GENOMIC CHARACTERISATION AND ANTIMICROBIAL RESISTANCE PROFILES OF *LISTERIA MONOCYTOGENES* ISOLATED FROM PIG FARMS

by

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DECLARATION

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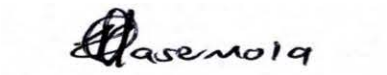
I declare that the above thesis is my work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references. I further declare that I have not previously submitted this work or part of it, for any degree or examination in any other higher education institution.

SIGNATURE:  **DATE** 08 July 2021

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DEDICATION

I dedicate this thesis to my son, Mpfunze.

ABSTRACT

Listeria monocytogenes is a zoonotic foodborne pathogen, transmissible from the natural agricultural environment to animals and humans. In recent years, the pig production industry has experienced a series of monetary losses as a result of the *L. monocytogenes* outbreak which threatened the economy of South Africa. This outbreak also had a detrimental effect on the health system of the country. In South Africa however, there is limited information regarding the genomic diversity of *L. monocytogenes*. Therefore, an overview of the genomic diversity of *L. monocytogenes* strains circulating at different levels of the pork production chain needs to be determined so as to be able to identify routes of contamination of the pathogen and thus improve meat safety. This study was aimed to determine the antimicrobial resistance patterns and population structure of *L. monocytogenes* isolated from pig farms in South Africa. Based on whole-genome sequence analysis, 77 isolates of *L. monocytogenes* were differentiated into four molecular serogroups with IIa (45.5%) being the most prevalent followed by IIc (26.0%), IVb (22.1%) and IIb (6.5%). Overall, 11 clonal complexes (CCs) were identified in this study, with the predominance being observed from; CC204 (23.4%), CC1 (19.5%) and CC2 (16.9%). Genetic elements associated with biocide, antimicrobial and heavy metal resistance were noted in 24.7 %, 48% and 11.7% of the isolates, respectively. *Listeria* pathogenicity island 1 and 3 that harbored clusters of virulence genes were present in 38.8% of the isolates. Five different plasmids were found in 68.9% of the isolates. This study has given baseline data on the genomic diversity of *L. monocytogenes* strains that are associated with biocides, heavy metal and antibiotics resistance genes. The data again demonstrated the genotypes of *L. monocytogenes* that are prone to contaminate the farm environment and possibly cause diseases in animals and humans.

Keywords: Serogroups; clonal complex; heavy metal; plasmids; *Listeria* pathogenicity

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LIST OF ACRONYMS

ActA	Actin Polymerizing Protein A
AFNOR	Association Françoise de Normalisation
ALOA	Ottaviani and Agosti Agar
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
β	Beta Haemolysis
BLEB	Buffered *Listeria* Enrichment Broth
BHI	Brain Heart Infusion broth
bp	Base Pair
BLA	Brilliance *Listeria* Agar
bglA	Beta-Glucosidase
Cat	Catalase
CAMP	Christiee-Atkins-Munch-Peterson
CC	Clonal Complex
CDC	Centres for Disease Control
CFU	Colony Forming Units
CFU g-1	Colony Forming Units per gram
cgMLST	Core genome Multilocus Sequence Typing
DAFF	Department of Agriculture Forestry and Fisheries
dapE	Succinyl Diaminopimelate Desiccinylase

Dat	D-amino Acid Aminotransferase
DNA	Deoxyribonucleic Acid
EC	Epidemic Clones
EUCAST	European Committee on Antibiotic Susceptibility Testing
FDA	Food and Drug Administration
H	Flagella Antigen
HIV	Human Immunodeficiency Virus
Hrs	Hours
Iap	Invasion associated protein
Inl	Internalin
ISO	International Organisation Standard
Ldh	L-lactate Dehydrogenase
lhkA	Histidine Kinase
LIPI	*Listeria* Pathogenecity Island
LLO	Listeriolysin O
LLS	Listeriolysin S
MLEE	Multilocus Enzyme Electrophoresis
MLST	Multilocus Sequence Typing
mpl	Metalloprotease
mPCR	Multiplex Polymerase Chain Reaction
MDR	Multidrug Resistance
NICD	Nationl Institute for Communicable Diseases
O	Somatic Antigen

PALCAM	Polymyxin – Acriflavine-LiCl-Ceftazidime-Aesculin-Mannitol agar
PCR	Polymerase Chain Reaction
PC-plc	Phosphatidylinositol – Specific Phospholipase
PFGE	Pulsed Field Gel Electrophoresis
Plc	Phosphatidylinositol-Specific Phospholipase
PMSC	Premature Stop Codon
PrfA	Positive Regulatory Factor A
qPCR	Quantitative Polymerase Chain Reaction
Rpm	Revolutions per minute
RTE	Ready-To-Eat
RNA	Ribonucleic Acid
SA	South Africa
SNP	Single Nucleotide Polymorphism
SSI	Stress Survival Islets
ST	Sequence Type
Spp	Species
TB	Tuberculosis
USA	United States of America
USDA	United States Department of Agriculture
USDA-FSIS	United States Department of Agriculture
UVM	University of Vermont –I formulation
VFDB	Virulence Factors Database

VNTR	Variable Number Tandem Repeat
WGS	Whole Genome Sequencing
wgMLST	Whole Genome Multilocus Sequence Typing
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1. General Introduction

Listeria monocytogenes is a ubiquitous foodborne bacterium commonly found in a different environmental niche such as soil, water and decaying vegetation (Piet et al. 2016). It causes a rare but severe infection known as listeriosis in humans and other animals (Smith et al. 2019). Animals mostly pigs are infected with *L. monocytogenes* through interaction with contaminated sources from nature such as soil and wastewater (Leong et al. 2014). Subsequently, this pathogen is introduced into the pork value chain through several mechanisms where it might cause disease in humans (Sofos and Geornaras, 2010). Animals infected with *L. monocytogenes* can shed the bacterium in their milk (Borucki et al. 2004), uterine discharges (Dennis, 1963) as well as faeces (Esteban et al. 2009). Although there are several sources of contaminations of *L. monocytogenes* within the pork value chain, natural environments in farm serve as principal source of meat contamination (Kim et al. 2012; Piet et al. 2016). Humans are infected by *L. monocytogenes* mostly through consumption of contaminated food of animal origin such as meat products (Thévenot et al. 2006), milk (Dalton et al. 1997), butter (Maijala et al. 1999) and cheese (Koch et al. 2010).

There have been several outbreaks of listeriosis which have been associated with pork and processed pork products worldwide (Smith et al. 2019). In SA, human listeriosis outbreaks and sporadic cases are mostly associated with consumption of contaminated pork meat and/or pork based ready-to-eat (RTE) such as cold meats, sausages and other deli-style meats (Smith et al. 2019). The 2017-2018 listeriosis outbreak in South Africa was linked to delicatessen meat refered

to as polony contaminated with *L. monocytogenes* from a meat processing plant in South Africa. This resulted in about 217 deaths linked to the disease outbreak and the hospitalization of 974 individuals excluding neonates. The human listeriosis outbreak in South Africa has negatively impacted the economy of the country placing a financial burden on the healthcare system as well as on the implicated industries. (Olanya et al. 2019). The global human infection of *L. monocytogenesis* is associated with a case fatality rate of 20 - 30% in high-risk groups of people (the elderly, infants and immunocompromised) and a hospitalisation rate of over 95% (de Noordhout et al. 2014).

The high case fatality rate of human listeriosis is exacerbated by certain strains that acquired resistance to commonly used antibiotics (Walsh et al. 2001; Safarpoor et al. 2013; Matle et al. 2019). Acquired antimicrobial resistance (AMR) strains of *L. monocytogenes* reduce efficiency of drugs that are used to control morbidity and mobility linked to severe human infections (Essack et al. 2017). This acquired resistance of *L. monocytogenes* strains is also associated with mobile elements such as prophages, transposons and plasmids. Several studies have shown *L. monocytogenes* strains containing plasmids and prophages that confer resistance to a wide range of antibiotics classes such quinolones and fluoroquinolones (Gømez et al. 2014). Antibiotics are widely used in food animals to treat and prevent infections as well as a food additive to promote growth.

Strains of *L. monocytogenes* isolated from the pig farm environment, pork meat and human clinical samples have been confirmed to be identical using pulsed-field gel electrophoresis (PFGE)

(Hellstrom et al. 2010). Furthermore, *L. monocytogenes* from agricultural soil have been found to be predominantly involved in cases of animal and human listeriosis (Kurpas et al. 2018). *Listeria monocytogenes* isolated from the tongues, tonsils and intestinal content of pigs have been linked to pig farm environment and diseases in human (Oorburg et al. 2013). The availability of data on the population structures and virulence potential of *L. monocytogenes* strains circulating in pig farms in the country is important for epidemiological purposes to determine areas in the farm that are prone to contamination by *L. monocytogenes* and prevent or reduce possible listeriosis outbreaks. Therefore, this research sought to investigate the genomic diversity of *L. monocytogenes* from the pig farm environment, pork or pork products and humans to understand their virulence potential and critical risk points within the pork value chain (Hilliard et al. 2018).

Listeria monocytogenes infections globally have been estimated to have caused over 3 million illnesses and 50,000 deaths annually (Havelaar et al. 2012). The burden of listeriosis in Africa is disproportionally high and WGS efforts however, have been concentrated in world regions that have much lower burdens of listeriosis. Even though WGS has been employed in South Africa for characterization of *L. monocytogenes* infections, these efforts have however been more centered on characterizing (e.g., sequence type investigations, virulence profiling) human clinical isolates. In South Africa, whole genome sequencing data derived non-human sources is limited. To our knowledge, the genomes sequenced in this study represent the largest collection of *L. monocytogenes* isolates genomes isolated from pig farms in South Africa.

1.1 Problem Statement

In the last few years, *L. monocytogenes* has emerged as a significant foodborne pathogen in South Africa. The pig production industry in the country was also affected by the listeriosis outbreak as pork was used in the production of some of the implicated food products (Smith et al. 2019; Thomas et al. 2020). These outbreaks pose serious challenges to public health and the economy especially because the pig production industry, contributes considerably to the growth and development plan of the country. For instance, the economic implications associated with 2017 – 2018 *L. monocytogenes* outbreaks to the pig industry include productivity losses, which were estimated at US $15 million (R212 million) (with the exchange rate being 1 United States Dollar = 14.15 South African Rands) (Olanya et al. 2019). Losses experienced by the processing industry because of cold meat recall were estimated at US $ 52. 9 million (R749. 41 million). There was also a 50% drop in consumer demand for pork and pork-based products, which impacted pig farmers as they experienced a 20 - 40% drop in pork prices. The export of pork processed meat products was suspended because of the outbreak resulting in an estimated loss of US $ 11. 3 million (R160. 08 million) in export revenue (Olanya et al. 2019). There was also a decline in the demand for pork meat and pork products and small businesses selling pork-based products such as cold meats also suffered a decline in income. Apart from economic losses, over 217 people died from the same outbreak with 42% of the cases being neonates who were infected during pregnancy or delivery. The cost evaluation of these deaths was US $260 million (R3. 7 billion), with hospitalisation costs estimated at US $10.4 million (R147 million) (Olanya et al. 2019).

The advent of antimicrobial resistance especially among bacteria that are pathogenic to humans poses a serious global threat. In the recent years, there have been several studies that have reported an increase in resistance levels amongst *L. monocytogenes* isolates which can have serious implications with regards to treatment of listeriosis infections due to this developing resistance to key antibiotics. However, the information of the AMR pattern and prevalence of *L. monocytogenes* has not fully been studied in the country. Furthermore, should this resistance to key antimicrobials persist, it will pose a serious challenge to human, animal and environmental health. The dissemination of antimicrobial resistant isolates through contaminated food is also more likely to increase as food animals reperesent a primary vector for transmission to humans (Gømez et al. 2014).

In South Africa, the bulk of *L. monocytogenes* infections with regards to whole-genome sequencing (WGS) efforts have focused on characterizing strains from human clinical isolates which have been associated with illnesses and/or outbreaks (Smith et al. 2017; Thomas et al. 2020). Whole genome sequencing based studies querying *L. monocytogenes* strains from farms in South Africa are limited (Matle at al. 2020), thus not much is known with regards to the lineages that are circulating in farms, animal products and animlas in the country. Whole genome sequencing has been increasingly employed to characterize *L. monocytogenes*, as it does not only replicate many important microbiological assays in silico (e.g., AMR, prediction of serotype), but can additionaly provide data used in the characterization of isolates (e.g., core- and whole-genome multi-locus sequence typing [MLST], identification of genome-wide single nucleotide polymorphisms [SNPs] and pan-genome characterization) (Smith et al. 2017; Thomas et al. 2020). Therefore, there is a

need to use WGS in the surveillance of *L. monocytogenes* in the country in order to determine the genetic diversity of the strains circulating among pig farms in South Africa.

1.2 Importance of the study

Listeria monocytogenes is ubiquitously found in a diverse agricultural environment and is the causative agent of severe foodborne disease. In South Africa, pork meat products are common vehicles of listeriosis and pig farms act as the main reservoir of *L. monocytogenes* as it is regularly detected in the faeces of clinically healthy pigs (Sarno et al. 2015). Despite the importance of pork meat products in causing human listeriosis, data on the epidemiology and ecology of *L. monocytogenes* in a pig farm environment is lacking in South Africa. This is because of the lack of targeted surveillance of *L. monocytogenes* at farms. The recent emergence of the *L. monocytogenes* outbreak that claimed human lives has highlighted a need for the country to strengthen national foodborne disease surveillance systems as a prerequisite to prevent similar occurences in the future and also ensure a food supply which is safe for their populations. This can be achieved through a consolidated collaborative approach between institutions of public and animal health sectors with one-health disease investigation and management strategies (Thomas et al. 2020).

The position of the current study is to provide baseline data on the occurrence and population structure of *L. monocytogenes* in commercial pig farms using WGS. Furrhermore, the use of WGS will assist in understanding the biology, phylogeny and ecology of *L. monocytogenes* contamination along the food value chain. This study will provide data that can close the gap that

exists in epidiomology and ecology of *L. monocytogenes* at pig farm primary food production level and contribute to building a much-needed national food safety and disease surveillance systems in the country. The data from our study will therefore provide baseline data that demonstrate the population structure of *L. monocytogenes* at pig farms. This data will contribute to (i) rapid detection and investigation of listeriosis outbreaks associated with pork products, (ii) help monitor trends of *L. monocytogenes*over over time in selected geographical areas, (iii) help determine control programme priorities in pig farms, (iv) help determine control programme effectiveness and (vi) determine which subtypes of *L. monocytogenes*are are prone to cause disease in humans. This information is needed to strengthen national foodborne disease surveillance systems in the country and possibly reduce or prevent deadly listeriosis outbreaks in the future.

1.3 Aim, Objectives and Hypothesis

The main aim of this study was to investigate the population structure and antimicrobial resistance profiles of *L. monocytogenes* strains isolated from commercial pig farms in five selected provinces of South Africa using whole genome sequencing technology.

The specific objectives of this study were:

a. To investigate the genomic lineages, molecular serogroups and clonal complex (CCs) of *L. monocytogenes* in pig farms from selected provinces of South Africa.

b. To investigate both the phenotypic and molecular anitimcrobial resistance profiles among *L. monocytogenes* isolates recovered in pig farms in five provinces of South Africa.

c. To investigate antibacterial biocide and heavy metal resistance genes in *L. monocytogenes* isolates in pig farms in five provinces South Africa

d. To determine prophages and plasmids in *L. monocytogenes* isolates from pig farms in South Africa.

e. To investigate the virulence genes and pathogenicity islands associated with *L. monocytogenes* in pig farms

f. To determine stress survival islet markers in *L. monocytogenes* isolates of South Africa

The hypotheses were:

a. It was hypothesized that the *L. monocytogenes* strains sequenced here will exhibit heterogeneous genomic characteristics as well as AMR patterns and prevalence.

1.4 Dissertation layout

This study comprises six chapters organised as follows:

Chapter 1: Introduction: Chapter 1 provides a brief background and an overview of the research which includes the problem statement, aim of the study, objectives and the importance of our study.

Chapter 2: Literature review: This chapter summarises existing literature on the prevalence of *L. monocytogenes* in pig and pork production value chain. It describes the global distribution of *L. monocytogenes* in humans and animals and gives a synopsis on various methods for isolation and subtyping of *L. monocytogenes* for epidemiological purposes.It also provides an overview of the antimicrobial profile and virulence traits of *L. monocytogenes* in humans.

Chapter 3: Research methodology: This chapter provides details on methods used for isolates selection, experiments and data analysis.

Chapter 4: Results: This chapter provides the collected results and its detailed description.

Chapter 5: Discussion: This chapter provides the explanation and interpretation of the results based on previous studies.

Chapter 6: Conclusion and recommendations: This chapter summarises the research findings based on the objectives, hypothesis and recommendations to the research problem.

CHAPTER 2: LITERATURE REVIEW

2.1 Characteristics of *Listeria monocytogenes*

Listeria monocytogenes is a saprotrophic bacterium that belongs to the genus *Listeria* which belongs to the phylum of Fermicutes that includes Gram-positive bacteria belonging to the genera *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus* and *Staphylococcu*s (Jamali et al. 2015). *Listeria monocytogenes* is a facultative anaerobic bacterium; it is catalase positive and oxidase and spot indole negative. It is motile at temperatures ranging between 20 and 25 ˚C because of peritrichous flagella but non-motile above 28 ˚C (de Vasconcelos Byrne et al. 2016). It has mechanisms that allow it to survive under high salt concentrations (10%), a wider range of pH (4.5 – 9.0) and a temperature range of 0 – 45 ˚C (de Vasconcelos Byrne et al. 2016). These properties make it possible for *L. monocytogenes* to be ubiquitous prevalent and has been reported in a variety of sources including water, soil, plants, vegetables, faeces, vegetables, dairy products, meat, animals and humans (Akrami-Mohajeri et al. 2018).

Listeria monocytogenes is grouped into 13 different serotypes which are: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e and 7. Out of these serotypes, only 1/2a, 1/2b, 1/2c and 4b are associated with diseases in animals and humans (Jamali et al. 2015). Serotype 1/2a is reported to be predominant in environmental and human isolates while 4b is linked to several human fatal listeriosis outbreaks (Chen et al. 2017). Serotypes 1/2b and 1/2c have shown an over-representation from food isolates and they have also been an increase of their presence in clinical cases (de Vasconcelos et al. 2008). The 13 serotypes of *L. monocytogenes* are further subdivided into six

molecular serogroups which are: IIa (1/2a, 3a), IIb (1/2b, 3b, 7), IIc (1/2c, 3c), IVa (4a, 4b), IVb (4ab, 4b, 4d, 4e), IVb-1 (atypical of IVb group) and L (Wieczorek and Osek, 2017).

Listeria monocytogenes is a highly heterogeneous bacterium that is grouped into four phylogenetic lineages (I - IV), seven epidemic clones (ECI-ECVI), multiple clonal complexes (CCs) and sequence types (STs) according to mutlilocus sequence typing (MLST) (Maury et al. 2019). *Listeria monocytogenes* strains belonging to lineage I comprised serogroups IIb and IVb and lineage II comprised of serogroups IIa and IIc are responsible for over 98% of human listeriosis cases worldwide (Lomonaco et al. 2015). Multilocus sequence typing is a technique used to analyse nucleotide sequence data from several conserved (usually 7) housekeeping genes to derive a combination of alleles known as a Sequence Type. The use of MLST in the present study has assisted in determining the lineages and CCs of *L. monocytogenes*. Clonal complexes are defined as a group of STs differing by only one allele from at least one other ST in the group regardless of its involvement in outbreaks. Different STs and CCs have been the cause of listeriosis infections in humans and animals (Smith et al. 2019).

The genome of *L. monocytogenes* is a circular chromosome with a size varying between 2.7 and 3.0 Mb (Hain et al. 2012). The nitrogenous bases of a DNA molecule are bound by hydrogen bonds and are Guanine or Cytosine (G-C) and Adenine or Thymine (A-T). The G-C pair is bound by three bonds, whereas A-T pairs are connected by two bonds (Schmitz-Esser et al. 2015). The G-C base pairs are more stable under higher temperatures than A-T base pairs, although the G-C content does not play a role in the stability of DNA. The percentage of these base pairs may refer to the

entire genome or a specific fragment of DNA or RNA, that is, a fragment of the genetic material that is part of a single gene, gene clusters or even a non-coding region. The genomic G-C content of *L. monocytogenes* is low, ranging between 36 – 39% (Hain et al. 2012). *Listeria monocytogenes* have a highly conserved but open pan-genome permiting limited integration of foreign chromosomal DNA (called mobile genetic elements) such as bacteriophages, plasmids, transposons, genomic islands and insertion sequences, which contribute to genome diversity through mutation, recombination and duplication (Kuenne et al. 2013). *Listeria monocytogenes* genomes contain some accessory genes in a highly variable chromosomal region or hypervariable hotspots (Kuenne et al. 2013).

2.2 *Listeria monocytogenes* in animals and farm environments

Listeria monocytogenes is is primarily an environmental bacterium that is associated with various natural niche such as soil, decaying vegetation, faeces and water. Many studies suggest that animals in the farms get *L. monocytogenes* through their interaction with these natural environments (Nightingale et al. 2004; Sauders et al. 2012). To support this notion, a study by Sauders et al. (2012) showed a prevalence of 8.7 % to 51.4 % for *L. monocytogenes* in soil samples from agricultural sites. This study also suggests that agricultural soil is a primary reservoir of *L. monocytogenes* as this bacterium can survive for years and even proliferate in this environment. Hellstrom et al. (2010) found genotypes of *L. monocytogenes* isolated from pig farms similar to those isolated from carcass samples. The same genotypes were also isolated in cut meats of the carcass indicating spreading of *L. monocytogenes* from the farm to the animal. Agricultural recycling of organic waste and fecal waste used as fertilizer can also be a source of contamination

of soil with *L. monocytogenes* (Vivant et al. 2013). Agricultural soil, therefore, plays a pivotal role in the transfer of *L. monocytogenes* to plants and animals and subsequently into the food chain (Kurpas et al. 2018). Furthermore, Korthals et al. (2008) suggested that the animal could acquire *L. monocytogenes* through bio-arosals generated from soil dust in the farm environment. Factors such as natural water and wastewater sources are also reported as a source of *L. monocytogenes* in farms. Many studies isolated *L. monocytogenes* in large numbers from natural water and wastewater sources near farming communities which serve as a source of animal contamination (Nightingale et al. 2004; Lyautey et al. 2007; Vivant et al. 2013; Linke et al. 2014).

2.3 Feed as a source of *Listeria monocytogenes* contamination in animal

Another important source of *L. monocytogenes* contamination in livestock animals is through consumption of feed particularly silages (Harakeh et al. 2009). Several studies have linked silages to many listeriosis outbreaks in different animals such as cattle, goats, pigs and sheep (Beloeil et al. 2003; Nightingale et al. 2004). Silage is a fermented preserved feed stored in a silo or airtight container used to feed animals in times of forage shortage due to seasonal changes during dry seasons (Nightingale et al. 2004). A silage that is therefore not well prepared can easily get contaminated supporting the growth of *L. monocytogenes* and can subsequently infect animals (Nightingale et al. 2004). In pig farms, there is also an association of animal infections with the eating of contaminated sausages and vegetables (Chen et al. 2019). Infected animals mostly pigs may be asymptomatic carriers of *L. monocytogenes* (Kanuganti et al. 2002). Faeces shed by both asymptomatic carriers and sick animals serve as a source of infection in ruminants and the cause of reintroduction of *L. monocytogenes* to the environment (Lekkas, 2016). Furthermore,

asymptomatic carrier animals are thought to transmit *L. monocytogenes* to humans through food as demonstrated by the molecular fingerprinting method (Piet et al. 2016).

2.4 Other sources of animal contamination by *Listeria monocytogenes*

Poor animal husbandry, lack of biosafety practices and people going in and out of the farm; have been reported as sources of *L. monocytogenes* in the farm. Furthermore, a wide range of environmental effects associated with global warming and climate change can play a vital role in the change of epidemiology and spread of *L. monocytogenes* in the farm (Chersich et al. 2018). For example, extreme temperatures associated with climate change are said to augment the replication cycle of *L. monocytogenes* and could cause a rapid rise in several bacteria in the environment (Chersich et al. 2018). Altered rainfall patterns and longer dry seasons can also influence the occurrence and spread of *L. monocytogenes* in the environment (Chersich et al. 2018).

2.5 Transmission of *Listeria monocytogenes* from the farm environment to animals

Farm environment and animal feed are considered an important source of contamination of *L. monocytogenes* in meat and meat products (Carpentier and Cerf, 2011). Food animals can harbour *L. monocytogenes* on their hide, intestine, external surface and hooves as a result of environmental contamination (Bolder and Mulder, 2007). The deep muscle tissues of healthy slaughtered animals contain very little if any bacteria at all. Processing at abattoirs can cause contamination of meat, particularly during dressing (removing of hooves and hides) and evisceration because of bacterial overload on these areas (Bolder and Mulder, 2007). Therefore, animals presented for slaughter at

the abattoir are the primary vehicle for initial contamination of meat with *L. monocytogenes* (Bolder and Mulder, 2007). In a study by Autio and co-works (2000), subtyping *L. monocytogenes* isolates from the pig farm and carcasses at meat processing facilities revealed a similar pulsotypes pattern. This suggested that *L. monocytogenes* from the pig farm had contaminated the carcasses as they were being processed . Besides animals, *L. monocytogenes* can also enter meat-processing facilities via raw material and people (Thévenot et al. 2006). Contamination of floor, floor drains and racks from animal waste has been reported as other sources of contamination of meat-processing facilities which subsequently get in the meat (Thévenot et al. 2006). Upon entering the abattoir, it is difficult to eradicate *L. monocytogenes* if proper monitoring and hygiene programmes are not implemented (Carpentier and Cerf, 2011). The survival of *L. monocytogenes* in the meat processing facilities is influenced by several complex factors such as their ability to grow under a tough condition like low temperature, extreme pH conditions and high salt concentrations (Carpentier and Cerf, 2011). Once *L. monocytogenes* contaminates the meat processing environment, it is persistent and can contaminate and re-contaminate carcasses, which subsequently cause listeriosis in humans.

