ANTAGONISTIC ACTIVITY OF SELECTED PLANT EXTRACTS AGAINST *Listeria monocytogenes* ISOLATED FROM RETAILED READY-TO-EAT FOODS

BY

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ABSTRACT

Listeriosis is caused by eating foods contaminated with *Listeria monocytogenes*. Antibiotic resistance in *Listeria monocytogenes* makes the treatment of listeriosis difficult hence, the need to explore other alternatives which natural plant products could offer. There is a dearth of information on the use of medicinal plants against *Listeria monocytogenes*. The aim of this study was to determine the antagonistic activity of selected plant extracts against *Listeria monocytogenes* isolated from ready-to-eat foods.

Four hundred and eleven retailed samples consisting of edible worms (124), salads (85), meat pies (94) and spiced snails (108) were purchased purposively along travellers' routes covering Ore to Port Harcourt (South South). Listerial isolation was done using *Listeria* selective agar. The isolates were characterised using standard cultural and molecular methods. Virulence markers such as *HlyA*, *Iap* and *InlA* were used to confirm the identity of *Listeria monocytogenes*. Effect of pH (2-9), NaCl (2-10%) and temperature (-4 to 70ºC) on growth of *Listeria monocytogenes* was determined and susceptibility to Augmentin, Cloxacillin, Ofloxacin and Ciprofloxacin was carried out by disk diffusion. Dried pulverised leaves of *Vernonia amygdalina* (*Va*), *Psidium guajuva* (*Pg*), and *Dacryodes edulis* (*De*) were extracted in ethanol and water. Anti-listerial potentials of different concentrations (100, 200, 400, 600, 800 and 1000mg/mL) of the extracts were determined *in vitro* using agar-well diffusion assay. Eighty-eight mice (18-20g) were randomly distributed into eleven equal groups for *in vivo* studies. Mice in groups 1 to 10 were orally infected with 1mL *Listeria monocytogenes* (10^7cfu/mL), then, aqueous concentrations (0.6, 0.8 and 1.0g/kg) of *Va*, *De* and *Pg* were orally administered as 1mL doses to groups 1, 2 and 3; 4, 5 and 6; and 7, 8 and 9, respectively. Group 10 served as the infected control, while group 11 was the uninoculated control. Histopathological changes in liver and assay of alanine aminotransferase (ALT) to determine the extent of liver damage was carried out using standard methods. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.01}$

Two hundred and forty five *Listeria* species were isolated from edible worms (115), salads (50), meat pies (35) and spiced snails (45) of which detection of the virulence markers

confirmed ninety species as *Listeria monocytogenes*. Optimum growth of *Listeria monocytogenes* was at pH 8, 2% NaCl and 37°C. Resistance by *Listeria monocytogenes* to Augmentin, Cloxacillin, Ofloxacin and Ciprofloxacin were 100.0%, 100.0%, 42.2% and 63.6%, respectively. Aqueous and ethanolic extracts of *Va* had higher *in vitro* anti-listerial activities against *Lm*EW94 at 100mg/mL (35.0±0.0; 35.0±0.0mm) compared to *De* (25.0±14.1; 25.0±1.4mm) and *Pg* (27.0±1.2; 27.0±1.3mm). Mortality (37.5%), apoptotic cells, cytoplasmic vacuolation and significant ALT (84.36±12.01U/L) increase were recorded only in infected but untreated mice (group 10). Liver histology of infected mice treated with 0.8g/kg *Va* (group 4) revealed mild kupfer cell with normal liver histological characteristics. Reduction in ALT concentration observed in infected mice treated with 0.6g/kg *Pg* (32.77±8.95U/L) signified improvement in liver health as the extracts had anti-listerial activities.

Extracts of *Vernonia amygdalina, Psidium guajuva* and *Dacryodes edulis* had *in vitro* and *in vivo* antagonistic activities against virulent strains of *Listeria monocytogenes*.

Keywords: *Listeria monocytogenes*, Ready-to-eat foods, *Vernonia amygdalina*, Antimicrobial plant extract

Word count: 500

CERTIFICATION

I certify that this work was carried out by Omonigho Ebakota DANIEL (Matric No. 146833) in the Department of Microbiology, University of Ibadan, Nigeria.

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DEDICATION

This work is dedicated to the Almighty God for His love, provision, protection, sound health and journey mercies throughout my programme.

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Omonigho Ebakota Daniel,

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TABLE OF CONTENTS

TABLE OF CONTENTS CONTD

TABLE OF CONTENTS CONTD

LIST OF TABLES

LIST OF TABLES CONTD

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

Abbreviation	Full meaning
AMLF	Amplified fragment length polymorphism
DNA	Deoxyribonucleic acid
EPS	Exopolysaccharides
FDA	Food and drug association
GHP	Good hygiene practices
GMP	Good manufacturing practices
HACCP	Hazard analysis and critical control points
LM	*Listeria monocytogenes*
MEE	Multilocus enzyme electrophoresis
MIC	Minimum inhibitory concentration
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
QAC	Quaternary ammonium compound
RAPD	Random amplified polymorphic DNA
REA	Restriction endonuclease analysis
RTE	Ready-to-eat

CHAPTER ONE

1.0 INTRODUCTION

The desire to consume healthy foods is a demanding request of consumers. On the average, a consumer tries to improve his/her daily diet based on personal preference or medical reasons. On the whole, consumers desire foods that are low in calories, fat, and sodium, high in fiber, and safe to consume. The safety of ready-to-eat products not only worries the general public, but is of great concern to persons in the industry. Processors use series of techniques including chlorine sprays, washing methods, organic acid sprays and steam treatment procedures to reduce the microbial load on products (Reagan *et al*., 1996; Kochevar *et al*., 1997; Bertsch *et al*., 2014). Still, these methods have not reduced the number of food recalls and food-borne outbreaks occurring as a result of microbial contamination of foods.

Listeria monocytogenes represents the *Listeria* species most commonly associated with disease in humans. As a facultative organism, *L. monocytogenes* can survive in soils and decomposing vegetation, however, once it gets into a human host, it can cause severe disease. The majority (99%) of the infections caused by *L. monocytogenes* are food-borne being ingestion of contaminated food especially contaminated ready-to-eat food products that do not undergo subsequent reheating (Swaminathan and Gerner-Smidt, 2007). This organism has great economical implications in the food industry due to recalls of contaminated food products and temporary shutdown of many food processing plants (Chen *et al*., 2014). The estimated annual cost of recalls related to *L. monocytogenes* for the food industry range from $1.2 billion to $2.4 billion in the US. *Listeria monocytogenes* is a foodborne pathogen due its ubiquitous nature, the ability to cross - contaminate equipments and foods during processing and its ability to cause listeriosis. In addition, *Listeria monocytogens* can colonize different surfaces and form biofilms that can adhere to equipments used for food production (Wong, 1998). *Listeria* can survive at

low pH, 1°C to 45°C temperatures, low water activity and refrigerated vacuum packed foods (Duffy *et al*., 1994; Buchanan *et al*., 2000).

L. monocytogenes can survive in different drying, freezing and heating conditions. They can tolerate sodium chloride content of 20% and are resistant to multiple antibiotics, making them difficult to treat (Charpentier *et al*., 1995; Jamali *et al*., 2013). The pathogenicity of *Listeria monocytogenes* depends on the serotype with 4b, 1/2b, 1/2c and 1/2a implicatecd in 98% human cases of listeriosis (Graves *et al*., 1999; Moreno *et al*., 2014). Numerous reports have implicated different food types such as milk and diary products, meat and animal products, raw vegetables and sea foods as sources of food borne listeriosis (Rodas-Suárez *et al*., 2006; De Nes *et al*., 2010; Sant'Ana *et al*., 2012). Life style changes have resulted in more foods eaten away from home which is obvious by the escalating numbers of food vending outlets and food hawkers. The hygienic practice involved in most food vending outlets still remain an issue of concern since contamination of foods can occur during harvesting, processing, equipments usage, transportation and human handling (Itohan *et al*., 2011). Reports on outbreaks of infections caused by *L. monocytogenes* in Nigeria are not well documented since the non-invasive form of the disease produces a self resolving gastroenteritis in healthy persons and this kind of gastroenteritis is similar to the ones caused by *Campylobacter* spp and *Salmonella* (FAO/WHO, 2004). Furthermore, there are no published data on the presence of *Listeria* on the larva of the palm weevil (*Rhynchophorus phoenicis*) or edible worm which is a popular proteinous snack in the South South region of Nigeria.

The occurance of *L. monocytogenes* in read-to-eat foods such as vegetables is a cause for concern since vegetables are often consumed in the minimally processed form in local dishes such as fresh salad and as an accompaniment to popular dishes such as rice (Fried, jellof and white rice).

There has been lots of interest recently in the role of complementary and alternative medicines for the treatment of various acute and chronic diseases (Green *et al*., 2010). The revival of interest in the use of African medicinal plants by many developing countries and the World Health Organization (WHO) has led to intensified efforts to explore the numerous plants with medicinal importance (El-kamali, 2009). Research has been geared towards finding scientific evidence for the claims of African herbs by

traditional healers. For many years, natural products in plants have been seen as a valuable source of medicinal agents with proven potential for treating diseases with hardly any side effects compared to the synthetic drug agent (Iwu *et al*., 1999). Different phytochemicals of different chemical classes have been reported to have inhibitory effects on several microorganisms *in vitro* (Cowan, 1999; Ayoola *et al*., 2006). These phytochemicals include terpenoids, essential oils, alkaloids, lectins, polypeptides, polyacetylenes, and phenolics, of which phenolics can be further divided into phenolic acids, flavonoids, quinones, tannins, coumarins, and simple phenols (Aiyegoro and Okoh, 2010). Knowledge of the phytochemical composition of plants and their anti-Listeria ability is desirable as such information would be valuable for the synthesis of complex chemical substances.

1.1 Statement of problem

Listeria monocytogenes, a high-risk emerging food-borne pathogen, has assumed lot of interest as a result of its association with several outbreaks of listeriosis across the world through implication of different variety of foods, both raw and processed. The presence and prevalence of these microorganisms in ready-to-eat foods (RTE) consumed in Nigeria has not been well studied.

1.2 Justification for Research

Preliminary studies have shown that *Listeria* species are prevalent in edible worms, salads, giant snails and meat pies. These RTE foods are cheap, very nutritious and consumed without heating by both the middle and high class individuals in Nigeria and frequently by traverler's as snack or part of food. Moreover, not much work has been carried out on *Listeria* in Nigeria because of the difficulty in differenciating the species. The presence of *Listeria monocytogenes* and its association with foods is important to create further awareness in order to reduce its colonization, transmission, cross-contamination and infections. A positive result from this research based on the study of the microorganism's physiological characteristics as well as *in vivo* antilisterial potential of different indigenous plants extracts would be of immense benefit as it would help control *L. monocytogenes*

1.3 Aims and Objectives

General Objective:

The aim of this study is to determine the antagonistic activity of selected plant extracts against *Listeria monocytogenes* isolated from ready-to-eat foods from the South South Nigeria.

Specific objectives

- Isolation and identification of *Listeria monocytogenes* from different ready-to-eat (RTE) foods using phenotypic and genotypic methods;
- Studing the physiology as well as the susceptibility pattern of selected isolates to different antibiotics;
- Investigation of the plasmid profile and virulent gene markers of identified isolates; and
- Determination of the antimicrobial capability and *in vivo* antilisterial potential of selected plants extract

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Listeria* in nature

All *Listeria* spp. are ubiquitous in nature and are generally dispersed in the environment. *Listeria* are commonly detected in foods, sewage, manure, animal feed, water, soil, vegetables as well as in animals and humans (Nightingale *et al*., 2004; Cocolin *et al*., 2005; Liu, 2008;). *Listeria* spp. are Gram-positive rods of 0.4-0.5 x 0.52 µm in size with rounded ends apperaing singly or in short chains, they are facultative anaerobes, oxidase negative, non spore-forming, catalase positive and non-capsulated bacteria (Holt *et al*., 1994). Periodically, there has been isolation of some catalase negative *Listeria* strains from clinical specimens (Swartz *et al*., 1991; Bubert *et al*., 1997; Annaleise *et al*., 2018). Microscopically, they appear singly as small Gram-positive rods and sometimes arranged in short chains. In direct smears they may be coccoid and so can sometimes be mistaken for Streptococci. *Listeria* spp. can grow in a wide temperature range (1-45°C) (Chavant *et al*., 2002). This endows the organism with a unique capacity to survive food processing and food storage conditions.

However, the most favourable growth of *Listeria* spp is between 30- 37°C with observed motility at low temperatures (20°C). Although, some strains of *Listeria* are non-motile at 37°C because they do not express flagellin proteins at this temperature (Way *et al*., 2004). *Listeria* spp. can be inactivated at temperatures greater than 60°C, making pasteurization suitable for eliminating this bacterium from several dairy products (Bubert *et al*., 1997; Chen *et al*., 2014). Alhough the most favourable pH for growth is 7.0, *Listeria* spp can grow between pH 4.5 and pH 9.2 (George and Lund, 1992). They are capable of multiplication in more than 10% (w/v) NaCl and at water of activity (a_w) below 0.93 (Farber *et al*., 1992).

2.2 *Listeria* Genus and Taxonomy

The *Listeria* genus was once classified into the family Corynebacteriaceae. It was named *Bacterium monocytogenes* by Murray *et al*. (1926), who isolated a 1–2 μm long and 0.5 μm wide round-ended Gram-positive rods from dead laboratory rabbits and guinea-pigs in Cambridge, United Kingdom (Farber and Peterkin, 1991). Following unusual deaths of gerbils in South Africa in the late 1920s, this bacterium was named *Listerella hepatolytica* by Pirie in honor of Lord Joseph Lister who determined that in order to prevent infections surgeons need to sterilize their instruments before each operation (Ryser, 1999). The strains isolated by Murray *et al.* (1926) showed resemblance to this bacterium and so was renamed *Listerella monocytogenes*. However, the generic name *Listerella* had previously been used for protozoa and in 1940, Pirie thus proposed a change of name to *Listeria monocytogenes*. This proposed name was accepted, even though the genus name already existed in botanical taxonomy, including an orchid named *Listeria*, and in zoology a diptera called *Listeria* (Seeliger, 1961).

Currently, it is widely accepted that the core phylogeny of *Listeria* consists of ten (10) species of *Listeria*: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. grayi*, *L.inocua*, *L. welshimeri*, *L. marthii*, *L. rocortiae*, *L. fleischmannii* and *L. weihenstephanensis* (Zhang *et al*., 2007; Lang-Halter *et al*., 2013). Among these species of *Listeria*, only *L. monocytogenes* and *L. ivanovii* are human and animal pathogens respectively, others are non-pathogenic. The organism is often classified along the following line: Genera: *Listeria*, Family: Listeriaceae, Order: Bacillales, Class: Bacilli, Phylum: Firmicutes and Genus: *Listeria*.

2.3 Characteristics and Classification of *Listeria* spp.

Listeria species appear as small rods ranging in size from 0.4 to 0.5 by 1-2μm, and sometimes are found to be arranged in short chains when viewed under the microscope. A coccoid appearance may be seen in direct smears. *Listeria* produces flagella at room temperature and exhibit a tumbling motion when examined in broth and a swarming motility can be observed in semi-soft agar at 30°C (Roberts and Wiedmann, 2003), but flagella are not produced at 37°C (Peel *et al*., 1988). *L. monocytogenes* and other *Listeria* species are catalase positive, indole negative, oxidase negative, Voges-Proskaüer (VP) positive and methyl red positive. They do not hydrolyse urea but utilize glucose and

aesculin with the production of acid but no gas. Species of *Listeria* can grow between -0.4°C and 50°C and utilize different sources of carbon, including C_5 and C_6 sugars (Farber and Peterkin, 1991). They can also survive and even proliferate under a variety of unfavorable conditions such as low temperatures and at high salt concentrations (up to 10% sodium chloride) by accumulating compatible solutes within the cell (Sleator and Hill, 2010). *Listeria monocytogenes* expresses β-hemolysin, which produces zones of clearing (hemolysis) on blood agar and they have been isolated from a wide range of environmental sources, including decaying vegetation, effluents, water, soil, human and animal feces and from different variety of foods. Domesticated ruminant animals participate in the maintenance of *Listeria* spp in the rural environment via a continuous fecal-oral enrichment cycle (Vazquez-Boland *et al*., 2001a).

2.4 Comparative genomics of *L. monocytogenes* and *L. innocua*

L. monocytogenes and *L. innocua* are genetically related and are often isolated from the same ecological habitat. If isolated together, *L. innocua* can overgrow *L. monocytogenes* in enrichment broth, which may preclude *L. monocytogenes* from being detected (Gnanou *et al*., 2005). The guanine + cytosine (G+C) content of *L. monocytogenes* strain-specific genes varies from 24 to 46% and the average G+C content for the entire genome is 38%. Similarly, many of the *L. innocua* specific surface proteins are located in regions with distinct G+C content.

Evidence suggests that the cluster for virulence gene (LIPI-1) exist in *L. monocytogenes, L. innocua, L. ivanovii, L. seeligeri,* and *L. welshimeri* and that this pathogenicity island can be lost in *L. innocua* and *L. welshimeri* kept in *L. seeligeri* genome in intact but inactivated form while been stabilized in *L. monocytogenes and L. ivanovii* (Johnson *et al.,* 2004; Schett *et al*., 2005). The first *L. monocytogenes* genome was sequenced in 2001 and its genome sequence was compared to the non-pathogenic *L. innocua* for explaination of the evolution of *Listeria* species and emergence of the pathogen (Glaser *et al*., 2001). The strains chosen were *L. monocytogenes* EGD-e (serotype 1/2a), a derivative of the first *L. monocytogenes* isolate which was isolated from a rabbit and *L. innocua* strain CLIP 11262 (serovar 6a). Both genomes encode many transporters, transcriptional regulators, putative surface and secreted proteins, consistent with the ability of both species to survive

under diverse environmental stresses and colonize various ecological niches (Glaser *et al*., 2001).

Nevertheless, 30 of the 133 surface proteins identified in *L. monocytogenes* EGD-e were absent from *L. innocua* and 20 of these 30 *L. monocytogenes* EGD-e specific surface proteins harbored typical surface proteins of Gram-positive bacteria (Glaser *et al*., 2001). This observation suggested that *L. monocytogenes* EGD-e may be able to better adapt to different ecological niches than *L. innocua.* In addition to surface proteins, a rich collection of putative regulatory proteins, and transport proteins, especially those of the phosphoenolpyruvate-dependent phosphotransferase (PTS) system may account for *L. monocytogenes* virulence and its ability to adapt, colonize, and respond to a wide variety of different environments and its virulence (Glaser *et al*., 2001). Comparative genomic analysis between the two genomes revealed 270 (9.5%) *L. monocytogenes* EGD-e specific genes while only 149 (5%) *L. innocua* specific genes exist (Gnanou *et al*., 2005). The distribution of these genes within the different functional categories shows that the pathogenic *L. monocytogenes* strain EGD-e possesses about twice as many cell wall, transport, and putative transcriptional regulatory proteins as its non-pathogenic counterpart (Glaser *et al*., 2001). *L. innocua* CLIP 11262 also carried a 100-kb plasmid (pLM100) not present in *L. monocytogenes* EGD-e. This plasmid harbored genes putatively encoding heavy metal resistance and shared strong similarity in several loci with plasmid pLM80 from the sequenced serotype 4b strain (H7858), which caused a multistate outbreak in 1998-1999 in the USA (Nelson *et al*., 2004).

Table 2.1: Physiological and biochemical characteristics of *Listeria* species

Specie	Xylose	Mannitol	Rhamnose	*S. aureus*	*R.equi*	ß-haemolysis	Moles % G+C
L. monocytogenes	-	-	+	+	-	+	37 -39
L.ivanovii	+	-	-	-	+	+	36 -38
L. seeligeri	+	-	-	(+)	-	(+)	36
L. innocua	-	-	V	-	-	-	37 -38
L. welshimeri	+	-	V	-	-	-	36
L. grayi	-	+	V	-	-	-	41 -42

Key; V: variable; (+): weak reaction; + : >90% positive; –: negative; R- *Rhodococcus*; S: *Staphylococcus*

Source: Collins *et al*. (1991).

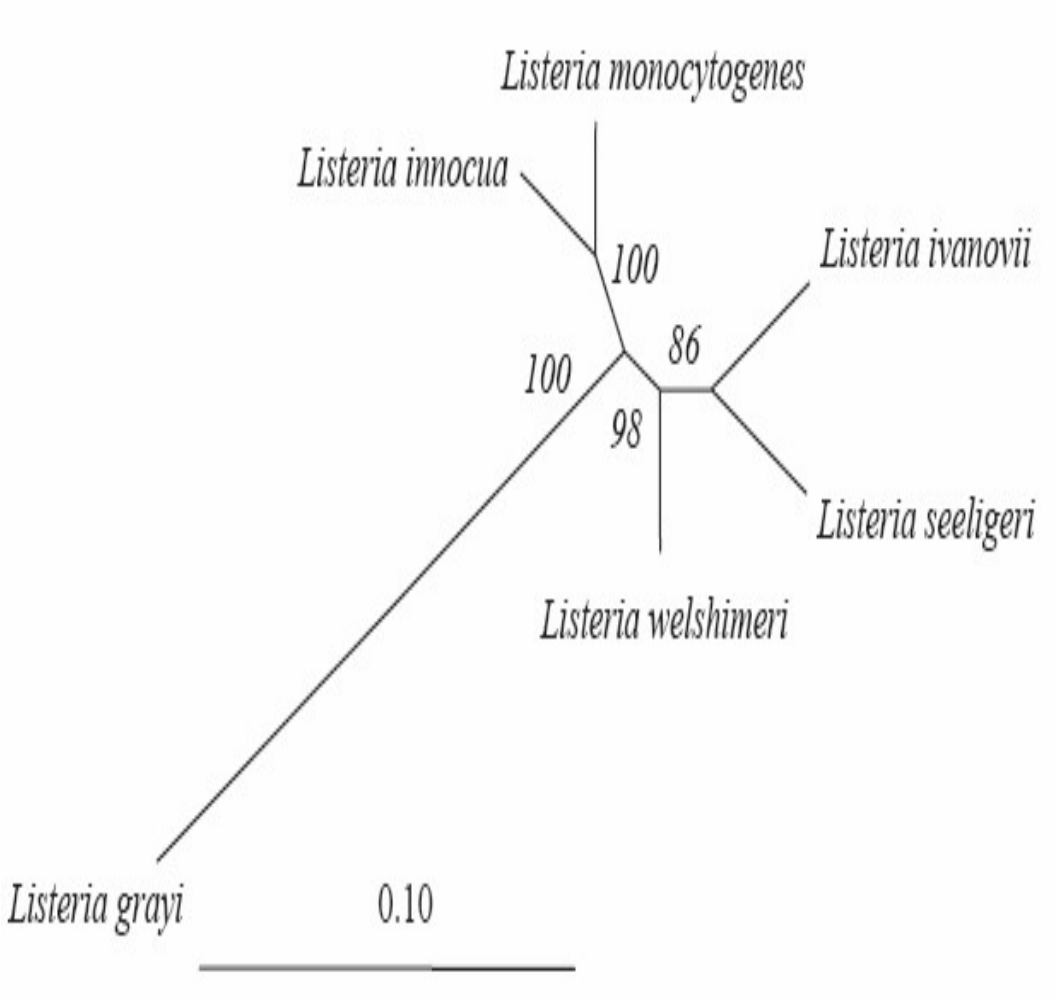


Fig.2.1: *Listeria* phylogenetic tree constructed from concatenated sequences of 16S and 23S rDNA, *prs, vclB, iap* and ldh

Source: Schimd *et al.* (2005)

2.5 Genetic diversity and Serotype of *Listeria monocytogenes*

L. monocytogenes strains display both genetic and serotypic diversity. Thirteen serotypes of *Listeria* have been recognized on the basis of somatic (O) and flagellar (H) antigens: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7. The virulence of *Listeria monocytogenes* has been reported to be dependent of the type of serotype with serotypes 1/2a, 1/2c, 1/2b and 4b involved in about 98% cases of documented human listeriosis (Altuntas *et al*., 2012). The 4a and 4c *L. monocytogenes* serotypes are rarely associated with listeriosis outbreaks despite its frequent recovery and isolation from different foods and environmental samples (Wiedmann *et al*., 1997; Abdollahzadeh *et al*., 2016). Although, majority of the isolated clinical strains of *L. monocytogenes* belong to serovars 1/2a, 1/2b, and 4b, the strains mostly associated with large outbreaks are serovars 1/2a and 1/2b (Kathariou, 2002). Serotype 1/2a is mostly prevalent in foods, while serotype 4b is prevalent in reported cases of human epidemics (Zang and Knabel, 2005). However, there exist some strain differences geographically with a global distribution of different combination of serotypes. Traditionally, serotyping (Seeliger and Hohne, 1979), phage typing, and Multilocus Enzyme Electrophoresis (MLEE) (Boerlin and Piffaretti, 1991) have been extensively used in order to subtype *L. monocytogenes* strains. Recently, molecular subtyping methods, such as Ribotyping (Gendel and Ulaszek, 2000), Pulsed-Field Gel Electrophoresis (PFGE) (Graves *et al*., 2009) as well as sequence-based methods such as Multilocus Sequence Typing (MLST) (Salcedo *et al*., 2003; Revazishvili *et al.,* 2004) have been adapted and are being increasingly used for subtyping purposes.

Phylogenetic analyses have shown that antigenic structure of this organism correlates well with the lineage or genomic division; serotype 4b, 1/2b, and 3b strains are found mostly in lineage I and serotype 1/2a, 1/2c, 3a, and 3c strains are found predominantly in lineage II while a third lineage, lineage III which has serotypes 4a and 4c was proposed due to the genetic distance of serotypes 4a and 4c based on ribotype and virulence gene polymorphism data (Wiedmann *et al*., 1997). Phylogenetic data suggest that some serotype 4b strains were included in lineage III and subgroups of these strains were reported to show unusual phenotypes compared to other serotype 4b strains, such as lack of the ability to ferment rhamnose and absence of putative virulence gene (*lmaA*) (Roberts *et al*., 1996).

Strains of *L. monocytogenes* in different lineages display significant differences in terms of prevalence in human and animal listeriosis. Phylogenetic analysis of polymorphisms in different housekeeping genes and the *prfA* virulence gene cluster indicates that the strains of lineage I seem to be strongly clonal, in contrast to strains of other lineages (Ward *et al*., 1998; Nightingale *et al*., 2004). Lineage II shows greater diversity and evidence of horizontal gene transfer. Genes from lineage III organisms were mostly similar to lineage I genes, in fact, both neighbor-joining and maximum-parsimony analyses of the combined *prfA* virulence gene cluster data strongly support a close genetic relationship between lineages I and III (Meinersmann *et al*., 2004; Ward *et al*., 2004; Nightingale *et al*., 2005). Furthermore, lineage I isolates tend to show greater cytopathogenicity in vitro than lineage II isolates (Pohl *et al*., 2006). *L. monocytogenes*, with lineage I strains containing isolates are associated with epidemics in humans, lineage II strains containing isolates are responsible for sporadic human disease (including serovars 1/2a and 1/2c) and lineage III strains comprising mostly animal pathogens (Nightingale *et al*., 2005).

2.6 Virulence of *Listeria monocytogenes*

Listeria monocytogenes and *L. innocua* both have a cluster of virulence gene located on an 8.2 kb pathogenic island on its genome. This cluster of virulence gene is regulated by the main positive regulatory factor A regulon (PrfA) (Chakraborty *et al*., 1992; Kreft *et al*., 1999). The location of this cluster is between the ldh and prs genes on the chromosome (Gouin *et al*., 1994). The virulence of *Listeria* has been reported to be dependent on the availability of this cluster and it contains mostly virulence genes involved in the intracellular cycle as well as the invasion of *Listeria.* The cluster encodes six genes, hly, prfA, plcB, plcA, mpl, actA, and three other small open reading frames (orfs) Z, Y, and X downstream of plcB (Kuhn and Goebel, 1999). The PrfA is an important gene essential for *L. monocytogenes* virulence regulating virulence and virulence-like genes (Chakraborty *et al*., 2000). The other virulence genes are the products of protein listeriolysin O (LLO) encoded by hly, a phosphatidylcholine-specific phospholipase C (PC-PLC) encoded by plcB, an actin polymerization protein (ActA) encoded by actA, a phosphatidylinositol-specific phospholipase C (PIPLC) encoded by plcA, a metalloprotease (mpl), and three other genes, Z, Y, and X whose functions in virulence of *L. monocytogenes* are presently unidentified. There are other virlence genes which are located outside of the virulence

cluster but have also been reported to be virulent (Kuhn and Goebel, 1999). These virulence genes are mostly concerned with production of surface proteins essential for internalization of the pathogen to the host cell. Examples are the inlC, inlB and inlA virulence genes which code for internalin C, B, and A respectively as well as the invasive iap gene which codes for p60.

2.6.1 Internalins

These are surface proteins found on *L. monocytogenes*. They exist in two forms, InlA and InlB. These internalins are used by the *Listeria* to invade mammalian cells via cadherins transmembrane proteins and Met receptors respectively (Hamon *et al*., 2007). The exact role of these proteins and their invasiveness *in vivo* is not well understood. Internalins are mainly surface exposed virulence factor present in a number of Gram-positive bacteria whose role ranges from recognition of cellular receptors to aid in pathogen entry to escape autophage (Babinet and Charles, 2008).

2.6.2 Listeriolysin O

Listeriolysin O (LLO) is a bacterial pore-forming toxin that can lyse the vacuolar membranes of host cells allowing escape of engulfed *L. monocytogenes*. LLO is an important virulent gene as loss of this gene would enable phagocytes of the host immune system to kill Listeriae within the vacuoles. LLO has been reported to be virulent in mice causing infections *in vivo* (Conlan and North, 1992; Beauregard *et al*., 1997).

Mutant *L. monocytogenes* which cannot secrete the listeriolysin toxin may be able to survive within vacuoles of non-professional phagocytes for a short time but they will not be able to multiply nor infect other cells as they cannot breakout from the vacuoles. In addition to the pore-forming function of LLO, LLO can cause infections of the spleen, dendritic cells and bone marrow resulting in apotosis (programmed cell death). Mutant bacteria which cannot secrete the listeriolysin toxin do not induce apoptosis while purified LLO toxin can induce apoptosis (Guzman *et al*., 1996). LLO also can act as an inflammatory stimulus by inducing endothelial cell activation (Drevets, 1998; Kayal *et al.,* 1999) and neutrophil activation (Sibelius *et al*., 1999). Listeriolysin O is encoded by the gene hly, which is part of a pathogenicity island called LIPI-1. Transcription of hly, as

well as other virulence factors of *L. monocytogenes* within LIPI-1 is triggered by the protein encoded by prfA gene

2.6.3 ActA protein

The Actin assembly-inducing protein (ActA) is a protein encoded assemblage used by *Listeria monocytogenes* to drive itself all the way through host cell (Kocks *et al*., 1992). ActA are also bacterial surface protein and the genes encoding them are actA or prtB. Once *L. monocytogenes* has escaped from the host vacuole with the aid of LLO toxins, it enters and multiplies in the cytoplasm. In order for *Listeria monocytogenes* to infect another cell, the ActA protein is required to induce polymerization of globular Act in molecules to form polarized Act in filaments. Listerial can move along these filaments to the membrane of the cell and cause portions of these cell membranes to bulge outwards forming structures called listeriopods (Kocks *et al.,* 1992; Moors *et al.,* 1999). These protuberances can then be engulfed by adjacent cells thereby allowing the spread of of *L. monocytogenes* without exposure to the host immune cells. ActA could possibly facilitate uptake of *L. monocytogenes* which do not produce internalins into certain types of host cells (Kocks *et al.,* 1992; Kuhn and Goebel, 1999).

2.6.4 Phospholipases

L. monocytogenes synthesizes two different phospholipases C; phosphatidylinositol specific phospholipase C (PI-PLC) and a broad-range or phosphatidylcholine-specific phospholipase C (PC-PLC). Both forms of phospholipases C are involved in the spread and invasivive ability of *L. monocytogenes*. Marquis *et al*. (1995) reported *L. monocytogenes* with mutatant phospholipases C genes were less virulent in mice compared to the wild type bacteria. PI -PLC have been reported to aid the escape of *L. monocytogenes* from the primary vacuole while PC-PLC is active during cell to cell spread of bacteria damaging the vacuolar membranes in some cell types such as epithelial cells (Smith *et al*., 1995). PC-PLC can act as a substitute for LLO, functioning in cell -to-cell spread of listeriae especially in the brain during cerebral listeriosis (Marquis *et al*., 1995; Schluter *et al*., 1998).

2.6.5 Clp proteases and ATPases

ClpC ATPase is a stress protein which aiding the disruption of the host vacuolar membrane (Rouquette *et al*., 1998). They are involved in the intracellular survival of listeriae modulating the expression of Act A protein and the internalins at the transcriptional level (Nair *et al*., 2000). The ClP (caseinolytic proteins) acts as chaperones which help in the accurate refolding or assembly of protein subunits. The CIP also act as proteolytic enzymes digesting proteins that cannot be altered conformationally. ATPaseClpE, another stress protein also plays a role in Listerial pathogenesis (Nair *et al*., 2000).

2.6.6. Protein p60

Protein p60 (iap gene) is murein hydrolase enzyme which catalyzes a reaction during the final stage of cell division of *L. monocytogenes*. It is usually present on the cell surface and can be secreted into the surrounding medium. Analyses of mutation in gene coding for this protein indicate that it is important for the phagocytosis of *L. monocytogenes* by some cell types (Kuhn and Goebel, 1999).

2.7 Listeriosis

Listeriosis can be defined clinically as when *L. monocytogenes* is isolated from foetus, blood, placenta and cerebrospinal fluid. Listeriosis in humans is rare, with less than ten cases per one million persons (Kela and Holmström, 2001). Although listeriosis is rare, it is of great importance due to the high mortality rate of about 20% reported worldwide (Du *et al*., 2017). Two distinct clinical manifestations of listeriosis exist; the invasive and the non-invasive form. The invasive form of listeriosis causes life-threatening disease in persons of specific risk groups. This risk groups include immune-compromised individuals (HIV/AIDS infection), drug abusers, pregnant women, people with low stomach acidity, cancer patients, newborn babies, alcoholics, patients with corticosteroid therapy and the elderly (McLauchlin *et al*., 2004). The clinical appearance of listeriosis includes meningitis (meningoencephalitis), septicemia, encephalitis and intrauterine or cervical infections in pregnant women (Abdul-Razzak and Bani-Hani, 2007). According to Mead *et al*. (1999), listerial infection acquired in early pregnancy could lead to

abortion, still birth or premature delivery. Listeriosis acquired late in pregnancy can be transmitted transplacentally leading to neonatal listeriosis.

The non-invasive listeriosis clinically appears like other bacterial gastroenteritis, which can self resolve within days (Frye *et al*., 2002). Most healthy individuals experience flu-like symptoms like those caused by other microorganisms. The time from exposure to serious form of listeriosis ranges from a few days up to 3 weeks, while gastrointestinal symptoms observed after exposure is greater than 12 hours (≥12hours). The fatality rate of the severe form of the disease is 30% (Allerberger, 2003). Eating of foods contaminated with *L. monocytogenes* is the principal route of transmission for listeriosis (Rasmaswamy *et al*., 2007). Food is then said to serve as an essential mode of transmission of listeriosis and to date, foods still plays a vital role in the spread of listeriosis. Although, other routes of listeriosis transmission apart from contaminated foods have been reported and indistinguishable strains have been isolated from epidemic cases and from those implicated in foods clearly showing the role of food in the epidemiology of listeriosis (Ertas and Seker, 2005).

There are reports of increasing number of *Listeria monocytogenes* from a few cells to 100 cells per gram after refrigeration of foods (Buchanan *et al*., 2000; Huss *et al*., 2000). The dose of *L. monocytogenes* that is infective is presently unknown, although, several reports above 100 viable cells are infective (Moreno *et al*., 2014). Infectivity is dependent on the strain of *L. monocytogenes* as well as the susceptibility of the host (Vazquez-Boland *et al*., 2001b). It is estimated by the Centers for Disease and Control (CDC) that roughly 2,500 cases of listeriosis are reported every year with 500 of the cases resulting in death (Mead *et al*., 1999). The incidence of *Listeria monocytogenes* is relatively low, but the consequences of infection may be severe. In Africa, listeriosis outbreak was recently reported in South Africa where 717 laboratory cases where reported to the National institute for communicable diseases (NICD) between January and December 2017(NCID, 2018). Also, cases of listeriosis have been reported from countries like Zambia where 85 cases of reported meningitis were due to *Listeria*, in Togo, 8 out of 342 healthy slaughtered animals were positive for *L. monocytogenes* (serovars 1/2a and 4b) isolated from the intestinal lymph nodes, and in Northern Nigeria 27% mortality due to *L. monocytogenes* (serovar; 4) was reported (Onyemelukwe *et al*., 1983). Moreover in

another study by Adetunji *et al*. (2003), the isolation rate of *L. monocytogenes* was reported to be 20% in a local cheese wara. Despite the increasing rate of listeriosis reported from several European countries in recent years (Allerberger and Wagner, 2010), and other outbreaks in the United States (Cartwright *et al.*, 2013), Canada (Clark *et al*., 2010; Taillefer *et al*., 2010) and China (Wang *et al*., 2012), the occurrence and prevalence of listeriosis in Nigeria is hardly reported.

2.8 Epidemiology and occurrence of *Listeria monocytogenes*

Although, pasteurization and several cooking methods used in different food industries can destroy *Listeria monocytogenes*, there are reports of post-processing contamination occurring due to the resilient nature of *Listeria monocytogenes*. The largest recall of 27.4 million pounds of fresh and frozen ready-to-eat turkey and chicken products occurred in October 2002 after a multi-state listeriosis outbreak (CDC, 2003). Eight states of USA reported a total of 53 culture-confirmed listeriosis cases from immunocompromised individuals, resulting in eight deaths and three stillbirths or miscarriages (CDC, 2003). In November 2000, there were five reported cases of stillbirths, three premature deliveries, and two infected newborns after consumption of homemade Mexican-style soft cheese in a Hispanic community in North Carolina (CDC, 2003). From May to November of the same year, ten USA states reported 29 cases of listeriosis from deli turkey meat, resulting in four deaths and three stillbirths or miscarriages (CDC, 2003). From January to June 2010, a total of 14 cases of laboratory-confirmed invasive listeriosis were reported to the Louisiana office of public health (OPH). Patients had reported eating hog head cheese (a meat jelly made from swine heads and feet). Isolates of *Listeria monocytogenes* from the blood samples of eight patients were identified as serotype 1/2a and had pulsed-field gel electrophoresis (PFGE) pattern combinations that were indistinguishable from one another. According to CDC in 2011, a total of 14 persons were infected with the *Listeria monocytogenes* outbreak strain in 11 states from the consumption of cheese. All 14 people were hospitalized, but three died. Also, in April 2014, the Centers for Disease Control and Prevention's outbreak reported that 28 people were infected with the outbreak strains of *Listeria monocytogenes* and five deaths occurred in connection with commercially produced, prepackaged caramel apples. From 2012–2016, the European Surveillance System reported between 1754 and 2555 *Listeria monocytogenes* cases from 30 EU/EEA

countries with United Kingdom, Germany and France accounting for 17%, 23% and 45% respectively (WHO, 2017)

2.9 Foods associated with *Listeria monocytogenes*

There are several sources for the occurrence of *L. monocytogenes* in food products. *Listeria monocytogenes* can contaminate foods through added ingredients from poultry and meat products. Air, brine solutions and ice which are used for chilling and refrigeration can also contaminate foods via food processing. This pathogen could also contaminate food products through contact (eg. shredders, filling and packaging equipment, utensils, slicers, dicers, and gloves) and also non-contact surfaces (cracked hoses, in-floor weighing equipment, conveyor belt rollers, equipment bearings, condensate drip pans, vacuum cleaners, on/off switches, etc). Food products could also get contaminated via the plant environment: drains, floors, walls, wet insulation, and door seals (ILSI, 2005). The main route of transmission of listeriosis is through the intake of contaminated foods. The International Life Sciences Institute in 2005 described high-risk foods capable for causing listeriosis as those with the following properties:

- Foods that have the potential for contamination with *L. monocytogenes*;
- Foods that can support the growth of *L. monocytogenes* to high numbers;
- Foods that are ready-to-eat;
- Food that require refrigeration; and
- Foods that is stored for an extended period of time (ILSI, 2005).

L. monocytogenes causes relatively few human disease cases, particularly compared to many other food borne pathogens (Ikeh *et al*., 2010). Ready-to-eat foods, cured meats (hot dogs, undercooked chicken), unpasteurized dairy foods (cheese and milk), seafood, salads, raw fish and unprocessed meats have been found to harbor *L. monocytogenes* (Schlech, 2000; Lennox *et al*., 2017). In Europe, the incidences of *L. monocytogenes* in cheeses from various countries were: Germany 9.2%, Italy 17.4%, France 3.3% and Austria 10% (Rudolf and Scherer, 2001). *Listeria* sp were reported frequently from ready-to-eat soft and semi-soft cheese. Several studies have also implicated *L. monocytogenes* in fish (Farber *et al*., 1991; McLauchlin *et al*., 2004), hotdogs and deli meats (Khelef *et al*.,

2006). High prevalence of *L. monocytogenes* have been reported from cold-smoked and gravad fish (Loncarevic *et al*., 1996, Keto and Rahkio, 1998), with higher prevalence in vacuum-packed fish products compared to non vacuum-packed fish products (Keto and Rahkio, 1998).

In the USA, CDC reported that as of April 20, 2015, a total of ten patients were infected with several strains of *Listeria monocytogenes* from four states: Kansas (5), Arizona (1), Texas (3) and Oklahoma (1). Illness onset dates ranged from January 2010 through January 2015 and all ten patients were hospitalized with three deaths were reported from Kansas. Human listeriosis was attributed to consumption of contaminated cantaloupe (CDC, 2011) and blue bell creameries single serving chocolate chip country cookie sandwich and the great divide bar ice cream (CDC, 2015). Also, *L. monocytogenes* contamination have been reported from several meat products, smoked fish, vegetables, and cooked marinated products (Meloni *et al*., 2009). Food surveys conducted in Malaysia detected of *L. monocytogenes* from several variety of foods including raw and RTE foods (Marian *et al*., 2012), raw salads (Ponniah *et al*., 2010), burger patties (Wong *et al*., 2011) and vegetarian burger patties (Wong *et al*., 2012).

Amongst the different kinds of foods consumed in Nigeria, fish is mostly associated with the occurrence of *Listeria*. *L. monocytogenes* contamination of fish reported has been largely on dried fish which is obtained by open air roasting of fresh fish. *Listeria* species isolated from the dried fish includes *L. monocytogenes, L.innocua, L.ivanovii* and *L. grayii* (Nwaiwu, 2015). *Listeria* has also been isolated in Nigeria from wara (Adetunji and Adegoke, 2008), Kunu (Nwachukwu *et al*., 2009), Kilishi (Yusuf and Tengku-Haziyamin, 2013), vegetables (Nwachukwu *et al*., 2010) and frozen poultry products (Adetunji and Odetokun, 2012).

2.10 Host defenses against *L. monocytogenes*

Animals defend themselves from bacterial attack by using cell mediated immunity, antibody mediated immunity and general non specific resistance factors. There has been no reports on the significant role of antibody mediated immunity in protecting against listeriosis rather, non specific host defenses such as stomach acidity are lethal to many *Listeria* (Audia *et al*., 2001) The normal gut microflora which inhabit all the available

niches in the intestine and use up all essential nutrients prevents bacteria such as *Listeria* from getting established. *L. monocytogenes* is known to be a poor competitor in the intestine and this probably explains the reduced mortality rate in healthy adults (Okamoto *et al*., 1994). Infection of epithelial and macrophage cells by *L. monocytogenes* strongly modulates the complex host cell signaling system affecting the production and activity of a number of non- specific proteins (Kuhn and Goebel, 1999). Cell -mediated immunity is probably the most important host defense against listeriosis with $CD4^+$ T helper lymphocytes activating macrophages (Czuprynski, 1994).

2.10.1. Neutrophils and macrophages

One way the immune system combat *L. monocytogenes* is by inflammatory response (Fleming and Campbell, 1997). During early stages of listeriosis, macrophages and neutrophils move to the spleen and liver destroying most of the *Listeria* cells which are from the small intestine. Although, some *Listeria* cells are able to escape by invading the hepatocytes, neutrophils are also recruited to the uero-placental unit and the central neverous system to kill *Listeria* cells that invade these organs (Guleria and Pollard, 2000). Macrophages and neutrophil can attach to and cause lyses of cells containing *Listeria* but some macrophages do not kill the *Listeria* cells allowing *Listeria* survival and escape. Macrophages that permit the survival of *Listeria* have been reported to contain too much or too little intracellular iron and have interleukin-10 on their surface. Interleukin-10 on macrophage surface is known to restrain the functions of macrophage (Fleming and Campbell, 1997).

2.10.2 Cytokines

Cytokines are small molecules including interferons (IFN), interleukins (IL), and tumor necrosis factor (TNF), which coordinates cells and activities of the immune system. IL-1, IL-2, IL-12, TNF- α, and IFN- γ are some of the cytokines that promote anti-listeria reactions. Other cytokines like the IL-4 and IL-10 have been reported to have a diminishing resistance to *L. monocytogenes* (Czuprynski, 1994). Interaction of endothelial cells with *L. monocytogenes* increases synthesis of IL-8 and granulocyte-macrophage colony stimulating factor (GM-SCF) which tract T- lymphocytes, monocytes and neutrophils. IL- 6, which induces T- cell differentiation and proliferation, is also a

cytokine produced in response to *Listeria*. Mutant *Listeria* cells which cannot synthesize listeriosin O do not provoke cytokine activity compared to wildtype cells (Rose *et al*., 2001).

