, 100% of *Aeromonas* isolates from Anambra samples were found to be positive for all virulence associated tests, except biofilm formation (70%). While 50% of Ebonyi isolates were positive for haemolysin, Enugu isolates were found to be negative. In other studies, 94% of 767 shellfish isolates were haemolysin positive and 59% of the haemolytic isolates were cytotoxic (Abbott *et al*., 1992); 86% of cat fish *Aeromonas* isolates were haemolytic (Wang and Silva, 1999). It has been reported that haemolysin and cytotoxin production were more frequent for *Aeromonas* species isolated from individuals with

diarrhoea than for those isolated from healthy individuals. However, the number of environmental isolates that exhibited haemolysin and cytotoxin production was larger than the number of human isolates that did (Kuhn *et al*., 1997). Cytotoxic activity of *Aeromonas* on Vero cells has occasionally been reported and associated with haemolysin activity (Isonhood and Drake, 2012).

All *Bacillus* isolates recovered from the three locations were found to be positive for gelatinase and lecithinase tests. On the other hand, all *Bacillus* isolates from Anambra and Ebonyi samples were positive for haemolysin, 50% of Enugu isolates were positive (Table 10). Haemolysin is one of the three toxins that have been implicated as aetiological agents of the diarrhoeal disease involving *Bacillus* (Beecher and MacMillan, 1991; Lund and Granum, 1996; Lund *et al*., 2000).

The use of antibiotics is considered the most important factor promoting the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine (Chaudhary *et al*., 2014). Antibiotic resistant bacteria from foods of animal origin, are ranked as the direct causative agents of foodborne diseases and stands for a possible source of drug resistance in human pathogens (Schlegelova *et al.* 2003). In this study, most *Citrobacter* isolates were resistant to ceftriaxone and amoxy/clavulanic acid compared to other antibiotics (Table 11). This corroborates with the findings from other studies that involved *Citrobacter* isolates from environmental samples (Sabrina-Hossain *et al*., 2017) and also from hospitalized patients (Arens *et al*., 1997).

The rise in drug resistance is becoming a serious problem in the treatment of shigellosis. Such rise is comparable to the extensive uncontrolled use of antibiotics in

developing countries (Fauci *et al*., 2008). In this study, 30%, 65%, 25% and 65% of the Enugu *Shigella* isolates were resistant to ampicillin, ceftazidime, cephalexin and ceftriaxone, respectively (Table 12). Nevertheless, there is a report that nalidixic acid was introduced to treat shigellosis caused by ampicillin and co-trimoxazole resistant strains (Iwalokun *et al*., 2001). Interestingly, *Shigella* isolates recovered in this study were sensitive to fluoroquinolones. These have been found effective in the treatment of shigellosis (Bhattacharya *et al*., 1987; Niyogi, 2007).

*E. coli* isolates across the three states were found to be more resistant to 3$^{rd}$ generation cephalosporins compared to other antibiotics (Table 13). There is a report from Belgium that about 35% of the *E. coli* strains isolated from live broilers are resistant to 3$^{rd}$ generation cephalosporins while over 60% of the broilers are found to be carriers of these 3$^{rd}$ generation cephalosporin-resistant *E. coli* after selective isolation (Depoorter *et al.,* 2012). Furthermore, *E. coli* isolates from Anambra (45%) and Ebonyi (30%) were resistant to streptomycin. Similarly, other studies reported that more than 50% of *E. coli* isolates from eggs and broiler chickens are resistant to streptomycin (Adesiyun *et al.,* 2007; Smith *et al.,* 2007). The possible explanation is that the snails may have picked up these antibiotic-resistant isolates from environments fertilized with chicken faeces and litters. This is supported by the fact that antibiotic-resistant *E. coli* strains have been isolated from broiler chicken faeces and litters (Diarrassouba *et al*., 2007). In addition, Johnson *et al.* (2007) reported that the poultry meat is one of the major sources of transferring antibiotic resistance.

Ebonyi (> 60%) and Enugu (> 40%) *Staphylococcus* isolates were resistant to chloramphenicol and norfloxacin in this study (Table 14). Thirty percent of Ebonyi isolates were found to be resistant to cloxacillin. In a similar study in Nigeria, 46.2% of *S. aureus*

isolates from poultry meat were reported to be resistant against chloramphenicol (Otalu *et al*., 2011). Efuntoye *et al*. (2011) found that *S. aureus* isolates recovered from snails had 100% resistance to augmentin, cloxacillin, cefuroxime and amoxycillin-clavulanic acid. However, in the present study, all *Staphylococcus* isolates were sensitive to 70% of antibiotics used in this study which indicates low prevalence of resistance to several antibiotics.

In this study, Anambra *Aeromonas* isolates were resistant to ceftazidime, ceftriaxone and nalidixic acid (Table 15). Also, Ebonyi isolates were resistant to ampicillin, ceftazidime and ceftriaxone, while Enugu isolates were resistant to ampicillin, amoxycillin/clavulanic acid and ceftriaxone. All isolates of *Aeromonas* were sensitive to 70% of antibiotics used in this study (Table 15). From literature, the majority of *Aeromonas* appear susceptible to tetracycline, aminoglycosides, 3$^{rd}$ generation cephalosporins and the quinolones (Janda and Abbott, 1998). The resistance to quinolones and nalidixic acid is considered to be chromosomally mediated, as a result of drug resistant isolates selective pressure (Goñi-Urriza *et al*., 2000).

The least prevalence of antibiotic resistance was observed among Anambra *Bacillus cereus* strains (Table 16). *B. cereus* is usually susceptible to aminoglycosides, chloramphenicol among others (Luna *et al*., 2007). However, Ebonyi (50%) and Enugu (65%) isolates tested in this study proved to be resistant to chloramphenicol. All *Bacillus* isolates were found to be sensitive to 60% of antibiotics used in this study. This suggests that health problems implicating *B. cereus* from these snails are likely to be successfully treated with most antibiotics.

Furthermore, the findings of this present study show that bacterial pathogens in these snails appear to possess the potential to acquire multidrug resistance as evidenced by the

antibiotic resistance patterns exhibited by some isolates (Table 17). These isolates may continue to progress with this trend if the source of the antibiotic resistant genes is not identified and checkmated. Scientific studies have come to the conclusion that soil is a major reservoir of antibiotic resistance genes (Martinez 2008; Finley *et al*., 2013; Fitzpatrick and Walsh, 2016). Another possible explanation connecting these snails and antibiotic resistant isolates could be based on the herbivore-feeding habit of snails, primarily on vascular plants (Rauth and Barker, 2002) and also their participation with other soil invertebrates in the decomposition of leaf litter (Hatziioannou *et al*., 1994). These plants may have been contaminated with antimicrobial resistant bacteria following sewage discharges or the use of irrigation water contaminated with faeces of humans and animals that are victims of antibiotics abuse (Bergogne-Berezin, 1997). This is important because formulated feeds for snails are not available in the market and it has become common practice for snail rearers to use vegetables, plant leaves and kitchen wastes to feed snails (Chah and Inegbedion, 2013).

The analysis of 16S rRNA gene sequences of ten selected isolates in this study revealed them to belong to species of the following genera: *Escherichia*, *Citrobacter*, *Staphylococcus, Aeromonas* and *Bacillus* (Table 18). *Escherichia fergusonii* has been shown to cause disease in humans (Funke *et al*., 1993; Bain and Green, 1999; Mahapatra and Mahapatra, 2005). It was recently isolated from vervets and baboons in South Africa and it was portrayed as a possible emerging pathogen of zoonotic importance (Glover *et al*., 2017).

