OCCURRENCE, DIVERSITY AND ANTIMICROBIAL SUSCEPTIBILITY OF *Listeria* SPECIES FROM RETAIL RAW BEEF AND GOAT MEAT IN PORT HARCOURT, NIGERIA

BY

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ABSTRACT

Listeriosis has emerged as a disease of public health importance because of its clinical severity, high fatality and challenges to its control including resistance to antibiotics. Outbreaks reported in developing countries including Nigeria were associated with consumption of meat and meat products. However, previous work on *Listeria* in Nigeria focused on environmental samples. Hence, the need to ascertain the presence of *Listeria* in meat and their response to antimicrobial agents. The study was designed to investigate the occurrence, diversity, virulence and antimicrobial resistance of *Listeria* species in raw beef and goat meat in Port Harcourt, Nigeria as well as control of *Listeria monocytogenes* using plant extracts.

A total of 240 meat samples (122 beef and 118 goat), comprising flesh (72), kidney (60), intestine (38) and liver (70) were purchased from Choba, Rumuokoro and Rumuokwuta markets. Bacteria were isolated from the meat and tested for the presence of *Listeria* spp using *Listeria* selective media. Isolates were identified using conventional and molecular methods. The isolates were screened for susceptibility to selected antibiotics using disk diffusion method. Virulence genes and plasmid were screened using molecular methods. *Thymus vulgaris* L., *Allium sativum* L., *Piper guineense* Schum and Thonn, *Monodora myristica* (Gaertn) Dunal, *Ocimum gratissimum* L. and *Xylopia aethiopica* (Dunal) A. Rich were separately extracted with ethanol and water. Effects of the extracts (0.1-5.0%) were determined against *L. monocytogenes* (1×10^8 cell/mL) in agar well diffusion, treated and untreated meat which served as control using standard methods. Data were analysed using descriptive statistics and ANOVA at $p=0.05$.

Eighty-one (33.8%) samples were positive for *Listeria* spp. *Listeria* isolates identified were *L. monocytogenes* (4), *L. innocua* (20), *L. ivanovii* (4), *L. seeligeri* (72), *L. welshimeri* (139), and *L. grayi* (71). All isolated *Listeria* were susceptible to gentamicin and vancomycin but resistant to amoxicillin (100%), augumentin (100%), cloxacillin (100%), tetracycline (88.5%), oxacillin (73.6%), erythromycin (43.7%), chloramphenicol (43.7%) and cotrimoxazole (33.3%). Virulence genes such as *inlJ* and *inlC* were detected in *L. monocytogenes*. Aqueous extract of *P. guineense* and *T. vulgaris* as well as ethanol extract of *T. vulgaris* inhibited *L. monocytogenes* growth at concentrations ranging from 1.0-5.0%, 0.5-5.0% and 0.1-5.0%, respectively. The reduction of *L. monocytogenes* attached to meat pieces varied during a 15 minutes immersion, ranging from 0.8 to 1.4 $\log_{10}$cfu/g. After 4-day storage at 30^{o}C, *L. monocytogenes* exhibited significant higher growth in control samples compared to extract treated samples with initial population in $\log_{10}$ cfu/g increasing from 5.4-6.6 to 9.1-10.2 and 5.3-6.5 to 8.5-9.9 respectively. There was no significant difference between control and treated samples stored at 10^{o}C for 15 days, although initial population increased from 5.4-6.6 to 8.5-8.8 $\log_{10}$cfu/g and 5.2-6.5 to 8.2-8.5 $\log_{10}$cfu/g respectively.

Presence of *Listeria* species was confirmed with *Listeria monocytogenes* having virulence characteristics. *Thymus vulgaris* had inhibitory potential against the growth

of *Listeria monocytogenes* in meat and growth media. Thus, *Thymus vulgaris* can be employed as a control agent of *Listeria* pathogen in meat processing.

Keywords: Listeriosis, *Listeria monocytogenes*, Antibiotic resistance, Plant extract

Word count: 484

DEDICATION

To my late father, Mr. Noel Ekeke Eruteya, for his love for education.

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CERTIFICATION

I certify that this work was carried out by Mr. O. C. Eruteya in the Department of Microbiology, University of Ibadan.

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CHAPTER ONE

INTRODUCTION

Meat is the major source of protein and valuable qualities of vitamins for most people in many parts of the World, thus they are essential for the growth, repair and maintenance of body cells and necessary for our everyday activities (Hassan *et al*., 2006). Due to the chemical composition and biological characteristics, meats are highly perishable foods which provide excellent source of nutrients for growth of many hazardous microorganisms that can cause infection in humans and spoilage of meat and economic loss (Kim and Rajagopal, 2001). The microorganisms of meat surfaces include *Listeria monocytogenes, Micrococcus* spp., *Staphylococcus* spp., *Clostridium* spp., *Bacillus* spp., *Lactobacillus* spp., *Brochotrix thermophacta*, *Salmonella* spp., *Escherichia coli, Serratia* spp. and *Pseudomonas* spp. (Bibek, 2005; Bhunia, 2008). These microbes would be inherent microflora in normal tissues of animals, air, environment or contamination due to unhygienic slaughtering, handling and processing (Zattola, 1972). Growth of food borne pathogens such as *Salmonella*, toxin-producing strains of *E. coli, L. monocytogenes, C. perfringens* and *S. aureus* are of concern with meat and poultry products (Forsythe, 2010).

Listeria monocytogenes is ranked first among foodborne pathogenic bacteria in terms of death rate, far ahead of *Campylobacter, Salmonella and Escherichia coli* 0157:H7 (Naghmouchi *et al*., 2007). This foodborne pathogen is responsible for non-invasive and invasive diseases in the elderly, pregnant women, neonates and immunocompromised populations (Jadhav *et al*., 2012). The invasive form can result in still births, abortions and premature deliveries. In neonates, it can lead to septicaemia, meningitis and is often fatal, whereas in immunocompromised populations it can cause meningitis, encephalitis, meningoencephalitis and septicaemia (Bell and Kyriakides, 2005). In contrast, the non-invasive form results in milder food poisoning like symptoms such as fever, headache and diarrhoea, often referred to as febrile gastroenteritis (Berrada *et al*., 2006).

Outbreaks and sporadic cases of listeriosis have been associated with contamination of different food items, including coleslaw, milk, pate, soft cheese, meat, and seafood products (Schlech, 2000).

The genus *Listeria* consists of a group of gram-positive bacteria of low GC content closely related to *Bacillus, Clostridium, Enterococcus, Streptococcus*, and *Staphylococcus*.(Va´zquez-Boland *et al*., 2001). The genus *Listeria* currently includes six species: *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri*, and *L. grayi*. Among these, *L. monocytogenes* is pathogenic to humans and animals, while *L. ivanovii* is pathogenic to animals and others are considered non-pathogenic (Va´zquez-Boland *et al*., 2001; Bhunia, 2008). Although *L. seeligeri* is considered non-pathogenic, it possesses a part of the virulence gene cluster, which is present in pathogenic *L. monocytogenes* and *L. ivanovii* (Bhunia, 2008). *L ivanovii* is implicated in abortion in animals (Schukken, *et al*., 2003). The other four *Listeria* species are essentially saprophytes that have adapted for survival in soil and decaying vegetation (Liu *et al*., 2007). *Listeria* spp. are ubiquitous in the environment and domestic animals, including cattle, pigs, sheep and fowl, can carry *L. monocytogenes* without exhibiting signs of disease (Uyttendaele *et al*., 1999).

In the past 25 years, *Listeria monocytogenes* has become increasingly important as a food-associated pathogen. Most European Union countries have an annual incidence of human listeriosis of between two and ten reported cases per million. Because of its high fatality rate, listeriosis ranks among the most frequent causes of death due to food-borne illness. *Listeria monocytogenes* infections are responsible for the highest hospitalization rates (91%) amongst known food-borne pathogens and have been linked to sporadic episodes and large outbreaks of human illness worldwide (Giantrancechi, et al., 2009). The ability to persist in food-processing environments and multiply under refrigeration temperatures makes *L. monocytogenes* a significant threat to public health. *Listeria monocytogenes* contamination is one of the leading microbiological causes of food recalls, mainly of meat, poultry, seafood and dairy products (Jemmi and Stephan, 2006).

1.1 Justification of the study

Listeriosis is one of the important emerging bacterial zoonotic infections worldwide. The major outbreaks of listeriosis have been associated with consumption of foods of animals' origin and fish. Reports indicate that listeriosis has emerged to be more

important in developed countries but is reported less frequently in developing countries. This may not be unconnected with lack of diagnostic facilities, limited resources together with the presence of other disease epidemics that claim more priority than listeriosis in developing countries, including Nigeria.

Large outbreaks of listeriosis have been reported in North America, Europe and Japan, but none in Africa and by extension Nigeria, and were attributed to seven major sources which include: raw meat, ready-to-eat (RTE) meat, raw milk, pasteurized milk, ice cream, fishery products and vegetables.

Despite efforts made by different regulatory agencies throughout the world, listeriosis is still one of the most serious food borne diseases of our society. Because of its clinical severity and high fatality, listeriosis is an infection of considerable public health concern.

Although most isolates of *L. monocytogenes* and other *Listeria* spp. are susceptible to antibiotics and natural antimicrobial agents active against Gram-positive bacteria, resistance has been reported in isolates from sporadic clinical cases, food or the environment. Treatment of infections due to opportunistic bacteria may become a problem, since many of these bacteria although generally showing low virulence are naturally resistant to numerous antibiotics and natural antimicrobial agents, indicating the necessity of monitoring the presence of virulent genes and antimicrobial susceptibility of the organisms to offset the paucity of knowledge in these regards.

The investigation was aimed at the prevalence, diversity, virulence gene determination as well as the antimicrobial resistant patterns of *Listeria monocytogenes* isolated from raw beef and goat meat from three major markets in Port Harcourt metropolis, Rivers State.

The data obtained will form the baseline for future studies and will provide regulatory agencies with valuable information needed for the establishment of public health policies for the diagnosis, control and prevention of listeriosis.

1.2 Scope of the study

This study was carried out in Port Harcourt Metropolis of Rivers State, a fast growing city in the Niger Delta region of Nigeria. Experimental samples of raw beef and goat meat will be purchased at random from three major markets in the densely populated

areas of Choba, Rumuokoro and Rumuokwuta. The purchased samples will be kept in sterile plastic bags and transported in ice container to the Microbiology laboratory, University of Port Harcourt. The microbiological analysis will be based on the characteristic appearance of *Listeria* on Fraser broth, polymixin acriflavin lithium chloride ceftazidime aesculin mannitol (PALCAM) agar, physiological and biochemical tests (Gram staining, catalase reaction, oxidase reaction, beta haemolysis on sheep blood agar and acid production from mannitol, rhamnose and xylose). Also, molecular characterization of *Listeria* isolates using polymerase chain reaction (PCR) and virulence determination on *L. monocytogenes* using oligonucleotide primers. The antimicrobial sensitivity pattern and the plasmid carriage within the *L. monocytogenes* strains will also be determined. Other microorganisms present in the meat samples will be enumerated using nutrient agar and characterized on the basis of the morphological, physiological and biochemical reactions.

1.3 Aim of study

The investigation was aimed at the occurrence, diversity, virulence gene determination as well as the antimicrobial resistant patterns of *Listeria monocytogenes* and other *Listeria* spp. from raw cow and goat meat in Port Harcourt metropolis, Rivers State.

1.4 Objectives of study

The objectives of this research include:

1. The determination of the prevalence of *L. monocytogenes* and other *Listeria* species in raw beef and goat meats in the area of study.
2. The identification of the serotypes of *L. monocytogenes* isolates from meat.
3. The determination of the presence of virulent genes in isolated *L. monocytogenes* using oligonucleotide primers
4. Antimicrobial susceptibility of *L. monocytogenes* and other *Listeria* species.
5. Determination of the presence of plasmid in *L. monocytogenes.*
6. Studies of effect of spices to control growth of *L. monocytogenes*.

CHAPTER TWO
LITERATURE REVIEW

2.1 The genus *Listeria*

Until 1960, *Listeria* was nearly exclusively isolated from pathological samples—thus the isolation of nearly only *L. monocytogenes* strains (Rocourt and Buchriester, 2007). Years later, with development of selective media, numerous strains were isolated from various environmental sources. Seeliger serotyped hundreds of strains collected between 1965 and 1980 and observed that they were nonhemolytic, characterized by particular antigenic factors (serovars 6a and 6b [formerly 4f and 4g] and undesignated serovars), and apparently nonpathogenic. They were named *L. innocua* in 1981 (Seeliger, 1981). Thus, simple phenotypic methods, serotyping and hemolysis, led to the demonstration that the species *L. monocytogenes* as defined in the eighth edition of *Bergey's Manual of Determinative Bacteriology* was heterogeneous, covering a number of different species (Buchanan and Gibbons, 1974).

The genus *Listeria* currently contains six species—*L. monocytogenes*, *L. ivanovii*, *L. innocua*,

L. welshimeri, *L. seeligeri*, and *L. grayi*—as evidenced by DNA homology values, 16S rRNA and DNA sequencing, chemotaxonomic properties, and multilocus enzyme analysis (Fig. 1.1), Based on DNA/DNA hybridization, DNA/DNA macroarray hybridization, 16S rRNA cataloguing, reverse transcriptase sequencing of 16S and 23S rRNA, sequencing of 16S–23S rRNA operon intergenic spacer region, and protein mapping, the genus embraces two closely related but obviously distinct lines of descent. One contains *L. grayi* and the other *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, and *L. seeligeri*. Within this line, the genus can be divided into two groups: *L. monocytogenes* and *L. innocua*, on the one hand, and *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* on the other (Collins *et al*., 1991; Sallen *et al*., 1996; Doumith *et al*., 2004a).

Listeria species are Gram-positive, rod-shaped, and nonspore-forming bacteria. They are 1–2 μm long and may exist as single or double cells (Bhunia, 2008). Occasionally, *Listeria* may display long chains depending on the growth conditions and

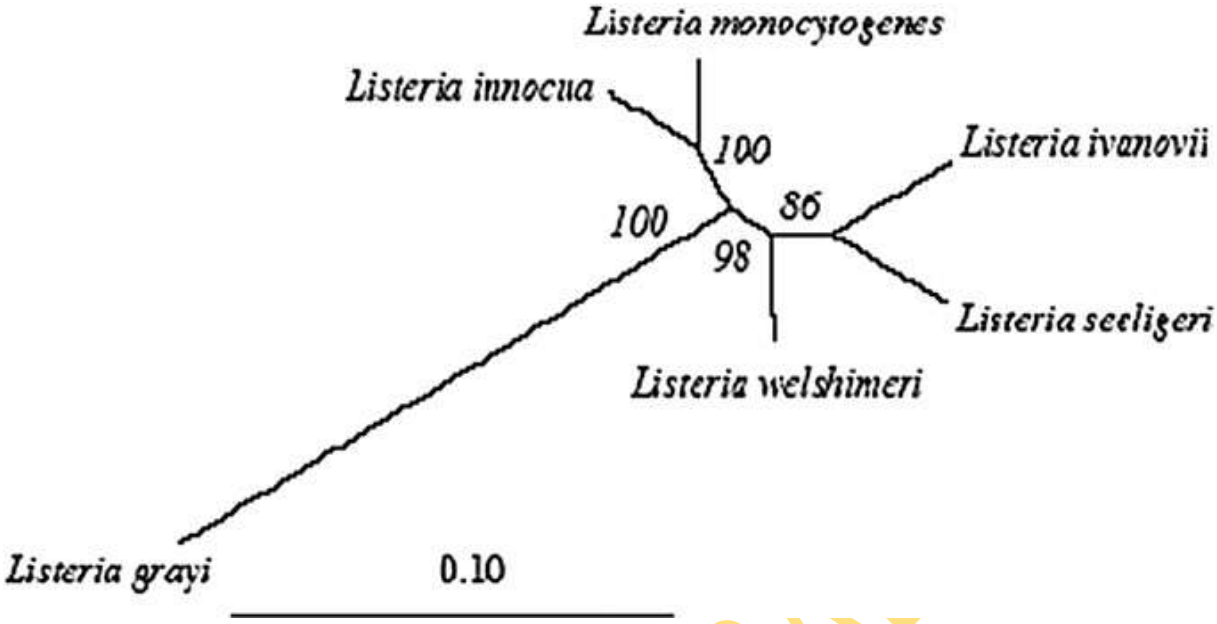


Fig. 1.1: Phylogenetic tree of the genus *Listeria*. This tree is based on the concatenated nucleic acid sequences of 16S and 23S rRNA, iap, prs, vclB, and ldh. The bar indicates 10% estimated sequence divergence.

Source: Schmid *et al*. (2005).

temperatures. *Listeria* is ubiquitous in the environment and domestic animals (cattle, pigs, sheep, and fowl) and survives in extreme environments, including broad pH ranges (4.1–9.6), high salts (10%), and in presence of antimicrobial agents (Fenlon *et al*., 1996; Bhunia, 2008). They are psychrophilic and grow at a wide temperature range (1–45 °C). Some species of genus *Listeria* produce hemolysins. *L. monocytogenes, L. ivanovii*, and *L. seeligeri* are hemolytic and produce a zone of β-hemolysis on blood agar plates (Bhunia, 2008).

All *Listeria* species are phenotypically very similar, but can be distinguished by combinations of the following tests: hemolysis, acid production from D-xylose, L-rhamnose, alpha methyl-D-mannoside, and mannitol (Rocourt *et al*., 1983). Phenotypic similarities are consistent with high genomic homologies between the different species (Rocourt *et al*., 1983; Collins *et al*., 1991; Sallen *et al*., 1996).

2.2 ***Listeria monocytogenes***

In 1926, E.G.D. Murray first reported the isolation of *Listeria monocytogenes* from rabbits and since then it was considered as an animal pathogen primarily causing "circling disease" in ruminants (cattle, sheep, and goats), pigs, dogs, and cats (Bhunia, 2008). The animals walk in a circle and exhibit uncoordinated posture, and are unable to stand without a support. However, in the late 1970s and early 1980s, this organism emerged as a foodborne pathogen causing numerous outbreaks in humans in North America (Bhunia, 2008).

Listeria monocytogenes has attracted the attention of a diverse group of investigators, including clinicians, food microbiologists, immunologists, and medical microbiologists. The reason for this broad degree of interest is due, in large part, to the fact that this facultative intracellular pathogen is highly amenable to experimental analysis and has a broad range of relevant biologic activities ranging from its growth in the environment, infection of many different animal species, and as an important human pathogen (Portnoy, 2007).

Listeriosis is a severe infection caused by *Listeria monocytogenes* particularly among the elderly, very young and immune-compromised individuals and has also been associated with late-term miscarriages in pregnant women (Farber and Peterkin, 1991; Mena *et al*., 2004; Gianfranceschi *et al*., 2009). *Listeria monocytogenes* was recognized as an animal pathogen more than 70 years ago (Murray *et al*., 1926), but has been regarded as a significant foodborne pathogen only in more recent years (Mena *et al*., 2004) with numerous foodborne listeriosis outbreaks reported all over the

world including the USA, Japan, New Zealand, Germany, England, France and other European countries over the past two decades (Warriner and Namvar, 2009).

Because of its clinical severity and the high incidence of fatalities, listeriosis is an infection of considerable public health concern (Vázquez-Boland *et al*., 2001; Gianfranceschi *et al*., 2009).

Listeria monocytogenes is a small (1-2µm in length and 0.5µm wide), facultatively anaerobic, nonsporulating, catalase-positive, oxidase-negative, gram-positive bacillus that grows readily on blood agar, producing incomplete β-hemolysis (Farber and Peterkin 1991; Low and Donachie, 1997; Bille *et al*., 2003). The bacterium possesses polar flagella and exhibits a characteristic tumbling motility at room temperature (25^{o}C) (Peel *et al*., 1988). Optimal growth occurs at 30–37^{o}C, but, unlike most bacteria, *L. monocytogenes* also grows well at refrigerator temperature (4–10^{o}C); and, by so-called cold enrichment, it can be separated from other contaminating bacteria by long incubation in this temperature range. The organism multiplies readily in aerobic and microaerophilic conditions at pH values as high as 9.6. Growth is absent or scanty in complete anaerobic conditions and multiplication is inhibited by pH values lower than 5.6 (Low and Donachie, 1997)

Listeria monocytogenes infections are responsible for the highest hospitalization rates (91%) amongst known food-borne pathogens and have been linked to sporadic episodes and large outbreaks of human illness worldwide (Jemmi and Stephen, 2006). Important characteristics of *L. monocytogenes* contributing to foodborne transmission are the ability to grow at refrigeration temperatures and in environments of reduced water-activity, high salinity and acidity, measures commonly used to control the growth of pathogens in foods (Foneesbech-Vogel *et al*., 2001; Mena *et al*., 2004; Chen *et al*., 2009). *Listeria monocytogenes* contamination is one of the leading microbiological causes of food recalls, mainly of meat, poultry, seafood and dairy products (Jemmi and Stephen, 2006).

2.3 Pathogenesis and Virulence of *L. monocytogenes*

Listeria monocytogenes is pathogenic for animals and humans without showing any significant host specificity. Infection most often begins after ingestion of food contaminated with the organism. The oral inoculum required to produce clinical infection is unknown; experiments in healthy mammals indicate that ≥109 organisms are required (Farber *et al*., 1991). The incubation period for invasive infection is not

well established, but evidence from a few cases related to specific ingestions points to a mean incubation period of 31 days, with a range from 11 to 70 days (Lober, 2007). Infection occurs in several steps:

a) Entry of the bacterium into the host

b) Lysis of the phagosomal vacuole

c) Multiplication in the cytosol

d) Direct cell-to-cell spread using actin-based motility.

Each step requires expression of specific virulence factors. Virulent *L. monocytogenes* organisms can cause disease without promoter organisms, but a 1987 outbreak in Philadelphia, for which no particular source was found, suggested that intercurrent gastrointestinal infection with another pathogen may enhance invasion in individuals colonized with *L. monocytogenes* (Schwartz *et al*., 1989). The major virulence genes are located in a cluster of genes on two different DNA loci and are mainly influenced by the positive regulatory factor A protein (Jemmi and Stephen, 2006).

Virulence factors which are thought to be important in the pathogenicity of *Listeria monocytogenes* strains have recently been characterized:

1) Internalins, encoded by different internalin genes *(inl)*, which take part in the invasion of epithelial cells and seem to be jointly responsible for the tissue tropism of *L. monocytogenes* (Dramsi *et al*., 1997; Schubert *et al*., 2002; Pentecost *et al*., 2006). The first members of this family to be characterized, *InlA* and *InlB*, encoded by the *inlAB* operon, were identified in *L. monocytogenes* by screening a bank of transposon-induced mutants for impaired invasiveness in Caco-2 cell monolayers (Vazquez-Boland *et al*., 2001). An element common to all internalins is a leucine-rich repeat (LRR) domain (Fig. 2.1) consisting of a tandem repeat arrangement of an amino acid sequence with leucine residues in fixed positions (Kajava, 1998). The typical LRR unit of internalins consists of 22 amino acids, with leucine or isoleucine residues at positions 3, 6, 9, 11, 16, 19, and 22 (— —L— —L— —L—L— —N—I— —I/L— —L). This sequence forms a novel; right-handed helix designated parallel b-helix, with a turn after every LRR unit, first identified in the pectate lyase of *Erwinia chrysanthemi* (Yoder *et al*., 1993; Heffron *et al*., 1998).

Internalin A (a 800-amino-acid protein), which contains LRR (leucine-rich repeats), (Fig 2.1) a typical N-terminal transport signal sequence and a cell wall anchor in the

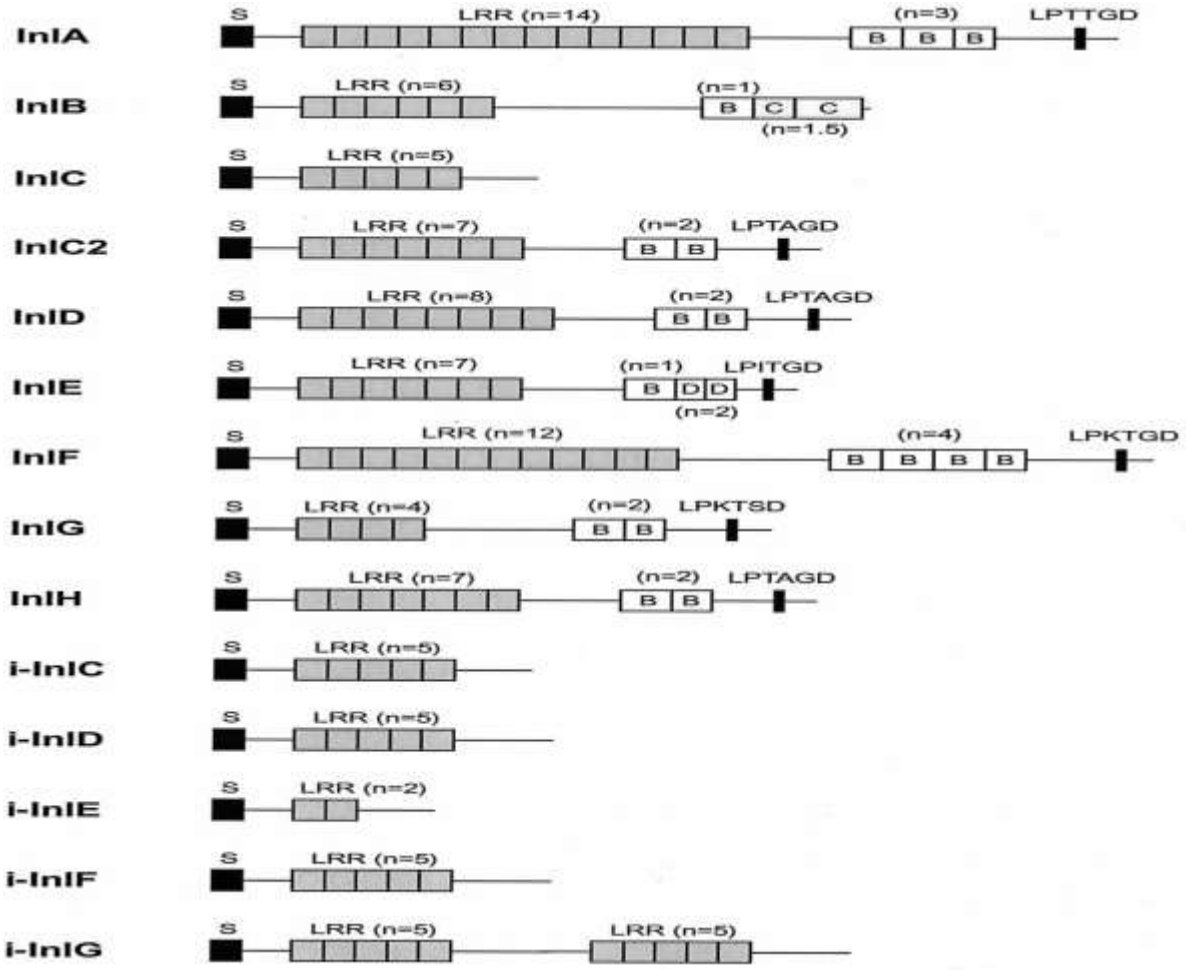


Fig. 2.1: Structure of the members of the internalin multigene family from *L. monocytogenes* and *L. ivanovii.* S, signal peptide; B, B-repeats; C, Csa domain repeats C-repeat; D, D-repeats.

