ISOLATION, CHARACTERIZATION AND ANTIBIOGRAM DETERMINATION OF LISTERIA SPECIES FROM CHICKEN MEAT SOLD IN ZARIA METROPOLIS, KADUNA STATE, NIGERIA

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[B.Sc. (Hons) Zoology, A.B.U, Zaria - 2010]
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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER DEGREE IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY,
FACULTY OF LIFE SCIENCES,
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ZARIA, NIGERIA

JANUARY, 2017

DECLARATION

I declare that the work in this dissertation entitled "Isolation, Characterization and Antibiogram Determination of *Listeria* Species from Chicken meat sold in Zaria metropolis, Kaduna State, Nigeria" has been performed by me in the Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

_____ _____

Sadisu MuhammadNUSA Date

CERTIFICATION

This dissertation entitled "ISOLATION, CHARACTERIZATION AND ANTIBIOGRAM DETERMINATION OF LISTERIA SPECIES FROM CHICKEN MEAT SOLD IN ZARIA METROPOLIS, KADUNA STATE, NIGERIA" by Sadisu Muhammad NUSA meets the regulations governing the award of the degree of Master of Science in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

_____	_____
Chairman, Supervisory Committee Prof. S. A. Ado	Date
_____	_____
Member, Supervisory Committee Prof. S. E. Yakubu	Date
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Head of Department Prof. I. O. Abdullahi	Date
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Dean, School of Postgraduate Studies Prof. K. Bala	Date

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DEDICATION

To my late mother Hajiya Fatima M. K. Nusa. May her soul rest in the bosom of Allah’s blessings.

ABSTRACT

This study was carried out to isolate, characterize and determine the antibiotic susceptibility pattern of *Listeria* species from chicken meat sold in Zaria metropolis, Kaduna State. A total of 254 samples (154 and 100 raw and processed chicken meat respectively) were collected from three selected districts. *Listeria* species isolation was performed according to standard procedures using *Listeria*primary and secondary enrichment broth,Oxford and PALCAM agar media. The isolates were identified using biochemical tests and confirmed using Microgen test kit. Antibiotic susceptibility test was carried out on the isolated *Listeria* species using disk diffusion method. From a total of 154 raw chicken meat samples analysed, 29 (18.8%) were positive for *Listeria* species. However, no *Listeria* species was isolated from 100 processed chicken meat samples analysed. No statistical significant differences ($P<0.05$) was observed in the distribution of *Listeria* species in raw chicken meat across the 3 locations.*Listeria* species identified in this study were *Listeria ivanovii* (44.8%), *Listeria grayi* (34.5%), *Listeria innocua* (13.7%), *Listeria seeligeri* (3.4%) and*Listeria murrayi* (3.4%).However, no*Listeria monocytogenes* was isolated from the samples. Oxford *Listeria* agar (100%) showed more efficiency in isolation of *Listeria* species than PALCAM agar (79.3%). The antibiotic susceptibility profile of *Listeria* species showed thatall the *Listeria*species isolated were resistant to ampicillin and cefoxitin. A total of 62% of the isolates were resistant to tetracycline and some of the *Listeria* spp. isolates also showed resistance to Vancomycin (24.7%), chloramphenicol (24.1%), erythromycin (24.1%), gentamicin (34.4%) and clindamycin (34.5%). None of the isolates was resistant to ciprofloxacin.Multiple Antibiotic resistance was also seen in all the *Listeria* species and multiple antibiotic resistance indices (MARI) was determined to be MARI≥0.2 for all the isolates. The rate at which raw chicken meat sold in Zaria

metropolis were contaminatedwith *Listeria* species is an indication that these poultry products could serve as a source of listerial infection for humans.

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LIST OF ABBREVIATIONS

CAMP	Christe, Atkins and Munch-Peterson test
CI	Confidence Interval
CLSI	Clinical and Laboratory Standards Institute
EC	European Commission
FDA	Food and Drug Administration
FSAI	Food Safety Authority of Ireland
ISO	International Organization for Standardization
LEB	Listeria Enrichment Broth
LPM	Lithium chloride/phenylethanol/moxalactam
MARI	Multi Antibiotic Resistance Indices
NaCl	Sodium Chloride
OIE	Office International des Epizooties
OXA	Oxford Agar
PALCAM	PolymyxinAcriflavine Lithium Chloride CeftazidimeAesculinMannitol
RTE	Ready-to-eat
TSAYE	Trypticase–soy agar containing yeast extract
TSBYE	Trypticase–soy broth containing yeast extract

CHAPTER ONE

1.0 INTRODUCTION

Listeriainfection caused by microorganisms of the genus *Listeria* occurs worldwide and in variety of animals and man (McLauchlin, 1997; Katarzyna *etal*., 2005). *Listeria monocytogenes* is one of the most important food-borne pathogens of humans. It is a Gram positive, rod-shaped, non-spore-forming, and facultative anaerobe (Vazquez-Boland *et al*., 2001; Sukhadeo and Trinad, 2009). It has the ability to cause severe diseases in humans and animals. *L.monocytogenes* is ubiquitous and can be found in food, water, soil, vegetables as well as animals and humans (Cocolin *et al*., 2005; Liu, 2008). *L.monocytogenes* has been the cause of outbreaks or sporadic cases of listeriosis in humans and has also been associated with invasive infections in more than 40 species of mammals and birds (Naightingale *et al*., 2005). Almost 99% of the *Listeria* infections have been attributed to the ingestion of contaminated food, principally ready to eat products (less than 0.1% of all food-borne illnesses) with a very high mortality (20 to 30%) (Mead *et al*., 1999; Ireton, 2006); mortality can be as high as 75%, in high risk persons (Khelef *et al*., 2006; Adzitey and Huda, 2010).

There are six species under the genus *Listeria*, notably: *L.monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L.seeligeri* and *L. grayi*. Out of these, only *L. monocytogenes* has been authentically reported by many researchers as human and animal pathogen. *L.ivanovii* has also been identified as pathogenic for animals but, mainly in sheep and cattle; and on rare occasions, *L. ivanovii* and *L. seeligeri* have been associated with human infections (Rocourt and Cossart, 1997).

Various foods and environmental samples have been implicated in the spread of *L. monocytogenes*. Thus the pathogen is repeatedly found in meat and meat products, raw

milk, and pasteurised dairy products, fish and fish products. Also, *L. monocytogenes* has been isolated from chopping boards, cleaning cloths, mincing machine, poultry meat and meat products (Mahmood *et al*., 2003), cooked meat, cured meats and smoked salmon, soft cheese and vegetables (Vitas *et al*., 2004; Ponniah *et al*., 2010), ready to eat foods (Aurora *et al*., 2009; Marian *et al*., 2012), sheep, goat, and cow milk (Rahimi *et al*., 2010), raw and pasteurized egg (Rivoal *et al*., 2010) and burger patties and vegetarian burger patties (Wong *et al*., 2011; Wong *et al*., 2012).

The high incidence of *L. monocytogenes* in raw chicken is a problem because of cross-contamination to other foods at home and the possibility of the microorganism surviving in processed chicken. Studies indicate that *Listeria* may survive marginal thermal processing of such products. In terms of survival in cooked chicken, *L. monocytogenes* has been isolated from prepared chicken sandwiches (Lieval *et al*., 1989) and from samples of ready-to-eat, precooked chicken (Kerr *et al*., 1990). The organism is considered hazardous in the food industry due to its ability to grow in low water activity (Nolan *et al*., 1992), low pH (Buchanan *et al*., 1993), as well as gas or vacuum-packaged products at refrigeration temperatures (Duffy *et al*., 1994), and all these measures are important in the control of food pathogens (Isaac, 2009)

Although the route of infection is primarily oral, the clinical manifestations of listeriosis are variable between groups and individuals (NFPA, 1999). In pregnant females, the infection is usually self-limited affecting only the uterine contents and causing miscarriage and stillbirth. The pregnant woman may, however, exhibit flu-like symptoms (NFPA, 1999; FSIS, 2001).In neonates infected in uterus, the infection causes a condition known as granulomatosis infantiseptium and lesions are common in the liver and placenta but can also be found in the brain, adrenal glands, spleen, kidney, lungs and the gastrointestinal tract (Ramaswamy *et al*., 2007). In non-pregnant adults

and children, listeriosis is principally an infection of immune-suppressed individuals and complications usually involve the central nervous system and the bloodstream, but may include pneumonia and endocarditis (NFPA, 1999; Sleator *et al*., 2009). The common symptoms of listeriosis include fever, watery diarrhoea, nausea, headache, and pains in joints and muscles (NFPA, 1999; Abdul-Razzak, 2007; Arun, 2008). Clinical manifestations of invasive listeriosis are usually severe and include abortion, sepsis, meningoencephalitis, neuro-encephalitis, chorioamnionitis, gastroenteritis and bacteraemia (Khelef *et al*., 2006; Sukhadeo and Trinad, 2009).

Detection of multi-drug resistant pathogenic bacteria in foods is considered as a public health risk worldwide. Excessive application of antibiotics in veterinary medicine may lead to distribution of antibiotic-resistant pathogens in the environment (Schwartz *et al*., 2003). Recently, multi-drug resistant isolates of *L. monocytogenes* have been detected in animals, different types of foods and human cases of listeriosis (Safdar and Armstrong, 2003; Marian *et al*., 2012; Jamali and Radmehr, 2013; Jamali *et al*., 2013).

1.1Statement of Research Problem

Listeria monocytogenes is a ubiquitous Gram positive bacterium of intracellular localization responsible for severe infections in humans and more than 40 animal species (Low and Donachie, 1997). *Listeriamonocytogenes* is included in the world Health Organisation's list of food borne pathogens (WHO, 2002). There have been several sporadic and endemic outbreaks worldwide indicating *Listeria* contaminated foods (Schlech *et al*., 1983; Fleming *et al*., 1985; Schwartz *et al*., 1989; Bula *et al*., 1995; CDC, 2002). Foods which are denoted as ready to eat (deli meats, salads), unpasteurized dairy foods (cheese and milk), cured and raw meats (hot dogs,

undercooked chicken), and items such as prepared seafood salads and even raw and unprocessed meats have been common foods implicated (Schlech, 2000).

While *L. monocytogenes* causes relatively few human disease cases, particularly compared to many other food borne pathogens (Mead et al., 1999), it appears to be commonly present in raw and RTE foods. The microbial contamination of carcasses occurs mainly during processing and manipulation such as skinning, evisceration, storage and distribution at slaughter houses and retail establishments (Gill, 1998; Abdalla *et al*., 2009). In developing countries, some traditional methods of handling, processing and marketing of meat undermine quality whereas poor sanitation leads to considerable loss of products as well as the risk of food-borne-diseases (Garcia de siles *et al*., 1997).

L. monocytogenes has been implicated in multiple large outbreaks worldwide. Each year in the United State, the incidence of listeriosis is estimated to range from 3.4 to 4.4 cases per million (Tappero *et al*., 1995; CDC, 2003). Annual projections in the United State indicate that approximately 2,500 cases of human listeriosis will occur where nearly 500 of these cases progress to death and 300 cases will require hospitalization (Stehulak, 1992; Mead *et al*., 1999; DBMD, 2001; FSIS-USDA, 2001; Ramaswamy *etal*., 2007). This projection may be underestimated by half due to asymptomatic symptoms occurring in healthy individuals who become infected, but show no clinical signs (Mead *et al*., 1999). Except for the major outbreaks, which involved hundreds of individuals, the incidence of listeriosis is difficult to determine. Estimate ranges from <2 to 12 per million of population. The Centre for Disease Control has estimated 1700 cases per year in the USA. Listeriosis cases in Africa, Asia and South America were reported (Low and Donachie, 1997).

During a one-year prospective study in Nothern Nigeria by Onyemelukwe *etal*. (1983), the clinical conditions in which *L. monocytogenes* was isolated from nineteen patients (six females and thirteen males) included meningitis, meningoencephalitis, spontaneous peritonitis, septicaemia, arthritis, pelvic infection and urethritis. All isolates were serotype 4 and a mortality of 27 percent was recorded. In humans, *L. monocytogenes* can affect the central nervous system, leading to death or generating neurological sequels, whereas the non-invasive form of the disease causes gastrointestinal syndrome. Although, illness could be develop in apparently healthful people, in addition, there are especially sensitive groups like the new-borns (Laciar *et al*., 2000), the pregnant women (Salazar *et al*., 2001), old and immunologically suppressed people (Torres *et al*., 2004). The mortality rate of this disease oscillates between 20 and 30% (Korkeala and Siitonen, 2003). Few cases of human infections caused by *L.ivanovii* have been detected (Ivanov, 1962). Three cases have been reported;a placenta infection in a post-childbirth (Elischerova *et al*., 1990), an AIDS patient (Cummins *et al*., 1994) anda 64 years old male patient with liver metastatic carcinoma (Snapir *et al*., 2006). A number of antibiotics have been suggested for the treatment of *L. monocytogenes*. Unfortunately, failures have been reported for all therapeutic programs because there is no consensus among various authors as to which antibiotic regimen is the most effective (McLauchlin and Nichols, 1994).

Previous published works on *Listeria* spp. in food and environmental samples in some states in Nigeria relates a high incident of the organism; 21% in plateau state (Chukwu, 2007), 58% in Sokoto (Salihu *etal*., 2008), almost 100% in Abia state (Nwachukwu *et al*., 2009), 78% in Nsuka, Enugu state (Ikeh *et al*., 2010), and average of 81% in Ogikwe, Imo state (Nkechi and Frank, 2012).

Listeriosis is considered a serious health problem due to severity of symptoms and its high mortality rate especially in pregnant women, neonates, and immune-compromised individuals. In spite of the mortality and the increase resistance of *Listeria* species to antibiotics, there are no enough reference compendium of the prevalence in different foods, distributional profiles across regions andantibiotic susceptibility profile of local isolates in Nigeria.

1.2Justification of the Study

Previous studies have confirmed the presence of *L. monocytogenes* in a wide variety of foodstuffs. Milk (mainly unpasteurised), dairy products (especially soft ripened cheeses), poultry meat and products and raw vegetables are considered to be the most frequently contaminated with listeriae (Anon, 2000).

The major source of ready to eat (RTE) chicken meat to the locals is sold by street hawkers in Nigeria. However, the hygiene status of street hawkers is questionable. Previous studies conducted in Nigeria (Salihu *et al*., 2008), and in other countries (Diaz-Lopez *et al*., 2011; Marian *et al*., 2012; Nyenje *et al*., 2012) found high prevalence of food borne pathogens in street foods.

Listeria spp. has been isolated from poultry meat and meat products in many countries around the world, although these foods have not been associated with documented outbreaks of human listeriosis (Abd El-Malek *et al*., 2010). The detection of *Listeria* spp. in chicken meat is a particular concern in terms of consumer safety, as these organisms are capable of growing on both raw and processed meat at refrigeration temperature (Abd El-Malek *et al*., 2010).

Knowledge of the distribution of *Listeria* species amongst raw and processed chicken meat across the regions in Nigeria is lacking. Therefore, the purpose of this study is to

establish the occurrence and antibiotic susceptibility profile of *Listeria* species in raw and processed chicken meat in some districts within Zaria metropolis of Kaduna State.

This study will perhaps be the first to be conducted in Zaria, North West of Nigeria, as there is no available published work to the best of my knowledge reported from the area of study. The data to be obtained will form the baseline for treatment, control and further future studies.

1.3 Aim of the Study

The aim of this study was to isolate, characterize and determine the antibiogram of *Listeria* species in raw and processed chicken meat sold in Zaria metropolis.

1.4Objectives of the Study

The objectives of this study were to:

1. Isolate and presumptively characterize*Listeria* species from chicken meat sold in Zaria using biochemical methods.
2. Confirm the isolates using Microgen test Kit.
3. Determine the efficiency of Oxford and PALCAM Listeria media for isolation of *Listeria* species
4. Determine the antibiotic susceptibility pattern of the *Listeria* species isolated.

CHAPTER TWO

2.0LITERATURE REVIEW

2.1 Distribution of *Listeria* species

Listeria is a widely distributed bacterium in nature and commonly found in soil, sewage, dust, water and causes listeriosis in humans and animals. Listeriosis is a relatively rare food-borne illness, but can be life threatening with high fatality rates. It is mainly associated with the consumption of processed foods that require no further cooking by the consumer (McLauchlin, 1996; Low and Donachie, 1997; Schlech, 2000), this includes Coleslaw (Schlech *et al*., 1983), milk (Fleming *et al*., 1985; Dalton *et al*., 1997), cheese (Linnan *et al*., 1988; Jensen *et al*., 1994; Bula *et al*., 1995; Goulet *et al*., 1995), butter (Lyytika¨inen *et al*., 2000), pate (McLauchlin *et al*., 1991; Kittison, 1992), ready to eat deli meats (Hurd *et al*., 2000), raw fruits, vegetables, salads and hot dogs.

L. monocytogenes is one of the ten phenotypically similar species of *Listeria* (i.e., *Listeria monocytogenes, L. innocua, L. ivanovii, L. welshimeri, L. seeligeri, L. grayi, L. rocourtiae, L. marthii, L. fleishmanii & L. weihenstephanensis*) (Khelef *et al*., 2006; McLauchlin and Rees, 2009; Bertsch *et al*., 2013; Halter *et al*., 2013). Among these ten species of *Listeria*, two species namely *L. monocytogenes* (pathogenic for human and animals) and *L. ivanovii* (pathogenic for animals) are usually associated with Listeriosis (Liu, 2006). *L. monocytogenes* is a cause of food-borne disease; it is linked to disproportionately high levels of morbidity and mortality (Weatherill, 2009; Clark *et al*., 2010). *L. monocytogenes* is carried within the intestinal tract of seemingly healthy animals.The pathogen confronts numerous stressful conditions in food processing environments and during infection of a host. It is capable to grow at refrigeration

temperature, at pH values of 5 and above, in high salt concentration (up to 10%) and are relative resistance to freezing and drying (Achemchem *et al*., 2006; Arslan and Ozdemir, 2008). *L. monocytogenes* and other *Listeria* spp. have also been isolated from a variety of raw and processed foods (Gudbjornsdottir *et al*., 2004).

Listeria is considered to be intolerant to the temperatures achieved during food processing, such as cooking and pasteurisation. *L. monocytogenes* in contaminated foods is associated with central nervous system (CNS) diseases, sepsis, endocarditis, focal infections, gastroenteritis and can cause still births and abortions (Zhou and Jiao, 2004). Non-invasive listeriosis occurs in healthy populations at low infection rates, usually causing only self-limiting gastrointestinal diseases (Doganay, 2003).

2.2 Taxonomy and General Characteristics of *Listeria* species

2.2.1 Taxonomy

Members of the genus *Listeria* have traditionally been classified into three typically hemolytic species (*Listeria monocytogenes*, *L. ivanovii*, and *L. seeligeri*) and two typically nonhemolytic species (*L. innocua* and *L. welshimeri*) (Seeliger and Jones, 1986). While *L. seeligeri* is considered nonpathogenic, it includes both hemolytic and nonhemolytic isolates (Volokhov *et al*., 2006; Den Bakker *et al*., 2010), with hemolytic isolates of this species containing a homologue of the main virulence gene cluster (i.e., the *prfA* cluster), which carries key virulence genes in *L. monocytogenes* and *L. ivanovii* (Gouin *et al*., 1994). An additional nonhemolytic species, *Listeria grayi*, has not been formally excluded as a member of the genus *Listeria* but has been shown to be very different from the other *Listeria* spp. (Boerlin *et al.,* 1991; Seeliger and Jones, 1986; Vaneechoutte *et al*., 1998).