2.10.3 T-cells

T- cells recognise and respond to antigens from *L. monocytogenes* which have been degraded by host proteasome and loaded onto major histocompatibility complex (MHC) proteins and presented by antigen presenting cells (APC) (Zenewicz and Shen, 2007). Antigen presenting cells (APC) such as macrophages gain listerial antigens by either direct phagocytosis of *L. monocytogenes* or phagocytosis of other cells (alive or dead) containing *L. monocytogenes*, a process known as 'cross presentation' (Heath and Carbone, 2001). Both cross presentation and direct presentation of bacterial antigens are thought to be important for T-cell priming during a primary response to *L. monocytogenes* infection (Goldfine and Shen, 2007). MHC class 1 proteins present foreign antigens from within infected cells to CD8 T-cells and are associated with the endogenous presentation pathway. MHC class 2 proteins present external antigens to CD4 T-cells and are associated with the exogenous presentation pathway (Szalay *et al*., 1994). T-cells confer immunity by essentially removing the protective intracellular niche of *L. monocytogenes*. T-cells can directly lyse infected cells or they can secrete IFN-γ to activate macrophages, which then deal with the liberated *L. monocytogenes*.

2.10.4 B-cells

B-cell mediated immunity is considered to play a minor role against *L. monocytogenes*. This is largely owing to the fact that *L. monocytogenes* is an intracellular parasite and the majorities of the bacteria live and spread with limited contact with the extracellular environment. Thus, *L. monocytogenes* specific antibodies would be of limited use in halting *L. monocytogenes* cell to cell spread. However, some studies have shown that B-cells can reinforce a T-cell dependent response against *L. monocytogenes* through mechanisms other than antibody formation (Stavru *et al*., 2011). Also, antibodies against *L. monocytogenes* virulence factors like listeriolysin O (LLO) can be used to target and

stop their function, subsequently trapping *L. monocytogenes* in the primary phagosome (Edelson and Unanue, 2001).

2.11 *Listeria monocytogenes* survival in ready-to-eat foods

2.11.1 Temperature

Listeria monocytogenes is able to grow and survival over varying range of storage temperatures. While the most favorable growth temperature is 30-37°C, lowest and highest limits for growth are 1°C and 45°C, respectively. Growth of *Listeria monocytogenes* has also been reported in chicken broth at temperatures as low as –0.1°C to –0.4°C (Walker *et al*., 1990). Due to the resilience nature *L. monocytogenes*, these bacteria have been found surviving and multiplying in refrigerated processed meat products including frankfurters, ham, chicken, bologna, sliced turkey and sausages stored at 4.4°C (McKellar *et al*., 1994).

2.11.2 Sodium chloride (NaCl) concentration

Listeria monocytogenes halotolerant ability has been reported with several strains resistant to varying concentrations of sodium chloride. For the prevention of these bacteria from foods, concentrations of NaCl higher that the inhibitory concentrations are used. Faleiro *et al.* (2003) reported the control of growth of *L. monocytogenes* in 10% NaCl within 72 hours at 25°C. Peterson *et al.* (1993) reported the control of *L. monocytogenes* contamination using NaCl and they observed the inhibition to a level of 10^2 cfu/g compared to the 10^6-10^8 cfu/g of *L. monocytogenes* in 6% NaCl at 5°C. Exposing *Listeria monocytogenes* to low levels of NaCl could act as a protective mechanism against acid shock (Faleiro *et al*., 2003).

2.11.3 Water activity

Low water activity (a_w) of many foods can to support the growth and multiplication of *L. monocytogenes*. Miller (1992) reported that the growth and survival of *L. monocytogenes* is dependent on the solute typa as well as the amount of water available. There are reports on the ability of *L. monocytogenes* Scott A growth in broth of 0.99 – 0.80 a_w. Below the minimum required a_w levels, cell death is proportional to the water activity.

2.11.4 pH

The ability of *L. monocytogenes* to grow and survive over varying pH depends on the a_w, nutrients, temperature of incubation as well as the composition of the products. Jay (2000) reported pH 6.0-8.0 as the range supporting optimum growth of *L. monocytogenes* while the growth of Listeria can occur between pH 2.0 to 9.6. Le Marc *et al.* (2002) also reported that combining low pH, high concentrations of weak organic acid, and low temperatures inhibited the growth *L. monocytogenes*.

2.11.5 Microbial competition

Competition in foods by other post-contaminants has been reported to inhibit the growth *L. monocytogenes*. These organisms contend for the existing nutrients thereby influencing the safety and stability of the product (Lawlor, 1999). *Pseudomonas* species which are aerobic psychotrophs are examples of bacteria that can out-compete *L. monocytogenes* growth in several foods. However, if the storage conditions are altered, these *Pseudomonas* species may be inhibited allowing *L. monocytogenes* to multiply to infectious levels prior to any spoilage signs occur (Marshall *et al*., 1991).

Lactic acid bacteria (LAB), which are generally regarded as safe (GRAS) bacteria have been reported to out-compete *L. monocytogenes* in foods. These organisms include the *Pediococci* sp, *Leuconostocs* sp and *Lactobacilli* spwhich use their secreted organic acids and bacteriocins to inhibit the growth of *L. monocytogenes*. LAB can lower the pH of the medium with their secreted organic acids making the environment unfavorable for *Listeria* (Farber *et al*., 1989; Degnan *et al*., 1992). The bacteriocins produced by some LAB might not inhibit *Listeria* in foods. Yousef *et al.* (1991) established the effectiveness of *Pediococci* sp as well as its pediocin for controlling *L. monocytogenes*.

2.12 Adherance and biofilm formation of *Listeria monocytogenes*

L. monocytogenes are able to form biofilms on different surfaces occurring as surface-attached communities of cells surrounded in an extra-cellular polysaccharide matrix (Nikolaev and Plakunov, 2007). Biofilms are significant as it makes *Listeria* grow, multiply, survive and resist varying physical and chemical agents intended for its elimination. With the ability to form biofilms, these bacteria survive extensively even with minimum nutrient conditions. Biofilm formation has great implication on hygiene as the

adhered communities of cells resist the processes used to remove contaminants from food contact surfaces (Oh and Marshall, 1995; Aarnisalo *et al.,* 2003). Biofilm adherence to surfaces occurs in two stages; the cells reversibly adhere to the surface through hydrophobic and electrostatic interactions, then, they then adhere irreversible by producing exopolysaccharides (EPSs) (Dunne, 2002).

The exopolysaccharides formed by the cell communities have been described to strengthen the cell attachment to the various surfaces (Costerton, 1999). *L. monocytogenes* has been reported to form biofilms on numerous types of food contact surfaces including rubber, plastic and stainless steel surfaces. The adhered cells demonstrated improved resistance to several cleaning agents, heat and disinfectants (Frank and Koffi, 1990; Ronner and Wong 1993; Oh and Marshall, 1995; Aarnisalo *et al*., 2003), all of which are used for sanitation in food processing industries. Small differences in adherence of *L. monocytogenes* between different food contact materials have also been reported. *L. monocytogenes* can adhere to stainless steel surfaces in less significant numbers than to rubber or polytetrafluorethylene (Sinde and Carballo, 2000) but adhere in higher numbers when compared to adherence on nylon (Blackman and Frank, 1996). Buna-N rubber has an inhibitory effect on *L. monocytogenes* and the number of cells that can adhere on Buna-N rubber has been reported to be small compared to adhered Listerial on stainless steel surfaces (Helke *et al*., 1993, Ronner and Wong 1993). *L. monocytogenes* adhesion for as little as 20 minutes and production of extracellular material within one-hour has been reported from several surfaces including rubber, polypropylene stainless steel and glass surfaces (Mafu *et al*., 1990; Wirtanen and Mattila-Sandholm, 1993; Chae and Schraft, 2000).

2.13 Resistance and adaptation of *Listeria monocytogenes* to disinfectants

Food processing industries use several disinfectants with different mode of action during sanitation. The minimum inhibitory concentration (MIC) has been used to show resistance of *L. monocytogenes* to several disinfectants. 7% to 26% of *L. monocytogenes* strains were reported to be resistant to quaternary ammonium compounds (QACs) (Mereghetti *et al*., 2000; Romanova *et al*., 2002). The action of an active efflux pump and the ability to modify its cell wall are the mechanisms used by *Listeria* stains to resist several disinfectant actions (Mereghetti *et al*., 2000). Numerous reports of listerial resistance to

hexamidine diisethionate, QAC benzalkonium chloride, and ethidium bromide exist (Lemaître *et al*., 1998).

Adaptation to disinfectant occurs when there is an increasing bacteria resistance to sub-lethal concentrations of the disinfectant (Aase *et al*., 2000). Six fold increase in *L. monocytogenes* observed with adaptation to benzalkonium chloride (Aase *et al*., 2000, Romanova *et al*., 2002). The action of an active efflux pump has been reported to be one of the adaptive resistance responses to QACs which are widely used during sanitation in food industries.. Cross-adaptation among disinfectants can occur due to specific or non-specific changes leading to increasing resistance to various physical and chemical agents with similar or different mechanism of action (McDonnell and Russell, 1999). Cross-adaptation has been documented in in *Serratia marcescens* between benzalkonium chloride and chlorhexidine gluconate and in *Pseudomonas aeruginosa* between chlorhexidine and QACs (Gandhi *et al*., 1993).

2.14 Antimicrobial resistance in *L. monocytogenes*

A positive outcome for listeriosis depends on the early administration of antibiotics. Charpentier and Courvalin (1999) showed that several *Listeria* strains as well as *L. monocytogenes* strains were susceptible to different antibiotics except streptomycin, cephalosporins, foemycin, tetracycline and erythromycin. The first choice of antibiotics treatment for listeriosis is the use of penicillin G, ampicillin combined with an aminoglycoside like gentamicin. The second choice therapy is sulphonamide combined with trimethoprim (Charpentier and Courvalin, 1999; Moreno *et al*., 2014).

Treatment of disease is usually by antibiotic therapy though some multidrug resistant strains have been isolated (Pesavento *et al.,* 2010). Advancement of bacterial resistance to several antibiotics is increasing due to increasing prescription of drugs clinically and the use of antibiotics as promoters in animal husbandry (Charpentier *et al.,* 1995). In 1988, the a multi-resistant strain of *L. monocytogenes* was isolated in France (Poyart-Salmeron *et al*., 1992) and since then, several strains of multi-resistant *L monocytogenes* have been isolated from different foods and environmental samples (Charpentier *et al.,* 1995). Antibiotic resistance in *L. monocytogenes* is generally due to acquisition of three types of movable genetic elements: self-transferable plasmids, mobilizable plasmids, and conjugative transposons (Charpentier and Courvalin, 1999; Conter *et al*., 2009). With the

increasing antibiotic resistance in *L. monocytogenes,* it would get to a time where almost all antibiotics will be rendered futile due to the different mechanisms used by *L. monocytogenes* to counter these agents. Resistance to tetracycline has been the most commonly reported resistance among strains of *L. monocytogenes* (Charpentier *et al.,* 1995; Charpentier and Courvalin, 1999; Jamali *et al*., 2013; Moreno *et al*., 2014). *tet*(P), *tet*(L), *tet*(M), *tet*(S), *tet*(K), and *tet*(O) are the six types of tetracycline-resistance genes reported in Gram positive bacteria, however, just *tet*(S) and *tet*(L) have been recognized in *L. monocytogenes* (Poyart-Salmeron *et al.,* 1992; Charpentier and Courvalin, 1999). Resistance by *Tet* (S) and *tet* (M) is by ribosomal protection, whereas the *tet* (L) gene codes for a protein which causes active efflux of tetracycline away from the bacteria. Efflux pumps have also been reported to be associated with fluoroquinolone resistance in *Listeria* (Godreuil *et al*., 2003). *Enterococci* and *Streptococci* represent a reservoir of resistance as *L. monocytogenes* can acquire resistance genes from them and transfer to them (Godreuil *et al*., 2003)

2.15 Regulatory Agencies and *L. monocytogenes*

The European Union Commission Regulation (EC) with No 2073/2005was commissioned in Europe and they proposed for *L. monocytogenes* in RTE foods (different from infant foods and foods intended for medical purposes) that;

(i) All through the shelf-life of food products, *L. monocytogenes* should be less than100 cfu/g.

(ii) *L. monocytogenes* should be absent in 25g of the food product after the final processing stage (Carrasco *et al*., 2007).

USDA-FSIS (Food Safety Inspection Service) stated that no detectable level of *L. monocytogenes* is permitted in RTE products (zero tolerance). The U.S. Food and Drug Administration (FDA) also maintain zero tolerance policy for *Listeria monocytogenes* in foods that are ready-to-eat, meaning that if *Listeria monocytogenes* should not detected in twenty five gram (25g) food sample or the food would be rendered adulterated as elucidated by section 21 U.S.C. 342(a)(1) Federal Food, Drug, and Cosmetic Act (Shank *et al*., 1996).

2.16 Laboratory isolation and identification of *Listeria monocytogenes*

The methods used for *Listeria* isolation in the laboratory must be able to recover at least one *Listeria* per 25g of food as specified by most regulatory agencies (Gasanov *et al*., 2005). This can be achieved by using any enrichment methods that suppress competing microflora due to the antimicrobial agents of the selective agars. The selective agents that are usually used in enrichment are nalixidic acid which inhibits the growth of gram negative bacteria, acriflavine for inhibition of other Gram positive bacteria; and cycloheximide for inhibition of fungi. The media used for isolation of *Listeria* also contains esculin. The inclusion of ferric ion in several *Listeria* media results in the formation of an intense black colour from the hydrolysis of esculin. This black colouration seen is as a result of the formation of a black precipitate from the interaction between ferric iron with 6, 7-dihydroxycoumarin and the product of esculin hydrolysis by β-D-glucosidase (Sacchetti *et al*., 2003). The most frequently used culture reference methods for the isolation of *Listeria* in foods are the standards as described by ISO 11290 (ISO, 1996), FDA-BAM *Listeria* isolation method from seafoods, vegetables and dairy products (Hitchins, 1998), USDA *Listeria* isolation method from environmental samples, poultry and meat samples (USDA-FSIS, 2002). The commercially available *Listeria* selective media include Nutrient Agar supplemented with esculine bile salt, Oxford Agar, Listeria Selective Agar and Palcam Agar.

Several chromogenic media have been used to facilitate easy identification of pathogenic *L. monocytogenes* from other species of *Listeria*. This is often based on production of acids during fermentation of specific sugars, secretion of enzymes by certain strains of *Listeria* and the inclusion of different antimicrobials the isolation media.

Chromogenic media is more accepted compared to other culture confirmation methods due to of its effortless preparation and interpretation of morphological characteristics. It allows for the presumptive identification and differentiation of *L. monocytogenes* for other *Listeria* species after 24 hours (Reissbrodt, 2004). The plcA gene cluster of virulence present in *L. welshimeri, L. seeligeri* and *L. monocytogenes* codes for phosphatidylinisitol phospholipase C (PIPL-C) synthesis, which is usually used for differentiating haemolytic species of *Listeria* from non-haemolytic species by forming a halo-like precipitation zone around haemolytic species. Ottaviani *et al*. (1997) combined this haemolytic detection

system with chromogenic substrate that detects β-D-glucosidase activity. The chromogenic media was referred to as "Agar *Listeria"* by Ottaviani and Agosti' (ALOA), as the turquoise blue colonies observed on the media are *Listeria* spp. while pathogenic *L. monocytogenes* and *L. ivanovii* are surrounded by a halo-like precipitation zone (Reissbrodt, 2004).

After selection of typical *Listeria* spp. colonies from either selective or differential agar plates, other biochemical test are used to further identify the *Listeria* spp. The biochemical tests consist of the Gram reaction, catalase, motility (inoculation into motility medium as well as observing wet mount samples under the phase-contrast microscope), haemolysis on blood agar and carbohydrate fermentation. The Christie–Atkins–Munch–Peterson (CAMP) another biochemical test used to identify Listeria species. CAMP test is easy to perform and interpret. It is carried out by streaking a β-haemolytic *Staphylococcus aureus* (ATCC strain 25923 or 49444, NCTC strain 1803 or 7428) and *Rhodococcus equi* (NCTC strain 1621, ATCC strain 6939) in a straight line parallel to each other on a sheep blood agar plate or on a double-layered agar plate with a small overlay of blood agar. The CAMP reaction is positive when there is an enhanced β-haemolytic zone at the intersection of the test and indicator strains. *Listeria monocytogenes* is positive when streaked parallel to *S. aureus* streak but negative with *R. equi*, while *L. ivanovii* gives the opposite reaction (Quinn *et al*., 1999). *L. ivanovii* can be differentiated from *L. monocytogenes* by its strong lecithinase reaction with or without charcoal in the medium, in comparison to *L. monocytogenes* which requires charcoal for its lecithinase reaction (Ermolaeva *et al*., 2003).

Serologically, strains of *Listeria* species are separated based on specific heat-stable somatic (O) and heat-labile flagellar (H) antigens. Fifteen (15) *Listeria* somatic (O) antigens exist with subtypes (I–XV) whereas flagellar (H) antigens of *Listeria* comprise of four subtypes (A–D) (Lukinmaa *et al*., 2003). The unique combinations of the O and H antigens of individual *Listeria* strains determine their serotype. At least 13 serotypes have been documented in *L. monocytogenes* (i.e. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7), and several serotypes in *L. seeligeri* (e.g. 1/2a, 1/2b, 3b, 4a, 4b, 4c and 6b), one

serotype in *L. ivanovii* (i.e. 5), and a few serotypes in *L. innocua, L. welshimeri and L. grayi* (1/2b, 6a and 6b) (Kathariou, 2002).

Several molecular typing methods have been used to characterize and identify strains of *L. monocytogenes* (Bille and Rocourt, 1996). These methods include Pulsed-Field Gel Electrophoresis (PFGE), Multilocus Enzyme Electrophoresis (MEE) typing, Random Amplification of Polymorphic DNA (RAPD), Restriction Endonuclease Analysis (REA) and ribotyping (Bille and Rocourt, 1996). DNA sequence-based typing and Amplified Fragment Length Polymorphism (AFLP) typing have also been used to type *L. monocytogenes* (Fonnesbech-Vogel *et al*., 2001; Cai *et al.*, 2002; Autio *et al*., 2003; Keto-Timonen *et al*., 2003). One of the best choices of typing of *L. monocytogenes* is the use of DNA macro-restriction analysis by is reproducible PFGE. This method is reproducible, has high discriminatory power and can be used for typing *L. monocytogenes* serotype 4b strains (Brosch *et al*., 1996). The method is based on the slicing the chromosomal DNA into large fragments with several restriction endonucleases. The sliced fragments can then be run in a pulsed-field gel electrophoresis allowing separation of the fragments. PFGE typing has been effectively used in identifying and tracing *L. monocytogenes* epidemics and related route of contamination in food processing plants (Ojeniyi *et al*., 1996; Unnerstad *et al*., 1996; Giovannacci *et al*., 1999; Miettinen *et al*., 1999; Lyytikäinen *et al*., 2000; Berrang *et al*., 2002). MEE typing has also been used in several epidemiological studies (Farber *et al*., 1991; Avery *et al*., 1996; Nesbakken *et al*., 1996; Buncic *et al*., 2001).

MEE is based on the gel electrophoresis separation of soluble metabolic enzymes resulting in several distinct electrophoretic types. The typeability of the isolates using this method is good, but its discriminatory power is not very high (Caugant *et al*., 1996). MEE typed isolates can further be typed with other methods to improve its discrimination (Rørvik *et al*., 2000). REA has a high discriminatory power and it is based on slicing chromosomal DNA into several fragments with commonly available restriction endonucleases. Depending on the type of restriction endonucleases used, numerous fragments can be generated which can complicate the interpretation of the patterns (Gerner-Smidt *et al*., 1996).

Ribotyping is based on the cutting the DNA using restriction endonucleases, after which separation is done by gel electrophoresis. Several fragments are obtained after electrophoresis using this method and then, a subset of the obtained fragments are visualized by hybridizing with a labeled ribosomal RNA. Ribotyping is a good typing method but its discriminatory power is limited (Swaminathan *et al*., 1996), although, the discriminatory power can be enhanced by using several different enzymes (Cesare *et al*., 2001). Automated ribotyping, which has been used in a number of epidemiological studies allows large amounts of isolates to be typed at the same time (Tkáčiková *et al*., 2000; Berrang *et al*., 2002; Aarnisalo *et al*., 2003). RAPD, a PCR-based typing method is used to amplify segments of the target DNA randomly by using several primers selected arbitrarily. RAPD has a high discriminatory power but achieving reproducible results is difficult (Wernars *et al*., 1996). RAPD has been useful for several epidemiological investigations (Giovannacci *et al*., 1999; Aguado *et al*., 2001; Fonnesbech-Vogel *et al*., 2001).

AFLP is also a PCR-based typing method used to digest the total bacterial DNA by restriction enzymes. An oligonucleotide adapter corresponding to the DNA sequence of the restriction site is ligated to the fragment. Subsets of fragments are then amplified and run in an electrophoresis machine (Guerra *et al*., 2002). The high discriminatory power of AFLP is comparable to that of PFGE (Fonnesbech-Vogel *et al*., 2001; Keto-Timonen *et al*., 2003). The partial or complete sequence of one or several genes can be achieved by DNA sequence-based typing methods. The data produced by these DNA sequence-based typing methods are explicit, sensitive and allows detection of a single nucleotide change (Cai *et al*., 2002). The differentiation and identification of *L. monocytogenes* from other *Listeria* species can also be achieved using PCR to target specific unique genes. The use of these PCR methods is more precise and less affected by natural differences than phenotypic methods (Liu, 2008).

2.17 Reducing the risk of listeriosis

The guidelines recommended for the prevention of listeriosis are similar to those used to help prevent other food borne illnesses, such as salmonellosis. In addition, there are

specific recommendations for persons at higher risk for listeriosis. FDA (2015) recommends the following;

- Fruits and vegetables should be washed thoroughly under running tap water before eating, cutting, or cooking. Even if the produce is peeled, it should still be washed before consumption.
- Uncooked meats and poultry products should be separated from RTE foods.
- Hands, knives, cutting boards and other utensils should be properly washed before and after handling and after preparing uncooked foods.
- The refrigerator should always be clean and set at 40°F or lower and the freezer 0°F or lower
- Raw foods from animal sources such as beef, pork, or poultry should be thoroughly cooked to a safe internal temperature.
- RTE foods should not be stored in the refrigerator beyond the use-by date
- Raw (unpasteurized) milk and foods with unpasteurized milk should not be consumed

Several control and management measures for *L. monocytogenes* in food chain are encompassed within good manufacturing practices (GMP), good hygiene practices (GHP), food safety/hygiene training and the Hazard Analysis Critical Control Point (HACCP) system.

2.18 Plants and Antilisteria Activity

Plants produce a vast diversity of secondary metabolites most of which are phytochemicals that have potential use in the pharmaceutical industry for new drug development purposes. Phytochemicals are naturally-occurring bioactive plant compounds that act as a natural defence system for the host plant and also provide colour, aroma, and flavor (Sharma *et al.*, 2012). Some phytochemicals have been shown to possess antimicrobial properties and these include terpenoids, essential oils, alkaloids, lectins, polypeptides, polyacetylenes, and phenolics, of which phenolics can be further divided into phenolic acids, flavonoids, quinones, tannins, coumarins, and simple phenols (Cowan, 1999). The terpenes are one of the largest and most diverse groups of plant secondary

metabolites. They include complex compounds that are formed by the cyclization of 2, 3-oxidosqualene (Ncube *et al.*, 2008). They include terpenoids and sterols as well as essential oils which carry the fragrance of the plant.

Terpenes possess antimicrobial properties and their mechanism of action is mainly through disruption of the bacterial membrane (Ncube *et al.,* 2008). Flavonoid modes of action usually involve formation of complexes with cell walls, binding to adhesins and inactivation of bacterial enzymes (Cowan, 1999). Tannin exert their antimicrobial action in different ways which include binding to proteins and adhesins, bacterial enzyme inhibition, substrate deprivation, formation of complexes with bacterial cell wall, membrane disruption, and metal ion complexation (Cowan, 1999). Alkaloids on the other hand intercalate into cell wall and/or DNA, causing leakage of bacterial cell contents or disruption of DNA synthesis, respectively, which eventually leads to bacterial death (Kumar *et al*., 1999).

A variety of herbs and spices have been tested for their efficacy in suppressing the growth of *L. monocytogenes* in culture media. Plant extracts that have shown to exhibit antilisterial activity include: hop extracts, eugenol, (Hao *et al*., 1998), pimento leaf, horseradish distillates (Ward *et al*., 1998), rosemary, cloves (Lis-Balchin and Deans, 1997), cinnamic acid, furanocoumarins and carvacol (Kim *et al*., 1995). *Garcinia kola, Moringa olifera*, *Origanum syriacum*, *Majorana hortensis*, *Rosmarinus officinalis*, c*ymbopogon citratus*, *Thymus vulgaris*, *Achillea millefolium*, *Calendula officinales* and *Artemisia annua* have also been reported to exhibit antilisterial activity (Blanco *et al.*, 2009; Bakr, 2013). Different commercial samples of plant essential oils and different varieties of the same herbs may exhibit differences in antilisterial potency because of varying amounts of phytochemicals. Plant extracts found to be effective against *Listeria* spp. in meat includes rosemary in ready-to-eat pork liver sausage, horseradish distillates on roast beef, and eugenol and pimento leaf on refrigerated cooked beef. It was observed that *L. monocytogenes* was usually less sensitive to these extracts in meat (compared to culture media) (Larson *et al*., 1996). In addition, the antimicrobial property of medicinal plants may differ depending on the form of added plants, such as fresh, dried, or extracted forms.

2.18.1 *Psidium guajava*

Psidium guajava (Myrataceae), a small tropical tree that grows up to 35 feet tall is widely grown for its fruit in tropics. *Psidium guajava* (Myrataceae) has about 133 genera and more than 3800 species. The bark and leaves of guava tree have a long history of medicinal uses that are still employed today (Nwinyi *et al*., 2009). The leaves and bark of the guava plant have been used to treat diarrhea, gastrointestinal disorders, toothaches, colds, and swelling. Guava is rich in fatty acids, tannins, vitamins, phenols, lectins, flavanoids, essential oils etc. A decoction of bark of leaves or a flower infusion is used tropically for wounds, ulcers and skin sores. Flowers are also mashed and applied to painful eye conditions such as sun strain, conjunctivitis or eye injuries. Commercially the fruit is consumed fresh or used in the making of Jams, Jellies, paste. Guava leaves are in the " Ducth-Pharmacopoea" for the treatment of Diarrhea. Flavanoids have been attributed as the main phytochemical responsible for guava's medicinal activity (Arima and Danno, 2002).

2.18.2 *Zingiber officinale*

Ginger (*Zingiber officinale*) is a medicinal plant that is widely used all over the world for a wide array of unrelated ailments including arthritis, cramps, rheumatism, sprains, sore throats, muscular aches, pains, constipation, vomiting, hypertension, indigestion, dementia, fever and infectious diseases (Ali *et al*., 2008). Ginger has direct anti-microbial activity and thus can be used in treatment of bacterial infections (Tan and Vanitha, 2004). Ginger belongs to Zingiberaceae family and the Zingiberaceous plants have strong aromatic and medicinal properties and are characterized by their tuberous or non-tuberous rhizomes (Chen and Knabel, 2007). Ginger is relatively inexpensive due to their easy availability and they are well tolerated by most people (Ali *et al*., 2008).

2.18.3 *Trema orientalis*

Trema orientalis is a species of flowering tree in the hemp family, Ulmaceae. It is known by many common names, including charcoal-tree, Indian charcoal-tree, pigeon wood, Oriental trema, and in Hawaii, where it has become naturalized gunpowder tree, or nalita (Dimo *et al*., 2006). It has a near universal distribution in tropical and warm temperate

parts of the world, with a range extending from South Africa, through the Middle East, the Indian subcontinent and southern China to Southeast Asia and Australia. *T. orientalis* is used in traditional medicine by the rural people and possesses various interesting pharmacological activities (Uddin *et al*., 2008). The root of the plant is used in the treatment of diarrhea, asthma and passing of blood in urine; the bark is used as poultice in muscular pain; the roots, barks and leaves are used to treat coughs, sore throats and epilepsy (Uddin *et al*., 2008). In African folk medicine, it is used in many diseases including dysentery, hypertension, etc (Iwe, 1993). Research has shown this plant is used as remedy for bronchitis, gonorrhoea, malaria, yellow fever, toothaches, and intestinal worms (Rulangaranga, 1991; Uddin *et al*., 2008). In recent studies, an aqueous extract from the bark of *T. orientalis* has been reported to reduce blood sugar levels infected diabetes mellitus animal model, and may be useful for treating this disease (Dimo *et al*., 2006).

2.18.4 *Heinsia crinita* (Atama)

Heinsia crinita is of the Family Rubiaceae, it is also known as "Bush apple" and called "Tonoposho", "Atama" and "Fumbwa" in Yoruba, Efik and Lingala dialects respectively. It is a scrambling shrub in secondary jungle, or small tree 8-13m high in under-storey of high evergreen forest (Ajibesin *et al*., 2008). The plant is common across the tropical region. Its scrambling shrub is persistent and has very conspicuous leafy calyxlobes, produces edible yellow or reddish fruits, sweet when ripe and pleasantly acidic (Ajibesin *et al*., 2008). *Heinsia crinita* with the local name "Atama" is common with south-eastern Nigerians and the leaf extract have been reportedly used in the treatment of skin rashes, head lice in children, umbilical hernia and various ailments such as cough, catarrh, sore throat, hypertension among others (Mahesh and Satish, 2008).

2.18.5 *Dacryodes edulis*

Dacryodes edulis, the African pear, is an evergreen fruit common to the central Africa and the gulf of guinea region (Omogbai and Ojeaburu, 2010). The bark decoction is used for treatment of dysentery and anemia. The decoction is also used as a gargle or mouthwash, for tonsillitis, and general oral hygiene (Ajibesin *et al*., 2008).

2.18.6 *Citrus aurantifolia* (Lime)

Citrus aurantifolia are used in traditional medicine to treat various diseases and it belongs to the family Rutaceae. *C. aurantifolia* is commonly called lime or bitter orange. Research studies done on the plant *Citrus aurantifolia* have shown bioactive activities for cold fevers, sore throats, sinusitis and bronchitis, as well as asthma (Pathan *et al*., 2012). Lime oil is mainly used as antidepressant and can be helpful for rheumatism arthritis and obesity (Dorman and Deans, 2000).

2.18.7 *Funtumia elastica* (silkrubber)

This is a medium-sized African rubber tree with glossy leaves, milky sap, and long woody seedpods. The bark is used in the traditional medicine of tropical Africa. The steroid, alkaloid, and conessine, which are found in *F. elastica*, have anti-bacterial properties in vitro (Zirihi *et al*., 2005). Crude extracts of *F. elastica* has been shown to inhibit the growth of many molds, including *Aspergillus*, *Penicillium*, and *Candida*.

2.18.8 *Gongronema latifolium* (Utazi)

Utazi, scientifically known as *Gangronema latifolium* is a shrub that climbs and has broad, heart-shaped leaves with characteristic bitter and slightly sweet taste, especially when eaten fresh. The stems are soft/hairy and yields milky latex or exudates. It belongs to the family of plants known as Asclepiadaceae and it is widespread in tropical rainforest of West African countries, such as Nigeria, Ghana, Côte d'Ivoire, Sierra Leone and Senegal, etc (Owu *et al*., 2012). In southern Nigeria, where it is commonly grown, the Yoruba know it as arokeke, the Igbos calls the plant utazi and the Efik and Ibibio call it utasi. Parts like fruits, seeds, leaves, root and bark contain essential oils, alkaloids, saponins, tannin, various minerals, vitamins and some essential amino acids. Research has shown that the whole plant exhibits analgesic, antitumor, broad spectrum antimicrobial (antibacterial, antifungal, antiparasitic and antiviral), antipyretic, antioxidant, anti-inflammatory, antiulcer, anti-sickling, anti-asthmatic, mild expectorant, hypoglycemic, hypolipidemic, hepatoprotective, digestive tonic and laxative properties (Owu *et al*., 2012). The leaves are believed to neutralise the intoxicating properties alcohol and its harmful effects on the liver (Akerele *et al*., 2008).

2.18.9 *Ocimum gratissimum* (Scent leaf)

Ocimum gratissimum also known as "alfavaca" is a common medicinal plant in the family Lamiaceae. It is an essential herbal medicine found in warm regions and in the tropics such as India and sub-Sahara Africa especially in Nigeria and Kenya (Aguiyi et *al*., 2000; Okigbo and Ogbonnaya, 2006; Lexa *et al*., 2008). In Nigeria, *O. gratissimum* is called "Efinrin" in Yoruba, "Nchoanwu" or "Ahuji" in Igbo, "Daidoya" in Hausa and "Aramogbo" in Edo (Effraim *et al*., 2000). It is naturally used in the treatment of different infections such as upper respiratory tract infections, diarrhoea, conjunctivitis, skin disease and pneumonia (Ilori *et al*., 1996; Nwinyi *et al*., 2009). *O. gratissimum* decoction is used for treatment of gonorrheal infection and mental illness in Congo (Abdulrahma, 1992). Ocimum oil which is present in these plants have been reported to have inhibitory activities against both Gram positive bacteria (*Listeria monocytogenes, Staphylococcus aureus*) and Gram negative bacteria such as *Escherichia coli*, *Shigella* spp., *Salmonella* spp and *Proteus* spp (Okigbo and Ogbonnaya, 2006). Antifungal activities of ocimum oil on *Trichophyton rubrum, T. mentagrophytes, Cryptococcus neoformans, Penicillium* spp. and *Candida albicans* have also been reported (Nwosu and Okafor, 1995; Akinyemi *et al*., 2004; Lopez *et al*., 2005). *O. gratissimum* has been reported to be valuable in the medication for people with HIV (Human Immuno deficiency Virus) and AIDS (Acquired Immuno Deficiency Syndrome virus) (Eyob *et al*., 2008).

2.18.10 *Vernonia amygdalina* (Bitter leaf)

Vernonia amygdalina, one of the species in the family Asteraceae, is a tropical shrub with height of 13mm, petiole leaf of about 6mm in diameter (Igile *et al*., 1995). *V. amygdalina* is indigenous to tropical Africa and cultivated all over sub-Saharan Africa (Bosch *et al*., 2005). *Vernonia amygdalina* commonly called bitter leaf in English due to its characteristic bitter taste and flavour, it is also called "shikawa" in Hausa, "oriwo" in Edo, "ewuro" in Yoruba and "olubu" in Igbo (Oboh and Masodje, 2009). The leaves are consumed as vegetable and condiments, after macerating and washing thoroughly to remove the bitterness (Mayhew and Penny, 1998). Hypoglycemic, antihelmitic, antitumourigenic, antimalarial and hypolipidaemic properties of *Vernonia amygdalina* have been reported (Abosi and Raserika, 2003; Izevbigie *et al*., 2004). In phyto-medicine,

the roots and leaves of these plants are used to treat stomach discomfort, fever, kidney disease and hiccups (Gill, 1992).

Many West African countries like Cameroon, Ghana and Nigeria use the stem and root as chewing sticks (Ogundare, 2011). It has also been documented that *V. amygdalina* has been used traditionally in blood clotting and has elicited a substantial reduction in the level of glucose in the blood at post-prandial time point (Fasola *et al.*, 2011). Newbold *et al*. (1997) showed that this plant has mild antimicrobial effect on rumen bacteria and protozoa while Kambizi and Afolayan (2001) proved that acetone extract of *V. amygdalina* possesses antibacterial activity towards *Escherichia coli*, *Bacillus cereus, Micrococcus kristinae, Staphylococcus aureus, Bacillus subtilis, Enterobacter cloacae* and *Bacillus pumilus.* Babalola and Okoh (1996) reported inhibition of *Bacillus circulans, Aerococcus viridans, Clostridium perfringens* and *Micrococcus* sp by *V. amygdalina.*

2.18.11 *Cassia alata*

Cassia is a native plant in Southeast Asia, Africa, Northern Australia and Latin America that is grown as ornamental plants with diverse medicinal uses (Parsons and Cuthbertson, 1992; Gritsanapan and Nualkaew, 2001). It is commonly known as “Rai dore” in Hausa, “Asuwon oyinbo” in Yoruba, ”Omirima” in Igbo and “Whu shil-shili” in Kilba (Timothy *et al*., 2012; Arbonnier, 2004). Several research have shown that *Cassia alata* contain antimicrobial substances that may be responsible for its reported activity in bacterial and fungal infections (Palanichamy and Nagarajan, 1990; Somchit *et al*., 2003; Timothy *et al*., 2012). Studies have reported the use of *C. alata* leaf for the treatment of intestinal parasitosis, haemorrhoids, syphilis, inguinal hernia, constipation, blennorrhagia and diabetes (Crockett *et al*., 1992; Caceres *et al*., 1993).

2.18.12 *Moringa oleifera*

The "*Moringa*" tree, belonging to monogeneric family Moringaceae (order Brassicales) is known as one of the world’s most valuable trees, as most part of the *Moringa* tree (root, ark, gum, leaf, pods, flowers, seeds and seeds oil) can be used for food, with high nutritional value, impressive range of medicinal uses or some other beneficial properties (Abalaka *et al*., 2012; Wadhwa *et al*., 2013). This tree is also known as the Ben oil tree, horseradish tree, mulangay, drumstick tree, nébéday, benzolive tree, moonga, kelor,

marango, saijhan or mlonge. The moringa tree is cultivated and used as a vegetable (leaves, pods flowers, roasted seeds), for spice (mainly roots), cooking and cosmetics oil (seeds) and as a medicinal plant (Rebecca, 2006). Important medicinal properties of *Moringa* include antipyretic, antiepileptic, antiinflamatory, antiulcerative, antihypertensive, cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial and antifungal activities ((Mehta *et al*., 2003; Nickon *et al*., 2003). In addition, *M. oleifera* seed possesses water purifying abilities (Kawo, 2007) and are well-known for their immune building capacity, anti-helminthic, detoxifying abilities as well as their inhibitory activities (Thilza *et al*., 2010). Also, Renitta *et al.* (2009) reported antimicrobial activity from the ethanolic extract of leaves, seeds and flowers of *Moringa oleifera* against microorganisms such as *Escherichia coli, Klebsiella pneumoniae*, *Enterobacter spp*, *Proteus mirabilis*, *Pseudomonas aeroginosa*, *Salmonella typhi*, *Staphylococcus aureus*, *Streptococcus* and *Candida albicans*.

2.19 RTE Foods Studied

2.19.1 Salads

Salad is a combination of fresh vegetables and cream milk that are low in fat and calories and are good source of dietary fiber and minerals (Adeshina *et al*., 2012). Salads can be referred as conventional when they are prepared from blend of diced lettuce, carrots, cucumber, cabbage, tomatoes, with the inclusion of pre-packaged salad cream and sometimes baked beans (Itohan *et al*., 2011). There has been increased consumption of salads vegetables due to reports on their nutritional as well as their medicinal value (Abdullahi and Abdulkareem, 2010). There has been several food borne outbreaks that are linked to the consumption of fresh vegetables salads (Doris and Seah, 1995). Contamination of salads results from the different vegetables used for its preparation making salads a vehicle of transmission of several diseases. Microbial contamination occurs during transport with containers, harvesting, contact with wild and domesticated animals, human handling and from vegetable harvesting and dicing equipments (Itohan *et al*., 2011). Moreover, the accessibility of potable water for adequate washing of these vegetables is insufficient in different areas resulting in washing these vegetables with dirty or contaminated water which could increase the microbial flora on these vegetables which

several people purchase and consume without further washing. *Listeria monocytogenes* and *Salmonella* sp have been isolated from salads making them hazardous to consumers (Little *et al*., 2007; Biniam and Mogessie, 2010; Iaren *et al*., 2013).

2.19.2 Giant African Land Snails

Snail is of the molluscan class Gastropoda that have coiled shells in the adult stage. When the word is used in its most general sense, it includes sea snails, land snails and freshwater snails. Land snail habitat occupies areas scuh as the tropical forest in southern Nigeria to the fringing riparian forests of Guinea Savannah (Odaibo, 2007; Ajayi *et al*., 2009). From November to March each year, Nigerian snails aestivate because of the hot dry weather. The two prominent snail's species are found abundantly in Nigeria. They are the edible giant land snails: *Achatina achatina* and *Archatina marginata* (Ajayi *et al*., 2009). They are found extensively in the Southern parts of Nigeria and the entire West African coastal area, central and South Africa, where the weather is favourable for their proliferation (Herbert and Kilburn, 2004). Snails have been reported to habour bacterial pathogens which are of public health implications. Adagbada *et al*. (2011) isolated *Staphylococcus auerus, Escherichia coli*, *Aeromonas spp, Vibro spp,* and *Pseudomonas spp* from *Achatina* species in Cross River and Akwa Ibom states of Nigeria. Also, Adegoke *et al*. (2010) isolated *Bacillus subtilis, Lactobacillus spp, Micrococcus luteus, Bacillus cerus, Escherichia coli, Staphylococcus aureus* from different species of snails (*Achatina fulica, Limcolaria species and Helix pomatia*) gotten from Uyo, Akwa Ibom State, Nigeria.

2.19.3 Meat pies

Meat pie is a pie with filling of meat and often other savory ingredients. Meat pie is a baked product consisting of a pastry casing enclosing meat or/and vegetable filling which may or may not contain mashed potato. Meat pies are made from flour and contain other ingredients such as fat and oils, potato and meat mostly beef (Eluma *et al*., 2015). The nutritive content of meat pies makes it suitable for microbial proliferation and spoilage. There is a great increase in the consumption of meat pies due to increasing numbers of working mothers, more school age children obtaining their own meals and refreshment and a highly mobile population (Falola *et al*., 2011). Bacteria isolated from meat pies

include *Staphylococcus auerus, Escherichia coli*, *Aeromonas spp, Pseudomonas,* and *Bacillus subtilis* (Nwachukwu and Nwaigwe, 2013).

2.19.4 Edible worm

The larva of palm weevil (*Rhynchophorus phoenicis* Fabr. Coleoptera: Curculionidae), is cherished as food among many communities in Nigeria and around the world. The larva is commonly called edible worm or maggot and it is sold or hawked along major roads and markets in Southern Nigeria. The larva is a delicacy in many parts of Nigeria and other countries in Africa where it is found (Ekrakene and Igeleke, 2007). Among the various ethnic nationalities in Nigeria, the Binis (Edo) call it 'orhu', the Esans 'okhin', the Ibibios (Akwa Ibom) 'nten', the Ibos 'eruru' or 'akwangwo' or 'nza', the Idomas (Benue) 'eko-ali', the Isoko 'odo', the Itsekiris 'ikolo', the Urhobos 'edon', the Bayelsas' " Bayelsa suya", the Yorubas 'awon' or 'ekuku'. The mode of preparation is known to vary from one geographical locality to another. In some places, it is boiled while others smoke, fry or simply eat it raw (Ekpo and Onigbinde, 2005). Although *L. monocytogenes* have not been reported from edible worms, Ogbalu and Williams, (2015) reported *Bacillus, Staphylococcus* sp*., Acinetobacter* sp., *Pseudomonas* sp. *Micrococcus* sp., *Proteus* sp., *Penicillium* sp., *Aspergillus* sp. and *Saccharomyces cerevisae* from raw larva sold at Taabaa Ogoniland, Rivers State. Other bacteria that have been isolated from *R. pheonicis* include; *Lactobacillus plantarum, Staphylococcus auerus, Bacillus subtilis, Pseudomonas aeruginosa* and *Proteus vulgaris* (Ekpo and Onigbinde, 2005).

Plate 2.1: Edible worms

Source: Martin (2015)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Area of Study

The study was carried out along Traveler's route of South South Nigeria. Sampling points covered Ore and parts of three (3) of the six (6) South South states of Nigeria (Plate 3.1). The choice of sampling point was based on the areas (both large and make shift restaurants) where travelers stop to eat and purchase foods for their loved ones at home. Furthermore, areas where hawkers hawk their food and snacks were also sampled. The Specific study areas are:

- Ore in Ondo State: geographical coordinates are 6° 45' 0" North, 4° 52' 0" East
- Sapele Road Benin city: geographical coordinates are 6° 34' 0"North 5° 63' 0" East
- Sapele round about: Located in Delta state Nigeria. Its geographical coordinates are 5° 52' 0" North, 5° 43' 0" East
- Effurun round about: Situated in Okpe, Delta, Nigeria, its geographical coordinates are 5° 33' 0" North, 5° 47' 0" East
- Ugheli- Patani road: Situated in Delta state, Nigeria, its geographical coordinates are 5° 30' 0" North, 5° 59' 0" East
- Postuku (after first bridge in Bayelsa state) : geographical location is 4° 45' 0 North, 6° 4' 59 East

3.2 Sample Collection

Sampling at each point was carried out without bias. The samples collected were Salads, Meat pies, snails, and edible worms. The sample size was four hundred and eleven (411). This included one hundred and twenty four (124) edible worm samples, eighty five (85) salads, one hundred and eight (108) snails and ninety four (94) meat pies. All RTE food samples in this study, except for salads were subject of high-temperature process (e.g. boiling for snails, baking for meat pies and roasting for edible worms).