Source: Vázquez-Boland *et al*. (2001)

C-terminal part, comprising the sorting motif LPXTG (Gaillard *et al*., 1991), and internalin B (a 630-amino-acid protein), which also carries an N-terminal transport signal, LLRs and so-called terminal glycine (G) tryptophan (W) modules but in contrast to InlA has no LPXTG motif are both shown to be involved in the internalization of *L. monocytogenes* by various mammalian nonphagocytic cells (Gaillard *et al*., 1991; Shen *et al*., 2000).

2) listeriolysin O, encoded by the gene *hlyA*, and phosphatidylinositol-specific phospholipase C (PI-PLC), encoded by the gene *plcA*, which take part in lysis of the phagosomes of the host cell and thus make the intracellular growth of *Listeria* cells possible (Portnoy *et al*., 1988; Marquis *et al*., 1995; Sibelius *et al*., 1999; Gaillot *et al*., 2000). Listeriolysin O (LLO), the major and first identified virulence factor of *L. monocytogenes* is a member of the cholesterol-dependent cytolysin (CDC) family of toxins (Tweten, 2005). These toxins are produced by numerous Gram-positive bacteria pathogens including *Streptococcus pneumonia* and *Bacillus anthracis*.

Among these pathogens, only *L. monocytogenes* is known to infect nonphagocytic cells and LLO, secreted by *L. monocytogenes*, is required for bacterial escape from endocytic vacuoles (Gaillard *et al*., 1987; Portnoy *et al*., 1992).

3) act A,encoding a secreted 639-amino-acid protein with a 610-residue mature form that is attached to the bacterial cell wall via its C-terminal region is involved in motility (Domann *et al*., 1992; Vazquez-Boland *et al*., 2001). Intercellular spread of *L. monocytogenes* is mediated by the surface-exposed actA protein induces the formation of polarized actin filament, along which the bacteria move to the cell membrane to form a bulge-out structure called listeriopod (Robbins *et al*., 1999; Cossart and Bierne, 2001; Seveau *et al*., 2007). The precise mechanism involved in actA-mediated invasion remains to be elucidated (Seveau *et al*., 2007). The central role of ActA in listerial intracellular motility and virulence was initially revealed by the unusual phenotype of an isogenic *actA* mutant of *L. monocytogenes* in infected tissue culture cells (Kocks *et al*., 1992).

4) Enzymes such as lecithinase, zinc metal protease and serine protease (Gaillot *et al*., 2000; Raveneau *et al*., 1992; Vazquez-Boland *et al*., 1992).

5) A fibronectin-binding protein, FbpA, has been described as a novel multifunctional *L. monocytogenes* virulence factor which seems to be involved in intestinal and liver colonization processes (Dramsi *et al*., 2004).

Several associated protein are critical for Listeria persistence in the intestinal tract, entering into the host cells for intracellular movement and cell-to-cell spread and evasion of the immune system (Table 2.1).

The pathophysiology of *Listeria* infection in humans and animals is still poorly understood, with most of the available information derived from interpretation of epidemiological, clinical, and histopathological findings and observations made in experimental infections in animals, particularly in the murine model (Vázquez-Boland *et al*., 2001). Despite being pathogenic at the species level, *L. monocytogenes* is in fact made up of a spectrum of strains or genotype with varying pathogenic potential; some of which are highly pathogenic and sometimes deadly while others are relatively avirulent and cause little harm in the host (Liu, 2006).

The virulence testing protocols developed to enhance the laboratory assessment of *L. monocytogenes* virulence include: in vivo bioassays, in vitro cell assays, and assays for key virulence-associated proteins and their corresponding genes (Liu *et al*., 2007). While the in vivo mouse virulence assay offers a comprehensive assessment of all known and uncharacterized virulence determinants of *L. monocytogenes*, its utility as a routine technique is questionable due to its expense and its use of animals (Liu *et al*., 2007). Cell cultures and laboratory animal tests cannot be used to ascertain the virulence or lack of virulence of *L. monocytogenes* strains in humans (Jacquet *et al*., 2002).

2.4 Clinical manifestation of *L. monocytogenes*

Individuals prone to symptomatic listeriosis include infants, elderly, pregnant women, and those with the underlying conditions which impair their immune functions, such as malnutrition, major surgery, low gastric acidity, and lack of physical fitness (Farber and Peterkin, 1991; Swaminathan and Gerner-Smidt,2007). Clinical manifestations of listeriosis range from gastrointestinal disturbances (non-bloody diarrhoea, nausea, and vomiting), to influenza-like illness with high fever, headache, and myalgia, and to serious septicaemia and meningitis (Bortolussi, 2008).

Mild impairment of cell-mediated immunity occurs during gestation, and pregnant women are prone to developing listerial bacteremia with an estimated 17-fold increase in risk (Weinberg 1984; Mylonakis *et al*., 2002). Twenty-two percent of perinatal infections with *L. monocytogenes* may have spontaneous abortions, stillborn foetuses, or newborns with meningitis (Rappaport *et al*., 1960; Bucholz and Mascola, 2001; Fayol *et al*., 2009; McClure and Goldenberg, 2009) as the organism proliferate in the

Table 2.1: Major virulence proteins in *Listeria monocytogenes*

Virulence factors	Size (kDa)	Receptor	Function
Protein regulatory factor (PrfA)	27		Regulation of virulence protein expression
Internalin (InlA)	88	E-cadherin(tight junction protein)	Responsible for invasion intointestinal epithelial cells and placenta during pregnancy
Internalin B (InlB)	65	Met (tyrosine kinase), gC1q-R/p32	Entry into hepatocytes and hepatic phase of infection
Virulence protein (Vip)	43	Gp96 (chaperone protein)	Invasion of epithelial cells
Listeria adhesion protein (LAP)	104	Hsp60 (chaperone protein)	Adhesion to intestinal epithelial cells
Autolysin amidase (Ami)	102	Peptidoglycan	Adhesion to host cells
p60 (cell wall hydrolase)	60	Peptidoglycan	Adhesion/invasion
Listeriolysin (LLO) *hlyA*	58-60	Cholesterol	A hemolysin aids in bacterial escape from phagosome inside the cell

Table 2.1 contd.

Virulence factors	Size (kDa)	Receptor	Function
Actin polymerization protein (ActA)	90	-	Nucleation of actin tail for bacterial movement inside the cytoplasm
Bile salt hydrolase (BSH)	36	-	Survival in gut
Phospholipase (*plcA* PI-PLC; *plcB* – PC-PLC)	29–33	-	Lyses of vacuole membrane
Metalloprotease (Mpl)	29	-	Helps synthesis of PLC

Source: Bhunia (2008)

Table 2.2: Clinical syndromes associated with infection with *Listeria monocytogenes*

Population	Clinical presentation	Diagnosis	Predisposing condition of circumstances
Pregnant women	Fever ± myalgia ± diarrhea Pretem delivery Abortion Stillbirth	Blood culture ± amniotic fluid culture	
Newborns:			
< 7 days old	Sepsis, pneumonia	Blood culture	Prematurity
≥ 7 days old	Meningitis, sepsis	Cerebrospinal fluid culture	
Nonpregnant adults	Sepsis, meningitis, focal infections	Culture of blood, cerebrospinal fluid, or other normally sterile site	Immunosuppression, advanced age
Healthy adults	Diarrhea and fever	Stool culture in selective enrichment broth	Possibly large innoculum

Source: Painter and Slutsker (2007)

placenta in areas that appear to be unreachable by usual defense mechanisms, and cell-to-cell spread facilitates maternal–fetal transmission (Bakardjiev *et al*., 2005).

Untreated bacteremia is generally self-limited; although if there is a complicating amnionitis, fever may persist in the mother until the fetus is aborted. Neonatal infection manifests like group B streptococcal disease in one of two forms (Lorber 1997): (1) early-onset sepsis syndrome usually associated with prematurity and probably acquired in utero; or (2) late onset meningitis, occurring at about 2 weeks of age in term infants, who most likely acquired organisms from the maternal vagina at parturition. Clinically, meningitis due to *L. monocytogenes* is usually similar to that due to more common causes; features particular to listerial meningitis are presented in Table 2.3. Nonpregnant adults with listeriosis most frequently present with sepsis, meningitis, or meningoencephalitis. Presenting symptoms in nonpregnant adults with central nervous system listeriosis may include fever, malaise, ataxia, seizures, and altered mental status.

An unusual form of listerial encephalitis involves the brain stem (Armstrong and Fung, 1993) and is similar to the unique zoonotic listerial infection known as circling disease of sheep (Gill, 1993). In contrast to other listerial CNS infections, this illness usually occurs in healthy older children and adults; neonatal cases have not been reported.

Many patients with invasive listeriosis give a history of antecedent gastrointestinal illness, often accompanied by fever. Although isolated cases of gastrointestinal illness due to *L. monocytogenes* appear to be quite rare (Schlech *et al*., 2005), at least seven outbreaks of food-borne gastroenteritis due to *L. monocytogenes* have been documented (Ooi and Lorber, 2005).

Although the incidence is low, the high mortality rates (about 30%) associated with listeriosis make *L. monocytogenes* one of the most deadly human food-borne pathogens (Schuppler and Loessner, 2010).

2.5 Listeria monocytogenes serotypes

Although *L. monocytogenes* is an important foodborne pathogen, the species encompasses a spectrum of strains with varied virulence and pathogenicity (Liu *et al*., 2007). There are 13 distinct O-antigenic patterns, which comprise the serovars; 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 4ab, and 7. Of which, 1/2a, 1/2b and 4b are responsible for 95 to 98% of the outbreaks and serotype 4b is considered the most virulent; a subpopulation of serotype 4b also exists and one or more of those subtypes are considered as epidemic clones (Jacquet *et al*.,2002; Katharious, 2002; Buchrieser,

2007; Bhunia, 2008). *L. monocytogenes* are grouped into three lineages based on their ribopatterns and their association with outbreaks, namely: lineage I (serotypes 1/2b, 3b, 3c, and 4b) from human clinical listeriosis, lineage II (serotypes 1/2a, 1/2c, and 3a) from food isolates and animal clinical cases, and less common lineage III (serotypes 4a and 4c, as well as some 4b) from animal and environmental specimens (Nadon *et al*.,2001; Bhunia, 2008; Jadhav *et al*., 2012) (Table 2.4).

There have been suggestions that *L. monocytogenes* subtypes and lineages differ in their association with specific host and non-host environments (Jemmi and Stephen, 2006).

Epidemiological data from different countries show that the majority of human outbreaks are associated with three *L. monocytogenes* serotypes (1/2a, 1/2b and 4b), despite the fact that there are 13 serotypes potentially capable of infecting humans (Wiedmann, 2002). This may reflect the greater adaptation of certain *L. monocytogenes* subtypes to food associated environments and human infection (Jemmi and Stephen, 2006). Serotypes 1/2a, 1/2b, and 1/2c are the types most frequently isolated from food or the food production environment. However, more than 95% of infections in humans are caused by the three serotypes 1/2a, 1/2b, and 4b. A majority of listeriosis outbreaks are caused by strains of serotype 4b. The rate of isolation of serotype 4b is higher among patients suffering from meningoencephalitis than in patients suffering from blood stream infection, indicating that strains of serotype 4b may be more virulent than other serotypes (Swaminathan and Gerner-Smidt, 2007).

2.6 Distribution of *L. monocytogenes* and *Listeria* spp.

2.6.1 Incidence of *Listeria monocytogenes* in environment

All members of the genus *Listeria* are widely distributed in nature as saprophytes, being found commonly in soil, decaying vegetation, and as part of the fecal flora of many mammals (Schuchat *et al*. 1991; Bille *et al*. 2003). Thus, farm animals and their environment may present an important source of food contamination and infections for humans (Brackett, 1988; Uyttendaele *et al*., 1999; Jemmi and Stephen, 2006). This

and 3a) from food isolates and animal clinical cases, and less common lineage III (serotypes 4a and 4c, as well as some 4b) from animal and environmental specimens (Nadon *et al*.,2001; Bhunia, 2008; Jadhav *et al*., 2012) (Table 2.4).

There have been suggestions that *L. monocytogenes* subtypes and lineages differ in their association with specific host and non-host environments (Jemmi and Stephen, 2006).

Table 2.3: Distinctive features of listerial meningitis compared with more common bacterial etiologies.

Feature Frequency	Percentage
Presentation can be subacute (mimics tuberculous meningitis)	~ 10
Stiff neck is less common	15–20
Movement disorders (ataxia, tremors, myoclonus) are more common	15–20
Seizures are more common	~ 25
Fluctuating mental status is common	~75
Positive blood culture is more common	75
Cerebrospinal fluid (CSF)	40
Positive Gram stain is less common	
Normal CSF glucose is more common	> 60
Mononuclear cell predominance is more common	~ 30

Source: Lorber (1997)

Table 2.4: Classification of *Listeria monocytogenes* based on genomic fingerprint patterns and association with epidemic outbreaks

Groups	Outbreaks	Pathogenic potential	Predominant serotypes
Lineage I	Epidemic clones and responsible for most outbreaks	High	1/2b, 3b, 4b, 4d, 4e
Lineage II	Sporadic listeriosis cases	Medium	1/2a, 1/2c, 3c, 3a
Lineage III	Rarely cause human disease	Low	4a, 4c
IIIA (Rham +ve)			4a (avirulent) and 4c (virulent)
IIIB (Rham −ve)			Virulent nonserotype 4a and nonserotype 4c; Serotype 7.
IIIC (Rham −ve)			Virulent 4c

Rham = Rhamnose fermentation property

Source: Bhunia (2008)

more are common in raw vegetables, raw milk, fish, poultry, and meats, including fresh or processed chicken and beef available at supermarkets or deli counters (Fleming *et al*., 1985; Johnson *et al*., 1988; Farber and Peterkin 1991; Okutani *et al*., 2004). Dairy products have received the most scrutiny (Brackett, 1988). Fruits and vegetables are believed to get this bacterium from soil and manure of animals. Seafood especially shellfish may also be contaminated and at least one outbreak of listeriosis has been linked to shellfish and raw fish (Brackett, 1988). From fresh produce, a number of *Listeria* spp. were also isolated (Heisick *et al*., 1989). The importance of food as a source of sporadic listeriosis is illustrated by two CDC studies in which 11% of all refrigerated food samples were contaminated, 64% of patients had at least one contaminated food, and, in 33% of instances, the patient and food isolates had identical strains (Pinner *et al*., 1992; Schuchat *et al*., 1992). The report of Ikeh *et al*. (2010) reveals the presence of *Listeria* in a number of foods in Nsukka region, South-East, Nigeria (Table 2.5)

2.7 Isolation and Identification of *Listeria* spp.

In time past, cold enrichment techniques were used for pathogen detection wherein Listeria strains were allowed to multiply at refrigeration temperatures (Lorber, 2007) as other bacteria would not be able to survive these temperatures, a technique often requiring several weeks. Currently, the isolation methods have necessarily employed enrichment in one or two stages before isolation on solid media. A number of procedures combining various enrichment and selection media have been tried. It is nearly impossible for one procedure to detect all the existing *L. monocytogenes* in food samples (Pini and Gilbert, 1988). It is difficult to enumerate this bacterium directly on agar media or by most-probable-number method (Lovett, 1988). The conventional isolation and identification procedure is presented in Figure 2.2.

2.7.1 Enrichment of *Listeria* spp.

The FDA method employs a single enrichment in a selective medium of trypticase soy broth with yeast extract, acriflavin, nalidixic acid, and cycloheximide. The sample in enrichment broth is incubated at 30^0C for 2 days. At 24 h and 2 days, streak the culture onto modified McBride's agar and onto lithium chloride-phenylethanol-moxalactam (LPM) agar (Lovett and Hitchins, 1989).

The USDA method is mainly based upon the Lee and McClain method (Lee and McClain, 1986). The sample is first enriched in the *Listeria* enrichment broth (UVM,

Table 2.5. Incidence of *Listeria* species in various food and environmental samples.

Sample	**Number of Samples Examined**	**Number of Positive Samples (%)**
Beef	20	16(80)
Fish	15	6(40)
Poultry	10	7(85)
Pork and Goat	7	0(0)
Soil	5	5(100)
Surface swabs	5	5(100)
Total	82	56(68)

Source: Ikeh *et al*. (2010)

Univ. of Vermont Medium) for 20-24 h at 30 C, and then transfered to the secondary enrichment in Fraser broth at 35C for 24-48 h. The UVM contains esculin, naladixic acid, and acriflavin and the Fraser broth contains of esculin, naladixic acid and lithium chloride. Blacken or darkened tubes resulting from esculin hydrolysis in the Fraser broth are to be streaked on MOX agar for isolation (McClain and Lee, 1988). Tubes that remain the original straw color are negative for *L. monocytogenes* (Fraser and Sperber, 1988). Modified Oxford Medium (MOX) is a highly *L. monocytogenes* selective medium containing ferric ammonium citrate, lithium chloride, 1% colistin soln. and Moxalactam (McClain and Lee, personal communication). Moxalactam, the broad-range cephalosporin antibiotic, proved to be highly useful, controlling not only the gram-negative bacteria but also most of the other gram-positives.

2.7.2 Isolation of *Listeria* spp.

The first selective agar medium used in the isolation of *L. monocytogenes* is McBride's agar which contains phenyl ethanol agar, glycine anhydride, lithium chloride, and sheep blood (McBride and Girard, 1960). Modified McBride's agar is also widely used with the sheep blood replaced by cycloheximide. A number of selective agars have been formulated (Lee and McClain, 1986; Loessner *et al*., 1988; Pucci *et al*., 1988; Bannerman and Bille, 1988; Buchanan *et al*., 1989); nevertheless, the LPM and MOX agars are widely accepted now.

A new selective medium was formulated by Al-Zoreky and Sandine. This medium contains the esculin, selective agents (acriflavin, ceftazidime, and moxalactam) plus agar base. Recognition of *Listeria* colonies is evident by black discoloration of the medium due to esculin hydrolysis without need for special illuminating equipment.

2.7.3 Confirmation of *Listeria* spp.

Confirmatory identification of *Listeria* spp. and *L. monocytogenes* consists of preliminary confirmation test, followed by biochemical test (Table 2.6). According to the USDA procedures, black colonies from MOX agar is streaked onto Horse blood overlay medium. Colonies with β-hemolysis, Gram positive, short rods, with tumbling motility proceed with biochemical identification. It is also easy to observe grey to blue colonies under fluorescent light on LPM agar.

2.8 Rapid methods for detection of *Listeria* spp.

The conventional method above is quite laborious as it requires randomly selecting a number of colonies obtained from a single sample and testing them which often results

in false presumptive (Freece *et al*., 2010). Certain other disadvantages associated with conventional methods include: extensive reliance on phenotype which is subject to changes under different environmental conditions; requirement for different chemicals, media and reagents; interference due to contaminating bacteria which can mask the presence of the target organism; and, atypical reactions given by atypical strains (Jadhav *et al*., 2012). The seriousness of this disease, and the efficiency and volume of today's food production and distribution networks highlight the need to develop rapid methods for detecting *L. monocytogenes* (Brehm-Stecher and Johnson, 2007).

Although no ideal detection method exists for *Listeria* (or for any analyte), consideration of characteristics that such a system would have may be useful in evaluation of existing methods or in development of new ones (Brehm-Stecher and Johnson, 2007). The ideal detection method would be: specific for the target analyte (*Listeria* or *L. monocytogenes*), sensitive (able to detect 1 CFU in a 25-g sample), rapid (substantially faster than cultural methods alone), reproducible, simple to use (with easily interpreted results), capable of direct detection in foods with minimal or no interference from the food matrix, able to distinguish between live and dead (or injured) cells, inexpensive (relative to expenses associated with traditional methods of detection), validated against standard techniques, automatable, and scalable according to testing needs (Brehm-stecher and Johnson, 2007).

The ability to rapidly detect listeriae in foods and in the food-processing environment may also enable more timely monitoring of critical control points, contributing to our ability to control *L. monocytogenes* in these environments and, ultimately, the incidence of disease (Wiedmann, 2002).

2.8.1 Rapid test kits and systems

Test kits and systems for rapid detection of both generic *Listeria* and *L. monocytogenes* are commercially available, and many of these have received AOAC approval. Assay formats include colorimetric DNA probe, latex bead-based lateral flow immunoassay, enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunofluorescence assay (ELFA), immunomagnetic separation (IMS), fluorescence *in situ* hybridization (FISH), and polymerase chain reaction (PCR). Most methods require selective enrichment for up to 48 h. One exception may be IMS-based methods, which can be used to selectively concentrate generic *Listeria* or *L. monocytogenes* from a sample without enrichment (Brehm-Stecher and Johnson, 2007).

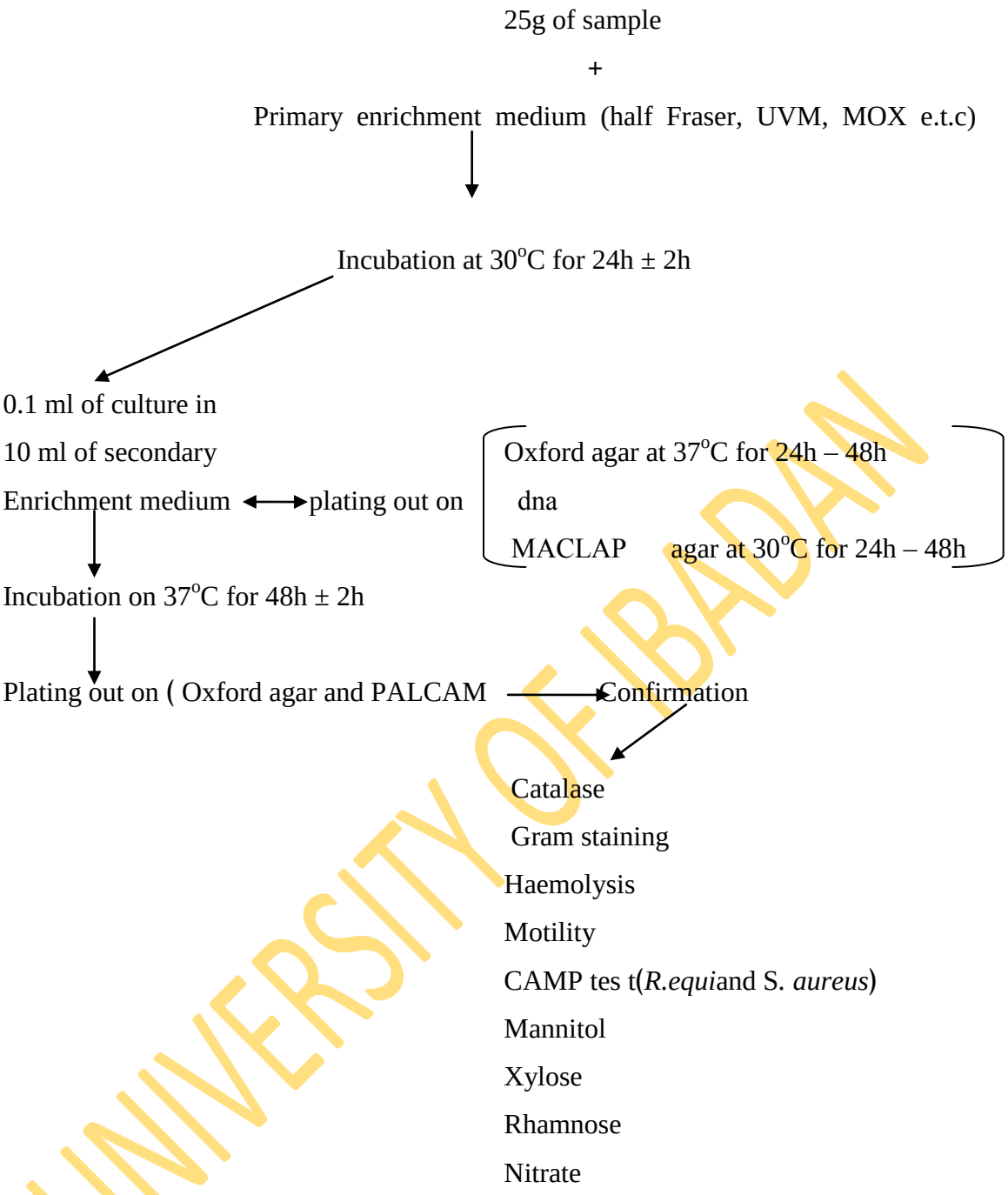


Figure 2.2. Procedure for the isolation and Identification of *Listeria spp*. and *L. monocytogenes.*

Source: Marinsek and Grebenc (2002)

Table 2.6. Biochemical identification of the species of the genus *Listeria*

			CAMP test		Production of acid from			
	Haemolysis on sheep blood	Nitrate reduced to nitrite	*S. aureus*	*R. equi*	D-mannitol	L-rhamnose	D-xylose	A-methyl D-mannoside
L. monocytogenes	+	-	+	-	-	+	-	+
L. ivanovii	++	-	-	+	-	-	+	-
L. innocua	-	-	-	-	-	V	-	+
L. welshimeri	-	-	-	-	-	V	+	+
L. seeligeri	V	-	+	-	-	-	+	V
L. grayi	-	+	NS	NS	+	-	-	NS

CAMP Christie, Atkinson, Munch-Peterson hemolysis assay; V= variable; NS= not stated;

+= Positive; - = Negative

Source: LQAD (2008)

Potential diagnostic targets for *Listeria* or *L. monocytogenes* include evolutionarily distinct nucleic acid sequences found in rRNA, mRNA, or chromosomal DNA. Additional targets may include structural components such as flagellar, somatic, or capsular antigens, or proteinaceous virulence factors such as ß-hemolysin or phospholipase C (Vázquez-Boland *et al*., 2001; Raybourne, 2002; Bhunia, 1997). The presence of these targets may be detected using a number of techniques, including PCR, DNA, or RNA hybridization, antibody-based approaches, or phenotypically, using diagnostic media (Brehm-Stecher and Johnson, 2007).

