Comparative genomics of *Listeria* species isolated from the meat processing chain in South Africa

By

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SUMMARY

COMPARATIVE GENOMICS OF *LISTERIA* SPECIES ISOLATED FROM THE MEAT PROCESSING CHAIN IN SOUTH AFRICA

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Keywords: *Listeria*, Whole genome sequencing, MLST, Antimicrobial resistance, Stress tolerance, Virulence factors, Mobile genetic elements.

Traditionally, foodborne pathogens are characterised using traditional methods such as serotyping and molecular typing assays. However, whole genome sequencing (WGS) conducted through next-generation sequencing (NGS) technology has recently emerged as a powerful tool for bacterial characterisation, including identification of whole genome-wide single nucleotide polymorphisms, core- and whole-genome multi-locus sequence typing, pan-genome characterisation and investigation of outbreaks caused by foodborne pathogens. Moreover, WGS provides many important microbiological

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assays in silico, including prediction of serotypes, virulence profiling, antimicrobial resistance, and stress tolerance associated with bacterial pathogens. The cost reduction of WGS has allowed it to become the new "gold standard" for the characterisation of bacterial pathogens and a viable alternative tool for diagnosing foodborne cases. In South Africa (SA), most WGS efforts have focused on characterising human clinical strains associated with illnesses and/or outbreaks. However, WGS-based studies querying foodborne bacterial pathogens isolated from non-human sources in SA are limited, and little is known regarding which lineages are circulating among animals, food, and the food processing industry in the country. As a result, this study was undertaken to perform a more detailed genomic analysis of *Listeria* species (spp.), including *Listeria monocytogenes, Listeria innocua,* and *Listeria welshimeri* isolated from animals, food, and the food processing environment in SA using WGS. Thus, the key findings of this study are presented here.

Based on WGS analysis, a total of 258 isolates of *Listeria* spp. were studied, including *L. monocytogenes* (*n* = 217), *L. innocua* (*n* = 38), and *L. welshimeri.* (*n* = 3). The *L. monocytogenes* were classified into two main lineage grouping: lineages I (*n* = 97; 44.7%) and II (*n* = 120; 55.3%). The lineage groups were further differentiated into IIa (*n* = 95, 43.8%), IVb (*n* = 69, 31.8%), IIb (*n* = 28, 12.9%), and IIc (*n* = 25, 11.5%) serogroups. The most abundant sequence types (STs) were ST204 (*n* = 32, 14.7%), ST2 (*n* = 30, 13.8%), ST1 (*n* = 25, 11.5%), ST9 (*n* = 24, 11.1%), and ST321 (*n* = 21, 9.7%). In addition, 14 clonal complexes (CCs) were identified with an over-representation of CC1, CC3, and CC121 in "Processed Meat-Beef", "RTE-Poultry", and "Raw-Lamb" meat categories, respectively. Furthermore, the *L. monocytogenes* strains that showed hyper-virulent potential were ST1, ST2, and ST204, and hypo-virulent potential were ST121 and ST321. The virulent potential was based on the presence or absence of major virulence factors such as *LIPI*-1, *LIPI*-3, *LIPI*-4, and the internalin gene family members, including *inlABCEFJ*.

Furthermore, amongst the presumed non-pathogenic spp. (*L. innocua and L. welshimeri*), the most common STs identified was ST537 (*n* = 22, 56%) followed by ST1085 (*n* = 6, 14.6%) in *L. innocua* isolates. The STs found in the *L. welshimeri*

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isolates were ST1005, ST1084, and ST168. WGS analysis points to the presumed non-pathogenic *Listeria* spp. is unlikely to cause disease manifestation compared to pathogenic species due to the low occurrence of virulence factors such as *inl*A, *inl*B, and *LIPI*-1. The findings of this study demonstrate a high level of genomic diversity among *Listeria* spp., especially *L. monocytogenes* isolates recovered across the meat value chain control points in SA.

This study undertook the detailed molecular characterisation of bacterial foodborne pathogens using WGS from non-human samples in SA. Examination, as well as characterisation of foodborne pathogens in both domestically and imported food and animal products, are very important endeavours that can help in the reduction of risk to public health. Moreover, understanding the population structure, antimicrobial profiles, and genomic characteristics of sequenced bacterial isolates can contribute to better control measures of this pathogen. In conclusion, the use of WGS has generated massive amounts of critical understanding of the genomic characteristics of foodborne pathogens circulating in the country's environment, food, and animal populations.

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Synopsis

This thesis explores the applications of WGS in food and food processing facilities to enhance understanding of the overall distribution of genomic elements that contribute toward the pathogenicity and survival of Listeria spp., including *L. monocytogenes, L. innocua, and L. welshimeri in food and food processing environment. This thesis further explores the development of a rapid online tool for sequence typing of Listeria* spp. *The insights from this work directly informed the incorporation of WGS into the national surveillance of Listeria* spp. *and clonal lineages in SA.*

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Declaration

I, **Thendo Mafuna**, declare that the thesis, which I hereby submit for the degree of Doctor of Philosophy (Bioinformatics) at the University of Pretoria, is my work and has not previously been submitted by me for a degree at this or any other tertiary institution.

This is to certify that:

- This thesis, entitled "***Comparative genomics of Listeria species isolated from the meat processing chain in South Africa***" comprises only my original work for this Ph.D. except where otherwise indicated;
- Due acknowledgment has been made in the text to all other material used;

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Dedication

This work is dedicated to my loving wife: Zwavhudi Laura Mafuna; my two beautiful children: Washu Sherilyn Mafuna and Womphulusa Edison Mafuna; my father: Mpfariseni Edison Mafuna; and mother: Azwihangwisi Lucy Mafuna for always believing in me and always wanting the best for me. To my siblings: Tshifhiwa Kutama, Lufuno Mafuna, and Zeldah Mushaathoni Mafuna, thank you for your support. You all have been my source of inspiration and strength. Thank you so much for the love and support you gave me.

I LOVE YOU!!!

GOD BLESS YOU

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God Bless You All, Thank You

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List of Abbreviations

ABI	Applied Biosystems
ADI	Arginine deiminase system
AMR	Antimicrobial resistance
ARC	Agricultural Research Council
As	Arsenic
BC	Benzalkonium chloride
BHI	Brain heart infusion (BHI)
BLAST	Basic Local Alignment Search Tool
BWA	Burrows-Wheeler Aligner
CC	Clonal complexes
Cd	Cadmium
CDS	Coding DNA sequence
cgMLST	Core-genome multilocus sequence typing
CNS	Central nervous system
CRISPRS	Clustered Regularly Interspaced Short Palindromic Repeat
DALRRD	Department of Agriculture, Land Reform, and Rural Development
DNA	Deoxyribose nucleic acid
EMBL	European Molecular Biology Laboratory

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FPE	Food processing industry
GAD	Glutamate decarboxylase
GUI	Graphical user interface
H	Flagellar
Inl	Internalin
LIPI	*Listeria* pathogenicity islands
MLST	Multilocus sequence typing
NGS	Next-generation sequencing technology
O	Somatic
OVI	Onderstepoort Veterinary Institute
PacBio	Pacific Biosciences
PFGE	Pulsed-field gel electrophoresis
PHASTER	PHAge Search Tool – Enhanced Release
PLSDB	Plasmids database
prfA	Positive regulatory factor A
QACs	Quaternary ammonium compound
RNA	Ribonucleic acid
SA	South Africa
SigB	Alternative sigma factor

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SNP	Single nucleotide polymorphism
spp	Species
SRA	Sequence Read Archive
SSI	Stress Survival Islet
ST	Sequence type
UK	United Kingdom
US	United States of America
VFDB	Virulence Factor database
wgMLST	Whole-genome multilocus sequencing typing
WGS	Whole-genome sequencing

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Glossary

Lineages	Temporal series of populations, organisms, cells, or genes connected by a continuous line of descent from ancestor to descendant.
Sequence types	Bacterial strains characterised by typing of multiple loci, using DNA sequences of internal fragments of multiple housekeeping genes.
serotypes	Distinct variation within a species of bacteria or virus or among immune cells of different individuals.
Clonal complexes	Groups of STs in which every ST shares at least five of seven identical alleles with at least one other ST in the group.

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CHAPTER ONE

CHAPTER ONE: LITERATURE REVIEW

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CHAPTER ONE: LITERATURE REVIEW

1.1 Introduction

Advances in medical technology, including the fast-growing field of genomics, are shaping scientific research and clinical medicine. The term "genomics" was first introduced in 1986 by Dr. Thomas Roderick and was initially intended as a term for the study of genomes comparison of various spp. as well as their genome evolution. The study of genomics involves the application of DNA sequencing and subsequent analyses through bioinformatics approaches to study the structure and functions of genes in organisms and the pathogens that cause disease in them.

In recent decades, genomics has already transformed microbiology and the study of prokaryotes through high-throughput NGS technologies. These NGS technologies have enabled us to easily identify bacterial pathogens, detect antibiotic resistance and virulence, and determine how they adapt to various environments using WGS data. However, despite the benefits of WGS to support traditional methods in diagnostic microbiology, there is still a limited application of genomics in agriculture, clinical and public health in SA.

1.2 Background

In recent years, foodborne pathogens have become a significant public health problem worldwide and their impact on health (significant morbidity and mortality rate) and the economy is increasingly recognized (Guerra *et al*., 2016; Abebe *et al*., 2020). In fact, foodborne pathogens are responsible for more than 600 million cases of illness worldwide (Abebe *et al*., 2020). The economic burden associated with foodborne pathogens, as reported by World Bank (2016) is estimated to cost US$ 95.2 billion annually in low- and middle-income countries. According to the World Health Organisation (WHO), 30% of the population suffers from foodborne diseases yearly in developed countries, and up to 2 million deaths per year are estimated in developing countries (Abunna *et al*., 2016; Abebe *et al*., 2020).

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Most foodborne pathogens have a zoonotic origin, and food products of animal origin are considered major vehicles of foodborne infections (Abebe *et al*., 2020). Food-producing animals are the primary reservoirs for foodborne pathogens, and animal food products have a high risk due to pathogens, natural toxins, adulterants, and other possible contaminants. The risk of foodborne diseases in humans is increasing as the consumption of food of animal origin increases (Zhao *et al*., 2021; Abebe *et al*., 2020). Furthermore, there have been growing concerns about foodborne pathogens that can acquire antimicrobial resistance (AMR) determinants in livestock environments, which can make infections in humans and animals more difficult and costly to treat (Founou, Founou & Essack, 2016).

There is evidence that foodborne pathogens are becoming a major concern in SA and contribute to public health challenges (Smith *et al*., 2019). In support of this notion, clinical reports are available showing that foodborne pathogens contribute to public health problems in Gauteng province. For example, besides the listeriosis outbreak during 2017-2018 that claimed over 100 lives in Gauteng, hospitals in the province experienced increased incidences of human foodborne illnesses associated with animal products (Smith *et al*., 2019). Therefore, with the increase in foodborne pathogens incidences that claimed human lives, a need arise to consolidate the collaborative approaches between institutions of public and animal health sectors with one-health disease investigation and management strategies. Resources and technical capabilities have to be mobilised to facilitate the surveillance of foodborne pathogens in humans and animals. As a result, enabling the identification of outbreaks, tracking the spread of diseases, and providing early warning for national as well as international human and animal health institutions (Schlundt *et al*., 2020). To this end, WGS is being increasingly employed to characterise foodborne pathogens from animals (e.g., livestock) and animal products, as WGS can not only replicate many important microbiological assays in *silico* (e.g., prediction of serotype, AMR), but provide additional data that can be used to characterise isolates (Carroll *et al*., 2021).

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1.3 The Genus *Listeria*

The *Listeria* genus consists of Gram-positive spp. belonging to the *Listeriaceae* family, which is widely distributed in nature (Buchanan*et al*, 2018). As of 2021, there are 26 recognized spp. and six subspecies in nature (https://lpsn.dsmz.de/genus/listeria). The *Listeria* spp. can be separated into two clades, including, *sensu stricto* (*L. monocytogenes* (Seastone, 1935), *L. innocua* (Seeliger, 1977), *L. ivanovii* (See, liger *et al*., 1984,) including subsp. *ivanovii* and *londoniensis* (Boerlin *et al*., 1992), *L. welshimeri* (Rocourt *et al*., 1983), *L. seeligeri* (Rocourt *et al*., 1983), *L. marthii* (Graves *et al*., 2010)) and *sensu lato* (*L. grayi* (Rocourt *et al*., 1992) including subsp. *grayi* and *murrayi* (Rocourt *et al*., 1992), *L. fleischmannii* (Bertsch *et al*., 2013) including subsp. *fleischmannii* and *coloradonensis* (den Bakker *et al*., 2013), *L. costaricensis* (den Bakker *et al*., 2014), *L. goaensis* (Núñez-Montero *et al*., 2018), *L. floridensis* (den Bakker *et al*., 2014), *L. aquatica* (den Bakker *et al*., 2014), *L. newyorkensis* (Weller *et al*., 2015), *L. cornellensis* (den Bakker *et al*., 2014), *L. rocourtiae* (Leclercq *et al*., 2010), *L. thailandensis* (Leclercq *et al*., 2019), *L. valentina* (Quereda *et al*., 2020), *L. riparia* (den Bakker *et al*., 2014), *L. booriae* (Weller *et al*., 2015), *L. weihenstephanensis* (Lang *et al*., 2013), *L. grandensis* (den Bakker *et al*., 2014)). Over the past decade, more than 15 new species have been discovered and added to the genus *Listeria*.

Within the genus *Listeria*, *L. monocytogenes* and *L. ivanovii* are the most important pathogens of human and animal (zoonosis) hosts, respectively (den Bakker *et al*., 2010). *L. monocytogenes* and *L. ivanovii* are genetically closely related to other non-pathogenic *Listeria* spp. *L. monocytogenes* is closely related to *L. innocua* and *L. marthii,* whereas *L. ivanovii* is closely related to *L. seeligeri,* which are non-pathogenic spp. although some of their isolates have a homologue of the main *Listeria* virulence gene cluster which can give rise to modern pathogenic and non-pathogenic *Listeria* spp. and strains. (Graves *et al*., 2010).

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1.4 Genetic diversity and epidemiological association between strains/lineages

Listeria monocytogenes were initially grouped into 13 serotypes based on the agglutination of somatic (O) and flagellar (H) antigens (Matle *et al*., 2020; Lachtara, Wieczorek & Osek, 2022). Of these serotypes, only three (1/2a, 1/2b, and 4b) were causing more than 90% of invasive human infections (Kim *et al*., 2018, Matle *et al*., 2020). Further grouping and differentiation on the strain level were conducted by molecular techniques such as pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (Burall *et al*., 2017). In *Listeria* spp., MLST was conducted by sequencing internal portions of seven housekeeping genes (Ragon *et al*., 2008; Mafuna *et al*., 2021). MLST showed that *L. monocytogenes* belong to a structured population consisting of four divergent lineages (I–IV), and the isolates belong to groups of genetically highly similar strains called clonal complexes (CCs) (Ragon *et al*., 2008; Lista & Fiore, 2022).

The three major genetic lineages are Lineages: I, II, and III, with lineage I comprising isolates belonging to serotypes 1/2b, 3b, 3c, and 4b, whereas lineage II comprises isolates belonging to serotypes 1/2a, 1/2c, and 3a; and lineage III comprising of isolates belonging serotypes 4a and 4c (Doumith *et al*., 2004; Chen *et al*., 2016; Nielsen *et al*., 2017). Furthermore, lineage I have the most epidemic strains implicated in causing multiple listeriosis outbreaks worldwide. Lineage II isolates are mostly overrepresented in foods, however, they have been isolated from human clinical cases as well. Lineage III isolates are mostly found in ruminants (Kathariou, 2002; Mohan *et al.,* 2021).

Furthermore, MLST of *L. monocytogenes* conducted by Kathariou (2002) and Nappi *et al.* (2005) allowed for the discovery of serotypes ½a, ½b, and 4b as the predominant causative agents of human listeriosis in different countries. Serotyping of *L. monocytogenes* isolates revealed that serotype ½ (½a, ½b, ½c) are more commonly found in food isolates than in human clinical cases. However, no specific association has been identified between a particular form of listeriosis and specific serotypes (Soni *et al*., 2014; Matle *et al*., 2020). In Europe and North America, most human listeriosis cases over the past 10 years (2000–2010) involved serotype 4b, and it was shown to be

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over-represented in perinatal listeriosis (Lacroix *et al*., 2014). However, MLST lacks the discriminatory power required for outbreak surveillance, even though it provides highly standardized pathogen genotypes and nomenclature. Examples of Genetic diversity of *Listeria* isolated in SA (Figure 2.7 and Appendix 2).

1.5 *Listeria monocytogenes*

Listeria was described for the first time by Murray (1924) as a result of the sudden deaths of six young rabbits and was named *Bacterium monocytogenes*. *L. monocytogenes* was then first detected by Nyfeldt (1929) in humans, and later that year, Gill (1937) described the same disease in sheep. Thereafter *L. monocytogenes* was recognised as a pathogen that causes sporadic human infections and was mostly encountered by individuals working on diseased animals (Lamont & Sobel, 2011). The first interest amongst food manufacturers started to emerge in the 1980s when several outbreaks occurred in Vacherin Mont d'Or in Switzerland in 1983–1987 and the United States (US) in 1983 (Klumpp & Loessner, 2013; Lekkas, 2016).

Listeria monocytogenes was then regarded as a major concern in the food and health industry throughout the world due to its ability to survive a wide range of harsh environmental conditions (Klumpp & Loessner, 2013; Lekkas, 2016). This bacterium can survive for long periods in frozen foods and can grow in pH 4.3 to 9.6 with optimal growth at neutral pH (Ricci *et al*., 2018). Furthermore, *L. monocytogenes* can form biofilm structures on various food contact surfaces including plastic and stainless steel (Osek, Lachtara & Wieczorek, 2022). This structure protects the pathogen from sanitizers used in the food industry as disinfectants (Osek, Lachtara & Wieczorek, 2022). This pathogen has isolated from a variety of environmental sources, because of its ubiquitous nature. These includes, soil, sewage, silage, water, waste effluent, and faeces of humans and animals and a variety of food products such as meat, chicken, smoked fish, unpasteurized dairy products, and vegetables (Matle *et al*., 2020). Unlike many other bacteria causing foodborne diseases, *L. monocytogenes* is an important foodborne pathogen that causes infectious disease in humans called listeriosis (Bintsis, 2017; Shamloo *et al*., 2019) Although it is widely distributed in the environment, its

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transmission to humans mainly occurs through consumption of contaminated food products during or after processing (Matle *et al.*, 2020; Zhao *et al.,* 2021). *L. monocytogenes* poses a serious risk to the food industry, particularly producers of pre-packaged ready-to-eat food products. This is due to its capability to form biofilms and the ability to thrive in harsh environmental conditions including high salt, low pH, and low temperature (Mazaheri *et al*., 2021; Raschle *et al*., 2021).

1.6 *Listeria innocua*

Listeria innocua is a ubiquitous bacterium widely distributed in the environment (Orsi *et al*., 2016). This bacterium is non-pathogenic to humans, although rare cases of *L. innocua* septicaemia and meningitis infections have been reported in humans and animals previously (Perrin *et al*., 2003; Moura *et al*., 2019). It has been reported that typical *L. innocua* is nonhemolytic. However, in Asia, North America, and Europe, atypical haemolytic *L. innocua* isolates have been reported in seafood (Johnson *et al*., 2004; Moreno *et al*., 2012; Milillo *et al*., 2012). This suggests that atypical hemolytic *L. innocua* isolates can spread worldwide. In 2004, the first atypical *L.* innocua strain (PRL/NW 15B95) was reported and considered hemolytic due to the presence of the *LIPI*-1 (Johnson *et al*., 2004). The atypical *L. innocua* isolates carrying the *LIPI*-3 were also identified and have been shown to exhibit hemolytic activity (Cotter *et al*., 2008; Moura *et al*., 2019).

1.7 *Listeria welshimeri*

Listeria welshimeri is a non-spore-forming, Gram-positive rod bacteria which are 0.5 to 2.0 µm in size. The first *L. welshimeri* strains to be isolated were SLCC5334, CIP8149 and *Welshimer* V8 from decaying plants (Hain *et al*.,2006). The virulent *Listeria* strains pathogenesis are due to the significant virulence determinants that are localized on a chromosomal locus between *prs* and *ldh,* the designated virulence gene cluster *LIPI*-1 (Palaiodimou *et al*., 2021; Mafuna *et al*., 2021). However, none of these virulence factors are found in the *L. welshimeri* genome, which suggests that these can be presumed to be non-pathogenic spp. (Palaiodimou *et al*., 2021).

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1.8 Human listeriosis

Listeria monocytogenes represents a major public health concern because of its ability to cause severe human illness with serious consequences. Its infection includes invasive listeriosis with meningitis, septicaemia, primary bacteriamia, endocarditis, non-meningitis central nervous system (CNS) infection, influenza-like illness, conjunctivitis, and severe non-invasive listeriosis with febrile gastroenteritis in susceptible individuals especially pregnant women, new-borns, elderly and immunocompromised individuals (Doganay, 2003; Matle *et al*., 2020; Zhang *et al*., 2021). Furthermore, severe infection with *L. monocytogenes* in humans causes stillbirth or abortion in pregnant women because this bacterium can be transmitted from the pregnant mother to foetus *in utero* (Buchanan *et al*., 2018). Severe invasive listeriosis is also associated with a high hospitalization rate (90%) and a fatality rate that can reach up to 20%-30% (Buchanan *et al*., 2018, Matle *et al*., 2020). Cases of listeriosis differ between countries and usually occur between 0.1 and 11.3 cases per million people (FAO/WHO, 2004).

The majority of foodborne listeriosis outbreaks have been linked to different food products, including various meat types (Figure 1). The first laboratory-confirmed invasive case of listeriosis occurred in 1988 due to the consumption of contaminated meat products (turkey franks) (Schwartz *et al*., 2018). Since then, most of the listeriosis causal products included processed, vacuum-packaged meat products (Jensen *et al*., 2016; Chen *et al*., 2017), pork tongue (Bozzuto, Ruggieri & Molinari 2010), sausages (Jensen *et al*., 2016), and polony (Smith *et al*., 2019). The most significant documented outbreaks of listeriosis in SA occurred between 2017 and 2018 and were associated with the consumption of ready-to-eat meat (RTE) products (Polony), with ST6 belonging to serotype 4b being the most predominant (Smith *et al*., 2019). Table 1 gives an overview of major foodborne listeriosis outbreaks because of meat products in the world.

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1.8.1 Listeriosis in South Africa

The high consumption rates of processed meat (ready-to-eat) and dairy products and the suitability of these products acted as vectors for transmission of *L. monocytogenes* that resulted in an outbreak of listeriosis in SA (Figure 1). Besides the effects on the health of consumers, such an occurrence caused economic losses through loss of consumer confidence in the brand and products thereof (Buchanan *et al*., 2018; Matle *et al*., 2020).

In SA, the outbreak of listeriosis occurred at the beginning of 2017. From 1 January 2017 up until 10 May 2018; 1,019 laboratory-confirmed cases of listeriosis have been reported to the National Institute for Communicable Diseases (NICD) from all SA provinces. The majority of cases came from three provinces: 581 (59%) from Gauteng, 118 (12%) from Western Cape, and 70 (7%) from KwaZulu-Natal provinces, with the remaining cases coming from the other provinces in SA (WHO, 2018; Allam *et al*., 2018). The outcome of the illness is known for 674 patients, of whom 183 (27%) of them died; this case fatality rate is comparable to other recorded listeriosis outbreaks worldwide. In this outbreak, 42% of the cases were neonates infected during pregnancy or delivery (WHO, 2018). Ninety-one percent of the *Listeria* strains isolated from ready-to-eat processed meat, and environmental samples from food processing plants belonged to *L. monocytogenes* ST6, which is one of the most virulent *Listeria* strains (Allam *et al*., 2018). Nine percent of the reported cases in the above mentioned outbreak were infected with other strains of *Listeria* spp. and not the predominant ST6 outbreak strain (WHO, 2018; Allam *et al*., 2018). This may indicate that more than one outbreak was ongoing in SA.