Although proposed at one time to represent a new genus, *Murraya* (Stuart and Welshimer, 1973; Stuart and Welshimer, 1974), *L. grayi* is currently considered a *Listeria* species (Collins *et al*., 1991). Two new nonhemolytic *Listeria* species (i.e., *Listeria rocourtii* and *L. marthii*) were reported in 2010 (Graves *et al*., 2010; Leclerq *et al*., 2010). The genus *Listeria* belongs to the class Bacilli and the order Bacillales,which also includes *Bacillus* and *Staphylococcus*. The genus *Listeria* currently contains 10 species: *L. fleischmannii, L. grayi, L. innocua, L. ivanovii, L. marthii, L. monocytogenes, L. rocourtiae, L. seeligeri, L. weihenstephanensis* and *L. welshimeri.L. denitrificans*, previously thought to be part of the *Listeria* genus, was reclassified into the new genus *Jonesia*(Collins *et al*., 1991).

Scientific Classification

Kingdom: Bacteria

Division: Firmicutes

Class: Bacilli

Order: Bacillales

Family: Listeriaceae

Genus: *Listeria*

Species: *L. aquatica, L. booriaeL. cornellensis, L. fleischmannii, L. floridensis, L. grandensis, L. grayi, L. innocua, L. ivanovii, L. marthii, L. monocytogenes, L. newyorkensis, L. riparia, L. rocourtiae, L. seeligeri, L. weihenstephanensis, L.welshimeri*

Pirie (1940)

2.2.2General characteristics

Listeria species

Listeria species are short Gram positive rods, 0.4-0.5 x 0.5-2.0µm, with rounded ends, occurring singly or in short chains and occasionally appearing filamentous. Members of the genus are facultative anaerobes, non-sporing, non-acid fast and do not possess a capsule. *Listeria* species are motile by peritrichous flagella (Fig 2.1) when grown at 20°C - 25°C and display a characteristic "tumbling" motility. The optimum growth temperature (but not for motility) is 30-37°C. Colonies on blood agar are non-pigmented and may resemble those of β-haemolytic streptococci. They are catalase positive, oxidase negative and ferment carbohydrates (Holt, 1994; Mitchell, 1996).

Listeria species are widely distributed in the environment; some species are pathogenic for humans and animals. The medically important species are:

L. monocytogenes

Microscopically, they appear as small rods, which are sometimes arranged in short chains. In direct smears they may be coccoid, and may be mistaken for streptococci. Longer rods may resemble *corynebacteria*. Haemolytic activity on blood agar has been used as a marker to distinguish *Listeria monocytogenes* from other *Listeria* species, but it is not an absolutely definitive criterion. Further biochemical characterization may be necessary to distinguish between the different *Listeria* species. *L. monocytogenes* is catalase positive and oxidase negative. *L. monocytogenes* is the agent of listeriosis, a serious infection caused by eating food contaminated with the bacterium. Listeriosis has been recognized as an important public health problem and the disease affects primarily pregnant women, neonates, elderly people, and those with weakened immune systems (UKSMI, 2014).

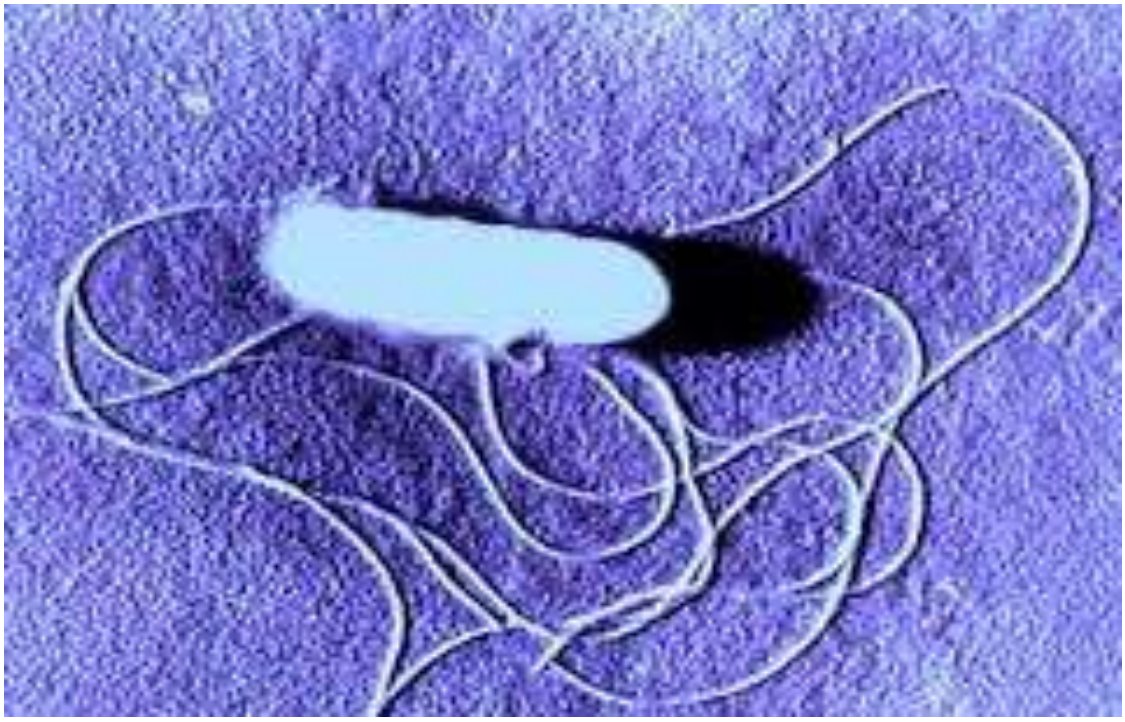

Fig 2.1: Microscopic view of *Listeria monocytogenes*under electron microscope (x 10^4)

L. ivanovii

Cells are small, motile rods. Colonies on tryptose agar are very small (0.5 to 1mm in diameter after 1 or 2 days of incubation at 37°C), regular, and smooth and appear bluish green when they are viewed by obliquely transmitted light. Colonies on sheep or horse blood (5%) agar are strongly β-haemolytic. Growth occurs at 4°C within 5 days. They are facultatively anaerobic. *L. ivanovii* are positive for catalase, Voges-Proskauer and methyl red tests, and aesculin hydrolysis. They are negative for oxidase, urea and gelatin hydrolysis; and reduction of nitrates. Neither indole nor H_2S is produced. Acid but no gas is produced from glucose and D-xylose. No acid is produced from D-mannitol, L-rhamnose, or α-methyl-D-mannoside. The species has also been isolated from healthy animals and human carriers and from the environment. Subsequently, this species has been divided into 2 subspecies. They are; *Listeria ivanovii* subsp. *ivanovii* and *Listeria ivanovii* subsp. *Londoniensis*(Boerlin *et al*., 1992). Most of the characteristics are similar to those of *L. ivanovii*, except that *L. ivanovii* subsp. *londoniensis* does not produce acid from ribose, but produces acid from N-acetyl-P-Dmannosamine after 18 to 24hr of incubation at 37°C4 (Seeliger *et al*., 1984).

L. seeligeri

Cells are small (0.4-0.8 x 0.5-2.5µm) rods which are motile by means of peritrichous flagella. Colonies on tryptose agar are similar to that of *L. welshimeri*. Growth occurs at 4°C within 5 days. They are facultatively anaerobic. They are positive for catalase, Voges-Proskauer and methyl red tests; and aesculin hydrolysis. They are negative for oxidase, reduction of nitrates, urea hydrolysis and indole production. Acid, but no gas, is produced from D-glucose and D- xylose. Acid is not produced from D-mannitol or L-rhamnose. Most strains do not produce acid from α-methyl-D-mannoside. They have

been isolated from plants, soil, and animal faeces (sheep) in Europe (Boerlin *et al*., 1992).

L. innocua

Cells are small rods occurring singly or in short chains. They are motile by means of peritrichous flagella. They are mesophilic, operating at an optimal temperature range of 30-37°C. *Listeria innocua* have a very complex metabolism. They are capable of metabolizing methane, sulphur and nitrogen, among many other organic and inorganic compounds. These organisms also carry out numerous biosynthetic pathways, including peptidoglycan synthesis. *L. innocua*, like other members of their genus, they are facultative anaerobes, which means that they can metabolism glucose (and other simple sugars) in under both aerobic and anaerobic conditions. Under the aerobic metabolism of glucose, they form lactic acid and acetic acid. However, under anaerobic conditions, the metabolism of glucose yields only lactic acid (Pine *et al*., 1989). This species is widespread in the environment and in food and has also been associated with one reported case of fatal bacteraemia (Perrin *et al*., 2003).

L. welshimeri

Cells are small (0.4 to 0.5 by 0.5 to 2.0µm) rods which are motile by means of peritrichous flagella. Colonies on tryptose agar are small (1 to 2mm in diameter after 1 or 2 days of incubation at 37°C), regular, and smooth with a blue-green colour when they are examined with obliquely transmitted light. Sheep erythrocytes are not haemolysed. Growth occurs at 4°C within 5 days. Metabolism is facultatively anaerobic. Acid, but no gas, is produced from D-glucose, D-xylose, and a-methyl-D-mannoside. Acid may or may not be produced from L- rhamnose. Acid is not produced from D-mannitol. They are positive for catalase, aesculin hydrolysis, Voges-Proskauer

and methyl red tests, and negative for oxidase, urea, gelatin hydrolysis, indole and H2S production as well as reduction of nitrates. They have been isolated from decaying plants and soil (Rocourt and Grimont, 1983).

L. grayi

According to Rocourt *et al*. (1992), *Listeria grayi* is an earlier heterotypic synonym of *Listeria murrayi* and so both were assigned to a single species, *Listeria grayi*. Cells are small (0.4-0.5 x 0.5-2µm) peritrichous rods which are motile. Colonies on tryptose agar are small (1-2mm in diameter after 1-2 days of incubation at 37°C), regular, and smooth. Growth occurs at 4°C within 5 days. Metabolism is facultatively anaerobic. They are positive for catalase, aesculin hydrolysis, Voges-Proskauer and methyl red tests and negative for the oxidase, urea and gelatin hydrolysis, H_2S and indole production. Reduction of nitrates to nitrites is variable. Acid, without gas, is produced from glucose, mannitol, and other sugars. Sheep erythrocytes are not haemolysed (UKSMI, 2014).

2.3 Historical Background of *Listeria monocytogenes* and *Listeria* species

The first documented case of *Listeria* was in 1924. In the late 1920s, two researchers independently identified *L. monocytogenes* from animal outbreaks. They proposed the genus *Listerella* in honor of surgeon and early antiseptic advocate Joseph Lister, but that name was already in use for a slime mold and a protozoan. Eventually, the genus *Listeria* was proposed and accepted. All species within the *Listeria* genus are gram-positive, not spore-forming, catalase-positive rods. The genus *Listeria* was classified in the family *Corynebacteriaceae* through the seventh edition of *Bergey's Manual of Systematic Bacteriology*. The 16S rRNA cataloging studies of Stackebrandt, *et al.* demonstrated that *L. monocytogenes* is a distinct taxon within the *Lactobacillus*-

Bacillus branch of the bacterial phylogeny constructed by Wöse. In 2004, the genus was placed in the newly created family *Listeriaceae*. The only other genus in the family is *Brochothrix* (Elliot *et al*., 1999)

Listeria monocytogenes was first described by E.G.D. Murray in 1924 based on six cases of sudden death in young rabbits, and published a description with his colleagues in 1926 (*Murray et al., 1926).* Murray referred to the organism as *Bacterium monocytogenes* before Harvey Pirie changed the genus name to *Listeria* in 1940 (Harvey, 1940). Although clinical descriptions of *L. monocytogenes* infection in both animals and humans were published in the 1920s, it was not recognized as a significant cause of neonatal infection, sepsis and meningitis until 1952 in East Germany (Potel, 1952).Listeriosis in adults would later be associated with patients living with compromised immune systems, such as individuals taking immunosuppressant drugs and corticosteroids for malignancies or organ transplants, and those with HIV infection (Schlech, 2001).*L. monocytogenes* was not identified as a cause of foodborne illness until 1981. However, an outbreak of listeriosis in Halifax, Nova Scotia, involving 41 cases and 18 deaths, mostly in pregnant women and neonates, was epidemiologically linked to the consumption of coleslaw containing cabbage that had been contaminated with *L. monocytogenes*-contaminated sheep manure (Schlech *et al*., 1983). Since then, a number of cases of foodborne listeriosis have been reported, and *L. monocytogenes* is now widely recognized as an important hazard in the food industry (Ryser and Marth, 1999).

2.4Intergeneric Relationship Between *Listeria* species

The genus *Listeria* is placed in the *Clostridium* sub branch of Gram-positive bacteria based upon the low G + C content of its genome (Rocourt *et al*., 1986; Cummins *et al*.,

1994). At one time it was thought to be closely related to *Erysipelothrix* although it differed from this genus in several respects and subsequent taxonomic studies have supported their separation (Davis *et al*., 1969; Stuart and Pease, 1972). Following the recent transfer of the non-pathogenic species *L. denitrificans* to a new genus, *Jonesia* (Rocourt *et al*., 1987a), *Listeria* now contains six species in addition to *L. monocytogenes*. The taxonomic position of two of them, *L. grayi* and *L. murrayi*, has been controversial (Seeliger and Jones, 1986) but recent genomic evidence confirms their place in *Listeria* (Rocourt *et al*., 1987b); neither of them is pathogenic for man. The remaining species, *L. innocua, L. seeligeri, L. welshimeri* and *L. ivanovii,* are essentially genomic in derivation from *L. monocytogenes* (Rocourt *et al*., 1983) and they are thus not easy to distinguish in routine laboratories.

Like *Brochothrix,* which they closely resemble, all *Listeria* species survive and grow slowly at low temperatures (1-5°C); this unusual ability is used to advantage for their selective isolation by 'incubation' in the refrigerator. This procedure is, however, slow so that selective and enrichment media such as Thallous Acetate-Nalidixic Acid Agar and Thiocyanate- or Thallous Acetate-Nalidixic Acid Broths (Kramer & Jones, 1969) should also be used. Both of these genera occupy a position between *Lactobacillus* and *Bacillus* and are more distantly related to *Streptococcus, Lactococcus, Enterococcus, Staphylococcus, Kurthia, Gemella,* and *Erysipelothrix.*

2.5 Phisiology and Metabolism of *Listeria* species

L. monocytogenes is catalase positive, oxidase negative and is able to survive between 0 and 45°C. The optimum growth temperature is around 30–37 °C. *L. monocytogenes* can grow at pH ranges between 4.5 and 9.0 (optimum pH between 6 and 8) and is able to multiply in food matrices at water activity (aw) values of 0.92 and in NaCl

concentrations of 12%, generally lethal to other microorganisms. *L. monocytogenes* is a ubiquitous organism, widely distributed in the environment: the principal reservoirs are soil, forage and water (Sauders and Wiedmann, 2007; Todd and Notermans, 2011; EFSA, 2014). Other reservoirs include healthy humans and animals (ILSI, 2005) or infected domestic and wild animals (EFSA, 2014). *L. monocytogenes* is a psychrotrophic bacterium, can multiply at low temperatures, both under aerobic and anaerobic conditions, adapt to disinfectants and adhere to various surfaces (Arevalos-Sánchez *et al*., 2012). *L. monocytogenes* is widespread in food processing facilities and has been isolated from different processing environments. Once introduced into the processing plants, it is able to survive and persist for a long time under adverse conditions (Farber and Peterkin, 1991; Gram *et al.*, 2007; Gandhi and Chikindas, 2007). The biofilm forming ability is an important cause for such persistence (Fonnesbech Vogel *et al*., 2001; Cruz and Fletcher; 2012).

The physiology and metabolism of *L. monocytogenes* affect the aetiology and pathogenicity of the organism. Such parameters are the facultatively anaerobic growth in the presence of respiratory enzymes and cofactors, catalase and super oxide dismutase which circumvent the macrophagic intracellular oxidative bursts. Others are the production of haemolysin phosphatase C, specific attachment to intestinal peyers patches and the crossing of placental and brain membrane barriers. These physiological activities, however, have relevance to the organism's virulence and growth as well as infectivity (Benedict, 1990).

2.6 Ecology of *Listeria* spp. in Environment and Food

Listeria species are unusual in that they can survive and multiply at both low and high temperatures. They can also endure a wide pH range of 4.4 to 9.8 (Brooks *et al*., 1989).

These facts help explain why they are ubiquitous in all types of environment. *Listeria* spp. are found in water, soil, vegetation, wild and domesticated animals, and humans. Cultivated land has less contamination of *Listeria* than uncultivated; sandy soil contains more *Listeria* than clay-like soil; untreated sewage water is a good breeding ground for *Listeria*. Since *Listeria* is also found in water sources, such as the sea, it is not surprising that fish, squids, crustaceans and other seafood have been found to contain the bacteria (Jemmi and Stephan, 2006). This could be harmful for humans if the contamination is from *L.monocytogenes* because they are the pathogenic species that cause disease (Buchrieser *et al*., 2003). Furthermore, human intervention, like effluents from food-processing plants, also increases the spread of *Listeria* into the environment (Jemmi and Stephan, 2006).

Soil has been suggested to be the natural reservoir of *Listeria* spp. since it is able to multiply there (Botzler *et al*., 1974; Dowe *et al*., 1997). *L.monocytogenes* has also been found in sludge from a fish farm (Jemmi and Keusch, 1994). Water environments such as coastal sea waters and rivers containing a high organic load have been found to carry *Listeria* spp. (Colburn *et al*., 1990).

Due to their ubiquitous presence, *Listeria* in general and *L. monocytogenes* in particular are also used as hygiene indicators in all stages of the food processing chain. *L. monocytogenes* has been isolated from various ready-to-eat products. In a study by Meldrum *et al*. (2010) the prevalence of *L. monocytogenes* was 4.1% in crustaceans (n=147), 6.7% in smoked fish (n=178), 2% in sushi (n=50) and 0.9% in green salad (n=335) samples in Wales. Wong *et al*. (2005) isolated *L. monocytogenes* from 1% of ham (n=104) and 1.7% of pate (n=60) samples in New Zealand. *L. monocytogenes* has also been isolated from dairy products. For example, *L. monocytogenes* was detected in 1.3% of fresh cheese samples in Spain (n=78), 0.2% of hard cheese samples in the

United Kingdom (n=1242) and 0.3% of ice creams in Italy (n=1734) (Busani *et al*., 2005; Cabedo *et al*., 2008; Little *et al.,* 2009). The prevalence of *L. monocytogenes* in bulk milk tank internationally is 1–60% (FSANZ 2009).

The presence of *L. monocytogenes* in ready-to-eat products is probably due to contamination occurring after the product has been processed. This contamination may occur during additional handling steps such as peeling, slicing and repackaging. Also, in the retail and food service environment, contamination may be transferred between ready-to-eat products (Lianou and Sofos, 2007). The type of handling that ready-to-eat meat receives may also influence the level of *L. monocytogenes* contamination. In a survey of retail packaged meats there was a significantly higher prevalence of *L. monocytogenes* reported in products cut into cubes (61.5%) (n=13), compared with sliced products (4.6%) (n=196) (Angelidis and Koutsoumanis, 2006).

The most numerous cases of listeriosis occur in urban areas without a clear association with animals, suggesting that food serves as a major vector of infection. Potentially contaminated foods include raw vegetables, unpasteurized milk, poultry, meat products from gourmet foods category, soft cheeses, and many other foods. Although marine foods have received little attention on the potential for contamination with *L. monocytogenes*, the disease has been linked to consumption of shrimp, crab meat, smoked rainbow salmon, and lobster (De Santis *et al*., 2007; Naim *et al*, 2009).