2.9 Molecular characterization of *Listeria* spp. by polymerase chain reaction (PCR)

2.9.1 Preparation of cultured *Listeria* for PCR

Molecular confirmation of *L. monocytogenes* from cultured isolates is relatively straightforward, with inhibitory compounds present in the original clinical, food and environmental specimens being largely eliminated during the culturing processes. Indeed, *L. monocytogenes* isolates can be readily detected in PCR without pretreatment (Jofre *et al*., 2005). However, it is generally true that prior processing helps further reduce unknown elements that may interfere with DNA polymerase, as use of untreated bacteria directly in PCR sometimes requires additional $MgCl_2$ for enhanced PCR sensitivity and reproducibility.

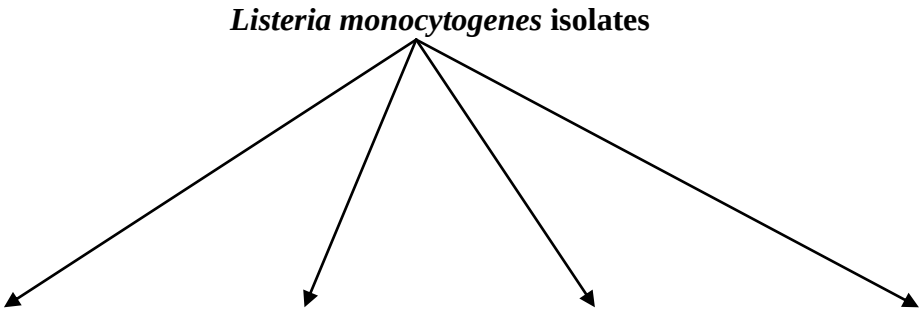
Boiling represents a simple and effective procedure to prepare *L. monocytogenes* cultured isolates for PCR amplification (Liu, 2008). However, this procedure was found to give variable results at times (Agersborg *et al*., 1997). Similarly, treating *L. monocytogenes* isolates with lysozyme and proteinase K followed by boiling does not always produce consistent outcome. On the other hand, heating *L. monocytogenes* in the presence of Triton X-100 tends to work better (Agersborg *et al*., 1997). Indeed, by using Triton X-100 and heat treatment before performing PCR targeting listeriolysin O gene, about 10 colony forming units (cfu) of *L. monocytogenes* were detectable (Agersborg *et al*., 1997). In addition, alkaline treatment also offers an easy and effective approach to prepare DNA template from *L. monocytogenes* cultured isolates. Through lysis of *L. monocytogenes* cells with NaOH and neutralization with Tris–HCl pH 7·0, approximately 10–50 fg of DNA (corresponding to 2–10 genome equivalents) were detected by PCR using ethidium bromide-stained agarose gels or Southern blots using non-radioactively labeled probes, respectively (Scheu *et al*., 1999). Furthermore, boiling *L. monocytogenes* cell pellets derived from enrichment broths in the presence

of 50 mM NaOH and 0.125% of SDS also proves a handy method for preparing PCR-ready DNA template (Rijpens and Herman, 2004). Another effective sample preparation method for *L. monocytogenes* isolates involves the use of polyethylene glycol (PEG), which is an organic solvent with a relatively low viscosity. In one recent report, by heating cultured bacteria in alkaline PEG (i.e., 60% PEG 200 and 20 mM KOH), DNA template was amplified by PCR without further neutralization (Chomczynski and Rymaszewski, 2006) (Figure 2.3).

Besides cultured isolates, these simple sample processing protocols have the potential to be applied to *L. monocytogenes* enriched broth culture (Rijpens and Herman, 2004). While it is well known that the enrichment broth culture frequently contains other microbial organisms, this will not cause a significant problem in view of the fact that the molecular diagnostic tests employed usually target *L. monocytogenes*-specific genes and they are unlikely to cross-react with non-*Listeria* organisms.

2.9.2 Polymerase chain reaction (PCR) procedure

PCR provides a method for exponential amplification of specific DNA sequences present in a sample or extracted from pure culture. Key components of the buffered PCR reaction mixture include oligonucleotide primers, deoxyribonucleotide triphosphates, the DNA template to be amplified, and a thermostable DNA polymerase usually in a 25µl or 50µl volume. The primers are designed to hybridize on either side of the target sequence, defining the region to be amplified (Border *et al*., 1990; Bubert *et al*., 1999; Norton, 2002; Liu *et al*., 2007). Additional components such as enzyme cofactors (e.g., Mg^{2+}) or additives intended to increase the specificity of the reaction or the thermal stability of the enzyme may also be present. PCR reactions are characterized by a three-step cycle involving (1) denaturation of the double-stranded template, (2) hybridization or annealing of primers to complementary regions of the target template, and (3) primer-directed synthesis of new DNA, also termed extension (Border *et al*., 1990; Bubert *et al*., 1999; Norton, 2002; Liu *et al*., 2007). DNA fragments generated in each cycle serve as templates for subsequent rounds of amplification and the number of templates present doubles with each cycle, leading to an exponential accumulation of the product (Border *et al*., 1990; Bubert *et al*., 1999; Norton, 2002; Liu *et al*., 2007). Typically, denaturation is carried out at 95^{o}C, annealing at 55^{o}C, and extension at 72°C, the temperature optimum for *Taq* polymerase. However, extension will still occur at non-optimal temperatures, and



Boiling	**Triton X100 and heat**	**Alkali and heat**	**Alkaline PEG and heat**
-Add a few colonies and 100µl of water -Vortex and heat at 100^{o}C for 10min -spin and use 5µ per 25µl of PCR mix -Outcome: PCR positive	-Add a few colonies and 100µl of Triton X-100 -Leave for 10min -Vortex and heat at 100oC for 10min -Spin and use 5µl per 25µl of PCR mix - Outcome: PCR positive	-Add a few colonies and 100µl of 50mM NaOH - Vortex and heat at 100oC for 10min -Add 10 µl of 1 M Tris pH 8.0 -Spin and use 5µl per 25µl of PCR mix - Outcome: PCR positive	-Add a few colonies and 100 µl of 60% PEG 200 and 20 mM KOH - Vortex and heat at 100oC for 10min - Add 10 µl of 1 M Tris pH 8.0 -Spin and use 5µl per 25µl of PCR mix - Outcome: PCR

Figure 2.3: Rapid preparation of *Listeria* isolates for molecular detection
Source: Liu (2008).

annealing and extension can sometimes be combined into a single step (Bassler *et al*., 1995).

A number of primers available for PCR-based detection of *L. monocytogenes* and other *Listeria* species have been tabulated in recent reviews (Border *et al*., 1990; Bubert *et al*., 1999). Commonly targeted genes in *L. monocytogenes* include the listeriolysin O gene, *hly* (Bassler *et al*., 1995; Bubert *et al*., 1997; Hudson *et al*., 2001; Aznar and Alarcón, 2002; Koo and Jaykus, 2002), and the gene for the invasion-associated protein p60, *iap* (Bubert *et al*., 1997; Bubert *et al*., 1999; Koo and Jaykus, 2002). Other targets include genes for aminopeptidase C (Winters *et al*., 1999), internalins A and B (Liu *et al*., 2007), phospholipase C (Cooray *et al*., 1994), a fibronectin-binding protein (Gilot and Content, 2002), and *lmaA* (also referred to as *Dth-18*), whose product is responsible for delayed-type hypersensitivity reactions in *L. monocytogenes* -immune mice (Schaferkordt and Chakraborty, 1997; Levin, 2003). Genus or species-specific 16S rRNA (Wang *et al*., 1991; Wang *et al*., 1992; Greisen *et al*., 1994) and 23S rRNA (Hudson *et al*., 2001; Rodríguez-Lázaro *et al*.,2004) sequences have also been targeted. Apart from detection, several PCR based methods have also been developed for characterization or typing of *L. monocytogenes* isolates (Franciosa *et al*., 2001; Koo and Jaykus, 2002). Successful PCR amplification is visualized by electrophoresis, with the appearance of a band correlated to the expected size of the fragment.

Foods contain proteins, fats, polyphenolic compounds, target or primer-degrading nucleases, and competitors of Mg2+ which could interfere with PCR reaction (Scheu *et al*., 1998; Norton, 2002; Rodriguez-Lazaro *et al*., 2005). Additionally, certain components of selective media used to enrich for *Listeria* may also have inhibitory activity, including acriflavin, bile salts, esculin, andferric ammonium citrate (Scheu *et al*., 1998). Apart from dilution, approaches for removal of PCR inhibitors include centrifugation, filtration, immunomagnetic capture of target cells, adsorption of cells to hydroxyapatite or metal hydroxides, surface adhesion of cells to polycarbonate membranes, spotting of food washes onto filters impregnated with chelators and denaturants, and chaotropic precipitation of DNA (Duffy *et al*., 1999; Hudson *et al*., 2001).

2.10 Treatment and Control of *Listeria monocytogenes* and listeriosis

The expanding population of highly susceptible individuals (ongoing epidemic of AIDS and use of immunosuppressive medications for treatment of malignancies), in combination with the high prevalence of the organism in foods, make it necessary to

control, prevent and reduce the risks of listeriosis (Vitas *et al*., 2004). However, the ability of *L. monocytogenes* to survive a wide range of adverse conditions, including acidic pH (O'Driscoll *et al*., 1996), low temperatures (Gill and Reichel, 1989), and high sodium chloride concentrations (Farber and Peterkin, 1991) make this organism difficult to control in food. Some adaptations include the increased production of cold shock proteins and cold acclimation proteins, the use of transporters and alternate regulatory proteins, and modifications in the lipid membrane to increase anteiso-C15:0 and shorten fatty acid chains for improved membrane fluidity (Chan and Wiedmann, 2009; Laksanalamai *et al*., 2010). Additionally, an accumulation of compatible solutes, such as carnitine and glycine betaine, that act as osmoprotectants and cryoprotectants is also observed during cold growth and under high-salt conditions (Chan and Wiedmann, 2009; Laksanalamai *et al*., 2010). This accumulation occurs through the use of transporters encoded by *betL*, *opuC*, and the *gbu* operon. Although *L. monocytogenes* is destroyed by pasteurization, several studies have reported its heat resistance and its ability to survive pasteurization due, in part, to the protective nature of the leukocyte in which the pathogen may be present (Doyle *et al*., 1987). Consequently, control of *L. monocytogenes* contamination represents a significant problem for the food industries, public health agencies, and government bodies (Ryan *et al*., 2009).

Strategies to reduce *L. monocytogenes* in foods and consequently listeriosis will depend on hygienic and sanitary production and processing practices (Adzitey and Huda, 2010). An effective control for *Listeria* has to target the farm, processing facilities and processing environment (Adzitey and Huda, 2010).

2.10.1 Antibiotics susceptibility of *Listeria* spp.

Due to the multiplicity of antibiotics, infections caused by drug-resistant bacteria did not represent a medical problem until the early 1980s (Charpentier and Courvalin, 1999). However, evolution of bacteria towards resistance has been considerably accelerated by the selective pressure exerted by over-prescription of drugs in clinical settings and their heavy use as growth promoters for farm animals (Charpentier and Courvalin, 1999). With bacteria having a remarkable ability to develop resistance to every antibiotic, it can be anticipated that even bacterial species such as *Listeria*, which were considered to be susceptible to ampicillin, aminoglycosides, tetracycline, macolides, vancomycin, carbenicillin, cephaloridine, chloramphenicol, erythromycin, furazolidone, methicillin, neomycin, novobiocin, oleandomycin, ticarcillin, azlocillin

and less susceptible to chlortetracycline, oxytetracycline, tetracycline, gentamicin, kanamycin, nitrofurantoin, pencillin G, streptomycin, will evolve towards multi-resistance (Hof *et al*., 1997; Charpentier and Courvalin, 1999; Swaminathan and Gerner-Smidt, 2007). Since the isolation of the first multiresistant strain of *L. monocytogenes* in France in 1988 (Poyart-Salmeron *et al*., 1990), *L. monocytogenes* strains resistant to one or more antibiotics have been recovered from food, the environment and sporadic cases of human listeriosis. Antibiotics to which some *L. monocytogenes* are resistant include tetracycline, gentamicin, penicillin, ampicillin, streptomycin, erythromycin, kanamycin, sulfonamide, trimethoprim, and rifampicin (Charpentier and Courvalin, 1999; Pesavento *et al*., 2010).

There is little information concerning *L. monocytogenes* antimicrobial resistance and especially little information about the isolates recovered from beef that has been determined by the microdilution method (Wieczorek *et al*., 2012).

Listeria infections are almost always treated with antibiotics, but antibiotic resistance in *L. monocytogenes* is being reported with increasing frequency for both food and clinical isolates (Walsh *et al*., 2001; Aureli *et al*., 2003; Safdar and Armstrong, 2003).

2.10.2 Natural antimicrobial (spices)

The meat industry at present uses chemical preservatives such as sodium acetate, sodium lactate and various nitrites to prevent the growth of *L. monocytogenes* in the products. Concerns over the safety of some chemical preservatives have prompted an increased interest in more natural alternatives (Menon and Garg, 2001; Singh *et al*., 2003). There is a need to find safe and effective replacements for chemical preservatives and treatments.

Natural extracts from herbs and spices have been long used in meats as flavoring agents as their constituents are generally recognized to be safe, either because of their traditional use without any documented detrimental impact or because of dedicated toxicological studies (Smid and Gorris, 1999).

Particular interest has been focused on the potential application of spice and herb extracts and essential oils (Singh *et al*., 2003). Spices and herbs have been used for thousands of centuries by many cultures to enhance the flavor and aroma of foods. Early cultures also recognized the value of using spices and herbs in preserving foods and for their medicinal value. Scientific experiments since the late 19th century have

documented the antimicrobial properties of some spices, herbs, and their components (Shelef, 1983; Zaika, 1988).

The antimicrobial properties of spices are desirable tools in the control of infections and in food spoilage (Aboaba, *et al.*, 2005). This property is very desirable as these spices are used as condiments in food preparation (Aboaba *et al*., 2011).

Plant essential oils (EOs) exhibit antimicrobial activity by interfering and destabilizing the operation of the phospholipids bilayer of the cell membrane, enzyme systems, and genetic material of bacteria (Kim *et al*., 1995).

The antimicrobial activities of plant extracts form the basis for many applications, including raw and processed food preservation, pharmaceuticals, alternative medicines and natural therapies (Lis-Balchin and Deans, 1997).

Many studies have reported that phenolic compounds in spices and herbs significantly contributed to their antioxidant and pharmaceutical properties (Cai *et al*., 2004; Shan *et al.*, 2005; Wu *et al*., 2006). Some studies claim that the phenolic compounds present in spices and herbs might also play a major role in their antimicrobial effects (Hara-Kudo *et al*., 2004). The most important of these bioactive constituents which are mainly secondary metabolites are alkaloids, flavonoids, tannins and phenolic compounds which are toxic to microbial cells. (Anyanwu and Nwosu, 2014).

Plant based antimicrobials have enormous therapeutic potentials as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials (Iwu *et al.*, 1999).

To reduce health hazards and economic losses due to foodborne microorganisms, the use of natural products as antibacterial compounds (Conner, 1993; Dorman and Deans, 2000) seem to be an interesting way to control the presence of pathogenic bacteria and to extend the shelf life of processed food. These compounds could be added during the food process.

In Nigeria, some spices are used for the preparation of certain type of soups which are delicacies and also recommended for fast relief of ailments such as malaria fever (Sofowora, 1993).

Only a few studies have been conducted to determine the antimicrobial activity of herbs on *L. monocytogenes* in actual food products (Hao *et al*., 1998; Yuste and Fung, 2002).

2.10.2.1 Thymus vulgaris L (Thyme)

Thymus vulgaris a member of the family lamiaceae, is a small shrubby plant with a strong spicy taste, which grows in several regions of the world (Leung and Foster, 1996).This plant is widely used as flavoring agents in food processing and in medicine for its expectorant, antiseptic, bronchiolytic, antispasmodic , antimicrobial and antifungal properties that make it popular as a medicinal herb and as a preservative for foods (Briozzo *et al*., 1989; Cosentino *et al*., 1999; Ramsewek, 2003).The main constituents of thyme include thymol (the main constituent of thyme 46.2%), carvacrol and flavonoids often thought to have strong inhibition activity against both Gram-positive and Gram-negative bacteria such as *Clostridium botulinum, Escherichia coli* and *Salmonella typhimurium* (Sivropoulou *et al*., 1996; Fan and Chen, 2001; Nevas *et al*., 2004). Other components include tannin, saponin and triterpenic acids (Alexanre and Godoy, 2008).

2.10.2.2 Xylopia aethiopica (Dunal) A. Rich (African guinea pepper)

Xylopia aethiopica is an angiosperm of the family Annonaceae, and grows predominantly in humid forest zones of West Africa (Oloyede and Aduramigba-Modupe, 2011).

Xylopia has been source of multiple biologically active compounds, specifically with microbicidal activity (Lopez *et al*., 2009).

The antimicrobial activity of the ethanolic, methanolic and aqueous extracts of *X. aethiopica*, fresh and dried fruits, leaf, stem bark and root bark essential oil and extracts were reported to show varying degrees of activity against *P. aeruginosa*, *B. subtilis*, *S. aureus*, *Klebsiella* sp., *E. coli*, *Proteus mirabilis*, *Candida albicans, Aspergillus niger* and *A. flavus*. However, they showed little activity against *K. pneumoniae* and had no observable activity against *E. coli*. The lack of activity against *E. coli* may be due to the fact that *E. coli*, a Gram negative bacterium, has an extra outer membrane that may be impermeable to the plant extract (Okigbo *et al*., 2005; Fleischer *et al*., 2008; Nweze and Onyishi, 2010; Sonibere *et al*., 2011).

2.10.2.3 *Monodora myristica* (Gaertn) Dunal (African nutmeg)

Morphologically, African nutmeg is a perennial edible plant of the Annanacea family; a berry that grows wild in the evergreen forests of West Africa (Burubai *et al*., 2009). The most economically imported parts are the seeds which are embedded in a white

sweet-smelling pulp of the sub-spherical fruit. The kernel, when ground is added to pepper soup as stimulant for women who just had babies.

African nutmeg has shown antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus* and *Candida albicans* (Odoh *et al*., 2004). Ethanol extract of African nutmeg, according to Dada *et al*. (2013) inhibited the growth of *Bacillus cereus, Staphylococcus aureus, Shigella dysenteriae, E. coli, Salmonella* spp., *Acinetobacter* spp. and *Aspergillus niger.*

***2.10.2.4 Allium sativum* L (garlic)**

Garlic is a member of the *Allium* genus; a branch of the liliaceae family that also includes onions, leeks, chives, and shallots (Rees *et al*., 1993; Saravanan *et al*., 2010). Garlic is among the oldest of all cultivated plants. It has been used as a medicinal agent for thousands of years. It is a remarkable plant, which has multiple beneficial effects such as antimicrobial, antithrombotic, hypolipidemic, antiarthritic, anti-coagulant, anti-hypertensive agent, anti-carcinogen, an immune system modulator, hypoglycemic and antitumor activity (Rees *et al*., 1993; Thompson and Ali, 2003; Kim *et al*., 2008).

Garlic, which is of great importance is often added to many foods especially those of meats due to its sharp odour, appetizer property and bitter taste and gives flavor to them (Goncagul and Ayaz, 2010). The anti-microbial activity of garlic exceeds that of most broad spectrum antibiotics, exhibiting anti-bacterial, anti-fungal and anti-viral properties (Rees *et al*., 1993)

Allicin, a most impressive broad-spectrum antimicrobial from garlic is seen today as an alternative to antibiotics, with antibacterial effects against a wide range of bacteria, including *Escherichia*, *Lactobacillus* sp., *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Streptococcus* spp., *Aeromonas hydrophilia*, *Klebsiella pneumonia*, *Bacillus cereus* and *Mycobacterium tuberculosis* but not against *L. monocytogenes* (Indu *et al*., 2006; Kumar and Sharma, 2009; Saravanan *et al*., 2010; Houshmand *et al*., 2013).

In laboratory studies, garlic has shown effective activities against influenza B and herps simple viruses, as well as *Candida, Aspergillus and Cryptococcus* (Goncagul and Ayaz, 2010).

2.10.2.5 *Piper guineense* Schum and Thonn (Brown pepper)

Piper guineesne belong to the family piperaceae, having more than 700 species throughout the tropical and subtropical regions of the world (Anyanwu and Nwosu, 2014).

The methanolic and ethanolic extracts of *Piper guineense* exhibited antibacterial activity against *Pseudomonas aeruginosa, E. coli, Staphylococcus aureus* and *Klebsiella* sp., *Proteus mirabilis, A. niger and C. albicans* (Sonibere *et al*., 2011; Osuola and Anyadoh, 2006).

Anyanwu and Nwosu (2014) reported a higher activity of ethanolic extract of brown pepper on *S. aureus, E.coli, P aeruginosa, B. subtilis, Candida albicans* and *Saccharomyces cerevisiae* than water extract.

2.10.2.6 *Ocimum gratissimum* L (Scent leaves)

Ocimum gratissimum L. is a shrub belonging to the family Lamiaceae. It is commonly known as Scent leaf or Clove basil and is found in many tropical and warm temperature countries such as India and Nigeria (Adebolu and Oladimeji, 2005; Okigbo and Ogbonnaya, 2006; Mbakwem-Aniebo *et al*., 2012). Its local names in some parts of Nigeria includes: Ncho-anwu, Ahuji (Igbo), Efinrin (Yoruba), Aramogbo or Ebavbokho (Edo) and Daidoya or Aai doya ta gida (Hausa) (Effraim *et al*., 2000; Owulade, 2004).

The medicinal uses of *O. gratissimum* in the treatment of upper respiratory tract infections, diarrhea, headache and conjunctivitis have been reported (Onajobi, 1986; Ilori *et al*., 1996).

Janssen *et al*. (1989) have reported the antimicrobial activity of four *Ocimum* species, namely *Ocimum canum, O. gratissimum, O. trichodon* and *O. urticifolium*.

The antibacterial activities of *O. gratissimum* have been reported against a number of gram positive (*Staphylococcus aureus, Bacillus spp.; Listeria spp.*) and Gram negative (*Esherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumoniae, Proteus mirabilis, Shigella flexineri*) bacteria. (Nakamura *et al*., 1999; Nguefack *et al*., 2004; Adebolu and Oladimeji, 2005; Mbata and Saikia, 2007; Matasyoh *et al*., 2008; Nwinyi *et al*., 2009; Alo *et al*., 2012) and dermatophytes (*Trichophyton, Microsporum, Epidermophyton*) (Mbakwem-Aniebo *et al*., 2012).

2.11 Plasmids in *L. monocytogenes*

Listeria plasmids are characterized by the presence of a number of diverse mobile genetic elements and a commonly occurring translesion DNA polymerase both of which have probably contributed to the evolution of these plasmids (Kuenne *et al*., 2010). The discovery of multiple genes involved in heavy metal resistance (cadmium, copper, arsenite) as well as multidrug efflux (MDR, SMR, MATE) have been reported in all listerial plasmids (Kuenne *et al*., 2010) and shown to promote bacterial growth and survival in the environment, and probably acquired as a result of selective pressure due to the use of disinfectants in food processing environment.

Plasmids have been sought in strains of *L. monocytogenes* causing epidemic disease have been attributed to nonplasmid-bearing strains (Schlech, 1988). An over-representation of plasmids in *L. monocytogenes* strains from food and the environment in comparison to those obtained from clinical cases have been reported (Lebrun *et al*., 1992; McLauchlin *et al*., 1997).

It was shown that plasmids were found more frequently (75%) in recurrent *L. monocytogenes* strains sampled from food/processing environments than in those from sporadic strains (35%) (Harvey and Gilmour, 2001). Plasmids were also more frequently associated with serogroup 1 strains compared to those from serogroup 4.

It was only in two cases that antibiotic resistance of *L. monocytogenes* could be traced to a plasmid (Poyart-Salmeron *et al*., 1990; Hadon *et al*., 1993). Also, Peterkin *et al*. (1992) and Vaz-Velho *et al*. (2001) reported their inability to detect plasmids in 79.5% (97 of 122) and 64.3% (36 of 56) *L. monocytogenes* on which plasmid profiling was performed respectively

The contribution of plasmids to the infectious process has not been examined and their evolutionary history is not yet well understood apart from homologies to other Gram-positive plasmids such as with plasmid pXO2 from *Bacillus anthracis* which is required for the pathogenic properties of this species (Nelson *et al*., 2004; Canchaya *et al*., 2010; Gilmour *et al*., 2010).

CHAPTER THREE

MATERIALS AND METHODS

3.1 **Area of study**

The study was carried out in Choba, Rumuokoro and Rumuokwuta markets of Obio-Akpor Local Government Area of Port Harcourt, Rivers State, Nigeria (Figure 3.1). The choice of the markets was based on the population distribution and economic activities of these areas.

3.2 **Sample collection**

A total of 240 raw beef and goat meat samples comprising 122 beef [flesh (36), intestine (20), kidney (32) and liver (34)] and 118 goat meat [flesh (36), intestine (18), kidney(28) and liver (36)] were randomly selected and purchased from meat vendors in three markets in Port Harcourt metropolis from March, 2011 to February, 2012. The samples were kept in ice box containing ice packs and immediately transported to the laboratory where they were kept in refrigerator (10^{o}C) until analyzed. The spices *Thymus vulgaris* L (thyme), *Allium sativum* L (garlic), *Piper guineense* Schum and Thonn (brown pepper), *Monodora myristica* (Gaertn) Dunal (African nutmeg), *Ocimum gratissimum* L (Scent leaves) and *Xylopia aethiopica* (Dunal) A Rich (African pepper) were purchased from retailers at the Choba and Aluu markets and identified at the University of Port Harcourt Herbarium. The *Listeria monocytogenes* PCM 2191 serovar 01/2 which served as positive control was obtained from the Polish Collection of Microorganisms, Poland.

3.3 **Isolation of *Listeria* spp**.

The techniques recommended by the United States Department of Agriculture (USDA) (LQAD, 2008) and the Health Products and Food Methods of the Government of Canada (Pagotto, *et al*., 2001) were employed using Fraser broth (Oxoid, England) and Polymixin acriflavin lithium chloride ceftazidime aesculin mannitol agar (PALCAM) (Oxoid, England).