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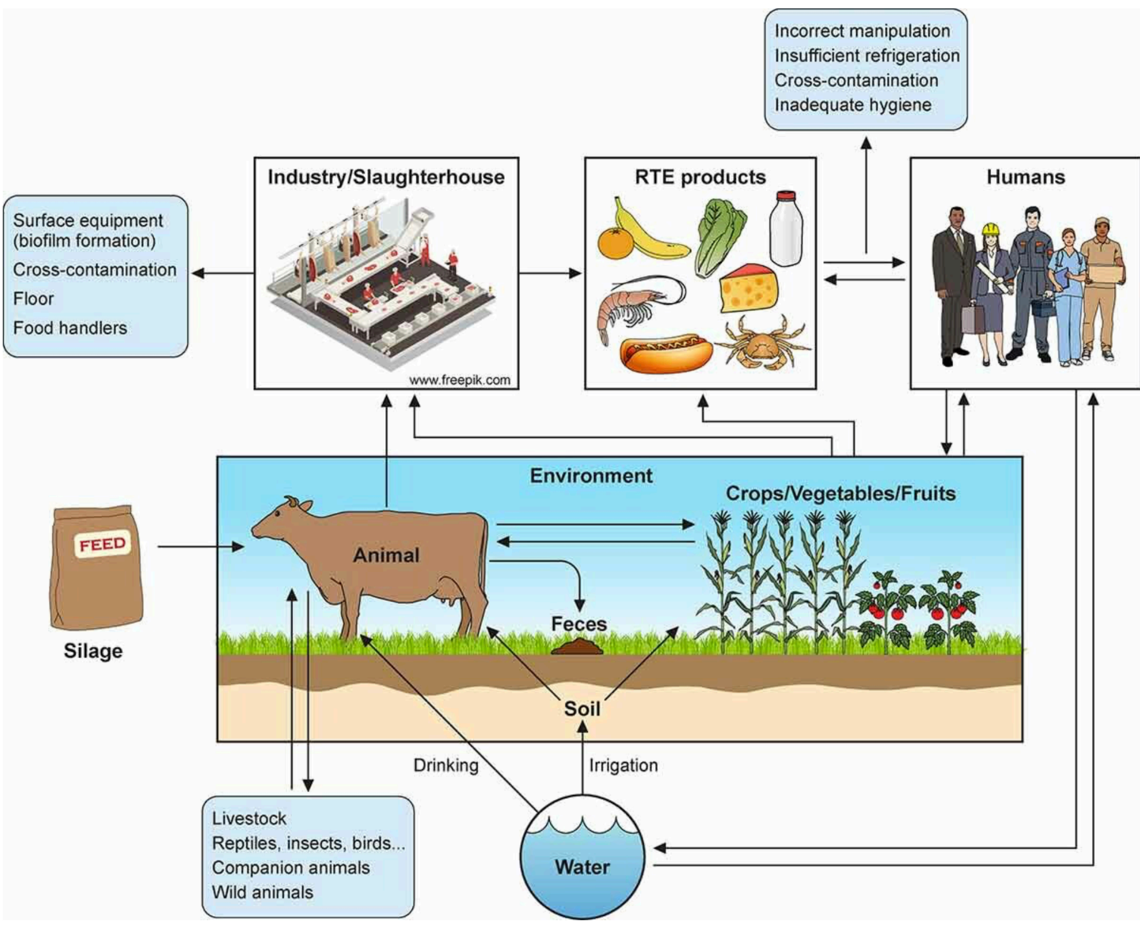

Figure 1. *L. monocytogenes* contamination sources. Transmission scenarios for *L. monocytogenes*. Potential transmission pathways indicated by arrows (Source: Quereda *et al*., 2021).

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Table 1. Major listeriosis outbreaks due to meat products in the world.

Year	Country	No. of cases (mortality)	Meat type	Serotype
1987–1989	UK	366 (ND)	Paté	4b
1900	Australia	9 (6)	Processed meats, paté	ND
1992	France	279 (85)	Pork tongue in jelly	4b
1993	France	38 (10)	Rillettes	4b
1996	Australia	5 (1)	Diced, cooked chicken	ND
1998–1999	USA	108 (14)	Hot dogs	4b
1999	USA	11 (ND)	Paté	ND
1999–2000	France	10 (3)	Rillettes	4b
1999–2000	France	32 (10)	Pork tongue in aspic	4b
2000	USA	30 (7)	RTE deli turkey meat	½a
2000	New Zealand	30 (ND)	RTE deli meats	½a
2001	USA	16 (ND)	Deli meats	½a
2002	USA	54 (8)	RTE deli turkey meat	4b
2006–2007	Germany	16 (ND)	RTE scalded sausage	4b
2011	Switzerland	6 (ND)	Cooked ham	½a
2013–2014	Denmark	41 (7)	Meat products	ND
2017–2019	South Africa	1036 (216)	Polony	4b (ST6)

ND = Not defined

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1.9 Stress tolerance, adaptation, and resistance in *Listeria*

Exposure of *Listeria* to stressful conditions prompts a response leading to phenotypes that may remain even after the stress disappears (Quereda *et al*., 2021). Such stress includes environmental stress, the acidic pH of the stomach, intestinal lumen microbiota, osmotic stress, and bile salts (Figure 2) (NicAogáin & O'Byrne, 2016; Quereda *et al*., 2021). *Listeria*'s ability to tolerate stress conditions is regulated by the alternative sigma factor (*SigB*), which induces hundreds of genes involved in the general stress response (Guerreiro *et al*., 2020). Stress tolerance of *L. monocytogenes* to harsh conditions is due to the Stress Survival Islet-1 (SSI), which has been linked to tolerance toward acidic, bile, gastric, and salt stresses, and the SSI-2, which is responsible for survival under alkaline and oxidative stresses (Mafuna *et al*., 2021).

1.9.1 *Listeria* tolerance to environmental stress in food and food-processing environments

Listeria has an outstanding capacity to adapt to stress conditions encountered in different environments due to SSI which contributes to the growth of *L. monocytogenes* in suboptimal conditions. This ability allows *L. monocytogenes* to proliferate in various food matrices with high salt concentrations, acidic pH, refrigeration temperatures, and germicidal blue light as well as the ability to persist in FPEs cleaned with disinfectants (NicAogáin & O'Byrne, 2016). *L. monocytogenes* may further exhibit tolerance to disinfectants exposure such as quaternary ammonium compound (QACs) and induce efflux pumps like *bcrABC* operon (Dutta *et al*., 2013). The mechanisms behind *Listeria's* ability to tolerate QACs depend mainly on the formation of biofilms and expression efflux pumps encoded by horizontally acquired genetic elements (Kovacevic *et al*., 2016; Kropac *et al*., 2019). The *L. monocytogenes* also induces helicases such as *lmo0866*, an RNA helicase homolog to DEAD-box protein A; and RNases, such as *lmo1027* upon exposure to cold environments (Hingston *et al*., 2017; Quereda *et al*., 2021). Exposure to high osmolality conditions in salt-preserved food leads to the development of the *mrpABCDEFG* operon (Burgess *et al*., 2016; Quereda *et al*., 2021). During osmotic shock, in order for *Listeria* to adapt to high salt concentrations, it

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induces *gbu* and beta which encode glycine-betaine transporters, and the carnitine ABC transporter *opuCA* (Burgess *et al*., 2016).

1.9.2 *L. monocytogenes* tolerance the acidic pH of the stomach

After ingesting contaminated food, *L. monocytogenes* move to the stomach, where it gets exposed to an extremely acidic pH which poses the first physicochemical antimicrobial host barrier (Cobb *et al*., 1996; Kvistholm *et al*., 2016; Quereda *et al*., 2021). For *L. monocytogenes* to survive the acidic pH, it uses regulatory systems that allow them to overcome the intracellular acidic pH of the gut and acidic pH of the food (Gahan & Hill, 2014). The regulatory systems that allow *L. monocytogenes* to survive in the stomach include the glutamate decarboxylase (GAD) system and arginine deiminase system (ADI) (Gahan & Hill, 2014; Quereda *et al*., 2021).

1.9.3 In the intestinal lumen *L. monocytogenes* competes with endogenous microbiota

L. monocytogenes must adapt to harsh intestinal conditions and coexist with host intestinal normal microbiota in the gut (Rolhion & Chassaing, 2016; Quereda *et al*., 2021). This intestinal microbiota depicts a sort of 'colonization resistance' protection against harmful foreign pathogens in the gut. This colonization resistance depends on mechanisms such as immune system maturation and growth inhibition of enteric pathogens by competition (Rolhion & Chassaing, 2016; Quereda *et al*., 2021). However, *L. monocytogenes* have developed ways to avoid such colonization resistance by the production of bacteriocins, enhancement of gut inflammatory response, and using alternative metabolic pathways like ethanolamine catabolism (Rolhion & Chassaing, 2016). In addition, the bacteriocins produced by *L. monocytogenes*, including *listeriolysin S* (*LLS)* and *Lmo2776*, either selectively kill or impair the growth of neighbour competing bacteria (Quereda *et al*., 2016). The *LLS* is encoded in the *LIPI*-3 island, which is mostly present in lineage I strains associated with clinical origin (Quereda *et al*., 2016). This gene cluster is overexpressed in pathogens that colonize the gut and once in the gut the bacteria show their bacteriocin activity (Quereda *et al*., 2016; Quereda *et al*., 2021).

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1.9.4 *Listeria* tolerates osmotic stress and bile salts in the intestines

L. monocytogenes is exposed to moderately high osmolality conditions in the host's intestines (Gahan & Hill, 2005). In order for this pathogen to survive these conditions, it increases the uptake of compatible solutes by overexpressing membrane transporters such as Gbu, OpuC, and BetL (Gahan & Hill, 2005). Other proteins with osmoprotectant activity include proline synthetase (ProAB), guanosine tetra-, and pentaphosphate (p) ppGpp synthetase RelA, RNA chaperone Hfq, proteases HtrA, and ClpC which can also be expressed in the stomach (Burgess *et al*., 2016). Additionally, most of these proteins are also involved in countering other stress conditions such as acidic or low-temperature conditions both in food and host (Burgess *et al*., 2016).

Bile is a complex mixture of bile acids, phospholipids, cholesterol, and biliverdin that contribute to a host-natural antimicrobial fluid (Cremers *et al*., 2014). For *L. monocytogenes* to tolerate bile acids, it expresses bile salt hydrolase (Bsh), which is the main factor contributing to bile tolerance (Begley *et al*., 2005).

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Figure 2. *L. monocytogenes* responds to stress encountered in the environment and within the host (Source: Quereda *et al*., 2021).

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1.10 Antimicrobial resistance in *Listeria*

1.10.1 Antimicrobial agents

Antimicrobial agents are natural, synthetic or semisynthetic substances used to treat or prevent bacterial infection in humans and animals, due to their bacteriostatic and bactericidal activities which inhibit growth and kill bacterial cells (Olaimat *et al*., 2018). There are currently antimicrobial agents to treat listeriosis in humans, however, there is no vaccine. Thus, early detection and diagnosis of listeriosis in humans is very important for the success of listeriosis antibiotic treatment (Caldero ń -Gonza ́lez *et al*., 2014).

The treatment of human listeriosis involves the use of antimicrobial agents including β-lactam (penicillin and ampicillin) or an aminoglycoside (gentamicin together with penicillin and ampicillin) (Olaimat *et al*., 2018). However, allergic reactions to penicillin have second choice therapy, is mostly used which consists of the combination of trimethoprim with a sulfonamide, such as sulfamethoxazole in co-trimoxazole (Alonso-Hernando *et al*., 2012). Additionally, there are other antimicrobial treatments of listeriosis using vancomycin which is used to treat pregnant women. Rifampicin, tetracycline, chloramphenicol, and fluoroquinolones are also used to treat listeriosis (Olaimat *et al*., 2018).

However, antimicrobial resistance in microorganisms including *Listeria* spp. is increasing, due to the development of resistant genes against the antimicrobials used in the clinical trial (O'Neill, 2015; WHO, 2018). Antimicrobial resistance is a major concern and threatens global public health, food security, and food development; because antimicrobial agents are becoming ineffective, which increases the rate of mortality, the recovery time in the hospitals, and medical costs (Olaimat *et al*., 2018; WHO, 2018).

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1.10.2 Mechanisms of antimicrobial resistance in *Listeria*

Listeria spp. acquires resistance to antimicrobial agents mainly via acquiring mobile genetic elements including self-transferable plasmids, mobilizable plasmids, and conjugative transposons (Moreno *et al*., 2014). Another mechanism of resistance is efflux pumps which are reported to be associated with fluoroquinolone resistance in *Listeria* (Wilson *et al*., 2018). Furthermore, it has also been reported that *L. monocytogenes* are acquiring resistant genes via mutations (Moreno *et al*., 2014).

1.10.2.1 Antimicrobial resistance mediated by conjugation

The process of transferring genetic material from one bacterial cell to the next is called conjugation (Verraes *et al*., 2013). *L. monocytogenes* use this conjugation mechanism as a major route of acquiring antimicrobial resistance (Wilson *et al*., 2018). It has been shown that *Listeria* receives most of the genetic materials from *Enterococci* and *Streptococci* (Wilson *et al*., 2018). Previous studies by Charpentier & Courvalin (1999) and Walsh *et al*. (2001) reported that plasmid pIP510 and pAMß1 which were normally found in *Streptococcus agalactiae* and *Enterococcus faecalis* can be acquired by *L. monocytogenes* through conjugation. The plasmid pIP510 and pAMß1 are known to encode resistance to lincosamides, streptogramins, macrolides, erythromycin, and chloramphenicol. Walsh *et al*., (2001) reported that a conjugative transposon that was initially found in *E. faecalis* can be acquired by *L. innocua* as well.

1.10.2.2 Active efflux of antimicrobials

In 2000 the first efflux mechanisms in *Listeria* were reported (Mata *et al*., 2000). One such multidrug efflux transporter in *Listeria* is *MdrL*. Another efflux pump is *Lde* which is associated with increased resistance to fluoroquinolone (Verraes *et al*., 2013). The presence of these two genes suggests that they are major contributors to antimicrobials resistance in *Listeria* spp. The *MdrL* infers resistance by pumping heavy metals, antimicrobials, and cefotaxime, while the *Lde* pump exhibit resistance to flouroquinolone (Mata *et al*., 2000; Godreuil *et al*., 2003).

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1.10.3 Antimicrobial resistance in RTE food

Antimicrobials are used in the food industry to prolong the shelf life of food products (Arshad & Batool, 2017). There is an increase in antibiotic-resistant bacteria worldwide due to the overuse of these drugs as growth promoters and in clinical settings to treat listeriosis (Chang et al., 2014). The first *L. monocytogenes* that had developed antimicrobial-resistant was reported in 1998 isolated from a patient in France (Charpentier & Courvalin 1999; Morvan *et al*., 2010). Thereafter, multiple *Listeria* spp. with antimicrobial resistance has been reported (Luque- Sastre *et al*., 2018). There is increased concern regarding antimicrobial resistance associated with RTE meat products. Studies by Kovacevic *et al*. (2013) and Gomez *et al*. (2014) indicated that there was an increase in antimicrobial resistance in *Listeria* isolates from RTE meat products when compared with FPEs. The repeated exposure to sub-lethal concentrations of antimicrobials such as ciprofloxacin has produced derivative strains with increased tolerance to other antibiotics and can also cause multidrug antimicrobial resistance (Kohanski *et al*., 2010). The over-use of antimicrobials to prevent foodborne pathogens has increased resistance in meat products.

1.10.3 Factors Influencing the antimicrobial resistance of *Listeria*

The extensive use of antimicrobials in humans and animals has greatly contributed to the progression and spread of antimicrobial resistance among foodborne pathogens, including *Listeria* spp. (Olaimat *et al*., 2018; Wilson *et al*., 2018). Antimicrobial resistance is known to develop in different ways in foodborne pathogens, including general physiology, mutation, and other types of genetic alteration (Wilson *et al*., 2018). During their adaption to environmental stresses, bacteria can become more resistant to antimicrobials (Wilson *et al*., 2018). Antimicrobial-resistant strains can be transferred between animals and humans through the food chain. Foodborne pathogens, including *L. monocytogenes* interact with low levels of antimicrobials in the food production chain. This interaction serves as a pre-exposure adaptation, allowing *L. monocytogenes* to become resistant to higher levels of antimicrobial drugs (Al-Nabulsi *et al*., 2015). *L. monocytogenes* encounters a wide range of environmental factors that influence

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antimicrobial resistance during food production and processing including physical factors such as heat, desiccation, high pressure, and irradiation; chemical factors such as oxidants, salts, and acids; and biological factors, such as microbial antagonism, which induces a cross-protection response that generates cells with increased resistance to different antibiotic resistance factors (Al-Nabulsi *et al*., 2015; Allen *et al*., 2016) (Figure 3).

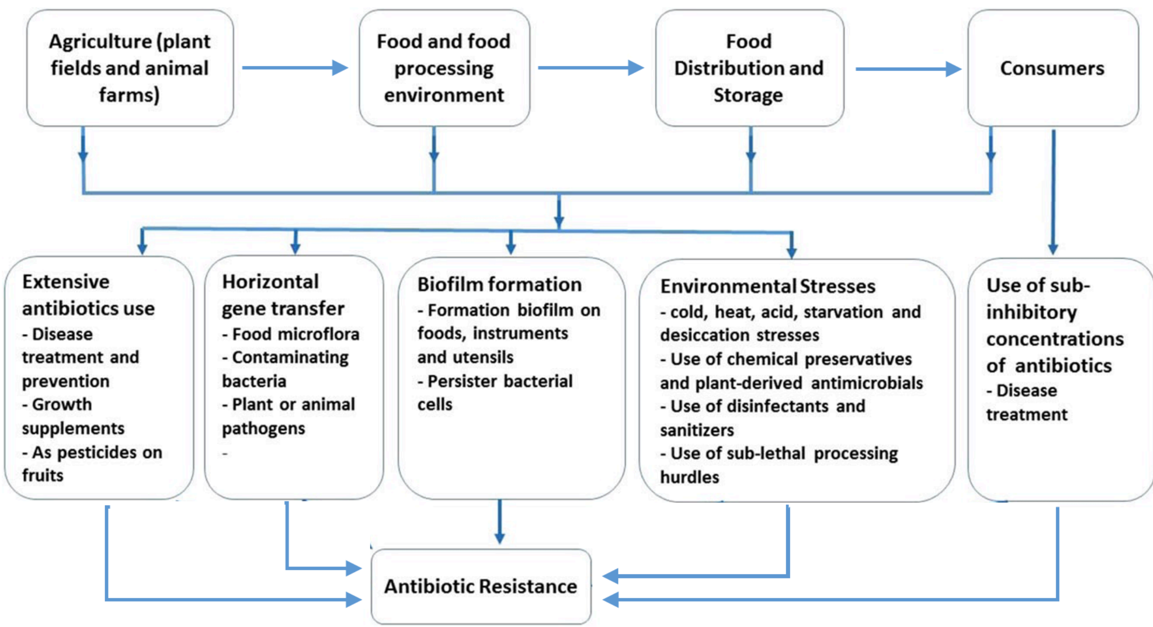

Figure 3. Food processing and agricultural factors influencing the antimicrobial resistance among *L. monocytogenes* food isolates.

1.11 Biocides and heavy metals resistance in *Listeria*

Industrial and agricultural activity may facilitate a toxic accumulation of heavy metals in the environment (Parsons *et al*., 2018). High tolerance to heavy metals such as cadmium (Cd) and arsenic (As) is a frequent trait in *Listeria* due to efflux pumps found in both chromosomes and plasmids (Ratani *et al.,* 2012; Parsons *et al*., 2018). Resistance to Cd is more prevalent in food-associated isolates (serogroup 1/2a and 1/2b) and persists in FPEs (Ratani *et al*., 2012; Parsons *et al*., 2018). On the other hand, resistance is more common in serotype 4b and isolates associated with listeriosis outbreaks (Lee *et al*., 2013; Parsons *et al*., 2018). The mechanism behind the increased

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tolerance to Cd and As in persistent but highly virulent strains are still elusive (Quereda *et al*., 2021).

1.12 Biofilms in *Listeria*

Listeria spp. has the unique ability to resist different stressful environments by forming a biofilm structure. Biofilm refers to clusters of bacteria attached to a surface and/or to each other and embedded in a self-produced matrix (Colagiorgi *et al*., 2017). The formation of biofilm increases *Listeria* spp. adaptation and promotes long-term survival in harsh environments. Biofilms are very difficult to remove, and once it forms. *Listeria* acquires resistance to antimicrobials, sanitizing agents, heat, pH, water, and nutrient availability (Colagiorgi *et al*., 2017). The ability of *Listeria* spp. to form biofilm is dependent on various environmental factors such as relative humidity, temperature, salinity, and surface type. Biofilm formation is also influenced by biofilm-associated protein, protein SecA2, and flagella (Guilbaud *et al*., 2015). It has been proved that *Listeria* can form a biofilm with other bacteria, increasing its biofilm structure and resistance to cleaning and sanitation (Colagiorgi *et al*., 2017). In the food processing industry, biofilm contributes to the majority of processing facilities contamination. However, *Listeria* spp. are capable of biofilm formation on various contact surfaces such as ceramic tiles, glass, stainless steel, and polyethylene surfaces (Figure 4) (Di Bonaventura *et al*., 2008; Guilbaud *et al*., 2015).

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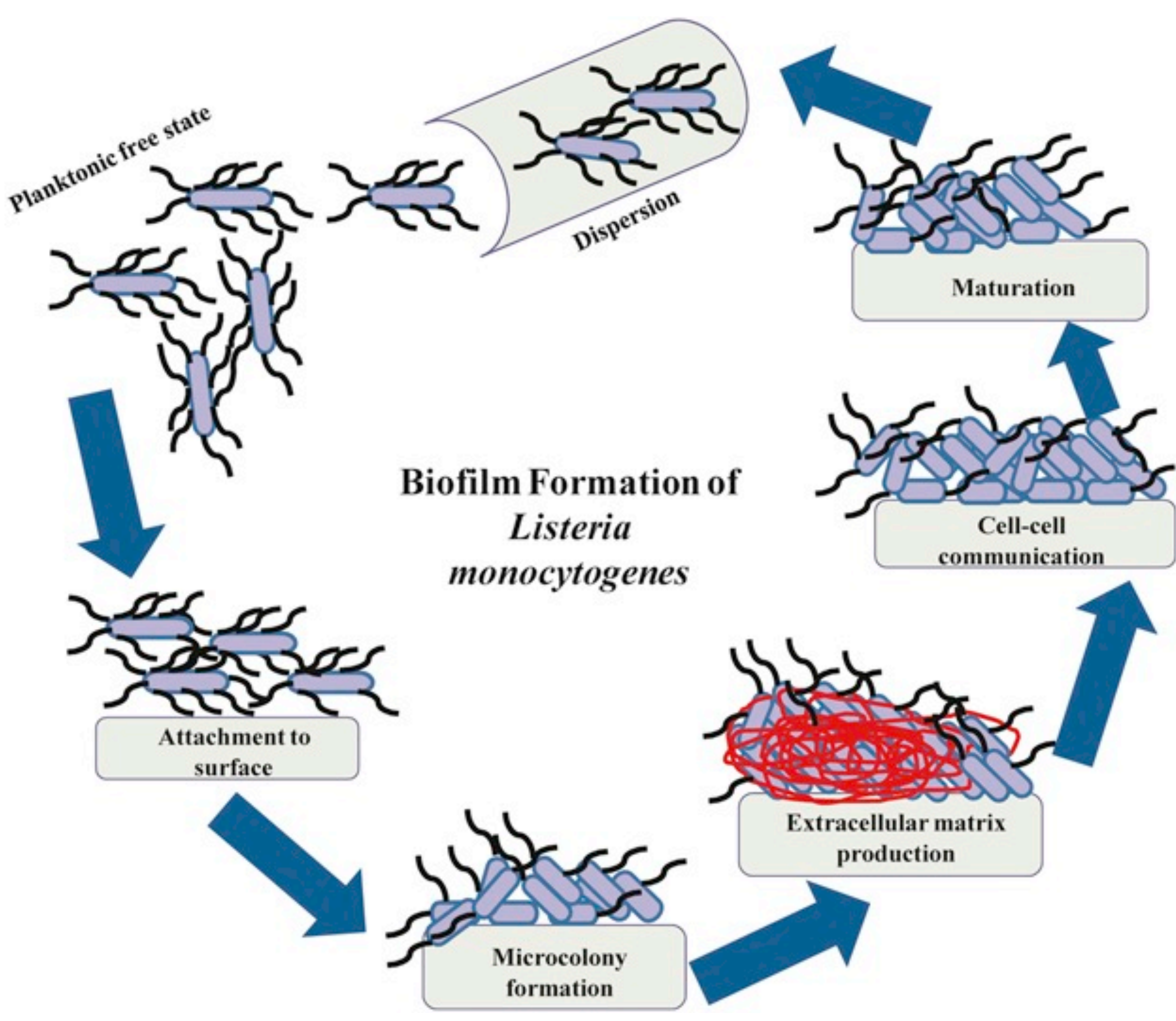

Figure 4. Biofilm formation of *L. monocytogenes* occurs in several stages, 1. Attachment to the surface, 2. Microecolony formation, 3. Extracellular matrix production, 4. Cell-cell communication, 5. Maturation, 6. Dispersion (Source: Oloketuyi & Khan, 2017).

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1.13 Pathogenicity of *Listeria*

The pathogenicity of *L. monocytogenes* is due to the expression of genes responsible for its ability to penetrate, proliferate and spread through host cells (Kaptchouang *et al*., 2020; Quereda *et al*., 2021). This pathogen can penetrate through barriers in the human body such as the intestinal epithelium, the blood-brain barrier, and the placenta (Chen *et al*., 2009; Carvalho, Sousa & Cabanes, 2014; Matle *et al*., 2020). The pathogenicity of *L. monocytogenes* has several stages including, 1. adhesion and invasion of host cells, 2. internalistion by host cells, 3. lysis of vacuole, 4. intracellular multiplication, and 5. intercellular spread to the adjacent cell (González-zorn *et al*., 2001; Chen *et al*., 2009). When contaminated food is ingested by an individual, *L. monocytogenes* survives exposure to high acidity, bile salts, non-specific inflammatory attacks, and proteolytic enzymes from the host system (Jeyaletchumi *et al*., 2012; Matle *et al*., 2020). Upon infection of the host cells by entering the host through intestinal epithelium, the bacterium is internalized in a membrane-bound vacuole. Once internalized, *L. monocytogenes* promotes its escape from the membrane-bound vacuole into the cytoplasm (where the bacteria replicates) by expression of *Listeriolysin* O and two *phosphatidylinositol-specific phospholipases* (*PlcA and PlcB*) (Moura *et al*., 2019). The intracytoplasmic *L. monocytogenes* use the actin of the host cell, in conjunction with their *ActA* protein, to promote their motility intracellularly (Vera *et al*., 2013; Cahoon & Freitag, 2014). After their uptake by adjacent cells, the bacteria escape from the double membrane-bound vacuole by secreting *Listeriolysin O* and the *phospholipases* (Vera *et al*., 2013; Cahoon & Freitag, 2014). The expression of several virulence factors prevents the killing of the host cell and allows the host cell cytoplasm to serve as a haven for bacterial survival and replication. Having developed a large arsenal of virulence determinants, *L. monocytogenes* is capable of infecting a large variety of cells, tissues, and organs. The life cycle of *L. monocytogenes* is shown in Figure 5.

