DETECTION OF ANTIBIOTIC RESISTANCE AND VIRULENCE GENES OF LISTERIA SPECIES ISOLATED FROM MEAT AND VEGETABLES SOLD IN PARTS OF KADUNA STATE, NIGERIA

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AUGUST, 2021

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DEPARTMENT OF MICROBIOLOGY,
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AUGUST, 2021

DECLARATION

I declare that the work in this thesis entitled **"Detection of Antibiotic Resistance and Virulence Genes of *Listeria* species Isolated from Meat and Vegetables Sold in Parts of Kaduna State, Nigeria"**has been carried out by me in the Department of Microbiology, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

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Signature Date

CERTIFICATION

This thesis entitled **"Detection of Antibiotic Resistance and Virulence Genes of Listeria Species Isolated from Meat and Vegetables Sold in Parts of Kaduna State, Nigeria"** by Chrinius HAMMUEL meets the regulations governing the award of the degree of Doctor of Philosophy of the Ahmadu Bello University, and is approved for contribution to knowledge and literary presentation.

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ABSTRACT

Listeriosis is cause by *Listeria* species andis one of the leading foodborne diseases in the world. Resistant species of*Listeria* species have been reported and this is a crucial global health challenge. This research was aimed at detecting resistance and virulence genes in *Listeria ivanovii* from meat and vegetables sold in parts of Kaduna State, Nigeria. A total of 400 samples were collected comprising of 25 each of beef, chicken, lettuce and cabbage from Samaru, Sabon Gari, Kakuri and Ungwan Boro markets between the months of September 2017- June 2018. The proximate compositions of the food samples were determined followed by total bacteria count by pour plate method. *Listeria* species were isolated using *Listeria* selective media and characterized using biochemical methods. The isolates of *Listeria* species were further confirmed using Microgen kits and polymerase chain reaction (PCR). The isolates were screened for *tetA*, *tetM* and *hlyA* genes coding for resistance and virulence respectively using PCR. One-way ANOVA was used to analysed the data.The results showed that the mean moisturelevel was highest in lettuce(94.44±0.18) while crude protein content was also found to be highest in chicken(22.48±0.56). The crude fibre was highest in cabbage(3.64±0.11) and lipid content was highest in beef(6.87±0.03). The mean percentage of carbohydrate content in the foods was highest in lettuce(3.15±0.08) than in chicken and beef. The total bacteria countin the food samples was high in all the food samples ranging from $1.25\times10^{7}\pm4.7\times10^{5}$ -$1.66\times10^{7}\pm4.7\times10^{5}$cfu/g. The prevalence of *Listeria*was 9.75%(39/400). *Listeria ivanovii* hadhighest prevalence of 8.0%(32/400)followed by *Listeriagrayi* [1.5%(6/400)] and *Listeria welshimeri* with a prevalence of 0.25%(1/400).*Listeria ivanovii* had higher prevalence of 9.0%(9/100) in Sabon Gari market while the prevalence of *Listeria grayi*was 2.0%(2/100) in Samaru and Sabon Gari markets. The prevalence of *Listeria welshimeri* was 1.0%(1/100) in Sabon Gari market. The percentage of *Listeria ivanovii* isolates resistant to tetracycline, ampicillin, oxacillin was 22%(7/32), 25%(8/32), 53%(17/32) respectively. The isolates were 100% susceptibility to ciprofloxacin and rifampicin. *Listeriaivanovii*and *Listeria grayi* were 50.0% (11/22) and 66.7%(2/3) multidrug resistant respectively. This study also revealed that 61.1%(11/18) of *Listeria ivanovii*isolates had MAR index above 0.20 significant level of the antibiotic resistant. The PCR showed that 57.1%(4/7) of the isolates were confirm to be *Listeria ivanovii*. Of the seven isolates of *Listeriaivanovii* considered for screening for resistance and virulence genes; 28.6%(2/7) from chicken

and beef had *tetA* gene, 42.86%(3/7) from beef, chicken and lettuce had *tetM* gene and 14.3%(1/7) from beef had haemolysin gene (*hlyA*). The majority of *Listeria ivanovii* with MAR indices of above 0.20 significant level indicate that most of the *Listeria ivanovii* originated from the environment where these antibiotics are highly abused. This finding calls for concern because it is an alert that the antibiotics might have been abused in the environment where the *Listeria* species were isolated.Foodborne infections caused by *Listeria* species in these environments can result to public health threat. There is a need for the relevant authority to educate the public on the hazards associated with indiscriminate use of antibiotics in the treatment of infections among human and animal population.

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ABBREVIATIONS DEFINITIONS, GLOSSARY AND SYMBOLS

et al	"and others"
PCR	Polymerase chain reaction
MARI	Multiple antibiotic resistance index
PrfA	Positive regulatory factor A
MR test	Methyl red test
VP test	Voges Proskauer test
DNA	Deoxyribonucleic acid
TBC	Total bacterial count
AIDS	Acquired immunodeficiency syndrome
LLO	Listeriolysin O
GMP	Good manufacturing practices
NAFDAC	National Agency for Food and Drug Administration and Control
WHO	World Health Organisation
16S rRNA	16 S(Svedberg) ribosomal ribonucleic acid
23S rRNA	23 S(Svedberg) ribosomal ribonucleic acid
MLST	Multilocus sequence typing
MVLST	Multi-virulence locus sequence typing
mRNA	Messenger ribonucleic acid
CDC	Centers for disease control and prevention
CLSI	Clinical laboratory standard institute
HIV	Human immunodeficiency virus
plcA	Phosphatidylinositol phospholipase A

plcB	Phosphatidylinositol phospholipase B
MID	Minimum infectious dose
CAMP test	Christie, Atkins and Munch-Petersen test
cAMP	Cyclic adenosine monophosphate
ELISA	Enzyme-linked immunosorbent assay
API	Application programming interface
LDE	Listeria drug efflux
SEM	Standard error of mean
g	gram(s)
min	minutes
h	hour(s)
^{o}C	Degree Celsius

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Listeria species are Gram-positive, non-spore forming, rods and facultative anaerobic bacteriawith peritrichous flagella that make them motile(Jamali *et al*., 2013). *Listeria* species cause a foodborne illness called listeriosis, which is an emerging zoonotic disease (Musa, 2016).*Listeria* species are ubiquitous and worldwide in distribution and have been isolated from a variety of sources, including soil, vegetables, meat, silage, faecal material, sewage, water as well as animals and humans (Dimic *et al*., 2010; Greenwood *et al*., 2012 and Jamali *et al*., 2013). These bacteria can grow over a wide temperature range of 1°C to 45°C, with an optimum growth temperature of 37°C. *Listeria* species can grow at pH values between 4.4 and 9.4 and at a water activity ≥0.92 with sodium chloride (NaCl) as the solute (Dimic *et al*., 2010).

There are eight species within the genus *Listeria*, which are *Listeria monocytogenes, Listeria ivanovii, Listeria innocua, Listeria seeligeri, Listeria welshimeri, Listeria grayi, Listeria murrayi* and *Listeriarocouritae*(Musa, 2016). Among these species, *L. monocytogene*s, *L. seeligeri* and *L. ivanovii* are associated with pathogenicity. *Listeria ivanovii* is an animal pathogen and is a rare cause of infection in human population, while *L. monocytogenes* causes infection in both human and animal populations (Momtaz and Yadollahi, 2013; Abdelrazeq *et al*., 2014). However, sporadic human infections due to *Listeria innocua* have also been reported (Abay *et al*., 2012). *Listeria monocytogenes* is capable of causing serious invasive illness(listeriosis) including manifestations of septicaemia, meningitis, pneumonia, and encephalitis in both humans and animals (Alzubaidy *et al*., 2013).

Listeria species have the potential to resist various environmental stresses, such as high salt concentration and or acidic solution, and can survive longer under stressful conditions than most other non-spore forming bacteria of foodborne disease concern (Tolga *et al*., 2010). They can survive for a long time in food processing plants and in foods at refrigeration temperatures. Although *Listeria* species commonly exist in raw foods of both plant and animal origin, they are also present in cooked foods due to post-processing contamination from food-processing environments; especially those that are cool and wet (Moreno *et al*., 2014).

Studies have provided sufficient evidence that *Listeria* species are resistant to various antibiotics such as rifampin, chloramphenicol, tetracycline, oxacillin, cefotaxime, ampicillin, erythromycin, gentamicin, and vancomycin (Pesavento *et al*., 2010). Lungu *et al*. (2011) reported that the first antibiotics resistant*L. monocytogenes* strain was reported in 1998; since then, an increasing number of resistant strains isolated from foods, animals and humans have been reported (Altuntas *et al*., 2012). The antibiotic resistance in *Listeria* species is mediated by diverse resistance genes that evolve because of antibiotic selection pressure exerted by the appropriate and/or inappropriate use of antibiotics (Founou *et al*., 2017).

The pathogenesis of *Listeria* species in humans or animals occur in several stages.These include adhesion and entry of the bacterium into the host by crossing internal barrier,lysis of the phagosomal vacuole, invasion of host epithelial cells, multiplication and damage of host digestive system and direct cell-to-cell dissemination even to brain and placenta in the case of pregnant women by actin-based motility (Lebreton and Cossart, 2017). Each step requires expression of specific virulence factors. The major virulence genes are located in a cluster of genes on two different

deoxyribonucleic acid (DNA) loci and are mainly influenced by the positive regulatory factor A protein (*PrfA*) (Price *et al*., 2018).

There are several groups of virulence factors, whichplay important role in pathogenicity of *Listeria monocytogenes* and *Listeria ivanovii* (Indrawattana *et al*., 2011). Among the multiple of these factors include: haemolysin protein (*hlyA*), phosphatidylinositol phospholipase(*plcA*), actin polymerisation protein (*actA*),invasive associated protein (*iap*), pleiotropic activator protein, exotoxin listeriolysin (LLO) protein, zinc metalloproteinase precursor, glycerealdehydes-3-phosphate protein (*gap*), peptidoglycan N-acetyl glucosamine protein and surface components (proteins) (Momtaz and Yadollahi, 2013; Lebreton and Cossart, 2017).

Healthy individuals who are infected have few or no symptoms; when symptoms are present, they usually consist of mild fever, muscle aches, nausea, or diarrhoea. Some may develop more severe symptoms such as meningitis, mental changes, brain abscesses, or death (Carrascal-Camacho *et al*., 2014). People with underlying risk factors such as depressed immune response (for example, pregnant woman and their foetus or newborn babies, cancer patients and HIV-AIDS patients) are at higher risk of developing the disease and some are more likely to have more severe disease (Gamboa-Marin *et al*., 2013; Abdelrazeq *et al*., 2014).

1.2 Statement of Research Problem

Meat are exposed to microorganisms during preparation, transport, preservation and distribution by vendors and can serve as vehicles of pathogens as reported by Chukuezi (2010) and Oluwafemi *etal*. (2013). Jamali *et al*. (2013) reported that the level of contamination of *Listeria* species especially *L. monocytogenes* and *L. ivanovii* in foods could pose a high public health risk to the consumer. Vegetables also serve as mediafor

the transmission of pathogens because most of them are irrigated with wastewater or water contaminated with animal faeces (Abakpa *et al*., 2011; Abakpa *etal*., 2013).

The global increase of foodborne infections with antibiotic resistant and virulent pathogens have been public health problems as reported by Eruteya *et al*. (2014). Diseases related to contamination of foods by pathogens constitute a major burden on human health (Alzubaidy *et al*., 2013). *Listeria* species have also been generally found to be responsible for most foodborne outbreaks among other food-associated pathogens in the globe (Momtaz and Yadollahi, 2013). Several virulent factors of *Listeria* species especially the*L*. *ivanovii* and *L*. *monocytogenes* in contaminated food are said to contribute to pathogenicity.

Antibiotic resistance in *Listeria* species is reaching an era where virtually all antibiotics will be rendered ineffective because of various mechanisms employed by the microorganisms to counteract therapeutic agents (Alzubaidy *et al*., 2013). The excessive exposure of these pathogens to antibiotics has also led to resistance or multidrug resistance, and is an important public health concerns since they may cause failure of therapeutic treatment (Al-Nabulsia*et al*., 2015).

Food contamination by *Listeria* species is one of the leading microbiological causes of food recalls in developed countries, mainly especially in meat, poultry, seafood and dairy products. Prevention and control measures are based on Hazard Analysis and Critical Control Point (HACCP) throughout food industry, and on specific recommendations for high-risk groups. Understanding how these microorganisms modify their cellular physiology to overcome stress is important in controlling *Listeria* species in food environments (Alsheik *et al*., 2013).

1.3 Justification

Microbiological quality of food is of concern since food can serve as a medium for transmission of pathogens in both developed and developing countries due to inadequate hygiene (Ssemanda *et al*., 2018). It is important to carry out this research because meat and vegetables have been found to be notable and consistent sources of *Listeria* species since the populace commonly consume them.Therefore, the study of the prevalence of *Listeria* species in our communities is very imperative to control their distributions(Osman *et al*., 2014). Foods generally serve as sources of pathogenic bacteria responsible for their pathogenicity in humans and animals (Kathariou, 2002; Sarowska *et al*., 2019).

Screening for antibiotic susceptibility of pathogens is crucial because there are substantial knowledge gaps particularly on the spread of antibiotic resistant organisms into the environment and their threats to human health (Arslan *et al*., 2017).The evaluation of antimicrobial susceptibility pattern of *Listeria* species from foods for human consumption is of importance to populace since antibiotic resistant pathogens are of significant public health concern (Pandove*et al*., 2013).

The results of the study would therefore add to the existing data for further studies of *Listeria* species in parts of Kaduna State. The data obtained from this research will add to the databasefor further studies and will be valuable information concerning *Listeria* species in the study area.

1.4 Aim and Objectives

The aim of this research work was todetectantibiotic resistance and virulence genes of *Listeria* species isolated from meat and vegetables sold in parts of Kaduna State, Nigeria.

1.5Objectives

The objectives of this study were to:

1. determine proximate composition of the selected foods,
2. determine the total bacterial count (TBC) in the food samples using pour plate method,
3. isolate and phenotypically characterise the *Listeria* species using biochemical tests,
4. determine the antibiotic resistance patternof the *Listeria* species isolated using single disc diffusion method,
5. confirm *Listeria*speciesand screen for tetracycline resistance andhaemolysin A (*hlyA*)genes using PCR.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of *Listeria* species

Gustav Hulphers who named the bacterium Bacillus hepatitis first discovered listeria species that cause listeriosis (tiger liver disease, silage sickness, leukocytosis, cheese sickness). *Listeria* was alsorecognised in 1891 in the tissue sample from German patients. It was isolated from the liver of rabbits in 1911 in Sweden and the diseases syndrome was recognised in sheep in Germany in 1925 (Musa, 2016).

Listeria was described as the causative agent of an epizootic listeriosis in rabbits and guinea pigs in 1924and were small Gram-positive rods named *Bacterium monocytogenes*. The monocytogenes was based on the pronounced mononucleosis evidence in infected rabbits. The same organisms in 1927 were later named *Listeriahepatolytica* due to liver marks in infected animals. In 1940, the bacterium(the causative agent of listeriosis) recognised as *Listeria monocytogenes*was named in honour of Lord Joseph Lister(Okere, 2014; Musa, 2016).

In the early 1980's *Listeria* was classified under anthropozoonoses, which was change to amphixenoses in late 1990's. The genus *Listeria* belongs to the clostridium sub-branch, together with *Staphylococcus, Streptococcus*, *Lactococcus* and *Bronchothrix*. Today, the genus *Listeria* contains at least eight species of which some are named after the scientist that discovered them (Leong *et al*., 2016).

2.1.1 Occurrence of *Listeria* species in humans in Nigeria

The first occurrence of prevalence of *Listeria* species in Nigeria was in 1965 at Lagos University Teaching Hospital where the levels of specific agglutinating antibodies of *Listeria* speciesin presumed healthy blood donors, staff and students were determined

(Nwaiwu, 2015). Although *Listeria* species were not isolated among 580 Nigerian students, it was discovered that the positive results provided by levels of somatic agglutinating and complement fixing antibodies were strong enough to provide evidence that there was wide occurrence of the organism in Nigeria and emphasised that complete proof would be the isolation of the organism from future clinical samples (Nwaiwu, 2015). *Listeria* species was finally isolated in Nigeria in 1969 as *Listeria leptomeningitis* in an adult female (Nwaiwu, 2015).

The first case of neonatal listeriosis was reported in 1982 where *Listeria monocytogenes* was isolated from in a 2-day old neonate who developed *Listeria* meningitisafter contracting the organism from the mother (Nwaiwu, 2015). Neonatal listeric septicaemia has also been documented in some Nigerian hospitals and has been of concern (Ogundare *et al*., 2016; Shobowale *et* al., 2016; Arowosegbe *et al*., 2017), even though, Nwadioha *et al.* (2013) found no *Listeria* species in a 3 year retrospective study of 1500 paediatric patients in Nigeria.

Bolarinwa *et al.* (2011) carried out a random microbiological screening of one hundred and sixty two (162) blood samples donated for transfusion at a Teaching hospital in Ile-Ife by performing colony morphology, Gram and spore stains and standard biochemical tests and found that *Listeria* species were among the Gram-positive organisms isolated. They pointed out that even though the organism was widely distributed in nature, it is rarely a body commensal in humans and suggested that environmental contamination, false-positive laboratory results, and skin contamination cannot be completely ruled out as reasons for *Listeria* detection (Nwaiwu, 2015).

2.2 Taxonomy of *Listeria* species

For many years after its discovery, the genus *Listeria* was monospecific, containing only the *L. monocytogenes*. Because of its ability to reduce nitrates, *L. denitrificans* was added in 1948; *L. grayi* was added in 1966 in honour of M.L. Gray, an American Microbiologist. *Listeriamurrayi* was added in 1971 (in honour of E.G.D. Murray, a Canadian Microbiologist) and *L. innocua* (named thus because of its harmlessness) in 1981 (Shantha and Gopal, 2014).

In 1985, *L. ivanovii* was added in honour of I. Ivanov, a Bulgarian Microbiologist; *L. welshimeri* was added in 1983 (in honour of H.J. Welshimer, an American Microbiologist) and *L. seeligeri* (in honour of H.P.R. Seeliger, a German Microbiologist) in 1983 (Musa, 2016). Members of the genus of *Listeria* have traditionally been classified into three typically haemolytic species (*Listeria monocytogenes*, *L. ivanovii*, and *L. seeligeri*) which have the ability to haemolyse blood agar and there are three typically nonhaemolytic species (*L. innocua*, *L. grayi* and *L. welshimeri*) (Romanolo *et al*., 2015).

As for the phylogenetic analysis of *Listeria*, the introduction of molecular biology methods allowed a better appreciation of the diversity within the genus of*Listeria*.The genus currently contains eight or more species viz:*L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L.murrayi*, *L. rocouritae* and *L. grayi* as evidenced by DNA homology values, 16S rRNA and DNA sequencing, chemotaxonomic properties, and multilocus enzyme analysis. Based on DNA/DNA hybridisation, DNA/DNA macroarray hybridisation, 16S rRNA cataloguing, reverse transcriptase sequencing of 16S and 23S rRNA, sequencing of 16S-23S rRNA operon intergenic

spacer region, and protein mapping, the genus embraces two closely related but obviously distinct lines of descent(Orsi and Wiedmann, 2016).

2.3 Distribution of *Listeria* species

Listeria species are highly adaptable, are very motile organisms, and therefore, can easily be spread in the environment. These organisms are found in the soil, vegetation, plants, mud, water, sludge, hospital dusts and in the intestinal tract of man, animals and birds (Al-Nabulsia *et al*., 2015). Their wide distribution in the environment may probably be due to their ability to grow at a wide range of temperature (4-45°C) and other adverse environemtal conditions. This feature makes them very difficult to be eliminated from food and dairy industries (Botticella *et al*., 2013; Orsi and Wiedmann, 2016).

It has been demonstrated that *Listeria monocytogenes* and other*Listeria* species can grow and form biofilms on several food processing surfaces including rubber, plastics, glass and stainless steel (Botticella *et al*., 2013). Biofilms of *Listeria*protect cells from the action of antimicrobials and sanitisers (Buchanan *et al*., 2017), potentially allowing long-term persistence of the microorganism in the food processing environment. This evidence suggests that *Listeria* species biofilms is a threat to food safety (Knudsen*et al*., 2016; Price *et al*., 2018). For this reasons it is critical to detect and remove *L. monocytogenes* biofilms in food processing environments in order to improve food safety (Botticella *et al*., 2013).

Listeria biofilms formation and attachment on objects or substrates involves factors such as motility, specific attachment structures, long and short-range attraction forces, hydrophobic forces and polysaccharides production (Botticella *et al*., 2013). *Listeria ivanovii* and *Listeria monocytogenes* can radically alter their metabolism in order to

utilise complex substrates by converting them into useable metabolites with the aid of bio emulsifiers in their biofilm colonies (Knudsen *et al*., 2016). These bioemulsifiers assist the organism in attaching themselves on surfaces in order to survive in nutrient-poor and otherwise hostile environments (Knudsen *et al*., 2016).

The attachment of *Listeria* species to a solid substrate is usually followed by microcolony formation. This microcolony then surrounds itself with extra-cellular polysaccharides (a glycocalyx). The glycocalyx, besides being a physical barrier, is strongly anionic and serves as protection to the microcolony. Those microcolonies of other *Listeria* species ultimately become a firmly attached biofilm (Botticella *et al*., 2013).

2.4 Epidemiology of *Listeria* species

Listeria affects so many of species in the Kingdom Animalia, these include sheep, goat, cattle, buffalo, horse, pig, camel, canine, rodent, wild animals, birds and humans. Small ruminants, especially sheep, are mostly affected (OIE, 2014). *Listeriamonocytogenes* has been isolated from meat and/or milk of goats, sheep, cattle, pig, chicken, quail, partridge, ostrich and buffaloes (Rahimi *et al*., 2012; Derra *et al*., 2013; Hasegawa *et al*., 2013; Kuan *et al*., 2013; Ndahi *et al*., 2013), fish and fishery products are also commonly associated with *Listeria* species(Dhama *et al*., 2015).

Since *Listeria*species are cosmopolitan, meaning they are found everywhere, they have also been isolated in so many food samples including vegetables and meat (Lambertz *et al*., 2012; Lambertz *et al*., 2013; Mateus *etal*., 2013; Poulsen and Czuprynski, 2013). In the northern hemispheres, *Listeria* species have a distinct seasonal occurrence, probably associated with seasonal feeding of silage with the highest prevalence between December and May. In animals e.g. sheep, the cases of abortions are more

common during February and March due to late pregnancies among their population. In human populations, the mortality rate due to listeriosis varies from 20 to 30%(Jahangir *et al*., 2011; Mateus *et al*., 2013, Dhama, *et al*., 2015).

There have been many reports regarding outbreaks of listeriosis in human and animal populations, which have been documented from different parts of the world. In human population, listeriosis is caused by all the 13 serotypes of *Listeria monocytogenes*, some of which include 1/2a, 1/2b, and 4b, and the rate of annual endemic disease varies from 2 to 15 cases per million of population (Munoz, 2012; Dhama *et al*., 2015). *Listeriamonocytogenes* isolates of human and foodborne origin, recovered during 20 years (1992-2012) from Argentina, southern America, corresponded to serotype 4b mostly (71%) while the rest 29% were to serotype 1/2b (Dhama *et al*., 2015).

A study carried out in a Spanish meat processing plant concluded the predominance of serotype 1/2a (36.8%), 1/2c (34%), 1/2b (17.9%) and 4b (11.3%) by multilocus sequence typing (MLST) characterisation (Martin *et al*., 2014, Dhama *et al*., 2015). Between particular forms of listeriosis, no direct links have been made but epidemiological association has been shown between perinatal listeriosis and serovars 1/2b, 3b and 4b (Dhama *et al*., 2015).

In Northern Italy, multi-virulence-locus sequence typing (MVLST) of *Listeria* species isolates revealed ruminants to be the natural reservoirs of these pathogens which could transmit the organism to humans, as a few earlier outbreaks in the 1980s were related tomanure of sheep and milk of cow (Rocha *et al*., 2013). Persons withimmunocompromised status such as pregnant women are more susceptible to listeriosis than others, around the world (Dhama *et al*., 2015). The gastrointestinal tract

of humans serves as a major reservoir of the pathogen and it can be recovered from faeces of pregnant women and normal human beings (Dhama *et al*., 2015).

The incidences of listeriosis(listeric diseases) appear to be increasing in European countries, especially among the elderly people (Hernandez- Milian and Payeras-Cifre 2014, Orsi and Weidmann, 2016). About 19 outbreaks of an invasive form of listeriosis were reported during 1991 to 2001 from Europe (Dhama *et al*., 2015). In Denmark, 299 of invasive cases of listeriosis have been reported from 1994 to 2003 (Hernandez-Milian and Payeras-Cifre, 2014; Dhama *et al*., 2015), 110 in Barcelona (Spain) from 1991 to 2005 and 58 cases in Valencia (Spain) from 2008 to 2010 (Suarez *et al*., 2007; Munoz *etal*., 2012; Dhama, *et al*., 2015).

In Sweden, 601 human isolates of *Listeria*specieshas been reported (Parihar *et al*., 2008; Dhama, *et al*., 2015). Overall incidence of listeriosis in the European Union has been reported to be 0.32 cases/100,000 people during 2011 with a case fatality rate of about 12.7% (Dhama, *et al*., 2015). Compared to 1996-1998, approximately 37% decrease in incidence was seen in the USA between 1996 to 2001 (Lorber, 2010; Dhama, *et al*., 2015), while Australia has seen a constant rise of incidence from 1991 to 2000, ranging from 0.2 to 0.4 cases/100,000 population (De-Noordhout *et al*., 2014; Dhama, *et al*., 2015).

In Asian countries, few cases of listeriosis have been reported, where 48 cases of listeriosis have been documented in Taiwan from 1996 to 2008 (Dhama *et al*., 2015) and 479 isolates of *Listeria* species have been isolated in China from 1964 to 2010 (Feng *et al*., 2013; Dhama *et al*., 2015). In India, the cases of listeriosis have been reported in patients who had a history of genital problems, abortion cases, child with abdominal pain and from cerebro-spinal fluid (Kaur *et al*., 2010; Dhama, *et al*., 2015).

In animals, Vaissaire (2000) reported 25% outbreaks of*Listeria* species from sheep and from cattle 60% in France during 1998-1999.

A study in Iran reported isolation of *Listeria* spp. from milk samples originating from cattle, buffalo, goat, sheep and camel (Rahimi *et al*., 2014). Incidence of animal listeriosis in India has been reported at varying times. Listeriosis outbreak in Nigeria affecting guinea pigs was documented to have almost 100% mortality (Chukwu, *et al*., 2006).

In Zaria, Nigeria, thirty five (35) isolates of *Listeria monocytogenes* have been reported in samples of animal milk and milk products (Usman *et al*., 2016b). *Listeria* species are widespread and truly ubiquitous in nature and commonly found in temperate zones. Rich sources include soil, manure/sewage, farm slurry, sludge, silage, animal feed, water and excreta/faeces of mammals and birds (Dhama, *et al*., 2015). They have also been isolated from walls, floors, drains, decaying vegetation, rivers, pasture herbage, factory effluents, of farms and other environments (Fieseler *et al*., 2014).

2.5 Mechanisms of Survival of *Listeria* spp. under some Adverse Environmental Conditions

2.5.1 Stress response of *Listeria* species

Microorganisms are able to ‘sense’ and adapt to constantly changing environments in which they find themselves (Dogbe, 2010). This characteristic is essential for the growth and survival of microorganisms. *Listeria* species are Gram-positive pathogens with the ability to adapt to a wide range of conditions such as refrigeration temperatures (2-45°C), acidic foods, high salt foods and within the host immune system (Dimic *et al*., 2010; O’Byrne and Utratna, 2010).

The responses to environmental insults are followed by the induction of specific patterns of stress response genes. Genes responsible for the stress response include *dnaJ*, *dnaK*, *grpE*, and *hrcA* (Camejo *et al*., 2011; Bergholz *et al*., 2012). The ability of *L*. *ivanovii* species to adapt to harsh environments makes these pathogens of food safety concern (Dogbe, 2010).

2.5.2 Survival at low and high temperatures

Refrigeration is one of the most common ways to increase the shelf life of foods, particularly foods which are consumed without any further processing (Dass, 2011; Orsi and Weidmann, 2016). The survival and growth of *Listeria* species at refrigeration temperatures (2-4°C) makes the control of this food borne pathogen difficult. Understanding the mechanisms behind the survival and growth of *Listeria* species under adverse conditions such as low temperature, acid stress and osmotic stress among others, could provide information to help develop more effective control methods (Bergholz *et al*., 2012; Wang and Shen, 2015). *Listeria* species does not survive at temperature beyond 45^{o}C. Listeria species are psychrotrophic bacteria, capable of growing at 30-37°C, as well as under refrigeration temperatures (Demic *et al*., 2010).