All RTE food samples were purchased fresh and transferred into sterile nylon bags. They were then transported to the laboratory for immediate analysis while samples that could not be subjected to immediate laboratory analysis were properly wrapped in aluminium foil in order to prevent microbial contamination and then stored in the refrigerator (4ºC) until the next day.

3.3. Isolation of *Listeria* species using *Listeria* Selective Agar

Prior to the evaluation of the presence of *Listeria* spp. in the food samples, the samples were crushed in a clean mortar with pestle. One (1) g of each sample was aseptically weighed and homogenized by vortexing with nine (9) mL of sterilized peptone water to produce a stock homogenate after which it was incubated at 37°C using Surgifield medical incubator (SM 9082A) for four (4) hours (Moreno *et al.,* 2012). Ten (10) fold serial dilutions were made aseptically from the homogenate after which one (1) mL of aliquot was pour-plated in duplicate onto *Listeria* selective agar (CM0856 Oxoid Oxford formulation containing supplement SRO140). The inoculated *Listeria* selective agar (LSA) plates were incubated at 37°C for 24-48h after which suspected *Listeria* colonies were counted and represented as cfu/g of the sample. All *Listeria* species hydrolyse aesculin as evidenced by a blackening of the medium hence discrete *Listeria* colonies appeared grey with black holo zones surrounding it. Pure isolates of *Listeria* sp. were then sub-cultured further biochemical testing.

3.4 Culture Preservation

Pure cultures of the *Listeria* isolates were sub-cultured onto Trypticase soy agar with 0.6% (w/v) yeast extract (TSAYE). Further growth was prevented by storing the culture at 4°C. At 4-week intervals, the isolates were transferred into fresh maintenance medium of the same composition.

Figure 3.1: Map showing location of sample areas in Nigeria

Table 3.1: Representative samples collected for *Listeria monocytogenes* isolation from parts of South South States in Nigeria

Sampling Site	Sample type	Sample Code	Sample size
Ore	Salads	LOSA	30
	Snails	LOSN	10
	Meat pie	LOMP	24
Sapele Road (Benin city)	Edible worm	LBEW	20
	Salads	LBSA	15
	Snails	LBSN	14
	Meat pie	LBMP	18
Sapele round about	Edible worm	LSEW	25
	Salads	LSSA	13
	Snails	LSSN	15
	Meat pie	LSMP	12
Effurun round about	Edible worm	LEEW	36
	Salads	LESA	12
	Snails	LESN	26
	Meat pie	LEMP	10
Effurun round about	Salads	LUSA	15
	Snails	LUSN	18
	Meat pie	LUMP	15
Postuku (After first bridge in Bayelsa)	Edible worm	LPEW	25
	Snails	LPSN	25
	Meat pie	LPMP	15
Total			411

3.5 Characterization of Isolates

3.5.1. Gram staining

This test was carried out to differentiate between Gram positive and Gram negative rods or coccal bacterial isolates. A smear of the 18-24h culture of each isolate was made on a clean glass slide, heat-fixed and stained with 2 drops of Crystal violet for 60 seconds, rinsed and stained with Iodine for 30 seconds. This was rinsed and decolorized with acetone, rinsed with tap water and finally counterstained with 2 drops of Safranin for 30 seconds. The slides were allowed to air-dry after which it was viewed under the microscope. The shape, colour and arrangement of the cells were observed (Adeshina *et al*., 2012).

3.5.2 Catalase Test

Catalase test is used to identify organisms that can produce the enzyme catalase (Alsheikh *et al*., 2013). A few drops of 3% Hydrogen peroxide were placed on a clean grease free slide, and then a loopful of the isolate was emusified on the hydrogen peroxide on the slide. Positive result was indicated by notable effervescence.

3.5.3 Sugar Fermentation Test

Sugar fermentation test used to distinguish between organisms that can ferment carbohydrate to produce acid (Collins *et al*., 1991). Isolates were grown in tryptone soy broth containing 0.5% of the carbohydrate (glucose, mannose, rhammose, fructose, lactose, maltose, D-xylose and D-mannitol), with bromo cresol purple as indicator, change in indicator from purple to yellow (acid) is indicative of positive reaction.

3.5.4. Oxidase test

Oxidase test was used to express the production of cytochrome C oxidase which is an enzyme that catalyzes the transport of electrons between electron donors in bacteria and a redox dye, tetra methyl-p-phenylenediamine (oxidase reagent) (Alsheikh *et al*., 2013). The

dye is usually reduced to a deep purple color. A smear from the bacterial isolate was made on Whatman No. 1 filter paper. This was flooded with 1% solution of tetramethyl-p-phenylenediamine hydrochloride. The formation of dark purple color within 5 -10 seconds was indicative of a positive oxidase reaction. Negative oxidase reaction revealed no color change.

3.5.5. Citrate utilization

The ability of the isolated *Listeria* sp. to utilize citrate during growth as a sole carbon and energy source was carried out using Simmon's citrate agar (Alsheikh *et al*., 2013). The slants were inoculated with the test organisms with the aid of a wire loop. Color change by the medium from green to blue was indicative of a positive result, while no color change indicated negative result.

3.5.6. Indole test

Indole test was used to determine the ability of the isolated *Listeria* sp. to degrade the amino acid tryptophan to indole. Tryptone soy broth (1%) was prepared and distributed to screw capped tubes and sterilized. After cooling, the tubes were inoculated and then incubated at 37°C for 48h. One (1) mL of Kovac's reagent was added to six (6) mL of the culture fluid. The mixture was thoroughly shaken for even mixing. The mixture was then allowed to stand until the reagent rose to the top. After a few minutes, the tubes was observed for a deep red colour which indicated indole production while the absence of a deep colour indicated a negative reaction (Yakubu *et al*., 2012).

3.5.7. Hydrogen Sulphide Test (H_2S)

Hydrogen Sulphide **(**H_2S) test was used to determine the capability of the isolated *Listeria* sp. to decompose organic or inorganic sulphur compounds to produce hydrogen sulphide (Yakubu *et al*., 2012). An inoculum from a pure *Listeria* culture was transferred aseptically to a sterile Kligler iron agar slant. The inoculated tube was incubated at 35-37°C for 24h. An alkaline slant-butt (red/yellow) indicates fermentation of dextrose only, an acid slant-butt (yellow/yellow) indicates fermentation of dextrose and lactose, an

alkaline slant-alkaline butt (red/red) indicates that neither dextrose nor lactose was fermented while a black precipitation in the butt indicates hydrogen sulphide production.

3.5.8. Haemolytic activity for *Listeria* spp.

The haemolytic activity of identified *Listeria* spp. was determined by streaking portions of the respective pure colonies on freshly prepared blood agar plates under aseptic conditions. The plates were incubated at 37°C for 48h and production of clear zones around the streaked colony was indicative of hemolytic activity (Yusuf and Tengkuhaziyamin, 2013).

3.6. OBIS mono for *L. monocytogenes*

The O.B.I.S. (Oxoid Biochemical Identification System) mono is a rapid colourimetric test intended for the detection of D- alanyl aminopeptidase (DALAase) in Gram-positive, catalase-positive, oxidase-negative short rod shaped bacteria, capable of growing on listeria primary isolation media (Ali and Moutaz, 2011). It is usually used to differentiate serologically *Listeria monocytogenes* from other *Listeria sp*. One ID601M O.B.I.S. mono test cards was removed from the pack, and then a discrete colony of the isolate was picked using a wire loop then placed on the O.B.I.S. mono test card. A drop of ID120 O.B.I.S. mono buffer was placed on the inoculated zone and the test card was placed in an ID090 OBIS reaction sleeve and incubated for 15m. After which, one drop of ID220 O.B.I.S. mono developing solution was placed onto the reaction zones. The purple colour observed within 20s is a positive reaction and it indicates the organism is not *Listeria monocytogenes*.

3.7 *Listeria* Serotyping

Serotyping was performed as described by manufacturer's instructions of commercially available *Listeria* antisera. DR1126 Oxoid Polyvalent antisera used were prepared against purified flagellin proteins from *Listeria monocytogenes* (antigens A, B and C) and *Listeria grayi* (antigen E). When the polyvalent antiserum was mixed with a suspension containing suspected *Listeria* spp the latex particles rapidly agglutinate forming visible clumps.

Absence of agglutination was indicative that the organism is not *Listeria* spp (Morobe *et al*., 2012).

3.8 Phosphatidylinositol- Specific Phospholipase (PI-PLC)

All *Listeria* isolates identified phenotypically were tested for PI-PLC activity using Rapid L-mono (Biorad). This medium is a chromogenic medium used for confirmation of *Listeria monocytogenes* based on Phosphatidylinositol specific phospholipase C (PIPLC) activity and non fermentation of xylose (Sang-Hyun *et al*., 2014). Overnight broth culture of each of the isolates was streaked on Rapid L-mono plates and incubated in Surgifield medical incubator (SM 9082A) at 37°C for 24-48h. Development of blue colonies surrounded by clear halo zone after incubation is presumptive for *Listeria monocytogenes* (Sang-Hyun *et al*., 2014).

3.9 16sRNA and phylogeny

3.9.1 Isolation of *L. monocytogenes* genomic DNA

Genomic DNA of *L. monocytogenes* isolate was extracted using QIAamp DNA Mini Kit (250) cat no 51306 with Qiagen DNA extraction protocol (Di-pinto *et al*., 2007). The bacteria was grown in tryptone Soy broth for 24h after which it was centrifuged at 20,000g for 5m then the supernatant was discarded leaving the cell pellets. 180µL of enzymatic lysis buffer AL (200ng/m lysostaphin: 200nM Tris HCl; pH 8.0; 2nM EDTA; 1.2% triton) was added to the cell pellet and 20µL of proteinase K was added then incubated at 37°C for 30m. Another 20µL of proteinase K and 200µl was added and mixed by vortexing and then incubated at 56°C for 30m and for a further 15m at 95°C after which it was briefly centrifuged to remove drops from the lid. 200µL buffer ATL was added to the samples; mixed by pulse vortexing for 15seconds at 70°C for 10m then 200µL of absolute ethanol was also added and mixed by pulse vortexing. The mixture obtained was then carefully transferred to the QIAamp mini spin column without wetting the rim and centrifuged at 6,000rpm for 1m after which the QIAamp mini spin column was placed in a clean 2mL collection tube and the filtrate was discarded. 500µL of buffer AW1 was added to the column, centrifuged at 6,000rpm for 1m after which the QIAamp mini spin column was placed in a clean 2mL collection tube and the filtrate was discarded.

The QIAamp mini spin column was carefully opened and 500µL of buffer AW2 was added to the column, centrifuged at 20,000rpm for 3m. The QIAamp mini spin column was placed in a clean 2mL collection tube and the previous flow through was discarded and centrifuged at 10,000rpm for 1m. The QIAamp mini spin column was placed in a new microcentrifuge tubes then 200µL buffer AE added, incubated at room temperature for 5m and centrifuged at 6,000rpm for 1m after which the DNA was stored at 4°C until ready for PCR amplification.

3.9.2 16sRNA Amplification

Extracted DNA templates were subjected to PCR using set (Forward and Reverse) of primers targeting 16SRNA of *L. monocytogenes*. PCR was performed with 27F-AGAGTTTGATCMTGGGTCAG and 1492R-GGTTACCTTGTTACGACTT, the primers allowed amplification of the 16SRNA genes of the *L. monocytogenes.* Thermocycling conditions used was 94 °C for 4m for initial denauration then 25 cycles, each at 96 °C for 10s, 50 °C for 5s and 60 °C for 4m and final extension at 72°C for7m .

3.9.3 Purifying Sequencing Extension Products by Isopropanol Precipitation

The 96-well reaction plate was removed from the thermal cycler and the amplified product transferred into 1.5mL microcentrifuge tube after which 400mL of 75% isopropanol was added, mixed by vortexing, left to stand at room temperature till precipitation took place. The tubes were spun for 30m at 16,000rpm, then, the supernatant were aspirated to new tubes and then 200mL 75% isopropanol were added to the tubes, vortexed, and centrifuged for 5m at 16,000rpm. The samples were re-suspended in injection buffer and then analysed with 3130xl genetic analyzer (Applied Biosystems) to give the sequences. MEGA6 was used to view and analyse the obtained data.

3.9.4 Generating consensus sequence

The bases were edited with MEGA6 software. The first and last twenty (20) bases were deleted from both forward and reverse sequence after which the reverse sequence in each case was made to complement the forward sequence by reverse complement then a pair-wise alignment was carried out on both forward and reverse sequence, and the consensus

sequence was obtained from the aligned sequence. The consensus sequence was pasted on blast page at National Center for Biotechnology Information (NCBI) website to obtain closely related strains.

3.9.5 Evolutionary relationship of strains

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) with MEGA 6 software. The optimal tree with the sum of branch length was = 3.19204511. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 9 nucleotide sequences. Codon positions included were first, second third and non-coding. The codon positions containing gaps and missing data were eliminated and the final dataset had a total of 1229 positions.

3.10 Physiological studies

3.10.1 Preparation of McFarland standard

To prepare 0.5 McFarland equivalent turbidity Standard, zero point five (0.5) mL of 1% barium chloride solution ($BaCl_2$) was added to 99.5mL of 1% sulphuric acid solution (H_2SO_4). The resulting standard was stored in the dark at room temperature.

3.10.2 Determination of inoculum size

Twenty-four hours (24h) broth cultures of Listeria isolates was diluted with sterile distilled water in plain tubes, vortexed, then centrifuged at 4000rpm for 5m and the suspension marched with 0.5 McFarland standard using a spectrophotometer at 600nm (Thermoscientific genesys UV-Visible spectrophotometer) representing approximately 108 cfu/mL (Azu and Onyeagba, 2007).

3.10.3 Growth at Different pH

This was carried out in tryptone soy broth. Here the pH of the medium was adjusted by preparing the broth in molarity sodium phosphate buffer at different pH (2,3,4,5,6,7,8 and 9). Then the suspension of each isolate in broth was transferred to 9mL broth at different

pH. After 24h of incubation, the broth was read using a Labda 25 UV/visible Spectrophotometer (Thermoscientific genesys UV-Visible Spectrophotometer) at 560nm.

3.10.4 Growth at Different Temperatures

The effect of temperature on the Listerial isolates was observed by inoculating 1mL of the suspension of each isolate into 5mL of freshly prepared tryptone soy broth. The broth was incubated at different temperatures (-4°C, 10°C, 37°C, 45°C, 60°C, and 70°C). After 24hours of incubation, the broth was read using a Labda 25 UV/visible Spectrophotometer (Thermoscientific genesys UV-Visible Spectrophotometer) at 560nm.

3.10.5 Growth at Different Biles concentration

The effect of bile on *Listeria* growth was observed by inoculating 1mL of the suspension of each isolate into 9mL of freshly prepared tryptone soy broth containing bile salt (Oxoid) at different concentrations: 0.2%, 0.3%, 0.4%, and 0.5% (w/v) after which inoculated tubes were incubated at 37°C for 24 hours and then growth was monitored using a Labda 25 UV/visible Spectrophotometer (Thermoscientific genesys UV-Visible Spectrophotometer) at 560nm.

3.10.6 Growth at Different NaCl concentration

The effect of NaCl on Listeria growth was observed by inoculating 1mL of the suspension of each isolate into 9ml of freshly prepared tryptone soy broth containing NaCl at different concentrations: 2%, 4%, 6%, 8% and 10% (w/v) after which tubes were incubated at 37°C for 24 hours and then growth was monitored using a Labda 25 UV/visible Spectrophotometer (Thermoscientific genesys UV-Visible Spectrophotometer) at 560nm.

3.10.7 Hydrophobicity potential of *L. monocytogenes* on different surfaces

Wood, glass, steel and aluminum were purchased and cut into squares 2cm by 2cm (Pawar and Chen, 2005). Prior to inoculation, the surfaces were properly washed and then air-dried before being autoclaved at 121°C, 15Psi for 15min. The *L. monocytogenes* cells

were inoculated into tryptone soy broth (TSB) after which the treated surfaces were dipped into tubes containing the TBS cells then incubated for 24hours, 48 hours and 72 hours. After incubation, *L. monocytogenes* attached to the different surfaces was quantified using Crystal Violet binding assay (Pawar and Chen, 2005).

The broth cultures were removed and the surfaces were rinsed thrice with sterile distilled water to remove loosely attached cells. The surfaces were then dried in an oven. The fixed cells on the surfaces were stained with 2 mL of 1% crystal violet for 15 minutes, and then excess stain was rinsed off by placing the glass vials under running tap water. The surfaces were then dried at 60°C for 5 minutes. The dye bound to the adherent cells was resolubilized with 33% (v/v) acetic acid. The optical density (O.D.) of each glass vial was measured at 595 nm using a Spectrophotometer (Thermoscientific genesys UV-visible Spectrophotometer) then the absorbance of the negative control was subtracted from the absorbance values to determine the actual value.

3.10.8 Screening for Amylase

Listeria sp. were screened for amylase production on tryptone soy agar plates containing 1% soluble starch (Borcan *et al*., 2014). The cultures were inoculated by streaking onto the starch plates after which the plates was incubated at 37°C for 48h after which the plates were flooded with Gram's iodine solution. Amylase producing isolates had clear zones around the point of streak.

3.10.9 Screening for Protease

Listeria sp. were screened for protease production on tryptone soy agar plates containing 1% skimmed milk (Matta *et al*., 1997). The cultures were inoculated by streaking on the plates after which the plates were incubated at 37°C for 48h. The plates were observed for clear zone around smears after incubation which indicated protease-producing isolates.

3.10.10 Screening for Lipase

A plate detection method was used to screen the *Listeria* isolates for lipase producing ability (Borcan *et al*., 2014). The medium used for screening contained: peptone 10 g/L; NaCl 5g/L; calcium chloride 0.1g/L; castor oil 1mL, agar 50g/L; Congo red 0.5g/L and distilled water. The sterile medium was poured, allowed to solidify; then, spot inoculated

with the *Listeria* isolates, and incubated at 37°C for 48h. Lipolysis was indicated by the appearance of clear zone of inhibition around the spot of inoculation.

3.10.11 Effect of different disinfectants/sanitizers on *L. monocytogenes*

The effect of different sanitizers/disinfectants against *L monocytogenes* was evaluated by means of agar-well diffusion assay. The disinfectants used were hypo, jik, Roberts's antiseptics, ethanol, dettol, izal and purit. Approximately, 0.1mL of the standardized inoculum was evenly spread with a swab stick onto the surface of the solidified agar plates. Once the plates had been aseptically dried, 5mm wells were punched into the agar with a sterile cork borer after which 0.2mL of the different concentrations: 1%, 2%, 5%, 10%, 25%, 50%, 75%, and 100% (v/v) of the disinfectants were placed into the wells and the plates were incubated at 37°C for 24hours. Antilisterial activity of the sanitizers/disinfectants was evaluated by measuring the diameter of zone of inhibition around the wells.

3.11 Antibiotic resistance analysis

The susceptibility of the different isolates to different antibiotics was evaluated by the disk diffusion method on Mueller-Hinton Agar (Oxoid Basingstoke, UK) (Aluyi *et al*., 2013). The antibiotics used were Ceftazidine (30µg CAZ), Cefuroxime (30µg CRX), Gentamicin (10µg GEN), Cloxacillin (10µg CXC), Ofloxacin (5µg OFL), Augumentin (30µg AUG), Nitrofuranton (200µg NIT), Ciprofloxacin (10µg CPX), Ceftrizone (30µg CRO), Amoxicillin (25µg AMX), Streptomycin (10µg STR), Pefloxacin (5µg PEF), Tetracycline (30 µg TET) and Erytromycin (5µg ERY). Pure cultures of *L. monocytogenes* were streaked on Muller-Hinton Agar after which the impregnated disk were aseptically placed on the inoculated plates and incubated at 37°C for 24 hours. After incubation the diameter zone of inhibition observed around each disk was measured in mm and interpreted in accordance with the Clinical and Laboratory Standards Institute Standards guidelines (CLSI, 2015)

3.12 Isolation of Plasmid DNA

QIA quick mini prep extraction kit (Qiagen) was used. The procedure was followed in accordance with the manufacturer's instructions. Five (5) mL of bacterial overnight culture was pelleted by centrifugation at 13,000rpm for 1 minute. The pellet was suspended in an RNAse containing buffer P1. P2 buffer was used to lyse the cells and this was neutralised with buffer P3. The sample was then gently mixed to avoid contamination with chromosomal DNA. The mixture was then centrifuged to remove all lysates at 13,000rpm for 2 minutes. The supernatant was removed and placed into spin columns and DNA was bound to this column membrane then washed with ethanol, which was subsequently removed entirely. Finally the DNA was eluted with 30 µl of elution buffer (EB).

3.13 Agarose gel preparation

Zero point eight percent (0.8%) agarose gel was prepared and two drops of ethidium bromide was added after which it was allowed to gel. Ten (10) µL of the isolated plasmid with the loading dye (NO70225 Bio lab England) was loaded in each well. Then 10µL of the 1kb DNA ladder (NO4685 Bio lab England) was loaded on the last well. The electrophoresis was ran at 100v for 40min after which bands were visualized with UV trans-illuminator.

3.14 Plasmid Curing

Plasmid curing was carried out on organisms that possessed plasmids by using sub-inhibitory concentration of 10% of Sodium Dodecyl Sulphate (SDS) as described by Yah *et al.* (2008). Antibiotic resistant isolates were grown in tryptone soy broth containing 10% SDS at 37^{o}C for 24h. After which the broth was agitated to homogenize the content and a loopful subcultured onto Mueller Hinton agar (MHA) plates. The plates were then screened for antibiotic resistance by the disk diffusion method as earlier described. Cured markers would be determined by comparison between the pre- and post-curing antibiograms of isolates. Loss of resistance after the plasmid curing was indicative of plasmid mediated resistance.

3.15 Detection of *L. monocytogenes* virulence and antibiotics resistant genes by PCR

3.15.1 Detection of hemolysin gene

Extracted DNA and *L. monocytogenes* ATCC19155 were subjected to PCR (Sharma and Mutharasan, 2013) using set (Forward and Reverse) of primers targeting haemolysin (hly) gene. HlyA (encoding lysteriolysin O) is a virulence gene that is required for escape from the vacuoles there by allowing *L. monocytogenes* to escape into the cytoplasm of the cell. It is also required for intracellular survival of invading *Listeria monocytogenes.* PCR was performed with F-CGGAGGTTCCGCAAAAGATG and R-CCTCCAGAGTGATCGATGTT primers (Inqaba Biotech, South Africa), the primers allowed amplification of 234 bp fragment of the hlyA gene of the *Listeria* genus. The tubes contained PCR PreMix (Inquaba, South Africa), which is premixed ready-to-use solution containing Tag DNA polymerase, dNTP, and MgCl. The reaction mixture was prepared in 0.2 mL PCR tubes with 25 µL reaction volumes (12.5 µL PreMix, 8.5 µL nuclease free water, 0.5 µL forward primer, 0.5 µL reverse primer and 3.0 µL template DNA) and carried out using the following conditions for thermocycling in a GeneAmp PCR system (Geneamp, Singapore) 94 °C for 4 minutes for initial denauration then 30 cycles, each at 94°C for 30 seconds, 52 °C for 1 minute, and 68°C for 1 minute 30 seconds and final extension at 68 °C for 7 minutes after which separation of the PCR products was done using ethidium bromide pre-stained 1.5% agarose gel in an electrophoresis tank and then PCR bands on gel were visualized under UV light.

3.15.3 Detection of Internalin gene (InlA)

Extracted DNA and *L. monocytogenes* ATCC19155 were subjected to PCR (Yung *et al*., 2003) using set (Forward and Reverse) of primers targeting Internalin gene (InlA) which is required for *L. monocytogenes* dispersion into non-phagocytic cells, such as epithelial cells. PCR was performed with F-AATCTAGCACCACTGTCGGG and R-TGTGACCTTCTTTTACGGGC (Inqaba Biotech, South Africa) (Ikeh *et al*., 2010). The primers allowed amplification of 733 bp fragment of the inlA gene of the *L. monocytogenes*. The PCR tubes contained inquaba PCR PreMix (Inquaba, South Africa), which is premixed ready-to-use solution containing Tag DNA polymerase, dNTP, and MgCl. The reaction mixture was prepared in 0.2 mL PCR tubes with 25 µL reaction volumes (12.5 µL PreMix, 8.5 µL nuclease free water, 0.5 µL forward primer, 0.5 µL

reverse primer and 3.0 µL template DNA) and carried out using the following conditions for thermocycling in a GeneAmp PCR system (Geneamp, Singapore) 94 °C for 4 minutes for initial denauration then 31 cycles, each at 94 °C for 45 seconds, 54 °C for 1 minute, and 68 °C for 1 minute and final extension at 68 °C for 8 minutes after after which separation of the PCR products was done using ethidium bromide pre-stained 1.5% agarose gel in an electrophoresis tank and then PCR bands on gel were visualized under UV light.

3.15.4 Detection of Invasive gene (Iap)

Extracted DNA and *L. monocytogenes* ATCC19155 were subjected to PCR (Yakubu *et al*., 2012) using set (Forward and Reverse) of primers targeting invasive gene (Iap) is required catalyzing a reaction during the final stage of *L. monocytogenes* cell division. PCR was performed with F-TTATACGCGACCGAAGCCAAC and R-CAAACTGCTAACACAGCTACTA (Inqaba Biotech, South Africa), the primers allowed amplification of 660 bp fragment of the iap gene of the *L. monocytogenes*. The reaction mixture contained PCR PreMix (Inquaba, South Africa), which is premixed ready-to-use solution containing Tag DNA polymerase, dNTP, and MgCl. The reaction mixture was prepared in 0.2 mL PCR tubes with 25 µL reaction volumes (12.5 µL PreMix, 8.5 µL nuclease free water, 0.5 µL forward primer, 0.5 µL reverse primer and 3.0 µL template DNA) and carried out using the following conditions for thermocycling in a GeneAmp PCR system (Geneamp, Singapore) 94 °C for 4 minutes for initial denauration then 31 cycles, each at 94 °C for 45 seconds, 54 °C for 1 minute, and 68 °C for 1 minute and final extension at 68 °C for 8 minutes after which after which separation of the PCR products was done using ethidium bromide pre-stained 1.5% agarose gel in an electrophoresis tank and then PCR bands on gel were visualized under UV light .

3.15.5 Detection of Resistant genes

Genes coding for erythromycin resistance (EryB), daptomycin resistance (Mpr F), and tetracycline resistance (Tet M and Tet A) were detected out using PCR (Mauro *et al*., 2009; Bertsch *et al*., 2014). PCR was performed with the following primers

tetM forward ----GTRAYGAACTTTACCGAATC

tetM reverse----- ATCGYAGAAGCGGRTCAC

tetA forward ---- TTGGCATTCTGCATTCACTC

tetA reverse----- GTATAGCTTGCCGGAAGTCG

erm(B) forward – GAAAAGGTACTCAACCAAATA

erm(B) reverse – AGTAACGGTACTTAAATTGTTTAC

mprF forward – TGCGGGTGGTCTTTACTTCC

mprF reverse – CGCGAGCAAGTGTGTTGAAA

The reaction mixture contained inquaba PCR PreMix (Inquaba, South Africa), which is premixed ready-to-use solution containing Tag DNA polymerase, dNTP, and MgCl. The reaction mixture was prepared in 0.2 mL PCR tubes with 25 µL reaction volumes (12.5 µL PreMix, 8.5 µL nuclease free water, 0.5 µL forward primer, 0.5 µL reverse primer and 3.0µL template DNA) and and carried out using the following conditions for thermocycling in a GeneAmp PCR system (Geneamp, Singapore) 94 °C for 4 minutes for initial denauration then 31 cycles, each at 94 °C for 45 seconds, 55 °C for 1 minute for Tet M and Erm B (60 °C for 1 minute for Tet A and Mpr F), 68 °C for 1 minute and final extension at 68 °C for 8 minutes after which after which separation of the PCR products was done using ethidium bromide pre-stained 1.5% agarose gel in an electrophoresis tank and then PCR bands on gel were visualized under UV light.

3.16 Antilisterial potential of different plants extracts

3.16.1 Sources of medicinal plants

The medicinal plants used in this research was *Psidium* guajuva Linn (guava), *Zingiber officinale* Roscoe (ginger), *Trema orientalis* (L.) Blume (charcoal tree), *Heinsia crinite* (Afzel) (atama), *Dacryodes edulis* (G. Don) Lam (African peer), *Citrus aurantifolia* (Christm.) Swingle (Lime), *Funtumia elastica* (Preuss) Stapf (silk rubber), *Gongronema latifolium* (utazi), *Vernonia amygdalina* Delile (bitter leaf), *Cassia alata* Linn (Cassia), *Moringa oleifera* Lam (moringa) and *Ocimum gratissimum* L. (scent leaf). These leaves were obtained from Emuhi farms at Ekpoma with geographical coordinate's

6°45′ North 6°08′ East Edo State, Nigeria. Identification of plants was done and confirmed by Dr. O. Olorunfemi of the Herbarium Unit, Department of Botany, University of Benin, Edo State, Nigeria. The leaves of these plants were dried at room temperature for about three (3) weeks then they were pulverized and stored in clean polythene bag until required.

3.16.2 Preparation of aqueous and ethanolic plant extracts

Fifty grams (50g) of each dried powdered leaves was soaked in water and ethanol for three days. At the end of the third day, the extracts were filtered and the filtrate was concentrated using rotary evaporator at 40°C. After complete evaporation, the extract was preserved aseptically at 4ºC until required (Ayoola *et al*., 2006).

3.16.3 Preparation of McFarland standard

To prepare 0.5 McFarland equivalent turbidity standard, 0.5mL of 1% barium chloride solution ($BaCl_2$) was added to 99.5mL of 1% sulphuric acid solution (H_2SO_4). The resulting standard was stored in the dark at room temperature (Azu and Onyeagba, 2007).

3.16.4 Determination of inoculum size

Pure cultures incubated for 24h was diluted with 1mL of sterile distilled water in plain tubes, vortexed, then centrifuged at 4000rpm for 5m and the suspension marched with 0.5 McFarland standard using a spectrophotometer at 600nm (Thermoscientific genesys UV-Visible spectrophotometer) representing approximately 10^8 cfu/mL (Azu and Onyeagba, 2007).

3.16.5 Preparation of plant extracts

Different concentrations (100mg/mL 200mg/mL, 400mg/mL, 600mg/mL, 800mg/mL and 1000mg/mL) of the different aqueous plant extracts was prepared by dissolving 0.1g, 0.2g, 0.4g, 0.6g, 0.8g and 1g the different extracts in 1mL sterile distilled water (Adeshina *et al*., 2012).

3.16.6 Agar well diffusion assay

Antimicrobial activities of the aqueous and ethanol extracts of the leaves were evaluated by means of agar-well diffusion assay (Adeshina *et al*., 2012). Twenty-five (25) mL of the molten Muller-Hinton agar was poured into sterile Petri dishes. Approximately, 0.1mL of the prepared inoculum was evenly spread with a hockey stick onto the surface of the solidified agar plates. Once the plates had been aseptically dried, wells were punched into the agar with a sterile cork borer (5mm) after which 0.2mL of the prepared extracts were placed into the wells and the plates were incubated at 37°C for 24h. Antimicrobial activity was evaluated by measuring the diameter zone of inhibition around the wells.

3.17 Phytochemical screening of different plants extracts

The ethanolic and aqeous extracts of the plants were tested for the presence of flavonoids, cardiac glycosides, reducing sugars, tannins, saponins, terpenoids, alkaloids, phenolics, resins and steroids

3.17.1 Test for Flavonoids

One (1) mL of extract (filtrate) in a test tube was mixed with 5ml of dilute ammonia and then 1mL of concentrated sulphuric acid (H_2S0_4) was added to the mixture. A yellow color was positive for flavonoids (Amadi *et al*., 2004).

3.17.2 Test for Tannins

One (1) mL of extract in a test tube was heated for five (5) minutes to boil. Thereafter, 2 drops of 15% ferric chloride was added, blue black coloration confirmed the presence of tannins (Trease and Evans, 1989).

3.17.3 Test for Cardiac glycosides

The test for cardiac glycosides was carried out using Killani's test (Sofowora, 1993). One (1) mL of extract in a test tube was mixed with 2mL glacial acetic acid, after which one (1) drop of 15% ferric chloride and 1mL of concentrated sulphuric acid was added to the mixture. Brown colouration formed at the interface indicates the presence of cardiac glycosides

3.17.4 Test for Saponin

The ability of saponins to produce frothing in aqueous solution was used as a screening test for saponins. One (1) mL of extract was mixed with 5mL of distilled water. The mixture was mixed vigorously and observed for frothing (Amadi *et al*., 2004).

3.17.5 Test for Steroids

One (1) mL of both extracts was placed in separate tubes after which 2mL of acetic acid and 2mL of concentrated sulphuric acid (H_2S0_4) was each added, mixed and observed for change of colour from violet to blue-green colouration which is a positive reaction for steroids (Sofowora, 1993).

3.17.6 Test for Terpenoids

The test for terpenoids was done using the Salkowski's test. One (1) mL of both extracts was mixed with 2mL chloroform and 3mL of concentrated H_2SO_4. Reddish brown colouration at the interface is positive for terpenoids (Sofowora, 1993).

3.17.7 Test for Alkaloids

One (1) mL of extract was mixed with 3 drops of Hager's reagents. Formation of yellow coloured precipitate is positive for alkaloids (Amadi *et al*., 2004).

3.17.8 Test for Reducing Sugars

One (1) mL of extract was added to boiling Fehling's solution A and B in a test tube. Colour change from blue to green was positive for reducing sugar (Trease and Evans, 1989).

3.17.9 Test for phenolics

Two drops of 5% ferric chloride was added to 5mL of each plant extracts in a test tube. A greenish precipitate was indicative of phenolics (Amadi *et al*., 2004).

3.17.10 Test for resins

Copper acetate solution (5mL) was added to 5mL of both plant extracts. The resulting solution was shaken vigorously and allowed to separate. A green coloured solution is evidence of the presence of resins (Amadi *et al*., 2004).

3.18 Life span of mice infected with *Listeria monocytogenes*

3.18.1 Test Procedure

The test was performed by the mice inoculation as described by Aurora *et al*. (2008). Thirty (30) weeks old sixteen white Swiss mice (Albany strain) of both sexes were procured. All the selected mice had weight between 18-20g.

3.18.2 Preparation of Test Inoculum and Mice Infectivity

Strain of *L. monocytogenes* was incubated in tryptone soy broth overnight and collected by centrifugation (14000rpm at room temperature for 1m) then re-suspended in sterile Phosphate Buffered Saline (PBS), pH 7.2 then the suspension marched with 0.5 McFarland standard using a Spectrophotometer (Thermoscientific genesys UV-visible spectrophotometer) at 600nm representing approximately 10^8 cfu/mL (Adetutu and Olorunnnisola, 2013).

The mice were divided into five groups of six mice each. The first group served as control and was fed with 1mL of sterile distilled water. Out of the remaining four groups, two groups were infected with 1mL each of the isolate orally and the other two groups were infected intra-peritoneally. After infection the mice were kept in separate cages in a clean and well disinfected room and were fed and given clean water. They were observed for a period of 5 days for clinical signs which included anorexia, fever, diarrhea, nervous signs and possible mortality. The number of death of infected mice was recorded and expressed as percentage mortality.

3.18.3 Postmortem Technique

Dead mice were dissected and organs removed while mice that were alive after 72h were sacrificed using chloroform then dissected using forceps scissors and scalpels. They were cut through the white line (linea alba), both thoracic and abdominal cavities were opened and the organs examined for gross lesions. Swollen stomach from infected mice was collected for re-isolation of pathogen. The organs collected were kept immediately in formalin. Blood from mice was also collected for full blood count (Amole *et al*., 2006).

3.18.4 Histopathology Studies

Samples of liver, kidney, heart and spleen from the control and infected groups were fixed in 10% neutral formalin for 24h, dehydrated in an alcohol-xylene series and embedded in paraffin wax. From each block, sections 2µm thick were prepared and stained with haematoxylin and eosin (HE) for histopathological examination.

3.19 *In vivo* assay of antilisterial potential of different aqueous plant extracts

Strains of *L. monocytogenes* was incubated in tryptone soy broth overnight and collected by centrifugation (14000 X g at room temperature for 1min.) then resuspended in sterile Phosphate Buffered Saline (PBS), pH 7.2 then the suspension marched with 0.5 McFarland standard using a spectrophotometer (Thermoscientific genesys UV-Visible spectrophotometer) at 600nm representing approximately 10^8cfu/mL. The mice were weighed and randomized into fourteen (14) groups of eight (8) mice each. Group 1 to group 10 were administered with 1mL each of the isolate orally after which group 1 to group 9 were then administered 1mL aqueous plant extract at different concentration daily (Effraim *et al*., 2000). After infection the mice were kept in separate cages in a clean and well disinfected room and were fed and given clean water. They were observed for a period of 8 days for clinical signs which included anorexia, fever, diarrhea, nervous signs and possible mortality. The number of death of infected mice was recorded and expressed as percentage mortality.

3.19.2. Postmortem Technique

Mice were sacrificed on the second, fourth and seventh day after treatment with aqueous leaf extracts (Effraim *et al*., 2000). They were sacrificed using chloroform then dissected using forceps scissors and scalpels. They were cut through the white line (linea alba), both thoracic and abdominal cavities were opened and the organs examined for gross lesions. The organs collected were liver spleen and stomach and they were kept immediately in formalin. Blood from mice was also collected for determination of assay of Alkaline Phosphatase (ALP) and assay of alanine aminotransferase (ALT) activity.

3.19.3 Assay of Alkaline Phosphatase (ALP)

Principle

Alkaline phosphatases are enzymes that split off the terminal phosphate group from an organic ester in alkaline solution. Alkaline Phosphatase in a sample hydrolyses para-nitrophenyl phosphate into para-nitrophenol and phosphate, in the presence of magnesium ions. The rate of absorbance increase of the ALP reaction mixture at 405nm due to liberation of para-nitrophenol is proportional to the alkaline phosphatase activity.

Þ-nitrophenylphosphate + H_2O $\xrightarrow{ALP}$ phosphate+ Þ-nitrophenol

Procedure

This test was carried out using Randox kits. In a cuvette, 0.02 mL of sample was mixed with 100mL of the reagent. The initial absorbance was read at 405nm, and subsequently after 1, 2 and 3 minutes. The mean absorbance per minute was used in the calculation:

ALP activity (IU/l) = 2760 × ΔA 405 nm/min;

Where: 2760 = Extinction coefficient; ΔA 405 nm/min = change in absorbance per minute for the homogenate sample (Klein, 1960; Hyder *et al*., 2013).

3.19.4 Assay of alanine aminotransferase (ALT) activity:

Principle

ALT catalyzes the reversible transamination of L-alanine and α-ketoglutarate to pyruvate and L-glutamate. ALT is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine.

α-ketoglutarate + L-alanine $\xrightarrow{\text{glutamic pyruvic transaminase (GPT)}}$ pyruvate + L-glutamate

Procedure

This test was carried out using as described by the manufacturers instruction in Randox kits. 0.5mL each of ALT substrate phosphate buffer was pipetted into two sets of test tubes labeled B (sample blank) and T (sample test). Zero point one (0.1) mL serum sample was added to sample test (T) only, mixed properly and incubated for 30 minutes in a water

bath regulated at 37^{o}C. After incubation, 0.5mL of 2, 4-dinitrophenylhydrazine was added to both tubes then allowed to stand for 20m at 25^{o}C. Then, 5.0mL NaOH solution was added to both test tubes, mixed thoroughly and absorbance of the test sample was read against sample blank at a wavelength of 546nm after 5m (Reithman and Frankel, 1957; (Klein, 1960; Hyder *et al*., 2013).

3.20 Statistical Analysis

All results were presented as Mean ± Standard Error of the Mean. A one way analysis of variance (ANOVA) was used to determine significant differences between Means. All data were analysed using SPSS 16.0 (Statistical Package for Social Sciences). Statistical significance was evaluated at $P<0.05$. Charts were plotted using Microsoft Excel, 2007.

Table 3.2: Plant extract concentrations administered to mice in different groups

Mice Groups	Plant extract
Group 1	0.6g/kg *Vernonia amygdalina* leaf
Group 2	0.8g/kg *Vernonia amygdalina* leaf
Group 3	1g/kg *Vernonia amygdalina* leaf
Group 4	0.6g/kg *Psidium guajuva* leaf
Group 5	0.8g/kg *Psidium guajuva* leaf
Group 6	1g/kg *Psidium guajuva* leaf
Group 7	0.6g/kg *D. edulis* leaf
Group 8	0.8g/kg *D. edulis* leaf
Group 9	1g/kg *D. edulis* leaf
Group 10	*Listeria monocytogenes* alone
Group 11	*Vernonia amygdalina* leaf alone
Group 12	*Psidium guajuva* leaf alone
Group 13	*D. edulis* leaf alone
Group 14	no organism nor leaf (negative control)

CHAPTER FOUR

4.0 RESULTS

Isolation and identification of *Listeria monocytogenes*

RTE foods form Ugheli-Patani road had *Listeria* counts ranging from 4.77-5.08 Log cfu/g, 2.36–2.70 Log cfu/g, 4.89–4.92 Log cfu/g and 2.54–2.72 Log cfu/g for edible worm, salads, snails and meat pie respectively. Also, RTE foods from Effurun round about had counts ranging from 5.35–5.45 Log cfu/g, 2.50–2.56 Log cfu/g, 5.01–5.18 Log cfu/g and 2.39–2.52 Log cfu/g for edible worm, salads, snails and meat pie respectively. Highest range of *Listeria* specie was 5.60–5.82 Log cfu/g from edible worms purchased from Postuku Bayelsa. Lowest range of *Listeria* species was 2.08-2.40 Log cfu/g in meat pies purchased from Ore.

Result of the biochemical characteristics of *Listeria* species isolated from RTE foods is shown in Table 4.2. All *Listeria* species were small to medium in size, having entire margins with slightly raised colonies, and they appear surrounded by zone of aesculin hydrolysis on *Listeria* selective agar. Their Gram's reaction test showed that the all the isolates were Gram positive rods appearing singly or in pairs and the rods were short and sometimes appearing as cocoid bacilli. The biochemical test carried out revealed that all *Listeria* isolates were catalase positive, indole negative, oxidase negative, citrate positive, negative for production of hydrogen sulphide. Only suspected *L. monocytogenes* and *L. ivanovii* isolates was positive to β-haemolysis on blood agar. All *Listeria* species were positive to *Listeria* antisera and only *L. monocytogenes* was negative to Oxoid Biochemical Identification System test (OBIS mono). All the *L. monocytogenes* fermented glucose, rhamnose, and fructose; none of them fermented lactose, mannose, maltose and galactose while all *L. grayi* fermented glucose, rhamnose, mannose, maltose and fructose, while none of them fermented lactose, xylose and galactose (Table 4.2).

Table 4.1: Occurrence of *Listeria* sp isolated from different RTE foods

Source of sample	Sample type	Number of Samples	Range of *Listeria* counts (Log cfu/g)
Ore	Salads	50	2.08 – 2.40
	Snails	10	3.60 – 4.72
	Meat pie	50	2.80 – 2.93
Sapele Road (Benin city)	Edible worm	20	5.54 – 5.74
	Salads	15	2.76 – 2.95
	Snails	14	5.07 – 5.18
	Meat pie	18	2.54 – 2.72
Sapele round about	Edible worm	30	5.35 – 5.48
	Salads	30	2.36 – 2.60
	Snails	30	4.85 – 4.92
	Meat pie	20	2.18 – 2.36
Effurun round about	Edible worm	40	5.35 – 5.45
	Salads	30	2.50 – 2.56
	Snails	30	5.07 – 5.18
	Meat pie	15	2.39 – 2.52
Ugheli-Patani road	Edible worm	18	4.77 – 5.08
	Salads	15	2.36 – 2.70
	Snails	18	4.89 – 4.91
	Meat pie	15	2.54 – 2.72
Postuku (After first bridge in Bayelsa)	Edible worm	40	5.60 – 5.82
	Snails	30	2.83 – 2.96
	Meat pie	15	4.25 – 4.39

Table 4.2: Biochemical characteristics of *Listeria* species isolated from RTE foods

Gram	Listeria antisera	OBIS mono	β-heamolysis	Aesculin hydrolysis	Catalase	Coagulase	Oxidase	Citrate	Indole	Hydrogen sulphite	Glucose	Lactose	Fructose	Rhammose	Xylose	Mannitol	Galactose	Maltose	Probable identity
+ Rods	+	+	-	+	+	-	-	+	-	-	A	-	A	-	-	A	-	-	*L.innocua*
+ Rods	+	+	-	+	+	-	-	+	-	-	A	-	A	A	-	A	-	-	*L. welshimeri*
+ Rods	+	+	-	+	+	-	-	+	-	-	A	-	A	A	-	A	-	A	*L. grayi*
+ Rods	+	+	-	+	+	-	-	+	-	-	A	-	A	-	A	-	-	-	*L. seelegeri*
+ Rods	+	+	+	+	+	V	-	+	-	-	A	-	A	-	A	-	-	-	*L. ivanovii*
+ Rods	+	-	+	+	+	V	-	+	-	-	A	-	A	A	-	-	-	-	*L.monocytogenes*

KEY: A=Acid, +=Positive, -=Negative β- Hea = β-heamolysis

The Percentage frequency of occurrence of *Listeria* species from RTE foods is shown in figure 4.1. *L. monocytogenes* had the highest frequency of occurrence of 36.37% while *L. ivanovii* had the lowest frequency of occurrence. *L. grayi*, *L. welshimeri*, *L. seeligeri* and *L. innocua* occurred 20.82%, 9.39%, 10.20% and 18.78% respectively.