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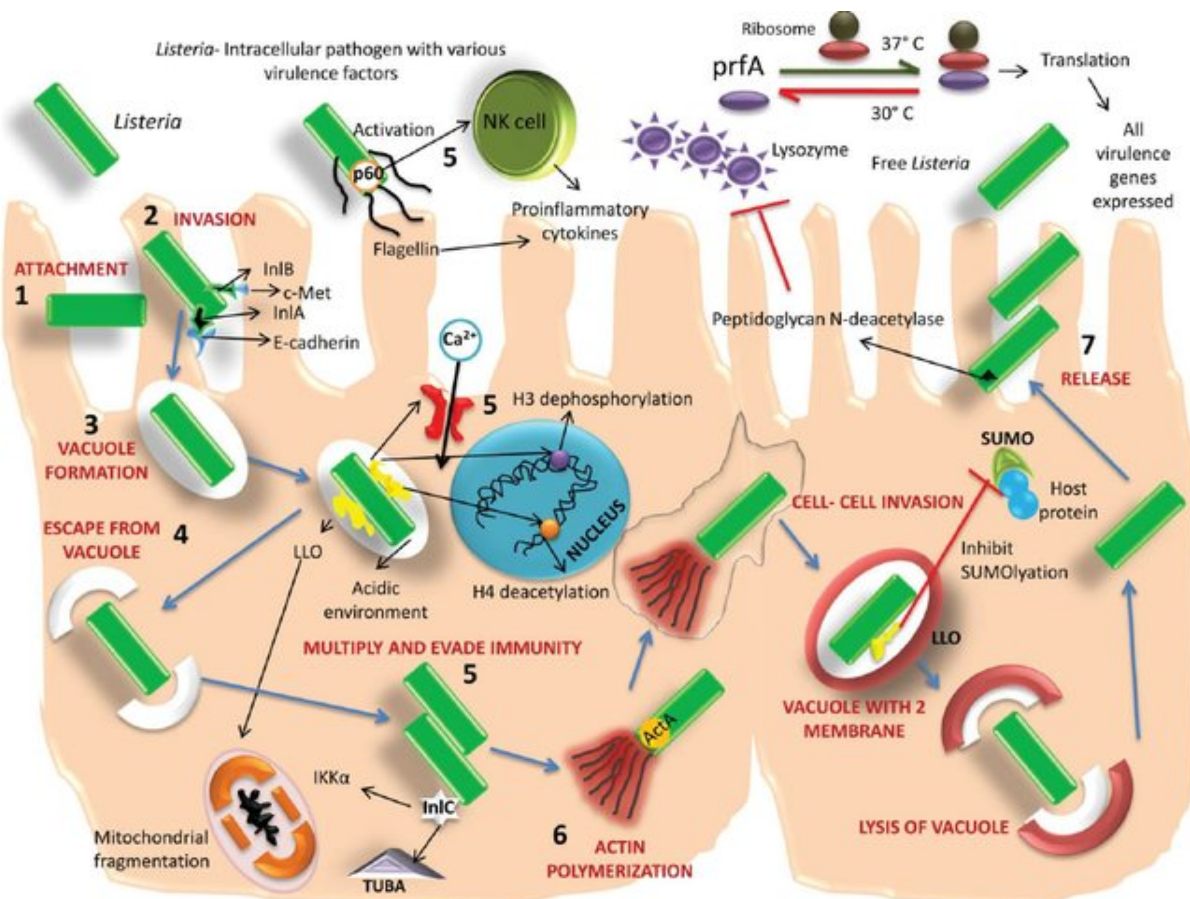

Figure 5. Virulence factors and pathogenesis of *Listeria.* (1) Attachment of *Listeria* to their receptor. (2) Invasion. (3) Phagocytic vacuole carries the organism inside. (4) *Listeria* escapes the vacuole by several mechanisms, including *LLO*. (5) *Listeria* evades host immunity by various mechanisms including *LLO*, Flagellin, and *InlC*. (6) Formation of actin tail propels the organism from one cell to another. (7) Finally, the release of the organism (Source: Jadhav, 2015).

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1.14 Virulence and Virulence Factors

The virulence of *Listeria* spp. including *L. monocytogenes* and *L. ivanovii* is mainly regulated by six genes, comprising *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB,* which are located in the *PrfA*-dependent virulent gene cluster known as *LIPI*-1 (Figure 6) (Rabinovich *et al*., 2012). The virulence of *Listeria* spp. is also dependent on genomic islands, *Listeria* pathogenicity islands (*LIPI*-1*, LIPI*-2*, LIPI*-3, and *LIPI*-4), and Internalins (*inl*) genes (Gilmour *et al*., 2010).

1.14.1 Internalins (*inl*)

Adhesion and invasion of host cells by *Listeria* spp. such as *L. monocytogenes* is the initial step in *Listeria*'s pathogenicity, which is mediated by internalin genes (Matereke & Okoh, 2020). The first internalins subfamily is large surface proteins (70–80 kDa), including Internalin A (*inlA*) and Internalin B (*inlB)* (Matle *et al*., 2020). The *inlA* is a surface protein required for adhesion and invasion into non-phagocytic cells, such as epithelial cells by *L. monocytogenes* (Matle *et al*., 2020; Matereke & Okoh, 2020). The *inlB* is another surface protein that plays a role in the invasion of hepatocytes in the liver by *L. monocytogenes* (Matle *et al*., 2020). The *inlA* and *inlB* expression differences are associated with mutations and poor invasion in *inlA*, which results in low invasion ability (Werbrouck *et al*., 2006; Jensen *et al*., 2016). The second group of surface proteins is small (25 kDa – 30 kDa) including *inlC, inlD, inlE, inlF, inlG,* and *inlH* (Vazquez-Boland *et al*., 2001; Matle *et al*., 2020). Other important internalins from the second group of surface proteins include *inlC* and *inlJ*. These proteins are involved in the post-intestinal dissemination of *L. monocytogenes (*Jensen *et al*., 2016). In addition to the internalins, another surface protein is *p104*, which plays a role in adhesion to intestinal cells.

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1.14.2 Listeriolysin O (*LLO*)

Listeriolysin O is a bacterial pore-forming toxin essential for the vacuole membrane lysis so that *L. monocytogenes* escape into the cytoplasm during the pathogenesis life cycle (Yu *et al*., 2018; Matle *et al*., 2020). This pore-forming surface toxin called *LLO* is produced by the *haemolysin* (*hly*) gene (Kyoui *et al*., 2014). The *L. monocytogenes* that cannot produce *LLO* are regarded as avirulent strains because the bacterium cannot be released to the cytoplasm to multiply and infect other cells (Pushkareva & Ermolaeva, 2010). The *LLO* is extremely sensitive to the environmental pH, with higher levels of expression observed under acidic pH levels (pH $<$ 6) and lower activity levels observed at neutral pH (Pushkareva & Ermolaeva, 2010). Another important virulence factor is a secondary haemolysin known as *Listeriolysin S* located in *LIPI*-3 and found explicitly in lineage I of *L. monocytogenes.* This haemolysin is induced only under oxidative stress conditions (Cotter *et al*., 2008).

1.14.3 Phospholipases (*plc*) and Metalloprotease

Phospholipases C secreted by *L. monocytogenes* to help in the lysis of vacuole membranes are *plcA* and *plcB* (Gouin *et al*., 1994; Vazquez-Boland *et al*., 2001; Matereke & Okoh, 2020). It has been reported that *plcA* helps the *L. monocytogenes* to exit from the primary vacuole, while *plcB* helps in the cell-to-cell spread of *listeria* (Matereke & Okoh, 2020). Maturation of *plcB* is dependent on a zinc *metalloprotease*, which is encoded by the *mpl* gene. *Metalloprotease* also helps *plcB, plcA*, and *hly* to disrupt the primary vacuoles after host cell invasion (O'Connor *et al*., 2010; Doyle *et al*., 2013; Matle *et al*., 2020).

1.14.4 Actin polymerising protein (*ActA*)

The *ActA* is a surface protein that facilitates the movement of *L. monocytogenes* to the cytoplasm by inducing the polymerisation of global actin molecules to actin filaments (Matereke & Okoh, 2020). The filaments are used for both inter- and intra-cellular movement by *Listeria* (Klumpp & Loessner, 2013). The *ActA* is a critical surface protein as it is involved in the pathogenicity and movement of *Listeria.* Previous studies

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(Mafuna *et al*., 2021; Matle *et al*., 2020) have indicated that all the virulent *L. monocytogenes* strains isolated in SA harboured the *ActA* gene. Another study performed by Doyle *et al*. (2013) showed lower virulence in *L. monocytogenes* serotypes 4a, 4c, 4d, and 4e is associated with low production levels of the *ActA* protein.

1.14.5 Invasion-associated protein (Protein p60)

The invasion-associated protein (*IAP*) is an extracellular protein *p60* that catalyzes a reaction during the final stage of *Listeria* cell division (Yu *et al*., 2018). It is usually found on the cell surface to promote *Listeria* adherence to the host cell. This *Iap* gene also plays a crucial role in the pathogenicity of the *Listeria* spp. (Quendera *et al*., 2016).

1.14.6 Positive regulatory factor A (*prfA*)

The *prfA* is a primary regulator of virulence factors/genes in the *Listeria* virulence gene cluster, as shown in Figure 6. However, other proteins function as regulators of this gene cluster in addition to *prfA* (Ryan *et al*., 2010). This additional regulator includes *VirR,* which is a response regulator necessary for *Listeria* virulence (Duroux *et al*., 2015). Furthermore, the expression of the *prfA* is controlled by sigma factor σB.

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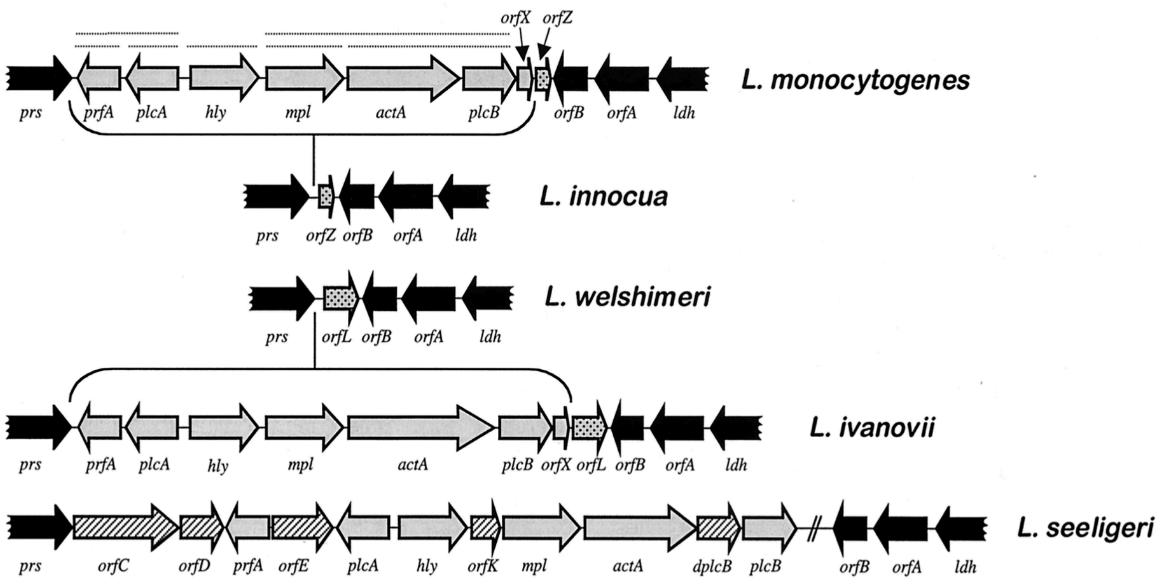

Figure 6. Physical and transcriptional organization of the central virulence gene cluster (*LIPI*-1) of *L. monocytogenes* and structure of the locus in other *Listeria* spp. Genes belonging to *LIPI*-1 are in grey, and the flanking loci are in black. Dotted lines above *L. monocytogenes LIPI*-1 genes indicate known transcripts (Source: Vazquez-Boland *et al*., 2001).

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1.15 The evolution of DNA sequencing technology

1.15.1 Early approaches to sequencing

The DNA sequence was first determined in the 1970s by Sanger and colleagues, Maxam and Gilbert, who introduced the concept of DNA sequencing by chain termination and fragmentation techniques, respectively. In 1977, two landmark articles were published on DNA sequencing technologies. The first article was on Sanger and colleagues' sequencing technique, commonly known as Sanger DNA sequencing, based on identifying DNA sequences by primed synthesis of DNA polymerase. The second article was Maxam and Gilbert's DNA sequencing method based on the chemical degradation DNA sequence in which terminally labelled DNA fragments were chemically cleaved at specific bases and separated by gel electrophoresis.

1.15.2 First-generation DNA sequencing

The Sanger sequencing method was responsible for introducing the first automated DNA, sequencers which uses fluorophore-labelled nucleotides that can produce approximately 1,000 bp sequences *(Smith et al.,* 1986). Compared to Maxam and Gilbert's method, the Sanger protocol was better due to its great availability, simplicity, high accuracy, and required less handling of toxic chemicals and radioisotopes. As a result, the following decade's Sanger protocol became the most widely used DNA sequencing protocol. Applied Biosystems (ABI) European Molecular Biology Laboratory (EMBL) company then optimised and commercialized the Sanger method (Ansorge *et al*., 1986). However, faster, higher-throughput, and cheaper technologies were required due to Sanger sequencing limitations such as low throughput, low sensitivity, time-consuming, and inability to perform parallel analysis of multiple targets (Shendure *et al*., 2017).

1.15.3 Second-generation DNA sequencing

Due to Sanger sequencing limitations, a series of novel Massively Parallel Sequencing (MPS) technologies were developed and termed next-generation technologies (Arsenic *et al*., 2015). These NGS technologies can simultaneously multiplex millions of

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sequencing reactions while avoiding the gel electrophoresis step to determine the nucleotide sequences. The first incarnation of NGS was a method known as pyrosequencing, which was adopted by 454 and later acquired by Roche (Margulies *et al*., 2005). The first commercial NGS platform was produced in 2005 by Roche. Another instrument developed was SOLiD sequencing, and later Ion Torrent by Life Technologies. However, the NGS commercial market has recently been dominated mainly by Illumina (Shendure *et al*., 2017).

Furthermore, NGS provides opportunities to analyse multiple genomes and transcriptomes in a highly efficient and timely manner at a much lower cost than Sanger-based sequencing methods (Buermans & den Dunnen, 2014). Applications of these technologies have already benefited various research areas (Buermans & den Dunnen, 2014). However, the higher-throughput methods have limitations. such as shorter sequences (Ulahannan *et al*., 2013).

1.15.4 Third-generation DNA sequencing

To overcome the shortcomings of NGS, a new approach that did not require the amplification step and can sequence long reads or sequences was sought (Levene *et al*., 2003). Pacific Biosciences (PacBio) developed a single-molecule real-time sequencing technology that uses fluorescent labels attached to the terminal phosphate (Jain *et al*., 2016). PacBio platforms can sequence longer reads than other NGS platforms (Koren & Phillippy, 2015). Recently, Oxford Nanopore developed nanopore sequencing technology that uses nano-scale pores in a lipid membrane that detect changes in voltage as strands of DNA (Jain *et al*., 2016). These devices can generate long reads that are around 1,000,000 bp in length and, in some cases longer than the genomes of some bacterial pathogens (Jain *et al*., 2016).

1.16. Bioinformatics

Bioinformatics is the burgeoning field that develops methods and software tools to effectively analyse biological data and addresses the application of computers to the collection, organization, analysis, manipulation, presentation, and sharing of biological

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data (Can, 2014; Gauthier *et al*., 2019). Bioinformatic processes are often used for initiatives that generate large data sets. The use of bioinformatics in understanding biological data usually involves the following steps: collecting statistics from biological data, building a computational model, solving a computational modelling problem, and testing and evaluating a computational algorithm (Can, 2014; Gauthier *et al*., 2019). The primary goal of bioinformatics is to increase the understanding of the biological processes and is also used in the analysis of various processes ranging from sequence analysis to structural bioinformatics and network and systems biology (Oulas *et al*., 2019). Analysis of biological data requires bioinformatic software tools, which run ranging from simple command-line tools to more complex graphical programs and stand-alone web services (Oulas *et al*., 2019).

The exponential increase in data generated through WGS has necessitated the development of computational software or tools to analyse the sequencing data (Oakeson *et al*., 2017). While commercial software options are becoming increasingly available, offering useability and standardization at the cost of financial expense and restricted application, much of the software development has been "open-source" software generated through the use of WGS in research contexts (Oakeson *et al*., 2017). These include both graphical user interface (GUI) and command-line tools designed to run on UNIX or Linux operating systems adopted in high-performance computing facilities. Common programming languages include basic Bash, Java, Perl, Python, and C (Ekmekci *et al*., 2016). The latter enables high-speed computation, but programs must be compiled during the installation process, while the other languages are translated as they are run. Perl and Python have been popular in recent years due to their ease of use and the availability of open-source suites of tools in each language developed specifically for the manipulation of biological sequence data; though similar implementations are available in other languages (Ekmekci *et al*., 2016). Another increasingly popular language is the statistical programming language R, for incorporating genomic data into statistical models for analysis, and visualizing results (Taosheng *et al*., 2017). In general, bioinformatic approaches to analysing microbial genomes have depended on the purpose of WGS genome characterisation, genome comparison, sample metagenomics, and the sequencing technology used.

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1.16.1 Sequencing data

Raw sequencing data can be retrieved in different formats from sequencing platforms (Cock *et al*., 2010). This can be in gzip-compressed FASTQ format, which includes identifiers for each sequencing read (Cock *et al*., 2010). Raw data generally contain sequence adapters – short unique sequences added during the library preparation process which are not part of the genome of the sequenced organism (Ewing *et al*., 1998; Head *et al*., 2014) (Figure 7).

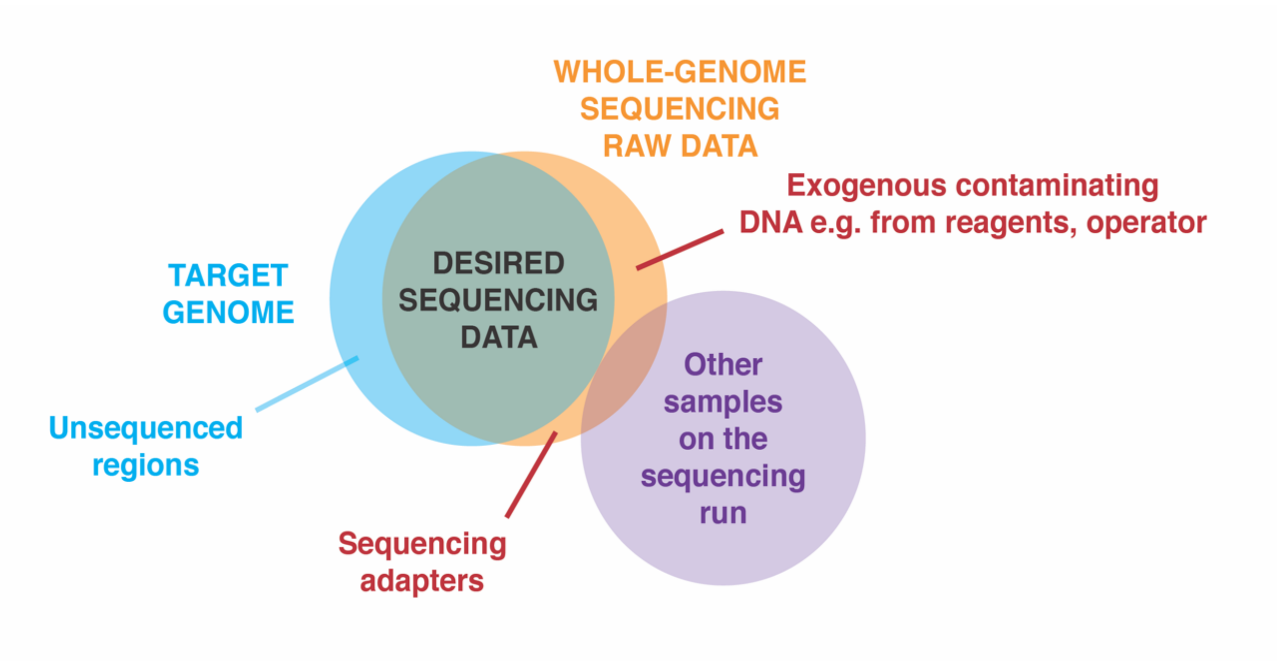

Figure 7. The raw whole genome sequencing data contents (Source: Kwong, 2017).

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1.16.2 Bioinformatics analysis

The exponential increase in WGS data requires the development of computational software or tools to analyse the sequencing data generated.

1.16.2.1 Genome characterisation

De novo genome assembly

The genome characterisation process to understand an organism's structural and functional biology starts with "*de novo*" genome assembly (Liao *et al*., 2015; Segerman, 2020). De novo assembly is a process of constructing short nucleotide sequences into longer ones (Liao *et al*., 2015). This process uses overlapping sequences to form a consensus. Genome assemblers can construct contiguous DNA sequences (contigs) using algorithms that incorporate theoretical sub-sequences known as *k*-mers, into De Bruijn graphs (Zerbino *et al*., 2008; Bankevich *et al*., 2012; Segerman, 2020).

Genome annotation

Following genome assembly, identifying, and annotating relevant genome sequence features allows for the visualisation of the genetic loci in the genome. This is achieved by using different automated genome annotators such as command-line tools (I.e. Prokka), web-based servers (I.e. RAST), and the NCBI prokaryotic genome annotation pipeline (Aziz *et al*., 2008; Chaudhuri *et al*., 2008; Seemann, 2014). The genome annotators can identify features such as RNA genes, coding DNA regions (CDS); and other features of interest, such as clustered regularly interspaced short palindromic repeat regions (CRISPRs) and prophages (UniProt Consortium, 2015).

Sequence searching

The most widely used algorithm or tool for searching for short nucleotide sequences in a genome is the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990; Camacho *et al*., 2009). This algorithm incorporates a heuristic approach for identifying similar sequences by constructing local alignments. Then the algorithm scores the similarity between the sequences for each of the alignments (Camacho *et al*., 2009).

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This algorithm has been the basis of other tools for genome characterisation, including *in silico* molecular typing, antimicrobial resistance, and virulence gene identification (Kent *et al*., 2002; Camacho *et al*., 2009).

1.16.2.2 Genome comparison

Bioinformatic genome comparison approaches compare two or more genomes and require whole-genome aligners (Dubchak *et al*., 2009; Edwards *et al*., 2013; Angiuoli *et al*., 2011; Rangwala *et al*., 2021). These genome aligners can be used to compare complete genome assemblies and detect large structural differences including acquisition or loss of mobile genetic elements (Angiuoli *et al*., 2011; Edwards *et al*., 2013; Rangwala *et al*., 2021). However, aligning large numbers of whole genomes remains computationally challenging (Armstrong *et al*., 2019).

1.17 Applications of microbial genomics in WGS studies

Whole genome sequencing is one of the primary investigation tools that has been explored in modern microbial genomics research (Kwong *et al*., 2015; Segerman, 2020). This tool is mainly applied in the structural and functional characterisation of the organism genome to identify genetic elements that may result in pathogenicity, adaptation, survival, antimicrobial resistance and virulence (Bertelli *et al*., 2013). Microbial genomics can identify genetic markers that may affect the treatment and prognosis of infections (Koser et al., 2012; Bertelli *et al*., 2013; Kwong *et al*., 2015). Currently, there are four main potential applications of WGS for bacterial pathogen characterisation in diagnostic and public health microbiology laboratories; species identification, strain typing, resistance detection, and virulence gene detection (Bertelli *et al*., 2013; Kwong *et al*., 2015).

1.17.1 Pathogen Identification and taxonomy

Bacterial identification has benefited from the use of microbial genomics in recent years. WGS plays a vital role in bacterial identification for an organism that cannot be detected using standard routine diagnostic methods (Hugenholtz *et al*., 2021). These organisms include organisms such as *Nocardia* and other *Actinomycetes* because this

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species require techniques such as 16S rDNA sequencing or specific nucleic acid probes to confirm identification as well as those that are viable but not culturable (Kwong *et al*., 2015; Hugenholtz *et al*., 2021).

1.17.2 Typing

Typing refers to the sub-classification of organisms beyond the spp. level (Sabat *et al*., 2013). Typing microbial pathogens is generally conducted for epidemiology surveillance, outbreak investigation, and infection control (Sabat *et al*., 2013). In recent years, studies have indicated the superior resolution of WGS over standard traditional typing methods in differentiating isolates (Kwong *et al*., 2015). WGS methods for typing include MLST. Additionally, new typing schemes based on WGS data have been developed and applied to pathogen surveillance, such as core-genome multilocus sequence typing (cgMLST) and whole-genome multilocus sequencing typing (wgMLST) for *L. monocytogenes*. However, these schemes have not yet been developed for all pathogens (Kwong *et al*., 2015; Mafuna *et al*., 2021).