2.7*Listeria*species in Chicken Meat and Meat Products

Poultry can become contaminated with *Listeria* spp. either environmentally or from healthy carrier birds during production in the farm (Skovgaard and Morgen, 1988). In poultry abattoir, processing plant, improper cleaning, disinfecting of environment and equipment, mishandling of the products may lead to *Listeria* contamination of poultry

carcasses and the final products (Uyttendaele, 1997; Loura *et al*., 2005). Contamination of RTE poultry products can occur after cooking by crosscontamination environmentally or via workers, surfaces and equipments (Osaili *et al*., 2011). Transmission of the resistant strains to human via contaminated food products may have public health consequences (Filiousis *et al*., 2009).

Charpentier and Courvalin (1999) have stated that the antimicrobial resistance of *Listeria* spp. is due to the acquisition of mobile genetic elements such as self-transferable and mobilizable plasmids and conjugative transposons.Many authors have also demonstrated a high prevalence of *L. monocytogenes*, and other *Listeria* spp. in meat and poultry product processing environments viz., in chilling and cutting rooms (Van de Elzen and Snijders, 1993) workers' hands (Kerr *et al*., 1995), and processing equipment (Lawrence and Gilmour, 1995)conveyor belt rollers (Tompkin, 2002) strongly suggesting that the processing environment represents a significant source of these organisms in finished products. While processed meat and poultry products are cooked to destroy *Listeria*, these bacteria can recontaminate the product while it is being handled, packaged or distributed (Tompkin *et al*., 1999; Lekroengsin *et al*., 2007). Studies involved knowing transmission routes which depended solely on isolating and counting the organism at different places along the processing line (Eklund *et al*., 1995;Lekroengsin*et al*., 2007).

2.8Occurrence of *Listeria* species in Animals in Nigeria

The early researchers on *Listeria* in Nigeria concentrated on the occurrence of the organism in human and this may be the possible reason why studies on the organism as it affects animals actually kicked off in the late eighties. At the moment, the most implicated animal is cattle but there are reports of*Listeria* in many other animals.Most

reported cases are from the north of the country where the majority of livestock production for food consumption is carried out (Nwaiwu, 2015). Oni *et al*. (1989) concluded that *L. monocytogenes* infection is widespread in domestic animals in Nigeria when they carried out a survey to determine the antibody prevalence to serotypes 1/2a, 1/2b, 1/2c, 3a and 4b in 1,190 serum samples from various animal sources in Kano and Kaduna States.

An outbreak of listeriosis was reported in a herd of cattle by Akpavie and Ikheloa (1992) from the south-western city of Ibadan. The organism was isolated in pure culture and the infected animals were associated with still birth, abortion and nervous signs before death. No micro abscesses in the brain were observed when histopathology was carried out but purulent meningitis was seen. In another unusual clinical case (Chukwu *et al*., 2006), the organism was isolated from specimens of blood and vaginal discharge of an African buffalo (Syncerus caffer) presented with septicaemia and abortion. The animal recovered after treatment and the authors pointed out the need for further investigation of listeriosis in wildlife since it was the first recorded case of *Listeria* infection found in wild life in Nigeria. In Sokoto State, Yakubu *et al*. (2012) found *Listeria* in 39 out of 192 raw milk samples collected from lactating cows identified in nomadic herds and small scale dairy farms. After biochemical characterization, 5 species of *Listeria* were found to be present with *Listeriainnocua* being the most abundant, followed by *L. ivanovii, L. monocytogenes, L. welshimeri* and *L. seeligeri*, respectively. The authors suggested that *Listeria* infection may have occurred as a result of unhygienic milking. Another report identified that poor methods of pasteurization contribute to high microbial counts in milk (Lawan *et al*., 2012). Occurrence of the organism has also been reported in other animals that produce milk (Nwaiwu, 2015).

This was established when an evaluation of 60 milk samples from West African dwarf and Red Sokoto breed of goats showed an incidence rate of 12% for *Listeria* (Adetunji and Olaoye, 2012). Animal droppings have been widely implicated in the occurrence of the organism. David and Odeyemi (2007) found *L. monocytogenes* in cattle faeces used for manure in Ado-Ekiti in Ekiti State while in a wider study, Umeh and Okpokwasili (2009) screened a total of 1000 fresh faecal samples of livestock from different animals namely cattle, sheep, goat, chicken and pigs for prevalence of *L. monocytogenes* and found that the organism occurred highest in faeces from cattle (30%) and lowest in pig faeces. Nwachukwu *et al*. (2012) called for veterinary surveillance of poultry droppings when they found high occurrence of *L. monocytogenes* in 3 farms in Okigwe, Imo State region of Nigeria. Particularly worrisome was the resistance of the isolated strains to chloramphenicol and ampicillin. The organism has been found in animals not normally associated with *Listeria*. Magaji *et al*. (2008) studied the microflora from the buccal cavity of 26 stray cats and found 3 *Listeria* spp. out of the 51 bacteria isolates identified in the study while Adeleke*et al*. (2012) collected cockroaches from residential areas and hospital vicinities to determine the microbial flora they harbour and found *L. monocytogenes* among other micro-organisms isolated.

2.9 Occurrence of *Listeria*species in Humans in Nigeria

Sequence typing confirmed that a predominant food borne *L. monocytogenes* clone caused human listeriosis cases and outbreaks in Canada from 1988 to 2010 (Knabel *et al*., 2012). Even though there have been cases of severe outbreaks of food infections with diarrhoea in Nigeria, there has been no outbreak attributed to a dominant strain or clone of *Listeria* which led to many people being admitted to the hospital for treatment. The organism has been reported in groups normally associated with *Listeria* e.g. neonates and pregnant women.

The first evidence of prevalence of *Listeria* in Nigeria was provided by Njoku-Obi and Njoku-Obi (1965) through serological evidence. They found it puzzling that no case of listeriosis was recorded in Nigeria as of 1965 and attributed this to non-recognition of the organism or confusion with other organisms that can decolourize the *Listeria* selective media. These authors studied the population served by Lagos University Teaching Hospital to ascertain if *Listeria* was common enough to warrant intensive efforts to isolate the organism. In their study, they determined the levels of specific agglutinating antibodies of *Listeria* in presumed healthy blood donors, staff and students. Although they did not isolate the organism in 580 Nigerians studied, the authors found that the positive results provided by levels of somatic agglutinating and complement fixing antibodies were strong enough to provide evidence that there was wide spread occurrence of the organism in Nigeria and emphasized that complete proof would be the isolation of the organism from future clinical samples.

The organism was finally isolated in Nigeria by Eyo *et al*. (1969) when they reported *Listeria leptomeningitis* in an adult female. The lady was successfully treated in the hospital with chloramphenicol and prednisone and to the best knowledge of the authors, this was the first proven case from Nigeria and the West African sub-continent as a whole (Nwaiwu, 2015). The first case of neonatal listeriosis was reported by Onyemelukwe and Lawande (1982) when they isolated *L. monocytogenes* of the same serotype from a 2-day old neonate who developed *Listeria meningitis* after contracting the organism from the mother. The mother and child were both effectively treated with ampicillin. Ako-Nai *et al*. (1999) suggested that *L. monocytogenes* was emerging as an agent in the aetiology of neonatal septicaemia in Nigeria when they isolated the organism among other dominant organisms in incidences of septicaemia in Ile-Ife. Furthermore, Adejuyigbe (2001) reported 5 positive cases of *L. monocytogenes* out of

66 septicemic neonates. However, studies carried out by Ojukwu *et al.* (2006) showed no isolation of the organism in 33 septicemic neonates out of 138 neonates screened and recently Nwadioha *et al*. (2013) found no *Listeria* in a 3 year retrospective study of 1500 paediatric patients. In another study with adults, Onyemelukwe *et al*. (1983) recorded a 27% mortality rate from 19 patients that tested positive for *L. monocytogenes* in a 1 year prospective study. The clinical conditions in which the organism was isolated included meningitis and meningoencephalitis.

Many years later, Emele (2000) screened 1097 cerebrospinal fluid samples submitted for analysis in Sokoto State and found that 0.4% of the samples were infected with *L. monocytogenes*. For other clinical samples, Esumeh and Odugbemi (1992) screened 420 faecal specimens from patients with acute gastroenteritis for *L. monocytogenes* and found no positive samples. However, they found the mannitol fermenting sub-species *L. grayii* in 4 samples. In their clinical study, Bolarinwa *et al*. (2011) carried out a random microbiological screening of 162 blood samples donated for transfusion at a teaching hospital in Ile-Ife by performing colony morphology, Gram and spore stains and standard biochemical tests and found that *Listeria* spp. was among the Grampositive organisms isolated. They pointed out that even though the organism was widely distributed in nature, it is rarely a body commensal in humans and suggested that environmental contamination, false positive laboratory results, and skin contamination could not be completely ruled out as reasons for *Listeria* detection. The disease has been noted to be largely undiagnosed and under reported in India (Barbuddhe *et al*., 2012) and this can be said to be the same situation in Nigeria (Nwaiwu, 2015).

2.10 Prevalence of *Listeria*species in NigeriaEnvironment

The diverse environment in Nigeria provides favourable conditions for *Listeria* to thrive and contaminate food sold in the open especially ready-to-eat (RTE) foods. The tropical weather is warm and humid all year round and many rural places are not very hygienic and have poor water sanitation. Environmental studies of Listeria in Nigeria have shown that the organism occurs in known *Listeria* sources such as soil, lakes and other sources that are not commonly mentioned in literature like veterinary surgical material (Tambuwal *et al*., 2009) and Naira currency notes (Kawo *et al*., 2009).*Listeria* from soil has also been identified by Ikeh *et al*. (2010) who examined soil samples from a field where cows and pigs were kept before slaughter and found *Listeria* in all samples examined. The authors attributed the presence of *Listeria* to faecal droppings from the animals while David and Odeyemi (2007) found the organism in soil used for farming and also named faecal dropping as the source.

A study of two anthropogenic lakes in Abia State in south east Nigeria by Nwachukwu *et al*. (2010) showed a prevalence rate of 91.67 and 79.17% for 24 samples analysed for each lake. The authors pointed out the ubiquity of the organism in nature and the organism's reputation as a water and foodborne bacterial pathogen as reasons for the high prevalence. Another study by Mawak *et al*. (2009) used the 2-step enrichment method to analyse natural water bodies including rivers, streams and ponds used for irrigation in Jos, Plateau State in the middle belt region of Nigeria. They found that four species of *Listeria* namely *L. monocytogenes, L.innocua, L.ivanovii* and *L. grayii* was present in 10 out of 30 samples analysed. Their report suggested that dry season farmers should be educated on measures that would reduce the hazards associated with *Listeria*.

The butcher's table from where fresh beef is sold has also been implicated as a vehicle for *Listeria*. Five surface swabs from butcher's tables taken by Ikeh *et al*. (2010) in Nsukka, south eastern Nigeria showed occurrence of *Listeria* in all the samples while Adetunji and Ishola (2011) enumerated *Listeria* on meat tables before and after sales of meat in Ibadan municipal abattoir in Nigeria. The latter found that there was an increase in *Listeria* count after meat sales. They attributed this to the wooden table used which may entrap bacteria and encourage cross contamination and highlighted that the results could reflect poor hygienic conditions of the meat tables (Nwaiwu, 2015).

2.11 Isolation and Identification of *Listeria* species

There is a variety of conventional and rapid methods currently available for the detection and identification of *L. monocytogenes* in samples from the food chain (primary production samples, feed, food samples, and environmental samples) and specimens from animal listeriosis. As low levels of *L. monocytogenes* could be difficult to detect, methods could also target *Listeria* spp. that have been used as bioindicators of the presence of *L. monocytogenes* in food and plant environmental samples. For animals and humans, conventional bacteriological methods are important for various reasons: their use results in a pure culture of the organism, which is useful for regulatory, epidemiological surveillance and outbreak management purposes (OIE, 2014). They remain the 'gold standards' against which other methods are compared and validated. These methods are usually very sensitive and they do not require sophisticated and expensive equipment, allowing widespread use (OIE, 2014). Some of the disadvantages of this group of methods include the relatively long period of time that the protocols require for completion, several 'hands-on' manipulations, the requirement for many different chemicals, reagents and media, the possibility of contaminating microorganisms masking the presence of the target ones, including overgrowth, the

potential overlook of atypical variants of the target organism and the relative subjectivity involved when interpreting bacterial growth on selective and differential agar plates (Andrews, 2002).

The isolation and identification of *L. monocytogenes* from samples from the food chain and specimens from animal listeriosis require the use of selective agents and enrichment procedures that keep the levels of contaminating microorganisms to reasonable numbers and allow multiplication of *L. monocytogenes* to levels that are enough for detection of the organism (OIE, 2014). In the early days of listerial clinical bacteriology, cold enrichment (Gray *et al*., 1948) was regularly used to this end, exploiting the ability of the organism to multiply at refrigeration temperatures (around 4°C), whereas contaminating bacteria would not multiply under these conditions. However, this procedure requires very long incubation times, often months, making it unacceptable for current investigations of food-borne outbreaks and sporadic cases, as well as for the implementation of effective hazard analysis critical control points (HACCP) programmes in food production and processing establishments. A number of selective compounds that allow growth of *L. monocytogenes* at normal incubation temperatures have been incorporated into culture media, shortening the time required for selective growth of the organism. Examples of these selective compounds include cycloheximide, colistin, cefotetan, fosfomycin, lithium chloride, nalidixic acid, acriflavine, phenylethanol, ceftazidime, polymixin B and moxalactam. Development of chromogenic media has allowed better isolation of this microorganism in samples from the food chain (OIE, 2014).

Conventional methods for the isolation of *L. monocytogenes* from samples from the food chain that have gained acceptance for international regulatory purposes include the United States Food and Drug Administration (FDA) method (Hitchins & Jinneman,

2011), the Association of Official Analytical Chemists (AOAC) official method (AOAC, 2012), the European Committee for standardization (CEN, EN) and the International Organization for Standardization (ISO) (ISO, 1996; 1998; 2005a; 2005b), the Nordic Committee on Food Analysis (NMKL) method (NMKL, 2007) and the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) method (USDA-FSIS, 2013a; 2013b). Depending on the nature of the sample, a particular method might be more suitable than others. The ISO Technical Committee ISO/TC 34, Agricultural Food Products, Subcommittee SC 9, Microbiology, in agreement with the EN Technical committee CEN/TC275, Food analyses, Working group 6, Microbiology from the food chain, claim that the EN ISO Standard 11290, parts 1 and 2 (ISO, 1996; 1998; 2005), can be used for the detection of *Listeria* species in a large variety of food and feed products but also in primary production and environmental samples. Although they recognise that this standard might not be appropriate in every detail in certain instances, they recommend that every effort should be made to apply this horizontal method as far as possible (OIE, 2014).

2.11.1 Enrichment and isolation of *Listeria* species

The principle of the EN ISO 11290 Part 1 amended method for the detection of *Listeria monocytogenes* (ISO, 2005a), covering all food chain and primary production samples, is given. Briefly, after preparation of the test portion and initial suspension, the first stage is inoculation of a selective primary enrichment medium containing one volume of lithium chloride and half a volume of both acriflavine and nalidixic acid (half-Fraser broth), which is also used as a dilution fluid for the test portion. Incubate the test portion at 30±1°C for 24±3 hours. The second stage is inoculation of the full-strength secondary liquid enrichment medium (Fraser broth) with a culture obtained in the first stage. Incubate the Fraser broth at from 35±1°C to 37±1°C for 48±3 hours. In the third stage,

samples from the cultures obtained in the first and second stages are plated out on the selective solid media.

For the detection of *L. monocytogenes* on foods, cultural methods such as those form the US food and Drug Administration (FDA) (Hitchins, 2001), the US Department of Agriculture (USDA) (McClain and Lee, 1989) and the International Standard Organization (ISO) (Anon, 1997) are commonly used. The method involve the use of Oxford (OX) or Modified Oxford (MOX) and either PALCAM or Lithium chloride phenylethanol moxalactam (LPM) selective differential agars. These plating media, but LPM, detect *Listeria* species by revealing esculinase (B-glucosidase) activity, a metabolic enzyme common to all *Listeria* species, but do not distinguish *L. monocytogenes* from other *Listeria* species (Leclercq, 2004). Therefore, typical colonies are selected from these selective differential media agars and submitted to further biochemical tests and haemolytic activity to determine the identity of the species (Jinneman *et al*., 2003).

Oxford agar contains lithium chloride, cycloheximide, colistin, acriflavine, cefotetan and fosfomycin as selective agents, and typical colonies of *Listeria* spp. are small, black and surrounded by a black halo. PALCAM agar contains Polimixin B, Acriflavin, Lithium chloride, ceftazidime, nalidixic acid,manitol and phenol red indicator.Colonies of *Listeria* species on PALCAM agar are grey–green and black–sunken centers with a black halo against a cherry red background (OIE, 2014).

As *Listeria* has specific nutritional requirements, the cultivation stage involving enrichment broths plays a key role in the recovery and multiplication of viable cells from the microorganisms present in the sample. However, in the stages following the enrichment, selective media can hamper microorganism recovery due to sensitivity to

the antibiotics in the media, resulting in their suppression by these inhibitory agents (Trabulsi and Althertum, 2008).

2.11.2 Efficacy of Oxford and PALCAM *Listeria* agar for detection of *Listeria* species

Two different selective agars, PALCAM and Oxford, recommended by the International Organization for Standardization (ISO) (1996) have been adopted in protocols for the detection/enumeration of *L. monocytogenes*. These methods incorporate added esculin which forms greyish-green to black colonies, sometimes evidenced by blackening of the media, aiding visualization and identification of colonies. However, this traditional method is unable to differentiate *L. monocytogenes* colonies form other *Listeria* species (Thalyta *et al*., 2012).

Studies have reported different efficacy of Oxford and PALCAM agar in suppressing accompanying microorganisms coupled with higher rates of isolation of *Listeria* spp. For instance, the study by Gunasinghe *et al*. (1994), comparing the performance of selective Oxford (Oxoid) and PALCAM (Merck) agars in varieties of Frankfurter sausages, salami and pate, found PALCAM agar to be superior and also noted more effective isolation of *Listeria*. Nayak *et al*. (2010), in studies of buffalo meat, noticed that PALCAM agar identified 100% of species analyzed and was in concordance with results on polymerase chain reaction (PCR). By contrast, Oxford agar showed a recovery rate of 60%. Capita (2001), examining cuts of raw poultry meat, found a significantly higher percentage of tested samples positive for *Listeria* spp. using PALCAM plating media compared with Oxford agar (95.0% versus 87.0%), respectively. However, Chukwu (2007) found no significant difference in their

efficiency, different rate in their effectiveness was observed and recorded, Oxford (100%) and PALCAM (99.5%).

Although no one medium has clearly emerged as superior, PALCAM medium appears to be preferred in Europe, whereas LPM and Oxford media are the most widely used in North America (Golden *et al*., 1988). Nevertheless, the use of more than one isolation media, employing different selective agents and systems of identifying colonies, is important to increase the chance of isolating the target organism. This procedure is recommended for detecting bacteria whose presence in foods, even at low amounts, can expose consumers to serious risk, as is the case for *L. monocytogenes* (Rodrigues *et al*., 2003).

2.11.3 Presumptive Confirmatory Test for *Listeria*

Typical *Listeria* spp. colonies, on the above selective/differential agar plates, are then selected for further identification to the species level, using a battery of tests. The tests include the Gram-staining reaction, catalase, motility (both in a wet mount observed under phase-contrast microscopy and by inoculation into semi-solid motility agar [0.2–0.4% agar] or U/Graigie's tube), haemolysis and carbohydrate use (Tables 2.1 and 2.2).