2.5.3 Changes in membrane composition

The lipids in the membranes of bacterial cells are in a fluid, crystalline state and this physical state is important to maintain membrane fluidity required for proper enzyme activity and transport. Changes in temperature lead to an alteration in the membrane lipid composition to maintain the ideal membrane fluidity (Parsons and Rock, 2013). A high proportion of iso and anteiso, odd-numbered, branched-chain fatty acids characterise the cell membrane of *Listeria* species (Barak and Muchova, 2013; Parsons and Rock, 2013). This keep the cell from adverse environment conditions.

When the temperature is reduced below optimum (7°C), changes that occur in the membranes of the listeriacells include an increase in the proportion of carbon (C)15:0 at the expense of C17:0, and the degree of unsaturated fatty acids, which helps enhance the fluidity of the membrane (Dogbe, 2010; Dass, 2011). This leads to fatty acid shortening (due to a decrease in C17:0) and a switch from iso to anteiso branching (i-C15:0 to a-C15:0) (Miladi *et al*., 2013). The shortening of fatty acid chain length decreases the carbon–carbon interaction between neighbouring chains in the cell membrane and this helps maintain the optimum degree of membrane fluidity for growth at low temperatures (Dogbe, 2010; Barak and Muchova, 2013).

2.5.4 Changes in gene expression and induction of proteins

In response to a temperature down shock, *Listeria* species (e.g. *Listeria monocytogenes* and *Listeria ivanovii*) produce cold shock proteins (Csps) and cold acclimation proteins (Caps) which are synthesised during balanced growth at low temperatures (Dogbe, 2010). Cold acclimation of a pathogen can be accompanied by changes in microbial gene expression (Dogbe 2010; Dass,2011; Miladi *et al*., 2013).

The increased expression of messenger ribonucleic acid (mRNA) for chaperone proteases such as GroEL, ClpP and ClpB indicates that these enzymes may be involved in the degradation of abnormal or damaged polypeptides that arise due to growth at low temperatures (Dogbe, 2010).

2.5.5 Survival under acid stress

Listeria speciesencounters a low-pH environment in acidic foods, during gastric passage and in the phagosome of the macrophage (Dass, 2011). The pathogen responds to and survives in these low-pH environments by utilizing a number of stress adaptation mechanisms. Exposure of *L. monocytogenes* to mild acidic pH of 5.5 (1 M lactic acid)

can induces the acid tolerance response (ATR), wherein the cells are resistant to severe acidic conditions (Dogbe, 2010; Bergholz *et al*., 2012; Wang and Shen, 2015).

2.5.6 Survival under osmotic stress

The response of microorganisms to osmotic stress involves both physiological changes and variations of gene expression patterns and is called osmoadaptation (Dogbe, 2010; Lebreton and Cossart, 2017). The use of salt to lower the water activity of foods is one of the traditional methods of food preservation; thus the ability of *Listeria* spp. to adapt and survive in high concentrations of salt makes it difficult to control the pathogen in foods preserved through salting (Dogbe, 2010). The organisms (*Listeria* spp) can live 100 days at 10.53% concentration of NaCl (Sarfraz *et al*., 2017).

One of the mechanisms used by *Listeria* spp. to tolerate salt stress is a change in its gene expression leading to an increased or decreased synthesis of various proteins. Similar to the two groups of proteins induced in response to cold shock (Csp and Cap), salt shock proteins (Ssp) and the stress acclimation proteins (Sap), are rapidly induced by *Listeria* spp. in response to osmotic stress and continue to be over expressed several hours after conditions return to normal (Dogbe, 2010; Wang and Shen, 2015).

2.6 Foods as Vehicles of *Listeria* species

Listeria species causing listeriosis have been associated with food samples such meat and meat products, milk and milk products, and various kinds of vegetables such as cabbage, corn, lettuce, sprouts, potatoes, cucumbers, parsley and watercress and other environmental samples (Ieren *et al*., 2013; Islam *et al*., 2016; Usman *et al*., 2016b). *Listeria* species found in ready-to-eat mixed foods and several outbreaks of*L. monocytogenes* infection associated with the ready-to-eat foods have been reported

from various parts of the world (Jeyaletchumi *et al*., 2010; Kramarenko *et al*., 2013; Ajayeoba *et al*., 2015; Oyinloye, 2016).

Contamination of foods by *Listeria* species can occur at any point in the food chain, including on farms, in food processing plants, in markets and in the home (Leong*et al*., 2016). *Listeria* species can be detected in a wide range of foods, including both raw and processed foods. Many foods such as soft cheeses, hot dogs, and seafood have been implicated in listeriosis outbreaks, but *Listeria* species also can be isolated from other foods such as beef, pork, fermented sausages, fresh produce and fish products (Alvarez-Ordonez *et al*., 2015; Eruteya *et al*., 2014; Afolabi *et al*., 2017; Amusan *et al*., 2017; Lennox *et* al., 2017).

Since *Listeria* species are found in soil and water, raw vegetables can become contaminated from the soil or from manure applied to soil to enhance the growth of the vegetables as reported for other pathogens by Abakpa *et al*.(2013). Animals (i.e., wildlife and domestic livestock) can be asymptomatic carriers of *Listeriaivanovii* and contaminate foods of animal origin such as dairy and meat through asymptomatic shedding in milk and faeces. Not only can *Listeria* species be isolated from raw foods (e.g. vegetables, uncooked meat), but can also be detected in processed foods such as soft cheeses and delicatessen meat (Leong *et al*., 2016).

2.7 Public Health Importance of Listeriosiss

Many foodborne zoonoses are of serious public health concerns with long-term sequel to various organs (Batz *et al*., 2013; Dhama *et al*., 2013b). Among these, listeriosis can cause severe and life-threatening complications (Dhama*et al*., 2011; Ta *et al*., 2012; Asakura *et al*., 2013; Dhama*et al*., 2013b; Kudirkiene *et al*., 2013). *Listeria* as an opportunistic intracellular pathogen that causes listeriosis has become an important cause of human foodborne infections worldwide (Gezali *et al*., 2016). Contamination

could be during preparation and it then multiplies during the storage process. (Gezali *et al*., 2016).

Listeriosis is now considered as an emerging foodborne zoonosis of increased public health significance. Fcactors driving this include poor refrigeration of foods for preservation, interest in organic and natural products (such as fresh vegetables), rearing of poultry and animals (which could be fed with contaminated feeds) towards better living (Milillo *et al*. 2012; Zhu *et al*., 2012; Dhama *et al*., 2013b). Unlike some other foodborne pathogens, *Listeria* speciescan multiply in contaminated refrigerated food (Gezali *et al*., 2016). *Listeriamonocytogenes* has also been isolated from seafoods, which poses life threatening to human population in the coastal areas of the world (Gawade *et al*., 2010).

Listeriosis can occur as sporadic, endemic and foodborne outbreak which causes septicaemic disease, meningoencephalitis, abortion and infection in other organs in human and animal populations. The majority of risks involve contamination of foods during processing and the potential of the organism to grow at refrigeration temperature (Zhu *et al*., 2012; Lambertz *et al*., 2013; Lamden *et al*., 2013; Viswanath *et al*., 2013).

Meningitis due to Listeria has been ranked at third position among the bacterial causes of meningitis in humans (Koopmans *et al*., 2014). Young ones in the human population, especially newborns, elderly persons, pregnant women, immunocompromised and immunologically immature individuals are generally at higher risk to acquire listeriosis (Martınez-Montero *et al*., 2013; Poulsen and Czuprynski, 2013; Sappenfield *et al*., 2013).

Studies from Australia and the USA have been documented that the elderly age group of over 60 years are more commonly succumb to listeriosis (CDC, 2013; Popovic *etal*.,

2014). The foods of animal origin including milk, meat and their products constitute the main culprits of listerial infection (Goh *etal*., 2012; Osman *etal*., 2014). In USA, the annual cost due to listeriosis was estimated to be US$ 2.3-22 billion, and the annual benefit of listeria food safety measures was $0.01-2.4 billion (Dhama *et al*., 2015). The cases due to listeriosis in 2010, was estimated as 23,150 illnesses, 5463 deaths and 172,823 disability-adjusted life-years globally. The proportion of perinatal cases was 20.7% (De Noordhout *etal*., 2014; Dhama *et al*., 2015).

Though incidences in these countries are low, mortality rate is higher (Gillespie et al. 2010; Dhama *et al*., 2015). In developed countries like the USA, England and Wales, approximately 20-25% of infections have been reported to lead to abortion and still birth (McLauchlin *et al*., 2004; Dhama *et al*., 2015). Fatal outbreak has been recorded in the USA during 2011, where 33 deaths were reported out of 147 infected persons (De-Noordhout *etal*., 2014). *Listeriamonocytogenes* infection, therefore, stands third next to *Clostridiumbotulinum* and *Vibriovulnificus* in the USA (Scharff, 2012; Dhama *et al*., 2015) as a result of food poisoning.

Epidemiological data regarding listeriosis infection in humans are not readily available from many developing countries (De-Noordhout *et al*., 2014), but genital listeriosis is very common in India. However, exact epidemiological data are not available due to under-reporting and poor diagnostic facilities. Studies regarding status of Listeria infection in various parts of developing countries are necessary to know the exact status of disease throughout the world (De-Noordhout *et al*., 2014; Dhama *et al*., 2015).

Occupational exposure to soil, vegetation and animals have also been reported, which may cause skin infections manifested by non-painful, non-pruritic, self-limited, localised, papulo-pustular or vesiculopustular eruptions (Zelenik *et al*., 2014; Dhama *et*

al., 2015). Humans can acquire listeriosis from contact with infected poultry/birds, consumption of listeric contaminated poultry meat or meat products (pre-cooked and ready-to-eat products) and food chain by faecal-oral route (Vivant *et al*., 2013; Dhama *et al*., 2015).

Improper/unhygienic food handling practices, contaminated water, flies and insects, and contaminated food materials are implicated in the spread of listeriosis (Dhama *et al*., 2011; Dhama *et al*., 2013a; Vivant *et al*., 2013; Dhama *et al*., 2015). Direct contact with animals/birds seems lesser significance in the transmission/spread of Listeria, excluding highly susceptible individuals. Person-to-person transmission is not generally observed. Proper cooking of the food is valuable in terms of killing the organism (Dhama *et al*., 2015).

2.8 Some of the species of *Listeria*

2.8.1 *Listeria monocytogenes*

Listeria monocytogenes cangrow at a wide temperature range of 1-45°C, with an optimum growth temperature of 37°C. The Gram-positiverod shape,non-spore forming, motile, aerobic to facultative anaerobic psychrotrophic bacterium is a ubiquitous, intracellular pathogen that invades body tissue and organs of both humans and animals (Nayak *et al*., 2015;Ibrahim, 2017). The bacterium has the ability to hydrolyse L-rhamnos, mannitol and D-mannoside

The invasive *L. monocytogenes* has been implicated within the past decade in several outbreaks in the foodborne illness listeriosis with a mortality rate of about 24%, mainly in immunecompromised persons (i.e. cancer, HIV, rheumatic diseases), pregnant women and their fetuses with symptoms of prenatal infection, abortion, neonatal death and septicaemia (Akano *et al*., 2014;Ibrahim, 2017).The infections caused by *Listeria*

monocytogenes can also be characterised by invasion of the central nervous system causing infections such as meningitis and meningoencephalitis.

Foodborne transmission is the main route of acquisition *Listeria monocytogenes* (Uwanibe *et al*., 2014; Saha *et al*., 2015; Pusztahelyi *et al*., 2016; Odu *et al*., 2017). *Listeriamonocytogenes* is generally quite susceptible to antibiotics but, resistant strains have been reported from foodstuffs and man (Ishola *et al*., 2016; Abatcha, 2017).*Listeriamonocytogenes* is widely distributed throughout the environment and has been isolated throughout various stages of food production and supply chain (Wilson *et al*., 2018). Incidences of listeriosis are frequently associated with the consumption of raw or ready-to-eat foodstuffs such as processed meat, soft cheese, milk products, salad, and seafood (Wilson *et al*., 2018).

2.8.2*Listeria ivanovii*

Listeria ivanovii (formerly known as *L. bulgarica* serotype 5 of *L. monocytogenes*), was first isolated in 1955 in Bulgaria from lambs with congenital listeriosis (Scortti*et al*., 2018). *Listeria ivanovii* is occasionally known to be associated with abortions in sheep and cows or septicaemia in sheep. This strain is motile, catalase positive and has beta-haemolytic activity of 7% sheep or horse blood and can hydrolyse aesculin (Scortti *et al*., 2018).

This strain cannot hydrolyse mannitol, L-rhamnose, or α-methyl-D-mannoside and is oxidase and urease negative (Javaid and Rashid,2018). *Listeria ivanovii* is of great economic importance, because of its pathogenicity to livestock and is widely distributed in nature. *Listeria ivanovii* has been isolated from various kinds of foods including meat, chickens, vegetables, milk and milk products. This organism can cause abortion and still birth in animals and has the ability to reduce the quality of livestock (Al-Jawad 2013; Javaid and Rahsid, 2018; Scortti *et al*., 2018).

Listeria ivanovii, like *L. monocytogenes*, is a typical facultative intracellular parasite which uses characteristic strategies to infect cells and tissues. This bacterium can gain access to vertebrate host via oral route and are capable of crossing the intestinal barrier and proliferating within macrophages and a variety of normally non-phagocytic cells (Alvarez-Ordonez*et al*., 2015). Detection of *L. ivanovii* in the faeces of human or human mesenteric adenitis suggests that the bacterium can cross the intestinal barrier in humans causing gastroenteritis and disseminate into bloodstream (Guillet *et al*., 2010; Wang *et al*., 2014).

Listeriaivanovii causes septicaemia diseases with enteritis and abortion in animals and rarely in humans. Beye*et al*. 2016 also reported a case of *Listeria ivanovii* strain G770 that caused a deadly aortic prosthesis infection in 78 years oldimmuno-compromised patient in Saudi Arabia. Therefore, *L. monocytogenes* is by far the leading cause of human listeriosis;*L. ivanovii* can also rarely lead tobacteraemia, abortion, still birth and cause infections among immune-compromised individuals (Barkallah *et al*., 2014).

Listeria ivanovii and *Listeria monocytogenes* have a very similar virulence genecluster. The virulence gene clusters of *Listeria ivanovii* and *Listeria monocytogenes* contains: (i) *pfrA* gene which encodes the positive master-regulator of most of the known virulence genes, (ii) *hly* gene which encodes the sulfhyldryl-activated pore forming listeriolysin necessary for bacterial escape from the phygosomes of host cells into the cytosol of the host, and (ii) *PlcA* and *PlcB* coding for two phospholipases facilitating the lyses of the host cell membranes (Javaid and Rashid, 2018). The gene *PlcA* codes for a phosphatidylinositol-specific phospholipase C, while *PlcB* encodes a phosphatidylcholine phospholipase C(Park*et al*., 2016).

In addition to the virulence genes cluster, other virulence genes have been identified to be scattered elsewhere in the genomes of *L. ivanovii* and *L. monocytogenes*. Most of these genes constitute a multigene family termed internalins and encode extracellular proteins containing varying numbers of 22 amino acids long lecucine-rich repeats (LRRs). Multiple internalins have been identified bothin *L. ivanovii* and *L. monocytogenes* (Durack *et al*., 2013).

Animal listeriosis cases due to *L. ivanovii* has been recorded in different continents, including Oceania (McAuley *et al*., 2014), North America (Grave *et al*., 2010; Rothrock *et al*., 2017), Europe (Atil*et al*., 2011; Abay *et al*., 2012; Bertrand *et* al., 2016) South America (Orsi and Weidmann, 2016), and Asia (Huang *et al*., 2015; Nayak *et al*., 2015). There has not been any document that reported animal listeriosis caused by *L. ivanovii* in Africa as at time of compiling this report.

Even though*Listeria ivanovii* has rarely been linked to human listeriosis, *L. ivanovii* isolation from humans with listeriosis symptoms has been reported in different continents, including Europe (Guillet *et al*., 2010) and Asia (Beye*et al*., 2016). Reports of human listeriosis caused by *L. ivanovii* have not been identified in Africa, South America, and North America (Orsi and Wiedmann, 2016).

2.8.3 *Listeria grayi*

Listeria grayi is an earlier heterotypic synonym of *Listeria murrayi* and so both were assigned to a single species, *Listeria grayi*. Cells are small (0.4-0.5 x 0.5-2μm) peritrichous rods which are motile. Colonies on tryptose agar are small (1-2mm in diameter after 1-2 days of incubation at 37°C), regular, and smooth. Growth occurs at 4°C within 5 days (Bertsch *et al*., 2013).

Listeria grayi is positive for catalase, esculin hydrolysis, Voges-Proskauer and methyl red tests and negative for the oxidase, urea and gelatin hydrolysis, H_2S and indole production. Reduction of nitrates to nitrites is variable. *Listeria gayi*produced acid from glucose without gas, mannitol, and other sugars. Sheep erythrocytes are not haemolysed by *Listeria grayi*(non-haemolytic pathogen)(Nusa, 2017).

The distribution of *L. grayi* is yet to be comprehensively elucidated. *Listeria grayi* has been isolated from several continents including Europe, Asia, Africa, South America and North America (Orsi *et al*., 2011). Some of the sources of these species have been freshwater fish and abattoirs. *Listeria grayi* is differentiated from other species by its ability to ferment mannitol (Orsi *et al*., 2011).

2.8.4 *Listeria welshimeri*

Listeriawelshimeri is a gram positive, facultative anaerobic, motile, non-spore-forming bacillus. Metabolism is facultative anaerobic. This strain of listeria does not hydrolyse D-glucose, D-xylose, a-methyl-D-mannoside, L-rhamnose, D-mannitol, but can hydrolyse aesculin, methyl red Voges-Proskauerand methyl red tests, it is positive for catalase and negative for oxidase, urea, gelatin hydrolysis, indole and H_2S production as well as reduction of nitrates (Nusa, 2017).*Listeriawelshimeri* is nonpathogenic and therefore non-haemolytic virulent organism (Yehia *et al*., 2016). It was first isolated from decayed vegetation in the United States by H.J.Welshimer, after whom the species was named.

2.9 Nature of Disease Caused by *Listeria* species

Listeriosis is food poisoning caused by eating foods contaminated with *Listeria monocytogenes*and *Listeria ivanovii*. *Listeria ivanovii*and *Listeria monocytogenes* are opportunistic pathogens, for which the minimum infectious dose (MID) is unknown

and depends on strain and victim susceptibility (Guillet *et al*., 2010). There are two types of diseases associated with Listeria; non-invasive and invasive listeriosis (Jeyaletchumi *et al*., 2010; Amusan *et al*., 2017).

Non-invasive lisetriosis is the milder form of the disease; the symptoms include diarrhoea, fever, headache and myalgia (muscle pain). Healthy adults and children sometimes are infected with *L. monocytogenes* (non-invasive listeriosis), but they rarely become seriously ill (Jeyaletchumi *et al*., 2010). The most common invasive listeriosis infections occur in pregnant women and their fetus, newborns, the elderly, and adults with impaired immune systems.

In pregnant women, the infection can result in miscarriage, premature delivery, serious infection of the newborn baby, or even stillbirth (Alsheikh, *et al*., 2013). Foetuses acquire the infection through the mother who has either colonised the organism in the gastrointestinal (GI) tract after consumption of contaminated foods or during childbirth. If a mother is carrying *Listeria* in the GI tractor the perianal region, the pathogen can contaminate the skin and respiratory tract of the child during birthing (O'Byrne and Utratna, 2010; Sudershan*et al*., 2014).Babies can be born with listeriosis, which are acquired through the placenta if their mothers eat contaminated food and get infected during pregnancy (Shindang *et al*., 2013).

In humans, central nervous system (CNS) infection by *Listeria* spp. presents primarily in the form of meningitis. This meningitis however is always associated with the presence of infections foci in the brain parenchyma especially in the brain stem (Surawanshi, 2014). *Listeria* species that cross the intestinal barrier are transported by the lymph or blood to the mesenteric lymph nodes, the spleen, and the liver. This initial step of host's tissue colonisation is rapid (Camejo*et al*. 2011).

The symptoms of listeriosis include meningoencephalitis, septicaemia, fever, muscle aches, encephalitis and sometimes nausea or diarrhoea. If infection spreads to the nervous system, symptoms such as headache, stiff neck, confusion, loss of balance, or convulsions can occur. But infected pregnant women may experience only a mild, flu-like illness (Alsheikh *et al*., 2013).

Listeriosis is characterised by invasion of the central nervous system, liver, spleen, blood, or placenta, causing symptoms such as meningitis, encephalitis and septicaemia (Wilson *et al*., 2018) as in Figure 2.1. Listeriosis cases may be sporadic, endemic and foodborne outbreak to induce septicaemic disease, meningoencephalitis, abortion and infection in other organs. The major risks involve in listeriosis is the contamination of foods during processing and the potential of the organism to grow at refrigeration temperature (Buzby and Roberts, 2009; Zhu *et al*., 2012; Lambertz *et al*., 2013; Lamden *et al*., 2013; Viswanath *et al*., 2013, Dhama *etal*., 2015).

Meningitis due to listeriosis has been ranked at third position among the bacterial causes of meningitis in humans (Koopmans *et al*., 2014). Therefore, newly born babies, elderly persons, pregnant women, HIV patients and other immunocompromised persons are generally at higher risk of acquiring this disease as reported by Martınez-Montero *et al*. (2013), Poulsen and Czuprynski (2013) and Sappenfield *etal*. (2013).

2.10 Entry and Colonisation of Host Tissues

2.10.1Crossing the intestinal barrier

The ingested listeria pathogen in contaminated foods has the ability to withstand adverse environment of the stomach of humans or animals before reaching the intestine (VaZquez-Boland *et al*., 2001, Becattini and Pamer, 2018). In the initial stages, the bacteria are detected mostly in the absorptive epithelial cells of the apical area of the villi. In later phases, theyare found mostly inside macrophages of the stroma of the villi

(VaZquez-Boland *et al*., 2001). This suggest that *Listeria* species can penetrate the host by invading the intestinal epithelium and this can take place through varieties of mechanism, including receptor-dependent entry into globlet and epithelial cells, trancytosis through M cellsand penetrating into underlying tissues (Lecuit *et al*., 2001; Kim *et al*., 2004; Becattini anmd Pamer, 2018).

Intestinal translocation of pathogenic listeriae occurs without the formation of gross macroscopic or histological lesions in the gut (VaZquez-Boland *et al*., 2001; Izar, 2013). This suggests that an epithelial phase involving bacterial multiplication in the intestinal mucosa is not required by *L. monocytogenes* for systemic infection. Indeed, a study using a rat ileal loop model of intestinal infection (VaZquez-Boland *et al*., 2001) has shown that *Listeria* species are translocated to deep organs very rapidly (within a few minutes), demonstrating that crossing of the intestinal barrier occurs in the absence of prior intraepithelial replication (Izar, 2013).

Thus, intestinal invasion and the ensuing febrile gastroenteritis syndrome probably result from extensive exposure of the intestine to pathogenic *Listeria* organisms. However, there may be possibility that some *L. monocytogenes* strains have a greater enteropathogenic potential and cause intestinal damage even at a lower dose.

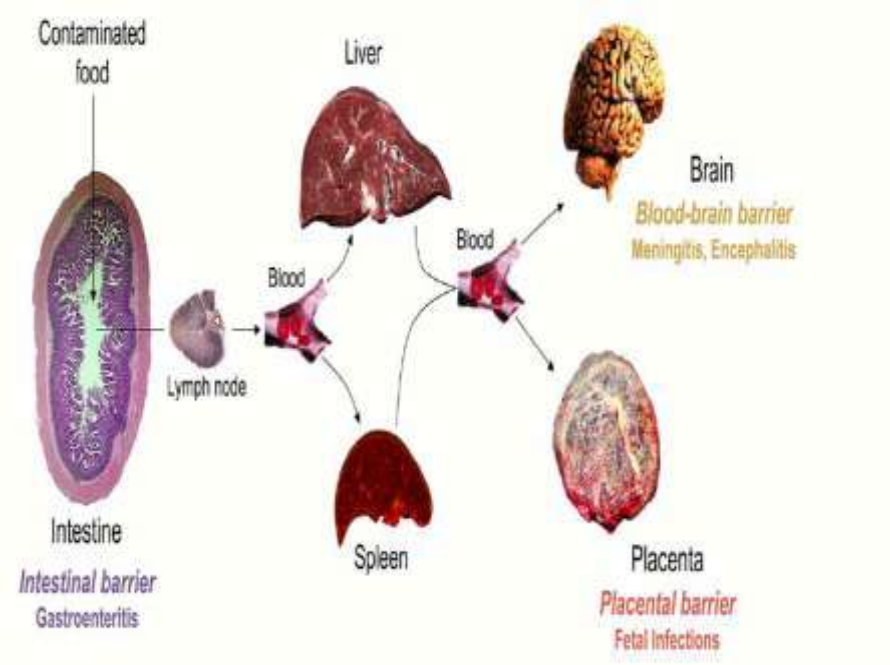

Fig. 2.1: Successive steps of listeriosis affecting different organs in human body (Dass, 2011).

Systemic spread of *L. monocytogenes* can have particularly severe consequences for selected cohorts of individuals, such as immunocompromised and cancer patients, pregnant women, young children and the elderly (Becattini and Pamer, 2018).

2.10.2 Multiplication in the liver

The *Listeria* organisms that cross the intestinal barrier are carried by the lymph or blood to the mesenteric lymph nodes, the spleen, and the liver as presented (VaZquez-Boland *et al*., 2001) This initial step of host tissue colonisation by *L. monocytogenes* is rapid. The unusually long incubation period required by *L. monocytogenes* for the development of symptomatic systemic infection after oral exposure in relation to that for other foodborne pathogens is therefore puzzling and indicates that listerial colonisation of host tissues involves a silent, subclinical phase, many of the events and underlying mechanisms of which are unknown (VaZquez-Boland *et al*., 2001).

Most of the bacterial load accumulates in the liver, presumably captured by the Kupffer cells that line the sinusoids (Izar, 2013). Kupffer cells are believed to initiate the development of antilisterial immunity by inducing the antigen-dependent proliferation of T lymphocytes and the secretion of cytokines. Not all *Listeria* cells are destroyed by tissue macrophages, and the surviving bacteria start to grow, increasing in numbers for 2 to 5 days in mouse organs (Izar, 2013).

The hepatocyte is the major site of bacterial replication in the liver. The characteristics of bacterial invasion of hepatocytes, as well as the consequences of such invasion, have not been fully evaluated (Santos*et al*., 2011). There are two possible ways for *L. monocytogenes* to gain access to the liver parenchyma after its intestinal translocation and carriage by the portal or arterial bloodstream: via Kupffer cells, by cell to cell spread, or by the direct invasion of hepatocytes from the disse space after crossing the

fenestrated endothelial barrier lining the sinusoids (Kanayam*et al*., 2015). *Listeria monocytogenes* has been shown to efficiently invade hepatocytes (Santo *et al*., 2011).

Direct passage from hepatocyte to hepatocyte would lead to the formation of infectious foci in which *L. monocytogenes* disseminates through the liver parenchyma without coming into contact with the humoral effectors of the immune system (Izaar,2013). During the early steps of liver colonisation, polymorphonuclear neutrophils are recruited at the sites of infection, forming discrete microabscesses (VaZquez-Boland *et al*., 2001).

Neutrophils have been shown to play an important role in controlling the acute phase of *Listeria* infection (Conlan and North, 1995; Izar, 2013). Hepatocytes respond to *Listeria* infection by releasing neutrophil chemo-attractants and exhibiting an increase in adhesion to neutrophils, resulting in microabscess formation (Conlan and North, 1995; Izar, 2013).

If the infection is not controlled by an adequate immune response in the liver, as may occur in immunocompromised individuals, unlimited proliferation of *L. monocytogenes* in the liver parenchyma may result in the release of bacteria into the circulation(VaZquez-Boland *e al*., 2001).

Listeria monocytogenes is a multisystem pathogen that can infect a wide range of host tissues, as indicated by its capacity to cause septicaemia involving multiple organs and by the variety of potential sites of localised listeriainfection. However, the principal clinical forms of listeriosis clearly show that *L. monocytogenes* has a pathogenic tropism towards the gravid uterus and the CNS (Pekova *et al*., 2017)

2.10.3 Invasion of the brain

The mechanism by which pathogenic *Listeria* speciesinfects the central nervous system (CNS) is still unknown.In humans, CNS infection by *Listeria* spppresents primarily in the form of meningitis (Lecuit *et al*., 2004, Tiri *et al*., 2018). This meningitis, however, is often associated with the presence of infectious foci in the brain parenchyma, especially in the brain stem (Kanayama *et al.*, 2015), suggesting *L. monocytogenes* has a tropism for nerve tissue(Tiri *et al*., 2018).