*Listeria monocytogene*s has been reported on meat and meat products from retail outlets and in the retail environment (Sauders et al. 2016). *Listeria monocytogenes* has also been present in floors, drains, sinks, gloves and hands of food handlers in retail environments posing as a source of contamination to meat at the retail level (Ferreira et al. 2014). Most human listeriosis cases reported worldwide were because of the consumption of meat products (Ferreira et al. 2014). The association between *L. monocytogenes* and livestock makes this bacterium on raw meat almost

unavoidable. The major factor responsible for the occurrence of contaminating pathogens in meat products is cross-contamination during the post-processing operations (e.g. slicing, chopping) at retail outlets (Brasileiro et al. 2016). Meat products particularly RTE become contaminated with *L. monocytogenes* before heat treatment and poses a great public health threat (Brasileiro et al. 2016). Ready-to-eat meat products consumed directly after purchase poses a high risk for consumers, especially if there is no further treatment such as cooking in reducing *L. monocytogenes* contamination (Jamali et al. 2013).

2.6 Listeriosis in animals and humans

2.6.1 Animals

Listeria monocytogenes affects many animal species and a large number of these animals are asymptomatic carriers shedding the bacterium in their feces. Healthy animals can also harbour *L. monocytogenes* (Nightingale et al. 2004) and subsequently cross-contaminating the rest of the carcass during slaughter. Listeriosis mostly affects ruminants causing meningititis encephalitis and uterine infections and on rare occasions septicemia (Hyden et al. 2016). The encephalitic form of the disease is commonly associated with neurological signs such as circling movements, excessive salivation and facial paralysis (Hyden et al. 2016). The occurrence of uterine infections usually results in late-term abortions (Nightingale et al. 2004). The septicemic form of the disease which rarely occurs is characterised by fever, depression and even death. Other clinical signs are infections of the eye, appetite loss and a decrease in activity (Kanuganti et al. 2002). In pigs, *L. monocytogenes* primarily presents as septicemia and piglets easily succumb to infection (Stein et al. 2018)). In pig neonates, *L. monocytogenes* can originate from the tonsils of a sow and enter the

intestinal tract of the piglet and becoming systematic (Rahman et al. 2002). Central nervous symptoms may also be observed in piglets which include in-coordination and progressive weakness which is usually followed by death. In sows, infection with *L monocytogenes* is characterised by abortions, stillbirths and an increase in weak piglets (Stein et al. 2018). Encephalitis because of infection with *L. monocytogenes* in pigs can sometimes occur which begins with a sudden refusal to eat and followed by central nervous signs which include incoordination, circling movements, partial paralysis and convulsions (Rahman et al. 2002)

2.6.2 Humans

Listeria monocytogenes cause two forms of listeriosis in humans: invasive and non-invasive infections (Buchanan et al. 2017). The non-invasive form of listeriosis can occur in healthy individuals as gastroenteritis and is a mild disease with symptoms like fever, vomiting, and diarrhoea (Vázquez et al. 2001). The invasive form of listeriosis can cause serious infections that affects immunocompromised individuals such as people with underlying diseases, elderly people and infants (de Noordhout et al. 2014). These high-risk groups of individuals may suffer severe illnesses like septicaemia, meningitis, meningoencephalitis and pregnant women may even suffer miscarriage or preterm birth (Vázquez et al. 2001). Although the invasive form of listeriosis occurs rarely, it has high mortality rates (20 - 30%), making it a great health threat (Zuber et al. 2019).
The clinical manifestation of both invasive and non-invasive listeriosis depends on several factors, which include the age and immune status of the person, mode of infection, virulence potential of ingested serotypes and number of viable cells ingested (Angelo et al. 2017). Buchanan and co-works (2017) showed that the incubation period of listeriosis varies significantly (1 - 70 days) and it can be long, thus making source tracing very difficult. Several studies suggest that incubation

periods of listeriosis are largely influenced by clinical manifestation, with the longest periods reported from pregnancy cases and central nervous system infections (Gilmour et al. 2010).

There has been a rise in human listeriosis outbreaks associated with pork and pork products that have been reported worldwide. Table 1 below gives an overview of the human lisiterisis linked to pork amd pork products during 1900 – 2018. The first laboratory-confirmed human listeriosis cases linked to pork meat products occurred in 1988 as a result of the consumption pork tongue contaminated by *L. monocytogenes* (Kanuganti et al. 2002). Since then, several other fatal human listeriosis outbreaks or sporadic cases involving different pork products such as pork tongue (De Valk et al. 2005) sausages (Winter et al. 2009) and ham (Jadhav, 2015) have been reported, with the largest documented outbreak occurring in South Africa between 2017 and 2018 as a result of polony. (Jensen et al. 2016; Smith et al. 2019).

Table 1: Human listeriosis cases linked to pork and pork products

Year	**Area**	**Pork related food type**	**No. of cases(deaths)**	**Serotypes**	**Reference**
1900	Australia	processed pork meat	9(6)	ND*	Watson et. al 1990
1992	France	jellied pork	279(85)	4b	Jacquet et al. 1993
1998-1999	United States	hot dogs	108(14)	4b	Jadhav, 2015
1999-2000	France	Rilletes	10(3)	4b	De Valk et al. 2005
1999-2000	France	pork tongue	32(10)	4b	De Valk et al. 2005

2000	New Zealand	deli meats	30	1/2a	Sim et al. 2002
2001	United States	deli meats	16	1/2a	Frye et al. 2002
2006-2007	Germany	scalded sausage	16	4b	Winter et al. 2009
2011	Switzerland	cooked ham	6	1/2a	Jadhav, 2015
2013-2014	Denmark	pork sausages	41(17)		Leong et al. 2016
2017-2018	South Africa	Polony	1036(216)	4b	Smith et al. 2019

*ND: no data

2.7 Treatment of human and animal listeriosis

2.7.1 Animals

Using antibiotics is the most viable solution when treating listeriosis infection in animals. The most commonly used antibiotics to treat listeriosis in animal includes oxytetracycline penicillin, ceftiofur, erythromycin and trimethoprim (OIE, 2014). This therapy works better at high drug dosage and in animals with early forms of the disease (Luque-Sastre et al. 2018). The duration that the antibiotic is administered to animals varies depending on the severity of the infection. (Luque-Sastre et al. 2018). Goats and sheep are highly susceptible to listeriosis and usually, when they display an acute form of the disease, death occurs in 4 – 48 hrs. Pigs and cattle have more tolerance to the disease with a survival of 4 – 14 days. Dexamethasone and chlortetracyclin are also considered very effective and beneficial in treating encephalitis but can cause abortions especially in cattle and sheep (Chopra et al. 2015; Dhama et al. 2015). Gentamicin has also been found effective for genital listeriosis in animals (Chopra et al. 2015). Ensuring clean animal housing,

administering fluids and electrolytes have been shown to minimise infection spread in listeriosis cases which are not severe (Luque-Sastre et al. 2018). In outbreaks, infected animals are segregated and use of silage which may have been the source of infection is discontinued (Dhama et al. 2015).

2.7.2 Humans

In general, human listeriosis is treated using beta-lactam antibiotics such as amoxicillin, penicillin or ampicillin together with aminogylcosides such as gentamycin. Individuals having allergic reactions to penicillin are treated with trimethoprim-sulfamethoxazole and patients not responding accordingly to standard antibiotic treatment are treated using tetracycline and erythromycin (Wilson et al. 2018). In certain cases, where there is less sensitivity to these drugs, other antimicrobials that can act against Gram positives are used (de Vasconcelos Byrne et al. 2016). Listeriosis does not have specific and distinguishable symptoms; it becomes difficult to initially treat it with appropriate antimicrobials (Hof, 2004).

2.7.3 Antimicrobial resistance in *Listeria monocytogenes*

Antimicrobial resistance of *L. monocytogenes* has been accelerated by the over-prescrption of antbiotics in veterinary medicine as well as their heavy use in livestock farms as growth promoters (Moreno et al. 2014). Some studies have reported *L. monocytogenes* strains resistant to first line antibiotics. For example, resistance of *L. monocytogenes* to gentamicin was reported in clinical strains in a study by Walsh et. al 2001, while Verraes et al. 2013 reported *L. monocytogenes* strains that were resistant to ampicillin. Listeria reistant to erythromycin, kanamycin, rifampin, sulphonamides and streptomycin have also been reported in clinical isolates from diffrenet countries (Moreno et al. 2014; Zhang et al. 2004).

Antimicrobial resistance of *L. monocytogenes* can be acquired through genetic elements. These elements include; self-transferable plasmids, mobilizable plasmids, and conjugative transposons (Moreno et al. 2014).

2.8 Socio-economic implications associated with human listeriosis

2.8.1 Impact of human listeriosis globally

In 2011, the USA reported a listeriosis outbreak that was associated with contaminated cantaloupes which infected 47 people and killing 33 (McCollum et al. 2013). It costed the USA government $35 million in food recall as cited by Desai et al. (2019). Another listeriosis outbreak that resulted in a 20-40% case fatality rate was reported in Canada in 2008 (Thomas et al. 2015). The outbreak was linked to delicatessen meat, which originated from an RTE meat processing plant resulting in 57 cases and 24 deaths. The estimated costs incurred by the Canadian government because of food recall and testing was 242 million Canadian dollars. The cost of losing life alone associated with this outbreak was estimated at $2.8 million (Thomas et al. 2015). In Europe, the biggest listeriosis outbreak was reported in Italy in 1997 (Tambo et al. 2018). The 1997 Italian outbreak was linked to cold corn and tuna salad and resulted in 1 500 confirmed cases with children being the most infected (Tambo et al. 2018). The cost per patient because of foodborne pathogens in Europe is highest in patients infected with listeriosis as compared to other foodborne pathogens (Mangen et al. 2015). In the Netherlands alone, it estimated that the cost of illness because of listeriosis was at an average cost of € 2.2 million per year (Mangen et al. 2015).

An outbreak in New Zealand involving corned silverside and ham from the same manufacturer reported 23 listeriosis cases. Of the 23 cases, 7 people were reported to have consumed silverside

and 16 consumed ham (Gadiel, 2010). They estimated the average treatment cost of the 23 cases was estimated at $ 32,300 per case (Gadiel, 2010).

2.8.2 Impact of human listeriosis in South Africa

Before the year 2017, there were a few human listeriosis cases that were documented in South Africa (Van Vollenhoven, 1999). However, in 2017, the country experienced the largest global outbreak of human listeriosis which infected over 1000 people and killing 217 with 27% case fatality rates (Thomas et al. 2020). The majority of cases and deaths were recorded in Gauteng (59%), Western Cape (12%), and KwaZulu-Natal (7%) provinces of South Africa (Smith et al. 2019; Olanya et al. 2019). Through WGSs, the source of the outbreak was traced to two cold meat production facilities located in Germiston (Gauteng province, South Africa) and Polokwane (Limpopo province, South Africa). The strain that was responsible for about 90% of outbreak cases was identified as sequence type 6 (ST6) (Chersich et al. 2018; Smith et al. 2019; Olanya et al.2019). The impact of the 2017-2018 listeriosis on the health system was enormous as the number of hospitalisations and mortality cases was greater as compared to other countries (Olanya et al. 2019). Various costs were incurred by the government and food industry which includes medical costs, food recalls and litigation costs amounting to a huge burden to the economy of the country. The pork industry in South Africa contributes about 2,15% to the agricultural economy and the industry is divided into 2 branches where 45% of pigs are produced for and sold to the fresh market while 55% is sold to the processed meat market (DAFF, 2019). This industry suffered a decline because of food recalls and the ban of processed meat imports from South Africa. The estimated costs associated with the ban of exported processed meat was US $ 7.8 million (Olanya et al. 2019).

2.9 Pathogenesis of *Listeria monocytogenes*

Listeria monocytogenes possess various virulence factors that help them to invade host cells, replicate in the host cell cytosol and spread to various cells. The virulence factors of *L. monocytogenes* are located at four different pathogenicity island designated as 1 to 4 (Chen et al. 2017). Pathogenicity island-1 harbour the most important virulence factors that are associated with disease-causing in a host and they include internalin, phosphatidylinositol–specific phospholipase (Gilmour et al. 2010), Listeriolysin O, actin polymerization protein and metalloprotease (Gilmour et al. 2010). These virulence factors are critical for *L. monocytogenes* to; adhere and invade host cells, cause lysis of host vacuole, for intracellular multiplication as well as for cell to cell spread (Vázquez–Boland et al. 2001) (Figure 1). Pathogenicity islands 2 - 4 often harbor factors that are linked to postintestinal infection (Vázquez–Boland et al. 2001).

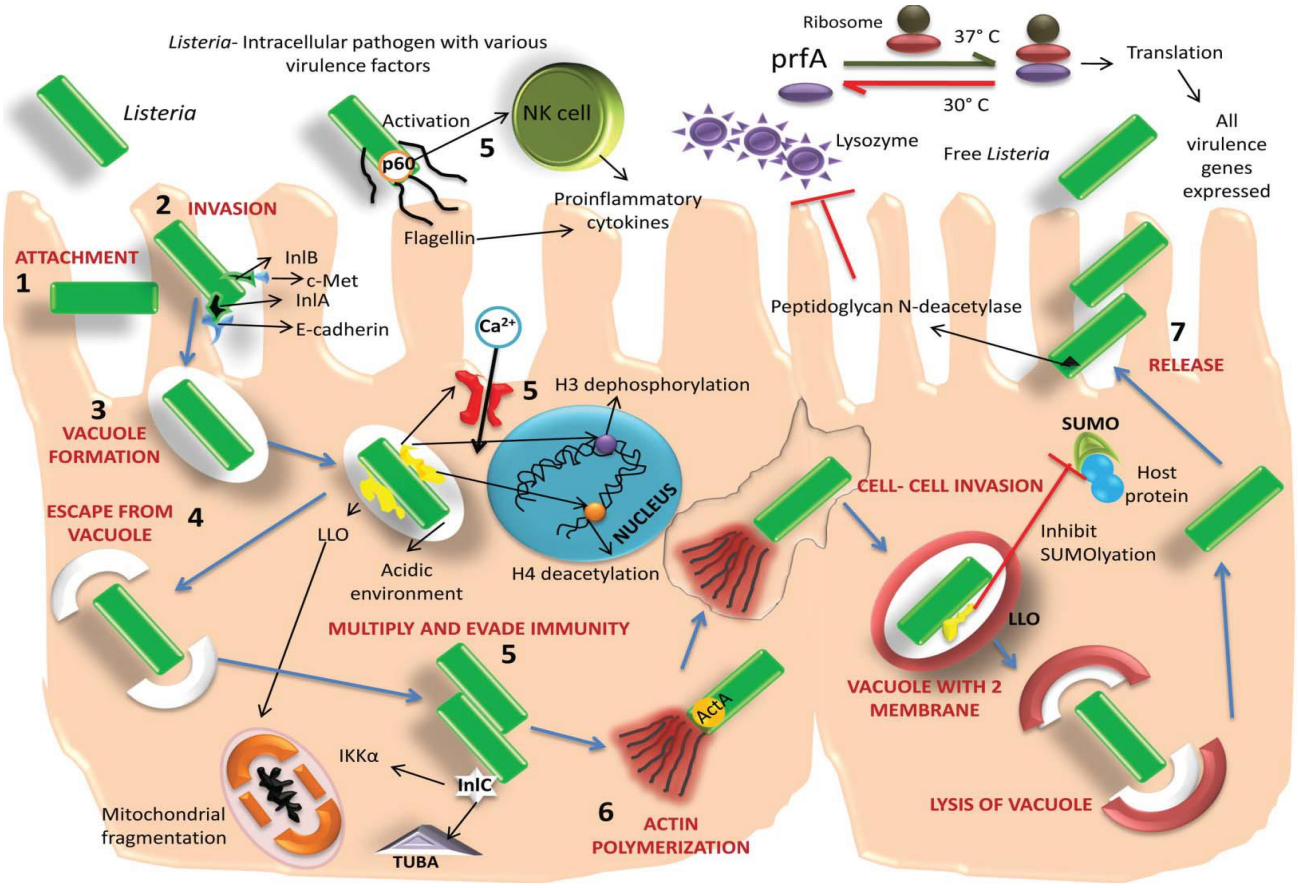

Figure 1: Schematic diagram on the pathogenesis of *Listeria monocytogenes* and its virulence factors adapted from Vázquez–Boland et al. (2001).

2.9.1 Adhesion and Invasion

The first step in the intracellular life cycle of *L. monocytogenes* is adhesion and invasion of the host cell which is facilitated by two subfamilies of internalin proteins namely InlA (70 - 80kDa) and InlB (25 - 30kDa) (Vázquez–Boland et al. 2001). These subfamilies of internalin proteins are also responsible for attachment of the bacterial cell to facilitate adhesion to the host and internalisation into a membrane bound vacoule (Vázquez–Boland et al. 2001). Internalin A facilitates the binding of *L. monocytogenes* with the protein E-cadherin for the invasion of epithelial cells (Vázquez–Boland et al. 2001). The binding of the bacterium with the E-cadherin protein promotes local cytoskeletal rearrangements in the host cell-stimulating uptake of *L. monocytogenes* by the epithelial cells. Internalin B facilities binding of the cellular receptor Met, which is a tyrosine kinase protein (Dhama et al. 2015). This binding allows the bacterium entry into the hepatocytes, fibroblasts and epithelial cells. The protein P60 is an invasion associated protein (Quendera et al. 2016) and is involved with the adherence of *L. monocytogenes* to the host cell (Quendera et al. 2016).

2.9.2 Lysis of host vacuole membrane

Once the *L. monocytogenes* has entered the host cell it can be entrapped into the vacuole membrane. It then utilises both the Listeriolysin O (LLO) and phosphatidylinositol phospholipses (Plc's) to destroy the vacuole membrane and escape into the cytoplasm. Failure to break the membrane will cause *L. monocytogenes* to be removed from the tissues (Pushkareva and Ermolaeve, 2010).

2.9.3 Intracellular multiplication

Once lysis of the membrane vacoule has taken place and *L. monocytogenes* has escaped into the cytoplasm; it proliferates before spreading to other cells (Dhama et al. 2015). This process is facilitated by several protein factors that have not been regarded as major virulence proteins. The Hexose phosphate translocase and lipoate protein ligase (Dhama et al. 2015) as well PlcA and PlcB have been identified as critical during the proliferation of this bacterium in the cytoplasm.

2.9.4 Cell to cell spread

After multiplication, the bacterium then moves to infect other cells with the help of surface proteins called actin polymerisation protein (ActA), phosphatidylinositol – specific phospholipase (PC-PLC) and zinc metalloprotease (Mpl). The actin polymerisation protein induces polymerization of globular actin molecules to form actin filaments. These actin filaments facilitate the movement of the bacterium both inter and intracellularly. Genes, which encode for actA, PC-PLC and Mpl are *actA, plcB,* and *mpl* respectively, are regulated by a positive regulatory factor A (prFA) (Dhama et al. 2015; Pushkareva and Ermolaeve, 2010).

2.10 Phenotypic methods of identification for *Listeria monocytogenes*

Culture-based methods are highly recommended for routine diagnosis of *L. monocytogenes* from environmental and food samples (Fox et al. 2016). There are different available culture-based methods for the isolation of *L. monocytogenes*. However, they follow a similar principle of sample enrichment in selective broth media and then plating on selective agar. Confirmation of presumptive culture colonies on agar is done through phenotypic identification using tests such as Gram staining, biochemical reactions and Christiee-Atkins-Munch-Peterson (CAMP) test (Piet et

al. 2016). Only four of these methods are internationally recognised for trading purposes. These methods include international organisation standards (ISO 11290), food and drug administration (FDA), food safety inspection services in the United States department of agriculture (USDA-FSIS) and the *Listeria* Precis method (Piet et al. 2016). These four internationally recognized methods entail, the primary and secondary enrichments of the samples in selective broth media followed by plating onto selective or differential media. These enrichment media contain different selective and enrichment supplements such as; acriflavine, ceftazidime, cefotetan, colistin, cycloheximide, fosfomycin, lithium chloride, moxalactam, nalidixic acid, phenylethanol and polymyxin B and (Jadhav, 2015). The purpose of these agents and antibiotics in the media is to inhibit the growth of Gram-negative bacteria that are usually present in food samples. The mechanism of action of these agents also varies, for example; cycloheximide inhibits protein synthesis while colistin disrupts the outer cell membrane of bacteria.

2.10.1 International Organization Standards (ISO 11290)

A food or enviromental environmental sample (25 g or 25 ml) is pre-enriched in 225 ml of half Fraser broth. The broth culture is then incubated for 24 hrs at 30 ˚C. A volume of 100 µl of the pre-enriched broth is then inoculated into 10 ml of Fraser broth and incubated for 24 hrs at 30 ˚C. A 10 µl of the enriched broth is streaked on the surface of Ottaviani and Agosti agar (ALOA) containing selective supplements and enrichment supplement as well as onto *Listeria* selective agar containing listeria selective supplement. Both cultures are then incubated at 37 ˚C for 48 hrs. Presumptive colonies of *Listeria* species are then determined and further biochemical tests are done for confirmation. Typical *Listeria* species colonies are blue-green with a halo on ALOA agar. On *Listeria* selective agar, presumptive *Listeria* colonies are brown in color surrounded by black

zones. (Law et al. 2015; Välimaa et al. 2015)

2.10.2 Food and drug administration (FDA)

A food or envirometal sample (25 g or 25 ml) is added into 25ml of Buffered *Listeria* Enrichment Broth (BLEB) containing nalidixic acid and acriflavine antibiotics, and incubated at 30 ˚C for 48 hrs. The BLEB cultured samples are then inoculated onto polymyxin – acriflavine-LiCl-ceftazidime-aesculin-mannitol agar (PALCAM) containing PALCAM selective supplement, with presumptive *Listeria* colonies appearing after 48 hrs incubation at 37 ˚C. Typical colonies of *Listeria* species are grey-green in color with a black sunken centre and a black halo (Zunabovic et al. 2011; Law et al. 2015; Välimaa et al. 2015).

2.10.3 Food safety inspection services in the United States Department of Agriculture (USDA-FSIS)

A 25 g or 25 ml of sample is pre-enriched in 225 ml of *the Listeria enrichment broth University* of Vermont-I (UVM I formulation) with *Listeria* primary selective enrichment supplemet and incubated for 24 hrs at 30 ˚C. One loopful (10µl) of the enriched broth is streaked on the surface of a *Listeria* chromogenic agar base and a second selective media such as *Listeria* selective agar which are incubated for 48 hrs at 37 ˚C. For secondary enrichment, 100µl of the enriched culture (UVM -I) is inoculated into 100 ml of UVM- II with *Listeria* secondary selective enrichment supplement. The plates are then incubated for 24 hrs at 30 ˚C. After incubation, a loopfull of the enriched culture is streaked on the surface of a *Listeria* chromogenic agar base and a second selective media and incubated for 48 hrs at 37 ˚C. Presumptive colonies are then determined and further biochemical tests are done for confirmation. Typical colonies of *Listeria* species are blue-

green with a halo on *Listeria* chromogenic agar and brown colored with a black zone on *Listeria* selective agar (Law et al. 2015; Välimaa et al .2015).

2.10.4 *Listeria* Precis method

Listeria Precis method has been approved by Association Françoise de Normalisation (AFNOR) as an alternative method to ISO 11290 because it gives results in only two days as compared to the other three methods which require 4 - 5 days (Leong et al. 2017). A sample (25 g or 25 ml) is pre-enriched in 225ml of One broth *Listeria* and incubated at 30 ˚C for 24 hrs. One loopful (10 µl) of the enriched broth is streaked on the surface of a Brilliance *Listeria* agar with brilliance listeria selective supplement and brilliance listeria differential supplemet. This is followed by incubation for 24 hrs at 37 ˚C. Presumptive colonies are then determined and further biochemical tests are done for confirmation. Typical colonies of *Listeria* species are blue-green with a halo (Leong et al. 2017).

2.10.5 Confirmation of suspect colonies from culture-based methods

Confirmation of suspect colonies from the above-listed culture-based methods is often carried out through biochemical tests. Biochemical tests include ability to produce haemolysis on blood agar plates, Gram stain, catalase test, motility test, Christie, Atkins and Munch-Peterson test with *Rhodococcus equi* and *Staphylococcus aureus* and carbohydrate utilisation tests. Rapid biochemical tests such as oxoid biochemical identification systemmicrobata and analytical profile index identification systems have been developed for the identification of *Listeria* spp including *L. monocytogenes* (Leong et al. 2017).

2.11 Molecular methods of identification for *Listeria monocytogenes*

There are different polymerase chain reaction (PCR) methods for the detection of *L. monocytogenes*, which include conventional nested PCR, real time PCR, and multiplex PCR (Gasanov et al. 2005; Law et al. 2014). In general, the detection of *L. monocytogenes* using PCR is based on targeting a specific gene(s). These methods have high sensitivity and specificity whilst saving time and money (Harakeh et al. 2009). In addition, PCR methods can detect *L. monocytogenes* at low concentrations. It detects the nucleic acid composition-dependent; therefore, it can detect dead cells of *L. monocytogenes* (Harakeh et al. 2009). For these reasons, PCR has demonstrated supremacy over culture-based methods.