Also, two isolates in this study were found to be different strains of *Citrobacter freundii* based on 16S rRNA gene sequence analysis (Table 18). *C. freundii* has been detected in fresh beef meat samples from local markets in Nigeria (Ukut *et al*., 2010). In India, a study on the bacterial diversity of the gastrointestinal tract of *Achatina fulica* using culture-

independent and culture-dependent methods reported the abundance of members of the genus *Citrobacter* (Pawar *et al*., 2012). *C. freundii* is ubiquitous in humans and animals. It has also been found to be prevalent in soil and water as a result of contamination from the waste materials of animals (De Padua *et al*., 2014; Sabrina-Hossain *et al*., 2017). It was proposed to be an emerging important foodborne pathogen since it has the ability to produce or acquire a number of heat-stable enterotoxins. It has been reported in an outbreak of gastroenteritis associated with consumption of chicken salad in the United States Air Force Academy (Warner *et al*., 1991), and egg shells in Korea (Chang, 2000). Moreover, *C. freundii* was detected in a sample obtained from a patient with pancreatic pseudocyst after an acute necrotizing pancreatitis (Lozano-Leon *et al*., 2011).

Another isolate of interest identified by 16S rRNA gene sequencing in this study was *Staphylococcus sciuri* (Table 18). It is considered one of the most ancestral and dispersed staphylococcal species, with a wide range of habitats that includes the skin of several animals as well as environmental reservoirs, such as soil (Kloos *et al*., 1976; Couto *et al*., 2000). Some studies have implicated *S. sciuri* in different infections such as endocarditis, peritonitis, septic shock, endophthalmitis, urinary tract infection, pelvic inflammatory disease, and wound infections (Hedin and Widerstrom, 1998; Horii *et al*., 2001; Stepanovic *et al*., 2003; Dakic *et al*., 2005a; Dakic *et al*., 2005b).

Two strains of *Bacillus cereus* were identified by 16S rRNA gene sequencing in the present study (Table 18). *B. cereus* is described as being of ubiquitous presence in nature and can be found in many types of soils, sediments, dust and plants (von Stetten *et al*., 1999; Schoeni and Wong, 2005) which are all present in the natural habitat of snails. The true

burden of illnesses caused by *B. cereus* is unknown probably because they commonly occur as sporadic cases, rather than in major outbreaks (Logan *et al*., 2011).

The PCR analysis to detect some toxin genes in *Staphylococcus* isolates revealed that *Staphylococcus sciuri* (0181EN) haboured *sea* gene, which was not detected in *Staphylococcus arlettae* (0180EN) as shown in Table 20. Although the occurrence of *sea* gene in coagulase-negative staphylococci such as *S. sciuri* is very rare, the need to screen for such gene among coagulase-negative staphylococci has been acknowledged in several articles since they are suggested to be reservoir of virulence genes for other bacteria (Blaiotta *et al*., 2004; Dakic *et al*., 2005a; Piechota *et al*., 2014). Staphylococcal Enterotoxin A (SEA) is the most commonly reported enterotoxin in foods, and also considered as the main cause of staphylococcal food poisoning, probably due to its extraordinarily high resistance to proteolytic enzymes (Balaban and Rasooly, 2000; Zargar *et al*., 2014). Since SEA is toxic even in low concentrations (0.6 ng/mL), detection of strains which harbor *sea* gene is important (Holeckova *et al*., 2002). The predominance of SEA in most foodborne disease outbreaks in different countries is well documented. For instance, about 90% of food poisoning isolates were reported to contain the *sea* gene in Korea (Argudín *et al*., 2010; Cha *et al*., 2006). Dakic *et al*. (2005b) did not detect genes encoding staphylococcal eneterotoxins in a large panel of 48 *S. sciuri* group isolates. Piechota *et al*. (2014) detected *sec* genes in 5 *S. sciuri* isolates obtained from cow's milk in Poland and reported the absence of *sea* gene in these isolates. Since staphylococcal enterotoxin genes are mostly carried on mobile genetic elements like plasmids (Fitzgerald *et al*., 2000), this strain of *S. sciuri* may have gained *sea* gene through genetic transfer and could possibly transfer this gene to other strains.

The diarrhoeal syndrome caused by *B. cereus* is generally attributed to at least three enterotoxins: haemolytic, non-haemolytic and cytotoxin K (Glasset *et al*., 2016; Owusu-Kwarteng *et al*., 2017). In the present study, PCR analysis of *Bacillus* isolates for toxin genes targeted some genes encoding haemolytic (*hbla* and *hblc*) enterotoxin complex, non-haemolytic (*nhea* and *nheb*) enterotoxin complex and cytotoxin K (*cytk*). The results indicated that *nheb* gene was the most frequently detected gene in all the three *Bacillus* isolates screened in this study (Table 21). Simailarly, Abbas *et al*. (2014) found that *nhea* gene was the most common gene detected in *B. cereus* isolated from milk and milk products in the market. In the present study, only *Bacillus* isolate 0186EN (*B. thuringiensis*) was found to possess four (*hbla, nhea, nheb* and *cytk*) out of the five toxin genes considered (Table 21). These findings align with other reports indicating that enterotoxin genes appear more predominant in *B. thuringiensis* than *B. cereus* (Hansen and Hendriksen, 2001; Prub *et al*., 1999; Rivera *et al*., 2000). In another study, it was observed that the toxicity of *B. thuringiensis* culture supernatants is generally at the same levels as those of *B. cereus* strains associated with food poisoning (Rivera *et al*., 2000). Also, while *Bacillus* isolate 0177EN (*B. cereus*) was found to possess *hbla* and *nheb* in this study, *Bacillus* isolate 0197AN (*B. cereus*) was found to habour *nhea* and *nheb*.

The pathogenicity of *B. cereus* diarrhoeal strains is not fully understood. From literature, the genetic studies carried out till date have been inconclusive and, regardless of the diseases they cause, all strains seem to carry genes encoding at least one of the known diarrhoeal toxins (Yang *et al*., 2005; Glasset *et al*., 2016). Mantynen and Lindstrom (1998) concluded that screening for the hemolysin *hbla* gene by the PCR method allows faster detection of enterotoxin production than testing with the RPLA enterotoxin test kit. It should

be noted that the occurrence of different combinations of enterotoxin genes in these *Bacillus* strains, observed in this study, suggests that virulence varies among strains.

Because of the high prevalence of bacterial pathogens observed among snails in this study, there was need to ascertain whether these pathogens persist along the stages of processing as performed at home. Snail samples from Enugu state were chosen for this study because of the high prevalence of selected pathogens recorded earlier in the present study (Table 3).

One challenge associated with processing of snail meat is the mucus or slime secreted by the snails (Gallo, 2002). In a nutshell, results indicated that bacterial counts in samples decreased along the stages of processing, and that desliming agents had various degrees of inhibitory effects on the counts of bacterial indicators and pathogens (Figures 20 – 27).