3.3.1 Primary selective enrichment

Twenty-five grams of each meat sample was transfered to a stomacher bag containing

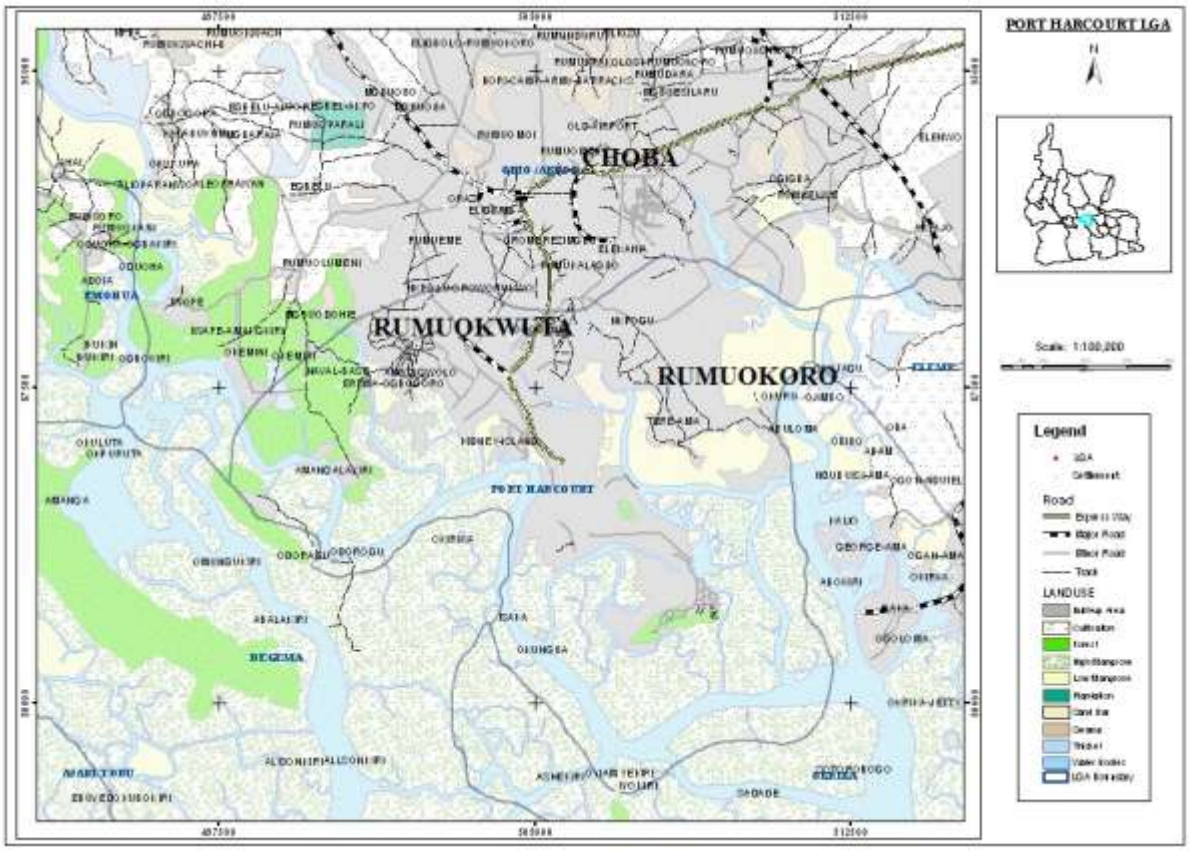


Fig. 3.1: Map of area of study.

225ml of sterile half-strenght Fraser broth. The mixture was homogenized using a stomacher (Lab-Blender, Seward Medical, London) at high speed for 1-2min. The test portion was incubated at 30^{o}C for 24h after 1ml was aseptically transfered into 9ml sterile normal saline. Ten-fold serial dilution was prepared before plating on Nutrient agar for isolation of other bacteria present in the meat. Distinct colonies on nutrient agar were streaked on fresh nutrient agar plate to obtain pure cultures and stored in nutrient agar slant for identification.

3.3.2 Secondary selective enrichment.

Full strength Fraser broth with full concentration of supplement was employed. From the pre-enrichment culture (half-strenght Fraser broth), 0.1ml was transferred into 10ml of full-strength Fraser broth and was incubated at 35^{o}C for 24-48h.

3.3.3 Isolation

From the culture obtained in Fraser broth showing evidence of darkening due to aesculin hydrolysis by *Listeria* spp., 0.1ml was transferred onto duplicate PALCAM plates. After spreading, plates were incubated at 37^{o}C for 24- 48h. The plates were examined for the presence of characteristic colonies presumed to be *Listeria* sp- 2mm greyish-green colonies with a black sunken centre and a black halo on a cherry-red background, following aesculin hydrolysis and mannitol fermentation. Five typical colonies were selected randomly from a pair of PALCAM plates for confirmation and subsequent identification.

3.4 Confirmation and identification.

Colonies suspected to be *Listeria* were transferred onto trypticase soy agar (Becton, Dickinson & Company, France) with 0.6% yeast extract (LAB M, UK) and incubated at 37^{o}C for 18 to 24h, before being subjected to the following standard biochemical tests: Gram staining, catalase reaction, oxidase reaction, beta haemolysis on sheep blood agar and acid production from mannitol, rhamnose and xylose. Confirmed isolates on the basis of criteria suggested by Seeliger and Jones (1986) were further identified using the polymerase chain reaction (PCR).

3.5 Polymarase chain reaction analysis.

3.5.1 Extraction of *Listeria* deoxyribonucleic acid (DNA)

DNA was extracted by the boiling method without Triton X-100 (Hitchins *et al.*, 2004). Cells were harvested by centrifugation (Eppendorf centrifuge 5418, Germany) of overnight brain heart infusion broth culture of *Listeria* in 2ml Eppendorf tube at 10,000rpm for 2min and the supernatants discarded. The pellets were re-suspended in

1ml sterile distilled water and re-centrifuged after vortexing (Vortexer 59A, Denville scientific INC, Taiwan) at 10,000rpm for 5min. The supernatants were again discarded and the pellets re-suspended in 200µL sterile water and vortexed. The suspensions were heated for 10min in a boiling bath (100^{o}C) (Grant GLS400, Grant Instrument, England). After cooling and vortexing, the mixtures were centrifuged at 10,000rpm for 5min. The supernatants were then transferred to a pre-labelled 1.5ml Eppendorf tube while the sediments were discarded. The DNA extracted were stored in deep freezer (-20^{o}C) until further analysis.

3.5.2 Identification by PCR.

Oligonucleotide primers described by Border *et al*. (1990) and Bubert *et al*. (1999) synthesized by Biomers.net GmbH, Germany as presented in Tables 3.1 and 3.2 respectively were employed.

Primers U1 and U2 are based on 16S rRNA sequences that is essentially considered throughout all bacteria and yields a product of 408 bp (base pair). Primer LI1 is also based on 16SrRNA sequence data and is specific to the genus *Listeria*. When used with U1 primer, yields a PCR product of 938 bp which is specific for *Listeria* spp. Primers LM1 and LM2 specific for *L. monocytogenes* are based on listeriolysin O gene sequence data and gives a characteristic PCR product of 702 base pairs (bp).

Primers monoA and Lis1B combination are for specific identification of all serotypes of *L. monocytogenes* yielding a PCR product of 660 bp (base pair); primers Ino2 and Lis1B are for all serotypes of *L. innocua*, PCR product 870 bp; primer Iva1 and Lis1B are for all serotypes of *L. ivanovii*, PCR product 1100 bp; primers Sel1 and Lis1B are for all serotype of *L. seeligeri*, PCR product 1100 bp; primers Wel1 and Lis1B are for all serotypes of *L. welshimeri*, PCR product 1050 bp; while primers combination Mugra1 and Lis1B are for all serotypes of *L. grayi*, PCR product 480 bp . Primers Siwi2 and Lis1B combination are jointly for *L. ivanovii*, *L. welshimeri* and *L. seeligeri*, PCR product 1200 bp.

The reactions involving U1, LI1, LM1 and LM2 were carried out in a final volume of 25µl, containing 2.5 µL 10×PCR buffer, 1.5 µL $MgCl_2$, 0.5µL dNTP (deoxynucleoside triphosphate), 0.25 µl each of appropriate primer, 0.1 µL AmphiTaq DNA polymerase (All products of Solis BioDyne, UK), 1.5µL of appropriate DNA preparation and 18.4µL sterile distilled water. Amplification following an initial denaturation at 95^{o}C for 3min was performed in 30 cycles, at 95^{o}C for 30s, 50^{o}C for 60s and 72^{o}C for 60s in a thermo cycler (Mastercycler-Eppendorf, Vapo-product,

Table 3.1: Sequences of oliginucleotide primers.

Primer	Derived from + or – strand	Sequence(5′-3′)	Location on 16SrRNA
U1	+	CAGCMGCCGCGGTAATWC	519-536
U2	-	CCGTCAATTCMTTTRACTTT	927-908
LI1	-	CTCCATAAAGGTGACCCT	1457-1440
LM1	+	CCTAAGACGCCAATCGAA	
LM2	-	AAGCGCTTGCAACTGCTC	

M denotes A or C; W denotes A or T; R denotes A or G

Source: Border *et al*. (1990)

Germany). A final extension was performed for 10min at 72^{o}C. A 8µl aliquot of PCR product mixed with a loading dye (10mM, EDTA, 10% glycerol, 0.015% bromo phenol blue and 0.017% sodium dodecyl sulphate (SDS), made up to 100ml) were checked in an ethidium bromide stained 1.5% agarose (Fermentas, Life Science, Germany) and the gel visualised in a UV transilluminator (GenoSens 1500, Clinx Science Instruments Co.Ltd, China). Reaction mixture with the DNA of *L. monocytogenes* PCM 2191 serovar 01/2 template served as positive control, while a reaction mixture without DNA template was incorporated as a negative control in each reaction.

The reactions involving MonoA, Iva1, Sel1, Wel1, Ino2, Mugra1, Siwi2, Lis1A and Lis1B were also carried out in a final volume of 25µL, containing 2.5 µL 10×PCR buffer, 1.5 µL $MgCl_2$, 0.5µL dNTP (deoxynucleoside triphosphate), 0.2 µL each of appropriate primer, 0.15 µL AmphiTaq DNA polymerase (All products of Solis BioDyne, UK), 1.5µL of appropriate DNA preparation and 18.45µL sterile distilled water. Amplification following an initial denaturation at 95^{o}C for 3min was performed in 30 cycles, at 95^{o}C for 30s, 58^{o}C for 60s and 72^{o}C for 60s in a thermo cycler (Mastercycler-Eppendorf, Vapo-product, Germany). A final extension was performed for 10min at 72^{o}C. A 8µL aliquot of PCR product mixed with a loading dye (10mM, EDTA, 10% glycerol, 0.015% bromo phenol blue and 0.017% sodium dodecyl sulphate(SDS), made up to 100 mL) were checked in an ethidium bromide stained 1.5% agarose (Fermentas, Life Science, Germany) and the gel read in a UV transilluminator (GenoSens 1500, Clinx Science Instruments Co. Ltd, China). Reaction mixture with the DNA of *L. monocytogenes* PCM 2191 serovar 01/2 template served as positive control, while a reaction mixture with no DNA template was incorporated as a negative control in each reaction.

3.6 Determination of species specific virulence genes of *Listeria monocytogenes.*

This was achieved using the primer combinations described by Liu *et al*. (2007). Table3.3. Primers in1A-F and in1A-R identifies *L. monocytogenes*; primers in1B-F and in1B-R identifies serotypes of *L. monocytogenes*; primers in1C-F and in1C-R, identifies virulence in *L. monocytogenes* and some *L. ivanovi*i while primers in1J-F and in1J-R identifies virulence in *L. monocytogenes*.

Table 3.2: Sequences of oliginucleotide primers.

Primer	Sequence (5′-3′)
Mono A	TTATACGCGACCGAAGCCAAC
Ino2	ACTAGCACTCCAGTTGTTAAAC
Mugra1	CCAGCAGTTTCTAAACCTGCT
Wel1	CCCTACTGCTCCAAAAGCAGCG
Iva1	CTACTCAAGCGCAAGCGGCAC
Siwi2	TAACTGAGGTAGCGAGCGAA
Sel1	TACACAAGCGGCTCCTGCTCAAC
Lis1A	ATGAATATGAAAAAAGGAAC
Lis1B	TTATACGCGACCGAAGCCAAC

Source: Bubert *et al*. (1999)

PCR was conducted in thermocycler (Mastercycler-Eppendorf, Vapo-product,Germany) in a volume of 25µL, containing 2.5 µL 10×PCR buffer, 1.5 µL $MgCl_2$, 0.5µL dNTP (deoxynucleoside triphosphate), 0.25 µL each of appropriate primer, 0.1µL AmphiTaq DNA polymerase(All products of Solis BioDyne,UK),1.5µL of appropriate DNA preparation and 18.65µL sterile distilled water. Amplification following an initial denaturation at 95^{o}C for 3 min was performed in 30 cycles, at 95^{o}C for 30s, 55^{o}C for 60s and 72^{o}C for 60s. A final extension was performed for 10min at 72^{o}C. A 8µl aliquot of PCR product mixed with a loading dye (10mM, EDTA, 10% glycerol, 0.015% bromo phenol blue and 0.017% sodium dodecyl sulphate(SDS), made up to 100mL) were checked in an ethidium bromide stained 1.5% agarose (Fermentas, Life Science, Germany) and the gel read in a UV transilluminator (GenoSens 1500, Clinx Science Instruments Co. Ltd, China). Reaction mixture with the DNA of *L. monocytogenes* PCM 2191 serovar 01/2 (Polish Collection of Microorganisms, Poland) template served as positive control, while a reaction mixture with no DNA template was incorporated as a negative control in each reaction.

3.7 Antibiotic sensitivity

Antibiotic sensitivity patterns of all the *Listeria monocytogenes* and other *Listeria* species confirmed biochemically and molecularly were performed by standard disk diffusion method on Mueller-Hinton agar (Titan, Biotech Ltd, Indian) following the procedures recommended by NCCLS (1997). Ten commonly used antibiotics (µg/disc) viz. augumentin (AUG) 30, amoxycilin (AMX) 25, erythromycin (ERY) 5, tetracycline (TET) 10, cloxacilin (CXC) 5, gentamycin (GEN) 10, cotrimoxazole (COT) 25, chloramphenicol (CHL) 30(Abtek^{R}), oxacillin (OXA) 1 and vancomycin (VAN) 30 (Oxoid) were tested. From an overnight culture in brain heart infusion broth, a 10^{8} cell/ml (0.5 MacFarland turbidity standards) bacterial culture was prepared in sterile saline, from which 0.1ml was inoculated onto Mueller Hinton agar, after which antibiotic discs were carefully and aseptically placed on the surface of the agar. The plates were incubated at 37^{o}C for 24h. Zone of inhibition was measured in millimeter.

3.8 Plasmid Isolation

The method described by Ehrenfeld and Clewell (1987) was employed with some modification. A 1.5mL sample of cells from a fresh overnight culture of each *L. monocytogenes* strain in BHI broth (LAB M, UK) was pelleted by centrifugation at

14,000 rpm for 2 min (Eppendorf centrifuge 5418, Germany) and suspended in 200µL of solution A [100mM glucose, 50mM Tris hydrochloride (pH 8), 10mM EDTA, containing 10mg of lysozyme per ml] and incubated for 30min at 37^{o}C. 400µL of a freshly prepared 1% sodium dodecyl sulphate in 0.2N NaOH was added to each tube and mixed by inverting tubes. The tubes were placed in an ice bath for 5min. Then 300µL of a 30% potassium acetate solution (pH 4.8) was added and the mixture maintained on ice for another 5min before a 5-min centrifugation at 14,000 rpm. The clear supernatant was transferred to a second tube and extracted once with a 1:1 (vol/vol) phenol-chloroform mixture. The aqueous phase was then precipitated with 500µL iso-propanol at room temperature. Nucleic acids were pelleted by a 15min centrifugation at 14,000rpm at room temperature. Pellet were left to dry before dissolving plasmid DNA with a 50µL 1×TE buffer (10mM Tris HCl (pH 8.0) and 1mM EDTA). Plasmids sizes were estimated by using the mobility plasmids of *Escherichia coli*. Agarose gels (1.5%) were stained with ethidium bromide (0.5µg/mL) and visualised in gel documentation system (GenoSens 1500, Clinx Science Instruments Co. Ltd, China).

3.9 Spices preparation and extraction procedure

The spices (100g) were air-dried for about 1 to 2 weeks and ground into fine powder using electric blender. The extraction was carried out by Soxhlet method for both the ethanol (absolute) and water. The fine powder (25g) was packed tightly in the Soxhlet extractor and 250mL of ethanol or aqueous used separately as solvent for extraction. The process was carried out for 6hours. The extract was then filtered and re-extracted under the same conditions to ensure complete extraction. The obtained extract were evaporated to dryness at 60^{o}C to get a dried product and stored in dried plastic bottles for further analysis at room temperature.

3.9.1 Preparation of crude ethanol extract

The method employed was that previously described by Akujobi *et al*. (2004). The spices extracts were diluted with 30% dimethylsulphoxide (DMSO) to obtain 0.1%, 0.5%, 1.0%, 2.5% and 5.0%. These were stored at 15^{o}C until required.

3.9.2 Preparation of crude aqueous extract

The method employed was that previously described by Zaika (1988). Spices dilution used were 0.1%, 0.5%, 1.0%, 2.5% and 5.0% (w/v) concentrations in sterile deionized water and stored at 15^{o}C overnight before being used.

Table 3.3: Identities and nucleotide sequence of *L. monocytogenes* internalin gene primers

Primer	Sequence (5′-3′)	Nucleotide position	Expected PCR product (bp)
in1A-F	ACGAGTAACGGGACAAATAG	94612-94631	
in1A-R	CCCGACAGTGGTGCTAGATT	95411-95392	800
in1B-F	TGGGAGAGTAACCCAACCAC	97957-97976	
in1B-R	GTTGACCTTCGATGGTTGCT	98839-98200	884
in1C-F	AATTCCCACAGGACACAACC	107306-107325	
in1C-R	CGGGAATGCAATTTTTCACTA	107822-107802	517
in1J-F	TGTAACCCGCTTACACAGTT	188989-189009	
in1J-R	AGCGGCTTGGCAGTCTAATA	189226-189207	238

Source: Liu *et al*. (2007).

3.10 Evaluation of antilisterial activity

Agar diffusion method was employed. From overnight broth cultures of the various *L. monocytogenes*, a 1× 10^8 cell/mL McFarland standard was prepared (by first centrifuging the overnight broth at 4,000 rpm for 10min and supernatant decanted. Two millilitre of sterile deionized water was then added; vortexed and centrifuged again at 4,000 rpm for 10 min. The resulting pellets was transferred to a physiological saline, while comparing with McFarland standards) and 0.1mL aseptically transferred to sterile Petri dishes before adding 20 mL molten Brian heart infusion agar cooled to 50^{o}C. The content was thoroughly mixed and then allowed to solidify. Six holes (5.0mm) were made in each plate using a cork borer and 0.2ml of the various spices concentrations of both the ethanol and aqueous extract transferred into each hole aseptically using a pipette. Plates were allowed to stand for prediffusion for 1h before incubation at 37^{o}C for 24h. Ethanol served as control for the crude ethanol extract of thyme. Zones of inhibition were measured and the average calculated.

3.11 **Meat studies**

For meat studies, 1.5g of beef (Plate 3.1) was weighed into glass Petri dish and autoclaved at 121^{o}C for 20min in order to mimic cooked meat. After cooling, the losses in mass were approximately 0.5g resulting in a 1g cooked beef pieces (Apostolidis *et al*., 2008).

3.11.1 Effects of thyme aqueous extract on attached *Listeria monocytogenes*

The meat pieces were transferred aseptically to a sterile beaker containing 20mL of cell suspension of *L. monocytogenes* (1× 10^8/mL) and allowed to stand for 10min. The meat pieces were then placed in a sterile Petri dish for 10min until the solution was reasonably absorbed into the cooked meat pieces. The inoculated meat tips were then immersed in spices water extract (1.0%) for intervals of 0, 5, 10 and 15min. The number of *Listeria* remaining on the meat tips was determined by homogenizing the meat pieces in 9mL sterile saline, followed by a 10-fold serial dilution and appropriate dilutions spread-plated in triplicates on brain heart infusion (BHI) agar. Incubation was at 37^{o}C for 24-36h (Chung *et al*., 1989; Apostolidis *et al*., 2008).

3.11.2 Effects of thyme aqueous extract on the growth of *Listeria monocytogenes* on meat

The meat tips were first transferred aseptically into 1.0% thyme aqueous extract for 10min after which they were transferred to the *Listeria* suspension (1×10^8/mL) in normal saline for 10min. the inoculated meat pieces were gently rinsed with sterile

saline solution, drained and placed in sterile Petri dishes singly. The plates were sealed with masking tape to reduce dehydration and incubated at 30^{o}C and 15^{o}C. *Listeria* counts were enumerated at 1, 2, 3 and 4 days for meat pieces incubated at 30^{o}C and at 0, 3, 6, 9, 12 and 15days for meat pieces incubated at 15^{o}C, by aseptically transferring the meat pieces to a stomacher bag containing 9ml sterile normal saline (0.85g/mL NaCl). Homogenization was done for 2min and 0.1mL of appropriate 10-fold serial dilutions spread plated on BHI agar. Incubation was at 37^{o}C for 24-36h. (Chung *et al*., 1989; Menon and Garg, 2001; Apostolidis *et al*., 2008).

3.12 **Phytochemical screening of *Thymus vulgaris***

The screening was for flavonoid, tannins, saponin and alkaloid.

3.12.1. Estimation of alkaloid

This was done following the method of Harborne (1973). Five grams of the sample was weighed into 250mL beaker and 200mL of 20% acetic acid in ethanol was added and covered. It was allowed to stand for 4h. This was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitate was complete. The whole solution was allowed to settle and the precipitate was collected on filter paper and washed with dilute ammonium hydroxide, filtered and residue dried and weighed. Percentage alkaloid was calculated

$$\frac{\text{Weight of filter paper and residue} - \text{weight of empty filter paper}}{\text{Weight of sample used}} \times 100$$

3.12.2. Estimation of flavonoid

The method described by Bohm and Kocipal-Abyzan (1994) was employed. Ten grams of the sample was extracted repeatedly with 100ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No 42 (125mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight. Percentage flavonoid was calculated.

$$\frac{\text{Weight of crucible and sample residue} - \text{weight of empty crucible}}{\text{Weight of sample used}} \times 100$$

Plate 3.1: Raw meat pieces before autoclaving (1.5g each)

3.12.3. Estimation of tannin

Tannin contents of extract were measured by Folin-Denis method (Stewart, 1974)

3.12.3.1. Preparation of standard tannic solution

Tannic acid (0.05g) was dissolved in 500mL distilled water

3.12.3.2. Preparation of Folin-Denis reagent.

Sodium tungstate (50g) and phosphomolybdic acid (10g) were dissolved in 300ml distilled water and later 25mL orthophosphoric acid was added to the solution. Mixture was refluxed for 2h, cooled and diluted to 500ml with distilled water.

3.12.3.3. Preparation of carbonate solution

Sodium carbonate (17 g) was dissolved in 100 mL of distilled water. Solution was allowed to stand overnight and then filtered through glass-wool.

3.12.3.4. Procedure

Sample (0.1g) was transferred into a 250mL conical flask and 50ml of distilled water was added. It was heated and boiled for 1h, filtered using a Whatman No. 44 filter paper into a 50mL volumetric flask. In a 50mL volumetric flask, 0.5ml sample extract, 2.5mL Folin-Denis reagent and 10ml sodium carbonate solution was added and made up to the mark with distilled water. Contents of flask was shaken and allowed to stand in water bath at 25^{o}C for 20min. the absorbance was measured at 760nm, using water as blank. The mg tannic acid in the sample was obtained by preparing a calibration curve from the standard reading.

$$\text{Soluble tannin (\%)} = \frac{\text{C (mg)} \times \text{extract volume (ml)}}{10 \times \text{aliquot (ml)} \times \text{sample weight}} \times 100$$

C= mg tannic acid obtained from the graph.

3.12.4. Estimation of saponin

Saponin contents of extract were measured by the method of AOAC (1990)

Sample (2g) was first defatted using acetone solvent by soxhlet continuous extraction method. The residue was further extracted with methanol solvent into a preweighed distillation flask by soxhlet continuous extraction. The extract was distilled to dryness and further placed in an air oven to eliminate all traces of methanol solvent. The flask was then re-weighed to obtain the weight of the saponin in the sample (Stewart, 1974).

$$\text{Saponin (\%)} = \frac{\text{Weight of flask and saponin residue} - \text{weight of empty flask}}{\text{Weight of sample defatted and extracted}} \times 100$$

3.13 **Morphological, Physiological and Biochemical Characterization of *Listeria* species and other Isolates from meat samples**

Eighteen to twenty four hours old pure cultures of bacteria were identified and characterized on the bases of their morphology, physiology and biochemical properties and by reference to Cowen and Steel's Manual for the Identification of Medical Bacteria (Barrow and Feltham, 1993).

3.13. 1 Gram staining

The Gram staining was done using crystal violet, Lugol's iodine, ethanol, and safranin. A smear of each isolate was made on a drop of normal saline on a grease-free slide. The smear was allowed to dry and then heat-fixed by passing over the Bunsen burner flame. The heat-fixed smear was flooded with crystal violet for 60seconds. The stain was washed off in gentle running tap water and then flooded with Lugol's iodine for 60 seconds. The Lugol's iodine was drained and the slide rinsed in running tap water and decolourized using 95% ethanol which was washed off immediately. The smear was counter-stained with safranin solution for 30 seconds after which the slide was blotted dry before observing under oil immersion objective of a light microscope. Gram- positive bacteria stained purple while Gram-negative bacteria appeared pink (Cheesbrough, 2000).

3.13.2 Spore staining

The spore staining was done using Malachite green solution and safranin. A smear of each isolate was made on a drop of normal saline on a grease free slide. The smear was allowed to dry and then heat-fixed by passing over the Bunsen burner flame. The heat-fixed smear was flooded with 5% Malachite green solution (w/v) and placed on the rim of a beaker of water heated over a Bunsen flame for 3-5minutes. The slide was then rinsed in gentle water and counter stained with safranin solution for 30 seconds after

which the slide was rinsed, blotted dry before observing under oil immersion objective of a light microscope. Spore stained green, while vegetative cells stained red.

3.13.3 Motility test

A colony of each isolate was stabbed into the motility medium in test tubes, approximately two-third of the depth and incubated at 37^{o}C for 18-24hours. Isolates which grew and formed a hazy zone around the stab line were recorded as positive, while isolates that formed a single line of growth that does not spread into the surrounding area were recorded as negative.

3.13.4 Catalase test

This determines the production of the enzyme which splits hydrogen peroxide into water and oxygen. An inoculum from the pure culture was emulsified on drops of hydrogen peroxide (3% v/v) on a clean glass slide. The rapid production of bubbles (effervescence) when bacteria were mixed with hydrogen peroxide solution was interpreted as positive test (i.e., the presence of catalase). Absence of effervescence showed catalase- negative bacteria (Cheesbrough, 2000).

3.13.5 Oxidase test

The oxidase test is used to assist in the identification of organisms that produce the enzyme cytochrome oxidase. A piece of Whatman No 1 filter paper in clean Petri dish was soaked with a few drops of freshly prepared oxidase reagent (1% tetramethyl-p-phenylene diamine dihydrochloride). The development of deep purple colour within 10 seconds was taken as oxidase-positive; while the absence of purple colouration indicated an oxidase-negative bacterium (Cheesbrough, 2000).