The neurotropism and special predilection of *L. monocytogenes* for the rhombencephalon are shown most clearly in ruminants, in which listerial CNS infection, in contrast to the situation in humans, develops mainly as primary encephalitis (Pekova *et al*., 2017).Although there is inflammatory lymphocyte or mononuclear cell infiltration of the meninges (Ghosh *et al*., 2018), this condition occurs as an extension of the brain process, and macroscopic lesions may not even be evident or may be restricted to basal areas, midbrain, and cerebellum. Unilateral cranial nerve paralysis is a characteristic of listerial rhombencephalitis in ruminants, leading to the well-known circling disease syndrome (VaZquez-Boland *et al*., 2001).

Brain lesions in listerial meningoencephalitis are typical and very similar in humans and animals (Ghosh *et al*., 2018). They consist of perivascular cuffs of inflammatory infiltrates composed of mononuclear cells and scattered neutrophils and lymphocytes. Bacteria are generally absent from these perivascular areas of inflammation. Parenchymal microabscesses and foci ofnecrosis and malacia are also typically present. Bacteria are relatively abundant in these lesions, within phagocytes or free in the brain parenchyma around the necrotic areas (Kanayama *et al*., 2015).

2.11 Virulence and Pathogenicity of *Listeria* species

Listeria ivanovii and *Listeria monocytogenes* have evolved sophisticated array of mechanisms that allow them to exploit the host. The pathogens can cause infections by attaching and invading the host cells, escaping from the host cell vacuole, spreading from cell to cell, crossing barriers and evading the host immune system as shown in Figure 2.2 (O'Byrne and Utratna, 2010; Dhama *et al*., 2015; Scortti *et al*., 2018). The ingested *Listeria* spp. with contaminated food can withstand the adverse environment of the stomach and evade the gastric acidity (Dhama *et al*., 2015).

When pathogenic *Listeria* species (*L. ivanovii* and *L. monocytogenes*) encounter human or animal cells after ingestion, they have the capacity to internalise themselves into both professional and non-professional phagocytes. Depending on the type of cell encountered, the interaction will require one or more of a group of virulence factors.An *Acta* gene mediates the actin-based motility in the host's cytosol and is required for maximum uptake of the bacterium into epithelial cells and to spread to adjacent cells (Alvarez-Ordonez, *et al*., 2015; Scortti *et* al., 2018). Several other factors affecting internalisation have been identified in both *L. ivanovii* and *L. monocytogenes*, but their mode of action has not been extensively elucidated (Dhama *et al*., 2015).

When the bacteria first enter the cell they are confined in a single membrane vacuole, which is lysed by the actions of the cholesterol dependent cytolysin LLO together with the action of phospholipases enzymes (*e.g.* PlcA and PlcB) (O'Byrne and Utratna, 2010). In the cytosol the bacterium utilises a hexose phosphate transporter (Hpt) to take up glucose-1-phosphate and other hexose phosphates from the cell. Hexose phosphate transporter is not essential for cytosolic replication, but is required for rapid cell

division (generation time of approximately 40 minutes) (Guillet *et al*., 2010; Lebreton and Cossart, 2017; Scortti *et al*., 2018).

To spread from an infected cell, pathogenic *Listeria* species utilise *ActA* to recruit cellular actin through the host factors, allowing the bacteria to be propelled through the cytosol (Lebretton and Cossart, 2017). When the bacteria reach the cell membrane it extends it into the adjacent cell, finally resulting in a double membrane enclosed vacuole from which *L. monocytogenes* escapes to perpetuate the infection without exposing itself to the extracellular environment. It should be noted that prolonged infection would eventually lead to death of infected cells.

2.11.1 Positive regulatory factor A (*PrfA*)

This protein is the main switch of a regulon including the majority of the known listerial virulence genes (Price *et al*., 2018). Positive regulatory factor A (*PrfA*) is the first identified and major regulator of virulence genes in *L. ivanovii* and *L. monocytogenes*. The virulence regulator protein of *Listeriaivanovii* is highly homologous to *PrfA from Listeria monocytogenes* (den Bakker, *et al*. 2013; Chen *et al*., 2017).

The protein encoded by the *PrfA* gene is a key factor for *L. monocytogenes* and *L. ivanovii* pathogenesis, and strains lacking this gene are avirulent (Chen *et al*., 2017). Positive regulatory factor A (*PrfA*) gene is a 237 amino-acid protein which belongs to the cyclic adenosine monophosphate (cAMP) receptor protein (CrP)-Fnr family of pleiotropic transcription regulators that can bind dexoxyribonuleic acid (DNA) as

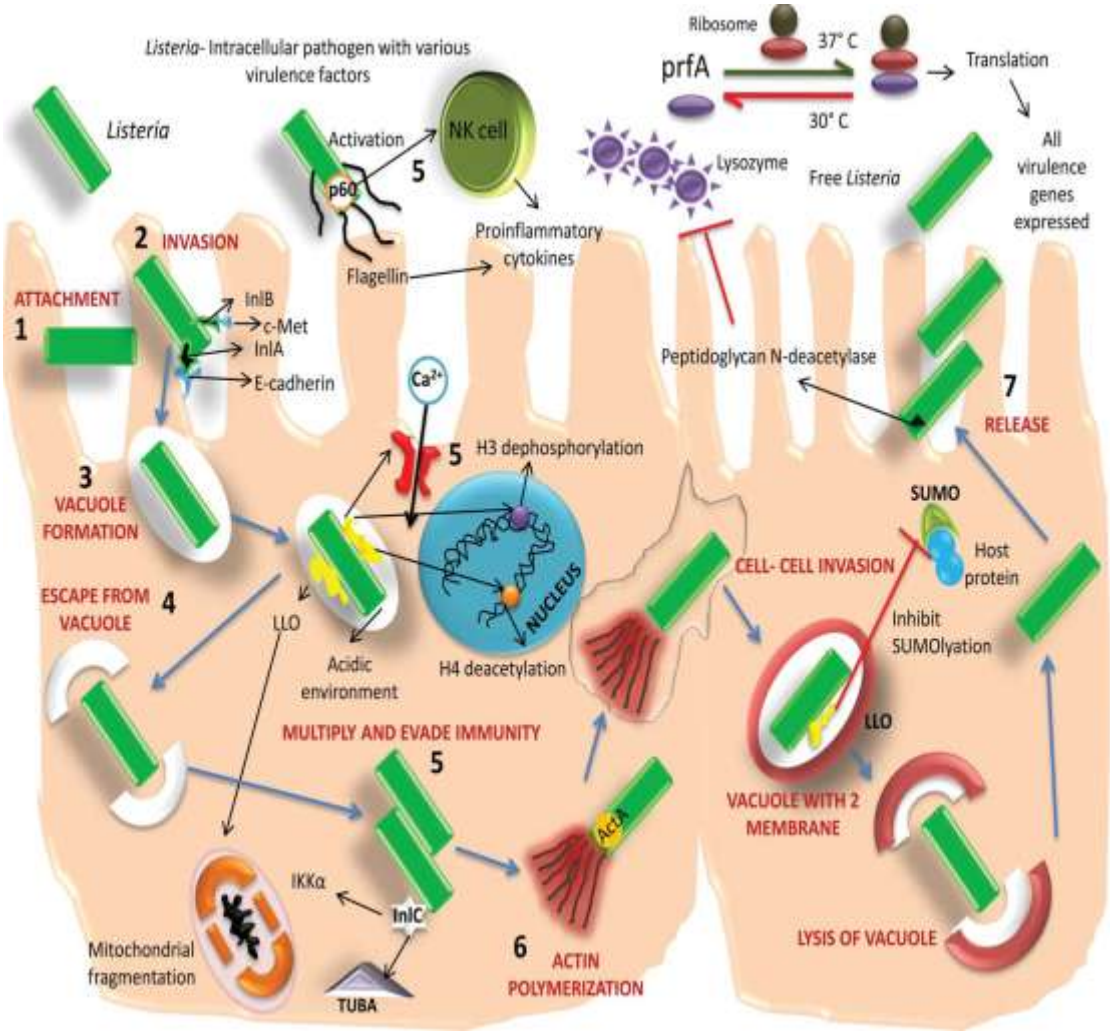

Figure 2.2: Virulence factors and pathogenesis of *Listeria* spp. (1) Inidicate the attachment of *Listeria*to their receptor. (2) Shows invasion. (3) Phagocytic vacuole carries the organism inside. (4) *Listeria* escapes the vacuole by several mechanisms including LLO. (5) *Listeria* evades host immunity by various mechanisms including LLO, Flagellin, and InlC. (6) Formation of actin tail propels the organism from one cell to another. (7) finally, release of the organism (Dhama *et al*., 2015).

dimers on specific sites in gene promoters and activate transcription (Good *et al*., 2016).

The C-terminal region of *PrfA* contains a helix-turn-helix (HTH) motif which allows *PrfA* to bind to a 14bp DNA palindromic sequence called the '*PrfA*-box', present in the -40 region of the target gene promoters (Good *et al*., 2016).

Positive regulatory factor A(*PrfA*) was initially identified for its ability to stimulate the expression of haemolysin gene,encoding the pore-forming toxin listeriolysin O (LLO),and was later found to regulate major genes supporting intracellular life. Haemolysin (*hly*)gene is co-expressed together with a *PrfA* dependent virulence cluster encompassing *PrfA* itself, as well as the genes encoding listeria-secreted phosphadidylinositol-specific (*plcA*).Positive regulatory factor A(*PrfA*) also activates other genes harbouring a *PrfA* box in their promoter region, and located elsewhere in the genome (Good *et al*., 2016; Portman *et al*., 2017).

Positive regulatory factor A(*PrfA*) gene is directly responsible for the transcription of 10 core virulence genes and indirectly affects the expression of over 140 others, many of which are essential for virulence (Lebreton and Cossart, 2017). The activity of *PrfA* is tightly regulated and only becomes activated upon entry into cells. This strict regulation is responsible for the transcript levels of the *PrfA*-dependent actin assembly-inducing protein *ActA* increasing over 200-fold in the host cytosol compared to its levels in broth cultures (Lebreton and Cossart, 2017).

A number of diverse factors influence *PrfA* activity, including temperature, osmolarity, and iron availability (Lebreton and Cossart, 2017).Positive regulatory factor A(*PrfA*) activity has also been linked to the nature of the sugar sources available to the bacteria (Portman *et al*., 2017). In the environment, the uptake of carbohydrates requires the

phosphoenolpyruvate phosphotransferase system (PTS) before they enter glycolysis; in contrast, in the host cell cytoplasm, bacteria can readily import phosphorylated hexoses, and use them in the pentose phosphate pathway (Portman*et al*., 2017).

During infection, the expression of the bacterial glutathione synthase gene (*gshF*) increases 10-fold; however, it is still not appreciated why *gshF* is unregulated in host cells or why exogenous glutathione (GSH) is insufficient to activate *PrfA* in traditional broth culture (Lebreton and Cossart, 2017; Portman *et al*., 2017).

2.11.2Haemolysin gene (*hlyA*)

Haemolysin gene is one of the most famous virulence factors found inpathogenic species of Listeria (*L. ivanovii* and *L. monocytogenes*), and is a key factor essential for their pathogenicity that involves escape of the pathogen into cytoplasm of the host as reported by O'Byrne and Utranta (2010) and Price *et al*. (2018).

The gene transcribed listeriolysin O (LLO) is a non-enzymatic, cytolytic, thiol-activated, cholesterol-dependent, pore-forming toxinprotein; hence, it is activated by reducing agents and inhibited by oxidizing agents. However, LLO differs from other thiol-activated toxins, since its cytolytic activity is maximised at a pH of 5.5. Listeriolysin is selectively activated within the acidic phagosomes (average pH ~ 5.9) of cells that have phagocytosed*L. monocytogenes* (Radoshevich and Cossart, 2017).

The listeriolysin toxin helps *L. monoctygenes* to lyse the phagocytic vacuole of the host. After LLO lyses the phagosome, the bacterium escapes into the cytosol, where it can grow intracellular (Radoshevich and Cossart, 2017). This allows the bacteria to live intracellular, where they are protected from extracellular immune system factors such as the complement system and antibodies. Upon release from the phagosome, the toxin has reduced activity in the more basic cytosol (Radoshevich and Cossart, 2017).

The expression of haemolysin (*hlyA*) geneis controlled by *PrfA* gene (the master regulator), when *L. monocytogenes* is growing intracellular. LLO is however not found in any abundance inside the cell cytosol, probably due to a combination of transcriptional control and a rapid degradation by host factors (Good*et al*., 2016).

2.12 Incubation Period of Listeriosis

The incubation period of listeriosis varies from 1 to 90 days (mean 30 days). There are two major clinical manifestations of listeriosis, sepsis and meningitis, which is often complicated by encephalitis (Musa, 2016). The onset of illness caused by listeriosis is typically marked by flu-like symptoms (fever and headache), and sometimes by nausea, stiff neck, confusion, weakness, vomiting and diarrhoea.

In some cases, these symptoms can develop into meningitis and septicaemia. Infection in pregnant women can lead to infection of the foetus, which can result in miscarriage, stillbirth, or the birth of an infected infant, although the mother usually survives the infection.The duration of illness varies from days to weeks (Musa, 2016).

2.13 Clinical Signs and Symptoms of Listeriosis

Clinically, listeriosis is one of the most recently recognised and least understood bacterial infections of man and animal. The clinical signs are not obvious especially in humans. However, a large number of patients with listeriosis often have underlying health conditions, which predispose them to listeriosis by interfering with T-cell-mediated immunity (Saha *et al*., 2015). In humans, symptomless faecal carriage is also common of pathogenic *Listeria* species (Suryawanshi, 2014).

The infection starts with sudden onset of fever, headache, nausea and vomiting, and other symptoms are followed by meningitis, pneumonia, septicaemia and endocarditis and localised abscesses (Suryawanshi, 2014; Saha *et al*., 2015; Oyinloye *et al*.,

2018).Invasive listeriosis is charaterised by the presence of the pathogen in the blood and in fluid of the central nervous system (Gezali *et al*., 2016).

The infection crosses the placenta and may lead to early-onset or late-onset neonatal listeriosis in the form of pneumonia, conjunctivitis, meningitis and otitis media (Saha *et al*., 2015). In pregnancy, abortion, stillbirth or premature labour may occur (Ikeh *et al*., 2010; Suryawanshi, 2014).

Listeriosis in animals is in two forms: the meningoencephalitis and visceral (like the liver, lungs, kidney, spleen and heart) (Suryawanshi, 2014; Saha *et al*., 2015; Scortti *et al*., 2018). The meningoencephalitic form involves neurological signs with dullness and somnolence. Other signs are dropping of saliva and lack of appetite and mastication, lateral deviation of the head with a tendency of cycling, paralysis with recumbency and death from respiratory failure (Chukwu, 2007).

The visceral form involves abortion with retained placenta. These presentations are often confused with other disease symptoms like hypocalcaemia, pregnancy toxaemia, meningitis and lopping ill in tick-infested areas (Suryawanshi, 2014). The symptoms of non-invasive listeriosis include fever, diarrhoea, muscle aches, nausea, vomiting, drowsiness, and fatigue. Non-invasive listeriosis is also known as listerial gastroenteritis or febrile listeriosis (Gezali *et al*., 2016)

2.14 Diagnosis of Listeriosis

Diagnosis is based on history, clinical signs, pathological lesions and detection of the pathogen. Definitive diagnosis can be made only after isolation and identification of the bacterium (Dhama *et al*., 2015). Isolation of *Listeria*spp is not much cumbersome as it can be readily isolated. However, difficulty may occur while recovering this pathogen from birds showing the encephalitic form of disease (OIE, 2014).

The ubiquitous nature, wide distribution and ability to survive for long periods outside the host's body present difficulty in concluding the source and spread of infection. Conventional methods for isolation of *Listeria*species, acceptable for international regulatory purposes, include the United States Food and Drug Administration (FDA) method, the Association of Official Analytical Chemists (AOAC) official method, the ISO 11290 standards, the United States Department of Agriculture (USDA)-Food Safety and Inspection Service (FSIS) method and the French Standards (OIE, 2014).

The preferred clinical samples for identification of the organism in culture include the brain tissue, lumbar cerebrospinal fluid (CSF), blood, liver, spleen, heart, aborted placenta and foetus, meconium of newborns, faeces, vomitus, and food/feed material (Scott, 2013; Dhama *et al*., 2015). Samples should be chosen according to clinical presentation: materials from the lesions in the liver, kidneys and/or spleen in case of septicaemic form; the spinal fluid, pons and medulla in case of encephalitic form; the placenta (cotyledons), foetal abomasal contents and/or uterine discharges in case of abortion. For isolation purposes, blood/tryptose agar or brain heart infusion medium are the best media (Dhama *et al*., 2015).

Traditional procedure for isolation of *Listeria* species from animal tissues includes direct plating of specimens on sheep blood agar or other rich culture media along with concomitant use of 'cold enrichment' technique and use of selective chromogenic listeria media. The organisms can be identified on the basis of Gram staining of smear/culture, motility, haemolysis, biochemical tests (catalase reaction), peroxide-anti-peroxide method, sugar utilisation, immunofluorescence test (Dhama *et al*., 2015; Usman *et al*., 2016a).

Immunohistochemical testing is mainly employed in locating antigen in lesions having few bacteria and is particularly employed to detect encephalitic listeriosis (Deb *et al*., 2013; Islam *et al*., 2016). Demonstration of listerial antigen in fixed tissues with lesions of septicaemic disease confirm listeriosis where culturing of organism is not feasible. Pathogenicity testing of *Listeria* isolates includes *in vitro* methods, viz., haemolysis on sheep blood agar, assay for phosphatidylinositol-specific phospholipase C (PI-PLC) activity, the CAMP test and *in vivo* tests, this include: inoculation of 3-week-old mice intra-peritoneally and 10- day-old chicken embryos through chorioallantoic membrane (CAM) route(Dhama *et al*., 2015).

Kaur *etal*. (2010) reported PI-PLC and polymerase chain reaction (PCR) as effective alternatives to in vivo pathogenicity test. Useful serodiagnostic tests are serum agglutination test, complement fixation test (CFT), haemagglutination (HA), haemagglutination inhibition (HI), antibody precipitation, growth inhibition test and enzyme linked immuosorbent assay (ELISA) (Dhama *et al*., 2013a; Benetti *et al*., 2014; OIE, 2014).

Detection of anti-haemolysin (LLO) antibodies by ELISA is employed for detection of septicaemic as well as abortion status of listeriosis. An avidin-biotin-based ELISA has been developed for detection of antibodies against LLO in milk samples of cattle with the assay showing potential as an epidemiological tool (Jeyaletchumi *et al*., 2010; Gorski*et al*., 2014; Leong *et al*., 2016).

Therefore, virulent markers/protein antigen, such as haemolysin gene, invasive associated protein, internalins (InlA, InlB, InlC, InlC2, Inl), actA protein (Jahangir *et al*., 2012), phospholipases C and autolysin p60 protein have been reported to be useful in diagnosis of listeriosis (Dhama *et al*., 2015). Among these, LLO has been

extensively found suitable for development of serological tests like ELISA in humans (Dhama *et al*., 2015). Protocols including conventional and non-conventional commercially available tests, e.g. Vitek, application programming interface (API), Microgen identification system, MICRO-ID (on the basis of the CAMP test), ELISAand nucleic acid assay kits have been developed for identification of *Listeria* species (Gorski *et al*., 2014).

For rapid detection of *Listeria* species in food matrices, lateral flow enzyme immune-chromatography together with an immunomagnetic separation has been developed recently (Cho and Irudayaraj, 2013). Molecular tools such as polymerase chain reaction (PCR), multiplex PCR and real-time PCR employing virulence-associated genes such as the *mpl* gene, *hlyA*, *prfA* gene and *ssrA* gene (Jin *et al*., 2012; Dhama *et al*., 2015) have been found rapid, specific, reproducible and reliable (Khan *etal*., 2013).

Several multiplex PCR assays have been developed for simultaneous detection of *Listeria*species (Lee *et al*., 2014). Multiplex real-time PCR assay based on molecular beacon chemistry was developed which could detect eight foodborne pathogens (Dhama *et al*., 2015). The combined use of ELISA, PCR and gene sequencing has been suggested for confirmatory diagnosis of listeriosis (Dhama*et al*., 2014; Verma *et al*., 2014).

2.15 Treatment of Listeriosis

The first and the best choice of treatment of listeriosis are β-lactam antibiotics (e.g. penicillin, ampicillin) alone (West and Tabansi, 2014) or in combination with aminoglycoside such as gentamicin, rifampin (Chen *et al*., 2010, Nyenje *et al*., 2012;Zeinali *et al*., 2017). The combination of trimethoprim with sulfanomide such as sulfamethoxazolein and co-trimoxazole is considered second choice of therapy.

Vancomycin and erythromycin are also used to treat bacteraemia in pregnant women diagnosed with listeriosis (Chen *et* al., 2010). However, various researchers (Nyenje, et al., 2012) have reported *Listeria* strains resistant to penicillin, ampicillin, erythromycin, streptomycin and tetracycline.

2.16 Mechanisms of Antibiotic Resistance among *Listeria* species

Antibiotic resistance in *Listeria* speciesis mainly due to acquisition of three types of movable genetic elements: self-transferable plasmids, mobilisable plasmids, and conjugative transposons (Van Meervenne *et al*., 2012). Efflux pumps were reported to be associated with fluoroquinolones (e.g. ciprofloxacin) resistance in *Listeria* species(Moreno*et al*., 2014; Wilson *et al*., 2018).

Enterococci and Streptococci, in particular, represent a reservoir of resistance genes for *Listeria* species. The gastrointestinal tract of humans is considered the most probable site where the acquisition, by *Listeria* spp., of conjugative plasmids and transposons from *Enterococcus-Streptococcus* takes place (Van Meervenne *et al*., 2012).Conjugation is a major mechanism used by *Listeria* species to acquire antibiotic resistance. The conjugative transfer of plasmids and transposons carrying antibiotic resistance genes from *Enterococcus* or *Streptococcus* to *Listeria*species and between *Listeria* species has been described by several studies (Van Meervenne *et al*., 2014; Wilson*et al*., 2018).

A broad-host-range plasmid, pIP510, first found in *Streptococcus agalactiae*, encodes resistance to chloramphenicol, macrolides, lincosamides and streptogramins. Plasmid pIP510 can be transfered by conjugation from *S. agalactiae* to *L. monocytogenes, L. murrayi*, and *L. grayi*. It replicates in *Listeria* species to promote its own transfer

between *Listeria* strains and back from *Listeria* speciesto *Streptococcus* species(Van Meervenne*et al*., 2012).

Transposon Tn916, initially discovered in *Enterococcusfaecalis*, is a broad-host-range conjugative transposon, encoding resistance to tetracycline and minocycline. Conjugative transfer of the Tn916-related transposon Tn1545, initially found in *S. pneumoniae*, was obtained from *E. faecalis* to *L. monocytogenes* in vitro and in vivo (Lungu*et al*., 2011). The conjugative transfer of a plasmid carrying the gene *vanA*, conferring resistance to glycopeptide antibiotics(e.g. vancomycin), from *E. faecalis* to *L. monocytognenes*, *L. ivanovi* and *L. welshimeri* has also been described (Lungu *et al*., 2011).

Most *Listeria* species or strains isolated from human and animal sources are believed to be resistant to tetracycline(Weldezgina and Muleta, 2016; Wilson *et al*., 2018). Conjugative plasmids and transposons originating from *Enterococcus-Streptococcus* are responsible for the emergence of resistance to tetracycline in *Listeria* speciesclinical isolates (Lungu*et al*., 2011). Plasmid pIP823, replicating by the rolling-circle (RC) mechanism, has a broad- host-range of both Gram-positive and Gram-negative bacteria.Chloramphenicol resistance can be conjugatively transferred through plasmids in the pC223 family (Uwanibe *et al*., 2014).

Antibiotic resistance mediated by efflux mechanisms was first reported in *L. monocytogenes* in 2000 (Scortti *et al*., 2018). The sequence of multidrug efflux transporter of *Listeria* (MdrL) protein is highly homological to the sequence of protein YfmO, a putative chromosomal multidrug efflux transporterof *B. subtilis*. An allele-substituted mutant of this gene in *L. monocytogenes* failed to pump out ethidium bromide and presented increased susceptibility to macrolides, cefotaxime and heavy

metals. Efflux pump in Listeria known as listeriadrug efflux (LDE) is associated with fluoroquinolone resistance in clinical isolates of *L. monocytogenes* (Buchanan *et al*., 2017).

The listeria drug efflux (LDE) protein showed 44% homology with pneumonia multidrug resistance (PMRA) of *Streptococcus pneumoniae*, which belongs to the major facilitator superfamily (MFS) of secondary multidrug transporters. The insertion inactivation of the gene *lde* results in increased susceptibility of fluoroquinolones (ciprofloxacin) in *Listeria* species (Lungu *et* al., 2011; Wilson *et al*., 2018).Antibiotic resistance in most bacteria can also be attributed to a mutation in an intrinsic chromosomal gene (Chen *et al*., 2010).

2.17 Preventive Measures of Listeriosis

Persons at increased risk, such as pregnant women and immunosuppressed adults, should be advised to avoid eating unpasteurised milk products and raw or partially cooked meat (Lekjing *et al*., 2017). Consumption of raw eggs may be a risk factor for listeriosis in some populations (Osman *et al*., 2014; Chen *et al*., 2017), and persons at risk should avoid these products. In addition, foods should be prepared without cross-contamination between raw and cooked foods, and vegetables should be washed carefully.

Foods prepared in a microwave oven may be unevenly heated, and sufficient standing time after cooking may be important to permit even distribution of heat by conduction without overcooking (Sreeja *et al*., 2016). Internal temperatures alone may not be adequate to ensure the safety of all microwaved foods, and new cooking guidelines may need to be established to minimise the risk of infection associated with eating microwaved foods (Zeinali *et al*., 2015).

Shredded vegetable salads and half-done vegetables should carefully be processed, because they harbour pathogens and serve also as source of listeria infection. Avoidance of under cooked meat is another preventive measure to prevent the spread of the pathogens in communities (Carrasco *et al*., 2008;Ajayeoba *et al*., 2015; Vallim *et al*., 2015). Therefore, reduction of nutritional exposure to high-risk or contaminated foods is the best ways to reduce the risk of listeriosis.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was conducted in Kaduna State, the North central part of Nigeria. Kaduna State is situated at 9^{o} 03' N and 11^{o} 32' N of equator and longitudes 6^{o} 05' E and 80^{o}38'E of the Greenwich meridian. These areas were located using Taiwan made Etrex® high-sensitive geographic positioning system (GPS) receiver. The entire population is around 6,066,562 according to 2006 national population census. The study area covered some markets including Unguwan Boro and Kakuri markets located in Central Kaduna. Other sampling locations included Samaru and Sabon Gari markets located in Sabon Gari Local Government Areain Northern Kaduna as presented in Figure 3.1 below. It was because of issue of insurgency Southern Kaduna was excluded in the study.

3.2 Determination of Sample Size

The sample size was determined using prevalence rate of 37.75% as reported by Eruteya *etal*. (2014) in Port Harcourt. The sample size was calculated using the formula as described by Ifeadike *et al*. (2012).

That is: $n = \frac{Z^2P(1-P)}{d^2}$

n = number of sample

p = prevalence rate of distribution of previous study = 37.75% = 0.3775

Z = standard normal distribution at 95% confident limit = 1.96

d = absolute desired precision of 5% = 0.05

Hence,

$n = \frac{1.96^2 \times 0.3775\ (1-0.3775)}{0.05^2} = 361$

The sample size for this study was increased to four hundred (400) samplesthis is to minimized statistical errors.

3.3 Study Design

The samples for this research included selected food samples such as raw beef (red meat), chicken, lettuce and cabbage. The choice of these food samples was informed by their daily consumption rate within the population. All the food samples were randomly collected from markets in each of the selected study areas.

3.4 Collection of Samples

In this research, 400 samples were collected; the samples were beef and chicken for meat products while vegetables included lettuce and cabbage. Hundred samples were apportioned evenly to the four locations of sampling. Thus, 100 of each sample were collected in a breakdown of 25 from each sample locations (Samaru, Sabon Gari, Kakuri and Ungwan Boro markets). The food samples were collected between the month of September, 2017 and June, 2018 and were transported in a sampling box containing ice pack to the Department of Microbiology, Ahmadu Bello University Zaria, for analysis.