At the end of desliming stage, the highest levels of reduction in concentrations of bacteria was achieved with wood ash, thereby improving the microbiological quality of snails: *Citrobacter* counts reduced by 3.85 Log CFU/g (Figure 22), *E. coli* counts reduced by 3.05 Log CFU/g (Figure 24), total aerobic plate counts reduced by 2.95 Log CFU/g (Figure 20), Coliform counts reduced by 2.78 Log CFU/g (Figure 21), Staphylococci counts reduced by 2.13 Log CFU/g (Figure 25), *Aeromonas* counts reduced by 1.99 Log CFU/g (Figure 26), *Shigella* counts reduced by 1.49 Log CFU/g (Figure 23) and *Bacillus* counts reduced by 1.00 Log CFU/g (Figure 27).

Wood ash, when added to the soil, has a high acid-neutralizing capacity which in turn increases the amount and quality of dissolved organic carbon (DOC). It has been reported that both increased pH and DOC quality affect the bacterial community in the soil (Steenari *et al*.,

1999; Holmberg and Claesson, 2001; Jokinen *et al*., 2006). Therefore, all of these observed effects on bacterial concentrations can be explained by the wood ash-induced increase in pH. Also, the finding that wood ash had the least effect on *Bacillus* counts (Figure 27) corroborates with the findings of Bang-Andreasen *et al*. (2017) who reported detrimental effects on culturable bacteria at a wood ash dose of 167 t $ha^{-1}$ and that spore forming bacteria represent the majority of the bacteria capable of surviving the high wood ash dose.

Alum is a salt made of a combination of an alkali metal such as sodium, potassium or ammonium and a trivalent metal; aluminum, iron or chromium. Potassium alum is commonly used to remove slime from snails in Nigeria (Amadi and Ngerebara, 2017). Alum has a property known as astringency, which refers to its ability to constrict body tissues and restrict the flow of blood (Olmez *et al*., 1998). This could explain the moderate detrimental effect it had on bacterial counts in this study (Figures 20 – 27). The results of the present study are in agreement with that of other studies that reported broad spectrum antibacterial activity by potassium alum (Dutta *et al.*, 1996; Ahmed, 2011; Bnyan *et al.*, 2014; Amadi and Ngerebara, 2017).

However, at the end of the washing stage, increase in mean bacterial counts was observed in most cases, especially in batch samples deslimed with wood ash, water from cassava retting and potassium alum. These findings could be because of a redistribution of contaminants on the carcass during the washing process (Prasai *et al*., 1995; Bell, 1997) and the reduction in concentration of the desliming agents at this stage . Even though *B. cereus* spores are not necessarily removed by regular washing (Andersson *et al*., 1995; Faille *et al*., 2002).

In this study, the reduction observed in all bacteria after the parboiling and cooking could be as a result of temperature reaching $85^0$C at the center of the snail meat due to heat applied at $100^0$C for 10 mins. It has been reported that for consumer safety, application of $90^0$C heat for 1.5 mins in the center and 99 – $100^0$C for 3–4 mins heat are ideal for mollusk and shellfish, respectively (Huss *et al*., 2000; Tzouros and Arvanitoyannis, 2000). Also a study in Greece found that the high levels of bacterial populations were considerably reduced after the appropriate processing of snails (Parlapani *et al*., 2014).

## CONCLUSION

This study has clearly demonstrated that edible snails displayed for sale in three selected markets in South East, Nigeria: Igboukwu, Abakaliki and Ogbete markets are neglected sources of bacterial pathogens in the food chain. This is because 300 samples of edible snails from these markets contained high loads of bacterial indicators and pathogens at high prevalence rate. Antimicrobial-resistant strains of bacterial pathogens, specifically *E. coli*, were found to be most resistant to 3$^{rd}$ generation cephalosporins compared to other antibiotics.

Also, different combinations of enterotoxin genes detected in the *Bacillus* strains suggests that virulence varies among strains. The use of wood ash as desliming agent can better improve the microbiological quality of snails compared to other agents. *Bacillus* isolates were found to be most resistant to the effect of any common desliming agent compared to other bacterial isolates studied. Also, this study has provided data on bacterial hazards associated with edible snails along the value chain which will be useful for formulation of food safety policy.

## RECOMMENDATIONS

**(a)** There is need to identify and protect, from human and animal interferences, the natural habitats of edible land snails.

**(b)** Human sewage, industrial and abattoir wastes should not be disposed in environments for rearing edible land snails.

**(c)** Appropriate food safety management system targeted torwards reduction of bacterial pathogens along the snail value chain should be formulated and implemented.

**(d)** Antimicrobial resistance surveillance programme should be extended to include food animals such as edible snails.

**(e)** Snails should be certified safe by veterinarians before sale in the market.

**(f)** Snail handlers and cooks should be educated on hygiene measures for processing and adequate cooking of edible snails to prevent dissemination of pathogens in homes.

## CONTRIBUTION TO KNOWLEDGE

This study has contributed to knowledge in the following ways:

**(a.)** The presence of important emerging pathgens in *A. achatina* which are *Escherichia fergusonii, Citrobacter freundii, Bacillus cereus, B. thuringiensis* and *Staphylococcus sciuri* has been affirmed via molecular studies,

**(b.)** Edible snails represent an important source of multi-drug resistant *E. fergusonii* and *C. freundii* in the food chain.

**(c.)** Edible snails habour *Staphylococcus sciuri*, *B. cereus* and *B. thuringiensis* strains which possess genes for enterotoxin production that are often implicated in foodborne disease outbreaks.

**(d.)** Common methods for culinary preparation of edible snails do not reduce bacilli counts in snail meat below allowable limits.

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## APPENDIX 1

**Descriptions of typical colonies of selected presumptive pathogens**

| Presumptive pathogen | Description of typical colonies |
|---|---|
| *Citrobacter* | Circular, smooth, raised, light pink colonies with black centres, 2 mm in diameter, mucoid surfaces and opaque on Salmonella-shigella agar after 24 hours of incubation. |
| *Shigella* | Circular, smooth, raised, colourless colonies, 2 mm in diameter, mucoid surfaces and transluscent on Salmonella-shigella agar after 24 hours of incubation. |
| *E. coli* | Circular, smooth, flat, greenish-metallic sheen colonies with dark centres, semi-mucoid surfaces, 1-3 mm in diameter and opaque on Eosin Methylene Blue agar after 24 hours of incubation. |
| *S. aureus* | Circular, smooth, raised, yellow colonies with yellow zones, 2-3 mm in diameter, mucoid surfaces and opaque on Mannitol Salt agar after 24 hours of incubation. |
| *Aeromonas* | Circular, smooth, slightly flattened yellow colonies with opaque centres and transluscent borders, 2-4 mm in diameter with mucoid surfaces on Thiosulfate Citrate Bile salt Sucrose agar after 24 hours of incubation. |
| *B. cereus* | Irregular, umbonate, entire, wrinkled, white or cream colonies with waxy aspects, 3-7 mm in diameter, sometimes smooth and mucoid surfaces, and opaque on Brain Heart Infusion agar after 24 hours of incubation. |

## APPENDIX 2

### Purification of Amplicon

1. Add 2 vol (20 µl) of absolute ethanol to the amplicon
2. Incubate at room temperature for 15 minutes
3. Spin down at 10,000 rpm for 15 minutes
4. Decant supernatant
5. Spin down at 10,000 rpm for 15 minutes
6. Add 2 vol (40 µl) of 70% ethanol
7. Decant supernatant
8. Air dry
9. Add about 10 µl of ultrapure water
10. Check for amplicon on 1.5% agarose