1.17.3 Resistance gene determination and Virulence profiling

WGS can also be used for genetic determinates of AMR identification (Ellington *et al*., 2017; Mafuna *et al*., 2021). WGS data can detect resistance genes such as plasmid-borne benzalkonium chloride (BC) resistance bcrABC cassette, efflux pump *Lde, and mdrL* found in *L. monocytogenes* (Mafuna *et al*., 2021). WGS data analyses can also readily detect acquired resistance genes, including encoding beta-lactamases and aminoglycoside modifying enzymes (Ellington *et al*., 2017). Characteristic point mutations in critical genes predict resistance phenotypes such as *rpoB* or *gyrA* can also be detected with WGS data (Ellington *et al*., 2017). Furthermore, WGS methods are used for surveillance of resistance mechanisms for pathogens such as *Neisseria gonorrhoeae*, where resistance has become a public health emergency (Kwong *et al*., 2015; Eyre *et al*., 2017).

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Virulence profiling can be conducted with the use of WGS data for the detection of virulence factors. For *L. monocytogenes,* important virulence factors include *LIPI*1-4 and internalins which are crucial in disease manifestation (Mafuna *et al*., 2021).

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1.18 Aims and objectives of the project

Listeria has evolved a plethora of genetic strategies that allow it to withstand stressful conditions very efficiently in food and FPEs. This outstanding ability is very important in this bacterium's surviving, adapting, and causing disease and outbreaks due to its antimicrobial resistance and virulence potential. As a result, *Listeria* spp. especially *L. monocytogenes* caught the attention of the food industry for its research and surveillance. Protocols for isolation and detection of this pathogen are available, with WGS being the gold standard for typing of *Listeria* spp. Despite the use of WGS as a surveillance tool and extensive research, *Listeria* outbreaks continue to occur worldwide. Therefore, extensive research or surveillance of this pathogen is necessary, especially in countries with a significant population that is immunocompromised due to human immunodeficiency virus (HIV), Tuberculosis (TB), malaria, and other infectious in African countries. Against this background, a comprehensive investigation to identify the population structure, mobile genetic elements, virulence factors, antimicrobials, metal, and biocide resistance of this pathogen is necessary to determine the evolution and survival of the *Listeria* spp. as well as the identification of outbreaks, tracking the spread of diseases. This will go a long way to providing early warning systems for national and international human and animal health institutions. To this end, we specified the following aims and objectives for the current project.

The aim of this study was to characterise the *Listeria* isolates isolated from meat, meat products, and food processing environment in South Africa and determine their antimicrobial resistance and virulence profiles using WGS.

To address this aim, the following research objectives were investigated:

1. To subtype and characterise *L. monocytogenes* isolates recovered at selected control points in the meat value chain in SA by means of WGS.

2. To use core genome-SNP analysis to determine the genetic relatedness of the most common *L. monocytogenes* strains in SA and to assess the genetic basis of the resistance, stress tolerance, genomic localization of the resistance genes in *L. monocytogenes* isolated from food products in SA; and identify key genomic

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features contributing to virulence potential of *L. monocytogenes* strains in the host.

3. To institute a comparison study to extend our understanding of the phylogenetic relatedness, stress resistance genes, virulence factors, and CRISPR-cas systems from the accessory genome of non-pathogenic *Listeria* spp. (*L. innocua* and *L. welshimeri*) isolated in SA compared to pathogenic *L. monocytogenes* strains.

4. To construct of an online tool for the prediction of *Listeria* sequence types using NGS raw data.

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CHAPTER TWO

Chapter Two: The population structure of *Listeria monocytogenes* in South Africa

Published Manuscript: Population Structure of Non-ST6 *Listeria monocytogenes* Isolated in the Red Meat and Poultry Value Chain in South Africa

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Article

Population Structure of Non-ST6 *Listeria monocytogenes* Isolated in the Red Meat and Poultry Value Chain in South Africa

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Abstract: Meat products have been implicated in many listeriosis outbreaks globally, however there is a dearth of information on the diversity of *L. monocytogenes* isolates circulating in food products in South Africa. The aim of this study was to investigate the population structure of *L. monocytogenes* isolated in the meat value chain within the South African market. Based on whole-genome sequence analysis, a total of 217 isolates were classified into two main lineage groupings namely lineages I (n = 97; 44.7%) and II (n = 120; 55.3%). The lineage groups were further differentiated into IIa (n = 95, 43.8%), IVb (n = 69, 31.8%), IIb (n = 28, 12.9%), and IIc (n = 25, 11.5%) sero-groups. The most abundant sequence types (STs) were ST204 (n = 32, 14.7%), ST2 (n = 30, 13.8%), ST1 (n = 25, 11.5%), ST9 (n = 24, 11.1%), and ST321 (n = 21, 9.7%). In addition, 14 clonal complex (CCs) were identified with over-representation of CC1, CC3, and CC121 in "Processed Meat-Beef", "RTE-Poultry", and "Raw-Lamb" meat categories, respectively. *Listeria* pathogenic islands were present in 7.4% (LIPI-1), 21.7% (LIPI-3), and 1.8% (LIPI-4) of the isolates. Mutation leading to premature stop codons was detected in *inlA* virulence genes across isolates identified as ST121 and ST321. The findings of this study demonstrated a high-level of genomic diversity among *L. monocytogenes* isolates recovered across the meat value chain control points in South Africa.

Keywords: *L. monocytogenes*; subtyping; serogroups; sequence types; clone complexes; pathogenic islands; lineages; *inlA*; sequencing

1. Introduction

The consumption of meat and meat-based products has increased in the last few years in South Africa (SA) [1]. This increase is primarily linked to human population growth, urbanization, higher disposable income, and a change in eating patterns as many people are adopting diets that contain high-quality animal proteins [2]. However, the chemical composition of meat predisposes it to bacterial

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contamination and serves as a vector for transmission of foodborne bacteria that can cause infection in humans and result in economic losses [3]. Occurrence of foodborne bacteria on meat can be due to poor animal management, slaughter practices, processing, storage conditions, and lack of meat safety knowledge [4]. Consumers need to be protected and provided with safe and wholesome products of animal origin. This can be achieved by practicing good farm animal management, proper personal hygiene, and routine surveillance of food products within the meat value chain [2]. Safe handling of meat is paramount to circumvent potential devastating effects on the health and economy of populations.

Listeria monocytogenes is a zoonotic foodborne bacterium that is responsible for causing a rare but potentially fatal disease known as listeriosis in humans and animals [5]. Human listeriosis has become a priority and economically important disease that contributes to public health challenges in SA and globally [6–9]. Over the years, the number of human listeriosis outbreaks and sporadic cases that emanated from various sources such as unmarked potatoes [10] and polony (Bologna sausage) [8,9] have been documented in SA. Furthermore, several studies in different geographical areas of SA have reported the presence of *L. monocytogenes* in a variety of meat products [4,11–13]. These studies revealed health challenges associated with *L. monocytogenes* as a result of high occurrence in food products.

The epidemic and sporadic cases of human listeriosis are commonly associated with consumption of contaminated food, particularly ready to eat (RTE) products [14]. Despite the low overall incidence of human listeriosis, this disease is linked to a high case fatality rate (20–30%) and hospitalization rates [6,15]. There is also evidence to suggest higher case fatality rates in pregnant women and individuals with neurolisteriosis [16]. The clinical manifestations of human listeriosis can range from self-limiting gastroenteritis that last a few days to more severe invasive and systematic illnesses that might be fatal in high-risk groups such as the elderly, infants, and immunocompromised people [17]. Therefore, a high percentage of the South African population is at risk as the elderly and other immunocompromised individuals contribute significantly to the total population.

The pathogenicity of *L. monocytogenes* is based on the production of the virulence factors, susceptibility of the host organism, and the virulence of a particular strain; hence, the exact infective dose and the safety margin of *L. monocytogenes* sequences is not well defined [18,19]. Several studies have provided detailed insights into the global population distribution and virulence potential of *L. monocytogenes* strains as well as the sources associated with important clonal complexes (CCs) and sequence types (STs). These have indicated an over-representation of CC1, CC2, CC4, and CC6 in clinical cases and the predominance of CC121 and CC204 in food sources [20–22]. In addition, the genomic characterization of the *L. monocytogenes* invasion protein (InlA) has shown a reduced virulence potential of some strains globally due to mutations associated with premature stop codons (PMSCs) [22,23]. The primary sources of *L. monocytogenes* CCs in the meat value chain are not well understood and limited data are available on the distribution of meat-associated with CCs and their virulence potential in the SA agriculture and meat value chain.

Before the 2017–2018 outbreak of listeriosis in SA, the disease was not required to be reported and as such was not under surveillance in the country; however, a national surveillance system has since been implemented, and all isolates from human patients are analyzed by means of whole-genome sequencing (WGS) [9]. In comparison, comprehensive data on the genome characterization of *L. monocytogenes* in the food products of animal origin value chains is still lacking in SA. Matle and co-workers [13] performed an extensive national baseline survey involving nine provinces of SA to determine the occurrence of *L. monocytogenes* strains in meat and meat products in abattoirs, meat processing plants, and retail outlets. Although this study provided important information on the extent of meat contamination, the need still existed to further investigate the genomic characteristics of *L. monocytogenes* isolated from meat products using WGS in SA. The aim of this study was to subtype and characterize *L. monocytogenes* isolates recovered at selected control points in the meat value chain in SA by means of WGS.

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2. Materials and Methods

2.1. Sample Information

The isolates used in this study were obtained from samples submitted between 2014 and 2019 at Agriculture Research Council-Onderstepoort Veterinary Research (ARC-OVR): Feed and Food laboratory, SA, as part of the Department of Agriculture, Land Reform, and Rural Development (DALRRD) Pathogen Profiling project number 21.1.1/VPH-01/OVI. The samples (n = 217) included raw meat (n = 55), processed meat (n = 126), RTE meat products (n = 15) and environmental samples collected from commercial pig farm environment during a listeriosis outbreak (n = 21). The samples originated from different animal protein sources such as beef, poultry, lamb, and pork and various food establishments (farm environments, butcheries, abattoirs, retail outlets and cold stores).

2.2. Isolates Categorisation

Considering the diversity of the samples from which the isolates originated, the isolates were grouped according to different categories based on the origin of the sample and the establishment of origin. The sample origin was defined as a concatenation of the type of meat product and the animal from which it was produced from. Samples collected from the farm environment were labelled as environmental samples. The number of isolates for each category is shown in Table 1.

2.3. Bacterial Strains and DNA Isolation

The isolates were preserved as lyophilized and were revived by inoculation into brain heart infusion (BHI) broth then incubated at 37 °C for 18–24 h. DNA was extracted from BHI broth culture using a High Pure PCR template preparation kit (Roche, Potsdam, Germany) according to manufacturer's instructions.

2.4. Genome Sequencing, Quality Control and de novo Assembly

Whole-genome sequencing (WGS) of the isolates was performed at the Biotechnology Platform, Agricultural Research Council, Onderstepoort, SA. DNA libraries were prepared using TruSeq and Nextera DNA library preparation kits (Illumina, San Diego, CA, USA), followed by sequencing on HiSeq and MiSeq instruments (Illumina, San Diego, CA, USA). Quality control including adapter removal of the raw data was done using BBDuk (version 37.90; https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/). SPAdes v.3.12.0 [23] was used to create a de novo assembly of each isolate.

Multi locus sequence type (MLST) profiles were obtained from the *Listeria* database hosted by the Pasteur Institute, France (http://bigsdb.pasteur.fr/listeria/) [24]. The MLST database contains 7 loci with a total of 2069 different alleles. A k-mer based mapping tool, stringMLST [25], was used to align reads against these profiles to determine the MLST for each sequenced sample using k-mers of length 21 and 35. To validate the k-mer based predictions, all de novo assembled isolates were analyzed using MLST v.2.18.0 [26]. Serotype determination was done in silico using stringMLST and validated with blastn v.2.10.0+.

Genomes of which the in silico determined sequence type and serogroup correlated with *L. monocytogenes* were annotated using Prokka v.1.14.0 [27]. The pan-genome composition was extracted using Roary [28] and a core genome phylogenetic tree constructed with IQ-TREE v.1.6.6 [29]. Pan-genome clusters were defined as follows: Core—genes present in all isolates; soft core—genes present in at least 95% of isolates; shell-genes present between 15% and 95% of isolates; cloud-genes in less than 15% of isolates. The core genome phylogenetic tree was visualized using ggtree v1.16.6 [30].

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Table 1. Number of isolates for each category together with the different STs and serogroups found in each category.

Establishment	Sample Origin	Number of Isolates	STs	CCs	Serogroups
Farm (*n* = 21)	Piggery Environment samples	21	ST5, ST7, ST9, ST31, ST155, ST288	CC5, CC7, CC9, CC31, CC155, CC288	IIa, IIb, IIc
Abattoir (*n* = 3)	Processed meat-Beef	1	ST9	CC9	IIc
	Raw-Pork	1	ST122	CC9	IIc
	Raw-Poultry	1	ST204	CC204	IIa
Butchery (*n* = 68)	Processed meat-Beef	53	ST1, ST2, ST3, ST5, ST7, ST9, ST87, ST121, ST155, ST204, ST321, ST820, ST876, ST1428	CC1, CC2, CC3, CC5, CC7, CC9, CC87, CC121, CC155, CC204, CC321	IIa, IIb, IIc, IVb
	Processed meat-Mixed	1	ST9	CC9	IIc
	Processed meat-Poultry	3	ST7, ST121, ST204	CC7, CC121, CC204	IIa
	Raw-Beef	1	ST378	CC19	IIa
	Raw-Pork	1	ST121	CC121	IIa
	Raw-Poultry	3	ST5, ST204, ST820	CC5, CC204	IIa, IIb
	[1] RTE-Beef	6	ST2, ST9, ST204	CC2, CC9, CC204	IIa, IIc, IVb
Cold store (*n* = 19)	Raw-Beef	1	ST9	CC9	IIc
	Raw-Poultry	18	ST1, ST2, ST5, ST7, ST9, ST121, ST155, ST204	CC1, CC2, CC5, CC7, CC9, CC121, CC155, CC204	IIa, IIb, IIc, IVb
Processing plant (*n* = 10)	Processed meat-Beef	2	ST2, ST9	CC2, CC9	IIc, IVb
	Processed meat-Pork	2	ST2, ST876	CC1, CC2	IVb
	Raw-Pork	2	ST2	CC2	IVb
	[1] RTE-Beef	1	ST204	CC204	IIa
	RTE-Pork	2	ST2, ST121	CC2, CC121	IIa, IVb
	[1] RTE-Poultry	1	ST3	CC3	IIb
Retail (*n* = 96)	Processed meat-Beef	63	ST1, ST2, ST5, ST7, ST9, ST121, ST204, ST321, ST876, ST1421, ST1428, ST1430	CC1, CC2, CC5, CC7, CC9, CC121, CC204, CC321	IIa, IIb, IIc, IVb
	Processed meat-Mixed	1	ST204	CC204	IIa
	Raw-Beef	2	ST1, ST204	CC1, CC204	IIa, IVb
	Raw-Lamb	2	ST121, ST321	CC121, CC321	IIa
	Raw-Pork	3	ST9, ST155, ST321	CC9, CC155, CC321	IIa, IIc
	Raw-Poultry	20	ST1, ST5, ST9, ST121, ST155, ST204, ST321	CC1, CC5, CC9, CC121, CC155, CC204, CC321	IIa, IIb, IIc, IVb
	[1] RTE-Beef	5	ST1, ST2, ST121, ST204, ST876	CC1, CC2, CC121, CC204	IIa, IVb

[1] RTE—Ready to Eat.

2.5. *Listeria* Pathogenicity Islands

The presence of *Listeria* Pathogenicity Island (LIPI) in the de novo assemblies was determined for LIPI-1, LIPI-3, and LIPI-4 using blastn v.2.10.0+ with a minimum percent identity of 95% and an e-value of 1×10^{-30}. All alleles for the abovementioned LIPI genes clusters were obtained from the *Listeria* database hosted by the Pasteur Institute, Paris, France (http://bigsdb.pasteur.fr/listeria/) [25].

2.6. Protein Sequence of *inlA* Genes

Protein sequences for the *inlA* genes were extracted from the annotated assemblies and aligned using all-versus-all blastp with an e-value of 1×10^{-30}. The results were filtered for 99% identify and clustered using the Markov clustering algorithm (MCL) [31] with an inflation parameter of 1.8. Protein sequences were inspected for truncation based on the reference protein length of 800 amino acids (AAs).

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2.7. Data Analysis

Analysis was done using R v.3.6.0 [32]. Proportion and association testing were done using Chi-Square tests and over-representation was indicated by a Pearson residual value of larger than 2. Diversity analysis according to ST occurrence within categories was done using the R package vegan v2.5-6 [33]. A distance matrix based on the ST count matrix was produced using vegan with the "bray" method invoked. Principle coordinate analysis was done using ape v5.3 [34] with the distance matrix as input.

3. Results

3.1. Typing Analysis

The isolates were grouped into different STs, 20 in total, and classified as either Lineage I or II (Figure 1). Eleven lineage I and nine lineage II STs were identified with lineage I accounting for 44.7% (n = 97) and lineage II accounting for 55.3% (n = 120) of the isolates. Five STs (25% of all STs) were found to be singularly represented in the isolates. The five most frequent STs were ST204 (n = 32, 14.7%), ST2 (n = 30, 13.8%), ST1 (n = 25, 11.5%), ST9 (n = 24, 11.1%), and ST321 (n = 21, 9.7%), respectively. The other identified STs are presented in Table 1. Fourteen CCs were identified of which six were in lineage I and eight in lineage II, with CC1 (n = 38, 17.5%) the most prevalent followed by CC204 (n = 32, 14.7%) and CC2 (n = 31, 14.3%). Four serogroups were identified in the 217 isolates, with serogroup IIa (n = 95, 43.8%) being the most prevalent, followed by IVb (n = 69, 31.8%), IIb (n = 28, 12.9%), and IIc (n = 25, 11.5%) (Figure 1).

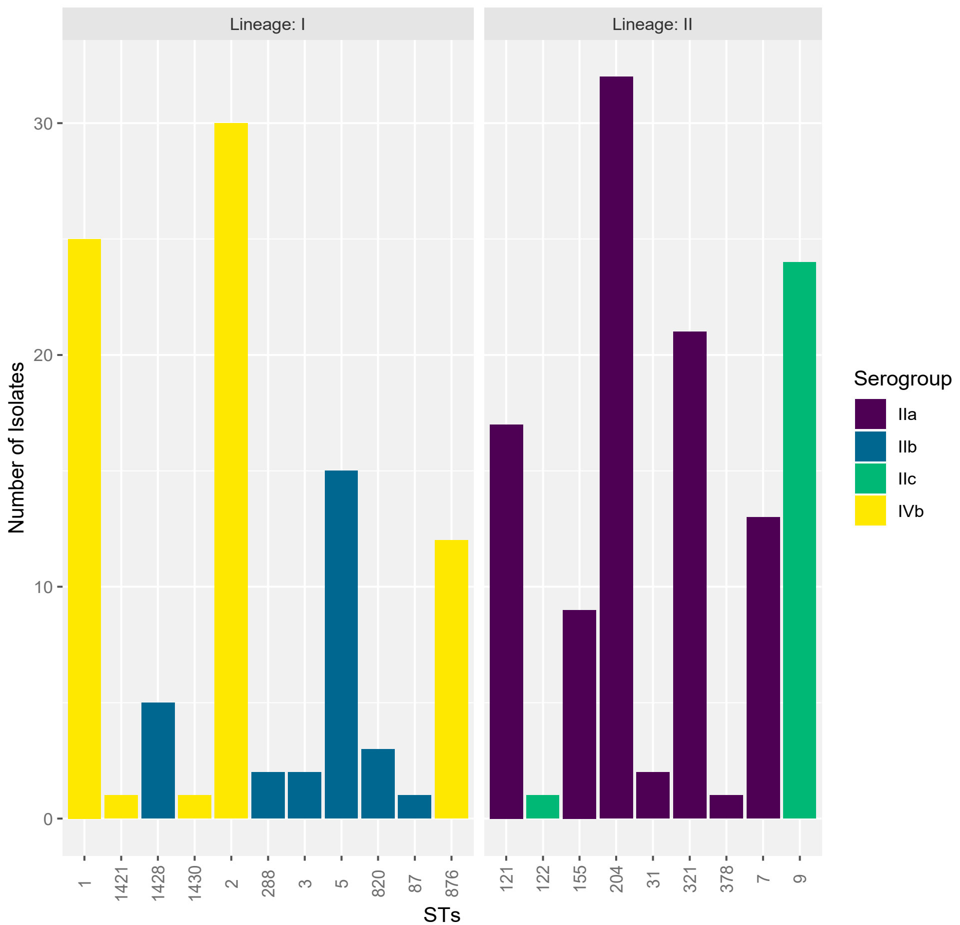

Figure 1. Lineage, serogroup, and sequence type distribution of *Listeria monocytogenes* isolates.

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The distribution of lineages, serogroups, CCs, and STs among the 217 isolates were tested using a Chi-Square goodness of fit test. The test results indicated that the serogroups (p-value = 1.283×10^{-13}), CCs (p-value = 5.761×10^{-24}) and STs (p-value = 3.327×10^{-32}) were not commonly distributed among the samples (Figure 2). In particular, serogroups IIa (lineage II) and IVb (lineage I) were found to be over-represented. The STs that exceeded the expected distribution were ST1, ST2, ST9, ST204, and ST321, which all belonged to serogroups IIa and IVb with the exception of ST9 in serogroup IIc.

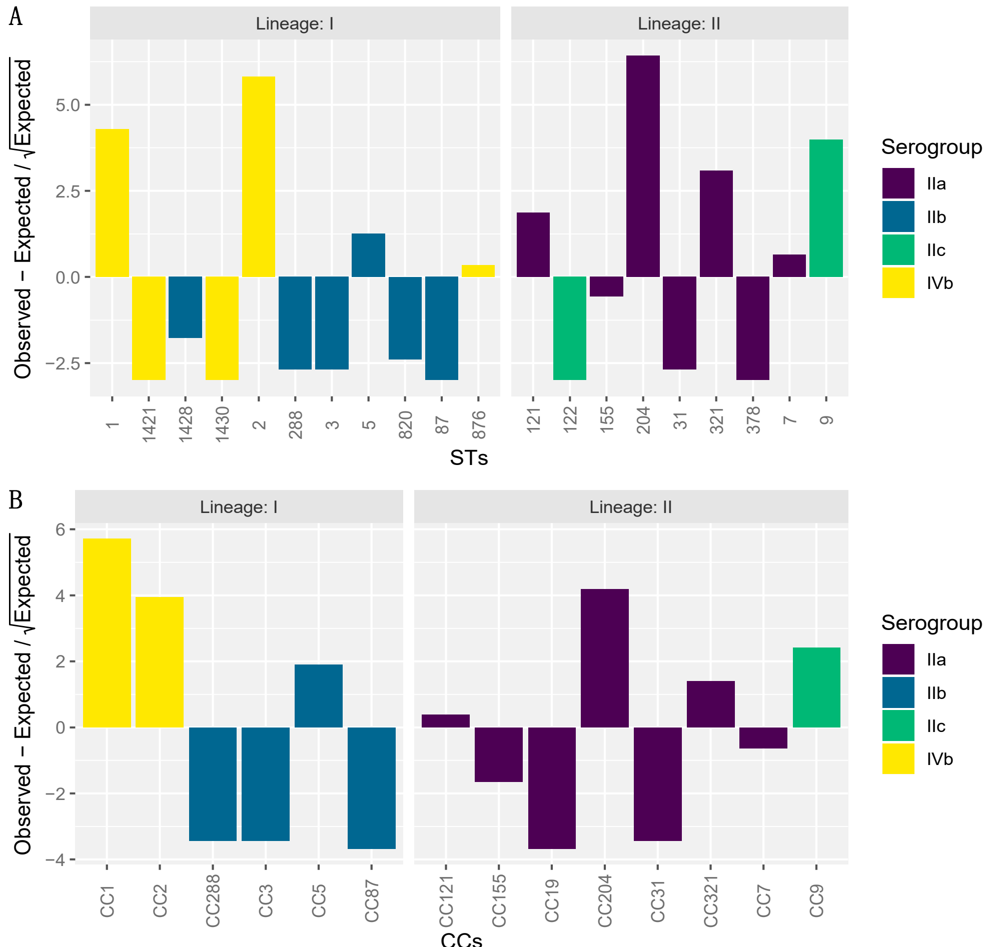

Figure 2. Hanging chi-gram indicating the deviation from expected occurrence across the *Listeria monocytogenes* isolates: (**A**) Sequence type; (**B**) clonal complex.

3.2. Samples Categories Analysis

Sample origin contained 12 categories (Table 1) and a Chi-Square test of independence were used to identify significant associations between the categories and the isolate typing results. Serogroup IIb in the "RTE-Poultry", IVb in the "Processed Meat-Beef", and IIc in the "Environmental Sample", "Abattoir" and "Farm" groups were found to be over-represented (p-value = 0.003). For the STs, over-representation was detected for "Processed Meat-Pork" (ST876), "Raw-Beef" (ST378), "Raw-Lamb" (ST121), "Raw-Pork" (ST122), "Raw-Poultry" (ST5), "RTE-Pork" (ST121), "RTE-Poultry" (ST3), and "Environmental Samples" (ST7, ST9, ST31, ST155, and ST288) (p-value = 1.771×10^{-11}). In the Establishment category, serogroup IIc was found to be significantly over-represented in Abattoirs and Farms (p-value = 0.009). Abattoirs were further found to be significantly associated with ST122;

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"Butcheries" with ST820; "Cold Stores" with ST121; Farms with ST7, ST9, ST31, ST155, and ST288; and "Processing Plants" with ST2 and ST3 (p-value = 8.602×10^{-13}).