To observe tumbling motility, a hanging drop preparation is made from a young broth culture, such as tryptone soya yeast extract broth, and incubated at room temperature for 8–24 hours. When semisolid motility agar is used after stab inoculation (about 1 cm) and incubation at 20–28°C, listeriae swarm through the medium, which becomes cloudy. At about 0.5 cm below the surface of the agar, a characteristic layer of increased growth is observed, like an umbrella. This occurs because of the better development of *Listeria* under aerobic conditions as opposed to strictly anaerobic conditions (OIE, 2014).

For haemolysing activity, horse and sheep blood-containing agar plates shall be used. After incubation at 37°C for 24 hours and inoculation by piercing the medium, *L. ivanovii* exhibits a wide zone of haemolysis. The haemolysis zone of *L. monocytogenes* is narrow, frequently not extending much beyond the edge of colonies. In this case, removal of the colonies could help interpretation. Rare strains of *L. monocytogenes* are not haemolytic (OIE, 2014). The CAMP/CAMP factor test and genetic tests may be required in certain circumstances (USDA, 2013).

2.11.4Confirmation of *Listeria* using microgen kit

Microgen Listeria ID is a rapid microwell based biochemical identification system capable of identifying individual *Listeria* species. The manufacturer claims this can be achieved from single colonies on selective agar plates and results are available after 18-24 hours. Microgen Listeria ID uses classical biochemical substrates in 11 of the 12 wells and an in-well micro-haemolysis test in well 12 to differentiate *Listeria innocua* from *Listeria monocytogenes*. For such a test to gain wide acceptance in laboratories the method must be reliable, easy-to-use and yield results that are comparable to the gold standard classical biochemical test methods (CMMAS, 2006).

Prior to the inoculation of the test strip, the following tests are recommended: Gram stain, oxidase, catalase, motility or alternatively use of a latex agglutination test e.g. Microscreen Listeria Latex Test. The test strip contain the following in its well Aesculin, Mannitol, Xylose, Arabitol, Ribose, Rhamnose, Trehalose, Tagatose. Glucose-1-Phosphate, Methyl-D-Glucose,Methyl-D-Mannose and Haemolysin (CMMAS, 2006).

2.12 Pathogenicity of *Listeria* species

Out of the six species of *Listeria*, only *L. monocytogenes* causes a disease called listeriosis in both human and animal hosts (Jemmi and Stephan, 2006). Most *Listeria monocytogenes* are pathogenic to both animals and humans to some degree; however, the bacterium has been reported to be carried in the intestinal tract of a small percentage of the human population without apparent symptoms (Rouquette *et al*., 1996). As with many different pathogens, the virulence of this bacterium varies depending on the particular strain and with the susceptibility of the victim. *Listeria monocytogenes* has been associated with ingested raw and contaminated foods, such as raw and pasteurized dairy products (Fleming *et al*., 1985), raw vegetables, raw meats and smoked fish (Rouquette *et al*., 1996). Because of its ability to grow at low temperatures, *Listeria monocytogenes* can be found growing in refrigerated foods as well (Rouquette *et al*., 1996).

Listeria monocytogenes causes the disease listeriosis in humans, with common manifestations in septicemia (Gray *et al*., 1966), corneal ulcer (Holland *et al*., 1987), pneumonia (Whitelock-Jones *et al*., 1989), meningitis (Dussurget *et al*., 2004), encephalitis (Dussurget *et al*., 2004), and cervical infections in pregnant women, which may have resulted in spontaneous abortion or stillbirth (Dussurget *et al*., 2004). Patient symptoms include influenza- like symptoms, and gastrointestinal symptoms of vomiting and diarrhea (Rouquette *et al*., 1996). When *Listeria monocytogenes* invades the gastrointestinal epithelium and then enters the host's monocytes, macrophages, or polymorphonuclear leukocytes, it becomes blood-borne and multiplies, both intracellularly and extracellularly. Intracellularly, it has access to the brain and transplacental migration to the fetus in pregnant women (Kazmierczak *et al*., 2005).

In animals infected with *Listeria monocytogenes*, such as mice, the bacteria first appear in macrophages and then spread to hepatocytes in the liver (Mansfield and Freitag, 2003). The bacteria stimulate a response that includes the production of gamma interferons, macrophage activating factors and a cytotoxic T cell response (Mansfield and Freitag, 2003). The pathogenesis of *Listeria monocytogenes* thrives on its ability to survive and multiply in phagocytic host cells. Virulence is thus associated with the ability of the bacterium to move within the cytoplasm of the host cells by polymerization of host cell actin (O'Neil, 2006). Secreting the enzyme invasion allows *Listeria monocytogenes* to penetrate host cells of the epithelial lining. The immune system can usually eliminate the infection before it spreads by using T lymphocytes specifically for *Listeria* antigens (Mansfield and Freitag, 2003). However, systemic disease may develop with a compromised immune system. Another virulence factor is the bacterium's ability to bind to epithelial cells by means of adherence to D-galactose receptors on the host cell (Kazmierczak *et al*., 2005).

Upon becoming ingested by a ruminent, *L. ivanovii* develop a characteristic intracellular life cycle that includes six virulence factors. These steps include: early escape from the phagocytic vacuole, multiplication in the host cell cytoplasm, directional intracytosolic motility by induction of actin polymerization at one pole of the bacterial cell, protrusion of centrifugally moving bacteria within the cytoplasmic evaginations, and phagocytosis of the pseudopod-like structures by neighboring cells, in which the cycle reinitiates (Vazquez-Boland *et al*., 2001) Successful infection of *L. ivanovii* within a host can lead to septicemic disease with enteritis, neonatal sepsis and abortion (Engelbrecht *et al*., 1998)

Listeria innocua, as its name suggests, is harmless to other organisms. It lacks the 10-kb virulence locus that is needed for pathogenicity (Jemmi and Stephan, 2006). Recently,

researchers discovered that four isolates of *Listeria* spp. produced inhibitory activities against the pathogen *L. monocytogenes*. One of the isolates, *Listeria innocua* 743, was "selected for further study" (Banerjee *et al*., 2001). It was revealed that the plasmid of *L. innocua* produced two compounds: a bacteriosin and a protein "involved in immunity toward other bacteriosins" (Banerjee *et al*., 2001). A bacteriosin is a type of antibiotic. It is a small peptide molecule with 30-60 amino acid residues. Bacteriosins are commonly produced by lactic acid bacteria and other bacterial species, but this is the first known case of *Listeria* spp. producing bacteriosin. This discovery has beneficial implications for controlling *Listeria monocytogenes*, which presents a danger as a food-borne pathogen. Bacteriosin can be used to inhibit the growth of *L. monocytogenes* in processed foods, thereby making the ingestion of such foods much safer for humans. Furthermore, the bacteriosin produced by *Listeria innocua* 743 revealed a very broad spectrum inhibition of *L. monocytogenes*, which means that the antibiotic can inhibit a wide variety of *L. monocytogenes* strains (Banerjee *et al*., 2001).

2.12.1 Virulence factors of *Listeria* species

Adhesion to the surface of mammalian cells; Close interaction between host cells and *L. monocytogenes* makes invasion possible.

Invasion of *L. monocytogenes* also contributes to its virulence. The pathogen can enter into macrophage cells or non-phagocytes. InlA and InlB are protiens that assist *Listeria* entry into non-phagocytes. InlA is a critical player for virulence.

Vacuole escape occurs before invasion of non-phagocytic cells and involves the lysis of vacuoles. Listeriolysin O (LLO) has critical role in the escape of *L. monocytogenes* from vacuoles. This secreted toxin is a cholesterol-dependent cytolysin

Motility and spread allows *Listeria* to spread once inside the host. ActA polymerizes actin filaments that forms a comet-like tail. This tail enables the pathogen to propel and move in the cytosol. The ability to propel itself within the cytosol allows the pathogen to invade neighboring cells (cell-to-cell spreading).

2.13 Antibiotic Susceptibility of *Listeria* species

Antimicrobial susceptibility testing (AST) refers to in vitro methods used to determine the susceptibility of a bacterium to an antimicrobial agent (Humphrey and Lightbown, 1952). CLSI, originally called The National Committee for Clinical Laboratory Standards, is an organisation in the United States of America that, through a subcommittee of volunteers who are experts in the field, writes and maintains methods for AST (CLSI, 2006; CLSI, 2013).

The following three methods have been shown to consistently provide reproducible and repeatable results when followed correctly (CLSI, 2008; Walker, 2007): i) disk diffusion, ii) broth dilution, iii) agar dilution.

Disk diffusion is straightforward to perform, reproducible, and does not require expensive equipment. Its main advantages are: low cost, ease in modifying test antimicrobial disks when required, can be used as a screening test against large numbers of isolates and can identify a subset of isolates for further testing by other methods such as determination of MICs

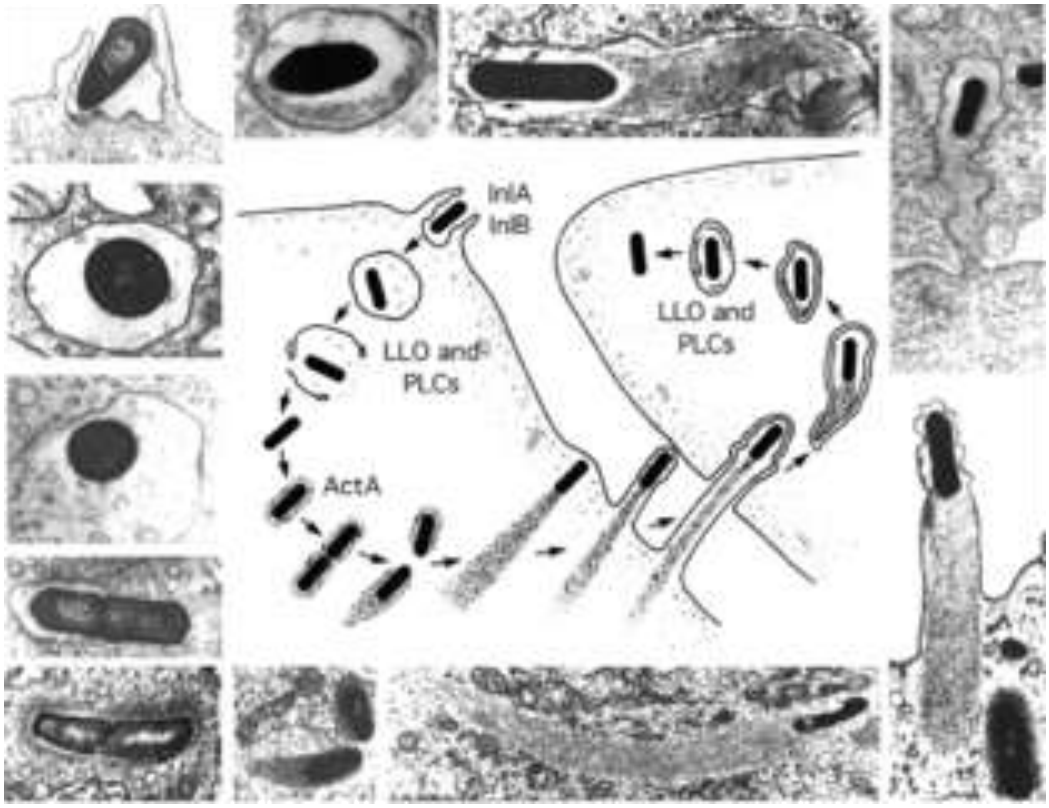

Fig 2.2: Mechanisms of pathogenicity of *Listeria* species

In the disk diffusion method (Bauer *et al*., 1966) a paper disk impregnated with a standard concentration of an antimicrobial agent is placed onto the surface of an agar medium onto which a bacterium has been lawn-inoculated at a standardized concentration of cells per mL. The antimicrobial agent diffuses through the agar resulting in a concentration gradient.

Listeria monocytogenes, as well as other *Listeria* spp., are usually susceptible to a wide range of antibiotics (Charpentier *et al*., 1995). However, evolution of bacterial resistance towards antibiotics has been accelerated considerably by the selective pressure exerted by overprescription of drugs in clinical settings and their heavy use as promoters in animals husbandry (Charpentier *et al*., 1995).Therefore, it was not unexpected when the first multiresistant strain of *L. monocytogenes* was isolated in France in 1988 (Poyart-Salmeron *et al*., 1990) and since then multi-resistant *L. monocytogenes* strains have been recovered from food, the environment and sporadic cases of human listeriosis (Charpentier *et al*., 1995). Antibiotics to which some *L. monocytogenes* strains are resistant to include tetracycline, gentamicin, penicillin, ampicillin, streptomycin, erythromycin, kanamycin, sulfonamide, trimethoprim, and rifampicin (Charpentier and Courvalin, 1999).

The first choice of treatment for listeriosis is a β-lactam antibiotic (e.g., penicillin or ampicillin), alone or in combination with an aminoglycoside (e.g., gentamicin). The association of trimethoprim with a sulfonamide, such as sulfamethoxazole in co-trimoxazole, is considered to be a second choice therapy (Bertrand *et al*., 2005; Safdar and Armstrong 2003; Conter *et al*., 2009). However, *Listeria* strains resistant to penicillin, ampicillin, erythromycin, streptomycin and tetracycline have been reported which dictates judicious use of these drugs (Bertrand *et al*., 2005; Charpentier *et al*., 1995; Srinivasan *et al*., 2005).

A current research conducted by the Department of Veterinary and Microbiological Sciences at North Dakota State University "examined the antimicrobial susceptibility of 86 *Listeria* spp. isolated from processed bison carcasses". Testing 25 antimicrobial agents on the isolates, it was ascertained that most (88-98%) exhibited resistance to bacitracin, oxacillin, cefotaxime, and fosfomycin. Tetracyline resistant was also common among the *Listeria* isolates (18.6%). Furthermore, the scientists discovered that there were "differences in resistance among *Listeria* spp.," with *L. innocua* showing the most resistance to the antibiotics and *L. monocytogenes* showing the least resistance (Li *et al*., 2007). The significance of this fact is that the high antimicrobial resistance displayed in *L. innocua* has the potential to transfer resistance to the low-resistance *L. monocytogenes*. This could be detrimental because high-resistance *L. monocytogenes* poses a more dangerous threat to public health (Li *et al*., 2007).

2.14 Listeriosis

Listeriosis is a disease caused by bacteria of the genus *Listeria*. *L. monocytogenes* is the pathogenic species in both animals and humans (McLauchlin and Jones, 1999). Few cases of human infections, however, are caused by *L. ivanovii* (Cummins *et al*., 1994; Lessing *et al*., 1994) and *L. seeligeri* (Rocourt *et al*., 1986).

Principally listeriosis causes intra-uterine infection, meningitis and septicaemia. Listeriosis during pregnancy manifests as a severe systemic infection in the unborn or newly delivered infant as well as a mild influenza-like bacteraemic illness in the pregnant woman. Pregnancy and neonatal cases comprise 10% to 20% of the listeriosis cases (McLauchlin *et al*., 2004). In adults and juveniles, the main presentations are as central nervous system infection and/or septicaemia. Most adult and juvenile cases occur amongst the immunosuppressed e.g. patients receiving steroid or cytotoxic

therapy or with malignant neoplasms. Other groups include patients with AIDS, diabetics, individuals with prosthetic heart valves or replacement joints and individuals with alcoholism or alcoholic liver disease. The incidence of infection increases with age, with the mean age of adult infection being over 55 years (McLauchlin *et al*., 2004). Mild non-invasive listeriosis, with gastroenteritis and fever, has also been reported in otherwise healthy individuals (Riedo *et al*., 1994; Miettinen *et al*., 1999; Aureli *et al*., 2000).

2.14.1 Primary routes of transmission

Foods can become contaminated with *L. monocytogenes* along the continuum from farm to fork, in the produce growing environment, during processing, or during handling and preparation in retail establishments and consumers' kitchens (ILSI, 2005).

The primary route of transmission is through the ingestion of contaminated food. The International Life Sciences Institute in 2005 described high-risk foods for causing listeriosis as those with the following properties:

1. Have the potential for contamination with *L. monocytogenes*.
2. Support the growth of *L. monocytogenes* to high numbers.
3. Are ready-to-eat.
4. Require refrigeration and
5. Are stored for an extended period of time(ILSI, 2005).

Although *L. monocytogenes* is the only member of the Listeria family that causes human illness, the presence of any member of the Listeria family in a food processing environment may indicate that conditions are favorable for *L.Monocytogenes*proliferation(ILSI,2005).

2.14.2 Symptoms and disease process

L. monocytogenes can cause mild, flu-like symptoms in healthy individuals when consumed at very high levels. A person with listeriosis has fever, muscle aches and occasional gastrointestinal symptoms such as nausea or diarrhea. If infection spreads to the nervous system, symptoms such as headache, stiff neck, confusion, loss of balance, or convulsions can occur. Infected pregnant women may experience only a mild, flu-like illness; however, infections during pregnancy can lead to miscarriage or stillbirth, premature delivery or infection of the newborn (Vishal, 2004).

2.14.3 Epidemiology and occurrence of *Listeria*

The incidence of listeriosis appears to be on the increase worldwide with a significant number of cases, especially in Europe. The annual endemic disease rate varies from 2 to 15 cases per million populations, with published rates varying from 1.6 to a high rate of 14.7 in France for 1986. *Listeria* has been isolated sporadically from wide variety of sources and listeriosis outbreaks that have occurred in the past have highlighted contaminated food as the main source of transmission. A wide range of foods such as salads, seafood's, meat, and dairy have been implicated in listeriosis (Huss *et al*., 2000). Usually the presence of *Listeria* species in food is thought to be an indicator of poor hygiene (Manani *et al*., 2006). A variety of ready- to–eat food products, such as frozen or raw vegetables, milk and milk products, meat and meat products and seafood support the growth of *Listeria monocytogenes*. These foods are considered of high risk due to the ability of *Listeria* to grow and survive in them (Kunene *et al*., 1999). However, there are other products, traditionally considered of low risk, which have recently been linked to listeriosis transmission, such as the large listeriosis outbreak reported in Italy due to the consumption of corn. Though no fatalities occurred, more than 1500 people were

affected (Aguado *et al*., 2004). There is no doubt that the susceptible population is increasing, as there is a steady increase in numbers and types of foods in which *L. monocytogenes* is isolated.

2.14.4 Prevention and control of listeriosis

In most cases, prevention relies on food safety. People at a high risk for listeriosis should thoroughly cook all food from animal sources, wash raw vegetables very well, and avoid eating or drinking unpasteurized milk products. All cooking tools, as well as the hands, should be washed after they have been in contact with raw food. Uncooked meats should be kept separate from vegetables, cooked foods and ready-to-eat foods (CFSPH, 2005).

High risk foods include soft cheeses such as feta, Brie, bleu cheese, Camembert and certain Mexican style cheeses (queso blanco, queso fresco, Panela), and deli meats. Susceptible people should avoid soft cheeses unless the label clearly states they are made from pasteurized milk. Delicatessen foods, as well as any leftover or ready-to-eat foods such as hot dogs, should be eaten only after they have been reheated to a high temperature. Other high-risk foods include refrigerated pâtés and meat spreads, and refrigerated smoked seafood, unless it has been cooked. Perishable and ready-to-eat foods should not be kept for long periods of time (CFSPH, 2005).

Guidelines have been published to prevent *Listeria* contamination in milk processing plants and other establishments. They are based on pasteurization and the prevention of cross-contamination between processed products leaving the plant and raw materials entering it Sanitation and hygiene during deliveries in ruminants and necropsies can decrease the risk of cutaneous disease in veterinarians and others who are occupationally exposed (CFSPH, 2005).

