Whole genome sequence analysis of virulent *Listeria* species from different sources

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Thesis accepted in fulfilment of the requirements of the degree Doctor of Philosophy in Biology at the North-West University

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Graduation: July 2024

DECLARATION

I, **Christ-Donald Kaptchouang Tchatchouang**, declare that the thesis entitled **Whole genome sequence analysis of virulent *Listeria* species from different sources**, submitted for the degree of Doctor of Philosophy in Biology (Molecular Microbiology) at the North-West University and the work contained herein is my own work in design and execution. It has not previously, in its entirety or part, been submitted to this or other universities for a degree. I further declare that all the materials contained herein have been duly acknowledged.

Signed at ……..... on this .....Day of 2023

_____

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Professor G Amagliani (Co-promoter)

DEDICATION

I would like to dedicate my PhD dissertation work to my family. From the bottom of my heart, I would like to thank my loving parents (Mr Tchatchouang Emmanuel and Mrs Tchatchouang Emilienne) for their endless love and encouragement. I also dedicate this work and give special thanks to my brothers, who have led by example. They have been my best cheerleaders.

.

ACKNOWLEDGEMENTS

First and foremost, I give thanks to the Lord Almighty for granting me a sound mind and a healthy body, which allowed me to finish this PhD study.

My sincere gratitude and appreciation go out to Prof. Collins N. Ateba, who served as my promoter and mentor throughout my graduate studies, for his indefatigable support, inspiration, and direction. I also appreciate the contributions from my co-promoter Prof G Amagliani from the University of Urbino, Carlo Bo, Italy.

To the Faculty of Natural and Agricultural Sciences, FNAS Dean, and the School of Biological Sciences staff of the Northwest University for their financial support, patience, and willingness to assist me at all times.

To my child, family, and friends who have helped me out in different ways while I've been doing this study.

SUMMARY

The World Health Organization has identified *Listeria monocytogenes* as one of four foodborne pathogens. It is a common Gram-positive facultative anaerobic bacillus. *Listeria monocytogenes* is the cause of the foodborne illness listeriosis, which has a mortality rate of 20–30% and is known to lead to serious complications like meningoencephalitis, sepsis, fetal infection, or miscarriage in pregnant women. It also affects young children, the elderly, and people with compromised immune systems. The purpose of this study was to determine the prevalence of virulent *Listeria* species in food and water samples collected from different sources in North-West, South Africa. A total of 207 samples of meat, milk, vegetables and water were collected and analysed for the presence of *Listeria* species using culture methods. The presumptive *Listeria* species detected were confirmed through species-specific PCR assay. The confirmed *Listeria* species were phenotypically screened for antibiotic resistance and biofilm-forming ability. Two multidrug-resistant and biofilm former *Listeria* species detected were subjected to whole genome sequence analysis to unravel their virulence, antimicrobial resistance, and biofilm genes. In this study, a total of 267 presumptive *Listeria* isolates were isolated. Out of which 53 (19.85%) were confirmed as the *Listeria* species, and these comprised 26 *L. monocytogenes*, 3 *Listeria* spp., 2 *L. welshimeri*, and 1 *L. thailandensis*. A large proportion of the *Listeria* species were resistant to erythromycin (76%), clindamycin (100%), gentamicin (100%), tetracycline (100%), novobiocin (100%), oxacillin (100%), nalidixic acid (100%), and kanamycin (100%). The isolates showed a range of multi-drug resistant (MDR) phenotypes, with the *L. monocytogenes* isolates showing the most E-DA-GM-T-NO-OX-NA-K MDR phenotypes. Additionally, 28% of the *L. monocytogenes* isolates were able to form biofilm at 4, 25, and 37 °C after 24, 48, and 72 h incubation period. Based on Whole Genome Analysis (WGS) analysis, the two isolates were confirmed to harbour virulent genes such as those responsible

for adherence, invasion, and toxin production. The genome sizes of both *Listeria* isolates were 3,341,013 and 3,558,124 bp and the GC contents were 37.35 and 36.66 % (LIN_NWU_CNKT and LIN_NWU_CNKT respectively). Both genomes possessed antibiotic-resistant genes, which confer resistance to drugs such as phosphonic acid, lincosamide, glycopeptide and tetracycline. Furthermore, several genes which are involved in biofilm formation were found in the genomes of the isolates. The frequent assessment of food and water for the presence of virulence, resistance, and biofilm-forming organisms is crucial to safeguard public health. This investigation detected *Listeria* species with virulence, multidrug resistance and biofilm producing abilities in food and water samples. These results imply that the *Listeria* spp. present in the North-West province of South Africa, possess virulent and resistant genes, which makes it of public health significance as it is found along the food chain.

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PUBLICATIONS FROM THIS THESIS

Kaptchouang Tchatchouang, C-D., Fri, J., De Santi, M., Brandi, G., Schiavano, G.F., Amagliani, G. and Ateba, C.N., 2020. Listeriosis outbreak in South Africa: a comparative analysis with previously reported cases worldwide. *Microorganisms*, *8*(1):135. https://doi.org/10.3390/microorganisms8010135

Kaptchouang Tchatchouang, C-D., Fri, J., Montso, P.K., Amagliani, G., Schiavano, G.F., Manganyi, M.C., Amagliani, G., and Ateba, C.N. 2022. Evidence of Virulent Multi-Drug Resistant and Biofilm-Forming *Listeria* Species Isolated from Various Sources in South Africa. *Pathogens*, 11(8):843.

CHAPTER ONE

INTRODUCTION

1.1 Background

Listeria species is Gram-positive which is classified as aerobic, microaerophilic and facultative anaerobic bacteria which comprises 17 species (Orsi and Wiedmann, 2016). The genus *Listeria* is classified under the family *Listeriacaea* and the optimum growth conditions of *Listeria* are up to 10% salt tolerance, pH 6-9 and 0 – 45 °C of ideal temperature with the optimal temperature being 30–37 °C (Lado *et al.*, 2007). Biochemical assays show that the cells are catalase positive, oxidase and morphological identification indicates chains or as single rods, non-sporulating, negative rod bacterial cells. A normal cell size measures 0.5 µm in diameter and 1 - 2 µm in length. *Listeria monocytogenes* is widely known as a significant human pathogen (Lakicevic *et al.,* 2015). Listeriosis is a serious disease with a mortality rate of 20%-30% caused by pathogenic *Listeria* species *(*Zhu *et al*., 2017*)*.

Listeriosis is a foodborne disease worldwide with a relatively high fatality rate (Murray *et al.*, 1926). Processed food may get contaminated during production from the raw product to the final consumer. This affects the public health and economic status of a particular country (Hussain and Dawson, 2013). Good manufacturing practices must be strictly followed to identify the critical control point where there is a high risk of contamination. Consumption of contaminated food is the main route of transmission to humans. Common symptoms included diarrhoea, abdominal pain, fever, vomiting and mild flu-like illness. Complications of the disease may lead to meningitis, meningoencephalitis or septicaemia. Immune-compromised individuals (pregnant women, HIV patients, cancer patients, infants, elders etc.) are highly at risk of contracting the disease. Pregnant

women infected with this bacterium can experience influenza-like illness (Anderson *et al.*, 2017). The bacteria can penetrate the placenta barrier and infect the fetus which can cause a miscarriage, stillborn and can affect brain development (Vázquez-Boland *et al.*, 2017). It is essential to identify where and when the food is contaminated in order to prevent future outbreaks.

Outbreaks of *Listeria* have been reported from hot dogs, meat, poultry, pasteurized and unpasteurized milk (Fleming *et al.*, 1985; Gudbjörnsdottir *et al.*, 2004; Lennox *et al.*, 2017), cheese (Goulet *et al.*, 2013; Bertsch *et al.*, 2013), vegetables, fruits (Ho *et al.*, 1986), plants, icecream (Forbes *et al.*, 1998), soil, water (Lang Halter *et al.*, 2013), sewage, and human samples (Noll *et al.*, 2018). A total of 820 listeriosis cases have been reported by the National Institute of Communicable Diseases (NICD) from January 2017 till 23 January 2018. The highest cases originated from the Gauteng province (59%), followed by the Western Cape (13%) and KwaZulu-Natal (7%). Samples were collected from blood (71%) and Cerebral Spinal Fluid (CSF) with 23% in the public and private healthcare sectors. Approximately 66% were diagnosed from the public and 34% from the private healthcare sectors within South Africa. The age distribution of listeriosis cases ranged from birth to 93 years old. The highest age group was neonate (≤28 days) with 96%. The gender distribution was 55% of female and 45% of male. Twenty-nine per cent of the cases were finally confirmed as listeriosis of which 34% died (NICD, 2018). Modern medicine has provided a wide range of antibiotics against Gram-positive bacteria including *Listeria* (Moellering Jr *et al.*, 1972).

In 1988, a patient suffering from meningoencephalitis was the first to present a multidrug-resistant *L. monocytogenes strain* in France (Poulsen *et al.*, 1988). Despite the multitude of antibiotics, *Listeria* species are resistant to some of the antibiotics like first-generation quinolones, fosfomycin, and third-generation cephalosporin. Due to the variable host range of *Listeria* species,

it has been reported to have resistance to antibiotics used for human and veterinary medicine. Antibiotic susceptibility testing of *Listeria* is fundamental to identifying the antibiotic resistance profile. Resistance of *Listeria* species will depend on the diversity of the sampling areas and environmental sources (Noll *et al.*, 2018). *Listeria* species can produce biofilm which increases the resistance of the bacteria (Doijad *et al.*, 2015).

Biofilms are communities of microorganisms (bacteria or fungi) that colonize an abiotic/biotic surface by producing extracellular polymeric substances (EPS). These enable the microorganisms to survive in unfavourable conditions. The population can exist in the environment as a single or multiple species within the matrix. The EPS within the complex matrix reinforce the bonding and makes the attachment irreversible. The EPS is generally made up of proteins, polysaccharides, and DNA (Colagiorgi *et al.*, 2016). Biofilm is problematic in various industries including food, manufacturing and medical. The risk of food contamination is higher when biofilm is formed, and it is difficult to eradicate communities of microorganisms. Hence, effective disinfectants and antibiotics are critical against the formation of biofilms. Pathogenic bacteria within the biofilm matrix may result in contamination of food products and food-borne outbreaks of disease (Chae and Schraft, 2000). The development of antibiotic resistance is a serious challenge to the treatment of bacterial illnesses using antibiotics; therefore, alternatives to antibiotics are highly sought after (Chiang *et al.*, 2018). Future solutions require an in-depth understanding of the factors influencing the emergence and spread of virulence and AMR in bacterial populations.

The culture-based technique is necessary for identification and enumeration as well as detection of viability, virulence, and AMR phenotypic profile of bacteria (Gill, 2017). However, the lengthy and tedious culturing process as well as unculturable bacteria limit the application of the culture-based technique. Given current resources and infrastructure, molecular techniques including

Whole Genome Sequence analysis (WGS) have been developed for rapid and high throughput detection of bacteria in research, public health laboratories, reference laboratories and hospital infection control-affiliated laboratories (Kwong *et al.*, 2015). The superior attributes of WGS analysis are largely associated with speedy and confirmatory power, which facilitates the urgent detection of bacteria (Montso *et al.*, 2022). Moreover, WGS bioinformatics can be used to confirm the identity, pathotype, virulence and AMR profile of bacteria (Davedow *et al.*, 2022).

1.2 Problem statement

Listeria species especially *L. monocytogenes* is a microorganism known to cause listeriosis. It is a ubiquitous bacterium, a potential pathogen and capable of carrying antibiotic resistance genes. Many sporadic cases of infection as well as outbreaks have been reported even in developed countries. Most of the outbreaks were linked to contaminated food and water consumption. South Africa has experienced severe outbreaks of listeriosis caused by the genus *Listeria* in 2017. There is insufficient information regarding the prevalence, occurrence, antimicrobial susceptibility patterns, and biofilm-forming ability of *Listeria* species. Therefore, this study was critical to investigate the genetic characterization of virulent *Listeria* species. The data generated will contribute to scientific knowledge for further investigation on the prevention and control of *Listeria* species.

1.3 Research aim and objectives

1.3.1 Aim

This study aimed to unravel the virulence, antimicrobial resistance, and biofilm profile of *Listeria* species isolated from different sources in North-West province of South Africa.

1.3.2 Objectives

The objectives of the study were to:

- isolate *Listeria* species from water, meat, vegetables, milk, and cattle faeces using culture methods.
- confirm the identity of the presumptive *Listeria* species isolated using species-specific PCR analysis.
- determine the antibiotic-resistant profile of the *Listeria* species isolated.
- determine the biofilm-forming potential of *Listeria* species isolated.
- unravel the virulence, antimicrobial resistance, and biofilm genes of the isolated *Listeria* species using whole genome sequence analysis.

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CHAPTER TWO

GENERAL LITERATURE REVIEW

2.1 History of *Listeria*

In the year 1926, *Listeria* was first linked to the unexpected deaths of six young rabbits and Murray for the first time called them *Bacterium monocytogenes* (Murray *et al.*, 1926). Afterwards, in honour of Sir Joseph Lister, Pirie in 1927 named the bacterium *Listerella hepatolytica*. Pirie was a pioneer in the field of antisepsis, and he fruitfully isolated the bacterium from the liver of numerous gerbils (*Latera lobenquiae*) in South Africa (Pirie, 1927). Three patients infected by the Epstein-Barr virus (EBV) were the primary confirmed human cases in the Kingdom of Denmark in 1929 (Buchner and Schneierson, 1968). In 1940, to harmonize the nomenclature of the bacterium, the name *Listeria monocytogenes* was adopted. A few years later, in the early 1960s, original research by Mackaness demonstrated that *L. monocytogenes* can survive and multiply in macrophages, and therefore, *Listeria has* been used in immunological research as a prototype intracellular parasite (Mackaness, 1962). Furthermore, there were investigations on the molecular mechanisms which focused on the virulence of *Listeria* earlier in the 1980s (Kathariou, 2002). Their research consideration was directed firstly into the haemolytic activity, a virulence marker which was observed in the pathogenic species. The result of these studies was the discovery of the hemolysin gene "*hly*", which describes the significant role played by haemolysin for pathogen phagosomal escape which is essential for intracellular bacterial propagation (Vazquez-Boland *et al.*, 1992). A few years later, haemolysin not only turned into the first *Listeria virulence* factor with its gene characterization but was also recognised as the first bacterial gene to have a critical function in the existence of a parasite contained by the cells of eukaryote host (Pizarro-Cerdá and Cossart, 2006). A study conducted by Glasser *et al.* (2001) on genomic comparisons of *Listeria*

species has also provided an increased understanding and knowledge of *Listeria* (Glasser *et al.*, 2001). To date, a body of research has been conducted in different areas (immunology, pharmacology, epidemiology) to enhance discoveries for the progress of science and to reduce as much as we can listeriosis outbreaks all over the world (Figure 1).

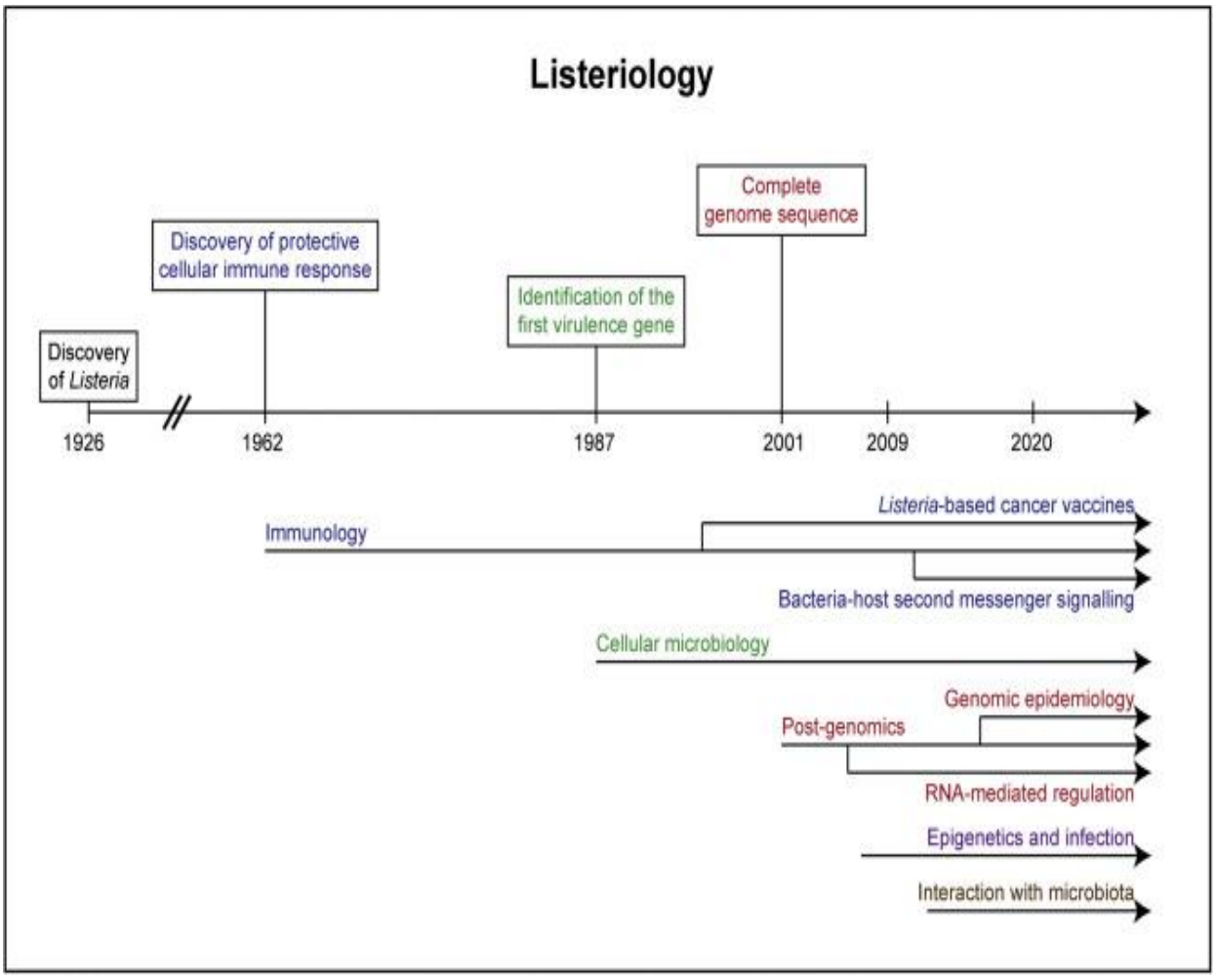


Figure 2.1: Listeriology timeline, showing the emergence of major areas of investigation (Lebreton *et al*., 2016).

2.2 *Listeria* species reservoirs and sources of food contamination

Listeria monocytogenes is described as a facultative anaerobic Gram-negative pathogen that is responsible for serious foodborne diseases in humans and other animal species, such as cattle, sheep, and goats (Nightingale *et al.*, 2004b). Most processed foods (e.g., pasteurization of milk) usually consist of raw food materials with inactivated *L. monocytogenes* which are time-temperature dependent. Ready-to-eat products are usually contaminated during food processing (Gudbjörnsdottir *et al.*, 2004; Nightingale *et al.*, 2004b; Sauders and Wiedmann, 2007). Furthermore, there is a strong correlation between human diseases and *Listeria monocytogenes* isolated from farm animals and the surrounding environment. Thus, the contamination of raw food occurs by composting from shedding animals (Ho *et al.*, 2007; Sauders and Wiedmann, 2007). Studies have proven that nearly 50% of *Listeria*-positive faecal specimens have been obtained from asymptomatic animals (livestock, goats, poultry, pigs and sheep) (Pearson and Marth, 1990; Orsi *et al.*, 2011). High levels of *L. monocytogenes* have been reported due to the ingestion of contaminated silage (Hinton, 2000). It was speculated that cattle farms may serve as natural sources of *L. monocytogenes* and, eventually, as a major source of contamination in the food manufacturing environment (Sauders and Wiedmann, 2007). Nonetheless, knowledge and understanding of the transmission of *L. monocytogenes* is limited, for instance, it is questionable if the infected animal affects the transmission of the *Listeria* pathogens or if the farm animals represent “dead-end” hosts.

L. monocytogenes transmission farms include preliminary contamination of agricultural crops and soil by wildlife, fowls or compost used to fertilize fields. Also, farm animals often encounter *L. monocytogenes* in the environment and crops through grazing. However, the infective dose of pathogenic *Listeria monocytogenes* may not be reached to cause an infection. Improperly

fermented silage (pH > 5.0 to 5.5) from contaminated crops and soil can permit successive intensification of *L. monocytogenes* numbers making silage feed a typical source of infection (Nightingale *et al.*, 2004b). Surface and underground waters, sewage sludge, and slaughter wastes can also serve as sources of *Listeria* (Farber and Peterkin, 1991; Ivanek *et al.*, 2006). The higher presence of *Listeria in* processing plants is due to the increased consumption of poultry meat. Animal-derived *L. monocytogenes* may discredit nourishment items, including crude drain, pasteurized drain, chocolate drain, and contaminate butter, delicate cheeses, meat, and poultry, and have been involved as sources of human listeriosis cases and flare-ups (Bryan, 1983; Glass and Doyle, 1989; Jay, 1996; Dangers *et al.*, 2018). Generally, *L. monocytogenes* is successfully inactivated during food processes such as pasteurization. It is therefore common that contamination of prepared ready-to-eat nourishment items by *L. monocytogenes* happens through cross-contamination of the wrapped-up item from the plant environment (Dangers *et al.*, 2018).

The study conducted by Jay (1996) has shown that approximately 16% of raw pork samples and 17% of raw poultry samples were contaminated by *Listeria* (Loessner *et al.*, 1997). Contamination due to handling post-heat treatment in the cutting room revealed *Listeria* spp. in vacuumed-packed cooked sliced ham, vacuumed-packed cooked meats, sausages and cured pork shoulder in a study conducted in Greece (Sergelidis *et al.*, 1997; Samelis and Metaxopoulos, 1999). In the food processing industry principally in the meat industry, the food-processing machines such as dicers and slicers in particular, are mostly associated with *Listeria*. Once on the equipment, the eradication of the contamination becomes very difficult which causes cross-contamination along the processing line (Lundén *et al.*, 2003).

Ready-to-eat (RTE) foods that are not heated before consumption have become a real concern in recent decades due to contamination with *L. monocytogenes.* Many food companies are adopting to manufacture quality RTE food with a low percentage of preservatives and sodium (Aarnisalo *et al.*, 2003) which is raising the population of bacteria growth and enhancing the possibility of the appearance of food outbreaks such as listeriosis. Different types of food such as soft cheese made with unpasteurized milk in 1984, hotdogs produced in processing plants in 1998 and sliced turkey meat produced from 2002 to 2003 were implicated in the three largest listeriosis outbreaks in the United States (Aarnisalo *et al.*, 2003; Kathariou *et al.*, 2003; Cartwright *et al.*, 2013; Kaptchouang Tchatchouang *et al.*, 2020).

All *Listeria* species are ubiquitous, and the bacterium is often described to demonstrate a plant saprophyte lifestyle. *Listeria is* commonly detected in the soil (Fenlon, 1999), water, manure, sewage (Watkins and SLEATH, 1981), vegetation (Weis and Seeliger, 1975; Wiedmann *et al.*, 1999a), animal feed (Wiedmann *et al.*, 1999b), and farm environments (Fenlon *et al.*, 1996b; Nightingale *et al.*, 2004c). *L. monocytogenes* has been confined from about 42 species of wild and domestic warm blooded animals, 17 avian species, counting residential and amusement fowl as well as shellfish, fish, oysters, ticks, and flies (Schuchat *et al.,* 1991). Also, this pathogen may be carried in the intestinal tracts of a small percentage of the human population without apparent symptoms (Grif *et al.*, 2003). Studies aimed at isolating *Listeria in* natural environments not associated with domestic livestock indicated that other *Listeria* spp. were detected at higher frequency than *L. monocytogenes* (MacGowan *et al.*, 1994). One such specifically reported a high incidence of *L. seeligeri* in samples collected from the environment. In another study where grass, leaves, stems, and roots were sampled, *L. monocytogenes* was detected in nine of 10 samples of

wilting grass; however, no *L. monocytogenes* was isolated from samples obtained from the roots or stems (Fenlon *et al.*, 1996b).

Listeria has also been shown to be common and present in surface samples from natural water sources, such as lakes, rivers and streams (Dijkstra, 1982). Next, animal feeds and improperly fermented ensiled feeds, in particular, have been associated with listeriosis outbreaks in sheep and cattle (Fenlon, 1985). The contaminated silage has been the outcome of poor silage quality such as inadequate moisture content, which may facilitate the survival and growth of *L. monocytogenes* (Grønstøl, 1979). The wide distribution of *L. monocytogenes* in nature allows this bacterium to be easily spread and cause infection. *Listeria monocytogenes* can cause infection by several transmission routes such as ingestion of contaminated foods (e.g. unpasteurized milk or contaminated ready-to-eat foods; (Fleming *et al.*, 1985; Linnan *et al.*, 1988), the transmission of the organism from mother to foetus in utero, directly to the foetus at the time of birth, or by direct contact with the organism which can cause lesions on the skin (McLauchlin *et al.*, 1990).

The ability of *L. monocytogenes* to survive and multiply in many non-host habitats and host species, and the number of possible transmission routes, makes this pathogen difficult to control. Contamination of foods by *L. monocytogenes* can happen at any point within the food chain, including on farms, in food processing plants, in retail establishments and the home (Nightingale *et al.*, 2004a). *L. monocytogenes* 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 *L. monocytogenes* also can be isolated from other foods such as beef, pork, fermented sausages, fresh produce and fish products (Rocourt and Bille, 1997). *Listeria* has been shown to survive within cultured buttermilk, butter and yoghurt; of which these specific foods primarily depend on adequate fermentation to yield a low product pH that does not support *Listeria*

growth. Many studies have shown that a wide variety of meats can become contaminated with *L. monocytogenes* and most contamination is observed in meat products and poultry. For example, Bailey *et al.* (1990) reported that between 12- 60% of raw chicken was contaminated with *L. monocytogenes* and young birds were colonized by this human pathogen at a higher rate.

Many studies have shown that the ability of *L. monocytogenes* to survive and grow on meat is dependent on temperature, pH of the meat, type of tissue and initial microflora already present on the meat's surface (Farber and Peterkin, 1991). Since *Listeria monocytogenes* is found in soil and water, raw vegetables can become contaminated from the soil or manure used as fertilizer (Schlech III *et al.*, 1983). Animals (i.e., wildlife and domestic livestock) can be asymptomatic carriers of *L. monocytogenes* and contaminate foods of animal origin such as dairy products and meats through asymptomatic shedding in milk and faeces. Not only can *L. monocytogenes* be isolated from raw foods (e.g. vegetables, uncooked meats), but it also can be detected in processed foods such as soft cheeses and delicatessen meats (Seeliger, 1958; Fenlon *et al.,* 1996a; Fenlon, 1999). Although Petran and Zottola (1989), it remains a significant problem in ready-to-eat foods for example frankfurters and delicatessen meats, that may become cross-contaminated by exposure in the food processing plant environment after cooking but before packaging (Tompkin, 2002).

In the food processing plant environment, one key reason that *Listeria* presents such a problem for many food manufacturers is simply because food processing conditions and the associated processing environment permit the growth of *Listeria.* This Gram-positive, facultative anaerobe, intracellular rod is capable of growth in a broad range of temperatures including refrigeration (e.g., 1 °C to 45 °C), a wide range of pH conditions (e.g., 4.3-9.5), relatively low water activity (> 0.90), and high salt concentrations (up to 10%), enabling survival and growth in many different foods and food-associated environments (Farber and Peterkin, 1991). Many studies have demonstrated

the ability of *L. monocytogenes* to colonize, multiply, and persist in the food processing environment as well as on food processing equipment over extended periods, showing the environmental survival characteristics of this foodborne pathogen (Lappi *et al.*, 2004; Kabuki *et al.*, 2004). Overall, *Listeria* is a very adaptable pathogen that is capable of survival even after freezing, surface dehydration, and spray chilling; however, *Listeria* can easily be killed with proper cooking (Seeliger and Jones, 1986; Junttila *et al.*, 1988). The nature of *Listeria* infection has been reported to cause serious side effects in animals and humans.

2.3 Transmission of the disease

Listeria monocytogenes is a facultative intracellular pathogen that can cause serious foodborne infection in humans and can cause invasive infections in numerous animal species, including ruminants (cattle, sheep, and goats) (Bagatella *et al.*, 2022). While contaminated animals and contaminated agrarian situations do not always directly result in human contaminations, animal-derived food products that have not been cooked recently (like raw drain) and raw plant root foods that have been contaminated by animal excrement or shedding present connections between human diseases and *L. monocytogenes* in domestic animals and domestic environments (Spanu and Jordan, 2020). Approximately 80- 90% of transmission of *Listeria* spp. is via the oral route, through the consumption of foods and water contaminated by animal faecal matter (den Bakker *et al.*, 2013; Jarvis *et al.*, 2016). However, they can also be transmitted between humans (from mother to fetus both in peripartum and uterus following maternal bacteremia). Other uncommon possible modes include infected animals to humans (Nelson *et al.*, 1985; Abay *et al.*, 2019; Goenka and Kneen, 2019). Transmission of *Listeria* from animal to human and its circulation in the environment is presented in Figure 2.

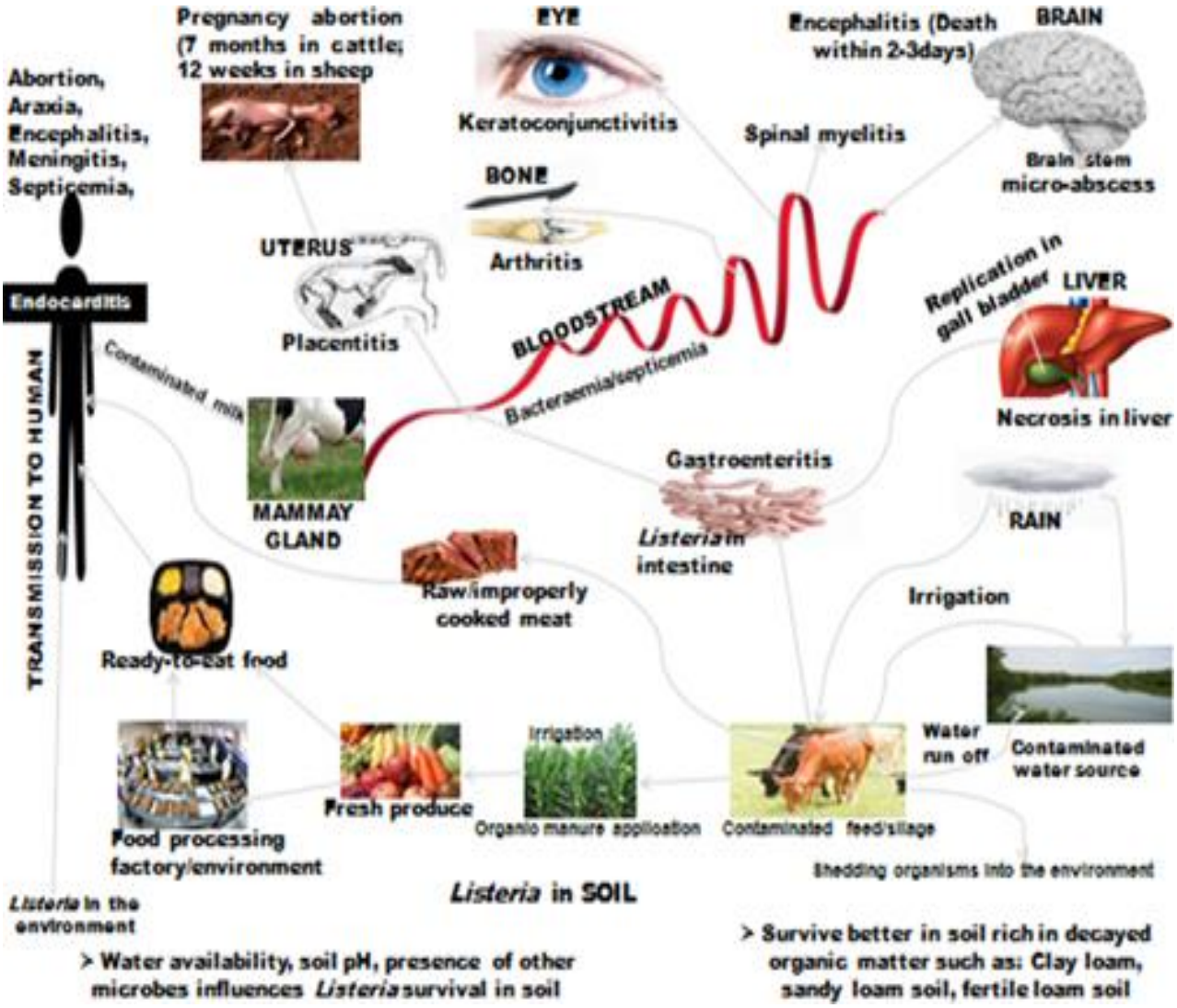


Figure 2.2: Transmission of *Listeria* species from animal to human and its circulation in the environment.

The infectious dose of *Listeria* may vary with the susceptible host and the strain causing infection (Golnazarian *et al.*, 1989). The approximate value of the infectious dose of *L. monocytogenes* for humans and primates is 10^6–10^7 CFUs, although a lower dose of 10^4 can cause listeriosis in persons with compromised immune systems as was the case of the outbreak associated with butter in Finland (Drolia and Bhunia, 2019). On the other hand, the World Health Organization (Organization, 2004) estimated 10^7–10^9 CFU as the infectious dose for healthy persons and 10^5–

10 CFU for high-risk individuals and the lethal dose value (LD_{50}) derived from the animal model for human listeriosis is 1.9×10^6 CFU. Due to the lack of a uniform benchmark on the exact value of infectious dose of this pathogen, the Zealand (2014) report stated that *Listeria monocytogene* should not be found in ready-to-eat food that supports its growth (not detected in 25 g) and a maximum value of 100 cfu/g for ready-to-eat food that will not support its growth. This limit was aimed at remedying the high death rate among vulnerable groups often associated with listeriosis outbreaks.

2.4 Clinical manifestations (signs and symptoms) of listeriosis

Listeriosis affects immunocompromised individuals such as newborns, or debilitated hosts, the elderly and pregnant women resulting in fatal consequences. Other immuno-compromised individuals who are highly at risk are cancer patients, transplant recipients, diabetics, and persons with AIDS (Schuchat *et al.*, 1992). Listeriosis infection causes fever, flu-like illness, mild, diarrhoea, abdominal pain and vomiting. A non-invasive form of listeriosis that manifests in immunocompetent people as febrile gastroenteritis, flu-like symptoms, and a shorter incubation period of 6 to 240 h may also occur. It consists of common symptoms like fever and muscle aches. After ingestion of the *Listeria,* within the first 5 days of the incubation, the bacteria may proliferate deeper in the tissues forming an invasive form of listeriosis and producing a systemic infection and the 21 days (3 weeks) represents the incubation period (FDA, 2009). The spread of the *Listeria bacterium* to the nervous system may cause headaches, stiff neck, loss of balance, confusion, or convulsions. In addition, listeriosis infection of the central nervous system may result in rhombencephalitis (brainstem encephalitis), encephalitis, or focal signs suggestive of brain abscess formation (Crum, 2002).

The disease conditions associated with *Listeria* infection could be categorized into non-invasive (febrile *Listeria* gastroenteritis) and invasive (infection caused at sterile part of the body such as the blood, liver and cerebral fluid) listeriosis (Zhu *et al.*, 2017). Non-invasive listeriosis manifests less severe symptoms like fever, diarrhoea, headache, and muscle pain (myalgia) which occur after a short incubation period and are often associated with ingestion of high doses of the pathogenic *Listeria* species by healthy persons (Halbedel *et al.*, 2020). Invasive listeriosis affects immunosuppressed individuals including cancer patients, pregnant women, acquired immunodeficiency syndrome (AIDS) patients, as well as young and elderly persons (Desai and Smith, 2017). It is usually characterized by the severity of symptoms and high death rate commonly associated with gastroenteritis, meningitis, pneumonia, septicaemia, and spontaneous abortion (Arslan and Baytur, 2019) (Vázquez-Boland *et al.*, 2001).