Analysis of CCs and over-representation in the various categories indicated 11 CCs, which were deemed to be associated with a certain category. In the sample origin category, the "Environmental samples" group displayed over-representation of various CCs, which were CC7, CC9, CC31, CC155, and CC288 (p-value = 2.222×10^{-16}). These CCs were further found to be over-represented in Farms (p-value = 2.658×10^{-8}). The "Processed Meat-Beef" category had an over-representation of CC1; CC9 in Abattoirs; and in the "Processing Plant" establishment, CC2 was more than what was expected. In the "RTE-Poultry" and "Processing Plant" categories CC3 was over-represented, CC5 in the "Raw-Poultry" and C19 in "Raw-Beef" categories with CC121 significantly abundant in "Raw-Lamb", "RTE-Pork", and "Import Cold Stores". Over-representation of serogroups, STs, and CCs, indicated by a Pearson residual value larger than 2, for all categories, is presented in Figure 3.

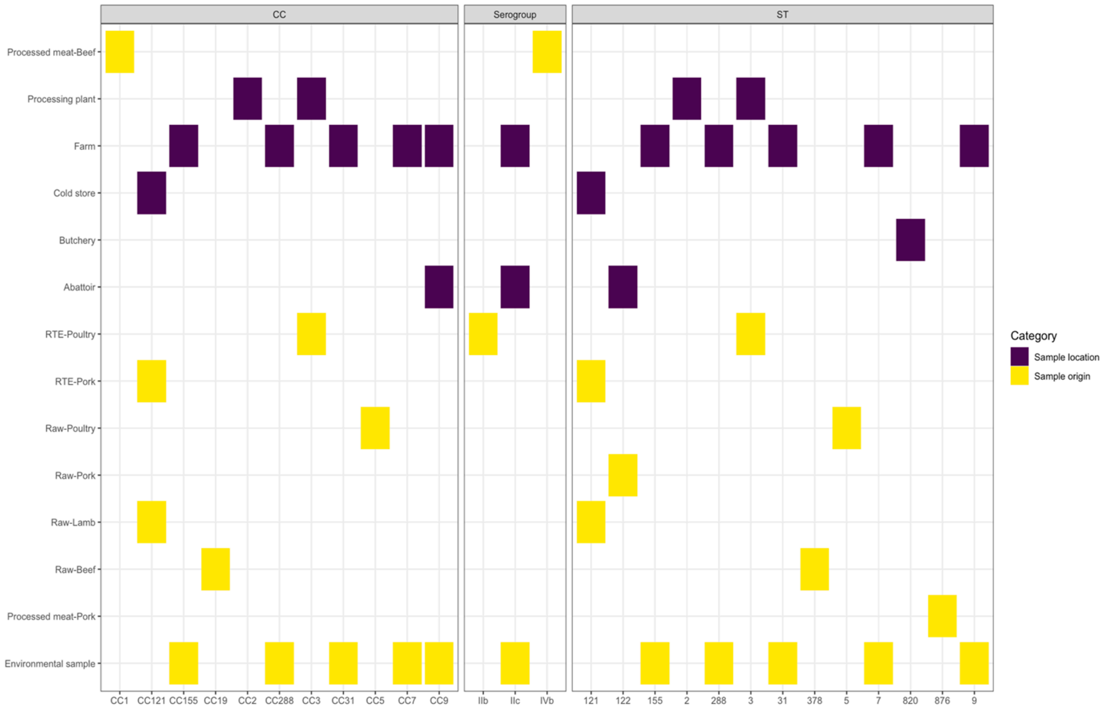

Figure 3. Over-representation of serogroups, clonal complexes (CCs) and sequence types (STs) across all categories.

3.3. ST Diversity Analysis

The ST results were transformed into a count matrix and diversity analysis done according to the sample collection categories. The frequency of STs per category is displayed in Figure 4. The values for four different diversity indices (Richness, Simpson, Shannon, and Inverse Simpson) are presented in Table 2 for both the categories "Sample origin" and "Sample location". Samples from the "Processed meat-Beef" category displayed the highest ST diversity, as indicated by all the indices, with "Raw-Poultry" having the second highest diversity. With regards to the "Sample location" it was found that the "Butchery" category had the highest ST diversity, closely followed by the "Retail" category. Results of clustering analysis and Principle coordinates analysis (PCOA) of the sample categories and the ST occurrence are displayed in Figure 5. In general, the "Sample origin" categories "Processed Meat-Beef", "Raw-Poultry", and "Environmental sample" formed a cluster with "Processed meat-Mixed" and "Raw-Beef" grouping together. In the "Sample location" category, "Butchery" and "Retail" grouped closely together.

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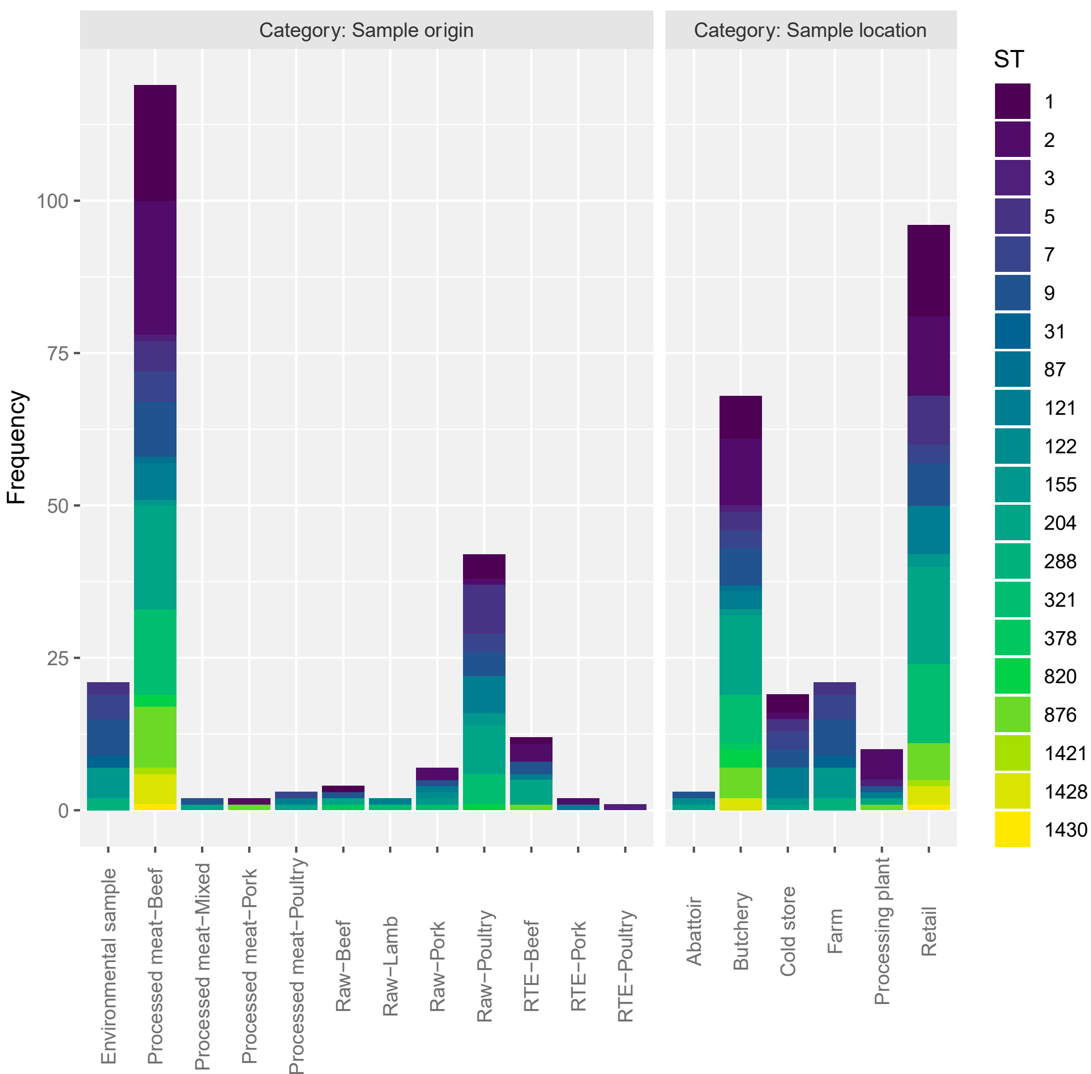

Figure 4. Frequency of STs per sample collection category.

3.4. Pathogenicity Islands

The *actA*, *hly*, and *mpl* genes, which form part of the LIPI-1 gene cluster were present in all the sequenced isolates. The complete gene cluster of LIPI-1 was present in 16 (7.4%) isolates all of which were found exclusively in "Raw-Poultry" and "Processed meat-Beef" categories obtained from "Butchery", "Cold Stores", and "Retail" Sample locations (Supplementary Table S1). These isolates presented CCs from lineage I (CC1, CC2 and CC5) and lineage II (CC9, CC121, CC155, and CC321). The complete LIPI-3 gene cluster was identified in 47 (21.7%) isolates (lineage I: 95.7%; lineage II: 4.3%), of which 35 (74.5%) originated from the "Processed meat-Beef" category. Four CCs (CC1, CC2, CC3, and CC288) belonged to lineage I and lineage II were represented only by CC204. A complete LIPI-4 gene cluster was detected in four isolates (1.8%) with the majority (75%) found in serogroup IVb (CC2 and CC87) and all in lineage I.

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Table 2. Diversity indices based on the occurrence of STs in the different categories.

Category	Samples	Richness	Shannon	Simpson	Inverse Simpson
Sample origin	Processed meat-Beef	16	2.357795795	0.884824518	8.682403433
	Processed meat-Mixed	2	0.693147181	0.5	2
	Processed meat-Pork	2	0.693147181	0.5	2
	Processed meat-Poultry	3	1.098612289	0.666666667	3
	Raw-Beef	4	1.386294361	0.75	4
	Raw-Lamb	2	0.693147181	0.5	2
	Raw-Pork	6	1.747868097	0.816326531	5.444444444
	Raw-Poultry	10	2.122400638	0.866213152	7.474576271
	[1] RTE-Beef	6	1.632630927	0.777777778	4.5
	RTE-Pork	2	0.693147181	0.5	2
	RTE-Poultry	1	0	0	1
	Environmental sample	6	1.687293537	0.798185941	4.95505618
Sample location	Abattoir	3	1.098612289	0.666666667	3
	Butchery	15	2.405603569	0.890138408	9.102362205
	Cold store	8	1.927544531	0.836565097	6.118644068
	Farm	6	1.687293537	0.798185941	4.955056179
	Processing plant	6	1.497866137	0.7	3.333333333
	Retail	13	2.30089177	0.885416667	8.727272727

[1] RTE—Ready to Eat.

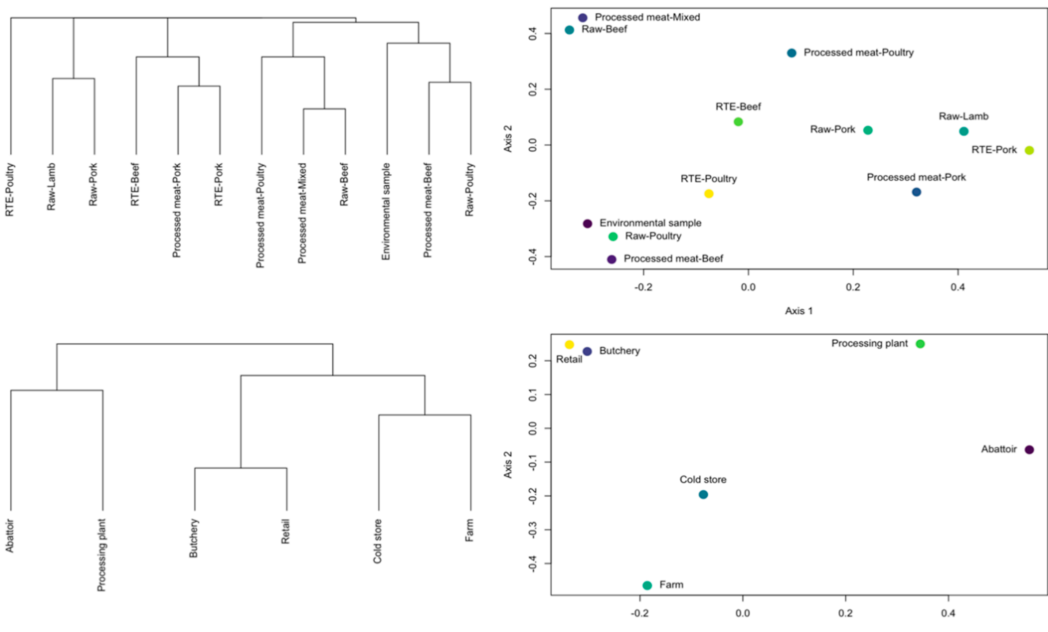

Figure 5. Hierarchical clustering dendograms and Principle coordinates analysis for the different sample categories.

3.5. Protein Sequence of *inlA*

Eight different *inlA* groups (1–8) were identified from the 217 sequenced isolates in this study (Figure 6 and Supplementary Table S2). Group 1 (size = 100) and group 2 (size = 93), harbored diverse STs, which all belonged to lineage II and lineage I, respectively. A total of 18 InlA protein sequences were found to be truncated with lengths range from 491–699 AAs. All the proteins in cluster 3 (size = 14, ST121) and cluster 6 (size = 2, ST121) were found to be truncated as well as the proteins of the singleton clusters 7 (size = 1, ST121) and 8 (size= 1, ST321). All truncated proteins belonged to isolates from

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lineage II, serogroup IIa, which were obtained across different establishments ("Butchery", "Retail", "Processing Plant", and "Cold Store") and sample origin ("Raw-Pork", "Raw-Lamb", "Raw-Poultry", "Processed Meat-Beef", "Processed Meat-Poultry", and "RTE-Beef") categories.

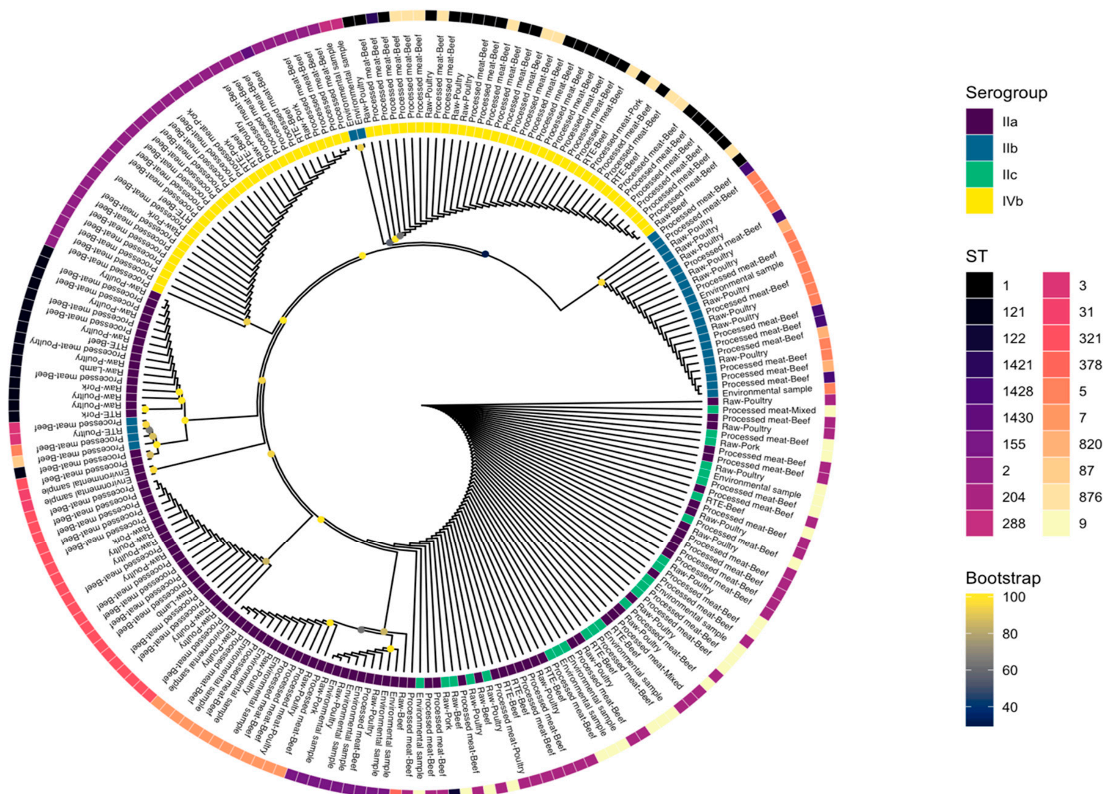

Figure 6. Phylogenetic analysis of *inlA* gene sequences obtained from isolates in this study.

3.6. Core Genome Phylogeny

In total, 22,790 genes were predicted across the 217 *L. monocytogenes* isolates. The partitioning of genes across the pan-genome was as follows: core – 1029 genes; soft core – 1141 genes; shell – 1711 genes; cloud – 18,909 genes. Phylogenetic analysis, based on the core genome, is displayed in Figure 7.

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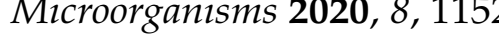

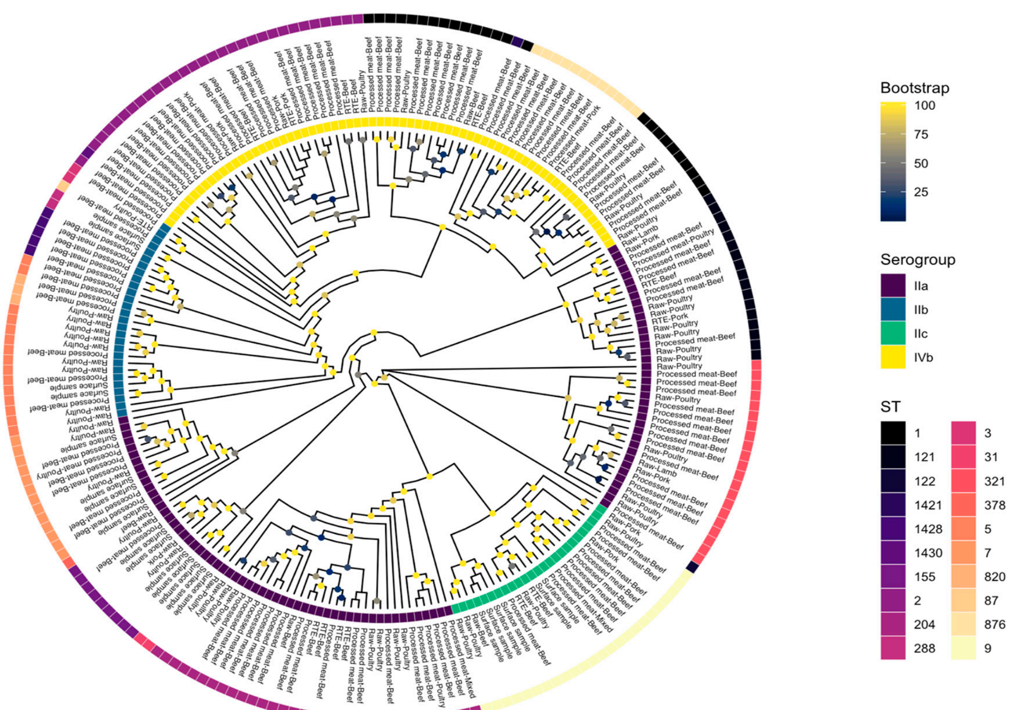

Figure 7. Phylogenetic analysis of *Listeria monocytogenes* isolates based on the core genome.

4. Discussion

To have a better understanding of population structure and genomic diversity of *L. monocytogenes* isolates in SA, a total of 217 isolates representing different meat and meat products as well as environmental samples were characterized using WGS. WGS is a very powerful tool for the characterization of *L. monocytogenes* as it allows an unprecedented subtyping resolution by using the entire genome to determine strain diversity and virulence traits [35–37]. The findings of the present study give a detailed overview into the genomic diversity of *L. monocytogenes* in the meat value chain that can inform food safety risk-based decisions and risk assessment.

The primary and universally acceptable method for characterization of *L. monocytogenes* isolates has been serotyping [38]. Serotyping has been used as a rapid tool for epidemiological investigations of listeriosis outbreaks and to understand the importance of certain serotypes in causing listeriosis in humans [39]. Analysis of the serotypes in this study revealed that all isolates belonged to four major serogroups IIa, IVb, IIb, and IIc (43.8%, 31.8%, 12.9%, and 11.5%, respectively). This is in general agreement with observations made in other countries where serogroup IIa, IIb, and IVb isolates were found frequently while serogroup IIc isolates were rarely found [39–41]. In Ireland, O'Connor et al. [42] analyzed 5869 of *L. monocytogenes* isolates from different foods and found that the most common serogroup was IIa (43.9%), followed by IVb (27.5%), IIb (16.1%), and IIc (12.2%).

In a comparative analysis of serogroups, a hierarchy among isolates was observed with IIa and IVb found to be over-represented. The high presence of serogroup IIa in this study was expected, as IIa has been previously identified as over-represented in food sources and environmental samples in different countries [22,41,43]. Although serogroup IIa is highly associated with contamination of food, it is important to mention that they can cause human infection in certain countries with a high number of susceptible individuals such as SA [44]. Over-representation of serogroup IVb in the present study is concerning as more than 80% of human infections globally are caused by *L. monocytogenes* strains in this serogroup [22,45,46]. Further analysis of serogroup distribution based on sample origin indicated over-representation (p-value = 0.003) of IIb in "RTE-poultry", IVb in "Processed meat", and IIc in

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"Environmental samples". This distribution provides critical information on the meat products that are prone to contamination by certain serogroups of *L. monocytogenes* and may subsequently help in good agricultural and hygiene practices, policy formation, and control measures of this bacterium in South Africa. For instance, implementing proper biosecurity and biosafety measures as good agriculture practice at farm level can play a critical role in minimizing the introduction and spread of different serogroups of *L. monocytogenes* on downstream processing steps across meat value chain.

MLST is a technique used to analyze nucleotide sequence data from a number of conserved (usually 7) housekeeping genes to derive a combination of alleles known as a ST. The application of this technique in the present study has served as tool also to determine the lineages and CCs of *L. monocytogenes*. CCs are defined as a group of STs differing by no more than one allele from at least one other ST in the group, regardless of its involvement in outbreaks [22]. Analysis of MLST data revealed the distribution of 20 different STs among all the sample isolates that belong to two main lineages, I or II. Similar descriptive differentiation of lineage I and lineage II isolates has been recorded in previous studies [22,47]. This suggests that lineage I and II isolates are important etiological agents common in the South African red meat and poultry value chain. The five largest ST groupings identified in this study, ST204 and ST321 (serogroup IIa), ST1 and ST2 (serogroup IVb), and ST9 (serogroup IIa), have previously been isolated from meat, meat products and production environments around the world [40,47–49]. However, this is the first detailed report on the distribution of STs along the livestock value chain in SA and as such it provides contemporary and applicable data. The predominant ST in the current study, ST204, has also been reported by Kwong et al. [50] and Ebner et al. [51], as the most common ST in meat-associated products in Australia and France. Other studies reported ST204 as a common persisting strain of *L. monocytogenes* that has been isolated from various sources such as food processing facilities [52], non-clinical isolates [22], and RTE food products [53]. ST1 and ST2 are regarded as the most common STs associated with food contamination and causing infection of humans and animal globally [54–56]. In a survey of food-producing facilities between 1996 and 2003 in Austria, ST1 and ST2 were the most predominant in meat-based products as cited by Ebner et al., [51]. Data on the occurrence and distribution of ST9 and ST321 in meat and meat products are lacking globally.

In comparison to the STs in the meat value chain, the non-ST6 sequence types reported from molecular epidemiology of human cases in South Africa are ST1, ST2, ST5, ST54, ST204, ST876, ST7, ST219, Unknown ST, Novel ST, ST101, ST1039, ST224, ST3, ST554, ST8, ST808, ST88, and ST87 in order of frequency [57]. ST6, ST132, ST155, ST2, ST204, ST3, ST5, ST533, ST602, and ST9 have been reported as the common environmental STs in SA can food production facilities [9,57]. Some of these STs (ST2, ST3, ST5, ST9, ST155, and ST204) have been reported in the present study and are induced with mechanisms that allow them to survive in food production environment and keep contaminating food products [5]. Therefore, there is need to link clinical isolates to food samples, since such epidemiological linkages are known to help further understand the key transmission routes and high-risk foods [22].

The absence of highly hypervirulent strains of *L. monocytogenes* ST6 was observed in the present study. In SA, ST6 strains have been associated with RTE products as samples cultured from a meat production facility's food contact and non-contact environmental surfaces yielded ST6 isolates, which, together with the isolates from the human patients, belonged to the same core-genome MLST cluster with no more than four allelic differences [9]. The absence of ST6 in the current study and the rapid decline in the incidence of *L. monocytogenes* ST6 infections in humans soon after a recall of the implicated RTE processed meat products suggests that polony (Bologna sausage) produced at a single facility was highly likely to be the outbreak source with the primary contamination originating from a confined primary source [9].