3.5 Determination of Proximate Composition of the Food Samples

3.5.1 Moisture content

Moisture content was determined according to the method of Der-Jiun *et al*. (2012). A clean crucible was dried to constant weight in a hot air oven at 105^{o}C and then cooled in desiccators and was weighed (W1). Two grams of the food sample was weighed into the previously labeled crucible and reweighed (W2). The container was dried in hot air oven at 105^{o}C to constant weight (W3). The percentage moisture content was calculated as:

$$\text{Percentage moisture content} = \frac{W_3\text{-}W_1 \times 100}{W_2\text{-}W_1}$$

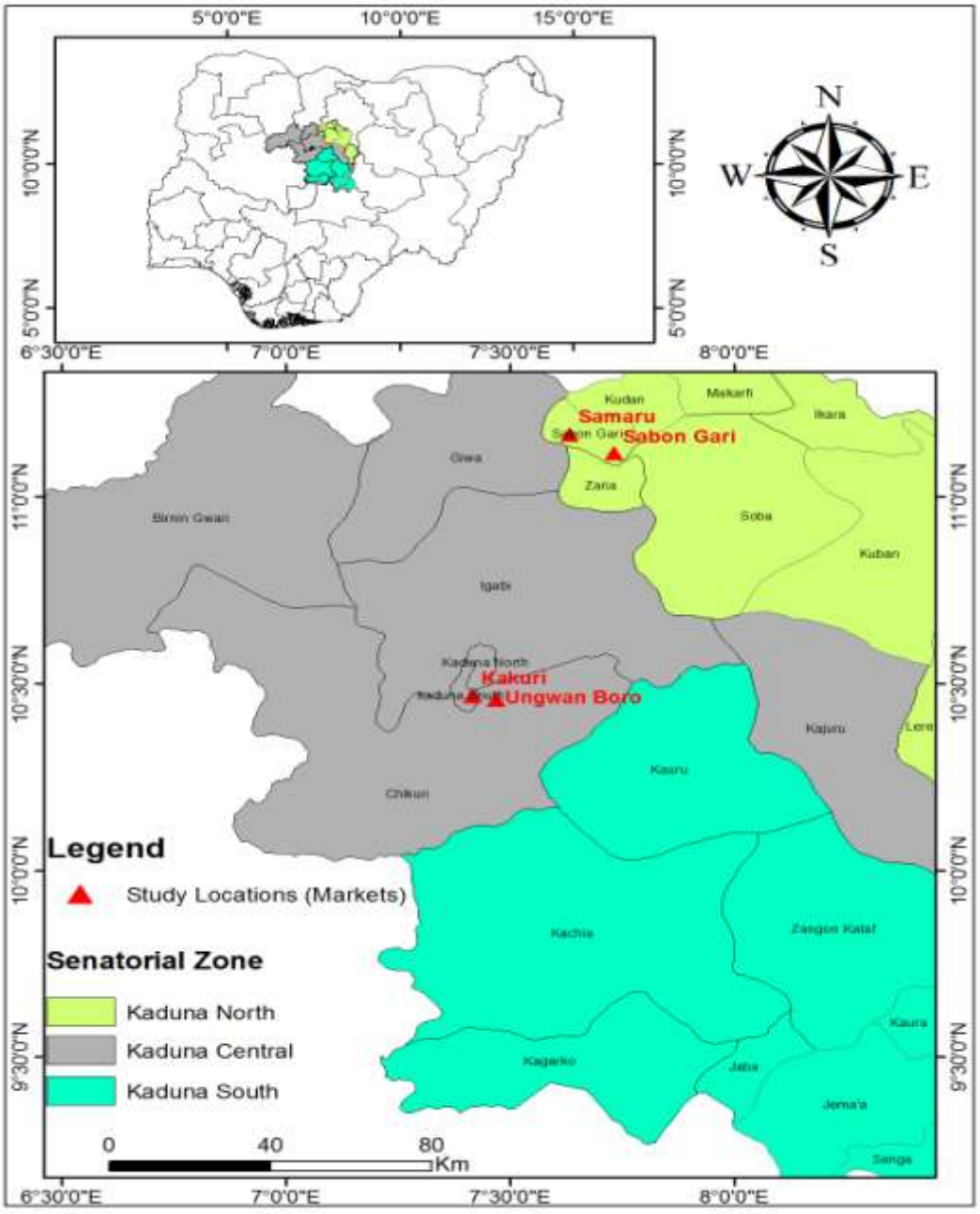

Figure 3.1: Map of Kaduna showing selected sampling locations (markets) (Bununu *et al*., 2015).

3.5.2 Ash content

Ash content was determined according to the method reported by Association of Analytical Chemists (AOAC)(2010) and Adeniyi *et al*. (2011). A porcelain crucible was dried in an oven at 100°C for 10 mins, it was cooled in a desiccator and weighed (W1). Two grams of the food was placed into the weighed crucible and was reweighed (W2). The sample washeated in the furnace at 500^{o}C for eight hours to ensure proper ashing. The crucible containing the ash was removed and cooled in desiccators and weighed (W_3). The percentage ash content was calculated as:

$$\text{Percentage ash content} = \frac{W_3 - W_1 \times 100}{W_2 - W_1}$$

3.5.3Crude protein

3.5.3.1*Protein Digestion*

The protein content was determined using the method adopted byBabalola and Akinsoyinu (2011).Briefly, 1.5g of defatted sample in an ashless filter paper was dropped into 300ml Kjeldahl flask. Twenty-five milliliters of H_2SO_4 and 3g of digesting mix catalyst (which was weighed separately into an ashless filter paper) was dropped into Kjeldahl flask. The flask wasthen transferred to Kjeldahl digestion apparatus. The sample was digested until a clear green colour was obtained. The digest was cooled and diluted to 100ml with distilled water.

3.5.3.2 *Distillation of the digest*

Twenty milliliters of the diluted digest was measured into a 500 ml Kjeldahl flask containing anti-bumping chips and 40 ml of 40% NaOH was slowly added by the side of the flask. A 250 ml conical flask containing a mixture of 50 ml of 2% of Boric acid and 4 drops of mixed indicator was used to trap the ammonia liberated. The conical flask and the Kjeldahl flask was then placed in the Kjeldahl distillation apparatus, with

the tubes inserted into the conical flask and the Kjeldahl flask. The flask was then heated to distill out the ammonia.The distillate was collected into boric acid solution. From the point when the boric acid turned green, 10 min was allowed for complete distillation of the ammonia present in the digest. The distillate was titrated with 0.1M HCl.

Calculation:

$$\text{Percentage N} = \frac{14 \times M14 \times Vt \times Tv \times 100}{\text{Weight of sample (mg)} \times V_s}$$

Where M = Actual molarity of acid, Tv = titre volume of acid used, Vt = Total volume diluted digest, Vs = Aliquot volume distilled.

The protein percentage was determined by conversion of nitrogen percentage of protein by using conversion factor (6.25)

Percentage crude protein = % Nitrogen (N_2) x 6.25

3.5.4Crude lipid content

A clean, dry 500ml round bottom flask, containing few anti-bumping granules was weighed (W_1) and 300ml of petroleum ether (40 - 60^{o}C) for extraction was poured into the flask fit with soxhlet extraction unit(AOAC, 2010). The extractor thimble containing twenty grams of the sample was fixed into the soxhlet extraction unit. The round bottom flask and a condenser were connected to the soxhlet extractor and cold water circulation was put on. The heating mantle was switched on and the heating rate was adjusted until the solvent was refluxed at a steady rate. Extraction was carried out for 6h. The solvent was recovered and the oil was dried in the oven at 70^{o}C for one hour (1h) (AOAC, 2010). The round bottom flask containing the oil was cooled in the desiccators and then weighed (W_2).

The lipid content was calculated thus:

Percentage crude Lipid Content = $\frac{W_2 - W_2 \times 100}{\text{Weight of sample}}$

3.5.5Carbohydrate content (by difference)

The total carbohydrate content was determined by difference. The sum of the percentage moisture, ash, crude lipid and crude protein was subtracted from 100 (Der-Jiun *et al.,* 2012).

Calculation:

Percentage total carbohydrate = 100 - (%Moisture + %Ash + %Fat + %Protein).

3.5.6 Crude fibre content

Two grams of the food was weighed into a round bottom flask, 100ml of 0.25M sulphuric acid solution was added and the mixture was boiled under reflux for 30 mins. The hot solution was quickly filtered under suction. The insoluble matter was washed several times with hot water until it was acid free. It was transferred into the flask and 100ml of hot 0.31M sodium hydroxide solution was added and the mixture was cooled under reflux for 30 mins and was quickly filtered under suction. The insoluble residue was washed with boiling water until it was base free. It was dried to constant weight in an oven at 100°C, cooled in desiccators and weighed (C_1). It was then incinerated in a muffle furnace at 550°C for 2 h. It was cooled in desiccators and reweighed (C_2) (AOAC, 2010). The crude fibre was calculated as:

The loss of weight on incineration = $\frac{C_1 - C_2 \times 100}{\text{Weight of original sample}}$

3.6 Total Bacterial CountDetermination

Total bacterial count was carried out using pour plate method as described by Kawo *et al.* (2012) and Owolabi and Ichokwu (2014). Twenty fivegram of the food sample was placed in sterile 225ml buffer peptone water to obtain a ratio of 1:10 and was thoroughly mixed, from which 1ml was transferred to the first test tube containing 9ml of buffer peptone water as diluent. This was repeated in other sets of the tubes containing 9ml of the buffer peptone water to dilute to 10^{-8}. The procedure was repeated for each sample. From the last two dilutions, 0.1ml each was dispensed to the center of Petri dishes. Prepared and cooled molten nutrient agar was poured into the Petri dish, gently swirl and allowed to solidified and incubated at 37^{o}C for 24h. At the end of incubation, plates with 30-300 colonies were counted using a colony counter and average was taken.

The colony forming unit was calculated as:

$$\text{CFU/ml} = \frac{\text{Mean count per plate} \times \text{dilution factor}}{\text{Volume of sample plated}}$$

3.7 Isolation of *Listeria*species

Twenty five gram of the food sample was inoculated into 225 ml of buffered bacteriological peptone water and incubated for 24h at ambient temperature. Twenty five millilitresof the buffered food sample was inoculated into 225 ml of *Listeria* selective enrichment broth medium to obtain a ratio of 1:10.*Listeria* enrichment supplement (Oxoid, SR0228) was added and incubated at 37^{o}C for 24h. A loopfull of the culture from the *Listeria* enrichment broth was subcultured onto Brilliance *Listeria* selective agar (Oxoid, CM1080) plates containing *Listeria* selective enrichment supplement (Oxoid, SR0142) and *Listeria* differential supplement (Oxoid, SR0228)and then was incubated at 37°C for 24h.

3.8 Gram Staining

Gram staining technique was carried out using a technique described by Cheesebrough (2009). From a 24hr culture, a colony was picked and emulsified in sterile distilled water on a slide and was heat fixed. The fixed smear was covered with crystal violet stain for 60 Sec. The stain was washed off rapidly with distilled water, and the slide was tilted off to remove the water and the smear was covered with Lugol‘s iodine for 60 Sec. The iodine was washed off with distilled water. Rapid decolourization was done with acetone-alcohol and washed immediately with distilled water. The smear was covered with Safranine for 60 Sec. The Stain was washed off with clean water and air dried. Morphology and Gram reactions of the isolates were determined by use of a microscope. Isolates, which appeared as Gram positive, rod shaped organisms, were further screened for biochemical tests such as catalase, oxidase, haemolysis, indole, motility, Voges-Poskauer (VP) test and were further identified using Microgen identification system.

3.9 Biochemical Characterisation of Presumptive *Listeria* isolates

The isolates of *Listeria* species were screened using some biochemical tests as described below.

3.9.1Catalase test

A drop of 3% hydrogen peroxide (H_2O_2) was placed on a clean dry slide. Then a fresh(24 h) colony of the *Listeria*species on nutrient agar medium was placed on the drop of hydrogen peroxide, positive result was indicated by air bubbles as a result of oxygen production, absence of air bubbles indicated negative result (Usman *et al*., 2016b).

3.9.2Oxidase test

A fresh (24h) purified colony was streaked on a piece of filter paper, and a drop of 1% colourless tetramethyl-p-phenylenediamine (TPD) reagent was added. A negative result was indicated by no colour change within 30 seconds.

3.9.3Haemolysis on sheep blood agar

A fresh (24h) pure colony was streaked on (7%) sheep blood agar, then wasincubated for 24 h at 37°C, a positive result was indicated by appearance of narrow zone of β-haemolysis around the colony (Usman *et al*., 2016a).

3.9.4Indole test

The colony of the 24h culture of the test organism was inoculated in a bijou bottle containing 3ml of sterile tryptone water. It wasincubated at 37°C for up to 48h and then was tested for production of indole by dispensing 0.5ml of Kovac's reagent. After it was gently mixed, a negative result showed no red colour in the surface layer within 10 mins (den Bakker *et al*., 2013).

3.9.5Methyl red (MR) test

A 24h culture of the bacterium was inoculated into a test tube of 3ml of glucose phosphate broth, which contained glucose and a phosphate buffer and was incubated at 37°C for 48h. The pH of the medium was tested for production of acid as a result of glucose fermentation by adding 5 drops of methyl red solution (0.04%). Positive result was indicated by development of red colour(MacFaddin, 2000).

3.9.6Voges-Poskauer (VP) test

A 24h culture of the bacterium was inoculated into a test tube of 3ml of glucose phosphate broth, which contain glucose and phosphate buffer and was incubated at 37°C for 48h. The culture was treated with 0.6ml of α-naphthol and shaken. Then 0.2ml

of 40% KOH was added to the broth. The tube was allowed to stand for 15mins positive result was indicated by colour appearance after allowed to stand for 1h; since maximum colour development occurs within one hour after addition of reagents (Suryawanshi, 2014**)**.

3.9.7Motility test

Small suspension of the pathogen from the 24h culture in *Listeria* enrichment broth was dropped on a slide and covered with a cover glass and then sealed with molten petroleum jelly to avoid drying. The preparation was examined microscopically for motile organisms using 10× and 40× objective lenses (Cheesbrough, 2009).

3.9.8Identification using microgen *Listeria* identification system

Twenty four hour cultured isolates of the *Listeria* spp were suspended or emulsified in a vial containing 2.5ml Listeria suspending medium. The suspension was prepared to form turbidity that match with 0.5 scale of McFarland's standard (1.5×10^8 cells/ml). The microwell test strip containing twelve different substrates was removed from the foil pouch and was placed in the holding frame and the lid was removed. With aid of sterile Pasteur pipette, 4 drops of the bacterial suspension was transferred to each well of the microwell strip. A drop of haemolysin reagent was added to well 12 for haemolytic activity. The lid was replaced onto the microwell test strip and was incubated at 37^oC for 24h. After the incubation, the lid was carefully removed from the microwell tests strip and the results were recorded. The tests on the report form which have been organised into triplets (sets of 3 reactions) were used to calculate the sum of the positive reactions; for each triplet forms a single digit of the octal code.The octal code was entered into the microgen identification system software version 1.2.5.26, which generated a report of the five most likely organisms from the database of *Listeria* species.

3.10 Genomic DNA Extraction

The genomic DNA of *Listeria ivanovii* was extracted using Bioneer extraction kit (South Korea). Briefly, a 24h culturewas carefully removed from a tryptone soy agar (TSA) and suspended into 200µL of 1 × PBS (phosphate buffered saline) at the concentration of 10^6 and was centrifuged for 5mins at 3000×g in a tube. The supernatant was carefully discarded without disturbing the pellet. The pellet was resuspended in 200µL of 1×PBS and proteinase K(20µL) and 10µL of RNase A was added and mixed thoroughly. The mixture was incubated in a tube for 2 mins at room temperature. Two hundred microliter of GB buffer was added to the sample and was mixedimmediately using votexing mixer. The mixture was incubated for 10 mins at60^oC using multi block heater. Four hundred microliter of absolute ethanol was added and mixed well by pipetting. The lysate was carefully transferred into upper reservoir of the binding column tube (fitted in a collection tube). The tube was centrifuged at 8000 rpm for 1 minute. The solution from the collection tube was discarded and the collection tube was reused. Five hundred microliter of WA1 buffer was added, the tube was centrifuged at 8000 rpm for 1 min, the solution was discarded from the collection tube and the collection tube was reused. Five hundred microliter (500µL) of W2 buffer was added and centrifuged at 8000 rpm for 1 min,it was centrifuged once more at 13000 rpm for 1 min to completely remove ethanol. The binding column tube was transferred to a new 1.5 ml tube for elution; fifty microliter of EA buffer was added into the binding column tube and allowed for 1 min at room temperature. It was centrifuged at 8000 rpm for 1 min to elute the DNA.

3.11Confirmation of *Listeria iavnovii* isolates using Polymerase Chain Reaction

The PCR reaction was carried using amplification of *Liv* gene (370bp) which code for putative glycosidase. The reaction was performed according to the method described by Miladi *et al*. (2013). The primers used are presented in Table 3.1. The reaction mixture (20µl) consisted of 2.0µl of each constituted primer (forward and reverse) (10pmol/µl), 0.8µl dNTP (10mMol), 3.0µl of 10× Taq polymerase (Prome®, USA), 1.8µl of $MgCl_2$ (Promega®, USA), 0.1µl of Taq polymerase (5U/µl) (Prome®, USA), 2.0µl of genomic DNA and the final volume was adjusted to 20µl by adding the remaining volume of nuclease free water. Amplification was proceeded which thermocycler (Gene AMP®PCR System 9700, AB Applied Biosystem, Singapore) with initial denaturation at 94^{o}C for 5 min, followed by 25 cycles of denaturation at 95^{o}C for 1 minute annealing temperature of primers was 52^{o}C for 1 minute and extension at 73^{o}C for 1 min. and final extension at 72^{o}C for 10 minutes.

3.11.1 Gel electrophoresis of *Liv* gene

The amplified PCR products (10µl) were resolved by electrophoresis in 1.5% agarose gel (Amresco Bioscience, USA) with Tris acetate electrophoresis buffer (TAE, 4.0mmol/l, Tris 1mmol/l EDTA, pH 8.0) at 105V for 35 minutethe gel was stained with ethidium bromide and finally visualised under UV transilluminator (BIO-RAD Molecular Imager Gel Doc™ Canada). The image was viewed in a computer for interpretations.

3.12 Standardisation of Inocula

The dilution of each of the suspension of the test isolates was prepared by picking a 24h colony of the isolates using sterile wire loop into sterile test tube containing sterile normal saline to form turbidity that match with 0.5 scale of McFarland's standard (1.5 × 10^8 cells/ml) (Coyle, 2005).

Table 3.1: Oligonucleotide primer sequence used for the amplification of *Liv, hlyA, tetA* and *tetM* genes.

Target gene	Primer sequence	Size(bp)	Reference
Liv gene	5'-GCT GAA GAG ATT GCG AAA GAA G-3' 3'-CAA AGA AAC CTT GGA TTT GCG G-5'	370	Zeinali *et al*., 2017
hlyA	5'-CCT AAG ACG CCA ATC GAA-3' 3'-AAG CGC TTG CAA GTC CTC -5'	702	Usman *et al*., 2016a
tetA	5'GGC GGT CTT CTT CTT CAT CAT GC-3' 3'CGG CAG GCA GAG CAA GTA GA-3'	501	Olowe *et al*., 2013
tetM	5'-GTG GAC AAA GGT AGA ACG AG-3' 3'-CGG TAA AGT TCG TCA CAC AC-5'	406	Hedayatianfard *etal*., 2014

3.13 Antibiotic Susceptibility Test

The antibiotic susceptibility pattern was determined using Kirby-Bauer-NCCLS modified single disc diffusion technique (Cheesbrough, 2009). The standardised inocula were inoculated by streaking on prepared Mueller-Hinton agar (Oxoid, CM0337) using sterile swab stick; the antibiotic disc was placed on the inoculated medium aseptically with the help of sterile forceps and incubated at 37°C for 24 h. The zones of inhibition cleared by each of the antibiotics against the test organisms were measured and the results were interpreted using the guideline from CSLI (2016) and all the results were recorded appropriately. Single antibiotic discs such as ampicillin (10 µg), vancomycin (30 µg), tetracycline (30 µg), clindamycin (2 µg), erythromycin (15 µg), ciprofloxacin (5 µg), rifampicin (5 µg), gentamicin (30 µg), amoxacillin (30 µg), chloramphenicol (30 µg), Cefoxitin (30 µg) and Oxacillin (1 µg) from Oxoid (England) each with expiry date of 4th February, 2021 were used.

3.14 Determination of Multiple Antibiotics Resistance (MAR) Index

Multiple antibiotics resistance was determined for each of the selected bacterial isolate using the formula MAR = X/Y where X is the number of antibiotics to which the test isolates displayed resistance to and Y is the total number of antibiotics to which the test organism has been evaluated for sensitivity (Tula *et al*., 2013).

3.15 Detection of *tetA*and *tetM*Genes using Multiplex Polymerase Chain Reaction

Multiplex PCR reaction was carried out to detect the presence of *tetA*(501bp) and *tetM*(406bp)genes in selected isolates of *Listeriaivanovii* that phenotypically exhibited resistance to tetracycline according to method described by Olowe *et al*. (2013) using

the primers (forward and reverse). The reaction mixture (25µl) for multiplex PCR contained 2.0µl of dNTPs (200µm/µl), 2.5 of 10× Taq buffer, 1.5µl of 25-Mm $MgCl_2$,0.5µl of each of oligonucleotide primers of tetA and tetM gene (25pm/µl) (reverse and forward), 0.75 Taq DNA polymerase (3U/µl) (Promega®, USA), 5.0µl of the template DNA (30ng/µl) and the volume was make up to 25µl using nuclease free water. The amplification was carrid out using a thermocycler (Gene AMP® PCR System 9700, AB Applied Biosystems, Singapore) with initial denaturation at 94^{o}C for 5 minutes followed by 30 cycles of denaturation at 94^{o}C for 45 Sec, annealing temperature of primers was 55^{o}C for 1 minute and extension at 72^{o}C for 1 minute and final extension at 72^{o}C for 5 minutes.

3.5.1 Gel electrophoresis of tetracycline resistance genes

The amplified PCR products (10µl) were resolved by electrophoresis in 1.5% agarose gel (containing 0.5µg/ml ethidium bromide) at 105V for 35 minute and finally visualized under UV transilluminator (Bio-Red Molecular Imager® Gel Doc™ Canada). The image was viewed in a computer for interpretations.

3.16 Detection of Haemolysin A (*hlyA*) Gene using Polymerase Chain Reaction

The PCR reaction was performed to detect presence of *hlyA* gene (720bp). The reaction was performed according to the method described by Usman *et al*. (2016a). The reactiobn mixture (20µl) for multiplex PCR contained 2.0µl of dNTPs (200µm/µl), 2.5 of 10× Taq buffer, 1.5µl of 25-Mm $MgCl_2$, 0.5µl of each of oligonucleotide primers of *tetA* and *tetM* gene (25pm/µl) (reverse and forward), 0.75 Taq DNA polymerase (3U/µl) (Promega®, USA), 5.0µl of the template DNA (30ng/µl) and the volume was make up to 20µl using nuclease free water. The amplification was carrid out using a thermocycler (Gene AMP® PCR System 9700, AB Applied Biosystems, Singapore)

with initial denaturation at 94^{o}C for 5 minutes, followed by 30 cycles of denaturation at 94^{o}C for 45 Sec, annealing temperature of primers was 55^{o}C for 1 minute and extension at 72^{o}C for 1 minute and final extension at 72^{o}C for 5 minutes.

3.6.1 Gel electrophoresis of *hlyA* gene

The amplified PCR products (10µl) were resolved by electrophoresis in 1.5% agarose gel (containing 0.5µg/ml ethidium bromide) at 105V for 35 minutes and finally visualized under UV transilluminator (Bio-Red Molecular Imager® Gel Doc™ Canada). The image was viewed in a computer for interpretations.

3.17 Data Analysis

The results obtained from this research were analyzed using the statistical package for social sciences (SPSS) Version 17.0 at 0.05 level of significance and 95% confidence interval. Analysis of variance (ANOVA) was used to check if there was significant difference in the mean percentage of proximate composition and the total bacterial count. The prevalence rate of the pathogen in the study areas was calculated in percentage. The results were recorded and presented in figures and tables.

CHAPTER FOUR

4.0 RESULTS

4.1 Proximate Composition of the Food Samples

The meanperecentage moisture content was found higher in lettuce (94.44±0.18) and the meanpercentage crude protein content was higher in chicken (22.48±0.56) and there was low mean percentage protein content in lettuce (0.45±0.07). The mean percentage protein content was higher in Chicken (22.46±0.56)and lettuce had lower mean protein content of 0.45±0.07.Cabbage had highestmean percentage content of crude fibre content (3.64±0.11). The meanpercentage content of ash was found higher in chicken (0.98±0.06).The mean percentage lipid contend of the food samples was higher in beef (6.87±0.23) and lower in lettuce (0.29±0.03) The mean percentage content of carbohydrate was higher in lettuce (3.15±0.08). There was significance difference in the proximate contents in the food sample from the selected locations except the ash content ($P < 0.05$) (Table 4.1).

The mean percentage moisture contentof beef from the four locations was 71.22±0.18, 70.17±0.23, 68.78±0.13 and 70.17±0.23 from Samaru, Sabon Gari, Kakuri and Ungwan Boro respectively. The mean percentage moisture content of cabbage from the four different locations was higher from Samaru (93.66±0.08). The mean percentage content of moisture in chicken was 71.93±0.23 (Samaru), 71.58±0.31 (Sabon Gari), 71.79±0.02 (Kakuri) and 71.68±0.31 (Ungwan Boro). The mean percentage moisture content in lettuce was 93.95±0.11 (Samaru), 94.40±0.16 (Sabon Gari), 94.83±0.12 (Kakuri) and 94.57±0.09 (Ungwan Boro). The data analysis using ANOVA showed that there was significant difference in the mean percentage moisture content in the food types from the different locations ($P = 0.001$) except in chicken (Table 4.2).

Table 4.1: Mean percentage proximate content of the selected foods

Parameter	Beef (±SEM)	Chicken (±SEM)	Cabbage (±SEM)	Lettuce (±SEM)	P-value
Moisture content	70.16±0.34	71.75±0.08	93.17±0.29	94.44±0.18	0.001*
Crude protein	21.56±0.76	22.48±0.56	0.54±0.05	0.45±0.07	0.001*
Crude fibre	0.00±0.00	0.00±0.00	3.64±0.11	0.78±0.02	0.001*
Ash content	0.88±0.09	0.98±0.06	0.86±0.03	0.92±0.03	0.523**
Lipid content	6.87±0.23	4.42±0.12	0.36±0.07	0.29±0.03	0.001*
Carbohydrate	0.48±0.09	0.49±0.07	1.44±0.14	3.15±0.08	0.001*

KEY: * = Significant at $P \leq 0.05$, SEM = Standard error of mean

Table 4.2: Mean percentage moisture content in the different food samples from different locations

Food type	No. of Sample	Percentage mean (±SEM) composition of moisture				
		Samaru	S/Gari	Kakuri	U/Boro	P-value
Beef	100	71.22±0.18	70.17±0.23	69.78±0.13	69.45±0.19	0.0001*
Cabbage	100	93.66±0.08	93.24±0.10	92.34±0.11	93.43±0.10	0.0001*
Chicken	100	71.93±0.23	71.58±0.31	71.79±0.02	71.68±1.19	0.9831**
Lettuce	100	93.95±0.11	94.40±0.16	94.83±0.12	94.57±0.09	0.0001*

KEY: * = Significant at $P \leq 0.05$, SEM = Standard error of mean

The meanpercentage ash content in each of the food samples from the four different locations as presented in Table 4.3 showed that beef from Samaru had higher ash content (1.15±0.05). Cabbage had higher mean percentage content of 0.96±0.01 from Ungwan Boro.The mean percentage ash content in chicken was 1.12±0.02 (Samaru), 1.00±0.05 (Sabon Gari), 0.81±0.02 (Kakuri) and 0.97±0.01 (Ungwan Boro). The mean percentage ash contnet in lettuce was 0.98±0.05, 0.92±0.03, 0.83±0.02 and 0.94±0.01 from Samaru, Sabon Gari, Kakuri and Ungwan Boro markets respectively. Chicken had higher percentage mean of ash than the other foods. The data analysis using ANOVA showed that there was significant difference in the mean percentage ash content in the food types from the different locations ($P < 0.05$)

The mean percentage protein content of protein in each of the food samples from the different locations in Table 4.4 indicates that, the protein content in beef was 19.59±0.17 (Samaru), 21.18±0.25 (Sabon Gari), 22.50±0.14 (Kakuri) and 22.97±0.20 (Ungwan Boro). The mean percentage protein content in cabbage from the four different locations was 0.69±0.04 (Samaru), 0.49±0.04 (Sabon Gari), 0.53±0.04 (Kakuri) and 0.45±0.00 (Ungwan Boro).