**The amplicon is now ready for sequencing reaction.**

## APPENDIX 3

## DATA ANALYSIS OF AEROBIC PLATE COUNTS OF BACTERIA IN SNAILS

| DATA SUMMARY ON AEROBIC PLATE COUNTS OF BACTERIA IN SNAILS | | | | | | |
|---|---|---|---|---|---|---|
| | Samples | | | | | |
| | 1 | 2 | 3 | | | Total |
| N | 100 | 100 | 100 | | | 300 |
| $\Sigma X$ | 874.4 | 888.3 | 932.2 | | | 2694.9 |
| Mean | 8.744 | 8.883 | 9.322 | | | 8.983 |
| $\Sigma X^2$ | 7654.554 | 7900.427 | 8698.53 | | | 24253.511 |
| Variance | 0.0889 | 0.0976 | 0.0865 | | | 0.1513 |
| Standard Deviation | 0.2981 | 0.3123 | 0.2941 | | | 0.3889 |
| Standard Error | 0.0298 | 0.0312 | 0.0294 | | | 0.0225 |

| STANDARD WEIGHTED-MEANS ANALYSIS | | | | | |
|---|---|---|---|---|---|
| ANOVA SUMMARY | | | | | |
| Source | SS | Df | MS | F | P |
| Treatment (Between groups) | 18.2042 | 2 | 9.1021 | 74.79 | <.0001 |
| Error | 24.1005 | 198 | 0.1217 | ****** | ****** |
| Ss/Bl | 2.9196 | 99 | ****** | ****** | ****** |
| Total | 45.2243 | 299 | ****** | ****** | ****** |

Ss/Bl = Subjects or Blocks depending on the design.

*TUKEY HSD TEST*

HSD[.05]=0.12; HSD[.01]=0.15
M1 vs M2 P<.05
M1 vs M3 P<.01
M2 vs M3 P<.01

M1 = Mean of Sample 1 (Anambra samples)
M2 = Mean of Sample 2 (Ebonyi samples)
M3 = Mean of Sample 3 (Enugu samples)

## APPENDIX 4

## DATA ANALYSIS OF COLIFORM COUNTS IN SNAILS

| DATA SUMMARY ON PLATE COUNTS OF COLIFORMS IN SNAILS | | | | | | |
|---|---|---|---|---|---|---|
| | Samples | | | | | |
| | 1 | 2 | 3 | | | Total |
| N | 100 | 100 | 100 | | | 300 |
| $\Sigma X$ | 741.4 | 763.7 | 749.9 | | | 2255 |
| Mean | 7.414 | 7.637 | 7.499 | | | 7.5167 |
| $\Sigma X^2$ | 5500.046 | 5846.023 | 5635.057 | | | 16981.126 |
| Variance | 0.0334 | 0.1378 | 0.1167 | | | 0.1038 |
| Standard Deviation | 0.1828 | 0.3713 | 0.3417 | | | 0.3222 |
| Standard Error | 0.0183 | 0.0371 | 0.0342 | | | 0.0186 |

| STANDARD WEIGHTED-MEANS ANALYSIS | | | | | |
|---|---|---|---|---|---|
| ANOVA SUMMARY | | | | | |
| Source | SS | Df | MS | F | P |
| Treatment (Between groups) | 2.5333 | 2 | 1.2666 | 10.93 | <.0001 |
| Error | 22.9514 | 198 | 0.1159 | ****** | ****** |
| Ss/Bl | 5.558 | 99 | ****** | ****** | ****** |
| Total | 31.0427 | 299 | ****** | ****** | ****** |

Ss/Bl = Subjects or Blocks depending on the design.

*TUKEY HSD TEST*

HSD[.05]=0.11; HSD[.01]=0.14
M1 vs M2 P<.01
M1 vs M3 nonsignificant
M2 vs M3 P<.05

M1 = Mean of Sample 1 (Anambra samples)
M2 = Mean of Sample 2 (Ebonyi samples)
M3 = Mean of Sample 3 (Enugu samples)

## APPENDIX 5

## DATA ANALYSIS OF VIABLE COUNTS OF PRESUMPTIVE *SALMONELLA* IN SNAILS

| DATA SUMMARY ON PLATE COUNTS OF PRESUMPTIVE *SALMONELLA* IN SNAILS | | | | | | |
|---|---|---|---|---|---|---|
| | Samples | | | | | |
| | 1 | 2 | 3 | | | Total |
| N | 100 | 100 | 100 | | | 300 |
| $\Sigma X$ | 638.3 | 723.7 | 637.2 | | | 1999.2 |
| Mean | 6.383 | 7.237 | 6.372 | | | 6.664 |
| $\Sigma X^2$ | 4074.999 | 5239.733 | 4064.584 | | | 13379.316 |
| Variance | 0.0074 | 0.0234 | 0.0439 | | | 0.1895 |
| Standard Deviation | 0.0859 | 0.153 | 0.2095 | | | 0.4353 |
| Standard Error | 0.0086 | 0.0153 | 0.021 | | | 0.0251 |

| STANDARD WEIGHTED-MEANS ANALYSIS | | | | | |
|---|---|---|---|---|---|
| ANOVA SUMMARY | | | | | |
| Source | SS | Df | MS | F | P |
| Treatment (Between groups) | 49.2554 | 2 | 24.6277 | 1114.38 | <.0001 |
| Error | 4.3693 | 198 | 0.0221 | ****** | ****** |
| Ss/Bl | 3.0225 | 99 | ****** | ****** | ****** |
| Total | 56.6472 | 299 | ****** | ****** | ****** |

Ss/Bl = Subjects or Blocks depending on the design.

*TUKEY HSD TEST*

HSD[.05]=0.05; HSD[.01]=0.06
M1 vs M2 P<.01
M1 vs M3 nonsignificant
M2 vs M3 P<.01

M1 = Mean of Sample 1 (Anambra samples)
M2 = Mean of Sample 2 (Ebonyi samples)
M3 = Mean of Sample 3 (Enugu samples)

## APPENDIX 6

## DATA ANALYSIS OF VIABLE COUNTS OF *SHIGELLA* IN SNAILS

| DATA SUMMARY ON PLATE COUNTS OF *SHIGELLA* IN SNAILS | | | | | | |
|---|---|---|---|---|---|---|
| | Samples | | | | | |
| | 1 | 2 | 3 | | | Total |
| N | 100 | 100 | 100 | | | 300 |
| $\Sigma X$ | 443.6 | 460 | 408 | | | 1311.6 |
| Mean | 4.436 | 4.6 | 4.08 | | | 4.372 |
| $\Sigma X^2$ | 1972.668 | 2126.06 | 1694.468 | | | 5793.196 |
| Variance | 0.0491 | 0.1016 | 0.3013 | | | 0.1969 |
| Standard Deviation | 0.2215 | 0.3188 | 0.5489 | | | 0.4438 |
| Standard Error | 0.0222 | 0.0319 | 0.0549 | | | 0.0256 |

| STANDARD WEIGHTED-MEANS ANALYSIS | | | | | |
|---|---|---|---|---|---|
| ANOVA SUMMARY | | | | | |
| Source | SS | Df | MS | F | P |
| Treatment (Between groups) | 14.1344 | 2 | 7.0672 | 40.73 | <.0001 |
| Error | 34.3569 | 198 | 0.1735 | ****** | ****** |
| Ss/Bl | 10.3895 | 99 | ****** | ****** | ****** |
| Total | 58.8808 | 299 | ****** | ****** | ****** |

Ss/Bl = Subjects or Blocks depending on the design.