3.13.6 Indole test

This test detects organisms that are able to break down the amino acid tryptophan with the release of indole. Test tubes containing 5ml of sterile peptone water were inoculated with pure colony and incubated at 37^{o}C for 48hour. After incubation, 1mL of Kovac's reagent was added and shaken gently.

A red ring in the surface layer within few minutes signified positive indole test while yellow colouration of the surface layer indicated a negative indole test (Cheesbrough, 2000).

3.13.7 Methyl red- Voges Proskauer (MRVP) test

This test is used to detect the ability of an organism to produce and maintain stable acid end products from glucose fermentation. A colony of each bacterial isolate was

inoculated into glucose phosphate broth, which contains glucose and a phosphate buffer and incubated at 37^{o}C for 48hours.

The methyl red test was carried out by adding 5drops of methyl red indicator to each tube and shaken gently. The development of a red/pink colour was taken as positive, while yellow colour was taken as negative.

For Voges Proskauer test, 0.6ml of alpha-naphthol was added to the test tubes and shaken before 0.2ml of 40% potassium hydroxide (KOH) was then added and shaken. On standing for 15min, tubes with evidence of red colour was taken as positive test; while those that did not show any colour change were taken as negative-VP test.

3.13.8 Urease test

The urease test detects organisms that can produce the enzyme urease. Five millilitres sterile Christensen's modified urea broth in test tubes was heavily inoculated and incubated at 37^{o}C for 24h. The tubes were then examined for characteristic pink colouration signifying positive result; and no pink colouration signified negative result.

3.13.9 Citrate utilization test

This test is based on the ability of an organism to use citrate as its only source of carbon. Colonies from pure culture plate were stabbed and streaked on pre-prepared slopes of Simmon citrate agar in bijou bottles and incubated at 35^{o}C for 48hours. Bright blue colouration signifies positive test, while the lack of change in the initial green colour signified negative citrate test (Cheesbrough, 2000).

3.13.10 Starch hydrolysis

A colony of each isolate was inoculated into prepared starch agar plate and incubated at 37^{o}Cfor 24h. Following incubation, the plates were flooded with iodine. Blue/ black colouration along line of streak indicates negative starch hydrolysis; while a clear zone along line of streak indicates positive starch hydrolysis (Cheesbrough, 2000).

3.13.11 Gelatin liquefaction

This test is used to detect the ability of bacteria to produce gelatinases that will hydrolyze (liquefy) gelatin, a solidifying agent in food. A colony of each isolate is stab-inoculated into nutrient gelatin tubes and incubated at room temperature for 48hours. Afterwards, tubes were placed in ice pack for 15minutes. After 15minutes, tubes were tilted gently to observe if gelatin has been hydrolyzed. If gelatin remains liquid, the result is positive but if the gelatin is solid, the result is negative.

3.13.12 Triple sugar iron agar (TSI) test

This test is for the determination of microorganisms that can produce hydrogen sulphide (H_2S) and ferment glucose and lactose with or without gas production.

The butt of the TSI-agar slant was stabbed inoculated and surface (slant) streaked using an inoculating needle and a loop and incubated at 37^{o}C and observed for 4days. Blackening of the medium indicated production of hydrogen sulphide. Acid production was indicated by a yellow colour while gas production was shown by a crack of split within the medium due to the tension resulting from gas production.

3.13.13 Nitrate reduction

Trypticase-nitrate broth is used to detect an organism's ability to reduce nitrate to nitrite or a further reduced nitrogenous compound such as nitrous oxide or nitrogen gas.

The isolates were each inoculated into 10mL trypticase-nitrate broth in test tubes and incubated at 37^{o}C for 48h. One milliltre of sulfanilic acid was added to each tube, followed by addition of 1ml of dimethyl 1-naphthylamine solution and shaken. Development of a red colour indicated a positive test. If no red colour develops, small amount of zinc powder is added. If a red colour develops, nitrate is present and the test is negative, if no red colour develops, nitrate has been reduced and the test is positive.

3.13.14 Sugar fermentation test

This test was performed to determine the ability of the isolates to metabolize the sugars (rhamnose, mannitol, xylose, glucose, lactose and sucrose) with a resultant production of acid or gas and acid. To 900mL of peptone water was added 10mL bromocresol purple as indicator and the mixture thoroughly mixed before dispensing 9mL into test tubes. Into each test tube was added Durham tube in an inverted position before sterilizing by autoclaving at 121^{o}C for 10min. After autoclaving and cooling, 1mL of sterile 10% of the various sugar solutions was added to the tubes before inoculation. Tubes were incubated at 37^{o}C for 48hours. Sugar fermentation was indicated by a change of colour from purple to yellow (acid produced), while gas production was indicated by collection of gas in the inverted Durham tubes.

3.14 **Statistical analysis**

The distribution of the *Listeria* species in the various meat types and parts, and the results of the inhibitory activity of thyme were subjected to analysis of variance (ANOVA); Duncan and Least Significant Difference (LSD) were used to determine

and compare means that differed at level of significance was set at p=0.05 using SPSS version 20.0 (Kinnean and Gray, 1999).

CHAPTER FOUR

RESULTS

4.1 Occurrence of *Listeria* species in sampled markets

The occurrence (%) of *Listeria* spp. from the three markets of Choba, Rumuokoro and Rumuokwuta is presented in Figure 4.1. The highest occurrence was from Rumuokwuta, 49.32% (36 of 73), followed by Rumuokoro, 32.10% (26 of 81), while the least was Choba, 22.09% (19 of 86).

4.2 Detection of *Listeria* species in raw beef and goat meats

Out of a total of 240 raw beef (122) and goat (118) meat samples analyzed, 81 (33.75%) were positive for *Listeria monocytogenes* and other *Listeria* species (Table 4.1). The 81 positive samples was made up of 48 (39.34%) and 33 (27.97%) for beef and goat meat respectively. The highest percentage of occurrence was from beef flesh (52.78%), followed by beef intestine (40.00%) while the least occurrence was from goat kidney (17.86%).

4.3 Darkening colouration of *Listeria* species in full-strenght Fraser broth

Macroscopic examination of full-strength Fraser broth and its supplement revealed a mild darkening colouration after 24h, but was pronounced after 48h of incubation; characteristic of all *Listeria* species, resulting from the hydrolysis of aesculin (Plate 4.1). No darkening colouration was observed in the control/uninoculated tube. The *Micrococcus* spp. isolated alongside *Listeria* spp. did not produce this darkening colouration when inoculated alone in the full-strenght Fraser broth.

4.4 Morphology of *Listeria* species on PALCAM agar

Morphological appearance of *Listeria monocytogenes* and other *Listeria* species on Polymixin acriflavin lithium chloride ceftazidime aesculin mannitol (PALCAM) agar plates showed 1-2mm greyish-green colonies with a black sunken centre and a black halo on a cherry-red background following aesculin hydrolysis characteristic off all *Listeria* spp. (Plate 4. 2) which are phenotypically very similar, only distinguishable by a combination of test and molecular methods.

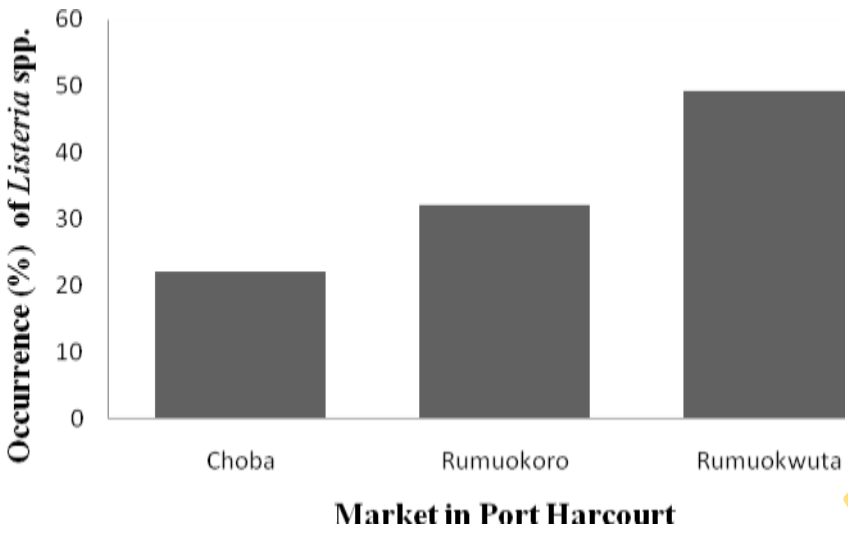


Fig. 4. 1: Occurrence (%) of *Listeria* spp. from the three markets in Port Harcourt

Table 4.1: Detection of *Listeria* species in raw beef and goat meat

Meat type	Number of samples		
	EXAMINED	POSITIVE	%
Beef flesh	36	19	52.78
Beef intestine	20	8	40.00
Beef kidney	32	9	28.13
Beef liver	34	12	35.29
Goat flesh	36	11	30.56
Goat intestine	18	5	27.78
Goat kidney	28	5	17.86
Goat liver	36	12	33.33
Total	240	81	33.75

Plate 4.1: A 48h secondary enrichment full-strength Fraser broth showing the characteristic darkening colouration by *Listeria* spp., following aesculin hydrolysis (A) and a control (B)

A

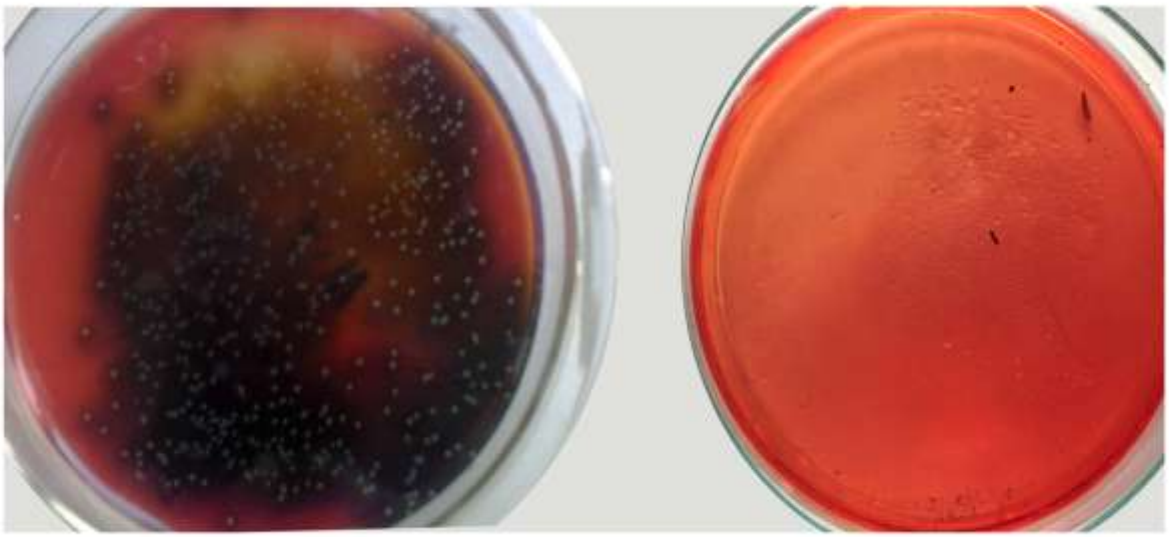


Plate 4.2: A 48-hour culture of *Listeria species* on PALCAM Agar plate showing typical diagnostic features of cherry pigmentation around the colonies and diffusion into the medium (A) and a control plate (B).

4.5 Morphology of *Micrococcus* species on PALCAM agar

PALCAM agar with its supplement added undoubtedly inhibited quite a number of bacteria isolated from the meat samples on Nutrient agar. Nevertheless, *Micrococcus sp*. grew alongside *Listeria* on some plates. *Micrococcus sp* on PALCAM is 2-4mm yellow colonies with a yellow halo as presented in Plate 4.3.

4.6 Physiological and Biochemical Characterization of *Listeria* species isolated from meat samples.

The summary of the physiological and biochemical tests confirming and differentiating the isolated *Listeria monocytogenes* and other *Listeria* species is presented in Table 4.2. They were all Gram-positive rods, catalase positive, oxidase negative and motile. However, variations were noticed with their haemolysis of sheep blood and sugar fermentation.

4 .7 Distribution of *Listeria* species in raw meat parts

The distribution of the varoius *Listeria* species following biochemical and molecular characterization in the meat parts sampled is presented in Table 4.3. The highest was from beef flesh, 74 (23.87%), followed by goat liver, 51(16.45%), next was beef liver, 46(14.84%) and the least was from goat kidney,17(5.48%). The occurence (%) of the various *Listeria* species show that *Listeria monocytogenes* and *L. ivanovii* occured 4 times each (1.29%), *Listeria innocua*, 20 (6.45%), *L. seeligeri*, 72(23.23%), *L. grayi*, 71 (22.90%) and *L. welshimeri*, 139 (44.84 %). The occurrence of *L. monocytogenes* and other *Listeria* species in the various meat types and parts were statistically significant at p=0.05. APPENDIX A.

4.8 Distribution (month, year) of occurrence of *Listeria* species in retailed meat samples

The percentage monthly distribution of all isolated *Listeria* species from March 2011 to February 2012 is presented in Figure 4.3. The highest occurrence was in July, 49(15.81%), followed by November, 47 (15.16%), next was August, 39(12.58%), while the least was in April, 6(1.94%). It is obvious from the results that the occurrence was more in the pick of the raining season months than the dry season months even when the dry season months were in different years.

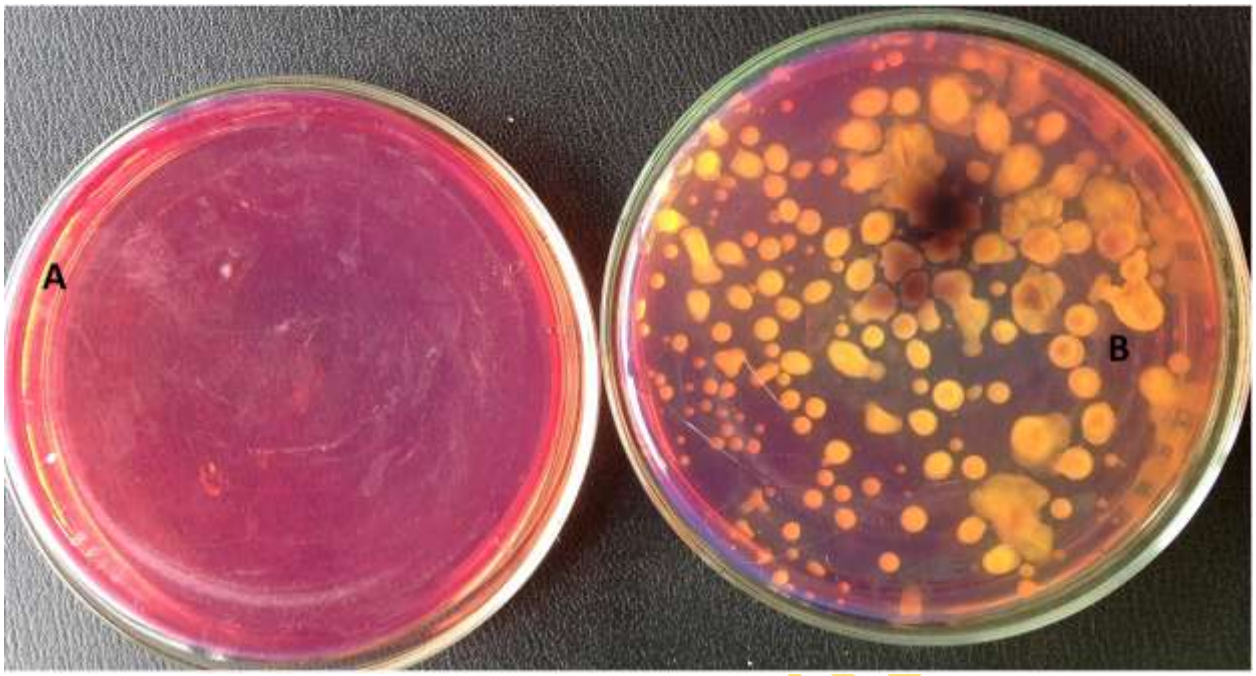


Plate 4.3: A 24-hour culture of *Micococcus* spp. on PALCAM agar plate showing 2-4mm yellow colonies with a yellow halo (B) and a control plate (A).

Table 4.2. Physiological and Biochemical Characterization of *Listeria* species isolated from meat samples.

Gram reaction/shape	Catalase	Oxidase	Haemolysis	Rhamnose	Mannitol	Xylose	*Listeria* Species
+ve/rod	+	-	+	+	-	-	*L. monocytogenes*
+ve/rod	+	-	-	v	-	-	*L. innocua*
+ve/rod	+	-	-	-	+	-	*L. grayi*
+ve/rod	+	-	+	-	-	+	*L. seeligeri*
+ve/rod	+	-	+w	-	-	+	*L. ivanovii*
+ve/rod	+	-	-	v	-	+	*L. welshimeri*

+= Positive; -= negative; v= variable; w= wide zone of haemolysis.

Table 4.3: Distribution of *Listeria* species isolated from various meat types/parts.

Listeria sp.	Number of *Listeria* species isolated from each meat type/parts								Total	Occurrence (%)
	BF	BL	BK	BL	GF	GI	GK	GL		
L. monocytogenes	0^{a}	0^{a}	0^{a}	0^{a}	3^{a}	1^{a}	0^{a}	0^{a}	4	1.29
L. innocua	5^{b}	3^{a}	1^{a}	3^{a}	1^{a}	0^{a}	1^{a}	6^{a}	20	6.45
L. seeligeri	18^{c}	1^{a}	13^{b}	8^{b}	9^{b}	4^{a}	7^{b}	12^{b}	72	23.23
L. ivanovii	2^{d}	0^{b}	1^{b}	1^{c}	0^{c}	0^{b}	0^{b}	0^{c}	4	1.29
L. grayi	14^{e}	8^{c}	10^{c}	11^{d}	9^{c}	6^{c}	1^{c}	12^{c}	71	22.90
L. welshimeri	35^{f}	17^{d}	16^{d}	23^{e}	11^{d}	8^{d}	8^{d}	21^{d}	139	44.84
Total	74	29	41	46	33	19	17	51	310	100.00

[a] Means with different superscripts for each *Listeria* species are not equal (p=0.05). Means with the same superscripts are equal. APPENDIX A
BF= beef flesh; BI= beef intestine; BK= beef kidney; BL= beef liver; GF= goat flesh; GI= goat intestine; GK= goat kidney; GL= goat liver.

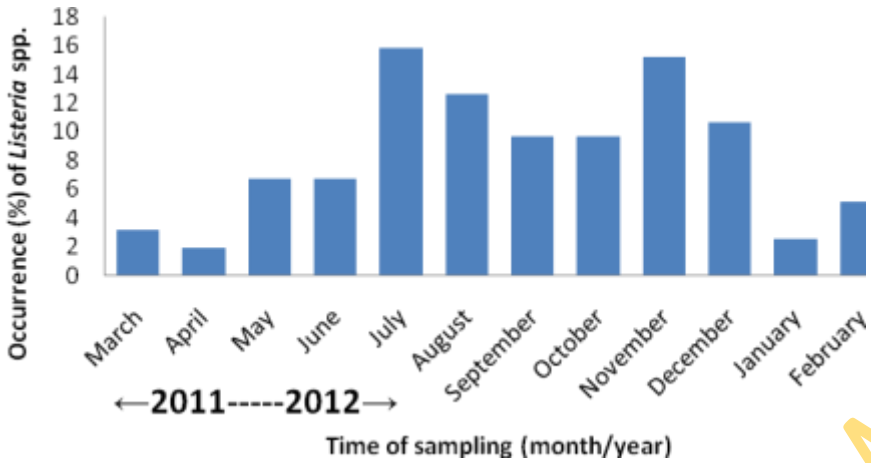


Fig. 4.3: Distribution (month/year) of occurrence of *Listeria* spp. in retailed meat samples purchased from 3 markets in Port Harcourt.

4.9 **Physiological and biochemical characterization of *Micrococcus* species and other bacteria isolated from meat samples on Nutrient agar.**

The physiological and biochemical characterization of *Micrococcus* sp. that grew on PALCAM and 48 other bacteria isolates from the beef and goat meat on Nutrient agarare presented on Table 4.4. They were identified tentatively as: *Escherichia coli,* 7 (14.58%); *Serratia marcenscens,* 2 (4.17%); *Staphylococcus* spp., 6(12.50%); *Micrococcus* spp., 15 (31.25%); *Cronobacter* spp., 8 (16.67%); *Enterococcus* spp., 4 (8.33%) and *Bacillus cereus,* 6(12.50%).

4.10 **Molecular characterization of *Listeria* species**

4.10.1 Gel chromatogram obtined using LI1 and U1 primer for all *Listeria* species

Plate 4.4 shows the gel chromatogram obtained when genomic DNA of *L. monocytogenes* PCM 2191serovar 01/2 strain and representative of each isolated *Listeria* species were subjected to PCR using LI1 and U1 primer combination for all *Listeria* species. Lanes: M, molecular weight standards; 1, *L.innocua;* 2, *L. welshimeri*; 3 *L. seeligeri*; 4, *L. grayi*; 5, *L. ivanovii*; 6, *L. monocytogenes*; 7, *L. monocytogenes* PCM 2191(positive control); *8,* control reaction (all reagent ingredients except chromosomal DNA).

4.10.2 Gel chromatogram obtained using LM1 and LM2 for *L. monocytogenes*

Plate 4.5 shows the gel chromatogram obtained when genomic DNA of *L. monocytogenes* PCM 2191 serovar 01/2 strain and representative of each isolated *Listeria* species were subjected to PCR using LM1 and LM2 primer combination specific for *Listeria monocytogenes*. A PCR product of 1,200bp was observed. Lanes: M, molecular weight standards; 1, control reaction (all reagent ingredients except chromosomal DNA); 2, *L. monocytogenes* PCM 2191 serovar 01/2; 3, *L.monocytogenes*; 4, *L.seeligeri*; 5, *L.ivanovii*; 6, *L.grayi*; 7, *L.innocua ;* 8, *L. welshimeri.*

4.10.3 Gel chromatogram obtained using Siwi2 and Lis1B primer for *L. seeligeri, L. ivanovii* and *L. welshimeri*

Plate 4.6 shows the gel chromatogram obtained when genomic DNA of *L. seeligeri,*

Table 4.4: Morphological, Physiological and Biochemical characterization of other isolates from retailed meat samples purchased from Choba, Rumuokoro and Rumuokwuta markets

Gram	Reaction	Shape	Spore	staining	Catalase	Oxidase	Citrate	Indole	Methyl red	Voges-Proskauer	H_2S	Starch	hydrolysis	Gelatin	liquefaction	Urease	activity	Nitrate	reduction	Glucose	Lactose	Sucrose	Mannitol	motility	Tentative identity
-		R	-		+	-	-	+	+	-		-		-						AG	A	A	AG	+	*Esherichia coli*
-		R	-		+	-	+	-	-	+		-		+/-						AG	-	AG	AG	+	*Serratia marcescens*
+		C	-		+	-	+	-	-	-		-		-		+/-		+		AG	AG		AG	-	*Staphylococcus* spp.
+		C	-		+	-	+	-	+	-		-		+		-		-		AG	-	A	-	-	*Micrococcus* spp.
-		R	+		+	-	+	-	-	+		-		+		-		+		AG	AG	AG	A	+	*Cronobacter* spp.
+		C	-		-	-	-	-	+	-		-		-		-		-		AG	AG	AG	-	-	*Enterococcus* spp.
+		R	+		+	+/-	+	-	-	+		-		+		+/-		+		A	A	-	A	+	*Bacillus cereus*

+ = Positive; - = Negative; A = Acid; G =Gas production; R = Rod; C= Cocci

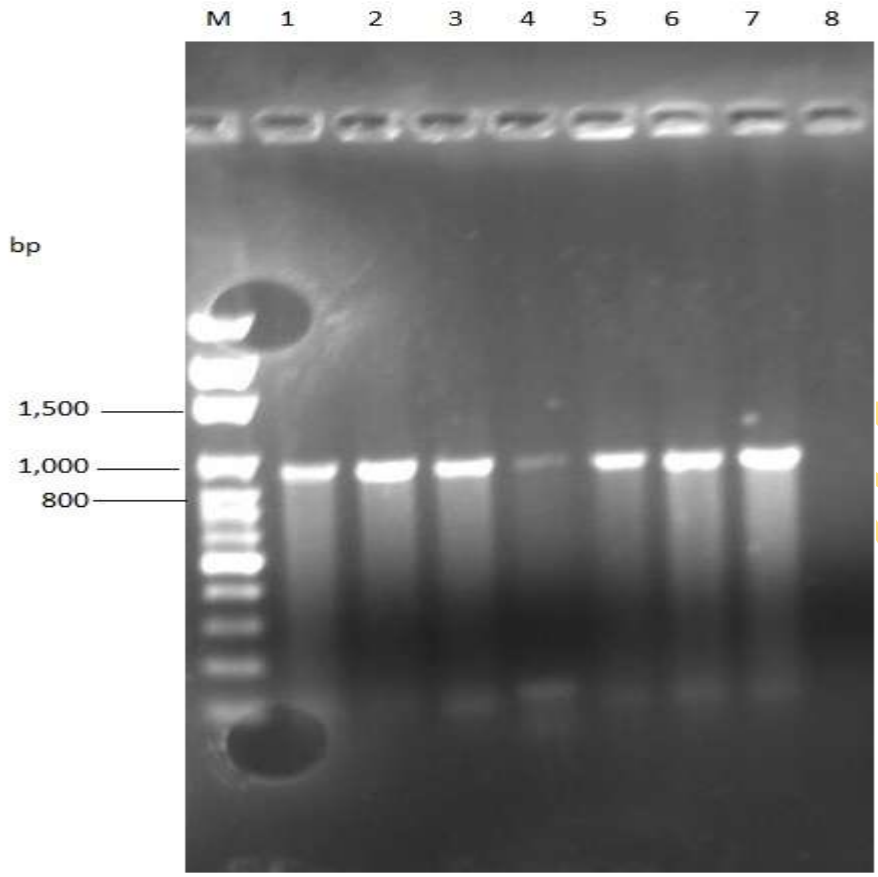


Plate 4.4: Gel chromatogram of genomic DNA of *L. monocytogenes* PCM 2191serovar 01/2 strain and representative of each isolated *Listeria* species (IS1-IS4). Lanes: M, molecular weight standards; 1, *L.innocua;* 2, *L. welshimeri*; 3 *L. seeligeri*; 4, *L. grayi*; 5, *L. ivanovii*; 6, *L. monocytogenes*; 7, *L. monocytogenes* PCM 2191(positive control)*; 8*, control reaction (all reagent ingredients except chromosomal DNA).