2.14.5 Treatment

When infection occurs during pregnancy, antibiotics given promptly to the pregnant women can often prevent infection of the fetus or new born. In general, isolates of *L. monocytogenes*, as well as strains of other species, are susceptible to a wide range of antibiotics except tetracycline, erythromycin, streptomycin, cephalosporins, and fosfomycin (Charpentier *et al*., 1995). The treatment of choice for listeriosis remains the administration of ampicillin, penicillin G combined with an aminoglycoside and gentamycin. The association of trimethoprim with sulphonamide, such as sulfamethaxazole in co–trimoxazole, is a second choice therapy (Charpentier *et al*., 1995). The most active agent in the combination is trimethoprim, which is synergized by sulfamethaxazole. Most isolates from clinical as well foodborne and environmental sources are susceptible to the antibiotics active against gram positive bacteria (Yucel *et al*., 2005).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study area was Zaria, one of the major cities in Kaduna State in Northern Nigeria, covering a total land area of 300 Km^2. It is made up of two local governments (Zaria and Sabon Gari LGAs) with 11 districts and a population of 408,198 people with annual growth rate of 3.3% (NPC, 2006).

The following are the recognised 11 districts in Zaria metropolis: Zaria City, Tudunwada, Samaru, Sabon Gari, Wusasa, PZ, Kongo, GRA-Zaria, Hanwa, Bussawa and Shika.

3.2 Study Design

The study was a cross-sectional study which was carried out on raw chicken meat and processed chicken meat (roasted) sold by various retail outlets and market places within the Zaria metropolis.

3.3 Sample Size

The sample size was calculated using the formula $N = Z^2 \times p \times q / d^2$

Where:

N = number of samples

Z = 1.96 at 95% confidence interval

p = recorded prevalence from literature

q = 1-p

d = allowable error

Using 21% prevalence recorded in Plateau State of Nigeria (Chukwu,2007) and 5% allowable error at 95% confidence level, the sample size was calculated as:

$N = (1.96)^2 \times 0.21 \times 0.79 / (0.05)^2$

=254

The sample sizewas calculated to be 254. This therefore, represents the sample size for the research with 154 collected as raw chicken meat and 100 as processedchicken meat.

3.4 Collection of Samples

Samples were randomly purchased from the various retail outlets and market places within the metropolis. Preference was given to Zaria, Sabon gari and Samaru markets due to their large sizes. The samples were collected into separate clean polythene bags and transferred to Microbiology Laboratory of the Ahmadu Bello University, Zaria for processing. All samples collected were processed immediately.A total of 154 raw chicken meat samples were collected with 70 from Sabon Gari, 54 from Zaria City and 30 from Samaru, while 30 roasted chicken meat samples were collected from Sabon Gari, 30 from Zaria City and 40 from Samaru to give a total of 100 roasted chicken meat Samples. This gave overall total of 254 chicken meat samples collected.

3.5 Sample Analysis

3.5.1 Isolation of *Listeria species* from chicken meat samples

Culture media preparation

Listeria Enrichment Broth (Fraser Iso 11290-1)

Listeria Enrichment Broth (LEB) base was suspended in a proportion of 28.7g/500ml of distilled water, mixed well and dissolved by heating with frequent agitation. The boiled medium was sterilized in autoclave at 121°Cfor 15min., cooled to 45-50°Cand one vial of Fraser *Listeria* selective supplement (Cat. 6001) for preparing full Fraser or half Fraser *Listeria* selective supplement (Cat.6002) reconstituted in 5ml of sterile distilled water was added. The medium was homogenized gently and dispensed into sterile containers.

Listeria Agar Base Oxford (Iso 11290-1)

Listeria agar base Oxford was suspended in a proportion of 27.8/500ml of distilled water, mixed well and dissolved by heating with frequent agitation. The medium was boiled for 1min until complete dissolved, sterilized in autoclave at 121°C for 15min., cooled to 45-50°Cand one vial of Oxford *Listeria* selective supplement (Cat. 6003) reconstituted in 5ml of sterile distilled water was added. The medium was homogenized gently and dispensed into sterile Petri dishes.

PALCAM Listeria Agar Base (Iso 11290-2)

PALCAM *Listeria* agar base was suspended in a proportion of 34.4/500ml of distilled water, mixed well and dissolved by heating with with frequent agitation. The medium was boiled for 1min until complete dissolution, sterilized in autoclave at 121°C for

15min., cooled to 45-50°C and one vial of PALCAM *Listeria* selective supplement (Cat. 6004) reconstituted in 5ml of sterile distilled water was added. The medium was homogenized gently and dispensed into sterile Petri dishes.

Trypton Soya Agar

Trypton Soya agar was suspended in a proportion of40g/litre of distilled water, boiled to dissolve, dispensed into sterile containers and sterilized by autoclaving at 121°C for 15min. The prepared medium was allowed to cool for use.

Trypton Soya Broth

Trypton Soya broth was suspended in a proportion of30g/litre of distilled water, boiled to dissolve, dispensed into sterile containers and sterilized by autoclaving at 121°Cfor 15min. The prepared medium was allowed to cool for use.

Isolation of *Listeria* Species

Isolation of *Listeria spp*. was carried out according to EN ISO Standard (ISO, 2005a). This involved the use of two steps (primary and secondary) enrichment, using half Fraser broth (UVM *Listeria* pre-enrichment broth) and Fraser broth (UVM *Listeria* full strength enrichment broth). The primary enrichment involved the addition of 25g chickenpart meat into 225ml of the prepared half Fraser broth. The test portion was pounded into pieces, measured and added to the measured prepared half Fraser broth and the mixture was homogenized by incubating using a shaker at 30°C for 24 h. After 24 h of the primary enrichment step, 0.1ml of the half Fraser broth culture was then inoculated in 10ml of the Fraser broth and incubated at 35°C for 48 h.

A positive Fraser broth culture (dark appearance of the culture) was streaked onto PALCAM and Oxford agar plates as selective culture media for *Listeria* isolates, while

a negative Fraser broth culture (showing straw colour of newly made broth) was re-incubated for another 24 h afterwhich no change was considered negative for *Listeria* species. Inoculated PALCAM and Oxford agar was incubated at 35°C for 48 hto allow full growth of weaker *Listeria*. Identification of the *Listeria* species was based on colour, size and colonial morphology. The colonies morphologically resembling *Listeria*were further subjected to confirmatory examination and biochemical tests using Gram staining, Catalase test, evaluation of Haemolysis type, motility test, indole test, Urease test, MR/VP and carbohydrates fermentation test. Presumptively characterized*Listeria* speciesstrains were sub-cultured onto Trypton Soya Agar (Merck, Darmstadt, Germany) slants and after 24 hrs of incubation, the slants were kept at 4°C for further studies.

Macroscopic examination of the culture

In Listeria Enrichment Broth (LEB):

Both the primary and secondary enrichment turns black coloration for positive outcome of *Listeria* species presence. If negative they remained straw color as they were prepared. Negative result for the secondary enrichment (full Fraser) was re-incubated for another 24 h,no change in appearance was finally considered negative for *Listeria*. The positive result for the secondary enrichment (full Fraser) was further preceded with culturing on Oxford agar and PALCAM agar.

On Oxford agar: Colonies of *Listeria* species were identified by their black or dark brownish sunken center appearance surrounded by a black-halo formation as a result of aesculin hydrolysis. This was compared with reference existing stock cultures of *Listeria* species.

On PALCAM agar: Colonies of *Listeria* species were identified by their grey–green and black–sunken centers with a black halo against a cherry red background. This was also compared with reference existing stock cultures of *Listeria* species.

3.5.2 Morphological and biochemical characterization of *Listeria*isolates

A single colony resembling *Listeria* species was sub-cultured onto a slant containing Trypton soy agar for further confirmatory tests as described bellow.

Gram Staining:

A colony from the cultured agar was put on a signed slide, emulsified with normal saline to make a smear, allowed to dry and heat fixed. Then Gram stainedto confirm the Gram reaction of the isolate.

Catalase Test:

A drop of 3% hydrogen peroxide (H_2O_2) was placed on a clean dry slide. Then a fresh colony of the growth *Listeria* from the agar medium was placed on the drop of hydrogen peroxide. Positive result was indicated by presence of air bubbles as a result of O_2 production.

Oxidase Test:

A fresh purified colony was streak on a piece of filter paper, and a drop of 1% colorless tetramethyl-p-phenylelediamine dihydrochrolide (TPD) reagent was added. A positive result was indicated by appearance of purple colour within 10-30 seconds.

Motility Test

After Gram's stain and catalase reactions, cells revealing characteristics of *Listeria* were further subjected to motilitytest. This was done using two methods,Motility under microscopy and motility in a semi-solid motility medium.

Motility under microscopy

The prepared Trypton Soya broth was divided in two parts for each isolate. The suspected colonies of the isolate were sub-cultured in both media. One was incubated at room temperature of 20-25°Cand the other at 37°Cfor 24 h. A drop of both cultured medium was placed on a clean glass slide and covered with a cover slip and a drop of oil immersion placed on top of the cover slip to be viewed under a light microscope with ×100 objective lens. A positive motility was observed by rapid movement and tumbling of the shot rod shaped bacteria due to the presence of peritrichous flagella. This motility only applied to the culture incubated at 20-25°Cand motility was not observed at 37°C.

Motility in a semi-solid motility medium

Using straight wire loop, suspected colonies were stabbed into the centre of the tube containing motility test medium to a depth of 5mm and incubated at room temperature for 24 h for one week. The organisms in the tube that showed umbrella shaped growth in the motility test medium near the microaerophllic subsurface of the medium were recorded as *Listeria*.

Urease test

Suspected isolates were inoculated heavily over the entire surface of the Urea agar slant. Caps were loosened before incubating overnight at 35–37°C. Urease-positive cultures

produced an alkaline reaction in the medium, evidenced by a pinkish-red color. Urease-negative organisms did not change the color of the medium, which was pale yellowish-pink. *Listeria* species are urease negative.

Indole test:

The test organisms were cultured in 3ml of peptone water containing tryptophan at 37ºC for 48h. One ml of diethyl ether was added, shaked well and allowed to stand until the ether rises to the top. Then 0.5 ml Kovac's reagent was gently run down the side of the test tube to form a ring in between the medium and the ether. Development of brilliant red colored ring indicated positive test (Cheesbrough, 2006). *Listeria* species are indole negative.

Methyl red/Voges –Proskauer(MR/VP)

Single colony from the pure culture of the test organism was inoculated into 8 ml of sterile MR-VP broth. After 48 h incubation at 37ºC, the cultured broth was devided into two portions. To one of the portion with 5 ml, 3 – 5 drops of methyl red solution was added and observed for color formation. Development of red or yellow color indicated methyl red positive or negative result respectively. To the other portion of 3 ml, 0.6 ml of 5% alphanapthol and 0.2 ml of 40% potassium hydroxide containing 0.3% creatine was added per ml of broth culture. Following well shaking, the broth was allowed to stand for 5-10 minutes to observe the color formation. Development of pink-red color indicated positive result for VP (Cheesbrough, 2006).*Listeria* species are MR-VP positive.

Haemolysis test

Suspected colonies were streaked on ordinary sheep blood agar plates for haemolytic reactions peculiar to *L. monocytogenes* and other *Listeria* species. The resultant haemolytic reactions were recorded.

Carbohydrates fermentation test

All suspected *Listeria*- like organisms were tested for their ability to ferment Mannitol, Xylose, and Rhamnose. Fifteen (15g) of Peptone water was weighed and dissolved in 1000ml of distilled water. 10% (100ml) of phenol red was added to the prepared peptone water as the indicator and divided into three separate conical flasks already containing one percent (1%) of each of the (Rhamnose, Xylose, and Mannitol) sugar added to it. The prepared suger solutions were then dispensed in Bijou bottles in 5ml each as labelled. The various sugar solutions were sterilized by free-steaming at 121°Cfor 5-10minutes to avoid complete decomposition. The sugars were then incubated at 37°C for 24 h for purity and sterility check after which they were ready for use.

The various sugar solutions were then inoculated with the suspected *Listeria* spp. isolates and incubated at 37°Cfor 24 h. When there was confusion in the change of colour as a result of the fermentation, the test portion was further incubated for 24 h for a clearer observation. The results were read and recorded.

3.5.3Confirmation of *Listeria* species using Microgen Kit

Microgen Listeria ID (MID 67) was used for the confirmation of the *Listeria*species isolated. Each Microgen Listeria-ID microwell test strip contains 11 dehydrated substrates for the performance of carbohydrate utilisation tests and one empty well for

the performance of a haemolysin reaction. The selection of the substrates included in the test panel is based on a combination of those substrates recommended in international standard methods plus additional tests which either confirm the isolate being tested as belonging to the genus *Listeria* (Aesculin Hydrolysis, Trehalose and Arabitol Fermentation and/or further enhance the differentiation of the various species comprising the genus.

Identification of isolates was achieved by recording the results visualised by a colour change after 18-24 h incubation (there were no reagents to be added on Day 2). These results were then analysed using the Microgen Identification System Software (MID-60).

A single well-isolated colony was selected from an 18-24 hour culture and emulsified in a vial of *Listeria* suspending medium (2.5ml) and mixed thoroughly to produce a homogenous suspension. The microwell test strip was removed from the foil pouch and placed in the holding frame then the lid was removed. Using a sterile Pasteur pipette 4 drops (approximately 100µl) of the bacterial suspension were transfered to each well of the microwell test strip. As a purity check, 1 drop of the organism suspension was transfered onto an appropriate non selective agar plate and the plate incubated aerobically at 35 - 37°C for 18 - 24 h. One drop of the haemolysin reagent was then added to well 12.

The lid was then replaced onto the microwell test strip and incubated at 35 - 37°C for 18- 24 h. After incubation the lid was removed from the microwell test strip, the change in colour of the well contents were observed, results interpreted using the table of test on the provided manual and recorded on the report forms provided. The use of the Microgen Standard Test kit for *Listeria* was as recommended by CMMAS (2006).

3.5.4 Antibiotic susceptibility of *Listeria* species

Antibiotic susceptibility was determined by the disk diffusion method. Standard discs were applied using a disc dispenser following the procedures recommended by the Clinical and Laboratory Standards Institute (CLSI, 2014). Fresh bacterial colonies of the confirmed*Listeria*isolates were separately grown at 37°Cin brain heart infusion broth (BHI) (Merk V4324393-947) for 24 h. The inoculum was standardized by adjusting to 0.5 McFarland turbidity standard($1.5x10^8$cfu/ml). One milliliter of the cell suspension prepared was applied onto the surface of Muller Hinton (MH) agar (BIO-RAD 2M2121) and then spread evenly using sterile cotton swab. Sterile cotton-tipped swab was used to inoculate the plate using the lawn inoculation technique by streaking back and forth from top of the plate to the bottom of the plate, turning the plate 60 degrees and repeated, then and turn another 60 degrees and repeated.

After spreading the inoculums evenly, paper discs impregnated with Ciprofloxacin (5µg), Vancomycin (30µg), Chloramphenicol (30µg), Erythromycin (15µg), Clindamycin (2µg), Ampicillin (10µg), Tetracycline (30µg), Cefoxitin (30µg), Gentamicin (10µg)were placed at an interval of 3 cm spacing from each other on the medium using sterile forceps and incubated for 18-24 hours at 37°C (Morobe *et al*., 2009). The antibiotics used were selected based on those use to prevent and control infectious diseases in poultry production (Abiola *et al*., 2005) and the common drugs used for the treatment of human infections (Wiggins *et al*., 1978; Winslow and Pankey, 1982). After incubation, the diameters (mm) of clear zones of inhibition around disks were measured using a ruler and compared with standards given by CLSI (2014)with*Staphylococcus aureus* ATCC 29213 used as control.

Determination of multiple antibiotics resistance index (MARI) of Listeria species

Multiple resistance and resistance pattern for the *Listeria* species isolates were also determined and the Multi antibiotic resistance calculated.

The Multi antibiotic resistance index of the *Listeria* species was determined using the formular described by Krumperman (1983):

$$\text{MARI} = \frac{\text{No. of antibiotics an isolate is resistant to}}{\text{Total No. of antibiotics used}}$$

3.6 Data Analysis

Data obtained from the study were subjected to descriptive statistical analysis through the use of statistical software package SPSS version 20.0. The descriptive data were reduced to percentages and means±SD. Calculated Chi-Square was used for analytical assessments at 95% confidence limit and 0.05 level of significance. Significant differences were considered when p-value obtained is ≤ 0.05.

CHAPTER FOUR

4.0 RESULTS

4.1Occurance of *Listeria* species in Chicken Meat Samples from Zaria Metropolis

The number of chicken meat samples positive for *Listeria* species based on the sampling locations is shown in Table 4.1. For raw chicken meat, Sabon Gari had the highest prevalence of 22.9% (16/70) positive for *Listeria* species, while 16.7% (9/54) of raw chicken meat collected from Zaria city had detectable *Listeria* species and 13.3% (4/30) analysed for Samaru indicated positivity. The total prevalence of *Listeria* species in raw chicken meat samples from all sampling locations was 18.8% (29/154). The difference in the distribution of detectable *Listeria* species in raw chicken meat samples across the sampling locations was not statistically significant at $p<0.05$ ($\chi^2= 1.501$, df= 2, p value= 0.472). No *Listeria* species was found in any of the 100 roasted chicken meat samples collected across the metropolis.

4.2 Isolation and Identification of *Listeria* species

The morphology (Gram reaction), biochemical characterization and suger fermentaion tests for presumptive identification of *Listeria* isolates were as shown in table 4.2. Other bacteria were invariably found in some of the experimental samples analysed. Gram negative shot rods arraged in single and clusters resembling *Enterobacteriacae* and Gram positive cocci resembling *Staphylococcus* spp were observed.

The result for the Microgen test kit of *Listeria* spp. is as shown in table 4.3. The presumptive identified *Listeria* isolates were confirmed based on Octal code and Percentage probability of every *Listeria* species.

Table4.1: Distribution of *Listeria* speciesin chicken meatacross the three locations in Zaria metropolis

Location	Raw chicken meat		Roasted chicken meat	
	No. of Samples Examined	**No. Positive (%)**	**No. of Samples examined**	**No. Positive (%)**
Sabon Gari	70	16 (22.9)	30	0 (0)
Zaria City	54	9 (16.7)	30	0 (0)
Samaru	30	4 (13.3)	40	0 (0)
Total	**154**	**29 (18.8)**	**100**	**0 (0)**

Raw chicken meat: $\chi^2 = 1.501$ df= 2 P= 0.472

Table 4.2: Morphological and biochemical characterization of *Listeria* spp

Morphology	Biochemical Characteristics						Sugar Ferm				
GR	**Cat**	**Oxd**	**Mot(25℃)**	**Ure**	**Ind**	**MR/VP**	**β Haem**	**Man**	**Xyl**	**Rha**	**Presumptive organism**
+SR/CB	+	-	+	-	-	+	+	-	+	-	*L. ivanovii*
+SR/CB	+	-	+	-	-	+	V	-	+	-	*L. seeligeri*
+SR/CB	+	-	+	-	-	+	-	-	-	V	*L. innocua*
+SR/CB	+	-	+	-	-	+	-	+	-	-	*L. grayi*
+SR/CB	+	-	+	-	-	+	-	+	-	V	*L. murrayi*
+Cocci	+	-	-	*	*	*	*	*	*	*	*Staphylococcus spp.*
-SR	*	*	*	*	*	*	*	*	*	*	*Enterobacteriacea*

KEY: GR= Gram reaction, Cat= Catalase test, Oxd=Oxidase test, Ure=Urease tset,
Ind= Indole test, MR/VP= Methyl red and Voges Poskure test, Mot=Motility test,
β Haem= Beta Haemolysis test, Man= Manitol test, Xyl= Xylose test,
Rha= Rhamnose test, Sugar Ferm= Sugar Fermentation test,
SR/CB= Shot rods or cocco bacilli, + = Positive, - = Negative, V= Variable
*= Not applicable

Table 4.3: Microgen Kit confirmation of *Listeria* spp.