The mean percentage protein content of chicken based on locations were 21.77±0.20, 21.47±0.78, 22.710.23 and 23.96±0.21 from Samaru, Sabon Gari, Kakuri, Ungwan Boro markets respectively. The mean percentage protein content in lettuce based on locations were 0.64±0.06 (Samaru), 0.46±0.04 (Sabon Gari), 0.40±0.03 (Kakuri) and 0.30±0.02 (Ungwan Boro). The chicken and beef had more protein content, cabbage and lettuce had the least protein content in the foods. The data analysis also proved that there was a significant difference in the protein content in each of the food samples based on location ($P < 0.001$).

Table 4.3: Mean percentage ash content in different food samples from different locations

Food type	No. of Sample	Percentage mean (±SEM) composition of ash				
		Samaru	S/Gari	Kakuri	U/Boro	P-value
Beef	100	1.15±0.05	0.83±0.05	0.71±0.02	0.84±0.02	0.0001*
Cabbage	100	0.81±0.03	0.83±0.02	0.85±0.02	0.96±0.01	0.0001*
Chicken	100	1.12±0.02	1.00±0.05	0.81±0.03	0.97±0.01	0.0001*
Lettuce	100	0.98±0.05	0.92±0.03	0.83±0.02	0.94±0.01	0.007*

KEY: * = Significant at $P \leq 0.05$, SEM = Standard error of mean

Table 4.4: Mean percentage protein content in different food samples from different locations

Food type	No. of Sample	Percentage mean (±SEM) composition of protein				
		Samaru	S/Gari	Kakuri	U/Boro	P-value
Beef	100	19.59±0.17	21.18±0.25	22.50±0.14	22.97±0.20	0.0001*
Cabbage	100	0.69±0.04	0.49±0.04	0.53±0.04	0.45±0.00	0.0004*
Chicken	100	21.77±0.20	21.47±0.78	22.71±0.23	23.96±0.21	0.0001*
Lettuce	100	0.64±0.06	0.46±0.04	0.40±0.03	0.30±0.02	0.0001*

KEY: * = Significant at $P \leq 0.01$, SEM = Standard error of mean

The mean percentage lipid content in beef from Sabon Gari was higher (7.30±0.07) and the mean percentage lipid content in beef from Ungwan Boro was 6.36±0.12. The mean percentage of lipid from cabagge was higher (0.55±0.04) and cabbage from Samaru had low lipid content (0.22±0.02). There was significance difference in the mean percentage lipid content proximate content in the food sample from the selected locations ($P < 0.05$)(Table 4.5).

The mean percentage lipid content in chicken was 4.74±0.12, 4.49±0.10, 4.22±0.13 and 4.28±0.10 from Samaru, Sabon Gari, Kakuri and Angwan Boro markets respectively. Lettuce had mean percentage lipid composition of 0.37±0.03 (Samaru), 0.24±0.04 (Sabon Gari), 0.24±0.02 (Kakuri) and 0.31±0.02 (Ungwan Boro). The statistical analysis showed that there was a significant difference in the mean composition of lipid in the food samples from the four different locations ($P < 0.05$).

The mean percentage carbohydrate content in beef from the sample locations was 0.73±0.10 (Samaru), 0.52±0.04 (Sabon Gari), 0.3±0.02 (Kakuri) and 0.38±0.03 (Ungwan Boro). Cabbage had mean carbohydrate content of 1.16±0.07, 1.54±0.07, 1.79±0.05 and 1.25±0.05 from Samaru, Sabon Gari, Kakuri and Ungwan Boro markets respectivel (Table 4.6). The mean percentage carbohydrate content of chicken was 0.47±0.06, 0.66±0.05, 0.52±0.03 and 0.31±0.02 from Samaru, Sabon Gari, Kakuri and Ungwan Boro respectively. Lettuce had carbohydrate mean content of 3.31±0.07 (Samaru), 3.18±0.09 (Sabon Gari), 2.94±0.10 (Kakuri) and 3.17±0.07 (Ungwan Boro). Lettuce had the highest content of carbohydrate with beef and chicken having the least.

Table 4.5: Mean percentage lipid content in different food samples from different locations

Food type	No. of Sample	Percentage mean (±SEM) composition of lipid				
		Samaru	S/Gari	Kakuri	U/Boro	P-value
Beef	100	7.23±0.11	7.30±0.07	6.60±0.12	6.36±0.12	0.0001*
Cabbage	100	0.22±0.02	0.28±0.02	0.55±0.04	0.38±0.02	0.0065*
Chicken	100	4.74±0.12	4.49±0.10	4.22±0.13	4.28±0.10	0.0001*
Lettuce	100	0.37±0.03	0.24±0.04	0.24±0.02	0.31±0.02	0.0024*

KEY: * = Significant at $P \leq 0.05$, SEM = Standard error of mean

Table 4.6: Mean percentage carbohydrate content in different food samples from different locations

Food type	No. of Sample	Percentage mean (±SEM) composition of carbohydrate				
		Samaru	S/Gari	Kakuri	U/Boro	P-value
Beef	100	0.73±0.10	0.52±0.04	0.30±0.02	0.38±0.03	0.0001*
Cabbage	100	1.16±0.07	1.54±0.07	1.79±0.05	1.25±0.05	0.0001*
Chicken	100	0.47±0.06	0.66±0.05	0.52±0.03	0.31±0.02	0.0001*
Lettuce	100	3.31±0.07	3.18±0.09	2.94±0.10	3.17±0.07	0.0204*

KEY: * = Significant at P ≤ 0.05, S/Gari = Sabon Gari, U/Boro = Ungwan Boro, SEM = Standard error of mean

The mean percentage content of fibre in each of the food samples from the four different locations is presented in Table 4.7. There was no fibre in the raw beef and chicken from the four different sampling locations. The mean perecentage fibre content in cabbage was appreciably higher in cabbage from Kakuri (3.94±0.07) and the mean percentage fibre content in lettuce was 0.78±0.03, 0.82±0.25, 0.77±0.02 and 0.73±0.02 from Samaru, Sabon Gari, Kakuri and Ungwan Boro markets respectively. Data analysis showed that there was a significant difference in the fibre content of cabbage from the four different locations (P = 0.0001) and no significant difference in the fibre content of lettuce from the four different sampling locations (P = 0.2835).

4.2 Mean Total Bacterial Count

The mean total bacterial count in food samples from Samaru market were $1.35\times10^{7}\pm5.510^{5}$cfu/g (beef),$1.42\times10^{7}\pm6.110^{5}$cfu/g (cabbage),$1.66\times10^{7}\pm4.7\times10^{5}$cfu/g (chicken) and $1.37\times10^{7}\pm5.7\times10^{5}$cfu/g (lettuce) as presented in Table 4.8.

The mean total bacterial counts of food samples from Sabon Gari market were $1.25\times10^{7}\pm4.5\times10^{5}$cfu/g (beef), $1.43\times10^{7}\pm4.710^{5}$cfu/g (cabbage), $1.35\times10^{7}\pm4.7\times10^{5}$cfu/g (chicken) and $1.30\times10^{7}\pm4.2\times10^{5}$cfu/g (lettuce). The food samples collected from Kakuri market had mean total bacterial count of $1.40\times10^{7}\pm5.3\times10^{5}$cfu/g (beef), $1.33\times10^{7}\pm2.6\times10^{5}$cfu/g (cabbage), $1.35\times10^{7}\pm4.7\times10^{5}$cfu/g (chicken) and $1.52\times10^{7}\pm4.9\times10^{5}$cfu/g (lettuce).

The mean total bacterial count of the meat and vegetables from Ungwan Boro market were $1.43\times10^{7}\pm3.1\times10^{5}$cfu/g (beef), $1.36\times10^{7}\pm3.5\times10^{5}$cfu/ml (cabbage), $1.43\times10^{7}\pm4.2\times10^{5}$cfu/g (chicken) and 1.5×107±5.8×105cfu/g (lettuce).

Table 4.7: Mean percentage crude fibre content in different food samples from differentlocations

Food type	No. of Sample	Percentage composition mean(±SEM) of fibre				
		Samaru	S/Gari	Kakuri	U/Boro	P-value
Beef	100	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	
Cabbage	100	3.51±0.04	3.62±0.06	3.94±0.07	3.48±0.07	0.0001*
Chicken	100	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	
Lettuce	100	0.78±0.03	0.82±0.25	0.77±0.02	0.73±0.02	0.2835**

KEY: * = Significant at $P \leq 0.05$, SEM = Standard error of mean

Table 4.8: Mean values of total bacterial count (TBC) in the food samples (n = 100)

Food type	No. of Samples	Mean (±SEM) total bacterial count(cfu/g)				
		Samaru	Sabon Gari	Kakuri	U/Boro	P-value
Beef	100	$1.35\times10^{7}\pm5.5\times10^{5}$	$1.25\times10^{7}\pm4.7\times10^{5}$	$1.40\times10^{7}\pm5.3\times10^{5}$	$1.43\times10^{7}\pm3.1\times10^{5}$	0.0402*
Cabbage	100	$1.42\times10^{7}\pm6.1\times10^{5}$	$1.43\times10^{7}\pm6.1\times10^{5}$	$1.33\times10^{7}\pm2.6\times10^{5}$	$1.36\times10^{7}\pm3.5\times10^{5}$	0.0419*
Chicken	100	$1.66\times10^{7}\pm4.7\times10^{5}$	$1.35\times10^{7}\pm4.7\times10^{5}$	$1.35\times10^{7}\pm4.7\times10^{5}$	$1.34\times10^{7}\pm4.2\times10^{5}$	0.0001*
Lettuce	100	$1.37\times10^{7}\pm5.7\times10^{5}$	$1.30\times10^{7}\pm4.2\times10^{5}$	$1.52\times10^{7}\pm4.9\times10^{5}$	$1.50\times10^{7}\pm5.8\times10^{5}$	0.0096*

KEY: * =Significant at $P \leq 0.05$

There was a significant difference in the mean values of total bacterial count in the food samples accoreding to locations ($P < 0.05$) except the food samples from Sabon Gari in which there was no significant difference in the mean total bacterial count ($P = 0.0587$). The mean total bacterial load in beef according to location was $1.35\times10^7\pm5.5\times10^5$cfu/g (Samaru), 1.25×10^7 $\pm4.7\times10^5$cfu/g (Sabon Gari), 1.40×10^7 $\pm5.3\times10^5$cfu/g (Kakuri), $1.43\times10^7\pm3.1\times10^5$cfu/g (Ungwan Boro). The mean total bacterial count in cabbage according to location was $1.42\times10^7\pm6.1\times10^5$cfu/g, $1.43\times10^7\pm6.1\times10^5$cfu/g, $1.33\times10^7\pm2.6\times10^5$cfu/g and $1.36\times10^7\pm3.5\times10^5$cfu/g from Samaru, Sabon Gari, Kakuri and Ungwan Boro markets respectively. The mean total bacterial count in chicken from Samaru, Sabon Gari, Kakuri and Ungwan Boro was $1.66\times10^7\pm4.7\times10^5$cfu/g, $1.35\times10^7\pm4.7\times10^5$cfu/g, $1.35\times10^7\pm4.7\times10^5$cfu/g and $1.34\times10^7\pm4.2\times10^5$cfu/grespectively.

The mean bacterial count of lettuce collected for this research was $1.37\times10^7\pm5.7\times10^5$cfu/g (Samaru), $1.30\times10^7\pm4.2\times10^5$cfu/ml (Sabon Gari), $1.52\times10^7\pm4.9\times10^5$cfu/g (Kakuri) and $1.50\times10^7\pm5.8\times10^5$cfu/g (Ungwan Boro). There was high population of bacteria in all the food samples selected for this research above 1.0×10^7cfu/g stipulated by the Codex Alimentarius Commission. The statistical analysis showed that there was a significant difference in the total bacterial counts of the meat and the lettuce ($P < 0.05$) except cabbage that showed that there was significant difference in the total bacteria count in cabbage ($P = 0.0419$).

4.3 Biochemical Characterisation of *Listeria* species using Microgen Kits

The biochemical characterisation of *Listeria* species using microgen kit based on sugar fermentation capacities of the isolates showed that 10 of *Listeria ivanovii* had percentage probability identification at 99.03% with octal code of 5752. Nine of the *Listeria ivanovii* had percentage probability identification of 95.31% with octal code of 5643. Five isolates of *L. ivanovii* were identified at 95.91% with octal code of 4657. Some isolates of *L. ivanovii* had

percentage probability identification at 99.96% (octal code 4454) and 99.87% (octal code 4645) respectively. Other isolates of *L. ivanovii* were identified at 99.79, 98.23 and 97.15% with octal codes 5757, 4641, 4653 and 5756 respectively. Four isolates of *Listeria grayi* and two isolates of the same species were identified at 96.47% (octal code 4646) and 99.55% (octal code 4643) respectively. *Listeria welshimeri* was identified at percentage probability of 94.32% with octal code of 4561 (Table 4.9).

4.4 Prevalence of *Listeria* species in the Food Samples

The distribution of *Listeria* species isolated from the food samples is presented in Table 4.10. Of the 400 samples collected, the overall prevalence of all the *Listeria* species in the food samples selected is 9.75%(39/400) with *Listeria ivanovii* having high total prevalence of 8.0%(32/400), *Listeria grayi* 1.5%(6/400) and *Listeria welshimeri* 0.25%(1/400). The prevalence of *Listeria* species in the food samples was appreciably highest in chicken 15.0%(15/100). The prevalence of the *Listeria* species in the food was low in beef 6.0%(6/100) and cabbage 6.0%(6/100). *Listeriaivanovii* was highest in chicken 12.0%(12/100). *Listeriagrayi* was isolated from chicken and lettuce but was not found in beef and cabbage.

The prevalence of *Listeria* species in food samples based on locations is presented in Table 4.11. *Listeria* species had a total prevalence of 10.0%(10/100) in Samaru, 12.0%(12/100) in Sabon Gari, 8.0%(8/100) in Kakuri and 9.0%(9/100) in Ungwan Boro. *Listeria ivanovii* had highest percentage prevalence of 9.0%(9/100) Sabon Gari. *Listeria grayi* had highest percentage prevalence of 2.0%(2/100) from Samaru and Sabon Gari markets.

Table 4.9: Phenotypic characterisation of *Listeria* spp. using microgen kit

Esc	Man	Xyl	Ara	Rib	Rha	Tre	Tag	G-1-P	M-D-Glu	M-D-Man	Hae	Octal code	Probability(%)	Inference organism(No.)
+	-	+	+	+	+	+	-	+	-	+	-	5752	99.03	*L. ivanovii* (10)
+	-	+	+	+	-	+	-	-	-	+	+	5643	95.31	*L. ivanovii*(9)
+	-	-	+	+	-	+	-	+	+	+	+	4657	95.91	*L. ivanovii* (5)
+	-	-	+	-	-	+	-	+	+	-	-	4454	99.96	*L. ivanovii* (2)
+	-	-	+	+	-	+	-	-	+	+	-	4646	96.47	*L. grayi* (4)
+	-	+	+	+	+	+	-	+	+	+	+	5757	99.79	*L. ivanovii* (1)
+	-	-	+	+	-	+	-	-	-	-	+	4641	98.23	*L. ivanovii* (1)
+	-	-	+	+	-	+	-	+	-	+	+	4653	97.21	*L. ivanovii* (1)
+	-	-	+	+	-	+	-	-	+	-	+	4645	99.87	*L. ivanovii* (2)
+	-	+	+	+	+	+	-	+	+	+	-	5756	97.15	*L. ivanovii*(1)
+	-	-	+	+	-	+	-	-	-	+	+	4643	99.55	*L. grayi* (2)
+	-	-	+	-	+	+	+	-	-	-	+	4561	94.31	*L. welshimeri* (1)

KEY: Esc = Esculin, Man = Mannitol, Xyl = Xylose, Ara = Arabitol, Rib = Ribose, Rha = Rhamnose, Tre = Trehalose, Tag = Tagatose, G-1-P = Glucose-1-Phosphate, M-D-Glu = Methyl-D-Glucose, M-D-Man = Methyl-D-Mannose, Hae = Haemolysin

Table 4.10: Prevalence of *Listeria* species in different food samples

Food Sample type	No.of samples	No. (%) of positive isolates of *Listeria* spp.	*Listeria* species			
			No.(%) of *L. ivanovii*	No.(%) of *L. monocytogenes*	No.(%) of *L. grayi*	No.(%) of *L. welshimeri*
Beef	100	6(6.0)	5(5.0)	0(0.0)	0(0.0)	1(1.0)
Chicken	100	15(15.0)	12(12.0)	0(0.0)	3(3.0)	0(0.0)
Cabbage	100	6(6.0)	6(6.0)	0(0.0)	0(0.0)	0(0.0)
Lettuce	100	12(12.0)	9(9.0)	0(0.0)	3(3.0)	0(0.0)
Total	400	39(9.75)	32(8.0)	0(0.0)	6(1.5)	1(0.25)

Table 4.11: Prevalence of *Listeria* species isolated from different sampling locations

Isolate	Location(%) (n = 100)			
	Samaru	S/Gari	Kakuri	U/Boro
Listeriaivanovii	8(8.0)	9(9.0)	7(7.0)	8(8.0)
Listeriamonocytogenes	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Listeriagrayi	2(2.0)	2(2.0)	1(1.0)	1(1.0)
Listeriawelshimeri	0(0.0)	1(1.0)	0(0.0)	0(0.0)
Total	10(10.0)	12(12.0)	8(8.0)	9(9.0)

S/Gari = Sabon Gari, U/Boro = Ungwan Boro

Listeriawelshimeri was isolated from Sabon Gari market (1.0%) (1/100). There was no *Listeriamonocytogenes* from any of the food samples.

4.5 Antibiotic Susceptibility Pattern of *Listeria* species

The antibiotic susceptibility studies of *Listeria* species showed that *Listeriaivanovii* highly resistant to oxacillin (34%) and 100% susceptible to ciprofloxacin and rifampicin. *Listeria grayi* isolates were 33% resistant to cefoxitin, clindamycin and oxacillin and were 100% susceptible to amoxicillin/clavulate, vancomycin, gentamicin, ciprofloxacin and rifampicin. *Listeria welshimeri* was susceptible to 9 of the 12 antibiotics (Table 4.12).

The antibiotic resistance patterns of the *Listeria* speciesas shown in Table 4.13 showed that: three isolates of *Listeria ivanovii* were resistant to clindamycin; sevenisolates were resistant to two different antibiotics. Fourisolates were resistant to three different antibiotics, another four isolates were resistant to four different antibiotics and oneof the isolate was resistant to five different antibiotics. One of the isolates of *Listeria grayi* was resistant to clindamycin, another isolate was resistant to three antibiotics and the other isolate was resistant to four antibiotics.

4.6 Multiple Antibiotic Resistances of the Isolates

The multiple antibiotic resistance (MAR) indices of the *Listeria* spp as shown in Table 4.14 indicates that isolates of *Listeria ivanovii* had MAR indices of 0.17, 0.25, 0.33 and 0.41. *Listeria grayi* had MAR indices of 0.25 and 0.33. The multi-drug resistance *Listeriaivanovii* isolates were 50% (11/22) resistant and *Listeriagrayi* isolates were 66.7%(2/3) were found to be multidrug resistant. In this study, 61.1%(11/18) *Listeria ivanovii* had MAR index above 0.20 antibiotic resistance significant level.

Table 4.12: Antibiotic susceptibility of *Listeria* species isolated from the selected foods

S/N	Antibiotics	*Listeria ivanovii* (n=32)			*Listeria grayi* (n=6)			*Listeria welshimeri* (n=1)		
		R(%)	I(%)	S(%)	R(%)	I(%)	S(%)	R(%)	I(%)	S(%)
1	Ampicillin (10 µg)	8(25)	-	24(75)	1(17)	-	5(83)	0(0)	-	1(100)
2	Tetracycline (30 µg)	7(22)	3(9)	22(69)	0(0)	1(17)	5(83)	0(0)	0(0)	1(100)
3	Erythromycin (15 µg)	4(13)	7(22)	21(66)	0(0)	2(33)	4(67)	0(0)	1(100)	0(0)
4	Amoxicillin/Clavulate(30 µg)	5(16)	-	27(84)	0(0)	-	6(100)	0(0)	-	1(100)
5	Cefoxitin (30 µg)	6(19)	-	26(81)	2(33)	-	4(67)	0(0)	-	1(100)
6	Chloramphenicol (30 µg)	0(0)	5(16)	27(84)	1(17)	0(0)	5(83)	0(0)	0(0)	1(100)
7	Vancomycin (30 µg)	0(0)	2(6)	30(94)	0(0)	0(0)	6(100)	0(0)	0(0)	1(100)
8	Clindamycin (2 µg)	10(31)	11(34)	11(34)	2(33)	4(67)	0(0)	0(0)	1(100)	0(0)
9	Gentamicin (10 µg)	2(6)	0(0)	30(94)	0(0)	0(0)	6(100)	0(0)	0(0)	1(100)
10	Oxacillin (1 µg)	11(34)	-	25(78)	2(33)	-	4(67)	0(0)	-	1(100)
11	Ciprofloxacin (5 µg)	0(0)	0(0)	32(100)	0(0)	0(0)	6(100)	0(0)	0(0)	1(100)
12	Rifampicin (5 µg)	0(0)	0(0)	32(100)	0(0)	0(0)	6(100)	0(0)	1(100)	0(0)

R = Resistant, I = Intermediate, S= Susceptible (CLSI, 2016)

Table 4.13: Antibiotic resistancepatterns of the *Listeria* species

	Listeriaivanovii		*Listeriagrayi*	
S/N	ResistancePattern	Frequency	Resistance Pattern	Frequency
1	DA	3	DA	1
2	TE	3	FOX, DA, OX	1
3	DA, OX	4	AMP, C, FOX, OX	1
4	E, DA,	1		
5	AMP, FOX	1		
6	AMP, AMC	1		
7	OX, FOX, E	1		
8	OX, AMP, DA	1		
9	AMP, AMC, TE	1		
10	OX, FOX, E	1		
11	CN, DA, E, TE	1		
12	OX, TE, CN, AMP	1		
13	AMC, AMP, OX, FOX	2		
14	FOX, OX, AMC, AMP, TE	1		

AMP = Ampicillin, TE = Tetracycline, E = Erythromycin, AMC = Amoxicillin/Clavulate(Clavulanic acid), FOX = Cefoxitin, C = Chloramphenicol, VA = Vancomycin, DA = Clindamycin, CN = Gentamicin, OX = Oxacillin

Table 4.14: Multiple antibiotic resistance (MAR) indices of the *Listeria* species

Organism	No. of multi-resistant isolates	Antibiotic combination	MAR index
Listeria ivanovii	7	2	0.17
	4	3	0.25
	4	4	0.33
	1	5	0.42
Listeria grayi	1	3	0.25
	1	4	0.33

4.7 Polymerase Chain Reaction Amplification of Target Genes

The agarose gel electrophore amplification of the 370bp *Listeriaivanovii* gene (Plate I) showed that amplicons in lanes 1, 2, 4 and 6 were positive for *Listeria ivanovii* as presented in Plate I. The agarose gel electrophoresis of multiplex PCR of amplified *tetA* (501bp) and *tetM* (406bp) genes in some of the isolates is presented in Plate II. Of the seven isolates of *Listeria ivanovii* considered for the screening for *tetA* gene, isolates from chicken and beef at lane 1 and 6 respectively had *tetA* resistance gene. Other amplicons shown in lanes 2(chicken), 4(lettuce) and 7(beef) are PCR products of *tetM* gene, which is also a common tetracycline resistance determinant among the isolates. Isolates from chicken (lane 3) and lettuce (lane 5) had none of the tetracycline resistance genes.

Out of the seven isolates of *Listeriaivanovii* considered for the screening for virulence genes using polymerase chain reaction, 14.3% isolate from beef at lane 6 had haemolysin gene (*hlyA*) with amplicon size of 720bp as presented in Plate III. Isolates from chicken (lanes 1-3), lettuce (lanes 4 and 5) and one of the beef sample (lane 7) had no haemolysin (*hlyA*) gene. The distribution of *tetA, tetM* and *hlyA* genes among the selected *Listeriaivanovii* indicate that 28.6% had *tetA* gene, 42.86% had *tetM* gene and 14.3% had *hlyA* gene (Table 4.15).

Plate I: Agarose gel electrophoresis of amplified *Liv* gene (370bp) of the isolates from the food samples.

Key: Lane M: DNA ladder (100bp), Lane 1, 2, 4 and 6 were the positive isolates, -ve: negative control.

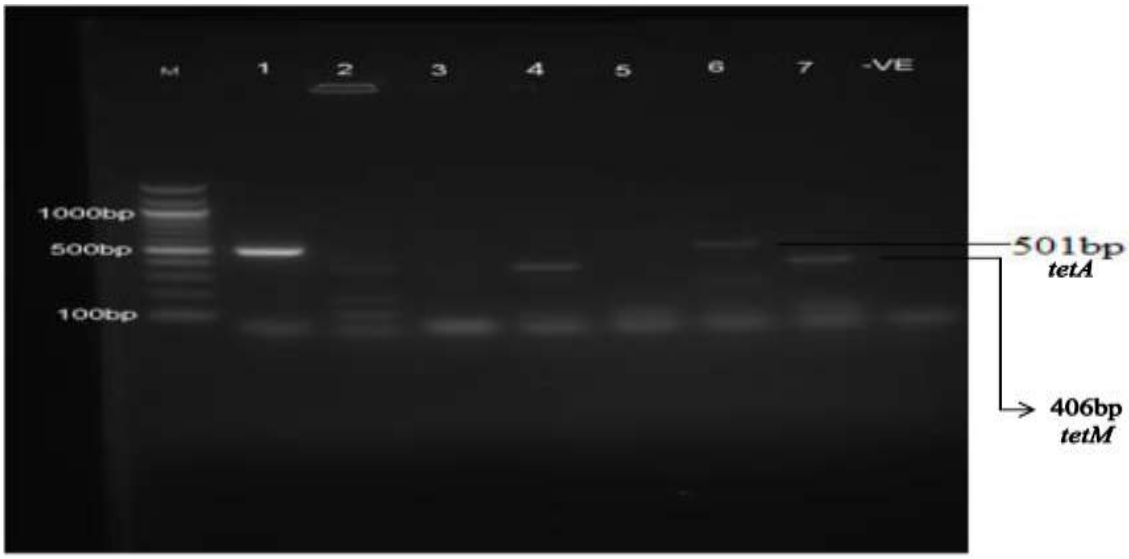

Plate II: Agarose gel electrophoresis of multiplex PCR of amplified *tetA*(501bp) and *tetM* (406bp) genesfrom some*Listeria ivanovii* isolates.

Key: M: DNA ladder (100 bp); -ve: negative control. Lane 1 and 6 show the presence of *tetA* gene; Lane 2, 4 and 7 show the presence of *tetM* gene Lanes 3, and 5 shows the absence of tetracycline resistance gene.

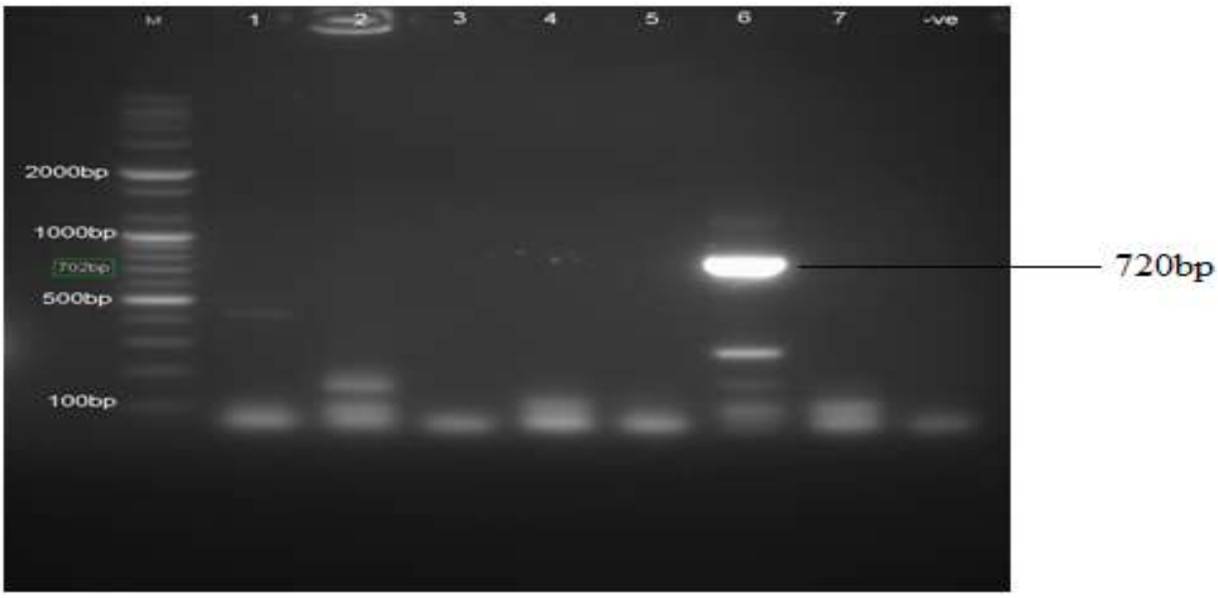

Plate III: Agarose gel electrophoresis of amplified *hlyA* gene (720bp) from some *Listeria ivanovii* isolates.