*TUKEY HSD TEST*

HSD[.05]=0.14; HSD[.01]=0.17
M1 vs M2 P<.05
M1 vs M3 P<.01
M2 vs M3 P<.01

M1 = Mean of Sample 1 (Anambra samples)
M2 = Mean of Sample 2 (Ebonyi samples)
M3 = Mean of Sample 3 (Enugu samples)

## APPENDIX 7

## DATA ANALYSIS OF VIABLE COUNTS OF *E. COLI* IN SNAILS

| DATA SUMMARY ON PLATE COUNTS OF *E. COLI* IN SNAILS | | | | | | |
|---|---|---|---|---|---|---|
| | Samples | | | | | |
| | 1 | 2 | 3 | | | Total |
| N | 100 | 100 | 100 | | | 300 |
| $\Sigma X$ | 714.8 | 694.4 | 565.3 | | | 1974.5 |
| Mean | 7.148 | 6.944 | 5.653 | | | 6.5817 |
| $\Sigma X^2$ | 5112.004 | 4825.01 | 3200.787 | | | 13137.801 |
| Variance | 0.0264 | 0.0313 | 0.052 | | | 0.4759 |
| Standard Deviation | 0.1625 | 0.1769 | 0.228 | | | 0.6899 |
| Standard Error | 0.0162 | 0.0177 | 0.0228 | | | 0.0398 |

| STANDARD WEIGHTED-MEANS ANALYSIS | | | | | |
|---|---|---|---|---|---|
| ANOVA SUMMARY | | | | | |
| Source | SS | df | MS | F | P |
| Treatment (Between groups) | 131.4441 | 2 | 65.722 | 1425.64 | <.0001 |
| Error | 9.1339 | 198 | 0.0461 | ****** | ****** |
| Ss/Bl | 1.7222 | 99 | ****** | ****** | ****** |
| Total | 142.3002 | 299 | ****** | ****** | ****** |

Ss/Bl = Subjects or Blocks depending on the design.

*TUKEY HSD TEST*

HSD[.05]=0.07; HSD[.01]=0.09
M1 vs M2 P<.01
M1 vs M3 P<.01
M2 vs M3 P<.01

M1 = Mean of Sample 1 (Anambra samples)
M2 = Mean of Sample 2 (Ebonyi samples)
M3 = Mean of Sample 3 (Enugu samples)

## APPENDIX 8

## DATA ANALYSIS OF VIABLE COUNTS OF *STAPHYLOCOCCUS* IN SNAILS

| DATA SUMMARY ON PLATE COUNTS OF *STAPHYLOCOCCUS* IN SNAILS | | | | | | |
|---|---|---|---|---|---|---|
| | Samples | | | | | |
| | 1 | 2 | 3 | | | Total |
| N | 100 | 100 | 100 | | | 300 |
| $\Sigma X$ | 100 | 474.6 | 466.2 | | | 1040.8 |
| Mean | 1 | 4.746 | 4.662 | | | 3.4693 |
| $\Sigma X^2$ | 100 | 2254.666 | 2207.85 | | | 4562.516 |
| Variance | 0 | 0.0224 | 0.3477 | | | 3.1827 |
| Standard Deviation | 0 | 0.1496 | 0.5897 | | | 1.784 |
| Standard Error | 0 | 0.015 | 0.059 | | | 0.103 |

| STANDARD WEIGHTED-MEANS ANALYSIS | | | | | |
|---|---|---|---|---|---|
| ANOVA SUMMARY | | | | | |
| Source | SS | df | MS | F | P |
| Treatment (Between groups) | 914.9939 | 2 | 457.4969 | 4411.74 | <.0001 |
| Error | 20.5368 | 198 | 0.1037 | ****** | ****** |
| Ss/Bl | 16.1032 | 99 | ****** | ****** | ****** |
| Total | 951.6339 | 299 | ****** | ****** | ****** |

Ss/Bl = Subjects or Blocks depending on the design.

*TUKEY HSD TEST*

HSD[.05]=0.11; HSD[.01]=0.13
M1 vs M2 P<.01
M1 vs M3 P<.01
M2 vs M3 nonsignificant

M1 = Mean of Sample 1 (Anambra samples)
M2 = Mean of Sample 2 (Ebonyi samples)
M3 = Mean of Sample 3 (Enugu samples)

## APPENDIX 9

## DATA ANALYSIS OF VIABLE COUNTS OF PRESUMPTIVE *VIBRIO* IN SNAILS

| DATA SUMMARY ON PLATE COUNTS OF PRESUMPTIVE *VIBRIO* IN SNAILS | | | | | | |
|---|---|---|---|---|---|---|
| | Samples | | | | | |
| | 1 | 2 | 3 | | | Total |
| N | 100 | 100 | 100 | | | 300 |
| $\Sigma X$ | 310.6 | 341.8 | 480.3 | | | 1132.7 |
| Mean | 3.106 | 3.418 | 4.803 | | | 3.7757 |
| $\Sigma X^2$ | 964.986 | 1171.866 | 2327.051 | | | 4463.903 |
| Variance | 0.0027 | 0.0363 | 0.2037 | | | 0.6261 |
| Standard Deviation | 0.0515 | 0.1905 | 0.4514 | | | 0.7913 |
| Standard Error | 0.0051 | 0.0191 | 0.0451 | | | 0.0457 |

| STANDARD WEIGHTED-MEANS ANALYSIS | | | | | |
|---|---|---|---|---|---|
| ANOVA SUMMARY | | | | | |
| Source | SS | df | MS | F | P |
| Treatment (Between groups) | 163.1793 | 2 | 81.5896 | 805.43 | <.0001 |
| Error | 20.0621 | 198 | 0.1013 | ****** | ****** |
| Ss/Bl | 3.964 | 99 | ****** | ****** | ****** |
| Total | 187.2054 | 299 | ****** | ****** | ****** |

Ss/Bl = Subjects or Blocks depending on the design.

*TUKEY HSD TEST*

HSD[.05]=0.11; HSD[.01]=0.13
M1 vs M2 P<.01
M1 vs M3 P<.01
M2 vs M3 P<.01

M1 = Mean of Sample 1 (Anambra samples)
M2 = Mean of Sample 2 (Ebonyi samples)
M3 = Mean of Sample 3 (Enugu samples)

## APPENDIX 10

## DATA ANALYSIS OF VIABLE COUNTS OF *BACILLUS* IN SNAILS

| DATA SUMMARY ON PLATE COUNTS OF *BACILLUS* IN SNAILS | | | | | | |
|---|---|---|---|---|---|---|
| | Samples | | | | | |
| | 1 | 2 | 3 | | | Total |
| N | 100 | 100 | 100 | | | 300 |
| $\Sigma X$ | 325.7 | 449 | 348.7 | | | 1123.4 |
| Mean | 3.257 | 4.49 | 3.487 | | | 3.7447 |
| $\Sigma X^2$ | 1062.035 | 2017.734 | 1217.561 | | | 4297.33 |
| Variance | 0.0124 | 0.0174 | 0.0166 | | | 0.3029 |
| Standard Deviation | 0.1115 | 0.132 | 0.1289 | | | 0.5504 |
| Standard Error | 0.0111 | 0.0132 | 0.0129 | | | 0.0318 |

| STANDARD WEIGHTED-MEANS ANALYSIS | | | | | |
|---|---|---|---|---|---|
| ANOVA SUMMARY | | | | | |
| Source | SS | Df | MS | F | P |
| Treatment (Between groups) | 85.9733 | 2 | 42.9866 | 2669.98 | <.0001 |
| Error | 3.1841 | 198 | 0.0161 | ****** | ****** |
| Ss/Bl | 1.4141 | 99 | ****** | ****** | ****** |
| Total | 90.5715 | 299 | ****** | ****** | ****** |