Depicted in Table 4.3 is the occurrence of *L. monocytogenes* isolated from different RTE foods. Of one hundred and fifteen (115) isolated *Listeria* spp. from edible worms only fifty five (55) representing 47.42% were identified as *L. monocytogenes*. For salads, snails and meat pies, there were 50, 45 and 35 isolated *Listeria* species respectively of which *L. monocytogenes* represented 30% of salads, 33.3% in snails, and 14.29% in meat pie. Highest positive sample of 47.82% *L. monocytogenes* was seen in edible worms and lowest percentage of 14.29% *L. monocytogenes* presence was seen in meat pies.

The molecular identification of *L. monocytogenes* isolated from RTE foods is shown in Table 4.4. *L. monocytogenes* LMEW70 with accession number KY053295 was similar to *L. monocytogenes* L1846, *L. monocytogenes* LMSN47 with accession number KY053298, LMSA55 with accession number KY053294, LMMP96 accession number KY053297, LMEW24 with accession number KY053300 was similar to *L. monocytogenes* L2625, LM850658, 19113, CFSAN023463 respectively.

Figure 4.2 shows the phylogenetic relationship *L. monocytogenes* isolated from RTE foods. *L. monocytogenes* LMSN70 is 87% related to *L. monocytogenes* OP1 and 96% related to *L. monocytogenes* NCTC 11801 (Figure 4.2.1). *L. monocytogenes* LMEW94 is 100% related to *L. monocytogenes* DSM 13 and *L. monocytogenes* NBRC 12200 and 71% related to *L. monocytogenes* BGSC 3A28 (Figure 4.2.2). *L. monocytogenes* LMSN 70 is more closely related to *L. monocytogenes* LMEW20 than to *L. monocytogenes* LMSA104 (Figure 4.2.3).

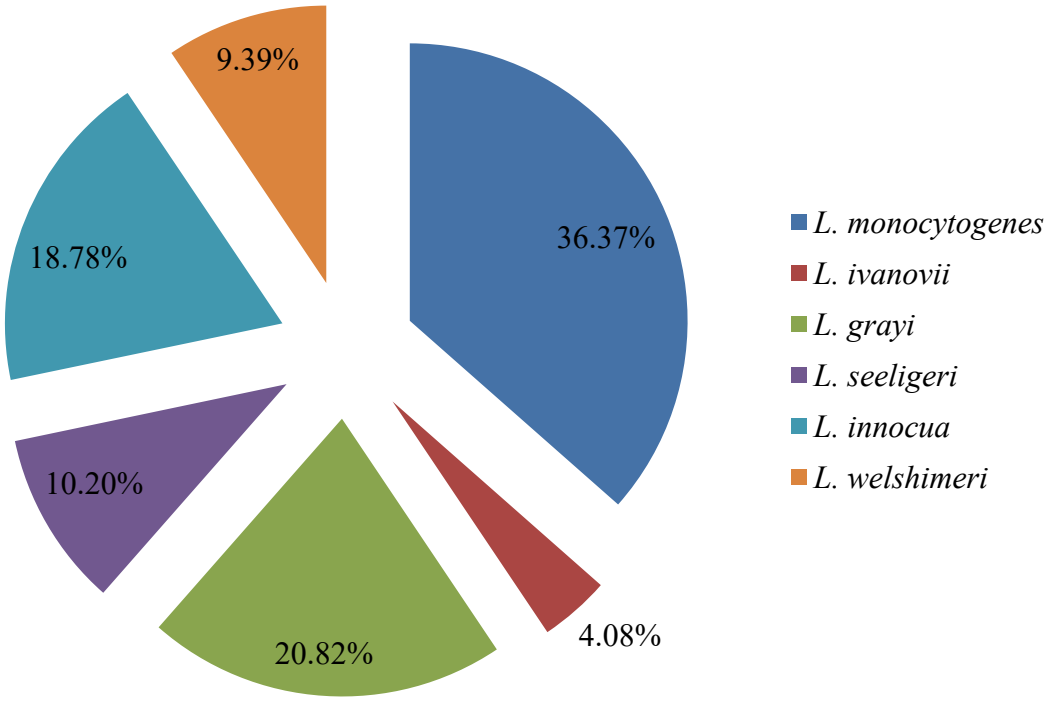


Figure 4.1: Percentage frequency of occurrence of *Listeria* species from RTE foods

Table 4.3: Occurence of *L. monocytogenes* isolated from different RTE foods

Sample	No. of *Listeria* sp. isolated	No positive for *Listeria monocytogenes* (%)
Edible worm	115	55 (47.82)*
Salads	50	15 (30)
Snails	45	15 (33.3)
Meat pie	35	5 (14.29)
Total	245	90 36.73)

*Figures in parentheses represent percentage of occurence

Table 4.4: Molecular identification of *L. monocytogenes* isolated from RTE foods

ISOLATE CODE	Accession No.	Relative Identity
LMEW20	KY053299	*L. monocytogenes J0161*
LMEW24	KY053300	*L. monocytogenes CFSAN023463*
LMEW70	KY053295	*L. monocytogenes L1846*
LMEW94	KY053296	*L. monocytogenes* La111
LMSN47	KY053298	*L. monocytogenes* L2625
LMSN108	KY053302	*L. monocytogenes* L2074
LMSA55	KY053294	*L. monocytogenes* LM850658
LMSA104	KY053301	*L. monocytogenes* JXH-150
LMMP96	KY053297	*L. monocytogenes* 19113

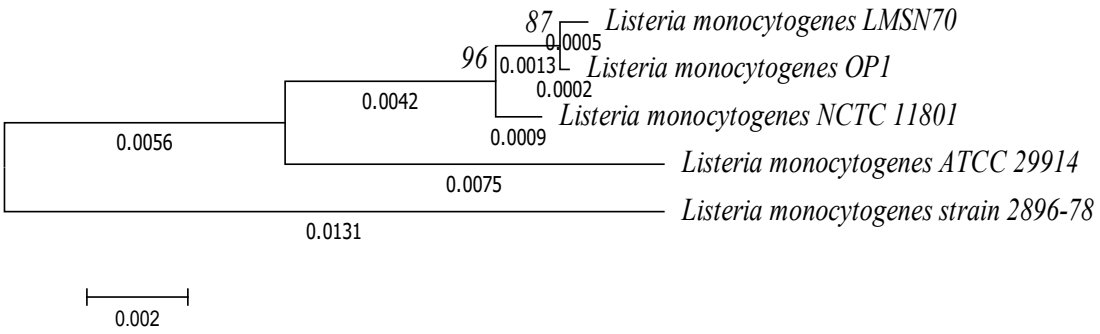


Figure 4.2.1: Phylogenetic relationship of *L. monocytogenes* LMSN70 isolated from RTE foods

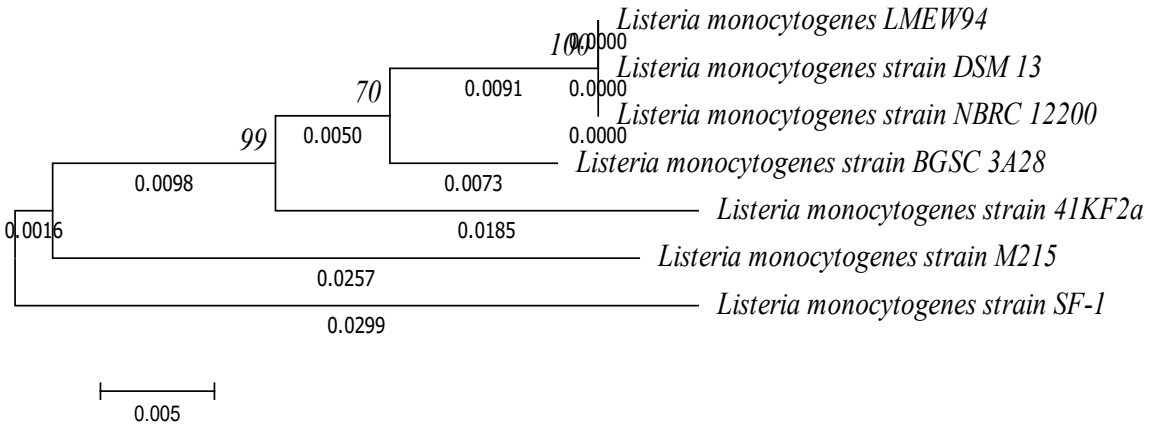


Figure 4.2.2: Phylogenetic relationship of *L. monocytogenes* LMEW94 isolated from RTE foods

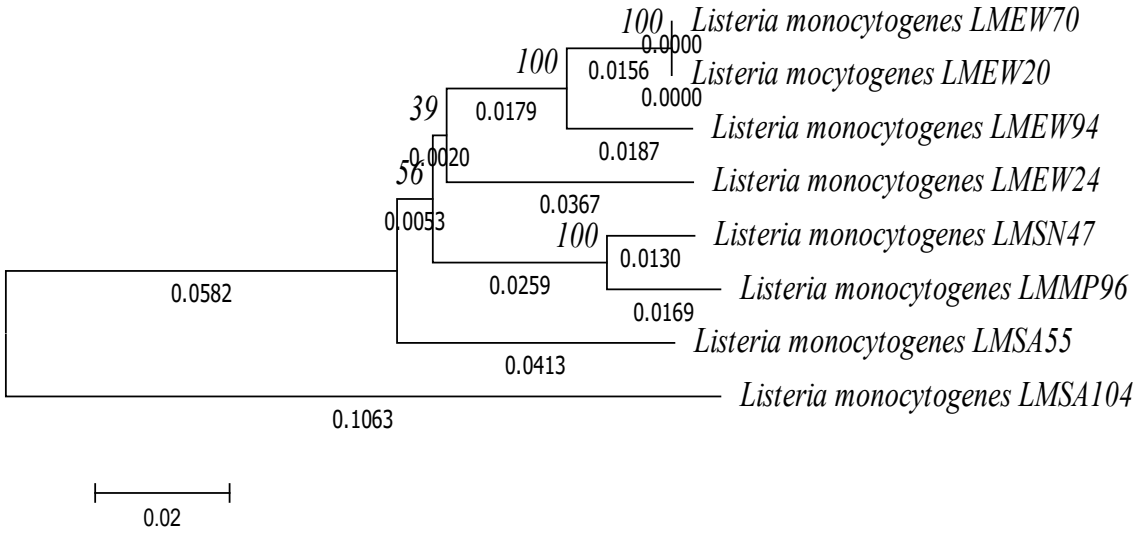


Figure 4.2.3: Phylogenetic relationship of different *L. monocytogenes* strains isolated from RTE foods

Effect of varying physiological parameters on the growth of *Listeria monocytogenes*

Effect of temperature on the growth of *L. monocytogenes* LMEW94 isolated from different RTE foods from South South Nigeria is shown in figure 4.3. *L. monocytogenes* LMEW94 was observed to grow between -10°C and 70°C within 48h period. Steady increase in growth was observed as temperature increased from -10°C with 37°C supporting the highest growth both at 24h and 48h period.

Depicted in figure 4.4 is the effect of pH on the growth of different *L. monocytogenes* isolated from different RTE foods from South South Nigeria. Listerial growth was observed to increase as pH was increased with pH 7 supporting highest growth of *L. monocytogenes* LMSN70 while pH 8 supported highest growth of *L. monocytogenes* LMEW20, LMSA55, LMEW94 AND LMLP104. Increase in the pH beyond pH 8 to pH 9 resulted in decreased growth

The effect of different NaCl concentration on the growth of *L. monocytogenes* isolated from different RTE foods from South South Nigeria is reported in figure 4.5. A gradual decrease in growth was observed with increase in NaCl concentration from two percent (2%) to ten percent (10%). Growth was highest at two percent (2%) NaCl concentration while lowest Listerial growth was observed at 10% NaCl concentration.

A gradual increase in growth was observed with increase in bile salt concentration from 0.2%-0.3% bile salt after which further increase in bile salt concentration resulted in decreased growth. Growth of *L. monocytogenes* LMEW20 was highest (1.324) at 0.3% bile salt (Figure 4.6)

L. monocytogenes LMSN70 showed increase in the hydrophobicity with increase in incubation time from 24h to 48h. Highest hydrophobic potential was observed on steel for steel while lowest hydrophobicity was observed with glass (Figure 4.7). Hydrophobic potential of *L. monocytogenes* can be described as steel > wood > aluminium > plastic > wood.

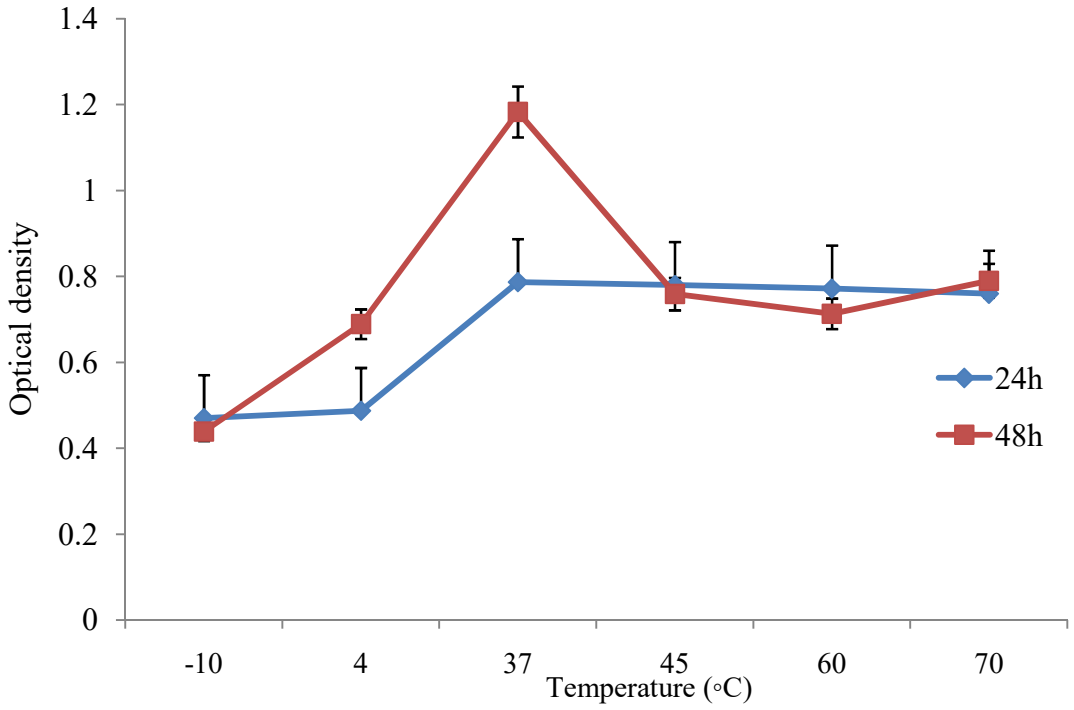


Figure 4.3: Effect of temperature on the growth of *L. monocytogenes* LMEW94 isolated from different RTE foods

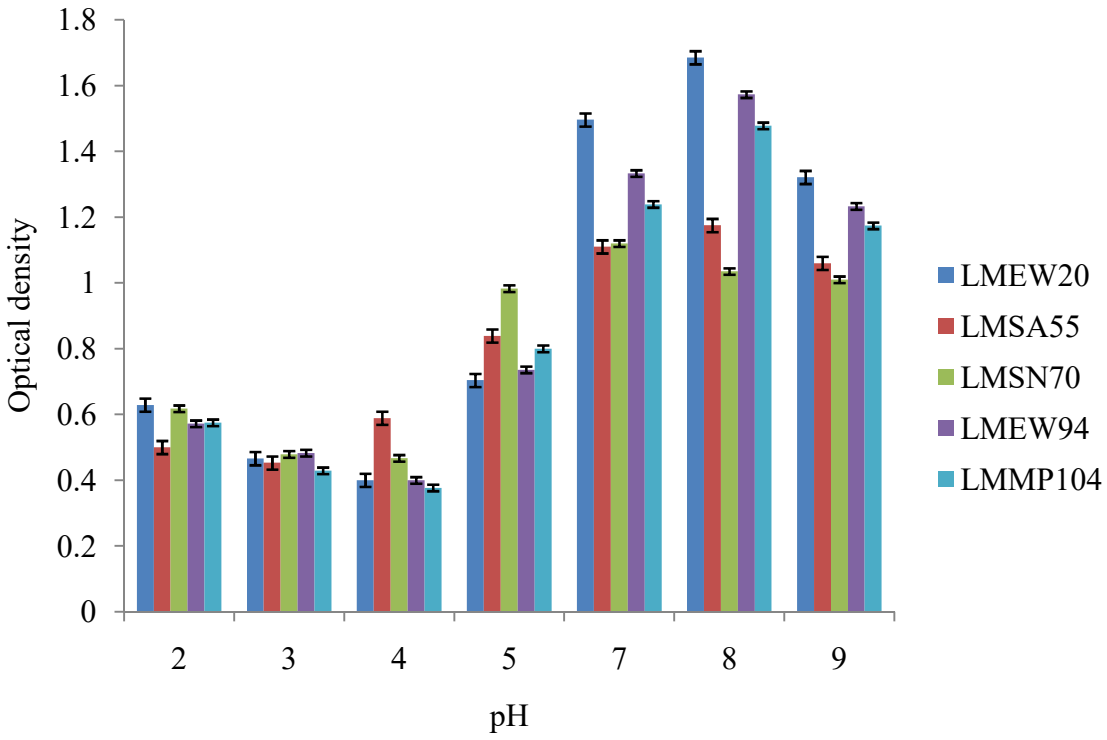


Figure 4.4: Effect of pH on the growth of different *L. monocytogenes* isolated from different RTE foods

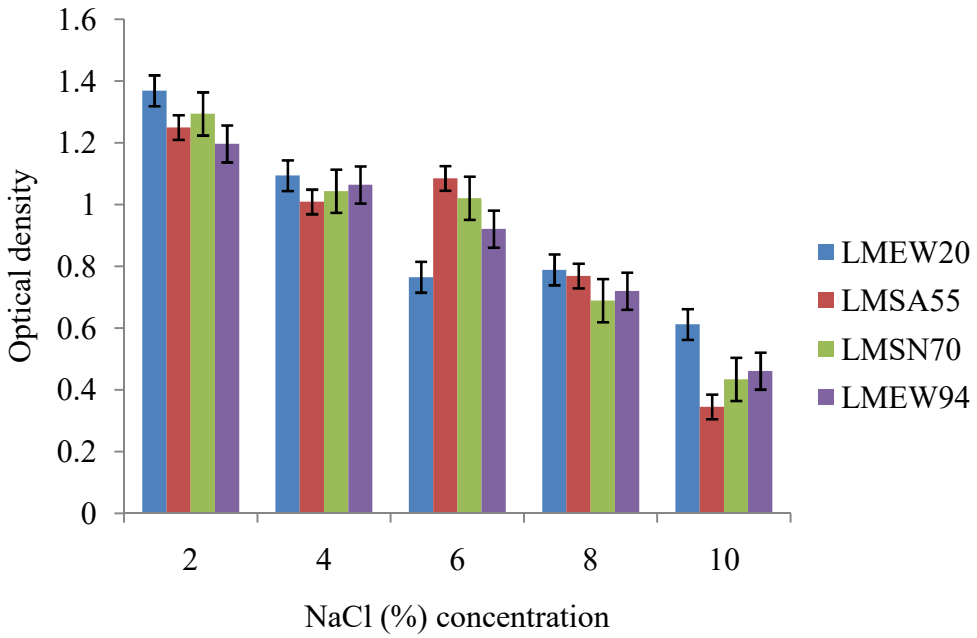


Figure 4.5: Effect of different NaCl concentration on the growth of *L. monocytogenes* isolated from different RTE foods

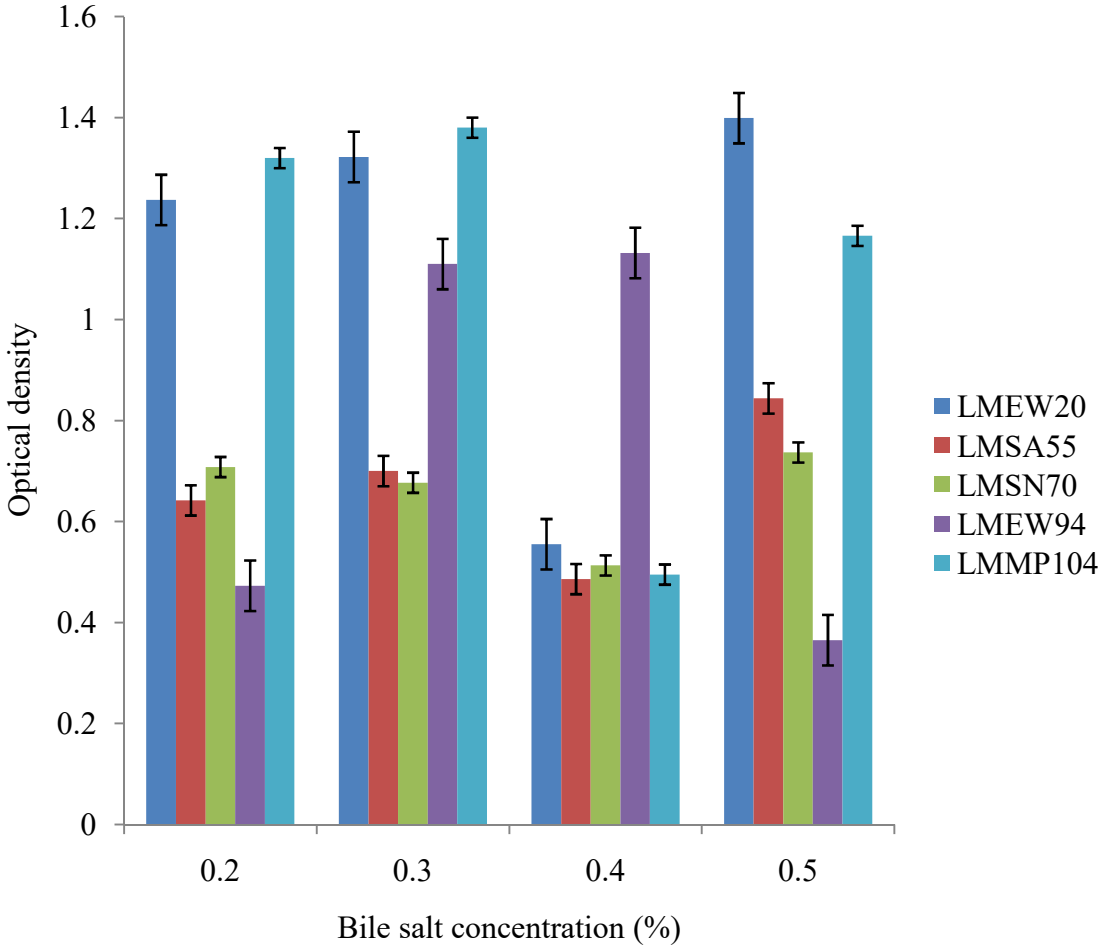


Figure 4.6: Effect of different bile concentration on the growth of *L. monocytogenes* isolated from different RTE foods

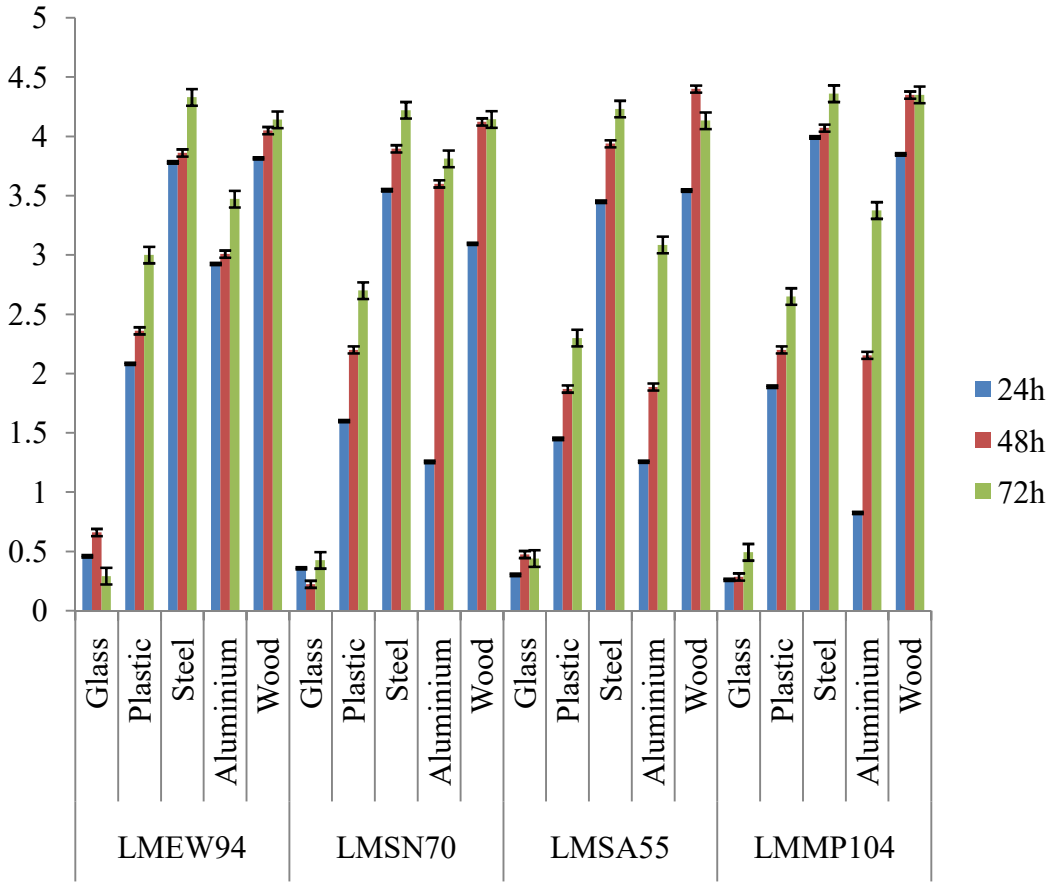


Figure 4.7: Hydrophobicity potential of *L. monocytogenes* isolated from different RTE foods

The result of screening for different enzymes in *L. monocytogenes* isolated from different RTE foods is shown in figure 4.8. The *L. monocytogenes* isolates were observed to produce more protease compared with lipase and amylase. Fifty-four percent (54%) of *L. monocytogenes* screened possessed protease while 13% possessed amylase and 33% possessed lipase.

Effect of disinfectants against *L. monocytogenes* isolated from different RTE foods is represented in Table 4.5, All *L. monocytogenes* were resistant to hypo, Robert antiseptics, izal and ethanol at 1%, 2% and 5%. All *L. monocytogenes* tested gave varying sensitivity to dettol at varying concentrations with 11 ± 1.41mm, 14 ± 2.12mm, 15 ± 2.12mm, 19 ± 1.41mm, 20 ± 0.00mm, 24 ± 1.41mm, 23 ± 1.41mm and 25 ± 0.70mm for *L. monocytogenes* LMSN70 at 1%,2%, 5%, 10%, 25%, 50%, 75% and 100% respectively. *L. monocytogenes* LMMP104 was senstitve to purit antiseptics at 50%, 75% and 100% with xone of inhibition 12 ± 0.00mm, 24 ± 0.00mm and 22 ± 5.22mm respectively.

The antibiotic susceptibility pattern of *L. monocytogenes* isolated from different RTE foods from South South Nigeria is as shown in Table 4.6. All *L. monocytogenes* was resistant to cloxacillin, amoxicillin and augumentin. *L. monocytogenes* LMSA108 was resistant to augumetin, nitrofurantoin and ceftriazone while been sensitive to ofloxacin, ciprofloxacin and pefloxacin. *L. monocytogenes* LMMP13 was resistant to augumentin, nitrofurantoin, amoxycillin, ceftriazone, cefuroxime, ceftazidine and cloxacillin sensitive to streptomycin, ofloxacin, tetracycline, pefloxacin, and erythromycin. *L. monocytogenes* LMMP13 had intermediate zone of inhibition to gentamycin and ciprofloxacin. *L. monocytogenes* LMMP14 was resistant to augumentin, ceftriazone, amoxicillin, ceftazidine and cloxacillin, intermediate zone of inhibition to streptomycin, nitrofurantoin and cefuroxime, and was sensitive to gentamycin, ofloxacin, ciprofloxacin, tetracycline, pefloxacin and erythromycin.

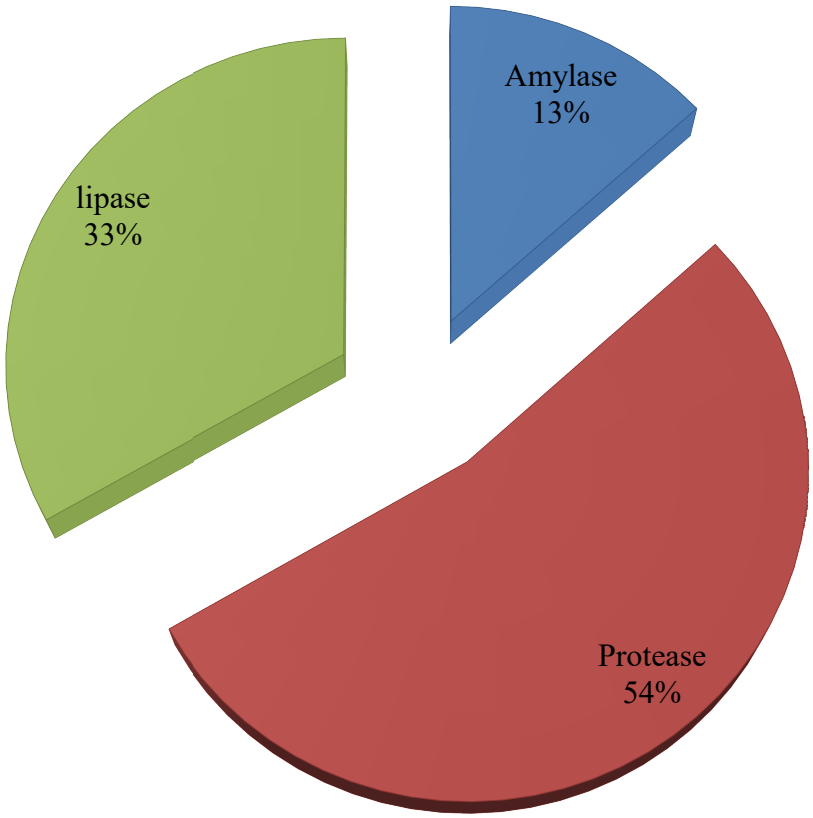


Figure 4.8: Profile of amylolytic, proteolytic and lipolytic enzymes from *L. monocytogenes* isolated from different RTE foods

Table 4.5: Effect of different disinfectants against *L. monocytogenes* isolated from different RTE foods

		Concentration/ Zone of inhibition (mm)							
Disinfectants	*L. monocytogenes*	1%	2%	5%	10%	25%	50%	75%	100%
Hypo	LMSN70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	LMEW94	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	LMMP104	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Jik	LMSN70	2 ± 1.41	3 ± 2.12	4 ± 2.82	4 ± 2.82	13 ± 12.20	18 ±10.60	22 ± 1.02	28 ± 1.60
	LMEW94	0.00	0.00	0.00	4 ± 1.22	5 ± 1.20	10 ±7.60	12 ± 1.02	20 ± 4.23
	LMMP104	2± 2.82	3 ± 7.42	4 ± 5.22	4 ± 4.87	10 ± 2.84	13 ±3.00	20 ± 2.24	25 ± 1.90
Roberts antiseptics	LMSN70	0.00	0.00	0.00	0.00	8 ± 2.83	11 ± 1.41	12 ± 0.00	14 ± 0.70
	LMEW94	0.00	0.00	0.00	0.00	6 ± 1.45	10 ± 0.00	13 ± 0.00	13 ± 0.00
	LMMP104	0.00	0.00	0.00	0.00	10 ± 7.22	12 ± 2.82	12 ± 0.00	14 ± 3.70
Dettol antiseptics	LMSN70	11 ± 1.41	14 ± 2.12	15 ± 2.12	19 ± 1.41	20 ± 0.00	24 ± 1.41	23 ± 1.41	25 ± 0.70
	LMEW94	12 ± 2.23	15 ± 3.34	16 ± 0.00	16 ± 1.22	20 ± 0.00	25 ± 6.24	22 ± 0.00	30 ± 1.82
	LMMP104	10 ± 0.00	12 ± 1.64	13 ± 2.12	20 ± 3.41	20 ± 0.00	22 ± 2.22	23 ± 1.41	20 ± 0.00

Disinfectants	*L. monocytogenes*	1%	2%	5%	10%	25%	50%	75%	100%
Izal	LMSN70	0.00	0.00	0.00	18±10.60	20 ± 1.41	25 ± 2.12	23 ± 4.24	25 ± 5.65
	LMEW94	0.00	0.00	0.00	10±0.00	15 ± 2.22	20 ± 4.12	25± 0.00	28 ± 2.24
	LMMP104	0.00	0.00	0.00	8 ± 2.60	10 ± 1.41	12 ± 0.00	18 ± 2.24	15 ± 1.70
Putrit antiseptics	LMSN70	0.00	0.00	0.00	0.00	11 ± 1.41	21 ± 1.41	22 ± 4.94	18 ± 6.26
	LMEW94	0.00	0.00	0.00	0.00	10 ± 2.23	20 ± 4.23	25 ± 2.23	13 ± 0.00
	LMMP104	0.00	0.00	0.00	0.00	12 ± 0.00	22 ± 0.00	22 ± 5.22	18 ± 1.23
Ethanol	LMSN70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	LMEW94	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	LMMP104	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 4.6: Antibiotic susceptibility pattern of *L. monocytogenes* isolated from different RTE foods

ISOLATE CODE	AUG	STR	NIT	GEN	CRO	OFL	AMX	CPX	TET	PEX	ERY	CRX	CAZ	CXC
LMEW2	R	I	I	S	R	S	R	S	S	S	R	I	R	R
LMEW6	R	R	R	I	R	I	R	S	R	I	R	R	R	R
LMSA10	R	S	R	I	R	S	R	I	I	S	R	S	R	R
LMMP13	R	S	R	I	R	S	R	I	S	S	S	R	R	R
LMEW14	R	I	I	S	R	S	R	S	S	S	R	I	R	R
LMEW16	R	R	R	I	R	I	R	S	R	I	R	R	R	R
LMMP18	R	R	S	I	R	S	R	S	I	I	I	R	R	R
LMSN19	R	I	R	I	R	S	R	S	S	S	I	I	R	R
LMEW20	R	I	I	S	R	S	R	S	S	S	R	I	R	R
LMEW22	R	R	R	I	R	I	R	S	R	I	R	R	R	R
LMSN 23	R	I	R	I	I	I	R	S	I	S	R	S	R	R
LMMP24	R	R	R	I	R	I	R	S	I	S	S	I	R	R
LMEW25	R	R	R	S	R	S	R	I	S	S	R	S	R	R
LMMP37	R	S	R	I	R	S	R	I	I	S	R	S	R	R
LMEW47	R	S	R	I	R	S	R	I	S	S	S	R	R	R

Table 4.6: Antibiotic susceptibility pattern of *L. monocytogenes* isolated from different RTE foods

ISOLATE CODE	AUG	STR	NIT	GEN	CRO	OFL	AMX	CPX	TET	PEX	ERY	CRX	CAZ	CXC
LMSN55	R	S	I	S	R	S	R	R	S	I	I	R	R	R
LMMP57	R	S	R	R	R	S	R	I	S	S	I	I	R	R
LMSN62	R	R	R	R	R	S	R	R	R	S	R	R	R	R
LMSA65	R	I	R	I	R	S	R	S	I	S	I	S	R	R
LMMP68	R	S	R	S	R	S	R	S	I	S	S	R	R	R
LMSN70	R	R	I	I	R	S	R	R	R	R	R	R	R	R
LMEW94	R	R	R	R	R	S	R	S	R	R	R	R	R	R
LMEW96	R	R	I	R	R	S	R	S	S	S	S	I	R	R
LMSA99	R	R	I	R	R	S	R	S	S	S	S	I	R	R
LMMP104	R	I	R	I	R	S	R	S	R	I	I	I	R	R
LMSA108	R	I	R	I	R	S	R	S	I	S	I	S	R	R
LMSA110	R	S	R	S	R	S	R	S	I	S	S	R	R	R
LMEW113	R	I	I	I	R	S	R	S	R	I	R	R	R	R

Key; R: Resistant, S: Sensitive, I: Intermediate, AUG: Augumentin, STR: Streptomycin, NIT: Nitrofurantoin, GEN: Gentamycin, CRO: Ceftriazone, OFL: Ofloxacin, AMX: Amoxycillin, CPX: Ciprofloxacin, TET: Tetracycline, PFX: Pefloxacin, ERY: Erythromycin, CRX: Cefuroxime, CAZ: Ceftazidine, CXC: Cloxacillin.

The antibiotic resistance/susceptibility spectrum of *L. monocytogenes* isolated from different RTE foods is as shown in Table 4.7. 100% of the *L. monocytogenes* were resistant to amoxicillin, cloxacillin, augumetin and ceftazidime while 18.88%, 20%, 57.77%, 24.44% and 13.33% of the *L. monocytogenes* were sensitive to nitrofurantoin, gentamicin, ofloxacin, erythromycin, and cefuroxime respectively. 50%, 66.66%, 53.33 and 27.77% of the *L. monocytogenes* had intermediate zone of susceptibility for Streptomycin, Pefloxacin, Erythromycin and Cefuroxime.

Table 4.8 shows the antibiotic resistance/susceptibility spectrum of *L. monocytogenes* isolated from different RTE foods. One hundred percent (100%) of the *L. monocytogenes* were still resistant to augumetin and ceftazidime while 22.22%, 31.11%, 80%, 30% and 24.44% of the *L. monocytogenes* were sensitive to nitrofurantoin, gentamicin, ofloxacin, erythromycin, and streptomycin respectively. 11.11%, 8.88%, 53.33% and 35.55% of the *L. monocytogenes* had intermediate zone of susceptibility for amoxycillin, cloxacillin, ceftriazone and cefuroxime.

Thirty-eight (42.22%) out of the 90 isolated *L. monocytogenes* haboured plasmids while 52 (57.78%) of the *L. monocytogenes* were without plasmid. The sizes of plasmid carried by the *L. monocytogenes* ranged from 30kbp to 100kbp. Twenty (22.22%) *L. monocytogenes* carried 50kbp plasmid while 2 (2.22%) the *L. monocytogenes* carried 100kbp plasmid (Table 4.9)

Table 4.7: Antibiotic resistance/susceptibility spectrum of *L. monocytogenes* isolated from different RTE foods

		Listeria monocytogenes spectrum		
Group	Antimicrobial agent	Susceptible (%)	Intermediate (%)	Resistant (%)
Nitrofurantoins	Nitrofurantoin	17 (18.88)	52 (55.55)	21 (23.33)
Aminoglycosides	Gentamicin	18 (20.00)	60 (66.66)	12 (13.33)
	Daptomycin	30(33.33)	10 (11.11)	50 (55.55)
	Streptomycin	15 (16.66)	45 (50.00)	30 (33.33)
Fluoroquinolones	Ofloxacin	52 (57.77)	38 (42.22)	NIL
	Ciprofloxacin	30 (33.33)	48 (53.33)	12 (13.33)
	Pefloxacin	18 (20.00)	60 (66.66)	12 (13.33)
ß- lactams	Amoxycillin	NIL	NIL	90(100)
	Cloxacillin	NIL	NIL	90(100)
	Augumentin	NIL	NIL	90(100)
Tetracycline	Tetracycline	25 (27.77)	35 (38.88)	30 (33.33)
Macrolides	Erythromycin	22 (24.44)	48 (53.33)	20 (22.22)
Cephems	Cefuroxime	12 (13.33)	25 (27.77)	43 (47.77)
	Ceftazidine	NIL	NIL	90(100)
	Ceftriazone	NIL	48 (53.33)	42 (46.66)

Table 4.8: Antibiotic resistance/susceptibility spectrum of *L. monocytogenes* isolated from different RTE foods from South South Nigeria after plasmid curing

		Listeria monocytogenes spectrum		
Group	Antimicrobial agent	Susceptible (%)	Intermediate (%)	Resistant (%)
Nitrofurantoins	Nitrofurantoin	20 (22.22)	60 (66.66)	10 (11.11)
Aminoglycosides	Gentamicin	28 (31.11)	60 (66.66)	2 (2.22)
	Daptomycin	30(33.33)	48 (53.33)	12 (13.33)
	Streptomycin	22 (24.44)	51 (56.66)	17 (18.88)
Fluoroquinolones	Ofloxacin	72 (80.00)	18 (20.00)	NIL
	Ciprofloxacin	35(38.88)	48 (53.33)	7 (7.77)
	Pefloxacin	26 (28.88)	52 (57.77)	12 (13.33)
ß- lactams	Amoxycillin	NIL	10 (11.11)	80(88.88)
	Cloxacillin	NIL	8 (8.88)	82(91.11)
	Augumentin	NIL	NIL	90(100)
Tetracycline	Tetracycline	29 (32.22)	42 (46.66)	19 (21.11)
Macrolides	Erythromycin	27 (30.00)	51 (56.66)	12 (13.33)
Cephems	Cefuroxime	18 (20.00)	32 (35.55)	40 (44.44)
	Ceftazidine	NIL	NIL	90(100)
	Ceftriazone	NIL	48 (53.33)	42 (46.66)

Table 4.9: Plasmid DNA harboured by *L. monocytogenes* isolated from RTE foods

Size of Plasmid (Kbp)	No. of *L. monocytogenes* harboring plasmid DNA (%)	No. of *L. monocytogenes* without plasmid DNA (%)
30	4 (4.44)	
35	8 (8.88)	
50	20 (22.22)	52 (57.78)
80	4 (4.44)	
100	2 (2.22)	
Total	38(42.22)	

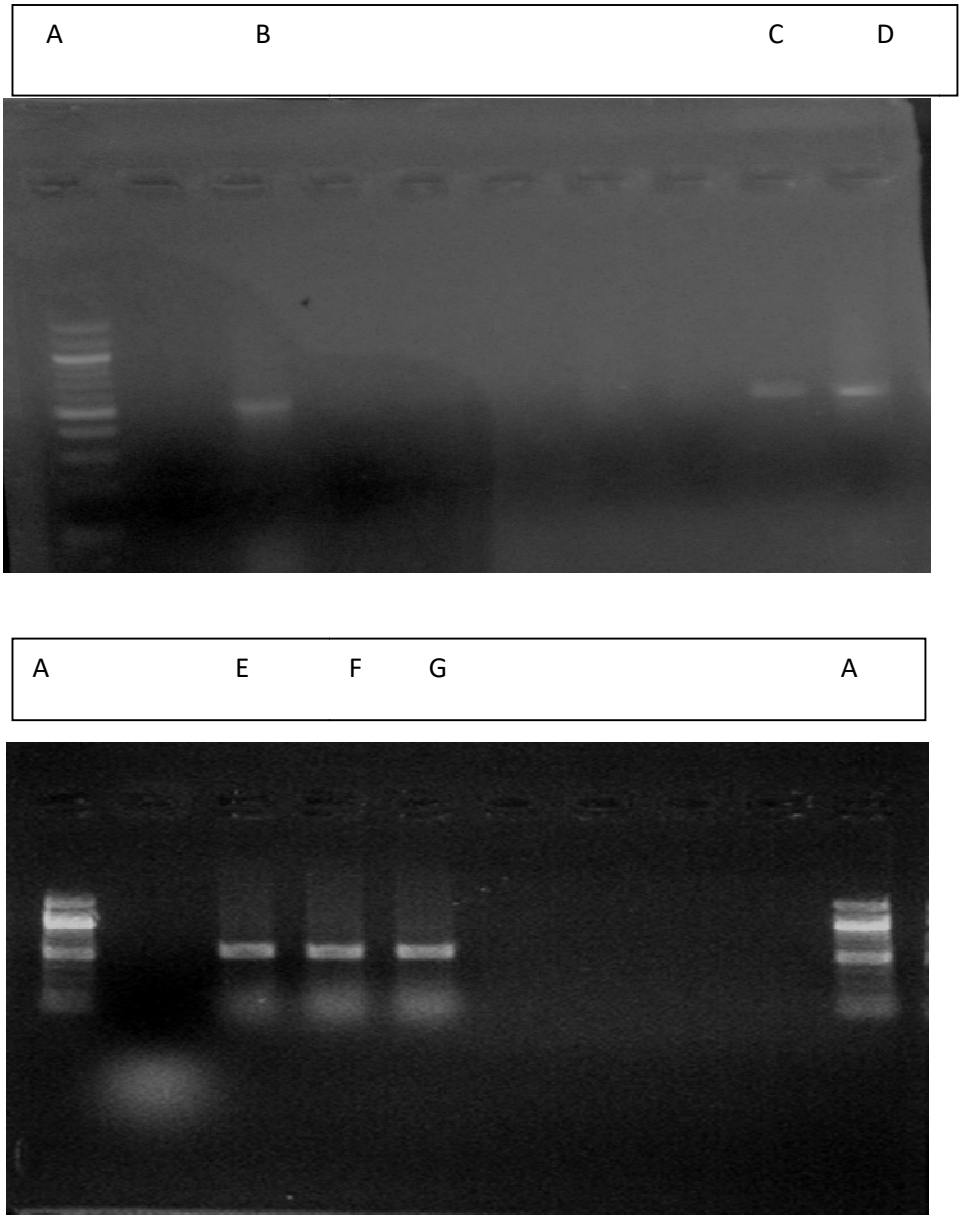


Plate 4.1: Agarose gel separation of plasmids isolated from *L. monocytogenes*

KEY;

Lane A: 100kbp Ladder

Lane B: *L. monocytogenes* LMSA55, Lane C: LMEW94, D: LMEW24, E: LMEW70, F: LMEW94, G: LMEW 106,

Determination of virulence gene markers and some antimicrobial resistant genes associated with *Listeria monocytogenes*

The picture of the agarose gel with amplified iap (encoding extracellular protein P60) gene from *L. monocytogenes* strains isolated from RTE foods is as shown in Plate 4.2. The band of the amplified isolates was 660bp and all *Listeria monocytogenes* isolated had amplified the IAP gene

The picture of the agarose gel with amplified Internalin A (InlA) gene from *L. monocytogenes* strains isolated from RTE foods is as shown in Plate 4.3. The amplified InlA gene size was 733bp and all *L. monocytogenes* isolated had amplified InlA virulent gene marker

Agarose gel with amplified listeriolysin O (hylA) gene from *L. monocytogenes* isolated from RTE foods is represented in Plate 4.4. All *L. monocytogenes* had the *HlyA* gene with the weight of the amplified gene being 234bp.