Unusual cases of lateral transmission of listeriosis from animals to humans have been reported. In these incidences, human disease symptoms have characteristics of localized cutaneous diseases as a substitute for systemic involvement (Vázquez-Boland *et al.*, 2001). In ruminant animals, *L. monocytogenes* mainly affects encephalitis and uterine diseases. Uterine diseases result in late-term abortions or septicaemia in neonates. Furthermore, animals with listeriosis show neurological symptoms (McLauchlin *et al.*, 1986; Pohl, 2005; Amene and Firesbhat, 2016). In uncommon cases, whereby the coordinated transmission of *L. monocytogenes* from animals to humans has been observed, infected persons presented with localized cutaneous instead of systemic disease. Such has resulted in skin lesions among veterinarians. Uterine contaminations are characterized by late-term premature births or septicaemia in neonates (Kaptchouang Tchatchouang *et al.*, 2020).

Furthermore, *L. monocytogenes* might affect the eyes and cause keratitis in ruminant animals; these signs have been associated with direct infection of the eyes with pathogenic *L. monocytogenes* existing in feeds, including silage (Hof, 2017). Shedding of *L. monocytogenes* in defecation material of infected animals; nonetheless, latent *L. monocytogenes* can exist in healthy animals (Wesley, 1999). Common symptoms experienced by infected pregnant women include headache, chills, gastroenteritis, or slight dizziness. A previous report shows that the average incubation time of listeriosis disease in pregnancy is 27.5 days, with a range from 17–67 days. Furthermore, it was stated that the symptomatic effect is similar to flu symptoms, showing fever, headache, and backache. Neonatal sepsis might also result from listeriosis in pregnancy commonly happens in the first trimester, and the effect is detected during the third trimester. Sepsis (bacteraemia) in pregnant women is a life-threatening condition caused by the body's reaction to listeriosis infection. This leads to spontaneous stillbirth, abortion, premature birth, or septicaemia, particularly in the newborn. Generally, the fetus or newborn is more vulnerable than the mother (Silver, 1998). According to the Food and Drug Administration (FDA, 2009), nearly 80% of perinatal and neonatal listeriosis infections may result in mortality. Healthy adults experience mild gastroenteritis; however, it is severe in immune-compromised individuals.

Gastroenteritis associated with *L. monocytogenes* shows numerous symptoms including fever (60–100% of cases), non-bloody diarrhoea (33–88%), arthromyalgia (20–100%) and headache (15–88%). Children with gastroenteritis experience fever and vomiting whereas adults experience diarrhoea and arthralgia. Immunocompromised populations, who are highly at risk, might develop meningitis, meningoencephalitis, or septicaemia. The colonization rate of pathogenic *Listeria is* estimated at 1–5% in healthy adults. Person-person contact increases the exposure of transient carriage of the pathogen per annum. A study reported that 2 out of 3 individuals encounter

incidents of faecal carriage that takes not more than 4 days and has an asymptomatic effect. Moreover, individuals with invasive *Listeria infection* exhibit 20–25% of faecal carriage.

Neonatal *L. monocytogenes* infections are due to endogenous transplacental transmission, breathing in contaminated amniotic fluid, or subsequent colonisation from a vaginal or maternal gastrointestinal carriage (Dyatlov and Lawrence, 2002). Therefore, the genital tract and blood are isolation sources of *Listeria pathogens* in the majority of the mothers, as shown in Figure 2.3. The premature newborn exhibits the onset of infection within 36 h, characterised as a transplacental neonatal disease. Neonates display sepsis (90%), respiratory distress or pneumonia (40%), meningitis (25%) and, often inflammatory granulomata also commonly referred to as granulomatosis infantiseptica. Furthermore, the infected newborn experiences a hasty maculopapular rash on the extremities. In some individuals, *Listeria monocytogenes* infection might spread to the bloodstream, resulting in bacteraemia. Bacteraemia also results in gastroenteritis and is commonly, associated with pregnancy or neonatal disease. Adult bacteremia may be a clinical reaction of HIV disease, steroid use, chronic renal infection, chemotherapy, some malignancy, or age older than 65 years. Acute meningitis and encephalitis due to *L. monocytogenes* are regarded as life-threatening complications of listeriosis. Complications like rhomboencephalitis affect the pons, midbrain, and/or cerebellum connected to the cranial nerve participation or cerebellar effects (ataxia, tremor), or the growth of hemiparesis. It has been reported that in neuroinvasive listeriosis cases, neck stiffness was experienced by 75% of patients, focal neurological symptoms in 30%, as well as 30% in seizures and coma in 7% (Tchatchouang *et al.*, 2020). The side effects of focal neurological effects include one or more cranial nerve participation (usually the 6th and 7th cranial nerves), aphasia, ataxia and hemiparesis.

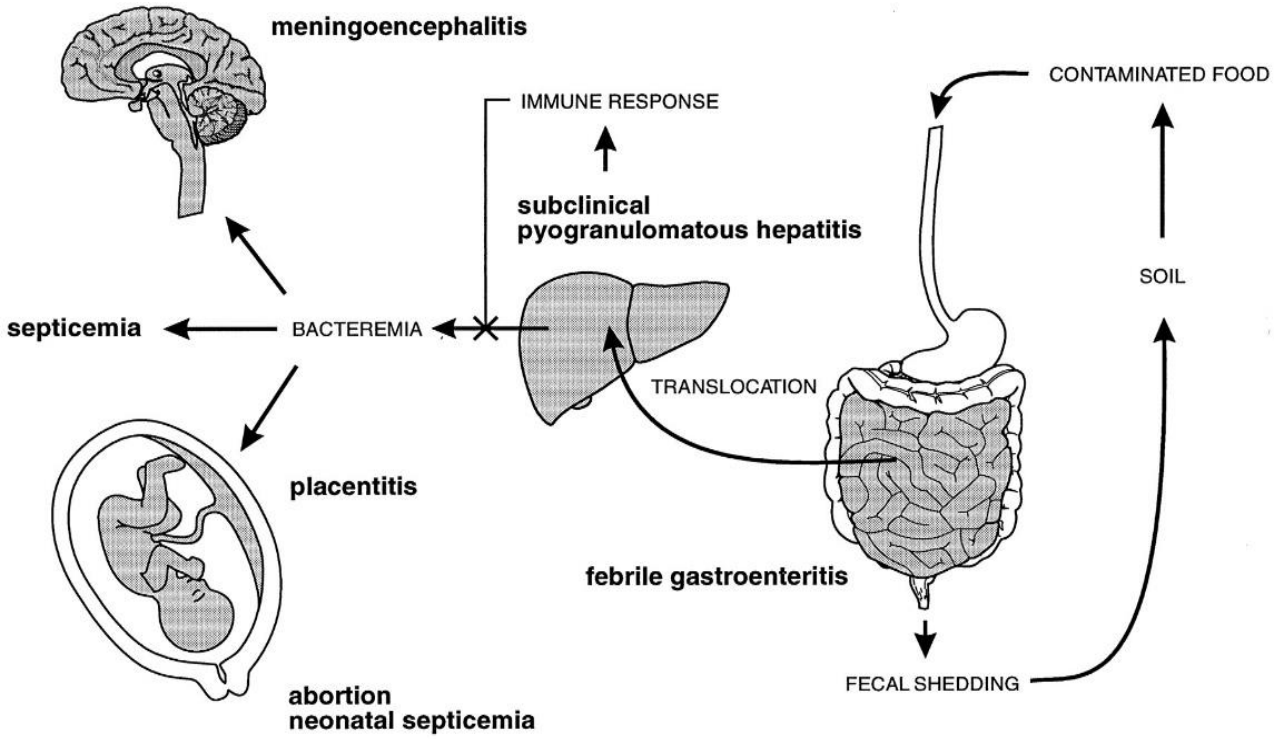


Figure 2.3: Schematic representation of the pathophysiology of *Listeria* infection (Vázquez-Boland, Kuhn, et al., 2001).

2.5 Pathogenicity and virulence of *Listeria*

After oral consumption infection with *L. monocytogenes* the pathogenic bacteria can cross the physiological barrier (intestinal barrier, the blood-brain barrier, and the fetoplacental barrier) of the individual. In the case where the pathogen crosses the intestinal barrier, it may be absorbed through the intestinal lumen, passing the epithelial cell which may spread to the blood system and mesenteric lymph nodes, especially in persons with suppressed immune system *L. monocytogenes* is then distributed to the liver and spleen colonizing especially the splenic and hepatic macrophages or epithelial cells. The accessory cells (dendritic cells) are the next cells targeted. Therefore the *L. monocytogenes* is capable of escaping the phagosomes and continuing to spread from cell to cell. Bacteria proliferation enables further colonization of more body cells except there is an effective adaptive immune response (Feng *et al.*, 2013). If it fails, it may lead to the spread

of the bacteria from the bloodstream to the brain and the placenta, causing a serious systemic infection. Using a mouse model for cellular immunity studies, *Listeria* is shown to express phospholipases (*PlcA* and *PlcB*) and pore-forming toxin listeriolysin O (*LLO*) to escape from the phagolysosome (Rudnicka *et al.*, 1997) to the cytoplasm to multiply with a generation time of one hour. *Listeria* has evolved a well-organized system that couples actin polymerization with intracellular mobility for the goal of adaptability. This is prepared to invade and infect nearby host cells. The protein *ActA* with actin filaments will reorganize into structures constructing comet tails at one distal end of the bacterial cells, thus permitting the bacteria to migrate nondirectionally (Domann *et al.*, 1992).

The actin filaments propel the bacteria until they get to the cytoplasmic membrane and produce filopodia or protrusions. Cell-to-cell spread occurs after it may be consumed by an adjacent cell. After the escape of the pathogen from the secondary double-membrane vacuole, the proliferation cycle restarts again. Similar to most bacteria, the virulence genes of *Listeria* are arranged into discrete genetic units called pathogenicity islands. The listed virulence factors are the crucial steps of *L. monocytogenes* intracellular parasitism and are physically connected with other virulence genes in a 9-kb chromosomal island named LIPI 1 (*Listeria* pathogenicity island 1) (Böckmann *et al.*, 1996). However, the pathogenesis of listeriosis is complex and involves numerous other significant genes, and factors that aid in the virulence of *L. monocytogenes* throughout the passageway through the hostile environment of the gut and the various steps of infection. The expression of diverse genes contributes to the pathogenicity of *L. monocytogenes* that assist in the penetration, proliferation and spreading of the cells. The above features are due to the hpt intracellular growth locus that is a specific transporter for phosphorylated hexoses, *InlA*, *Listeria pathogenicity* island-1 (*LIPI-1*), *InlAB* internalisation locus and *InlA* and *InlB* surface proteins

encoded by *InlAB* internalisation locus. They cluster with their respective receptors on the host cell surface using their Leucine-Rich Repeat (*LRR*) domains (*InlA* with human E-cadherin, a calcium-dependent intercellular adhesion glycoprotein, although *InlB* and the hepatocyte growth factor receptor *Met* work cooperatively to facilitate bacterial internalization and invasion into host cells (Camejo *et al.*, 2011).

Listeriolysin O (*LLO*) which is a pore-forming toxin and two phospholipases C (*PlcA* and *PlcB*) which are encoded on *LIPI-1*, bind to digest the phagocytic vacuole envelop of host cells. The hpt intracellular growth locus cluster with a component of a complex system (hexose-6-phosphate) and actin assembly inducing protein (*ActA*), to play a significant part in the intracellular bacterial growth, cell-to-cell migration, and actin polymerisation. The virulence of *L. monocytogenes* is generally managed by six genes (*PrfA, PlcA, hly, mpl, actA* and *plcB*) belonging to the PrfA-dependent virulent gene groups and some virulence-related genes positioned at the exterior of the gene cluster. Stress survival islet 1 (*SSI-1*) is also *L. monocytogenes* genes that are responsible for cell survival under unfavourable conditions such as acidic pH and elevated salt concentrations, particularly within the food industry (Sokolovic *et al.*, 1993). *L. monocytogenes* and *L. ivanovii* possess positive regulatory factor A, *PrfA* which regulates the expression of diverse virulence factors. Furthermore, they consist of the *hlyA* gene that encodes a 60-kDa sulfhydryl-activated pore-forming listeriolysin O (*LLO*) that is critical to assist in the release of invading bacterial cells from the phagosomes of host cells into the host cytosol (Fig 2.4) (Sokolovic *et al.*, 1993).

The *LLO* is primarily accountable for clearing bacterial cells from the initial and secondary intracellular vacuoles. *hlyA* and *LLO* are the principal gene targets for molecular identification of *L. monocytogenes* particularly in food samples, on-farm microbiological control initiatives, and

infection in pregnant women their corresponding protein target for serological diagnosis of ovine listeriosis. Lysis of host cell membranes may be assisted by *plcA* and *plcB* encoded for phosphatidylinositol-specific phospholipase C and phosphatidylcholine phospholipase. Research shows that *InlA* and *InlB* internalins which are cell wall proteins are essential for adhesion and invasion of non-phagocytic host cells, however, might be expressed or repressed in certain environmental circumstances, therefore influencing the virulence of *L. monocytogenes*. (Cossart and Bierne, 2001 ; Vázquez-Boland *et al.*, 2001; Dussurget *et al.*, 2004; Shi *et al.*, 2013), Figure 2.4.

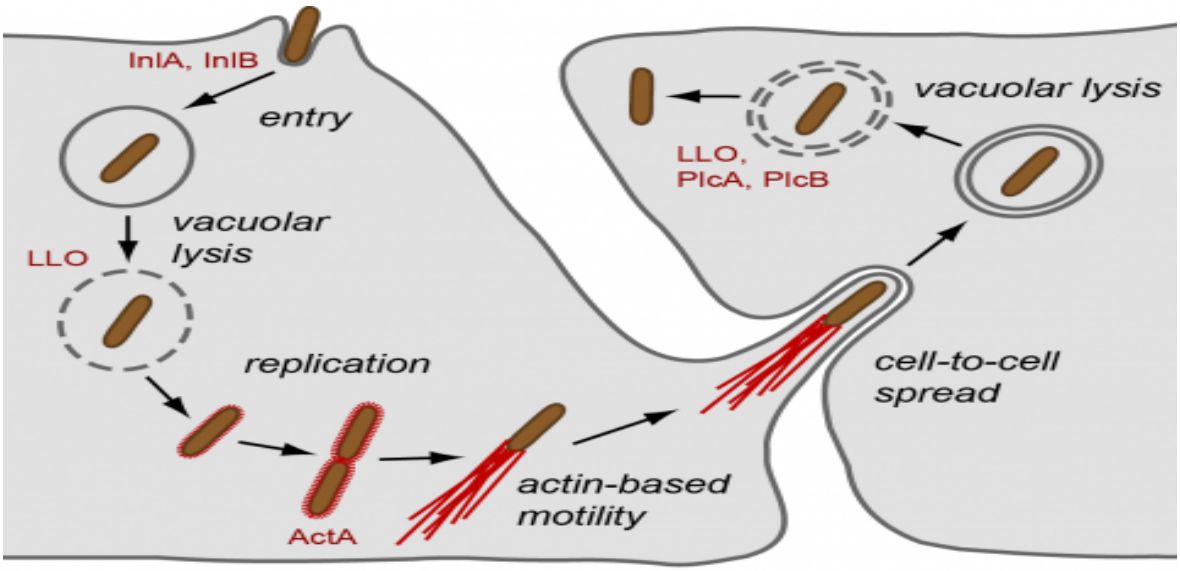


Figure 2.4: Virulence factors and pathogenesis of *L. monocytogenes* (source image: Pasteur Institute)

2.6 Epidemiology of listeriosis and outbreaks reported worldwide

The Centers for Disease Control and Prevention (CDC) estimated 1600 cases of listeriosis with 260 mortalities per 100,000 population in the United States from 2009 to 2010 (CDC, 2012). In the United States, *L. monocytogenes* is one of the most infectious food-borne pathogens

accountable for 28% of mortality rates with 92% representing the highest rate of hospitalization (Scallan *et al.*, 2011). Pregnant women are considered as most vulnerable individuals, 20 times more likely to be infected by *Listeria monocytogenes* resulting in listeriosis than other healthy populations due to its invasive nature (Cossart and Bierne, 2001) reported that approximately one-third of Listeriosis cases are pregnancy-associated cases. A study conducted by Mead *et al*. (2006) highlighted that about 2,500 cases of clinical listeriosis in human beings (500 human losses) occur per annum in the United States (Mead *et al.,* 2006). Furthermore, *L. monocytogenes* contaminated food that was found from animal origin, raw milk, or pasteurized milk (Pitt *et al.*, 1999; Miettinen *et al.*, 2001), is considered a cause of human listeriosis cases and outbreaks (Lovett *et al.*, 1987; Van Kessel *et al.*, 2004; Magalhães *et al.*, 2015; Allata *et al.*, 2017). In Nova Scotia, there were reported cases of 42 human listeriosis in an outbreak involving contaminated coleslaw. The source of the listeriosis was due to the untreated sheep compost used on the farm as fertilisers which previously had ovine listeriosis (Swaminathan and Gerner-Smidt, 2007). In 1999, Mead *et al*. (2006) reported that roughly 2,500 clinical human obtrusive listeriosis (counting 500 passings) happen each year in the United States (Mead *et al.*, 2006).

Numerous studies have reported serious life-threatening outbreaks of *L. monocytogenes* globally. According to the Centers for Disease Control (CDC, 2008), approximately 3.4 per million to 4.4 per million incidence cases of listeriosis occur in the United States every year (Tappero *et al.*, 1995). In addition, the incidence of listeriosis fluctuates from 3.5 individuals per million in Bristol, England increasing to 6 to 7 individuals per million in Denmark. *L. monocytogenes* has been isolated from various sources such as raw or contaminated milk, contaminated vegetables, and RTE meats such as hot dogs or pate. A significant number of listeriosis outbreaks are sporadic or transpire in small groupings, determining the food source accountable for sporadic cases or small

clusters is challenging, and the food attribution of listeriosis remains to be fully elucidated (Slutsker and Schuchat, 1999). After the occurrence of the cluster of infections, epidemiological studies are conducted to avoid further infection as well as to identify the liable food source and food processing plant where that food was produced. An outbreak of foodborne infection might be identified when a group of two or more infections are related to the same or similar isolate of a specific foodborne pathogen and the same food source (CDC, 2019).

Identification of the small clusters is essential and must be studied to identify the probable common source accountable for the outbreak as well as to prevent further diseases. Fortunately, human listeriosis is rare; nevertheless, it has an extended incubation period of 7-60 days, which creates problems in linking the disease to a specific food product (Farber and Peterkin, 1991). In the United States, twelve severe listeriosis outbreaks happened between 1970 and 2002. Thus, leading to 466 listeriosis infections, whereby only two of these twelve outbreaks affected more than 100 cases each (USDA, 2003). Moreover, raw eggs were involved in one of the outbreaks, and the remaining eleven outbreaks were related to the consumption of RTE products. One of the biggest outbreaks was caused by contaminated Mexican-style soft cheese resulting in 142 cases (Linnan *et al.*, 1988). Another severe listeriosis outbreak involved contaminated frankfurters and deli meats affecting 101 persons with 21 mortalities (CDC, 1998; CDC, 1999; Mead *et al.*, 2006). Furthermore, it was established that two RTE products were isolated from the same manufacturing plant, and renovation occurred at the time; therefore, this construction quite possibly could have led to post-processing contamination of the RTE product (Mead *et al.*, 2006).

Leading listeriosis outbreak sources were reported to be contaminated dairy products, which have resulted in four known outbreaks, meat has also been involved in three known outbreaks, while eggs and vegetables have each caused a single outbreak (McLauchlin *et al.*, 1990; USDA, 2003;

Ryan *et al.*, 2010; Tchatchouang *et al.*, 2020). To address the issue there is improved surveillance, awareness of listeriosis and molecular subtyping, and accountable food sources, thus effortlessly identifying and tracing back to a specific brand or plant. In other countries except the United States, 18 outbreaks affected >1,000 listeriosis cases happened between 1970 and 2000, and were from a single food source which was identified in 17 of the outbreaks. Listeriosis food source includes dairy products, especially butter and cheese, which were involved in six outbreaks. Furthermore, meat products like pâté, pork tongue, and RTE ham were responsible for five outbreaks. Seafood such as smoked mussels, cold-smoked trout, and raw fish were involved in four outbreaks. Where else, in raw vegetables and cabbage affected two outbreaks (McLauchlin *et al.*, 1990; Ryan *et al.*, 2010; Tchatchouang *et al.*, 2020).

In 1978, Australia recorded several food sources including unpasteurized milk and vegetables accountable for the listeriosis outbreak (Allerberger and Guggenbichler, 1989). Dairy and RTE meat products (frankfurters, deli meat, pâté, and pork tongue) are considered the leading sources of listeriosis. However, common sources of listeriosis outbreaks reported in other countries except for the United States were soft cheeses and mould-ripened cheeses (USDA, 2003). Related studies were from case-control investigations of sporadic listeriosis cases, whereby un-reheated frankfurters, undercooked chicken, soft cheeses, and foods purchased at a deli counter were contaminated with the listeriosis bacterium (Schwartz *et al.*, 1988; Schuchat *et al.*, 1992). Pinner *et al.* (1992), corroborate by reporting that contaminated foods were RTE foods and foods from which serotype 4b was isolated. Nearly 70% of global outbreaks reported serotype 4b. Listeriosis outbreaks reported locally and internationally have been revealed to be alike in that the *L. monocytogenes* strains were responsible for the outbreaks with an affinity towards serotype 4b than any other serotype. In addition, other sources of food and food products were accountable for

the mortality rates, which seem to be similar when comparing global outbreaks of listeriosis (Mead *et al.,* 1999).

2.7 Listeriosis outbreak in South Africa

Recently, South Africa was facing life-threatening outbreaks of listeriosis. Tchatchouang *et al.* (2020) reported that this outbreak was considered the most severe listeriosis outbreak to date. Between the 01st of January 2017 to 23rd of January 2018, the National Institute of Communicable Diseases (NICD) reported 820 cases of listeriosis. Furthermore, the lowest cases were reported in Kwa Zulu-Natal (7%) and Western Cape (13%) however, Gauteng Province recorded the highest cases (59%). Sample collection (blood (71%) and Cerebral Spinal Fluid (CSF) 23%) originate from the patients hospitalised in the public and private health care sectors with almost 66% of diagnostic analysis from public and private healthcare sectors 34%. The age varied from newborn to 93 years, with a large proportion (96%) of cases being neonates (≤28 days). Moreover, females (55%) were more affected than their male counterparts (45%) (NICD, 2018). Human listeriosis outbreaks are not frequent, however, listeriosis infection in humans can be life-threatening and cause a high mortality rate. In addition to several factors mentioned earlier, the supply chain is not the only source responsible for outbreaks of listeriosis, several reports have shown that current antibiotics and disinfectants are ineffective due to the dramatic increase in microbial resistance which also plays a role.

2.8 Antimicrobial therapy of *Listeria infection*

In the clinical setting, listeriotic treatment is primarily the β-lactams class such as penicillin and ampicillin, with or without gentamicin (Olaimat *et al.*, 2018). *L. monocytogenes*, as well as other *Listeria* spp., are usually susceptible to a wide range of antibiotics (Hof and Hefner, 1988).

However, the evolution of bacteria towards resistance has been considerably accelerated by the selective pressure exerted by the over-prescription of drugs in clinical settings and their heavy use as promoters for growth in farm animals (Charpentier *et al.*, 1995). Isolation of the first multi-resistant strain of *L. monocytogenes* was in France in 1988 (Poyart-Salmeron *et al.*, 1990) and since then multi-resistant *L. monocytogenes* strains have been recovered from food, the environment, and sporadic cases of human listeriosis (Hadorn *et al.*, 1993; Charpentier *et al.*, 1995). Antibiotics to which some *L. monocytogenes* strains are resistant include tetracycline, gentamicin, penicillin, ampicillin, streptomycin, erythromycin, kanamycin, sulfonamide, trimethoprim, and rifampicin (Charpentier *et al.*, 1999). Tetracycline resistance has been the most frequently observed among *L. monocytogenes* isolates (Charpentier *et al.*, 1995; Charpentier *et al.*, 1999).

Another alternative is vancomycin and trimethoprim/sulfamethoxazole therapy for patients who are penicillin-allergic (Sereno *et al.*, 2019). According to the World Health Organization (WHO), there is a rapid spread of antimicrobial resistance (AMR), a growing concern in global public health necessitating all government sectors and society to take action due to the rapid development of AMR, therapeutic options for the prevention and treatment of various nosocomial and community-acquired infections (World Health Organization, 2020) are limited. Hospitals have become the breeding ground for the development of resistant pathogens especially as it is a set up made of weak immune patients who have close contact with each other allowing the spread of antimicrobial-resistant strains. Hence, a significant number of patients hospitalized suffer from multi-infectious diseases and reuse the same medication enhancing microbial resistance (Weinstein, 2001).

Also, antibiotics are used in the veterinary sector in food-producing animals usually for therapy, and preventive treatment, as growth promoters which also increases the emergence of resistant strains (Allen *et al.*, 2013). Bacterial *L. monocytogenes* is no exception. In 1988 resistant strains of the *L. monocytogenes* were isolated and detected (Farber *et al.*, 1988). Subsequently, numerous studies since then have reported countless resistant strains from patients, food/food processing environments and animals/animal farms (White *et al.*, 2002). Moreover, *L. monocytogenes* multidrug-resistant strains have also been reported. *L. monocytogenes* is intrinsically resistant to first-generation Quinolones, Monobactams, Fosfomycin, and broad-spectrum Cephalosporins (Troxler *et al.*, 2000). Antibiotic resistance and particularly multi-resistance have led to the ineffectiveness of treatment. The consequences of treatment failure have a direct upturn in human morbidity, mortality, and a raise in healthcare costs (Allen *et al.*, 2013; Allen *et al.*, 2016).

2.8.1 Antibiotic therapy against listeriosis in humans and food animals

From the start, the high lethality of listeriosis is hard to comprehend, since practically all strains of *L. monocytogenes* are powerless to virtually all normal anti-microbial agents. Remedial achievement is blocked by the specific natural conduct of these microorganisms, which are facultative intracellular microbes (Sheehan *et al.*, 1994). This implies that in specific circumstances, the microscopic organisms might be protected from the extracellular environment that contains high concentrations of the antibacterial substance. In general, isolates of *L. monocytogenes* are susceptible to a wide range of anti-microbials (Hood and Zottola, 1997; Srinivasan *et al.*, 2005) aside from the new cephalosporins and fosfomycin. Most isolates from a clinical setting must be sensitive to these anti-toxins. Consequently, one can foresee the sensitivity to anti-infection agents of particular isolates with a high likelihood, which implies that sensitivity testing is not obligatory for each situation.

Resistance to specific anti-toxins, for example, streptomycin and kanamycin, which do not assume a part in treatment, and erythromycin, gentamicin, trimethoprim, and rifampin, has been identified uniquely in a couple of isolates from food (Hof *et al.*, 1997). The equivalent is valid for clinical isolates, in which resistance to streptomycin tetracycline, erythromycin, or chloramphenicol has seldom been recognized (Hof *et al.*, 1997; Haaber *et al.*, 2017). This anti-toxin obstruction is encoded by plasmids in the majority of the cases, albeit chromosomally encoded resistance from antibiotics (tetracycline) has likewise been recommended (Saw, 2015; Kaushik *et al.*, 2022). Since enterococcal plasmids encoding opposition determinants are effectively moved *in vitro* to *Listeria* species, one could envisage that such a plasmid move may happen in nature substantially more regularly, for instance, in the human stool, not animals' (Roberts and Schwarz, 2016). It is likely that MICs and MBCs *in vitro* on artificial media are not adequate to anticipate the restorative estimation of medication in the treatment of listeriosis.

Medication for listeriosis was known to be ampicillin. Nonetheless, in 1984, obstruction towards this medication was accounted for (Rapp *et al.*, 1984; Rivero *et al.*, 2003). Improper use of these antibiotics in animals has been proposed as one reason for the improvement of opposition against ampicillin in human listeriosis through animal-inferred nourishments (Srinivasan *et al.*, 2005; Mathew *et al.*, 2007; Tiwari *et al.*, 2020). Gentamicin has been discovered compelling in the treatment of bovine genital listeriosis (Chopra *et al.*, 2015). *Listeria* from food, the environment, animals, and human sources has been discovered to be impervious to regularly utilized anti-biotic agents, including gentamicin, cotrimoxazole and ofloxacin (Low and Donachie, 1997; George *et al.*, 2005; Rahimi *et al.*, 2012; Soni *et al.*, 2013; Ndahi *et al.*, 2014). Since chemotherapy with antibiotics is the solitary accessible technique for the therapy of listeriosis, prudent utilization of

these medications in clinical and veterinary practices alongside observing anti-microbial weakness is vital (Okada *et al.*, 2011; Barbosa *et al.*, 2013). Cell-mediated immunity is the predominant host protection approach against *Listeria* species and relies mostly on the action of cytotoxic T-cells. Eventually, this is linked to listeriosis and conditions involving impairments of cell-mediated immunity (Theisen and Sauer, 2017).

The bacterial intracellular lifecycle disrupts the effectiveness of chemotherapy drugs including the lack of development of anti-*Listeria* immunity in the host post-infection. A study reported that an anti-listeriolysin O (*LLO*) counteracted monoclonal antibody thus, expanding host resistance to disease in mouse models (Thakur *et al.*, 2019). Furthermore, it was proven that anti-*LLO* antibodies interrupt the development of *Listeria* species inside the macrophages, a procedure which was utilized for serodiagnosis of the disease in humans and animals, although it cannot distinguish between a current and previous infection (Theisen and Sauer, 2017). Current research suggests that antimicrobial chemotherapy is the most suitable and feasible listeriosis treatment. Penicillin-based antibiotics are considered to be the most efficacious which must diffuse through the host cell, sustain a high intracellular concentration, and attach to (Van de Velde *et al.*, 2009). Even when encephalitis symptoms are severe, death may occur; therefore, successful treatment depends on the initial treatment and high drug doses administered to animals with encephalitis. Treatment for sheep and goats has no implicit effect on the animals until neurological involvement or chronic cases appear. The Merck Veterinary Manual (Dhama *et al.*, 2015) stipulates that a dose of 10 mg/kg body weight per day of chlortetracycline administered for 5 days intravenously in cattle is the most potent treatment in encephalitis cases. The intramuscular administration of penicillin G at a rate of 44,000 U/kg body weight per day for 1 to 2 weeks, however, makes it advised that the initial injection be followed by intravenous administration of the same dose. The

period for antimicrobial treatment depends on the degree of infection. Furthermore, in serious cases, it is advised that treatment continues for up to 1 week after the clinical signs have disappeared. Housekeeping, and supportive care, together with fluids and electrolytes, form part of therapy. In some cases, a high dose of dexamethasone (1 mg/kg, given intravenously) is useful and advisable however others dispute it because it may lead to abortion in the last two trimesters in cattle and after day 135 in sheep.

Gentamicin antibiotics are effective against bovine genital listeriosis (Dhama *et al.*, 2015). Other treatments of listeriosis in livestock are trimethoprim/sulfamethoxazole and erythromycin. In birds, an encephalitic antibiotic was ineffective however, erythromycin, penicillin, trimethoprim/ sulfamethoxazole, gentamicin, and tetracycline were able to treat septicemic forms of the disease. The focus of the chemotherapeutic approaches to treat human listeriosis is subjective to a specific individual and the correlating diagnosis. The frequently used treatment is ampicillin or penicillin G mixed with an aminoglycoside, usually gentamicin (Penduka *et al.*, 2014). A substitute treatment is a mixture of trimethoprim/sulfamethoxazole. Janakiraman (2008) reported on various treatment alternatives for human listeriosis, as illustrated in Table 2.1.

Table 2. 1**:** Treatment selections for Listeriosis infection.

Type of patient	**First-line defence**	**Second Line defence**
Bacteremia	Ampicillin ≥ 6 g per day IV	
Bacteremia patient >50 years	Ampicillin ≥ 6 g combined with gentamicin is added for 14 days	Erythromycin grams per day, or TMP/SMX (trimethoprim/sulfamethoxazole) 200–320 mg of TMP component, or vancomycin 1 g intradermally (tid) for 14 days.

Listeriosis in Pregnant women	Ampicillin ≥ 6 g per day IV administration is prescribed for 7-14 days or IV erythromycin 4 g per day	
Acute meningitis	Ampicillin ≥ 6 g per day IV	
Acute meningitis above 50 years	Ampicillin 6 g per day is combined with gentamicin IV	TMP/SMX 200–320 mg of TMP
Endocarditis	Ampicillin ≥ 6 g per day	
Endocarditis above 50years	Ampicillin ≥ 6 g per day with gentamicin by IV; 1 g tid vancomycin and gentamicin 2.5 mg/kg/d are administered.	
Brain abscess	Ampicillin 14 g per day	Ampicillin 14 g per day plus gentamicin 2.5 mg/kg/d; TMP/SMX 200–320 mg of TMP component for 4 to 6 weeks
Joint/bone infection	Ampicillin ≥ 6 g/d IV with gentamicin	
Joint/bone infection patient > 50 y no prosthesis	Ampicillin ≥ 6 g/d IV with gentamicin, and lifelong oral amoxicillin or TMP/SMX in each case for 6 weeks	
Joint/bone infection patient > 50 y with prosthesis	Ampicillin ≥ 6 g/d IV with gentamicin, and lifelong oral amoxicillin or TMP/SMX in each case for 2 weeks	

2.8.2 Mechanisms of antibiotic resistance in *Listeria monocytogenes*

Conjugative transposons, mobilizable plasmids and self-transferable plasmids are the main mobile genetic elements responsible for antibiotic resistance in *L. monocytogenes* (Charpentier *et al.*,

1999). In addition, efflux pumps have been documented to also have a contributing effect (Godreuil *et al.*, 2003). In 1983, Evans and Macrina first reported Plasmid pIP501 that was resistant to streptogramins, macrolides, chloramphenicol and lincosamides in *Streptococcus agalactiae.* Furthermore, Plasmid pIP501 was initially transferred to *L. monocytogenes* by conjugation, then back to *Streptococcus* (Perez-Diaz *et al.*, 1982). Plasmid *pAMb1* was identified as a wide-host-range plasmid of *Enterococcus faecalis*, exhibiting resistance to the antibiotic erythromycin. It was later transferred *E. faecalis* to *L. monocytogenes* through conjugation, which reproduced in the new host and moved using conjugation between *L. monocytogenes* strains and from *L. monocytogenes* back to *E. faecalis* (Flamm *et al.*, 1984). Biavasco *et al.* (1996) reported a plasmid with a *vanA* gene cluster, conferring glycopeptide resistance, moved from *Enterococcus faecium* to *L. monocytogenes*, *Listeria welshimeri*, and *Listeria ivanovii.* Plasmid pIP823 was regarded as having various hosts such as *E. coli, L. monocytogenes* and *E. faecalis*. In addition, the plasmid migrated amongst *L. monocytogenes* and *E. coli*, and amongst *L. monocytogenes* and *E. faecalis* (Charpentier *et al.*, 1999). In the food environment, the migration of antibiotic resistance from lactic acid bacteria to *Listeria* through conjugation was studied (Toomey *et al.*, 2009). In vitro study showed the movement of erythromycin resistance from the lactic acid bacteria to *Listeria*. Conjugative transposon *Tn916* with a wide range of hosts, consisting of *tetM* tetracycline resistance gene, and was first discovered in *E. faecalis* (Franke and Clewell, 1981). Plasmid *Tn916* moved from *E. faecalis* to *Listeria innocua* through conjugation, propelling it to other *Listeria* strains and then back to *E. faecalis* (Vicente *et al.*, 1988; Celli and Trieu-Cuot, 1998). A *Tn916*-related transposon demonstrated a move from *E. faecalis* to *L. monocytogenes* both *in vitro* and *in vivo* (Doucet-Populaire *et al.*, 1991; Poyart-Salmeron *et al.*, 1990).