Key: M: DNA ladder (100bp); -ve: negative control; Lane 6 shows the presence of *hlyA* gene; Lanes 1, 2, 3, 4, 5 and 7 shows the absence of *hlyA* gene.

Table 4.15: Distributions of *tetA*,*tetM*and *hlyA* genes in *Listeria ivanovii* isolated from the food samples

Sample type	No. of Isolates Examined	No. of positive *hlyA* gene (%)	No. of positive *tetA* gene (%)	No. of positive *tetM* gene (%)
Beef	2	1(50)	1(50)	1(50)
Chicken	3	0(0.0)	1(33.3)	1(33.3)
Lettuce	2	0(0.0)	0(0.0)	1(50)
Total	7	1(14.3)	2(28.6)	3(42.86)

CHAPTER FIVE

5.0 DISCUSSION

Moisture content in foods is one of the major intrinsic factors thatplay important role in distribution of microorganisms in foods and the environment. In this research, there was appreciable high moisture content in all the food samples from the four sampling locations. This is one of the factors that can encourage the multiplication and survival of *Listeria* species in the food samples (Byrne *et al*., 2016; Tayyeb *et al*., 2017).

Moisture is a medium that carries molecules into cytoplasm and out of cell through permeable membrane (Adeniyi *et al*., 2011). Moisture isan important factor that is mostly considered in preservation of foods from microbial spoilage. It has also been noted that the amount of moisture in the vegetables affects the nutrient concentration in the vegetables (Nwachukwu and Idah, 2015).

In this research, the significant difference in the moisture content in the food samples ($P < 0.05$) might be due to nature of the food samples since the proximate contents in foods are generally not the same as also reported by Hussain *et al*. (2016).The moisture content reported here in the raw beef corroborates the report of Fakolade (2012) who reported moisture content of beef as 76.58±1.75; Tayyeb *et al*. (2017) reported 93.02±0.63 of moisture content in cabbage. The findings in this research also corroborates the work of Alam *et al*. (2017) who reported 19.66±0.70 (protein), 3.73±0.38 (lipid) and 1.06±0.04 (ash) in raw beef.

Ashing of food reveals its mineral constituents that are either required in large or small amount by the microorganisms (Hogg, 2005). The presence of minerals in foods even though it was quantified in this research can play an important role in the distribution of *Listeria* species in the food samples. The mineral content can support decomposition or

spoilage of foods while some of them can serve as cofactors in production of enzymes in bacteria (Hogg, 2005).

The mean percentage ash content of beef as shown in Table 4.5 which ranged from 0.71±0.02 to 1.15±0.05 and was lower than the mean ash content of 1.5±0.26 in beef as reported by Fakolade (2012). Shehzad *et* al. (2014) also reported percentage mean content of ash in beef to be 1.10±0.1. Tayyeb *et al*. (2017), reported 0.67±0.19 as the mean percentage content of ash in cabbage. In this research, the values ranged from 0.81±0.03 to 0.96±0.01. The composition of ash in chicken (0.88±0.03 to 0.98±0.02) agrees with 0.93±0.01 ash content in chicken as reported by Devi *et al*. (2017). Nwachuckwu and Idah (2015) reported lower mean ash content (0.37±0.09) in lettuce. The significant difference in the ash content in the food samples could be as a result of difference in the nutrient composition in the chicken, beef and vegetables as reported by Butnariu and Butu (2014) as well as the composition of soils that are raised on or planted in.

The protein content was higher in chicken and beef than in the cabbage and lettuce and thiscould be due to the fact that proteins are higher in foods of animal origin than in foods of plant origin. Proteinplay an important role in the replication of these bacteria in food and environmenteven though they are a less useful source of energythan carbohydrates and lipids. Protein content in foods are metabolised by bacteria to peptides and amino acids; these then can undergo deamination resulting in a compound that is able to enter either directly or indirectly, the tricarboxylic acid (TCA) cycle that could finally aid in growth and replication of the *Listeria* species in the selected foods (Hogg, 2005).

The growth and replication of *Listeria* spp. in foods high in protein content can deteriorate and render such foods unfit for consumption. The percentage mean protein composition of the raw beef from the four locations ranged from 19.59±0.17 - 22.97±0.20 whichcorroborates the percentage mean protein content of 22.89±1.10 in raw beef meatas reported by Omojola, *et al*. (2014).The difference between the percentage mean valuesof the protein in chicken that ranged from 21.47±0.78 - 23.96±0.21 is similar to the mean percentage protein composition of 19.57±0.43 in chicken reported by Devi *et al*. (2017).

The mean percentage protein in cabbage (0.45±0.00 -0.69±0.04) in this research is lower than the mean percentage protein value of 1.80±051 in cabbage reported by Tayyeb*et al*. (2017).Caunii *et al*. (2010) reported more protein composition in lettuce than the percentage mean protein value ranged 0.30±0.02-0.64±0.06 as obtained in this work. The significant difference in the percentage mean composition of the protein values in the foods may be due to variation in the types of food sampling location.

The lipid content in the food samples was appreciably higher in beef and chicken than cabbage and lettuce; this is not surprising because lipid is higher in foods of animal origin than of plant origin as reported by Butnari and Butu (2014). The significant difference in mean percentage values of lipid in each of the foods from different locations could also be due to variations in feeds for poultry and cattle, use of fertilizers and the nature or the composition of soil for example soil mineral compositon, soil water retention capacity and availability of humus (Israel *et al*., 2020). Lipid contentof foods can be metabolised by *Listeria* species for energy and are the major component of cell walls and membranes as reported by Hogg (2005). In microorganisms, a class of

enzymes called lipases hydrolyses lipids to their constituent parts. The fatty acids produced enter the cyclic β-oxidation pathway.

The percentage mean lipid composition of beef samples as shown in Table 4.6which ranged from 6.36±0.12 -7.30±0.07 was higher than the 2.058±0.60 reported by Shehzad*et al*. (2014). The lipid composition ofchickens ranging from 4.22±0.13 - 4.47±0.12 is slightly higher that lipid content of 3.27±0.22 as reported by Chepkemoi *et al*. (2013). de Oliveira *et al*. (2016) reported an even higher lipid content(6.4±0.60) in chicken. Cabbage and lettuce recorded low lipid ranges of 0.22±0.02 - 0.55±0.04 and 0.24±0.02 -0.37±0.03 respectively. This could be due to difference in the locations where these works were conducted as reported by Israel *et al*. (2020).

The carbohydrate content of cabbage and lettuce was high compared to carbohydrate content of beef and chicken. This could be because carbohydrate is high in foods of plant originthan in foods of animal origin as reported by Januskevicius *et al*. (2012). Therefore, this could be the reason why vegetables have fermentable more sugars than animals. Microorganisms metabolise simple sugars or glucose through *Embden-Meyerhof* or glycolytic pathway to pyruvate, which is fed into tricarboxylic acid (TCA) cycle (Hogg, 2005). *Listeria* species in the food samples metabolise carbohydrate as primary source of energy and substrate for cell replication, development of cell wall (peptidoglycan), capsular materials and other parts of cell structure (Lawan *et al*., 2013).

The percentage mean carbohydrate content of food samples corroboratesthe report of Elbakheet *et al*. (2017),who reported carbohydrate content in beef from 0.30%. Ogbede *et al*. (2015) reported 4.52±0.22 as percentage mean carbohydrate content of cabbage,

which is higher than the 1.16±0.07 -1.79±0.05 reported in this work. The percentage mean carbohydrate content of lettuce does not agree with the 6.04±0.22 carbohydrate content reported by Nwachukwu and Idah (2015).

It is not also surprising that fibre was detected in the selected vegetables because plants are the major sources of fibres. Plant fibre is a good source of carbohydrate that plays important role in microbial cell structure and function. The percentage mean fibre composition of cabbage content (3.48±0.07-3.94±0.07) in this work is in agreement with the mean percentagefibre content reported by Tayyeb *et al*. (2017). In their work, they reported 3.42±1.12 as the percentage mean fibre composition in cabbage. The percentage mean composition of fibre in lettuce ranged from 0.73±0.02 - 0.82±0.25 against the report of Nwachukwu and Idah (2015) who documented 1.25±0.10 as percentage mean composition fibre of lettuce.

The absence (0.00±0.00) of fibre in the fresh beef and chicken meat is in agreement with report of Adeniyi *et al*. (2011). The significant difference in the mean values of fibre in cabbage from different locations ($P < 0.05$) could be because of difference in the location and nutritional values or effect of environmental factors such as ths soil pH, fertility and topograph. There was no significant difference in the percentage mean fibre composition in lettuce ($P = 0.2835$). This could be because lettucesfrom the selected locations have the same nutritional constituents as reported by Israel *et al*. (2020).

The result of the total bacterial count showed that the raw beef, chicken and the vegetables from different sample locations were highly contaminated with high mean bacterial counts ranging from $1.25\times10^{7}\pm4.7\times10^{5}$cfu/g - $1.66\times10^{7}\pm4.7\times10^{5}$cfu/gacross

the food samples. This could be because of high level of contaminations from different sources as reported by Kolawale-Joseph *et al.*(2011) and Iliyasu *et al*. (2017).

High total bacteria count in cabbage and lettuce in this research is agreement with the report of Abdullahi and Abdulkareem (2010), this connotes high level of contamination. These results corroborate the report of Chuku *et al*. (2016) from Lafiya in Nigeria, where they reported the meant bacteria countsranging from 1.2×10^{7} - 6.11×10^{8}cfu/g in raw beef.

The significant difference in the mean values of bacterial counts in the food samples ($P < 0.05$) could be because of differences in the level of contamination of each of the food samples.It might be due to nature of nutrients and other factors in the food that enhance the growth of microorganisms such as pH and temperatue. Many factors influence the bacterial load in foods, including direct or indirect contamination from the environment, such as from soil, water, compost and feces (Zhu *et al*., 2017).

In developing nations like Nigeria, continued use of untreated water and use of manure as fertilizers on farmlands for the production of fruits and vegetables is on the increase; these could be sources of pathogens (Tiimub *et al*., 2012). Sprinkling of vegetables in the market with probablycontaminated water to keep them fresh or handling by infected marketers could also be source of contamination (Uzeh and Adepoju, 2013; Chukwuma, 2016).

Certain outbreaks of gastroenteritis have been linked to consumption of contaminated vegetables as reported by Uzeh and Adepoju (2013). Vegetables that are used as salad have been implicated as a cause of food poisoning and thus, they are hazardous to the

health of the consumers (Chukwuma, 2016). This could be linked to the fact that most of these vegetables are consumed without being subjected to thorough washing.

Raw meat has served as a vehicle for the transmission of various diseases, especially in developing countries where hygienic standards are mostly compromised. The bacterial load in the raw beef may be due to contamination basically by unhygienic practices engaged by butchers, unclean water, contaminated containers, as well as slaughter slabs (surfaces) as reported by Chuku *et al*. (2016). Cross contamination can occur from the use of slaughtering tools without any form of sterilisation. (Rouger *et al*., 2017). People handling meat at different stages can also be source of contamination (Peter *et al*., 2016).

In this research,the overall prevalence of 9.75% was established for *Listeria* species in all the selected food samples and locations. The presence of the *Listeria* species in the fresh beef could be because of cross contamination in the slaughterhouse and during distribution or marketing. It is generally assumed that meat cannot be free from *Listeria* species because of the nature of slaughterhouse and distribution of that allows greater chance of contamination (Islam *et al*., 2016). Thus, meat need to be cooked well to eliminate these pathogens as reported by Alsheik *et al*. (2013).

Atil *et al.* (2011) reported total prevalence of 6.4% as positive isolates of *Listeria* species from animals, food and environmental samples.Sarker and Ahmed, (2015) also reported 7.5% as total prevalence of *Listeria* species in food samples. The absence of *Listeria monocytogenes* (0.0%) in the food samples from the four (4) locations in this findings corroborates the earlier report of Okonkwo *et al*. (2014) from Onitsha main market in Anambra State where they also could not isolate the pathogen in food

samples they collected. This does not rule out the possible presence of the pathogen in the locations where the samples were collected.

The high occurrence of the *Listeria* species in the chicken could be because of unhygienic technique used in the removal of the intestinal parts during processing and by contact with contaminated surfaces in the slaughterhouse. Therefore, the contamination could come within or outside the chicken (Suleiman *et al*., 2017). The raw chicken beingthe major source of *Listeria* species corroborates the findings of Hussain *et al*. (2016), that poultry is ranked first as one of the major sources of food listeriosis.

The contamination of lettuce by the *Listeria* species could perhaps be attributed to the fact that lettuce has high percentage of moisture composition and the high level of fermentable sugar such as glucose favourable for the replication of these organisms (Ieren *et al*., 2013). Secondly, it is close to the ground, which is usually in contact with the soil and the use of chicken and animal dung to compost the soil for growing of vegetables by farmers(Mohammed and Kawo, 2014).

The occurrence of *Listeria* species in cabbage could be attributed to the fac that the plant is close to ground or soil top (which is a reservoir of foodborne pathogens) and might have been contaminated by various source of pollutions(Mohammed and Kawo, 2014). Even though the lower leaves of the cabbage, which are directly in contact with the soil are normally cut off during harvest but one cannot rule out contamination during harvest or during transportation and distribution (Mohammed and Kawo, 2014).

The presence of *Listeria* species in the samples could be due to that fact that the organisms have been reported to occur widely spread in nature and therefore can

contaminate vegetables in an environment (Ieren *et al*., 2013; Dahshan *et al*., 2016; Peter *et al*; 2016).*Listeria* species can contaminate the vegetables from human faeces and animal or chicken dung applied to farmlands as compost manure (Abakpa *et al*., 2013)

In this research, *Listeria ivanovii* was the major contaminant of all the food samples (8.0%). It is not surprising that *Listeria ivanovii* was the major *Listeria* species isolated in all the samples; because it could be because it is a zoonotic pathogentherefore both humans and animals help in spreading the pathogen.*Listeria ivanovii* infection in ruminants is associated with eating spoiled silage of hay, as happens with *L. monocytogenes*, suggesting foodborne origin. *Listeriaivanovii* has been isolated from various foods including meat, milk and vegetables (Guillet*et al*., 2010).

The distribution of *Listeria ivanovii* in vegetables could be as result of human activities.These include application of animal and chicken droppingsby farmers on farmlands as well as irrigation of the farmlands with pollutedwater as reported by Odjadjare *et al*. (2011); Tiimub *et al*. (2012); Abakpa *et al*. (2013) and Ssemanda *et al*. (2018).

The prevalence of *Listeria ivanovii* in the food samples is in agreement with the report of Guillet *et al*. (2010), that *Listeria ivanovii* is isolated occasionally from animals, vegetables, poultry and environmental sources. Ndahi *etal*. (2013) also discovered higher prevalence of *Listeriaivanovii* (6.3%) among *Listeria* species isolated from meat and meat productsin Zaria. High prevalence of *Listeria ivanovii* in this research is in agreement with work earlier reported by Alsheik *et al*. (2013) who reported 19.4% from raw broiler chicken in Sudan. *Listeria grayi*,*Listeria seeligeri*, *Listeria*

*innocua*and *Listeria welshimeri* were also isolated from meat products, although the latter occurred with the lowest frequency among *Listeria* spp. The occurrence of *Listeriaivanovii* in the food samples can pose a potential health hazard it is an enteric opportunistic pathogen (Dahshan *et al*., 2016).

The antibiotics which the *Listeria* species were considerable resistant to in this work were tetracycline, ampicillin, amoxacillin, oxacillin, erythromycin, clindamycin, cefoxitin and gentamicin, which are extensively used in Nigeria. The wide variation of antibiotic resistance patterns between the isolates of *Listeria* species may also be due abuse to of these antibiotics (Falodun *et al*., 2016). Such multidrug resistance has serious implications for the empiric therapy of infections caused by these pathogensand for possible co-selection of antimicrobial resistance mediated by multidrug resistance plasmids.

The presence of antibiotic-resistant *Listeria* species in the food samples has an important health implication, since there is frequent and uncontrolled use of antibiotics in both veterinary and human medicine (Zeinali *et al*., 2017). Antibiotic-resistance does not only interfere with effective treatment measures, the antibiotic resistance gene pool in bacteria facilitates horizontal transfer of these genes among different bacteria that pose huge threat to humankind (Bertsch *et al*., 2014; Sarker and Ahmed, 2015).

Foodborne pathogens including *Listeria* species are showing trends towards resistance to standard available therapies. Antibiotic resistant *Listeria* species have been recovered from food environment and sporadic cases of human listeriosis (Chen *et al*., 2010). Ingestion of antibiotics is known to provide selective pressure ultimately leading to a higher prevalence of resistant bacteria (Olowe *et al*., 2013).

The high susceptibility of *Listeria* species to amoxicillin/clavulate, gentamicin, chloramphenicol, vancomycin and rifampicin in this research proved that the combinations of these antibiotics might be the choice for the treatment of listeriosis (Wilson *et al*., 2018). The susceptibilities of the isolates of *Listeria* species to vancomycin, ciprofloxacin, clindamycin and chloramphenicol in this research is in agreement with the research reported by Moreno *et al*. (2014). This could be due to the fact that they might not have any mechanisms either phenotypically and genotypically to resist the activity of antibiotics.

The resistant *Listeria* speciesin vegetables might have found their way to the vegetables through the application of poultry or animal droppings(containing the *Listeria* resistant strains) on farmlands and use of contaminated irrigated water on vegetables. The primary cellular mechanisms of resistance include active transport of the antibiotics out of bacterial cell via efflux pumps, permanent reduction of cell membrane, modification of the antibiotic target site and inactivation of the antibiotic through enzymatic degradation plays roles in the resistance of the pathogens (Wilson *et al*., 2018).

Detection of multidrug resistant pathogenic bacteria in foods is therefore considered as a public health threat (Jamali *et al*., 2013; Falodun *et al*., 2016). Excessive application of antibiotics in both human and veterinary medicine may lead to distribution of antibiotic-resistant pathogens in foods and environment. Multiple antibiotic resistances in *Listeria* species might be attributed to antimicrobial selective pressure and gene transfer mechanisms between and among *Listeria* species and close relatives such as *Enterococcus*, *Staphylococcus* and *Streptococcus* species (Usman *et al*., 2016b).

Multidrug- resistant pathogens can have cause more severe illness and prolonged fever than the susceptible ones due to possible association of the resistance plasmid with the

virulence genes (Olowe *et al*., 2013; Odu and Okonko, 2017). This kind of association is very plausible as the pathogenic bacteria are more likely to encounter antibiotics than the less virulent organisms.*Listeriaivanovii* isolates were 50% (11/22)mutli-drug resistant and *Listeria* grayi isolates were 66.7%(2/3) multidrug resistant this may be due to abuse of antibiotics. Foodborne infections cause by the *Listeria* species in this environment might become public health a challenge.

Multiple antibiotic resistance (MAR) index value lower than 0.20 indicates that the organism originated from a lower risk source in which the antibiotics are seldom or never used (Bilung *et al*., 2018). Multiple antibiotic resistance (MAR) index value higher than 0.20 indicates that the isolates of*Listeria* spp.originated from a higher risk source which is greatly exposed to antibiotics. In this study, 61.1%(11/18) *Listeria ivanovii* had MAR index above 0.20 significant level, suggesting that majority of *Listeria ivanovii* isolates originated from the environmentwhere these antibiotics are highlyabuse, this calls for concern because it is an alert that the antibiotics might have been abused in environment where *Listeria* species were isolated.

Multiple antibiotic resistance (MAR) of *Listeria* spp. in the vegetables could be as a result of the usage of animal waste as fertilizer which might contain antibiotic residues used to prevent or treat animal diseases and promote animal growth as reported by Hu *et al*. (2010) and Okpo *et al*. (2018). Veterinary drugs are still administered by non-registered Veterinarians and farmers often purchase and administer the drugs without proper prescription leading to multidrug resistant of these pathogens among animal populations (Okpo *et al*., 2018).

Tetracycline is one of the most frequent antibiotics used in Veterinary Medicine. Therefore, the detection of the tetracycline resistance genesin this work could be due to

indiscriminate use of the antibiotic in parts of Kaduna State as reported by Zeinali *et al*. (2017). Tetracycline resistance in *Listeria* species might also be acquired through the horizontal gene transfer by plasmid and transposons from the same or different species as reported by Wilson *et al*. (2018). The *tetM* gene is one of the most common resistance genes express by *Listeria* species against tetracycline and is widely distributed among various Gram positive (Hadayatianfard *et al*., 2014) bacteria.

The absence of*tetA*and *tetM*genes in some of the isolates could be becauseof loss of the plasmid harbouring this gene since antibiotic resistance genes are plasmid-mediated genes. In addition, the *Listeria* species may resist antibiotics by efflux pump, which expels drugs fromcell at a high rate. Must efflux pumps are multidrug transporters that efficiently pump a wide range of antibiotics, contributing to multidrug resistance (Santajit and Indrawattana, 2016).

The absence of *tetA*and *tetM* genes in some of the isolates could also be associated with modification of drug target site or prevention of the drug to reach the target site and modification of antibiotic molecule by the *Listeria* species. Decreased permeability could be another method employed by the isolates (Bassetti *et al*., 2013). Mutations can result in antibiotic resistance in bacteria and resistant bacteria can survive antibiotics by natural selection (Olowe *et al*., 2013; Wilson *et al*., 2018)

Manifestation of tetracycline resistance has also been decisively link to increased selective pressure caused by extensive use of antibiotics as growth promoter in livestock or in medical treatment of humans or animals (Wilson *et al*., 2018). Resistance to tetracycline could also be due to frequent use of the antibiotic in the treatment of animals and poultry, which could lead to frequent exposure of these pathogens (Zeinali *et al*., 2017).

The presence of haemolysin A (*hlyA*) gene in multidrug-resistant zoonotic bacteria such as *L. ivanovii*is of great concern to clinicians in treatment of patients infected by such an organism (Ndahi *et al*., 2013). Pathogenic *Listeria* spp (like *L. ivanovii*) in foods habour virulence geneswhich can pose serious danger to consumers of the food samples if not properly cooked or processed. This is prevalent in different meat products such as ready-to-eat meat products (Ndahi *et al*., 2013).

The presence of haemolysin A (*hlyA*) gene in the *L. ivanovii*signifies the ability of *Listeriaivanovii* to cause diseases in human and animal populations.*Listeria ivanovii* can cause severe foetal-placental infections associated with outbreaks of neonatal death or sporadic abortion in sheep and cattle. *Listeria ivanovii* is capable of adhering to and penetrating human amnion-derived cells, lysing the phagosomal membrane, polymerising and recognising the host cell action in the form of tails, replicating in host cell cytososl after phagosomal escape and spreading from cell to cell (O'Byrne and Utratna, 2010; Alvarez-Ordonez *et al*., 2015; Chen *et al*., 2017).

Listeriaivanovii strains are comparable to *L. monocytogenes* in their ability to invade epithelial and endothelial cells in human cell and are more efficient than *L. monocytogenes* (Alvarez-Ordonez *et al*., 2015),even though, the clearance of *L. ivanovii* in human blood cell (septicaemia) is faster than *L. monocytogenes*. Therefore, *Listeria ivanovii* is one of the foodborne pathogens that can causes infections of varying degrees in humans (Guillet *et al*., 2010).

The absence of *hlyA* gene in some of the isolates of *Listeria ivanovii*might be because they are non-pathogenic strains as reported by Bergholz *et al.* (2012), Alessandria *et al*. (2013), Ndahi *et al*. (2013) and Cordero *et al*. (2016). The isolates may have other

genes such as sphingomyelin (*smcL*) that codes for sphingomyelinase C, which also has haemolytic activity (Wang *et al*., 2014).

The detection of*hlyA*gene (702bp) in this research corroborates the report of Abdelrazeq *et al*. (2014) and Usman *et al*. (2016a) even though the prevalence is lower. The report of these findings is similar to the report of Javaid and Rashid (2018) that only two species of *Listeria* have been recognised as pathogenic: *Listeria monocytogenes* and *Listeria ivanovii*.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

The proximate compositions of beef, chicken, lettuce and cabbage in this research revealed that the moisture content was found in appreciable amount in the foods with lettuce having the highest mean percentage (94.44±0.18). The average crude protein in chickenhad the highest protein content (22.48±0.56).Cabbage had the highest crude fibre content with average value of 3.64±0.11. An average ash content of 0.98±0.06 was found in chicken. Beef had highest lipid content with mean value of 6.87±0.23 and carbohydrate content was higher in lettuce with average mean value of 3.15±0.18. These valuable nutrrients can support microbial growth and replications in the food samples.

It was evident from the findings in this study that, the mean percentage of the total bacterial count in food samples were high ranging from $1.2\times10^{7}\pm4.7\times10^{5}$ to$1.66\times10^{7}\pm4.7\times10^{4}$.The bacterial load in these food samplesdid not satisfy the microbiological standards of 1.0×10^{7}cfu/g stipulated by the Codex Alimentarius Commission.The total prevalence of *Listeria* species in the food samples was 9.75%(39/400).Out of these *Listeria* species,32(8.0%), 6(1.5%), and 1(0.25%) were *Listeria ivanovii,Listeria grayi* and *Listeria welshimeri* respectively.

The antibiotic susceptibility of the *Listeria* species showed that *Listeria ivanovii* isolates were more resistant to tetracycline, ampicillin, clindamycin and oxacillin and were all susceptible to ciprofloxacin and rifampicin. All the isolates of *Listeriagrayi* weresusceptible to amoxicillin/clavulate, vancomycin, gentamicin, ciprofloxacin and rifampicin.

The multi-drug resistance Listeria species showed that *Listeriaivanovii*(61.1%) and *Listeriagrayi* (50.0%) had MAR index greater that 0.20 index significant value. This could be due to the fact that these Listeria species might be isolated from the

environment where the antibiotics indiscriminately used.The polymerase chain reaction showed that of the seven isolates selected 28.6% had tetA genes and 42.86% had tetM genes.

The PCR result also indicated that out of the seven isolates of *Listeriaivanovii* considered for detection of virulence genes, one of the isolates from beef had haemolysin gene (*hlyA*). The presence of *hlyA* gene in *Listeria* species is associated with their pathogenicity.The prevalence of these *Listeria* species in lettuce and cabbage can be of great concern because they are usually consumed raw and susceptible people(immune-compromised individuals) can be at a risk.

6.2 Recommendations

i. There is a need for relevant authorities to seriously engage the butchers on proper hygienic practices during meat processing such as the avoidance of use of unclean water for washing, contaminated containers, as well as contaminated slaughter surfaces and slabs for sales of meatamong others that could serve as sources of *Listeria* species.
ii. Cabbage and lettuce should thoroughly be washed before consumption to avoid foodborne infectionas cause by *Listeria* species since they can be eating raw. There is a need for handlers of raw foods to be educated on the importance of adherence to hygiene.
iii. It is recommended that the meat be properly cooked to reduce the microbial load to acceptable limit to avoid foodborne infections
iv. There is a need for a relevant authority to educate the public on the hazards associated with indiscriminate use of antibiotics in the treatment of infections among human and animal population.

v. It is important for the National Agency for Food and Drug Administration and Control (NAFDAC) enforce safety standards for foods retailed in Nigeria, and strengthen the regulation of retailed meat by conducting periodic quality control checks to ensure compliance to established safety standards.

vi. It is hereby, recommended that more research be conducted on the prevalence of *Listeria* species in Kaduna state to avoid outbreak of listeriosis cause by the *Listeria* species associated with foods.

vii. Futher research on the impacts and dynamics of genetic antibiotic resistance determinants of *Listeria* species in the study areas should further be conducted.