Diversity analysis performed according to the sample collection categories ("Sample origin" and "Sample location") showed that isolates from "Processed Meat-Beef" and "Butchery" categories harboured more heterogonous STs (ST1, ST2, ST3, ST5, ST7, ST9, ST87, ST121, ST155, ST204, ST321, ST820, ST876, and ST1428) of *L. monocytogenes*. It was also observed that isolates from "Raw-Poultry" and "Retail" categories harbored the second highest diversity of STs (ST1, ST5, ST9, ST155, ST204, and ST321).

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The clustering observed between "Processed Meat-Beef/Raw-Poultry" and "Butchery/Retail" categories based on the ST occurrence is highly comparative with several previous studies that recorded more diversity in isolates from RTE products [20,58]. Although, human infections caused by *L. monocytogenes* are commonly linked to RTE products, the findings of the present study are important in the South African context as raw-meat and processed-meat products are part of the raw materials for RTE.

The isolates in the present study were also classified into 14 CCs that represent two typical groups of *L. monocytogenes* CCs. The first group (infection-associated isolates) includes isolates (CC1, CC2, CC4, and CC6) that belong to lineage I and have a strong link to clinical cases (also known as hypervirulent strains) while the second group (food-associated isolates) represent isolates (CC7, CC9, CC121, CC155, and CC204), which belong to lineage II and are predominantly found in the food production environment [58]. The distribution of infection-associated isolates revealed the presence of CC1 and CC2, which were found to be over-represented in the "Raw-Beef" and "Processing Plant" categories, respectively. This over-representation of CC1 and CC2 clones in meat samples has been reported in different studies globally, which suggest their adaptation to diverse food products [25,59]. The distribution of food-associated isolates of *L. monocytogenes* CCs revealed a significant over-representation of CC7, CC155, CC9, CC121, and CC204, which all belong to lineage II. CC7 and CC155 were mostly found in isolates recovered from farm and environmental samples. CC7 isolates have been globally reported from diverse sources such as animal (wild, poultry, ruminants and fish), abattoir floor, compost, animal food products (milk, cheese, meat), and animal feeds (hay, silage) suggesting the possibility that it might persist in varying environments [39,60–62]. CC155 isolates were frequently detected in food samples in Eastern Asia [63], animals in Switzerland [64], and to a lesser extent in clinical cases in France, New Zealand, Greece, and Netherlands [65]. In the present study, CC9 and CC121 were significantly abundant in samples from "Raw-Lamb" and "Cold Stores (raw imported meat samples) categories and over-represented in "RTE-Pork" meat. Other studies also reported CC9 and CC121 as being significantly over-represented in food of animal origin and food-processing facilities around the world [47,52,66,67]; however, in this study, no significant association of CC204 isolates were observed with respect to environmental samples as well as meat and meat products isolates.

In the current study, although there was a bias towards specific lineages and CCs, there was considerable variation on pathogenic islands known to contribute to *L. monocytogenes* virulence among the isolates. The complete LIPI-1 gene cluster was detected in 16 isolates, recovered exclusively from "Raw-Poultry" and "Processed meat-Beef" categories. The presence of LIPI-1 in these meat products indicate an increased potential risk to cause infection in humans as LIPI-1 harbor a cluster of genes, *prfA*, *plcA plcB*, *hly*, *mpl*, and *actA*, that are very important in the infectious cycle of *L. monocytogenes* [68]. LIPI-3 consists of eight genes (llsAGHXBYDP) and this complete gene set was detected in 47 isolates, of which the majority (95.7%) were from lineage I (CC1, CC2, CC3, and CC228) and originating from the "Processed meat-Beef" category. This island encodes for hemolysin listeriolysin S, which is known to contribute to the survival of *L. monocytogenes* in human polymorphonuclear neutrophils [69]. Therefore, the presence of LIPI-1 and LIPI-3 islands in isolates from the "Processed meat-Beef" category, increases the risk for certain members of the population, such as the elderly, acquiring listeriosis in SA. LIPI-4 island is often implicated in placental and central nervouss system infections [69,70] and was found in four isolates in the present study. LIPI-4 has previously been identified mostly in CC4 isolates, however results of this study indicate its presence in CC2 and CC87 isolates, which is consistent with a recent report of this island in CC2 and CC87 isolates cultured in China [70]. The presence of LIPI-4 islands in hypervirulent CC2 and CC87 *L. monocytogenes* strains in beef and pork products must be a consideration in public health risk management.

The *inlA* gene encodes a surface protein that is responsible to facilitate the invasion of human intestinal epithelial cells by *L. monocytogenes* [71]. However, truncation of the *inlA* gene due to premature stop codons (PMSCs) has been associated with reduced invasiveness in some *L. monocytogenes* STs that possess them [71]. Analysis of the inlA protein sequence from isolates in this study identified 18 isolates, all from ST121 (n = 17) and ST321 (n = 1), having PMSCs, both of which were lineage II STs.

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Although the ST121 and ST321 PMSC mutation has previously been reported [22,64], this is the first report in SA.

5. Conclusions

Characterization of *L. monocytogenes* isolates from 2014–2019 using WGS has provided valuable insights into strain diversity and virulence potent of isolates found in meat products consumed in SA. This is also the largest study to report baseline data on the presence of *L. monocytogenes* serogroups, lineages, STs, and CCs across meat value chain in SA. This study confirmed the heterogeneous distribution of *L. monocytogenes* CCs across different meat and meat products with evidence of over-representation of certain CCs, which share similarities with those previously linked with human listeriosis outbreaks in other geographical areas. This study again illustrated meat products which are prone to contamination by diverse strains of *L. monocytogenes* within a specific point in the value chain. This study highlights the association of multiple STs of *L. monocytogenes* to different meat products in SA and identifies virulence traits as well as genetic mutations of certain subgroups found in food products. Therefore, the information generated here can be used in food safety risk assessment, management and protecting public health.

Future work is still required to compare the WGS dataset produced from this study with clinical isolates from the same timeframe and geographic regions, to identify clusters and determine potential linkages to human listeriosis cases and outbreaks, taking into consideration temporal, microbiological, and epidemiological evidence.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/8/1152/s1, Table S1: *Listeria* pathogenic islands metadata, Table S2: InlA protein sequence clusters metadata.

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Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER THREE

Chapter Three: Whole Genome-based characterization of *Listeria monocytogenes* isolates

Published Manuscript: Whole Genome-Based Characterization of *Listeria monocytogenes* Isolates Recovered From the Food Chain in South Africa

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Whole Genome-Based Characterization of *Listeria monocytogenes* Isolates Recovered From the Food Chain in South Africa

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Listeria monocytogenes is an important foodborne pathogen which has the ability to adapt and survive in food and food processing facilities where it can persist for years. In this study, a total of 143 *L. monocytogenes* isolates in South Africa (SA) were characterized for their strain's genetic relatedness, virulence profiles, stress tolerance and resistance genes associated with *L. monocytogenes*. The Core Genome Multilocus Sequence Typing (cgMLST) analysis revealed that the most frequent serogroups were IVb and IIa; Sequence Types (ST) were ST204, ST2, and ST1; and Clonal Complexes (CC) were CC204, CC1, and CC2. Examination of genes involved in adaptation and survival of *L. monocytogenes* in SA showed that ST1, ST2, ST121, ST204, and ST321 are well adapted in food processing environments due to the significant over-representation of Benzalkonium chloride (BC) resistance genes (*bcrABC* cassette, *ermC, mdrL* and *Ide*), stress tolerance genes (SSI-1 and SSI-2), Prophage (φ) profiles (LP_101, vB LmoS 188, vB_LmoS_293, and B054 phage), plasmids profiles (N1-011A, J1776, and pLM5578) and biofilm formation associated genes. Furthermore, the *L. monocytogenes* strains that showed hyper-virulent potential were ST1, ST2 and ST204, and hypo-virulent were ST121 and ST321 because of the presence and absence of major virulence factors such as LIPI-1, LIPI-3, LIPI-4 and the internalin gene family members including *inlABCEFJ*. The information provided in this study revealed that hyper-virulent strains ST1, ST2, and ST204 could present a major public health risk due to their association with meat products and food processing environments in SA.

Keywords: cgSNP, cgMLST, AMR, virulence profiles, Benzalkonium chloride resistance, stress tolerance, plasmids, prophages

INTRODUCTION

Listeria monocytogenes remains a considerable public health concern due to its complex ecology and ability to survive in various harsh environmental conditions posed in the food processing facilitates (Ferreira et al., 2014; Hurley et al., 2019; Chen et al., 2020). Assessing the genetic diversity of *L. monocytogenes* is critical in understanding the epidemiology, ecology, and pathogenicity of this

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pathogen. *Listeria monocytogenes* consists of three major evolutionary lineages including lineages I, II, and III, as well as a rare lineage IV (Chen et al., 2020). These lineages represent 13 recognized serotypes of *L. monocytogenes* which are further grouped into four PCR-serogroups: IIa (1/2a and 3a), IIc (1/2c and 3c), IIb (1/2b and 3b), and IVb (4b, 4d, and 4e) (Doumith et al., 2004; Chen et al., 2020). Molecular typing of *L. monocytogenes* strains can also be done using Multilocus Sequence Typing (MLST), which is based on the sequence variants of seven housekeeping genes to determine their ST and CC. Recently, the cgMLST typing method that takes into account the sequence variation of 1,748 *L. monocytogenes* core genes, has been used to improve isolates discrimination and allowing a standardized comparison with isolate databases for outbreak investigations and surveillance of listeriosis (Moura et al., 2016, 2017).

The adaptation and survival of *L. monocytogenes* in the food processing facilities occur mainly through their ability to proliferate in low temperature, pH and osmotic stress (Takahashi et al., 2014), as well as resistance to sanitation agents and formation of biofilm (Hurley et al., 2019). The control of *L. monocytogenes* in the food processing facilities is mostly based on application of quaternary ammonium compounds (QACs) biocides, such as BCs (Zacharski et al., 2018; Maury et al., 2019). However, the evolution of *L. monocytogenes* resistant to the BCs has been reported in several studies and has become a serious global concern (Zacharski et al., 2018; Korsak et al., 2019). These BC resistances are associated with several efflux resistance genes including *bcrABC* cassette, *Ide, mdrL, qacH, qacA, qacE*Δ*1-sul*, and *emrE* which have been reported in various serotypes, ST and CC of *L. monocytogenes* isolated from diverse sources (Kovacevic et al., 2016; Korsak et al., 2019). Furthermore, another key adaptation of *L. monocytogenes* in the environment is the ability to tolerate toxic metals such as arsenic and cadmium (Jesse et al., 2014; Nunes et al., 2016). As result, the co-occurrence of toxic metals and biocide resistance genes in *L. monocytogenes* contribute to the selection of different resistance genotypes and phenotypes that can cause human listeriosis (Angelo et al., 2017; Parsons et al., 2018).

However, despite antibiotic treatment including β-lactam antibiotic such as amoxicillin, penicillin, or ampicillin, and aminoglycosides, such as gentamycin, listeriosis is responsible for mortality rate of 20–30% world-wide (Wang et al., 2015; Wilson et al., 2018). There are reports on *L. monocytogenes* isolates resistant to one or more antibiotics primarily cephalosporins, oxacillin and fosfomycin, particularly in Southern and Western regions of Asia (Sugiri et al., 2014; Wang et al., 2015). The genetic basis of antibiotic resistance in *L. monocytogenes* is associated with different genes such as genes encoding for efflux pumps, particularly for the major facilitator superfamily (*Ide*); erythromycin ribosome methylase (erm) genes (*ermA, ermB*, and *ermC*); tetracycline resistance genes (*tetA, tetK,* and *tetL*); *fosX,* and *lmrB* (Wilson et al., 2018). The role of mutations in DNA gyrase topoisomerase II (*gyrA* and *gyrB*), topoisomerase IV (*parC* and *parE*) in the development of antibiotic resistance by *L. monocytogenes* was also pointed out by Moreno et al. (2014) and Wilson et al. (2018). The virulence potential of this bacteria is mainly contributed by virulence genes such as *prfA, plcA, hly, mpl, actA, plcB, inlA, inlB*, and *lspA* (Chen et al., 2019).

Several studies in SA have reported the presence of *L. monocytogenes* in food products (Matle et al., 2019; Smith et al., 2019; Thomas et al., 2020). Matle et al. (2019, 2020) conducted a national survey to determine the occurrence and population structure of *L. monocytogenes* strains in meat and meat products isolated from retail, meat processing facilities and abattoirs in SA. Although, this study provides crucial information on meat contamination with *L. monocytogenes*, further investigations are still required to determine the hyper-virulent strains, antibacterial resistance genes, stress tolerance capabilities of *L. monocytogenes* in SA food products. Thus, the objectives of this study were to: (1) use core genome-SNP analysis to determine the genetic relatedness of the most common *L. monocytogenes* strains in SA; (2) assess the genetic basis of the resistance, stress tolerance, genomic localization of the resistance genes in *L. monocytogenes* isolated from food products in SA; and (3) identify key genomic features contributing to virulence potential of *L. monocytogenes* strains in the host.

MATERIALS AND METHODS

Isolates Selection, Genome Assembly, and Annotation

A subset of 152 isolates were selected from a total of 217 isolates from our previous study (Matle et al., 2020). The isolates were selected based on quality of the raw reads and *de novo* assembly in order to avoid false prediction of genes of interest in the present study. Briefly, the raw read quality was assessed with FastQC v.0.11.9 (Andrews, 2010) and the adapters and low-quality reads were trimmed using Trimmomatic v.0.39 (Bolger et al., 2014). SPAdes v.3.13.1 program (Bankevich et al., 2012) was used to create *de novo* assembly of each isolate. The resulting genome assembly were further quality assessed with QUAST v.5.0.2 (Gurevich et al., 2013) and annotated using Prokka v.1.13.7 (Seemann, 2014).

About nine isolates showed poor *de novo* assembly statistics and they were only included in the MLST analysis and subsequently removed from further statistical analysis (**Supplementary Table 1**). The large scale MLST analysis of *L. monocytogenes* isolates including the isolates of the present study were published by Matle et al. (2020). The cgMLST analysis was also performed using chewBBACA v.3.0 (Silva et al., 2018) only on the isolates used in the present study (a subset of 217 isolates). The cgMLST typing was run with an external schema adapted from BIGSdb-*Lm* platformhttps://bigsdb.pasteur.fr/listeria[1] (Jolley and Maiden, 2010; Moura et al., 2016). The allele calling on the target genomes were performed with chewBBACA Allele Calling algorithm using the *Listeria_monocytogenes*.trn training file based on the reference strain *L. monocytogenes* EGD-e (acc. No. NC003210). The cgMLST results of these isolates were included as **Supplementary Figures 1**, **2**.

[1]https://bigsdb.pasteur.fr/listeria

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Core Genome Single-Nucleotide Polymorphism

A reference-based variant calling analysis was performed using the Snippy v.2.6[2]. 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 and 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 and 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). The total number of SNPs from both inside and outside recombination events were determined with Gubbins (Croucher et al., 2014) using the core alignment file produced by Snippy v2.5.

Prediction of Virulence Factors, Antimicrobial Resistance, and Stress-Related Genes

Genome assemblies were screened for the presence/absence of genes rendering resistance to antimicrobials, biocides, and heavy metals; and also stress tolerance genes and virulence factors as well as biofilm formation associated genes. ABRicate v0.8.10 was used for this screening with the minimum identity and coverage cut-offs values set by default settings. All alleles for stress tolerance, virulence factors and resistance genes were retrieved from the *Listeria* database hosted by the Pasteur Institute, Paris, France[1]. The biofilm formation associated genes were also retrieved from NCBI (**Supplementary Table 3**). Other databases used for analyses of virulence factors and resistance genes with ABRicate v0.8.10 were CARD v2.0.3 (Jia et al., 2017), BacMet database (Pal et al., 2014) and Virulence Factor database (VFDB) (Chen et al., 2016). Virulence factors and resistance genes identified by ABRicate v0.8.10 were validated by blastn v.2.10.0+.

Plasmid Reconstruction

Plasmids of the *L. monocytogenes* strains were *de novo* predicted using MOB-suite software (Robertson and Nash, 2018). The MOB-recon algorithm was used to identify plasmid contigs from the draft genomic assemblies. The BLAST-based MOB-recon tool uses markers from sequence databases of known replicons and relaxases in conjunction with a reference database of clustered plasmids provided by MOB-suit software. Finally, the PLSDB web-resource (Galata et al., 2019), a comprehensive large-scale database comprising 13,789 (November 2018) complete sequences of bacterial plasmid, was used for a large-scale comparative analysis to retrieve plasmid records similar to the herein assembled plasmids. The PLSDB database was interrogated using ABRicate v0.8.10[3] with minimum identity and coverage cut-offs values set by default settings.

Prediction of Prophages

In order to identify putative prophages, genome assemblies were searched by 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 and the percentages of CDSs that match a certain phage. Intact and questionable regions with sequence lengths over 20 kbp were used for the prophage profiling.

Statistical Validation

Statistical validation of the results was performed using R v.3.6.0[4] Distribution and association testing were done using Chi-Square tests and over-representation was indicated by a Pearson residual value larger than 2. Additional analysis was done using in-house python scripts.

RESULTS

The Core-SNP Phylogenetic Clustering of the Most Common *L. monocytogenes* STs in SA

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 three distinct clusters with SNP difference ranging up to 41 SNPs in the core parts of the genomes of these strains (**Figure 1**). Moreover, the ST1 and ST2 were grouped in two distinct clusters with SNP difference ranging up to 27 and 34 SNPs, respectively (**Figure 1** and **Supplementary Table 2**). These results indicate that SA *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 maximum two SNPs core genome difference between these isolates (**Figure 1** and **Supplementary Table 2**). Another observation from these results was that ST clustering did not follow the specific isolation sources. In general, the core-SNP phylogenetic tree displayed a good congruence to the cgMLST phylogenetic tree as it is demonstrated in **Figure 2**. Discrepancies between trees in many cases could be resolved by reordering of the clusters without influencing topologies of the trees.

[2]https://github.com/tseemann/snippy

[3]https://github.com/tseemann/ABRicate

[4]https://www.R-project.org/

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FIGURE 1 | Core-SNP phylogeny showing genetic relatedness of the *L. monocytogenes* strains in SA. **(i)** A section pruned from the original tree showing the South African genetically related ST204 strains. **(ii)** A section pruned from the original tree showing the South African genetically related ST2 strains. **(iii)** A section pruned from the original tree showing the South African genetically related ST1 strains.

Antimicrobial Resistance and Biofilm Formation Genes

The antimicrobial resistance genes were identified in all the isolates of *L. monocytogenes*. These genes include *fosX*, *lin*, *norB*, and *mprF* which confer resistance, respectively, to fosfomycin, lincosamides, quinolones and cationic peptides that disrupt the cell membrane such as defensins (**Figure 3**). Genes *tetM* and *tetS* that confer resistance to tetracycline were infrequent among isolates. The *tetM* was found only in ST2 and ST9 belonging to serogroups IIb and IVb of lineage I. The *tetS* was observed only in one isolate belonging to ST2 from serogroup IVb of lineage II. Tetracycline resistance genes *tetM* and *tetS* were detected in isolates originated from beef and poultry meat samples obtained from retail and butchery (**Figure 3**).

Biofilm formation associated genes including *inlL, prfA, actA, lmo0673, bapL, recO, lmo2504,* and *luxS* which play a significant role in survival and persistence of *L. monocytogenes* were analyzed and detected in (n = 72, 47%; n = 149, 98%; n = 72,

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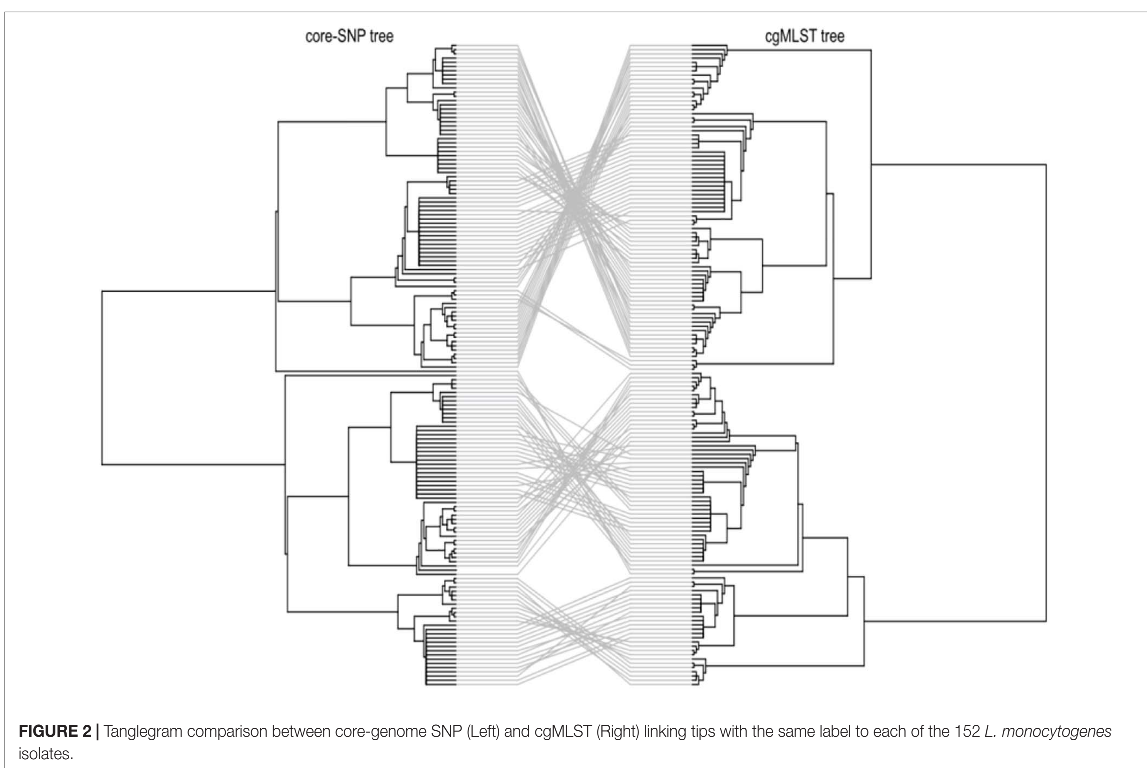

FIGURE 2 | Tanglegram comparison between core-genome SNP (Left) and cgMLST (Right) linking tips with the same label to each of the 152 *L. monocytogenes* isolates.

47%, *n* = 78, 51%; *n* = 6, 3.9%; *n* = 82, 53%; *n* = 130, 86%; and *n* = 145, 95%) of the isolates, respectively (**Supplementary Table 3**). The *L. monocytogenes* strains which harbored majority of these genes except for *lmo0673* and *bapL* genes were ST204 and ST321 both belonging to serogroup lla. The well-known ST1 and ST2 isolates which are associated with clinical human listeriosis appear to have less overall biofilm formation associated genes and were also missing the *actA* gene, an important biofilm formation gene. More than 90% of these isolates harbored *prfA, lmo2504,* and *luxS genes.* However, none of the isolates harbored all 8 genes associated with biofilm formation (**Supplementary Table 3**). Interestingly, *bapL* gene was only specific for ST121 which also harbored most of these genes, but also lacked *actA* gene in the sequenced genomes.

Benzalkonium Chloride Resistance and Stress Tolerance Genes

The chromosome-borne BC resistance genes including *mdrL* and *Ide*, which are the major facilitator superfamily efflux pumps of *L. monocytogenes* conferring resistance to BC were present in (*n* = 143, 100%) and (*n* = 124, 86.7%) of the isolates, respectively (**Figure 3**). In many cases, these genes were found in chromosomal inserts of the plasmid-borne BC resistance *bcrABC* cassette (*n* = 55, 38%). The presence of the *bcrABC* cassette was characteristic for ST204 and ST321 all belonging to serogroup IIa of lineage II ($p < 0.05$). Another plasmid-borne BC resistance *ermC* gene was present in (*n* = 58, 40%) of the isolates and was over-represented in ST321 belonging to serogroup IIa of lineage II ($p < 0.05$; **Figure 3**). No specific over-representation of *Ide*, *bcrABC* cassette and *ermC* was observed in isolates from beef or poultry meat samples ($p > 0.05$). However, the *bcrABC* cassette and *ermC* were significantly over-represented in the isolates from butchery and retail samples ($p < 0.05$; **Figure 3**).

The stress survival islets (SSI-1 and SSI2), which are known to be responsible for proliferation of *L. monocytogenes* under stressful conditions in food processing facilities, were present in (*n* = 86, 55%) and (*n* = 11, 7.7%) of the isolates, respectively. The SSI-1 was found to be significantly over-represented in ST9, ST204 and ST321 belonging to serogroups IIa and IIc of lineage II ($p < 0.05$; **Figure 3**). The SSI-2 was found to be significantly over-represented in ST121 belonging to serogroup IIa of lineage II ($p < 0.05$; **Figure 3**). Islets SSI-2 were over-represented with $p < 0.05$ in the isolates obtained from meat samples from meat processing plants and cold stores in contrast to the distribution of islets SSI-1 showing nor statistically reliable preferences regarding different sources of isolation of *L. monocytogenes* (**Figure 3**).