2.8.2.1 Resistance mediated by efflux pumps

Efflux pumps are proteins encoded by bacteria in both Gram-positive and Gram-negative bacteria. They possess various functions including the removal of toxic substrates such as antibiotics from inside the cells to the exterior environment (Van Bambeke *et al.*, 2000). They permit the transportation of antibiotics of various classes, which may be related to multiple drug resistance (Webber and Piddock, 2003). *Listeria monocytogenes* has been reported to contain two efflux pumps. Ethidium bromide (EtBr), heavy metals, and antibiotics (macrolides and cefotaxime) may all be extruded by an efflux pump with the designation *MdrL*. A further efflux pump is known as *Lde* is linked to fluoroquinolone resistance as well as, to a lesser extent, to resistance to acridine orange and *EtBr*. The efflux pumps *MdrL* and *Lde* have been well described in *L. monocytogenes* (Mereghetti *et al.*, 2000). *MdrL* mediates drug resistance to macrolide antibiotics, cefotaxime, heavy metals and as well as ethidium bromide (Mata *et al.*, 2000). *Lde* is primarily resistant to the DNA intercalating dyes acridine orange and ethidium bromide as well as fluoroquinolone (Godreuil *et al.*, 2003).

2.8.2.2 Acquired resistance to some antibiotics

The movement of plasmids enterococcal and streptococcal as well as transposons encoding for antibiotic resistance genes have been transferred by conjugation from Enterococcus-Streptococcus to *Listeria* including other *Listeria* (Charpentier *et al.*, 1999; Mahendra *et al.*, 2020). Plasmid *pAMβ1* which is considered widespread in *Enterococcus faecalis* has developed resistance to erythromycin and moved by horizontal gene transfer conjugation from *E. faecalis* to *L. monocytogenes* at 10^{-4} to 10^{-8} (Hof, 2017). *Listeria monocytogenes BM4210*, was originally obtained from an 84-year-old patient with meningoencephalitis (Charpentier *et al.*, 1999) in 1988 at the Hospital of Dijon and the strain was resistant to tetracycline (MIC, 128 μg/ml)-minocycline

(MIC, 8 μg/ml), chloramphenicol (MIC, 64 μg/ml), streptomycin (MIC, 256 μg/ml) and erythromycin (MIC, 2,048 μg/ml). Later it was discovered that *Listeria monocytogenes* BM4210 encoded for resistance determinant plasmid *pIP811*, which was frequently carried by *Enterococcus* and *Streptococcus* than in *Listeria* and *Staphylococcus*. The movement of *pIP811* by conjugation occurred more between strains of *L. monocytogenes* and/or *Enterococcus-Streptococcus* than in *Staphylococcus* strains. Several studies have shown strong structural similarity between *pIP811* and *pAMβ1*, which are widely distributed resistance plasmids from *Enterococcus* and *Streptococcus* species (Hraoui *et al.*, 2011).

Moreover, they encode for resistance to erythromycin, chloramphenicol, and streptomycin were homologous to *cat221* (encoding a chloramphenicol acetyltransferase), *aad6* (encoding a 6-N-aminoglycoside nucleotidyltransferase) and *ermAM* (encoding an rRNA methylase), frequent in *Enterococcus* and *Streptococcus*. They are footprints *to L. monocytogenes BM4210* multiple antibiotic resistance acquired under natural conditions of a plasmid from Enterococcus-Streptococcus (Charpentier *et al.*, 1999). The new tetracycline resistance gene *tet(S)*, was shown to be tetracycline-minocycline resistant by ribosomal protection. *Listeria monocytogenes BM4210* encoded for *tet(S)* gene, which was detected in two multi-resistant *L. monocytogenes* clinical strains (Clermont *et al.*, 1997). It was reported that multi-resistant *L. monocytogenes* isolates were obtained in Bordeaux and Zurich and were resistant to chloramphenicol, tetracycline-minocycline and erythromycin, but not to streptomycin. The strain from Bordeaux encoded for plasmid *pUBX1*, which harbour resistance genes and was self-transferable by conjugation to *L. monocytogenes*, *E. faecalis*, *S. agalactiae*, and *Staphylococcus aureus*. The other strain from Zurich harbour plasmid *pWDB100*, was self-transferable to *L. monocytogenes*, *Staphylococcus epidermidis* and *Enterococcus*. Tetracycline resistance trait in *L. monocytogenes* obtained from humans, as three

self-transferable multi-resistant plasmids from *L. monocytogenes* encoding *tet(S)* gene (Poroś-Głuchowska and Markiewicz, 2003).

From a total of 1,288 clinical strains obtained in 1967 and 1981, 1983 and 1986, and 1987 and 1990, 33 isolates were resistant to tetracycline in the United Kingdom. *Listeria* resistant to tetracycline was discovered in the food and environmental sources. A total of 1,040 *Listeria* strains was obtained from food and environmental sources and investigation showed that they were resistant to tetracycline (MIC, 64 to 128 μg/ml) and minocycline (MIC, 4 to 32 μg/ml): 37 *L. monocytogenes* strains, 22 *Listeria* strains, and 2 *L. welshimeri* strains; one strain of *Listeria* was resistant to tetracycline-minocycline and streptomycin. Several clinical *L. monocytogenes* isolates were shown to be resistant to streptomycin (Charpentier *et al.*, 1999). A minimum inhibition concentration (MIC) of 30 μg/ml was shown against streptomycin in 195 *Listeria* species isolated from several sources (human samples, food, and environment) collected globally.

Resistant traits of *L. monocytogenes* shown in 1991 in Italy, where 98 strains isolated from food, only two *L. monocytogenes* isolates were resistant to streptomycin, sulfamethoxazole, and kanamycin, one isolate was resistant to rifampin, sulfamethoxazole, streptomycin, and kanamycin, and one isolate was found to be resistant to four antibiotics and also chloramphenicol and erythromycin. The same study identified 85 strains were *Listeria* isolated food-borne sources, in which one isolate was resistant to erythromycin, and another two strains were resistant to streptomycin and sulfamethoxazole. *L. monocytogenes* and *Listeria* isolates, which were resistant to erythromycin encoded for the *ermC* gene. This gene moved by conjugation to *Listeria* and *E. faecalis* without a link to a conjugative plasmid (Poroś-Głuchowska and Markiewicz, 2003). Plasmid *pRYC16* has a broad spectrum in *Listeria*, which possesses a conjugative cryptic replicon. In other studies, plasmid could transfer *pDB1*, which is a nonconjugative plasmid of *Bacillus*

subtilis, from *L. grayi* to *L. monocytogenes* (Charpentier *et al.*, 1999). In a study conducted on *Listeria* transconjugants, there was a jump of vancomycin resistance in *L. monocytogenes, L. ivanovii, L. welshimeri*, and *L. seeligeri* with a frequency higher than that in the primary matings. Multiple antibiotic resistance in *L. monocytogenes* has migrated in *Enterococcus, Streptococcus*, and *Staphylococcus* as proven in three clinical strains, of which two originated from France and the other from Switzerland (Poroś-Głuchowska and Markiewicz, 2003).

2.8.2.3 Intrinsic resistance to others

L. monocytogenes usually requires low concentrations of antibiotics and other antimicrobial agents in the agricultural and food sectors. In this regard, this forms part of the pre-exposure adaptation that enables *L. monocytogenes* to develop resistance to higher concentrations of antibiotics or antimicrobial drugs. Several studies have shown that *L. monocytogenes* increase against the antibiotics nisin and lacticin 3147, since short-chain fatty acids have been utilized as preservatives and are naturally occurring in the gastrointestinal tract (Kwon and Ricke, 1998; Van Schaik *et al.*, 2002). Adaptation to severely low pH challenges allows *L. monocytogenes* to tolerate mild acidic pH (Davis *et al.*, 1996). However, this strategy evoked the development of resistance of *L. monocytogenes* against some antibiotics (Phan-Thanh and Montagne, 1998; Alonso-Hernando *et al.*, 2009). In addition, the transmission of resistance to the subtypes of *L. monocytogenes* that were susceptible before were resistant to more than one antimicrobial drug (Walsh *et al.*, 2001; Srinivasan *et al.*, 2005). For instance, a research study conducted by Lemaitre *et al.* (1998) proved the transfer of antibiotic resistance from *L. monocytogenes* to *S. aureus*, thus the study of Srinivasan *et al.* (2005), as well as Walsh et al. (2001), displayed that the transfer transmission from *E. coli* to *S. aureus* and *L. monocytogenes* while *Streptococci* and *Enterococci* constitute the storage of antibiotic resistance genes for *L. monocytogenes*.

L. monocytogenes is a facultative bacterium and persists in the digestive tract because humans and animals are appraised necessary anaerobic habitat (Sleator *et al.,* 2009). It has been demonstrated that the transfer of resistance genes from *enterococci* and *streptococci* to *L. monocytogenes* is done in the gut of humans and animals (Doucet-Populaire *et al.,* 1991). Although, little research has been run to explain the place of anaerobiosis on the response of *L. monocytogenes* to different conditions, within antibiotic stress or antibiotic resistance transfer and acquisition. Lack of nutriment permit *L. monocytogenes* cells to resist successfully different bacteria (Lou and Yousef, 1996; Lou and Yousef, 1996; Mendonca *et al.,* 2004); thus *L. monocytogenes* can grow in lower or high temperature (0 to 45 °C), lack of nutrients can be a profit in the sector of processing plant that might converse defence against antibiotics. A study conducted by Lou and Yousef (1996) proves that the overheating shock of *L. monocytogenes* cells for 1 hour at 45 °C raises the protection of this bacteria to sodium chloride and ethanol. Even though there is no documentation on the response of the heat of heat-adapted *L. monocytogenes* to antibiotic stresses. More research needs to be conducted on the response of low-temperature-adapted cells to different habitat stresses, within antibiotics. The resistance relationship between metal ions and antibiotic are more related and the usual plasmid might be involved. For instance, the *robA* plasmid from a cyclohexane tolerant mutant expressed carried by an *E. coli* strain raises the tolerance to solvents and resistance to antibiotics and heavy metals such as mercury, cadmium, and silver (Nakajima *et al.*, 1995).

Penicillinase plasmids harboured by *S. aureus* isolates were discovered by Silver and Misra (1988) to be resistant to mercury. Furthermore, Hayashi *et al.* (1993) outlined that *Vibro* strains that were resistant to guide nickel sulfate, cobalt chloride, sodium arsenate and acetate were also resistant to aminobenzylpenicillin. The characterization of *L. monocytogenes* isolated from a turkey-

processing plant conducted by Mullapudi *et al.* (2008) shows resistance to arsenic, cadmium and benzalkonium chloride (BC). The entire benzalkonium chloride (BC) resistant isolates were also resistant to cadmium, while no relationship was found between BC and arsenic. The outcome of this research stipulates that the processing plant might represent storage for *L. monocytogenes* resistance to disinfectants or metals and increase the chance of usual genetic components or mediation of antibiotics resistance mechanism. Concerning the field of metal ion and antibiotic resistance in *L. monocytogenes*, there is a real demand for more studies to investigate this issue. In addition, there are many bacteria discovered in seafood due to contact with heavy metals and deep research must be done to clarify the transfer of resistance. Definitively, it is critical to know the important role that the preservation factors besides habitat stress factors on the effectiveness of specific cells to antibiotics.

2.8.3 Antimicrobial resistance in the food chain (include information from primary production/farming)

Various studies reported *L. monocytogenes* antibiotic-resistant isolates found in the food, food-producing and food-processing environments. Cheese and pork were contaminated with *Listeria* strains that are resistant to multiple antibiotics, however, more than 80% of the strains of food origins were sensitive to penicillin G and ampicillin, although a significant number were resistant to cephalosporins cefotaxime and cefoxitin was almost 100% (Rota *et al.*, 1996). A total of 0.6% of *L. monocytogenes* strains obtained from retail foods were resistant to more than one antibiotic besides the 19.5% of *Listeria* strains, indicating that the capability to obtain antibiotic resistance may be species-related. *L. monocytogenes* strains were isolated from poultry carcasses, and shown to possess resistance towards more than one antimicrobial agent, suggesting that poultry are a probable source for antibiotic-resistant foodborne diseases (Antunes *et al.*, 2002). Srinivasan *et al.*

(2005) reported *L. monocytogenes* strains were resistant to more than one antimicrobial agent from dairy farm areas, thus all strains were resistant to cephalosporin C, trimethoprim, and streptomycin. Conventionally and organically grown poultry was contaminated with *L. monocytogenes*, which was resistant to the antibiotics. Furthermore, it was established that conventionally grown poultry was more resistant as compared to organically grown poultry, due to a lack of exposure to antibiotics (Miranda *et al.*, 2008). A comparison study on eggs from hens raised either conventionally or organically conducted in Germany showed all strains were susceptible to the tested antibiotics (Schwaiger *et al.*, 2010). *L. monocytogenes* was isolated from a turkey and established that 59% were resistant to ceftriaxone, 90% were resistant to oxacillin and 3% of the strains were resistant to ciprofloxacin (Lyon *et al.*, 2008). Ready-to-eat foods were contaminated with *L. monocytogenes* and displayed high levels of resistance to ampicillin, methicillin, and gentamycin (Pesavento *et al.*, 2010). With respect to the above, it might be concluded that gradually, changes are arising in the natural environment and the occurrence of antibiotic resistance in the *L. monocytogenes*. Espaze and Reynaud (1988) anticipated the emerging problem of *Listeria* species-resistant strains in food-producing and food-processing territories would have escalated. Numerous reports showed that antibiotic resistance movements and exchanges occur in *L. monocytogenes* (Perez-Diaz *et al.*, 1982; Flamm *et al.*, 1984; Vicente *et al.*, 1988; Biavasco *et al.*, 1996; Celli and Trieu-Cuot, 1998) and the foods environment might be a possible breeding ground.

2.8.4 Techniques for detecting antibiotic resistance in *Listeria* species

Antibiotics can be defined as antimicrobial agents that inhibit bacterial growth by their bactericidal or bacteriostatic action. Nonetheless, some antibiotics can be bacteriostatic and bactericidal

depending on concentration. Usually, the evaluation of antibiotic resistance can be determined using phenotypic as well as genotypic methods. Phenotypic methods involve the determination of the inhibition potentials of antibiotics and methods used include agar and broth dilution method (Wiegand *et al.*, 2008), as well as filter paper disc (Kirby-Bauer) method (Bauer, 1966) and the dilution method (minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (Brown and Young, 1947; Bradshaw *et al.*, 1990; Owuama, 2017). On the other hand, the detection of genes encoding resistance properties by molecular techniques such as PCR has been proven to be a sound and reproducible method in the detection of resistance traits in bacterial species including *Listeria* species.

The identification of antimicrobial resistance using PCR is conducted by targeting genes that the *Listeria* strain might carry. Specific primers are used to target resistance genes such as those that encode for tetracycline efflux pump *(tetA-tetE, tetG,)*, resistance to aminoglycoside (aadA), streptomycin (strA,strB), beta-lactamase–ampicillin (a*mpC*), dihydropteroate synthetase type II (sulII)); dihydropteroate synthetase type I (*sul1*) of folate pathway antibiotics and erythromycin esterase type II (*ereB*). Other resistance gene targets include the nonenzymatic chloramphenicol-resistance protein gene (*cmlA*), chloramphenicol transporter florfenicol export protein gene (*floR*), vanillate o-demethylase oxygenase subunit gene (*vanA, vanB*), penicillin-binding protein gene *(penA*). The adenine methylase gene, *ermA* confers resistance to erythromycin and erythromycin resistance methylase (*ermB*). Data from a genome-wide analysis of the *Listeria monocytogenes* gene encoding lmo0762, a homolog of *hflX*, were reported by Duval *et al.* (2018). In addition, *Listeria hflXr* forms part of the antibiotic resistance genes group. It is worth noting that the presence of these genes could be deduced from their whole genome sequence data. A study conducted by Haubert *et al.* (2018), revealed a multidrug-resistant isolate of *Listeria*

monocytogenes serotype 1/2a, named *Lm16* isolate. These isolates presented a particularity of being highly resistant to disinfectants, heavy metals, and antimicrobials. The whole-genome shotgun method has been used to sequence the *Listeria* isolate with the Illumina MiSeq platform. The assembly and analysis of the new genome have propelled the presence of mutations in the genes *efTu* and *gyrA* and three efflux pumps (*mepA*, *msrA*, and *norB*) prove resistance to antimicrobials. It has given us the opportunity to identify a new feature resistance gene in *Listeria monocytogenes* foodborne.

2.8.5 Epidemiological data of antimicrobial resistance in *Listeria* species (isolates from food and confirmed cases linked to humans consuming contaminated food)

Human listeriosis is a rare disease and occurs as cases of invasive infections, which could be related to outbreaks or as sporadic diseases by consumption of contaminated food. Approximately 0.1–1.1 cases per 105 population were reported globally in nonperinatal cases of invasive listeriosis. In addition, CNS diseases were estimated at 47% of infected individuals with a 36% mortality rate. In North America and Western Europe, *Listeria monocytogenes* was allocated to be the third or fourth bacteria to cause bacterial meningitis. *L. monocytogenes* have been isolated from stool samples of low percentages of asymptomatic, healthy patients. A total of 3.5% of stool samples were isolated from three patients over 12 months using PCR and recovered from culture in 1.15% (Drevets and Bronze, 2008). Paduro *et al.* (2020) conducted molecular epidemiology surveillance on *Listeria monocytogenes* in Chile over a ten-year duration. *Listeria monocytogenes* strains were isolated from consumption of contaminated food products that caused serious outbreaks of public healthcare concern. Furthermore, *L monocytogenes* serotypes 1/2a, 1/2b and 4b were predominantly isolated from food products thus showing epidemiological surveillance of *L. monocytogenes* in Chile.

Ranjbar and Halaji (2018), reported human listeriosis as a sporadic foodborne infection with epidemiological association due to consumption of contaminated food products from Iran. Data revealed that 10% were isolated from human specimens, with 4% from food samples and *L. monocytogenes* serotypes were 4b, 1/2a, and 1/2b. There has been a swift epidemiological trend of *Listeria monocytogenes* outbreaks and sporadic cases from 1996 to 2018 towards an increase in incidence related to foods and an increase in international incidences. In addition, the rise involved high-risk patients including pregnant women and immunocompromised patients (Desai *et al.*, 2019). Jennison *et al.* (2017) conducted extensive epidemiological research on 224 *L. monocytogenes* strains isolated from various food sources as well as human samples from Australia. An in-depth investigation showed that *L. monocytogenes* serogroups *IIA*, *IIB*, and *IVB* consisted of 94% of all strains, with *IVB* isolated from most clinical strains. In addition, *L. monocytogenes* serogroup *IIA* was predominant in almost all dairy and meat strains. In 2018–2019, Germany recorded one of the most massive invasive listeriosis outbreaks in Europe caused by contaminated blood sausage. Invasive listeriosis is considered to be a life-threatening uncontrollable foodborne disease in humans. Listeriosis incidence is increasing worldwide, but some countries have implemented molecular surveillance programs to improve the recognition and management of listeriosis outbreaks. In Germany, routine whole-genome sequencing-, core genome multilocus sequence typing, and single nucleotide polymorphism calling are used for subtyping of *Listeria monocytogenes* isolates from listeriosis cases and suspected foods. An uncommon molecular cluster of *L. monocytogenes* strains in large groupings was shown such as 134 highly clonal, benzalkonium-resistant sequence type 6 strains obtained from 112 notified listeriosis cases. Epidemiologic features showed that blood sausage was contaminated with *L.*

monocytogenes which was highly associated with clinical strains. Epidemiologic studies are crucial in assisting in identifying the outbreak source or vehicle (Halbedel *et al.*, 2020).

2.9 Biofilm

The misuse of antibiotics is not the sole reason for the exponential proliferation in the development of microbial resistance. Another factor is the ability of microbial cells to form biofilms. Formation of biofilm develops resistance by low permeability of cell walls, generic resistance (phenotype changes) and reduced oxygen concentration at the base of the biofilm (Høiby, 2002; Lambert, 2002). Furthermore, the diffusion of the antibiotics through the biofilm layer may be postponed or prevented due to the charge interaction between the EPS and the antimicrobial agent (Spoering and Lewis, 2001). Biofilms are microorganisms enclosed in self-produced extracellular polymeric substances (EPS) (Shi and Zhu, 2009). Extracellular polymeric substances (EPS) consist mainly of proteins, polysaccharides and DNA (Colagiorgi *et al.*, 2016). Moreover, biofilm formation serves as a protection and survival strategy during unfavourable environmental conditions. They are ubiquitous, occurring as single or multi-species structures (Srey *et al.*, 2013). Biofilms ensure the survival of microbial cells in food processing environments by allowing persistent cells to endure. In due course, contamination of food in the production chain may occur. Biofilm formation consists of several main stages, (a) adhesion to the surface, (b) colonization, (c) biofilm maturation and (d) detachment (Chen and Wen, 2011). Surface adhesions and cell-to-cell communication signalling pathways are the key components to successful biofilm formation.

2.9.1 Adhesion of planktonic cells to surface

Initially, microbial cells sense some advantageous environmental parameters that enable the adhesion of planktonic cells (Lindsay and Von Holy, 2006). This step may be active or in a passive

manner depending on the bacterial cells´ physicochemical properties (Van Houdt and Michiels, 2010). *L. monocytogenes* have been reported to adhere to surfaces (Shi and Zhu, 2009). It is the initial contact between a conditioned surface and planktonic microbial cells. It is a reversible stage. The planktonic cells come in proximity to the surface of interest. Electrostatic forces, van der Waals forces and hydrophobic interactions are certain attractive or repulsive inter-surface forces created (Dunne, 2002). The net sum of attractive or repulsive forces will regulate the strength of adhesion. Planktonic cells attach to the surface and exhibit several attachment behaviours. Adequate time is compulsory for the initial attachment. Various microbial interactions with the surface might happen by sedimentation and Brownian motion (Leon-Morales *et al.,* 2004). Secondary adhesion on the other hand is the irreversible attachment phase that occurs after the reversible phase. The bond between the surfaces occurs on a molecular level. Irreversible attachment retains the active motility preventing gliding, swimming, darting and sliding (Kolari, 2003). Microbial cells no longer move perpendicular to the attachment surface. Loosely bounded microbial cells secrete exopolysaccharide material that forms a complex with the surface materials. The EPS stimulate membrane-bound sensory proteins of the cells, while the exopolysaccharide matrix attaches the cells to the surface and each other (Dunne, 2002).

2.9.2 Colonization

Planktonic cells grow and reproduce to form microcolonies. Before this, the microbial cells should be irreversibly attached to the substrate, so that the colonization and maturation take place. The micro colonies will form communities on the substratum resulting in an escalation of the overall density and complexity of the biofilm (Kumar and Anand, 1998). The aggregated microcolonies are seen as an organizational unit in a biofilm and opening water channels. This will be a multi-layered structure that matures into a biofilm. The mature biofilm will self-produce extracellular

polymeric substances or exopolysaccharides (EPS) which will enclose microbial cells forming a biofilm-forming matrix. The EPS ensures that the bond is strengthened, and the attachment irreversible. Nonetheless, biofilm growth is restricted by available nutrients and the accumulation of waste products (Hugo and Russell, 1998).

2.9.3 Detachment

The biofilm cycle is finalized; thus, cell shedding begins, releasing cells into the immediate environment. Individual daughter cells or clumps of cells might detach from the environment by turbulent shear forces or dispersion and dissolution. These active processes are highly controlled by the attached cell communities. Subsequently, the planktonic cells can return and resume biofilm formation again (Lindsay and Von Holy, 2006). All the different steps of biofilm are represented in Figure 2.5.

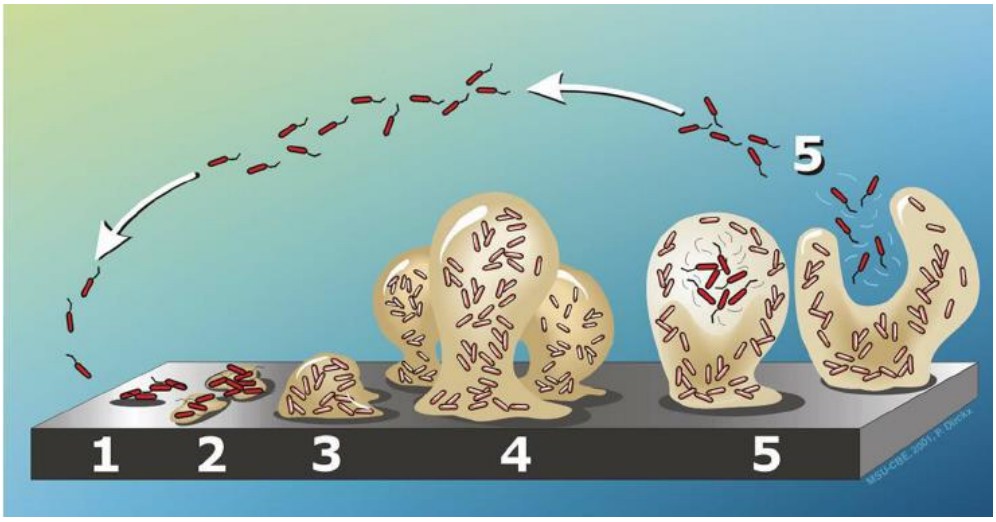


Figure 2.5: Process of biofilm formation. 1: Attachment of cells, 2: Cells proliferate, and extra polymeric substances (EPS) are formed, 3: A three-dimensional structure forms, 4: Maturation and 5: Detachment of cells with recolonization (Van Houdt and Michiels, 2005).

2.9.4 Biofilm formation by *Listeria* species

Listeria species are considered one of the leading foodborne pathogens responsible for serious foodborne outbreaks. Several studies have reported *Listeria* species on food-contact surfaces such as stainless steel, low-density polyethylene (LDPE), polyvinyl chloride (PVC), polyester (PET), and rubber. Like other bacteria, the formation of biofilms plays an important role in antibiotic resistance. Figure 2.6 illustrates the antibiotic resistance in the community and cellular research has shown bacteria including *Listeria* species adhere better to hydrophilic materials like stainless steel and glass rather than to hydrophobic surfaces such as polystyrene (Bonsaglia *et al.*, 2014). Hood and Zottola, (1997), confirmed that *L. monocytogenes* V7 strains were capable of attaching to stainless steel surfaces but did not form biofilms. Thus, illustrating the ability of this genus to form biofilm on various surfaces (Davies, 2003). Chae and Schraft, (2000), proved that *L. monocytogenes* strains attached to glass coupons under static conditions and all strains were able to form biofilms within 24 h. Beresford *et al.*, (2001) showed that *L. monocytogenes* attached and colonized a broad range of materials used in food processing including stainless steel, different polymers, and aluminium.

Furthermore, he proved that the number of cells adhered to coupons increased during the first two h of contact, regardless of the nature of the material. *L. monocytogenes* have been isolated in the food processing environment and formed biofilms on both biotic and abiotic surfaces. Nonetheless, *L. monocytogenes* have been reported to form only attached monolayers on surfaces (Hua *et al.*, 2019). *L. monocytogenes* thrives in moist environments reporting the highest incidence (Wong, 1998). Krysinski *et al.*, (1992) found that *Listeria* was able to attach to cleaned and sanitized stainless steel, polyester and polyesters/polyurethane. Moreover, it was resistant to detergent and these surfaces showed different resistance to sanitizing agents. Arizcun *et al.* (1998) studied

various decontamination procedures to remove *L. monocytogenes from* glass surfaces. In addition, found that the most effective decontamination procedure at conditions of 55 ℃, with high osmolality (10.5% sodium chloride) together with acetic acid (pH 5.4). After bacteria were detached, cells would be more susceptible to sanitizing conditions. Finally, studies found that different sanitation protocols should be utilized when using different materials (Rodriguez *et al.*, 2008).

Colagiorgi *et al.* (2016) reported that the morphology of *L. monocytogenes* has been altered from rod to coccoid during biofilm formation and grows more slowly than planktonic cells. The extracellular matrix of *L. monocytogenes* comprises polysaccharides, proteins and DNA. Polysaccharides are key components of the biofilm matrix, and the polysaccharide teichoic acid (TA) has been related to the biofilm-forming ability because it is found both in the cell wall and in the EPS (Colagiorgi *et al.*, 2016). Biofilm-associated protein (*BapL*) and flagellin (*FlaA*) have been theorized to have a role in the initial attachment to surfaces (Lemon *et al.*, 2008). Upon investigation, extracellular DNA (eDNA) established in the matrix plays an essential role in holding the structure with polysaccharides and proteins. Research into the architecture of *Listeria* biofilm has shown that *L. monocytogenes* produces a honeycomb-like structure, although flat unstructured mono- and multilayers have also been described (Reis-Teixeira *et al.,* 2017). The organized structures ensure that the microbes can withstand environmental stressors including dehydration, and treatment with antimicrobial and sanitizing agents (Folsom *et al.,* 2006). However, biofilms might be a source of contamination and storage for pathogenic or spoilage bacteria (Sommer *et al.,* 1999).

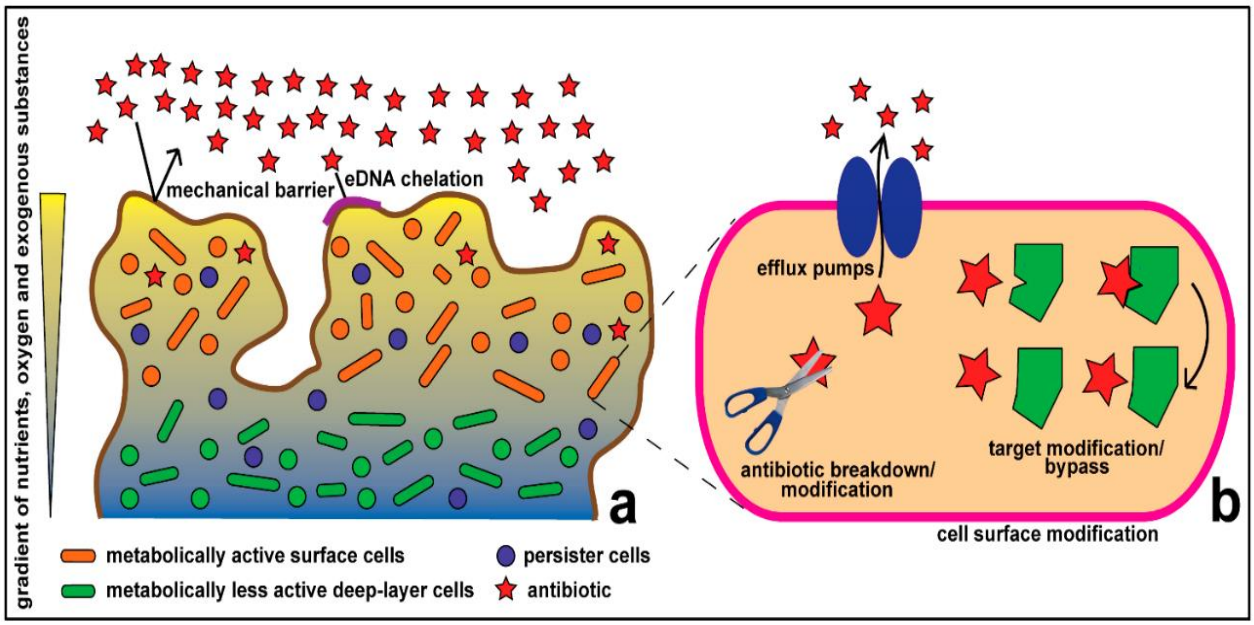


Figure 2.6: Antibiotic resistance at the community (a) and cellular (b) levels (Penesyan *et al*., 2015).

2.9.5 The impact of biofilm formation on the food industry: A case of *Listeria* species

Listeria species are ubiquitous foodborne bacterial pathogens in the environment and are known to be high-risk contaminants in food preparation and processing environments. The food processing industry is considered as an area whereby *L. monocytogenes* as well as other food pathogens contaminate the food. Figure 2.7 contextualizes various essential routes for cross-contamination of pathogenic *Listeria* species in the food preparation and processing industry such as the food matrix, open surface, construction material, static or flow system, cleaning solutions and protocol (Whitehead *et al.*, 2015). The formation of biofilm by *L. monocytogenes* ensures persistence on equipment and environments of food industries and, therefore, plays a crucial role in food contamination and equipping the bacterial pathogen to survive in unfavourable conditions. Da Silva and De Martinis (2013) reported several outbreaks of listeriosis cases in some food manufacturing plants in Brazil. However, the number of cases in the outbreaks was said to be

underdiagnosed and/or underreported. Liu *et al.* (2020), isolated 131 *L. monocytogenes* strains from beef processing plants specifically beef abattoirs in China. Some 67.2% (n=88 strains) were shown to be powerful biofilm producers at ideal environmental conditions, in particular a temperature of 25 °C and pH 7.4. This study supports the fact that the food industries such as the beef plants in China, are considered at high risk for contamination by pathogenic *L. monocytogenes*, a strong biofilm producer. Chaturongkasumrit *et al.* (2011) studied the construction material of the conveyor belt surfaces utilized in most food processing lines. The conveyor belt surfaces were rough and produced from polyester urethane (PSU).

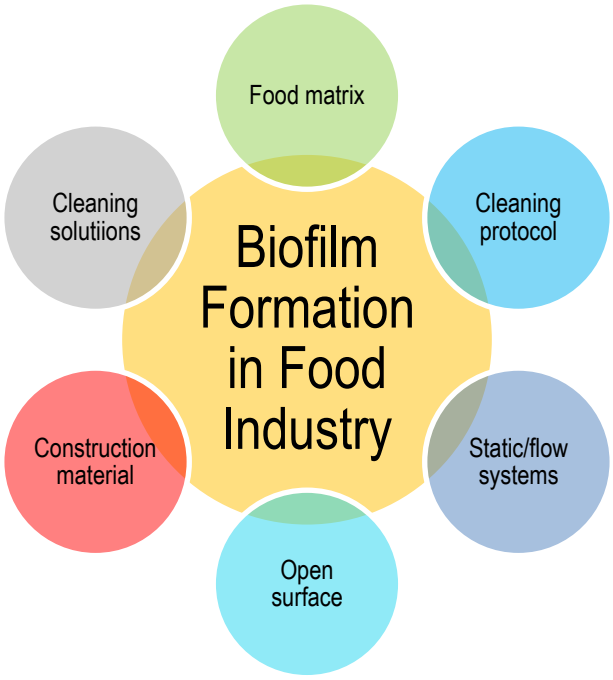


Figure 2.7: Various factors affecting biofilm formation in the food industry (adapted from (Whitehead et al., 2015).

Two strains were used *Listeria monocytogenes* (ATCC19114 and ATCC51782) and induced to form biofilm on the PSU conveyor belt surface in the chicken meat plant in Thailand. Later, the surface was cleaned and sanitized. The results showed that the biofilm reduced significantly after high concentrations of cleaning and sanitizing. However, the complete reduction was not

accomplished. With respect to the above, there is a high impact of biofilm formation on the food industry and construction material is an essential factor. *L. monocytogenes* ATCC 19117 was able to adhere to the AISI 304 stainless steel surface swiftly. The bio transfer potential was prominent in all the biofilm formation stages. Surface-adhered together with bio transfer potential proves that *L. monocytogenes* is a possible threat to the food industry, hence listeriosis outbreaks in food processing and production plants (Oliveira *et al.*, 2010). In South Africa, the listeriosis outbreak caused 216 fatalities in 2018 with 1060 cases. The impact financial was averaged at R3, 79 billion; hospitalization was at R1, 5 billion and production losses at R2, 19 billion. Table 2.2 highlights various impacts of biofilm formation on patients, the government, and the food industry.

Table 2.2: Effect of biofilm formation on patients, government, and the food industry (adapted from (Devleesschauwer *et al.*, 2018).