Reactions														
Esc	**Man**	**Xyl**	**Ara**	**Rib**	**Rha**	**Tre**	**Tag**	**G-1-P**	**M-D-M**	**M-D-G**	**Haem**	**Octal code**	**% Probabilty**	**Inference Organism (No.)**
+	-	+	+	-	-	+	-	+	+	-	+	5455	99.77	*Listeria ivanovii* (8)
+	-	+	+	+	-	+	-	-	+	-	+	5645	96.69	*Listeria ivanovii* (3)
+	-	+	+	+	-	+	-	-	-	-	+	5641	99.93	*Listeria ivanovii* (2)
+	-	+	+	-	-	+	-	-	+	-	-	5444	93.46	*Listeria seeligeri* (1)
+	-	-	+	-	+	+	-	-	+	+	-	4546	93.95	*Listeria innocua* (1)
+	-	-	+	-	-	+	-	-	+	+	-	4446	97.86	*Listeria innocua* (3)
+	+	-	+	+	-	+	-	-	-	+	-	6642	100	*Listeria grayi* (6)
+	+	-	+	+	-	+	-	-	-	-	-	6640	100	*Listeria grayi* (3)
+	+	-	+	-	-	+	-	-	-	-	-	6440	99.95	*Listeria grayi* (1)

Esc= Esculin, Man= Manitol, Xyl= Xylose, Ara= Arabitol, Rib= Ribose, Rha= Rhamnose, Tre= Trehalose, Tag= Tagatose, G-1-P= Glucose-1- Phosphate, M-D-G= Methyl-D-Glucoside, M-D-M= Methyl-D-Manoside, Haem= Haemolysis

Table 4.4shows the occurrence of each *Listeria* spp. from the analysed samples based on the sampling locations. *Listeria monocytogenes* was not detected in any of the samples analysed from all the sampling locations. However,*L. ivanovii* was detected highest among positive samples from Zaria city with a prevalence of 55.6% (5/9)while *L. innocua* was the least detected in 11.1% (1/9) of samples analysed for Zaria city. Samples analysed for Sabon Gari had a prevalence of 43.8% (7/16) for *L. Ivanovii,* which was the highest while only 1 out of 16 samples (6.3%) was detected for both *L. Seeligeri* and *L. murrayi*. Futhermore,out of 4 samples analysed for *Listeria* in Samaru, 3 (75%) had detectable *L.innocua* with 1 (25%) detected as *L. ivanovii*.

The percentage occurance of each *Listeria* spp. amongst the positive samples was as shown in Fig. 4.1. Out of the 29 positive samples for *Listeria* spp., *Listeria ivanovii* has the highest frequency of occurrence having 13(44.8%). It was followed by *Listeria grayi* with the rate of 34.5%(10). *Listeria innocua* was found to be 4(13%). Only 1 (3.4%) of both *Listeria seeligeri* and *Listeria murayi* was detected.

Table 4.4: Occurance of various *Listeria* species in raw chicken meat sold in Zaria metropolis.

N= 154

	Sampling locations/ Occurance[%]			
***Listeria* spp.**	**Sabon Gari**	**Zaria city**	**Samaru**	**Total**
L.monocytogenes	0 (0.0) [0.0]	0 (0.0) [0.0]	0 (0.0) [0.0]	0
L. ivanovii	7 (53.8) [43.7]	5 (38.5)[55.6]	1 (7.7) [25]	13
L. seeligeri	1 (100) [6.3]	0 (0.0) [0.0]	0 (0.0) [0.0]	1
L. welshimeri	0 (0.0) [0.0]	0 (0.0) [0.0]	0 (0.0) [0.0]	0
L. grayi	4 (40) [25.0]	3 (30) [33.3]	3 (30) [75]	10
L. innocua	3 (75) [18.7]	1 (25) [11.1]	0 (0.0) [0.0]	4
L. murayi	1 (100) (6.3)	0 [0.0] (0.0)	0 [0.0] (0.0)	1
Total	16	9	4	29

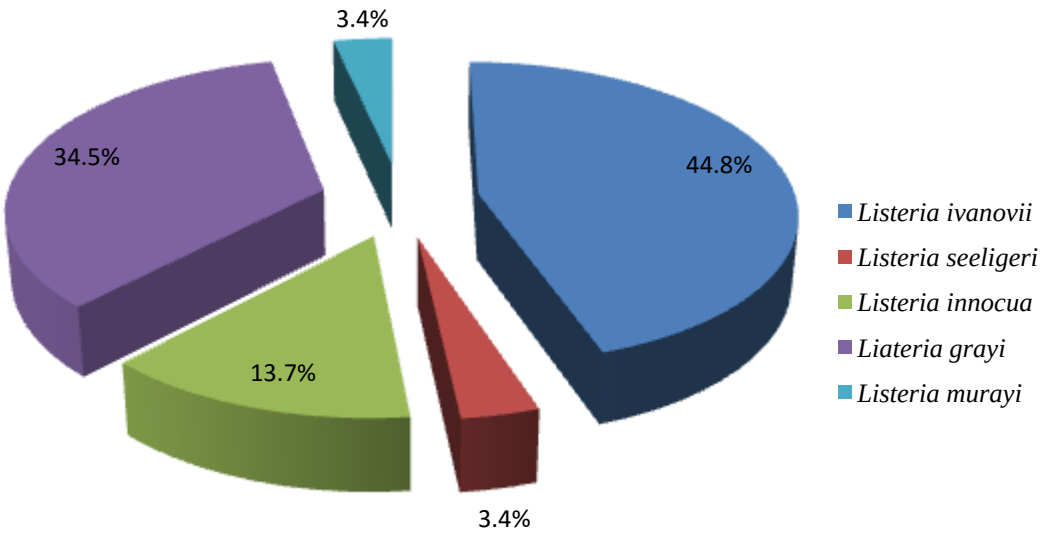

Fig 4.1: Percentage occurrence of each *Listeria* spp. amongst the positive samples.

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The distribution of *Listeria ivanovii, Listeria grayi* and *Listeria inoccua* in raw chicken meatacross the three locations in Zaria metropolis is as presented in Table 4.5. Seven of the raw chicken meat samples were positive for *Listeria ivanovii* out of the 70 samples analysed in Sabon Gari representing 10%, while 5 of the 54 raw chicken meat samples analysed in Zaria City was detected positive for *Listeria ivanovii* (9.3%) and 1/30 raw chicken meat samples showed positivity for *Listeria ivanovii* (3.3%). In total, *Listeria ivanovii* had a prevalence of 8.4% (13/154) in raw chicken meat analysed across the three locations.

*Listeria grayi*was found in 4 of the 70 raw chicken meat samples analysed in Sabon Gari representing 5.7%, while 3 of the 54 raw chicken meat samples showed positivity for *Listeria grayi* (5.6%) and 3 of the 30 raw chicken meat samples were detected positive for *Listeria grayi*. This gives a total of 10 raw chicken meat samples positive for *Listeria grayi* out of the 154 analysed representing 6.5%.

None of the raw chicken meat samples analysed for Samaru was positive for *Listeria innocua,* but 3 of the 70 raw chicken meat samples analysed for Sabon Gari indicated positive for *Listeria innocua*representing 4.3% and only 1 of the 54 samples analysed for Samaru shows positivity for *Listeria innocua* (1.9%). This gives a total of 2.6% prevalence (4/154) for*Listeria innocua* in raw chicken meat samples analysed across the three locations

Table 4.5: Distribution of *Listeria ivanovii, Listeria grayi* and *Listeria inoccua* in rawchicken meatacross the three locations in Zaria metropolis

Location	No. Samples Examined	No. Positivefor *L. ivanovii*(%)	No. Positive for *L. grayi* (%)	No. Positive for *L. inoccua* (%)
Sabon Gari	70	7 (10)	4 (5.7)	3 (4.3)
Zaria City	54	5 (9.3)	3 (5.6)	1 (1.9)
Samaru	30	1 (3.3)	3 (10)	0 (0.0)
Total	**154**	**13 (8.4)**	**10 (6.5)**	**4 (2.6)**

***L. ivanovii*:**	χ^2= 1.279	df= 2	P= 0.527
***L. grayi*:**	χ^2= 0.756	df= 2	P= 0.685
***L. inoccua*:**	χ^2= 1.707	df= 2	P= 0.425

Table 4.6 shows the distribution of *Listeria seeligeri and Listeria murrayi* in raw chicken meatacross the three locations in Zaria metropolis respectively. Only 1 of the 70 raw chicken meat samples analysed for Sabon Gari were positive for both *Liseria seeligeri* and *Listeria murrayi* representing 1.4%, while none of the 54 and 30 raw chicken meat samples analysed for Zaria City and Samaru respectivelywere found positive for *Listeria seeligeri* and *Listeria murrayi*. This gives a total of 0.6% (1/154) raw chicken meat samples analysed for both *Listeria seeligeri* and *Listeria murrayi*.

4.3 Efficiency of the Selective Agars Used

Of the two solid media used for the isolation of*Listeria* species, all of the 13 (100%)*Listeriaivanovii* grew on Oxford *Listeria* agar while 8 (61.5%) of them grew on PALCAM *Listeria* agar. *Listeriaseeligeri* showed no growth on PALCAM agar except on Oxgford *Listeria* agar. This was represented in Fig 4.2. Thisresults in a 100% growth of the *Listeria* species on Oxford agar and 79.3% on PALCAM agar.

4.4Antibiotic Susceptibility Pattern of the *Listeria* species

Antibiotic susceptibility test of *Listeria* species isolated from chicken meats sold in Zaria metropolis are as shown in Table 4.7. All *Listeria ivanovii* isolated were found to be resistant to ampicillin (100%), Cefoxitin (100%) while 61.5% showed resistance to tetracycline, but all were sensitive to ciprofloxacin. All of the 4 *Listeria innocua* isolated were resistant to ampicillin (100%), Cefoxitin (100%), tetracycline (100) and Vancomycin (100%) but were all susceptible to chloramphenicol (100%).

Table 4.6: Distribution of *Listeria seeligeri*and *Listeria murrayi*in raw chicken meat across the three locations in Zaria metropolis

Locations	No. of Samples Examined	No. Positivefor *L. seeligeri*(%)	No. Positive for *L. murrayi* (%)
Sabon Gari	70	1 (1.4)	1 (1.4)
Zaria City	54	0 (0.0)	0 (0.0)
Samaru	30	0 (0.0)	0 (0.0)
Total	**154**	**1 (0.6)**	**1 (0.6)**

***L. seeligeri*:**	χ^2= 1.208	df= 2	P= 0.547
***L. murrayi*:**	χ^2= 1.208	df= 2	P= 0.547

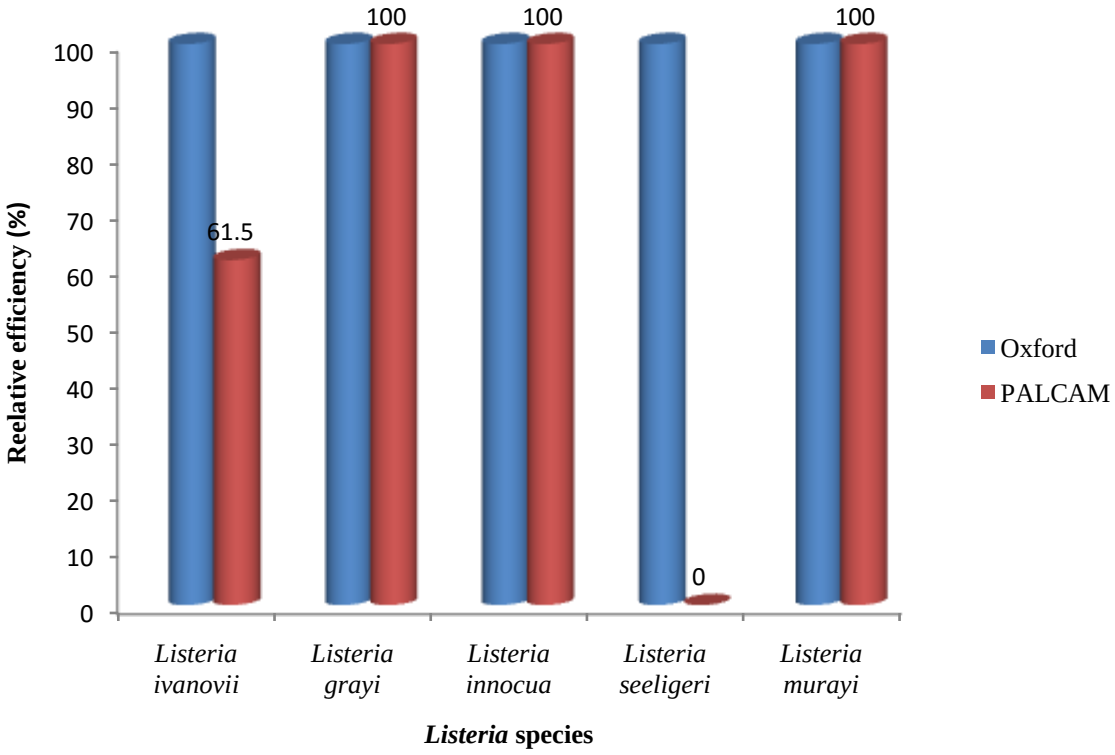

Fig 4.2: Relative efficiency of the two selective solid media used for isolation of*Listeria* species

Table 4.7: Antibiotic susceptibility of the various *Listeria* species isolated from chicken meat sold in Zaria metropolis

Antibiotics	L. ivanovii (n=13)			L. grayi n=10			L. innocua (n=4)			L. seeligeri and L. murrayi (n=2)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Ciprofloxacin 5µg	11(84.6)	2(15.4)	0 (0)	10(100)	0(0)	0(0)	2(50)	2(50)	0(0)	2(100)	0(0)	0(0)
Vancomycin 30µg	2(15.4)	9(69.2)	2(15.4)	1(10)	8(80)	1(10)	0(0)	0(0)	4(100)	0(0)	0(0)	2(100)
Chloramphenicol 30µg	10(76.9)	0(0)	3(23.1)	7(70)	0(0)	3(30)	4(100)	0(0)	0(0)	1(50)	0(0)	1(50)
Erythromycin 15µg	10(76.9)	0(0)	3(23.1)	7(70)	0(0)	3(30)	3(75)	0(0)	1(25)	2(100)	0(0)	0(0)
Clindamycin 2µg	7(53.8)	3(23.1)	3(23.1)	7(70)	0(0)	3(30)	1(25)	0(0)	3(75)	1(50)	0(0)	1(50)
Ampicillin 10µg	0(0)	0(0)	13(100)	0(0)	0(0)	10(100)	0(0)	0(0)	4(100)	0(0)	0(0)	2(100)
Tetracycline 30µg	0(0)	5(38.5)	8(61.5)	0(0)	4(40)	6(60)	0(0)	0(0)4(100)		0(0)	2(100)	0(0)
Cefoxitin 30µg	0(0)	0(0)	13(100)	0(0)	0(0)	10(100)	0(0)	0(0)4(100)		0(0)	0(0)	2(100)
Gentamicin 10µg	10(76.9)	0(0)	3(23.1)	4(40)	0(0)	6(60)	4(100)	0(0)	0(0)	1(50)	0(0)	1(50)

KEYS: S= Susceptible, I= Intermediate, R= Resistance

All of the 29 *Listeria* spp. isolated from the chicken meat samples were resistant to ampicilin (100%) and cefoxitin (100%). Also, eighteen (62%) of the isolates were found resistant to tetracycline. Some*Listeria* spp. isolateswere sensitive to Vancomycin (75.3%), chloramphenicol (75.9%), erythromycin (75.9%), gentamicin (65.6%) and clindamycin (65.5%) whileall the isolates showed sensitivity to ciprofloxacin. This is as shown in Fig. 4.3.

All the isolates showed resistance to at least one antibiotic (table 4.8). Four of the isolates were resistant to 2 antibiotics, 4, 10, 6, 4, and 1 showed resistance to 3, 4, 5, 6, and 7 antibiotics respectively. Eighteen resistant phenotypes were obtained with varying combinations of 2, 3, 4, 5, 6 and 7 antibiotics. The predominant MAR phenotypes were Amp,Fox and Va,Amp,Fox having 13.8% and 10.3% respectively. The multiple antibiotic resistance indices of the isolates were also determined as shown in Fig 4.4 and all the isolates showed MARI $\geq$ 0.2.

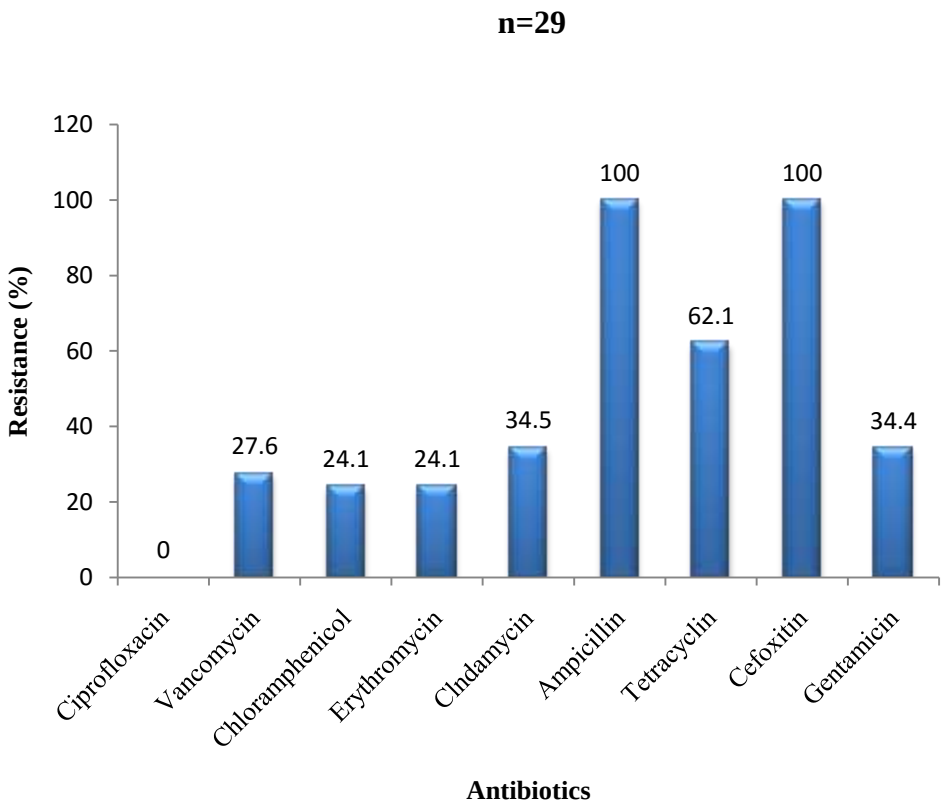

Fig 4.3: Distribution of the antibiotic resistance of the*Listeria* isolates.