2.11.1 Conventional PCR

Conventional PCR is one of the most commonly used PCR methods for detecting of bacterial pathogens including *Listeria* spp (Law et al. 2015). With conventional PCR, two single-stranded synthetic oligonucleotides or specific primers are used for amplification of a target DNA sequence in a temperature-controlled 3 step cycle using a thermal cycler. These three steps include the denaturation of the double-stranded DNA into single stranded DNA, annealing of the two primers to their complementary DNA and synthesis of the DNA. The PCR amplification products are separated by agarose gel which is stained with a DNA stain and visualised on the gel as bands (Law et al. 2015). In conventional PCR, a single target of DNA can get amplified to up to 106 copies in 3 hrs. Conventinal PCR has been used to detect *Listeria* spp and also to differentiate *L. monocytogenes* from other similar species. The species specific 16S rRNA and 23S rRNA conventional PCR have previously been used in the identification of *L. monocytogenens* (Jadhav,

2015). This type of PCR has also been utilised to identify *L. monocytogenes* through detection of *hly* and *actA* virulence genes (Law et al. 2015). The main drawbacks of conventional PCR are labourers and time consuming and are also ineffective for high throughput analysis (Jadhav, 2015). This method also cannot differentiate between live or dead cells (Truter, 2015).

2.11.2 Multiplex PCR

Multiplex PCR (mPCR) is a more rapid method of detection in comparison to conventional PCR since it allows for the detection of multiple genes belonging to the same species through simultaneous amplification (Chen et al. 2017). In mPCR, multiple gene targets are amplified by using several sets of specific primers in a single reaction (Liu et al. 2006). Multiplex PCR which was developed by Doumith et al. (2004) allows subtyping of *L. monocytogenes* into four molecular serogroups (1/2a, 1/2b, 1/2c and 4b). This is achieved through simultaneously detecting different genes of each serogroup (Jadhav, 2015). As with Conventional PCR, mPCR cannot distinguish between live or dead cells (Truter, 2015).

2.11.3 Real Time PCR or Quantitative PCR (qPCR)

In Real Time PCR, a fluorescent dye, hydrolysis probe and oligonucleotide hybridisation probes are used to monitor the accumulated PCR product (Law et al. 2015). Commercially available kits have been developed for this PCR method for the detection of *L. monocytogenes*. Examples of these commercially available kits include Probelia *Listeria monocytogenes* PCR System (Bio-Rad), TaqMan *Listeria monocytogenes* Detection Kit (Applied Biosystems), LightCycler *Listeria monocytogenes* Detection Kit (Roche), GeneVision Rapid Pathogen Detection System for *Listeria monocytogenes* (Warnex) and iQ-Check *L. monocytogenes* kit (Bio-Rad Laboratories) (Law et al.

2015; Liu et al.2012). Real Time PCR does not require post PCR processing, thus minimising the risk of cross-contamination and allowing high throughput analysis (Law et al. 2015).

2.12 Subtyping methods for *Listeria monocytogenes*

Listeria monocytogenes is a genetically heterogeneous organism that has been evolutionarily divided into different serogroups, epidemic clones, lineages and clonal complexes (Chen et al. 2016). A variety of subtyping methods such as serotyping, multilocus enzyme electrophoresis (MLEE), pulse field gel electrophoresis (PFGE), random amplified polymorphic, Multiple-locus variable number of tandem repeat analysis (MLVA), Multilocus sequence typing (MLST) and Whole genome sequencing (WGS) have been developed to type this bacterium (Salcedo et al. 2003; Chenal-Francisque et al. 2011). Subtyping methods are often used for monitoring and surveillance of listeriosis outbreaks as well as for source tracking (Jensen et al. 2016). Subtyping methods are also important for determining the population genetics and taxonomy of *L. monocytogenes* and to differentiate a hypervirulent strain from hypovirulent strains (Moura et al. 2017). Because *L. monocytogenes* is an important foodborne pathogen of public health concern, subtyping of isolates from food and human cases is imperative to establish epidemiological links for surveillance or investigations of outbreaks (Gilmour et al. 2010).

2.12.4 Whole genome sequencing (WGS)

Whole genome sequencing is a molecular method that can characterise foodborne pathogens at a molecular level; by so doing, this method sheds light on the biology, transmission and distribution of the bacteria (Franz et al. 2016). Whole genome sequencing of bacterial genome differentiates strains very well and provides data regarding their phylogeny, serotype classification, virulence

and resistance profiles (Burall et al. 2016). This sequencing method is increasingly being recognised as a powerful technological tool that can be used for comparing isolates during outbreaks investigations (Kwong et al. 2016). Therefore, WGS is one of the genomic typing methods that surpasses other typing methods because it is more sensitive, specific and high resolution to cluster compared to other methods (Fagerlund et al. 2016). The WGS has also gained interest when comparing isolates from a clinical specimen with food and environmental samples. This is because WGS provides more data compared to conventional typing methods such as PFGE (Burall et al. 2016). Methods such as WGS in recent years have emerged to be preferred methods for epidemiological surveillance and investigation of outbreaks (Fagerlund et al. 2016). According to Franz et al. (2016), through WGS the whole DNA make-up of the organism is revealed; this enables extensive typing of microorganisms with higher resolution in relation to other typing methods such as PFGE and MLST. Therefore, WGS has been reported as a new gold standard for typing of *L. monocytogenes* (Lekkas, 2016).

2.12.1 Serotyping

Serotyping of *L. monocytogenes* uses a slide agglutination test that is based on somatic (O) and flagellar (H) antigens as described by Seeliger and Hohnes scheme (Camargo et al. 2016; Liu et al. 2006). Fifteen O-antigens are designated as (I-XV) and four H- antigens designated (A-D), the combination reaction between the O and H antigens differentiate *L. monocytogenes* into 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e and 7) (Camargo et al. 2016). Although this method is the first-line of subtyping *L. monocytogenes*, it is time-consuming, not user friendly and highly subjective. It also has limited discriminatory power and low reproducibility levels (Henri et al. 2016). Cross-reaction between different serotypes can occur

because of antigen sharing (Camargo et al. 2016). Animals are required for the production of the antisera and there is a scarcity of antisera manufacturers' worldwide (Hyden et al. 2016).

2.12.2 Pulse field gel electrophoresis (PFGE)

Pulse field gel electrophoresis is a method that has been used for many years to determine relatedness between isolates from different sources to identify a source of disease (Burall et al. 2016). This method has been viewed as the gold standard for subtyping *L. monocytogenes* during outbreak investigations (Martin et al. 2014). The principle of PFGE is based on breaking DNA into fragmented patterns through restrictive enzymes *Asc*I and *ApaI* (Nyarko and Donelly, 2015). Pulse field gel electrophoresis does however have limitations, which include the inability to be conclusive and provide evolutionary data on *L. monocytogenes*. Furthermore, it cannot distinguish among certain serotypes of *L. monocytogenes* (Martin et al. 2014). Pulse field gel electrophoresis is however time-consuming, it requires highly skilled personnel and precise standardization is required for inter-laboratory comparisons (Chenal-Francisque et al. 2013; Liu, 2006).

2.12.3 Multilocus sequence typing (MLST)

The multilocus sequence is a typing method that provides information on the variation in housekeeping genes in *L. monocytogenes* (Henri et al. 2016). Housekeeping genes are important genes that are required for metabolic processes and survival of the bacteria, therefore these genes are ideal to use for detection (Wu et al. 2016). Multilocus sequence typing characterizes isolates using fragments of seven housekeeping. Gene regions are sequenced and assigned an allele profile. Each isolate will be characterized by the alleles at each of the 7 loci, constituting a sequence type (Wu et al. 2016). Housekeeping genes used for *L. monocytogenes* in MLST are *abcZ* (transporter),

bglA (beta-glucosidase), cat (catalase), d*apE* (succinyl diaminopimelate desiccinylase), *dat* (D-amino acid aminotransferase), *ldh* (L-lactate dehydrogenase) and *lhkA* (histidine kinase) (Wu et al. 2016). This method detects variation that has slowly developed within the amplified housekeeping genes; this technique further allows fast and dependable comparisons of results obtained from different laboratories across the world with the use of worldwide web databases (Zhang et al. 2004; Wang et al. 2015). Multilocus sequence typing was developed to defy ambiguity in the DNA fragment-based typing method such as PFGE (Zhang et al. 2004), hence it's used as "a reference method for global epidemiology and population biology of bacteria and its applications to *L. monocytogenes* effectively allowing isolates comparison across laboratories" (Chenal-Francisque et al. 2011). Multilocus sequence typing has also been recognised as a tool to investigate the population structure of *L. monocytogenes* (Jadhav, 2015). Multilocus sequence typing can be used for researching bacterial population genetics and is therefore useful for typing *L. monocytogenes* isolates (Martin et al. 2014). Multilocus sequence typing has many advantages over other typing methods as it has allowed different laboratories to compare and exchange information on isolates. Besides, the comparison of the DNA fragments is not ambiguous and there is an electronic transfer of data of the nucleotide sequences that is also an advantage because the data is readily available for comparison between laboratories (Salcedo et al. 2003).

2.12.5 Other subtyping methods for *Listeria monocytogenes*

There are many subtyping methods which have been used to differentiate strains of *L. monocytogenes* at subspecies level (Gasanov et al. 2005; Nyarko and Donelly, 2015). These methods include; phage typing, multilocus enzyme electrophoresis, amplified fragment length polymorphism, multilocus number of variable tandem repeat analysis, random amplified

polymorphic DNA, ribotyping and repetitive extragenic palindrome PCR. Some of these methods however have several limitations and posses a low discriminatory power for surveillance of outbreak investigations. These methods were not discussed in detail in this review, and as such are listed in Table 2.

Table 2: Advantages and disadvantages of subtyping techniques used for *Listeria monocytogenes* strains

Subtyping	**Basis of discrimination**	**Advantages**	**Disadvantages**	**Reference**
Phage typing	Based on the interaction of *L. monocytogenes* with a defined set of bacteriophages resulting in host cell lysis.	This method can be used for a large number of samples	Not all strains are typable using this method; particularly serotypes 1/2. It has poor discriminatory power	Gasanov et al. 2005; Nyarko and Donelly 2015
Multilocus Enzyme Electrophoresis (MEE)	Based on the difference in electrophoteric mobility of enzymes which permits differentiation of the strains of *L. monocytogenes.*	Ease of usage Easy to interpret All strains are typable by MEE.	Poor discriminatory power Labor intensive Non-producibility in inter-lab comparisons was identified.	Jadhav ,2015; Gasanov et al. 2005; Camargo et al. 2016

Amplified fragment length polymorphism (AFLP)|	Based on the selective amplification of fragments from genomic DNA and gel analysis of the amplified products	High reproducibility High sample throughput	Complex method requiring high skilled individuals. Requires an automated sequencer.	Zunabovic et al. 2011
Multilocus number of variable tandem repeat analysis (MLVA)	Based on the analysis of the difference in the number of tandem repeats at a particular locus on a genome.		Tandem repeats do not always yield reproducible results within serotypes.	Nyarko and Donelly 2015; Zunabovic et al. 2011
Random amplified polymorphic DNA (RAPD)	PCR based method which uses a single primer to anneal to locations on the target DNA to yield a genetic profile.	Ease of usage More rapid method of subtyping Less expensive	Lower discriminatory power	Nyarko and Donelly 2015; Camargo et al. 2016
Ribotyping	Based on the digestion of chromosomal DNA using enzymes to yield smaller DNA fragments which are followed by hybridisation of ribosomal RNA.	Can be used with automation Highly reproducible Robust	Lower efficiency in differentiating strains of serotypes 1/2b and 4b.	Gasanov et al. 2005; Camargo et al. 2016
Repetitive Extragenic Palindrome (REP- PCR)	Based on the binding of short REP elements using oligonucleotide primers.	Rapid Inexpensive	Lower efficiency in differentiating strains of serotypes 1/2b and 4b.	Nyarko and Donelly 2015

2.13 Conclusion

Listeria monocytogenes is among the major foodborne bacterium globally and it has commanded majority of research and surveillance which focus mostly on food products of animal orgin. However, there is limited studies that have investigated the gemonic diversity of this bacterium on pig farms in most countries including South Africa, hence, the current study was prompted. Moreover, there is rapid increase in the technological development in methods for detection, identification and subtyping for *L. monocytogenes*. To this end, WGS has been proposed as a new gold standard for typing of *L. monocytogenes* due to its high resolution power and reduction in sequencing costs. Despite this proposal, South Africa has not fully embraced the use of WGS in non-human clinical isolates of *L. monocytogenes*. Therefore, the population structure of *L. monocytogenes* strains circulating in pig farms in the country is currenly unknown. Despite the extensive research and advancement made on this bacteria, sporadic cases and high profile outbreaks linked to *L. monocytogenes* are still being reported, and are aggravated by the high number of immunocompromised individuals in most countries due to HIV, TB, malaria and other infectious diseases associated with poverty including in South Africa. In most of these African countries that is considered to have a significant population of individuals that are immunocompromised, there is no data on the prevalence and genomic characteristics of *L. monocytogenes.* Therefore, passive surveillance systems are critical in those countries as they will not only determine prevalence, but will also be used for population structure studies of *L. monocytogenes*. The increased AMR threat associated with *L. monocytogenes* strains is mostly in line with a global pattern and prevalence of foodborne pathogens.

CHAPTER 3: RESEARCH METHODOLOGY

3.1 Sample collection

Listeria monocytogenes isolates used in this study were obtained from samples submitted at Agricultural Research Council - Onderstepoort Veterinary Research (ARC-OVR): Feed and Food laboratory in South Africa. The samples were received from veterinarians, farmers and veterinary public health officers for isolation and identification of *L. monocytogenes* during the 2017 – 2018 listeriosis outbreak. These samples were collected from various environmental sites from commercial pig farms. The Feed and Food laboratory kindly provided the *L. monocytogenes* isolates used in this study at no cost. Whole Genome Sequencing of the isolates was performed at the Agricultural Research Council - Biotechnology Platform (ARC-BTP).

3.2 Isolates used in the study

At the time of this study, the ARC-OVR: Feed and Food Laboratory had 296 isolates of *L. monocytogenes* in a depository which were isolated from various environmental samples. Of these isolates, 77 from commercial pig farms were selected for our study. The selection of isolates included farms that supply pigs to abattoirs and production facilities where the meat is converted into cold meat since the listeriosis outbreak was linked to pork products. The selected isolates were from five provinces that were regarded as high risk during the 2017 – 2018 outbreak (NICD, 2018). Notably, the laboratory did not have isolates from KwaZulu Natal which was also classified as a high-risk province. The selected provinces demonstrated a high occurrence of *L. monocytogenes* in pork and pork meat during the 2014 - 2016 national survey (Matle et al. 2019). The isolates

represent five different provinces of South Africa which includes Gauteng (n = 30), Limpopo (n = 10), Free State (n = 10), Mpumalanga (n = 12) and Western Cape (n = 15) (Figure 2). The isolates were randomly selected to include various pig farms from the provinces where possible.

3.3 Experimental procedures

3.3.1 Bacterial strains

The isolates stored in lyophilised cooked meat broth were revived by inoculation into Brain Heart Infusion (BHI) broth (Thermofisher, Johannesburg, South Africa) then incubated at 37 ºC for 18 - 24 hrs. The incubated BHI broth samples were then streaked on 5% sheep blood agar (ThermoFisher, South Africa) followed by incubation at 37 ºC for 18 - 24 hrs. To ascertain whether the revived isolates were *L. monocytogenes*, preliminary identification tests were conducted by morphological examination using Gram stain, catalase, oxidase, spot indole reactions and the macrobata identification systems.

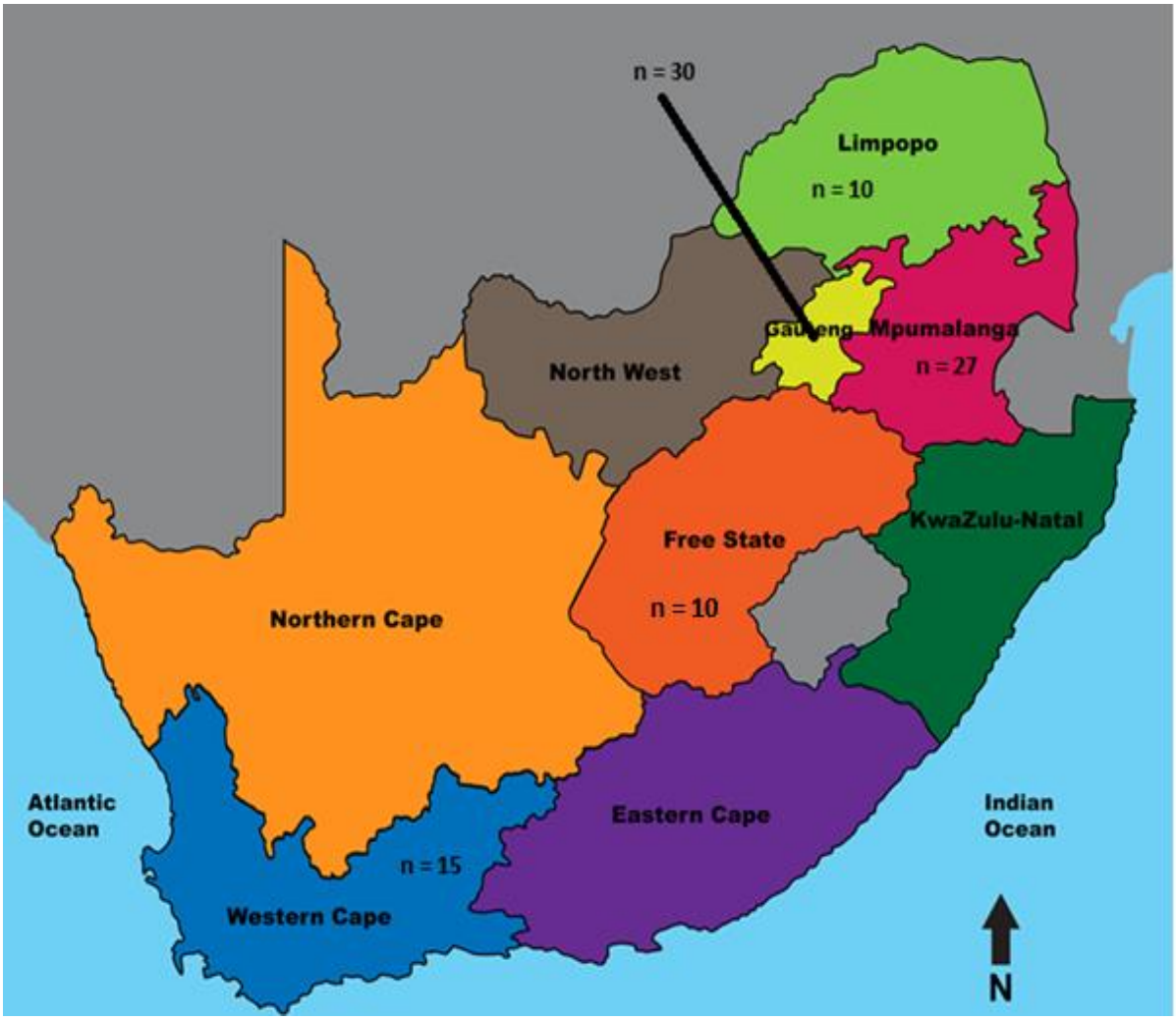

Figure 2: Location representing pig farms from which samples were collected in South Africa

3.3.2 DNA extraction

DNA extraction was performed using the the High Pure PCR Template preparation kit (Roche, Germany) protocol from a 24 hrs culture of *L. monocytogenes* grown on blood agar. A 200 µl of binding buffer and 40 µl Proteinase K were then added to the bacterial suspension and incubated at 70 °C for 10 min. Following incubation, a 100 µl of isopropanol was added. A high pure filter

tube (filter tube) was inserted into a collection tube. The sample was then added into the high pure filter tube assembly and centrifuged for one minute at 13 000 rpm. After centrifugation, the high pure filter tube was removed from the collection tube and the collection tube was discarded. The high pure filter tube was placed in a new collection tube, this was followed by the addition of a 500 µl of inhibitor removal buffer into the high pure filter tube. The mixture was centrifuged for one minute at 13000 rpm and the collection tube discarded following centrifugation. The high pure filter tube was again inserted into a new collection tube and 500 µl wash buffer was added and the mixture centrifuged at 13 000 rpm for one minute. This was repeated twice. To extract the DNA, the high pure filter tube with DNA was inserted into a clean centrifuge tube and 200µl of pre-warmed elution buffer was added to the filter tube and centrifuged at 13 000rpm for one minute. The micrcentrifuge tube containing the extacted DNA was stored at -80ºC for further analysis. The quantity and purity of the DNA was assessed using Qubit flourimetric quantitation (Thermofischer Scientific, Waltham, MA, USA).

3.3.3 Genomic DNA sequencing of *Listeria monocytogenes*

The sequence libraries from extracted DNA template were created using TruSeq and Nextera DNA library preparation kits (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. The sequence libraries were generated through fragmentation of extracted genomic DNA, which was followed by ligating NexteraXT and TruSeq adapters to both fragmented ends (3" and 5"). The quality and sizes of the fragment were assessed on the Perkin Elmer LabChip GX. After a quality check, the fragmented DNA was pooled and the KAPA library quantification was used to perform library quantification. Paired-end (3" and 5") sequencing was performed on HiSeq instruments (Illumina, San Diego, CA, USA) as recommended by the manufacturer on the average

genome coverage of 2 x 300. The cycle parameters used for sequencing were: read 1 : 101, index read 1 : 8, index read 2 : 8 and read 2 : 101. An RTA version 1.17.21.3 was used for the generation of base call files. Before assembly, FASTQ files were evaluated for quality using FastQC v0.70 (Andrews, 2010). Sequencing reads were trimmed using Trimmomatic v0.33 (Bolger et al. 2014) and BBDuk (version 37.90; https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/) for removal of adapters and low-quality reads. Reads with a quality score of <20 and length shorter than 70 bp as well as Phred mean the quality of less than 24 were trimmed at the 5'-end and 3'-end and poor quality reads were then discarded. Similarly, if the post-trimmed yield was less than 150 megabases, the sample was also discarded. The identity of the sample was confirmed using an A kmer (a short string of DNA of length k) based approach (https://github.com/phe-bioinformatics/kmerid); it was also used to ensure the sequence was free from contamination. De novo assembly of trimmed paired-end libraries was done using SPAdes v.3.12.0 (Bankevich et al. 2012).

3.3.4. Core-genome MLST in silico subtyping

The core genome MLST (cgMLST) analysis was performed using chewBBACA version 3.0. Core genome MLST typing was run with an external schema adapted from the BIGSdb-Lm platform (https://bigsdb.pasteur.fr/listeria) (Jolley and Maiden,2010; Moura et al. 2017). The cgMLST scheme comprising 1,748 highly conserved core loci representing 62% of coding regions from the *L. monocytogenes* EGD-e reference strain. The allele calling on the target genomes was performed with a chewBBACA allele calling algorithm using the *L. monocytogenes* training file-based reference *L. monocytogenes* EGD-e (acc. No. NC003210) as training for Prodigal and removing all paralogous loci. For PCR serogroup and whole genome MLST (wgMLST) determination,

seven-gene MLST scheme profiles were performed in silico for all the isolates using stringMLST (Gupta et al. 2017).

3.3.5 Screening for the presence of antimicrobial resistance, antibacterial biocide and metal resistance genes, prophages and virulence factors.

Genome assemblies were screened for the presence/absence of genes encoding antimicrobial resistance, virulence factors, biocide, heavy metal resistance and plasmids using ABRicate version 0.8.10 (https://github.com/tseemann/ABRicate) with the ResFinder database (Zankari et al. 2012), NCBI Bacterial Antimicrobial Resistance Reference Gene Database (Feldgarden et al. 2019), CARD version 2.0.3) (Jia et al. 2017), Virulence Factor database (VFDB) (Chen et al. 2016), megares database (Lakin et al. 2017), BacMet database (Pal et al. 2014) and PLSDB database (Galata et al. 2019) as input with minimum identity and coverage cut-offs of >90 and validated with blastn v.2.10.0+. To identify the putative prophage, genomes assemblies were submitted through the URL API to the PHASTER (PHAge Search Tool –Enhanced Release) server (Arndt et al. 2016). This application scores prophage regions as “intact”, “questionable” or “incomplete” based on several criteria such as the number of CDSs homologous to certain phages, the percentages of CDSs that match a certain phage.

3.3.6 Core genome single-nucleotide polymorphism

A reference-based variant calling analysis was performed using Snippy v.2.6 (https://github.com/tseemann/snippy). The annotated genomes were mapped against the complete reference genome of *L. monocytogenes* EGD-e (acc. No. NC003210) with the Burrows-Wheeler Aligner (BWA) v.0.7.12 using default settings (Li & Durbin, 2009). After mapping, the average

depths were determined with SAMtools v.1.3 (Li et al. 2009). The variants were called using Freebayes v.0.9.20 (Garrison & Marth, 2012) with the following parameters: minimum base quality of 20, minimum read coverage of 10X, and 90% read concordance at a locus for a variant to be reported. A calling of core genome single nucleotide polymorphisms (SNPs) was produced in Snippy v2.5 to infer a high-resolution phylogeny using Fasttree v.2.1.10 (Price *et al*., 2010).