Impact of biofilm formation		
Individual	Food industry	Government
Death Hospitalization Illness Quality of life Production loss Medical cost Caregiver costs Self-protection	Recall of products Food plant closing Cleaning and sanitization Product liability Reputation costs Regulation compliance Discarding products Loss of time Company shutdown Preventative measures	Laboratory testing and reporting Outbreak response Public health surveillance Recall assistance Inspection Regulatory enforcement Food safety impact

2.9.6 Current food safety interventions to counteract the impact of biofilm

Biofilm formation has become an issue of food safety, particularly in the food processing and production industry. Thus, various approaches have been utilized to inhibit and control the biofilm formation on food contact surfaces and equipment to prevent the initial adherence and colonization on the surface, obstruct molecular signals that modulate the growth of biofilms, and break the EPS. These include conventional cleaning and sanitizing of food surfaces and equipment using toxic compounds, for instance, acetic acid, sodium hypochlorite, hydrogen peroxide, lactic acid and quaternary ammonium (Da Silva and De Martinis, 2013). Regarding the above-mentioned, it is essential to obtain ideal conditions such as temperature, exposure time, mechanical removal, appropriate concentrations and a combination of chemicals may be needed (Sofos, 2009). Nonetheless, to date, it is well-known that these conventional approaches of cleaning and sanitizing work best against sessile bacterial cells rather than when they are in a community protection by the EPS Table 2.2 shows modern-day strategies used in controlling biofilms.

Table 2.3: The modern approaches to control biofilms (Da Silva and De Martinis, 2013).

Strategies	**Applications**	**Advantages**	**Limitations**
Enzyme-based detergents	EPS removal and biofilm dispersion	Improve disinfectant efficacy and antimicrobial action	High cost, low commercial accessibility, technology and production are mostly patent-protected
Bacteriophages	Cell lysis and EPS disintegration by	Provide a natural, highly specific,	Technology for this needs further development

	enzymes (e.g., Listex P100)	nontoxic and feasible approach	
Microbial interactions and metabolites	Interspecies competition Bacteriostatic or bactericidal activity Biofilm dispersion and quorum sensing inhibition Anti-adhesive properties and induction of solid surface physicochemical modifications	Interferes with the biological activities of pathogenic bacteria. Alone or as part of synergistic procedures could provide a new line of efficient biofilm control strategies	Food and clinical applications may offer some risks for consumers and hospital patients

According to the World Health Organization (WHO), the control of *L. monocytogenes* is necessary at all phases in the food chain preventing it in the final food product. Because *L. monocytogenes* are ubiquitous and can withstand normal preservative methods, including smoke, salt and even acidic conditions, not forgetting the low refrigeration temperatures (around 5 °C), WHO and the Food and Drug Administration, FDA (https://www.fda.gov/files/food/published/Draft-Guidance-for-Industry--Control-of-*Listeria* -monocytogenes-in-Ready-To-Eat-Foods-%28PDF%29.pdf) highly recommend the utilization and implementation of Good Hygienic Practices (GHP) and

Good Manufacturing Practices (GMP) at all stages of the food chain. WHO also recommends that pathogenic *L. monocytogenes* should be eliminated by pasteurization and cooking. Generally, the WHO's five keys to safer food which should form part of the food safety management system based on the principles of Hazard Analysis Critical Control Points (HACCP) include keeping everything clean, differentiating between raw and cooked, proper cooking, keeping it safe temperatures, using uncontaminated water and raw materials. (https://www.who.int/news-room/fact-sheets/detail/listeriosis). The South African Government, guidance on controlling and preventing *L. monocytogenes* is in line with the WHO and FDA (https://www.gov.za/faq/health/what-listeriosis-en-how-can-it-be-prevented#). In this study, we have selected bacteriophages as an alternative to combat pathogenic, resistant, biofilm-forming *L. monocytogenes.*

2.9.7 Synthesis of literature review

The extensive literature review of the study was condensed and used to produce a review article that appears in chapter three of this thesis.

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CHAPTER THREE

LISTERIOSIS OUTBREAK IN SOUTH AFRICA: A COMPARATIVE ANALYSIS WITH PREVIOUSLY REPORTED CASES WORLDWIDE

(This chapter has been published in the journal "Microorganisms" Volume 8, Issue 1.

https://doi.org/10.3390/microorganisms8010135)

Abstract

Listeria species are Gram-positive, rod-shaped, facultative anaerobic bacteria, which do not produce endospores. The genus *Listeria* currently comprises 17 characterised species of which only two (*L. monocytogenes* and *L. ivanovii*) are known to be pathogenic to humans. Food products and related processing environments are commonly contaminated with pathogenic species. Outbreaks and sporadic cases of human infections have resulted in considerable economic loss. South Africa witnessed the world's largest listeriosis outbreak characterised by a progressive increase in disease cases from January 2017 to July 2018. Of the 1060 laboratory-confirmed listeriosis cases reported by the National Institute of Communicable Diseases (NICD), 216 deaths were recorded. Epidemiological investigations indicated that ready-to-eat processed meat products from a food production facility contaminated with *L. monocytogenes* were responsible for the outbreak. Multilocus sequence typing (MLST) revealed that a large proportion (91%) of the isolates from patients were sequence type 6 (*ST6*). Recent studies have reported a recurrent occurrence of small outbreaks of listeriosis with more severe side effects in humans. This review provides a comparative analysis of a recently reported world's most severe listeriosis outbreak in

South Africa with those previously encountered in other countries worldwide. In this chapter, specific emphasis has been made on the transmission of the pathogen, clinical symptoms of the disease and its pathogenicity. The review is expanded to elaborate on the major outbreaks of listeriosis reported in various parts of the world, the sources of contamination, morbidity and mortality rates as well as the cost implications. Based on the data generated during the South African outbreak, listeriosis was added to the South African list of mandatory notifiable medical conditions. Surveillance systems have been strengthened in the South African food chain in order to assist in preventing and facilitating early detection of both sporadic cases and outbreaks of infections caused by these pathogens in humans.

Keywords: Food chain, foodborne disease, *Listeria monocytogenes*, listeriosis, South African outbreak, sequence.

3.1 Introduction

Listeria was reported for the first time in 1924 by Murray and colleagues due to the sudden deaths of six young rabbits and was named *Bacterium monocytogenes* (Murray *et al.*, 1926). Subsequently, in 1927 Pirie named the bacterium *Listerella hepatolytica* in honor of Sir Joseph Lister, a pioneer in the field of antisepsis after successfully isolating it from the liver of several gerbils (*Latera lobenquiae*) in South Africa (Pirie, 1927a). In 1929, three patients suffered from infectious mononucleosis, due to *Listeria* and were the first confirmed human cases in Denmark (Schmidt and Nyfeldt, 1938). In 1940, the name *Listeria monocytogenes* was finally adopted to harmonize the nomenclature of the bacterium.

Listeria species are Gram-positive, catalase-positive, oxidase-negative, facultative anaerobic bacteria. Morphological identification presents cells as chains or single rods, non-sporulating,

possessing peritrichous flagella with tumbling motility (Bell, 2005). *Listeria* measures approximately 0.5 µm in diameter and 1-2 µm in length. The genus is classified under the family *Listeriacaeae* which comprises 17 species including *L. monocytogenes, L. ivanovii, L. seeligeri, L. welshimeri, L. grayi, L marthii, L. rocourtiae, L weihenstephanensis L. fleischmannii, L. floridensis, L. aquatica, L. cornellensis, L. riparia, L. grandensis, L. booriae* and *L. newyorkensis* (Bell and Kyriakides, 2012; Weller *et al.*, 2015). *Listeria* species are capable of tolerating up to 10% salt, a pH of 6 - 9 and temperatures of 0 - 45 °C with an optimal temperature range of 30-37 °C (George *et al.*, 1988; Lado *et al.*, 2007). *Listeria* species are widespread, however, among them, *L. monocytogenes* and *L. ivanovii* are human pathogens, and causative agents of listeriosis (Orsi *et al.*, 2011; Lakicevic *et al.*, 2015). Listeriosis caused by *L. monocytogenes* is a severe disease with a mortality rate of 20-30% (Zhu *et al.*, 2017b). This foodborne disease affects numerous individuals worldwide with a high fatality rate (Murray *et al.*, 1926).

The pathogen is a major threat to the food industry as it can survive and proliferate in extreme conditions such as high salinity, acidity, and refrigeration temperatures (Likotrafiti *et al.*, 2013). Due to its ubiquitous nature, it has been found in diverse natural environments such as plants, soil, silage, animals, sewage, and water. Contaminated food including vegetables, dairy, poultry, pre-cooked or frozen meat, pork, seafood and several ready-to-eat (RTE) foods are implicated as sources of infection due to their association with sporadic and epidemic cases worldwide (Fenlon, 1999; Marshall and Levy, 2011). In the modern, fast-paced lifestyle, the population is consuming more ready-to-eat (RTE) and ready-to-cook food products that entail minimal processing (Davis and Stewart, 2002; Suresh, 2013). There is, therefore, a high risk of food contamination which can cause severe clinical health effects in humans.

3.2 Potential sources of contamination with *Listeria* species

Listeria monocytogenes is the causative agent of multiple listeriosis outbreaks worldwide (Farber and Peterkin, 1991) associated with a variety of foods including milk and other dairy products, vegetables, salads, meat products and ready-to-eat foods (i.e. smoked salmon). Outbreaks caused by contaminated fishery products have also been reported (Tham *et al.*, 2000). Poor manufacturing practices might result in food contamination by this pathogenic bacterium in the food supply chain (Wirtanen *et al.*, 2000). A previous report showed that factors such as poor sanitary practices, contaminated processing environments and temperature abuse during overstay storage in retail outlets led to *Listeria*-contaminated vacuum-packed (VP) smoked fish products (Tocmo *et al.*, 2014). Numerous organizations worldwide (WHO, FAO, Codex Alimentarius, etc.) have been applying the policy of "Zero Tolerance" for *L. monocytogenes* in RTE processed foods to reduce the high risk of food contamination and consequently reduce the spread of infection to the public (Molins *et al.*, 2001; Todd, 2004). From these sources, the bacterium can propagate, disseminate and contaminate food products, a source of infection to animals and humans. Due to the severity of the disease, stringent procedures must be established to detect the critical control point where contamination is at its maximum level. It is worth noting that approximately 20-30% of both sporadic and outbreak *L. monocytogenes* cases are fatal (Gasanov *et al.*, 2005; Alessandria *et al.*, 2010).

3.3 Transmission of the disease

The reservoirs of infection are the soil and the intestinal tracts of asymptomatic animals, including wild and feral mammals, birds, fish, and crustaceans (Fenlon, 1985a). Infected animals can shed *L. monocytogenes* in their faeces (Esteban *et al.*, 2009), milk (Borucki *et al.*, 2004) and uterine discharges (Dennis, 1963). It is also found in aborted foetuses and occasionally in the nasal

discharges and urine of symptomatic animals (Barr and Anderson, 1993). Soil or faecal contamination results in its presence on plants and in silage (Fenlon, 1999). Most infections are acquired by ingestion, but *Listeria* can also be spread by inhalation or direct contact (Ho *et al.*, 1986). Venereal transmission might also be possible (Wiedmann *et al.*, 1999b). In ruminants, listeriosis typically occurs after the consumption of contaminated silage or other feed (Fenlon *et al.*, 1996a). For humans, contaminated food sources include raw meat and fish, unpasteurized dairy products, and uncooked vegetables. *L. monocytogenes* has also been found in foods contaminated after processing, particularly soft cheeses, deli cold cuts, sliced or grated cheese, and ice cream. The infective dose for oral transmission is unknown but it is thought to depend on the bacterial strain and the host susceptibility (Schuchat *et al.*, 1991; Hazards *et al.*, 2018). Processed food may get contaminated during the production process from the raw product to the final consumer. Due to the significant increase in the level of contamination and the risk of exposure of food to *L. monocytogenes*, food security and safety are of global concern (Hoc, 2007). Emphasis is now placed on the negligence of food production/processing facilities and rethinking consumer habits towards the consumption of convenient foods (Rocourt and Bille, 1997). A major issue facing the chain of production is the fact that *Listeria* pathogen can survive under low temperatures resulting in increased cases and high mortality rates due to the infection. It is worth mentioning that listeriosis due to *L. monocytogenes* is not only prominent in humans but also life-threatening with various lethal symptoms (Schuchat *et al.*, 1991; Lamont *et al.*, 2011). Healthy people seem to consume most *Listeria*-contaminated foods without any clinical signs; however, in susceptible persons, the infective dose is probably about 10-100 organisms (Hof and Hefner, 1988; Buchanan *et al.*, 2017). Vertical transmission is the usual source of infection in newborn human infants and ruminants; infections are transmitted either transplacentally or from an infected birth canal.

Humans can also be infected by direct contact with infected animals during calving, lambing, or necropsies. Cases have been reported after contact with sick birds or the carcasses of asymptomatic poultry (McLauchlin, 1987; Nightingale *et al.*, 2004b). *L. monocytogenes* is relatively resistant to freezing, drying and heat. It can tolerate a pH from 3.6 to 9.5, and a sodium chloride content of up to 10%. A pH of greater than 5 (e.g., in spoiled silage) favours the growth of this organism, however, it has also been found in silage with a pH less than 4 (Ryser *et al.*, 1997; Vasseur *et al.*, 2001; Venkitanarayanan *et al.*, 2002).

3.4 Clinical signs and symptoms of listeriosis

Listeriosis is a fatal infection that mostly affects pregnant women, newborns, the elderly, and immunocompromised or debilitated hosts. In symptomatic individuals, common symptoms include diarrhoea, abdominal pain, fever, vomiting, mild and flu-like illness. Pregnant women may also experience chills, headaches, slight dizziness, or gastroenteritis (Craig *et al.*, 1996; Goulet *et al.*, 2013; Vázquez-Boland *et al.*, 2017). Sepsis (bacteraemia) in pregnant women may eventually lead to abortion, stillbirth, premature birth, or septicaemia in the newborn. *L. monocytogenes* infection might result in side effects on the central nervous system such as encephalitis, rhombencephalitis (brainstem encephalitis), or focal signs suggestive of brain abscess formation (Vázquez-Boland, Kuhn, *et al.*, 2001). Although common symptoms of relatively mild gastroenteritis are observed in healthy adults, it is severe in immune-compromised patients. Complications of the disease include meningitis, meningoencephalitis, or septicaemia with immune-suppressed individuals highly at risk (Anderson *et al.*, 2017).

3.4.1 Asymptomatic carriage of *Listeria monocytogenes*

Listeria colonizes at least 1-5% of healthy adults. Moreover, every year, humans experience multiple exposures to persons with transient carriage of the pathogen (Goulet *et al.*, 2013). Research has shown that 2 out of 3 people experience episodes of faecal carriage which take less than 4 days and are asymptomatic. Approximately 20-25% of faecal carriage is displayed by people who come in contact with invasive *Listeria* disease (Humphries and Linscott, 2015).

3.4.2 Gastroenteritis due to *Listeria monocytogenes*

Listeria-infected humans may experience gastroenteritis. There are several side effects associated with gastroenteritis, such as fever (60-100% of cases), non-bloody diarrhoea (33-88%), arthromyalgia (20-100%) and headache (15-88%). Fever and vomiting are common in children with gastroenteritis, while diarrhoea and arthralgia are common in adults. The incubation period ranges from 6 h to 10 days and the symptoms span from 1-3 days, although they can last for up to one week. Hospitalisation caused by gastroenteritis due to listeriosis is usually prominent in children and the elderly. Blood cultures from gastroenteritis patients may yield *Listeria* (Opperman and Bamford, 2018).

3.4.3 Listeriosis in pregnancy

Listeriosis infection in pregnant women presents a serious side effect because of its invasive nature. A previous study reported the mean incubation period of listeriosis in pregnancy as 27.5 days, with a range of 17-67 days. Major side effects include mild flu-like symptoms, fever, backache, and headache, and some might lead to neonatal sepsis. *Listeria* infection in pregnancy usually occurs at the early stages, and the side effects are observed during the third trimester. The

disease often results in poorer neonatal outcomes. Serious side effects associated with pregnant women are spontaneous abortion, stillbirth, or preterm birth (Okpala and Ifeoma, 2019).

3.4.4 Neonatal infections due to *Listeria monocytogenes*

Listeria infection in neonates occurs through endogenous transplacental transmission, inhalation of infected amniotic fluid, or following colonization from the maternal gastrointestinal or vaginal carriage. A study reported the isolation of *Listeria* pathogens from the genital tract and blood cultures of the majority of mothers. The premature offspring experienced the onset of the disease within 36 h, which represented transplacental neonatal infection. Neonates exhibited sepsis (90%), respiratory distress or pneumonia (40%), meningitis (25%), and sometimes with inflammatory granulomata (granulomatosis infantiseptica). The appearance of a rash occurs with maculopapular or papulovesicular lesions on the trunk or extremities. Between 5-30 days postnatal period, the late-onset disease progresses and presents with the expansion of non-specific symptoms, sepsis and meningitis (Visintine *et al.*, 1977; Dyatlov and Lawrence, 2002).

3.4.5 Bacteremia due to *Listeria monocytogenes*

Listeria monocytogenes can disseminate to the blood leading to bacteremia, one of the clinical presentations in some *Listeria*-infected persons. Bacteremia due to *Listeria* might lead to gastroenteritis, and in most cases, it is associated with pregnancy or neonatal infection. Bacteremia in adults might be a clinical effect of HIV infection, chronic renal disease, steroid use, underlying malignancy, chemotherapy, or age >65 years (Maayan *et al.*, 1981; Jurado *et al.*, 1993).

3.4.6 Meningitis due to *Listeria monocytogenes*

Acute meningitis and encephalitis have been regarded as some of the severe symptoms of listeriosis. Other symptoms include rhomboencephalitis, which is the involvement of the midbrain, pons and/or cerebellum linked to cranial nerve involvement or cerebellar signs (ataxia, tremor), or the development of hemiparesis. The incubation period of meningitis is estimated to be 0-21 days, with an average of 10 days. Studies have shown that cases of neuroinvasive listeriosis, and neck stiffness were shown in 75% of cases, focal neurological signs in 30%, seizures in 30% and coma in 7%. Focal neurological symptoms comprised of single or multiple cranial nerve involvement, (most commonly the 6th and 7th cranial nerves) hemiparesis, ataxia, and aphasia (Lavetter *et al.*, 1971; Brouwer *et al.*, 2006).

3.5 Pathogenicity factors and virulence potential

The pathogenicity of *L. monocytogenes* is due to the expression of different genes which are responsible for its ability to penetrate, proliferate and spread in cells (Orsi and Wiedmann, 2016). These capabilities are attributed to their *inlAB* internalization locus, *Listeria* pathogenicity island-1 (*LIPI-1*), and *hpt* intracellular growth locus, respectively (Vázquez-Boland, Kuhn, *et al.*, 2001). *InlAB* internalization locus encodes two surface proteins: *InlA* and *InlB* that bind their corresponding receptors on the host cell surface, via their Leucine-Rich Repeat (*LRR*) domains, that is *InlA* binds human E-cadherin, a calcium-dependent intercellular adhesion glycoprotein, while *InlB* binds hepatocyte growth factor receptor Met (Shen *et al.*, 2000) and together mediate bacterial internalization and invasion into host cells. *LIPI-1* encodes a pore-forming toxin listeriolysin O (*LLO*) and two phospholipases C (*PlcA* and *PlcB*), which cooperate to lyse the phagocytic vacuole membrane of host cells (Dussurget *et al.*, 2004). The hpt intracellular growth locus encodes hexose-6-phosphate transporter and actin assembly-inducing protein (ActA), which

play an important role in intracellular bacterial growth, cell-to-cell spread and actin polymerization (Vázquez-Boland, Kuhn, *et al.*, 2001).

The virulence of *L. monocytogenes* is mainly regulated by six genes (*PrfA*, *PlcA*, *hly*, *mpl*, *actA* and *plcB*) residing in *PrfA*-dependent virulence gene cluster and other virulence-related genes located outside this gene cluster (Vázquez-Boland *et al.*, 2001). Furthermore, *L. monocytogenes* carries a gene cluster of five genes termed stress survival islet 1 (*SSI-1*), contributing to the survival of cells under suboptimal conditions, including low pH and high salt concentrations, in food environments (Ryan *et al.*, 2010).

In summary, the virulence gene clusters of *L. monocytogenes* and *L. ivanovii* contain *PrfA* which encodes the positive master regulator of most of the known virulence genes while *hlyA* encodes the sulfhydryl-activated pore-forming listeriolysin O (*LLO*) essential for bacterial escape from the phagosomes of host cells into the host cytosol. Therefore, *LLO* is the main virulence feature for the discharge of bacteria from the primary and secondary intracellular vacuoles (Soni *et al.*, 2017). The species-specificity properties of the *hly*A gene and the encoded protein *LLO* allowed their use as molecular targets for *L. monocytogenes* detection in food (Amagliani *et al.*, 2016) and on-farm microbiological control (Amagliani *et al.*, 2012), and for serological diagnosis of ovine listeriosis (Amagliani *et al.*, 2006). Moreover, anti-*LLO* antibody detection could be useful to screen previous or ongoing *L. monocytogenes* infection in pregnant women (Massacesi *et al.*, 2019). On the other hand, the *PlcA* and *plcB* code for two phospholipases phosphatidylinositol-specific phospholipase C, and phosphatidylcholine phospholipase respectively that assist in the lysis of host cell membranes. Soni *et al.* (2017) stated that the virulence genes comprising members of the internalin protein family (*inlA, inlB*), encode cell wall proteins that are important for attachment and invasion of the non-phagocytic host cells while the other virulence genes are linked with the infection

events; they adjust the metabolic activities and help the pathogen to respond to stress. The *hlyA* gene encodes a 60-kDa haemolysin protein, permitting *L. monocytogenes* to escape from vacuoles by dissolving phagosome membranes. Böckmann *et al.* (1996) stated that the phosphatidylinositol-specific phospholipase C protein is encoded by *PlcA* and its function is similar to that of the haemolysin protein. These virulent genes can be expressed or repressed by a few environmental attributes which can affect the virulence of *L. monocytogenes* (Shi *et al.*, 2013).

Listeria monocytogenes by definition, is a bacterial pathogen which brings about food poisoning, meningitis as well as rapid abortions in pregnant women (Robbins *et al.*, 1999). This bacterium attacks the host via the epithelial lining of the gut. After staying in the host cell, the bacterium prompts a local cytoskeletal rearrangement which brings about the uptake of the bacterium into a primary vacuole. Using *ActA* gene, a pore-forming haemolytic protein, and other aspects, the bacterium escapes from the vacuole into the host cytoplasm, where it splits and makes use of proteins of the host cytoskeleton to acquire intracellular actin-based motility. This means of motility is vital for useful spread within the cell from the cytoplasm of one infected host cell to another. This mechanism of intercellular spread in endothelial tissue has been speculated to describe how *L. monocytogenes* invades the trans-placental and blood-brain barriers (Robbins *et al.*, 1999).

L. monocytogenes achieves intercellular spread by capturing the host cell's actin-based motility components to force itself into the host cell's plasma membrane and infect adjacent cells. The membrane-associated bacterial protein *ActA* is essential and appropriate for such motility. Distributed in a gradient along the bacterial surface, *ActA*, therefore, nucleates actin filaments and further catalyses their elongation (Smith *et al.*, 1996).

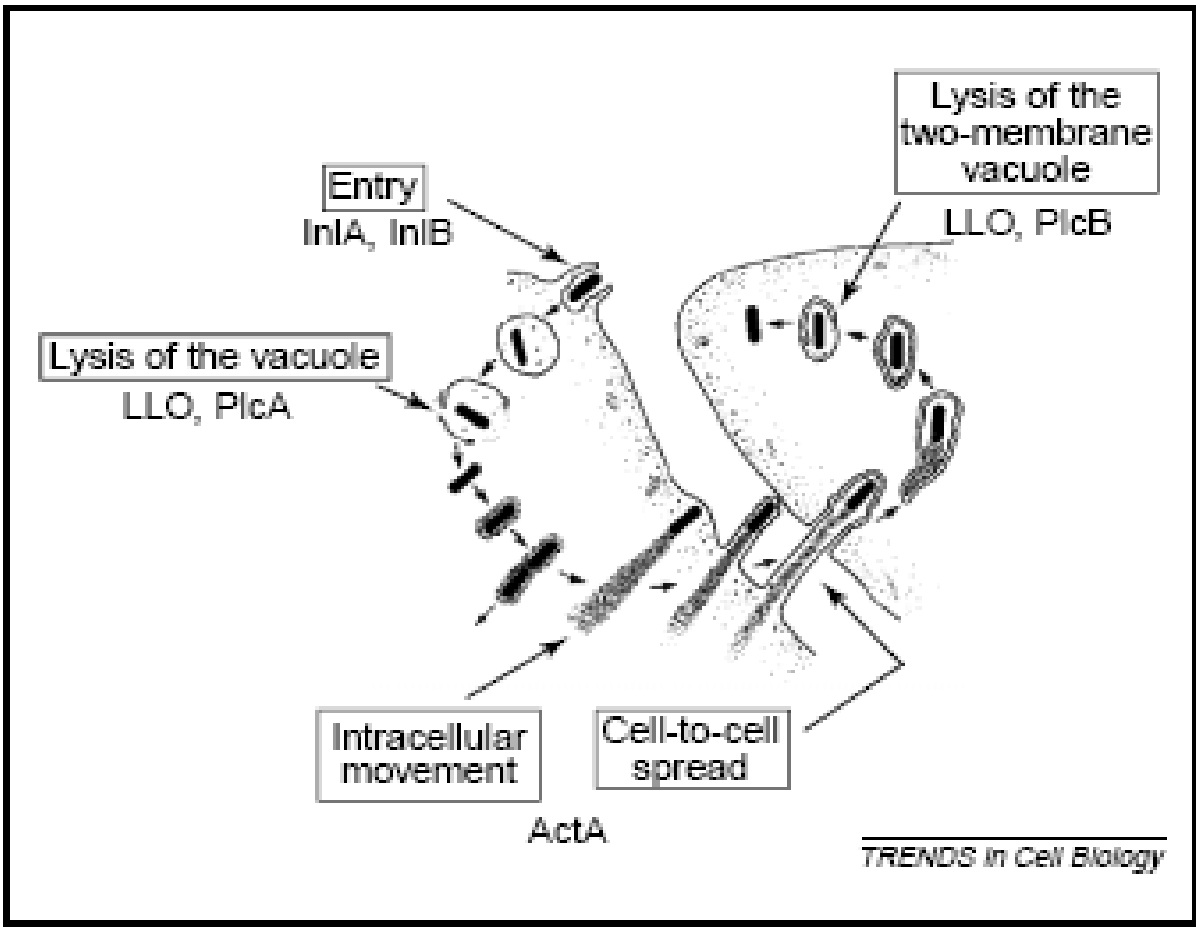


Figure 3.1: The infection process of a host cell by *L. monocytogenes* (Cossart et al., 2003).

3.6 Antibiotic resistance in *Listeria monocytogenes*

The first *L. monocytogenes* strains resistant to antimicrobials were detected in 1988 (Poyart-Salmeron *et al.*, 1990). Since then, an increasing number of strains resistant to one or more antibiotics, isolated from humans, animals, and food (Noll *et al.*, 2018), including food processing environments (Haubert *et al.*, 2016), and dairy farms (Srinivasan *et al.*, 2005), have been reported in recent years.

Although *L. monocytogenes* is considered vulnerable to a wide range of antibiotics, intrinsic antibiotic resistance to first-generation quinolones, fosfomycin, monobactams and broad-spectrum cephalosporins has been described (Hof, 2003). Moreover, the clinical use of antibiotics contributed to the selection of resistant strains, particularly for those antibiotics commonly used to treat listeriosis. β-lactams (penicillin and ampicillin), with or without gentamicin, are the main antibiotics considered for listeriosis treatments; vancomycin and trimethoprim/sulfamethoxazole can be used as alternative therapy for penicillin-sensitive patients (Pagliano *et al.*, 2017).

Veterinary use of antimicrobials in food-producing animals, which are commonly administered for disease therapy and prophylaxis and as growth promoters could also contribute to the emergence of resistant strains (Lungu *et al.*, 2011). Antibiotic resistance and particularly multidrug-resistance represent severe public health problems since they are most often associated with treatment failures. Therefore, monitoring changes in the antibiotic resistance of *L. monocytogenes* due to the continuing emergence of resistant strains, particularly by integration of phenotypic and genotypic techniques, is needed.

3.7 Outbreak of listeriosis

3.7.1 Listeriosis outbreaks in the United States

In 1998, an outbreak occurred in 10 states linked to the consumption of hot dogs and possibly refrigerated, processed deli meat. Forty patients contaminated by a single strain, *L. monocytogenes* were identified in early August 1998 from the states of Ohio (13 cases); New York (12); Tennessee, Massachusetts, and West Virginia (three each); Michigan (two); and Connecticut, Oregon, Vermont, and Georgia (one each). Out of the forty infected patients, information could only be accessed from 38 which included six newborns and 32 adults (median age: 69 years; range:

18-88 years) with 55% of patients being females. Of these, deaths were recorded in one foetus and three elderly (CDC Centers for Disease Control Prevention., 1998). The same year on December 22, Bil Mar Food recalled the production lots of EST P261 or EST 6911 of hot dogs and meat products that might be contaminated. This recall comprised the Ball Park, Bil Mar, Bryan Bunsize and Bryan 3-lb Club Pack, Grillmaster, Hygrade, Mr. Turkey, Sara Lee Deli Meat, and Sara Lee Home Roast brands (Gaysinsky *et al.*, 2007).

Listeria monocytogenes was also isolated from 29 patients in 10 states between May 17 and November 26th, 2000. Of these, the majority of cases, 26 (90%) occurred from 15th July to 26th November 2000, while fewer cases 3 (10%) were isolated in the earlier months. The 29 patients were distributed as follows: New York (15 cases); Georgia (3); Connecticut, Ohio, and Michigan (2 each); California, Pennsylvania, Tennessee, Utah, and Wisconsin (1 each). 21 patients were elderly (range 29–92 years) with a median age of 65 years, and 8 were perinatal. Three miscarriages or stillbirths and 4 deaths were reported (CDC, 2000). Since then, outbreaks or sporadic cases of the infection have been reported almost every year. Table 3.1 summarizes listeriosis outbreaks in the USA from 1976-2019.

Table 3.1: Some Listeriosis outbreaks in the USA 1976- 2019.

Year	**Number of cases (no. of death, no. of miscarriages)**	**Food**	**References**
1976	20	Raw salad	Ho *et al.*, (1986)
1983	49	Milk	Fleming *et al.*, (1985)
1985	142 (41)	Soft cheese	Linnan *et al.*, (1988)
1989	9 (1)	Shrimps	Riedo *et al.*, (1994)

1998-1999	108 (14; 4)	Hot dog	CDC (1999)
2000	29 (4; 3)	Turkey meat	CDC (2000)
2001	12	Mexican style cheese	CDC (2001)
2002	46 (7; 35)	Fresh and frozen ready-to-eat turkey and chicken	CDC (2002)
2007	311 (52)	Milk	CDC (2007)
2007	5 (3)	Dairy milk	CDC (2008)
2010	10 (5)	Pre-cut celery	Gaul *et al.*, (2012)
2011	147 (33)	Cantaloupe	Buchanan *et al.*, (2017)
2012	22 (4)	Ricotta salata Cheese	CDC (2012)
2014	5 (2)	Mung bean sprouts	CDC (2015b)
2014	4	Stone fruit	Chen *et al.*, (2016)
2010-2015	10 (3)	Ice cream	CDC (2015a)
2014-2015	5 (3)	Caramel apples	CDC (2015c)
2016	9 (3)	Frozen vegetables	CDC (2016c)
2016	2 (1)	Raw milk	CDC (2016b)
2016	19 (1)	Packaged salads	CDC (2016a)
2017	8 (2)	Soft Raw Milk Cheese Made by Vulto Creamery	CDC (2017)
2018	4	Pork Products	CDC (2018a)
2018	4 (1)	Deli Ham	CDC (2018b)
2019	8 (1)	Deli-Sliced Meats and Cheeses	CDC (2019)

3.7.2 Listeriosis outbreaks in the European Union

Listeriosis cases in the EU were reported in 12 countries (667 cases) in 1999 which rose to 1583 cases distributed in 23 countries in 2006; a total of 667 cases were reported in 12 countries in 1999, 586 in 2000(13 countries), 872 in 2001(13 countries), 909 in 2002 (15 countries), 1070 in 2003 (16 countries), 1264 in 2004 (22 countries), 1427 in 2005 (19 countries) and 1583 in 2006 (23 countries). This report shows that human listeriosis cases are on the increase each year. Table 3.2 presents human-reported cases of listeriosis in the EU from 1999- 2006.

Table 3.2: Human cases of listeriosis reported in Europe in 1999–2006 (Denny and McLauchlin, 2008).

Country	**Number of confirmed cases**							
	1999	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**
Austria	13	14	9	16	8	19	9	10
Belgium	64	48	57	44	76	70	62	67
Cyprus								1
Czech Republic						16	15	78
Denmark	44	39	38	28	29	41	46	56
Estonia	1					2	2	1
Finland	46	18	28	20	41	35	36	45
France	275	261	187	218	220	236	221	290
Germany	31	33	216	240	256	296	510	508
Greece	1	2	3	5		3		6
Hungary						16	10	14
Ireland		7	7	6	6	11	11	7
Italy	17	13	31			25	51	51
Latvia		36		16	8	5	3	2

Lithuania					2	1	2	4
Luxembourg								4
Malta								0
Netherlands			16	32	52	55	96	64
Poland				31	5	10	22	28
Portugal						38		
Slovakia				7	6	8	5	12
Slovenia					6	1		7
Spain	32	35	57	49	52	100	68	78
Sweden	27	46	67	39	48	44	35	42
United Kingdom	116	115	156	158	255	232	223	208
EU Total	**667**	**586**	**872**	**909**	**1070**	**1264**	**1427**	**1583**
Bulgaria		2000						6
Iceland		14						
Liechtenstein		48						
Norway			18	17	18	21	14	27

Based on the European outbreak case definition, as of 6 March 2018, a multi-country foodborne outbreak was verified in five countries, involving 32 confirmed cases and six deaths due to or with the infection (Table 3.3) (EFSA *et al.*, 2018).

- Austria reported two confirmed cases from 2016. Both cases were males, aged over 85 years at symptom onset, one case was fatal.
- Denmark reported four confirmed cases with sampling dates in January 2017, May 2017 and February 2018 (two cases). Three cases were female patients, and one was a male. Their age ranged between 37 and 74 years. One case was fatal (Helwigh and Müller, 2018).

- Finland reported 14 confirmed cases with sampling dates from September 2016 to January 2018. Nine cases were females, and five were males. Their age ranged between 22 and 92 years. Two cases were fatal.

- Sweden reported six confirmed cases with isolates. Of these, five were females while one was a male with an age range between 70 and 94 years. Two cases were fatal.

- The United Kingdom reported six confirmed cases between 2015 and 2018 with an age range of 22-84 years. Four cases were males and two females.

Table 3.3: Listeriosis verified cases in five European countries between 2015 and 2018.

Country	**Confirmed cases (No. of deaths)**				**Total number of Cases**	**Total number of deaths**
	2015	**2016**	**2017**	**2018**		
Austria	0	2 (1)	0	0	2	1
Denmark	0	0	2	2 (1)	4	1
Finland	0	3	10 (2)	1	14	2
Sweden	0	3 (1)	3 (1)	0	6	2
United Kingdom	1	2	2	1	6	0
Total	**1 (0)**	**10 (2)**	**17 (3)**	**4 (1)**	**32**	**6**

3.7.3 Listeriosis outbreaks in Australia

Listeria monocytogenes has become an invasive pathogen in foods and processed food products. In Australia, the year 2003 had an outbreak with the highest mortality (30 %) [99]. In 2009, there was contamination of chicken wraps sold on domestic flights while in 2010, *L. monocytogenes* contaminated melons and caused a multijurisdictional outbreak, with approximately 70 cases reported (Figure 3.2). The highest number of listeriosis cases was reported in 2014 and the most prevalent group affected were patients 80 years and above. In 2018, the isolated *L. monocytogenes* strain was serotype 4b strain ST240 and affected 59% of females.