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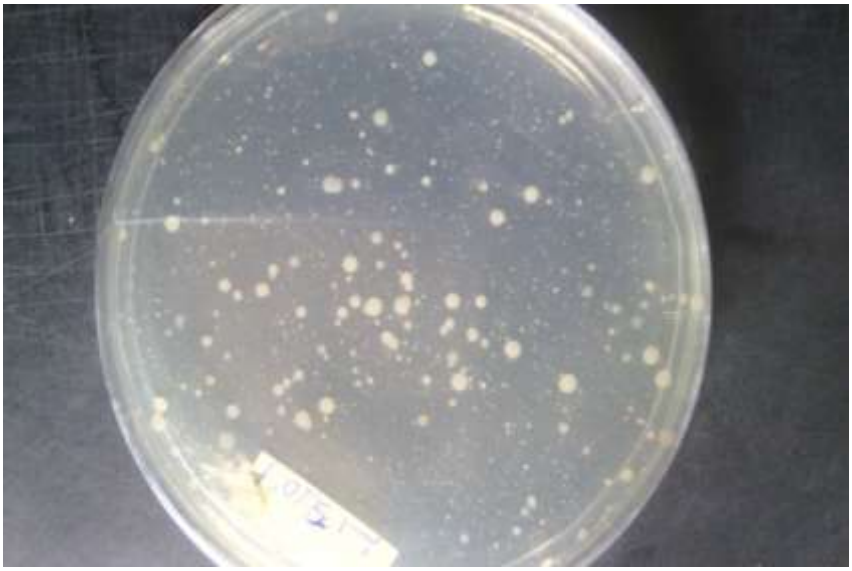
Appendices

Appendix I: Biochemical characteristics of presumptive *Listeria* spp. isolates

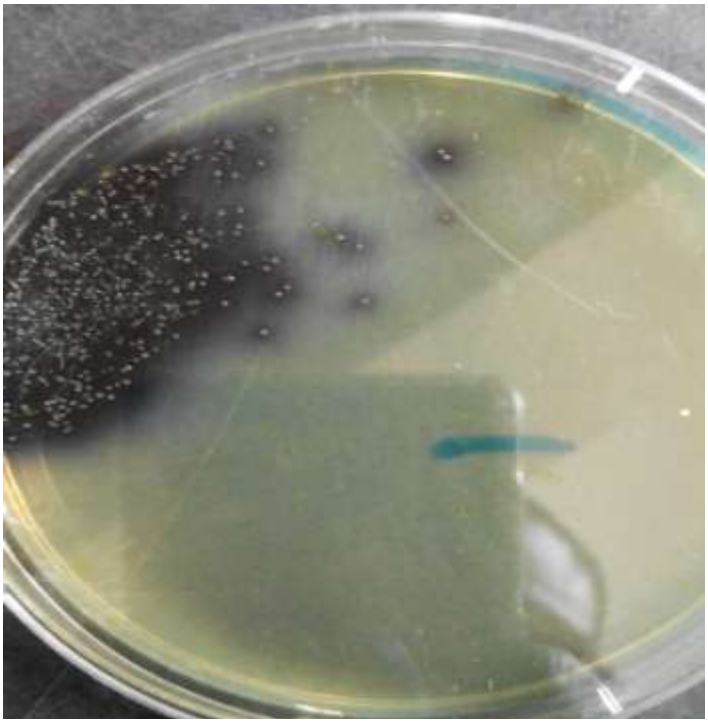
S/N	Isolate code	Gram staining	Haem	Oxidase test	Catalase test	Indole test	MR test	VP test	Motility	Inference
1	BSM	+	+	-	+	-	+	+	+[a]	*Listeria* spp
2	ChSM	+	+	-	+	-	+	+	+	*Listeria* spp
3	ChSM	+	-	-	+[d]	-	+	+	+[a]	*Listeria* spp
4	CaSM	+	+	-	+	-	+	+	+[a]	*Listeria* spp
5	LSM	+	+	-	+	-	+	+	+	*Listeria* spp
6	BSG	+	-	-	+[d]	-	+	+	+	*Listeria* spp
7	ChSM	+	+	-	+	-	+	+	+	*Listeria* spp
8	ChSG	+	+	-	+	-	+	+	+	*Listeria* spp
9	LSG	+	+	-	+	-	+	+	+	*Listeria* spp
10	ChSM	+	+	-	+	-	+	+	+	*Listeria* spp
11	ChSG	+	+	-	+	-	+	+	+	*Listeria* spp
12	ChSG	+	+	-	+	-	+	+	+	*Listeria* spp
13	ChSM	+	-	-	+[d]	-	+	+	+[a]	*Listeria* spp
14	BSM	+	+	-	+	-	+	+	+	*Listeria* spp
15	BSG	+	+	-	+	-	+	+	+	*Listeria* spp
16	ChSM	+	+	-	+	-	+	+	+	*Listeria* spp
17	BSG	+	+	-	+	-	+	+	+	*Listeria* spp
18	CaSG	+	+	-	+	-	+	+	+	*Listeria* spp
19	ChSG	+	-	-	+[d]	-	+	+	+[a]	*Listeria* spp
20	CaSM	+	+	-	+	-	+	+	+	*Listeria* spp
21	CaSG	+	+	-	+	-	+	+	+	*Listeria* spp

22	LSM	+	+	-	+	-	+	+	+	*Listeria* spp
23	LSG	+	+	-	+	-	+	+	+[a]	*Listeria* spp
24	LSM	+	+	-	+	-	+	+	+	*Listeria* spp
25	LSG	+	+	-	+	-	+	+	+	*Listeria* spp
26	ChSG	+	-	-	+[d]	-	+	+	+	*Listeria* spp
27	ChKK	+	+	-	+	-	+	+	+	*Listeria* spp
28	ChKK	+	+	-	+	-	+	+	+	*Listeria* spp
29	ChUB	+	+	-	+	-	+	+	+	*Listeria* spp
30	ChKK	+	-	-	+[d]	-	+	+	+	*Listeria* spp
31	ChUB	+	+	-	+	-	+	+	+	*Listeria* spp
32	CaKK	+	+	-	+	-	+	+	+	*Listeria* spp
33	ChUB	+	+	-	+	-	+	+	+	*Listeria* spp
34	LKK	+	+	-	+	-	+	+	+[a]	*Listeria* spp
35	LKK	+	+	-	+	-	+	+	+	*Listeria* spp
36	LUB	+	+	-	+	-	+	+	+	*Listeria* spp
37	ChUB	+	-	-	+[d]	-	+	+	+	*Listeria* spp
38	CaUB	+	+	-	+	-	+	+	+[a]	*Listeria* spp
39	BUB	+	+	-	+	-	+	+	+	*Listeria* spp

Note: + = Positive reaction, - = negative reaction d = positive but weak reaction, a = weak motility, Haem = haamolysis on sheep blood agar, MR = methyl red test, VP = Voges Proskauer test

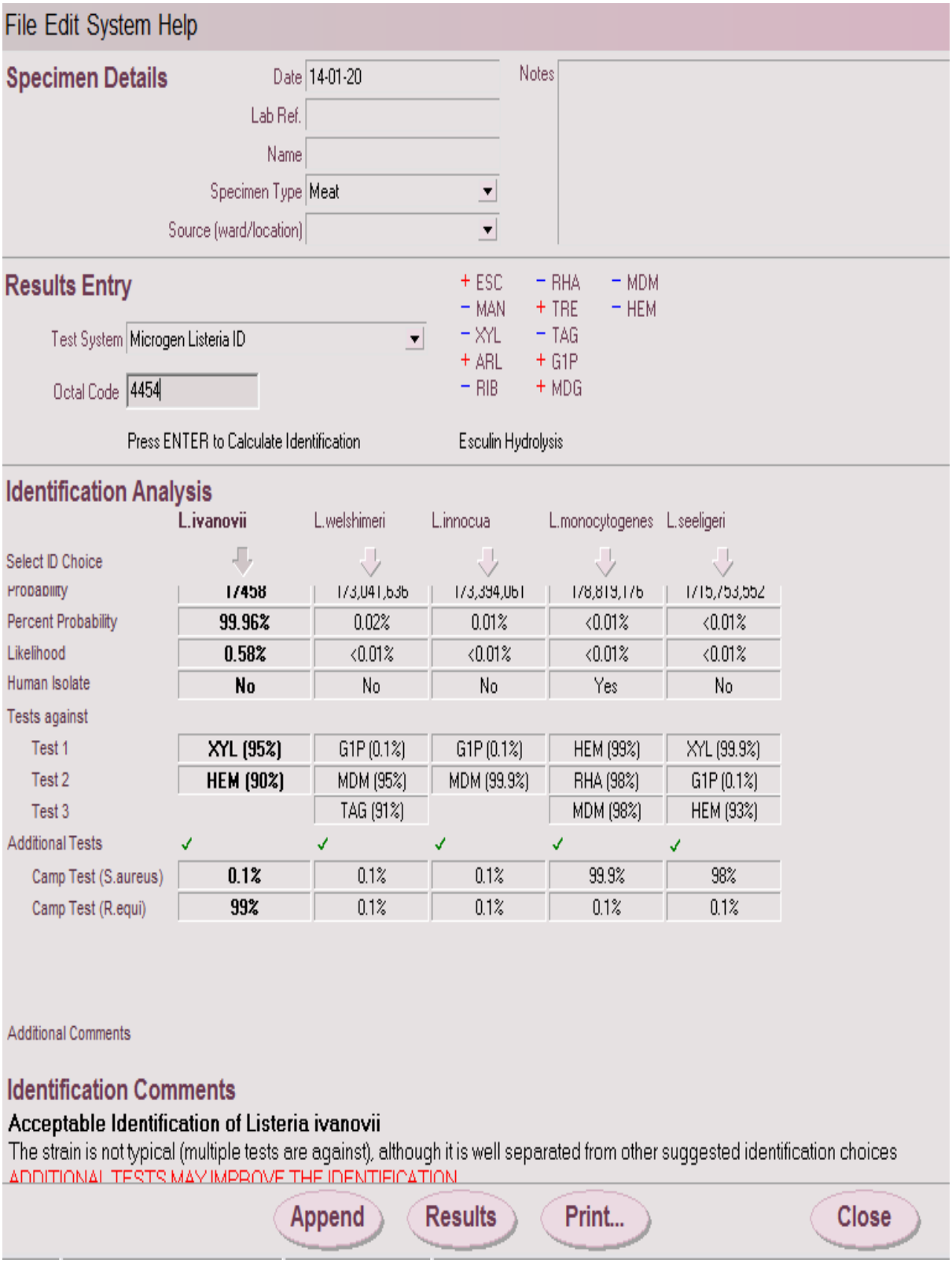


Appendix II: Plate showing total bacterial count



AppendixIII: Plate showing colonies of *Listeria* species on Listeria selective medium

Apendix IV: Identification of Listeria species using Listeria ID kit system



File Edit System Help

Specimen Details

Date: 14-01-20
Lab Ref.:
Name:
Specimen Type: Meat
Source (ward/location):
Notes:

Results Entry

Test System: Microgen Listeria ID
Octal Code: 4643

Press ENTER to Calculate Identification

+ ESC	− RHA	+ MDM
− MAN	+ TRE	+ HEM
− XYL	− TAG	
+ ARL	− G1P	
+ RIB	− MDG	

Esculin Hydrolysis

Identification Analysis

	L.grayi	L.ivanovii	L.monocytogenes	L.innocua	L.welshimeri
Select ID Choice					
Probability	**1/52,056**	1/14,660,184	1/56,114,776	< 1/100,000,000	< 1/100,000,000
Percent Probability	**99.55%**	0.35%	0.09%	<0.01%	<0.01%
Likelihood	**<0.01%**	<0.01%	<0.01%	<0.01%	<0.01%
Human Isolate	**No**	No	Yes	No	No
Tests against					
Test 1	**HEM (0.1%)**	MDM (0.1%)	RIB (0.1%)	RIB (0.1%)	RIB (0.1%)
Test 2	**MAN (97%)**	XYL (95%)	MDG (99.9%)	MDG (99.9%)	HEM (0.1%)
Test 3		MDG (95%)	RHA (98%)	HEM (0.1%)	MDG (98%)
Additional Tests	✓	✓	✓	✓	✓
Camp Test (S.aureus)	**0.1%**	0.1%	99.9%	0.1%	0.1%
Camp Test (R.equi)	**0.1%**	99%	0.1%	0.1%	0.1%

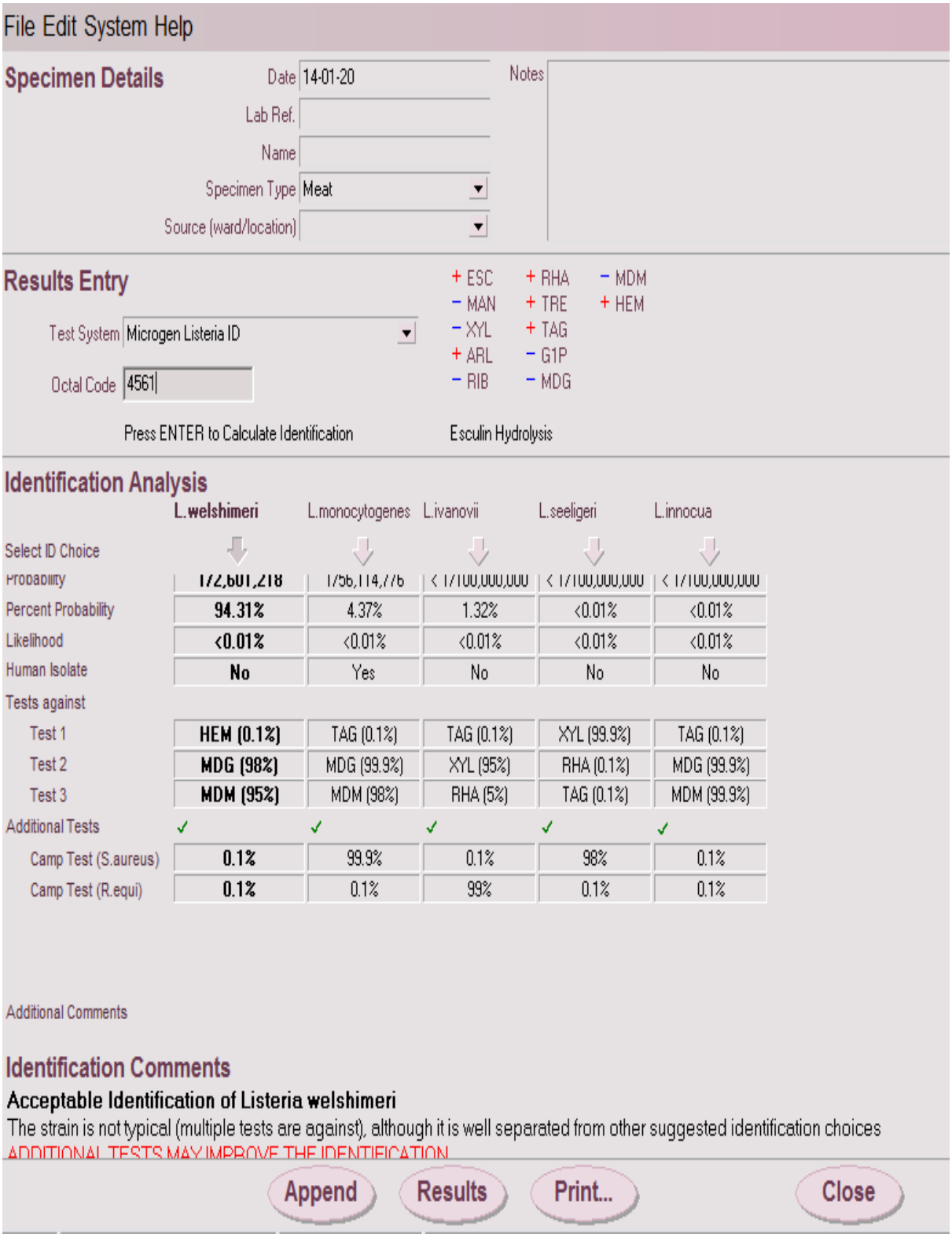
Additional Comments

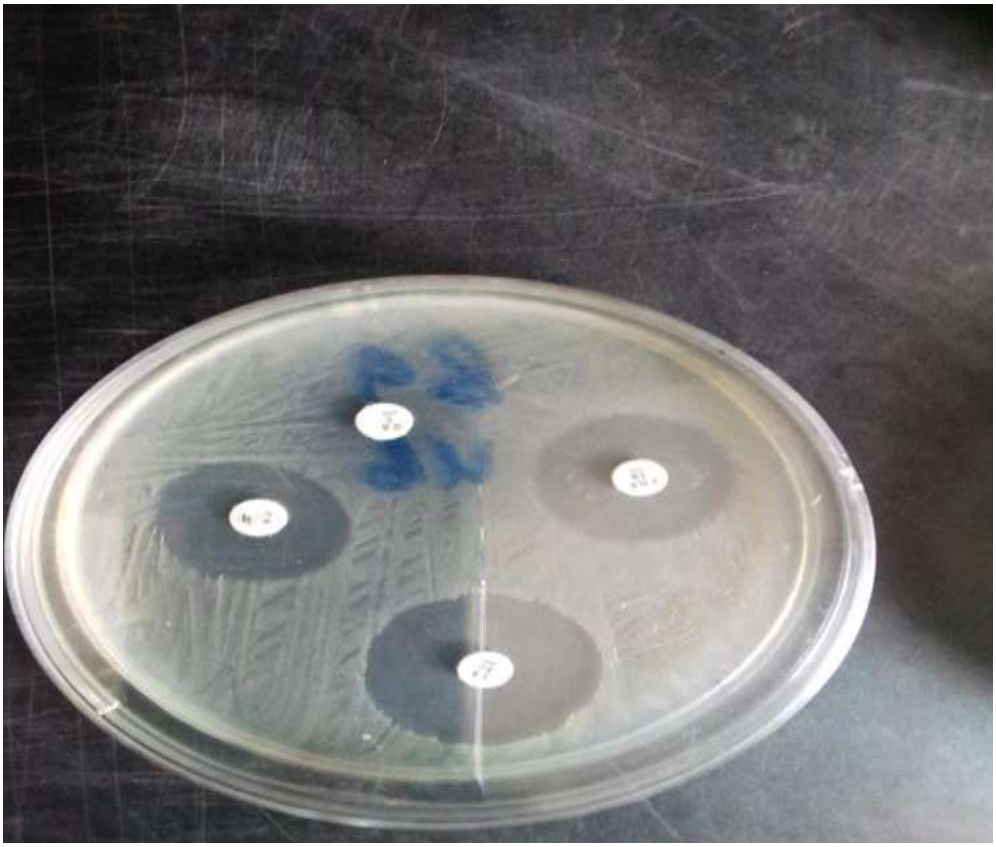
Identification Comments

Acceptable Identification of Listeria grayi

The strain is not typical (multiple tests are against), although it is highly separated from other suggested identification choices

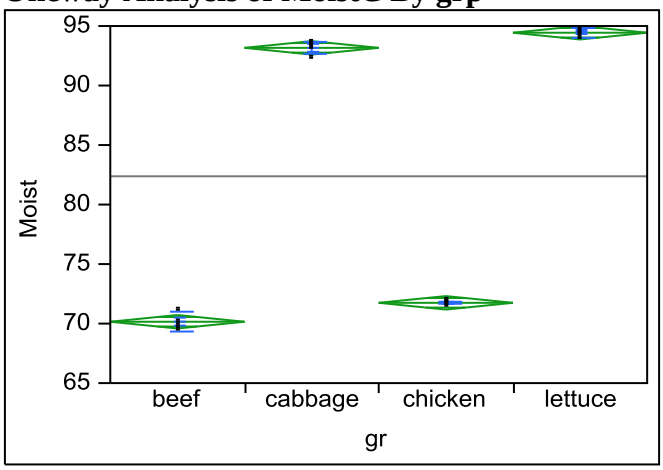
Append | Results | Print... | Close


File Edit System Help
Specimen Details
Date 14-01-20
Lab Ref.
Name
Specimen Type Meat
Source (ward/location)
Notes
Results Entry
Test System Microgen Listeria ID
Octal Code 4561
Press ENTER to Calculate Identification
+ ESC + RHA − MDM
− MAN + TRE + HEM
− XYL + TAG
+ ARL − G1P
− RIB − MDG
Esculin Hydrolysis
Identification Analysis
L.welshimeri L.monocytogenes L.ivanovii L.seeligeri L.innocua
Select ID Choice
Probability 172,601,218 1756,114,776 < 171UU,UUU,UUU < 171UU,UUU,UUU < 171UU,UUU,UUU
Percent Probability 94.31% 4.37% 1.32% <0.01% <0.01%
Likelihood <0.01% <0.01% <0.01% <0.01% <0.01%
Human Isolate No Yes No No No
Tests against
Test 1 HEM (0.1%) TAG (0.1%) TAG (0.1%) XYL (99.9%) TAG (0.1%)
Test 2 MDG (98%) MDG (99.9%) XYL (95%) RHA (0.1%) MDG (99.9%)
Test 3 MDM (95%) MDM (98%) RHA (5%) TAG (0.1%) MDM (99.9%)
Additional Tests ✓ ✓ ✓ ✓ ✓
Camp Test (S.aureus) 0.1% 99.9% 0.1% 98% 0.1%
Camp Test (R.equi) 0.1% 0.1% 99% 0.1% 0.1%
Additional Comments
Identification Comments
Acceptable Identification of Listeria welshimeri
The strain is not typical (multiple tests are against), although it is well separated from other suggested identification choices
ADDITIONAL TESTS MAY IMPROVE THE IDENTIFICATION
Append
Results
Print...
Close



AppendixV: Plate showing the antibacterial susceptibility of *Listeria* species

Fit Y by X Group
Oneway Analysis of MoistC By grp



Oneway Anova
Summary of Fit

Rsquare	0.998452
Adj Rsquare	0.998065
Root Mean Square Error	0.520573
Mean of Response	82.37625
Observations (or Sum Wgts)	16

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grp	3	2097.2290	699.076	2579.657	<.0001*
Error	12	3.2519	0.271		
C. Total	15	2100.4810			

Means for Oneway Anova

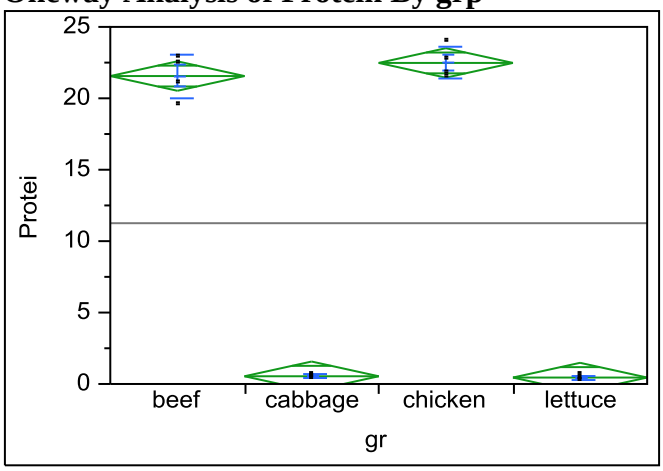
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Beef	4	70.1550	0.26029	69.588	70.722
Cabbage	4	93.1675	0.26029	92.600	93.735
Chicken	4	71.7450	0.26029	71.178	72.312
Lettuce	4	94.4375	0.26029	93.870	95.005

Std Error uses a pooled estimate of error varianc

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
Beef	4	70.1550	0.768570	0.38429	68.932	71.378
Cabbage	4	93.1675	0.577776	0.28889	92.248	94.087
Chicken	4	71.7450	0.150222	0.07511	71.506	71.984
Lettuce	4	94.4375	0.369989	0.18499	93.849	95.026

Oneway Analysis of Protein By grp



Oneway Anova
Summary of Fit

Rsquare	0.994233
Adj Rsquare	0.992791
Root Mean Square Error	0.946875
Mean of Response	11.25688
Observations (or Sum Wgts)	16

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grp	3	1854.7871	618.262	689.5840	<.0001*
Error	12	10.7589	0.897		
C. Total	15	1865.5459			

Means for Oneway Anova

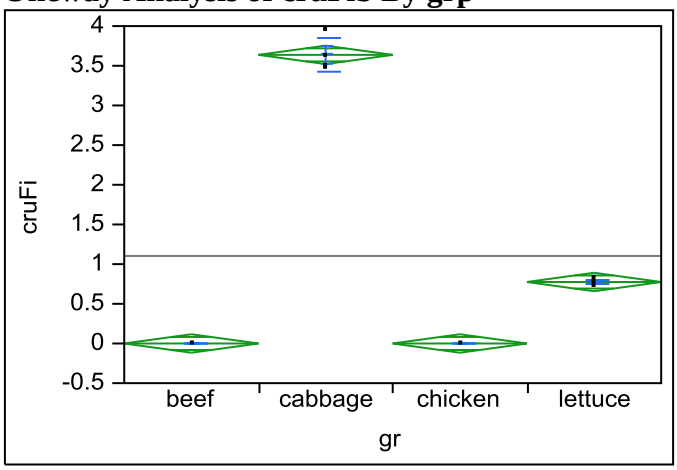
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Beef	4	21.5600	0.47344	20.53	22.592
Cabbage	4	0.5400	0.47344	-0.49	1.572
Chicken	4	22.4775	0.47344	21.45	23.509
Lettuce	4	0.4500	0.47344	-0.58	1.482

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
Beef	4	21.5600	1.51625	0.75812	19.147	23.973
Cabbage	4	0.5400	0.10520	0.05260	0.373	0.707
Chicken	4	22.4775	1.12064	0.56032	20.694	24.261
Lettuce	4	0.4500	0.14283	0.07141	0.223	0.677

Oneway Analysis of cruFib By grp



Oneway Anova
Summary of Fit

Rsquare	0.996195
Adj Rsquare	0.995243
Root Mean Square Error	0.106839
Mean of Response	1.103125
Observations (or Sum Wgts)	16

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Grp	3	35.857969	11.9527	1047.139	<.0001*
Error	12	0.136975	0.0114		
C. Total	15	35.994944			

Means for Oneway Anova

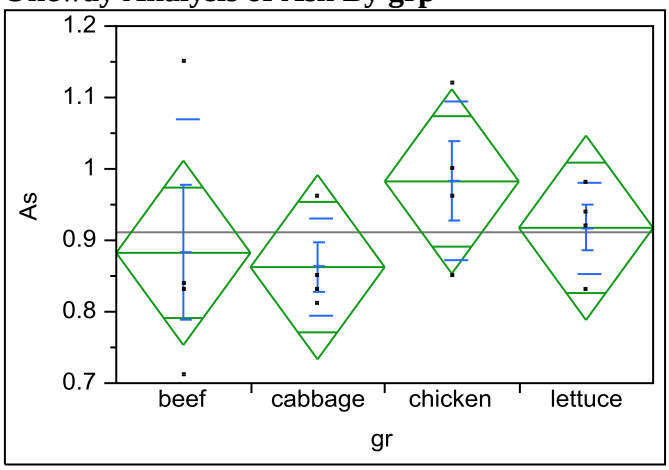
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
Beef	4	0.00000	0.05342	-0.116	0.1164
Cabbage	4	3.63750	0.05342	3.521	3.7539
Chicken	4	0.00000	0.05342	-0.116	0.1164
Lettuce	4	0.77500	0.05342	0.659	0.8914

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
Beef	4	0.00000	0.000000	0.00000	0.0000	0.0000
Cabbage	4	3.63750	0.210456	0.10523	3.3026	3.9724
Chicken	4	0.00000	0.000000	0.00000	0.0000	0.0000
Lettuce	4	0.77500	0.036968	0.01848	0.7162	0.8338

Oneway Analysis of Ash By grp



Oneway Anova
Summary of Fit

Rsquare	0.164748
Adj Rsquare	-0.04406
Root Mean Square Error	0.118568
Mean of Response	0.91125
Observations (or Sum Wgts)	16

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	0.03327500	0.011092	0.7890	0.5230
Error	12	0.16870000	0.014058		
C. Total	15	0.20197500			

Means for Oneway Anova

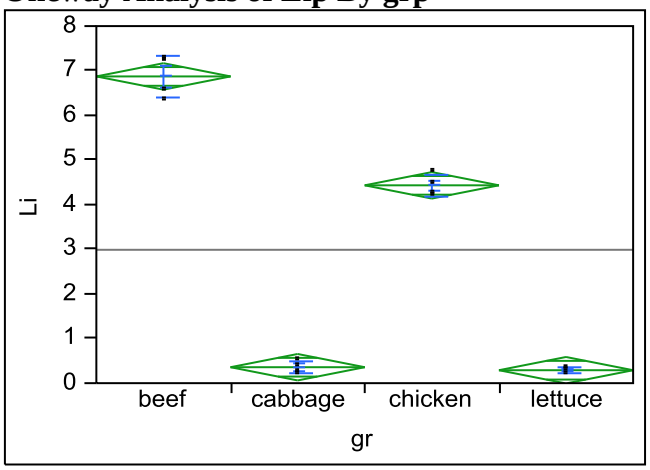
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
beef	4	0.882500	0.05928	0.75333	1.0117
cabbage	4	0.862500	0.05928	0.73333	0.9917
chicken	4	0.982500	0.05928	0.85333	1.1117
lettuce	4	0.917500	0.05928	0.78833	1.0467