3.3.7 Phenotypic antimicrobial susceptibility test

All tested isolates of *L. monocytogenes* were subjected to antimicrobial susceptibility test (AST) using Kirby Bauer Disk diffusion method as previously described by Matle et al. (2019). The isolates were tested against 16 antibiotic discs representing 11 classes as listed in Table 3. These classes of antibitics were chosen as they are the most commonly used in livestock farming and in human clinical settings (Moyane et al. 2013). The overnight pure cultures of *L. monocytogenes* on BHI agar were inoculated into the sterile saline solution and diluted to the equivalent concentration of 0.5 McFarland standard. A 100 µl of the diluted culture was then spread aseptically on Mueller-Hinton agar plates which was supplemented with 5% sheep blood agar (ThermoFisher, SA). The plates could stand for 15 min at room temperature before the antibiotic discs were inserted. Six discs were placed per inoculated plates; the plates were then incubated for 24 hrs at 35 °C. After incubation, the zone of inhibition around individual inoculated discs was determined and interpreted as resistance or susceptible using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines for *L. monocytogenes*. *Listeria monocytogenes*ATCC 19111 and *Escherichia coli*ATCC 25922 reference strains were used as a positive and negative control, respectively. Results were interpreted using the EUCAST as

described for *L. monocytogenes*. Guidelines for *Staphylococcu*s species were used for interpretation of results in cases of unavailable breakpoints for *Listeria* species, as they have almost similar biochemical profiles.

Table 3: List of antibiotics used in the antimicrobial resistance test

Classes	Antibiotics	Disk Concentration (µg)
β-Lactams	Ampicillin (AMP)	30
	Penicillin (P10)	10
Cephalosporin	Cephalothin (KF)	30
Aminoglycosides	Amikacin (AK)	30
	Gentamicin (CN)	10
	Streptomycin (S)	25
	Kanamycin (K)	30
Lincosamides	Clindamycin (DA)	10
Sulfonamides	Trimethoprim (W)	5
Carbepenems	Ertapenem (ETP)	10
	Meropenem (MEM)	30
Glycopeptides	Vancomycin (VA)	10
Tetracycline	Tetracycline (TE)	30
Macrolides	Erythromycin (E)	15
Phosphonic Acids	Fosfomycin (FOS)	30
Polypeptides	Nalidixic Acid (NA)	30

3.4 Statistical analysis

ANOVA was used to determine the difference in the antimicrobial susceptibility of isolates and to determine association among the clonal complexes and provinces. A statistically significant difference was considered at $p<0.05$. Kappa value (coefficient of agreement) was also determined to establish whether a relationship exists between phenotypes and molecular antimicrobial resistance profiling. Data were analysed using STATA 10 software (Stata Corporation, College Station, TX, U.S.). The diversity analysis according to the clonal complex was determined using the R package vegan v2.5-6 (Oksanen et al. 2018).

3.5 Ethical consideration

The ARC: OVR granted us permission to conduct this study on their facilities and ethics clearance (Reference no. 202/CAES_HREC/034) was obtained from the ethics committee of the College of Agriculture and Environmental Sciences, University of South Africa.

3.6 Limitation of the research

The limitation of this study was firstly, the inability to do genomic comparison between our isolates in this study and that of clinical and non clinical isolates in the country. This is due to the fact that all sequenced isolates from South Africa which are available in public database belong to sequence type 6 (ST6). Secondly, the number of isolates that were sequenced could not allow us to determine statistical significance among the distribution of sequence types and clonal complexes in the different provinces of South Africa.

3.7 Data Availability

The 77 genome sequences of *L. monocytogenes* isolates were deposited at the ARC: Bacterial Internal Central Database under the accession numbers from BICD1266 to BICD1343. Once the IP issues are resolved, the isolates will be deposited into National Centre for Biotechnology Information (NCBI)/GenBank

CHAPTER 4: RESULTS

4.1 Genome sequencing, assembly, and annotation characteristics

The sequenced isolates of *L. monocytogenes* in this study have shown common characteristics associated with the genus *Listeria*. These characteristics include the low genomic guanine (G) and cytosine (C) (G-C) content of 37.2% on average with a size ranging from 2.9 to 3.2 base pairs (bp). De novo assembly of the isolates was between 23 to 234 contigs with N50 ranging from 197, 8751 bp and 637,980 bp (Appendix 1).

4.2 Population structure of *Listeria monocytogenes* elucidated by typing analysis

4.2.1 Molecular serogroups and lineage

The sequenced isolates in this study were grouped into four different molecular serogroups (IIa, IIb, IIc and IVb), which belong to either lineage I or II. Lineage I accounted for 28.6% (n = 22) while lineage II for 71.4% (n = 55) of the isolates. Of the four identified molecular serogroups, IIa (45.5%; n = 35) has been the most prevalent followed by IIc (26.0%; n = 20), IVb (22.1%; n = 17) and IIb (6.5%; n = 5) (Figure 3). With regards to the molecular serogroups identified within the provinces as shown in Table 4, Gauteng province reported serogroup IIa and serogroup IVb representing majority of the isolates with 14.3% (n=11) and 18.8% (n=14), respectively. The second highest occurrence of isolates also belonging to serogroup IIa and serogroup IVb were from Mpumalanga representing 7.8% (n=6) of the isolates. Limpopo and Western Cape showed similar results with serogroups IIa and IIc representing 6.5% (n=5) and 2.6% (n=2) of isolates from both provinces. It was observed that the occurrence of isolates belonging to serogroup IIa was

significantly low in the Free State (2.6%; n=2) as compared to the other provinces where it was represented in 6.5-14.3% of the isolates.

4.2.2 Clonal complex

Eleven distinct clonal complexes (CCs) which corresponded with 11 sequence types (STs) were identified (Table 4). The identified CCs also belonged either to lineage I or II with lineage II harboring diverse strains representing seven CCs (CC7, CC9, CC87, CC121, CC155, CC204, and CC321). Lineage I strains were differentiated into four CCs (CC1, CC2, CC3 and CC5) (Table 4). The most commonly reported CCs in the present study was CC204 (23.4%; n = 18) followed by CC1 (19.5%; n = 15), CC2 (16.9%; n = 13); CC5 (10.5%; n =8); and CC9 and CC121 (9.1%; n = 7 each) respectively. Other CCs with low frequency of detection are reported in Table 4 and include CC3 (n = 2; 2.6%) and CC321, CC87, and CC155 (n = 1; 1.3%) each.

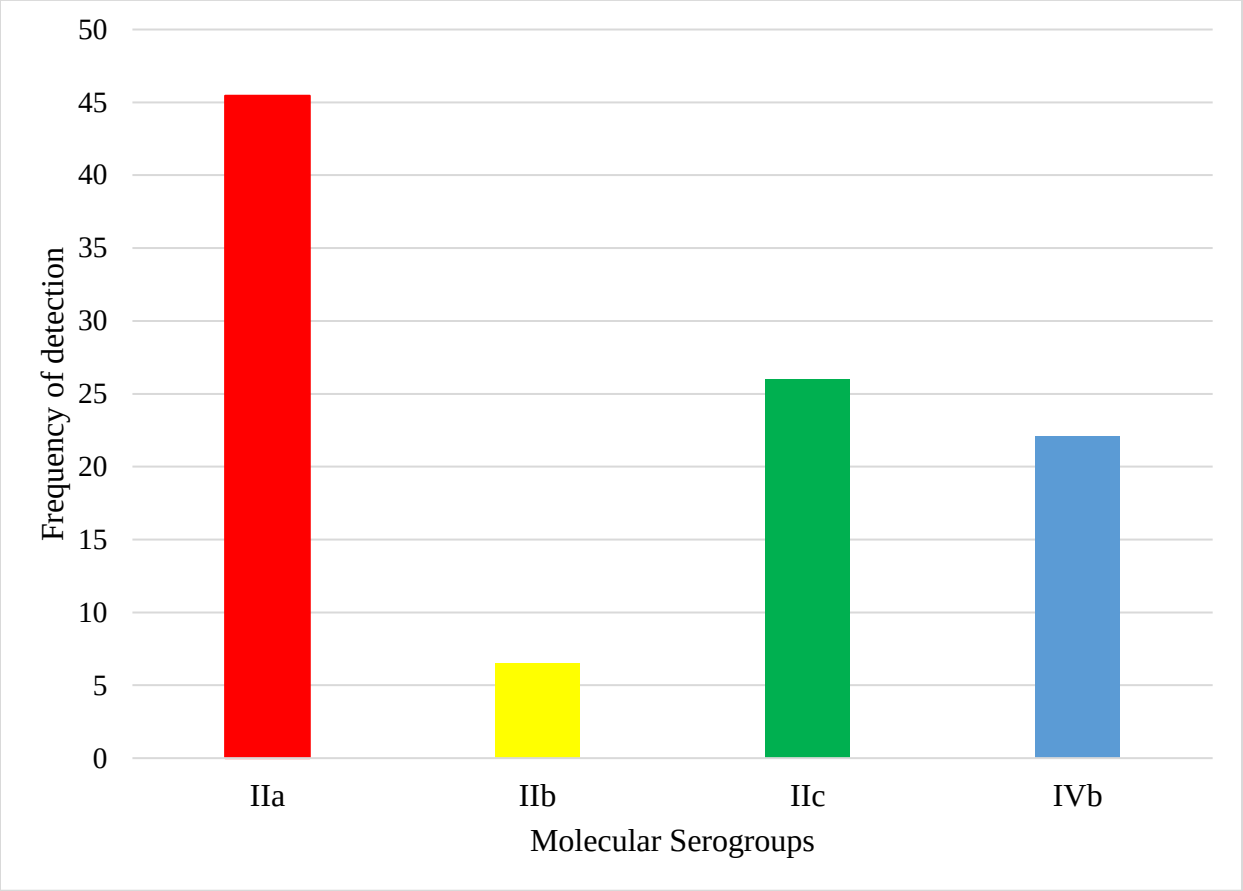

Figure 3: Frequency of occurrence of molecular serogroups of *Listeria monocytogenes*

4.2.3 Analysis of clonal complex diversity by provinces

The distribution of *L. monocytogenes* strains according to the provinces was also investigated in the present study. Testing for association among the CCs and provinces shows a significant dependence (p-value = 0.0002) suggesting that distribution of *L. monocytogenes* strains may vary based on geographical area. Ten of the eleven reported CCs (CC7, CC121, CC155, CC204, ST321, CC5, CC9, CC1, CC2 and CC87) in this study were detected in isolates from Gauteng province with CC1 (23.3%; n = 7) and CC2 (20%; n = 6) being the most predominant. Gauteng province

also reported three unique CCs namely CC87, CC155 and CC321 that represent serogroup IVb (lineages I) and IIa (lineage II) respectively (Table 4). A similar pattern of a high occurrence of CC1 (30%; n = 3) and CC2 (20%; n = 2) was also observed in Free State. Limpopo province reported six different CCs (CC121, CC204, CC5, CC9, CC1 and CC2) with overrepresentation of CC204 (40%; n = 4). Mpumalanga province also reported six different CCs (CC121, CC204, CC5, CC1, CC2 and CC3) with CC121 (33.3%; n = 4) being the most commonly found. Mpumalanga only reported CC3, which represents serogroup IVb. In Western Cape Province, CC204 (33.3%; n = 5) was overrepresented in most of the isolates while the distribution of other CCs remains similar at a very low (13.3%; n = 2) detection rate and they include CC7, CC5, CC1, CC2, and CC3.

Table 4: Frequency of occurrence for *Listeria monocytogenes* molecular lineage and serogroup, sequence types and clonal complex per province of South Africa

Linegae	Sero-group	Sequence type	Clonal Complex	Provinces (number of strains) (%)					
				GP (n = 30)	L (n = 10)	MP (n = 12)	WC (n = 15)	FS (n =10)	Total (n = 77)
II	IIa	ST7	CC7	2 (6.7)	0 (0)	0 (0)	2 (13.3)	0 (0)	4 (5.6)
		ST121	CC121	2 (6.7)	1 (10)	4 (33.3)	0 (0)	0 (0)	7 (9.1)
		ST155	CC155	1 (3.3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.3)
		ST204	CC204	5 (16.7)	4 (40)	2 (16.7)	5 (33.3)	2 (20)	18 (23.4)
		ST321	CC321	1 (3.3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.3)
	IIb	ST5	CC5	4 (13.3)	1 (10)	2 (16.7)	0 (0)	1 (10)	8 (10.4)
I	IIc	ST9	CC9	1 (3.3)	2 (20)	0 (0)	2 (13.3)	2 (20)	7 (9.1)
	IVb	ST1	CC1	7 (23.3)	1 (10)	2 (16.7)	2 (13.3)	3 (30)	15 (19.5)
		ST2	CC2	6 (20)	1 (10)	2 (16.7)	2 (13.3)	2 (20)	13 (16.9)
		ST87	CC87	1 (3.3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.3)
		ST3	CC3	0 (0)	0 (0)	2 (16.7)	0 (0)	0 (0)	2 (2.6)

GP: Gauteng; L: Limpopo; MP: Mpumalanga; WC: Western Cape; FS: Free State.

4.3 Resistance profiling of *Listeria monocytogenes*

4.3.1 Overall phenotypic antimicrobial resistance

Table 5 display the phenotypic antimicrobial resistance (AMR) results of all tested *L. monocytogenes*isolates in this study. All (100%) tested isolates displayed resistance against nalidixic acid and fosfomycin. Forty-five percent (n = 35) of the isolates were resistant to gentamicin followed by ampicillin and amikacin with 42.9% (n = 33), kanamycin 33.8 % (n = 26) and ertapenem 32.5% (n = 25). Twenty-four percent (n = 19) of the isolates were resistant to tetracycline while 23.4% (n = 18) were resistant to penicillin and 20.8% (n = 16) to cephalothin and 19.5% (n = 15) to meropenem. The lowest resistance was observed against erythromycin 4.5% (n = 3). It was observed that all (100%) isolates were susceptible to clindamycin and streptomycin. Distribution of phenotypic AMR resistance of *L. monocytogenes* by province revealed a significant ($p \leq 0.05$) resistance of the tested isolates toward gentamycin and cephalothin by isolates from the Western Cape. The isolates from Gauteng and Mpumalanga were highly resistant ($p \leq 0.05$) to kanamycin and cephalothin, respectively.

Table 5: Distribution of antimicrobial resistance profile for *Listeria monocytogenes* isolates by provinces

Classes	Antibiotics	Resistant isolates (%)	Number of resistant isolates (%)					p value
			GP (n =30)	L (n = 10)	MP (n = 12)	FS (n = 10)	WC (n = 15)	
B-Lactams	AMP	33 (42.9)	15 (50)	3 (30)	2 (16.7)	6 (60)	7 (46.7)	0.302
	P10	18 (23.4)	10 (33.3)	1 (10)	3 (25)	2 (20)	2 (13.3)	0.037
Cephalosporin	KF	16 (20.8)	1 (3.3)	0 (0)	2 (16.7)	1(10)	12 (80)	*0.001
Aminoglycosides	AK	33 (42.9)	18 (60)	0 (0)	2 (16.7)	6 (60)	7 (50)	0.039
	CN	35 (45.5)	8 (26.7)	2 (20)	4 (33.3)	7 (70)	14 (93.3)	*0.004
	S	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	-
	K	26 (32.5)	22 (73.3)	1 (10)	0 (0)	1 (10)	2 (13.3)	*0.0002
Lincosamides	DA	0 (0)	0 (0)	0 (0)	0 (0)	(0)	0 (0)	-
Sulfonamides	W	16 (20.8)	2 (6.7)	1 (10)	10 (83.3)	1 (0)	1 (6.7)	*0.002
Carbepenes	EPT	25 (32.5)	10 (33.3)	4 (40)	6 (50)	1 (0)	4 (26.7)	0.161
	MEM	15 (19.5)	7 (23.3)	3 (30)	0 (0)	0 (0)	5 (33.3)	0.143
Glycopeptides	VA	0 (0)	(0)	0 (0)	0 (0)	0 (0)	0 (0)	-
Tetracycline	TE	19 (24.7)	8 (26.7)	3 (30)	2 (16.7)	2 (20)	4 (26.7)	0.032
Macrolides	E	3 (3.9)	2 (6.7)	0 (0)	1 (8.3)	0 (0)	0 (0)	0.279
Phosphonic Acids	FOS	77 (100)	30 (100)	10 (100)	12 (100)	10 (100)	15 (100)	0.206
Polypeptides	NA	77 (100)	30 (100)	10 (100)	12 (100)	10 (100)	15 (100)	0.206

P: Penicillin; AMP: Ampicillin; KF: Cephalothin; CN: Gentamycin; S: Streptomycin; K: Kanamycin; AK: Amikacin; TE: Tetracycline; DA; Clindamycin; E: Erythromycin; MEM: Meropenem; ETP: Ertapenem; VA: Vancomycin; W: Trimethoprim GP: Gauteng; L: Limpopo; MP: Mpumalanga; WC: Western Cape

4.3.2 Phenotypic multi-drug resistance (MDR) patterns

Multi-drug resistance (MDR) (resistance to four or more antimicrobial agents) (Falagas and Karageorgopoulos, 2008) based on phenotypic testing was also investigated. Overall, 11 predominant MDR patterns were observed in this study for MDR (Table 6). The predominant MDR pattern was observed against four antimicrobial agents namely NA-FOS-AMP-KF (70.2%; n = 47). However, the majority of the isolates showed an MDR pattern ranging between 5 to 8 antibiotics per isolate. It was also noticed that about 1.5% (n = 1) of all tested isolates showed the highest MDR pattern to 14 of the 16 antimicrobial agents.

Table 6: Antimicrobial Resistance Patterns

MDR pattern number	Antimicrobial Resistance Patterns	Isolates no.
4	NA-FOS-AMP-KF	47
5	NA-FOS-KF-AK	38
6	NA-FOS-P10-KF-AK-TE	27
7	NA-FOS-CN-VA-TE-K-EPT	19
8	NA-FOS-P10-KF-AK-CN-K-MEM-VA-TE	12
9	NA-FOS-AMP-P10-K-EPT-MEM-TE	7
10	NA-FOS-AMP-KF-AK-CN-K-EPT-MEM	3
11	NA-FOS-AMP-P10-KF-AK-CN-K-EPT-MEM-TE	4
12	NA-FOS-AMP-P10-KF-AK-CN-K-EPT-MEM-TE	2
13	NA-FOS-AMP-P10-KF-AK-CN-K-W-EPT-MEM-TE	1
14	NA-FOS-AMP-P10-KF-AK-CN-K-W-EPT-MEM-TE-E	1

NA: Nalidixic acid; FOS: Fosfomycin; P10: Penicillin; AMP: Ampicillin; KF: Cephalothin; CN: Gentamycin; S: Streptomycin; K: Kanamycin; AK: Amikacin; TE: Tetracycline; DA; Clindamycin; E: Erythromycin; MEM: Meropenem; ETP: Ertapenem; VA: Vancomycin; W: Trimethoprim

4.3.3 Identification of genotypic antibiotics resistance

4.3.3.1 Resistance genes profile

The molecular antimicrobial resistance profiles for all sequenced *L. monocytogenes* isolates in this study revealed the presence of genes encoding resistance to a wide range of antibiotics. However, the observed genotypic AMR profiles were similar, with a slight difference among a few of the isolates. Intrinsic AMR genes of *L. monocytogenes* (*FosX, lin, mprF, norB* and *mgrA*) were detected in all isolates (Figure 4). The *tetM* gene was detected in 3.9% (n = 3) of the isolates which belong to CC9 (66.7%; n = 2) and CC2 (33.3%; n = 1) (Table 7), the isolates were from Gauteng (n = 2) and Limpopo (n = 1) provinces, respectively (Figure 5). The *tetS*gene was only detected in 1.3% (n = 1) of the isolates belonging to CC2, which originated from Gauteng. The isolates in this study were also found to carry *qnrA* 36.4 % (n = 28), *qnrB* 3.9% (n = 3) and *qnrS* 18.2% (n = 14) genes which confer resistant to quinolone. The distribution of quinolone resistant genes varied among the CCs with *qnrA* being predominantly found in CC204 (60.7%; n = 17) followed by CC1 (17.9%; n = 5) while *qnrS* in CC204 (35.7 %; n = 5) and CC7 (42.9%; n = 6) respectively (Table 7). All *qnrB* genes were only detected in isolates from CC5, which originated from Mpumalanga (66.7%; n = 2) and Gauteng (33.3%; n = 1) (Figure 5). The *qnrA* gene was mostly reported in isolates from Gauteng (n = 15) followed by Western Cape (n = 7), Free State (n = 4) and Limpopo (n = 2) provinces. The *qnrS* gene was detected mostly in isolates from Limpopo (n = 6) followed by Gauteng (n = 4).

The genes which encodes resistance for ampicillin (*ampC)* and penicillin (*penA)* were detected in 23.4% (n = 18) and 20.8% (n =16) of the isolates respectively. The *ampC* gene was commonly

reported in CC1 (50%; n = 9) originated mainly from Gauteng. The *penA* gene was distributed evenly across different CCs and predominately reported in isolates from Mpumalanga (n = 8). Streptomycin resistance genes (*strA* and *strB*) were reported in 42.9% (n = 33) and 18.2% (n = 14) of the isolates correspondingly (Table 7). The *strA* gene was present in CC2 (44.1%; n = 15), and CC204 (29.4%; n = 10) while *strB* was found in CC7 (28.6%; n = 4). Both streptomycin resistance genes were predominately detected from isolates originating from Western Cape and Mpumalanga provinces. *lmrB* gene was found in 11.7% (n = 9) isolates which belong to CC1 (44.4%; n = 4) and CC2 (55.6%; n = 5) while *lmrD* gene was present in 2.6% (n = 2), which were detected in CC9. *lmrB* and *lmrD* genes originated mostly from isolates from Limpopo (n = 6) and Gauteng (n = 2) provinces in that order (Figure 5).

The *aad6* gene which is responsible for resistance of *L. monocytogenes* to aminoglycosides was present in 58.4% (n = 45) of the isolates with a high detection rate in CC204 (22.2%; n = 10) follow by CC7 (17.8%; n = 8) and CC1 (15.6%; n = 7). This gene was detected mostly in isolates from Gauteng (n = 17) followed by Mpumalanga (n =10) and Limpopo (n =8) provinces. Three major trimethoprim encoding genes were detected in this study with *dfrI* (27.3%; n = 21) being the most prevalent followed by *dfrII* (15.6%; n =12) and *dfrIII* (6.5%; n =5). The distribution of these genes varied among the CCs and provinces. Genes encoding for erythromycin and vancomycin were detected in less than 3.9% (n = 3) of the isolates in this study (Table 7).