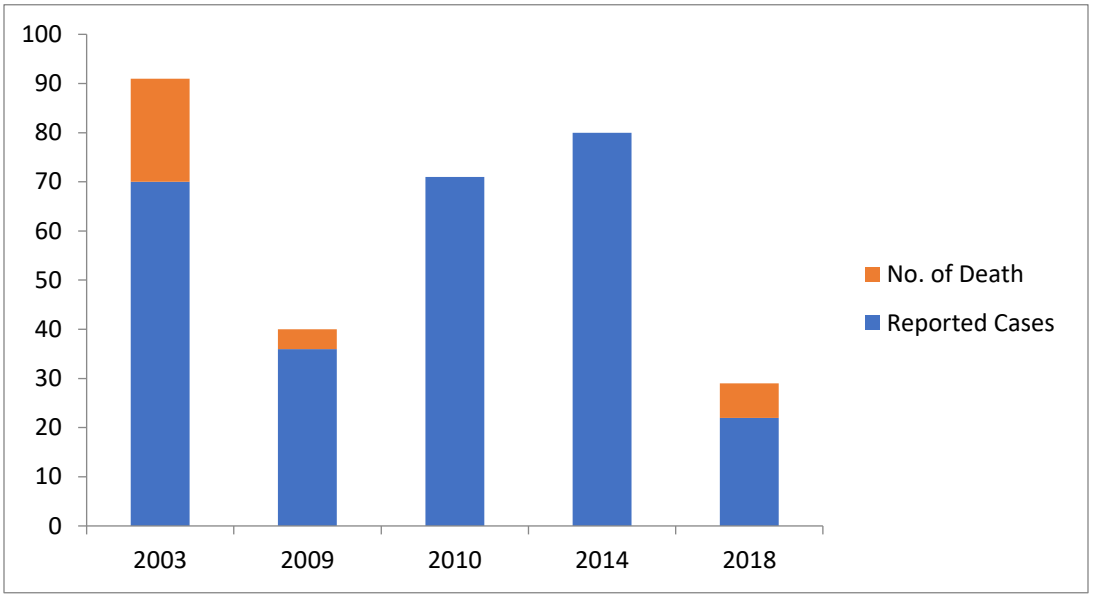


Figure 3.2: Outbreak of listeriosis in Australia during 2003, 2009, 2010, 2014 and 2018.

3.7.4 Listeriosis outbreaks in Asia

For the past few decades, Asia has experienced severe outbreaks of listeriosis. Several reports in Asian countries such as Japan (Okutani *et al.*, 2004; Makino *et al.*, 2005), Thailand (Bangkok)

(Indrawattana *et al.*, 2011) and Taïwan (Hsieh *et al.*, 2009; Huang *et al.*, 2011) have been documented for clinical listeriosis cases. Table 3.4 shows that *L. monocytogenes* targets the immune-compromised individuals such as pregnant women, and infants. This is displayed in outbreak reports in China from 1964 and 2010 (Feng *et al.*, 2013).

Table 3. 4: Reporting periods of clinical listeriosis cases for each subgroup in China, 1964–2010.

Reporting period	No. of cases				
	Immune-competent patients	Immune-compromised Patients	Pregnant women	Neonates	Total
1964–1970	1	0	0	1	2
1971–1980	1	0	0	0	1
1981–1990	8	2	0	0	10
1991–2000	12	7	5	9	33
2001–2010	27	12	25	37	101
Total	48	21	30	47	147

3.7.5 Listeriosis outbreaks in Nigeria

Nigeria is regarded as the "Giant of Africa" due to its large population and growing economy. Regardless of this, there have been limited reports documented and surveillance systems of listeriosis outbreaks. However, researchers have successfully isolated *Listeria* in humans, animals, the environment and food samples in Nigeria. *Listeria* was shown to affect pregnant women and infants while ruminants dominated reports of incidences in animals. Table 3.5 illustrates the various sources of *Listeria* in Nigeria including humans, fish, water, animals, soils, and the environment (Nwaiwu, 2015).

Table 3.5: illustrates the various sources of *Listeria* in Nigeria including humans, fish, water, animals, soils, and the environment.

Source	**Origin**	**References**
Human	Patient with meningitis, septicaemia	Onyemelukwe *et al.*, (1983); (Emele, 2000; Chukwu, (2007)
	Neonatal and mother	Onyemelukwe and Lawande, (1982)
	Neonates	Ako-Nai *et al.*, (1999)
	Blood samples	Bolarinwa *et al.*, (2011)
	Faecal specimen	Esumeh and Odugbemi, (1992)
Animals	Cow faeces	Lawan *et al.*, (2013)
	African buffalo	Chukwumuhammad *et al.*, (2006)
	Guinea pig	Chukwuogo *et al.*, (2006)
	Pig, dog, cattle, goat, horse, camel, chicken	(Oni *et al.*, 1989; Eruteya *et al.*, 2014)

	Animals dropping	Akpavie and Ikheloa (1992)
	Raw cattle milk	Yakubu *et al.*, (2012)
	Raw goat milk	Adetunji and Olaoye (2012)
	Domestic cats	Magaji *et al.*, (2008)
	Cockroach	Adeleke *et al.*, (2012)
Environment	Butcher'stable	Adetunji and Isola (2011)
	Lake	Nwachukwu *et al.*, (2010)
	Irrigation water	Mawak *et al.*,(2009)
	Nairacurrency notes	Kawo *et al.*, (2009)
	Veterinary surgical material	Tambuwal *et al.*, (2009)
	Soil	David and Odeyemi (2007)
	Fish	Shinkafi and Ukwaja (2010)

Food	Vegetables	Ikeh *et al.,* (2010)
	Dried beef *Kilishi*	Yusuf and TengkuHaziyamin (2013)
	Kunu drink	Nwachukwu *et al.,* (2009)
	Wara soft cheese	Nwachukwu *et al.,* (2009)
	Fermented food (*Gari* etc…)	Ijabadeniyi (2007)
	Frozen poultry	Adetunji and Odetokun (2012)

3.7.6 Listeriosis outbreaks in South Africa

Currently, South Africa is experiencing severe outbreaks of Listeriosis. To date, this is the highest outbreak of *Listeria* in history affecting a single nation. A total of 820 listeriosis cases were reported by the National Institute of Communicable Diseases (NICD) from January 2017 till 23 January 2018. The highest number of cases originated from Gauteng province (59%), followed by the Western Cape (13%) and Kwa Zulu-Natal (7%). Samples were collected from blood (71%) and cerebrospinal fluid (CSF) with 23% in the public and private healthcare sectors. Approximately 66% were diagnosed from the public and 34% from the private healthcare sector. The age distribution of cases ranged from birth to 93 years with the majority (96%) of cases being neonates (≤28 days). The gender distribution was 55% of females and 45% of males (NICD,

2018b). Figure 3 illustrates the evolution of confirmed listeriosis cases during the period of 01 January 2017 to 17 July 2018 in South Africa (NICD, 2018a).

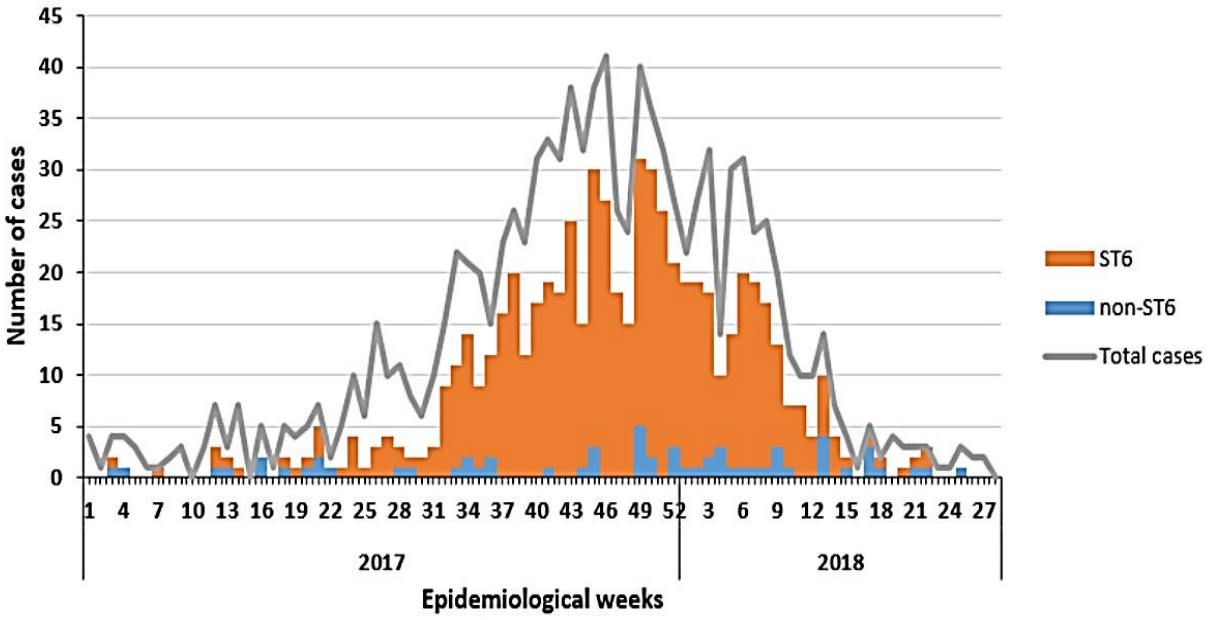


Figure 3.3: Epidemic curve of laboratory-confirmed listeriosis cases by date of clinical specimen collection (N=1 060) and sequence type (ST) (n=636), South Africa, 01 January 2017 to 17 July 2018 (NICD, 2018a).

Fortunately, human listeriosis is rare. However, there is a high case of fatality if disease ensues. With the very long incubation period which can range from 5 days up to 60 days, attributing the illness to a specific food product becomes difficult (Farber and Peterkin, 1991). In addition to several factors mentioned earlier that contribute to the outbreaks of listeriosis in the supply chain, resistance of microorganisms to antibiotics and disinfectants also plays a role (Schirone *et al.*, 2019).

3.7.7 Estimated Cost of the listeriosis outbreak in South Africa

Food spoilage caused by microbial pathogens results in massive economic loss to both the company responsible and the country. In developing countries such as South Africa, listeriosis

outbreaks are poorly documented (NICD, 2018a), including the major outbreak in 2017-2018, which caused a deadly epidemic. Approximately, 1060 cases were reported with 216 deaths (NICD, 2018a). Processed meat product (Polony) produced by Tiger Brands Limited (South Africa) was identified as the responsible source. The accountable factories were Tiger Brand of Polokwane in Limpopo and Germiston in Johannesburg (van der Vyver, 2018). The economic loss was projected to be US $ 260 million (R 3, 79 billion) in terms of lethality, while hospitalization cost associated with the one-month recovery of affected patients by the disease was at US $ 10.4 million (R1, 5 billion). Furthermore, more than US $ 15 million (R2, 19 billion) has been attributed to loss due to productivity and export of food processing companies due to the outbreak (Olanya *et al.*, 2019).

3.8 Conclusion

In conclusion, this review confirms that South Africa has recorded the highest cases of listeriosis outbreaks with a high mortality rate. *L. monocytogenes* was successfully isolated from contaminated possessed meat (Polony). South Africa is considered the economic capital of Africa and is developing exponentially. South Africa also has strong regulations on food safety management and laws which are aligned with international standards. Despite this, the recent outbreak of listeriosis has shown some limitations in food safety policies. The Department of Health in South Africa has recently reviewed the list of notifiable diseases to include listeriosis.

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CHAPTER 4

CHARACTERIZATION OF VIRULENCE, ANTIBIOTIC RESISTANCE AND BIOFILM-FORMING GENES IN *LISTERIA* SPECIES FROM DIFFERENT SOURCES

(Sections of this chapter have been published in the journal "Pathogens" 2022, 11(8), 843. Doi: 10.3390/pathogens11080843)

Abstract

The current study was undertaken with the primary objective of isolating and identifying *Listeria* species from different sources (milk, vegetables, meat, water, and faecal samples) in various locations in Mafikeng, South Africa. A total of 207 samples encompassing milk, vegetables, meat, water and faecal materials were collected aseptically from local markets, farms, rivers, and boreholes. All samples were subjected to half Fraser broth and Fraser broth respectively as pre-enrich and enrichment broth followed by the streaking on Oxford agar. Morphological, physiological, and biochemical assays were conducted to identify up to the genus level. Presumptive positive *Listeria* species isolates were further subjected to polymerase chain reaction using different genes; *16S rRNA, prs, Lmo0733,* and *namA* as a confirmatory tool to identify *Listeria* species. From the 207 samples, the highest prevalence was observed in meat samples with 51% presumptive positive for *Listeria* species followed by 37% water samples, 9% and 3% respectively from vegetables and faecal samples. Milk samples tested negative. The presumptive samples were obtained from Mafikeng 44 (37%), followed by Zeerust 25 (21%), Lichtenberg 16

(14%), Marikana 11 (9%), Potchefstroom 7(6%), Klerckdorp, Lichtenburg (Korfontein) and Vryburg respectively with 5 (4%). A total of 267 isolates were identified as presumptive *Listeria* spp. The morphological and biochemical features show small rod-shaped, Gram-positive, facultatively anaerobic, catalase-positive, and oxidase-negative with a tumbling motility using flagella. Out of 267 isolates, 53 isolates were presumptive *Listeria* species, thus contributing to 19.8%. A total of 25 (47%) isolates were identified as *L. monocytogenes*. The primer *namA* a species-specific gene for *Listeria ivanovii* was evaluated and no isolates were amplified. *Listeria monocytogenes* remains a serious foodborne pathogen with severe public health issues causing fatalities and hospitalization. The findings of this study have shown that a large proportion of *Listeria* species isolates were resistant to most of the antibiotics 66.67% to 100%, excluding ampicillin, trimethoprim + sulfamethoxazole, erythromycin and norfloxacin. The MAR phenotype E-DA-GM-T-NO-OX-NA-K appeared in three different locations on different samples which can be due to the use of antibiotics in those areas. All the isolates were amplified for gene *prfA* (100%), followed by *hlyA* gene in 17 out of 25 (68%) and *actA* gene detected in 14 isolates (56%). However, *plcB* genes were detected in 5 isolates. Our findings reveal that strong biofilm attachments were formed at 4 °C for 24 hrs, 48 hrs and 72 hrs in all isolates (n=25; 100%), followed by moderate biofilm formation for 4 isolates (16%) at 72 hrs and weak biofilms at 72 hrs for 3 (12%) *L. monocytogenes* isolates. In addition, PCR-based biofilm formation genes showed in 18 out of 25 (72%) were amplified for *flaA*, followed by 14 (56%) for *luxS*. In conclusion, our study reveals that *L. monocytogenes* carried virulence genes with multi-antibiotics resistance (MAR) and a strong biofilm formation. The current control majors for *L. monocytogenes* have proven to be ineffective in decreasing the occurrence and resistance of this pathogen in South African food and water sources.

Keywords: Isolation, molecular identification, *Listeria* species, *Listeria monocytogenes.*

4.1 Introduction

The genus *Listeria* encompasses six known species: *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi*. In addition, only two are pathogenic: *Listeria monocytogenes* and *L. ivanovii* (Orsi andWiedmann, 2016). The clinical symptoms of the diseases are characterized by spontaneous abortions, meningoencephalitis, septicaemia, gastroenteritis, and serious infections in newborns. *Listeria monocytogenes* infection is a public health issue due to the increment in the fatality rate, hospitalization rate and neonatal death rate (Zhu *et al.*, 2017b). The main route of the infection is the consumption of contaminated food.

Once infected animals begin to shed the bacteria, cells of *Listeria* species may occur in the faeces, milk, and uterine discharges (Djønne, 2007). This subsequently leads to the contamination of soil or environmental sources resulting it their presence on plants and silage. High levels of morbidity and mortality have been associated with *Listeria* contamination throughout history and in different parts of the globe (Mateus *et al.*, 2013). Rapid and reliable identification of pathogenic bacteria including *Listeria* species is essential for the proper control, monitoring, and prevention point source contaminations that may be result in even epidemic cases (Váradi *et al.*, 2017).

The control and prevention of listeriosis rely on consumption and the effectiveness of antibiotics. However, *L. monocytogenes* have been susceptible to certain antibiotics such as quinolones, fosfomycin, and expanded spectrum cephalosporins which are also effective against other Gram-positive bacteria. *L. monocytogenes* is generally sensitive to a wide spectrum of antibiotics (Morvan *et al.*, 2010). The high death rate was due to the inefficacy of antibiotic treatments that were administrated to the patients. Adverse implications of the use of antibiotics in animals and

humans include the possible development of antibiotic-resistant zoonotic pathogens in food and their spread as contaminants in food (Zhang *et al.,* 2023). Recent studies have shown that there has been a development of resistance to antibiotics, which are currently on the market. Pharmacological studies focused on the synergistic association of high doses of aminopenicillin (ampicillin or amoxicillin) and gentamicin as an alternative. Other substitutes include rifampin, vancomycin, linezolid, and carbapenems, trimethoprim, which were commonly utilized in case of intolerance of beta-lactams (Lu *et al.,* 2021). *L. monocytogenes* is an opportunistic bacterium whereby some strains have been reported to possess virulence genes and pathogenicity. Virulence measures the pathogenicity of a microorganism and also takes into account virulence factors that incorporate the traits of the bacterial strain, which results in the risk/safety assessment (Mazaheri *et al.*, 2021). Furthermore, pathogenicity relies on the immune status of the host, the nature of the bacterial isolates, and the number of organisms in the exposure (Freitak *et al.*, 2007).

Previous studies have shown the capability of *Listeria monocytogenes* to form biofilm on a broad range of surfaces including stainless steel, polypropylene, glass, or rubber (Krysinski *et al.*, 1992; Ronner and Wong, 1993; Dygico *et al.*, 2020). *L. monocytogenes* colonize these surfaces producing monolayer biofilms, which later mature to pose a challenge in controlling the pathogenic bacteria (Cappitelli *et al.*, 2014; Gray *et al.*, 2018; Lee *et al.*, 2019). Furthermore, *L. monocytogenes* subtypes attach and colonize surfaces extensively and have increased antimicrobial resistance than the wild-type isolates. Numerous studies have agreed that *L. monocytogenes* can colonize diverse processing plant surfaces (Chavant *et al.*, 2002; Møretrø and Langsrud, 2004; Valderrama and Cutter, 2013; Truchado *et al.*, 2022). Due to the above reason, *L. monocytogenes* is considered one of the most problematic pathogenic bacteria posing serious food safety issues. Many countries have adopted a zero-tolerance policy toward *L. monocytogenes*

within their legislation, ensuring good hygienic standards in the food production industry (Warriner and Namvar, 2009; Farber *et al.*, 2021; Zhang *et al.*, 2021). In this current chapter, PCR-based technologies were utilized to determine the identity and virulence profiles of *L. monocytogenes*. Furthermore, antimicrobial resistance as well as biofilms were investigated from various food and water sources.

4.2 Materials and Methods

4.2.1 Purchasing of culture media and reagent

Culture media is the basis of most microbiological examinations. Without high-quality media, the ability to achieve accurate, and reproducible microbiological test results reduces. All culture media utilized in this study were obtained from Merck (Biolab, South Africa) and the reagents were purchased from Sigma–Aldrich (Kempton Park, South Africa).

4.2.2 Ethical clearance

In the present study, since animal faecal samples were used for microbiological analysis ethical clearance (NWU-00733-18-A9) was obtained from the Mafikeng Ethics Committee.

4.2.3 Area of samples collection

In the present study, samples were collected randomly throughout Northwest Province. Table 4.1 and Figure 4.1 indicate the different locations selected based on the various geological climatic conditions.

Table 4. 1: Geological location of sampling site and source of sampling.

Study Areas	**Geological coordinates**	**Sampling source**
Mafikeng	-25° 51' 54.79" S, 25° 38' 39.16" E	water, meat, vegetable, animal faeces
Zeerust	-25° 32' 13.02" S, 26° 04' 30.43" E	water, meat, vegetable, animal faeces
Rustenburg	25° 39' 16.0128" S, 27° 15' 21.0744" E	water, meat, vegetable, animal faeces
Lichtenburg	-26° 09' 7.20" S, 26° 09' 34.85" E	water, meat, vegetable, animal faeces
Vryburg	-26° 57' 23.72" S, 24° 43' 42.24" E	water, meat, vegetable, animal faeces
Potchefstroom	-26° 43' 0.01" S, 27° 06' 0.00" E	water, meat, vegetable, animal faeces

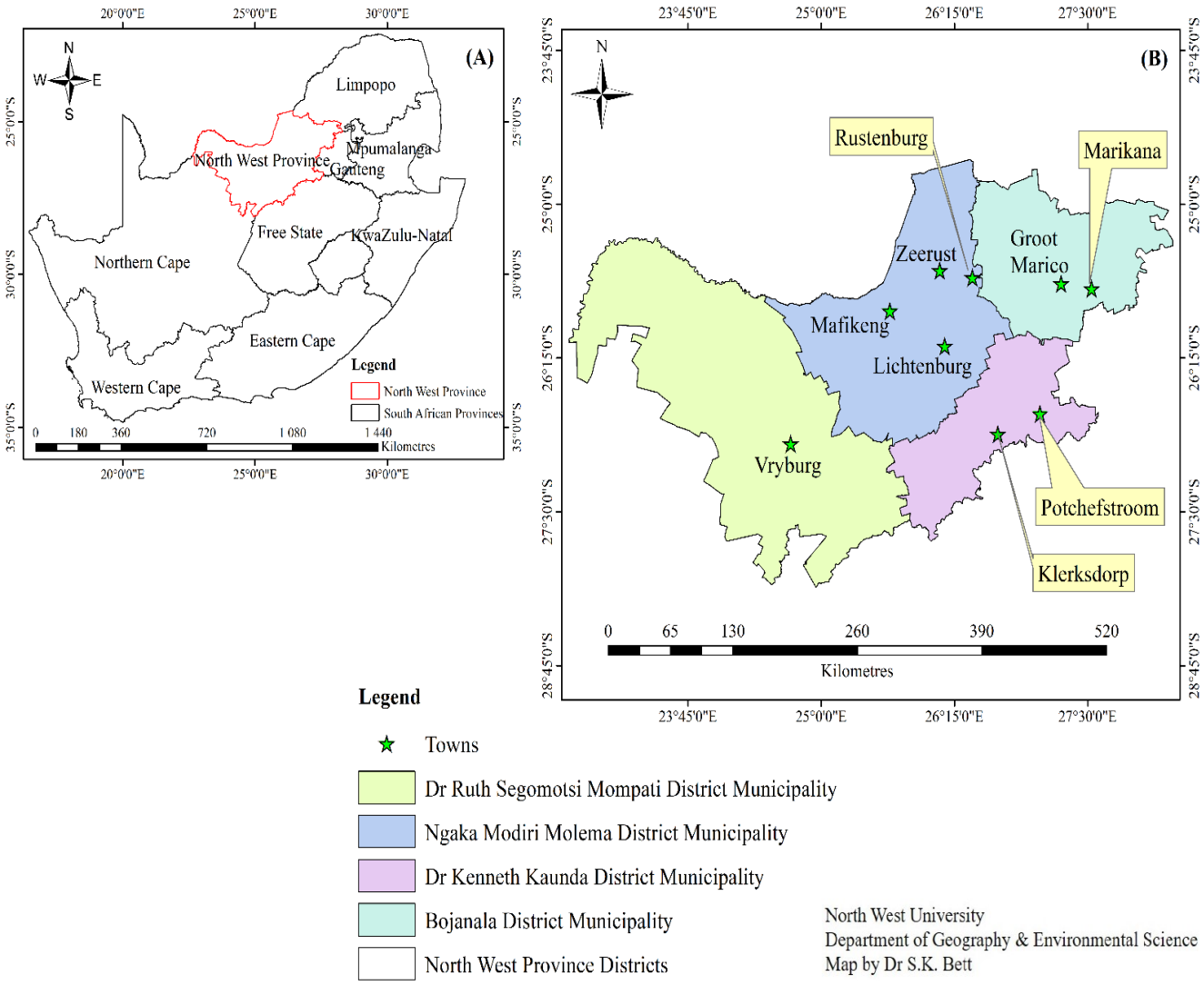


Figure 4.1: Location map showing the sampling area using the geolocation.

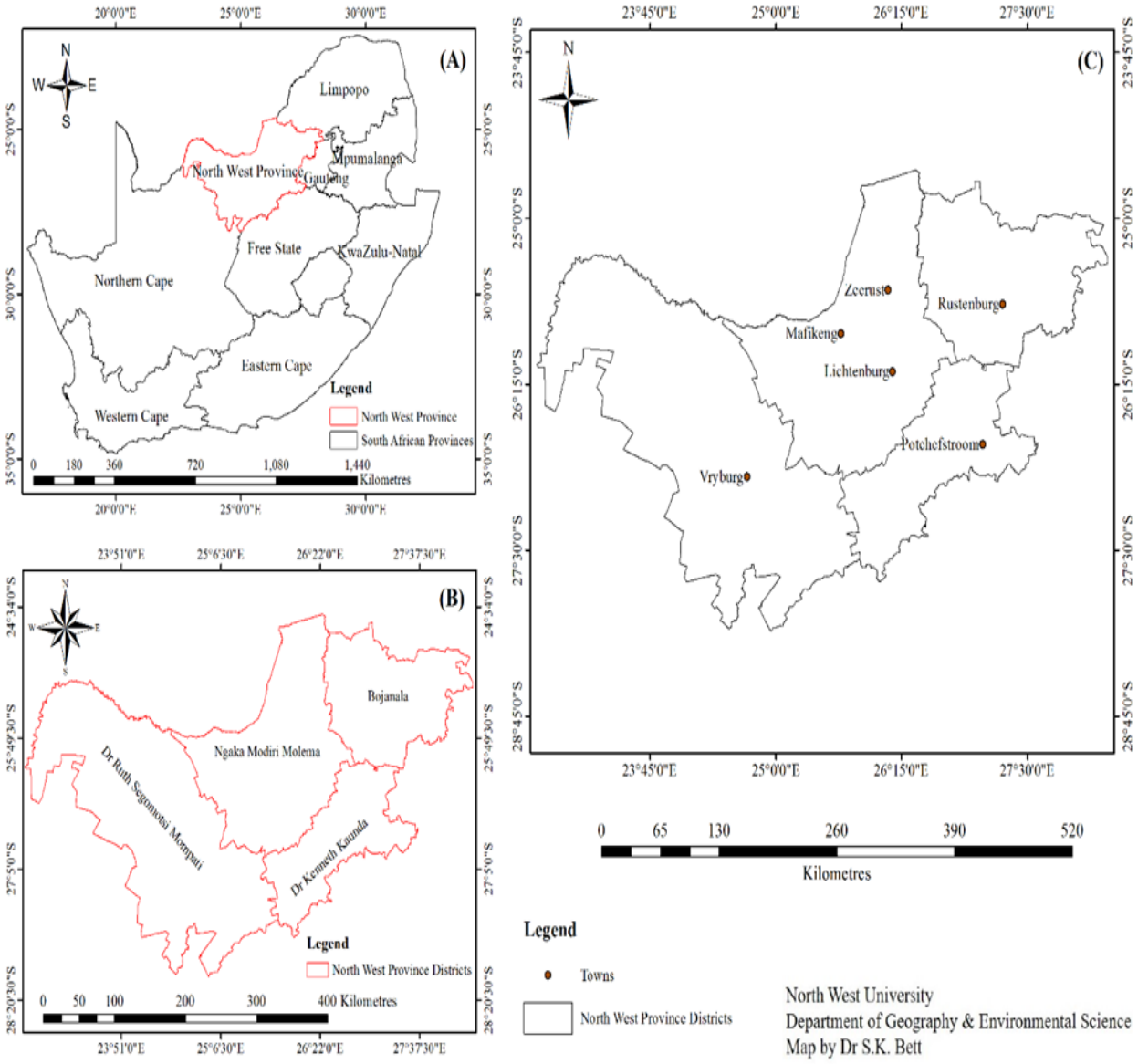


Figure 4.1: Location map showing the sampling area using the geolocation (Continued).

4.2.4 Sample collection

A total of 207 samples encompassing milk, vegetables, meat, water and faecal were collected aseptically from local markets, farms, rivers, and boreholes. All samples were stored at 4 °C in the cold room before analysis in the Department of Microbiology laboratory, Mafikeng, North West Province.

4.2.5 Isolation of *Listeria* species

Listeria spp. was isolated from milk, vegetables, meat, water, and faecal samples. Animal faecal samples were obtained from commercial farms in North-West province. Sterile arm-length gloves were used for the direct collection of faeces from the rectums of animals to avoid cross-contamination. The samples were transported immediately to the laboratory inside cooler boxes filled with ice at 4°C. Isolation of *Listeria* spp. was done as described by ISO 11290-1 (The International Organization of Standardizations (ISO, 1996) (ISO, 2004). Half Fraser broth (Neogen LAB 211, Acumedia, UK) was used as a primary selective enrichment medium. Ferric ammonium citrate (Acumedia, UK) was added as a supplement. Then, 25 mg food sample was added to 225 ml half Fraser broth (Neogen LAB 211, Acumedia, UK) in a stomacher bag and gently homogenized for two minutes and incubated at 30 °C for 24 h. Similarly, 25 ml milk was sampled, and the pH was adjusted to neutral and was thoroughly mixed with a 1:10 ratio to half Fraser broth and was incubated at 30 °C for 24 h. On the second day, about 0.1 ml of culture was transferred to a tube containing 10 ml of Fraser broth (enriched with acriflavin hydrochloride 0.025 g/l of distilled water and nalidixic acid 0.02 g/l of distilled water and ferric ammonium citrate). The inoculated medium was incubated at 37 °C for 48 h. After incubation, a loopful of inoculum was taken from the broth and streaked onto pre-dried sterile plates of Oxford agar (Neogen LAB 122, Acumedia, UK). Plates were incubated for 24 - 48 h at 37 °C and after incubation plates were examined for grey-green colonies with black background, typical for *Listeria* spp. From each plate randomly five typical presumptive colonies were taken and re-streaked on Oxford agar plates to purify the isolates.

4.2.6 Bacterial preservation

Presumptive isolates that satisfy the preliminary identification tests were stored for future use and to achieve this 1 mL of 20 % (v/v) glycerol was aliquoted into 1.5 µL sterile Eppendorf tubes. A single pure colony for each isolate was transferred into the tubes using sterile disposable plastic loops. The contents of the tubes were homogenized by vortexing and stored at -80 °C.

4.2.7 Control strain

Listeria monocytogenes ATCC 19111 was used as a quality control strain in the present study.

4.2.8 Identification by morphological and biochemical tests

The purified presumptive isolates were identified at the species level based on haemolytic patterns and biochemical analysis, as previously described protocol (Rorvik *et al*., 1995). Gram staining, motility, haemolysis, catalase, and CAMP tests were also performed. Rhamnose (AES Lab., Combourg, France), Xylose (AES Lab., Combourg, France) and Mannitol (Merck, Darmstadt, Germany) fermentation was evaluated, and positive reactions (acid formation) was indicated by a yellow colour within 24 to 48 h (ISO, 1996; OIE, 2008).

4.2.9 Molecular characterization of *Listeria* species

4.2.9.1 Genomic DNA extraction from presumptive isolates

Genomic DNA was extracted from all presumptive isolates using the Zymo Research Genomic DNA™ Tissue MiniPrep Kit supplied by Inqaba Biotec Industries (Pty) Ltd Pretoria, South Africa according to the manufacturer's instructions. The DNA samples were stored at -20°C for further molecular identification and characterization.

4.2.9.2 Determination of the concentration of DNA extracted

The quality and quantity of DNA extracted were determined using a UV visible spectrophotometer (model S-22, Boeco, Germany) at wavelengths of 260 nm and 280 nm. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of the DNA extracted (Sambrook *et al.*, 1989).

4.2.9.3 PCR amplification of 16S rRNA gene fragment

The 16S rRNA gene fragments are sections of prokaryotic DNA found in all bacteria and archaea that possess unique sequences in each bacterium (Song *et al.*, 2005). These sequences have high discriminatory powers making them very useful for the identification of different bacterial species. Bacterial 16S rRNA gene fragments were thus amplified as an internal control for all presumptive isolates. DNA samples from all presumptive isolates were used for amplification of 16S rRNA gene fragments in the isolates using the protocol of Korzeniewska and Harnisz (2013). The PCR reactions were performed using a pair of universal 16S rRNA oligonucleotide primers 27F: AGAGTTTGATCATGGCTCAG and 1492R: GGTACCTTGTTACGACTT (Lane, 1991). Amplifications were performed using a DNA thermal cycler (model- Bio-Rad C1000 Touch™ Thermal Cycler) obtained from Life Science Group, USA). The reactions were prepared in 25 µL volumes made up of 12.5 µL of 2X DreamTaq Green Master Mix (0.4mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4mM $MgCl_2$ and loading buffer), 0.25 µL of each primer, 1 µL of template DNA and nuclease-free sterile water. All the PCR reagents used in this study were obtained from Thermo Fisher Scientific Inc., USA and were supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. The following PCR conditions were used during the amplification of 16S rRNA genes: an initial denaturation at 94 °C for 3 minutes, followed by 25 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute and extension at 72

°C for 2 minutes. A final extension step was performed at 72 °C for 10 minutes. DNA from *L. monocytogenes* (ATCC 19111) was included in this experiment as a positive control.

4.2.9.4 Identification of *Listeria* species using PCR

The PCR reactions were performed using the primers *prs* and *Lmo0733.* Amplifications were performed using a DNA thermal cycler (model- Bio-Rad C1000 Touch^{TM} Thermal Cycler) obtained from Life Science Group, USA). The reactions were prepared in 25 µL volumes made up of 12.5 µL of 2X DreamTaq Green Master Mix (0.4mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4mM MgCl2 and loading buffer), 0.25 µL of each primer, 1 µL of template DNA and nuclease-free sterile water. All the PCR reagents used in this study were obtained from Thermo Fisher Scientific Inc., USA and were supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. The following PCR conditions were used during the amplification of *16S rRNA* genes: an initial denaturation at 94 °C for 3 minutes, followed by 25 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute and extension at 72 °C for 2 minutes. A final extension step was performed at 72 °C for 10 minutes. DNA from *L. monocytogenes* (ATCC 19111) was included in this experiment as a positive control. The identification of *Listeria* species was mainly based on species-specific primers (Table 4.2). The *prs* and the *Lmo0733* are respectively the genes for the identification of *Listeria* species and *Listeria monocytogenes.*

Table 4.2: Specific primers of *Listeria* species identification (Cao *et al.*, 2018).

Gene	Primers	Sequence (5'-3')	Amplicon size (bp)
prs	*prs*F	ACGCATGTTGTTCGCAC	101
	*prs*R	TGGAAGAGCGATGGAGTT	

Lmo0733	*Lmo0733*F *Lmo0733*R	TGTCCAGTTCCATTTTTAAC TTGTTGTTCTGCTGTACGA	416

4.2.10 Gel electrophoresis

All the PCR amplicons were resolved by electrophoresis on a 1% (w/v) agarose gel. A 1 kb molecular weight marker was included in each gel and was used to confirm the relative sizes of amplicons. A ChemiDoc Imaging System (Bio-RAD ChemiDocTM MP Imaging System, UK) was used to capture the images**.**

4.2.11 Antimicrobial Susceptibility of *Listeria monocytogenes*

The Kirby-Bauer disc diffusion technique was used to determine the antimicrobial susceptibility profile of the isolates (Bauer *et al*., 1966). A total of 12 antibiotic discs ordered from Mast Diagnostics, UK were used to determine the antimicrobial susceptibility profile of the isolates. Ampicillin (AMP), 10 µg; Erythromycin (E), 15 µg; Kanamycin (K), 30 µg; Norfloxacin (NOR), 10 µg; Novobiocin (NB), 30 µg; Tetracycline (TET), 30 µg; Oxacillin (OX), 5 µg; Clindamycin (DM), 2 µg; Nalidixic Acid (NA), 30µg; Gentamicin (GM), 15µg; Meropenem (MEM), 10 µg and Trimethoprim + Sulfamethoxazole (SXT), 1.25 + 23.75 µg. The selection of some of these antibiotic discs was done due to the fact that they are extensively used as prophylactics in both beef and dairy cattle farming in South Africa. Mueller Hinton plates were placed into an incubator aerobically for 24 h at 37 °C and the reading of the plates after 24 h was done following the Clinical Laboratory Standards Institute (CLSI, 2016). The result interpretations were based on the antibiotic growth inhibition zone diameter size compared with standard reference values in a way to classify the isolates as sensitive, intermediate or resistant to a precise antibiotic. For quality

control during the process of antimicrobial susceptibility test, *Listeria monocytogenes* ATCC 19115, and *Listeria ivanovii* ATCC 19119 were used as references.