Table 4.8: Multiple resistance patterns of the *Listeria* species isolated

Antibiotics Combination	No. of Isolates	Resistance pattern	Percentage resistance (%)
2	4	Amp, Fox	13.8
3	3	Va, Amp, Fox	10.3
	1	Amp, TE, Fox	3.4
4	2	Amp, TE, Fox, CN	6.9
	2	Va, Amp, TE, Fox	6.9
	2	C, Amp, Fox, CN	6.9
	2	DA, Amp, TE, Fox	6.9
	1	C, DA, Amp, Fox	3.4
	1	C, E, Amp, Fox	3.4
5	2	E, Amp, TE, Fox, CN	6.9
	2	Va, DA, Amp, TE, Fox	6.9
	1	Da, Amp, TE, Fox, CN	3.4
	1	Va, Amp, TE, Fox, CN	3.4
6	1	E, DA, Amp, TE, Fox, CN	3.4
	1	Va, C, Amp, TE, Fox, CN	3.4
	1	Va, E, DA, Amp, TE, Fox	3.4
	1	Va, C, DA, Amp, Fox, CN	3.4
7	1	C, E, DA, Amp, TE, Fox, CN	3.4

KEY: Va= Vancomycin, C= Chloramphenicol, E= Erythromycin, DA= Clindamycin, Amp= Ampicillin, TE= Tetracyclin, Fox= Cefoxitin, CN= Gentamicin

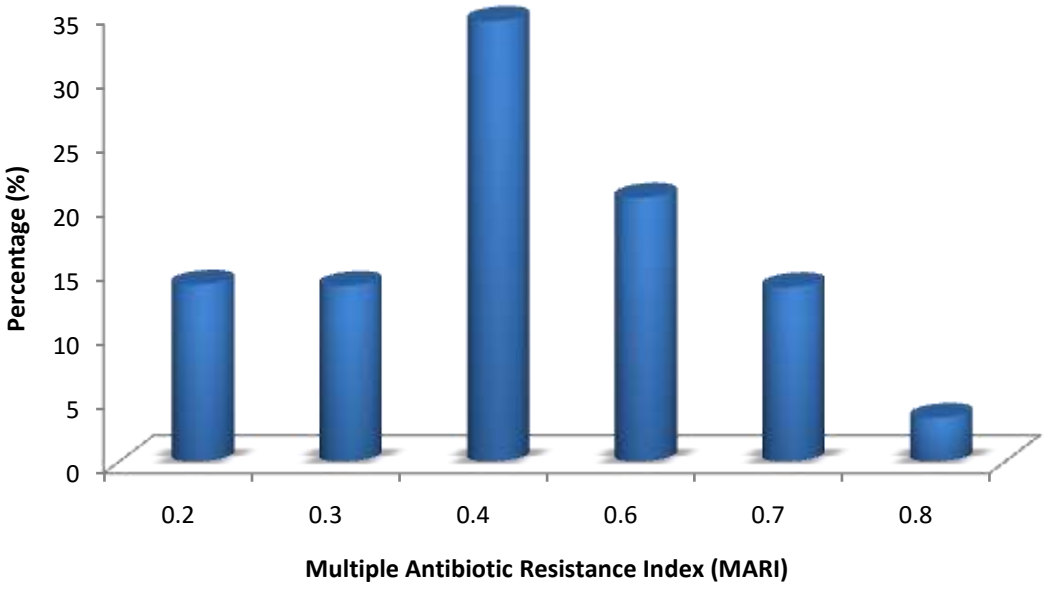

Fig4.4: Multiple Antibiotics Resistance Indices and the Percentage of Isolates Involved.

***NOTE; MARI ≥0.2 Depicts the source as high risk.**

CHAPTER FIVE

5.0 DISCUSSION

The overall occurance of *Listeria* spp. from the study was 29 (18.8%) out of the 154 samples of raw chicken meat across the three locations from which they were purchased is as shown in Table 4.1. This prevalence is considered relatively high and is in agreement with many published works in the country and across the world. Mahmood *et al*. (2003) recorded 23.75% for poultry meat and poultry meat products but 12.5 for fresh poultry meat. Rahimi *et al*. (2012) reported 16.9% from 160 samples of chicken in Iran. Alzubaidy *et al*. (2013) reported 20% from 70 samples of raw poultry meat in Erbil and Koya cities. Rafie *et al.* (2013), isolated 20% from chicken, 30% from turkey, 25% from ostrich, making a total of 23%, Kalorey *et al*. (2005), reported 14.9% in India, and Daniel*et al.* (2015), in Makurdi of Benue state, Nigeria recorded overall contamination of 14.75% for frozen and fresh chicken meat samples (120), but only 4 (6.67%) of the 60 samples of fresh poultry meat were contaminated with *Listeria* species.

Other works found a higher prevalence of*Listeria* spp. ranging from 30-100% (Katarzyna*et al*., 2005;Salihu*et al*., 2008; Ikeh*et al.*, 2010;Nwachukwu*et al*., 2009 and Alsheikh *et al*., 2013). Others had a lower prevalence such as Kanarat *et al.* (2011)who conductedthe study from 2004-2009 to investigate the prevalence of *Listeria monocytogenes*in the chicken production chain in Thailand recorded 59 (2.5%) and 2 (0.2%) samples of fresh frozen chicken meat and RTE chicken products respectively.Mohsen *et al.* (2013), from Iran recorded 10 (6.6%) positive from 150 samples for *Listeria* species. The variation in the prevalence may arise from the way poultry meat carcases are being handled at their slaughter houses, the set-up of the

slaughter house and the hygienic status as it has been suggested that presence of *Listeria* spp. in fresh poultry meat carcases is mostly as a result of contamination from the environment and handling mechanics.

No *Listeria* spp. was detected in any of the RTE chicken meat (roasted) purchased from the metropolis as shown in Table 4.1. In this case, before roasted chicken meat is sold to consumers, it is properly heat-treated which may be enough to kill all *Listeria* species. It has been suggested that thermal treatment used in processing procedures was effective because Listeriae are usually killed after heating at 67.5°C for 60-70 seconds (Alzubaidy *et al.*, 2013). However, the heat-treatment of the roasted poultry meat does not warrant the safety of the consumer as other bacterial contaminants were detected such as *Enterobacteriaceae* and *Staphylococcus spp*.

Fromthe result obtained in Table 4.1, the difference in distribution of the *Listeria* spp. In raw chicken meat across the three locations, Sabon Gari (22.9%), Zaria city (16.7%), and Samaru (13.3%) was not statistically significant ($p<0.05$). This shows that the presence of *Listeria* spp. in chicken meat sold in Zaria is not affected by their locations. These may be due to the fact that all the poultry meat processing plants set up in Zaria metropolis are almost alike in both their hygienic status and ways of handling.

From the 29 (18.8%) positive samples of *Listeria* spp. detected, *Listeria ivanovii* was the highest recorded at the rate of 44.8% (13), followed by *Listeria grayi* 34.5% (10). *Listeriainnocua* was found at the rate of 13.8% (4). Only 1 (3.4%) was detected for both *Listeriaseeligeri* and *Listeriamurrayi* as seen in Figure 4.1. This distribution varies with the reports of many authors who found *Listeriainnocua* to be the highest detected from poultry meat and poultry meat product in Nigeria and across the world. Katarzyna *et al*. (2005) detected 43 (61.4%) out of 70 samples of raw poultry meat in Poland and

recorded *Listeria innocua* highest with prevalence of 63%, followed by *Listeria welshimeri* with 16%,*Listeria monocytogenes* 14%, *Listeria grayi* 5% and *Listeria seeligeri* 2%. He found no *Lisetria ivanovii* in his work. Other authors that detect *Listeria innocua* and *Listeria monocytogenes* with highest prevalence were (Capita *et al*., 2001; Salihu *et al.*, 2008; Abd El-Malek *et al*., 2010; Mohsen *et al*., 2012; Alzubaidy *et al*., 2013). Some published work such as Eruteya *et al*. (2014) detected *Listeria welshimeri*[138 (44.5%)] as the highest out of 310 raw red meat parts samples positive for *Listeria* species in Port-Harcourt, Nigeria, followed by *Listeria seeligeri*[72 (23.2%)] and *Listeria grayi*[71 (22.9%)], then *Listeria innocua*[20(6.5%)]. *Listeria ivanovii*[4(1.3%)] and *Listeria monocytogenes*[4 (1.3%)] were the lowest detected. Daniel *et al*. (2015) got [17 (14.7%)]*Listeria* spp. out of 120 samples of frozen and fresh Chicken Sold in Makurdi, Nigeria, where he recorded *Listeria grayi* [10 (58.8%)] as the highest, followed by *Listeriainnocua*[3(17.65)], then *Listeria ivanovii*[2(11.76%)] and *Listeria welshimeri*[2(11.76%)]. However, some authors were in agreement with the distribution of *Listeria* spp. in chicken in this research. Alsheikh *et al*. (2013) recorded 95 (38%) for *Listeria* spp. out of 250 retail broiler Chickens Ready to Eat Meat Products in Sudan and found *L. ivanovi* (54.7%) as the highest occurring *Listeria* species followed by*L. monocytogenes* (35.78%), then *L. grayi* (4.21%), *L. welshimeri* (3.15%). and *L. seeligeri* (2.10%).

The variation in the distribution of the *Listeria* spp. amongst the authors may largely result from geographical distribution, methods of isolation, and the kind of media employed. Researches from various geographical locations revealed varied results in the isolation of *Listeria* species. Asian and European countries have done a lot of work on the presence of *Listeria* spp. in poultry meats and found a high prevalence compared to countries in Africa especially Nigeria whose research in*Listeria* spp. in poultry meats

has been rarely done and relatively less number of *Listeria* spp. have been found which could be related to cold nature of the Asian and European countries. This was as stated by Nwaiwu (2015) who wrote an overview on *Listeria* spp. in Nigeria and related that despite the world-wide reports of outbreaks of food-borne listeriosis, the occurrence of *Listeria* is still not widely reported in Nigeria. This is possibly due to lack of a large cold storage food chain and the absence of a comprehensive surveillance system for food-borne pathogens.

No *Listeria monocytogenes* was detected in the present research as shown in Table 4.4. This varies with a lot of similar works done especially in other countries like the Asian, European and other African countries. Similar researches revealed variable number of *Listeria monocytogenes*e.g in India (Kalorey *et at*., 2005) reported 8.5%, Pakistan (Mahmood *et al*., 2003) recorded 5% Malaysia (Jamali*et al*., 2013) revealed 23.5%, turkey (Atil*et al*.,2011) recorded 16.4%, Poland (Katarzyna *et al*., 2005) showed 14% prevalence, spain (Gómez *et al*., 2015) had 22.7% prevalence, Ethiopia (Molla *et al*., 2004) recorded 5.1% prevalence, Sudan (Alsheikh *et al*., 2013) recorded 12.8%. Although research works in Nigeria reported high prevalence of *Listeria monocytogenes*, they were mostly detected in experimental samples different from poultry meat. Salihu *et al*. (2008) recorded 29 (25%) positive for *L. monocytogenes* out of the 115 samples analysed from smoked fish in Sokoto, Nigeria. Chukwu (2007) reported a prevalence rate of 8.7% (78) for *Listeria monocytogenes* in animal droppings and environmental samples in areas of Plateau state, Nigeria.

Some published works in Nigeria on occurance of *Listeria* species in Poultry meat revealed 0% prevalence for *Listeriamonocytogenes*. Daniel *et al*. (2015) whose work on contamination and antibiotic susceptibility profile of *Listeria* species in frozen and fresh chicken sold in Makurdi, Nigeria recorded 10 (8.3%) *Listeria grayi*, 3 (2.5%) *Listeria*

innocua, 2 (1.7%) *Listeria ivanovii*, 2 (1.7%) *Listeriawelshimeri* and no *Listeria monocytogenes* found out of the 120 samples analysed. Similarly, Lucy *et al*. (2014) research work on isolation of *Listeria monocytogenes* from raw vegetables and meats sold in open market in Onitsha meropolis, Anambra State, Nigeria shows no *Listeria monocytogenes* in any of the experimental samples but found *E. coli* and *Staphylococcus aureus* as contaminants.

This trend of results seen in Nigeria compared to other countries may be associated with geographical location. Preferential selection of colonies may also be considered as a reason base on the kind of selective media used as colonies of *Listeria monocytogene*s and most of the *Listeria* species are almost alike in size and colour on Oxford and Palcam *Listeria* selective agars.It has also been revealed that the ability of *Listeria innocua* to produce bacteriosin revealed a very broad spectrum inhibition of *L. monocytogenes*, which means that the antibiotic can inhibit a wide variety of *L. monocytogenes* strains (Banerjee *et al*., 2001). Overall, the presence of the *Listeria* species was found to be significant at 95% Confidence interval as seen in Table 4.4.

Although high prevalence of *Listeria* spp. was reported in Port-Harcort, Nigeria in raw cow and goat meat by Eruteya *et al*. (2014) with 310 positive samples, only 4 (1.29%) was recorded positive for *Listeria monocytogenes*.

The two solid selective media (Oxford *Listeria* agar and PALCAM agar) used in the study for the isolation of *Listeria* species were found to be effective but at different rate as presented in Figure 4.2. Oxford *Listeria* agar was observed to be more effective at 100% rate while PALCAM *Listeria* agar gave 79.3% efficiency. Chi-squarestatistical tool was used and their was statistical significant difference in efficiency of Oxford and PALCAM Listeria media in isolation of *Listeria* species. Although, Chukwu (2007)

found no statistical significant difference in their efficiency, different rate in their effectiveness was observed and recorded, Oxford (100%) and PALCAM (99.5%). There are other selective media used for isolation of *Listeria* such as Chromogenic agar, but Oxford Listeria agar has been suggested as the most effective for isolation of all *Listeria* species. Its Limitation is the difficulty in differentiating *Listeriamonocytogenes* from other *Listeria* species. The difference in the effectiveness of the two solid media may have resulted from the conponents of the supplements used.

Although all these solid media were effective in isolation of *Listeria* spp., limitations were observed as they also allow other contaminants like Gram negative rods resembling *enterobacteriacea* and Gram psositive cocci resembling *Staphylococcus* species to thrive. This was also reported by Lucy *et al*. (2014) who found no *Listeriamonocytogenes* on the selective media used but other contaminants such as *E.coli* (26%) and *Staphylococcus aureus* (10%) out of the 150 samples studied. However, the major limitations observed in this study was seeing colonies exhibiting similar characteristics with *Listeria* spp. on the selective media such as black colonies with black hallow on Oxford listeria agar and PALCAM listeria agar but were found to be gram negative rods and gram positive cocci after the Gram stain that are not *Listeria* after preceding tests.

All the *Listeria species* isolates from chicken meat samples collected in the study were observed to be resistant to ampicillin (100%) as seen in Table 4.10 and Figure 4.3. This is similar to the findings of Ieren *et al*. (2013) in Zaria and Yakubu *et al*. (2012) where they observed a resistance of 92.9% and 100% to ampicilin by *Listeria monocytogenes* respectively but varied with the work of Morobe *et al*. (2009) in Botswana who found 100% susceptibility of the *Listeria monocytogenes* isolates to ampicillin.The high level of resistance by the *Listeria* species to ampicillin is a pointer that there is misuse of this

drug within the study region. The susceptibility of *Listeria* spp. to ciprofloxacin (86.2%), chloramphenicol (75.9%) and erythromycin (75.9%) was similar to the work of Lida *et al*. (2011) who recorded ciprofloxacin (66.7%), chloramphenicol (88%) and erythromycin (100%) susceptibility. The study also shows that some organisms show multiple drug resistance and all of the species showed resistance to at least one (1) of the antibiotics. This is comparable to the findings of Jamali*et al*. (2015) who reported multiple drug resistance by some of the *Listeria*species isolated.

The variation in the susceptibility of these organisms to antibiotics may be connected to their previous exposure to the antibiotics and thereby varying the degree of resistance. In addition to this, the Gram reaction of the organisms also influences their susceptibility to the antibiotics used (Buhlmann *et al*., 2008). The multiple antibiotic resisance index (MARI) was calculated and the isolates all showed MARI of $\geq$ 0.2 in Figure 4.4. An observation of MARI $\geq$0.2 means that the isolate is high risk, which could be a source where antibiotics are in constant abuse, and the act is bringing about high selective pressure (Suresh *et al*., 2000).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In this study, *Listeria* spp. Have been found to also be a major contaminants of chicken meats sold in Zaria metropolis, Kaduna state, Nigeria and it has been established that poultry meat contamination is basically from the environment. Absence of *Listeria* spp. in processed chicken meat (roasted) indicates that further heat-treating the meat before selling to consumers eliminate the organisms but was not enough to eliminate some other bacteria such as *Enterobacteriacea* and *Staphylococcus species*.

Although *Listeria monocytogenes* was not isolated from chicken meats sold in Zaria, the study does not authentically suggest the non-existence of the bacterium from the environment. However, the presence of it could be significantly low compared to other *Listeria* species and may likely pose no health risk in Zaria.

It was observed that Oxford *Listeria* agar had no limit to the type of *Listeria* specie capable of growing on it making it to perform better than PALCAM *Listeria* agar. The use of the selective media was also seen as the best method of isolating *Listeria* spp., since it limit the growth of other bacterial cells and help to distinct *Listeria* spp. from the ones capable of growing.

The study also showed that all *Listeria* spp. isolated from chicken meats sold in Zaria metropolis were resistant to ampicilin which has been a common antibiotic used in the

community, but were susceptible to a number of the antibiotics used;Ciprofloxacin,vancomycin, chloramphenicol, erythromycin, clindamycin, tetracycline andgentamicin. Multiple drug resistance was also observed in most of the *Listeria* species which poses threat to antimicrobial therapy in the areas of effectiveness of the antimicrobial agents in therapy.

6.2 Recommendations

1. Prevalence studies on the occurrence of *Listeria* spp. among poultry meat and on possible dangers of poultry meat will be needed to elucidate the epidemiology of listeriosis in Nigeria.
2. Improvement of hygienic conditions at slaughter process particularly effective disinfection procedures has been recommended to reduce the risk to human health.
3. Slaughter houses should be sensitized on the need for proper hygienic practices during processing of the meat and housing of the birds.
4. The public should be sensitized to avoid eating meat products that microbiological standards have not been applied in their production to avoid outbreaks.
5. *Listeria* species resistance to antibiotics should be further studied because of the implications of its resistance mechanisms for other Gram positive bacteria.