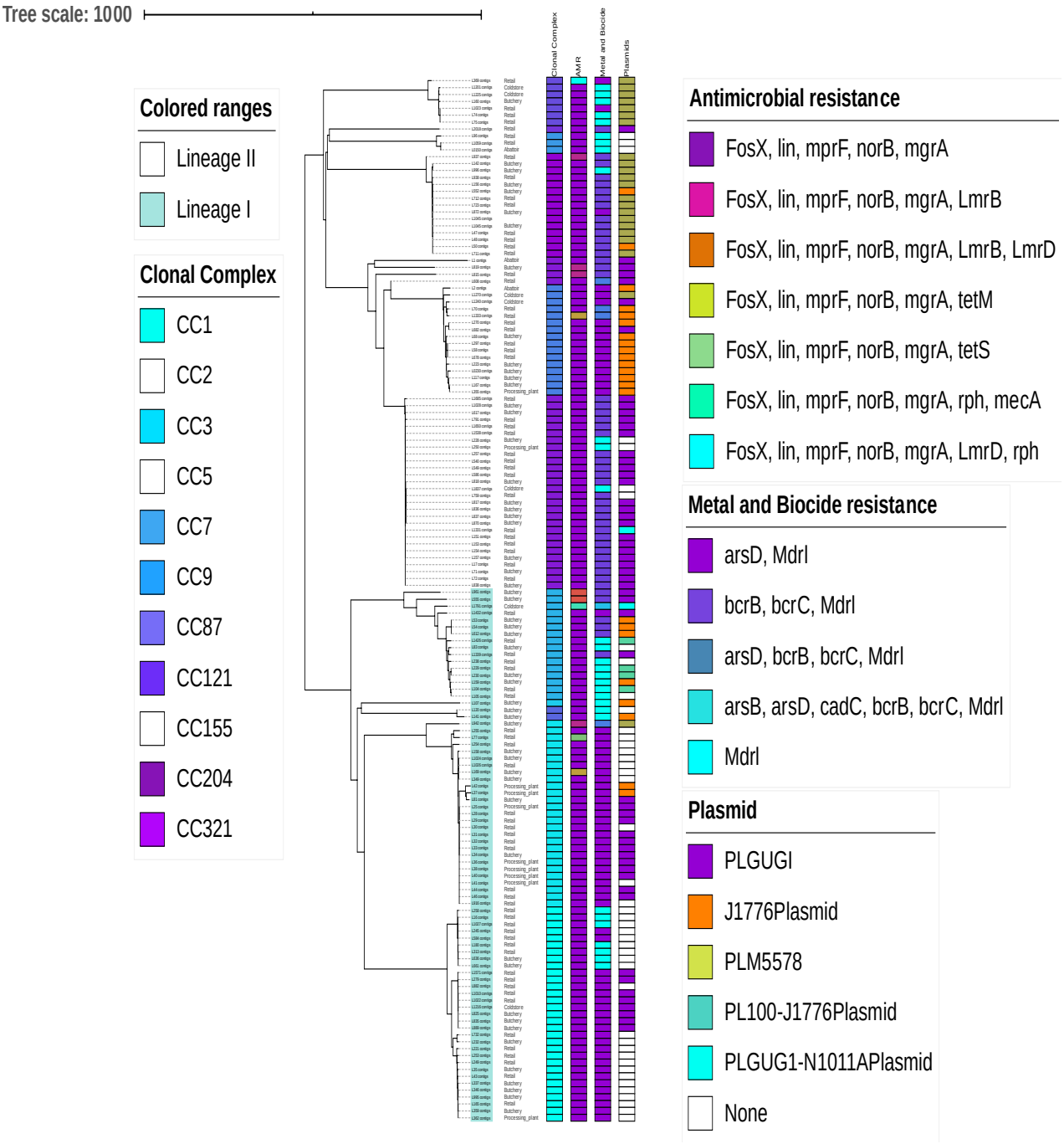

Figure 4: core-genome MLST phylogenetic clustering of antimicrobial, metal and biocide resistance genes and plasmids according to Clonal Complexes of the *isolated Listeria monocytogenes* isolates

Table 7: Distribution of antimicrobial resistant genes per clonal complexes

Function	**Genes**	**Number of isolates (%)**	**Clonal complex (%)**										
			CC1	CC2	CC3	CC5	CC7	CC9	CC87	CC121	CC155	CC204	CC321
Tetracycline	*tetM*	3 (3.9)	0 (0)	1(33.3)	0 (0)	0 (0)	0 (0)	2(66.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	tetS	1 (1.3)	0 (0)	1(100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Quinolone	*qnrA*	28 (36.4)	5(17.9)	2(7.1)	1(3.6)	1(3.6)	0 (0)	2(7.1)	0 (0)	0 (0)	0 (0)	17(60.7)	0 (0)
	qnrB	3 (3.9)	0 (0)	0 (0)	0 (0)	3(100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	qnrS	14 (18.2)	1(7.1)	1(7.1)	0 (0)	0 (0)	6(42.9)	0 (0)	0 (0)	0 (0)	0 (0)	5(35.7)	0 (0)
ß-lactamase & Metallo-ßlactamase Proteins	*ampC*	18 (23.4)	9(50)	4(22.2)	0(0)	0(0)	2(11.1)	0(0)	1(5.6)	0(0)	0 (0)	1(5.6)	1(5.6)
	penA	16 (20.8)	3(18.8)	3(18.8)	1(6.3)	1(6.3)	1(6.3)	1(6.3)	1(6.3)	1(6.3)	1(6.3)	2()	1(6.3)
Streptomycin	*strA*	33 (42.9)	2(5.9)	15(44.1)	0 (0)	1(2.9)	1(5.9)	1(2.9)	1(2.9)	1(2.9)	1(2.9)	10(29.4)	0 (0)
	strB	14 (18.2)	2(14.3)	2(14.3)	1(7.1)	1(7.1)	4(28.6)	0 (0)	0 (0)	0 (0)	1(7.1)	2(14.3)	1(7.1)
Lincomycin	*lmrB*	9 (11.7)	4(44.4)	5(55.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	lmrD	2 (2.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2(100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Aminoglycoside N3-Acetyltransferase	*aad6*	45 (58.4)	7(15.6)	5(11.1)	2(4.44)	6(13.3)	8(17.8)	3(6.7)	0 (0)	2(4.4)	1(2.2)	10(22.2)	1(2.2)
Trimethoprim	*dfrI*	21(27.3)	3(14.3)	2(9.5)	1(4.8)	0 (0)	5(23.8)	0 (0)	0 (0)	0 (0)	0 (0)	10(47.6)	0 (0)
	dfrII	12(15.6)	5(41.7)	1(8.3)	1(8.3)	0 (0)	4(33.3)	0 (0)	0 (0)	0 (0)	0 (0)	1(8.3)	0 (0)
	dfrIII	5(6.5)	1(20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3(60)	1(20)
Vancomycin	*vanA*	2 (2.6)	0 (0)	0 (0)	1(50)	0 (0)	1(50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	vanB	2 (2.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2(100
Erythromycin	*ermA*	3 (3.9)	1(33.3)	0 (0)	0 (0)	1(33.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1(33.3)	0 (0)
	ermB	1 (1.3)	0 (0)	0 (0)	0 (0)	0 (0)	1(100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Multidrug resistance factors		6 (7.8)	1(16.7)	1(16.7)	0 (0)	1(16.7)	1(16.7)	0 (0)	0 (0)	0 (0)	0 (0)	2 (33.3)	0 (0)

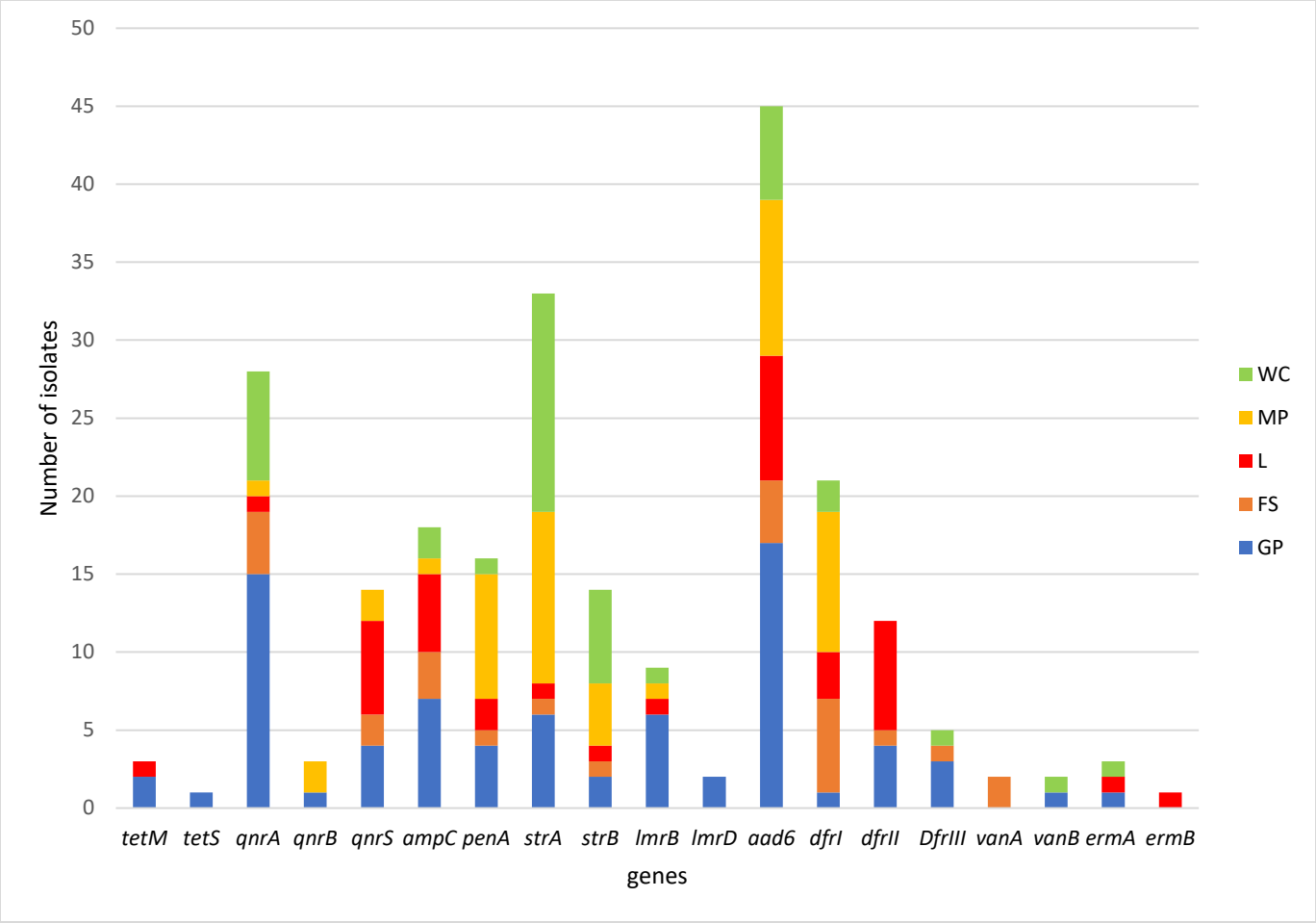

Figure 5: Distribution of antimicrobial resistant genes by provinces in South Africa

4.3.3.2 Hetero-resistance of *Listeria monocytogenes*

Heterogeneous resistance was observed from three isolates against trimethoprim and kanamycin antibiotics. Two of the isolates belonging to serogroup IIa representing CC7 and CC204 displayed hetero-resistance towards trimethoprim (Figure 6) while one isolate from the same serogroup, IIa representing CC155 displayed hetero-resistance to kanamycin (Figure 7). The genes encoding resistance against trimethoprim and kanamycin were not detected from WGS against these three isolates, hence they are classified as hetero-resistant.

Figure 6: Hetero-resistant against trimethoprim

Kanamycin

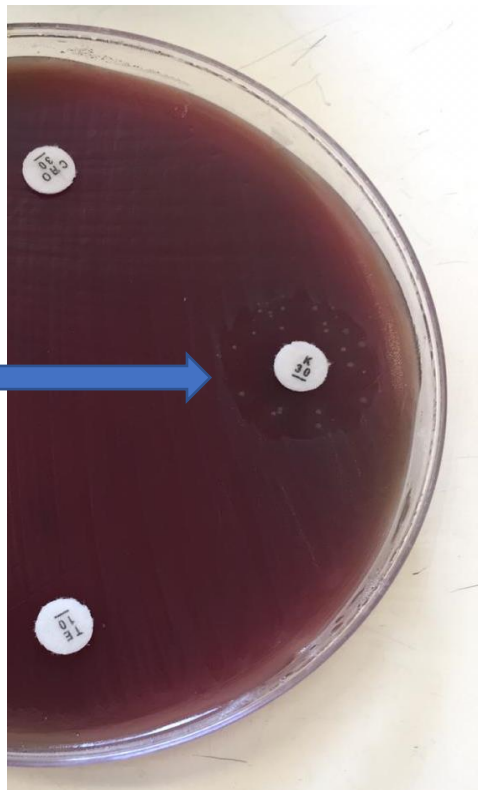

Figure 7: Hetero-resistant against kanamycin

4.3.3.3 Comparison of phenotypic and genotypic antimicrobial resistance profiling

The coefficient agreement of tested antibiotics was calculated to determine the correspondence between phenotypic and genotypic resistance profiling results. It was observed that of the 16 tested antibiotics, there was poor agreement reported between phenotypic and genotypic resistance except for streptomycin, gemtamycin, penicillin and ampicillin with Kappa agreement ranging from fair to good (Table 8).

4.3.3.4 Antibacterial biocides resistance profile

The sequenced isolates in this study were also examined for the presence of biocides that are known to confer resistance in *L. monocytogenes*. The *mdrL* and *lde* genes, which confer resistance to benzalkonium chloride (BC), were detected in all sequenced isolates. The cassette *bcrABC* genes were detected in 24.7 % (n = 19) of the isolates. This cassette was mostly detected from lineage II (CC204 and CC321) and for lineage I, was only detected in CC5 (Figure 4).

Table 8: Kappa agreement between phenotypic and genotypic resistance profiling of *Listeria monocytogenes*.

Antibiotics	Agreement	Expected agreement	Kappa	Standard error	Z	p-value	Strength of agreement
Streptomycin	39.91%	21.75%	0.301	0.029	12.84	< .0001	Fair
Gentamycin	92.90%	80.99%	0.602	0.080	0.83	<. 0001	Good
Penicillin	48.40%	21.76%	0.300	0.027	11.90	< .0001	Fair
Tetracycline	98.29%	92.20%	0.184	0.829	0.083	< .0001	Poor
Vancomycin	80.97%	41.93%	0.199	0.068	14.0.3	< .0001	Poor
Trimethoprim	81.38%	21.08%	0.199	0.837	11.90	< .0001	Poor
Ampicillin	70.01%	52.90%	0.301	0.025	11.87	< .0001	Fair
Erythromycin	80.81%	21.85%	0.201	0.833	11.78	< .0001	Poor
Kanamycin	81.30%	47.03%	0.172	0.842	11.87	< .0001	Poor

The interpretation of kappa: < 0.2= Poor; > 0.2 ≤ 0.4=Fair; > 0.4 ≤ 0.6=Moderate; > 0.6 ≤ 0.8=Good and > 0.8 ≤ 1= Very good (McHugh, 2015).

4.3.3.5 Heavy metal resistance profiles

The presence of heavy metals revealed *arsD and arsB* arsenic resistance genes in 48% (n = 37) and 11.7% (n = 9) of the sequenced isolates, respectively. The *arsD* gene was detected mostly in isolates belonging to CC1 and CC2 from lineage I and CC9 from lineage II while *arsB* gene was mostly found in CC204 (Figure 4). Examination of the presence of other heavy metals revealed a low presence (less than 2%) of genes encoding for cadmium (*CadC*), iron (*FrvA*), zinc, copper (*CopC*), aluminium (*CorA* and *CzcD*), lead (*Pbr*), and cobalt (*CorA*) resistance. It was observed

that there was no specific correlation that has been observed between heavy and biocide resistance profiles among the sequenced isolates in this study. Interestingly, one isolate from CC5 (Gauteng) was found to carry multiple genes which include *arsB, arsD*, *cadC, bcrB, bcrC* and *mdrL*.

4.4 Investigation of mobile elements profiling

4.4.1 Distribution of plasmids among environmental isolates of *Listeria monocytogenes*

The distribution of plasmids among sequenced isolates was investigated which showed the presence of four different *L. monocytogenes* plasmids in 68.9% (n = 53) of the isolates. The most predominate plasmids in this study was pLGUG1 (50.7%; n = 39) followed by N1011A (9.1%; n = 7), J1776 (5.2%; n = 4) and pLI100 (3.9%; n = 3) (Figure 8). The phylogenetic clustering of the plasmids according to their associated CCs showed pLGUG1 was found in CC204 belonging to lineage I and CC1 as well as CC2, which both belong to lineage II (Figure 4). The J1776 plasmid was found in CC9 from lineage II. The co-occurrence of pLGUG1 pLI100 and J1776 plasmids were found in CC5 from lineage I and and CC204 from lineage II. Furthermore, lineage II isolates had more plasmids occurrence and diversity compared to lineage I (Figure 8). The distribution of plasmids by provinces revealed the presence of PLGUGI in the entire provinces while J1776 was found only in Gauteng and Limpopo provinces (Table 9). PLI100 and N1011A were exclusively found in Mpumalanga province.

4.4.2 Distribution of prophages of *Listeria monocytogenes*

The prophage (φ) profiles of the 77 *L. monocytogenes* isolates were characterised using the PHASTER tool for identification and annotation of putative prophage sequences. A total of 8 unique intact, questionable or incomplete prophages regions were present across different *L.*

monocytogenes CCs isolates. The most commonly identified *Listeria* prophages were: vB LmoS 188 [NC_028871], LP 030 2 [NC_028929], LP-101 [NC_024387], LP 030 2 [NC_021539], A600 [NC_009815], A500 [NC_009810], A118 [NC_003216] and B054 [NC_009813]. PHASTER identified unique sets of prophages characterising *L. monocytogenes* strains from each clonal group, which were presented across different CCs. Within the major clonal groups, vB_LmoS 293, vB LmoS 188, LP 030 2 and A118 (Intact phages), were present in CC1, vB LmoS 188, LP 030 2 and LP 030 2 (Intact phages) were present in CC2; LP-101, A118 and vB_LmoS 293 (Intact phages) were present in CC204. Other important putative prophages detected in all the study isolates are presented in Table 9.

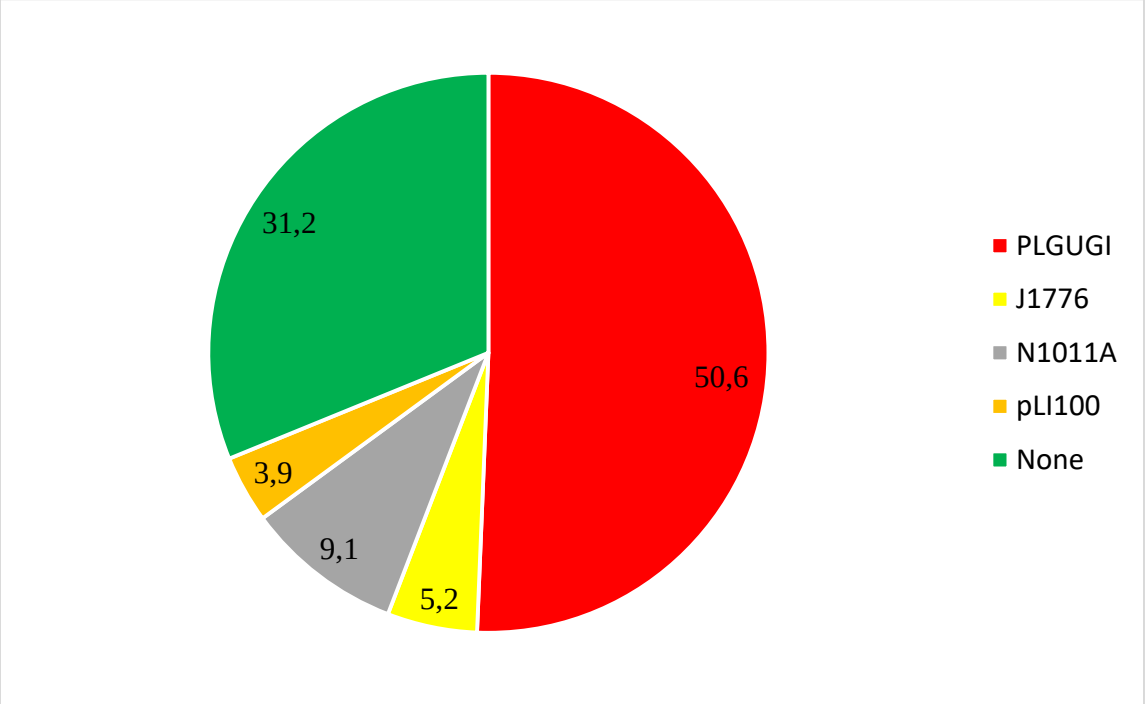

Figure 8: Frequency of detection of *Listeria monocytogenes* plasmids

Table 9: Predicted prophages *Listeria monocytogenes* strains

Province	Number of Sample	CC	Prophages			Plasmids
			Intact	Questionable	Incomplete	
Western Cape	15	CC1 CC2 CC9 CC204	vB_LmoS_188 [NC_028871]	A118 [NC_003216]	A118 [NC_003216] A006 [NC_009815]	pLGUG1
Limpopo	10	CC1 CC2 CC5 CC9 CC121 CC204	LP_101 [NC_024387]; B054 [NC_009813]; vB_LmoS_188 [NC_028871]	A118 [NC_003216]	A118 [NC_003216]; A006 [NC_009815]	pLGUG1 J1776plasmid
Mpumalanga	12	CC1 CC5 CC121 CC204	vB_LmoS_293 [NC_028929]; vB_LmoS_188 [NC_028871]; [A118_NC_003216]; [LP_101_NC_024387]	A118 [NC_003216]	A118 [NC_003216]; vB_LmoS_188 [NC_028871]	pLGUG1 pLI100 N1011A
Free State	10	CC1, CC2, CC9, CC5, CC204	vB_LmoS_188 [NC_028871]	A118 [NC_003216]	A118 [NC_003216]; A006 [NC_009815]; vB_LmoS_188 [NC_028871]	pLGUG1
Gauteng	30	CC1, CC2, CC5, CC7, CC9, CC87, CC121, CC155, CC204, CC321	vB_LmoS_188 [NC_028871]; vB_LmoS_293 [NC_028929]; LP_030_2 [NC_021539]; LP_101 [NC_024387]	A118 [NC_003216]; B025 [NC_009812]; LP_101 [NC_024387]	A500 [NC_009810]; vB_LmoS_188 [NC_028871]; A118 [NC_003216]; A006 [NC_009815], LP_030_3 [NC_024384]; vB_LmoS_293 [NC_028929]	pLGUG1 J1776

4.5 Investagation of Stress Survival Islets

In the current study, Stress survival islet 1 (SSI-1) was detected in 9.1% (n = 7) isolates which all belonged to CC1 of lineage I. The SSI-2 was found only in two isolates which belong to CC121 and CC204 of lineage II, respectively.

4.6 The assessment of virulence profiles across different clonal complex

The presence of four *Listeria* pathogenicity islands 1 to 4 (LIPI-1 to LIPI-4) was investigated in the present study. A complete LIPI-1, which harbour virulence gene cluster comprising six genes (*prfA, plcA, hly, mpl, actAplcB*) were present in 38.9% (n = 30) of the isolates and 93% of them were from lineage II across different CCs. The complete LIPI-3 consisting of eight genes (*llsA, llsB, llsD, llsG, llsH, llsP, llsX, llsY*) was found in 19.5% (n = 15) of the isolates belonging to CC1 (40%; n = 6), CC2 (13.3%; n = 2) and CC3 (6.7%; n = 1) from lineage I respectively. Moreover, complete LIPI-3 was detected in 2 isolates from CC204 which belong to lineage II. The *IIsA* gene which is located in LIPI-3 was only found in CC121 isolates (Figure 9). *Listeria* pathogenicity islands 1 and 2 were not found in all the isolates. Further analysis of virulence factors in this study indicated that surface protein genes, *aut, inlF* and *gtcA* genes were found in about 89% lineage II isolates but absent from CC1 and CC2 from lineage I isolates except for *gtcA.* The virulence surface protein gene *Vip* was mostly present in lineage I across different CCs, but absent in CC204 and CC321 from lineage II isolates (Figure 10). Furthermore, the internalin's protein gene *inlF* and *InlJ* and the *actA* genes were absent in CC121 isolates from lineage II. Other important putative virulence factors detected in these isolates are presented in Figure 9.

4.7 Core-genome MLST and core-SNP phylogenomic clustering of *Listeria monocytogenes* clonal complex

To compare the genome of sequenced isolates, the isolates were mapped and aligned against the *L. monocytogenes* EGD-e reference genome to generate an alignment with core SNPs and a phylogenetic tree consistent with the cgMLST phylogenetic tree. Both the core-genome MLST and core-SNP phytogenic trees showed denealiation into lineage I and II and all the distinct CCs formed individual clusters within the lineages (Figure 10). Core-SNP phylogenetic tree revealed clustering of predominate CCs (CC204, CC1 CC2) into a single large branch compared. Interestingly, L1_contigs comprised CC204 from lineage II changed position and clustered with lineage I isolates in the core-SNP tree. The two phylogenetic trees displayed a good congruence between the two phylogenetic trees but there were differences in the two phylogenies predominantly caused by the inversion of clusters, which is explained by differences between the internal nodes. A visual comparison of the two phylogenetic trees indicated that most isolates were grouped into the same clusters whether analysed with core-SNPs or cgMLST (Figure 10).

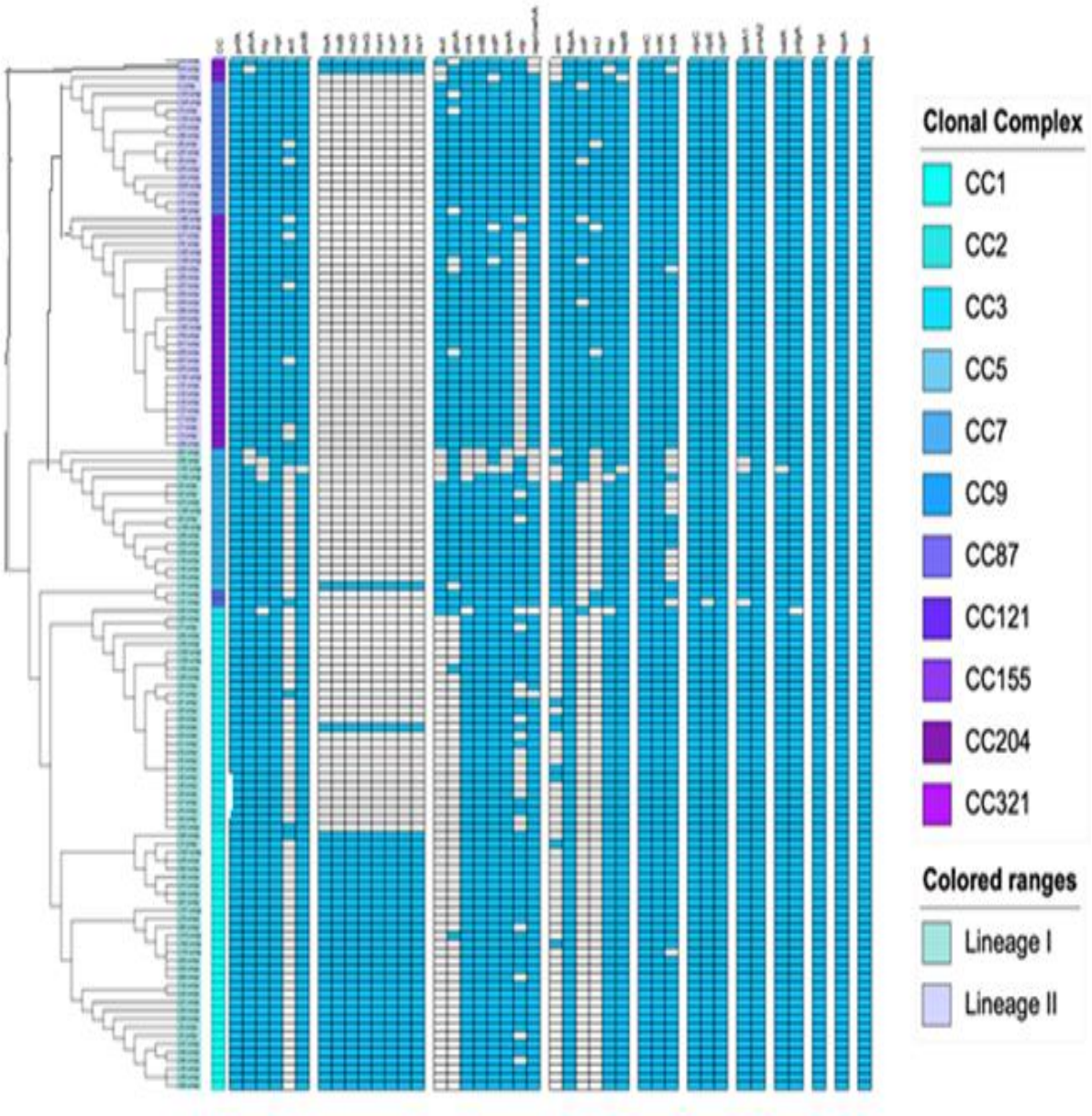

Figure 9: Core-genome MLST phylogenetic clustering of putative virulence factors across different *Listeria monocytogenes* Clonal Complexes.