4.2.12 Detection of Resistance Genes of *Listeria monocytogenes*

The isolates confirmed to be *Listeria* spp. that were presenting resistance to more than two antibiotics were considered as multidrug resistance and were tested on harboring of the *Erm (A)*, *Erm (B1)*, *Erm (B2)*, *Erm (C)*, *Erm (T)*, *BLATEM*, *Drf1*, *Drf5*, *Drf12*, *dfr1S*, *dfr5S*, *dfr7s*, *dfr12s*, *dfr17s*, *Tet A*, *Tet B*, *Ant (3'')*, *Sul1*, *Sul2* and *Pse1* antibiotics resistance genes (Tchatchouang *et al.*, 2022), listed in table 4.3. The primer sequences, PCR conditions and amplicon sizes are all grouped in table 4.3. The preparation of all PCR reactions was done within a mixture of 12.5 µL of 2X DreamTaq Green Master Mix, 11µL of RNasenuclease-free PCR water, 0.5µM of each primer (Forward and Reverse), and 1 µL of template DNA for a total of 25µL standard volume. DNA thermal cycler (model- Bio-Rad C1000 Touch^{TM} Thermal Cycler, Singapore) was used for amplification and all PCR products were well saved at 4°C prior to electrophoresis. All the PCR Amplicons were checked by electrophoresis on a 2% (w/v) comprising 0.001µg/mL ethidium bromide. A DNA marker (Fermentas, USA) with a size of 100 bp DNA molecular weight was incorporated in each gel as the measurement of the sizes of the amplicons and all the agarose gel were captured in images using a ChemiDoc Imaging System (Bio-Rad ChemiDoc^{TM} MP Imaging System, UK) including a Gene Snap software, version 6.0022.

Table 4.3: Resistance Genes of *Listeria* spp. tested in this study.

Target genes	Primer sequences (5'-3')	T°C	Amplicon Size (bp)
erm(A)	F: AAGCGGTAAACCCCTCTGAG	52	441
	R: TCAAAGCCTGTCGGAATTGG		
erm(B)	ermB1-F: CATTTAACGACGAAACTGGC	60	425
	ermB1-R: GGAACATCTGTGGTATGGCG		
	ermB2-F: GAAAAGGTACTCAACCAAATA	59	639
	ermB2-R: AGTAACGGTACTTAAATTGTTTAC		
erm(C)	F: ATCTTTGAAATCGGCTCAGG	49	295
	R: CAAACCCGTATTCCACGATT		
erm(T)	F: TATTATTGAGATTGGTTCAGGG	55	395
	R: GGATGAAAGTATTCTCTAGGGATTT		
blaTEM	F: TTC TTG AAG ACG AAA GGG C	60	1190
	R: ATG GTG AGT GGA ACG AAA AC		
dfr1	F: TGGTAGCTATATCGAAGAATGGAGT	52	425
	R: TATGTTAGAGGCGAAGTCTTGGGTA		
dfr5	F: AGCTACTCTTTAAAGCCTTGACGTA	55	341
	R: GTGTTGCTCAAAAACAACTTCG		
dfr12	F: GAGCTGAGATATACACTCTGGCACT	60	155
	R: GTACGGAATTACAGCTTGAATGGT		
dfr1s	F: ATGGAGTGCCAAAGGTGAAC	52	241
	R: TATCTCCCCACCACCTGAAA		
dfr5s	F: TCATTAATGGCTGCAAAAGC	55	460

	R: CCTTTTGCCAAATTTGATAGC		
dfr7s	F: TCTGCAACGTCAGAAAATGG	55	404
	R: TGCTCAAAAACCAAATTGAAA		
dfr12s	F: TTTATCTCGTTGCTGCGATG	60	457
	R: TAAACGGAGTGGGTGTACGG		
dfr17s	F: GAAAATATCATTGATTTCTGCAGTG	55	465
	R: TTTTTCCAAATCTGGTATGTATAATTT		

4.2.13 PCR amplification of virulence genes of *Listeria* species

Inqaba Biotechnical Industries Pty Ltd, Pretoria, South Africa synthesized the primers for the detection of Actin gene (*ActA*), p60 gene (*iap*), Phosphatidylinositol Phospholipase C gene (*PlcA, PlcB*), Internalin gene (*InlA, InlB, InlC, InlJ*), regulatory gene (*prfA*), and the Hemolysin gene (*hlyA*) The details of the primers are presented in Table 4.3. The methodology used in this study was in the literature (Furrer *et al.*, 1991; Notermans *et al.*, 1991; Paziak-Domańska *et al.*, 1999; Suárez *et al.*, 2001; Chico-Calero *et al.*, 2002) and the PCR was standardized for the finding of virulence linked genes namely *inlA, inlB, inlC, inlJ, actA, hlyA, plcA, plcB* and *iap* of *Listeria* spp. with proper adjustments. All the PCR products were held at 4 °C till electrophoresis was done for separation after being amplified using a DNA thermal cycler (model- Bio-Rad C1000 Touch™ Thermal Cycler, Hercules, California, USA). The preparation of the PCR reaction was done as 25µL standard volumes including 12.5 µL of 2X DreamTaq Green MasterMix, 0.5µM of each primer, 1 µL of template DNA and RNasenuclease-free PCR water. The *Listeria* species isolates were screened for the presence or absence of virulence-associated gene(s) by using the standard

PCR protocols for the detection of *actA, hly A, inlA, inlB, inlC* and *inlJ, iap, PrfA,* plcA and *plcB* genes as per the methodology described previously (Table 4.4) (Liu *et al.*, 2007; Xu *et al.*, 2009).

Table 4.4: Primers for the amplification of the virulence genes of *Listeria.*

Gene	**Primers**	**Sequence (5'-3')**	**Amplicon size (bp)**	**Reference**
inlA	*inlA*F	CCTAGCAGGTCTAACCGCAC	256	(Coroneo *et al.*, 2016)
	*inlA*R	TCGCTAATTTGGTTATGCCC		
inlB	*inlB*F	TGATGTTGATGGAACGGTAAT	272	Du *et al.*, (2017)
	*inlB*R	CTCGTGGAAGTTTGTAGATGC		
inlC	*inlC*R	AATTCCCACAGGACACAACC	517	Liu *et al.*, (2007)
	*inlC*F	CGGGAATGCAATTTTTCACTA		
inlj	*inlj*R	TGTAACCCCGCTTACACAGTT	238	Liu *et al.*, (2007)
	*inlj*F	AGCGGCTTGGCAGTCTAATA		
actA	*actA*F	CCAAGCGAGGTAAATACGGGA	650	Lomonaco *et al.*, (2012)
	*actA*R	GTCCGAAGCATTTACCTCTTC		
PrfA	*prfA*F	ACCAATGGGATCCACAAGA	467	Bubert *et al.*, (1999)
	*prfA*R	CAGCTGAGCTATGTGCGA		

hlyA	*hlyA*F *hlyA*R	ATCATCGACGGCAACCTCGGA GAC CACCATTCCCAAGCTAAACCA GTGC	404	Du *et al.*, (2017)
PlcA	*plcA*F *plcA*R	CTCGGACCATTGTAGTCATCTT CACTTTCAGGCGTATTAGAAAC GA	326	Lomonaco *et al.*, (2012)
plcB	*plcB*F *plc*BR	AATATTTCAATCAATCGGTGGC TGA GGGTAGTCCGCTTTCGCTCTT	289	Du *et al.*, (2017)
iap	*Iap*F *iap*R	ACAAGCTGCACCTGTTGCAG TGACAGCGTGTGTAGTAGCA	131	Kaur *et al.*, (2007)

4.2.14 Biofilm formation of *Listeria monocytogenes* associated with antimicrobial resistance

4.2.14.1 Microtiter plate 96 well assay

To be able to achieve the biofilm formation test of *Listeria* species a couple of reagents and materials were used such as phosphate buffer saline tablets (Merck, South Africa), sterile 96 well Eppendorf polystyrene flat-bottom microtitre plate (Lasec "Pretoria SA" (PTY) LTD), analytical grade absolute ethanol (95%) and Crystal violet (Merck, Cape town South Africa). The types *Listeria* used were isolated from food and water samples around North-West Province, South

Africa. *Listeria monocytogenes* ATCC 19115 and *Pseudomonas aeruginosa* (ATCC 27853) were used as a positive control, none inoculated media broth (negative control). To grow the biofilm, a microtiter plate assay was used as earlier described by Borucki *et al*. (2003) and Castelijn *et al*. (2013) with few modifications. The samples stored at -80°C as glycerol stocks were reconstituted, plated on tryptic soy agar added with 0.6% yeast extract (TSAYE) for 24 h at 37 °C and a single colony was inoculated into 15ml of tryptic soy broth supplement with 0.6% yeast extract (TSBYE) and incubate at 37 °C overnight. Approximately 10 µL of the overnight culture was used to inoculate 96-well plates containing 190 µL of Broth Heart Infusion (BHI) per well. Each isolate was loaded in a set of triplicates into the well. The first three wells consisted of the negative control comprising of the BHI and the two positive controls which were *Pseudomonas aeruginosa* (ATCC 27853) suspension (positive control; strong biofilm producer) and the second positive control (*Listeria monocytogenes* ATCC 19115) which was 190µL of BHI in the well and 10µL of the overnight culture all in triplicate. The microtiter plate 96-wells were incubated at 3 different temperatures (4, 25 and 37 °C) and each set of the 3 temperatures was incubated for 24 h, 48 h and 72 h. After the incubation period, the 96 well plates were rinsed three times with PBS (300µL, pH 7.4) to remove unattached planktonic microbial cells and excess PBS were removed by blotting the plate using a clean paper towel. Once the microtiter plate was dried, 200µL of crystal violet was added to the wells to stain the biofilm formed by the cells on the surface of the well. After one hour, the crystal violet was rinsed three times using 300µL of PBS and blotted for 30-60 minutes. Some 95% ethanol (200µL) was added to each well for fixation of the biofilm formed and was incubated at room temperature for an hour. The spectrophotometer was used to read the optical density (OD630nm). The cut-off value of the negative control was determined as three standard deviations (SD) above the mean OD of the negative control. As stipulated by Stepanovi´c *et al*.

(2000), biofilm formation was categorized into four groups according to the ODs obtained: OD ≤ ODc, non-adherent; ODc < OD ≤ 2 x ODc, weak biofilm formation; 2 x ODc < OD ≤ 4 x ODc, moderate biofilm formation; and 4 x ODc < OD, strong biofilm formation.

4.2.14.2 Genotypic identification of *luxS* and *flaA* genes in *Listeria* species by PCR

The polymerization chain reaction was utilized to detect the biofilm-forming genes of *Listeria* species The forward and reverse primers were designed by Inqaba Biotechnology (Pretoria South Africa). The primers were *luxS* (*lmo 1288*) with forward primer 5'GGA AAT GCC AGC GCT ACA CTC TTT 3', reverse primer 5'ATT GCA TGC AGG AACTTC TGT CGC 3' and *flaA* (*lmo 0689*) with and forward 5'GCG CAA GAA CGT TTA GCA TCT GGT 3' and reverse 5' TTG AGT AGC AGC ACC TGT AGC AGT 3' with the amplicons size of 208 and 363 bp, respectively (Warke *et al.*, 2017). The preparation of all PCR reactions was done within a mixture of 12.5 µL of 2X DreamTaq Green Master Mix, 11µL of RNasenuclease-free PCR water, 0.5µM of each primer (Forward and Reverse), and 1 µL of template DNA for a total of 25µL standard volume. The conditions of the PCR are stated in Table 4.5. DNA thermal cycler (model- Bio-Rad C1000 Touch^{TM} Thermal Cycler, Singapore) was used for amplification and all PCR products were well saved at 4 ℃ prior to electrophoresis.

Table 4.5: Primers for the amplification of the biofilm genes of *Listeria* species.

Biofilm genes			
GGA AAT GCC AGC GCT ACA CTC TTT	*luxS*	208	94°C for 2 min, 35 cycles,30 sec at 94°C, 30 sec at 58°C and 1 min, extension at 72°C and final extension of 7 min at 72°C
ATT GCA TGC AGG AACTTC TGT CGC			

GCG CAA GAA CGT TTA GCA TCT GGT	*FlaA*	363	94°C for 2 min, 35 cycles,30 sec at 94°C, 58°C for 1 min, extension at 72°C and final extension of 7 min at 72°C

4.3 Results

4.3.1 Isolation and Identification of *Listeria* spp.

In the present study, 207 samples (59 water, 104 foods, and 44 faecal samples) were collected in North-West Province and tested for the presence of *Listeria* species. Some 118 (57%) of the 207 samples were suspected to be positive for *Listeria* species. Morphological features and biochemical tests (catalase +, rod, gram +, beta hemolysis, H2S, gas, oxidase, methyl red +). *Listeria monocytogenes* hydrolyzes esculetin into esculetin, forming a black complex with iron (III) ions. Therefore, *Listeria monocytogenes* form brownish-green colonies with black halos on Oxford media. Of the 118 samples suspected to be positive for *Listeria* species, a total of 267 isolates with different morphotypes from different samples were obtained and their identities were determined through genus specific PCR. After successful DNA extraction, the bacterial 16S rRNA gene fragment was amplified from all isolates with a 1492 bp obtained. *Listeria*-specific genus primers (*prs*) were used to identify the sequence specific for *Listeria* in the isolates generating a 370 bp amplicon. Of the total of 267 isolates, 53 (19.85%) were positive for *Listeria*, comprising 14 (5.24%) from water samples, 36 (13.48%) from food samples, and 3 (1.12%) from faecal samples. In addition, a PCR-specific for the *Listeria monocytogenes Lmo737* gene was used to confirm the identities of *L. monocytogenes* (Table 4.6, Figure 4.2). Of the 53 (19.85%) positive *Listeria* spp., 25 (9.36%) *L. monocytogenes* were detected, and these comprised 14 (5.24%) from water samples and 11 (4.12%) from food samples (Figure 4.3) with an amplicon size of 691 bp.

Other species like *L. welshimeri*, *L. seeligeri* and *L. grayi* did not amplify. Sequence data of the bacterial 16S rRNA amplicons were subjected to BLAST search on the NCBI platform (as a nucleotide FASTA format) and the data were compared to those of previously deposited nucleotides in the NCBI library. In this study, 26 of the 53 nucleotide sequences were *Listeria monocytogenes*, 3 are *L. monocytogenes*, 2 are *L. welsimeri*, 1 is *Listeria tyrandesis*, and the rest (21) are uncultured *Listeria*. From the results of the nucleotide blast search, most of the sequences showed less than 95% homology in the database, and 8 showed more than 95% similarity. The sequence was clean and consistent with the 1998-2016 Chromas Lite software version 2.5.0.

Table 4.6: Different sources of *L. monocytogenes* isolates, and also the positive result for *prs* and *Imo737* genes.

Samples	**Presumptive positive samples to *Listeria* species**	**Number of presumptive *Listeria* species isolated**	**Positive isolates to *prs* genes specific for *Listeria* species**			**Positive isolates to *Listeria monocytogenes (lmo737)***		
207	118	267	Water	Food	Faecal	Water	Food	Faecal
			14	36	3	14	11	0
TOTAL			53			25		

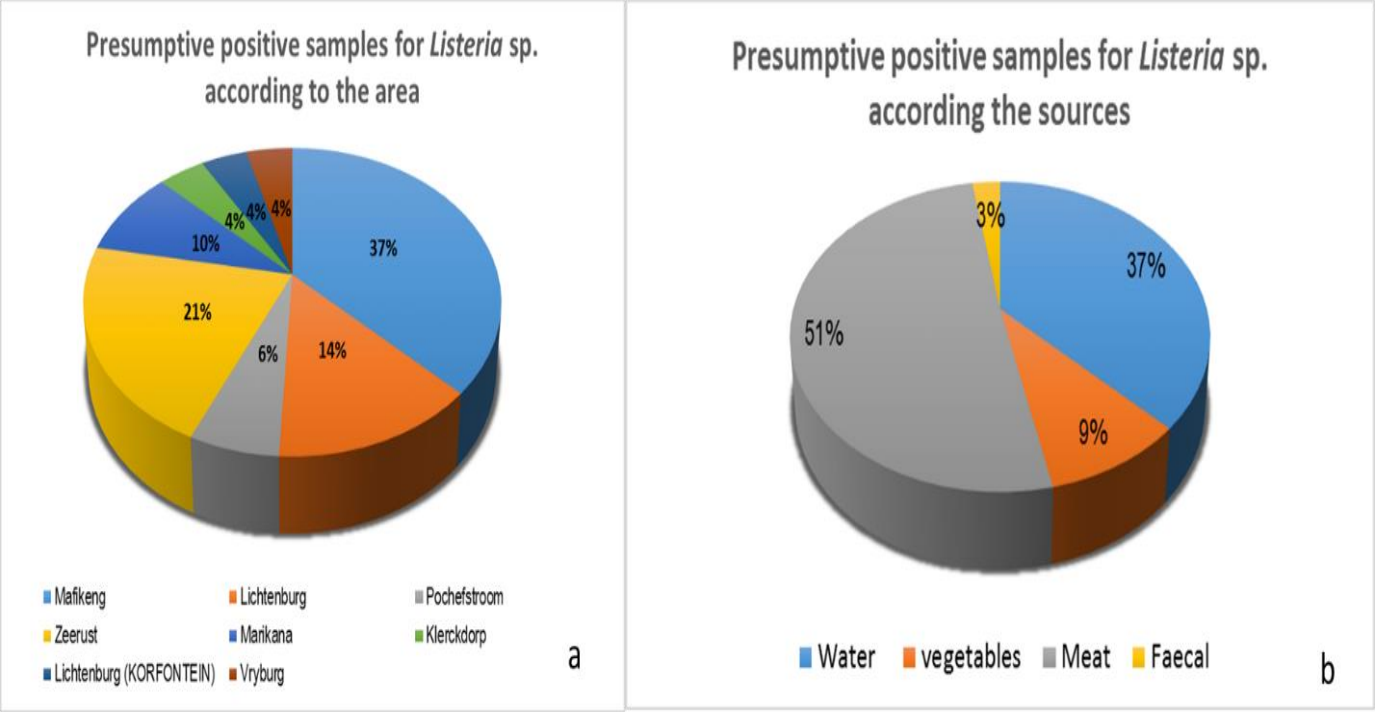


Figure 4.2: Presumptive positive for *Listeria* species according to the (a) area and the (b) source.

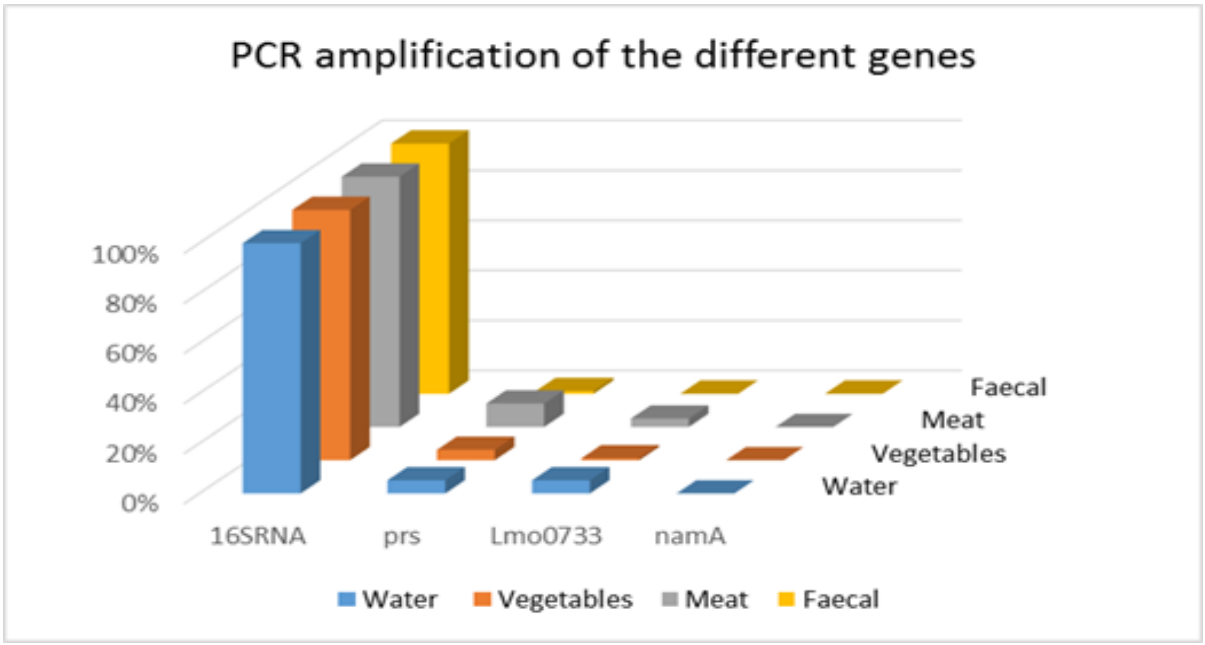


Figure 4.3: PCR amplification of different genes.

4.3.2 Antibiotic resistance

4.3.2.1 Antibiotic-resistance profile

Another main goal was to determine the antibiotic resistance of *Listeria monocytogenes* obtained from various samples and locations. Data generated from PCR identification showed that a total of 25 isolates were positive for *Listeria monocytogenes*. A total of 12 antibiotic discs were used for antibiotic resistance and 25 isolates of *Listeria monocytogenes* were screened with the *L. monocytogenes* control strain (ATCC 19115). Our results show that 66.67% to 100% of *Listeria monocytogenes* isolates are resistant to most antibiotics except ampicillin, trimethoprim + sulfamethoxazole, erythromycin, and norfloxacin. Norfloxacin was sensitive to all *L. monocytogenes* isolates except one isolate from minced meat obtained in Lichtenberg. All the *L. monocytogenes* isolates except for one isolate found in borehole water from Mahikeng were resistant to Kanamycin. A total of seven antibiotics (kanamycin, novobiocin, tetracycline, oxacillin, clindamycin, nalidixic acid, and gentamicin) proved to be the most resistant. Water samples showed *Listeria monocytogenes* isolates that were more resistant than food samples (Table 4.7).

Table 4.7: Antimicrobial resistance outline of *Listeria monocytogenes.*

Samples	Sampling Site	AP	TS	E	MEM	DA	GM	NOR	T	NOV	OX	NA	K
FOOD													
Meat	Lichtenburg N=4	25	0	50	25	100	0	100	100	100	100	100	100
Mince	Lichtenburg N=2	0	50	100	50	100	100	50	100	100	100	100	100
	Zeerust N=2	50	50	100	50	100	100	0	100	100	100	100	100
Cucumber	Zeerust N=2	0	50	50	50	100	100	0	100	100	100	100	100
Lettuces	Zeerust N=1	0	0	100	0	100	100	0	100	100	100	100	100
WATER													
Borehole	Lichtenburg N=3	33.33	33.33	100	100	100	100	100	100	100	100	100	100
		0	33.33	66.67	66.67	100	100	0	100	100	100	100	100
	Vryburg N=3	20	80	60	0	100	100	0	100	100	100	100	20

	Mafikeng N=5												
Dam	Mafikeng N=2	0	0	100	0	100	100	0	100	100	100	100	100
	Zeerust N=1	0	0	100	0	100	100	0	100	100	100	100	100

4.3.2.2 Multiple Antibiotic Resistant phenotypes of *Listeria monocytogenes*

The phenotypes of antibiotic resistance detected by *Listeria monocytogenes* and isolated from meat, vegetables and water are shown in Table 4.8. In the dam's water isolates, the MAR phenotypes EDAGMTNOOXNAK and EDAGMTNOOXNAK were 100% superior to Zeerust and Mafikeng, respectively. However, the MAR phenotypes TSDAGMTNOOXNAK, TSEDAGMTNOOXNA, APDAGMTNOOXNAK, TSEDATNOOXNAK, and TSEDAGMTNOOXNAK in Mafikeng well water were as low as 20%. In vegetables, the MAR phenotype EDAGMTNOOXNAK was 100% dominant in Zeerust. From the meat and minced meat sector, the MAR phenotypes EDAGMTNOOXNAK, TSEMEMDAGMNORTNOOXNAK, APTSEMEMDAGMTNOOXNAK and EDAGMTNOOXNAK were dominant in Lichtenberg and Zeerust. Nonetheless, MAR phenotypes MEMDAGMTNOOXNAK and APDAGMTNOOXNAK were inferior at 25% in Lichtenburg. Our MAR phenotypes data is displayed in detail in (Table 4.8).

Table 4.8: Multiple Antibiotic Resistant profile of *Listeria monocytogenes.*

Meat			
	Phenotype	Number of observed	Percentage
Lichtenburg (n=4)	E-DA-GM-T-NO-OX-NA-K	2	50%
	MEM-DA-GM-T-NO-OX-NA-K	1	25%
	AP-DA-GM-T-NO-OX-NA-K	1	25%
Minced meat			

Lichtenburg (n=2)	E-DA-GM-T-NO-OX-NA-K	1	50%
	TS-E-MEM-DA-GM-NOR-T-NO-OX-NA-K	1	50%
Zeerust (n=2)	AP-TS-E-MEM-DA-GM-T-NO-OX-NA-K	1	50%
	E-DA-GM-T-NO-OX-NA-K	1	50%
Cucumber			
Zeerust (n=2)	TS-E-MEM-DA-GM-T-NO-OX-NA-K	1	50%
	DA-GM-T-NO-OX-NA-K	1	50%
Lettuce			
Zeerust (n=1)	E-DA-GM-T-NO-OX-NA-K	1	100%
Water (Borehole)			
Lichtenburg (n=3)	AP-E-MEM-DA-GM-T-NO-OX-NA-K	1	33.33%
	TS-E-MEM-DA-GM-T-NO-OX-NA-K	1	33.33%
	E-MEM-DA-GM-T-NO-OX-NA-K	1	33.33%
Vryburg (n=3)	E-MEM-DA-GM-T-NO-OX-NA-K	1	33.33%
	TS-E-DA-GM-T-NO-OX-NA-K	1	33.33%
	MEM-DA-GM-T-NO-OX-NA-K	1	33.33%
Mafikeng (n=5)	TS-DA-GM-T-NO-OX-NA-K	1	20%

	TS-E-DA-GM-T-NO-OX-NA	1	20%
	AP-DA-GM-T-NO-OX-NA-K	1	20%
	TS-E-DA-T-NO-OX-NA-K	1	20%
	TS-E-DA-GM-T-NO-OX-NA-K	1	20%
Water (Dam)			
Zeerust (n=1)	E-DA-GM-T-NO-OX-NA-K	1	100%
Mafikeng (n=2)	E-DA-GM-T-NO-OX-NA-K	2	100%

4.3.3. Antibiotic resistance genes

All 25 *Listeria monocytogenes* isolates with PCR-specific genes were tested for antibiotic resistance. Of the 20 antibacterial genes, only 10 were amplified and no others were detected. The best detections were *ant (3'')* La 92% (23/25), *dfr1S* 84% (21/25), *Sul1* 80% (20/25), *erm (C)* 80% (20/25). The lowest was *tetA* and *tetB*, which was 8% (2/25) (Figure 4.4). Sample 4B was isolated from 25 *Listeria monocytogenes* isolates and carried 7 resistance genes from 10 amplified resistance genes. Isolates 39W, 43W, and 37W did not amplify the resistance gene but were also isolated from water.

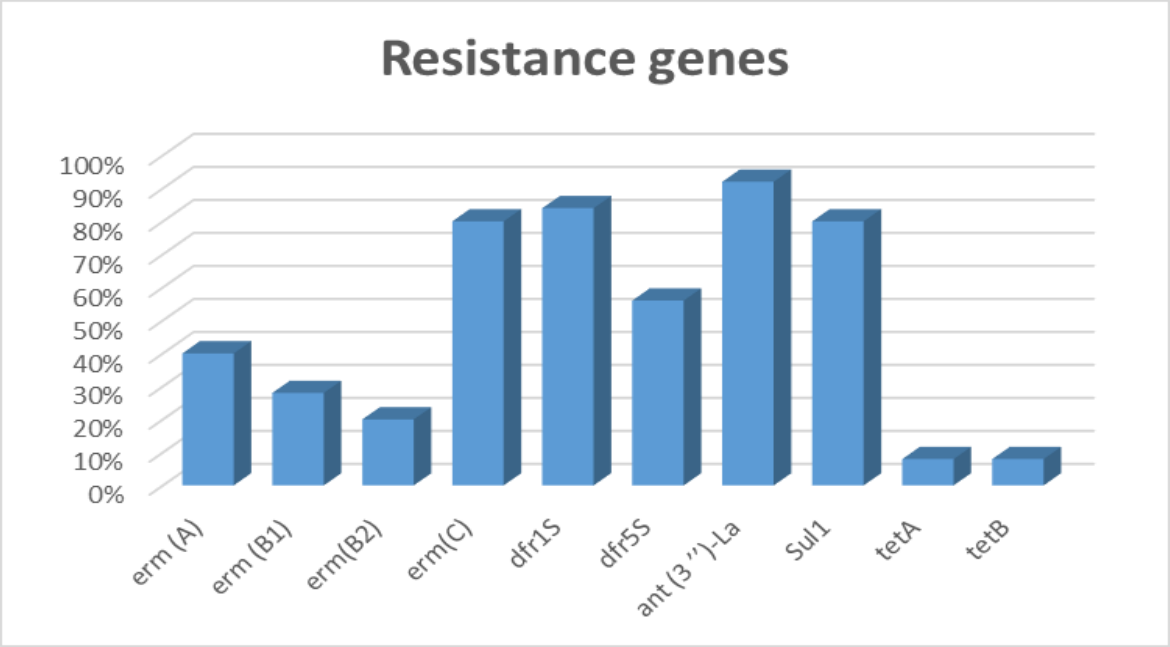


Figure 4.4: Detection of resistance gene of *Listeria monocytogenes*.

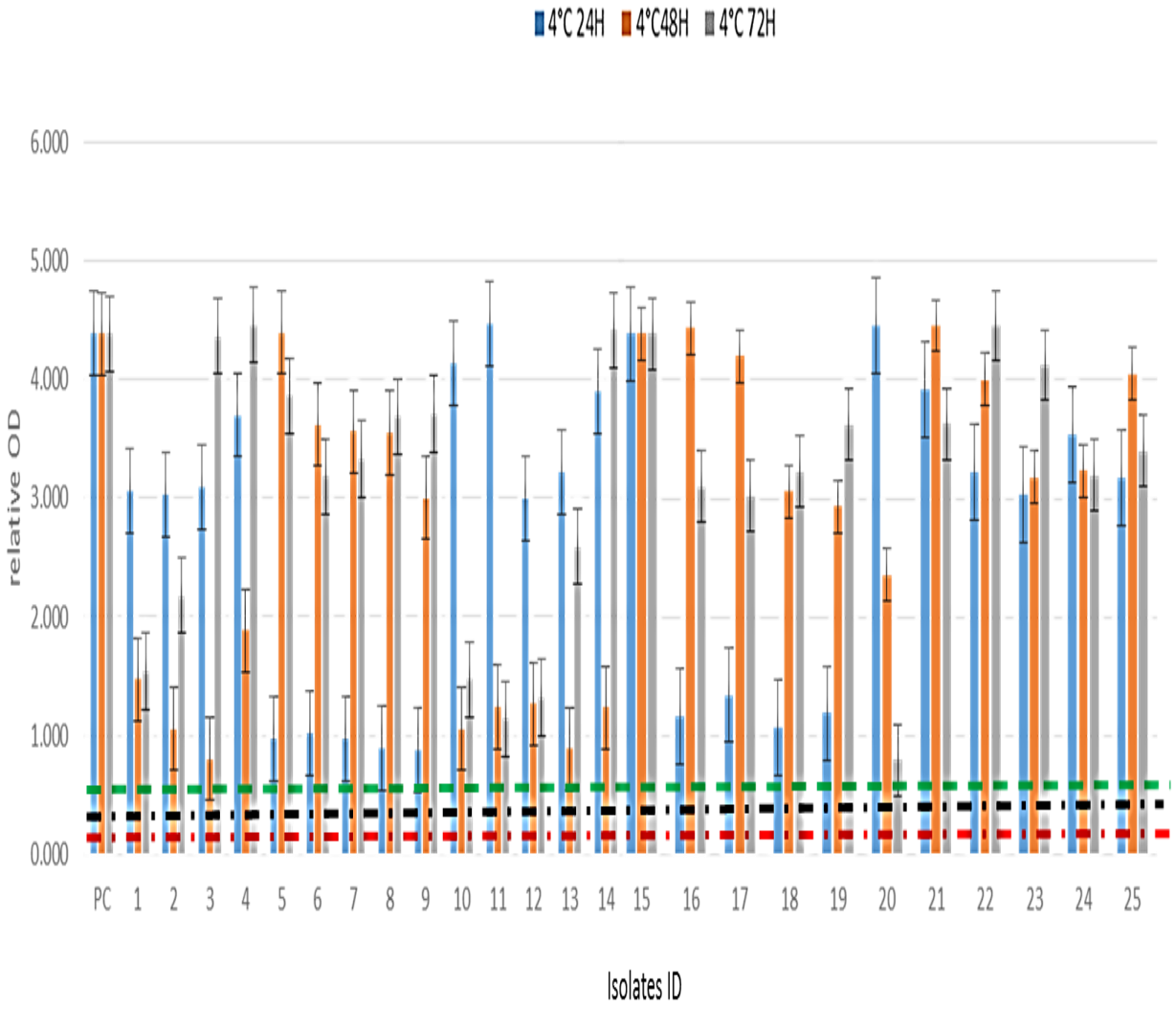
4.3.4 Virulence gene of *Listeria monocytogenes*

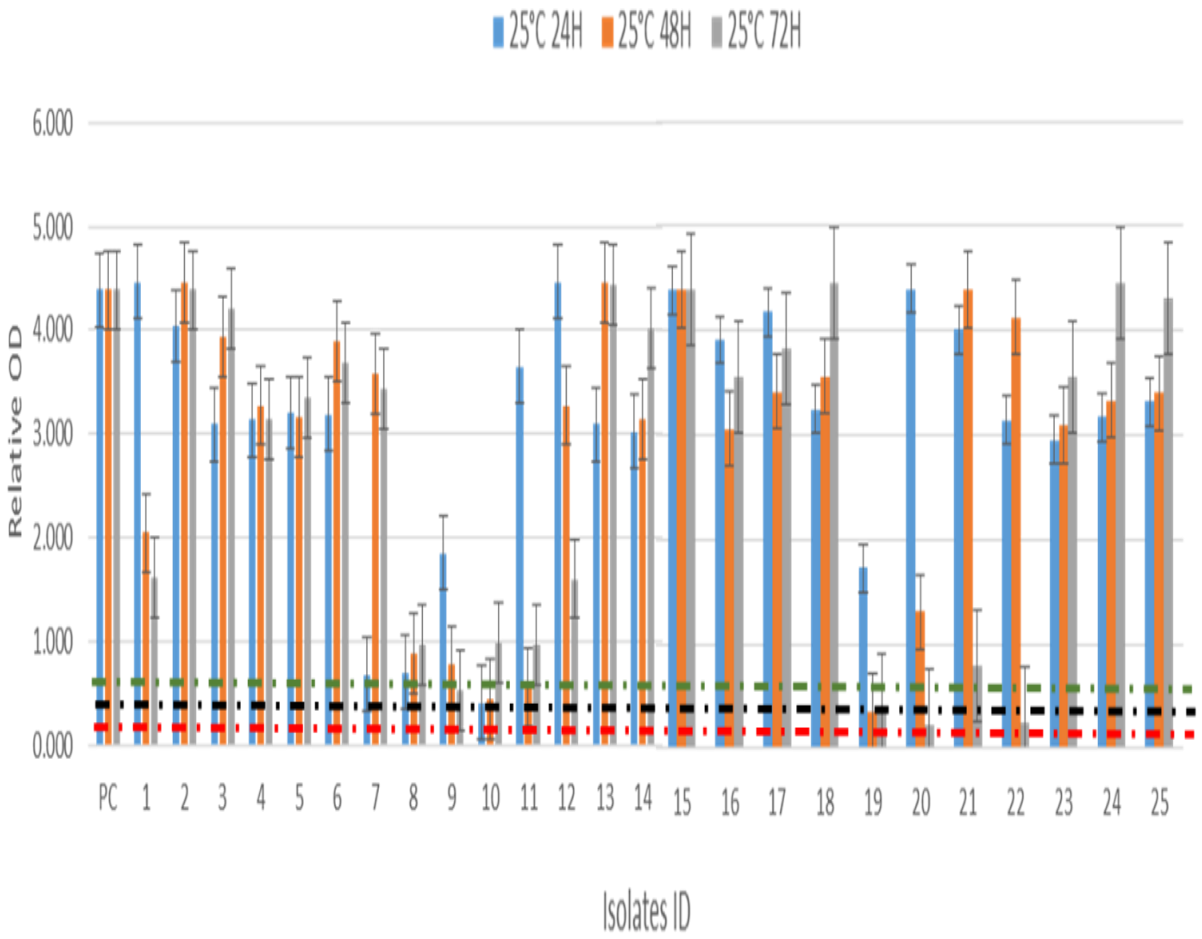
The gene *PrfA* had the highest prevalence detected in 25 out of 25 (100%) *L. monocytogenes* isolates followed by the *hlyA* gene in 17 out of 25 (68%) and the *actA* gene detected in 14 isolates (56%). The smallest proportion (20%) expressed the presence of *PlcB* genes in only 5 isolates. Out of 25 *Listeria monocytogenes* isolates, only 3 isolates (samples 4A, 7, 8) carried 8 virulence genes isolated from food samples. A total of 5 virulence genes (*actA, hlyA, inlC, inlJ, PrfA*) were reoccurring as a common trend in this study. Out of 25, 12 isolates amplified only one virulence gene, the *PrfA* virulence gene was detected in all isolates.