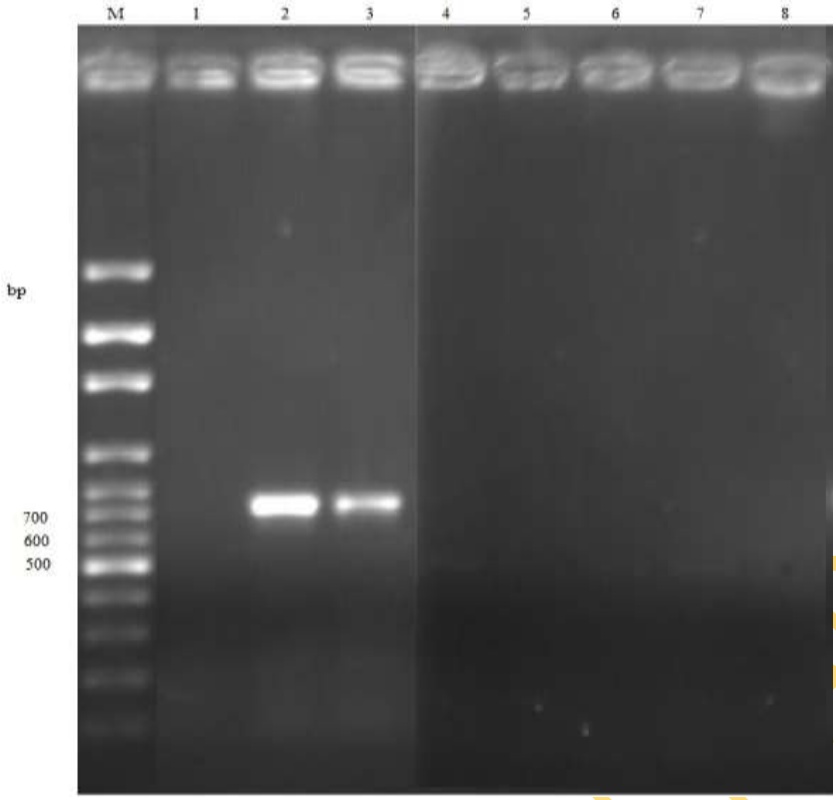


Plate 4.5: Gel chromatogram of genomic DNA of *L. monocytogenes* PCM 2191 serovar 01/2 strain and representative of each isolated *Listeria* species (IS1- IS4). Lanes: M, molecular weight standards; 1, control reaction (all reagent ingredients except chromosomal DNA); 2, *L. monocytogenes* PCM 2191 serovar 01/2; 3, *L. monocytogenes*; 4, *L. seeligeri*; 5, *L. ivanovii*; 6, *L. grayi*; 7, *L. innocua ;* 8, *L. welshimeri*

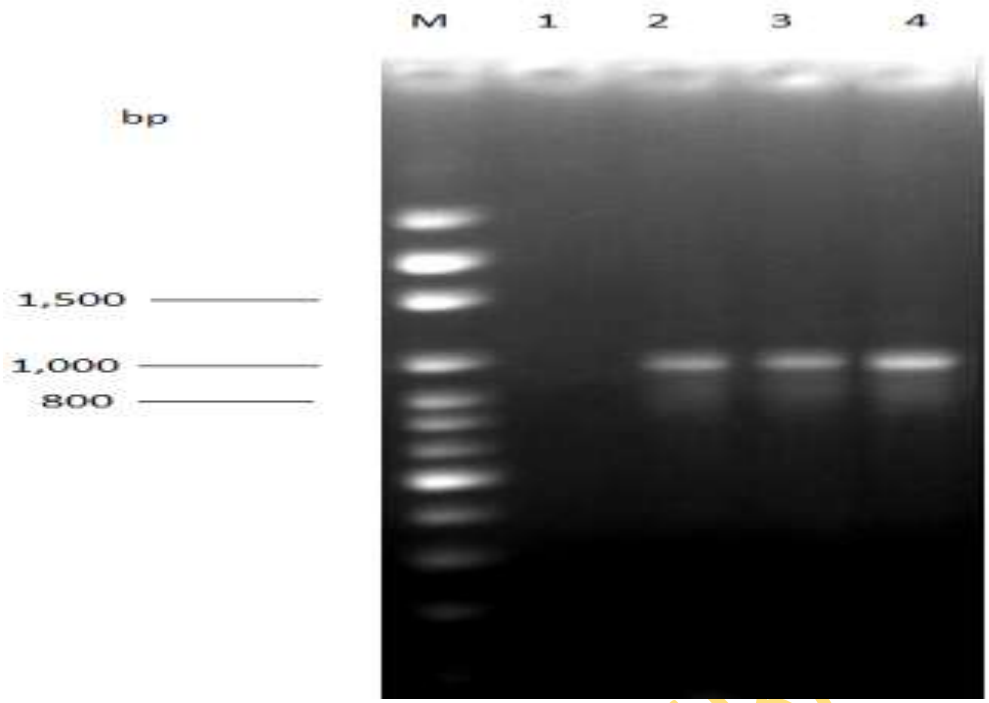


Plate 4.6: Gel chromatogram of genomic DNA of *Listeria seeligeri, L. ivanovii* and *L. welshimeri*. Lanes: M, molecular weight standards; 1, control reaction (all reagent ingredients except chromosomal DNA); 2, *L. ivanovii*; 3, *L.welshimeri*; 4, *L.seeligeri* .

L. ivanovii and *L. welshimeri* were sujected to PCR using Siwi2 and Lis1B primer combination for *Listeria seeligeri, L. ivanovii* and *L. welshimeri.* A PCR product of 1.200 bp was observed. Lanes: M, molecular weight standard; 1, control reaction (all reagents except chromosomal DNA); 2, *L. ivanovii; 3, L. welshimeri; 4, L. seeligeri*

4.11 Determination of species and virulence of *L. monocytogenes*

4.11.1 *Listeria monocytogenes* species-specific recognition using in1A-F and in1A-R and, *in1B-F* and *in1B-R* primer combination

Plate 4.7: shows the gel chromatogram obtained when total genomic DNA from *L. monocytogenes* PCM 2191 serovar 01/2 strain and isolated *Listeria monocytogenes* were subjected to electrophoresis using in1A-F and in1A-R primer combination for *L.monocytogenes* species-specific recognition. A PCR product of 800bp was observed for only the *L. monocytogenes* PCM 2191 serovar 01/2 strain. Lanes: 1, *L. monocutogenes* PCM 2191 serovar 01/2 strain; 2-5, isolated *L.monocytogenes*; 6, control reaction (all reagent ingredients except chromosomal DNA); M, molecular weight standard.

Plate 4.8 shows the gel chromatogram obtained when total genomic DNA from the *L. monocytogenes* PCM 2191 serovar 01/2 strain and isolated *Listeria monocytogenes* were subjected to PCR using *in1B-F* and *in1B-R* primer combination for *L.monocytogenes* species-specific recognition. A PCR product of 884bp was observed for only the *L. monocytogenes* PCM 2191 serovar 01/2 strain. Lanes: M, molecular weight standards; 1, control reaction (all reagent ingredients except chromosomal DNA); 2, *L. monocytogenes* PCM 2191 serovar 01/2 strain;3-6,isolated *L.monocytogenes* .

4.12 Determination of virulence in *L. monocytogenes* using *in1J-F* and *in1J-R* and, *in1C-F* and *in1C-R* primer combination

Plate 4.9 shows the gel chromatogram obtained when total genomic DNA from *L. monocytogenes* PCM 2191 serovar 01/2 strain and isolated *Listeria monocytogenes* were subjected to PCR using in1J-F and in1J-R primer combination for virulence determination of *L.monocytogenes*. Lanes: M, molecular weight standards; 1, control reaction (all reagent ingredients except chromosomal DNA); 2, *L. monocytogenes*

PCM 2191 serovar 01/2; 3-6, isolated *L.monocytogenes*. Three of the isolated *L. monocytogenes* (lanes: 3,4 and5) along side the reference *L. monocytogenes* PCM 2191 serovar 01/2 strian (lane 2) had the expected 238 base pair (bp); confirming the presence of the virulence gene.

Plate 4.10 shows the chromatogram obtained when total genomic DNA from the *L. monocytogenes* PCM 2191serovar 01/2 strain and isolated *Listeria monocytogenes* were subjected to PCR using *in1C-F* and *in1C-R* primer combination for virulence determination of *L.monocytogenes*. Lanes: 1, *L. monocytogenes* PCM 2191 serovar 01/2; 2-5, isolated *L.monocytogenes*; 6, control reaction (all reagent ingredients except chromosomal DNA); M, molecular weight standards. Two of the isolated *L. monocytogenes* (lanes: 4 and 5) along side the reference *L. monocytogenes* PCM 2191 serovar 01/2 strian (lane 1) produced the expected 517 base pair(bp) , indicative of the presence of the virulence gene.

4.13 **Antibiotic susceptibility of *Listeria* species**

The antibiotic sensitivity pattern of a strain of *Listeria monocytogenes* from goat meat in Port Harcourt on Mueller Hilton agar is presented in Plate 4.11. Varying zones of inhibition were observed against gentamycin (GE), chloramphenicol (CH), tetracycline (TE), erythromycin (ER) and vancomycin (V). Zones of inhibition were not observed against amoxicillin (AM), augumentin (AU) and cloxacillin (CX), oxacilline (O), and cotrimoxazole (CT).

The distribution of antibiotic resistant *Listeria* isolates against the ten antibiotics employed in this study is presented in Table 4.8. All the isolates and *L. monocytogenes* PCM 2191 serovar 01/2 were susceptible to gentamycin (GEN) and vancomycin (VAN), and resistant to amoxicillin (AMX), augumentin (AUG) and cloxacilin (CXC). Varying resistance was observed against tetracycline (88.5%), oxacillin (73.6%), erythromycin (43.7%), chloramphenicol (43.7%) and cotrimoxazole (33.3%).

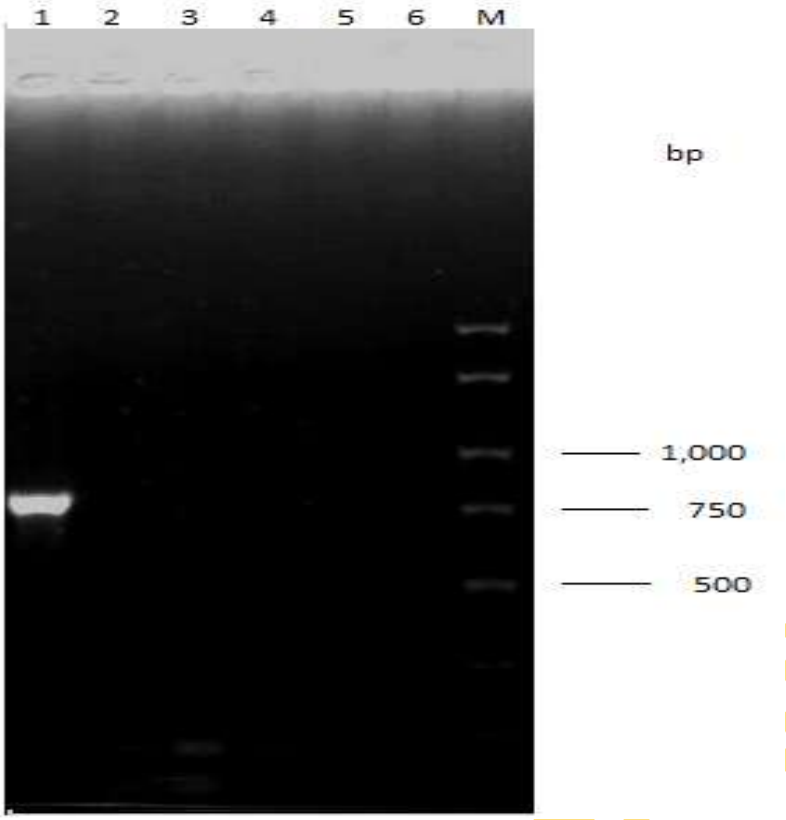


Plate 4.7: Gel chromatogram of genomic DNA of *L. monocytogenes* PCM 2191 serovar 01/2 strain and isolated *Listeria monocytogenes* (IS1-IS4). Lanes: 1, *L. monocytogenes* PCM 2191 serovar 01/2 strain; 2-5, isolated *L.monocytogenes*; 6, control reaction (all reagent ingredients except chromosomal DNA); M, molecular weight standards.

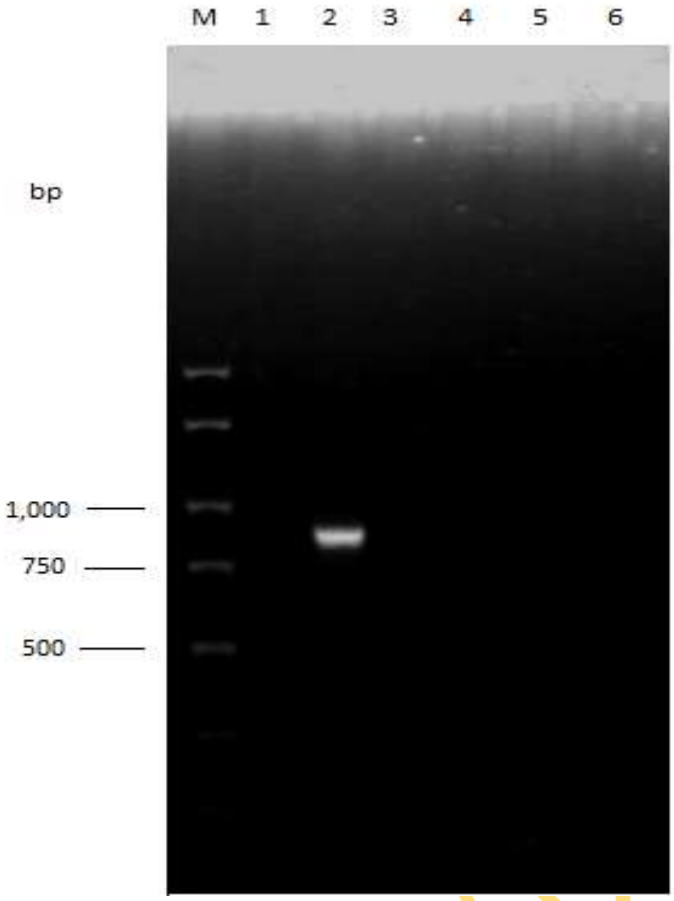


Plate 4.8: Chromatogram of genomic DNA of the *L. monocytogenes* PCM 2191 serovar 01/2 strain and isolated *Listeria monocytogenes* (IS1-IS4). Lanes: M, molecular weight standard; 1, control reaction (all reagent ingredients except chromosomal DNA); 2, *L. monocytogenes* PCM 2191 serovar 01/2; 3-6, isolated *L.monocytogenes* .

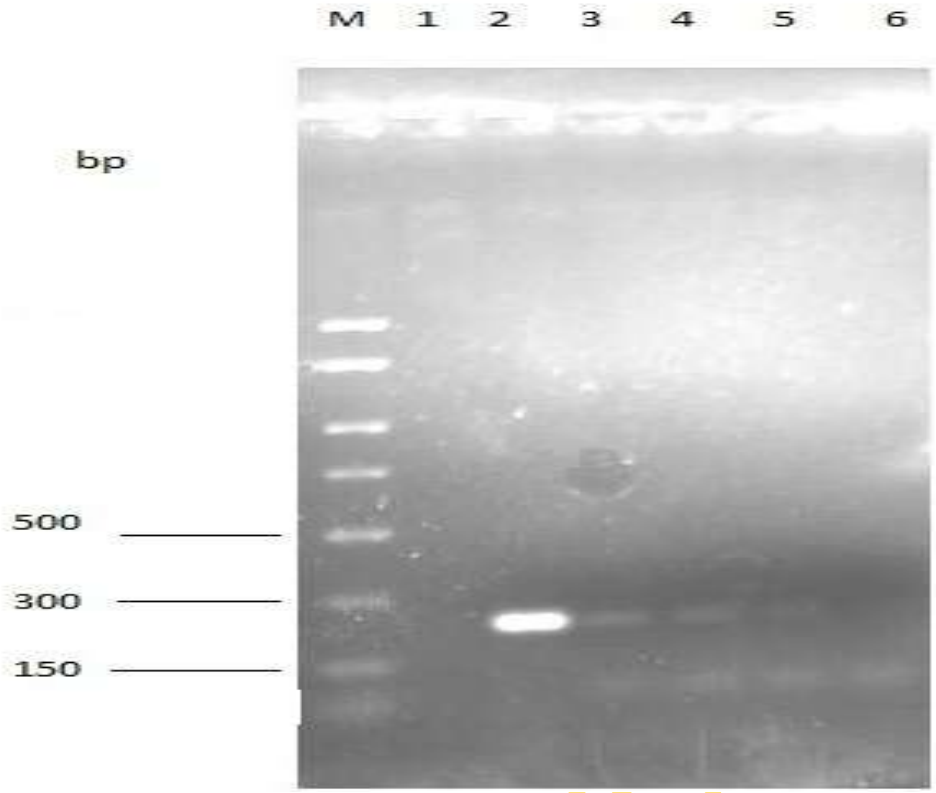


Plate 4.9: Gel chromatogram of genomic DNA of *L. monocytogenes* PCM 2191 serovar 01/2 strain and isolated *Listeria monocytogenes* (IS1-IS4). Lanes: M, molecular weight standards; 1, control reaction (all reagent ingredients except chromosomal DNA); 2, *L. monocytogenes* PCM 2191 serovar 01/2; 3-6, isolated *L.monocytogenes*.

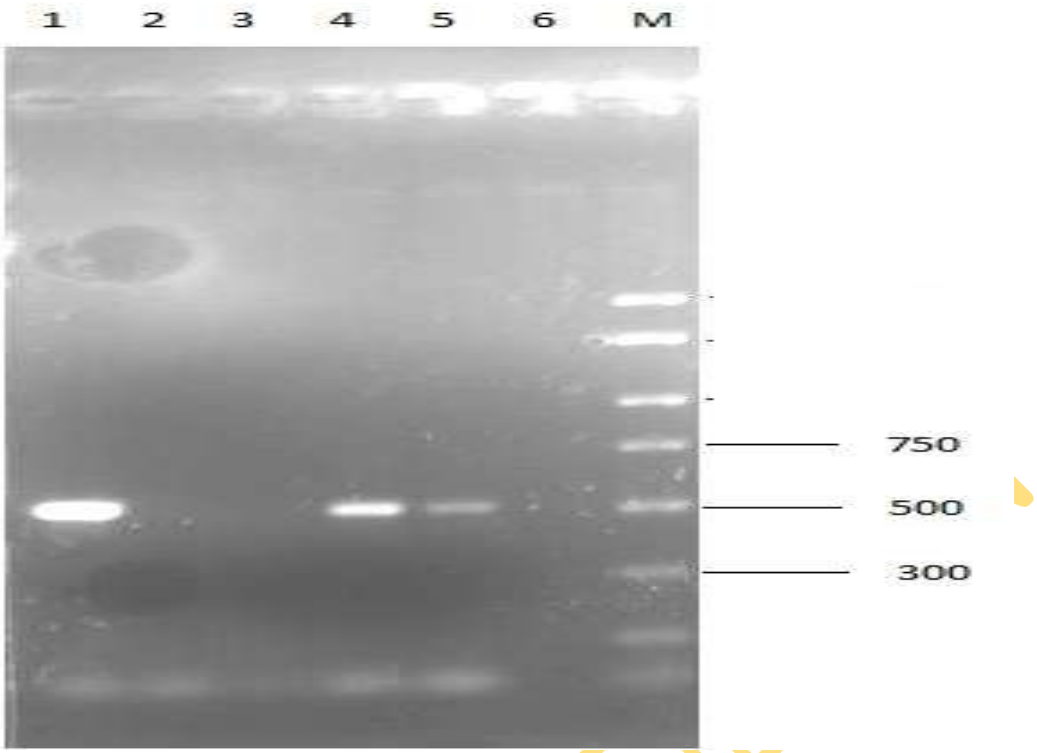


Plate 4.10: Gel chromatogram of genomic DNA of *L. monocytogenes* PCM 2191serovar 01/2 and isolated *Listeria monocytogenes* (IS1-IS4). Lanes: 1, *L. monocytogenes* PCM 2191 serovar 01/2 strain; 2-5, isolated *L. monocytogenes*; 6, control reaction (all reagent ingredients except chromosomal DNA); M, molecular weight standards.

Plate 4.11: Antibiotic sensitivity pattern of a *Listeria monocytogenes* isolated from raw goat meat on Mueller Histon agar. V, vancomycin; O, oxacillin; TE, tetracycline; ER, erythromycin; AM, amoxicillin; AU, augumentin; CH, chloramphenicol; CT, cotrimoxazole; GE, gentamycin; CX, cloxacillin.

Table 4.5: Distribution of antibiotic resistant *Listeria* isolates against ten antibiotics according to species.

Listeria species	No. tested	AUG 30µg/disc	AMX 25µg/disc	ERY 5g/disc	TET 10µg/disc	CXC 5µg/disc	GEN 10µg/disc	COT 25µg/disc	CHL 30µg/disc	OXA 1µg/disc	VAN 30µg/disc
L. monocytogenes	5	5	5	5	5	5	0	1	2	4	0
L. innocua	20	20	20	20	20	20	0	4	0	8	0
L. ivanovii	4	4	4	2	4	4	0	1	2	0	0
L. seeligeri	72	72	72	27	51	72	0	27	40	70	0
L. grayi	71	71	71	32	65	71	0	35	35	64	0
L. welshimeri	139	139	139	50	125	139	0	35	57	83	0
Total	311	311	311	136	270/305	311	0	103/309	136	229	0
% resistance		100	100	43.7	88.5	100	0	33.3	43.7	73.6	0

AUG, augumentin; AMX, amoxacylin; ERY, erythromycin; TET, tetracycline; CXC, cloxacillin; GEN, gentamycin; COT, cotrimoxazole; CHL, chloramphenicol; OXA, oxacilline and VAN, vancomycin.

4.14 **Plasmid profile of *L. monocytogenes***

The plasmid profile of *L. monocytogenes* PCM 2191 serovar 01/2 and isolated *Listeria monocytogenes* is presented in Plate 4.12. Lanes: M, molecular weight standards; 1, *L. monocytogenes* PCM 2191 serovar 01/2; 2-5, isolated *L.monocytogenes* . There was no evidence of the presence of plasmids indicating that the resistance of the isolates to selected antibiotics may not be plasmid-mediated.

4.15 **Anti-listerial activity of crude aqueous and ethanol extract of thyme**

The inhibitory effect of the crude ethanol extract of thyme on *L. monocytogenes* isolated from raw goat meat in Port Harcourt is presented in Plate 4.13. All the concentrations (0.1%, 0.5%, 1.0%, 2.5% and 5.0%) employed inhibited the growth of the strains. No inhibition was observed in the control.

The results of the anti-listerial effects of ethanol extract of thyme are presented in Table 4.6. All the concentrations employed inhibited *L. monocytogenes* PCM 2191 serovar 01/2 and the four *L. monocytogenes* strains isolated from raw goat meat with the zones of inhibition decreasing with a decrease in concentration of the spice.

The results of the anti-listerial effects aqueous extract of thyme are presented in Table 4.7. Only concentrations ranging from 0.5% to 5.0% had inhibitory effects on *L. monocytogenes* PCM 2191 serovar 01/2 and four *L. monocytogenes* strains isolated from raw goat meat in Port Harcourt. No inhibition was observed with 0.1% concentration.

Table 4.8 shows the anti-listerial effect of aqueous extract of brown pepper against *L. monocytogenes* PCM 2191 serovar 01/2 and four *L. monocytogenes* strains isolated from raw goat meat in Port Harcourt. No inhibition was observed with the 0.1% and 0.5% concentrations. The inhibition zones for concentrations 5.0, 2.5 and 1.0 were in decreasing order.

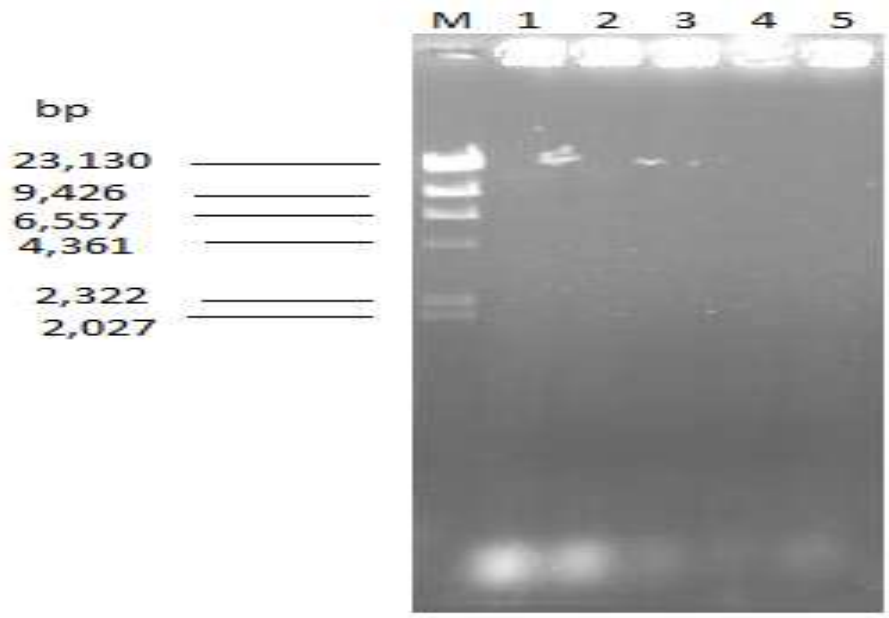


Plate 4.12: Plasmid profile of *L. monocytogenes* PCM 2191 strain and isolated *Listeria monocytogenes* (IS1-IS4). Lanes: M, molecular weight standards; 1, *L. monocytogenes* PCM 2191 serovar 01/2; 2-5, isolated *L.monocytogenes*

Plate 4.13: Inhibition zones produced by ethanol extract of thyme against *Listeria monocytogenes* at concentrations ranging from 0.1 to 5.0% (w/v). CT= control (ethanol); 1= 5.0%; 2=2.5%; 3=1.0%; 4=0.5% and 5=0.1%.

Table 4.6: Sensitivity of a strain of *L. monocytogenes* to different concentrations of crude ethanol extract of thyme.