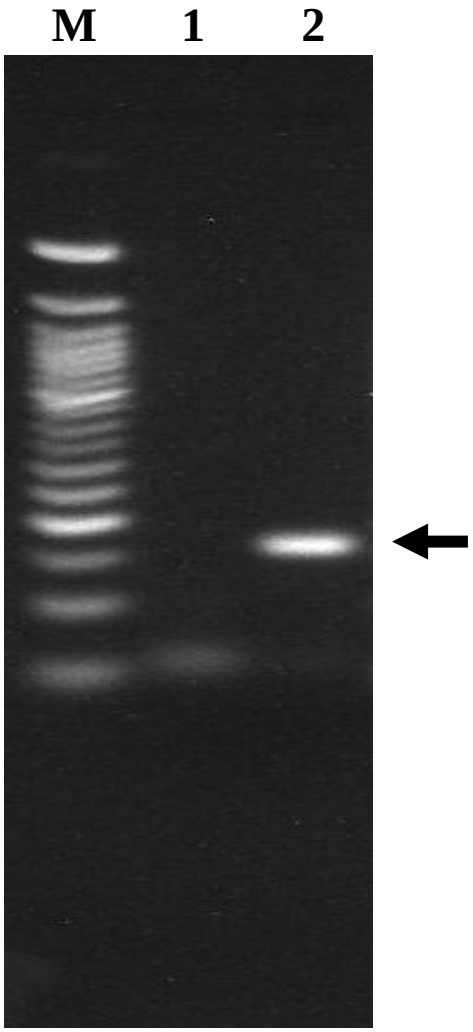
Ss/Bl = Subjects or Blocks depending on the design.

*TUKEY HSD TEST*

HSD[.05]=0.04; HSD[.01]=0.05
M1 vs M2 P<.01
M1 vs M3 P<.01
M2 vs M3 P<.01

M1 = Mean of Sample 1 (Anambra samples)
M2 = Mean of Sample 2 (Ebonyi samples)
M3 = Mean of Sample 3 (Enugu samples)