Antimicrobial resistance genes in *L. monocytogenes* isolated from RTE foods from South South Nigeria is shown in Table 4.10. Ten (10) *L. monocytogenes* representing 20% were positive for daptomycin resistance gene (mprF). Of 30 *L. monocytogenes* strains tested for tetracycline resistance genes, 15 (50%) carried tetA while 10 (33.30%) had tetM genes. 6(30%) of *L. monocytogenes* were positive for erythromycin resistant genes (ermB).

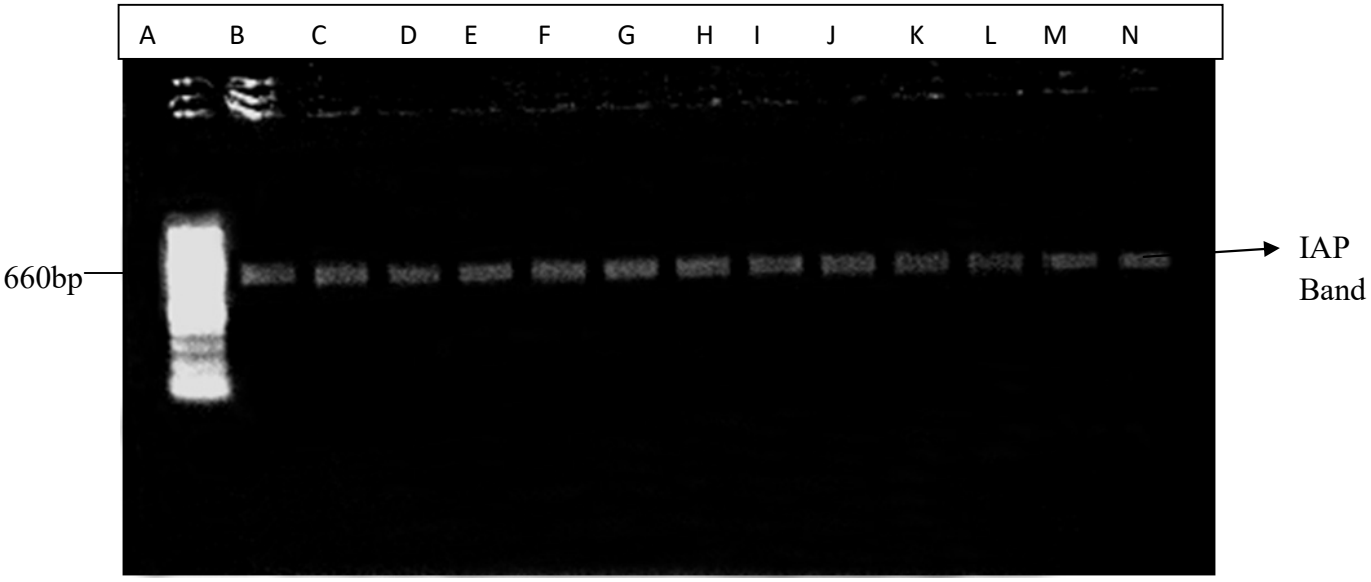


Plate 4.2: Agarose gel separation of amplified Extracellular protein P60 (Iap) gene from *L. monocytogenes* isolated from RTE foods

KEY;

Lane A: 100bp Ladder

Lane B: *L. monocytogenes* ATCC19155

Lane C: LMEW20, D: LMEW24, E: LMEW70, F: LMEW94, G: LMSN47,

H: LMSN108, I: LMSA55, J: LMSA104, K: LMMP104 L: LMEW 106,

M; LMSA38, N: LMMP 106

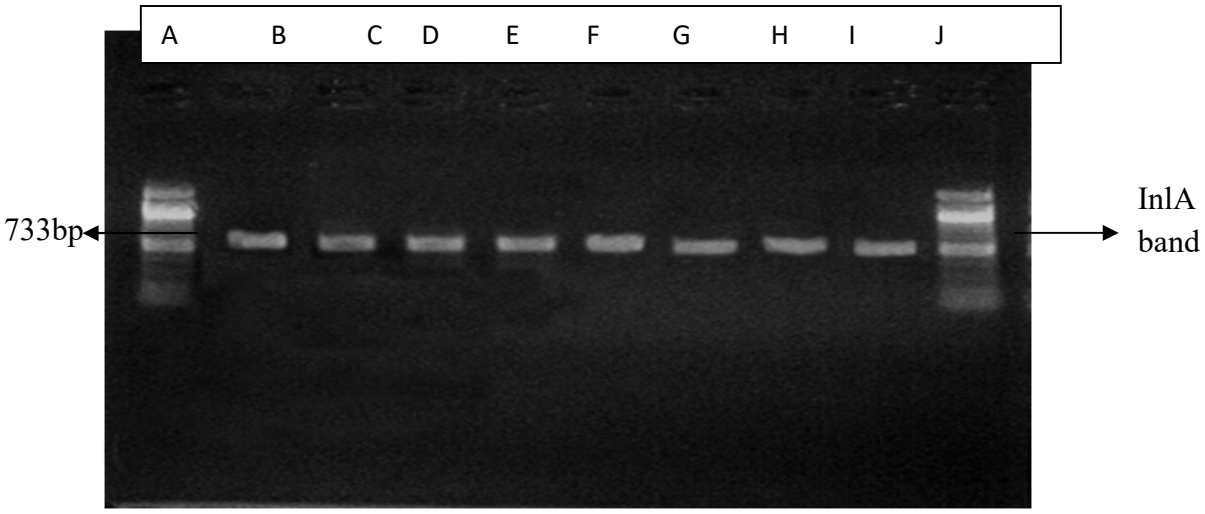


Plate 4.3: Agarose gel separation of amplified Internalin A (InlA) gene from *L. monocytogenes* isolated from RTE foods

KEY;

Lane A and J: 100bp Ladder

Lane B: *L. monocytogenes* ATCC19155

Lane C: LMEW20, D: LMEW24, E: LMEW70, F: LMEW94, G: LMSN47,

H: LMSN108, I: LMSA55, J: LMSA104,

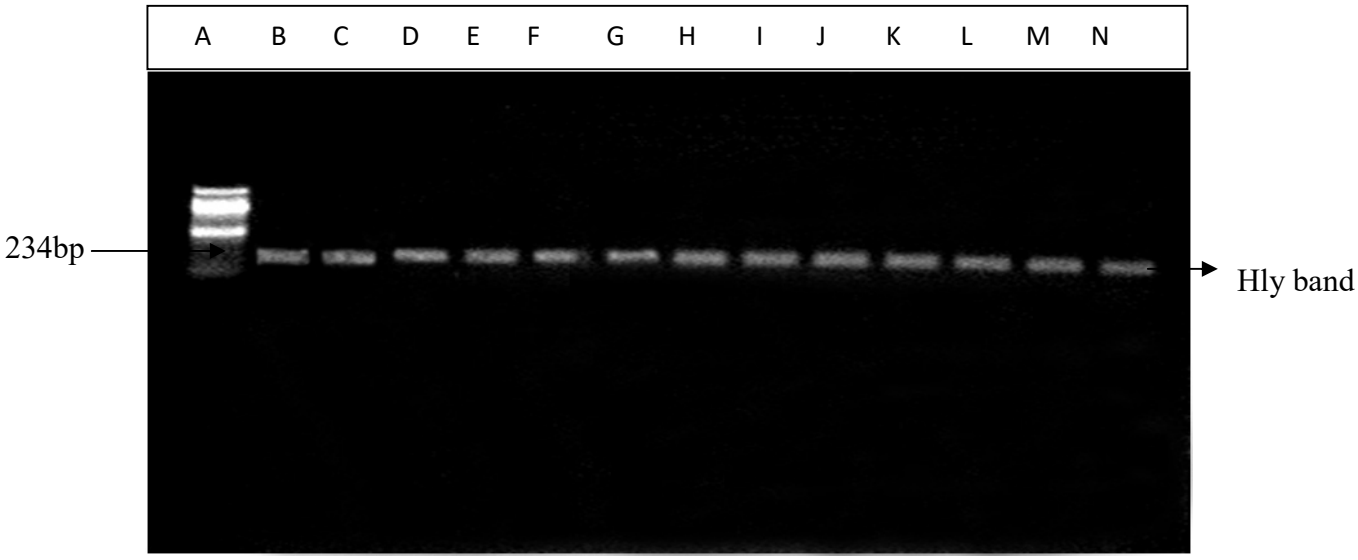


Plate 4.4: Agarose gel separation of amplified listeriolysin O (HylA) gene from *L. monocytogenes* isolated from RTE foods

KEY;

Lane A: 1kbp Ladder

Lane B: *L. monocytogenes* ATCC19155

Lane C: LMEW20, D: LMEW24, E: LMEW70, F: LMEW94, G: LMSN47,

H: LMSN108, I: LMSA55, J: LMSA104, K: LMMP104 L: LMEW 106,

M; LMSA38, N: LMMP 106

Table 4.10: Antimicrobial resistance genes in *L. monocytogenes* strains isolated from RTE foods

Antibiotic resistance gene markers	Number of *L. monocytogenes* tested	Proportion of *L. monocytogenes* carrying the resistance genes
Tetracycline (tetA)	30	15 (50%)
Tetracycline (tetM)	30	10 (33.30%)
Erythromycin (ermB)	20	6 (30%)
Daptomycin (mprF)	50	10 (20%)

Determination of *in vivo* virulence of *Listeria monocytogenes*

Shown in Figure 4.9 is the life span of mice inoculated with *L. monocytogenes* isolated from different RTE foods. Mice infected with *L. monocytogenes* LMSN70 orally exterminated one (1) within 3days while when infected interperitoneally, it exterminated two (2) mice. For *L. monocytogenes* LMEW94, when it was infected orally and interperitoneally, it exterminated four (4) mice within three (3) days each from the group while none died in the control group.

The packed cell volume of mice infected with *L. monocytogenes* LMSN70 was 35% compared with the control 42%. The lymphocytes of mice infected with *L. monocytogenes* LMSN70 (11.9 g/dl) and LMEW94 (11.9 g/dl) was lower than the control (14.0 g/dl). The lymphocyte of mice infected with *L. monocytogenes* LMSN70 (52%) and LMEW94 (68%) was higher than the control (30%) (Table 4.11).

Phytochemical screening and *in vitro* antilisterial potential of different aqeous and ethanolic plant extracts

The phytochemical screening of the different ethanolic plants extracts is shown in table 4.12. *Zingiber officinale* and *Ocimum gratissimum* had alkaloids, saponins, steroids, terpenoids, cardiac glycoside, reducing sugar, phenolics, resins, flavonoids, and tannins present in varying amount. Cardiac glycoside was absent in *Trema orientalis*, *Heinsia crinite*, *Dacryodes edulis*, *Citrus aurantifolia* and *Funtumia elastic* while tannins was absent in *Cassia alata and terpenoids* absent in *Moringa oleifera.*

Zingiber officinale, *Moringa oleifera and Ocimum gratissimum* had alkaloids, saponins, steroids, terpenoids, cardiac glycoside, reducing sugar, phenolics, resins, flavonoids, and tannins present in varying amount. Cardiac glycoside was present in *Psidium guajuva*, *Trema orientalis*, *Citrus aurantifolia* and *Funtumia elastic* but absent in *Heinsia crinite* and *Dacryodes edulis*. Alkaloids and tannins was absent in *Cassia alata and terpenoids* while steroids were absent in *Trema orientalis*, *Heinsia crinite*, *Funtumia elastica* and *Gongronema latifolium* (Table 4.13)

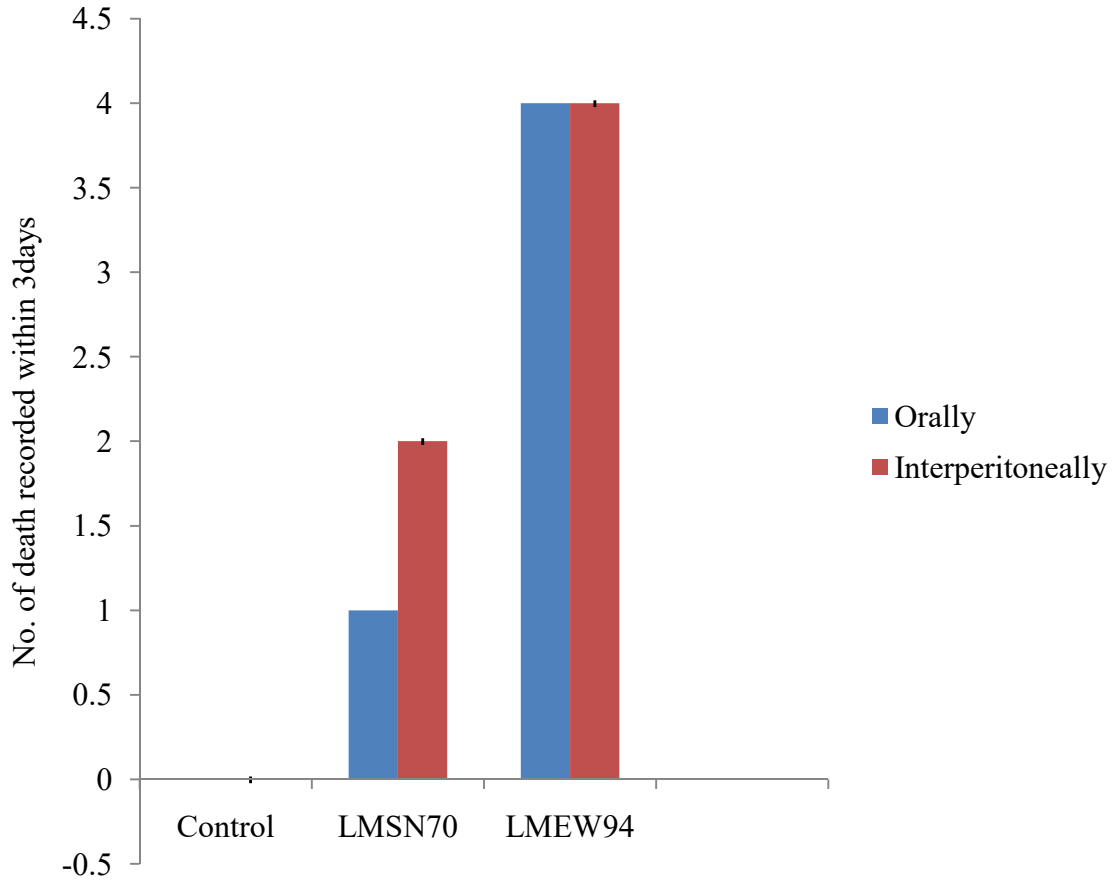


Figure 4.9: Life span of mice inoculated with *L. monocytogenes* isolated from different RTE foods

Table 4.11: Full blood count of mice infected with *L. monocytogenes* isolated from different RTE foods

	PCV (%)	Hb (g/dl)	RBC ($x10^{12}$/l)	MCH (pg)	MCV (pf)	MCHC (g/dl)	WBC ($x10^{9}$/l)	Neut (%)	Lymph (%)
Control	42	14.0	5.5	28	72	33.1	12.0	29	30
LMSN70	35	11.9	4.8	25	81	33.3	20.4	62	52
LMEW94	33	11.0	3.9	25	84	33.3	25.6	70	68

KEY: PCV: Packed cell volume; Hb: Haemoglobin; RBC: Red blood cells; WBC: White blood cell; MCH: Mean corpuscular haemoglobin; MCV: Mean corpuscular volume; MCHC: Mean corpuscular hemoglobin concentration; NEUT: Neutrophil; LYMPH: Lymphocyte

Table 4.12: Phytochemical screening of different ethanolic plant extracts

Scientific names	Common name	Alk	Sap	Ste	Tep	C. gly	R. sug	Phe	Res	Flav	Tan
Psidium guajuva	Guava	+	+++	+	-	+	+	+	++	+++	++
Zingiber officinale	Ginger	++	+++	+	++	++	+	+++	++	++	+
Trema orientalis	Charcoal tree	++	++	+	-	-	++	+	+	++	++
Heinsia crinite	Atama	++	+	++	++	-	+	++	+++	++	++
Dacryodes edulis	African pear	+++	++	++	+++	-	+	+	+	+	+
Citrus aurantifolia	Lime	++	+++	++	-	-	+	-	++	++	++
Funtumia elastic	Silk rubber	++	+++	+	+	-	+	+	+++	+	++
Gongronema latifolium	Utazi	+++	++	++	+	+	+	+	+	++	+
Ocimum gratissimum	Scent leaf	+++	+++	++	++	++	++	+++	++	++	++
Vernonia amygdalina	Bitter leaf	++	++	+	+	++	+	++	++	+++	+++
Cassia alata	Cassia	++	++	++	++	+	++	+++	++	++	-
Moringa oleifera	Moringa	+++	+++	+++	-	++	+	++	+++	+	+

Key: Alk- Alkaloids, Sap-Saponins, Ste- Steroids, Tep- Terpenoids, C. gly: Cardiac glycoside, R. sug: Reducing sugar, Phe: Phenolics, Res: Resins, Flav- Flavonoids, Tan: Tannins, +++ = highly present, ++ = moderately present, + = slightly present, - = absent

Table 4.13: Phytochemical screening of different aqueous plant extracts

Scientific names	Common name	Alk	Sap	Ste	Tep	C. gly	R. sug	Phe	Res	Flav	Tan
Psidium guajuva	Guava	+++	++	++	-	++	++	+++	+++	++	+
Zingiber officinale	Ginger	+++	++	+	+	+	++	++	+	++	++
Trema orientalis	Charcoal tree	+++	+	-	-	+	+	++	++	+++	++
Heinsia crinite	Atama	++	+	-	-	-	++	+	+++	++	++
Dacryodes edulis	African pear	++	+	+	+	-	++	++	+++	++	++
Citrus aurantifolia	Lime	++	++	+++	-	++	+	+	++	++	++
Funtumia elastic	Silk rubber	+	++	-	-	+	+	+	+	++	+
Gongronema latifolium	Utazi	+++	++	-	-	-	+	+	+++	++	++
Ocimum gratissimum	Scent leaf	++	++	++	+	+	++	++	++	+++	+
Vernonia amygdalina	Bitter leaf	++	+++	+	+	+	++	++	+	+++	+++
Cassia alata	Cassia	_	+	+	+	+	++	++	++	+	_
Moringa oleifera	Moringa	++	+	++	+	+	++	+	++	+	++

Key: Alk- Alkaloids, Sap-Saponins, Ste- Steroids, Tep- Terpenoids, C. gly: Cardiac glycoside, R. sug: Reducing sugar, Phe: Phenolics, Res: Resins, Flav- Flavonoids, Tan: Tannins, +++ = highly present, ++ = moderately present, + = slightly present, - = absent

The antilisterial activity of different ethanolic leaf extracts against *L. monocytogenes* isolated from RTE foods is shown in Table 4.14. Guava leaves had antilisterial activity against *L. monocytogenes* LMSN70 with 30 ± 2.00mm, 45 ± 14.12mm, 50 ± 7.07mm, 57 ± 0.00mm, 60 ± 0.00mm and 65 ± 0.00mm at 100 mg/mL, 200 mg/mL, 400 mg/mL, 600 mg/mL, 800 mg/mL and 1000 mg/mL respectively. Ginger leaves did not show any antilisterial activities at concentration less than 600mg/mL but had 40 ± 0.00mm, 25 ± 4.23mm and 20 ± 7.23mm at 1000mg/mL for *L. monocytogenes* LMSN70, LMEW94, LMMP104 respectively. Bitter leaves had antilisterial activity against *L. monocytogenes* LMEW94 with 35 ± 0.00mm, 50 ± 0.00mm, 60 ±2.33mm, 64 ± 2.23mm, 70 ± 0.00mm, and 35 ± 0.00mm at 100 mg/mL, 200 mg/mL, 400 mg/mL, 600 mg/mL, 800 mg/mL and 1000 mg/mL respectively.

The antilisteria activity of different aqeous leaf extracts against *L. monocytogenes* isolated from RTE foods is shown in Table 4.15. African pear leaves had antilisterial activity against *L. monocytogenes* LMSN70 with 25 ± 14.14mm, 40 ±0.00mm, 48 ±0.00mm, 50 ± 7.00mm, 55 ± 4.23mm and 63 ± 0.00mm at 100 mg/mL, 200 mg/mL, 400 mg/mL, 600 mg/mL, 800 mg/mL and 1000 mg/mL respectively. Ginger leaves did not show any antilisterial activities at concentration less than 600mg/mL but had 17 ± 0.00mm, 25 ± 4.23mm and 20 ± 7.23mm at 1000mg/mL for *L. monocytogenes* LMSN70, LMEW94, LMMP104 respectively. Bitter leaves had antilisterial activity against *L. monocytogenes* LMEW94 with 35 ± 0.00mm, 50 ± 0.00mm, 60 ±2.33mm, 64 ± 2.23mm, 70 ± 0.00mm and 65 ± 0.00mm at 100 mg/mL, 200 mg/mL, 400 mg/mL, 600 mg/mL, 800 mg/mL and 1000 mg/mL respectively.

Table 4.14: Antilisteria activity of different ethanolic leaf extracts against *L. monocytogenes* isolated from RTE foods

		Ethanolic leaf extract concentration (mg/mL) / Zone of Inhibition (mm)					
Common name		100	200	400	600	800	1000
Guava Leaves	LMSN70	30 ± 2.00	45 ± 14.12	50 ± 7.07	57 ± 0.00	60 ± 0.00	65 ± 0.00
	LMEW94	27 ± 1.25	38 ± 2.53	48 ± 5.04	60 ± 0.00	60 ± 0.00	65 ± 4.23
	LMMP104	33 ± 0.00	40 ± 2.14	47 ± 0.00	60 ± 0.00	60 ± 0.00	62 ± 2.28
Ginger leaves	LMSN70	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	30 ± 4.23	40 ± 0.00
	LMEW94	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	10 ± 0.00	25 ± 4.23
	LMMP104	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	15 ± 1.23	20 ± 7.23
African pear leaves	LMSN70	25 ± 14.14	40 ±0.00	40 ±0.00	50 ± 7.00	55 ± 4.23	40 ± 0.00
	LMEW94	25 ± 14.14	40 ±0.00	40 ±0.00	50 ± 7.20	55 ± 4.23	40 ± 0.00
	LMMP104	25 ± 14.14	40 ±0.00	40 ±0.00	50 ± 7.00	55 ± 0.00	40 ± 0.00
Lime leaves	LMSN70	20 ± 3.23	25 ± 7.00	25 ± 7.07	30 ± 14.14	35 ± 7.07	55 ± 5.03
	LMEW94	15 ± 0.00	20± 1.23	20 ± 0.00	20 ± 7.53	30 ± 4.23	35 ± 2.00
	LMMP104	20 ± 7.10	25 ± 7.00	30 ± 0.00	33 ± 3.13	35 ± 7.07	40 ± 5.03
Silk rubber leaves	LMSN70	55 ±7.10	65 ± 3.00	70 ±0.00	60 ± 2.53	50 ± 0.00	40 ±0.00
	LMEW94	30±0.00	45 ± 1.00	55 ±0.00	70 ± 0.00	50 ± 0.00	40 ±0.00
	LMMP104	45 ±0.00	55 ± 0.00	65 ±0.00	72 ± 2.33	70 ± 0.00	40 ±0.00
Bitter leaf	LMSN70	30 ± 0.00	50 ± 0.00	55 ±7.07	63 ± 3.45	70 ± 7.07	40 ± 0.00
	LMEW94	35 ± 0.00	50 ± 0.00	60 ±2.33	64 ± 2.23	70 ± 0.00	35 ± 0.00
	LMMP104	33 ± 0.00	50 ± 0.00	62 ±0.00	66 ± 7.03	70 ± 0.00	43 ± 0.00

Table 4.14: Antilisteria potential of different ethanolic leaf extracts against *L. monocytogenes* isolated from RTE foods contd

		Ethanolic leaf extract concentration (mg/mL) / Zone of Inhibition (mm)					
Common name		100	200	400	600	800	1000
Moringa leaf	LMSN70	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
	LMEW94	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
	LMMP104	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Cassia leaf	LMSN70	10 ± 0.00	22 ± 8.43*	25 ± 1.45	32 ± 0.00	38 ± 2.56	37 ± 3.04
	LMEW94	8 ± 0.00	15 ± 0.00	20 ± 7.12	25 ± 0.00	30 ± 0.00	32 ± 0.00
	LMMP104	7 ± 0.00	12 ± 2.43	18 ± 2.24	26 ± 0.00	34 ± 2.42	34 ± 2.04

*Each valueis a Mean of duplicate determinants ± Standard Error

Table 4.15: Antilisteria activity of different aqeous leaf extracts against *L. monocytogenes* isolated from RTE foods

		Aqeous leaf extract concentration (mg/mL) / Zone of Inhibition (mm)					
Plants		100	200	400	600	800	1000
Guava Leaves	LMSN70	15 ± 7.07	25 ± 7.07	30 ± 0.00	33 ± 4.94	40 ± 0.00	45 ± 0.00
	LMEW94	27 ± 1.25	38 ± 2.53	48 ± 5.04	50 ± 0.00	52 ± 0.00	50 ± 4.23
	LMMP104	17 ± 0.00	23± 2.14	27 ± 0.00	36 ± 0.00	48 ± 0.00	50 ± 2.28
Ginger leaves	LMSN70	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	13 ± 4.23	17 ± 0.00
	LMEW94	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	10 ± 0.00	25 ± 4.23
	LMMP104	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	15 ± 1.23	20 ± 7.23
African pear leaves	LMSN70	25 ± 14.14	40 ±0.00	48 ±0.00	50 ± 4.23	55 ± 4.23	63 ± 0.00
	LMEW94	25 ± 14.14	40 ±0.00	40 ±0.00	50 ± 7.00	59 ± 4.23	65 ± 0.00
	LMMP104	22± 3.53	42± 4.50	65 ± 0.00	60 ± 0.00	70 ± 0.00	72 ± 3.52
Lime leaves	LMSN70	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
	LMEW94	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
	LMMP104	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Silk rubber leaves	LMSN70	10 ± 0.00	20 ± 0.00	30 ± 0.00	40 ± 14.12	50 ± 7.07	55 ± 14.14
	LMEW94	15±0.00	25 ± 1.00	28±0.00	40 ± 0.00	50 ± 0.00	40 ±0.00
	LMMP104	17 ±0.00	19 ± 0.00	25 ±0.00	42 ± 2.33	54 ± 0.00	53 ±0.00
Bitter leaf	LMSN70	30 ± 0.00	50 ± 0.00	55 ±7.07	63 ± 3.45	70 ± 7.07	50 ± 0.00
	LMEW94	35 ± 0.00	50 ± 0.00	60 ±2.33	64 ± 2.23	70 ± 0.00	65 ± 0.00
	LMMP104	33 ± 0.00	54 ± 0.00	62 ±0.00	66 ± 7.03	69 ± 0.00	73 ± 0.00

Table 4.15: Antilisteria potential of different aqeous leaf extracts against *L. monocytogenes* isolated from RTE foods

		Aqeous leaf extract concentration (mg/mL) / Zone of Inhibition (mm)					
Plants		100	200	400	600	800	1000
Moringa leaf	LMSN70	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
	LMEW94	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
	LMMP104	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Cassia leaf	LMSN70	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
	LMEW94	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
	LMMP104	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00

*Each valueis a Mean of duplicate determinants ± Standard Error

Determination of the *in vivo* antilisterial potential of selected plant extracts

Histopathological examination of the organs (liver and spleen) is depicted in plate 4.4 to picture 4.6. Some of liver of the infected mice was characterized with apoptptic cells which were seen as cells with esinophilic cytoplasm with dense nuclei (Picture 4.4) others showed vacoulation of the cytoplasm as a result of fatty change (Picture 4.5). The spleen of the infected mice had normal splenic architecture with occasional giant cells containing multiple nuclei and abundant cytoplasm (Plate 4.6)

Plate 4.7 shows the histology of liver of mice treated and untreated with 10^7cfu/mL *L. monocytogenes* and 0.8g/kg aqueous leaf extract combination. Histology of mice liver fed with *L. monocytogenes* and bitter leaf extract combination revealed visible centrioles with well fenestrated sinusoidal space with mild kupfer cell activation. The histology of liver of mice fed with *L. monocytogenes* and guava leaf extract combination also revealed sinusoidal space with mild kupfer cell activation

The histology of mice spleen treated and untreated with 10^7cfu/mL *L. monocytogenes* and 0.8g/kg aqueous leaf extract combination is depicted in Plate 4.8. Histology of mice spleen fed with *L. monocytogenes* and bitter leaf extract combination revealed prominent lymphoid follicles with centrally to eccentrically located blood vessels with the follicles (white pulp) consisting of aggregates of lymphocytes and congested dilated blood vessels. The histology of mice spleen fed with normal diet and water showed normal splenic architecture.

Histology of mice stomach fed with *L. monocytogenes* and guava leaf extract combination revealed mild neutrophilic infiltration in the gastric mucosa, submucosa, and lamina muscularis of the non glandular region of the stomach while histology of mice stomach fed with normal diet and water had no neutrophilic infiltration in the gastric mucosa, submucosa, and lamina muscularis (Plate 4.9).

Test Control

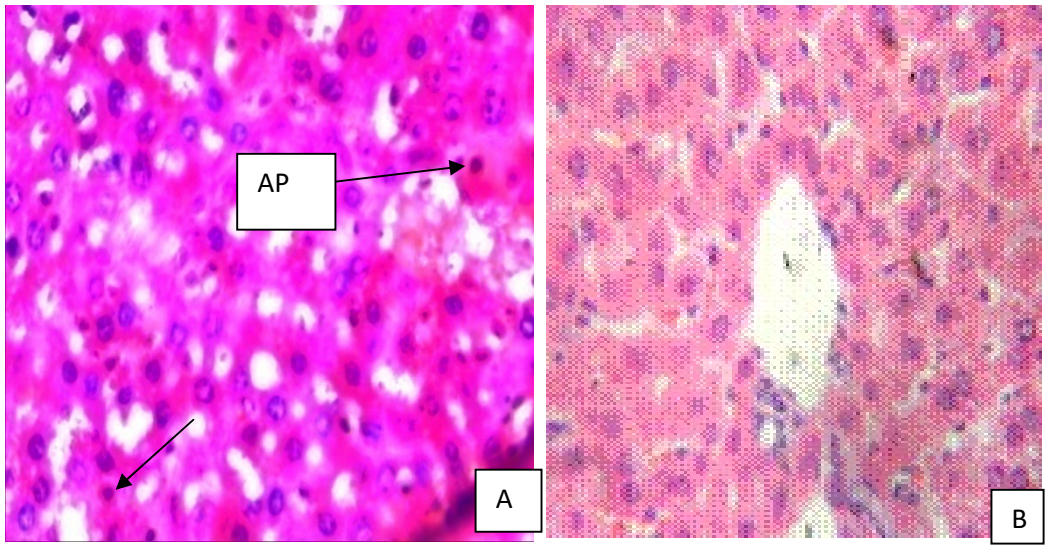


Plate 4.5: Histology of liver of mice treated and untreated with *L. monocytogenes* isolated from RTE foods

Haematoxylin and Eosin x 400 magnification

A: Histopathological section of liver of mice fed with *L. monocytogenes* showing apoptotic cells (AP) with intensely eosinophilic cytoplasm and small dense nuclei.

B: Histopathological section of liver of mice fed with normal diet and water showing normal cellular architecture

Test Control

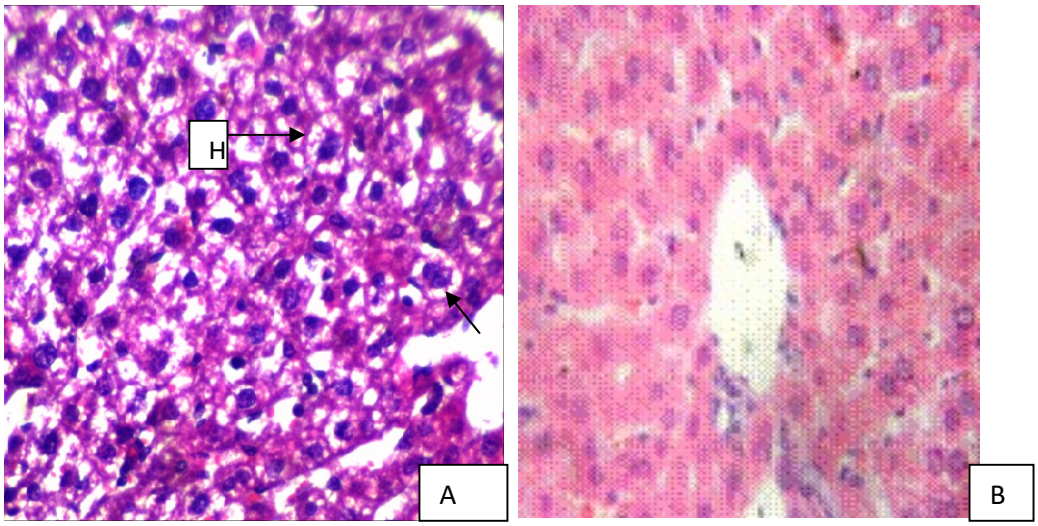
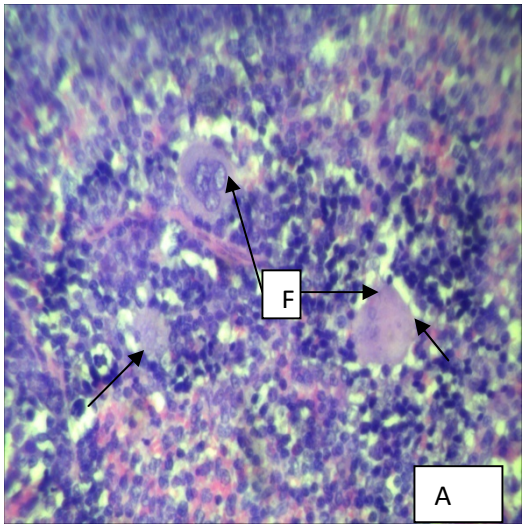


Plate 4.6: Histology of liver of mice treated and untreated with *L. monocytogenes* isolated from RTE foods

Haematoxylin and Eosin x 400 magnification

A: Histopathological section of liver of mice fed with *L. monocytogenes* showing vacoulation of the cytoplasm (H)

B: Histopathological section of liver of mice fed with normal diet and water showing normal liver architecture



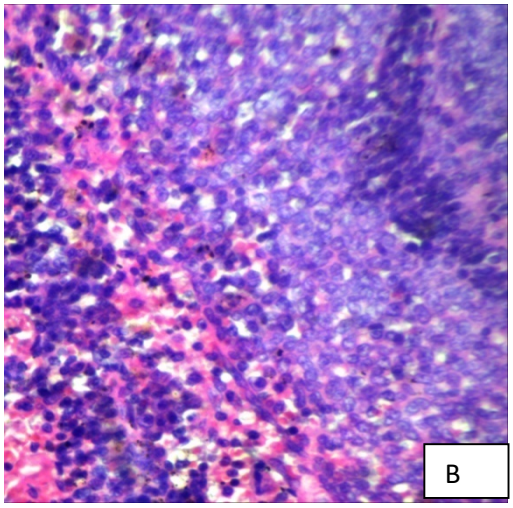


Plate 4.7: Histology of spleen of mice treated and untreated with *L. monocytogenes* isolated from RTE foods

Haematoxylin and Eosin x 400 magnification

A: Histopathological section of spleen of mice fed with *L. monocytogenes* showing normal splenic architecture with occasional giant cells (F) containing multiple nuclei and abundant cytoplasm.

B: Histopathological section of spleen of mice fed with normal diet and water showing normal splenic architecture

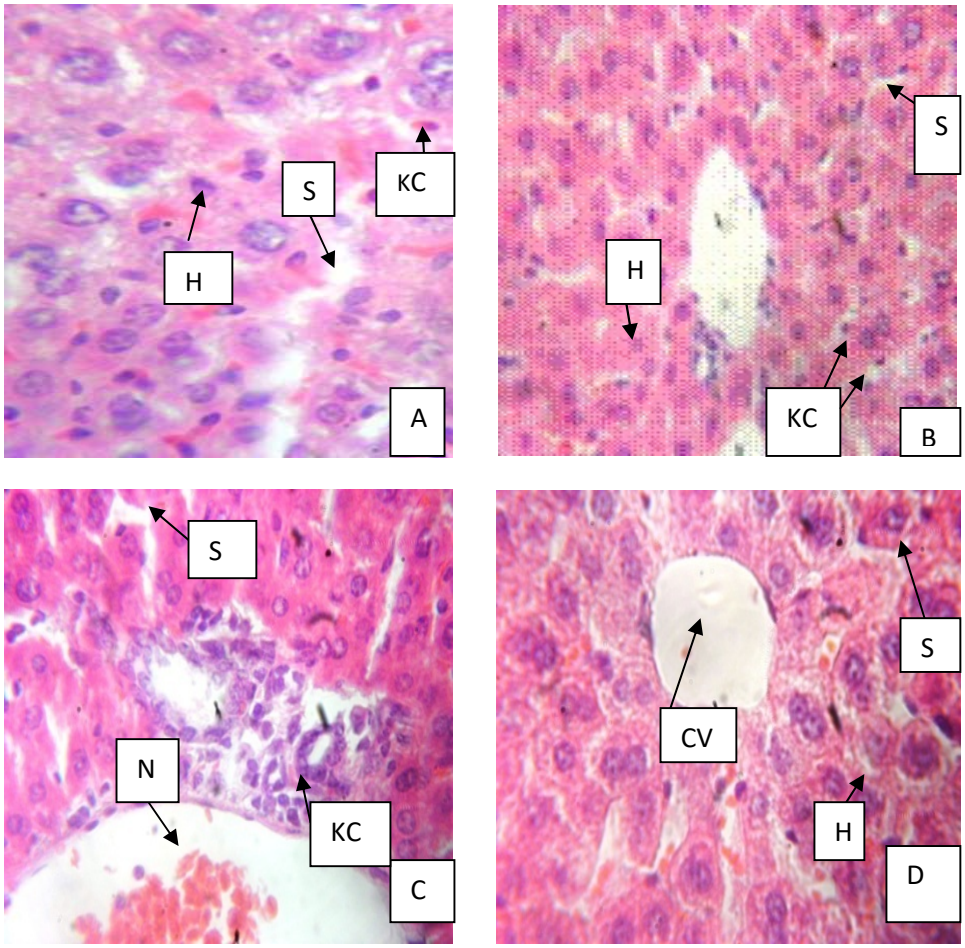


Plate 4.8: Histology of liver of mice treated and untreated with 10^7cfu/ml *L. monocytogenes* and 0.8g/kg aqueous leaf extract combination

A: Histology of liver of mice fed with *L. monocytogenes* and bitter leaf extract combination. Histology reveals visible centrioles with well fenestrated sinusoidal space (S). The hepatocytes (H) appear distinct with mild kupfer cell activation (KC).

B: Histology of liver of mice fed with *L. monocytogenes* and guava leaf extract combination. Histology reveals sinusoidal space (S). The hepatocytes (H) appear distinct with mild kupfer cell activation (KC).

C: Histology of liver of mice fed with *L. monocytogenes* and *D. edulis* leaf extract. Liver histology reveals central vein (CV), sinusoids (S) and hepatocytes (H) with mild kupfer cell activation (KC) and neutrophillic infiltrates (N).

D: Histology of liver of mice fed with normal diet and water. Normal liver architecture with prominent central vein (CV), sinusiodal space (S) and hepatocytes (H).

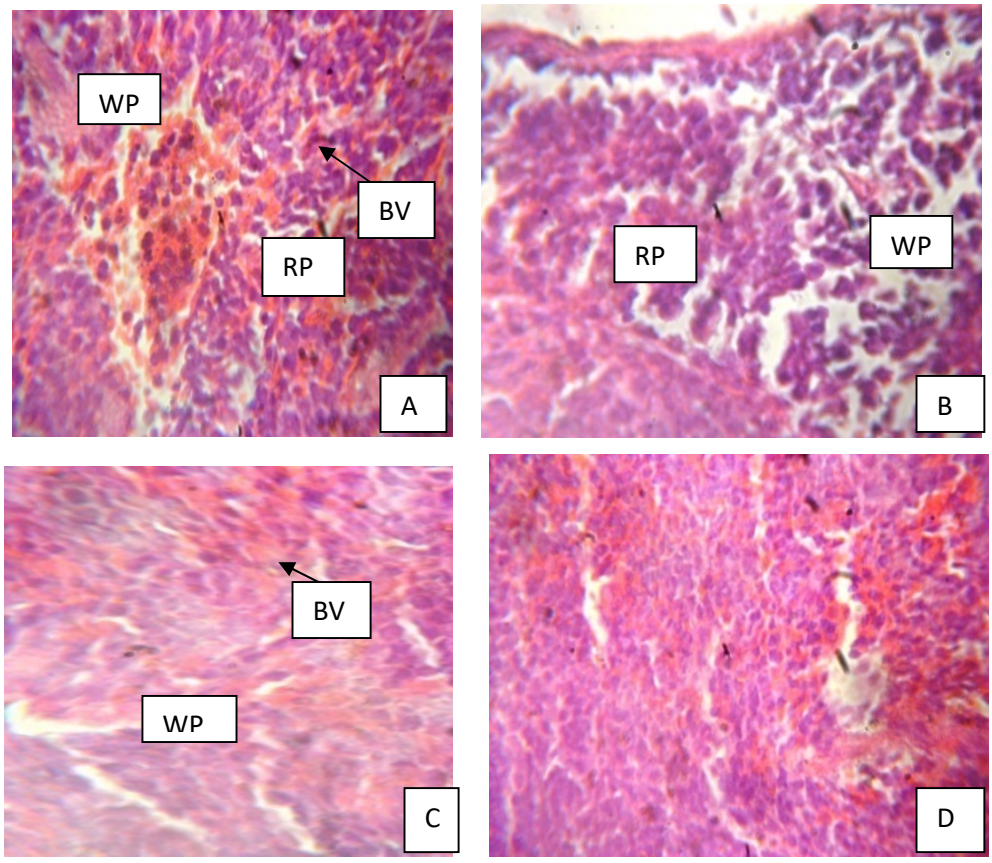


Plate 4.9: Histology of mice spleen treated and untreated with 10^7cfu/ml *L. monocytogenes* and 0.8g/kg aqueous leaf extract combination

A: Histology of mice spleen fed with *L. monocytogenes* and bitter leaf extract combination. Section of the spleen shows prominent lymphoid follicles with centrally to eccentrically located blood vessels (BV). The follicles (white pulp) (wp) consist of aggregates of lymphocytes. The red pulps (RP) are prominent and shows a normal configuration. Congested dilated blood vessels are present.

B: Histology of mice spleen fed with *L. monocytogenes* and guava leaf extract combination. Section of the spleen shows prominent loose lymphoid follicles with centrally to eccentrically located blood vessels. The follicles (white pulp) (WP) consist of aggregates of lymphocytes. The red pulps (RP) are prominent and shows a normal configuration.

C: Histology of mice spleen fed with *L. monocytogenes* and *D. edulis* leaf extract. Section of the spleen shows prominent lymphoid follicles with centrally to eccentrically located blood vessels (BV). The follicles (white pulp) consist of aggregates of lymphocytes. The red pulps are prominent and shows a normal configuration.