Concentration of extract (%)	Zones of inhibition (mm)				
	PCS	IS1	IS2	IS3	IS4
5.0	18.0 ±1.0	16.0 ±0.0	15.0 ±0.0	16.0 ±0.0	15.0 ±0.0
2.5	16.0 ±1.0	15.0 ±0.0	14.5 ±0.0	14.0 ±0.0	14.0 ±0.0
1.0	14.0 ±0.0	12.0 ±0.0	13.0 ±0.0	13.0 ±0.0	12.5 ±0.0
0.5	12.0 ±0.0	11.0 ±0.0	12.0 ±0.0	12.0 ±0.0	10.0 ±0.0
0.1	11.0 ±0.0	11.0 ±0.0	11.5 ±0.0	10.0 ±0.0	9.0 ±0.0
Control	NI	NI	NI	NI	NI

PCS= *L. monocytogenes* PCM 2191 serovar 01/2; IS1- IS4= *L. monocytogenes* isolated from raw goat meat in Port Harcourt; NI= No inhibition. Mean values are obtained from triplicate measurement using descriptive statistical analysis. APPENDIX B

Table 4.7: Sensitivity of strains of *L. monocytogenes* to different concentrations of aqueous extract of thyme.

Concentration of extract (%)	Zones of inhibition (mm)				
	PCS	IS1	IS2	IS3	IS4
5	16.0 ±0.0	15.0 ±0.0	14.0 ±0.0	14.0 ±0.0	13.0 ±0.0
2.5	12.0 ±1.0	12.0 ±0.0	12.0 ±0.0	13.0 ±0.0	11.0 ±0.0
1.0	11.0 ±1.0	10.0 ±0.0	10.0 ±0.0	10.0 ±0.0	9.0 ±0.0
0.5	9.0 ±0.0	8.0 ±0.0	7.0 ±0.0	8.0 ±0.0	8.0 ±0.0
0.1	NI	NI	NI	NI	NI

NI= No inhibition; PCS= *L. monocytogenes* PCM 2191 serovar 01/2; IS1- IS4= *L. monocytogenes* isolated from raw goat meat in Port Harcourt. Mean values are obtained from triplicate measurement using descriptive statistical analysis.

APPENDIX B

Table 4.8: Sensitivity of strains of *L. monocytogenes* to different concentrations of aqueous extract of brown pepper.

Concentration of extract (%)	Zones of inhibitions (mm)				
	PC	IS1	IS2	IS3	IS4
5	16.0 ±0.0	15.0±0.0	14.0 ±0.0	14.0 ±0.0	13.0 ±0.0
2.5	12 ±1.0	12.0 ±0.0	12.0 ±0.0	13.0 ±0.0	11.0 ±0.0
1.0	8.0 ±0.0	7.0 ±0.0	5.0 ±0.0	6.0 ±0.0	5.0 ±0.0
0.5	NI	NI	NI	NI	NI
0.1	NI	NI	NI	NI	NI

NI= No inhibition; PCS= *L. monocytogenes* PCM 2191 serovar 01/2; IS1- IS4= *L. monocytogenes* isolated from raw goat meat in Port Harcourt. Mean values are obtained from triplicate measurement using descriptive statistical analysis.

APPENDIX B

4.16 **Autoclaved meat pieces**

The pieces of meat after the autoclaving are presented in Plate 4.14. The meat pieces initially weighed 1.5g, but after autoclaving weighed 0.9 to 1.0g each.

4.17 **Effects of aqueous extract of thyme on *L. monocytogenes* attached to meat pieces**

The effect of thyme during the attachment of *L. monocytogenes* PCM 2191 serovar 01/2 and four *L. monocytogenes* strains isolated from raw goat meat in Port Harcourt, is shown in Figure 4.4. There was a noticeable decrease in the number of cells attached to the meat as the exposure time increases. The effect was pronounced with an isolated *L. monocytogenes* (IS3) compared to the *L. monocytogenes* PCM 2191 serovar 01/2 (PCS).

4.18 **Effects of aqueous extract of thyme on *L. monocytogenes* growth in meat pieces**

The effect of 1.0% aqueous extract of thyme on the growth of *L. monocytogenes* PCM 2191 serovar 01/2 and four *L. monocytogenes* strains isolated from raw goat meat pieces stored at 30^{o}C is shown in Table 4.9. The numbers of *L. monocytogenes* on the thyme treated meat pieces were consistently lower than the untreated pieces. There was a significant difference in the mean count (Log_{10}CFU/g) of the untreated and treated meat pieces at p=0.05.

The effect of 1.0% aqueous extract of thyme on the growth of *L. monocytogenes* on meat pieces stored at 10^{o}C is shown in Table 4.10. There was no significant difference between mean count (Log_{10} cfu/g) of the untreated and treated (p=0.05), although initial population increased from 5.40-6.62 to 8.48-8.81 log_{10} cfu/g and 5.20-6.46 to 8.23-8.49 log_{10} cfu/g respectively.

4.19 **Phytochemical screening of thyme**

The occurrence (%) of the phytochemical components (alkaloid, flavonoid, tannin and saponin) of the ethanol and aqueous extracts of *Thymus vulgris* (thyme) is presented in Table 4.11. The occurrence of the components was generally higher in ethanol extract than the aqueous extract.

Plate 4.14: Meat pieces, after autoclaving.

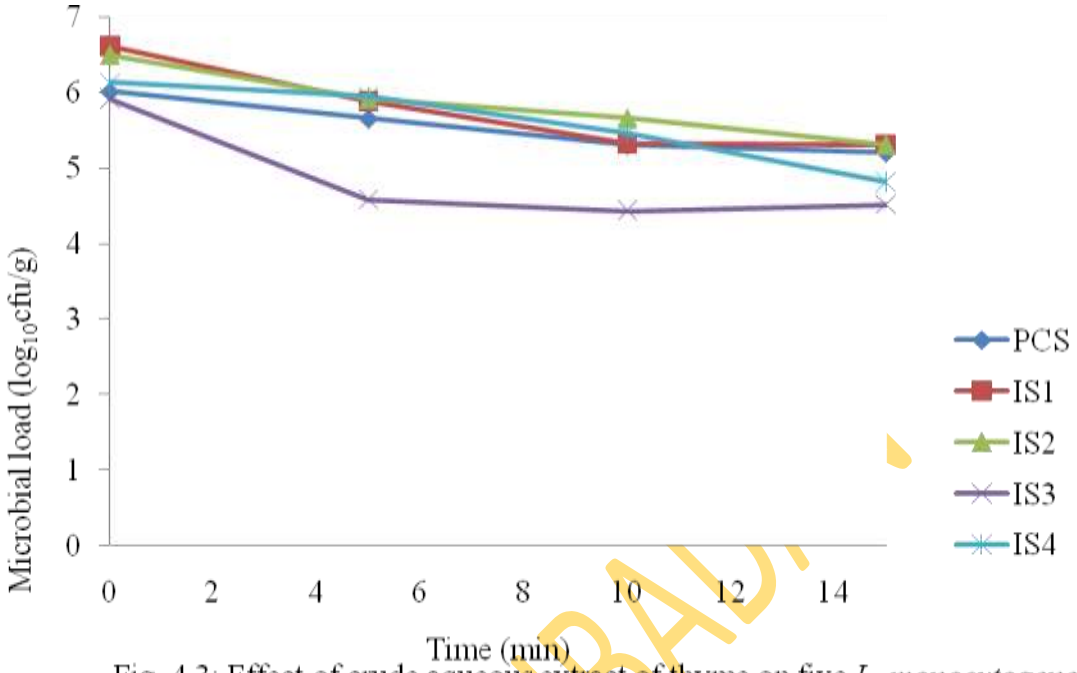


Fig. 4.3: Effect of crude aqueous extract of thyme on five *L. monocytogenes* attached to meat pieces within 15 min.

PCS= *L. monocytogenes* PCM 2191 serovar 01/2; IS1- IS4=Isolated *L. monocytogenes* isolated from raw goat meat in Port Harcourt.

Table 4.9: Effect of 1.0% aqueous extract of thyme on growth of five strains of *L. monocytogenes* on meat pieces stored at 30^{o}C for 4 day

Organism's code	Treatment	Mean count (Log_{10}CFU/g)/Time/days				
		0	1	2	3	4
PCS	Control	5.48^{a}	8.41^{d}	8.68^{f}	9.00^{c}	9.51^{d}
	Treated	5.45^{a}	8.04^{c}	8.36^{b}	8.41^{b}	9.30^{b}
IS1	Control	6.11^{b}	8.38^{d}	8.58^{e}	9.32^{d}	9.46^{c}
	Treated	6.00^{b}	8.11^{d}	8.49^{d}	9.04^{c}	9.34^{b}
IS2	Control	6.60^{d}	8.08^{c}	9.43^{g}	9.48^{d}	10.15^{c}
	Treated	6.40^{c}	8.23^{d}	8.32^{b}	8.48^{b}	9.20^{b}
IS3	Control	6.49^{d}	7.38^{b}	8.48^{c}	8.59^{b}	9.08^{b}
	Treated	6.46^{c}	7.20^{b}	8.43^{c}	8.49^{b}	8.53^{a}
IS4	Control	5.36^{a}	6.11^{a}	7.86^{a}	8.04^{a}	9.97^{e}
	Treated	5.28^{a}	7.60^{b}	7.81^{a}	7.90^{a}	9.87^{e}

PCS= *L. monocytogenes* PCM 2191 serovar 01/2; IS1- IS4= *L. monocytogenes* isolated from raw goat meat in Port Harcourt.

[a] Means with different superscripts for each *L. monocytogenes* are not equal (p=0.05). Means with the same superscripts are equal. APPENDIX C

Table 4.10: Effect of 1.0% aqueous extract of thyme on growth of *L. monocytogenes* on meat pieces stored at 10^{o}C for 15 days

Organism's code	Treatment	Mean count (Log CFU/g)/Time/days 0	3	6	9	12	15
PC	Control	5.51±0.12^{c}	7.59±0.12^{c}	7.66±0.12^{c}	7.95±0.12^{c}	8.38±0.16^{c}	8.57±0.14^{c}
	Treated	5.43±0.10^{c}	7.20±0.13^{c}	7.40±0.11^{c}	7.60±0.12^{c}	8.30±0.13^{c}	8.49±0.12^{c}
IS1	Control	6.04±0.23^{bc}	7.32±0.13^{c}	7.62±0.15^{c}	7.85±0.14^{c}	8.23±0.15^{c}	8.48±0.14^{c}
	Treated	6.00±0.55^{bc}	7.15±0.21^{bc}	7.56±0.39^{bc}	7.71±0.33^{b}	8.04±0.33^{bc}	8.46±0.33^{bc}
IS2	Control	6.62±0.30^{abc}	7.86±0.19^{bc}	7.98±0.22^{bc}	8.11±0.10^{bc}	8.38±0.17^{bc}	8.54±0.13^{bc}
	Treated	6.38b±0.17^{abc}	7.69±0.21^{bc}	7.73±0.19^{abc}	8.04±0.31^{abc}	8.28±0.30^{abc}	8.46±0.31^{abc}
IS3	Control	6.52±0.80^{abc}	7.71±0.74^{abc}	7.92±0.74^{abc}	8.32±0.72^{abc}	8.51±0.74^{abc}	8.61±0.73^{abc}
	Treated	6.46±0.26^{abc}	7.32±0.29^{abc}	7.75±0.28^{abc}	7.98±0.26^{abc}	8.00±0.30^{abc}	8.23±0.28^{abc}
IS4	Control	5.40±0.92^{ab}	6.38±0.92^{ab}	7.49±1.0^{ab}	7.78±1.10^{ab}	8.34±1.09^{bc}	8.81±1.10^{ab}
	Treated	5.30±0.49^{a}	6.20±0.49^{a}	7.41±0.48^{a}	7.52±0.56^{a}	8.28±0.55^{a}	8.32±0.56^{a}

PCS= *L. monocytogenes* PCM 2191 serovar 01/2; IS1- IS4= *L. monocytogenes* isolated from raw goat meat in Port Harcourt.

[a] Means with different superscripts for each *L. monocytogenes* are not equal (p=0.05). Means with the same superscripts are equal. APPENDIX D

Table 4.11: Percentage occurrence of Phytochemical components of Thyme extracts

Extract	Alkaloid (%)	Flavonoid (%)	Saponin (%)	Tannin (%)
TEE	13.3	39.2	47.0	0.5
TAE	8.9	39.3	51.2	0.6

TEE= Thyme Ethanol Extract and TAE= Thyme Aqueous Extract

CHAPTER 5

DISCUSSION

The importance of raw meat and meat products as a vehicle for the transmission of various diseases, especially in countries where hygienic standards are not strictly enforced has been well documented (Liu *et al*., 2007). It is not surprising that *L. monocytogenes* and other *Listeria* spp. were found in meat considering its widespread occurrence in nature, especially in plants, and their associations in nature with other bacteria that are well established in meats thus it is reasonable to find it in meats (Low and Donachie, 1997).

Overall, 33.75% (81 of 240) of all meat samples in this study were contaminated with *Listeria* species. The 81 positive samples were made up of 48 (59.26%) and 33(40.74%) for beef and goat meat, respectively. The highest percentage of occurrence was in beef flesh (52.78%), followed by beef intestine (40.00%); while the least occurrence was in goat kidney (17.86%). This incidence of *Listeria* in the meat samples examined is in agreement with the report of a 0 to 68% prevalence of *Listeria* in fresh meat in Portugal (Mena *et al*., 2004). This can be attributed to the consumption of contaminated silage or other feeds, fecal contamination during evisceration and from handlers and slaughterhouses (Border *et al*., 1990; Nørrung *et al*., 1999; Molla *et al*., 2004; Nightingale *et al*., 2004).

The percentage occurrence of *Listeria* spp. from the selected markets revealed highest occurrence in Rumuokwuta (49.32%), followed by Rumuokoro (32.10%) while the least was Choba (22.09%). Coincidentally, three of the isolated *L. monocytogenes* are from Rumuokwuta samples while one was from Choba samples

The isolated *Listeria* species, presented the darkening characteristics in full-strength Fraser broth containing supplement, following aesculin hydrolysis. On Polymixin acriflavin lithium chloride ceftazidime aesculin mannitol agar (PALCAM) agar, the colonies were about 2mm, greyish-green with a black sunken centre and a black halo on a cherry-red background as previously reported by Pagotto *et al*. (2001). Their morphology, physiological and biochemical reactions showed that they were all Gram-positive rods, catalase positive, oxidase negative and motile. However, variations were

noticed with their haemolysis of sheep blood and sugar fermentation. All other bacteria isolated from the meat samples on nutrient agar, did not grow on PALCAM agar with supplement, except *Micrococcus* spp. which grew alongside *Listeria* in some plates. The colonies which were yellowish-green with a yellow halo ranged from 2-4mm depending on the duration of incubation (Pagotto *et al*., 2001).

The distribution of the *Listeria* species following biochemical and molecular characterization in the meat parts sampled showed that the highest was from beef flesh,74 (23.87%), followed by goat liver, 51 (16.45%), next was beef liver, 46 (14.84%) and the least was from goat kidney, 17 (5.48%). The occurrence of *L. monocytogenes* and other *Listeria* species in the various meat types and parts were statistically significant (p=0.05).

In this study, *L. welshimeri was* the predominant *Listeria* species isolated from the cow and goat meat samples; other *Listeria* species were less common. This result differs from previous reports (Jay, 1996; Low and Donachie, 1997; Pagotto *et al*., 2001; Okutani *et al*., 2004; Molla *et al*., 2004; Kasek-Paszkowska *et al*., 2005), wherein *L. monocytogenes* and *L. innocua* were most often reported, with most investigators reporting one or the other to be most predominant and *L. welshimeri* coming third or next after *L. innocua*, followed by *L. seeligeri* and lastly *L. ivanovii* in meat among these five related species. *Listeria monocytogenes* accounted for 4(1.29%) of the 310 *Listeria* isolated. None of these *L. monocytogenes* was from the cow samples. Previous studies in Nigeria, India, Serbia and Bangkok, reported the inability to isolate *L. monocytogenes* in raw goat meat, beef and other meat type sampled (Ikeh *et al*., 2010; Molla *et al*., 2004; Ramaswamy *et al*., 2007; Pesavento *et al*., 2010; Indrawattana *et al*., 2011; Rahimi and Farzinezhadizadeh, 2012). However, a number of authors reported a 4.65% and 6.4% (Bulgaria), 5.1% (Ethiopia), 6.66% (India), 17.7% (Portugal), 20% (Greek), 31% (Denmark) and 35% (Spain) prevalence of *L. monocytogenes* from raw beef, goat meta and other meat types (Seeliger and Jones, 1986; Wiedmann *et al*., 1999; Uyttendaele *et al*.,1999; Vazquez-Boland *et al*., 2001; Vitas *et al*., 2004; Swaminathan and Gerner-Smidt, 2007; Ivanović *et al*., 2009); making it obvious that the occurrence of *L. monocytogenes* varies from one place and one author to the other.

The distribution of the *Listeria* species shows that the highest occurrence was in July (15.81%), closely followed by November (15.16%), next was August (12.58%) while the least was in April (1.94%). This is comparable with a high incidence of *Listeria* in

beef and goat meat during fall in Iran (Yucel *et al*., 2005), since the months of July and August are the peak of the raining season in Port Harcourt, Nigeria. It is obvious from the result that the occurrence was more in the peak of the raining season than in the dry season even when these dry season months were in different years.

The inability of the Lis1A and Lis1B, and MonoA and Lis1B primer combinations to produce the expected PCR product for all *Listeri*a species and *L. monocytogenes* respectively, resulted in the use of primer combination; LI1 and U1 and LM1 and LM2 targeting the more conserve genomes of all *Listeria* and *L. monocytogenes* respectively. However, the others produced the expected PCR product, amongst them Siwi2 and Lis1B for *L. seeligeri, L. ivanovii* and *L. welshimeri*, the most predominant of the *Listeria* species isolated.

A total of 48 other isolates from the cow and goat meat on nutrient agar were identified as *Escherichia coli*, 7 (14.58%); *Serratia marcenscens*, 2 (4.17%); *Staphylococcus* sp., 6(12.50%); *Micrococcus* sp., 15 (31.25%); *Cronobacter* sp.8 (16.67%); *Enterococcus* sp., 4 (8.33%) and *Bacillus cereus*, 6(12.50%) According to Bibek (2005) and Bhunia (2008), the microorganisms of meat surfaces include: *Listeria monocytogenes, Micrococcus* spp., *Staphylococcus* spp., *Clostridium* spp., *Bacillus* spp., *Lactobacillus* spp., *Brochotrix thermophacta*, *Salmonella* spp., *Escherichia coli, Serratia* spp. and *Pseudomonas* spp. *Listeria monocytogenes* is ranked first among these foodborne pathogenic bacteria in terms of death rate, far ahead of *Campylobacter, Salmonella and Escherichia coli* 0157:H7 (Naghmouchi *et al*., 2007).

With *L. monocytogenes* comprising a diversity of strains of varying pathogenicity, the ability to precisely track the strains involved in listeriosis outbreaks and speedily determine their pathogenic potential is critical for the control and prevention of further occurrences of this deadly disease (Liu, 2006). The phenotypic subtyping methods are generally less sensitive, have low differentiation ability and are not easy to reproduce, whereas the genotypic approaches are more sensitive and reliable (Shuckken *et al*., 2003).

The result of this investigation shows the inability of the genomic DNA of the isolated *L. monocytogenes* to form the expected band of 800bp and 884bp with the *inlA* and *inlB* respectively. The genomic DNA of *L. monocytogenes* PCM 2191 serovar 01/2 however, produced the 800bp and 884bp bands with the *inlA* and *inlB* primers. Liu *et al.* (2007) reported the failure of serotypes 4a-4e strains to produce expected band of 884bp with *inlB* primers whereas, the 36 strains tested produced the 800bp band with

inlA primers both individually and in multiplex. These findings of Liu *et al.* (2007) was in agreement with earlier report by Poyart *et al.* (1996), that *inlA* were consistently present in 68 *L. monocytogenes* they examined, regardless of the origin, serovar and virulence of the isolates. They also reported that the size of the amplified DNA fragments of repeats A and B of inlA were constant in all 68 isolates of *L. monocytogenes*. However, during routine confirmation of serotype designations of serotype 4b *L. monocytogenes* by multiplex PCR, Lee *et al* (2011) observed certain isolates with atypical profiles.

The *inlJ* gene primers produced the expected band of 238bp with *L. monocytogenes* PCM 2191 serovar 01/2 and 3 of the 4 isolated *L. monocytogenes*. The putative internalin gene *lmo*2821, designated *inlJ* has been confirmed to be a novel internalin gene directly involved in *L. monocytogenes* virulence (Sabet *et al*., 2005). It is present in *L. monocytogenes* strains/serotypes that are capable of causing human listerial outbreaks and mouse mortality but absent in avirulent, non-pathogenic strains/serotypes (Donmith *et al*., 2004b; Liu, 2004). According to Liu (2006), *inlJ* represents the target of choice for laboratory differentiation of virulent from avirulent *L. monocytogenes* strains. Liu *et al.* (2007) has observed some uncommon *L. monocytogenes* strains/serotypes (lineage IIIB strains F2-086 and R2-142) with virulence potential but lacking the *inlJ* gene.

The *inlC* gene primers produced the expected band of 517bp with *L. monocytogenes* PCM 2191 serovar 01/2 and 2 of the 4 isolated *L. monocytogenes*. According to Engelbrecht *et al.* (2006), internalin gene *inlC* is a known virulence protein in *L. monocytogenes* with a role in the post-intestinal stages of listerial infection. Only 1 of the 4 isolated *L. monocytogenes* and the *L. monocytogenes* PCM 2191 serovar 01/2 produced the expected 517 bp and 238 bp bands with the internalin *inlC* and *inlJ* primers respectively.

The presence of the *inlC* and/or *inlJ* genes in a given *L. monocytogenes* strain implies its potential virulence and its ability to cause mouse mortality via the intraperitoneal route; it does not necessarily indicate the certainty of the strains harbouring these genes to produce disease in humans via the conventional oral ingestion (Liu *et al*., 2007). This notwithstanding, any strain of *L. monocytogenes* should be considered potentially pathogenic for humans (Jacquet *et al*., 2002). The inability of the genomic DNA of *L. monocytogenes* isolated from goat meat to produce the expected 800bp and 884bp with the internalin *inlA* and/or *inlB* gene primers respectively remains to be unraveled since

they produced the 517bp and/or 238bp with the internalin *inlC* and *inlJ* gene primers and one with both primers.

Although most isolates of *L. monocytogenes* and other *Listeria* spp. are susceptible to antibiotics active against Gram-positive bacteria, resistance has been reported in isolates from sporadic clinical cases, food or the environment (Charpentier and Courvalin, 1999).

In this study, the antibiotic sensitivity of *L. monocytogenes* PCM 2191 serovar 01/2 alongside 86 strains of *Listeria* spp. isolated from the raw cow and goat meat was evaluated using the standard disk diffusion method. The results revealed that all the randomly selected *Listeria* strains, including *L. monocytogenes* PCM 2191 serovar 01/2 were sensitive to gentamicin (GEN) and vancomycin (VAN), and resistant to amoxicillin (AMX), augumentin (AUG) and cloxacillin (CXC). Varying resistance was observed against tetracycline (88.5%), oxacillin (73.6%), erythromycin (43.7%), chloramphenicol (43.7%) and cotrimoxazole (33.3%), giving credence to past reports of multiple-antibiotic resistance strains in Nigeria and other countries (David and Odeyemi, 2007; Umeh and Okpokwasili, 2009; Rahimi and Farzinezhadizadeh, 2012; Shini *et al*., 2012; Enurah *et al*., 2013; Vinothkumar *et al*., 2013). The findings of this study were in agreement with reports that 100% Listeria isolates were sensitive to vancomycin and gentamicin (Safdar and Armstrong, 2003; Swaminathan and Gerner-Smidt, 2007; Zhang *et al*., 2007; Arslan and Özdemir, 2008; Indrawattana *et al*., 2011) but not to augumentin (amoxicillin-clavulanate) which was reported to have inhibited the growth of 47 *Listeria* isolates tested by Arslan and Özdemir (2008). Yan *et al*. (2010) reported that 1 of 90 *L. monocytogenes* tested were each resistant to gentamicin and vancomycin. A number of authors have reported varying levels of resistance of *Listeria* spp. and *L. monocytogenes* to tetracycline, oxacillin, gentamycin, chloramphenicol, augumentin, cotrimoxazole and erythromycin (Srinivasan *et al*., 2005; Lyon *et al*., 2008; Umeh and Okpokwasili, 2009; Pesavento *et al*., 2010; Yan *et al*., 2010; Indrawattana *et al*., 2011; Rahimi and Farzinezhadizadeh, 2012; Wieczorek *et al*., 2012; Shini *et al*., 2012; Enurah *et al*., 2013; Vinothkumar *et al*., 2013) comparable to the result of this study.

David and Odeyemi (2007) reported highest resistance of *L. monocytogenes* to cloxacillin, while Shini *et al*. (2012) reported a 100% resistance of *L. innocua* to cloxacillin.

The occurrence of resistant strains is usually attributed to the acquisition of transposons or plasmids-carrying resistance determinants and transfer occurs mainly in the digestive tracts of animals and humans, frequently *Enterococcus* spp. and *Streptococcus* spp. (Charpentier *et al*., 1995; Charpentier and Courvalin, 1999); but the plasmid profile of *L. monocytogenes* PCM 2191 serovar 01/2 and the four isolated *L. monocytogenes* in this study proved otherwise, suggesting a possible sub-lethal antibiotic dose or selective antibiotic pressure-induced resistance (Witte, 1998; Mazel *et al*., 2000). Peterkin *et al*. (1992) and Vaz-Velho *et al*. (2001) reported their inability to detect plasmids in 79.5% (97 of 122) and 64.3% (36 of 56) *L. monocytogenes* on which plasmid profiling was performed, respectively; while Schlech (1988) reported that large outbreaks of *L. monocytogenes* epidemic have been attributed to nonplasmid-bearing strains. Biavasco *et al*. (1996) has reported the transfer of vancomycin resistance from enterococci to *L. monocytogenes* by conjugation under laboratory conditions. The multiple-antibiotic resistance observed in the non-pathogenic strains poses a threat of transferring same to other bacteria or *L. monocytogenes*. The range of antibiotics to which resistance was observed in the study is of a great concern since antibiotics such as erythromycin, tetracycline and chloramphenicol were previously effective in the treatment of listeriosis (Hof, 1991).

Many spices and herbs exert antimicrobial activity due to their essential oil fractions. A number of scientists have reported the in-vitro antimicrobial activity of crude extract and essential oils from oregano, thyme, sage, rosemary, clove, coriander, garlic, onions and other spices against both bacteria and molds (Shelef *et al*., 1980; Rees *et al*., 1993; Kumar and Berwal, 1998; Sağdıç, 2003; Nasar-abbas and Halkman, 2004; Indu *et al*., 2006; Shan *et al*., 2007; Celikel and Kavas, 2008); but little information exist on the in-vivo antimicrobial efficacy of plant essential oils against foodborne pathogens in meats (Aureli *et al*., 1992; Burt and Reinders, 2003). The use of natural preservatives such as spices and herbs to inhibit growth of serious psychotropic pathogens such as *L. monocytogenes* is of great interest to the meat industry (Solomakos *et al*., 2008).