The Assessment of Virulence Factor Genotypes Across Different Serogroups, STs and Isolation Sources

A total of 68 putative virulence factors were present across the *L. monocytogenes* isolates. The presence and integrity of *Listeria* pathogenicity islands LIPI-1, LIPI-2, LIPI-3, and LIPI-4 were

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FIGURE 3 | Core-genome MLST phylogenetic grouping of AMR, stress tolerance, biocide resistance genes, plasmids and prophages across the 152 *L. monocytogenes* isolates. The heat map shows the presence (blue) or absence (white) of genes involved in *L. monocytogenes* resistance and virulence. The isolation source MC and EC labeling on the heat map indicate the Meat category and Establishment category, respectively.

investigated in our previous published study (Matle et al., 2020) and the *Listeria* pathogenicity islands results for the present isolates were included as **Supplementary Figure 3**. The internalin gene family members including *inlABCEFJK* were present in more than 90% of the isolates. The *inlD* and *inlG* were present in 88 and 47% of the isolates, but absent in ST9 and ST1 which were part of the most abundant ST identified (**Figure 4**). Other important virulence factors detected in genomes of *L. monocytogenes* isolates include adherence virulence factors such as *ami, fbpA, lap,* and *lapB*, which were present in 54.6, 98.68, 91, and 98% of the isolates; invasion virulence factors *aut*, *gtcA*, *lpeA* and *vip*, which were present in 97, 43, 95, and 72% of the isolates; as well as intracellular survival factors *lplA1*, *prsA2* and *svpA*, which were present in 98, 98.6, and 98.6%, respectively. The *ami*, *gtcA* and *vip* genes were over-represented, respectively in ST204, and ST321; ST1, ST2, ST876; ST1, ST2, ST9, and ST876 ($p < 0.05$; **Figure 4**).

The Distribution of *L. monocytogenes* Plasmids Between Different Serogroups, STs and Isolation Sources

A total of four unique plasmids that contribute to the resistance of *L. monocytogenes* to antibiotics were identified in 71% of the tested isolates. Of the four unique plasmids, the most frequent was plasmid N1-011A (n = 52, 36.34%), followed by plasmids

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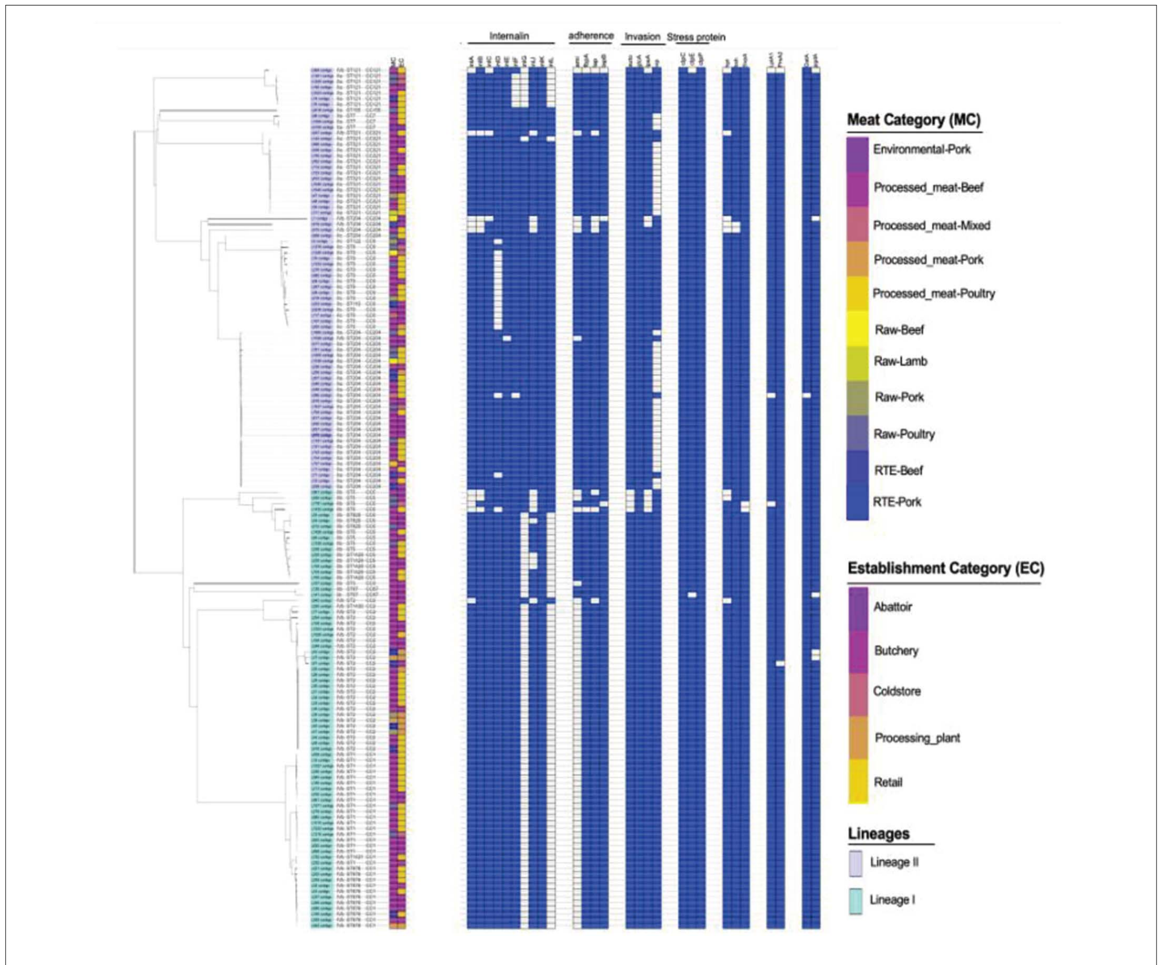

FIGURE 4 | Core-genome MLST phylogenetic grouping of putative virulence factors across the 152 *L. monocytogenes* isolates. The heat map shows the presence (blue) or absence (white) of genes involved in *L. monocytogenes* virulence. The isolation source MC and EC labeling on the heat map indicate the Meat category and Establishment category, respectively.

J1776 (n = 28, 19.6%), pLM5578 (n = 25, 16.8%), and pLI100 (n = 4, 2.6%) across all the study isolates (**Figure 3**). Plasmid N1-011A was significantly over-represented in ST2 belonging to serogroup IVb of lineage I, and was also over-represented in ST204 belonging to serogroup IIa of lineage II ($p < 0.05$). Plasmid J1776 was over-represented in ST2 belonging to serogroup IVb of lineage I, and was also over-represented in ST9 belonging to serogroup IIc of lineage II ($p < 0.05$). Plasmid pLM5578 was over-represented in ST121 and ST321 belonging to serogroup IIa of lineage II ($p < 0.05$). Plasmid pLI100 was observed only in four isolates belonging to ST1428 of serogroup IIb from lineage I, which also contained plasmid J1776 (**Figure 3**). The significant association of the plasmids with different isolation sources showed that for the meat category: plasmids N1-011A, J1776, and pLM5578 showed no statistically reliable association with the source of isolation of the pathogen ($p > 0.05$). Contrary, as to the establishment categories: plasmids J1776 and pLM5578 showed a significant association with the retail and butchery category ($p < 0.05$). However, that was not the case with plasmid N1-011A showing no significant associations with either source or establishment categories ($p > 0.05$; **Figure 3**).

Prophage (φ) Profiles of *L. monocytogenes* Isolates

Prophage (φ) profiles of the *L. monocytogenes* genomes sequenced in this study were determined using the PHASTER tool for identification and annotation of putative prophage sequences. A total of nine different intact, questionable, or incomplete prophages regions were found across different *L. monocytogenes* isolates (**Figure 3**). The intact prophage LP_101 [NC_024387] (n = 53, 37%) was the most prevalent followed by vB LmoS 188 [NC_028871] (n = 45, 31.46%), vB_LmoS_293 [NC_028929] (n = 18, 12.58%), and B054 [NC_009813] (n = 14,

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9%). The LP_101 phage was over-represented in ST121, ST204, and ST321 belonging serogroups IIa of lineage II and also in ST9 belonging to serogroup IIc of lineage II ($p < 0.05$). Phage vB LmoS 188 was over-represented in ST1 and ST2 belonging to serogroup IVb of lineage I ($p < 0.05$). Phage vB_LmoS_293 was over-represented in ST1 belonging to serogroup IVb of lineage I ($p < 0.05$). Phage B054 was over-represented in ST204 belonging to serogroup IIa of lineage II ($p < 0.05$). The significant association of the prophages with different isolation sources showed that for the meat category showed no statistically reliable association with the source of isolation of the pathogen or establishment categories ($p > 0.05$).

DISCUSSION

The application of the MLST based approach provided important information on the distribution and grouping of genetically related *L. monocytogenes* strains in the SA food processing environment. In the present study, a total of four serogroups represented by 19 STs belonging to 11 different CCs which are a group of closely related STs were identified and classified to lineage I and lineage II using the cgMLST analysis (**Supplementary Figures 1**, **2**; Matle et al., 2020). The current study revealed that the most prevalent serogroups among SA isolates were IVb and IIa, which have also been found to be over-represented in food sources in other countries and were causative agents of more than 80% of global *L. monocytogenes* infections in human (O'Connor et al., 2010; Jamali et al., 2015; Lee et al., 2018). The most prevalent STs were ST204 and ST321 belonging to lineage II, which were mainly found in foods and food processing environments. Other common isolates were ST1 and ST2 belonging to lineage I, which are highly associated with clinical human listeriosis and demonstrate an enhanced pathogenetic potential (Maury et al., 2019; Matle et al., 2020; Palma et al., 2020). The *L. monocytogenes* strains and variants reported in the present study have been shown to be globally distributed and able to survive and persist for months and even years in food-processing environments and to be kept in contaminating food products in food processing environments for long time (Knudsen et al., 2017; Harrand et al., 2020; Matle et al., 2020).

The current study showed a paraphyletic variability of isolates ST1, ST2, and ST204, which differed by up to 41 SNPs in their core genome sequences contrasting them from ST321 isolates, which showed a significant level of conservation of their core genome with not more than two SNPs difference between them. It shows that ST1, ST2, and ST204 variants potentially are more dangerous in generating unusual genetic variants of the pathogen causing disease outbreaks. A study by Li et al. (2017) also reported a significant genetic variability of different *L. monocytogenes* isolates from foods demonstrated by SNP calling. Grouping of isolates by their core-SNP displayed a good congruence with cgMLST clustering; however, it should be noted that the strains grouped into clusters by these two methods still may show quite different pathogenicity potentials due to absent or present of different resistance and virulence genes located within chromosomes, plasmids and prophages (Li et al., 2017; Blanc et al., 2020).

Recent studies on antimicrobial resistance of *L. monocytogenes* have typically reported low levels of antimicrobial resistance in isolates from the food production environments. These reports were based on several studies performed in SA, Europe and Asia (Li et al., 2016; Matle et al., 2019; Wilson et al., 2018). The present study has reported that various antibiotic resistance genes, including *fosX*, *lin*, *mprF*, *norB*, and *mgrA*, were present in all the isolates including the strains from food processing environments. This global trend to a wider distribution of the antibiotic resistant genes in *L. monocytogenes* population was reported in a recent publication by Wilson et al. (2018). The repertoire of resistance genes typical for *L. monocytogenes* is enriching other genes, particularly by tetracycline resistance genes *tetM* and *tetS* found in a few isolates belonging to ST2 and ST9 of serogroups IVb and llb, which were isolated from butchery and retail. These genes have been detected previously in *L. monocytogenes* strains isolated from food and environmental samples (Escolar et al., 2017; Olaimat et al., 2018). Although, tetracycline is believed to be the most frequent resistance trait in *L. monocytogenes* isolated from human and food processing environments, the present study found tetracycline resistance genes only in few *L. monocytogenes* isolates, which most likely were acquired by *L. monocytogenes* with conjugative plasmids and transposons originating from *Enterococcus* or *Streptococcus* as result of horizontal gene transfer (Olaimat et al., 2018).

The key factors of adaptation and survival of *L. monocytogenes* in the food processing environments is the ability to develop resistance to QACs, such as BC, through the activity of efflux pumps encoded by *qacH* and genes of the *brcABC* cassette (Horlbog et al., 2018) and biofilm formation. The present study identified several chromosome-borne BC resistance genes, *mdrl* and *ide*, that confer tolerance to BCs in all the isolates. A study by Conficoni et al. (2016) also reported the presence of *mdrl* and *ide* in isolates from meat-processing environment that agrees with the present study. Several other genes, such as *ermC emrE, qacH,* and *bcrABC* cassette, also are responsible for tolerance to BC, a very common compound of sanitizers which is used in food industry (Kovacevic et al., 2016; Muhterem-Uyar et al., 2018; Kurpas et al., 2020). The present study identified *bcrABC* cassettes in 38% of isolates and the plasmid-borne *ermC* gene in 40% of the isolates belonging to serogroup IIa (ST121, ST204, and ST321) of lineage II, which suggests that these isolates are well adapted to survival in the food-processing environment where QACs are commonly used as sanitizers. Indeed, it was shown in the current study that these genes were over-represented in retail and butchery. Identification of drug resistance genes performed in this study may not be comprehensive due to inability to complete whole genome sequences of the isolates. Particularly, several well-known *Listeria* resistance genes such as *emrE* (Kovacevic et al., 2016) and *qacH* carried with Tn6188 (Horlbog et al., 2018) were not found when the sequences were searched against the BacMet database. Additionally, nucleotide sequences of these genes were obtained from the database of *Listeria* genes hosted at http://bigsdb.pasteur.fr/listeria/ and

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blasted against the assembled contigs of the *Listeria* isolates. This search didn't retrieve any significant matches. Either these genes were absent in the sequenced genomes, or they were fragmented in the contigs sequences. The SSI-1, which has been linked to tolerance toward acidic, bile, gastric, and salt stresses, was present in 55% of the isolates and was found to be significantly over-represented in ST9, ST204, and ST321 belonging to IIa serogroup of lineage II ($p < 0.05$). The SSI-2, which is responsible for survival under alkaline and oxidative stresses (Harter et al., 2017), was found to be significantly over-represented in ST121 from lineage II isolated from processing plant and cold store categories ($p < 0.05$). These results corroborate with a previous study (Hurley et al., 2019) showed that SSI-2 was only found in ST121, whereas SSI-1 was distributed in various STs from both lineages I and II. Co-occurrence of BC resistance genes with the stress response genes revealed by the current study implies a serious hygiene management concern. The only available data with regard to the resistance of *L. monocytogenes* to disinfectants applied in food production environments refer to genotypic resistance to QACs. Dilution in the environment and biodegradation result in QAC concentration gradients and as a result, the microorganisms are frequently exposed to sub inhibitory concentrations of QACs. The low-level resistance to QACs in *L. monocytogenes* may contribute to its environmental adaptation and persistence (Martínez-Suárez et al., 2016). Therefore, a need exists to evaluate the use of QACs disinfectants groups and the occurrences of resistance in food production facilities in SA and worldwide. Moreover, the present study also showed that ST204 and ST321 appear to have high ability of biofilm formation capacity which contribute to *L. monocytogenes* adaptation and survival in food processing environment. These results corroborate with a previous study (Pasquali et al., 2018; Stoller et al., 2019) showed that these strains have high biofilm forming capacity under specific environmental conditions. Pasquali et al. (2018) showed that the biofilm formation associated *actA* gene was truncated in all ST121 isolates. Similar trend was observed in the present study were *actA* gene was not detected in all the ST121, ST1, and ST2 isolates. This *actA* gene is known to be responsible for polymerization of actin which is important for motility of *L. monocytogenes* within the host cell as well as in the first steps of biofilm formation (Travier et al., 2013; Pasquali et al., 2018).

The pathogenic potential of a given *L. monocytogenes* strains is determined by the functionality of a large number of genes known as "virulence factors," all of which have different roles at various stages of the infection cycle. The present study assessed for the presence of 115 putative virulence markers that could be used to predict the level of potential virulence of *L. monocytogenes* isolates. It was suggested to classify isolates of this species as putatively hypo-virulent, with unknown virulence potential, and putatively hyper-virulent (Hurley et al., 2019). A total of 68 virulence markers were identified across the isolates suggesting that most virulence markers are ubiquitous across *L. monocytogenes* strains in SA. Intact LIPI-1, which harbor Prf-A dependent virulence cluster genes that are critical in the infectious cycle of *L. monocytogenes*, was mostly presented in ST1 and ST876 isolates from serogroup IVb belonging to lineage I, and also in ST9, ST204, and ST321 from serogroups IIa and IIc belonging to lineage II (**Supplementary Figure 3**; Matle et al., 2020). LIPI-1 has been reported to be the first identified pathogenicity island in *L. monocytogenes* distributed across different *L. monocytogenes* strains (Chen et al., 2020). In the present study, the LIPI-3, which is associated with enhancing the virulence capabilities of *L. monocytogenes,* was found ubiquitous in ST1 from serogroup IVb belonging to lineage I, but was also present in 2 isolates from lineage II belonging to ST204 (**Supplementary Figure 3**; Matle et al., 2020). The LIPI-3 Island carries a gene encoding the hemolytic and cytotoxic factor known as listeriolysin S, which contributes to the intracellular survival of *L. monocytogenes* in human polymorphonuclear neutrophils (Clayton et al., 2011; Hurley et al., 2019). Painset et al. (2019) and Chen et al. (2020) reported similar findings and revealed that LIPI-3 is ubiquitous to lineage I, which was also observed in the present study. Hyper-virulent strains have also been shown to possess the recently described pathogenicity island LIPI-4 that confers hyper-virulence by enhancing the invasion of the CNS and placenta (Grad and Fortune, 2016; Maury et al., 2016). The LIPI-4 Island was identified in the present study in 3.2% of the isolates belonging to serogroup IIb and IVb (ST2 and ST87) from lineage I (**Supplementary Figure 3**; Matle et al., 2020). While isolates of ST1, ST2, ST204, and ST321 generally were characterized with an abundance of virulence genes. However, the known adhesion and invasion related genes, *aut, inlF, inlJ,* and *vip*, were not found in genomes of these microorganisms which suggests a possible limitation of the invasiveness and virulence of this *L. monocytogenes* strains (Lindén et al., 2008; Martins et al., 2012). The *inlA gene* was found in more than 90% of the isolates in the current study. A recently published work on the same *L. monocytogenes* isolates revealed the truncation of the gene *inlA* due to premature stop codon, which has been associated with reduced invasiveness in some *L. monocytogenes* strains (Matle et al., 2020). This mutation may serve as a marker of hypo-virulence. Analysis of translated *inlA* protein sequence from isolates in this study identified 18 isolates, all from ST121 and ST321 of lineage II having this mutation reported for the first time for SA isolates (Matle et al., 2020).

This study suggested an important role of virulence plasmids of *L. monocytogenes* to confer increased tolerance to multiple stress condition in food processing environments. Blasting of nucleotide sequences of the found plasmids against NCBI database revealed homology of these plasmids at more than 90% similarity with the virulence plasmid N1011A, pLM5578, J1776, and pLI100 common for *L. monocytogenes* isolates (Palma et al., 2020). The majority of plasmids N1011A and pLM5578 isolates also carried *bcrABC* cassette suggesting a high correlation between the presence of these plasmids and BC tolerance in *L. monocytogenes* strains. Plasmid N1011A was associated with the most common isolates of serogroups IVb and IIa (ST204 and ST2), while pLM5578 was associated with serogroup IIa (ST121 and ST321) suggesting an importance of this plasmids in contribution to survival of hyper-virulent *L. monocytogenes* strains in the food processing environments (Kuenne et al., 2010). Furthermore, Kropac et al. (2019) showed that small

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plasmid pLMST6 which harbor *emrC* gene confers increased BC tolerance in *L. monocytogenes.* Plasmid PLMST6 was not detected in the present study. In addition to the virulence plasmids, nine prophages were distributed across the *L. monocytogenes* isolates from different sources. Analysis of the genetic repertoire of these prophages suggested their possible involvement in virulence and resistance. ST1, ST2, ST204, and ST321 displayed the highest numbers of prophages per genomes. This shows that adaptation of *L. monocytogenes* to specific environmental niches in food processing industry and short-term evolution of both distantly and closely related *L. monocytogenes* strains have been linked to the diversification of these prophages (Harrand et al., 2020; Palma et al., 2020).

CONCLUSION

The findings of this study that was based on NGS sequencing of *L. monocytogenes* isolates revealed the overall contribution of plasmids, prophages chromosomal genes toward pathogenicity and adaptation to meat processing and storage environment. The study showed that ST1, ST2, ST121, ST204, and ST321 were the most frequent among isolates and well adapted to survive in food processing environments in SA. Several hyper-virulent strains were revealed among isolates belonging to ST1, ST2, and ST204, which could present a major public health risk due to their association with meat products and food processing environments in SA, whereas hypo-virulent isolates from both lineage I and II belonged to ST121 and ST321. The information provided in this study is important for enhancing our understanding of the adaptation and survival of this pathogen in the food-processing environments. Also, the obtained results will aid in developing new approaches to assess the virulence potential of *L. monocytogenes* isolates and the efficacy of using BC disinfectants in food-processing facilities in SA.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available in the NCBI Sequence Read Archive (SRA) repository, accession number: PRJNA720786.

ETHICS STATEMENT

Ethical approval was obtained from University of Pretoria, Faculty of Natural and Agricultural Sciences Research Ethics Committee (NAS324/2020). All methods in this study were approved by University of Pretoria, Faculty of Natural and Agricultural Sciences Research Ethics Committee, and performed in accordance with the relevant guidelines and regulations.

AUTHOR CONTRIBUTIONS

RP, IM, KM, and OR: conceptualization. OR and RP: supervision. TM: writing original draft preparation, methodology, bioinformatics, and statistical analysis. RP, OR, and IM: manuscript review and editing. IM and KM: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.669287/full#supplementary-material

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CHAPTER FOUR

Chapter Four: Comparative genomics of *Listeria* species isolated from meat and FPE

Published Manuscript: Comparative Genomics of *Listeria* Species Recovered from Meat and Food processing facilities

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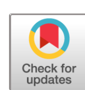
RESEARCH ARTICLE



Comparative Genomics of *Listeria* Species Recovered from Meat and Food Processing Facilities

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ABSTRACT *Listeria* species (spp.) are contaminants that can survive in food, on equipment, and on food processing premises if appropriate hygiene measures are not used. Homologous stress tolerance genes, virulence gene clusters such as the *prfA* cluster, and clusters of internalin genes that contribute to the pathogenic potential of the strains can be carried by both pathogenic and nonpathogenic *Listeria* spp. To enhance understanding of the genome evolution of virulence and virulence-associated properties, a comparative genome approach was used to analyze 41 genome sequences belonging to *L. innocua* and *L. welshimeri* isolated from food and food processing facilities. Genetic determinants responsible for disinfectant and stress tolerance were identified, including the efflux cassette *bcrABC* and *Tn6188_qac_1* disinfectant resistance determinant, and stress survival islets. These disinfectant-resistant genes were more frequently found in *L. innocua* (12%) than in *L. welshimeri* (2%). Several isolates representing the presumed nonpathogenic *L. innocua* still carried virulence-associated genes, including *LGI2*, *LGI3*, *LIPI-3*, and *LIPI-4* which were absent in all *L. welshimeri* isolates. The mobile genetic elements identified were plasmids (*pLGUG1* and *J1776*) and prophages (PHAGE_Lister_vB_LmoS_188, PHAGE_Lister_LP_030_3, PHAGE_Lister_A118, PHAGE_Lister_B054, and PHAGE_Lister_vB_LmoS_293). The results suggest that the presumed nonpathogenic isolates especially *L. innocua* can carry genes relevant to the strain's virulence and stress tolerance in the food and food processing facilities.

IMPORTANCE This study provides genomic insights into the recently expanded genus in order to gain valuable information about the evolution of the virulence and stress tolerance properties of the genus *Listeria* and the distribution of these genetic elements pertinent to the pathogenic potential across *Listeria* spp. and clonal lineages in South Africa (SA).

KEYWORDS sequence type, virulence profiles, benzalkonium chloride resistance (BC), stress tolerance, plasmids, prophages, plasmid analysis

The genus *Listeria* consists of 26 spp., of which many of the spp. have been described recently (1, 2). Of these spp., *L. monocytogenes* and *L. ivanovii* are of primary concern to humans and ruminant animals (3, 4). *Listeria* spp. can be found ubiquitously in the environment, with *L. innocua* reported as the most isolated *Listeria* spp. (3, 4). *L. monocytogenes* are regarded as food and food processing environments (FPE) contaminants. Hence, the incidence of listeriosis is mainly linked to the consumption of contaminated foods (5, 6). Contracting *L. monocytogenes* by immunocompetent individuals tends to cause gastrointestinal symptoms that are transient in nature and often disappear within a short period of time (7). Furthermore, *Listeria* spp. are often used as indicator organisms for environmental sampling and when detected provide a signal that conditions favorable for *L. monocytogenes* growth or survival could exist (8, 9). Using a broad indicator group, such as *Listeria* spp.,

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increases the chances of finding these niches and controlling them in an effective manner (8, 9).

Of recent, it has been reported that atypical hemolytic *L. innocua* can actively cross the intestinal epithelium and spread systematically to the human liver and spleen. However, it has limited virulent potential compared with virulent strains of *L. monocytogenes* (10). In addition, in rare cases, the virulent strains of *L. innocua* (11, 12), *L. ivanovii* (13, 14), *L. welshimeri* (15), *L. grayi* (16, 17), and *L. seeligeri* (18) have been reported in human clinical cases, mainly in immunosuppressed individuals (11, 12, 18). The *Listeria* spp. causing infection in humans have been reported to possess *L. monocytogenes* internalin genes (*inl*A and *inl*B) which encode proteins required for invasion of different cell types as well as incomplete pathogenicity island-1 (*LIPI*-1) comprising of *prf*A, *hly*, and *plc*A genes (12, 19). These strains possess different combinations of *L. monocytogenes LIPI*-1, *inl*A, and *inl*B genes (19). Moura et al. (10) and Rossi et al. (19) investigated the genetic analysis of virulence characters highlighting that food can be a source of potentially pathogenic strains of *Listeria* spp. belonging to spp. generally considered to be innocuous. They reported the *L. monocytogenes inlA* and *hly* virulence determinants can be harbored not only by atypical *L. innocua* strains, but also by *L. welshimeri* and *L. seeligeri* isolates. They concluded that spp. identification is not sufficient to estimate the risk associated with the presence of *Listeria* spp. in food, and both contamination prevention and the identification of contamination sources should be extended to all *Listeria* spp. (10, 19).