D: Histology of mice spleen fed with normal diet and water. Normal splenic architecture is shown in the histology

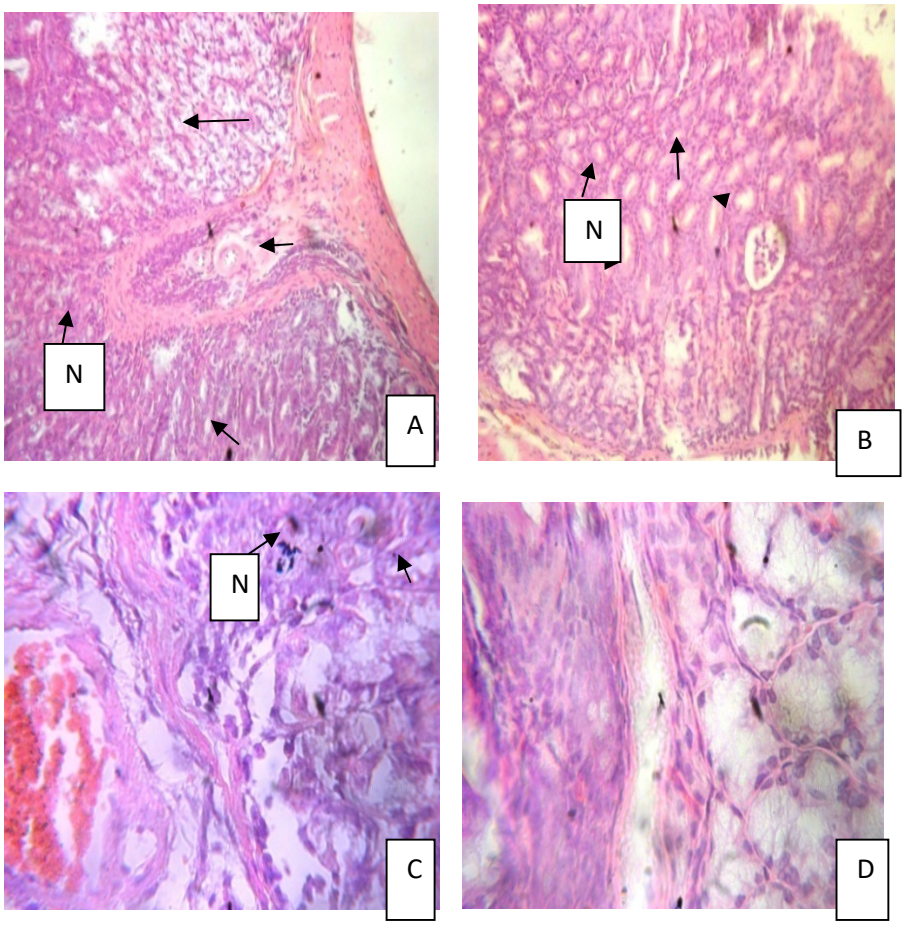


Plate 4.10: Histology of mice stomach treated and untreated with 10^7cfu/ml *L. monocytogenes* and 0.8g/kg aqueous leaf extract combination

A: Histology of mice stomach fed with *L. monocytogenes* and bitter leaf extract combination. Stomach histology revealed mild neutrophilic infiltration (N) in the gastric mucosa, submucosa, and lamina muscularis of the non glandular region of the stomach

B: Histology of mice stomach fed with *L. monocytogenes* and guava leaf extract combination. Stomach histology revealed mild neutrophilic infiltration (N) in the gastric mucosa, submucosa, and lamina muscularis of the non glandular region of the stomach

C: Histology of mice stomach fed with *L. monocytogenes* and *D. edulis* leaf extract. Inflammation of the fundic mucosa alleviated, but infiltration of macrophages and lymphocytes as well as neutrophils (N) was persistently observed in submucosa and lamina muscularis and fundus.

D: Histology of mice stomach fed with normal diet and water No neutrophilic infiltration was observed in the gastric mucosa, submucosa, and lamina muscularis.

Plate 4.10 shows the histology of mice liver treated and untreated with 10^7cfu/mL *L. monocytogenes* and different concentrations of aqueous bitter leaf extract combination. Histology of mice liver fed with *L. monocytogenes* and 0.6g/kg bitter leaf extract combination reveals mild vascular erosion around the central vein while the histology of mice liver fed with *L. monocytogenes* and 0.8g/kg bitter leaf extract combination revealed visible centrioles with well fenestrated sinusoidal space with mild kupfer cell activation. The histology of mice liver fed with normal diet and water revealed normal liver architecture with prominent central vein, sinusiodal space and hepatocytes.

The histology of mice spleen fed with *L. monocytogenes* and 0.6g/kg bitter leaf extract combination reveals prominent lymphoid follicles with centrally to eccentrically located blood vessels. The histology of mice spleen fed with *L. monocytogenes* and 0.8g/kg bitter leaf extract combination shows prominent red pulps and a normal configuration while the histology of mice spleen fed with *L. monocytogenes* and 1g/kg bitter leaf extract combination reveals enlarged lymphoid follicles in the white pulp with reactive terminal centres containing scanty lymphocytes and tangible body macrophages surrounded by a darker mantle zone (Plate 4.11)

Histology of mice stomach fed with *L. monocytogenes* and 0.6g/kg bitter leaf extract combination showed thick mucosa with fundic gland mucosa and noticeable neutrophillic infiltrates while the histology of mice stomach fed with *L. monocytogenes* and 0.8g/kg bitter leaf extract combination revealed mild neutrophilic infiltration in the gastric mucosa, submucosa, and lamina muscularis of the non glandular region of the stomach (Plate 4.12). There was reduced neutrophilic infiltration in the mucosa of fundus and the submucosa or lamina muscularis of non glandular region, fundus, and pylorus when fed with *L. monocytogenes* and 1g/kg bitter leaf extract combination.

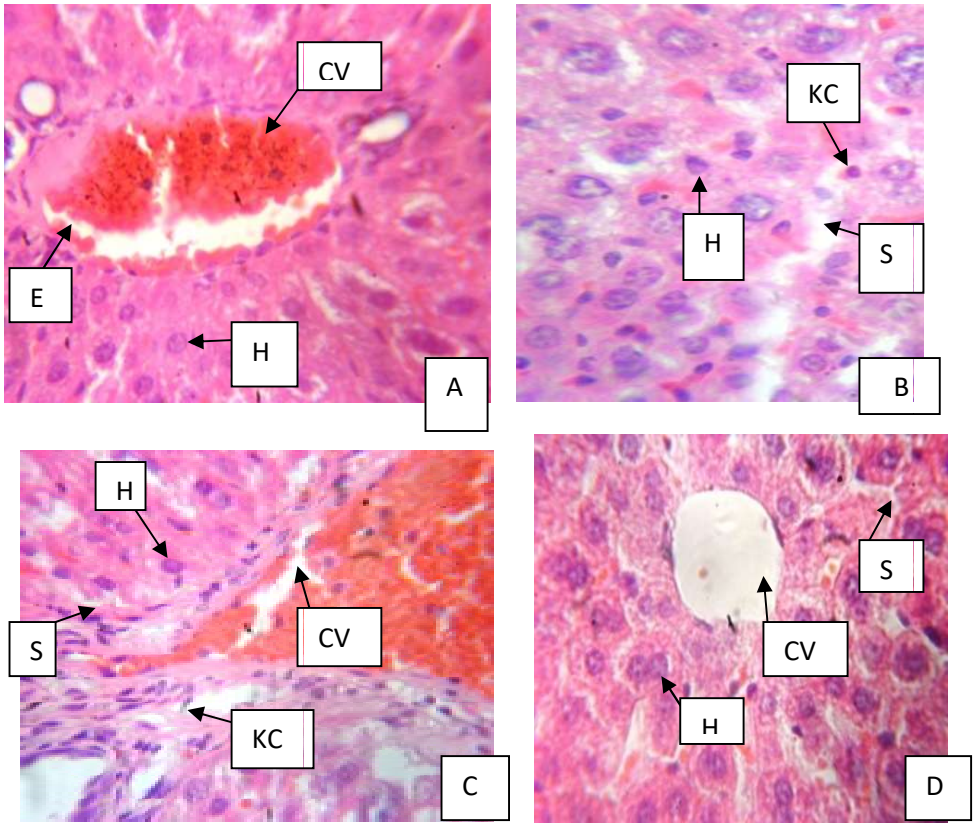


Plate 4.11: Histology of mice liver treated and untreated with 10^7cfu/ml *L. monocytogenes* and different concentrations of aqueous bitter leaf extract combination

A: Histology of mice liver fed with *L. monocytogenes* and 0.6g/kg bitter leaf extract combination. Histology reveals mild vascular erosion (E) around the central vein (CV)

B: Histology of mice liver fed with *L. monocytogenes* and 0.8g/kg bitter leaf extract combination. Histology reveals visible centrioles with well fenestrated sinusoidal space (S). The hepatocytes (H) appear distinct with mild kupfer cell activation (KC).

C: Histology of mice liver fed with *L. monocytogenes* and 1g/kg bitter leaf extract combination. Histology reveals Liver histology reveals central vein (CV), sinusoids (S) and hepatocytes with mild kupfer cell activation (KC)

D: Histology of mice liver fed with normal diet and water. Normal liver architecture with prominent central vein (CV), sinusiodal space (S) and hepatocytes (H).

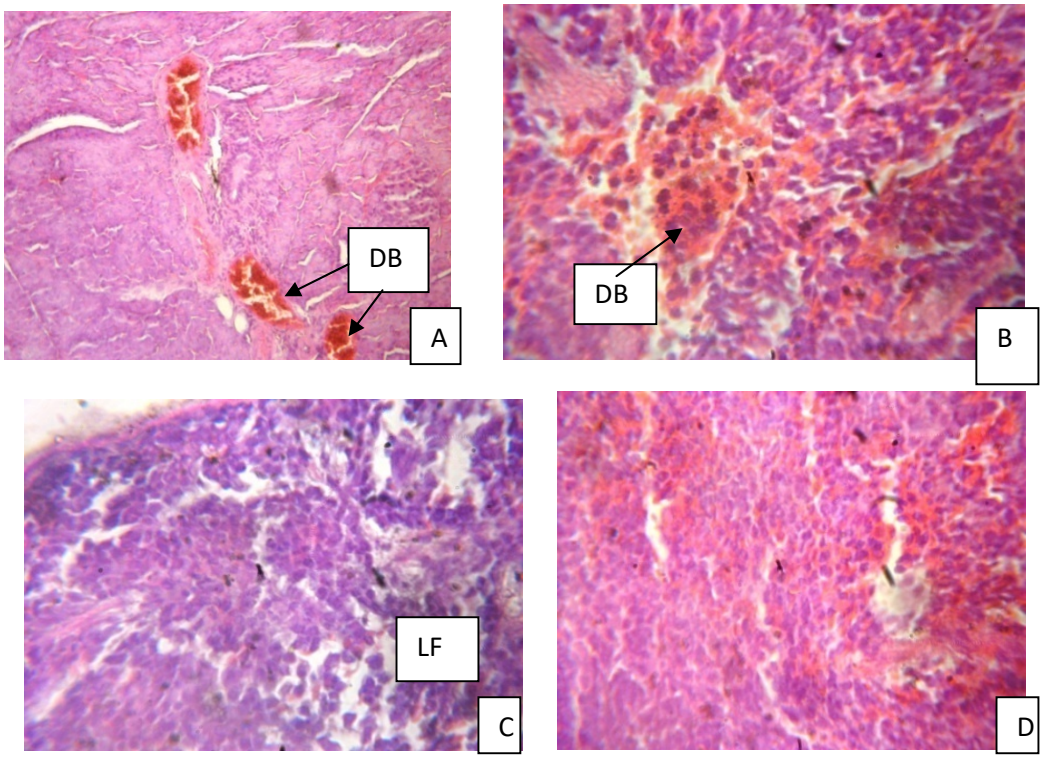


Plate 4.12: Histology of mice spleen treated and untreated with 10^7cfu/ml *L. monocytogenes* and different concentrations of aqueous bitter leaf extract combination

A: Histology of mice spleen fed with *L. monocytogenes* and 0.6g/kg bitter leaf extract combination. Section of the spleen shows prominent lymphoid follicles with centrally to eccentrically located blood vessels. The follicles (white pulp) consist of aggregates of lymphocytes. The red pulps are prominent and shows a normal configuration. Congested dilated blood (DB) vessels are present

B: Histology of mice spleen fed with *L. monocytogenes* and 0.8g/kg bitter leaf extract combination. Section of the spleen shows prominent lymphoid follicles with centrally to eccentrically located blood vessels. The follicles (white pulp) consist of aggregates of lymphocytes. The red pulps are prominent and shows a normal configuration. Congested dilated blood vessels (DB) are present.

C: Histology of mice spleen fed with *L. monocytogenes* and 1g/kg bitter leaf extract combination Spleen histology reveals enlarged lymphoid follicles in the white pulp with reactive terminal centres containing scanty lymphocytes and tangible body macrophages surrounded by a darker mantle zone.

D: Histology of mice spleen fed with normal diet and water. Normal splenic architecture is shown in the histology

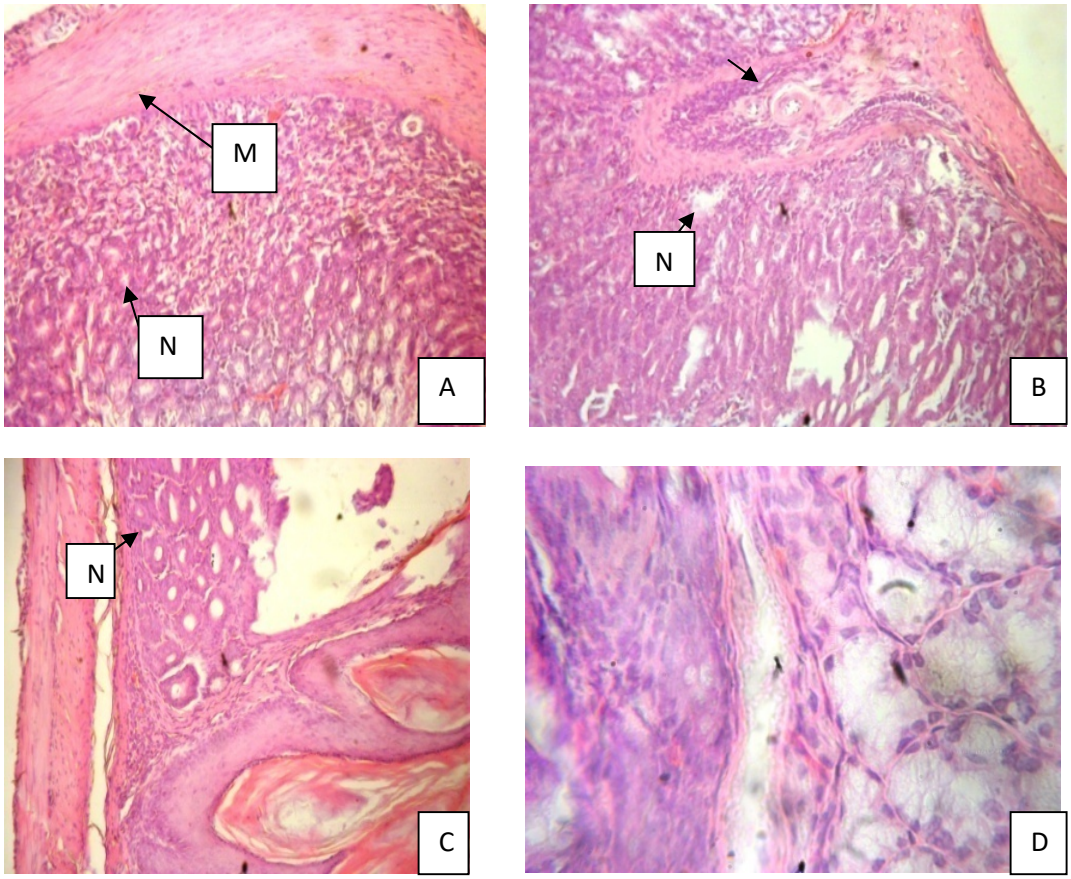


Plate 4.13: Histology of mice stomach treated and untreated with 10^7cfu/ml *L. monocytogenes* and different concentrations of aqueous bitter leaf extract combination

A: Histology of mice stomach fed with *L. monocytogenes* and 0.6g/kg bitter leaf extract combination. Histology shows thick mucosa (M) with fundic gland mucosa. The thick gastric pit into the mucosal with noticeable neutrophillic infiltrates

B: Histology of mice stomach fed with *L. monocytogenes* and 0.8g/kg bitter leaf extract combination. Stomach histology reveals mild neutrophilic infiltration in the gastric mucosa, submucosa, and lamina muscularis of the non glandular region of the stomach

C: Histology of mice stomach fed with *L. monocytogenes* and 1g/kg bitter leaf extract combination. Histology reveals marked reduced neutrophilic infiltration in the mucosa of fundus and the submucosa or lamina muscularis of non glandular region, fundus, and pylorus

D: Histology of mice stomach fed with normal diet and water No neutrophilic infiltration was observed in the gastric mucosa, submucosa, and lamina muscularis.

The comparative diffeences in alanine aminotransaminase concentration in albino mice fed with different aqeous leaf extract and *L. monocytogenes* rations LMEW94 rations on different days is shown in Table 4.16. Highest mean value was observed with *L. monocytogenes* alone with ALT concentration of 78.02 ± 5.87 on the 2nd day, 88.08 ± 3.22 on the 4th day, 88.01 ± 0.00 on the 7th day and mean ALT concentration of 84.34 ±12.01. 0.6g/kg bitter leaf with *L. monocytogenes* combination had mean ALT concentration of 26.33 ± 9.20 while 0.8g/kg *D. edulis* leaf with *L. monocytogenes* combination had mean ALT concentration of 38.30 ± 10.21. 18g/kg *D. edulis* leaf with *L. monocytogenes* combination had ALT 53.05 ± 0.66, 41.02 ± 4.56 and 25.10 ± 3.00 on the 2nd, 4th and 7th day respectively.

Comparative diffeences in alkaline phosphatase concentration in albino mice fed with different aqeous leaf extract and *L. monocytogenes* LMEW94 rations on different days is shown in table 4.17. 0.6g/kg *D. edulis* leaf with *L. monocytogenes* ration had ALP concentration of 176.64 ± 3.03, 74.52 ± 0.00 and 154.42 ± 2.00 on the 2nd, 4th and 7th days respectively. 0.8g/kg bitter leaf with *L. monocytogenes* ration had mean ALP concentration of 248.40 ± 0.00, 135.24± 3.33 and 132.56 ± 6.05 on the 2nd, 4th and 7th day respectively while 1g/kg guava leaf with *L. monocytogenes* ration had mean ALP concentration of 274.08 ± 0.08, 151.80 ± 9.04 and 176.56 ± 1.09 on the 2nd, 4th and 7th day respectively.

Table 4.16: Comparative differences in alanine aminotransferase concentration in albino mice fed with different aqueous leaf extract and *L. monocytogenes* LMEW94 rations on different days

		Alanine aminotransferase (ALT) (U/L)			
Groups	Combination of groups	2nd day	4th day	7th day	MEAN ± SE
group 1	0.6g/kg bitter leaf+ *L.monocytogenes*	21.65 ± 0.07*	12.25 ± 1.50	43.10 ± 0.00	26.33 ± 9.20[a]↑
group 2	0.8g/kg bitter leaf + *L.monocytogenes*	28. 56 ± 4.56	20.04 ± 6.00	17.40 ± 2.33	22.00 ± 0.33 [a]
group 3	1g/kg bitter leaf + *L.monocytogenes*	37.05 ± 4.44	25.50 ± 3.70	20.10 ± 1.07	27.55 ± 12.41
group 4	0.6g/kg guava leaf + *L.monocytogenes*	17.01 ± 7.54	48.06 ± 5.03	33.04 ±1.56	32.77 ±8.95
group 5	0.8g/kg guava leaf + *L.monocytogenes*	17.20 ± 0.77	14.09 ± 4.02	15.01± 0.00	15.63 ±0.88[a]
group 6	1g/kg guava leaf + *L.monocytogenes*	8.04 ± 7.07	21.00 ± 0.00	12.05 ± 0.00	13.75 ±3.84[a]
group 7	0.6g/kg *D. edulis* leaf + *L.monocytogenes*	21.09 ± 3.35	52.06 ± 3.05	33.04 ± 2.02	35.52 ±9.02[b]
group 8	0.8g/kg *D. edulis* leaf + *L.monocytogenes*	57.04 ± 2.07	35.21 ± 8.56	22.05 ± 0.00	38.30 ±10.21[b]
group 9	1g/kg *D. edulis* leaf + *L.monocytogenes*	53.05 ± 0.66	41.02 ± 4.56	25.10 ± 3.00	39.72 ±16.86[b]
group 10	*L.monocytogenes*	78.02 ± 5.87	88.08 ± 3.22	88.01 ± 0.00	84.36 ±12.01[c]
group 14	Negative control	54.03 ± 0.25	45.04 ± 0.07	34.00 ± 1.33	44.40 ±5.78[b]

*Each value is a Mean of triplicate determinations ± Standard Error; ↑Means with the same alphabet is significant at 0.05

Table 4.17: Comparative diffeences in alkaline phosphatase concentration in albino mice fed with different aqeous leaf extract and *L.monocytogenes* LMEW94 rations on different days

		Alkaline Phosphatase (ALP) (U/L)			
Groups	Combination of groups	2nd day	4th day	7th day	MEAN ± SE
group 1	0.6g/kg bitter leaf+ *L.monocytogenes*	121.44 ± 2.56	215.28 ± 7.07*	150.64 ± 5.00	162.45 ± 4.86[a]↑
group 2	0.8g/kg bitter leaf + *L.monocytogenes*	248.40 ± 0.00	135.24± 3.33	132.56 ± 6.05	172.06 ± 3.13[a]
group 3	1g/kg bitter leaf + *L.monocytogenes*	245.80 ± 7.77	124.84 ± 3.56	111.04 ± 3.26	160.56 ± 2.53[a]
group 4	0.6g/kg guava leaf + *L.monocytogenes*	267.08 ± 7.08	124.84 ± 0.00	127.60 ± 1.86	173.17 ± 2.98[a]
group 5	0.8g/kg guava leaf + *L.monocytogenes*	250.52 ± 0.57	107.64 ± 3.19	163.40 ± 5.29	173.74 ± 3.01[a]
group 6	1g/kg guava leaf + *L.monocytogenes*	274.08 ± 0.08	151.80 ± 9.04	176.56 ± 1.09	200.81 ± 3.40[a]
group 7	0.6g/kg *D. edulis* leaf + *L.monocytogenes*	176.64 ± 3.03	74.52 ± 0.00	154.42 ± 2.00	135.19 ± 1.67[a]
group 8	0.8g/kg *D. edulis* leaf + *L.monocytogenes*	248.52 ± 8.03	200.36 ±7.75	124.23 ± 2.93	203.03 ± 6.23[a]
group 9	1g/kg *D. edulis* leaf + *L.monocytogenes*	247.24 ± 4.00	204.56 ± 3.25	167.7 ± 0.00	206.5 ± 2.43[a]
group 10	*L.monocytogenes*	295.96 ± 7.07	305.63 ± 3.56	323.45 ± 4.15	308.34 ± 4.92[b]
group 14	Negative control	256.68 ± 7.04	223.43 ± 2.25	143.45 ± 3.60	207.85 ±33.60[a]

*Each value is a Mean of triplicate determinations ± Standard Error; ↑Means with the same alphabet is significant at 0.05

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

L. monocytogenes are widely distributed in nature and members of these aerobic, non-spore forming bacilli have been recovered in several countries from different environments including food, water, soil, silage, animal fecal matter and sewage (Vaid *et al*., 2010). Following globalization, there is increasing consumption of ready-to-eat foods all over the world. It is astonishing that *L. monocytogenes* has been documented as an essential opportunistic human food-borne pathogen due to increased refrigeration use as primary preservation means, food production changes, processing, distribution, and changes in the eating behavior of people towards the ease of ready-to-eat foods (Vazquez-Boland *et al*., 2001a). In this study, a total of 90 *L. monocytogenes* were isolated and characterized from edible worms, salads, meat pies, and cooked spiced snails purchased from different vendors located at different parts of Nigeria's South South. This finding represents a major threat to human safety as these foods are relatively cheap and are consumed by many people of various ethnic groups making them prone to listerosis; an infection commonly associated with consumption of *L. monocytogenes* contaminated foods.

The presence of *Listeria monocytogenes* in food samples utilized in this research could be attributed to poor sanitary conditions of the food vendors, the use of unclean utensils, unhygienic conditions of the production area and the nature and source of water supplied for washing. *Listeria monocytogenes* is likely introduced into food production facilities due to their inherent properties that allow them to survive and effectively compete with other microbes in food processing environments. They have the ability to grow at low pH, refrigeration temperatures, areresistant to freezing, high salt concentrations and can adapt to stresses that exist at times in food production facilities (Lou and Ousef, 1999).

Moreover, the tropical weather in Nigeria is warm and moist all year round and this favours the growth of *Listeria* in foods since most foods are sold openly especially ready-to-eat (RTE) foods that are sometimes hawked. Environmental ecology of *Listeria* in Nigeria have been reported from soil, lakes, veterinary surgical instruments (Tambuwal *et al*., 2009), Naira notes (Kawo *et al*., 2009), kilishi, a sun-dried beef (Moshood and TengkuHaziyamin, 2013), kunu, a fermented drink (Nwachukwu *et al*., 2009), wara, a soft cheese prepared from coagulating fresh cows' milk (Adetunji and Adegoke, 2008), vegetables including salads (Pondei and Ogbonna, 2004) and frozen poultry (Adetunji and Odetokun, 2012).

Snails are consumed by the average and wealthy Nigerians as part of a meal or as snacks. Snails are mainly consumed because they have been found to contain low fat. The results of this work revealed *L. monocytogenes* was found in snail samples and these *L. monocytogenes* are known to cause gastro-intestinal infections (Ajayi *et. al*., 2009). Contamination of snails can occur from the spices used for preparation, handling and from the intestinal tract of the snail during preparation. Although *L. monocytogenes* was not isolated from snails in previous work, other bacteria of public health importance have been isolated from snails. Adagbada *et al*. (2011) isolated *Staphylococcus aureus, Escherichia coli, Aeromonas* sp, *Vibrio* sp and *Pseudomonas* sp from *Achatina* species in Cross River and Akwa Ibom states of Nigeria. Adegoke *et al*. (2010) also isolated *Staphylococcus aureus* and *Escherichia coli* from different species of snails (*Achatina fulica*, *Limcolaria* species and *Helix pomatia)* and Agbonlahor *et al.* (1994) recorded the occurrence of *Proteus* sp (10.4%), *Escherichia coli* (5.7%), *Pseudomonas aeruginosa* (4.2%), *Salmonella* spp (0.3%) and *Yersinia* spp (0.6%) from snails.

Salads contain raw vegetables which are usually consumed with salad cream or mayonnaise. The vegetables used for preparation of salads can get contaminated from the soil during harvesting, transportation, handling, from surfaces, utensils, wash water used during their preparation and salad dressings which are usually high in nutrients. Vegetables for salad can also get contaminated from faecal droppings which are used as manure in most farmlands in Nigeria. David and Odeyemi (2007) reported the presence of *L. monocytogenes* in soils used for farming and attributed its presence to the faecal

dropping from farm animals. Most times these salads are refrigerated before sale to consumers and these refrigerators can be a potential source of Listerial contamination. Sergelidis *et al*. (1997) demonstrated that colonization of refrigerators by *L. monocytogenes* is a possible source of food contamination. McLauchlin (1996) also reported that foods known to be able to transmit *Listeria* have commonly been highly processed with increased shelf life at refrigeration temperatures. Also, majority of those foods are consumed without prior re-heating. Results from the current work are in conformity with the results of Jeyaletchumi *et al*. (2010) who isolated *L. monocytogenes* from salad vegetables, Kuan *et al*. (2017) who isolated *L. monocytogenes* from vegetable farms and Pinto *et al*. (2010) who reported a frequency of 27% of *L. monocytogenes* in mayonnaise in deli salads sold in Italy. Furthermore, Jamali *et al*. (2013) isolated *L. monocytogenes* from lettuce and *L. ivanovii* was isolated from balangu, cabbage and lettuce samples. The high occurrence of the *L. monocytogenes* in lettuce, a vegetable usually added to salads could perhaps be attributed to the fact that lettuce is close to ground, and it is regularly in contact with the farmland, soils and manure when growing (Nwachukwu *et al*., 2010). Monge and Arias-Echandi, (1999) reported cabbage as the base vegetable for the occurrence of *L. monocytogenes* in salads and attributed its presence to high levels of fermentable sugars such glucose which are readily utilized by *L. monocytogenes* (Dogbe, 2010).

Edible worms are very proteineous and nutritious snacks. These worms are smoked, dried or fried, staked, and then wrapped in nylon before been displayed at different busy points for purchase. Edible worms may get contaminated during or after processing, transportation and sales. They may also get contaminated during handling by the vendors at sales point. Other bacteria of public health importance aside from *L. monocytogenes* have been isolated. Ikenebomeh and Elohor (2005) reported the isolation of *S. auerus*, *B. cereus* and *E. aerogenes* from roasted and fresh edible worms (*Rhynchophorus phoenics*) larvae collected from 5 locations in Delta and Edo states of Nigeria. Ekrakene and Igeleke (2007) also isolated *Staphylococcus aureus* (100%), *Bacillus cereus* (30%), *Escherichia coli* (20%), *Enterococcus faecalis* (45%) and *Pseudomononas aeruginosa* (35%) from roasted larva of the palm weevil *Rhynchophorus phoenicis* [F] from Edo and Delta States of Nigeria.

Meat pies are snacks that are commonly consumed in Nigeria. They range in sizes, price and richness in composition. Most of them contain mashed potatoes, mashed meat and spices. *L. monocytogenes* may contaminate meat pies if they are not baked properly at sufficient temperatures. Wong *et al*. (2011) observed that *L. monocytogenes* was not detected after 6 min cooking of chicken burger patties but *L. monocytogenes* was found when the cooking was done in 4 min. Also, contamination may arise from the meat used for preparation, and from handling and exposure at sales points. This finding is supported by Morobe *et al*. (2009) who reported the presence of *L. monocytogenes* in meat pies (1.8%) and Ahmed *et al*. (2017) who also reported the presence of *L. monocytogenes* in minced meat used for meat pies

The cultural and biochemical properties of the isolates agreed with the Bergey's Manual of Systematic Bacteriology. Survival on simple laboratory medium implied the isolates being chemoheterotrophs which became metabolically active and versatile using up a variety of simple organic compounds. The use of selective medium for *Listeria* sp. isolation in this research was to eliminate other bacteria that are found in the same food environment thereby making the isolation easier.

In this study, *L. monocytogenes* isolates were observed to be capable of growing between -10°C- 70°C. The ability to grow at low and high temperatures demonstrates their ability to adapt to seasonal weather changes an indicator they would be present all year round in environments and in foods. Their ability to grow at low temperatures indicates that they would be able to contaminate foods held at refrigeration temperatures while their ability to grow between 45°C- 70°C also indicates that if foods are not sufficiently heated, *L. monocytogenes* would be present in the final products. Similar report has been observed by Lado and Yousef, (2007) who observed that -1.5 and 45°C temperature range supported the growth of *L. monocytogenes*, with 30–37°C as the optimal temperature while above 50°C was fatal to *L. monocytogenes*.

L. monocytogenes in this study were were able to grow between pH 2 and pH 9. The ability to grow in both acidic and alkaline has been reported by several authors. *L. monocytogenes* strains have been reported to possess an arginine deiminase (ADI) pathway and an agmantine (AgDI) deiminase system that contributes to pH homeostasis

(Ryan *et al*., 2008; Smith *et al*., 2013). This ADI system is not present in non-pathogenic species like *L. innocua* and the ADI system operates through the catabolism of arginine to produce ornithine and ammonium ions (NH) which protect the cells at low pH.

L. monocytogenes isolates in this study were capable of growing at bile concentration of between 0.2% and 0.5%. Bile may improve colonization of the human gastrointestinal tract (GI) by *L. monocytogenes* and may also be an important mechanism by which the bacterium can survive in the gallbladder. Bile has also been shown to affect various physiological and metabolic properties such as motility, invasion, biofilm formation and toxin production (Cormac *et al*., 2014). *L. monocytogenes* strains express a bile salt hydrolase (BSH), an enzyme that has the potential to detoxify individual conjugated bile acids and contributes to Listerial survival in the GI tract (Dussurget *et al*., 2002; Begley *et al*., 2005). *Listeria monocytogenes* cholecystitis (colonization of the gallbladder) has been reported, which suggests an inherent tolerance of very high levels of bile (Hardy *et al.*, 2004). The authors demonstrated that *L. monocytogenes* strain LO28 was able to tolerate concentrations of bovine, porcine and human bile well in excess of those encountered *in vivo*. Confluent growth was observed on agar plates supplemented with 15% oxgall, 15% bovine bile, 2% porcine bile or 15% human bile and Listerial cells could survive and even grow in broth supplemented with 30% oxgall (Begley *et al*., 2002). A research by Olier *et al*. (2004) observed the bile tolerance of fifty human asymptomatic carriage, clinical, food and environmental isolates of *L. monocytogenes* and demonstrated that all *L. monocytogenes* isolates could grow in broth supplemented with 5% porcine bile salts suggesting that *L. monocytogenes* strains are inherently bile resistant.

L. monocytogenes in this study were capable of growing at sodium chloride (NaCl) concentration between 2% and 10%. *Listeria monocytogenes* can tolerate sodium chloride stress by changing its gene expression leading to an increased or decreased synthesis of various proteins such as the Ctc protein (Duche *et al*., 2002). The Ctc protein is induced in response to salt stress (Duche *et al*., 2002). Similar findings by Hudson, (1992) reported *L. monocytogenes* survivival for 6 hours at 10°C in solutions containing 6, 16, or 26% NaCl and concluded that salt has protecting ability and it can partially protect *L. monocytogenes* from several stresses. Linton *et al*. (1995) reported a correlation between NaCl

concentration and heat resistance. They observed that increasing the concentration of NaCl from 0 to 4% in an inoculated infant formula proportionally increased the resistance of *L. monocytogenes* to heat. Other research has focused on the concentration of NaCl acting as a protective cell mechanism. Faleiro *et al*. (2003) observed that exposing *L. monocytogenes* to low NaCl levels protects the cell against acid shock up to pH 3.5.

L. monocytogenes isolates were observed to produce more protease compared with lipase and amylase in this study. Enzyme production is one of the mechanisms for survival as their ability to produce enzymes explains their survival and growth in various substrate-dependent environments. Protease production by *L. monocytogenes* has been reported by Shalinisen and Satyararayana (1993) and Shumi *et al*. (2004). Report have showed that 32 of 122 strains of *L. monocytogenes* gave positive result for esterase/lipase activity and the authors observed that there was no correlation between the enzyme activities and the serotype of the organisms tested (Shumi *et al*., 2004). Reports have also shown amylase production from *L. denitrificans* (Mahmud and Anwar, 2009).

L. monocytogenes isolates in this study was capable of adhering at varying degree to glass, steel, aluminum and wood. Biofilm production is a way that helps microorganism to survive and grow for an extended period of time (Swaminathan, 2001). The biofilm that is produced on the different surfaces of food processing lines and instruments can be transferred to food products thereby causing contamination to the final product. *L. monocytogenes* resistance can increase after formation of biofilm as the formation of biofilm makes the bacterium 1000 times more resistant to environment stresses, antimicrobials and disinfectants (Chapman, 2003). It has been reported that the physicochemical properties of the surface (E.g. nutrient composition, surface charge, pH hydrophobicity, composition of nutrient and surface temperature) can influence the formation of biofilms and consequently the adherence capacity of microorganisms (Chmielewski and Frank, 2006). Duncan-Hewitt (1990) reported that the hydrophobic effect of the surface may be the most important driving force for the adhesion of most pathogenic bacteria.

The level of biofilm produced by different microorganisms has been associated with the virulence characteristic of such isolates (Hood and Zottola, 1995; Gulsun *et al*., 2005).

Higher biofilm producers have been observed to be more virulent than lower biofilm producers. Similarly, Milanov *et al*. (2009) observed that *L. monocytogenes* showed attachment on glass surface regardless of strain. Chae and Schraft, (2000) and Chae *et al.* (2006) also observed similar attachment of *L*. *monocytogenes* cells on glass surfaces for 3 h incubation at 37°C. *L. monocytogenes* strains were reported to form biofilms on plastic surfaces (Djordjevic *et al*., 2002; Stepanovic *et al*., 2004; Tomicic *et al*., 2016). Truelstrup and Vogel, (2011) and Moltz and Martin, (2005) showed that *L. monocytogenes* produced biofilm on stainless steel coupons and Gamble and Muriana, (2007) observed that *L. monocytogenes* strains adhered equally well to glass, plastic, rubber, and stainless steel. All the strains showed a slight raise in the quantities of attached cells over 48 and 72hours and no reduction in the amount of biofilm was observed during testing hours in this study. Increase in biofilm formation with longer incubation period demonstrated in this study has also been reported by Moltz and Martin, (2005) in stainless steel chips. Also, significant difference ($P < 0.05$) in biofilm formation was observed between 24 and 48 h incubation period and between 24 and 72 h incubation periods for all the *Listeria* strains.

All *L. monocytogenes* isolated in this work were resistant to cloxacillin, augumetin, amoxicillin and ceftrizone but they exhibited varying degree of sensitivity to gentamycin, tetracycline, ofloxacin, pefloxacin, streptomycin, ciprofloxacin, erythromycin, cotrimozazole, nitrofuratin and ceftazidine. The resistance of *L. monocytogenes* strains in this study to common drugs of choice in Nigeria can be attributed to abuse of drugs use in animal husbandry, mutation and appearance of new strains (Adetunji and Adegoke, 2008). The results in this study are consistent with reports of emergence of resistance in *Listeria* sp. and the need for further antimicrobial studies to determine their resistance mechanisms and their potential as reservoirs of transferable resistance genes. The little resistance of *L. monocytogenes* to ciprofloxacin and gentamicin reported in this study is supported by Yakubu *et al*. (2012) and Rahimi *et al*. (2012) who reported over 80.0% susceptiblity of different *L. monocytogenes* to several antimicrobial agents. There are, however, increasing reports of *Listeria* strains isolated from various sources being resistant to penicillin, ampicillin, tetracycline, streptomycin, clindamycin, oxacillin and vancomycin. Research has recorded up to 92.9% resistance to ampicillin (Issa *et al*., 2011) and Yakubu *et al*.

(2012) reported 100% ampicllin resistance from *L. monocytogenes* isolated from processed meats and milk.

Although all strains of *L. monocytogenes* from this study were augumentin resistant, Umoh *et al*. (1990) reported all strains as augumentin-susceptible suggesting that genetic differences may occur within the strains of *L. monocytogenes*. According to Heger *et al*. (1997) and Troxler *et al*. (2000), newer generations of cephalosporins, like ceftriaxone, had no *in vitro* effect on *L. monocytogenes* and attributed the resistance to the lack of specific penicillin-binding proteins 3 and 5 in the *Listeria* cytoplasmic membrane. Moreover, resistance to tetracycline and ciprofloxacin has been reported in *L. monocytogenes* isolated from foods (Walsh *et al*., 2001; Navratilova *et al*., 2004). The acquired resistance to ciprofloxacin may be due to the acquisition of the *Listeria* drug efflux (Lde) gene that could pump the drug out of the cell before its concentration is high enough to be bactericidal (Godreuil *et al*., 2003).

Resistance to several antibiotics was observed in this study with 100.0% of the isolates observed to be resistant to more than two antimicrobial agents and 64.3% of the isolates were resistant to more than four antimicrobial agents. This multidrug resistance observed is supported by Yakubu *et al*. (2012) whose dairy isolates of *L. monocytogenes* was resistant to more than two antimicrobial with 20% of the isolates multidrug resistant. Lotfollahi *et al*. (2011) also reported multidrug resistance in *L. monocytogenes* isolated from human urine, rectal swab, vaginal swab, blood, and placental bit.

The *iap* gene has been demonstrated to be a reliable PCR target for differentiation of *Listeria* spp. Similar use of iap genes has been reported by (Bubert *et al*., 1999; Wagner *et al*., 2000; Usman *et al*., 2016). Bubert *et al*. (1992) characterized the *iap* gene from different *Listeria* spp. and demonstrated the presence of conserved regions at the 5′ and 3′ ends and a species-specific internal region. The ‘iap’ gene of *L. monocytogenes* encodes the major extracellular protein (P60), which has been shown to be basically an essential murein hydrolase required for adherence/invasion of the organism to the targeted eukaryotic cell (Wisniewski and Bielecki, 1999). It has been shown that the corresponding iap gene portion is also hypervariable in length in different isolates belonging to the same

serotypes, thereby, can help in identification of different strains of *L. monocytogenes* (Klein and Juneja, 1997).

InlA, also known as Internalin is essential for *L.monocytogenes* entry into the human intestinal epithelial cell line (Gaillard *et al.*, 1991). InlA plays a fundamental role in the invasion of *L. monocytogenes* into many cells. It induces entry by binding host cell adhesion molecule ecadherin, which is predominantly localised at the basolateral surface of enterocytes and is briefly exposed at the surface of extrusion of apoptic cells (Ireton, 2007).

There is evidence which confirms that LLO (listeriosin) is the primary virulence factor associated with *L. monocytogenes* infection, in particular escape from the primary phagosome (Kayal and Charbit, 2006). LLO is responsible for escape from the primary phagosome formed by initial internalisation event, also the double membrane secondary phagosome associated with cell to cell infection (Schnupf and Portnoy, 2007). These findings commensurate with the published work for detection of LLO in *L. monocytogenes* (Paziak-Domanska *et al*., 1999; Quendera *et al*., 2016).

Plasmid profile showed that the isolates carried plasmid in this study and the presence of the plasmid could be the reason for multi resistance to antibiotics observed in this work. Plasmids in *Listeria* species have been studied extensively (Lebrun *et al*., 1992) and plasmids of different sizes, which encode a few or many genes, have been isolated from various *Listeria* species (Peterkin *et al*., 1992). Akya *et al.* (2012) reported that plasmid carriage was independent of bacterial serotype and suggested that large plasmids may not be important for virulence and persistent infections. The observed plasmid reported in this study is in conformity with the reports of Lebrun *et al*. (1992) who tested *L. monocytogenes* strains and observed that 28% were found to harbor 32, 43, 81 and 106-kb plasmids of which one strain harbored two plasmids. Also, Enujiugba (2009) reported the presence of plasmids of molecular sizes ranging from 1.4kbp to 4.5kbp among the acute diarrhoea causing bacteria species which were resistant to ampicillin, chlramphenicol and tetracycline.

For tetracycline resistance in this study, 15 (50%) of the isolates carried tetA while 10 (33.30%) carried tetM genes. tetM is known to confer resistance by ribosomal protection, while tetA confer resistance by efflux (Charpentier and Courvalin, 1999). Similar report has revealed the presence of tetM and tetA genes in 91.7% and 83.3% respectively in *Listeria* that are tetracycline resistant (Jamali *et al*., 2015). The prevalence rate of the tetM and tetA, in the present study also agrees with the reports of Poyart- salmeron *et al*., 1992; Charpentier *et al*., 1995; Charpentier and Courvalin, 1999 and Pourshaban *et al*., 2002 who reported high frequency of tetracycline resistance genes from several strains of *L. monocytogenes*

Daptomycine resistant genes were also detected in this study. This findings agrees with the reports of Moreno *et al*. (2014) where the only resistance genes that were detected in *L. monocytogenes* were related to fluoroquinolone and daptomycin resistance. Daptomycin resistance has already been described in *Staphylococcus* spp. and *Enterococcus* spp. to involve four genes (including *mprF*) that are homologs in *L. monocytogenes* (Palmer *et al*., 2011). Although few studies have tested *Listeria* for susceptibility to daptomycin, clinical isolates of *L. monocytogenes* has been reported to demonstrate a high daptomycin MIC (4 mg/L).

Erythromycin resistant genes were also detected in this study. Similar research by Roberts *et al*. (1996) reported a unique isolate resistant to erythromycin and carrying an *erm*(C) gene. In Listeria spp., two genes for resistance to macrolide-lincosamide-streptogramin B (MLS_B), *erm*(B) and *erm*(C), have been reported (Charpentier and Courvalin, 1999). These *erm* (*e*rythromycin *r*ibosome *m*ethylase) genes encode methyltransferases that modify 23S rRNA in bacteria. Srinivasan *et al.* (2005) reported their inability to detect genes responsible for erythromycin resistance in 38 *Listeria* isolates from dairy farms in spite of observed phenotypic resistance to the antibiotic.

In this current study, all observed phenotypic antibiotics resistance profiles of the *L. monocytogenes* isolated were not confirmed by detection of corresponding antibiotics resistance genes. For instance, 30(33.3 %) isolated *L. monocytogenes* were resistant to tetracycline phenotypically, however, tetA resistance gene was only detected in fifteen

(15) of these isolates (50%). Similar results have been reported by Srinivasan *et al*. (2005); Jamali *et al*. (2015). This discrepancy in phenotypic and genotypic expression of antibiotics resistance suggests that mutation in ribosomal protein gene or decreased outer membrane permeability can have effect on antimicrobial resistance phenotypes (Farmer *et al*., 1992). The observations generally suggest that the presence of antimicrobial resistance genes in Listerial isolates do not always correlate with phenotypic antibiotic resistance, and indicates that other mechanisms such as decreased outer membrane permeability, activation of efflux pump, or mutation in a ribosomal protein may have contributed to the antimicrobial resistance phenotypes observed in this study (Charpentier and Courvalin, 1999) .