4.3.5 Biofilm formation

4.3.5.1 Biofilm formation

Biofilm formation was assessed by a microtiter plate assay at OD 630 nm using crystal violet staining and classified as non-adhesive, weak, moderate, or strongly adherent. In addition, the experiments were performed within 72 h at various temperatures of 4, 25, and 37 °C. All *Listeria monocytogenes* isolates produced biofilms on the surface of the microtiter. Biofilm formation was terrible between 24 and 48 h. In the current study, a microtiter plate assay was used to determine the biofilm volume of the *Listeria monocytogenes* isolate. *Listeria monocytogenes* isolates were able to form biofilms on the surface of polystyrene plates at different temperatures. Our data showed that the strongest biofilm adhesions were observed at 4 °C, at 24 h, 48 h, and 72 h at 4 °C for all isolates (n = 25; 100%). At 25 °C, considered room temperature, a total of 3 (12%) *Listeria monocytogenes* isolates produced weak biofilms after 72 h. Medium biofilm was formed from 4 isolates (16%) after 72 h. Strong biofilm formation was demonstrated by 18 (72%) isolates after 24 h, 48 h and incubation time. At 37 °C, biofilm formation was strong after 24 h of incubation and decreased after 72 h ($P < 0.05$), as shown in Figure 4.5.


4°C 24H
4°C 48H
4°C 72H
6.000
5.000
4.000
3.000
2.000
1.000
0.000
relative OD
PC 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Isolates ID


25°C 24H
25°C 48H
25°C 72H
6.000
5.000
4.000
3.000
2.000
1.000
0.000
Relative OD
PC 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Isolates ID

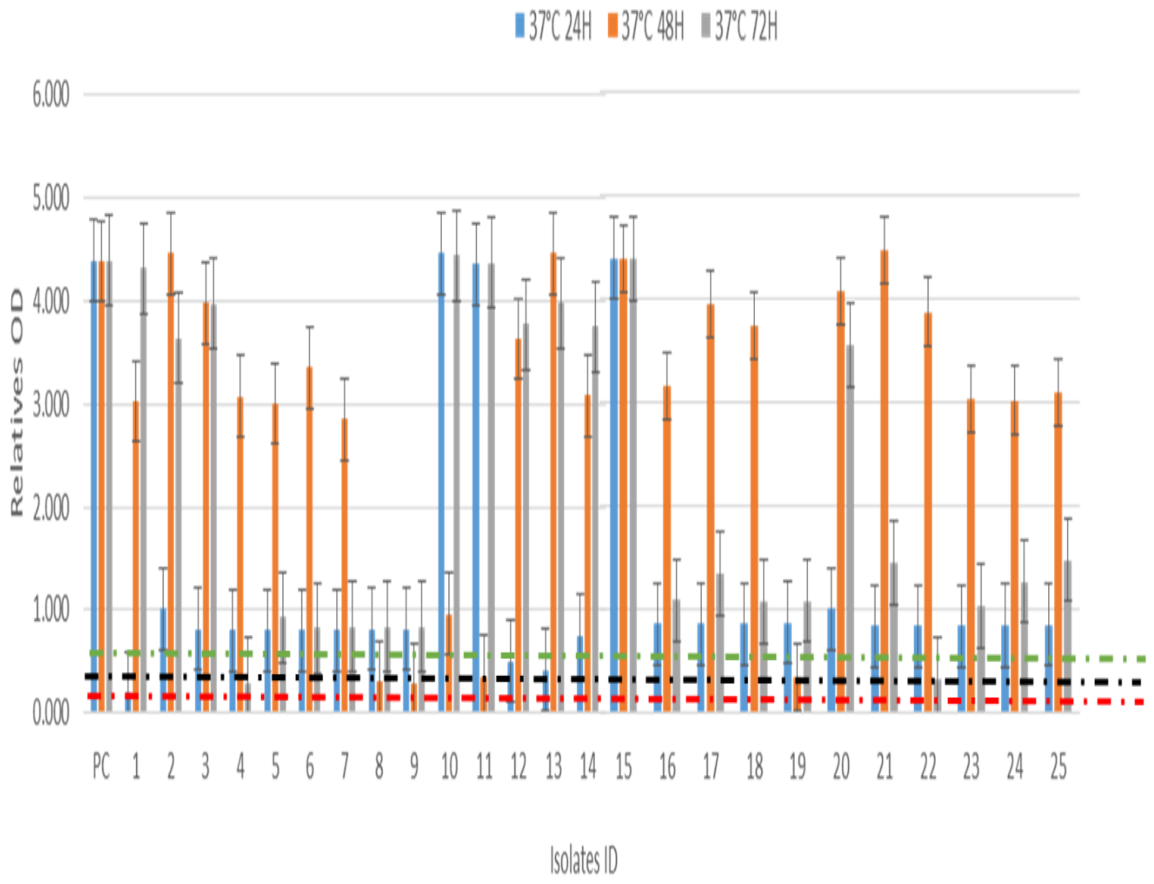


Figure 4.5: Biofilm formation of *L. monocytogenes* isolates on 96-well microtiter plate at different temperatures

4.3.5.2 Biofilm Formation Genes

PCR was used to determine the presence of the biofilm gene. Of the 25 isolates identified by PCR as *Listeria monocytogenes*, 14 were positive for luxS (56%) and 18 (72%) were positive for *flaA*. A total of 9 (36%) isolates from food samples contained the *luxS gene*, followed by 5 (20%) isolates containing the *FlaA* gene from water and food samples. A total of 9 isolates (5 from food samples and 4 from water samples) were simultaneously positive for the *flaA* and *luxS* genes. Figure 4.6 shows the agarose gel electrophoresis for the detection of amplified *luxS* gene and *FlaA* gene bands.

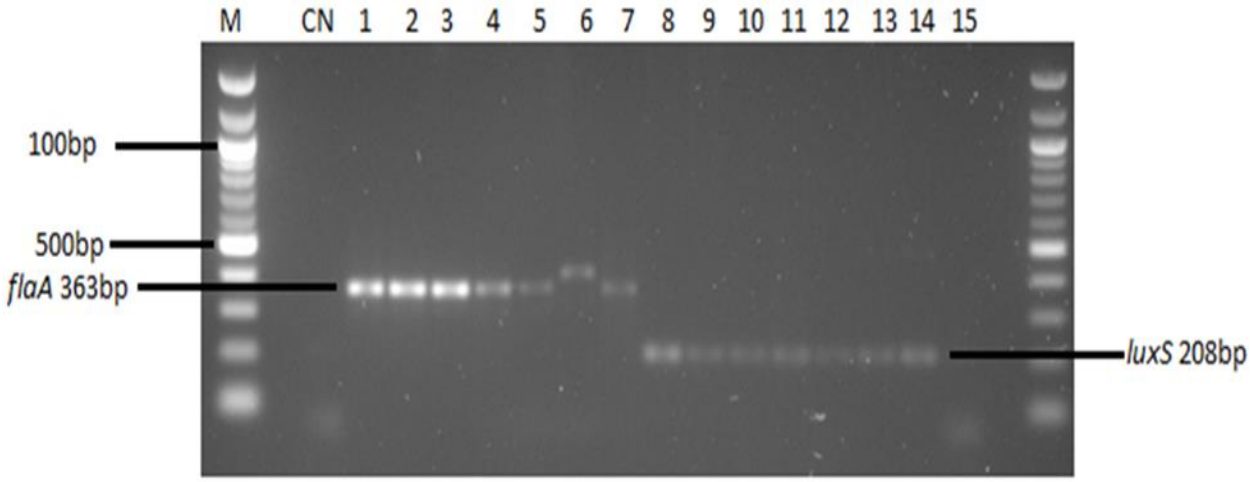


Figure 4.6: Biofilm formation genes amplification of *L. monocytogenes* isolates using PCR. Lane1, DNA marker100 bp. Lane 2, Negative control. Lane 3-9, Positive amplification of *flaA* gene. Lane 10-15, Positive amplification of *luxS* gene

4.4 Discussion

In this study, the main purpose was to confirm the presence of *Listeria monocytogenes*. From various sources that are related to the food chain, that is, infecting humans and resistance to antibiotics. This study was inspired by the 2017 outbreak of listeriosis in South Africa. In the current study, a total of 267 isolates were obtained from a variety of sources and their identity was confirmed using PCR-based techniques. Of the 267 isolates, 53 (19.85%) were positive for *Listeria*, of which 14 (5.24%) were water samples, 36 (13.48%) were food samples, and 3 (1.12%) were faecal samples. 53 (n = 53; 19.85%) are positive for the genus *Listeria*, 25 (9.36%) are identified as *Listeria monocytogenes*, 14 (5.24%) from water samples and 11 (4.12%) from food samples. In addition, three were *L. monocytogenes*, two were *L. welshimeri*, one was *Listeria thailandesis*, and the rest (21) were unidentified *Listeria* species. In another study by Odjadjare and Okoh (2010), wastewater from a South African sewage treatment plant was collected and a total of 48 samples were *Listeria*-positive species. A total of 17 isolates

(30%) were *Listeria monocytogenes* containing *L. ivanovii*, consisting of 11 (19.6%) and 1 (1.8%), was *Listeria monocytogenes*. *L. grayi* was 4 (7.14%), and *L. innocua* was 1 (1.8%). Due to the seriousness of this outbreak, several studies have been conducted on various sources to detect *Listeria* species (Matle *et al.*, 2019; Matle *et al.*, 2020; Rip and Gouws, 2020 Makumbe *et al.*, 2021).

Many studies have been reported on the prevalence and incidence of *Listeria monocytogenes* in meat, meat products, water, and vegetables. These products typically require multiple treatment steps before consumption and may be contaminated with *Listeria monocytogenes* at the post-treatment stage, indicating poor hygiene practices in retail food processing. Listeriosis is one of the most problematic food-borne diseases in some countries of the world and causes serious outbreaks at all stages of the food manufacturing process (Zhu *et al.*, 2017a; Desai *et al.*, 2019; Muchaamba *et al.*, 2019; Lüth *et al.*, 2020). *Listeria monocytogenes* can grow in both raw and cooked meat at refrigerated temperatures (Saraiva *et al.*, 2018; Yehia *et al.*, 2020). An early study by Yücel *et al.* (2005) reported the prevalence of *L. monocytogenes* in meat samples. Of the 146 samples, 70 (54.10%) were positive for *L. monocytogenes*. It was detected in minced meat *Listeria* had the highest incidence (86.4%). In addition, *Listeria monocytogenes* was isolated from 9 of 79 samples (6.16%). Further investigation revealed other species, 68 samples (46.57%) of *L. innocua*, 1 sample (0.68%) of *L. welshimeri*, 1 sample (0.68%) identified as *L. murrayi*. *Listeria monocytogenes* was the most frequently isolated species in various meat samples. Our results show a similar prevalence, with 57% reported in the current study.

Our results show that the majority of *Listeria monocytogenes* isolates are resistant to most antibiotics (66.67 to 100%), except for ampicillin, trimethoprim + sulfamethoxazole,

erythromycin, and norfloxacin. It was shown in another study that chloramphenicol showed the highest effect on the *Listeria monocytogenes* isolate with the lowest resistance (3.70%), and nalidixic acid showed the lowest effect with 90.74% resistance. Multiple antibiotic resistance patterns were most prominent with nalidixic acid/cloxacillin (35.2%), nalidixic acid/colistin (31.5%), and cloxacillin/colistin/nalidixic acid (29.6%). However, the lowest dose was 5.6% for chloramphenicol/nitrofuran/cotrimoxazole (Enurah *et al.*, 2013), which is inconsistent with our findings. Heidarzadeh *et al.* (2021) found that contrary to our results, *tetM* was detected at a high rate of 70% of tetracycline-resistant isolates. About 27.27% of isolates have *dfrD*. The *ermB* gene was detected in 83.33% of erythromycin-resistant isolates (Heidarzadeh *et al.*, 2021). It is said to be drug-resistant to *L. monocytogenes* and is rarely observed. Nevertheless, over the last two decades, resistance to one or more common antibiotics found in both clinical and environmental sources have increased. In general, resistance genes are inherited by transmissible plasmids and conjugate transposons. This is no exception to *Listeria monocytogenes*, which is resistant to antibiotics. In this study, the MAR phenotype EDAGMTNOOXNAK occurred in three different locations in different samples. This may be due to the use of antibiotics in this area. Continued abuse and improper use of antibiotics lead to the development of antibiotic resistance, especially multidrug-resistant (MDR) strains (Morvan *et al.*, 2010; Makhubalo *et al.*, 2016; Tshitshi *et al.*, 2020). Our findings showed multiple resistance to water and food samples, even though studies could not prove a direct transmission route for resistant *Listeria monocytogenes*.

Another purpose of this study was to determine the pathogenic genes for *Listeria monocytogenes*. All isolates amplified the *prfA* gene (100%), followed by the *hlyA* gene in 17 of 25 (68%) and the *actA* gene in 14 isolates (56%). However, the *plcB* gene was detected in 5

isolates. In contrast to our results, Du *et al.* (2017), reported that the *plcB* gene was amplified in all isolates and *inlB*, *actA*, *plcA*, and *iap* were amplified in 71.4-90.5% of isolates. The *prfA* gene encodes the protein that transcribes the listeriolysin gene (*lisA*). This underscores the importance of the *prfA* gene in the pathology of *Listeria monocytogenes*. In addition, *Listeria monocytogenes PrfA* had two important components: response to environmental stress conditions and its ability to infect the host (Gaballa *et al*., 2019). This may explain the fact that pathogenic genes (*actA*, *hlyA*, *inlC*, *inlJ*, *prfA*) are retained as a general trend in this study. However, *prfA* has been isolated from food, water, and human clinical samples (López *et al.*, 2013; Soni *et al.*, 2013; Kanki *et al.*, 2015) and has been described by many researchers.

We investigated the biofilm formation of 25 strains of *L. monocytogenes* isolated from various sources such as food (meat, minced meat, lettuce and cucumber) and water (boreholes and dams). *Listeria monocytogenes* can grow over a wide temperature range (245 °C). For this reason, three different temperatures are evaluated: 37 °C (corresponding to the optimum growth temperature range of 37 °C), 25 °C (representing room temperature), and 4 °C (representing refrigeration temperature) (George *et al.*, 1988; Lado *et al.*, 2007; Tchatchouang *et al.*, 2020). Our results show that strong biofilm adherence was formed in all isolates (n = 25; 100%) at 24, 48, and 72 h at 4 °C, and after 72 h, 4 isolates (16%) show that a moderate biofilm was formed from 3 (12%) of *Listeria monocytogenes* isolates at 72 h with a weak biofilm. In addition, PCR-based biofilm genes showed that 18 of 25 (72%) were amplified with *flaA*, followed by 14 (56%) with *luxS*. A total of 9 (36%) isolates from food samples contained the *luxS* gene, followed by 5 (20%) isolates containing the *flaA* gene from water and food samples. Similar results were reported by Lee *et al.* (2019).

A total of 58 *Listeria monocytogenes* isolates produced strong biofilm communities at 37 °C and 10 °C. The *Listeria monocytogenes* isolate used in this study was obtained from food sources and food processing equipment. In addition, stress tolerance studies such as the effects of salt and temperature were performed on *Listeria monocytogenes* isolates (Lee *et al.*, 2019). Recent studies have shown that *Listeria monocytogenes* produced biofilms at optimal neutral pH and 37 °C, with supplemental data showing that temperature is important for *Listeria monocytogenes* biofilm formation. It was concluded that the effects of various factors on biofilm production depend on the load (Fan *et al.*, 2020). In Egypt, especially in the city of Sohag, 295 (n = 295) ice cream samples were collected from aborted women. Vaginal swabs, stool and urine were collected to examine the pathogenicity of *Listeria monocytogenes*. Biofilm studies were performed by PCR detection of biofilm genes such as *LuxS* and *flaA*. The *LuxS* gene was detected in 81.8%, followed by *flaA* in 72.7% of ice cream. In aborted females, the *LuxS* gene was amplified by 81.8%, followed by *flaA* (63.6%). This contradicts our results (Hassanien and Shaker, 2021). In another study by Warke *et al.* (2017) evaluating the potential of *Listeria monocytogenes* biofilms, 23 of 38 (60.52%) were amplified for the *luxS* gene and 1 (2.63%) were positive for the *flaA* gene (Warke *et al.*, 2017).

For the past 20 years, researchers have shown the effectiveness of biofilms in the food industry. Microbial communities produced on abiotic or biological surfaces trapped in extracellular polymer substances (EPS) known as biofilms have been shown to increase microbial resistance. *Listeria monocytogenes* is no exception, as these biofilms are due to mutual contamination and can protect and contain pathogenic or perishable bacteria (Djordjevic *et al.*, 2002). In the presence of biofilm formation, it is more resistant to antibiotics than planktonic cells

(Manganyi *et al.*, 2015; Maje *et al.*, 2020). Therefore, it presents a problem with the control of *Listeria monocytogenes* from food sources, water and food processing surfaces and equipment.

4.5 Conclusion

In summary, our study has shown that *Listeria monocytogenes* has become a problematic food-borne pathogen worldwide. In South Africa, the outbreak of food-derived *Listeria monocytogenes* had serious consequences of high mortality and enormous economic losses. Our findings show *L. monocytogenes* has a pathogenic gene and is resistant to several antibiotics. In addition, further investigation showed strong biofilm formation by the *Listeria monocytogenes* strain. This clearly shows that *Listeria monocytogenes* has all the properties that contribute to pathogenicity, but it is difficult to control because of its resistance. Detailed studies are recommended to demonstrate the prevalence, pathogenicity, antibacterial properties, and biofilm formation of *Listeria monocytogenes* in South African meat products, water, and vegetables. Current precautions are inadequate to prevent such serious outbreaks. Therefore, effective alternatives to control and prevent resistant pathogenic *Listeria monocytogenes* are needed to reduce the risk of listeriosis.

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CHAPTER FIVE

WHOLE-GENOME SEQUENCE PROFILING OF *LISTERIA* SPECIES FROM DIFFERENT SOURCES: GENETIC DIVERSITY AND PUBLIC HEALTH SIGNIFICANCE

Abstract

Humans require nutrients to help drive cellular processes and thus ensure survival. However, consuming food that is contaminated with pathogenic microorganisms could lead to disease and even death in individuals with symptoms of diarrhoea, headache, vomiting, nausea, and abdominal cramps are reported as notable complications of listeriosis. Foodborne outbreaks have become an issue of severe public health concern owing to increased globalization and active food trade among countries. In addition, the frequent and increasing incidence of foodborne disease outbreaks associated with antimicrobial-resistant (AMR) bacterial pathogens remains a serious public health concern. Moreover, data generated during the South African foodborne outbreak caused by AMR *Listeria monocytogenes*, resulted in the inclusion of listeriosis into the South African list of mandatory notifiable medical conditions. Future solutions in managing the increasing threat caused by AMR foodborne pathogens to humans, require frequent surveillance of food products using techniques with high throughput and discriminatory potentials. This is aimed at ensuring that clinically significant virulent and AMR pathogens as well as their associated signature fingerprints in the food chain are quickly established to prevent local, multistate, and multinational foodborne outbreaks. Thus, this study assessed the pathotype, virulome and resistome of *Listeria* species previously isolated from different sources in North-West Province South Africa using whole genome sequencing

(WGS). Two multidrug-resistant *Listeria* species (LIN_NWU_CNKT and LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000)) were isolated from food and water samples respectively. Based on WGS analysis, the isolates were confirmed as *Listeria innocua* with genomes that are closely related to previously isolated human pathogens. The genomes of these two isolates harboured virulent genes such as those responsible for adherence, invasion, and toxin production. In addition, the genomes carried antibiotic-resistant genes, which confer resistance to drugs such as cephalosporin, tetracycline, phenicol, macrolide, rifamycin, carbapenem, fluoroquinolone, peptide, and aminoglycoside. Furthermore, mobile genetic elements were detected in their genomes. These findings highlight a crucial need to enforce standard operating procedures in food processing to reduce the spread of AMR and foodborne outbreaks.

5.1 Introduction

The constant increase in human population continues to pose significant challenges to access to safe, nutritious, and healthy food. The sustainable development goals, particularly goal 3: good health and well-being for all presents a view that food safety is a critical element to improve and strengthen global health, and ensure sustainable development (Founou *et al.*, 2021). Moreover, the desire to increase production to meet demand strains natural resources and introduces pollution of the environment thus affecting food safety and food security (Tilman *et al.,* 2011). Against this background, attaining an efficient, and resilient food system is highly required for effective management of food security (FAO, 2017). This highly coordinated approach requires the involvement of decision-makers and all stakeholders and adherence to effective and efficient measures on farms, factories, slaughterhouses, supermarkets, street vendors and kitchens (WHO, 2015). In addition, the need for urgent

identification and tracking of foodborne pathogens in the food chain especially during outbreak situations is crucial. Traditional bacterial identification and typing methods including serotyping, antibiotic and phage typing are primarily based on biochemical characteristics thus affecting their sensitivity and specificity (Sloan *et al.,* 2017). To address these challenges, recent years have seen the development and use of more reliable molecular-based tools that provide timeous, accurate and highly effective data for the identification and tracking of bacteria contaminants in the food chain (Zhao *et al.,* 2014; Zhao *et al.,* 2016). These typing tools also provide data that are very useful and epidemiologically important.

Amongst the molecular typing methods, whole genome sequencing (WGS) has revolutionized the field of genomics, enabling researchers to unravel the intricate genetic makeup of microorganisms and gain invaluable insights into their biology and evolution (Fuentes-Pardo and Ruzzante, 2017). With WGS, it is possible to delve into the genomic composition of pathogens thus uncovering vital information regarding their ecology, transmission and adaptability (Li *et al.*, 2021). WGS offers a comprehensive analysis of the entire genetic content of an organism, unveiling the complete sequence of its genome, including genes, regulatory elements, and other structural features (Fuentes-Pardo and Ruzzante, 2017).

Listeria species, especially *Listeria monocytogenes*, possess multiple stress response mechanisms that enable them to overcome varying environmental conditions such as temperature, osmotic pressures and pH thus they can easily survive within different stages of the food chain (Berrang *et al.,* 2000; Milillo *et al.,* 2012; Saldivar *et al.,* 2018). Moreover, the potential of *Listeria* species in *L. monocytogenes* to present severe and challenging pathological conditions in the elderly, immunocompromised individuals, and pregnant women (Ryser and Marth, 2007; Milillo *et al.,* 2012; Silk *et al.,* 2013) amplifies its status as a notorious foodborne

pathogen. Although *Listeria* species do not occur in the approved WHO global priority pathogens list, the clinical significance of *Listeria* species in South Africa is enhanced by the recent occurrence of the World's most severe listeriosis outbreak (Thomas *et al.,* 2020). Therefore, investigating the genetic foundations of virulence and antimicrobial resistance among *Listeria* species is crucial to safeguarding public health (Shoai-Tehrani *et al.,* 2019). Through the application of WGS to *Listeria* species, researchers can gain a deeper understanding of the genetic diversity and functional elements within this species, providing insights into its potential as a pathogen (Gwida *et al.,* 2020; Li *et al.,* 2021). WGS analysis of *Listeria* species has generated information on evolutionary relationships between different strains from similar or different populations (Gwida *et al.,* 2020). These evolutionary trends are achieved through comparative genomics approaches, thus making it possible for single nucleotide polymorphisms (SNPs) and structural variations among strains to be identified (Gwida *et al.,* 2020; Li *et al.,* 2021). However, some studies reported that the enhancement of phenotypic traits such as virulence or adaptation to different environments with data on these genetic differences may be useful in enabling the identification of key genetic determinants involved in pathogenesis and persistence of bacterial pathogens (Fuentes-Pardo and Ruzzante, 2017; Li *et al.,* 2021). The potential of WGS analysis to facilitate the tracking of *Listeria* species across different geographical regions and industries, aiding in the identification of potential sources and routes of contamination (Gwida *et al.,* 2020) makes it an incredible tool for epidemiological investigations.

In addition to enhancing our understanding of the evolutionary dynamics of *Listeria* species, WGS analysis plays a crucial role in advancing food safety and quality (El Zowalaty *et al.*, 2019). The comprehensive genomic data obtained through WGS can be utilized to establish robust surveillance systems for monitoring the presence and spread of *Listeria* species within the food production chain (El Zowalaty *et al.*, 2019; Gwida *et al.*, 2020). High-resolution genotyping methods, such as single nucleotide variant (SNV) analysis and core genome multilocus sequence typing (cgMLST), enable the accurate identification of the source of *Listeria* contamination in food products and the tracing of its transmission pathways (El Zowalaty *et al.*, 2019; Gwida *et al.*, 2020). This information empowers regulatory bodies and food manufacturers to implement targeted interventions, preventing outbreaks and safeguarding public health.

Antibiotic resistance constitutes one of the most serious public health threats to humans worldwide (WHO, 2014). The global health challenges as well as the economic and societal costs posed by AMR threaten the management of infections in humans and animals (Baker, 2015). Although the detection of the AMR profiles of bacterial pathogens has been achieved through culture-based antimicrobial susceptibility testing, the reliability of this method is affected by its reliance on visually obtained data that does not provide any information on the mechanisms of resistance (Argimón *et al.*, 2020). Moreover, bacterial cells belonging to a similar clone may display identical resistance profiles (Argimón *et al.*, 2020). WGS therefore allows for the identification and characterization of antimicrobial resistant genes and other genomic elements that may confer resistance to commonly used antimicrobials (Li *et al.*, 2021; Ramadan *et al.*, 2023). By deciphering the resistance mechanisms, WGS analysis provides insights into the potential transfer of resistant genes to other bacteria, including *Listeria* species,

and generates vital data that may be useful in the development of effective strategies to combat antimicrobial resistance (Gwida *et al.*, 2020). With this attribute, WGS has become an essential tool for drug development (Roemer and Boone, 2013). This study assessed the pathotype, virulome and resistome of *Listeria* species previously isolated from different sources in the North-West Province of South Africa using whole genome sequencing (WGS).

5.2 Materials and Methods

5.2.1 Bacterial strains

Two *Listeria* species (LIN_NWU_CNKT and LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000)) previously isolated from food (processed meat and mincemeat) and water samples subjected to WGS analysis. Details of the isolation, identification, antimicrobial resistance and virulence gene determinants and the biofilm-forming potentials of the isolates are shown in Kaptchouang *et al.* (2022) presented in Chapter Four of the thesis. The notable resistance profiles of the multidrug-resistant genes are *ant (3 '')-La*, *dfr1S*, *sul1*, *ermC*, *tetA* and *tetB* with virulence genes including *prfA*, *hlyA*, *actA* and *plcB*. The presence of these resistance and virulence determinants in these isolates highlights their potential public health significance and thus a serious concern when present in the food chain.

5.2.2 Genomic DNA extraction

Pure frozen cultures of multidrug-resistant *Listeria* species were revived by sub-culturing on a *Listeria* isolation medium (Oxford formulation, Acumedia). The plates were incubated aerobically at 37 °C for 24-48 h. Pure greenish-grey colonies were inoculated in 15 mL TSB (Merck, South Africa) and incubated aerobically at 37 °C for 24 h. Bacteria cells were harvested through centrifugation at 10000 rpm for 10 minutes and the supernatant was

discarded. Genomic DNA was extracted from the cells using the Research Genomic DNA™– Tissue MiniPrep Kit (Zymo Research, ZR Corp. Irvine, United States). The DNA was quantified using a NanoDrop™ Lite spectrophotometer (Thermo Fischer Scientific, Walton, MA, United States). DNA samples with A260/A280 ratios ranging from 1.80 to 2.0 were classified as high-purity DNA and used for sequencing.

5.2.3 Sequencing and Library Preparation

The MGI Universal DNA library prep kit was used to prepare the libraries following the manufacturer's instructions. The MGI DNBSEQ-G400 was used to perform the paired-end sequencing for a total of 312 cycles. Kbase online platform (https://kbase.us/) (version 2.1) was used to analyse and assess the quality of the raw reads (Arkin *et al.*, 2018).

5.2.4 Trimming and Assembly

The FastQC version 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to verify the sequence quality of the raw reads. Poor-quality sequences and adapters were removed using Trimmomatic (v0.36) (Bolger *et al.*, 2014). SPAdes 3.13.0 was used to do a *de novo* assembly of the trimmed (high-quality) reads (Prjibelski *et al.*, 2020).

5.2.5 Genome Annotation and Comparative Analysis

The draft assembled genomes of the two *Listeria* were annotated using Prokka (v1.12) (Seemann, 2014) on the Kbase platform (https://kbase.us/) and checked with rapid annotation using the subsystem technology (RAST) server (version 2.0) for comparison (Aziz *et al.*, 2008; Brettin *et al.*, 2015). The circular genome maps of the isolates were generated by uploading the annotated genome on the circular genome visualization online database

(http://wishart.biology.ualberta.ca/cgview/) (Stothard and Wishart, 2005). Reference genomes from NCBI were used through PATRIC algorithms to construct a phylogenetic tree. The reference and representative genomes that are most comparable to the target *Listeria* strains in this study were discovered using Mash/MinHash (Ondov *et al.,* 2016). PGfams were selected from these genomes in order to determine how the interested target genomes fit into the phylogeny. Thereafter, the protein sequences from these families were aligned with the Multiple Sequence Comparison by Log-Expectation (MUSCLE 3.8.31 "default version") software. The nucleotides for each of these sequences were mapped to obtain the protein alignment (Edgar, 2004). The combined set of amino acid and nucleotide alignments was concatenated into a data matrix to provide the support values for the phylogenetic tree. Then, this matrix was evaluated using RaxML (Stamatakis, 2014) and rapid bootstrapping (Stamatakis *et al., 2008*). The annotated genome sequences were submitted to GenBank to obtain accession numbers.

5.2.6 Detection of pathotype, virulome, resistome, and mobilome harboured by the isolates

The specific isolate contigs were searched against the PathogenFinder version 1.1 database (https://cge.food.dtu.dk/services/SerotypeFinder/) on the genomic epidemiology online platform (https://www.genomicepidemiology.org/services/) to determine the group to which the isolates belong based on pathogenicity (Cosentino *et al*., 2013). The contigs of the isolates were compared using the virulence factors of bacterial pathogens (VFDB) online database (http://www.mgc.ac.cn/VFs/), in order to determine the virulence genes harboured in the genomes of these isolates. Additionally, the VirulenceFinder (version 2.0) database (https://cge.food.dtu.dk/services/VirulenceFinder/) was used to identify acquired virulence

genes. The AMR genes carried in the genomes of the isolates were determined by submitting the contigs to the online resistance gene identifier (RGI) program (https://card.mcmaster.ca/analyze/rgi) (Zankari *et al.,* 2012). This application predicts antibiotic resistome(s) from protein or nucleotide data based on homology and SNP models using reference data from the Comprehensive Antibiotic Resistance Database (CARD).

5.3 Results

5.3.1 The genomic deoxyribonucleic acid (DNA) and sequence quality

The genomic deoxyribonucleic acid (DNA) extracted from the two isolates (LIN_NWU_CNKT and LIN5_NWU_CNKT) were of good quality with A260/280 and A260/230 ratios of 1.80 and 2.00, respectively. Also, the assembled genomes were of good quality based on the statistical comparison to other genomes within these same species.

5.3.2 Genomic assembly features of *Listeria innocua*

The genomes of the two isolates (LIN_NWU_CNKT and LIN5_NWU_CNKT) were assembled using SPAdes. The genome lengths for the LIN_NWU_CNKT and LIN5_NWU_CNKT were 3341013 and 3558124 bp, respectively. NCBI GenBank platform received the genomic sequences of the two isolates (LIN_NWU_CNKT and LIN5_NWU_CNKT) and ascension number JAVCAR000000000 was assigned to LIN5_NWU_CNKT while the ascension number for LIN_NWU_CNKT is yet to be released. Table 5.1 provides a summary of the information obtained from the genomes after assembly and annotation. The genomes for the strains LIN_NWU_CNKT and LIN5_NWU_CNKT had 3,769 and 3,722 protein-coding sequences respectively, 48 versus 45 transfer RNA sequences, and 4 versus 2 ribosomal RNA sequences, respectively. The G+C contents for the isolates were

37.35% and 36.66% for LIN_NWU_CNKT and LIN5_NWU_CNKT, respectively. Isolate LIN_NWU_CNKT had 518 contigs compared to 92 for LIN5_NWU_CNKT.

Table 5.1: Genomic assembly report of LIN_NWU_CNKT and LIN5_NWU_CNKT isolates.

Features	LIN_NWU_CNKT	LIN5_NWU_CNKT
Genome size (bp)	3341013	3558124
DNA G+C content	37.35%	36.66%
Number of contigs	518	92
Contig N50	3	1
Contig L50	311534	3,558,124
CDS	3769	3,722
tRNA	48	45
rRNA	4	2
Partial CDS	0	0
Miscellaneous RNA	0	0
Chromosomes	0	0
Ascension Number	Not yet released	JAVCAR000000000

5.3.3 Genomic annotation of strains LIN_NWU_CNKT and LIN5_NWU_CNKT

5.3.3.1 Protein features on LIN_NWU_CNKT and LIN5_NWU_CNKT

Data from the annotation revealed the presence of hypothetical proteins (886 for LIN_NWU_CNKT and 1,069 for LIN5_NWU_CNKT and proteins with assigned functions (2,883 for LIN_NWU_CNKT and 2,653 for LIN5_NWU_CNKT (Table 5.2). The proteins with functional assignments included those having Kyoto Encyclopedia of Genes and Genomes

(KEGG) pathway maps, gene ontology (GO) assignments and enzyme commission (EC) numbers. Two categories of protein families are included in the annotation with PATRIC: genus-specific protein families (PLfams) and cross-genus protein family (PGfams) families. In Table 5.2, the examined strains' protein characteristics are described in detail.

Table 5.2: Proteins features of LIN_NWU_CNKT and LIN5_NWU_CNKT

Proteins features	**LIN_NWU_CNKT**	**LIN5_NWU_CNKT**
Hypothetical proteins	886	1,069
Proteins with functional assignments	2,883	2,653
Proteins with EC number assignments	947	827
Proteins with GO assignments	772	670
Proteins with pathway assignments	647	561
Proteins with PATRIC genus-specific family (PLfam) assignments	3,328	3,070
Proteins with PATRIC cross-genus family (PGfam) assignments	3,666	3,616

5.3.3.2 Subsystem analysis of LIN_NWU_CNKT and LIN5_NWU_CNKT genomes

According to Overbeek *et al*. (2005), a subsystem is a collection of proteins that work together to carry out a particular biological function or structural complex. The subsystems unique to each of the examined genomes were outlined by PATRIC (Figure 5.1). The genes involved in various cellular functions were tallied up and allocated to the appropriate subsystems.