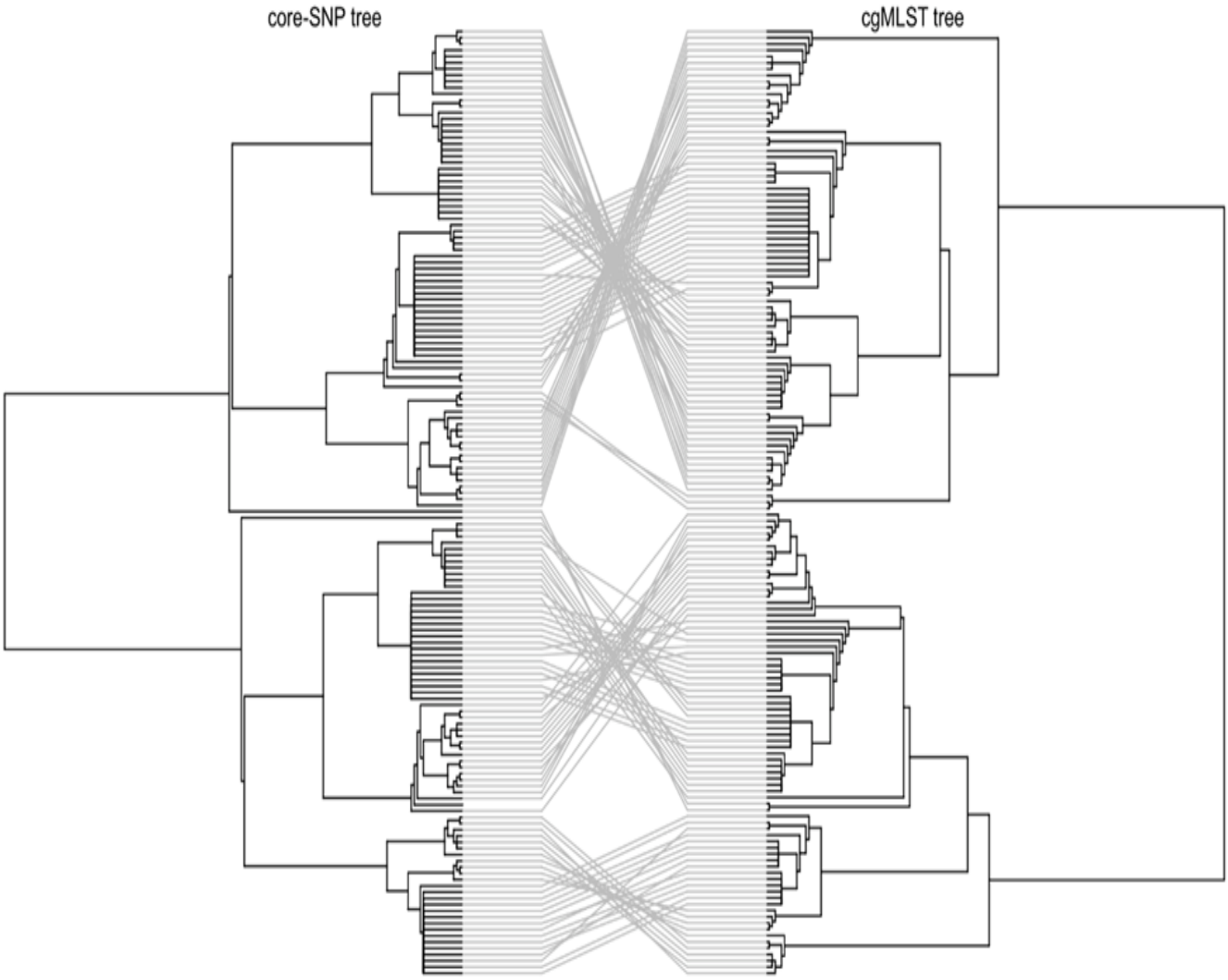

Figure 10: Tanglegram comparison between core-genome SNP phylogeny (Left) and cgMLST (Right) linking tips with the same label to each other of 77 isolates from the *Listeria monocytogenes* isolates

4.8 The core-SNP phylogenetic clustering of the most common *L. monocytogenes* Sequence types

To investigate the genetic relatedness of the most common *L. monocytogenes* strains in SA, the isolates were mapped against the *L. monocytogenes* EGD-e reference genome and aligned, generating an alignment with core SNPS and a phylogenetic tree. The core-SNP analysis showed that the most frequent ST204 was grouped in 3 distinct clusters with SNP difference ranging up to 41 SNPs in the core parts of the genomes of these strains. Moreover, the ST1 and ST2 were grouped in 2 distinct clusters with SNP differences ranging up to 27 and 34 SNPs, respectively (Figure 11). These results indicate that South African *L. monocytogenes* isolates belonging to ST1, ST2 and ST204 were generally paraphyletic mixes of diverse genetic variants. Contrary, the strains belonging to ST321 were highly monophyletic and showed a maximum 2 SNPs core genome difference between these isolates (Figure 11). In general, the core-SNP phylogenetic tree displayed a good congruence to the cgMLST phylogenetic tree as is demonstrated in Figure 10.

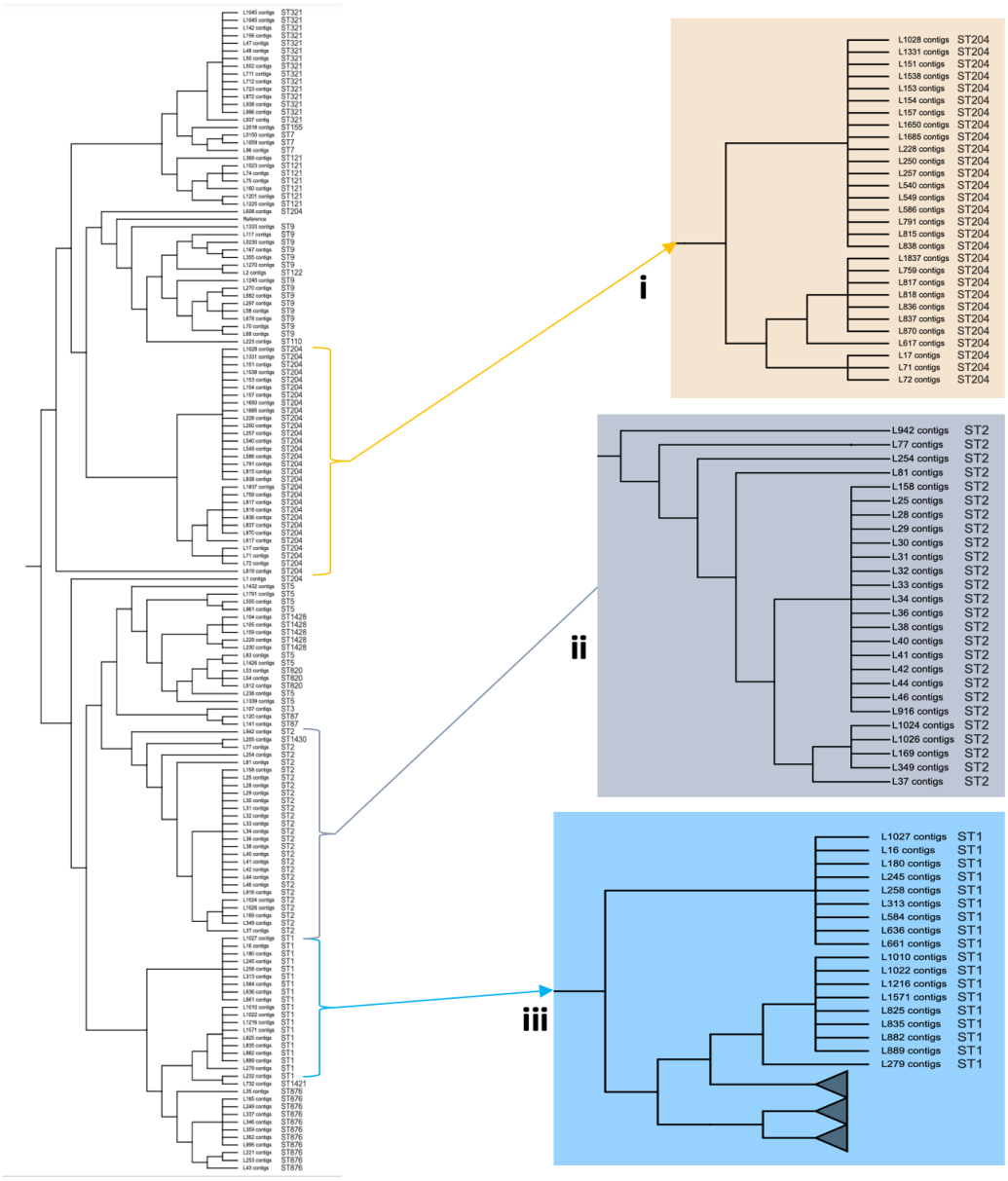

Figure 11: core-SNP phylogeny showing genetic relatedness of the *L. monocytogenes* strains in SA. (i) A section pruned from the original tree shows the South African genetically related ST204 strains. (ii) A section pruned from the original tree shows the South African genetically related ST2 strains. (iii) A section pruned from the original tree shows the South African genetically related ST1 strains.

CHAPTER 5: DISCUSSION

5.1 General features of sequenced isolates of *Listeria monocytogenes*

Listeria monocytogenes is a well-studied foodborne bacterium, primarily because of its severe public health and economic implications. Despite these implications, there is still no data published on the occurrence and population structure of *L. monocytogenes* in pig farms in South Africa. Therefore, the present study used WGS technology to characterise *L. monocytogenes* strains through determination of lineages, molecular serogroups, clonal complexes, various resistance genes and mobile genetic elements of 77 isolates collected from the environment of pig farms in 5 provinces of South Africa.

5.2 Molecular serogroups and lineages of *Listeria monocytogenes*

5.2.1 Molecular serogroups

The current study reported four different molecular serogroups of *L. monocytogenes* from the sequenced isolates with a predominance of serogroup IIa (45.5%) followed by IIc (26.0%), IVb (22.1%) and IIb (6.5%). This distribution of *L. monocytogenes* molecular serogroups in the present study is inconsistent with previous studies, which are reported in sows and livestock farms in other countries. A study by Boscher et al. (2012) reported molecular serogroups distribution as follows: IIa (41%), IIb (36%), IVb (21%) and (2%) in pig farms from France. Felix et al. (2018) found serogroup IIa and IIc in 41.2% and 1.2% of the samples isolated pig farms in France. However, Megumi et al. (2014) reported the predominance of serogroup IIb (40.5%), IIa (36.9%) and IVb (21.6%) respectively in livestock farms from Japan. In Korea, serogroup IVa (3a and 4ab) were

found to be overrepresented in pig farms (Oh et al. 2016). Although the current study did not investigate factors influencing the presence of the serogroups identified, a study by Beloeil et al. (2003) reported that differences in the occurrence and distribution of *L. monocytogenes* serogroups among different countries are largely influenced by soil type, type of animal feed, inefficient biosecurity measures on the farm and adaptability of certain serogroups to a specific environment, which could also be true in the present study.

The current study provides useful information to understand the dominant serogroups considering that there are no previous data on the serogroups of *L. monocytogenes* isolated from South African pig farms. It is also important to mention that Matle et al. (2020) reported similar serogroups in pork meat from the abattoir and retail outlets across nine provinces of South Africa, demonstrating pig farms as a possible source of contamination of *L. monocytogenes.* Comparative analysis of molecular serogroups revealed the overrepresentation of serogroup IIa in all the isolates in the current study. The high presence of serogroup IIa in this study was not surprising as IIa has been previously identified as over-represented in environmental samples in different studies (Shimojima et al. 2015; Braga et al. 2017; Lee et al. 2018). This serogroup is commonly associated with contamination of food products such as cheese, milk and meat and has been reported to cause listeriosis infections in some countries (Leong et al. 2016). Few studies globally have described *L. monocytogenes* serougroup IIa in 0 – 51% from the environment of pigs farms, which may reflect a difference in farm management methods (Braga et al. 2017).

5.2.2 Lineages

Further analysis of sequenced data revealed that all isolates in this study belong to two main lineages, I or II. Both the lineages harbour *L. monocytogenes* serotypes 1/2a (lineage II) and serotypes 1/2b and 4b (lineage I) which are commonly associated with human clinical cases (Renato et al. 2011). Our results, therefore, suggest that lineage I and II isolates are important and possibly common in pig farms in South Africa with potential to spillover to humans. Although similar descriptive differentiation of lineage I and II isolates in pig farms has been previously reported in many countries, their presence in a farm environment is linked to high profile human listeriosis cases (Haase et al. 2014; Lee et al. 2018). For instance, the 2017-2018 human listeriosis outbreaks in South Africa was associated with *L. monocytogenes* serotype 4b belonging to lineage I (Smith et al. 2019). Similarly, the human listeriosis outbreak that occurred in New Zealand in 2000 was associated with serotype 1/2a which belongs to lineage II (Sim et al. 2002).

5. 3 Clonal complexes of *Listeria monocytogenes*

Using MLST based approach in the current study revealed important data on the occurrence and distribution of different CCs of *L. monocytogenes* from the environmental isolates. Eleven different CCs were identified using cgMLST analysis. The most prevalent CCs were CC204 and CC9 belonging to lineage II and CC1, CC2, CC5 belonging to lineage I, whilst CC7, CC3, CC87, CC155 and CC321 were less prevalent in this study. Most CCs identified in our study have global distribution (Martín et al. 2014) and have been isolated from different sources, which include environmental samples (Fagerlund et al. 2016), meat and meat-producing facilities (Jennison et al. 2017).

The CC204 was the most predominant clonal complex in the current study. This predominance was not surprising as previous studies have reported CC204 to be more common in environmental samples from pig farms (Stein et al. 2018). For instance, Kwong et al. (2016) and Ebner et al. (2015) reported CC204 as the most commonly detected clonal complex of *L. monocytogenes* in pork products and pig farms in Australia and Switzerland correspondingly. The reason for the common presence of CC204 in the environmental samples of pig farms is due to its primary association with soil (Fox et al. 2016). This might be the underliying reason for CC204 isolates to colonise a broad range of environmental niches including pig farms (Fox et al. 2016). The CC204 has also been linked with human clinical infection in various countries including France, Australia and South Africa (Fox et al. 2016; Jennison et al. 2017; Thomas et al. 2020). However, its significance is mostly observed in food processing facilities such as abattoir. In food facilities, CC204 can survive for months or years and keep on contaminating food resulting in possible product contamination having serious economical costs to the facilities (Fox et al. 2016).

The CC1 was the second most predominant accounting for 19.5% of the isolates in our study. This clonal complex is regarded as the most commonly distributed globally with predominancy in the environment, food and clinical isolates (Chen et al. 2016). The presence of CC1 in the current study was not surprising as its frequency has been reported in farm environments in different countries (Wang et al. 2012; Rückerl et al. 2014; Henri et al. 2017). In a nationwide survey of *L. monocytogenes* conducted in Austria between 1996 and 2003, CC1 was found as the most common genotype in the agricultural sector including in livestock farms (Ebner et al. 2015). Papic et al. (2019) revealed that 25% of isolates recovered from sheep and pig farms in their study belonged

to CC1. The CC1 has also been linked to rhomboencapahalitis in cattle, sheep and pigs from various farms in China (Yerlikaya et al. 2019) and France (Maury et al. 2019). It has also been extensively isolated in pigs and pork products. Sarno et al. (2015) reported it from the tonsils of slaughtered fattening pigs in Switzerland.

The CC1 is a hyper-virulent strain of *L. monocytogenes* because of its strong association with human listeriosis infection including maternal neonatal listeriosis (Maury et al. 2016). The CC1 has also been linked to severe human listeriosis outbreaks such as the 2008 outbreak which occurred in China because of jellied pork consumption (Chen et al. 2016). Its presence in our isolates from pig farms suggests an important public health risk if this clonal complex is introduced into the pork meat value chain because of failure in biosafety and biosecurity measures in farms (https://www.biosecurity.govt.nz/dmsdocument/26084/direct). This CC has the potential to threaten food security as it has been reported to cause the death of many animals (Ebner et al. 2015).

The CC2 was the third most occurring genotype in this study. As with CC1, this clonal complex has a wide global occurrence and several previous authors demonstrated the presence of CC2 in over 30 countries (Chenal- Francisque et al. 2011; Wang et al. 2012; Rückerl et al. 2014; Maury et al. 2016; Henri et al. 2017). In France, Félix (2018) reported CC2 in 15% of isolates from natural pig environments, which was slightly lower than that reported in our current study. A study in North America showed CC2 isolates to be significantly associated with food (Lee et al. 2018) followed by clinical and environmental isolates as compared to other CCs in the same study.

Furthermore, Amajoud (2018) and Matle et al. (2020) found CC2 to be overly represented in isolates from food products in Morocco and South Africa respectively. *Listeria monocytogenes* CC2 strains are hypervirulent with a strong association with a non-invasive form of listeriosis in humans and animals (Jennison et al. 2017). This CC has been linked to pork meat listeriosis outbreaks in countries such as the United Kingdom and Italy (Aurelli et al. 2000; Duranti el al. 2018).

The hypervirulent CC5 was reported as the fourth (10.4%) of isolates prevalent genotype of *L. monocytogenes* in the current study. This hypervirulent strain has also been reported globally as circulating in different geographical regions (e.g., Austria, Canada, Australia, Switzerland, Finland, China, South Africa and Chile) and different origins (animals, human and environment) (Chenal-Francisque et al. 2011; Chen et al. 2019; Felix et al. 2018; Smith et al. 2019). The CC5 has been linked to several multi-state listeriosis outbreaks in the United States such as the imitation crabmeat outbreak in 1996; cantaloupe outbreak in 2011; Hispanic style cheese and stone fruits in 2014 and the ice cream outbreak in 2015) (Schmid et al. 2014; Wang et al. 2015; Buchanan et al. 2017; Meier et al. 2017). The presence of CC5 in our isolates poses a serious public health threat considering its virulence and association with human cases from various sources (Felix et al. 2018). The public health threats associated with CC5 are exacerbated by its ability to proliferate and survive in the presence of commonly used detergents in the abattoirs (Cherifi et al. 2018). This presents a new food safety challenge for the industry.

The CC9 and CC121 were both reported as the fifth most prevalent genotype in the current study

accounting for 9.1% isolates. This finding is constant with the global occurrence of CC9 in environmental samples from livestock farms (Parisi et al. 2010; Chenal-Francisque et al. 2011; Maury et al. 2016). The CC9 has been reported as the most frequently isolated *L. monocytogenes* genotype in Spain with a detection rate of 50% in environmental samples from different agricultural sites (Martín et al. 2014). A previous study that sequenced 680 *L. monocytogenes* isolates in Norway reported that 10% of the isolates from the meat industry and farms belonged to CC9 (Møretrø et al. 2017). The CC9 is also known to persist in food processing environments (Ebner et al. 2015) and has been found to circulate from all levels within the pork production chain (Felix et al. 2018). This shows that this CC may possess certain traits allowing it to persist in various food including the food processing environment and allowing it to have a high tolerance for stress resistance (Maury et al. 2016). Because of its strong association with contamination of food and food production facilities, this CC is classifield as a hypovirulent strain and can cause infection only in immunocompromised patients (Maury et al. 2016).

The findings of our study regarding CC121 in the isolates agree with those previously reported in different countries (Chenal-Francisque et al. 2011; Martin et al. 2014; Wang et al. 2015; Parisi et al. 2010). However, Maury et al. (2016) reported an overrepresentation of CC121 in food sources compared to environmental isolates (92.9% vs. 7.0%) which was higher than that reported in our study. Zuber and co-workers (2019) found CC121 as the most persistent and the second common, occurring CC in pork meat processing facilities in Europe. While a study by Ebner et al. (2015) reported CC121 as the most prevalent CCs in pork meat products in Switzerland. Similarly, Parisi et al. (2010) and Martin et al. (2014) also reported the persistence of CC121 in meat processing

facilities. These studies do not report the presence CC121 in pig farms but pork products. Our studies provide a new set of data on CC121, which can be used for future reference. Henri et al. (2016) suggested that although CC121 strains are better able to persist in the food processing environment, they are less virulent to humans. The CC121 strains are reported to be hypovirulent and have a strong association with meat products (Maury et al. 2019).

Of the 77 sequenced isolates in the current study, 5.6% belonged to CC7. A study of pork meat production value chain in France reported 1.2% of the isolates from the farm belonged to CC7, which was lower than what was reported in our study (Felix et al. 2019). Other studies in China and Austria reported CC7 in less than 1% of the isolates from RTE pork-based products and farm soil samples, respectively (Wang et al. 2012; Linke et al. 2014). The reason for the variation of results between these studies and might be because of the number of samples tested as they have tested over 1000 samples. No clinical data has suggested the pathogenesis of CC7 in humans and this could be because of its rare occurrence (Wang et al. 2012; Linke et al. 2014). Therefore, our study provides important data on the occurrence of CC7 on pig farms which can be useful in the future for a better understanding of this genotype.

The CC3 was also determined at low levels with only 2.6% of isolates sequenced in this group. Similar to our study, Chen et al. (2020) also reported lower levels of CC3 in ready-to-eat meat-based products from China. Wang et al. (2012) also found CC3 to be the least common clonal complex and found in only 3.8% of isolates from meat-based products belonging to CC3. Contradictory to our study, CC3 was ranked amongst the most prevalent CCs occurring globally

in Australia (Chenal- Francisque et al. 2011). This clonal complex was also amongst the most frequently occurring in clinical cases from China (Wang et al. 2012). The CC3 has been reported to be hypervirulent and has also been associated with the 1994 outbreak from Illinois as a result of chocolate milk (Wang et al. 2012).

CC87, CC155 and CC321 were reported in less than 1.3% isolates in our study. Contradictory to these findings, CC87 and CC155 were the most prevalent in various studies from China. The reasons for this could be due to the difference in sampling techniques, hygienic conditions of the farms' tests, different microbiological methods and geographical differences (Amajoud et al. 2018). Wong et al. (2018) reported CC87 in 63.7% of the pork products. A study by Chen et al. (2019) found this clonal complex as the third most prevalent (15.3%) in isolates from pig farms. Another study in China reported 29. 2% and 17% of *L. monocytogenes* isolates from environmental and RTE pork food were assigned to CC87 (Chen et al. 2020). CC87 has been rarely reported in other countries except for China and Spain, where two unrelated epidemiological outbreaks occurred in 2013 and 2014 (Perez-Trallero et al. 2014). There is a lack of data on CC87 in farm samples due to absent surveillance systems in pig farms from many countries (Perez-Trallero et al. 2014). CC155 has frequently been found in food samples in Eastern Asia (Chen et al. 2019), animals and farm environment in Switzerland and a few clinical cases in France, Greece, New Zealand and Netherlands (Chenal-Francisque et al. 2011). Although CC87 and CC155 have been reported to be less virulent in humans, their predominance in food requires further analysis as they can emerge as hypervirulent strains (Maury et al. 2019). There is limited information on CC321 and because of that, we are unable to discuss it.

5.3.1 Distribution of *L. monocytogenes* clonal complexes in different provinces of South Africa

The CCs reported in the present study were not equally distributed among five different provinces. Provinces showed co-existence of multiple CCs, which suggests a possibility of several sources of contamination (Lee et al. 2018). For example, the isolates from Gauteng province showed the co-existence of seven CCs followed by Limpopo and Mpumalanga showing co-existance of six. Both the Western Cape and Free State revealed co-existance of five CCs. The reasons for the co-existence of multiple CCs in the same geographical location are poorly understood but previous studies around the world have also reported similar findings (Chen et al. 2019; Smith et al. 2019). However, Chen et al. (2019) stated that the co-existence of multiple CCs in the same geographical location can be associated with animal movement because of trading which can cause the introduction of new clonal complexes in non endemic areas. This could be true in our study as there is a lot of animal movement in between provinces particularly in Gauteng where there is a higher influx of animal migration due to trade and economical reasons (DAFF, 2018).

5. 4 Phenotypic antibiotic resistance profile of *Listeria monocytogenes*

5.4.1 Phenotypic AMR by province

Distribution of phenotypic AMR resistance of *L. monocytogenes* by province revealed a significant ($p \leq 0.05$) resistance of the tested isolates toward gentamycin and cephalothin by isolates from Western Cape while isolates from Gauteng and Mpumalanga were highly resistant ($p \leq 0.05$) to kanamycin and cephalothin respectively. Gentamycin, kanamycin and cephalothin are sold over the counter in South Africa and farmers have direct access to them without the prescription from

a vetenarian (Henton, 2011). The significance in resistance levels which was observed against these antibiotics in Western Cape, Gauteng and Mpumalanga suggest possible over-use in pig farms from those provinces. Therefore, there is a need to do future investigations on the behavior and attitudes of the farmers in those provinces against the use of antibiotics. This is very important as such significant levels of resistance in *L. monocytogenes* isolates can be transferred to other bacterial species and cause serious challenges (Henton, 2011). There were no studies relating to the distribution of AMR of *L. monocytogenes* by province and therefore, a very small comparison could be made.