Mice were chosen as the animal model in this study because of its similarities to humans in disease manifestations. The inability of the neutrophiles to kill the *L. monocytogenes* injected to the mice in this study does not relate to poor ingestion of the organism by the macrophage, but neutralizing properties of *L. monocytogenes* to all defense mechanisms of the macrophages with the aid of listeriosin O (LLO) and other virulent genes that makes the organism evade the immune system (Weiss and Schaible, 2015). Histopathology of mice in this study showed that *L. monocytogenes* strains had effect on the liver and spleen with vaculation of the hepatocytes and giant cells containing multiple nuclei in spleen. Similar study has shown that histopathological changes were observed in the liver, heart and the kidneys. The authors explained that the liver was mostly affected with mild degeneration of the hepatocytes (Ikeh *et al.*, 2010). Though no effect was seen in the heart and kidney of mice in this study, Ikeh *et al*. (2010) reported mild pericarditis in the region of the coronary groove of the infected mice heart and frank hemorrhage and occasional mononuclear infiltration of the cortical interstitium of the kidney. Kennedy and Miller, (1992) also showed coagulative necrosis and infiltration by macrophages and neutrophils in the infected mice organs and observed that majority of *L. monocytogenes* isolates were virulent both in mice with LD_{50} varying from $10^{3.86}$ to $10^{6.74}$ and in embryonated eggs with LD_{50} varying from $10^{1.23}$ to $10^{3.35}$

Herbal medicines are valuable as alternatives in the primary and complementary health care system. Unfortunately, there are diverse medicinal plants in the environment

that their inhibitory activities are yet to be discovered. Although, a lot of plants are continuously screened for their antimicrobial effects, the main factors that determine the antimicrobial activity are the type and combination of plant phytochemicals, quantity used, pH, type of microorganism, and temperature of the environment (Gull *et al*., 2012). The solvent used to extract the components of the medicinal plant also affect antimicrobial activity of the extract (Niranjan *et al*., 2013). Although several reports had been published that describe the antibacterial properties of different herbs and spices (Gull *et al*., 2012) only a few has focused on antilisterial activity. The antilisteria activity observed in this study could attributed to the presence of secondary metabolites like, tannins, saponins, terpenes and flavonoids which were detected and have previously been reported to have antibacterial activity. Omoya and Akharaiyi, (2012) reported zone of inhibition of ethanol and methanol extracts of *Z. officinale* against different bacteria of public health importance. *Heinsia crinita* which had no antilisteria activity in this study was also reported by Enyi-Idoh *et al*. (2013) having no zone of inhibition on *Salmonella typhii. P. gujava* leaf extract was reported to have invitro activity against *L. monocytogenes* with 70% aqueous extract of *P. guajava* leaf having 25mm*;* methanol extract of *P. guajava* leaf had 30mm*;* ethylacetate extract of *P. guajava leaf* had 27mm and hexane extract of *P. guajava* leaf *had no* in-vitro effect (Omoregie *et al*., 2010).

Vuddhakul *et al*. (2007) observed that garlic extracts inhibited the growth of *V. parahaemolyticus*, *E. coli* and *S. aureus.* Although *Trema orientalis* had no antilisterial activity in this study, crude methanol extract of *Trema orientalis* has been observed to have antimicrobial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Plesiomonas shigelloides*, *Shigella dysenteriae*, and *Vibrio cholerae* while the aqueous extract showed antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Vibro cholerae*, *Shigella flexner*, *Shigella sonnei* and *Pseudomonas aeruginosa* (Sarder, 2008). There are reports on the antibacterial activities of *Dacryodes edulis* against coliforms, *Escherichia coli, Salmonella typhi, Staphyloccocus aureus* and *Streptoccocus faecalis* with zone of inhibition of 30mm, 35mm, 32mm, 34mm and 35mm respectively (Nwokonkwo, 2014**).** Furthermore, *Citrus aurantifolia* has been reported to have antimicrobial activities against *Klebsiella pneumoniae*, *Pseudomonas sp*, *Aspergillus. niger*, *Aspergillus fumigatus* and *Mucor sp*.

L. monocytogenes in this study was observed to be susceptible to common disinfectants/antiseptics at varying concentration tested. The antimicrobial properties of chloroxylenol, the main chemical constituent of dettol and other chlorinated phenols have been extensively studied. The antimicrobial effectiveness of sodium hypochlorite which is the main component of bleach is based on its high pH (hydroxyl ions action) which interferes in cytoplasmic membrane integrity with irreversible enzymatic inhibition, biosynthetic alterations in cell metabolism and phospholipid destruction observed in lipidic peroxidation (Estrela *et al*., 1995). Estrela *et al*. (2002) reported that the minimum concentration of 1% NaCl is required for inhibiting *B. subtilis, S. aureus, E. faecalis* and *P. aeruginosa*, while *C. albicans* was inhibited at 0.1% NaCl. Similar reports has shown Darasan 214 to be effective in reducing the number of *L. monocytogenes* cells in suspension and attributed its antilisterial activity to the presence of quaternary ammonium compounds, which have been found to be most effective in destroying freely suspended cells. Furthermore, Darasan 7058 whose main component is chlorine was also observed to be effective against *L. monocytogenes* as chlorine damages the cell membrane resulting in leakage of cell components (Earnshaw and Lawrence, 1998). Chlorine also forms substitution products with proteins and amino acids, and has been reported to be efficient in reducing the number of free-living, unattached cells of *L. monocytogenes* (Tuncan, 1993). The antimicrobial properties of Dettol, Izal, Z-germicide, Jik, have also been described by El-Mahmood and Doughari, (2009); Thomas *et al*. (2012); Otokunefor and Usoh, (2009) and Iruoha *et al*. (2011).

Data has been accumulated on the phamacological properties of different leaf extracts at different concentrations. Some research has focused on the hepatoprotective nature of leaf extacts using albino mice or rat model. Kupfer cells were observed in the photomicrograph *L. monocytogenes* and the leaves (bitter leaf, grava leaf and *D. edulis* leaves) used in this study. Kupfer cells constitute 80–90% of the tissue macrophages present in the body (Manfred *et al.*, 2006). They reside within the lumen of the liver sinosoids and are therefore constantly exposed to the gut- derived bacteria, microbial toxins and bacteria endotoxins known to activate macrophage. Upon activation, kupfer cells release various products including cytokines, nitric oxide, prostanoides and reactive

oxygen species (Bilzer *et al*., 2006). Therefore, kupfer cells seen in these photomicrograph may intimately be involved in the liver's response to *L. monocytogenes* cells or its toxins.

Similar research by Mebratu *et al.* (2013) demonstrated that the treatment of mice at 400 and 800mg/kg of aqueous leaf extract of *V. bipontini* did not alter hematological and some biochemical composition of blood. The findings in this research are also supported by previous research by Nwanjo (2005) who demonstrated that treatment with aqueous *V. amygdalina* extract did not cause any pathologic lesions in the liver and kidneys even at 800mg/kg and attributed the absence of any significant effect on the organs was due to absence of cyanogenic glycoside in *V. amygdalina* leaf which is usually responsible for histopathological changes. Amole *et al*. (2006) further showed that microscopic observation of the tissue sections of liver and kidney showed no morphological abnormalities as compared to the controls after chronic oral administration of aqueous *V. amygdalina* leaf extract.

The lamina portion of the normal gastric mucosa may contain a few scattered neutrophils. However, infiltration of the epithelium by neutrophils represents a pathologic tissue response and is considered an active component of gastritis. Moreover, neutrophils play a negative role in normal tissue repair as they produce bioactive substances capable of accelerating tissue damage, including oxygen radicals (Dovi *et al*., 2004), digestive enzymes and pro-inflammatory cytokines (Alzoghaibi *et al*., 2005). The result in this study showed mild to moderate neutrophil infiltration in the gastric mucosa, submucosa, and lamina muscularis of the non-glandular region of the stomach with the *L. monocytogenes* and the leaves (bitter leaf, grava leaf and *D. edulis* leaves) combination used in this study. The presence of marked reduced neutrophilic infiltration in the mucosa of fundus and the submucosa or lamina muscularis of non glandular region, fundus, and pylorus of *L. monocytogenes* and 1g/kg bitter leaf extract combination reveals gastroprotection. Kobayashi *et al*. (2001) observed inhibition of neutrophil infiltration as well as protection of gastric mucosa and while Shimizu *et al*. (2000) observed that improved healing of gastric ulcers in animal model resulted from the reduced infiltration of neutrophil into ulcerated gastric tissue while increase in neutrophil infiltration into ulcerated gastric tissue delayed the healing of stomach ulcers (Fujita *et al*., 1998). The

gastroprotective properties of the plant may be due to the enhanced antioxidant defence system which is known to be an embodiment of scavengers which removes free radicals predisposing the stomach to inflammation (Ajeigbe *et al*., 2014).

Histopathological investigation of spleen did not exhibit any abnormalities in mice treated *L. monocytogenes* and the leaves (bitter leaf, grava leaf and *D. edulis* leaves) combination implying protection of the spleen against the effect of *L. monocytogenes*

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities are commonly measured clinically as a part of a diagnostic evaluation of hepatocellular injury to determine liver health (Wang, 2012). The amount of these biochemical makers in circulation during analysis can enable prediction of the extent of hepatocellular damages. Elevated activities of AST, ALP and ALT in serum are indicative of cellular leakage, loss function integrity of cell membranes in liver (Rajesh and Latha, 2004) or damage to the hepatocytes (Sood, 2006). The evaluations demonstrated by *V. amygdalina*, *Psidium guajava*, and *D. edulis* leaf in antilisterial potency is invariably reflected in the hepatoprotective activities and this could be attributed to the phytochemical compounds of the leaves. The presence of phytochemicals in plants has been reported to able to inhibit different bacteria species and produce antioxidant potentials able to protect organs from reactive oxygen species (ROS) generated during bacterial infections. The inoculation of the combination of *L. monocytogenes* and different concentrations of *V. amygdalina*, *Psidium guajava*, and *D. edulis* into albino mice in this study caused different reactions in the AST, ALT and ALP activities.

The non-significant increase or decrease ($p < 0.05$) of ALT and ALP levels in the serum of mice treated with *L. monocytogenes* and different plant extracts in this study may indicate the radical-scavenging activity of the plant extracts and that there may not be enzyme leakage into the bloodstream of the mice. This result in this study shows that the extracts protected the mice against the *L. monocytogenes* and at same time did not have any apparent toxicity on the liver indicating development of hepatoprotection. This also indicates that on extension of treatment with the extracts, normal architectural structures

of liver organ may be retained (Akharaiyi *et al*., 2015). The results in this study is supported by Adetutu and Olorunnisola, (2013) and Yahya *et al*. (2013) who reported hepatoprotective abilities of different plant extracts. Similar results also suggests that *D. edulis* seeds can offer some levels of hepatoprotection possibly due to their content of flavonoids which exerts a membrane-stabilizing action that protects the liver cells from injury (Schnell *et al*. 2003). Also, Roy *et al*. (2006) reported that the use of *P. guajava* leaves lowered high serum AST and ALT levels in experimental models with induced liver injury. Similarly, the hepato-protective potentials of various extracts of *V. amygdalina* in experimental animals has been recorded by Ijeh and Obidoa, (2004), Iwalokun *et al.*(2006) and Arhoghro *et al.*(2009) as no significant effect on the serum levels of the liver marker enzymes assayed (AST, ACP, ALT, ALP and Bilirubin) was observed

5.1 Contribution to Knowledge

- This research had brought to our knowledge the presence of *L. monocytogenes* in RTE edible worms, meat pies, salads and spiced snails.
- It has also reported the use of both conventional and molecular methods to correctly identify *Listeria monocytogenes.*
- It has a highlighted the physiological attributes of *L. monocytogenes* as well as its susceptibility pattern to different antibiotics.
- This research has shown that some of the phenotypic antibiotic resistance displayed is carried by plamids of varying sizes.
- This research has reported that plants especially bitter leaf, African pear leaf and guava leaf can promote strategies towards elimination of notable food-borne pathogens like *Listeria monocytogenes*.

5.2 Conclusion

The presence of virulent *Listeria monocytogenes* in ready-to-eat foods was established. The virulent strains of *Listeria monocytogenes* exhibited varying *in vitro* suceptibility to different concentrations of ethanol and aqueous plant extracts. Plants such as *Vernonia amygdalina, Dacryodes edulis* and *Psidium guajuva* showed promising use against

Listeria monocytogenes in vivo as its inclusion resulted in significant reduction of alanine aminotransaminase (ALT) liver marker.

5.3 Recommendation

Further surveillance of the prevalence of *L. monocytogenes* in other RTE foods should be carried out to enable the recognition of contaminated foods. There is also the need for co-ordinated research between universities and relevant institutions or agencies as regards to *L monocytogenes* and a cooperative effort of public health agencies, physicians, veterinarians, scientists, the food industries, regulatory agencies, research institutes and consumers to prevent or control this organism. Nigerian Government agencies like National Food and Drug Administration (NAFDAC) and Federal Environmental Protection Agency (FEPA) should include *L. monocytogenes* as reportable pathogen of public health importance in foods and in environmental hygiene and this organism should be a food-borne organism of interest when checking the microbiology quality of foods and other confectionaries meant for human consumption in Nigeria. Good sanitary measures among individuals, food vendors, and personnel working in the industries cannot be over emphasized as it will help to curb the menace of potential listeriosis in Nigerian population/community.

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APPENDIX A

Results from a test of molecular clocks using the Maximum Likelihood method

	ln*L*	Parameters	(+*G*))	(+*I*)
With Clock	-13770161.368	13	n/a	n/a
Without Clock	-15033.672	20	n/a	n/a

The molecular clock test was performed by comparing the ML value for the given topology with and without the molecular clock constraints under Tamura-Nei (1993) model. The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level ($P = 0$). The analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1229 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

ONEWAY VAR00002 of aqeous extracts against *Listeria monocytogenes*

/STATISTICS DESCRIPTIVES

/MISSING ANALYSIS

/POSTHOC=TUKEY ALPHA(0.05).

Descriptive

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	12	15.0000	20.22600	5.83874	2.1490	27.8510	.00	50.00
2	12	15.0000	23.93172	6.90849	-.2055	30.2055	.00	70.00
3	12	22.9167	23.39953	6.75486	8.0493	37.7840	.00	60.00
4	12	24.1667	25.39088	7.32972	8.0341	40.2993	.00	70.00
5	12	29.1667	28.74918	8.29917	10.9003	47.4330	.00	80.00
Total	60	21.2500	24.29733	3.13677	14.9733	27.5267	.00	80.00

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1825.000	4	456.250	.760	.556
Within Groups	33006.250	55	600.114		
Total	34831.250	59			

Post Hoc Tests

Multiple Comparisons

VAR00002
Tukey HSD

(I) aqeous	(J) aqeous	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.00000	10.00095	1.000	-28.2059	28.2059
	3	-7.91667	10.00095	.932	-36.1226	20.2893
	4	-9.16667	10.00095	.889	-37.3726	19.0393
	5	-14.16667	10.00095	.620	-42.3726	14.0393
2	1	.00000	10.00095	1.000	-28.2059	28.2059
	3	-7.91667	10.00095	.932	-36.1226	20.2893
	4	-9.16667	10.00095	.889	-37.3726	19.0393
	5	-14.16667	10.00095	.620	-42.3726	14.0393
3	1	7.91667	10.00095	.932	-20.2893	36.1226
	2	7.91667	10.00095	.932	-20.2893	36.1226
	4	-1.25000	10.00095	1.000	-29.4559	26.9559
	5	-6.25000	10.00095	.970	-34.4559	21.9559
4	1	9.16667	10.00095	.889	-19.0393	37.3726
	2	9.16667	10.00095	.889	-19.0393	37.3726
	3	1.25000	10.00095	1.000	-26.9559	29.4559
	5	-5.00000	10.00095	.987	-33.2059	23.2059
5	1	14.16667	10.00095	.620	-14.0393	42.3726
	2	14.16667	10.00095	.620	-14.0393	42.3726
	3	6.25000	10.00095	.970	-21.9559	34.4559
	4	5.00000	10.00095	.987	-23.2059	33.2059

Homogeneous Subsets

VAR00002

Tukey HSD

aqeous	N	Subset for alpha = 0.05 1
1	12	15.0000
2	12	15.0000
3	12	22.9167
4	12	24.1667
5	12	29.1667
Sig.		.620

Means for groups in homogeneous subsets are displayed.

ONEWAY VAR00002 of ethanolic extracts against *Listeria monocytogenes*
/STATISTICS DESCRIPTIVES
/MISSING ANALYSIS
/POSTHOC=TUKEY ALPHA(0.05).

Oneway

Descriptives

Z_Inh

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	12	22.5000	22.61335	6.52791	8.1322	36.8678	.00	60.00
2	12	29.1667	28.43120	8.20738	11.1023	47.2310	.00	70.00
3	12	34.1667	27.45520	7.92563	16.7225	51.6109	.00	70.00
4	12	32.5000	26.67140	7.69937	15.5538	49.4462	.00	70.00
5	12	32.5000	26.67140	7.69937	15.5538	49.4462	.00	80.00
Total	60	30.1667	25.87399	3.34032	23.4827	36.8506	.00	80.00

ANOVA

Z_Inh

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1040.000	4	260.000	.372	.828
Within Groups	38458.333	55	699.242		
Total	39498.333	59			

Post Hoc Tests

Multiple Comparisons

Z_Inh

Tukey HSD

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-6.66667	10.79539	.972	-37.1132	23.7799
	3	-11.66667	10.79539	.816	-42.1132	18.7799
	4	-10.00000	10.79539	.885	-40.4465	20.4465
	5	-10.00000	10.79539	.885	-40.4465	20.4465
2	1	6.66667	10.79539	.972	-23.7799	37.1132
	3	-5.00000	10.79539	.990	-35.4465	25.4465
	4	-3.33333	10.79539	.998	-33.7799	27.1132
	5	-3.33333	10.79539	.998	-33.7799	27.1132
3	1	11.66667	10.79539	.816	-18.7799	42.1132
	2	5.00000	10.79539	.990	-25.4465	35.4465
	4	1.66667	10.79539	1.000	-28.7799	32.1132
	5	1.66667	10.79539	1.000	-28.7799	32.1132
4	1	10.00000	10.79539	.885	-20.4465	40.4465
	2	3.33333	10.79539	.998	-27.1132	33.7799
	3	-1.66667	10.79539	1.000	-32.1132	28.7799
	5	.00000	10.79539	1.000	-30.4465	30.4465
5	1	10.00000	10.79539	.885	-20.4465	40.4465
	2	3.33333	10.79539	.998	-27.1132	33.7799
	3	-1.66667	10.79539	1.000	-32.1132	28.7799
	4	.00000	10.79539	1.000	-30.4465	30.4465

Z_Inh

Tukey HSD

		Subset for alpha = 0.05
Conc	N	1
1	12	22.5000
2	12	29.1667
4	12	32.5000
5	12	32.5000
3	12	34.1667
Sig.		.816

Means for groups in homogeneous subsets are displayed.

16sRNA Sequence of *L. monocytogenes* isolated from various RTE foods

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
LM 55           AGTCGAGCGA ATGACGAGAA GCTTGCTTCT CTGATTTAGC GGCGGACGGG
LM 57           CCGGTTACGT TCCAACTGAT TAGAAGCTTG CTTCTCTGAT GTTAGCGGCG
LM 70           GTCGAGCGGT AACAGGGGAA GCTTGCTTCT CGCTGACGAG CGGCGGACGG
LM 94           CTGCTATCTG CAAGTCGAGC GGACCGACGG GAGCTTGCTC CCTTAGGTCA
LM 96           GCACTTCGGC GGCTGGCTCA CAGTCGAGCG GACAGAAGGG AGCTTGCTCC
LM 48           TCGAGCTGGG GTCTATTGAG TGGGTTTCTG TCTTAGGGGG TACGGGGCGG
LM 47           AGTTCGAGCG AACAGATGAG AAGCTTGCTT CTCTGATGTT AGCGGCGGAC
LM 20           GRCGCTATCT GCAGTCGAGC GAACAGATAA GGAGCTTGCT CCTTTGACGT
LM 22           GWCGAGCGAA TGGATTAAGA GCTTGCTCTT ATGAAGTTAG CGGCGGACGG
LM 23           KCGRRCGYTG TGAKTRSASC TYGTTTTATG CCSTKTACGK AGTMAGSTGA
LM 24           AKCTTGCTCT TATGAAGTTA GCGGCGGACG GGTGAGTAAC ACGTGGGTAA
LM 37           CKCCCAACCA ATGGACTAAT AGCTTGCTCT TCTGTAGTTA GCGGCGGACG
LM 104          CGAGCGGTAA CAGGGGAAGC TTGCTTCTCG CTGACGAGCG GCGGACGGGT
LM 108          GWCGAGCGTA CAGATAAGGA GCTTGCTCCT TTGACGTTAG CGGCGGACGG
LM 110          TCTGGGGGGC CTTTTTACAT GGCGAGGGCG TAGCGAACAG AATAGGAGCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
LM 55           TGAGTAACAC GTGGGCAACC TGCCCTACAG ATCGGGATAA CTCAGGGAAA
LM 57           GACGGGTGAG TAACACGTGG GTAACCTACC TATAAGACTG GGATAACTCC
LM 70           GTGAGTAATG TATGGGGATC TGCCCGATAG AGGGGGATAA CCACTGGAAA
LM 94           GCGGCGGACG GGTGAGTAAC ACGTGGGTAA CCTGCCTGTA AGACTGGGAT
LM 96           CGGATGTTAG CGGCGGACGG GTGAGTAACA CGTGGGTAAC CTGCCTGTAA
LM 48           GAAATTGTTG CCCACTATAG ATGGGTCACT TCAGATACGG GTCTATTCAG
LM 47           GGGTGAGTAA CACGTGGGTA ACCTACCTAT AAGACTGGGA TAACTCCGGG
LM 20           TAGCGGCGGA CGGGTGAGTA ACACGTGGGT AACCTACCTA TAAGACTGGA
LM 22           GTGAGTAACA CGTGGGTAAC CTGCCCATAA GACTGGGATA ACTCCGGGAA
LM 23           TKCYAKYGAT TTCASCTGCC TTAASGTGGG AYRTCTMCGG TRAACSSRGG
LM 24           CCTGCCCATA AGACTGGGAT AACTCCGGGA AACCGGGGCT AATACCGGAT
LM 37           GGTGAGTAAC ACGTGGGTAA CCTGCCCATA AGACTGGGAT AACTCCGGGA
LM 104          GAGTAATGTA TGGGGATCTG CCCGATAGAG GGGGATAACT ACTGGAAACG
LM 108          GTGAGTAACA CGTGGGTAAC CTACCTATAA GACTGGAATA ACTCCGGGAA
LM 110          TGCTCCTTTT GACGTTAGGC GGCGGACGGG TGAGTAAACA CGTGTGTAAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
LM 55           CCTGGGCTAA TACCGGATAA TCCTTCGAAT CACATGTTTT GAAGTTGAAA
LM 57           GGGAAACCGG GGCTAATACC GGATAATATT TTGAACCGCA TGGTTCAATA
LM 70           CGGTGGCTAA TACCGCATAA TCTCTTAGGA GCAAAGCAGG GGAACTTCGG
LM 94           AACTCCGGGA AACCGGGGCT AATACCGGAT GCTTGATTGA ACCGCATGGT
LM 96           GACTGGGATA ACTCCGGGAA ACCGGAGCTA ATACCGGATA GTTCCTTGAA
LM 48           TTTATTTTTA GCCCGTTTTC TCATAGTATA AACGCTTCGG GTGGTCATAA
LM 47           AAACCGGGGC TAATACCGGA TAATATTTTG AACCGCATGG TTCAATAGTG
LM 20           ATAACTCCGG GAAACCGGGG CTAATGCCGG ATAACATATA GAACCGCATG
LM 22           ACCGGGGCTA ATACCGGATA ACATTTTGAA CCGCATGGTT CGAAATTGAA
LM 23           CTAMTACGTG GTYAKCAKYK YGWAYCGTAT GGTTCGCATT TGTTAGGYKT
LM 24           AACATTTTGA ACCGCATGGT TCGAAATTGA AAGGCGGCTT CGGCTGTCAC
LM 37           AACCGGGGCT AATACCGGAT AACATTTTGA ACCGCATGGT TCGAAATTGA
LM 104          GTGGCTAATA CCGCATAATC TCTAAGGAGC AAAGCAGGGG ACCTTCGGGC
LM 108          ACCGGGGCTA ATGCCGGATA ACATATAGAA CCGCATGGTT CTATAGTGAA
LM 110          CTACCTATAA GGACTGGAAT AACTCCGGGT AACCGGGGCT TAATGCCGGG
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
LM 55           GGCGCTTCGG CGTCACTGTA GGATGGGCCC GCGGTGCATT AGCTAGTTGG
LM 57           GTGAAAGACG GTTTCGGCTG TCACTTATAG ATGGACCCGC GCCGTATTAG
LM 70           TCCTTGCGCT ATCGGATGAA CCCATATGGG ATTAGCTAGT AGGTGGGGTA
LM 94           TCAATCATAA AAGGTGGCTT TTAGCTACCA CTTACAGATG GACCCGCGGC
LM 96           CCGCATGGTT CAAGGATGAA AGACGGTTTC GGCTGTCACT TACAGATGGA
LM 48           GATGTACCGA GCGTTGCGCT GTTGCAAGAA CGGCTTCCTT GTGACGCTCG
LM 47           AAAGACGGTT TCGGCTGTCA CTTATAGATG GACCCGCGCC GTATTAGCTA
LM 20           GTTCTATAGT GAAAGATGGT TTTGCTATCA CTTATAGATG GACCCGCGCC
LM 22           AGGCGGCTTC GGCTGTCACT TATGGATGGA CCCGCGTCGC ATTAGCTAGT
LM 23           RTYCGRYTRT KKCTTAWSGA SGGACCCGCA GKGKAYCTTM GTCTAGWTGG
LM 24           TTATGGATGG ACCCGCGTCG CATTAGCTAG TTGGTGAGGT AACGGCTCAC
LM 37           AAGGCGGCTT CGGCTGTCAC TTATGGATGG ACCCGCGTCG CATTAGCTAG
LM 104          CTTGCGCTAT CGGATGAACC CATATGGGAT TAGCTAGTAG GTGGGGTAAT
LM 108          AGATGGTTTT GCTATCACTT ATAGATGGAC CCGCGCCGTA TTAGCTAGTT
LM 110          ATTAACATAT AGAAACCGCA TGGGTTCTAT AGTGAAAAGA TGGTTTTGCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
LM 55           TGGGGTAACG GCCTACCAAG GCAACGATGC ATAGCCGACC TGAGAGGGTG
LM 57           CTAGTTGGTA AGGTAACGGC TTACCAAGGC GACGATACGT AGCCGACCTG
LM 70           ATGGCTCACC TAGGCGACGA TCCCTAGCTG GTCTGAGAGG ATGATCAGCC
LM 94           GCAKTAGCTA GTTGGTGAGG TAACGGCTCA CCAAGGCGAC GATGCGTAGC
LM 96           CCCGCGGCGC ATTAGCTAGT TGGTGAGGTA ACGGCTCACC AAGGCGAMGA
LM 48           TAGGGAGTGT GAGGGCGCCG GCCCACCGTG CG........ ..........
LM 47           GTTGGTAAGG TAACGGCTTA CCAAGGCGAC GATACGTAGC CGACCTGAGA
LM 20           GTATTAGCTA GTTGGTAAGG TAACGGCTTA CCAAGGCGAC GATACGTAGC
LM 22           TGGTGAGGTA ACGGCTCACC AAGGCAACGA TGCGTAGCCG ACCTGAGAGG
LM 23           TGARGYMACG GCTCACCGTG ARGGTCAATS GAWRCGTRGM STACCTGTGA
LM 24           CAAGGCAACG ATGCGTAGCC GACCTGAGAG GGTGATCGGC CACACTGGGA
LM 37           TTGGTGAGGT AACGGCTCAC CAAGGCAACG ATGCGTAGCC GACCTGAGAG
LM 104          GGCTCACCTA GGCGACGATC CCTAGCTGGT CTGAGAGGAT GATCAGCCAC
LM 108          GGTAAGGTAA TGGCTTACCA AGGCGACGAT ACGTAGCCGA CCTGAGAGGG
LM 110          ATCACTTATA GATGGGACCC GCGCCGTATT AGCTAGTTGG GTAAGGTAAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
LM 55           ATCGGCCACA TTGGGACTGA GACACGGCCC AAACTCCTAC GGGAGGCAGC
LM 57           AGAGGGTGAT CGGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG
LM 70           ACACTGGGAC TGAGACACGG CCCAGACTCC TACGGRRGGC AGCAAGTGGG
LM 94           CGACCTGAGA GGGTGATCGG CCACACTGGG ACTGAGACAC GGCCCAGACT
LM 96           TGCGTAGCCG ACCTGAGAGG GTGATCSGCC ACACTGGGAC TGAGACACGG
LM 48           .......... .......... .......... .......... ..........
LM 47           GGGTGATCGG CCACACTGGA ACTGAGACAC GGTCCAGACT CCTACGGGAG
LM 20           CGACCTGAGA GGGTGATCGG CCACACTGGA ACTGAGACAC GGTCCAGACT
LM 22           GTGATCGGCC ACACTGGGAC TGAGACACGG CCCAGACTCC TACGGGAGGC
LM 23           GKGCAGAKMG GCCASASYGG CACTAATACA CCTCCCAYRY KWAKRCGGTR
LM 24           CTGAGACACG GCCCAGACTC CTACGGGAGG CAGCAGTMGG GAATCTTCCG
LM 37           GGTGATCGGC CACACTGGGA CTGAGACACG GCCCAGACTC CTACGGGAGK
LM 104          ACTGGGACTG AGACACGGCC CAGACTCCTA CGGGAGGCAG CAGTGGGGAA
LM 108          TGATCGGCCA CACTGGAACT GAGACACGGT CCAGACTCCT ACGGGAGGCA
LM 110          GGCTTACCAG GGCGACGATA CGTAGCCGAC TGAGAGGGTG ATTCGGCCAC
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
LM 55           AGTAGGGAAT CTTCCACAAT GGACGAAAGT CTGATGGAGC AACGCCGCGT
LM 57           GAGGCAGCAG TAGGGAATCT YCCGCAATGG GGCGAARGCC TGACGGAGGC
LM 70           GAATATTGCA CAATGGGCGC ARGCCTGATG CAGGCCATGC CGCGTGTATG
LM 94           CCTACGGGAG GCAGCASTAG GGAATCTTCC GCAATGGACG AAAGTCTGAC
LM 96           CCCAGACTCC TACGGGAGGC AGCAGTAGGG AATCTTCCGC AATGGACGRA
LM 48           .......... .......... .......... .......... ..........
LM 47           GCAGCAGTAG GGAATCTTCC GCAATGGGCG AAAGCCTGAC GGAGCMACGC
LM 20           CCTACGGGAG GCAGCAGTAG GGAATCTTCC GCAATKGGCG AAMGCCTGAC
LM 22           AGCAGTAGGG MATCTTMCGC AATGGACGAA AGTCTGACGG AGCAACGCCG
LM 23           WWMWGCAGTA GGRRATCWTC CGCRATGGAC RMMAGTCTGR CGRAGCARCG
LM 24           CAATGGACGA AAGTCTGACG GAGCAACGCC GCGTGAGTGA TGAAGGCTTT
LM 37           CAGCAGTAGG GAATCTTCCG CAATGGACGA AAGTCTGACG GAGCRACRCA
LM 104          TATTGCACAA TGGGCGCAAG CCTGATGCAG CMWTGCCGCG TGTATGAAGA
LM 108          GCAGTAGGGA ATCTTCCGCA WTGGGCGAAA GCCTGASSGA GCAACGCCGC
LM 110          ACTGGAACTG AGACACGGTC CAGACTCCTA CGGAGGCAGC AGTAGGGATC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
LM 55           GAGTGATGAA GGTTTTCSGA TCGTAAAACT CTGTTGTAAG GGAAGAACAA
LM 57           AACCGCCGCG TGAGTGATGA AGKTCTTCGG GATCGTAMAA CTCTGTTGTW
LM 70           AAGAAGGCCT TAGGGTTGTA AAGTACTTTC AGTCGGGAGG AAGKCGTTGA
LM 94           GGAGCAACGC CGCGTGAGTG ATGAAGGTTT TCGGATCGTA AAACTCTGTT
LM 96           AGTCTGACGG AGCAACGCCG CGTGAGTGAT GAAGGTTTTC SGATCGTAAA
LM 48           .......... .......... .......... .......... ..........
LM 47           CCGCGTGGAG TGATGMAGGT MTTCGGATCG TAAMACTCTG TWGTTAGGGA
LM 20           SGAGCAACSC CGCKTGAGTG ATGAAGKGTT TCGGCTCGTA AMACTCTGTT
LM 22           CGTGAGTGAT GAAGGCTYTC GGRTCGTAAA ACTCTGTTGT TAGGGAAGAM
LM 23           MCKMKTSWGT KMTGTAACKG MYYTCGRGKC GYRAAACTCT GYTGTKAGGG
LM 24           CGGGTCGTRM AACTCTGTTG TTAGGGAAGA ACAAGTGCTA GTTGAATAAG
LM 37           CGCKTGAGTG ATGAAGGCTT TCGGRTCGTA AAACTCTGTT GTTAGGGAAG
LM 104          AGGCCCTAGG GATTGTAAAG TACTTTCAGT CGGGAGGAAG GCGTTGATGC
LM 108          GTGAGTGATG AAGGGTTTCS GCTCGTAAMA CTCTGTTATT ACGGSAAGMA
LM 110          CTCCGCAATG AGCGAAGCCT GACGGATCTC GCCGCTGATT TAGAATGATT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        410        420        430        440        450
LM 55           GTACGTTARG AAATGAACGT ACCTTGACGG TACCTTATTA GAAAGSCACG
LM 57           AGGGAAAGAA CMAATTTGTT AGTAACTGAW CAAGTCTTGA CGGTACCTAA
LM 70           TGCTAATATC ATCAACGATT GACGTTACCG ACAGAAGAAG CACSGGCTAA
LM 94           GTTARGGAAG AACAAGTACC GTTCGAATAG GGCGGTACCT TGACGGTACC
LM 96           GCTCTTGTTT GTTAGGGAAG AACAAGTGCA AGAGTAACTG CTTGCACCTT
LM 48           .......... .......... .......... .......... ..........
LM 47           MGAMCAAATW TGTTAGTAAC TGAACAAGTC TTGACGGTAC CTAACCAGAA
LM 20           ATTAGGGAAG AACATATGTG TAAGTAACTG TGCACATCTT GACGGTACCT
LM 22           CAMGTGCTAG TTGAATARGC TGGCACCTTG ACGGTACCTA ACCAGAAAGC
LM 23           ARSAACAAGT GMTAGTTGAA TAMSCTGGYA GTCCWYGACS GTACCTAACS
LM 24           CTGGCACCTT GACGGTACCT AACCAGAAAG CCACGGCTAA CTACGTGCCA
LM 37           AACMAGTGCT AGTTGAATWA GCTGGCACCT TGACGGTACC TAACCAGAAA
LM 104          TAATATCATC ARCGATTGAC GTTACCGACA GAAGAAGCAC CGGCTAACTC
LM 108          CATATGTGTA AGTAACTGTG CACATCTTGA CSGTACCTAA TCAGAAAGSC
LM 110          CGACTCGTAA TGCTGTTATA GGAGATCTTT TTTTTAGTTA CGGGG.....
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        460        470        480        490        500
LM 55           GCTAACTACG TGCCAGCAGC CGCGGTAATA CGTAGGTGGC AAGCGTTGTC
LM 57           CCAGAAAGCC ACGGCTAACT ACGTGCCAGC AGCCGCGGTA ATACGTAGGT
LM 70           CTCCGTGCCA GCAGCCGCGG TAATACGGAG GGTGCAAGCG TTAATCGGAA
LM 94           TAAYCAGAAA GCCACGGCTA ACTACGTGCC AGCAGCCGCG GTAATACGTA
LM 96           GACGGTACCT AAYCAGAAAG CCACGGCTAA CTACGTGCCA GCAGCCGCGG
LM 48           .......... .......... .......... .......... ..........
LM 47           AGCCACGGCT AACTACGTGC CAGCAGCCGC GGTAAATACG TAGGTGGCAA
LM 20           AATCAGAAAG CCACGGCTAA CTACGTGCCA GCAGCCGCGG TAATACGTAG
LM 22           CACGGCTAAC TACGTGCCAG CAGCCGCGGT AATAACGTAG GTGGCAAGCS
LM 23           ATGARWGCYA MGGSTWRSWR SGTKYCAGCA GCCGCGKTAA GTRCKKARGT
LM 24           GCAGCCGCGG TAATACGTAG GTGGCAAGCG TTATCCGGAA TTATTGGGCG
LM 37           GCCACGGCTA ACTACGTGCC AGCAGCCGCG GTWATACGTA GGTGGCAAGC
LM 104          CGTGCCAGCA GCCGCGGTAA TACGGAGGRT GCAAGCGTTA ATCGGAATTA
LM 108          ACGGCTAACT ACGTGYCAGC AGCCGCGGTA ATACGTAGGT GGCAAGCGTW
LM 110           .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        510        520        530        540        550
LM 55           CGGATTTATT GGGCGTAAAG CGCGCGCAGG TGGTTTCTTA AGTCTGATGT
LM 57           GGCAAGCGTT ATCCCGGAAT TATTGGGCGT AAAGCGCGCG TAGGCGGTTT
LM 70           TTACTGGGCG TAAAGCGCAC GCAGGCGGTT GATTAAGTTA GATGTGAAAT
LM 94           GGTGGCAAGC GTTGTCCGGA ATTATTGGGC GTAAAGCGCG CGCAGGCGGT
LM 96           TAATACGTAG GTGGCAAGCG TTGTCCGGAA TTATTGGGCG TAAAGGGCTC
LM 48           .......... .......... .......... .......... ..........
LM 47           GCGTTATCCG GTAWTAWTGG GCGTAAAGCG CGCGTAGGCC GGTTTCTTAA
LM 20           GTGGCAAGCG TTATCCGGAA TTATTGGGCG TAAAGCGCGC GTAGGCGGTT
LM 22           KTATCCCGGA ATTATTGGGC GTAAAGCGCC GCGCCAGGTG GTTTTCTTAA
LM 23           GKMAMGCKTT AWCCRSWMYK MTTGGGCGWR AASSGCGCGC AGGGTTGARW
LM 24           TAAAGCGCGC GCAGGTGGWT TCTTAAGTCT GATGTGAAAG CCCACGGMTC
LM 37           GTTATCCGGA ATTATTGGGC GTAAAGCGCG CGCAGGTGGT TTCTTAAGTC
LM 104          CTGGGCGTAA AGCGCACGCA GGCGGTTGAT TAAGTTAGAT GTGAAATCCC
LM 108          ATCCGGGAAT TATTGGGCGT AAAGCGCGCG TAGGCGGTTW CTTAAGTCTG
LM 110          .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        560        570        580        590        600
LM 55           GAAAGCCCAC GGCTCAACCG TGGAGGGTCA TTGGAAACTG GGGAACTTGA
LM 57           CTTAAGTCTG ATGTGAAAGC CCACGGCTCA ACCCGTGGAG GGGTCATTGG
LM 70           CCCCGGGCTT AACCTGGGAA TGGCATCTAA GACTGGTCAG CTAGAGTCTT
LM 94           TTCTTAAGTC TGATGTGAAA GCCCCCGGCT CAACCGGGGA GGGTCATTGG
LM 96           GCAGGCGGTT TCTTAAGTCT GATGTGAAAG CCCCCGGCTC AACCCGGGGA
LM 48           .......... .......... .......... .......... ..........
LM 47           GTCTGATGTG AAAGCYCACG GCTCAACCCG TGGAGGGTCA TTGGAWACTG
LM 20           TCTTAAGTCT GATGTGAAAG CCCACGGCTC AACCGTGGAG GGTCATTGGA
LM 22           GTCTGATGTT GAAAGCCCCA CGGCTCAACC GTGGAGGGTC ATTGGAAACT
LM 23           TTCTWAAGTC TGAWKTGAMR GSSSMCGGCY CAACCGTKGA GRGTCATTGG
LM 24           AACCGTGGAG GGTCATTGGA AACTGGGAGA CTTGAGTGCA GAAGAGGAAA
LM 37           TGATGTGAAA GCCCACGGCT CAACCGTGGA GGGGTCATTG GAAACTGGRR
LM 104          CGGGCTTAAC CTGGGAATGG CATCTAAGAC TGGTCAGCTA GAGTCTTGTA
LM 108          ATGTGAAAGC YCACGGCTCA ACCGTGGAGG GTCATTGGAA ACTGGGAAAC
LM 110          .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        610        620        630        640        650
LM 55           GTGCAGAAGA GGATAGTGGA ATTTCCAAGT GTAGCGGTGA AATGCGTAGA
LM 57           AAACTGGGAA ACTTGAGTGC AGAAGAGGAG AGTGGAAATT CCATGTGTAG
LM 70           GTAGAGGGGG GTAGAATTCC ATGTGTAGCG GTGAAATGCG TAGAGATGTG
LM 94           AAACTGGGGA ACTTGAGTGC AGAAGAGGAG AGTGGAATTT CCACGTGTAG
LM 96           GGGTCATTGG AAACTGGGAA ACTTGAGTGC AGAAGAGGAG AGTGGAATTT
LM 48           .......... .......... .......... .......... ..........
LM 47           GGAAACTTGA GTGCAGAAGA GGAGAGTGGA ATTTCCATGT GTAGCGGTGA
LM 20           AACTGGGAAA CTTGAGTGCA GAAGAGGAAA GTGGAATTTC CATGTGTAGC
LM 22           GGGAGGACTT GAGTGCAGAA GAGGARAGTG GAATTCCATG TGTAGCGGTG
LM 23           AAACTGGGWG WYTTRAKTCG MAGMARMGGR AAGTGRAMCT TACCAGGTST
LM 24           GTGGAATTCC ATGTGTAGCG GTGAAATGCG TAGAGATATG GAGGAACACC
LM 37           GACTTGAGTG CAGAAGAGGA RAGTGGAATT CCATGTGTAG CGGTGAAATG
LM 104          GAGGGGGGTA GAATTTCCAT GTGTAGCGGT GAWATGCGTA GAGATGTGGA
LM 108          TTGAGTGCAG AAGAGGAAAG TGGAATTCCA TGTGTAGCGG TGAAATGCGC
LM 110          .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        660        670        680        690        700
LM 55            GATTTGGAGG AACACCAGTG GCGAAGGCGA CTGTCTGGTC TGTAACTGAC
LM 57            CGGTGAAATG CGCAGAGATA TGGAGGAACA CCAGTGGCGA AGGCGGCTCT
LM 70            GAGGAATACC GGTGGCGAAG GCGGCCCCCT GGACAAAGAC TGACGCTCAG
LM 94            CGGTGAAATG CGTAGAGATG TGGAGGAACA CCAGTGGCGA AGGCGACTCT
LM 96            CCACGTGTAG CGGTGAAATG CGTAGAGATG TGGAGGAACA CCAGTGGCGA
LM 48            .......... .......... .......... .......... ..........
LM 47            WATGCGCAGW GATATGGWGG WACACCAGTG GCGWWGGCGG CTCTCTGGTC
LM 20            GGTGAAATGC GCAGAGATAT GGAGGAACAC CAGTGGCGAA GGCGACTTTC
LM 22            AAATGCGTAG AGATATGGAG GAACACCCAG TGGGCGAAGG CGRCTTTCTG
LM 23            TGYCAKCTKK TGAMAWSCKT RGAGATRKGG MKKWWCTWYC RGKGGCAGAR
LM 24            AGTGGCGAAG GCGACTTTCT GGTCTGTAAC TGACACTGAG GCGCGAAAGC
LM 37            CGTAGAGATA TTGGAGGAAC ACCAGTGGCG AAKGCGACTT TCTGGTCTGT
LM 104           GGAATACCGG TGGCGTAWGG CGGCCCCCTG GACAAAGACT GACGCTCAGG
LM 108           AGAGATATGG AGGAACACCA GTGGCGAAAG GCGACTTTCT GGTCTGTAAC
LM 110           .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        710        720        730        740        750
LM 55            ACTGAGGCGC GAAAGCGTGG GGAGCAAACA GGATTAGATA CCCTGGTAGT
LM 57            CTGGTCTGTA ACTGACGCTG ATGTGCGAAA GCGTGGGGAT CAAACAGGAT
LM 70            GTGCGAAAGC GTGGGGAGCA AACAGGATTA GATACCCTGG TAGTCCACGC
LM 94            CTGGTCTGTA ACTGACGCTG AGGCGCGAAA GCGTGGGGAG CGAACAGGAT
LM 96            AGGCGACTCT CTGGTCTGTA ACTGACGCTG WGGAGCGWAA GCGTGGGGWG
LM 48            .......... .......... .......... .......... ..........
LM 47            TGTAACTGAC GCTGWTGTGC GWAAGCGTGG GGATCAAACA GGATTAGATA
LM 20            TGGTCTGTAA CTGACGCTGA TGTGCGAAAG CGTGGGGATC AAACAGGATT
LM 22            GTCTGTAACT GACACTGAGG CGCGAAAGCG TGGGGAGCAA ACAGGATTAG
LM 23            KGMSASGTGG TKCATGGTCT GTAACTGWCA GCTCGWGKCG YGARAKSKTG
LM 24            GTGGGGAGCA AACAGGATTA GATACCYTGT TAGTYCACGC CGTAAACGAT
LM 37            AACTGACACT GAGGCGCGGA AAGCGTTGGG GAGCAAACAG GATTAGATAC
LM 104           TGCGTAAWGC GTGGGGAGCA AACAGGATTT AGATACCCTG GTAGTCCACG
LM 108           TGACGCTGAT GTGCGAAAGC GTGGGGATCA WACAGGATTA GATACCCTGG
LM 110           .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       760        770        780        790        800
LM 55           CCACGCCGTA AACGATGAGT GCTAAGTGTT AGGGGGTTTC CGCCCCTTAG
LM 57           TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGAGTGCTA AGTGTTAGGG
LM 70           TGTAAACGAT GTCGATTTGA AGGTTGTTCC CTTGAGGAGT GGCTTTCGGA
LM 94           TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGAGTGCTA AGTGTTAGAG
LM 96           CGATACAGGA TTAGATACCC TGGTAGTCCA CGCCGTAAAC GATGAGTGCT
LM 48           .......... .......... .......... .......... ..........
LM 47           CCCTGGTAGT CCACGCCGTA AWCGATGAGT GCTAAGTGTT AGGGGGTTTC
LM 20           AGATACCCTG GTAGTCCACG CCGTAAACGA TGAGTGCTAA GTGTTAGGGG
LM 22           ATACCCTGGT AGTYCMCGCC GTWAACGATG AGTGCTAAGT KTWAGAGGGT
LM 23           GGTTRAGTCC CCMAACAGGA KYMSATACCC TKATTTTTAG TYSMCRYCRT
LM 24           GAGTGCTAAG TGTTAGAAKG GTTTTCSGCC CTTCTAGTGC TGMAGTTWAC
LM 37           CCTGGTAGTC CACGCCGTAA ACGATGAGTG CTAAGTGTTA GAGGGTTTCC
LM 104          CTGTAAACGA TGTCGATTTG GWKGTTGTGC CCTTTGAKGC GTGGCTTCCG
LM 108          TAGTCCACGC CGTAAACGAT GAGTGCTAAG TGTTAGGGGG TTTCCGCCCC
LM 110          .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       810        820        830        840        850
LM 55           TGCTGCAGCT AACGCATTAA GCACTCCGCC TGGGGAGTAC GACCGCAAGG
LM 57           GGTTTCCGCC CCCTTAGTGC TGCAGCTAAC GCATTAAGCA CTCCGCCTGG
LM 70           GCTAACGCGT TAAATCGACC GCCTGGGGAG TACGGCCGCA AGGTTAAAAC
LM 94           GGTTTCCGCC CTTTAGTGCT GCAGCAAACG CATTAAGCAC TCCGCCTGGG
LM 96           AAGTGTTAGG GGGTTTCCGC CCCTTAGTGC TGCWGCTAAC GCATTTTAWG
LM 48           .......... .......... .......... .......... ..........
LM 47           CGCCCCTTAG TGCTGCAGCT AAACGCATTT AWGCACTCCG CCCTGGGGAG
LM 20           GTTTCCGCCC CTTAGTGCTG CAGCTAACGC ATTAAGCACT CCGCCTGGGG
LM 22           TTCCGCCCTT TAGTGCTGAA GKTAACGCMT TAAGCACTCC GCCTGGGGAG
LM 23           WAACGWTGKG YRCTTWAGKG TKACTSMSGG TKWCMARCCS KWKRRWGSTG
LM 24           GCAWTWAWGC ACTCCGCCTG GGGAGTACCG RCCGCAAGGC TGAATACTCA
LM 37           GCCCTTTAGT GCTGMAGYTA ACGCATTWAA GCACTCCGCC TGGGGAGTAC
LM 104          GWGCTAACGC GTTWAATCGA CCGCCTGGGG TAGTTACGGC CGCAAGGTTA
LM 108          TTAGTGCTGC AGCTAACGCA TTTAAGCACT CCGCCTGGGG AGTACGACCG
LM 110          .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       860        870        880        890        900
LM 55           GTTGAAACTC AAAGGAATTT GACGGGGGCC CGCACAAGCG GTGGAGCATG
LM 57           GGAGTACGAC CGCAAGGTTG AAACTCAAAG GAATTGACGG GGACCCGCAC
LM 70           TCAAATGAAT TGACGGGGGC CCGCACAAGC GGTGGAGCAT GTGGTTTAAT
LM 94           GAGTACGGTC GCAAGACTGA AACTCAAAGG AATTGACGGG GGCCCGCACA
LM 96           CACTCCGCCT GGGGGAGTAC GGTCGCAAGA CTGATAACTC ATAAGGTAWT
LM 48           .......... .......... .......... .......... ..........
LM 47           TACGACCGCA AAGGTTGATA ACTCATARGG AWTTGACGGG GWCCCGCACA
LM 20           TAGTACGACC GCAAGGTTGA AACTCAAAGG AATTGACGGG GTACCCGCAC
LM 22           TACGGCCGCA AGGCTGAWAC TCAAAGGAAT TGACGGGTGG CCCGCACMAG
LM 23           KRGWTRACGT CAAWTYAKCA CTSCSCCTKR TGACCTGKRC RRCCSCYRWG
LM 24           ATAGGWATTG ACGGGGGCCC GGCACYAGCG GTKGAGCATG KTGTTTWATT
LM 37           GGCCGCAAGG CTGAAACTCA AARGWATTGA CGGGGGCCCG CACCAGCGGT
LM 104          AATACTCATA WTGAATTGAC GGGGGCCCGC ACAWGCGGTG GAGCATTGTG
LM 108          CAAGGTTGAW ACTCAAAGGA ATTGACGGGG WCCCGCACAA GCGKTGGAGC
LM 110          .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        910        920        930        940        950
LM 55           TGGTTTAATT CGAAGCAACG CGAAGAACCT TTACCAGGTC TTGACATCCC
LM 57           AAGCGGTGGA GCATGTGGTT TAATTCGAAG CAACGCGAAG AAYCTTACCA
LM 70           TCGATGCAAC GCGAAGAAYC TTACYTACTC TTGACATCCA GAGAACTTAG
LM 94           AGCGGTGGAG CATGTGGTTT AATTCGAAGC AACGCGAAGA ACCTTACCAG
LM 96           TGACGGGGGC CCGCACAATG CGGTGGAGCA TGTGGTTTAW TTCCGAAGCW
LM 48           .......... .......... .......... .......... ..........
LM 47           WGCGGTGGWG CATGTGGTTT AATTCGAAGC AACGCGAAGA ACCTTACCAA
LM 20           AAGCGGTGGA AGCATGTGGT TTAATTCGAA GCAACGCGWA GAACCTTACC
LM 22           CGGTGGAGCA TGTGGYTTWA TTYGWAGCAA CGACGAAGAA CSTTACMCRG
LM 23           CTCSAATGRC YSRTAMRAAK WGACGGCAWG MCCGCACKAG CGKGRAGCAT
LM 24           TCGTAWGCAA CGCGAAGAAC CTTTACMAGG GTCTYGACWT CSTCTTGACC
LM 37           GGAGCATGTG GTTTAATTCG AAGCWACGCG GAGWACCTTA CCAGGTCYTG
LM 104          GTTTGAWTTC GATGCAACGC GAAGAACCTT ACCKACTCTT GACATCCAGA
LM 108          ATGTGGTTTA ATTCGAAGCA WCGCGAAGWA CCTTACCAAW TCTTGACATC
LM 110          .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        960        970        980        990       1000
LM 55           AATGACCGTC CTAGAGATAG GATTTTCCCT TCGGGGACAT TGGTGACAGG
LM 57           AATCTTGACA TCCTTTGACC GCTCTAGAGA TAGAGTCTTC CCCTTCGGGG
LM 70           CAGAGATGCT TTGGTGCCTT CGGGAACTCT GAGACAGGTG CTGCATGGCT
LM 94           GTCTTGACAT CCTCTGACAA CCCTAGAGAT AGGGCTTCCC CTTCGGGGGC
LM 96           ACGCGAAGAA CCTTACCAGG TCTTGACATC CTCTGACAAC CCTAGAGATA
LM 48           .......... .......... .......... .......... ..........
LM 47           ATCTTGACAT CCTTTGACCG CTCKAGAGAT AGAGTCTTCC CCTTCGGGGG
LM 20           AAATCTTGAC ATCCKTTGAC CACTCTAGAG ATAGAGTTTT CCCCTTCGGG
LM 22           TCYTGACMTC STYTGACCAC CCTAGAAGAT ARGGCTTTTC CYTYGGGRGC
LM 23           RTGGATCTMT TAAAYCSTTK CATCRGWWMG RACKTWYAGS YTGMCAWCTC
LM 24           ACCCTAARAG ATAGGGCTTC TCCTTCGGGA GCCAGAGTGA CMGGTGRTGC
LM 37           ACATCSTCTG AMCAYCCTAG AGATWRGGCT TCTSCTTCGG GAGCAGAGTG
LM 104          GAACTTAGCA GAGATGCTTT GGTGCCTTCG GGAACTCTGA GACAGGTGCT
LM 108          CTTTGACCAC TCTAGAGATA GARCTTTCCC CTTCGGGGGA CAAAGTGACA
LM 110          .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1010       1020       1030       1040       1050
LM 55           TGGTGCATGG TTGTCGTCAG CTCGTGTCGT GAGATGTTGG GTTAAGTCCC
LM 57           GACAAAGTGA CAGGTGGTGC ATGGTTGTCG TCAGCTCGTG TCGTGAGATG
LM 70           GTCGTCAGCT CGTGTTGTGA AATGTTGGGT TAAGTCCCGC AACGAGCGCA
LM 94           AGAGTGACAG GTGGTGCATG GTTGTCGTCA GCTCGTGTCG TGAGATGTTG
LM 96           GGGCTTTCCC TTCGGGGACA GAGTGACAGG TGGTGCATGG TTGTCGTCAG
LM 48           .......... .......... .......... .......... ..........
LM 47           ACAAAGTGAC AGGTGGTGCA TGGTTGTCGT CAGCTCGTGT CGTGAGATGT
LM 20           GGACAAAGTG ACAGGTRGTG CATGGTTGTC GTCAGCTCGT GTCGTGAGAT
LM 22           ARAGTGACAG GTGGTGCATR GTTGTTGTCA GCTCGTGTCG TGAGATGTTG
LM 23           TGACACCTMM ARKAAGGCKS RMATCGSWSS TAATSRCRGA TSWSCATGMY
LM 24           ATGKYTGTCG GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC
LM 37           ACAGGTGGTG CATGGTTGTC GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA
LM 104          GCATGGCTGT CGTCAGCTCG TGTTGTGAAA TGTTGGGTTA AGTCCCGCAA
LM 108          GGTGGTGCAT GGTTGTCGTC AGCTCGTGTC GTGAGATGTT GGGTTAAGTC
LM 110          .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1060       1070       1080       1090       1100
LM 55           GCAACGAGCG CAACCCTTAT TCTTAGTTGC CATCATTTAG TTGGGCACTC
LM 57           TTGGGTTAAG TCCCGCAACG AGCGCAACCC TTAAGCTTAG TTGCCATCAT
LM 70           ACCCTTATCC TTTGTTGCCA GCGATTCGGT CGGGAACTCA AAGGAGACTG
LM 94           GGTTAAGTCC CGCAACGAGC GCAAMCCTTG ATCTTAGTTG CCAGCATTCA
LM 96           CTCGTGTCGT GAGATGTTGG GTTAAGTCCC GCAACGAGCG CAACCCTTGA
LM 48           .......... .......... .......... .......... ..........
LM 47           TGGGTTAAGT CCCGCAACGA GCGCAACCCT TAAGCTTAGT TGCCATCATT
LM 20           GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTTAAGCTTA GTTGCCATCA
LM 22           GGTTAAATCC CGCAACGAGC GCAACCCTTG ATTTTAGTTG CCAKCATTTA
LM 23           SCRGTMARYY CGTTCSYGGC CCWTGTACYS AYMRCCCGTC SYACCACGAG
LM 24           GWGCGCAACC CTTGATTTTA GTTGCCATCA TTTAGTTGGG CACTTTAAGG
LM 37           GTCCCGCCAC GAGCGCAACC CTTGATTTTA GTTGCCATCA TTTAGTTGGG
LM 104          CGAGCGCAAC CCTTATCCTT TGTTGCCAGC GATTCGGTCG GGAACTCAAA
LM 108          CCGCAACGAG CGCAACCCTT AAGCTTAGTT GCCATCATTA AGTTGGGCAC
LM 110          .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1110       1120       1130       1140       1150
LM 55           TAAGGAGACT GCCGGTGACA AACCGGAGGA AGGTGGGGAT GACGTCAAAT
LM 57           TAAGTTGGGC ACTCTAGGTT GACTGCCGGT GACAAACCGG AGGAMGGTGG
LM 70           CCGGTGATAA ACCGGAGGAA GGTGSGGATG ACGTCAAGTC ATCATGGCCC
LM 94           GTTGGGCACT CTAAGGTGAC TGCCGGTGAC AAACCGGAGG AAGGTGGGGA
LM 96           TCTTAGTTGC CAGCATTCAG TTGGGCACTC TAAGGTGACT GCCGGTGACA
LM 48           .......... .......... .......... .......... ..........
LM 47           AAGTTGGGCA CTCTAGGTTG ACTGCCGGTG ACAAACCGGA GGAAGGTGGG
LM 20           TTAAGTTGGG CACTCTAGGT TGACTGCCGG TGACAAACCG GAGGAAGGTG
LM 22           GTTGGGCACT TTAAGGGGAC TGCCGGTGAC AAACCGGAGG AAGGTGGGGA
LM 23           AGKTYTACTS AYCGKAWGYM KRTGRKGWAC CCTYTMGA.. ..........
LM 24           TGACTGCCGG TGACCAACCG GAGGAAGGTG GGGAAGACGT CAAATCATCA
LM 37           CACTCTAAGG TGACTGCCGG TGACAAACCG GAKGAAGGTK GGGATGACGT
LM 104          GGAGACTGCC GGTGMTAAAC CGGAGGAAGG TGGGGATGAC GTCAAGTCAT
LM 108          TCTAGGTTGA CTGCCGGTGA CAAACCGGAG GAAGGTGGGG ATGACGTCAA
LM 110          .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1160       1170       1180       1190       1200
LM 55           CATCATGCCC CTTATGACCT GGGCTACACA CGTGCTACAA TGGACGATAC
LM 57           GGATGACGTC AAATCATCAT GCCCCTTATG ATTTGGGCTA CACACGTGCT
LM 70           TKACGAGTAG GGCTACACAC GTGCTACAAT GGCGTATACA AAGAGAAGCG
LM 94           TGACGTCAAA TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA
LM 96           AACCGGAGGA AGGTGGGGAT GACGTCAAAT CATCATGCCC CTTATGACCT
LM 48           .......... .......... .......... .......... ..........
LM 47           GATGACGTCA AATCATCMTG CCCCTTATGA TTTGGGCTAC ACACGTGCTA
LM 20           GGGATGACGT CAAATCATCA TGCCCCTTAT GATTTGGGCT ACACACGTGC
LM 22           AGACGTCAAA TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA
LM 23           .......... .......... .......... .......... ..........
LM 24           TGCCCCTTAT GACCTGGGCT ACACACGTGC TACAATGGGC GGTACAAAGA
LM 37           CAAATYAKCA TGCCCCTTAT GACCTGGGCT ACACACKTGC TACAATGGGC
LM 104          CATGGCCCTT ACGAGTAGGG CTACACACGT GCTACAATGG CGTATACAAA
LM 108          ATCATCATGC CCCTTATGAT TTGGGCTACA CACGTGCTAC AATGGACAAT
LM 110          .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1210       1220       1230       1240       1250
LM 55           AAAGAGTCGC AAACTCGCGA GGGTAAGCTA ATCTCATAAA ATCGTTCTCA
LM 57           ACAATGGATA ATACAAAGGG CAGCGAATCC GCGAGGCCAA GCAAATCCCA
LM 70           ACCTCGCGAG AGCAAGCGGA ACTCATAAAG TACGTCGTAG TCCGGATTGG
LM 94           ATGGGCAGAA CAAAGGGCAG CGAAGCCGCG AGGCTAAGCC AATCCCACAA
LM 96           GGGCTACACA CGTGCTACAA TGGACAGAAC AAAGGGCTGC GAGACCGCAA
LM 48           .......... .......... .......... .......... ..........
LM 47           CAATGGATAA TACAWAGGGC AGCGAATCCG CGAGGCCAAG CAAATCCCAT
LM 20           TACAATGGAC AATACAAAGG GCAGCTAAAC CGCGAGGTCR TGCAAATCCC
LM 22           ATGGGCGGTA CAAAGAGCTG CCAAGCCGCG AGGGTGAGCT AATCTCATAA
LM 23           .......... .......... .......... .......... ..........
LM 24           GCTGCCAGGC CGCGAGGTTG AGCTAATTTC ATAAAACCGT TTTCAGTTCG
LM 37           GGTACAAAGA GCTGCAAGGC GCGAGGTGAG CTAATTTCAT AAAACCTTTT
LM 104          GAGAAGCGAC CTCGCGAGAG CAAGCGGAAC TCATAAAGTA CGTCGTAGTC
LM 108          ACAAAGGGCA GCTAAACCGC GAGGTCATGC AAATCCCATA AAGTTGTTCT
LM 110          .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1260       1270       1280       1290       1300
LM 55           GTTCGGATTG TAGGCTGCAA CTCGCCTGCA TGAAGCCGGA ATCGCTAGTA
LM 57           TAAAATTATT CTCAGTTCGG ATTGTAGTCT GCAACTCGAC TACATGAAGC
LM 70           AGTCTGCAAC TCGACTCCAY GAAGKCGGAA TCGCTAGTAA TCSTAGATCA
LM 94           ATCTGTTCTC AGTTCGGATC GCAGTCYGCA ACWSGACTGC GTGAAGCTGG
LM 96           GGTTTAGCCA ATCCCACAAA TCTGTTCTCA GTTCGGATCG CAGKCTGCAA
LM 48           .......... .......... .......... .......... ..........
LM 47           AAAATTATTC TCAGTKCGGA TTGTAGTCTG CAACTCGACT ACATGAAGCY
LM 20           ATAAAGWTGT TCTCAGTTCG GATTGTAGTC TGCAACTCGA CTACAYGAAG
LM 22           AACCGTTTTC AGTTCGGATT GTTGRCTGCA ACTTGCCTAC ATGAAGCCGG
LM 23           .......... .......... .......... .......... ..........
LM 24           GATTGTAGGC TGCAACTTGC CTACCTGAAG CCGGAATCGC TAGTAATCGC
LM 37           CATTGGATTG GTCAATTGCT CAGACGGACC TGAACCGTAC AGCCGGAAGT
LM 104          CGGATTGGAG TCTGCAACTC GACTCCATGA AGTCGGWATC GCYAGTAATC
LM 108          CAGTTCGGAT TGTAGTCTGC AACTCGACTA CATGAAGCTG GMATCRCYAG
LM 110          .......... .......... .......... .......... ..........