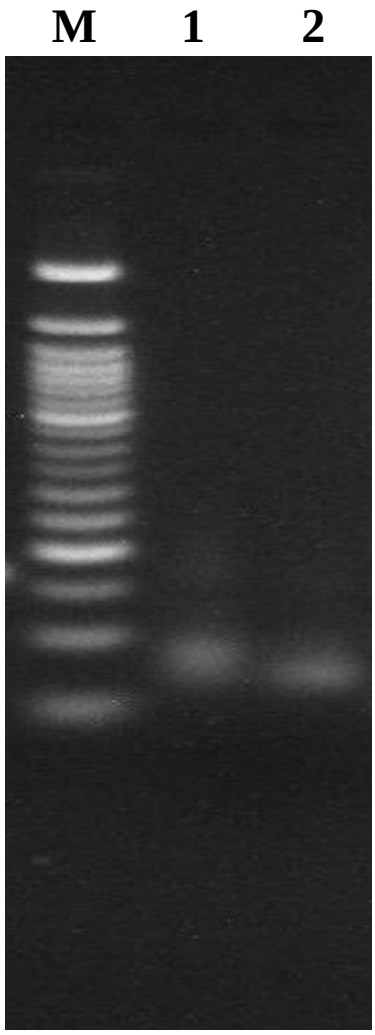
**APPENDIX 11**




**(M) Ladder (50 bp); (1) 0180EN; (2) 0181EN.**

**Gel image from the PCR analysis of *Staphylococcus* isolates showing the *sea* gene**

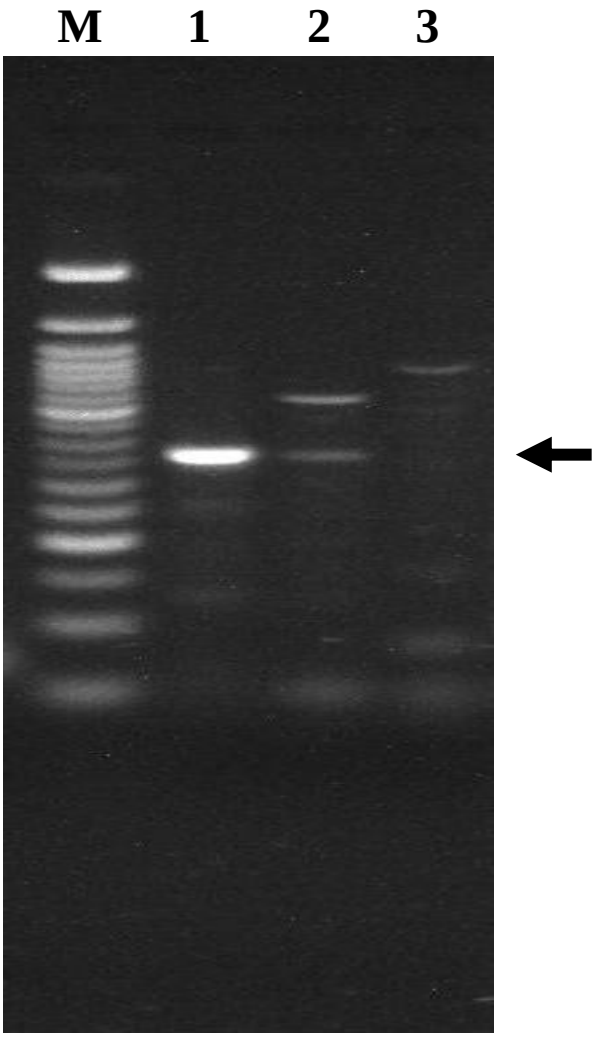
**APPENDIX 12**




**(M) Ladder (50 bp); (1) 0180EN; (2) 0181EN.**

**Gel image from the PCR analysis of *Staphylococcus* isolates for *exhc* gene**

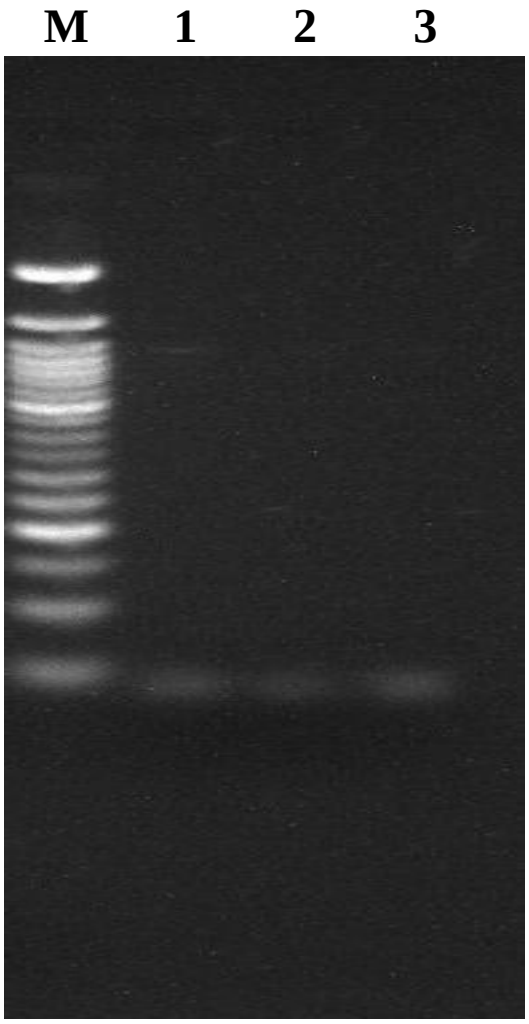
**APPENDIX 13**




**(M) Ladder (50 bp); (1) 0186EN; (2) 0177EN; (3) 0197AN.**

**Gel image from the PCR analysis of *Bacillus* isolates showing the *hbla* gene**

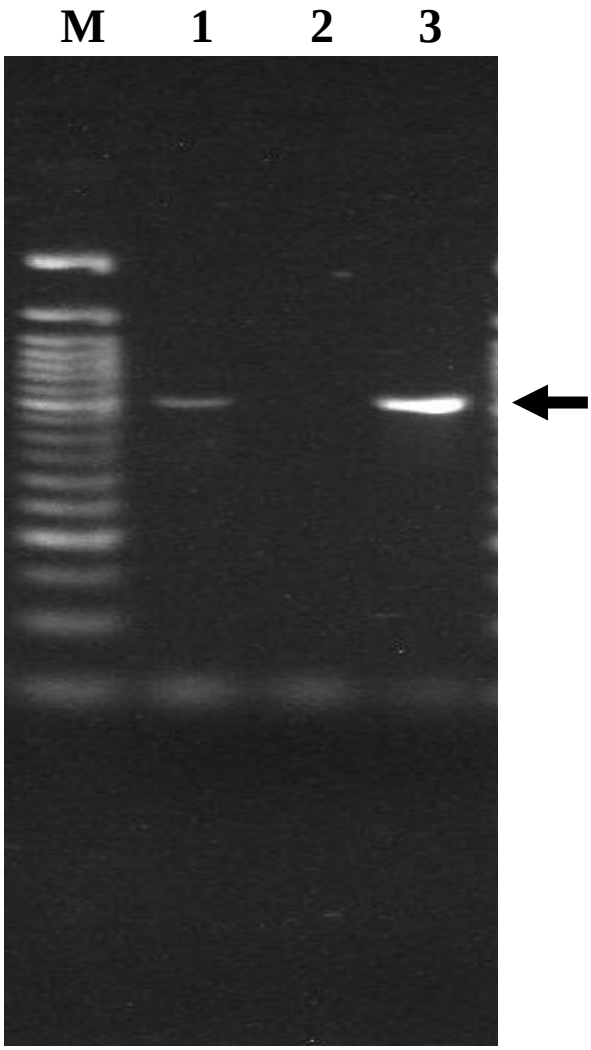
**APPENDIX 14**




**(M) Ladder (50 bp); (1) 0186EN; (2) 0177EN; (3) 0197AN.**

**Gel image from the PCR analysis of *Bacillus* isolates for *hblc* gene**

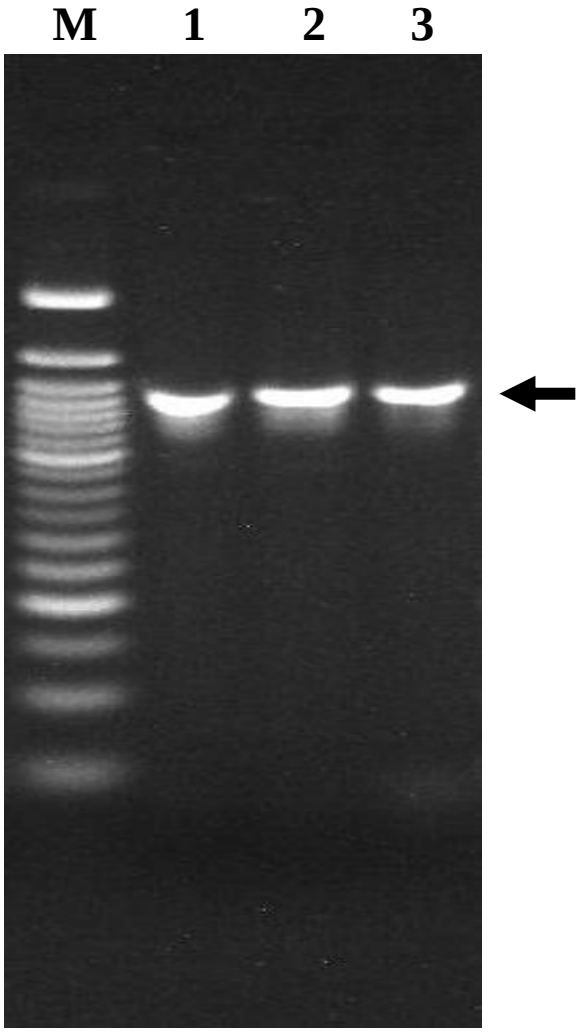
**APPENDIX 15**




**(M) Ladder (50 bp); (1) 0186EN; (2) 0177EN; (3) 0197AN.**

**Gel image from the PCR analysis of *Bacillus* isolates showing *nhea* gene**

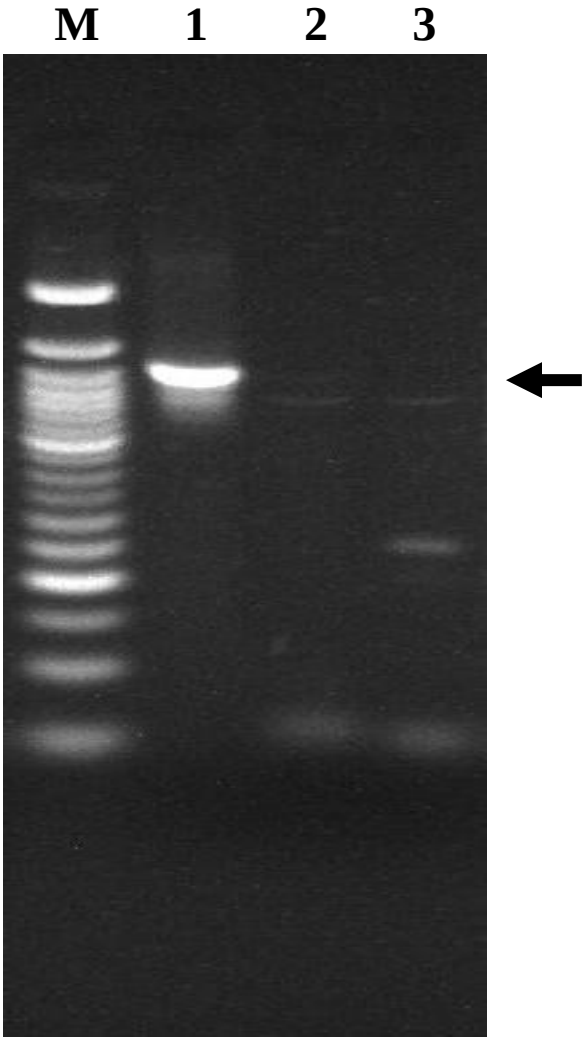
**APPENDIX 16**




**(M) Ladder (50 bp); (1) 0186EN; (2) 0177EN; (3) 0197AN.**

**Gel image from the PCR analysis of *Bacillus* isolates showing *nheb* genes**

**APPENDIX 17**




**(M) Ladder (50 bp); (1) 0186EN; (2) 0177EN; (3) 0197AN.**

**Gel image from the PCR analysis of *Bacillus* isolates showing *cytk* gene**

## APPENDIX 18

**Data on Mean Aerobic Plate counts of bacteria in snail meat during processing**

| **Desliming agents** | **Shucking (Log cfu/g)** | **Evisceration (Log cfu/g)** | **Desliming (Log cfu/g)** | **Washing (Log cfu/g)** | **Parboiling (Log cfu/g)** | **Cooking (Log cfu/g)** |
|---|---|---|---|---|---|---|
| Ash | 9.31 | 8.93 | 5.98 | 6.89 | 2.7 | 2.44 |
| Cassava water | 8.79 | 8.02 | 7.04 | 7.35 | 3.09 | 3.12 |
| Garri | 9.4 | 8.65 | 7.72 | 7.22 | 3.68 | 3.45 |
| Lime | 9.56 | 9.15 | 8.16 | 6.81 | 3.6 | 2.67 |
| Potassium Alum | 9.02 | 8.22 | 6.92 | 6.56 | 3.81 | 2.91 |

## APPENDIX 19

### Data on Mean coliform counts in snail meat during processing

| **Desliming agents** | **Shucking** (Log cfu/g) | **Evisceration** (Log cfu/g) | **Desliming** (Log cfu/g) | **Washing** (Log cfu/g) | **Parboiling** (Log cfu/g) | **Cooking** (Log cfu/g) |
|---|---|---|---|---|---|---|
| Ash | 7.79 | 7.01 | 4.23 | 5.63 | 0 | 0 |
| Cassava water | 7.32 | 6.94 | 6.65 | 6.73 | 0 | 0 |
| Garri | 7.3 | 6.8 | 6.71 | 6.07 | 0 | 0 |
| Lime | 7.62 | 7.06 | 6.84 | 6.04 | 0 | 0 |
| Potassium Alum | 6.86 | 6.42 | 5.4 | 4.84 | 0 | 0 |

## APPENDIX 20

**Data on Mean counts of *Citrobacter* in snail meat during processing**

| **Desliming agents** | **Shucking** (Log cfu/g) | **Evisceration** (Log cfu/g) | **Desliming** (Log cfu/g) | **Washing** (Log cfu/g) | **Parboiling** (Log cfu/g) | **Cooking** (Log cfu/g) |
|---|---|---|---|---|---|---|
| Ash | 7.31 | 6.51 | 2.66 | 3.95 | 0 | 0 |
| Cassava water | 6.82 | 6.27 | 5.92 | 6.42 | 0 | 0 |