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APPENDIX I

Culture media preparation for isolation and identification of *Listeria*species

Fraser listeria enrichment broth

Formular in g/L

Sodium Chloride	20g
Disodium Hydrogen Phosphate	12g
Tryptone	5g
Proteose Peptone	5g
Yeast Extract	5g
Beef Extract	5g
Lithium Chloride	3g
Monopotassium Phosphate	1.35g
Esculin	1g
Ammonium Ferric	0.5g
Nalidixic acid	0.02g
Acriflavine	0.025g

Listeria agar base oxfordIso 11290-1

Formular g/L

Peptones	23g
Lithium Chloride	15g
Esculin	1g
Ferric Ammonium Citrate	0.5g

Sodium Chloride	5g
Maize Starch	1g
Bacteriology Agar	10g

Oxford listeria selective supplement ISO 11290-1 (Cat. 6003)

Composition: each vial for 500ml

Cyclohexamide	200mg
Colistin Sulfate	10mg
Fosfomycin	5mg
Acryflavine Hydrochloride	2.5mg
Cefotetan	1g

Listeria agar base PALCAMISO 11290-2

Formula in g/L

Peptones	23g
Lithium Chloride	15g
Mannitol	10g
Yeast extract	3g
Esculin	0.8g
Glucose	0.5g
Ferric Ammonium Citrate	0.5g
Phenol Red	0.08g
Sodium Chloride	5g
Maize starch	1g

Bacteriological Agar	10g

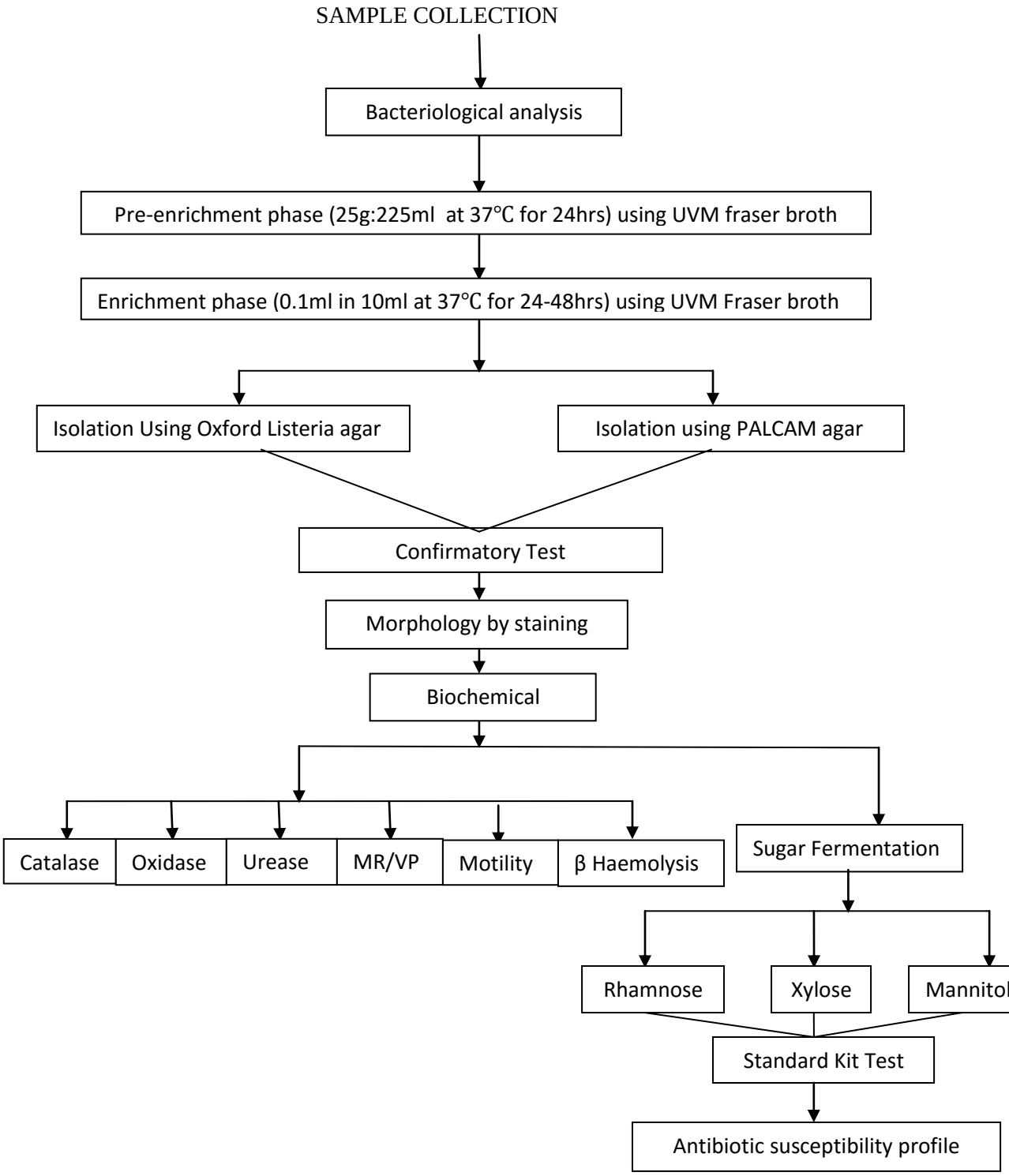
Palcam listeria selective supplement (Cat. 6004)

Composition: 1 vial for 500 ml of the medium

Ceftazidime	10mg
Polymyxin B Sulfate	5mg
Acryflavine	2.5mg

APPENDIX II

Flowchart showing the process for detection and enumeration of *Listeria*species



APPENDIX III

Plates for isolation and identification of *Listeria* species

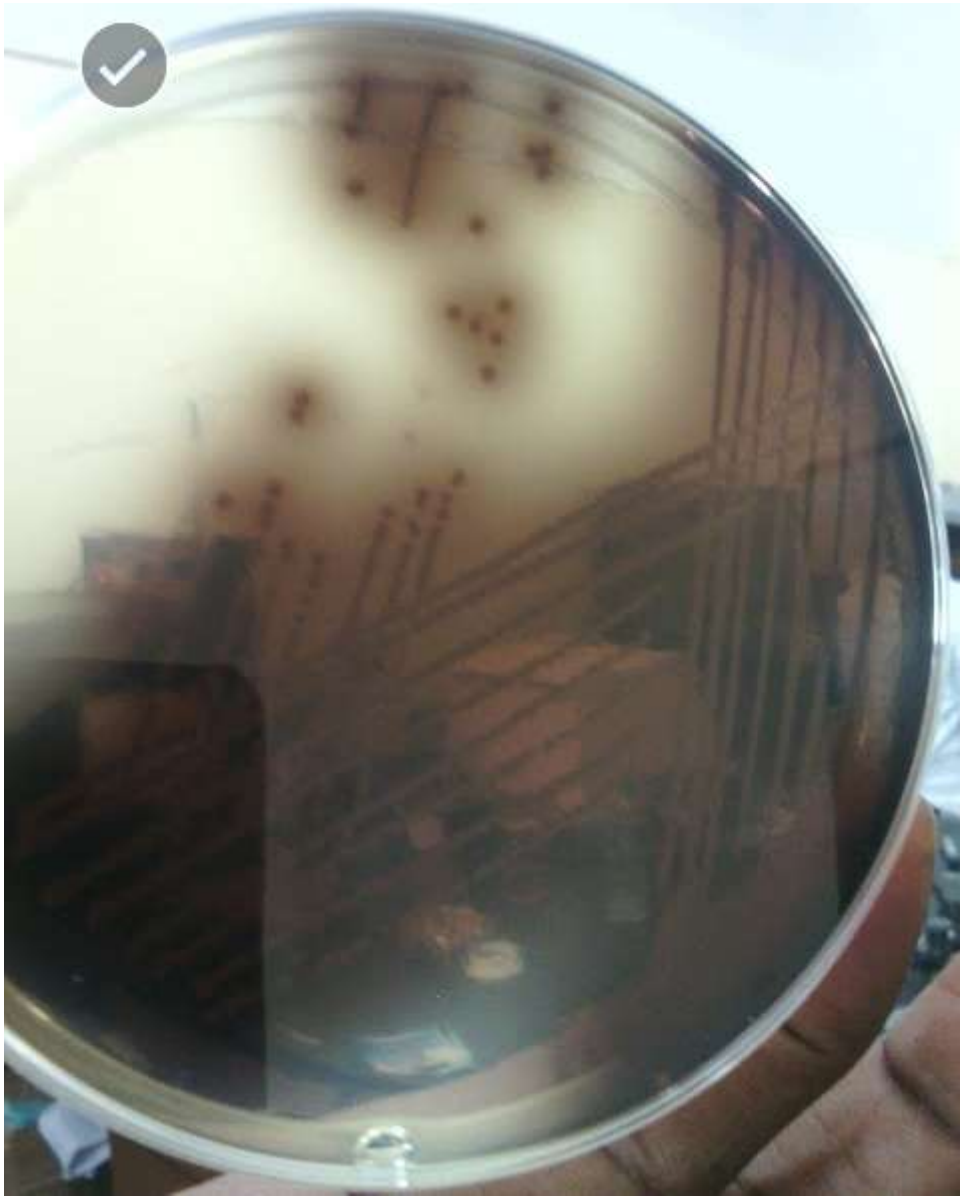
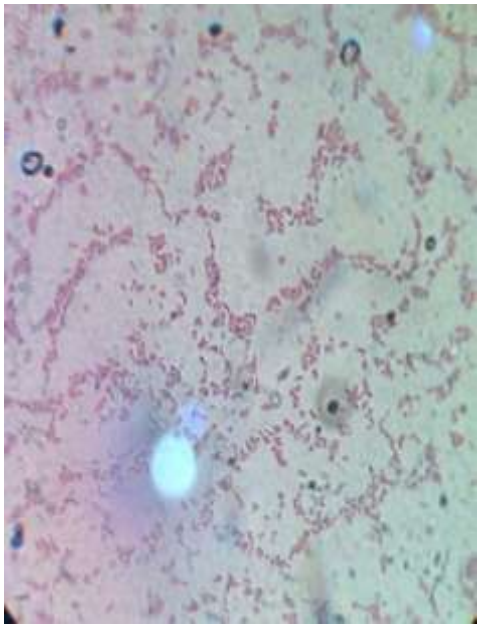
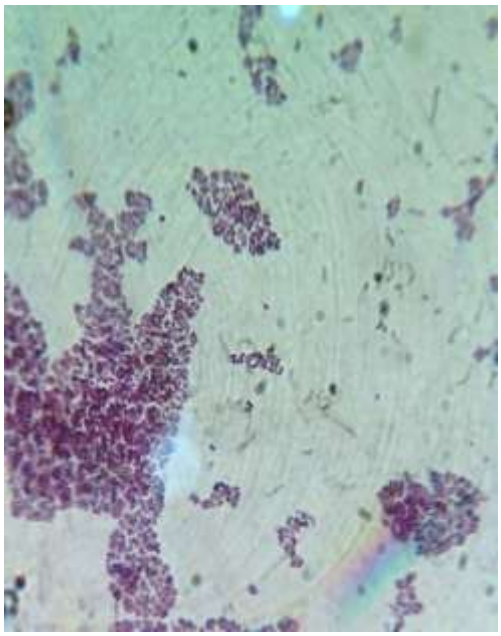


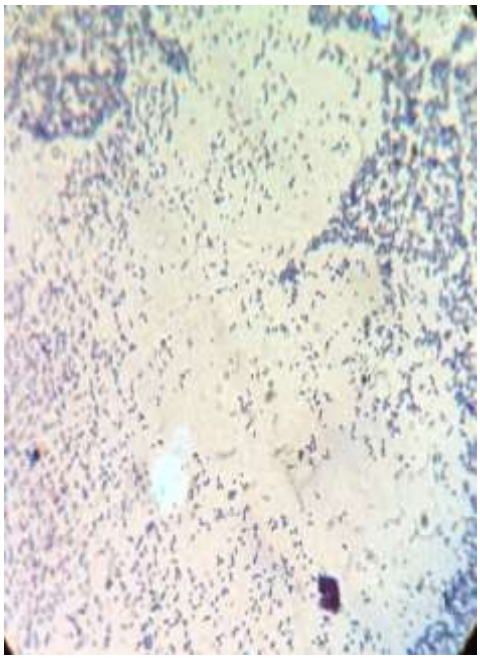
Plate I: A 48hrs culture of*Listeria* spp on Oxford *Listeria* agar showing blackening of the surrounding medium of the dark-brown colonies as a result of esculin hydrolysis

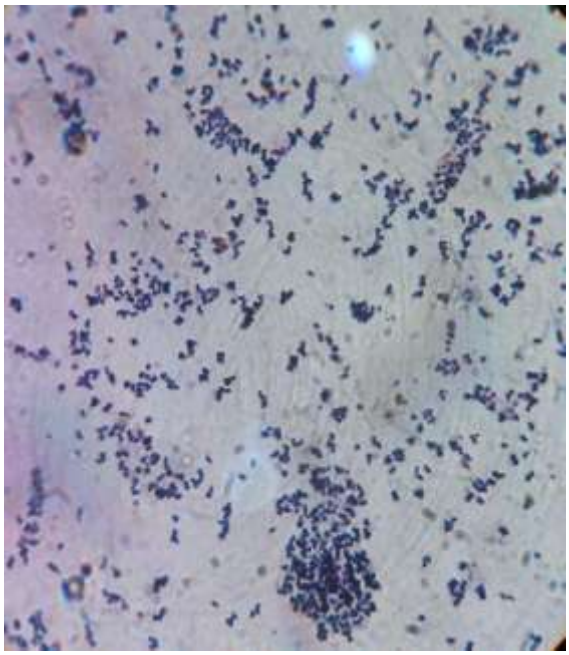
Plate II: A 48hrs culture of *Listeria* spp. showing the gray green colonies surrounded by black hallow medium due to esculin hydrolysis by the colonies





(a) Gram negative shot rods (*enterobacteriacea*) (b) Gram positive cocci (*Staphylococcus*)





(c) Shot rods (d) Coccoid form

Plate III: Gram-stained images as viewed under ×100 light Microscope with oil immersion showing variable forms of *Listeria* spp.

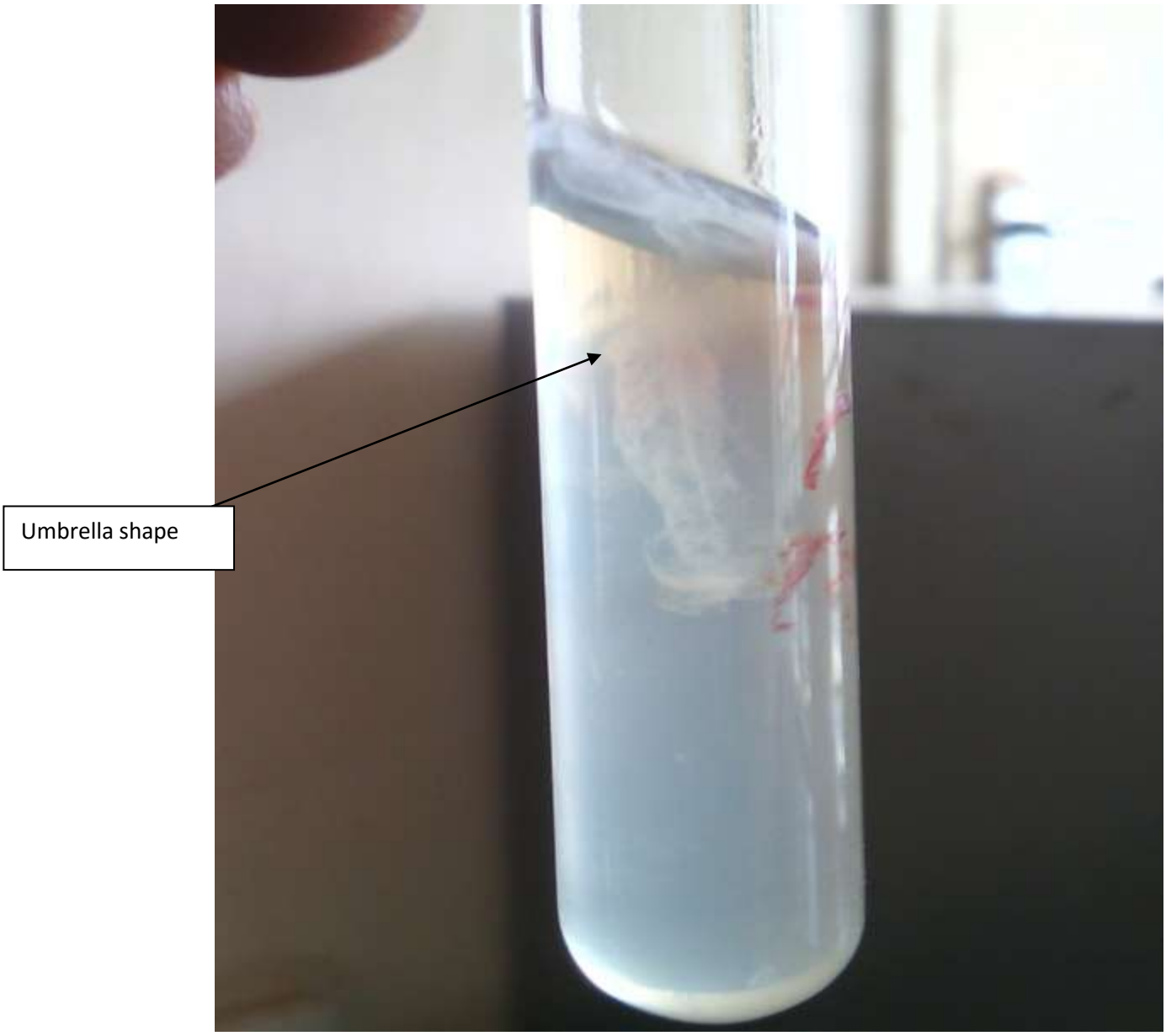


Plate IV: Typical umbrella shape motility of *Listeria* spp in a semi-solid medium at 25℃

Plate V: Microgene Test Kit for Listeria; from top to buttom; *Listeria ivanovii*, *Listeria innocua*, *Listeria seeligeri* and *Listeria grayi*.

APPENDIX IV

Statistical Analyses

Statistical Analysis of the General Distribution of *Listeria* species from raw chicken meat Samples Examined

H0 = The Observation of *Listeria* species among the raw chicken meat samples has no effect on the environment.

	Sampling location			
***Listeria* spp.**	**Sabon gari**	**Zaria city**	**Samaru**	**Total**
L. ivanovii	7	5	1	13
L. seeligeri	1	0	0	1
L. grayi	4	3	3	10
L. innocua	3	1	0	4
L. murayi	1	0	0	1
∑x	16	9	4	G = 29
x	3.2	1.8	0.8	

$$SS = \sum x^2 - \frac{G^2}{N} = 7^2+1^2+4^2+3^2+1^2+5^2+0^2+3^2+1^2+0^2+1^2+0^2+3^2+0^2+0^2+4^2 - \frac{29^2}{5\times3} = 121 - 56.07$$

$$= 64.93$$

$$SST = \frac{\sum T^2}{n} - \frac{G^2}{N} = \frac{16^2}{5} + \frac{9^2}{5} + \frac{4^2}{5} - \frac{29^2}{15} = (51.2 + 16.2 + 3.2) - 56.07 = 70.6 - 56.07 = 14.53$$

$$SSB = \frac{\sum B^2}{n} - \frac{G^2}{N} = \frac{13^2}{3} + \frac{1^2}{3} + \frac{10^2}{3} + \frac{4^2}{3} + \frac{1^2}{3} - \frac{29^2}{15} = (56.3 + 0.3 + 5.3 + 33.3 + 0.3) - 56.1$$

= 95.5 – 56.07 = 39.43

SSE = SS – (SST + SSB)

= 64.93 – (14.53 + 39.43)

= 11

Summary Table for Analysis of Variance (ANOVA)

Sources of Variation	Sum of Square	Degree of Freedom	Mean of Square	F
Treatments	14.53	2	7.25	5.27
Blocks	39.43	4	9.85	6.54
Error	11	8	1.38	

Looking at the critical values of F distribution at P = 0.05 and 2/8 and 4/8 degrees of freedom are 4.46 and 3.34.

Since 5.27>4.46 and 6.54>3.34 this leads to the reject of the null hypothesis. The results obtained from the statistical analysis reveals that the occurrence of the *Listeria* species in the experimental samples is significant.

* SS= Sum of square

SST= Sum of square treatment

SSB = Sum of square block

SSE = Sum of square error.

APPENDIX V

Values of antibiotic susceptibility test of*Listeria* species

			Inhibition Zone Daimeter to nearest mm		
Antibiotics	**Disc Code**	**Disc Potency**	**Resistance**	**Intermediate**	**Susceptibility**
Ciprofloxacin	Cip	5g	≥21	16 – 20	≤15
Vancomycin	Va	30g	≥17	15 – 16	≤14
Gentamicin	CN	10g	≥15	13 – 14	≤12
Cefoxitin	Fox	30g	≥22	-	≤21
Chloramphenicol	C	30g	≥18	13 – 17	≤12
Erythromycin	E	15g	≥23	14 – 22	≤13
Ampicilin	Amp	10g	≥29	-	≤28
Tetracyclin	TE	30g	≥19	15 – 18	≤14
Clindamycin	DA	2g	≥21	15 – 20	≤14