5.4.2 Resistance of *Listeria monocytogenes* to antibiotics

I. Nalidixic acid and Fosfomycin

All tested isolates displayed phenotypic resistance against nalidixic acid and fosfomycin antibiotics. This finding was not surprising as *L. monocytogenes* is is known to have natural (intrinsic) resistance against nalidixic acid and fosfomycin (Troxler et al. 2000). This natural resistance is because of nalidixic acid and fosfomycin that have large molecular compounds that cannot permeate the cell wall of *L. monocytogenes* (Guérin et al. 2014). However, several studies have reported the susceptibility of *Listeria* species toward nalidixic acid and fosfomycin (Karageorgopoulos et al. 2012; Guérin et al. 2014). The new susceptibility pattern of *Listeria* species against these antibiotics is linked to mutation because of modification in topoisomerase (Guérin et al. 2014; de Vasconcelos Byrne et al. 2016). Therefore, the phenotypic antibiotic test was performed in our isolates to determine whether the observation of the susceptibility pattern of *Listeria* species against nalidixic acid and fosfomycin was true in South African isolates.

II. Gentamicin, amikacin, ampicillin, kanamycin, ertapenem, tetracycline, penicillin, cephalothin, trimethropim and meropenem.

Between 19-45% of isolates were resistant to meropenem, cephalothin, penicillin, tetracycline, ertapenem, kanamycin, ampicillin, amikacin and gentamicin respectively. These findings can be a cause of concern considering that *L. monocytogenes* is known to be susceptible to a wide range of antibiotics used against Gram-positive bacteria (Walsh et al. 2001; Moreno et al. 2014; Striper Dehkordi et al. 2013; Şanlıbaba et al. 2018). There have been several studies reporting antibiotics resistance in *L. monocytogenes* isolates from food, the environment and human beings (Walsh et al. 2001; Safarpoor Dehkordi et al. 2013). This development in antibiotic resistance can be a result of the over-prescription of antimicrobials for veterinary medicine and the over-use of antimicrobials growth promoters in animal feed (Moreno et al. 2014). The spreading of antimicrobial resistance can also be attributed to the increase in global trade and travel between countries and continents, making antimicrobial resistance a huge threat to public health (Moreno et al. 2014). Strains of *L. monocytogenes* which are resistant to antibiotics can reduce the efficacy of antimicrobial agents used in combating and controlling illnesses associated with human listeriosis infections (Essack et al. 2017). Pig environments contaminated with antimicrobial resistant strains *L. monocytogenes*is are detrimental to public health and the economy (Essack et al. 2017).

- Gentamicin

The resistance level observed in the current study of 45% against gentamycin is alarming as it can lead to treatment complications having serious public health and economic implications.

Gentamycin is a first-line drug of choice to treat human listeriosis globally as recommended by the World Health Organisation (WHO, 2014). The gentamycin resistance level reported in our study was higher than that recorded in pig farms from Ireland (0.1%) (Walsh et al. 2001). However, it was lower than that previously reported by Gómez et al. (2014) who reported 74.4% in isolates from pig farms in the USA. The high levels observed in our isolates are because of the over-use of gentamycin in livestock production as a growth promoter (Henton et al. 2011).

- Amikacin

The present study revealed that 42.9 % of the isolates were resistant to amikacin. There has been a wide variation in the reported occurrence of *L. monocytogenes* resistance to amikacin in various livestock farms around the world (Wieczorek et al. 2012; Maktabi et al. 2015). For example, Aksoy et al. 2018 reported amikacain resistance of 6.7% for *L. monocytogenes* isolates, whilst. Safdar and Armstrong (2003) reported no resistance to the same antibiotic in isolates from the USA. This variation in resistance to amikacin is associated with its extensive use in the veterinary settings in different countries to treat suspected sepsis in febrile leukopenia (Al-Nabulsi et al. 2015a). This high resistance level in amikacin is associated with its extensive use in the veterinary settings to treat suspected sepsis in febrile leukopenia (Al-Nabulsi et al. 2015a).

- Ampicillin

The phenotypic resistance to ampicillin was also high with a resistance of 42.9%. We frequently use this antibiotic in humans to treat bacterial infectious diseases (Carryn et al. 2003). In fact, ampicillin is a first-line antibiotic for the treatment of both animal and human listeriosis (Chen et al. 2016). The high resistance level of *L. monocytogenes* towards ampicillin reported in our study

is concerning. These concerns emanate from the fact that if listeriosis cannot be treated with first-line antibiotics such as ampicillin, other probably more expensive methods for treatment will have to be employed increasing health care costs (Carryn et al. 2003). The findings of our studies are like those reported in Brazilian pig farms and pork abattoirs (Moreno et al. 2014).

- Kanamycin

The present study revealed 33.8% of the isolates to be resistant to kanamycin. Similar to amikacin, this antibiotic is recommended as an alternative drug to gentamycin in the treatment of listeriosis. Their recommendation is simply because they all belong to the aminoglycosides antibiotic class. However, there is no data on the active use of this antibiotic in the treatment of human or animal listeriosis. The importance of our findings is that it serves as a future reference on the resistance of *L. monocytogenes* isolates from pig farms to this antibiotic in the country. The reported resistance levels in the present study to kanamycin are higher than that of Al Nabulsi et al. (2014) and Wu et al. (2015), who reported resistance of 6.7% and 3.2% respectively. The variation in the results is because in South Africa kamamycin is heavily used to treat bovine tuberculosis and in the agricultural sector (Henton, 2011).

- Ertapenem

In the present study, 32.5% of the isolates were resistant to ertapenem. This antibiotic a broad-spectrum activity against gram positive bacteria and is used in humans as a last resort antibioticto to treat patients with severe bacterial infections or those suspected of having bacterial infections resistant to most antibiotics (Lemaire et al. 2005). The resistance level reported in our study suggests that ertapenem might not be successfully used as an alternative therapy in South African

L. monocytogenes isolates. The resistance level of ertapenem recorded in our study contradicts the findings of Olaniran et al. (2015) which reported 0% resistance in 78 *L. monocytogenes* isolates against these antibiotics in livestock farms.

- Tetracycline

The present study reported only 24% of isolates to be resistant to tetracycline. Tetracyclines are broad spectrum antibiotics used in treating a wide range of bacterial infections in humans (Bahrami et al. 2012). Tetracyclines are also commonly used in animals as growth promoters in animal feed (Chopra et al. 2015; Bahrami et al. 2012). The reported resistance level against tetracycline in our study is in line with other global trends (Moyane et al. 2013; Peter et al. 2016; Akrami-Mohajeri et al. 2018). However, our finding shows that the extensive use of tetracycline in pig farms as a feed additive can be a serious problem in the future in the country. The results in our study were lower than those reported from *L. monocytogenes* isolates by Wang et al. (2012) who reported 26.9%, Fallah et al. (2013) who reported 34.7%, Garedew et al. (2015) who reported 37.5%, Jamali et al. (2015) who reported 27.9% and Obaidat et al. (2015) reporting 64.4%. However, a study by Şanlıbaba et al. (2018) reported resistance levels of 5.9% which was lower than what was found in our study.

- Penicillin

In the present study, the phenotypic level of resistance to penicillin was 23.4%. Penicillin is a first-line antibiotic for treating listeriosis and is also one of the most frequently used antibiotics in human health (Chen et al. 2016; Moyane et al. 2013). This antibiotic is also used as an additive in livestock feed (Moyane et al. 2013). The results of our study show low levels of resistance compared to what was reported by Moreno et al. (2014) in Brazilian pig farms and pork abattoirs.

Other studies also reported higher resistance levels with Prazak et al. (2002) and Garedew et al. (2015) reporting 81% and 66.7% respectively. The low resistance level reported in our study is because of a reduction in using penicillins in livestock in South Africa.

- Cephalothin

In our study, 20.8% of isolates were resistant to Cephalothin. Cephalothin is effective in treating human bacterial infections such as skin infections and respiratory tract infections. These antibiotics are effective against gram positive bacteria and act by disrupting the synthesis of protein (Shahbaz, 2017). The development of resistance reported in the current study is a cause for concern as cephalothin is usually used to treat bacterial infections of an unknown aetiology like *L. monocytogenes* (Krawczyk-Balska et al. 2015). The findings in our study about this antibiotic can serve as a future reference on the resistance of *L. monocytogenes* to cephalothin. Few studies are investigating the resistance of isolates of *L. monocytogenes* to cephalothin in pig farms and our studies contribute to this body of knowledge. A study by Yan et al. (2010) reported that less than 1% of *L. monocytogenes* displays resistance against ephalothin in China. Another study in Ethiopia by Garedew et al. (2015) showed that most of the *L. monocytogenes* isolates are susceptible to cephalotin.

- Trimethroprim

The current study showed that 20.8 % of the *L. monocytogenes* isolates were resistant to trimethoprim. This antibiotic is grouped under a class of antibiotics known as sulfonamides. Sulfonamides have a wide variety of clinical uses and were one of the first antibiotics introduced in clinical medicine. This class of antibiotics works against positive bacteria including *L.*

monocytogenes by hindering the synthesis of folic acid required by some bacteria for cell growth (Yousef et al. 2018). Trimethoprim is a second-line drug of choice to treat listeriosis in people with allergies to a penicillin (de Vasconcelos Byrne et al. 2016). The reported resistance level against trimethoprim in this study is an indication that this antibiotic is still effective for listeriosis. Similar findings were reported by Ieren (2014) while Ennaji et al. (2008) and Şanlıbaba et al. (2018) recorded resistance levels of 17.7% and 18.6% respectively toward trimethoprim in *L. monocytogenes* isolates from various environmental sources.

- Meropenem

Phenotypic resistance to meropenem was 19.5%, which was higher than that reported by Haubert et al. (2015) and Sanlibaba et al. (2018) of 10% and 5.9 % respectively. This variation is mostly because meropenem is among the over-prescriped drug in the country to treat a wide spectrum of gram positive bacteria. An invitro study by Stepanovic et al. (2004) showed that meropenem is highly effective against listeriosis. Therefore, our results suggest a possibility that the use of this drug can be helpful in the treatment of listeriosis in the country.

III. Clindamycin, streptomycin and vancomycin

It was also observed that all (100%) isolates were susceptible to clindamycin, streptomycin and vancomycin. These antibiotics are used in treating bacterial infections in both human and animal medicine. They are also important in treating infections caused by multidrug-resistant gram positive bacteria (Sarkar & Haldar, 2019). In fact, they are considered a last-line defence antibiotic against gram positive bacterial diseases including listeriosis (Temple & Nahata, 2000). The absence of phenotypic resistance to clindamycin, streptomycin and vancomycin in our study shows

that this antibiotic can still be used to treat listeriosis. This absence of phenotypic resistance in our isolates can be attributed to the banned use of clindamycin, streptomycin and vancomycin as growth promoters in the country (Henton, 2011). The low resistance levels against these antibiotics have also been previously reported in other countries (Haubert et al. 2015; Sanlibaba et al.2018). A study by Bertschet al. (2014) reported resistance to clindamycin in 27% of isolates from food products while Şanlıbaba et al. (2018) observed no resistance (0.0%) to this antibiotic from meat isolates in Turkey. The high susceptibility levels reported in our study against streptomycin are in agreement with a study by Maktabi et al. 2015 and Kovacevic et al. 2013.

5.4.3 Multidrug resistance

The investigation of MDR revealed 11 patterns with four antimicrobial agents (NA-FOS-AMP-KF) being the most predominant. Moreover, the majority of the isolates showed an MDR pattern ranging between 5 to 8 antibiotics per isolate with one isolate having the highest pattern to 14 antimicrobial agents. *Listeria monocytogenes* can become resistant through the acquisistion of mobile genetic elements rendering the antibiotic ineffective or from mutations increasing the expression of intrinsic resistance mechanisms.

The extensive use of antimicrobial agents for the promotion of growth and as prophylactics in livestock in South Africa could be a reason for high MDR levels (11 patterns) observed in most antibiotics tested. In fact, multidrug resistant bacteria that are zoonotic, have been shown to develop as a result of their usage in food animals. This high resistance may also be because of the over-use of antibiotics because of poor regulatory control as a majority of farmers' access livestock antibiotics without prescription. The high MDR occurrence in the current study is of concern as

the efficacy of antibiotics used in disease control may be reduced, therefore constituting a public health risk. This can cause a serious problem in patients who are immunocompromised as they are more susceptible to infection (Olaniran et al. 2015) especially in South Africa where over 30% of the population is immunonocompromised and burdened by diseases such as TB and HIV (https://www.tbfacts.org/tb-statistics-south-africa[accessed:14 April.2017]). Therefore, there is a serious need to aggressively introduce public awareness campaigns on farmer education on the use of antibiotics in the country.

5.5 Genotypic antibiotic resistance profile of *Listeria monocytogenes*

5.5.1 Resistance genes profiles

The application of WGS revealed the presence of five intrinsic resistance genes in all sequenced *L. monocytogenes* isolates. The genes included *fosX* (resistance to fosfomycin), *lin* (lincosamides) and *mprF*, *norB* as well as *mgrA* (floroquinolones). This finding complements the phenotypic resistance against fosfomycin and nalidixic acids. The mechanism of the intrinsic resistance genes has been well documented and discussed in section 5.4.1 (I) in the current study. Other studies globally have reported similar results in *L. monocytogenes* recovered in livestock farms, animal and food products (Troxler et al. 2000; Guérin et al. 2014). Tetracycline resistance genes *tetS* and *tetM* were detected in 1.3 % and 3.9% of the sequenced isolates respectively. Molecular mechanisms of tetracycline resistance in *L. monocytogenes* occurred because of efflux, ribosome mutation and enzymatic inactivation which is facilitated by genes such as *tetS, tetM* and others (Yan et al. 2016). These findings were highly surprising as tetracycline has been the antibiotic with the most frequently acquired resistance in environmental, food and clinical isolates of *L.*

monocytogenes (Yan et al. 2016). Moreover, this antibiotic is approved as growth promoters in South Africa which have been considered a driver of resistance among bacteria towards tetracycline (Henton, 2011). The low presence of *tetS* and *tetM* genes in our isolates suggests that tetracycline is not extensively used in veterinary therapy and feed additives in the tested farms. Second, it means there is little if any genetic material transferred under natural conditions originating from other bacteria (enterococci and streptococci) which harbour a high prevalence of tetracycline genes (Baquero et al. 2020). The implication of the low presence of resistance genes reported in our study means that tetracycline should be effective in treating listeriosis in the country. The finding of this occurrence of *tetS* and *tetM* genes was inconsistent with the rate reported elsewhere among *L. monocytogenes* isolates from animal farm and pork products (Emmanuelle and Courvalin, 1999). Although *tetS* and *tetM* genes are very low in our isolates, they are located on mobile DNA elements which are linked with MDR in gram-positive bacteria (Wilson et al. 2018). Therefore, with active efflux pumps, these genes may be transferred to humans and present a hazard to public health.

Mechanisms of quinolones resistance in *L. monocytogenes*includes two categories of mutation and acquisition of resistance-conferring genes such as*qnrA, qnrB* and *qnrS* (Rodríguez-Martínez et al. 2011). In the present study, quinolones resistance genes *qnrA, qnrB* and *qnrS* were identified in 36.4%, 3.9% and 18.2% of isolates respectively which presented *L. monocytogenes* strains classified to clonal complexes CC1, CC5, CC7 and CC204. A similar occurrence (37%) for *qnrA* and a low detection rate for *qnrB* and *qnrS* were observed in *L. monocytogenens* isolates from livestock farms (Porel et al. 2005) and animals including pigs (Porel et al. 2005). The increasing

occurrence of plasmid mediated quinolone gene "*qnrA* and *qnrB*" is alarming because of their capacity for transferability to other bacteria affecting humans (Rodríguez-Martínez et al. 2011). Second, quinolones are the last alternative antibiotic for of treatment human diseases such as salmonellosis and listeriosis. The drivers of quinolones resistance in *L. monocytogenes* strain are as a result of the accumulation of mutations in the genes *(gnr*) coding for the target bacterial enzymes of the DNA gyrase and DNA topoisomerase IV (Rodríguez-Martínez et al. 2011).

The genes that encode resistance toward streptomycin including *strA* and *strB* were observed in 42.9% and 18.2% of isolates respectively. The *strA* gene has been recovered in 44% of *L. mononcytogenes* from livestock farms in Turkey (Srinivasan et al. 2005) and in 81.1% of *L. mononcytogenes* in meat products (Matle et al. 2019). The gene *strB* was also detected in 84.6% of *L. monocytogenes* isolates from meat products in South Africa (Matle et al. 2019). In France, *strA* and *strB* occurred in 50% of *L. monocytogenes* isolates from humans (Morvan et al. 2010) while in the USA, these genes were recovered in 90.9% of the *L. monocytogenes* strains isolated from animals respectively (Srinivasan et al. 2008). All these studies showed that *strA* and *strB* genes are most commonly observed in *L. monocytogenes* isolates resistant to streptomycin. Streptomycin has been approved as a growth promoter in pig industries in the country and the high level of these genes in our isolates suggests possible environmental contamination with resistant *L. monocytogenes*. Both streptomycin resistance genes were predominantly detected from isolates originating from Western Cape and Mpumalanga provinces. This can be attributed to the over-use of streptomycin in animal feed because of the huge pig population in those provinces (DAFF, 2015). Furthermore, *aadB* gene for aminoglycoside can confer resistance to streptomycin

(Carpentier and Courvalin, 1999). *The aad6* gene was found in 58.4 of the isolates with a high detection rate in CC204 (22.2%) followed by CC7 (17.8%) and CC1 (15.6%). More than 170 aminoglycoside-resistance-encoding genes have been described. However, the *aad6* gene is the only aminoglycoside-resistance-encoding gene that has been described in *L. monocytogenes* and *L. innocua* (Lungu et al. 2011). The *aad6* genes have also been reported *in L. monocytogenes* clonal complexes including CC204, CC7 and CC1 (Yan et al. 2016) which have been reported in the current study. Therefore, our results are not surprising as there are within the global range of *aadB* gene in *L. monocytogenes*.

The genes responsible for resistance to ampicillin (*ampC*) and penicillin (*penA*) were detected in 23.4% and 20.8% of the isolates respectively. These findings are highly inconsistent with other previous studies that failed to detect the genes responsible for ampicillin and penicillin resistance in *L. monocytogenes* around the world (Srinivasan et al. 2005; Davis & Jackson 2009.) A study in Poland showed that 9.5% and 5.1% of isolates carried *ampC* and *penA* genes respectively (Mackiw et al. 2016). In the Czech Republic, Argentina and Brazil, *ampC* and *penA* genes were not detected in *L. monocytogenes* from livestock farms (Haubert et al. 2016; Prieto et al. 2016). The findings of our study have serious public health implications considering ampicillin and penicillin are firstline drug of choice to treat listeriosis.

The *lmrB* and *ImrD* genes which are chromosomally-encoded efflux pump that confers resistance to Lincosamides in *L. monocytogenes* were found in 11.7% and 2.6% of the isolates respectively. This finding is highly surprising as most strains of *L. monocytogenes* display high resistance to

cephalosporins especially the third and fourth generations such as Lincosamides (de Vasconcelos Byrne et al. 2016). Three major trimethoprim encoding genes were detected in this study with *dfrI* (27.3%) being the most prevalent followed by *dfrII* (15.6%) and *dfrIII* (6.5%). The distribution of these genes varied among the CCs and provinces. Resistance to trimethoprim-sulfamethoxazole is mostly associated with the dihydrofolate reductase (dhfrs) enzyme that facilitates the breaking down of dihydrofolate to tetrahydrofolate in prokaryotic and eukaryotic cells (Morvan et al. 2010). There are over 15 *dhfrs* genes that confer trimethoprim-sulfamethoxazole resistance in bacteria but only three genes (*dhfr I-III*) are considered important for gram-positive bacteria (Morvan et al. 2010). The presence of these three genes (*dhfr I-III*) in the current study suggests their role in trimethoprim-sulfamethoxazole resistance.

Genes (*ermA* and *ermB*) encoding for erythromycin were detected in less than 3.9% of the isolates while vancomycin *(vanA* and *vanB*) genes were found in 3.9% of the isolates in this study. Listeria is normally sensitive to erythromycin and vancomycin (Hof, 2004). Similar to our study, Srinivasan et al. (2005) and Yücel et al. (2005) found genes encoding to erythromycin and vancomycin at a lower frequency. Their isolates were from animal and food sources. Bae et al. (2014) recovered strains of *L. monocytogenes* from various food products such as vegetables, seafood, meat and dairy foods which also harboured a low frequency of *ermA* and *ermB* genes as well as *vanA* and *vanB* genes. Arslan and Özdemir (2008) found less than 2% of *L. monocytogenes* harbouring resistant genes to erythromycin and vancomycin. Shi et al. (2015) found six *Listeria* strains isolated in their study from various RTE foods which included cooked meat and cold vegetable dishes from China were not carrying genes encoding resistance to erythromycin and

vancomycin. The reason for a low resistance of genes to erythromycin and vancomycin is that these drugs are used under strict conditions in many countries including South Africa.

5.6 Hetero-resistance of *Listeria monocytogenes*

Three isolates in the current study displayed heterogeneous resistance against trimethoprim and kanamycin. This means there are subpopulations in the isolates from CC7, CC204 and CC155 that were confirmed to demonstrate the ability to grow in a medium containing high concentrations of trimethoprim and kanamycin. The heterogeneous resistance to the trimethroprim and kanamycin observed in our isolates could be a result of the transient phenotypic changes being quickly activated upon contact with the antibiotics (Martinez-Servat et al. 2018). The heterogeneous resistance to trimethroprim and kanamycin and the clinical significance, thus, require more experimental and clinical research.

5.7 Comparison of phenotypic and genotypic antimicrobial resistance profiling

The coefficient agreement test between phenotypic and genotypic resistance profiling results revealed poor Kappa agreement (Table 8) against cephalothin, kanamycin, amikacin, tetracycline, clindamycin, erythromycin, meropenem, ertapenem, vancomycin and trimethoprim. This poor Kappa agreement in the current study suggests that phenotypic resistance observed in our isolates was induced by other mechanisms other than genes as previously reported in the other studies (Ruiz-Bolivar et al. 2011; Jamali et al., 2015). Kappa agreement ranging from fair to good (Table 8) between phenotypic and genotypic resistance was reported against streptomycin, penicillin, ampicillin and gemtamycin. This result indicates that for each phenotypic resistance recorded

against the above-mentioned antibiotics, there was at least one corresponding resistance gene. Futhermore, the phenotypic resistance observed against penicillin, ampicillin and gemtamycin was encoding by corresponding genes. The *ampC*, *penA* and *aad6* genes which are responsible for penicillin, ampicillin and gemtamycin respectively are located on mobile DNA elements which are linked with MDR in gram-positive bacteria (Alekshun et al. 2007). Therefore, with activated efflux pumps, these genes may be transferred to humans and present a hazard to public health.

5.9 Heavy metal resistance profiles

5.9.1 Arsenic resistance

The current study also investigated the presence of heavy metal in *L. monocytogenes*isolates from pig farms, which revealed the presence of *arsD* and *arsB* genes in 48% (n = 37) and 11.7% (n = 9) in sequenced isolates respectively. These genes are responsible for encoding resistance in *L. monocytogenes* towards arsenic. Arsenic has been used in the production of broad-spectrum antimicrobial agents such as arsinothricin which are last-line antibiotics (Lee et al. 2018). The resistant genes detected in our isolates encode tolerance in *L. monocytogenes* strains against antibiotics containing arsenic and lead to serious clinical complications because of lack of alternative therapy (Lee et al. 2018). Kuenne et al. (2013) and Lee et al. (2018) reported *arsD* and *arsB* genes in a range of 10 - 49% from *L. monocytogenes* strains in livestock farms which is consistent with the findings of our study. The *arsD* and *arsB* genes were reported in isolates belonging to CC1, CC2 and CC9. These findings suggest that CC1, CC2, and CC9 strains may have added advantage to survive in environments with arsenic or in the presence of antibiotics that contain arsenic. Considering the hypervirulence nature of CC1, CC2 and CC9, this resistance can

be a serious problem. Our findings are in agreement with a study by Lee et al. (2018) which associated arsenic resistance primarily with CC1, CC2, CC4 and CC9 in *L. monocytogenes*. Moreover, the high number of isolates harbouring *arsD* genes in our study is not surprising, particularly in environmental isolates as it has been previously reported (Lee et al. 2018).

5.9.2 Cadmium resistance

Less than 2% of the sequenced isolates in the present study harboured *cadC* genes. The function of *cadC* gene as previously reported in *L. monocytogenes* was to confer resistance to cadmium (Mullapudi et al. 2008). The lower resistance levels of the *L monocytogenes* isolates in the current study to cadmium reflect the relatively low propensity of our strains having transposon (Tn5422), which is mostly harboured on plasmids and responsible for tolerance to cadmium (Lebrun et al. 1994). The low detected levels of genes conferring resistance and the absence of three known resistant determinants (*cadA1*, *cadA2*, *cadA3*) to cadmium reported in the present study are consistent with previous studies. For example, Mullapudi et al. (2008) reported over 70% of the isolates represent different *L. monocytogenes* serotypes to harbour genes conferring resistance to cadmium in Turkey.