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1310       1320       1330       1340       1350
LM 55           ATCGCGGATC AGCATGCCGC GGTGAATACG TTCCCGGGCC TTGTACACAC
LM 57           TRGAATCGCT AGTAATCGTA GAKCAGCATG CTACGGTGAA TACGTTCCCG
LM 70           GAATGCTACG GTGAATACGT TCCCGGGCCT TGTACACACC GCCCGTCACA
LM 94           AATCGCTAGT AMTCGCGGAT CAGCATGCCG CGGTGAATAC GTTCCCGGGC
LM 96           CTCGACTGCG TGAAGCTGGA ATCGCTAGTA ATCGCGGATC AGCATGCCGC
LM 48           .......... .......... .......... .......... ..........
LM 47           GGAATCGCTA GTAATCGTAG ATCAGCATGC TWCGGTGAAT ACGTTCCCGG
LM 20           CTGGAATCGC TAGTAATCST AGATCAGCAT GCTACSGTGA ATACGTTCCC
LM 22           AATCGCTTGT AATCGCGGAT CAGCCAGCCG CGGTGAATAC GTTCCCGGGC
LM 23           .......... .......... .......... .......... ..........
LM 24           GGATCAGCCA GCCGCGGTGA ATACGTTCCC GGGCCTTGTA CACCCCGCCC
LM 37           CCGCCTTCCA CCCGCCCCYA GATTTAYCGA GCGGAGAC.. ..........
LM 104          GTAGATCAGM ATGCTACGGT GAATACGTTC CCGGGCCTTG TACASAYCGC
LM 108          TAATCGTAGM TCAGCATGCT ACGGTGAATA CGTTCCCGGG TCTTGTACAC
LM 110          .......... .......... .......... .......... ..........
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       1360       1370       1380       1390       1400
LM 55           CGCCCGTCAC ACCACGAGAG TTTGTAACAC CCGAAGTCGG TGGGGTAACC
LM 57           GGTCTTGTAC ACACCGCCCG TCACACCACG AGAGTTTGTA ACACCCGAAK
LM 70           CCATGGGAGT GGGTTGCAAA AGAAGTAGGT AGCTTAACCT TCGGGA....
LM 94           CTTGTACACA CCGCCCGTCA CACCACGAGA GTTTGTAACA CCCGAAGTCG
LM 96           GGTGAATACG TTCYCGGGCC TTGKACACAM CGCCCGTSAC WCCACGAGAG
LM 48           .......... .......... .......... .......... ..........
LM 47           GTCTTGTACA CACCGCCCGT CACACCACGA GAGTTTGTAA CACCCGAAGC
LM 20           GGGTCTTGTA CACACCGCCC GTCACAYCAC GWGAGTTTGT AACACYCKAA
LM 22           CTTGTACACC CCCCCCCTCC CCCCCCGAGA GTTTGTAACC CCCGAAGTCG
LM 23           .......... .......... .......... .......... ..........
LM 24           GTCACCCCCC GAGAGTTTGT AACCCCCGAA GTCGGTGGGG TAACCTTTTT
LM 37           .......... .......... .......... .......... ..........
LM 104          CCGTCACACC ATGGGARTGG GTTGCAAAAG AAGTAGGTAG CTTAAYCTCG
LM 108          ACCGCCCGTC ACACCACGAG AGTTTGTAAC ACCCGAAGCC GGTGGAGTAA
LM 110          .......... .......... .......... .......... ..........


                ....|....| ....|....| ....|....| ....|.
                       1410       1420       1430
LM 55           GTAAGGARC. .......... .......... ......
LM 57           CCGGTGGAGT AACCTTTTAG AGCT...... ......
LM 70           .......... .......... .......... ......
LM 94           GTGAGGTAAC CTTTGGAGCC AGCCGCCGAA AGGGGC
LM 96           TKTGCAACAC CCGAAGTCGG TGAGGTAACC TTTA..
LM 48           .......... .......... .......... ......
LM 47           CGGTGGAGTA ASCTTTTAGG AGCCA..... ......
LM 20           GCCGGTGGAG TAACCATTTA TGGAG..... ......
LM 22           GTGSGGTWAC CTTTGG.... .......... ......
LM 23           .......... .......... .......... ......
LM 24           G......... .......... .......... ......
LM 37           .......... .......... .......... ......
LM 104          .......... .......... .......... ......
LM 108          CCMTYTATGA GCTAGCCGTC GAAGGGGA.. ......
LM 110          .......... .......... .......... ......
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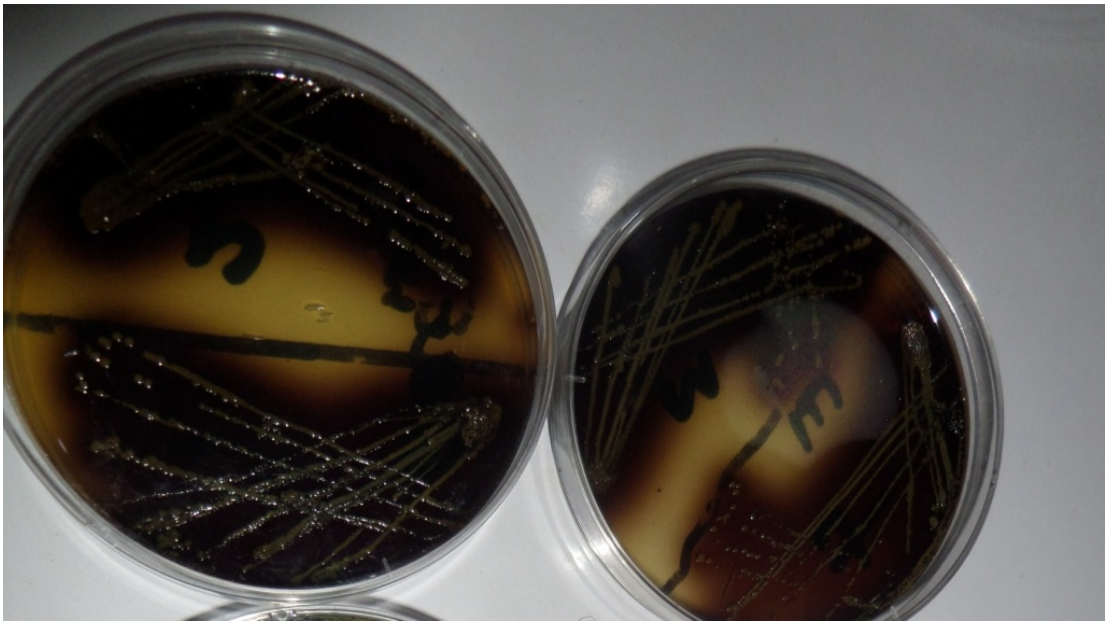
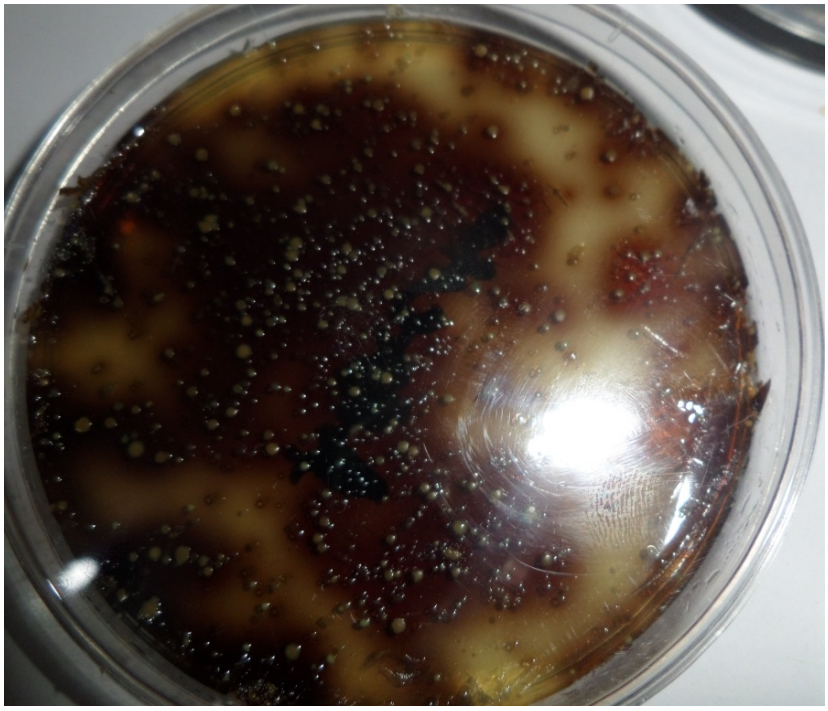
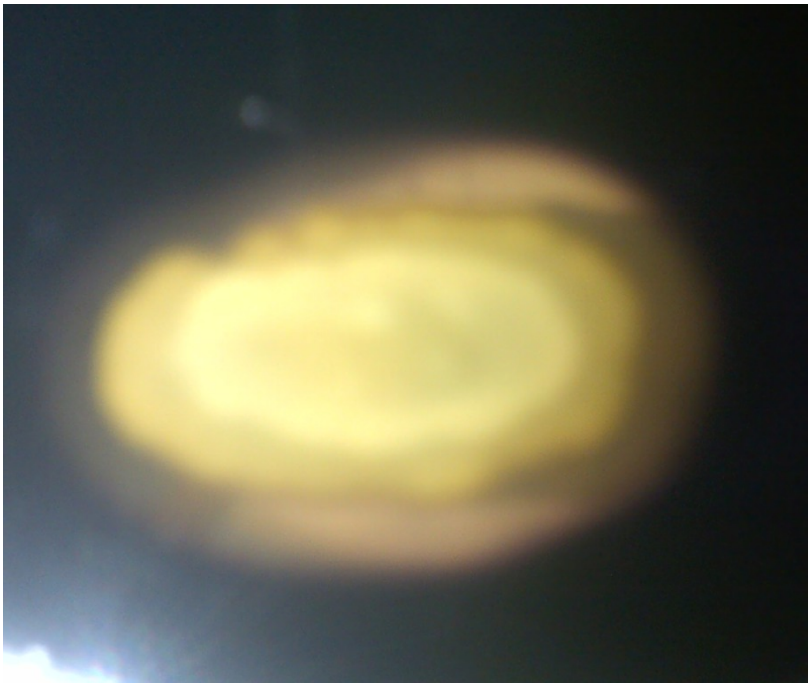
APPENDIX B

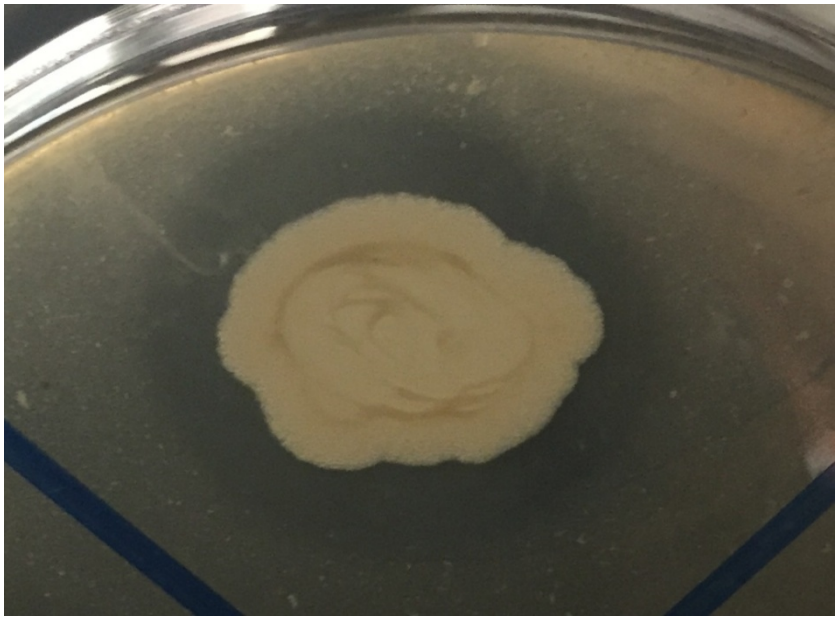




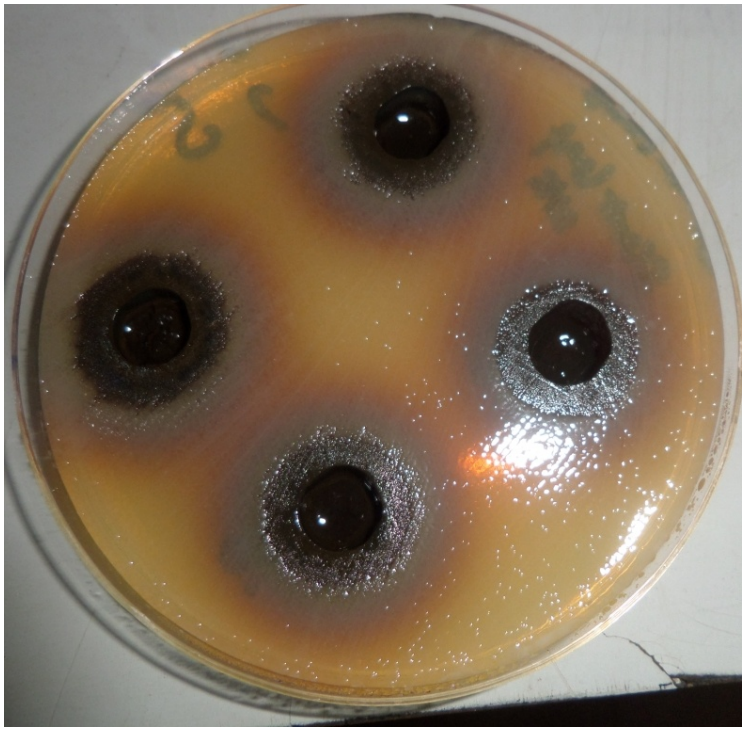
Listeria sp on Listeria Selective Agar (Oxford Formulation)

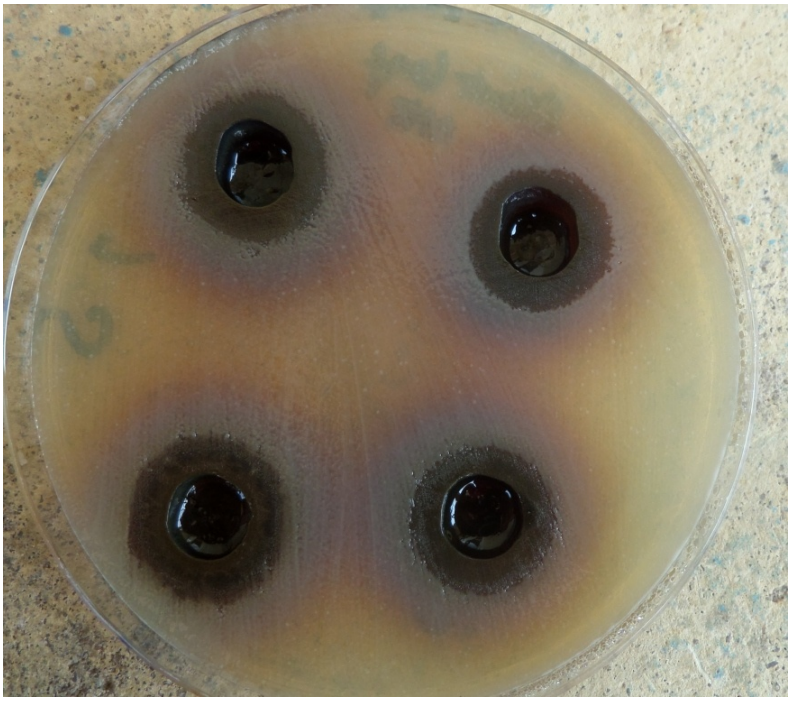


Amylase Screening from *Listeria monocytogenes* using starch as substrate

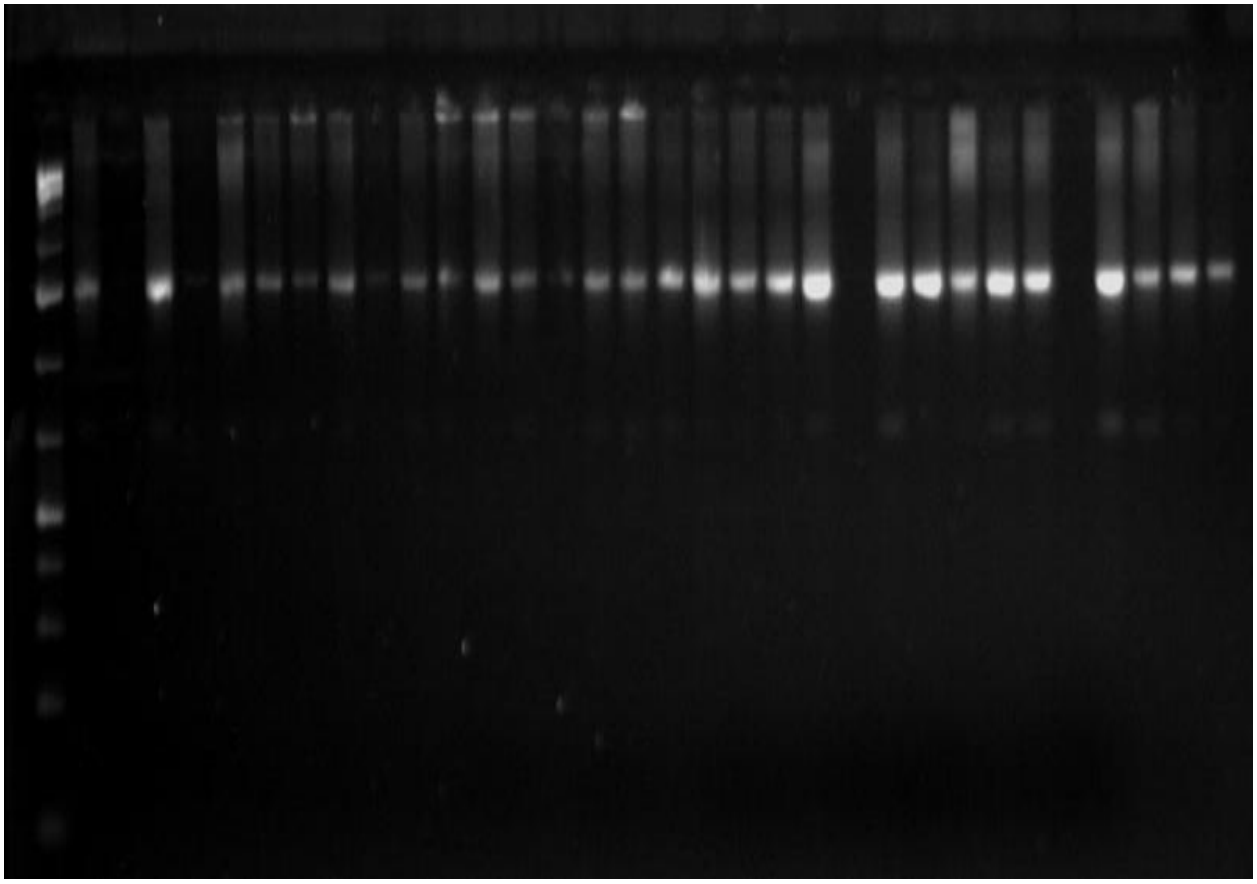


Protease Screening from *Listeria monocytogenes* using skimmed milk as substrate

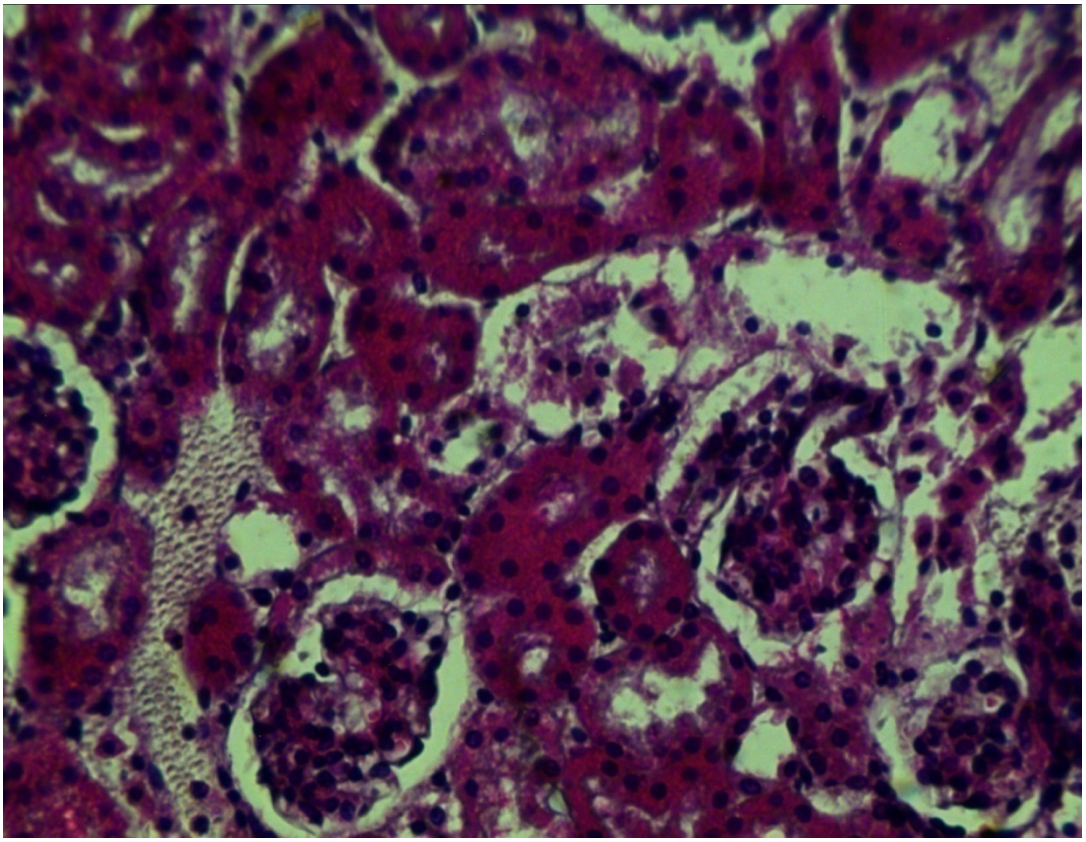




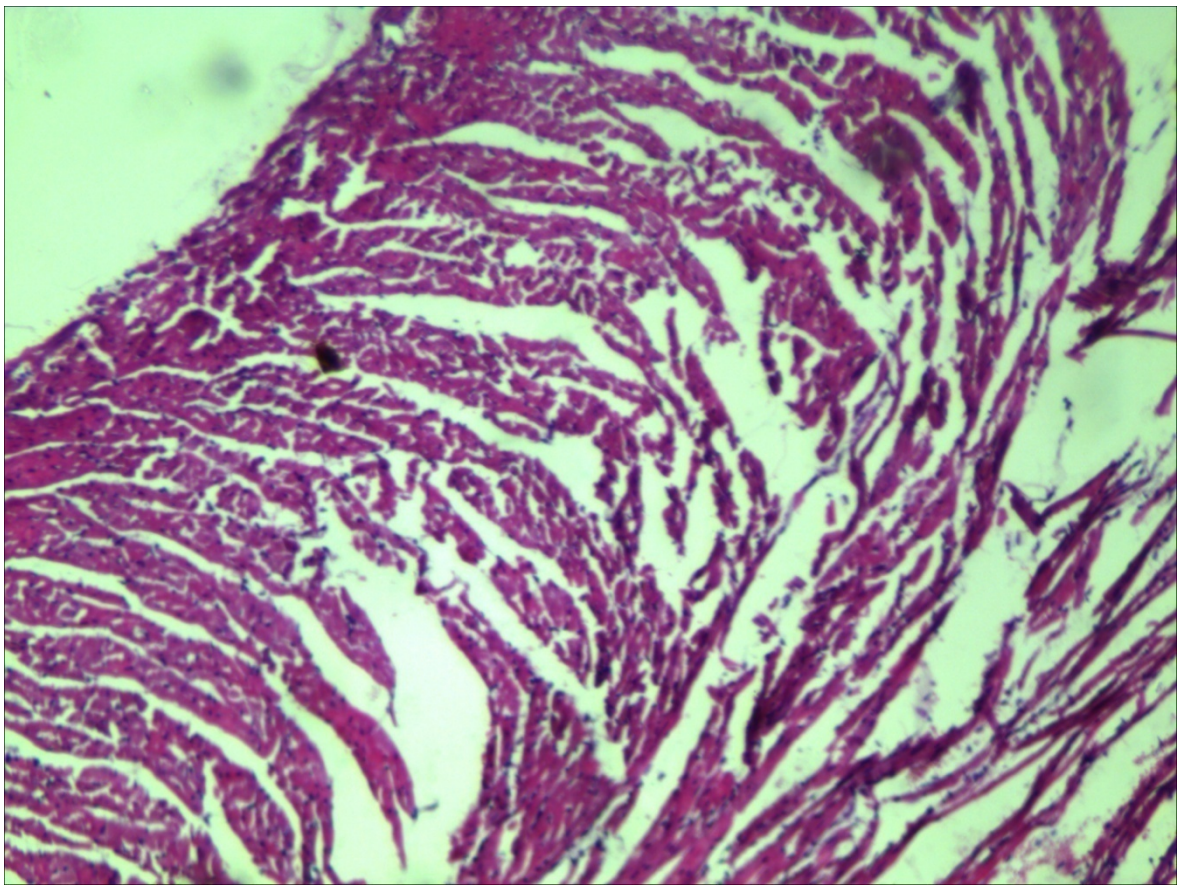
Picture showing zones of inhibition by different concentration of plant extracts against *Listeria monocytogenes*



Agarose gel showing purified *L. monocytogenes* DNA template before sequencing



Picture showing normal kidney architecture of mice infected with *L monocytogenes*



Picture showing normal heart architecture of mice infected with *L. monocytogenes*

APPENDIX C

Tryptone Soy Broth (Oxoid CM129)

Formula per litre	
Pancreatic Digest of soybean meal (Oxiod L44)	3.0g
Pancreatic Digest of Caesarea (Oxoid L42)	17.g
Sodium Chloride	5.0g
Debasic potassum phosphate	2.5g
Dextrose	2.5g
pH	7.3
Distilled water	1,000ml

Listeria Selective Agar (Oxford Formulation)

Formula per Litre	
Cohembia blood agar base	39g
Aesculin	1.0g
Ferric ammonium citrate	0.5g
Lithium Chloride	15.0g
pH	7.2 ± 0.2

Listeria Selective Supplement (Oxford Formulation SR140E)

Formula per vial	
Cycloheximide	200mg
Colistin sulphate	10mg
Acriflavine	2.5mg
Cefotatan	1.0mg
Fosfomycin	5.0g

Injection Buffer

XTerminator™ Solution (store at 4°C)

SAM™ Solution

Buffer for Agarose gel

Tris base	4.85g
EDTA	0.37g
Na Acetate	1.64g

Dissolve in 100mL water and add 900mL distilled water