The results of the agar diffusion antilisterial activity of the crude ethanol and aqueous extract of the six spices, namely: *Thymus vulgaris* L (thyme), *Allium sativum* L (garlic), *Piper guineense* Schum and Thonn (brown pepper), *Monodora myristica* (Gaertn) Dunal (African nutmeg), *Ocimum gratissimum* L (scent leaves) and *Xylopia aethiopica* (Dunal) A Rich (African pepper) against the *Listeria monocytogenes* PCM 01/2 and the isolated *L. monocytogenes* showed that only the thyme and brown pepper

inhibited the growth of *L. monocytogenes*. While both the ethanol and aqueous extract of thyme inhibited the growth of the *Listeria* strains, only the aqueous extract of brown pepper had inhibitory effects on the *Listeria* strains. However, Anyanwu and Nwosu (2014) reported a higher activity of ethanol extract of brown pepper on *S. aureus, E.coli, P aeruginosa, B. subtilis, Candida albicans* and *Saccharomyces cerevisiae* than aqueous extract while Aboaba *et al*. (2011) reported the contrary against *S. aureus, E.coli, P aeruginosa*, amongst other bacteria strains. All the concentrations (0.1 – 5.0%) of the thyme ethanol extract inhibited the *L. monocytogenes* strains; this was not the case with the aqueous extracts of both thyme and brown pepper with minimum inhibitory concentrations of 0.5% and 1.0% respectively. There was significant difference (p=0.05) between the zones of inhibition for all the concentrations employed This finding with thyme is in agreement with previous report by Al-Muhna (2010) that both the aqueous and ethanol extracts of thyme had excellent inhibitory effect against *L. monocytogenes* at concentrations varying from 25mg/mL to 100mg/mL. Celikel and Kavas (2008) reported that of five plant essential oils of thyme, myrtle, laurel, sage and orange, tested against *E. coli, L. monocytogenes, S. aureus* and *Candida albicans* at concentrations of 5-20µL/disk, the essential oils of thyme showed the highest inhibition, while orange was the lowest. Marino *et al*. (1999) reported that thyme essential oils had a significant bacteriostatic activity against the nine strains of Gram-negative bacteria and six Gram-positive bacteria tested. Singh *et al*. (2003) reported that when thyme, clove, pimento, rosemary and sage essential oils were tested against different strains of *L. monocytogenes*, thyme oil was the most inhibitory producing clear zones of inhibition at concentrations of 7.8mL/l and 15.6mL/l. Abdollahzadeh *et al*. (2014), also reported that of thyme, cinnamon and rosemary; thyme essential oil showed the greatest antimicrobial activity against *L. monocytogenes*. The essential oils from other *Thymus sp*. notably: *Thymus eriocalyx* and *Thymus x-porlock* tested on agar plates and in broth tubes according to Rasooli *et al*. (2006) showed very strong anti-*listeria* properties. The findings with garlic is in agreement with earlier report that garlic extract showed excellent anti-bacterial activity against 20 serogroups of *Escherichia coli*, 8 serotypes of *Salmonella* and *Aeromonas hydrophila*, except *L. monocytogenes* (Indu *et al*., 2006). According to Kumar and Berwal (1998), *L. monocytogenes* was the least sensitive of *Staphylococcus aureus, Salmonella typhi* and *Escherichia coli* while Kim *et al*. (2008) reported that garlic shoot juice at concentrations of 2.5%, 5.0% and 10.0% had strong and similar pattern

of anti-listerial effect as nisin against *L. monocytogenes.* Mbata and Sailia (2007) have reported antilisterial activity of *O. gratissimum* at concentration of 250mg/mL. A number of authors have reported the antibacterial activity of *O. gratissimum* against *Escherichia coli, Salmonella typhi, Klebsiella pnuemoniae, Staphylococcus aureus* (Adebolu *et al*., 2005; Nwinyi *et al*., 2009; Alo *et al*., 2012). Although, there are no reported cases of antilisterial activity of *Piper guineense, Xylopia aethiopica* and *Monodora myrisrica*, there are however, reported anti-bacterial activity of these spices against *Pseudomonas aeruginosa, Bacillus subtilis* and *Staphylococcus aureus, Esherichia coli, Enterococcus feacalis, Klebsiella* sp.,*Salmonella* sp., *Shigella* sp., *Enterobacter* sp. (Konning *et al*., 2004; Eruteya and Odunfa, 2009; Nweze and Onyishi, 2010; Aboaba *et al*., 2011; Oloyede and Aduramigba-Modupe, 2011; Ilusanya *et al*., 2012). It has been reported that inactivity of plants extracts may be due to age of plants, extraction solvent, method of extraction and time of harvesting of plant materials (Okigbo and Ajale, 2005; Okigbo *et al*., 2005). On the basis of these results and reports of Solomakos *et al*. (2008); Smith-Palmer *et al*. (2001); Menon and Garg, (2001); Ghasemi-Pirbalouti *et al*. (2010) and Abdollahzadeh *et al*. (2014), 1% thyme aqueous extract which may appeal to consumers and not negatively impacting the taste of the meat or exceeding acceptable flavor and odour thresholds was selected for further study on its inhibitory ability on growth of *L. monocytogenes* in meat pieces.

In this study, the final population of *L. monocytogenes* population on meat pieces dipped in 1% crude aqueous extract of thyme (v/w) for 0-15min. The reduction in the *L. monocytogenes* populations varied during immersion for 15min was reduced. The greatest decrease was observed in IS3 (from 5.92 to 4.51 $\log_{10}$ cfu/g), followed by IS4 (from 6.13 to 4.81 $\log_{10}$ cfu/g) while the least was in PCS (from 6.01 to 5.20 $\log_{10}$ cfu/g) resulting in a reduction ranging from 0.81 to 1.41 $\log_{10}$ cfu/g at the end of 15min. This finding was in agreement with the report of Mytle *et al*. (2006), in their work with 1.0% clove extract against various *L. monocytogenes* strains.

The results of the effects of 1.0% crude aqueous extract of thyme on the growth of *L. monocytogenes* in meat pieces indicated that all strains survived and grew at both 10^{o}C for 15days and 30^{o}C for 4 days; though to different degrees. Samples treated with thyme extract displayed a population of *L. monocytogenes* lower than the control samples, during the storage at both temperatures as previously observed by Abdollahzadeh *et al*. (2014) working at a temperature of 4^{o}C.

After a 4-day storage at 30^{o}C, *L. monocytogenes* populations exhibited higher growth rate in the control samples for all cases compared to the treated samples with the initial population of 5.36-6.60 $\log_{10}$ cfu/g in control increasing to 9.08-10.15 $\log_{10}$ cfu/g and 5.28-6.46 $\log_{10}$ cfu/g of treated samples increasing to 8.53-9.87 $\log_{10}$cfu/g. Significant differences (p=0.05) were bserved between the control and the treated samples from day 1 to day 4 of storage.

For samples stored at 10^{o}C, the initial recorded population of 5.40-6.62 $\log_{10}$cfu/g of *L. monocytogenes* in control samples increased to 8.48-8.81 $\log_{10}$ cfu/g while the treated samples increased from 5.20-6.46 $\log_{10}$ cfu/g to 8.23-8.49 $\log_{10}$ cfu/g. There was no significant difference (p=0.05) between the control and the treated samples all through the 15-days storage period

The inability of the crude aqueous extract of thyme to yield a significant inhibition of *L. monocytogenes* in meat pieces stored at 30^{o}C and particularly at 10^{o}C as evident in the agar diffusion and attachment test is of great concern. This may not be unconnected with the ability of *L. monocytogenes* to survive and grow to significant numbers on refrigerated meat product, making post-process contamination a significant concern for ready-to-eat (RTE) meat (Grau and Vanderlinde, 1992; Solomakos *et al*., 2008); the stability of thyme extract and/or oil during food storage and processing (Farbood *et al*.,1976; Smith-Palmer *et al*., 2001; Abdollahzadeh *et al*., 2014); the intrinsic properties of food (fat, protein, water content, antioxidants, preservatives, pH, salt, and other additives) and the extrinsic determinants (temperature, vacuum packaging, gas, air, and characteristics of microorganisms) which can influence bacteria sensitivity (Hao *et al*., 1998; Burt, 2004).

Effect of essential oils (EO) is known to be reduced in a food matrix system as a result of interaction with the different components of food, thus requiring much larger concentrations to reduce the bacterial populations (Farbood *et al*., 1976; Smith-Palmer *et al*., 2001). According to Hao *et al*. (1998), the inhibitory effect of spice and herb extract would be overcome by microorganisms with prolonged storage time.

Little information exists on the in-vivo antimicrobial efficacy of plant EOs against food-borne pathogens in meat matrix (Solomakos *et al*., 2008). Research shows that a greater concentration of EO is needed in in-vivo antibacterial assays than in-vitro to achieve the same effect in foods (Abdollahzadeh *et al*., 2014). The intrinsic properties of food (fat, protein, water content, antioxidants, preservatives, pH, salt, and other

additives) and the extrinsic determinants (temperature, vacuum packaging, gas, air, and characteristics of microorganisms) can influence bacterial sensitivity (Burt, 2004).

It has been reported that antimicrobial properties of crude spice extract are due mostly to the presence of active compounds such as, alkaloids, phenols, glycosides, steroids, essential oils, quinines, saponins, iridoids, coumarins and tannins (Ebana *et al*., 1991; Nilforoushzadeha *et al*., 2008).

Phytochemical screening revealed the presence of the following components in both the crude aqueous extract of thyme: (alkaloids (13.3%), flavonoids (39.2%), saponins (47.0%) and tannins (0.5%) and crude ethanol extract of thyme: (alkaloids (8.9%), flavonoids (39.3%), saponins (51.2%) and tannins (0.6%). Flavonoid and saponins have been detected in thyme and reported as bioactive components responsible for its antimicrobial activity (Hossain *et al*., 2013). Dahiya and Purkayastha (2012) reported that flavonoid and saponins from extracts of neem, tulsi and aloe vera serve as major active compounds against methicillin-resistant *Staphylococcus aureus*. If spice extract and essential oils are expected to be widely applied as antibacterial agents in food, the sensory impacts can alter the taste of food or exceed acceptable flavour and odour thresholds (Abdollahzadeh *et al*., 2014). The aqueous extracts show promise and form a primary platform for further phytochemical and pharmacological studies for use as alternative therapy.

CONCLUSION AND RECOMMENDATION

The study was aimed at investigating the prevalence, diversity, virulence and antimicrobial resistance of *Listeria* spp. in retailed raw beef and goat meat in Port Harcourt and the control of *L. monocytogenes* using plant extracts.

This study has demonstrated the presence and distribution of *L. monocytogenes* and other *Listeria* species in raw meat examined in Port Harcourt, with the strain *L. monocytogenes* carrying the virulent genes. An increased antimicrobial resistant was demonstrated in line with worldwide pattern indicating a potential public health risk. Although the degree of inhibition observed in the meat study is difficult to have direct application, which is true with most natural antimicrobial, the study is presenting a possible frontier for harnessing. The high percentage of the isolation of *Listeria* suggests the need for improved food safety through proper hygienic measures during processing of food and meat products. It seems an impossible task protecting food

samples from such defilement, but with proper handling and processing, the challenge can be practically controlled to a very large extent.

Contribution to knowledge

- The data provide the first molecular insight into the *Listeria* spp. isolates from meat in Port Harcourt and elucidate a potential risk of people contracting listeriosis.
- The occurrence of *L. monocytogenes* virulent strains resistant to antibiotic previously employed in listeriosis treatment.
- The potential application of thyme for the control of listeriosis.

The recommendations were as follows:

- The investigation of the distribution of *Listeria* in commonly implicated foods should be intensified in Nigeria.
- The use of both conventional and molecular method for its characterization should be encouraged.
- More work should be done in the area of control of *Listeria monocytogenes* in food systems.

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APPENDICES

APPENDIX A

Mean separation using scheffe for distribution of Listeria species from various meat types and parts.

Beef flesh

	Listeria species isolated from samples	N	Subset for alpha = .05					
		1	2	3	4	5	6	1
Duncan(a)	*L. monocytogenes*	3	.0000					
	L. ivanovii	3		2.0000				
	L. innocua	3			5.0000			
	L. grayi	3				14.0000		
	L. seeligeri	3					18.0000	
	L. welshimeri	3						35.0000
	Sig.		1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Beef intestine

	Listeria species isolated from samples	N	Subset for alpha = .05			
		1	2	3	4	1
Duncan(a)	*L. monocytogenes*	3	.0000			
	L. ivanovii	3	.0000			
	L. seeligeri	3	1.0000			
	L. innocua	3		3.0000		
	L. grayi	3			8.0000	
	L. welshimeri	3				17.0000
	Sig.		.125	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Beef kidney

	Listeria species isolated from samples	N	Subset for alpha = .05			
		1	2	3	4	1
Duncan(a)	*L. monocytogenes*	3	.0000			
	L. innocua	3	1.0000			
	L. ivanovii	3	1.0000			
	L. grayi	3		10.0000		
	L. seeligeri	3			13.0000	
	L. welshimeri	3				16.0000
	Sig.		.125	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Beef liver

	Listeria species isolated from samples	N	Subset for alpha = .05				
		1	2	3	4	5	1
Duncan(a)	*L. monocytogenes*	3	.0000				
	L. ivanovii	3	1.0000				
	L. innocua	3		3.0000			
	L. seeligeri	3			8.0000		
	L. grayi	3				11.0000	
	L. welshimeri	3					23.0000
	Sig.		.159	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Goat flesh

	Listeria species isolated from samples	N	Subset for alpha = .05			
		1	2	3	4	1
Duncan(a)	*L. ivanovii*	3	.0000			
	L. innocua	3	1.0000			
	L. monocytogenes	3		3.0000		
	L. seeligeri	3			9.0000	
	L. grayi	3			9.0000	
	L. welshimeri	3				11.0000
	Sig.		.159	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Goat intestine

	Listeria species isolated from samples	N	Subset for alpha = .05			
		1	2	3	4	1
Duncan(a)	*L. innocua*	3	.0000			
	L. ivanovii	3	.0000			
	L. monocytogenes	3	1.0000			
	L. seeligeri	3		4.0000		
	L. grayi	3			6.0000	
	L. welshimeri	3				8.0000
	Sig.		.125	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Goat kidney

	Listeria species isolated from samples	N	Subset for alpha = .05			
		1	2	3	4	1
Duncan(a)	*L. monocytogenes*	3	.0000			
	L. ivanovii	3	.0000			
	L. innocua	3		1.0000		
	L. grayi	3		1.0000		
	L. seeligeri	3			7.0000	
	L. welshimeri	3				8.0000
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Goat liver

	Listeria species isolated from samples	N	Subset for alpha = .05			
		1	2	3	4	1
Duncan(a)	*L. monocytogenes*	3	.0000			
	L. ivanovii	3	.0000			
	L. innocua	3		6.0000		
	L. seeligeri	3			12.0000	
	L. grayi	3			12.0000	
	L. welshimeri	3				21.0000
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

APPENDIX B:

P control

	concentration	N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a]	0.1	3	11.0000			
	0.5	3	12.0000			
	1.0	3		14.0000		
	2.5	3			16.0000	
	5.0	3				18.0000
	Sig.		.082	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Isolate 1 water

	conc for pg water	N	Subset for alpha = 0.05				
			1	2	3	4	5
Duncan[a]	.10	3	.0000				
	.50	3		8.0000			
	5	3			10.0000		
	2.50	3				12.0000	
	0.1	3					15.0000
	Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

P control pg water

N	Subset for alpha = 0.05
	1
3	.0000
3	.0000
3	
3	
3	
	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

APPENDIX C

Mean separation using scheffe for the effect of 1.0% aqueous extract of thyme on L. monocytogenes stored at 30^0C for 4 days

Homogeneous Subsets

Day 0 for 30^oC

	stored at 30 degrees	N	Subset for alpha = 0.05			
			1	2	3	4
Scheffe[a]	IS4 test	3	5.2800			
	IS4 control	3	5.3600			
	pcs test	3	5.4500			
	pcs control	3	5.4800			
	IS1 test	3		6.0000		
	IS1 control	3		6.1100	6.1100	
	IS2 test	3			6.4000	6.4000
	IS3 test	3			6.4600	6.4600
	IS3 control	3				6.4900
	IS2 control	3				6.6000
	Sig.		.677	.988	.064	.677

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Day 1 for 30^oC

	stored at 30 degrees	N	Subset for alpha = 0.05			
			1	2	3	4
Scheffe[a]	IS4 control	3	6.1100			
	IS3 test	3		7.2000		
	IS3 control	3		7.3800		
	IS4 test	3		7.6000	7.6000	
	pcs test	3			8.0400	8.0400
	IS2 control	3			8.0767	8.0767
	IS1 test	3				8.1100
	IS2 test	3				8.2300
	IS1 control	3				8.3800
	pcs control	3				8.4100
	Sig.		1.000	.198	.068	.285

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Day 2 for 30°C

	stored at 30 degrees	N	Subset for alpha = 0.05						
			1	2	3	4	5	6	7
Scheffe[a]	IS4 test	3	7.8100						
	IS4 control	3	7.8600						
	IS2 test	3		8.3200					
	pcs test	3		8.3600					
	IS3 test	3			8.4300				
	IS3 control	3			8.4800	8.4800			
	IS1 test	3				8.4900			
	IS1 control	3					8.5800		
	pcs control	3						8.6800	
	IS2 control	3							9.4300
	Sig.		.129	.370	.129	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Day 3 for 30°C

	stored at 30 degrees	N	Subset for alpha = 0.05			
			1	2	3	4
Scheffe[a]	IS4 test	3	7.9000			
	IS4 control	3	8.0400			
	pcs test	3		8.4100		
	IS2 test	3		8.4800		
	IS3 test	3		8.4900		
	IS3 control	3		8.5567		
	pcs control	3			8.9333	
	IS1 test	3			9.0400	
	IS1 control	3				9.3200
	IS2 control	3				9.4800
	Sig.		.160	.122	.489	.069

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Day 4 for 30°C

	stored at 30 degrees	N	Subset for alpha = 0.05				
			1	2	3	4	5
Scheffe[a]	IS3 test	3	8.5300				
	IS3 control	3		9.0800			
	IS2 test	3		9.2000	9.2000		
	pcs test	3		9.3000	9.3000	9.3000	
	IS1 test	3		9.3400	9.3400	9.3400	
	IS1 control	3			9.4533	9.4533	
	pcs control	3				9.5067	
	IS4 test	3					9.8700
	IS4 control	3					9.9700
	IS2 control	3					10.1500
	Sig.		1.000	.089	.105	.303	.052

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

APPENDIX D

Mean separation using scheffe for the effect of 1.0% aqueous extract of thyme *on L. monocytogenes* stored at 10^{O}C for 15 days

Homogeneous Subsets

Day 0 for 10^{O}C

	Stored at 10 degree	N	Subset for alpha = 0.05		
			1	2	3
Scheffe[a]	IS4 test for 10 degree	3	5.9833		
	IS4 contro for 10 degree	3	6.3600	6.3600	
	IS3 control for 10 degree	3	6.8633	6.8633	6.8633
	IS3 test for 10 degree	3	7.4033	7.4033	7.4033
	IS2 test for 10 degree	3	7.6233	7.6233	7.6233
	IS2 control for 10 degree	3	7.7000	7.7000	7.7000
	IS1 control for 10 degree	3		8.0333	8.0333
	IS1 test for 10 degree	3		8.0367	8.0367
	pcs control for 10 degree	3			8.3633
	pcs teest for 10 degree	3			8.4167
	Sig.		.119	.137	.207

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Day 3 for 10^{O}C

	Stored at 10 degree	N	Subset for alpha = 0.05		
			1	2	3
Scheffe[a]	IS4 test for 10 degree	3	5.8900		
	IS4 control for 10 degree	3	6.2133	6.2133	
	IS3 control for 10 degree	3	7.2433	7.2433	7.2433
	IS3 test for 10 degree	3	7.3700	7.3700	7.3700
	IS2 test for 10 degree	3		7.6367	7.6367
	IS1 test for 10 degree	3		7.8300	7.8300
	IS2 control for 10 degree	3		7.8667	7.8667
	IS1 control for 10 degree	3			8.1800
	pcs control for 10 degree	3			8.3100
	pcs teest for 10 degree	3			8.4033
	Sig.		.113	.053	.369

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Day 6 for 10^{O}C

	Stored at 10 degree	N	Subset for alpha = 0.05		
			1	2	3
Scheffe[a]	IS4 test for 10 degree	3	5.9367		
	IS4 contro for 10 degree	3	6.2867	6.2867	
	IS3 control for 10 degree	3	7.0533	7.0533	7.0533
	IS3 test for 10 degree	3	7.3867	7.3867	7.3867
	IS2 test for 10 degree	3	7.6300	7.6300	7.6300
	IS2 control for 10 degree	3		7.7833	7.7833
	IS1 test for 10 degree	3		7.9333	7.9333
	IS1 control for 10 degree	3			8.1067
	pcs control for 10 degree	3			8.3367
	pcs teest for 10 degree	3			8.4100
	Sig.		.068	.083	.248

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Day 9 for 10^{O}C

	Stored at 10 degree	N	Subset for alpha = 0.05		
			1	2	3
Scheffe[a]	IS4 test for 10 degree	3	6.0700		
	IS4 contro for 10 degree	3	6.5100	6.5100	
	IS3 control for 10 degree	3	7.2267	7.2267	7.2267
	IS3 test for 10 degree	3	7.6233	7.6233	7.6233
	IS2 test for 10 degree	3	7.6367	7.6367	7.6367
	IS2 control for 10 degree	3		7.9733	7.9733
	IS1 test for 10 degree	3		8.1700	8.1700
	IS1 control for 10 degree	3			8.3800
	pcs teest for 10 degree	3			8.4833
	pcs control for 10 degree	3			8.6533
	Sig.		.149	.105	.241

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Day 12 for 10^{O}C

	Stored at 10 degree	N	Subset for alpha = 0.05		
			1	2	3
Scheffe[a]	IS4 test for 10 degree	3	6.0433		
	IS4 contro for 10 degree	3	6.4967	6.4967	
	IS3 control for 10 degree	3	7.2133	7.2133	7.2133
	IS2 test for 10 degree	3	7.6233	7.6233	7.6233
	IS3 test for 10 degree	3	7.6367	7.6367	7.6367
	IS2 control for 10 degree	3		7.9667	7.9667
	IS1 test for 10 degree	3		8.1500	8.1500
	IS1 control for 10 degree	3			8.3667
	pcs teest for 10 degree	3			8.4433
	pcs control for 10 degree	3			8.6533
	Sig.		.141	.113	.239

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Day 15 for 10^{O}C

	Stored at 10 degree	N	Subset for alpha = 0.05		
			1	2	3
Scheffe[a]	IS4 test for 10 degree	3	6.0567		
	IS4 contro for 10 degree	3	6.5033	6.5033	
	IS3 control for 10 degree	3	7.2200	7.2200	7.2200
	IS2 test for 10 degree	3	7.6300	7.6300	7.6300
	IS3 test for 10 degree	3	7.6300	7.6300	7.6300
	IS2 control for 10 degree	3		7.9700	7.9700
	IS1 test for 10 degree	3		8.1600	8.1600
	IS1 control for 10 degree	3			8.3733
	pcs teest for 10 degree	3			8.4633
	pcs control for 10 degree	3			8.6533
	Sig.		.148	.108	.239

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

APPENDIX E

Occurrence of *Listeria* spp. from the three markets

Market	Number of samples purchased	Number positive	Occurrence (%)
Choba	86	19	22.093
Rumuokoro	81	26	32.099
Rumuokwuta	73	36	49.315
Total	240	81	

APPENDIX F

Monthly distribution of *Listeria* spp.

Month/Year	Number of *Listeria* detected	Occurrence (%)
March, 2011	12	3.87
April, 2011	6	1.94
May, 2011	21	6.77
June, 2011	21	6.77
July, 2011	49	15.81
August, 2011	39	12.88
September, 2011	30	9.68
October, 2011	29	9.35
November, 2011	47	15.16
December,2011	33	10.65
January, 2012	8	2.58
February, 2012	15	4.84
Total	310	

APPENDEX G

Effects of thyme water extract on five strains of *L. monocytogenes* attached to meat pieces within 15min

Organism/Incubation time	0	5	10	15
PCS	6.01	5.65	5.30	5.20
IS1	6.61	5.88	5.33	5.31
IS2	6.49	5.91	5.65	5.30
IS3	5.92	4.58	4.43	4.51
IS4	6.13	5.94	5.46	4.81

APPENDIX H

COMPOSITION AND PREPARATION OF MEDIA

Polymycin acriflavin lithium chloride ceftazidine aesculin mannitol (PALCAM) agar

Composition	**g/L**
Columbia blood agar base	39.0
Yeast extract	3.0
Glucose	0.5
Aesculin	0.8
Ferric ammonium citrate	0.5
Mannitol	10.0
Phenol red	0.08
Lithium chloride	15.0
pH (25^{o}C)	7.2±0.2

PALCAM selective supplement

Composition	**mg/L**
Polymycin B	10.0
Acriflavin hydrochloride	5.0
Ceftazidine	20.0

Directions

Suspend 34.5g in 500mL distilled water. Bring gently to the boil to dissolve completely. Sterilize by autoclaving at 121^{o}C for 15min. cool to 50^{o}C and aseptically add PALCAM selective supplement, reconstitute as directed. Mix well and pour into sterile Petri dishes.

Fraser broth

Composition	g/L
Proteose peptone	5.0
Tryptone	5.0
'Lab-Lemco' powder	5.0
Yeast extract	5.0
Sodium chloride	20.0
Di-sodium hydrogen phosphate	12.0
Potassium dihydrogen phosphate	1.35
Aesculin	1.0
Lithium chloride	3.0
pH (25^{o}C)	7.2 ±.02

Fraser selective supplement

Composition	Per litre
Ferric ammonium citrate	0.5g
Nalidixic acid	20.0mg
Acriflavin hydrochloride	25.0mg

Directions

Suspend 28.7g in 500mL distilled water and mix well to dissolve completely. Sterilize by autoclaving at 121^{o}C for 15min. cool to below 50^{o}C, and aseptically add the contents of one vial of Fraser selective supplement reconstituted as directed in the product insert. Mix well and distribute in sterlie

Tripticase soy broth

Composition	**g/L**
Pancreatic digest of casein	17.0
Pancreatic digest of soya bean	3.0
Sodium chloride	5.0
Dipotassium phosphate	2.5
Dextrose	2.5
pH	7.3±0.2

Directions

Suspend 40.0g in 1000mL distilled water. Heat to boiling to dissolve completely and dispense into appropriate container. Sterilize by autoclaving at 121^{o}C for 15min.