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
beef	4	0.882500	0.187861	0.09393	0.58357	1.1814
cabbage	4	0.862500	0.067020	0.03351	0.75586	0.9691
chicken	4	0.982500	0.111467	0.05573	0.80513	1.1599
lettuce	4	0.917500	0.063443	0.03172	0.81655	1.0185

Oneway Analysis of Lip By grp



Oneway Anova
Summary of Fit

Rsquare	0.992974
Adj Rsquare	0.991217
Root Mean Square Error	0.272033
Mean of Response	2.988125
Observations (or Sum Wgts)	16

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	125.49862	41.8329	565.2932	<.0001*
Error	12	0.88802	0.0740		
C. Total	15	126.38664			

Means for Oneway Anova

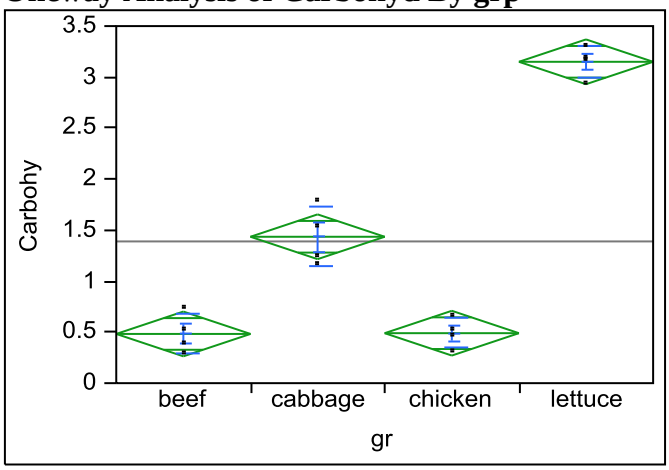
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
beef	4	6.87250	0.13602	6.576	7.1689
cabbage	4	0.35750	0.13602	0.061	0.6539
chicken	4	4.43250	0.13602	4.136	4.7289
lettuce	4	0.29000	0.13602	-0.0064	0.5864

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
beef	4	6.87250	0.464570	0.23228	6.1333	7.6117
cabbage	4	0.35750	0.144309	0.07215	0.1279	0.5871
chicken	4	4.43250	0.235425	0.11771	4.0579	4.8071
lettuce	4	0.29000	0.062716	0.03136	0.1902	0.3898

Oneway Analysis of Carbohyd By grp



Oneway Anova
Summary of Fit

Rsquare	0.974924
Adj Rsquare	0.968655
Root Mean Square Error	0.201448
Mean of Response	1.389375
Observations (or Sum Wgts)	16

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	18.932719	6.31091	155.5129	<.0001*
Error	12	0.486975	0.04058		
C. Total	15	19.419694			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
beef	4	0.48250	0.10072	0.2630	0.7020
cabbage	4	1.43500	0.10072	1.2155	1.6545
chicken	4	0.49000	0.10072	0.2705	0.7095
lettuce	4	3.15000	0.10072	2.9305	3.3695

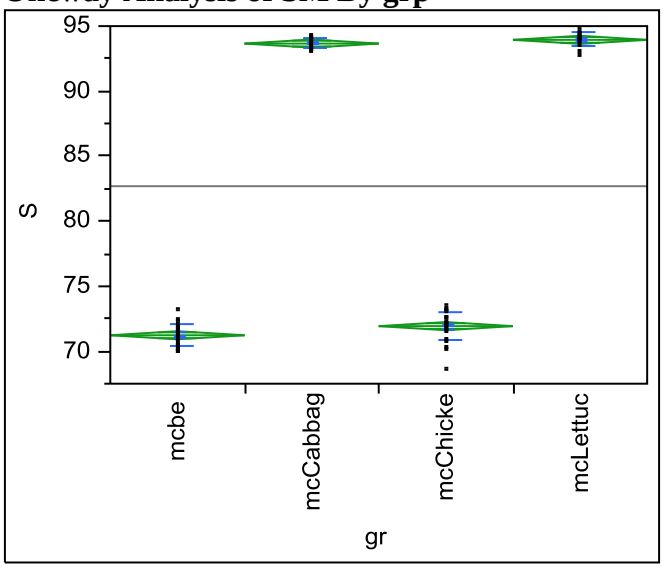
Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
beef	4	0.48250	0.188392	0.09420	0.1827	0.7823
cabbage	4	1.43500	0.286880	0.14344	0.9785	1.8915
chicken	4	0.49000	0.144453	0.07223	0.2601	0.7199
lettuce	4	3.15000	0.153840	0.07692	2.9052	3.3948

MOISTURE CONTENT

Fit Y by X Group
Oneway Analysis of SM By grp



Oneway Anova
Summary of Fit

Rsquare	0.995184
Adj Rsquare	0.995033
Root Mean Square Error	0.789288
Mean of Response	82.6886
Observations (or Sum Wgts)	100

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	12357.061	4119.02	6611.852	<.0001*
Error	96	59.806	0.62		
C. Total	99	12416.867			

Means for Oneway Anova

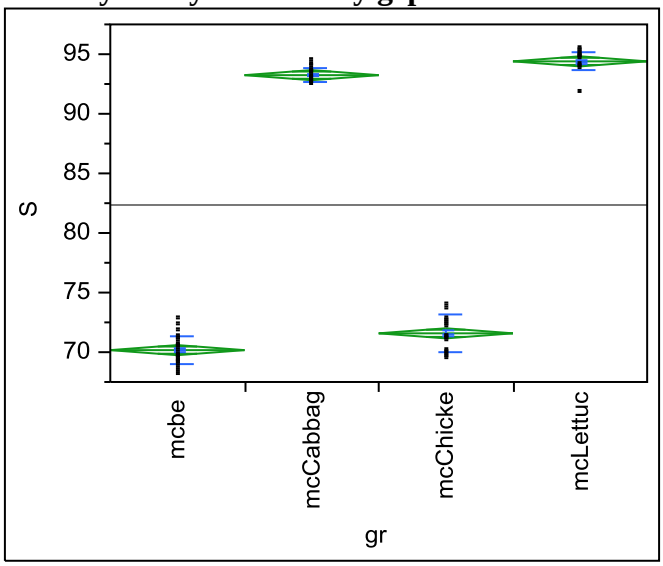
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
mcbeef	25	71.2248	0.15786	70.911	71.538
mcCabbage	25	93.6580	0.15786	93.345	93.971
mcChicken	25	71.9264	0.15786	71.613	72.240
mcLettuce	25	93.9452	0.15786	93.632	94.259

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
mcbeef	25	71.2248	0.87799	0.17560	70.862	71.587
mcCabbage	25	93.6580	0.39485	0.07897	93.495	93.821
mcChicken	25	71.9264	1.13222	0.22644	71.459	72.394
mcLettuce	25	93.9452	0.53217	0.10643	93.726	94.165

Oneway Analysis of SG By grp



Oneway Anova
Summary of Fit

Rsquare	0.991746
Adj Rsquare	0.991488
Root Mean Square Error	1.069932
Mean of Response	82.35
Observations (or Sum Wgts)	100

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	13203.645	4401.22	3844.682	<.0001*

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Error	96	109.896	1.14		
C. Total	99	13313.542			

Means for Oneway Anova

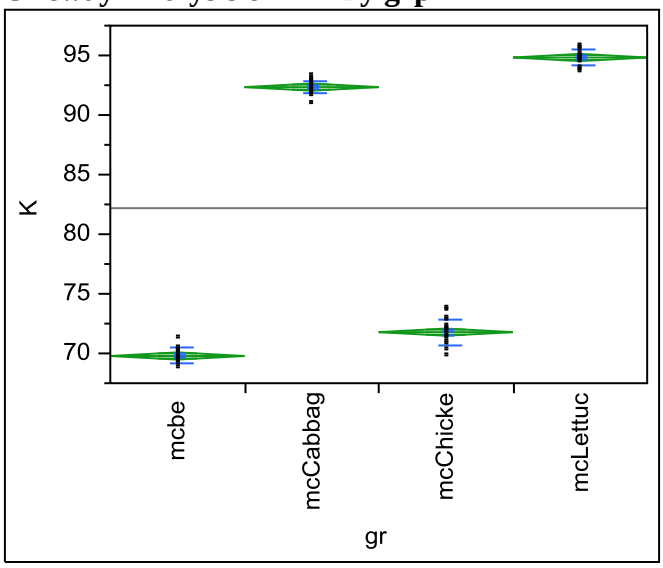
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
mcbeef	25	70.1724	0.21399	69.748	70.597
mcCabbage	25	93.2440	0.21399	92.819	93.669
mcChicken	25	71.5824	0.21399	71.158	72.007
mcLettuce	25	94.4012	0.21399	93.976	94.826

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
mcbeef	25	70.1724	1.14560	0.22912	69.700	70.645
mcCabbage	25	93.2440	0.52241	0.10448	93.028	93.460
mcChicken	25	71.5824	1.53279	0.30656	70.950	72.215
mcLettuce	25	94.4012	0.80266	0.16053	94.070	94.733

Oneway Analysis of KK By grp



Oneway Anova
Summary of Fit

Rsquare	0.995811
Adj Rsquare	0.99568
Root Mean Square Error	0.75831
Mean of Response	82.185
Observations (or Sum Wgts)	100

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	13123.724	4374.57	7607.501	<.0001*
Error	96	55.203	0.58		
C. Total	99	13178.927			

Means for Oneway Anova

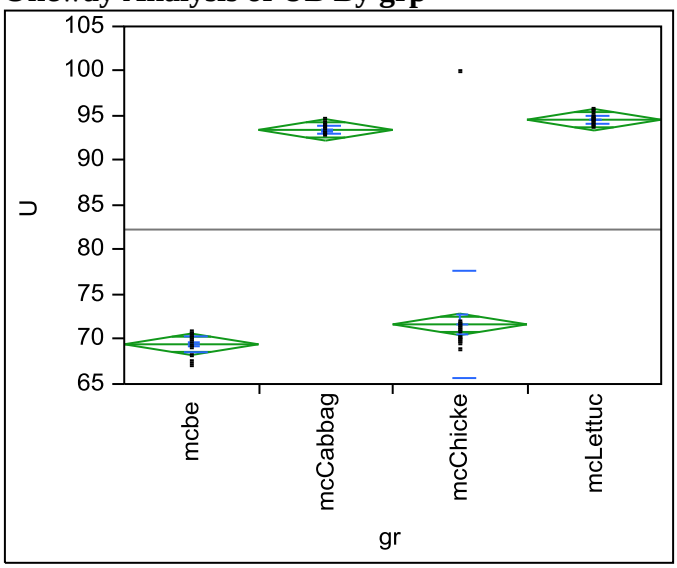
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
mcbeef	25	69.7844	0.15166	69.483	70.085
mcCabbage	25	92.3424	0.15166	92.041	92.643
mcChicken	25	71.7852	0.15166	71.484	72.086
mcLettuce	25	94.8280	0.15166	94.527	95.129

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
mcbeef	25	69.7844	0.67031	0.13406	69.508	70.061
mcCabbage	25	92.3424	0.55109	0.11022	92.115	92.570
mcChicken	25	71.7852	1.08032	0.21606	71.339	72.231
mcLettuce	25	94.8280	0.61646	0.12329	94.574	95.082

Oneway Analysis of UB By grp



Oneway Anova
Summary of Fit

Rsquare	0.94005
Adj Rsquare	0.938176
Root Mean Square Error	3.029335
Mean of Response	82.2806
Observations (or Sum Wgts)	100

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	13814.211	4604.74	501.7765	<.0001*
Error	96	880.979	9.18		
C. Total	99	14695.191			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
mcbeef	25	69.4452	0.60587	68.243	70.648
mcCabbage	25	93.4320	0.60587	92.229	94.635
mcChicken	25	71.6760	0.60587	70.473	72.879
mcLettuce	25	94.5692	0.60587	93.367	95.772

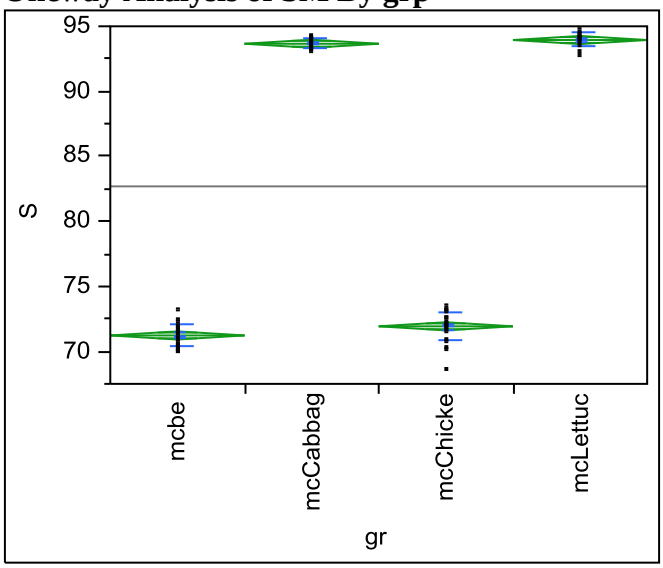
Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
mcbeef	25	69.4452	0.96601	0.1932	69.046	69.844
mcCabbage	25	93.4320	0.49815	0.0996	93.226	93.638
mcChicken	25	71.6760	5.94212	1.1884	69.223	74.129
mcLettuce	25	94.5692	0.46618	0.0932	94.377	94.762

LIPID CONTENT

Fit Y by X Group
Oneway Analysis of SM By grp



Oneway Anova
Summary of Fit

Rsquare	0.995184
Adj Rsquare	0.995033
Root Mean Square Error	0.789288
Mean of Response	82.6886
Observations (or Sum Wgts)	100

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	12357.061	4119.02	6611.852	<.0001*
Error	96	59.806	0.62		
C. Total	99	12416.867			

Means for Oneway Anova

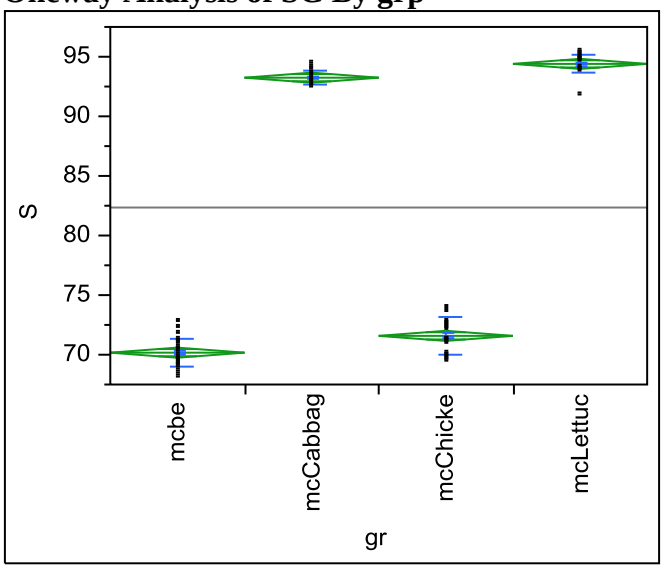
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
mcbeef	25	71.2248	0.15786	70.911	71.538
mcCabbage	25	93.6580	0.15786	93.345	93.971
mcChicken	25	71.9264	0.15786	71.613	72.240
mcLettuce	25	93.9452	0.15786	93.632	94.259

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
mcbeef	25	71.2248	0.87799	0.17560	70.862	71.587
mcCabbage	25	93.6580	0.39485	0.07897	93.495	93.821
mcChicken	25	71.9264	1.13222	0.22644	71.459	72.394
mcLettuce	25	93.9452	0.53217	0.10643	93.726	94.165

Oneway Analysis of SG By grp



Oneway Anova
Summary of Fit

Rsquare	0.991746
Adj Rsquare	0.991488
Root Mean Square Error	1.069932
Mean of Response	82.35
Observations (or Sum Wgts)	100

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	13203.645	4401.22	3844.682	<.0001*
Error	96	109.896	1.14		
C. Total	99	13313.542			

Means for Oneway Anova

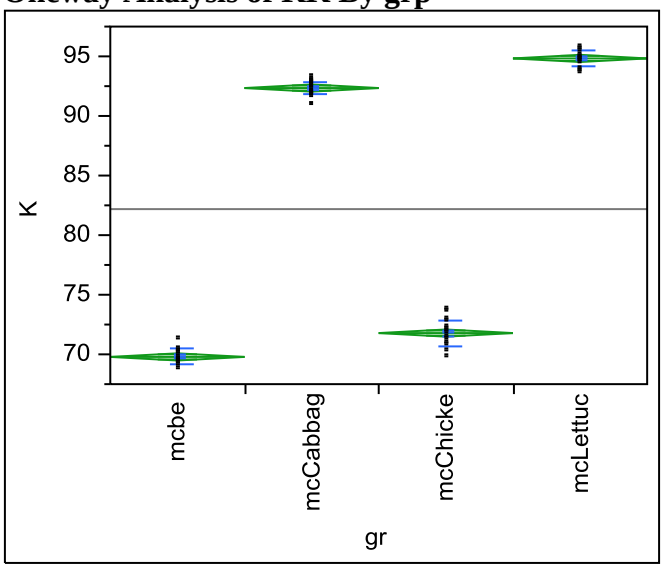
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
mcbeef	25	70.1724	0.21399	69.748	70.597
mcCabbage	25	93.2440	0.21399	92.819	93.669
mcChicken	25	71.5824	0.21399	71.158	72.007
mcLettuce	25	94.4012	0.21399	93.976	94.826

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
mcbeef	25	70.1724	1.14560	0.22912	69.700	70.645
mcCabbage	25	93.2440	0.52241	0.10448	93.028	93.460
mcChicken	25	71.5824	1.53279	0.30656	70.950	72.215
mcLettuce	25	94.4012	0.80266	0.16053	94.070	94.733

Oneway Analysis of KK By grp



Oneway Anova
Summary of Fit

Rsquare	0.995811
Adj Rsquare	0.99568
Root Mean Square Error	0.75831
Mean of Response	82.185
Observations (or Sum Wgts)	100

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	13123.724	4374.57	7607.501	<.0001*
Error	96	55.203	0.58		
C. Total	99	13178.927			

Means for Oneway Anova

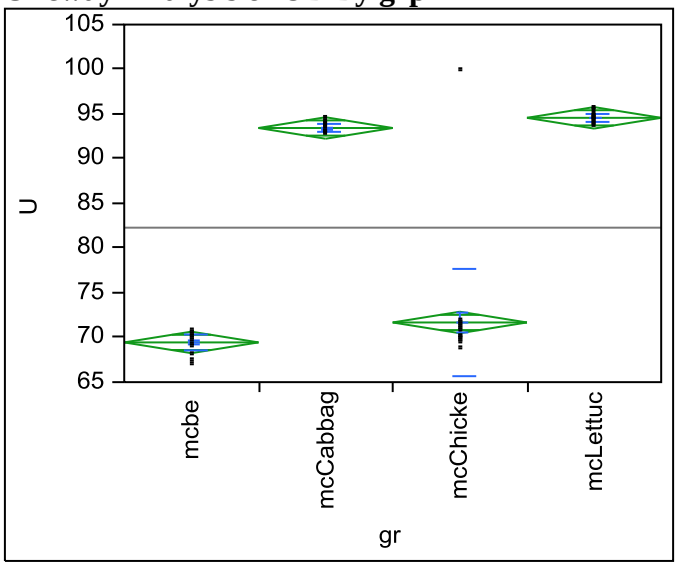
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
mcbeef	25	69.7844	0.15166	69.483	70.085
mcCabbage	25	92.3424	0.15166	92.041	92.643
mcChicken	25	71.7852	0.15166	71.484	72.086
mcLettuce	25	94.8280	0.15166	94.527	95.129

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
mcbeef	25	69.7844	0.67031	0.13406	69.508	70.061
mcCabbage	25	92.3424	0.55109	0.11022	92.115	92.570
mcChicken	25	71.7852	1.08032	0.21606	71.339	72.231
mcLettuce	25	94.8280	0.61646	0.12329	94.574	95.082

Oneway Analysis of UB By grp



Oneway Anova
Summary of Fit

Rsquare	0.94005
Adj Rsquare	0.938176
Root Mean Square Error	3.029335
Mean of Response	82.2806
Observations (or Sum Wgts)	100

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	13814.211	4604.74	501.7765	<.0001*
Error	96	880.979	9.18		
C. Total	99	14695.191			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
mcbeef	25	69.4452	0.60587	68.243	70.648
mcCabbage	25	93.4320	0.60587	92.229	94.635
mcChicken	25	71.6760	0.60587	70.473	72.879
mcLettuce	25	94.5692	0.60587	93.367	95.772

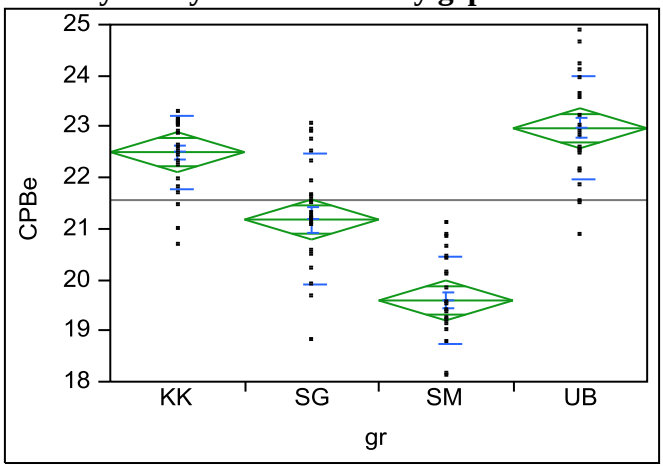
Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
mcbeef	25	69.4452	0.96601	0.1932	69.046	69.844
mcCabbage	25	93.4320	0.49815	0.0996	93.226	93.638
mcChicken	25	71.6760	5.94212	1.1884	69.223	74.129
mcLettuce	25	94.5692	0.46618	0.0932	94.377	94.762

CRUDE PROTEIN

Fit Y by X Group
Oneway Analysis of CPBeef By grp



Oneway Anova
Summary of Fit

Rsquare	0.647688
Adj Rsquare	0.636678
Root Mean Square Error	0.987521
Mean of Response	21.56
Observations (or Sum Wgts)	100

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	172.10830	57.3694	58.8286	<.0001*
Error	96	93.61890	0.9752		
C. Total	99	265.72720			

Means for Oneway Anova

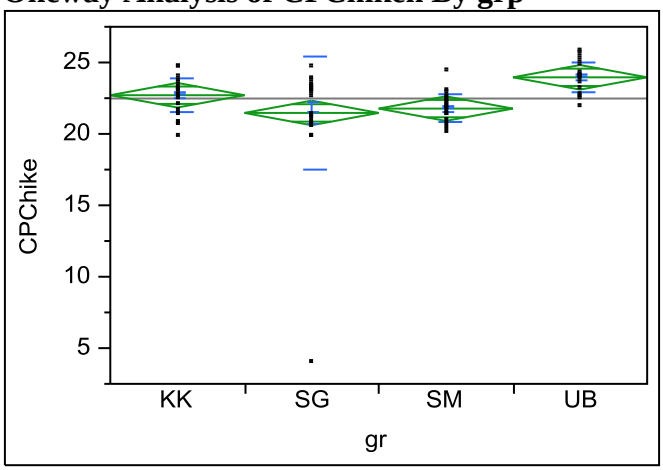
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
KK	25	22.5008	0.19750	22.109	22.893
SG	25	21.1788	0.19750	20.787	21.571
SM	25	19.5924	0.19750	19.200	19.984
UB	25	22.9680	0.19750	22.576	23.360

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
KK	25	22.5008	0.72270	0.14454	22.202	22.799
SG	25	21.1788	1.27474	0.25495	20.653	21.705
SM	25	19.5924	0.85082	0.17016	19.241	19.944
UB	25	22.9680	1.01471	0.20294	22.549	23.387

Oneway Analysis of CPChiken By grp



Oneway Anova
Summary of Fit

Rsquare	0.172952
Adj Rsquare	0.147107
Root Mean Square Error	2.167715
Mean of Response	22.4784
Observations (or Sum Wgts)	100

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	94.33450	31.4448	6.6918	0.0004*
Error	96	451.10284	4.6990		
C. Total	99	545.43734			

Means for Oneway Anova

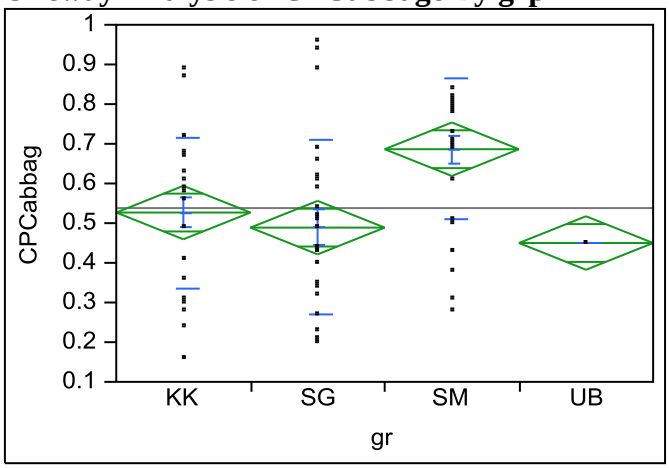
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
KK	25	22.7100	0.43354	21.849	23.571
SG	25	21.4692	0.43354	20.609	22.330
SM	25	21.7720	0.43354	20.911	22.633
UB	25	23.9624	0.43354	23.102	24.823

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
KK	25	22.7100	1.16437	0.23287	22.229	23.191
SG	25	21.4692	3.91534	0.78307	19.853	23.085
SM	25	21.7720	0.99850	0.19970	21.360	22.184
UB	25	23.9624	1.05512	0.21102	23.527	24.398

Oneway Analysis of CPCabbage By grp



Oneway Anova
Summary of Fit

Rsquare	0.225723
Adj Rsquare	0.201526
Root Mean Square Error	0.169895
Mean of Response	0.538
Observations (or Sum Wgts)	100

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	0.8078160	0.269272	9.3289	<.0001*
Error	96	2.7709840	0.028864		
C. Total	99	3.5788000			

Means for Oneway Anova

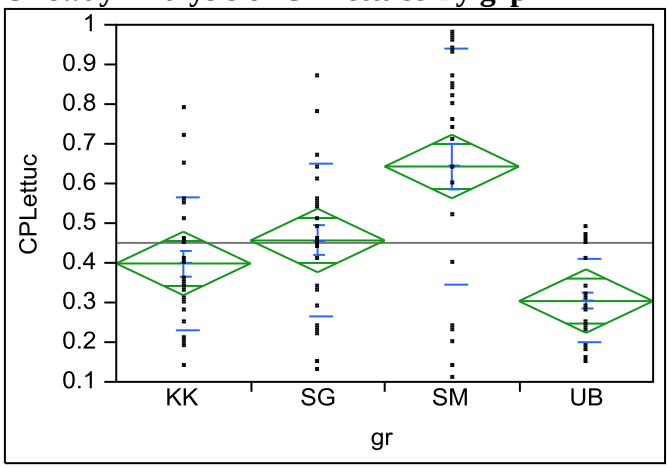
Level	Number	Mean	Std Error	Lower 95%	Upper 95%
KK	25	0.526800	0.03398	0.45935	0.59425
SG	25	0.488800	0.03398	0.42135	0.55625
SM	25	0.686400	0.03398	0.61895	0.75385
UB	25	0.450000	0.03398	0.38255	0.51745

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
KK	25	0.526800	0.189884	0.03798	0.44842	0.60518
SG	25	0.488800	0.219247	0.04385	0.39830	0.57930
SM	25	0.686400	0.177009	0.03540	0.61333	0.75947
UB	25	0.450000	0.000000	0.00000	0.45000	0.45000

Oneway Analysis of CPLettuce By grp



Oneway Anova
Summary of Fit

Rsquare	0.281483
Adj Rsquare	0.259029
Root Mean Square Error	0.201876
Mean of Response	0.4503
Observations (or Sum Wgts)	100

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
grp	3	1.5326990	0.510900	12.5362	<.0001*
Error	96	3.9123920	0.040754		
C. Total	99	5.4450910			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
KK	25	0.398400	0.04038	0.31826	0.47854
SG	25	0.456400	0.04038	0.37626	0.53654
SM	25	0.642800	0.04038	0.56266	0.72294
UB	25	0.303600	0.04038	0.22346	0.38374

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
KK	25	0.398400	0.166500	0.03330	0.32967	0.46713
SG	25	0.456400	0.191874	0.03837	0.37720	0.53560
SM	25	0.642800	0.296008	0.05920	0.52061	0.76499
UB	25	0.303600	0.104199	0.02084	0.26059	0.34661