5.9.3 Other heavy metals

Other heavy metal-resistance genes for iron (*FrvA*), zinc, copper (*CopC*), aluminium (*CorA* and *CzcD*), lead (*Pbr*) and cobalt (*CorA*) resistance were also detected in a few of our isolates. These genes are known to help *L. monocytogenes* to survive and multiply in agricultural soil. However, they do not have any known clinical significance (Ryan et al. 2000). Moreover, these genes have been commonly detected in *L. monocytogenes* isolates from countries with intensive pig farming

such as China (Hingston et al. 2013)

5.9.4 Co-existence of biocide and heavy metal resistance genes

Interestingly, one isolate from CC5 (Gauteng province) was found to carry multiple resistance genes (*arsB, arsD, CadC, bcrB, bcrC* and *mdrL*) to biocide and heavy metal. Several other studies in the USA revealed that *L. monocytogenes* isolates which were implicated in the 1998-1999 hot dog-related listeriosis outbreak harboured multiple resistance genes *(arsB, arsD, CadC, bcrB, bcrC* and *mdrL*), BC, cadmium and arsenic (CDC, 1999). The co-occurrence of heavy and biocide resistance genes in *L. monocytogenes* contributes to the selection of different resistance genotypes and phenotypes that can cause human listeriosis (Angelo et al. 2017; Parsons et al. 2019). Therefore, the co-existence of resistance genes to BC, arsenic and cadmium even though is relatively low in our isolates, may still represent an important adaptation for *L. monocytogenes* in the pork value chain and subsequently cause disease in humans. Apart from CC5, there was no specific correlation that has been observed between heavy and biocide resistance profiles among the sequenced isolates in this study.

5.8 Antibacterial biocides resistance profile

The current study reported the presence of *mdrL* and *lde* genes in all the sequenced isolates. The *mdrL* and *lde* genes are known to confer resistance of *L. monocytoegenes* to qouaternary ammonium compounds such as benzalkonium chloride (BC) (Mata et al. 2000). The BCs are widely used as disinfectants in the agriculture and food industry to ensure the bacteriological safety of food products (Yu et al. 2018). The control of *L. monocytogenes* in the food processing industry is mostly based on the application of BC (Zacharski et al. 2018). Therefore, the presence of *mdrL*

and lde genes in our isolates is very alarming. Should they be transferred into the food processing facilities, they will continue to grow and multiply in the presence of cleaning detergents and keep recontaminating the food products (Yu et al. 2018). The *mdrL* and *lde* genes in our isolates can also lead to cross-adaptation of *L. monocytogenes* to other disinfectants (tertiary alkylamine, sodium hypochlorite), heavy metals and antimicrobial agents (macrolides) (Mata et al. 2000; Romanova et al. 2006). Therefore, the detection of these genes in our isolates suggests that *L. monocytogenes* can grow and multiply in the presence of the detergent, which poses a serious public and economic problem for the pork industry in the country. Previous studies have reported these genes in *L. monocytogenes* isolates from livestock farms (Meier et al. 2017), agricultural environment (Aase et al. 2000), food-producing facilities (Haubert et al. 2019) and meat-processing environments (Conficoni et al. 2016) in different countries.

Further investigation in antibacterial biocides resistance revealed the presence of cassette bcrABC in 24.7 % of the isolates. This cassette is also known to confer tolerance of *L. monocytogenes* isolates to BC through efflux pumps (Elhanafi et al. 2010). Bacterial efflux pumps are membrane proteins that can be specific for one substrate or transport a broad range of structurally dissimilar compounds from the cytoplasm to the exterior of a cell (Rodríguez-Martínez et al. 2013). The resistance to BC which is associated with efflux genes such as bcrABC cassette has been reported in different *L. monocytogenes* strains isolated from diverse sources (Korsak et al. 2019; Kovacevic, et al. 2016). In fact, cassette bcrABC in *L. monocytogenes* isolates is reported to range from 10% to as much as 46% in various countries such as Switzerland, Norway, Turkey and Spain (Mullapudi et al. 2008; Ebner et al. 2015; Ortiz et al. 2016). The detection rate of 24.7% of cassette *bcrABC*

genes reported in *L. monocytogenes* isolates from the current study is within the range as previously recorded in other countries (Elhanafi et al. 2010; Rodríguez-Martínez et al. 2013). Our findings highlight the need for regular surveillance of biocide susceptibility since there are indications that farmers are possibly using detergents with BC to clean their farms which can pose a serious problem for the food industry in the future in South Africa.

5. 10 Mobile elements profiling

5.10.1 Distribution of plasmids

The distribution of plasmids among sequenced isolates was investigated which showed the presence of four different *L. monocytogenes* plasmids across 68.9% of the isolates with a predominance of pLGUG1 (50.7%) followed by N1011A (9.1%), J1776 (5.2%) and pLI100 (3.9%) plasmids. The plasmids are known to provide *L. monocytogenes* strains with a growth advantage when exposed to selective conditions such as high acids and salt and oxidative, osmotic pressure and heat stress (Kuenne et al. 2010). Therefore, this suggests that 68.9% of our isolates have the potential to survive and multiply under harsh conditions in diverse environmental niches posed at food processing facilities. The implication of this is that should our isolates be transferred into food processing facilities due to poor hygiene practices, it will be very difficult to get rid of them. The presence of plasmids in strains of *L. monocytogegenes* have been reported to also carry genes conferring resistance to heavy metals, sanitizers and a wide range of antibiotics (Elhanafi et al. 2010; Katharios-Lahwermeyer et al. 2012; Rakic-Martinez et al. 2011). Furthermore, similar to our study *L. monocytogenes* have been reported to harbor plasmids with frequencies as high as 79% (Dykes et al. 1994).

Plasmid pLGUG1 was found in 50.7% of isolates and it harbored bcrBC genes which showed a correlation to BC tolerance in *L. monocytogenes* strains in the present study. This suggests that the pLGUGI plasmid is responsible for the resistance of our isolates to biocides such as BC. The pLGUG1 was highly associated with the hypervirulent clonal complexes CC1, CC2 and CC204 in the current study. This is important as pLGUG1 plasmid contributes to the survival and proliferation of *L. monocytogenes* in agricultural farms and food processing environments (Kuenne et al. 2010) and subsequently causes disease in humans. The plasmid J1776 was found in 5.2% of the isolates which belong to CC9 from lineage II. This plasmid was found to harbour arsenic resistance encoding the arsD gene. This suggests that the J1776 plasmid is primarily responsible for the arsenic resistance of our isolates. The co-occurrence of pLGUG1 and J1776 plasmids were found in CC5 from lineage I and CC204 from lineage II which are consistent with earlier findings that arsenic and BC resistance is chromosomally mediated in *L. monocytogenes* (Kuenne et al. 2010). This is also the reason to have observed the co-existence of biocide and heavy metal resistance in our isolates as discussed in section 5.9.4. Of the two remaining plasmids, N1011A and pLI100 which were reported in 9.1% and 3.9% of the isolates respectively have no clinical significance in *L. monocytogenes* as such as there was no previous literature found where we could refer to with regards to the findings in our study.

It was observed that isolates from lineage II had more plasmids occurrence and diversity as compared to lineage I. The distribution of plasmids by provinces revealed the presence of PLGUGI in all provinces while J1776 was found only in Gauteng and Limpopo provinces. PLI100 and N1011A were exclusively found in Mpumalanga province. The variation in the distribution of plasmids between different lineages of *L. monocytogenes* and provinces is due to the segregation

kinetics of naturally occurring plasmids or horizontal gene transfer (Jamrozy. et al 2017). This implies certain strains of *L. monocytoegenes* in our study could have lost or gained plasmids through the above mechanisms, hence the variation. The implication of this is that lineage II isolates that come from Gauteng and Limpopo provinces will display more tolerance to biocides, heavy metals and AMR.

5.10.2 Distribution of prophages

Detection of prophages through the PHASTER tools has identified eight prophages (unique intact, questionable or incomplete) across different CCs in the present study. Prophages are viruses that kill bacteria (Kutter and Sulakvelidze, 2005) and they enhance the survival of *L. monocytogenes* strains in various environments (Vu et al. 2019). The commonly found prophages in the current study belong to either *Siphoviridae* (A006, A118, B054, LP-101, LP-030- 2 and LP-030-3) and *Myoviridae* (vBLmoS188 and LmoS293). *Siphoviridae* and *Myoviridae* are bacteriophages in the order, *Caudovirales*. The *Myoviridae* have long contractile tails and the *Siphoviridae* have long noncontractile tails (Maniloff and Ackermann 1998). Another study has also reported the isolation of similar *L. monocytogenes* prophages in food value chain from Turkey and the USA (Vongkamjan et al. 2012) and Zhang et al (2012) also reported the isolation of a small fraction of three to four similar prophages as the foundation in our study. Therefore, the findings of our study support the notion that the identified prophages have global occurrence and are linked to survival evolution and persistence of *L. monocytogenes* isolates in the agricultural value chain (Kuenne et al. 2013;). They have also been found to play a significant role in the virulence and pathogenicity of certain strains of *L. monocytogenes* (Vu et al. 2019). The present study also revealed that CC1, CC2 and CC204 displayed a high number of prophage regions per genome, which shows that

genetically related strains isolated from the same farm environment harbour high conserved prophage regions in terms of region and CDS identified. Matle et al. (2019), reported CC1, CC2 and CC204 to harbour major *Listeria* prophages in South African meat products.

5.10.3 Investagation of Stress Survival Islets

In the current study, stress survival islet 1 and 2 (SSI-1 and SSI-2) were detected in 9.1% and 2.3% of the isolates respectively. These findings were not surprising since these stress survival islets are uncommonly reported in environmental isolates. SSI-1 is mostly associated with human isolates where it helps *L. monocytogenes* to grow under bile, salt, acidic and gastric stress conditions (Harter et al. 2017). The SSI-2 is mostly present in isolates from food and food processing environments and helps these pathogens to proliferate under alkaline and oxidative stress conditions (Hilliard et al. 2018). Furthermore, the current study reported that isolates from SSI-1 all belonged to CC1 of lineage I while isolates from SSI-2 belonged to CC121 and CC204 of lineage II. Similar findings have been reported globally (Ryan et al. 2010; Hilliard et al. 2018). The similarities are because CC1 strains are hypervirulent with a strong association with human listeriosis while CC121 and CC204 are known to persist in food facilities for long periods. The persistence of C121 and CC204 is linked to the presence of SSI-2 which seems to be true in the current study.

5.11 Assessment of virulence profiles across different clonal complex

The pathogenic potential of a given *L. monocytogenes* strain is determined by the functionality of a large number of genes known as “virulence factors”, all of which have different roles in the various stages of the infection cycle. The present study assessed for the presence of 115 putative

virulence markers with 41 of them identified across the isolates. However, LIPI 2 was not found in all the isolates which are not uncommon as this LIPI is mostly associated with clonal complexes which were not reported in the current study. Futhermore, a complete LIPI-1, which harbours the virulence gene cluster consisting of six genes were present in 38.9% of the isolates and 93% of them were from lineage II across different CCs. These findings are similar to those reported by Kirchner and Higgnis, (2008) and Martins et al. (2012). *Listeria* pathogenicity island consists of six genes which are *hly, mpl, actA, plcA, plcB* and *prfA* which are responsible for key processes in the life cycle of *L. monocytogenes* and divided into three transcriptional units (Linden et al. 2008). The *hly* monocistron is considered as the centre; downstream is where *mpl, actA* and *plcB* are found and *plcA* and *prfA* are located upstream (Chen et al. 2020). Therefore, the occurrence of LIPI-1 in 93% of the isolates from lineage II suggests a high degree of pathogenicity and also that most virulence markers are ubiquitously distributed in *L. monocytogenes* from this lineage.

The present study also reported, the presence of complete LIPI-3 in 19.5% of the isolates belonging to CC1 (40%), CC2 (13.3%) and CC3 (6.7%) from lineage I respectively. Chen et al. (2020) and Painset, (2019) also reported similar findings which revealed the presence of LIPI-3 in lineage I isolates. Fox et al. (2016) found the CC204 in isolates from Ireland and Australia harboured LIPI-3. The LIPI-3 consists of eight genes (*llsA, llsB, llsD, llsG, llsH, llsP, llsX, llsY*) which are responsible for enhancing the virulence capabilities of *L. monocytogenes* by hemolysis of red blood cell and facilitating colonisation of the host intestine (Quereda et al. 2017). *Listeria monocytogenes* strains that possess LIPI-3 are reported to have added advantage to adhere and colonise the intestine cell suggesting high likelihood of causing diseases in humans (Quereda et

al. 2017). This is the reason for their detection in hypervirulent clonal complexes (CC1, CC2 and CC3) in the current study. However, few isolates from CC121 and CC204 in lineage II harboured complete LIPI-3 which is uncommon as genetic material can be exchanged by different clonal complexes of *L. monocytogenes* (Su et al. 2019). The implication of this is the observation of high pothegenicity in CCs which are normally hypovirulent such as CC121 and CC204 (Lee et al. 2018).

Furthermore, the present study indicated the presence of surface protein genes namely *aut, inlF*, and *gtcA* which were found in 89% of lineage II isolates and absent from CC1 and CC2 from lineage I isolates except for *gtcA*. However, virulence surface protein gene *vip* was present in most of lineage I across different CCs but absent in CC204 and CC321 from lineage II isolates. The *aut, inlF, gtcA*, *inlJ* and *vip* are responsible for the adhesion and invasion of *L. monocytoegenes* to host cells (Martins et al. 2012). Therefore, the absence of the autolytic activity of *aut, vip*, and *inlF* genes in certain clonal complexes from our isolates could affect the invasiveness and render the pathogen avirulent (Kirchner and Higgnis, 2008; Linden et al. 2008 and Martins et al. 2012).

The internalin's protein gene *inlF* and *InlJ* as well as the *actA* genes were absent in CC121 isolates from lineage II. Internalin and *actA* are responsible for early invasiveness and facilitation of *L. monocytogenes* to cross the intestinal epithelium through interaction with the host-cell receptor E-cadherin (Su et al. 2019). The absence of these genes in CC121 is not uncommon as this strain is mostly associated with environmental contamination with little or no clinical significance.

The results of LIPI and surface proteins suggest that lineage II isolates seem more pathogenic than

isolates from lineage I. This is uncommon and inconsistent with previous studies as lineage I mostly house hypervirulent strains of *L. monocytogenes* (Dhama et al., 2015; Quendera et al., 2016). The inconsistency may be because virulence factors can be encoded on the mobile and intergrative genetic elements such as bacteriophages, plasmids, pathogenicity islands, intergrative and conjugative elements and conjugative transposons (Boyd, 2012). Therefore, the bacteria can gain or lose some of these mobile and intergrative genetic elements which can render them virulent or avirulent.

5.12 Core-genome MLST and core-SNP phylogenomic clustering of *Listeria monocytogenes* clonal complex

In comparison, the cgMLST and core-SNP phylogenetic tree displayed good congruence but there were differences in the two phylogenies which may be due to the absence and presence of different resistance genes including AMR, virulence, biocide and heavy metal genes and mobile genetic elements. The cgMLST has the advantage of using the allele as the central unit in MLST schemes; it has a reduction effect of recombination due to accessory genes or homologous recombination of small DNA segments. SNP calling analysis considers each SNP in the recombinant segment as an additional mutation (Blanc et al. 2020). However, as the cgMLST scheme includes accessory genes, the presence/absence of these genes might have an impact on the resulting phylogeny (Blanc et al. 2020) and a similar trend was observed in the present study as the phylogeny seems to be impacted by the presence of genes.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

In this study, which is arguably among the first in South Africa, we investigated AMR profiles and population structure of *L. monocytogenes* strains circulating in the environment of pig farms. Although this study does not provide a comprehensive review on the national population distribution of *L. monocytogenes* strains in pig farms, it is a step in the right direction to address the food safety and epidemiological challenges caused by this pathogen across the pork meat value chain. This study gives baseline data on the genomic diversity of *L. monocytogenes* strains in terms of the occurrence of lineages, serogroups and clonal complex and their association with resistance to biocides, heavy metals and antibiotics. This data has demonstrated the genotypes of *L. monocytogenes* that are prone to contaminate the farm environment and possibly cause diseases in animals and humans. The baseline data generated in this study using WGS technology will contribute to future epidemiological studies that can help in preventing or reducing pork-related listeriosis outbreaks which recently emerged in the country. Furthermore, it contributes to the global data on the population structure of *L. monocytogenes* from South Africa.

The investigation on the resistance profiles of our isolates confirmed the heterogenous distribution of *L. monocytogenes* strains with evidence of over-representation for phenotypic and genotypic AMR to commonly used antibiotics. Data on antimicrobial-resistant patterns will be useful in the empirical management of animal and human listeriosis. This study again illustrated that pig farm environments are highly contaminated with diverse strains of *L. monocytogenes*, which are

resistant to different biocides and heavy metals used in cleaning detergents in food facilities. The over-use of antibiotics in the agricultural sector especially in veterinary medicine is the leading contributor of bacteria that are antibiotic-resistant being transferred in human pathogens through the food chain. There is, therefore, a need to put much emphasis on monitoring the antimicrobial resistance of *L. monocytogenes* in both humans and animals as a result of the emergence of certain strains to commonly used antibiotics although the majority of the strains were susceptible.

The data provided on the genetic mobile elements such as plasmids and virulence factors and SNPs of *L. mononcytogenes* strain is very critical for enhancing the understanding of ecology, evolution divergency, adaption and survival of *L. monocytogenes* in pig farms. Besides, it is important for epidemiological purposes and source tracking of *L. monocytogenes* related outbreaks. Therefore, it can be concluded that the data provided by this study can be used to close the gap that exists in the epidemiology and ecology of *L. monocytogenes* at pig farms and contribute to building national food safety and foodborne bacterial pathogen surveillance systems in the country.

6.2 RECOMMENDATIONS

From the results of this study, it can be recommended that there is a need for targeted nationwide surveillance of *L. monocytogenes* at the farm level. The presence of biocide genes also warrants further investigation into the misuse or incorrect use of BC-containing detergents in the agricultural sectors. Due to a significant drop in the price of Whole Genome Sequencing, it is recommended that all isolates of *L. monocyotogenes* across the food value chain be sequenced. The Whole Genome Sequencing should be adopted as the new gold standard for biotyping of *L.*

monocytogenes in the country.

6.3 FUTURE STUDIES

Future work will still need to be done to compare the Whole Genome Sequencing dataset of isolates in the current study with that from food and clinical isolates within the same time frame and geographic location. This comparison will identify clusters and determine whether there are any linkages between what is circulating on pig farms and the human listeriosis cases while observing the microbiological and epidemiological evidence.

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APPENDIX 1: Genomic characteristics of *Listeria. monocytogenes* isolates used in this study

Isolates name	Province	Genome size (bp)	Contigs	GC content (%)	N50
L2	Gauteng	3101432	127	37.97	1978,751
L58	Gauteng	2994 232	38	37.82	480372
L36	Gauteng	2997211	48	37.90	63798
L38	Gauteng	3101293	37	37.88	411134
90	Gauteng	3020685	40	37.84	411134
L41	Gauteng	3107793	67	37.97	160236
L47	Gauteng	3034949	113	37.82	73231
L540	Gauteng	2940398	147	37.90	410112
L549	Gauteng	2951840	104	37.88	238911
L552	Gauteng	3109248	73	37.84	162650
L586	Gauteng	3031652	81	37.90	239271
1187	Gauteng	3110054	49	37.88	80387
1198	Gauteng	2980380	23	37.84	69620
269-C	Gauteng	3039820	60	37.90	139275
L0069	Gauteng	2997337	45	37.88	72786
L0072	Gauteng	3106983	113	37.84	116356
L0073	Gauteng	2989973	58	36.90	76253
L0075	Gauteng	3108304	85	37.82	118472
L0076	Gauteng	3133595	234	37.90	192247
L0150	Gauteng	3109458	89	37.82	124978
L0156	Gauteng	3082882	127	37.90	118082
L117	Gauteng	3110310	75	37.82	462476
30-C	Gauteng	3109110	58	37.82	475758
2330-C	Gauteng	2956449	64	37.82	586317
090-C	Gauteng	3091184	82	37.82	470065

11-C	Gauteng	3112177	27	37.82	489995
30-C	Gauteng	3109148	30	37.82	292306
71-C	Gauteng	3109709	29	37.82	264984
L678	Limpopo	3109446	30	37.84	438630
L676	Limpopo	3003035	28	37.84	90017
L0157	Limpopo	3113478	46	37.84	127207
L617	Limpopo	3115273	51	37.84	189129
L584	Limpopo	3114504	46	37.84	74560
L362	Limpopo	2949539	125	37.84	220145
L0752	Limpopo	2940398	62	37.84	146187
L0751	Limpopo	2951840	113	37.84	192840
L0750	Limpopo	3109248	124	37.84	235624
L0185	Limpopo	2999028	36	37.84	152931
L0812	Mpumalanga	2997842	55	37.84	586189
L0811	Mpumalanga	2999075	51	37.84	167699
L0790	Mpumalanga	3045313	54	37.84	123020
L0762	Mpumalanga	3111187	61	37.84	470065
L0748	Mpumalanga	3108853	102	37.84	192840
L0738	Mpumalanga	2987321	65	37.84	292306
L0737	Mpumalanga	2997596	84	37.90	264984
L0186	Mpumalanga	2997211	97	37.82	185501
L1778	Mpumalanga	3101393	109	37.90	11089
L1223	Mpumalanga	3020685	52	37.90	90017
L1214	Mpumalanga	3107793	98	37.90	438630
L1207	Mpumalanga	2949539	127	37.84	192840
L1225	W. Cape	3114504	92	37.84	438630
L916	W. Cape	2951840	113	37.82	587182
L1837	W. Cape	3114504	46	37.90	5786101
L1791	W. Cape	2940398	62	37.82	591893
L1270	W. Cape	2949539	125	37.90	146187
L1240	W. Cape	2963720	121	37.82	192840
L938	W. Cape	3095241	48	37.90	127207
L1216	W. Cape	3100524	98	37.82	189129

L1201	W. Cape	3015090	121	37.90	146187
L1870	W. Cape	2949539	112	37.82	150682
L1854	W. Cape	2902538	89	37.90	489995
L1852	W. Cape	3114504	88	37.82	118972
1848	W. Cape	2940398	154	37.90	150802
1845	W. Cape	2951840	98	37.89	146187
1789	W. Cape	3109248	48	37.92	5786101
1329	Free State	2999844	58	37.86	586189
1300	Free State	2999066	55	37.92	167699
1389	Free State	3045327	57	37.90	123020
1157	Free State	3111298	66	37.89	470065
8001	Free State	3108963	75	37.87	192840
1072-2	Free State	2987452	94	37.85	292306
8800	Free State	2997691	109	37.87	264984
9852	Free State	2997211	219	37.88	185501
6207	Free State	3101390	62	37.90	11089
3985	Free State	3020706	103	37.91	90017
1200	Free State	3107810	100	37.89	438630
5879	Free State	3100981		37.91	
5200	Free State				
01L-028	Free State				

APPENDIX 2: ETHICS CLEARING LETTER

UNISA university of south africa

UNISA-CAES HEALTH RESEARCH ETHICS COMMITTEE

Date: 14/02/2020

NHREC Registration # : REC-170616-051
REC Reference # : 2020/CAES_HREC/034
Name : Ms PM Masemola
Student #: 50163353

Dear Ms Masemola

Decision: Ethics Approval from 13/02/2020 to completion

Researcher(s): Ms PM Masemola
50163353@mylife.unisa.ac.za

Supervisor (s): Prof FT Tabit
tabitft@unisa.ac.za; 011-471-2796

Dr I Matle
matlei@arc.agric.za; 012-529-4137

Working title of research:

Genomic characterisation and antimicrobial resistance profiles of *Listeria monocytogenes* isolated from pig farms

Qualification: MSc Life Science

Thank you for the application for research ethics clearance by the Unisa-CAES Health Research Ethics Committee for the above mentioned research. Ethics approval is granted until the completion of the project, **subject to submission of yearly progress reports and further clarification. Failure to submit the progress report will lead to withdrawal of the ethics clearance until the report has been submitted.**

Due date for progress report: 28 February 2021

Please note the points below for further action:

1. What is the motivation for the selected sample size? The researcher should indicate how it was determined.

More detail is required on the statistical analysis. What type of ANOVA will be used? The researcher should include the experimental design in the research proposal, as the design will

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APPENDIX 3: PROPOSAL APPROVAL LETTER

UNISA university of south africa

Department of Life and Consumer Sciences
School of Agriculture and Life Sciences
College of agriculture and Environmental Sciences
Private Bag X6
Florida
1710

To: P.M Masemola (Student no: **501-633-53**)

Subject: Outcome of your research proposal

It gives me great pleasure to inform you that your MSc research proposal titled:" **Genomic characterization and antimicrobial resistance profiles of Listeria monocytogenes isolated from pig farms"** has been approved.

Comments and suggested improvements were provided by the review committee. These comments will be communicated to you by your supervisor.

Good luck with the rest of your studies.

Best regards

Lebelo Date 17/1/2020

Prof SL Lebelo
COD: Department of Life and Consumer Sciences

APPENDIX 4: LANGUAGE EDITING CERTIFICATE

APPENDIX 4: LANGUAGE EDITING CERTIFICATE

ZANEZ EXPERT EDITING

Registered with the South African Translators' Institutes (SATI)
Reference number 1000363

SACE REGISTERED

27 February 2021

Report on Master Thesis: PUSELETSO MASELEPE MASEMOLA (50163353)

Thesis title: GENOMIC CHARACTERISATION AND ANTIMICROBIAL RESISTANCE PROFILES OF *LISTERIA MONOCYTOGENES* ISOLATED FROM PIG FARMS

This serves to confirm that I edited substantively the above document including a Reference list. I returned the document to the author with some tracked changes intended to correct errors and clarify meaning. It was the authors' responsibility to attend to these changes.

Yours faithfully

Dr. K. Zano

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