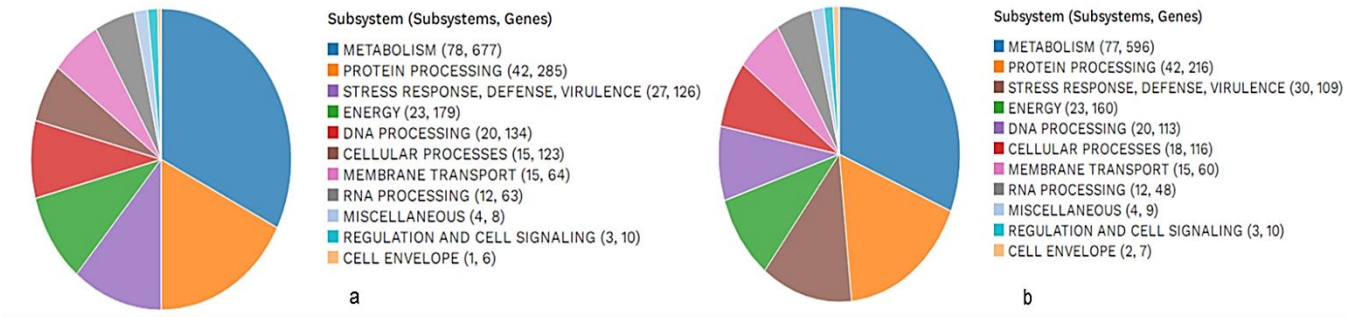


Figure 5.1: Subsystem analysis of *Listeria innocua* strain (a) LIN_NWU_CNKT and (b) LIN5_NWU_CNKT

In contrast to strain LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000) (b), which revealed 109 genes belonging to 30 subsystems involved with the same mechanisms, strain LIN_NWU_CNKT (a) displayed 126 genes belonging to 27 subsystems that play a role in stress response, defence, and virulence mechanisms. Additionally, as compared to strain LIN_NWU_CNKT, strain LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000) did not have any additional genes or subsystems (Figure 5.1). A circular graphical representation of the distribution of the bacterial genome annotations in a circular pattern is shown in Figure 5.2.

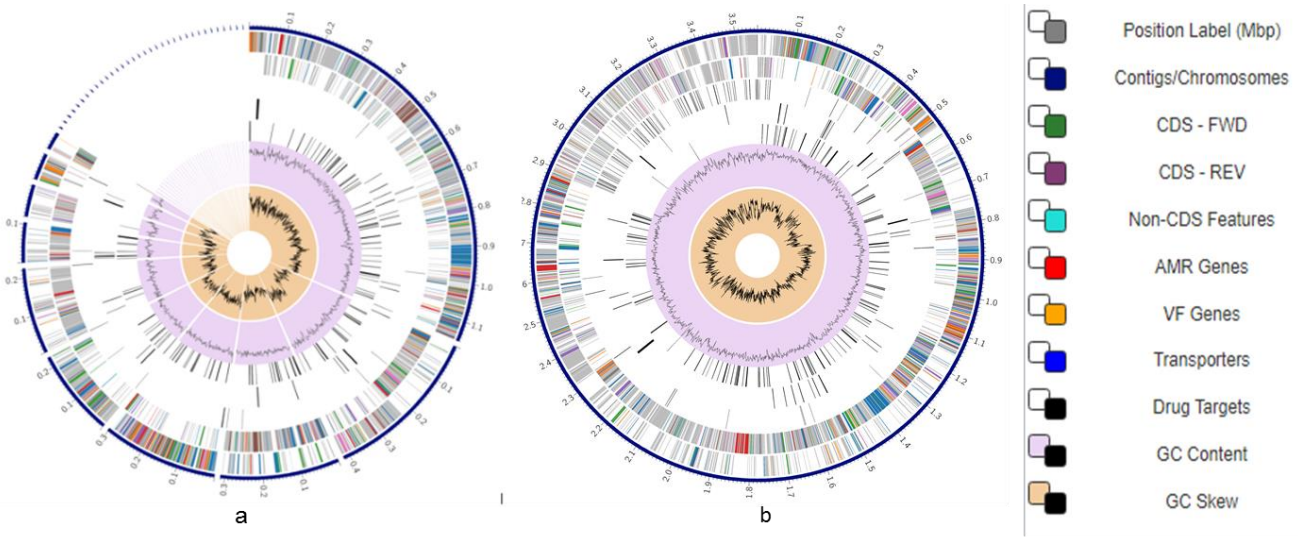


Figure 5.2: The distribution of the annotated genomes of strains LIN_NWU_CNKT (a) and LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000) (b) in a circular graphical display. This display comprises the contigs, CDS on the forward and reverse strands, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to known virulence factors, GC content, and GC skew, in that order from outer to inner rings. The subsystem to which these genes belong is indicated by the colours of the CDS on the forward and reverse strands (see legend).

5.3.3.3 Virulence genes detected LIN_NWU_CNKT and LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000) genomes

The genomes of both isolates contained multiple virulence genes, according to the results from the virulence factors of bacterial pathogens online platform (Table 5.3). Some of the virulence genes identified in LIN_NWU_CNKT and LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000) comprised genes involved in adhesion (*dltA*, *fbpA*, *ami*, *inlJ*, *lap* for both isolates and *srtC1* for LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000)), bile resistance (*bsh* for LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000)), invasion (*hpt*, *aut*, *iap*/*cwhA*, *gtcA*, *inlA* just to name a few for both strains), iron uptake (*hbp2* for LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000)), peptidoglycan modification (*oatA* and *pdgA* for both isolates), surface anchoring proteins (*lgt*, *lspA*, and *srtA*

for both strains and *strB* for LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000)) and enzyme secretory systems (*plcA* for LIN_NWU_CNKT and *stp* for LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000)). Generally, the genome of isolates LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000) possessed a large proportion of virulence genes when compared to that of LIN_NWU_CNKT. These findings highlight, on a "One Health Perspective", the public health implications of the investigated genomes and the potential pathogenicity of the isolates thus suggesting the need for strict control measures in the food chain.

Table 5.3: Virulence genes detected in LIN_NWU_CNKT and LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000).

Virulence factors class	Virulence factors	Related genes	
		Listeria innocua (LIN_NWU_CNKT)	*Listeria innocua* (LIN5_NWU_CNKT (ACESSION NUMBER JAVCAR000000000))
Adherence	D-alanine-polyphosphoribitol ligase	*dltA*	*dltA*
	Fibronectin-binding protein	*fbpA*	*fbpA*
	GW autolysin	*Ami*	*ami*

	Internalin J (LPXTG protein)	*inlJ*	*inlJ*
	Listeria adhesion protein	*Lap*	*lap*
	Ebp pili (Enterococcus)	-	*srtC1*
Bile resistance	Bile-salt hydrolase	-	*bsh*
Enzyme	PI-PLC	*plcA*	-
	Serine-threonine phosphatase	-	*stp*
Immune modulator	InlC	*inlC*	-
	InlK	-	*inlK*
	LntA	*lntA*	-
Intracellular survival	Lipoate protein ligase A1	*lplA1*	*lplA1*
	Oligopeptide-binding protein	*oppA*	*oppA*
	Post-translocation chaperone	-	*prsA2*
	Sugar-uptake system	*Hpt*	-
Invasion	Autolysin (GW protein)	*Aut*	*aut*
	Cell wall hydrolase	*iap/cwhA*	*iap/cwhA*

	Cell wall teichoic acid glycosylation protein	*gtcA*	*gtcA*
	Internalin A (LPXTG protein)	*inlA*	*inlA*
	Internalin B (GW protein)	-	*inlB*
	Internalin P	*inlP*	-
	Lipoprotein promoting entry protein	-	*lpeA*
	Virulence protein (LPXTG protein)	*Vip*	-
Iron uptake	Hemoglobin binding protein	-	*hbp2*
Nucleation-promoting factor	ActA	*actA*	-
Peptidoglycan modification	OatA	*oatA*	*oatA*
	PdgA	*pdgA*	*pdgA*
Regulation	AgrA/AgrC	*agrA*	*agrA*
		agrC	*agrC*
	CheA/CheY	*cheA*	*cheA*

		cheY	*cheY*
	LisR/LisK	*lisK*	*lisK*
		-	*lisR*
	Positive regulatory factor	*prfA*	-
	VirR/VirS	*virR*	*virR*
		virS	*virS*
Surface protein anchoring	Lipoprotein diacylglyceryl transferase	*Lgt*	*lgt*
	Lipoprotein-specific signal peptidase II	*lspA*	*lspA*
	Sortase A	*srtA*	*srtA*
	Sortase B	-	*srtB*

5.3.3.4 Antimicrobial Resistant (AMR) genes of *Listeria innocua* strain LIN_NWU_CNKT and LIN5_NWU_CNKT

Analysis of the genomes revealed the presence of several AMR genes that were verified by the results derived from the online antibiotics RGI database. The genomes of both the LIN_NWU_CNKT and JAVCAR00000000 strains possessed phosphonic acid resistance genes, lincosamide resistant genes, glycopeptide resistance genes, and tetracycline resistance genes. Details of the classes of antibiotics and the corresponding drugs to which resistance determinants were detected in the genomes are shown in Table 5.4. The mechanisms of

resistance for the antibiotic-resistant genes that were identified in these genomes are associated with either target antibiotic efflux pumps [*tet(45)* and *tet(M)*], alteration of the target antibiotic binding site (*vanT* and *vanY*) or antibiotic inactivation enzymes (*FosX*, *Fosl* and *lin*).

Table 5.4: Antibiotic resistance detected in strains LIN_NWU_CNKT and LIN5_NWU_CNKT

Drug Class	**AMR Gene**	**AMR Gene Family**	**Resistance Function**	**LIN_NWU_ CNKT**	**LIN5_NWU_CNKT**
phosphonic acid antibiotic	*FosX*	fosfomycin thiol transferase	antibiotic inactivation	+	+
lincosamide antibiotic	*Lin*	lincosamide nucleotidyltransferase (LNU)	antibiotic inactivation	+	+
glycopeptide antibiotic	*vanT* gene in *vanG* cluster	glycopeptide resistance gene cluster, vanT	antibiotic target alteration	+	+
glycopeptide antibiotic	*vanY* gene in *vanB* cluster	vanY, glycopeptide resistance gene cluster	antibiotic target alteration	+	+
tetracycline antibiotic	*tet(45)*	major facilitator superfamily (MFS) antibiotic efflux pump	antibiotic efflux	-	+

tetracycline antibiotic	*tet(M)*	major facilitator superfamily (MFS) antibiotic efflux pump	antibiotic efflux	-	+
phosphonic acid antibiotic	*Fosl*	fosfomycin thiol transferase	antibiotic inactivation	-	+

5.3.3.5 Phylogenetic assessment of nucleotide sequences of strains LIN_NWU_CNKT and LIN5_NWU_CNKT

In a comparison of LIN_NWU_CNKT and LIN5_NWU_CNKT isolates with other *Listeria* strains, the two isolates showed significant resemblance to other strains of the same species obtained from diverse sources, as indicated by the alignment of the 16S rRNA sequences (Figure 5.3). The PATRIC methods employ NCBI reference genomes to create a phylogenetic tree. Mash/MinHash was used to identify the representative and nearest reference genomes to our strains of interest (Ondov *et al.*, 2016). To ascertain the evolutionary position of the investigated genomes of interest, PGfams were chosen from these genomes. Nucleotides for each of the protein sequences from these families were mapped to the protein alignment after the sequences were aligned using MUSCLE (Edgar, 2004). To ascertain the evolutionary position of our genomes of interest, PGfams were chosen from these genomes. Nucleotides for each of the protein sequences from these families were mapped to the protein alignment after the sequences were aligned using MUSCLE (Edgar, 2004). In order to produce the support values in the phylogenetic tree, the joint set of amino acid and nucleotide alignments were

concatenated into a data matrix. This matrix was then analyzed using fast bootstrapping (Stamatakis *et al.*, 2008) using RaxML (Stamatakis, 2014).

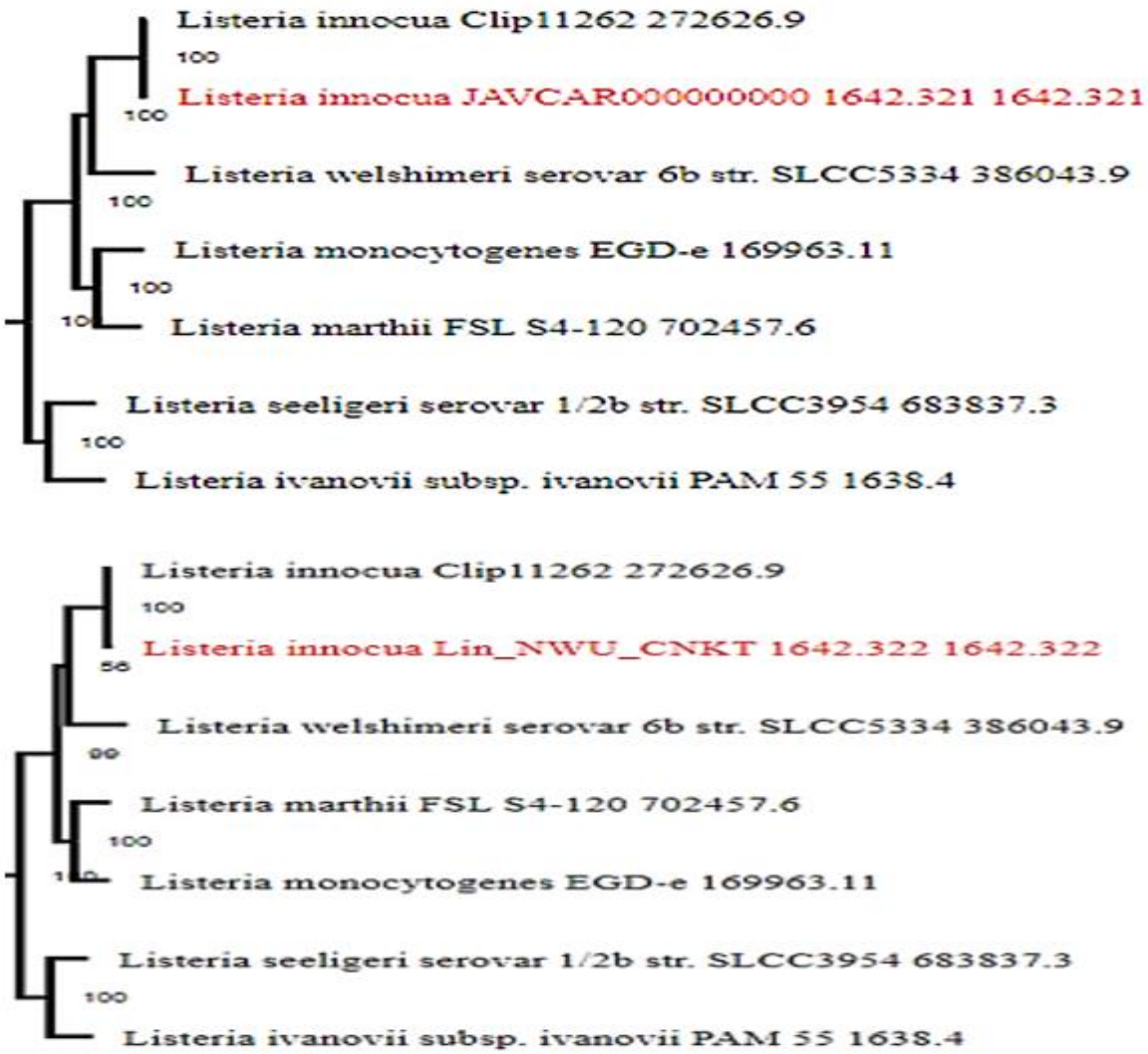


Figure 5.3: Phylogenetic tree determining the relationship between strain LIN_NWU_CNKT (a) and LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000) (b) and other *Listeria* isolates of the same species.

5.4 Discussion

Food safety which involves the implementation of strategies and processes to safeguard the food supply chain from contamination with microbial and chemical pollutants is considered an important public health issue worldwide (Radovanovic, 2011; Uyttendaele *et al.,* 2016). Despite the fact that many countries throughout the world may tend to increasingly be operating interdependently with respect to their available food supply chains and food safety, the increasing occurrence of foodborne outbreaks especially due to increased globalization highlights the need for strict control systems (Scallan *et al.*, 2011).

Foodborne pathogens including but not limited to *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* species, and *Clostridium perfringes* are considered a significant threat to food safety (Scallan *et al.*, 2011; Zhao *et al.,* 2017). Moreover, with the drive by the WHO to ensure "Good health and well-being for all" through the sustainable development goals (particularly Goal 3), it is reported that food safety is a critical element to assist in realizing this aim (Founou *et al.*, 2021). Given that food supply chains operate across multiple local, national and international borders, the involvement of decision-makers and all stakeholders as well as adherence to effective and efficient measures on farms, meat processing facilities, retail supermarkets, street vendors and homes are critical (WHO, 2015). Moreover, the utilization of highly reliable, sensitive and specific identification assays coupled with typing methods with high recognition abilities and high repetition rates are important to ensure consistency for epidemiological purposes (Belkum *et al.,* 2007).

The lengthy and tedious process for isolating and identifying bacterial pathogens through culturing and reliance on biochemical assays coupled with the difficulty of isolating unculturable bacteria present a significant challenge that limits the application of the culture-based techniques (Zhao *et al.,* 2014; Zhao *et al.,* 2016). To curb these setbacks, recent years have seen the development and utilization of various molecular identification and typing methods that have several advantages including improved sensitivity and specificity, high speed and accuracy as well as increased potential to differentiate between bacteria strains based on differences in fingerprinting profiles (Zhao *et al.,* 2014; Zhao *et al.,* 2016). Whole genome sequencing techniques have been developed for rapid and high throughput detection of bacteria in research, public health laboratories, reference laboratories and hospital infection control-affiliated laboratories (Kwong *et al.*, 2015). The superior attributes of WGS analysis are largely associated with speedy and confirmatory power, which facilitates the urgent detection of bacteria (Kwong *et al.,* 2015). Moreover, WGS bioinformatics analysis provides within a short time the identity, pathotype, virulence and AMR profile of bacteria contaminants (Davedow *et al.*, 2022).

This study reveals the features that are present in the genomes of two *Listeria innocua* strains LIN_NWU_CNKT and LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000) and also associated with their pathogenicity and multidrug resistance profiles. The isolation of these isolates from food products and water, respectively indicates their significant public health significance given the “One Health” concept. South Africa experienced the World’s most severe outbreak of listeriosis in 2017 caused by the consumption of polony that was contaminated with *Listeria monocytogenes* (Desai *et al.*, 2019). Despite the limited information on the prevalence, occurrence, antimicrobial susceptibility patterns, biofilm-forming ability

and the occurrence of foodborne outbreaks associated with other *Listeria* species, such as *L. innocua*, evidence of its genetic relatedness to *L. monocytogenes* (Johnson *et al.*, 2004; Clayton *et al.,* 2014) highlights the need for untargeted investigations. Moreover, the association of *L. innocua* with some excessively "rare" complications such as septicemia and meningitis in humans (Perrin *et al.,* 2004; Favaro *et al.,* 2014) and ruminants (Walker *et al.,* 1994; Rocha *et al.,* 2013) amplified their public health implications. In addition, to the detection of several virulence factors in the *L. innocua* genomes, the isolates were classified as human pathogens (Cosentino *et al.*, 2013) and may cause diseases in susceptible hosts if consumed in food and water. In this study, the virulence genes encoding for *PrfA, ActA, Lisk, InlA, InlB, InlJ, AggA* were detected in the genomes of the isolates. Similarly, observations have been reported by El Zowalaty *et al*. (2019), Gwida *et al*. (2020), Li *et al*. (2021).

Data generated from the virulence factors of bacterial pathogen databases confirmed that the LIN_NWU_CNKT and LIN5_NWU_CNKT (ACCESSION NUMBER JAVCAR000000000) genomes possessed a wide range of virulence genes. The virulence factors harboured by the two isolate genomes include adherence, invasion, iron uptake, secretory system and toxin. Several classes of extracellular organelles of bacteria that facilitate attachment and motility have been associated with virulence factors leading to pathogenicity (Pakbin *et al.*, 2021). These extracellular organelles include flagella, pili and curli fibres which can be classified as adherence virulence factors. Also, bacteria produce toxins as a long-term survival strategy in the environment. However, the toxin's mode of action has been associated with virulence factors causing pathological damage to susceptible hosts (Wang *et al.*, 2021; Ghazaei, 2022). Bacterial toxins can damage the cell membrane, inhibit protein synthesis, destroy blood cells, and activate the immune response, leading to disease (Ghazaei, 2022).

Several AMR genes mediating resistance to antibiotics belonging to different drug classes were detected in the genomes of the two isolate sequences. The AMR genes detected in the two isolates include *fosX, lin, vanT,* and *tet* covering resistant to phosphonic acid, lincosamide, glycopeptide, and tetracycline, respectively. The presence of several AMR genes in the genomes of the two isolates poses a threat to public health due to the limited choice of antibiotics to treat any disease caused by these bacteria. This highlights the crucial need for the development of a novel alternative therapeutic agent.

5.5 Conclusions and Recommendations

The current findings revealed that the two isolate genomes possessed several virulence genes. Furthermore, whole genome sequence analysis revealed that the two isolate sequences were human pathogens. In addition, the two isolates harboured several antimicrobial resistance genes as well as different mobile genetic elements. Therefore, continuous monitoring of pathogenic bacteria and enforcement of control measures to minimize food contamination in the farm environment and processing stage is imperative to safeguard human health.

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CHAPTER SIX

GENERAL DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.1 General discussion

The World Health Organization has identified *Listeria monocytogenes* as one of the major foodborne pathogens (WHO, 2015). *Listeria* species, especially *Listeria monocytogenes* is the cause of the foodborne illness known as listeriosis (Reda *et al.*, 2016; Thakur *et al.*, 2018). Listeriosis has been reported to have a mortality rate of 20–30% and is known to lead to serious complications including meningoencephalitis, sepsis, fatal infections, or miscarriage in pregnant women (Thakur *et al.*, 2018; Desai *et al.*, 2019). The disease presents significant health challenges in young children, the elderly, and immuno-compromised individuals (Reda *et al.*, 2016).

A number of sporadic cases of infection and outbreaks have been reported even in countries with more advanced public health policies (Cartwright *et al.*, 2013; Tchatchouang *et al.*, 2020). Most outbreaks have been linked to the consumption of contaminated food and water (Cartwright *et al.*, 2013; Desai *et al.*, 2019). South Africa experienced the World's most severe outbreak of listeriosis in 2017 caused by the consumption of polony that was contaminated with *Listeria monocytogenes* (Desai *et al.*, 2019). Despite the limited information on the prevalence, occurrence, antimicrobial susceptibility patterns, and biofilm-forming ability and the occurrence of foodborne outbreaks associated with other *Listeria* species, such as *L. innocua*, evidence of its genetic relatedness to *L. monocytogenes* (Johnson *et al.*, 2004; Clayton

et al., 2014) highlights the need for untargeted investigations. Moreover, the association of *L. innocua* with many excessively "rare" complications such as septicaemia and meningitis in humans (Perrin *et al.,* 2004; Favaro *et al.,* 2014) and ruminants (Walker *et al.,* 1994; Rocha *et al.,* 2013) amplified their public health implications.

This study was inspired by the 2017 outbreak of listeriosis in South Africa. The purpose of this study was to determine the prevalence of virulent *Listeria* species in food and water collected from different sources in South Africa. A total of 207 samples of meat, milk, vegetables, and water were collected and analysed for the presence of *Listeria* species using culture methods. The presumptive *Listeria* species detected were confirmed through species-specific PCR assay. The confirmed *Listeria* species were phenotypically screened for antibiotic resistance and biofilm-forming ability. The multidrug-resistant and biofilm former *Listeria* species detected were subjected to whole genome sequence analysis to unravel their virulence, antimicrobial resistance, and biofilm gene.

In this study, a total of 267 presumptive *Listeria* spp. were isolated. Out of the 267, some 53 (19.85%) were confirmed to be *Listeria* species, and these were comprised of 26 *L. monocytogenes*, 3 *Listeria*, 2 *L. welshimeri*, and 1 *L. thailandensis*. A large proportion of the *Listeria* species were resistant to erythromycin (76%), clindamycin (100%), gentamicin (100%), tetracycline (100%), novobiocin (100%), oxacillin (100%), nalidixic acid (100%), and kanamycin (100%). The isolates showed a range of multi-drug resistant (MDR) phenotypes, with the *L. monocytogenes* isolates showing the most E-DA-GM-T-NO-OX-NA-K MDR phenotypes. Additionally, 28% of the *L. monocytogenes* isolates were able to form biofilm at 4, 25, and 37 °C of 24, 48 and 72 h incubation. Many studies have been reported on the prevalence and incidence of *L. monocytogenes* in meat, meat products, water, and vegetables.

These products typically require multiple treatment steps before consumption and may be contaminated with *L. monocytogenes* at the post-treatment stage, indicating poor hygiene practices in retail food processing. Listeriosis is one of the most problematic food-borne diseases in some countries of the world and causes serious outbreaks at all stages of the food manufacturing process (Desai *et al.*, 2019; Malakar *et al.*, 2019; Zhang *et al.*, 2021). *L. monocytogenes* can grow in both raw and cooked meat at refrigerated temperatures (Muchaamba *et al.*, 2019). Therefore, continuous monitoring of pathogenic bacteria and enforcement of control measures to minimize food contamination in the farm environment and food processing are imperative to safeguard human health (Zhang *et al.*, 2021). Also, further research is needed to develop a novel alternative therapeutic agent to combat multidrug-resistant bacteria.

Although the culture-base technique is necessary for identification and enumeration as well as detection of viability, virulence and antimicrobial resistant (AMR) phenotypic profile of bacteria (Gill, 2017), whole genome sequence (WGS) analysis is useful to determine their genetic profile (Montso *et al.*, 2022). Based on WGS analysis, the isolates were confirmed to harboured virulent genes such as those responsible for adherence, invasion, and toxin production. Several classes of extracellular organelles of bacteria that facilitate attachment and motility have been associated with virulence factors leading to pathogenicity (Pakbin *et al.*, 2021). These extracellular organelles include flagella, pili, and curli fibres which can be classified as adherence virulence factors. Also, bacteria produce toxins as a long-term survival strategy in the environment. However, the toxin's mode of action has been associated with virulence factors causing pathological damage to susceptible hosts (Wang *et al.*, 2021; Ghazaei,

2022). Bacterial toxins can damage the cell membrane, inhibit protein synthesis, destroy blood cells and activate the immune response, leading to disease (Ghazaei, 2022).

In addition, the genomes of the sequence isolates possessed antibiotic-resistant genes, which confer resistance to drugs such as phosphonic acid, lincosamide, glycopeptide, and tetracycline. The presence of several AMR genes in the genome of the two isolates poses a threat to public health due to the limited choice of antibiotics to treat any disease caused by these bacteria. This highlights the crucial need for the development of a novel non-antibiotic therapeutic agent.

6.2 Conclusion

In conclusion, the literature review has shown that some *Listeria* species have become problematic food-borne pathogens worldwide. In South Africa, the outbreak of foodborne disease witnessed as a result of pathogenic *Listeria* species had serious consequences of high morbidity, mortality, and enormous economic losses. This study shows the presence of *Listeria* species in some food and water samples collected around South Africa. A larger proportion (53%) of the *Listeria* species detected were among the human pathogens including *Listeria monocytogenes.* Additional investigation showed that the *L. monocytogenes* strains detected in this study can form biofilm. Further study confirmed that all the *L. monocytogenes* isolated were multidrug-resistant phenotypically. Finally, whole genome sequence analysis revealed several virulome, resistome, and mobile genetic elements harboured in the isolates. This clearly shows that *L. monocytogenes* has all the properties that contribute to pathogenicity, but it is difficult to control because of its resistance ability.

6.2 Recommendations for future research work

Frequent surveillance studies are recommended to demonstrate the prevalence, pathogenicity, antibacterial properties, and biofilm formation of *Listeria monocytogenes* in food and water samples to safely guide public health. Also, the current precautions available to mitigate foodborne disease outbreaks are inadequate due to the increasing incidence of antimicrobial resistance development among *Listeria monocytogenes*. Therefore, effective alternatives to control and prevent resistant pathogenic *Listeria monocytogenes* are needed to reduce the risk of listeriosis.

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APPENDICES

APPENDIX 1: ETHICS CERTIFICATE

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ETHICS APPROVAL LETTER OF STUDY

Based on approval by the **Faculty of Natural and Agricultural Sciences Ethics Committee (FNAS-REC)**, the Faculty of Natural and Agricultural Sciences Ethics Committee hereby **approves** your study as indicated below. This implies that the North-West University Senate Committee for Research Ethics (NWU-SCRE) grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Whole Genome Sequence Analysis of Virulent Listeria Species from Different Sources
Study Leader/Supervisor: Prof CN Ateba
Student: CD Kaptchouang Tchatchouang

Ethics number:	N	W	U	-	0	0	7	3	3	-	1	8	-	A	9
	Institution				Study Number						Year			Status	

Status: S – Submission; R – Re-Submission; P – Provisional Authorisation; A – Authorisation

Application type: Single **Risk Category:** **Minimal**
Commencement date: 01/02/2018
Expiry date: 31/08/2024
Approval of the study is initially provided for a year, after which continuation of the study is dependent on receipt and review of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation.

Special in process conditions of the research for approval (if applicable):

- The following documentation are archived by FNASREC and should be complete and kept up to date:
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  - Signed approval from the scientific committee indicating the proposed risk category
- All researchers involved in the study should submit signed NWU code of conduct statements annually.
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- All research involving human interaction should follow best ethical practise and keep documents as proof. This includes informed consent, questionnaires, incorporation of risk-benefit, and responsible data management.
- Any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the FNASREC. Ethics approval is required BEFORE approval can be obtained from these authorities.

APPENDIX 2: LANGUAGE EDITING CERTIFICATE

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Whole genome sequence analysis of virulent Listeria species

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APPENDIX 3. PUBLICATION 1

microorganisms

MDPI

Review

Listeriosis Outbreak in South Africa: A Comparative Analysis with Previously Reported Cases Worldwide

Christ-Donald Kaptchouang Tchatchouang [1], Justine Fri [1], Mauro De Santi [2,3], Giorgio Brandi [2,3], Giuditta Fiorella Schiavano [3], Giulia Amagliani [2] and Collins Njie Ateba [1,4,*]

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Received: 22 November 2019; Accepted: 27 December 2019; Published: 17 January 2020



Abstract: *Listeria* species are Gram-positive, rod-shaped, facultative anaerobic bacteria, which do not produce endospores. The genus, *Listeria*, currently comprises 17 characterised species of which only two (*L. monocytogenes* and *L. ivanovii*) are known to be pathogenic to humans. Food products and related processing environments are commonly contaminated with pathogenic species. Outbreaks and sporadic cases of human infections resulted in considerable economic loss. South Africa witnessed the world's largest listeriosis outbreak, characterised by a progressive increase in cases of the disease from January 2017 to July 2018. Of the 1060 laboratory-confirmed cases of listeriosis reported by the National Institute of Communicable Diseases (NICD), 216 deaths were recorded. Epidemiological investigations indicated that ready-to-eat processed meat products from a food production facility contaminated with *L. monocytogenes* was responsible for the outbreak. Multilocus sequence typing (MLST) revealed that a large proportion (91%) of the isolates from patients were sequence type 6 (ST6). Recent studies revealed a recurrent occurrence of small outbreaks of listeriosis with more severe side-effects in humans. This review provides a comparative analysis of a recently reported and most severe outbreak of listeriosis in South Africa, with those previously encountered in other countries worldwide. The review focuses on the transmission of the pathogen, clinical symptoms of the disease and its pathogenicity. The review also focuses on the major outbreaks of listeriosis reported in different parts of the world, sources of contamination, morbidity, and mortality rates as well as cost implications. Based on data generated during the outbreak of the disease in South Africa, listeriosis was added to the South African list of mandatory notifiable medical conditions. Surveillance systems were strengthened in the South African food chain in order to assist in preventing and facilitating early detection of both sporadic cases and outbreaks of infections caused by these pathogens in humans.

Keywords: Food chain; foodborne disease; *Listeria monocytogenes*; Listeriosis; South African outbreak; sequence type 6

1. Introduction

Listeria was reported for the first time in 1924 by Murray and colleagues due to the sudden deaths of six young rabbits and was named *Bacterium monocytogenes* [1]. Subsequently, in 1927, Pirie named

APPENDIX 4. PUBLICATION 2

pathogens

MDPI

Article

Evidence of Virulent Multi-Drug Resistant and Biofilm-Forming *Listeria* Species Isolated from Various Sources in South Africa

Christ-Donald Kaptchouang Tchatchouang [1], Justine Fri [1], Peter Kotsoana Montso [1], Giulia Amagliani [2], Giuditta Fiorella Schiavano [3], Madira Coutlyne Manganyi [4], Giulia Baldelli [2], Giorgio Brandi [2] and Collins Njie Ateba [1,*]

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Abstract: Listeriosis is a foodborne disease caused by *Listeria monocytogenes* species and is known to cause severe complications, particularly in pregnant women, young children, the elderly, and immunocompromised individuals. The aim of this study was to investigate the presence of *Listeria* species in food and water using both biochemical and species-specific PCR analysis. *L. monocytogenes* isolates were further screened for the presence of various antibiotic resistance, virulence, and biofilm-forming determinants profiles using phenotypic and genotypic assays. A total of 207 samples (composed of meat, milk, vegetables, and water) were collected and analyzed for presence of *L. monocytogenes* using species specific PCR analysis. Out of 267 presumptive isolates, 53 (19.85%) were confirmed as the *Listeria* species, and these comprised 26 *L. monocytogenes*, 3 *L. innocua*, 2 *L. welshimeri*, and 1 *L. thailandensis*. The remaining 21 *Listeria* species were classified as uncultured *Listeria*, based on 16SrRNA sequence analysis results. A large proportion (76% to 100%) of the *L. monocytogenes* were resistant to erythromycin (76%), clindamycin (100%), gentamicin (100%), tetracycline (100%), novobiocin (100%), oxacillin (100%), nalidixic acid (100%), and kanamycin (100%). The isolates revealed various multi-drug resistant (MDR) phenotypes, with E-DA-GM-T-NO-OX-NA-K being the most predominant MDR phenotypes observed in the *L. monocytogenes* isolates. The virulence genes *prfA*, *hlyA*, *actA*, and *plcB* were detected in 100%, 68%, 56%, and 20% of the isolates, respectively. In addition, *L. monocytogenes* isolates were capable of forming strong biofilm at 4 °C (%) after 24 to 72 h incubation periods, moderate for 8% isolates at 48 h and 20% at 72 h ($p < 0.05$). Moreover, at 25 °C and 37 °C, small proportions of the isolates displayed moderate (8–20%) biofilm formation after 48 and 72 h incubation periods. Biofilm formation genes *flaA* and *luxS* were detected in 72% and 56% of the isolates, respectively. These findings suggest that proper hygiene measures must be enforced along the food chain to ensure food safety.

Keywords: *L. monocytogenes*; virulence; multi-drug resistance; biofilm formation; food safety

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Citation: Kaptchouang Tchatchouang, C.-D.; Fri, J.; Montso, P.K.; Amagliani, G.; Schiavano, G.F.; Manganyi, M.C.; Baldelli, G.; Brandi, G.; Ateba, C.N. Evidence of Virulent Multi-Drug Resistant and Biofilm-Forming *Listeria* Species Isolated from Various Sources in South Africa. *Pathogens* **2022**, *11*, 843. https://doi.org/10.3390/pathogens11080843

Academic Editors: Juhee Ahn, Kidon Sung and Saeed Khan

Received: 9 June 2022
Accepted: 18 July 2022
Published: 27 July 2022

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1. Introduction

Listeria species are Gram-positive bacteria that belong to *Listeriacaeae* family. They are widely distributed in nature and capable of causing listeriosis in humans and animals [1]. In terms of pathogenicity, *L. monocytogenes* and *L. ivanovii* are the most pathogenic species, and they are responsible for several outbreaks in humans [2]. Their ubiquitous nature allows them to strive in various environments, particularly water, soil, plants, animals, silage,