| Garri | 6.83 | 6.37 | 5.46 | 5.09 | 0 | 0 |
| Lime | 6.48 | 6.15 | 5.58 | 3.2 | 0 | 0 |
| Potassium Alum | 5.47 | 4.78 | 2.9 | 3.49 | 0 | 0 |

## APPENDIX 21

**Data on Mean counts of *Shigella* in snail meat during processing**

| **Desliming agents** | **Shucking (Log cfu/g)** | **Evisceration (Log cfu/g)** | **Desliming (Log cfu/g)** | **Washing (Log cfu/g)** | **Parboiling (Log cfu/g)** | **Cooking (Log cfu/g)** |
|---|---|---|---|---|---|---|
| Ash | 6.42 | 4.77 | 3.28 | 4.26 | 0 | 0 |
| Cassava water | 4.43 | 3.64 | 2.54 | 2.22 | 0 | 0 |
| Garri | 4.99 | 3.75 | 3.64 | 2.65 | 0 | 0 |
| Lime | 4.66 | 4.39 | 2.71 | 0.1 | 0 | 0 |
| Potassium Alum | 4.8 | 4.33 | 2.84 | 2.69 | 0 | 0 |

## APPENDIX 22

**Data on Mean counts of *Staphylococcus* in snail meat during processing**

| **Desliming agents** | **Shucking** (Log cfu/g) | **Evisceration** (Log cfu/g) | **Desliming** (Log cfu/g) | **Washing** (Log cfu/g) | **Parboiling** (Log cfu/g) | **Cooking** (Log cfu/g) |
|---|---|---|---|---|---|---|
| Ash | 5.6 | 4.69 | 2.56 | 4.41 | 0 | 0 |
| Cassava water | 6.32 | 6.09 | 4.9 | 4.82 | 0 | 0 |
| Garri | 5.47 | 5.1 | 4.9 | 3.48 | 0 | 0 |
| Lime | 6.33 | 5.85 | 4.63 | 4.09 | 0 | 0 |
| Potassium Alum | 6.38 | 6.19 | 5.8 | 5.3 | 0 | 0 |

## APPENDIX 23

**Data on Mean counts of *E. coli* in snail meat during processing**

| **Desliming agents** | **Shucking** (Log cfu/g) | **Evisceration** (Log cfu/g) | **Desliming** (Log cfu/g) | **Washing** (Log cfu/g) | **Parboiling** (Log cfu/g) | **Cooking** (Log cfu/g) |
|---|---|---|---|---|---|---|
| Ash | 7.59 | 6.6 | 3.55 | 4.9 | 0 | 0 |
| Cassava water | 6.8 | 6.46 | 5.54 | 5.26 | 0 | 0 |
| Garri | 6.91 | 6.49 | 6.1 | 5.37 | 0 | 0 |
| Lime | 7.28 | 6.81 | 6.29 | 5.24 | 0 | 0 |
| Potassium Alum | 6.57 | 6.2 | 5.01 | 3.96 | 0 | 0 |

## APPENDIX 24

**Data on Mean counts of *Aeromonas* in snail meat during processing**

| **Desliming agents** | **Shucking** (Log cfu/g) | **Evisceration** (Log cfu/g) | **Desliming** (Log cfu/g) | **Washing** (Log cfu/g) | **Parboiling** (Log cfu/g) | **Cooking** (Log cfu/g) |
|---|---|---|---|---|---|---|
| Ash | 5.51 | 4.69 | 2.7 | 3.62 | 0 | 0 |
| Cassava water | 5.32 | 5.19 | 4.5 | 3.96 | 0 | 0 |
| Garri | 5.32 | 5.21 | 4.15 | 4.63 | 0 | 0 |
| Lime | 3.75 | 3.24 | 0.1 | 0.1 | 0 | 0 |
| Potassium Alum | 3.91 | 3.73 | 3.4 | 2.57 | 0 | 0 |

## APPENDIX 25

**Data on Mean counts of *Bacillus* in snail meat during processing**

| **Desliming agents** | **Shucking (Log cfu/g)** | **Evisceration (Log cfu/g)** | **Desliming (Log cfu/g)** | **Washing (Log cfu/g)** | **Parboiling (Log cfu/g)** | **Cooking (Log cfu/g)** |
|---|---|---|---|---|---|---|
| Ash | 4.74 | 4.66 | 3.66 | 2.71 | 2.72 | 2.64 |
| Cassava water | 4.3 | 3.77 | 3.37 | 3.52 | 3.31 | 2.53 |
| Garri | 4.79 | 4.67 | 4.46 | 4.28 | 3.97 | 3.24 |
| Lime | 5.23 | 5.2 | 4.18 | 3.5 | 2.97 | 2.76 |
| Potassium Alum | 5.54 | 5.23 | 4.86 | 4.49 | 4.09 | 3.14 |