Traditionally, foodborne pathogens are characterized using traditional methods such as serotyping and molecular typing assays (9). However, whole-genome sequencing (WGS) has recently emerged as a powerful tool for bacterial characterization and investigation of outbreaks caused by foodborne pathogens including *Listeria* spp. (20–22). WGS-based studies querying the presumed nonpathogenic *Listeria* spp. strains isolated from food and FPE sources in South Africa (SA) are limited. Furthermore, little is known regarding their virulent potent and which sequence types (STs) are circulating among animals, food, and the food processing industry in the country. Hence, genomic insights into the recently recognized expanded diversity of the genus *Listeria* are necessary to improve our understanding of the evolution of virulence and virulence-associated properties of these potentially dangerous pathogens. This knowledge will also enhance the ability to develop and implement testing and food control procedures for the pathogenic and the presumed nonpathogenic *Listeria* spp. This study aimed to institute a comparison study in order to extend our understanding of the phylogenetic relatedness, stress resistance genes, virulence factors, and CRISPR-cas systems from the accessory genome of the presumed nonpathogenic *Listeria* spp. (*L. innocua and L. welshimeri*) isolated in SA compared with pathogenic reference *L. monocytogenes* strains and including the *L. monocytogenes* from our previous study (23).

RESULTS

General and specific genomic features of the Listeria species genomes isolated from food and food processing environments. An overview of the genetic subtypes and genome characteristics of the isolates in this study is presented in Table 1. The *L. monocytogenes* EDG-e, CLIP80459, and F2365 genomes ranged from 2.91 to 2.94 Mbp. The *L. innocua* genomes ranged from 2.79 to 3.032 Mbp. Finally, the *L. welshimeri* genome from 2.78 to 2.86 Mbp. The GC content was lowest among *L. welshimeri* (36.22% to 36.33%), followed by *L. innocua* (37.26% to 37.44%), and finally *L. monocytogenes* (37.98% to 38.06%). The total pangenome size between the three *Listeria* spp. contained 11,782 genes across the 44 *Listeria* isolates. The partitioning of genes across the pan-genome was as follows: core, 1,165; softcore, 87; shell, 1,869; and cloud, 8,661 genes. The *L. innocua* pangenome consisted of 9,346 genes, of which 2,296 genes were core to all strains. The *L. welshimeri* pangenome had 3,234 genes, and 2,492 genes were core to all strains. The *L. monocytogenes* pangenome had 3,481 genes, and 2,255 genes were core to all strains. Genome comparison is summarized in a Venn diagram (Fig. 1) and a core genome phylogenetic tree (Fig. 2).

The most common ST identified in *L. innocua* strains were ST537 (n = 22, 56%) followed by ST1085 (n = 6, 14.6%). The STs found in the *L. welshimeri* strains were ST1005, ST1084, and ST168 (Table 1; Fig. 2).

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TABLE 1 General and specific genomic features of the *Listeria* spp. genomes isolated from food and processing environments

Isolates	Sequence types (ST)	Species	Genome size (mbp)	No. of contigs	GC (%)	Sample type	Meat category	Establisment category
L0171	ST132	*L. innocua*[a]	2.98	14	37.30	Beef patties	Processed meat-beef	Retail
L1034	ST1005	*L. welshimeri*[a]	2.81	21	36.22	Russian wors chicken	Processed meat-poultry	Retail
L1036	ST1085	*L. innocua*[a]	2.88	13	37.34	beef wors	Processed meat-beef	Retail
L11	ST537	*L. innocua*[a]	2.88	33	37.46	Vienna	RTE-pork	Abattoir
L1221	ST1610	*L. innocua*[a]	3.03	42	37.29	Chicken leg Quarter	Raw-poultry	Cold store
L13	ST537	*L. innocua*[a]	2.88	24	37.47	Vienna	RTE-poultry	Processing plant
L1335	ST1480	*L. innocua*[a]	2.88	15	37.26	Chicken wing	Raw-poultry	Retail
L14	ST537	*L. innocua*[a]	2.88	17	37.47	Polony	RTE-poultry	Processing plant
L145	ST637	*L. innocua*[a]	2.90	17	37.46	Minced meat	Processed meat-beef	Butchery
L15	ST537	*L. innocua*[a]	2.88	18	37.47	Polony	RTE-poultry	Processing plant
L166	ST1085	*L. innocua*[a]	2.88	13	37.34	Beef patties	Processed meat-beef	Butchery
L18	ST537	*L. innocua*[a]	2.88	19	37.47	Wors	Processed meat-beef	Retail
L181	ST1085	*L. innocua*[a]	2.88	15	37.33	Beef wors	Processed meat-beef	Retail
L186	ST1085	*L. innocua*[a]	2.88	15	37.33	Beef mince meat	Processed meat-beef	Retail
L19	ST537	*L. innocua*[a]	2.88	20	37.47	Mince	Processed meat-beef	Retail
L21	ST537	*L. innocua*[a]	2.88	15	37.47	Wors	Processed meat-beef	Retail
L22	ST537	*L. innocua*[a]	2.88	19	37.47	Wors	Processed meat-beef	Retail
L23	ST1084	*L. welshimeri*[a]	2.78	17	36.26	Mince	Processed meat-beef	Retail
L24	ST537	*L. innocua*[a]	2.88	16	37.47	Beef minced meat	Processed meat-beef	Butchery
L241	ST599	*L. innocua*[a]	2.79	14	37.42	Pork Russian	Processed meat-pork	Butchery
L3	ST537	*L. innocua*[a]	2.88	17	37.47	Patties	Processed meat-pork	Retail
L4	ST537	*L. innocua*[a]	2.88	18	37.47	Vienna	RTE-pork	Processing plant
L505	ST1085	*L. innocua*[a]	2.92	52	37.31	Pork Russian	Processed meat-pork	Butchery
L519	ST1085	*L. innocua*[a]	2.88	17	37.33	Beef mince	Processed meat-beef	Retail
L52	ST448	*L. innocua*[a]	2.87	15	37.37	Wors	Processed meat-beef	Butchery
L57	ST537	*L. innocua*[a]	2.88	17	37.47	Mince	Processed meat-beef	Retail
L59	ST537	*L. innocua*[a]	2.88	16	37.48	Patties	Processed meat-beef	Retail
L6	ST537	*L. innocua*[a]	2.89	25	37.48	Polony	RTE-pork	Processing plant
L60	ST537	*L. innocua*[a]	2.88	18	37.48	Beef biltong	RTE-Beef	Retail
L61	ST537	*L. innocua*[a]	2.88	16	37.47	Beef biltong	RTE-beef	Retail
L62	ST168	*L. welshimeri*[a]	2.86	18	36.33	Wors	Processed meat-beef	Retail
L63	ST537	*L. innocua*[a]	2.88	22	37.47	Mince	Processed meat-beef	Retail
L64	ST537	*L. innocua*[a]	2.88	17	37.47	Beef biltong	RTE-beef	Retail
L69	ST537	*L. innocua*[a]	2.88	16	37.47	Patties	Processed meat-beef	Retail
L7	ST537	*L. innocua*[a]	2.88	17	37.46	Patties	Processed meat-beef	Processing plant
L735	ST599	*L. innocua*[a]	2.79	172	37.41	Beef mince	Processed meat-beef	Retail
L755	ST637	*L. innocua*[a]	2.88	79	37.46	Beef mince	Processed meat-beef	Butchery
L8	ST537	*L. innocua*[a]	2.88	18	37.47	Mince	Processed meat-beef	Retail
L80	ST537	*L. innocua*[a]	2.88	16	37.47	Beef biltong	RTE-beef	Butchery
L84	ST132	*L. innocua*[a]	2.96	25	37.30	Beef patties	Processed meat-beef	Butchery
L85	ST132	*L. innocua*[a]	2.96	27	37.30	Minced meat	Processed meat-beef	Butchery
EDG-e	ST35	*L. monocytogenes*[b]	2.94	1	37.98	N/A	N/A	N/A
Str.CLIP80459	ST4	*L. monocytogenes*[b]	2.91	1	38.06	N/A	N/A	N/A
Str.F2365	ST1	*L. monocytogenes*[b]	2.91	1	38.04	N/A	N/A	N/A

[a]Nonpathogenic strains.
[b]Pathogenic strains.

Phylogenetic analysis of the presumed nonpathogenic Listeria species. The phylogenetic analysis, based on 44 *Listeria* spp. core genes showed that *L. monocytogenes* (EDG-e, Str.CLIP80459, and Str.F2365) were closely related and shared a more significant ($P < 0.05$) number of genes mainly with *L. innocua* ($n = 1{,}452$) in comparison with the number of genes shared with *L. welshimeri* ($n = 1{,}214$) (Fig. 1). The number of genes shared exclusively between *L. innocua* and *L. welshimeri* was 1,222 genes from the pangenome. The phylogenetic analysis showed that *L. welshimeri* cluster was distinct from *L. monocytogenes* and *L. innocua* clusters, as displayed in Fig. 2. Within the *L. innocua* clade, the diversity within the strain in this clade is based on the presence or absence of orthologous genes (Fig. 2).

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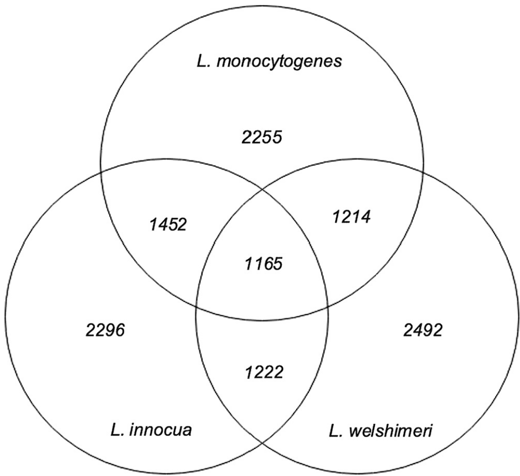

FIG 1 Pangenome analysis of the *Listeria* spp. isolates included in the current study. Grouped by spp. (*L. monocytogenes*, *L. innocua*, and *L. welshimeri*). Numbers represent gene coding loci associated with one or more species.

Stress resistance determinants in the presumed nonpathogenic Listeria species. The plasmid-borne benzalkonium chloride (BC) resistance *bcrABC* cassette and *Tn6188_qac_1* were the two disinfectant resistance determinants identified among the study isolates. The *bcrABC* cassette was only present in ST637 belonging to *L. innocua* and ST168 belonging to *L. welshimeri* (Fig. 2). The *Tn6188_qac_1* gene was only present in *L. innocua* harboring ST132. The *cadA* and *cadC* genes that confer resistance to cadmium were only identified in *L. monocytogenes* belonging to ST35 (Fig. 2).

Listeria Genomic island 2 (*LGI2*), which encodes a large arsenic resistance operon (*arsD1A1R1D2R2A2B1B2*) was present in all the *L. innocua* isolates in the present study and showed 100% identity with *LGI2* recovered from https://bigsdb.pasteur.fr/listeria/ and NCBI databases. The *Listeria* genomic island 3 (*LGI3*) was identified in *L. innocua* belonging to ST1085, ST132, ST637, and ST448 (Fig. 2). The *LGI2*-3 were only identified in *L. innocua* strains that harbored other resistance genes, and none of these islands were found in *L. welshimeri* (Fig. 2). The *L. monocytogenes* stress survival islets (SSI-1 and SSI-2) that encode resistance to stress conditions such as temperature, pH, and osmotic stress were identified in the present study. The SSI-2 was identified in all *L. innocua* isolates analyzed, whereas SSI-1 was only present in 1 isolate belonging to *L. welshimeri* ST168 (Fig. 2).

Virulence determinants in the presumed nonpathogenic Listeria species. The distribution of essential virulence *determinants* in the genus *Listeria* is shown in Fig. 2. *Listeria* pathogenicity islands *LIPI*-1 to *LIPI*-4, which contributes to pathogenesis and increase the bacteria's virulence, were investigated. The *LIPI*-1, *LIPI*-3, and *LIPI*-4 were identified in the pathogenic *Listeria* spp. (EDG-e, Str.CLIP80459, and Str.F2365) whereas complete *LIPI*-1 was not detected in *L. innocua* and *L. welshimeri* isolates (Fig. 2). The complete sequences of *LIPI*-3 consisting of eight genes (*llsA*, *llsB*, *llsD*, *llsG*, *llsH*, *llsP*, *llsX* and *llsY*) was found in all *L. innocua* ST132 isolates (Fig. 2). The complete sequences of *LIPI*-4 consisting of five genes (LM9005581_70009 to LM9005581_70014) were found in all *L. innocua* isolates, but this pathogenic island was not found in any *L. welshimeri* isolates (Fig. 2). Genes of internalin synthesis, including *inlABCEFJK*, were found exclusively in the *L. monocytogenes* genomes (Fig. 2).

Mobile genetic elements in the presumed nonpathogenic Listeria species. Two plasmids (*pLGUG1* and J1776) were identified in the present isolates and showed more than 95% identity and coverage to their corresponding plasmids from PLSDB (24) (Fig. 2). The plasmid found in *L. welshimeri* was *pLGUG1*. The J1776 plasmid was only present in *L. innocua* isolates belonging to ST1085, ST132, ST637, ST1610, and ST448. A total of five intact prophages were also identified in the present isolates and showed 95% identity and coverage against the

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FIG 2 Presence of selected stress tolerance, virulence genes, genomic islands, and plasmids among *Listeria* spp. isolates in this study. The branch length represents the evolutionary time between two nodes. The heatmap to the right of the phylogeny denotes whether the Benzalkonium chloride resistance genes, virulence factors, SSI, plasmid replicons, and prophages were present or absent in the *Listeria* genomes. The presence is indicated by blue and absence is indicated by white. The phylogeny was constructed and annotated using IQ-TREE and visualize by iToL.

PHASTER server reference sequences (25) (Fig. 2). The most common prophages identified in the present isolates were PHAGE_Lister_vB_LmoS_188 and PHAGE_Lister_LP_030_3, and both prophages were detected in all *L. innocua* ST537 isolates. The prophages detected in *L. welshimeri* were PHAGE_Lister_vB_LmoS_188 in ST1005 and PHAGE_Lister_LP_030_3 in ST1084, respectively. Other known prophages identified were PHAGE_Lister_A118, PHAGE_Lister_B054, and PHAGE_Lister_vB_LmoS_293 detected in *L. innocua* belonging to ST448, ST132, ST1610, and ST1480 isolates (Fig. 2). The plasmids and prophages identified in the presumed nonpathogenic strains were absent in *L. monocytogenes* (EDG-e, Str.CLIP80459, and Str.F2365).

The presence of CRISPR-cas systems in the presumed nonpathogenic Listeria species. The presence of CRISPR-cas systems (a system that degrades foreign genetic elements) in *Listeria* spp. were investigated. A total of three CRISPR-cas system types, including 12 different cas genes, were present in the study isolates (Table 2). The CAS-Type IIA system was present in 11 *L. innocua* strains belonging to ST537, ST637, ST132, ST599, and ST1610. The CAS-Type IIA system in *L. innocua* strains comprises of the following cas genes: Cas3_0_I, Cas3_1_I, Cas9_1_II, Cas1_0_II, Cas2_0_I-II-III, and Csn2_0_IIA (Table 2). The CAS-Type IB system was present in 15 *L. innocua* strains with cas genes Cas2_0_I-II-III-V, Cas3_0_I, Cas5_0_IB, Cas7_1_IB, Cas8a1_0_IB, Cas6_0_I-III, Cas5_0_IB, and Cas10_1_IIIB belonging to ST132, ST448, ST637, ST1085, ST637, and ST1610 (Table 2). The CAS-Type IIC system (Cas9_1_II, Cas1_0_II, Csn2_0_IIA) was only present in 1 *L. innocua* strain belonging to ST 1610. *L. welshimeri* ST1005 was found with only CAS-Type IIA system consisting of

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TABLE 2 Different types of CRISPR-cas systems types and Cas genes detected in *Listeria* spp. genomes[a]

Types	Cas genes	Isolates	Sequence types (ST)
CAS-type IIA	*Cas3_0_I, Cas3_1_I, Cas9_1_II, Cas1_0_II, Cas2_0_I-II-III, Csn2_0_IIA*	*ST537*	*L. innocua*
	Cas9_1_II, Csn2_0_IIA	*ST637, ST132, ST599, ST1610*	
CAS-type IB	*Cas2_0_I-II-III-V, Cas3_0_I, Cas5_0_IB, Cas7_1_IB, Cas8a1_0_IB, Cas6_0_I-III, Cas5_0_IB*	*ST132*	*L. innocua*
	Cas10_1_IIIB	*ST448, ST637, ST1085, ST637, ST1610*	
CAS-type IIC	*Cas9_1_II, Cas1_0_II, Csn2_0_IIA*	*ST1610*	*L. innocua*
-	Cas3_0_I, Cas3_1_I	*ST637*	*L. innocua*
-	Cas2_0_I-II-III	*ST1610*	*L. innocua*
CAS-type IIA	Cas2_0_I-II-III-V, Cas3_0_I, Cas5_0_IB, Cas7_1_IB, Cas8a1_0_IB, Cas6_0_I-III	*ST1005*	*L. welshimeri*
CAS-type IIA	-	*ST35*	EDG-e[b]
-	-	*ST4*	Str.CLIP80459[b]
-	-	*ST1*	Str.F2365[b]

[a]-, not defined.
[b]*L. monocytogenes*.

cas genes Cas2_0_I-II-III-V, Cas3_0_I, Cas5_0_IB, Cas7_1_IB, Cas8a1_0_IB, and Cas6_0_I-III (Table 2).

Comparative genomic analysis of resistance genes in the presumed nonpathogenic species and *Listeria monocytogenes* strains isolated from SA. The current presumed nonpathogenic isolates were compared with our previously published by (26, 27) pathogenic *L. monocytogenes*, isolated between 2014 and 2019 from food and FPEs. Benzalkonium chloride resistance markers, including the plasmid-borne BC resistance *bcrABC* cassette and *Tn6188_qac_1* detected in the present study (Fig. 2) were also detected in our *L. monocytogenes* genomes from the previous study (26) except for the *Tn6188_qac_1*. For SSI markers, SSI-1 which was observed only in one isolate belonging to *L. welshimeri* in the present study (Fig. 2), was also observed in the *L. monocytogenes* ($n = 86$, 55%) from our previous study (26). However, the SSI-2 which was observed in all the present *L. innocua* isolates (Fig. 2), was only observed in ($n = 11$, 7.7%) of the *L. monocytogenes* (26). For the virulence genes, the *LIPI*-1 which was observed in ($n = 16$, 7.4%) of the *L. monocytogenes* isolates (27) was absent in all the presumed nonpathogenic strains in the present study. The internalin gene family members, including *inlABCEFJK* which were present in 90% of the *L. monocytogenes* (26) isolates were absent in all the present presumed nonpathogenic strains. However, *LIPI*-3 and *LIPI*-4 which were observed in the present *L. innocua* strains (Fig. 2), were also observed in ($n = 47$, 21.7%) and ($n = 4$, 1.8%), respectively, in our previous *L. monocytogenes* (27). The plasmids and prophages identified in the presumed *nonpathogenic* strains were also observed in *L. monocytogenes* strains (26) except for the *pLGUG1* plasmid.

DISCUSSION

This study presents the intrinsic genetic attributes of pathogenic and presumed nonpathogenic *Listeria* spp. (*L. innocua* and *L. welshimeri*) isolated from FPE in SA through characterization of virulence factors, stress tolerance genes, and phylogenetic relationships between the isolates. In the present study, a total of eight and three unique STs belonging to *L. innocua* and *L. welshimeri* were detected in the selected isolates, respectively. The most common STs in the *L. innocua* were ST537, ST1085, and ST132. The STs identified in *L. welshimeri* were ST1005, ST1084, and ST168. This MLST results tally with two studies conducted in China (28) and Ireland (6) which showed the STs detected in the presumed nonpathogenic *Listeria* spp. were ST537, ST1005, ST1084, and ST168 belonging to *L. innocua* and *L. welshimeri*, respectively. The pangenome and phylogenetic analysis revealed that *L. monocytogenes* share more genes mainly with *L. innocua* ($n = 1{,}452$), than with *L. welshimeri* ($n = 1{,}214$). This is supported by the likelihood of this spp. being closer to each other in evolutionary terms (8). Similar findings were observed by den Bakker et al. (3) and Palaiodimou et al. (6) *Listeria innocua* is a nonhemolytic *Listeria* spp. found in similar environments common to *L. monocytogenes*,

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and the high number of genes shared between the two spp. compared with *L. welshimeri* could have been a result of horizontal gene transfer (29).

The ability of *Listeria* strains to adapt and survive in food and FPE is based on the development of resistance to disinfectant (QACs) such as BCs as well as biofilm production. For these bacteria to further thrive in the environment, they must tolerate many stressful conditions that are unfavorable to their survival (6, 25, 26, 30). In fact, previous studies have suggested disinfectant resistance markers such as the *bcrABC* cassette, *emrC*, *emrE*, *qacA*, *qacC*, *qacH*, and *qacE*Δ*1* determinants have been previously identified in *Listeria* spp. These markers are associated with resistance to QACs, a class of disinfectant used in the food processing facility (6, 26, 31). The disinfectant resistance determinants such as bcrABC cassette were identified in ST637, ST168 belonging to *L. innocua* and *L. welshimeri*, respectively, and *Tn6188_qac_1* in *L. innocua* harboring ST132 in the present study. These genes were also detected in our *L. monocytogenes* from our previous study (26). These known disinfectant-resistant determinants point to an associated survival and persistent contamination dynamics in the food and FPEs by these strains (ST637, ST132, and ST168) (6, 8, 29). Additionally, none of the heavy metal resistance markers (cadmium and arsenic) which provide resistance to stress conditions were observed in the present presumed nonpathogenic *Listeria* spp. isolates.

Previous studies revealed genomic islands could contain genes that improve the fitness of a strain (23, 32). The presence of these islands might lead to increased FPEs survival and pathogenic potential of the strain. Of the genomic islands identified in *Listeria* spp., *LGI1* and *LGI3* have been associated with survival and persistence in food and FPEs (23, 32), and *LGI2* potentially provides an increased survival, persistence, and virulence potential (32–34). *LGI2* was identified in all the *L. innocua* isolates, while *LGI3* was identified in *L. innocua* isolates belonging to ST1085, ST132, ST637, and ST448. The islands can potentially cause increased virulence and environmental fitness within FPEs. These isolates can maintain the arsenic and cadmium resistant determinants along with various metabolism, transport, stress resistance, transposon, and regulatory genes (32).

The SSI-1, which has been linked to tolerance toward acidic, bile, gastric, and salt stresses (35–37), was identified in a single isolate belonging to *L. welshimeri* ST168 and this SSI was also identified in 55% of our previously published *L. monocytogenes* isolates (26). The SSI-2, responsible for survival under alkaline and oxidative stresses, was observed in all *L. innocua* isolates analyzed. The *L. monocytogenes* harbored 7.7% of the SSI-2 (32) and none of SSI-2 was found in *L. welshimeri*. Palaiodimou et al. (6) reported similar findings exhibiting *L. welshimeri* and *L. monocytogenes* harbored SSI-1 compared with *L. innocua* strains, which carried SSI-2 only. The presence of SSI-2 indicates an increased ability of *L. innocua* strains to survive under alkaline and oxidative stresses in FPEs (6, 36).

The expression of the most key *Listeria* spp. virulence factors identified to date is under the control of the *prfA* virulence cluster or the *Listeria* pathogenicity island (*LIPI*) which encodes several essential proteins for intracellular survival and motility (3, 37). The *LIPI*-1 (*Prf-A* dependent virulence cluster genes that are critical in the infectious cycle of *L. monocytogenes*) and *LIPI*-2 (the former encoding the primary virulence gene locus in *L. monocytogenes* and the latter encoding virulence factors in *L. ivanovii*) were not detected in the current isolates. The *LIPI*-3 pathogenicity island encodes *Listeriolysin S* and is associated with increased *L. monocytogenes* virulence capabilities ubiquitous in ST132 belonging to *L. innocua* and was also detected in 21% of our previous *L. monocytogenes* (27). The *LIPI*-4 pathogenicity island, which is associated with *L. monocytogenes* hype-virulence by enhancing the invasion of the central nervous system and placenta, was detected in all the *L. innocua* isolates in the present study and was also found in 1.8% of the *L. monocytogenes* from our previous study (27). None of the *L. welshimeri* strains harbored *LIPI*-1 to *LIPI*-4 found in other *Listeria* spp. The findings correspond with the results of a study conducted in Ireland that did not detect any of the *LIPI* in *L. welshimeri* (6). However, the same study did not detect *LIPI*-3 in their study isolates but this genomic island was detected in the present *L. innocua* isolates and in *L. monocytogenes*. A study by Matle et al. (27) reported *LIPI*-4 was identified in all their *L. innocua* studied. The internalin gene family members (*inlABCEFJK*) were not detected in any presumed nonpathogenic *Listeria* strains analyzed in the present isolates. This internalin family

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of proteins has proven roles in virulence and host-pathogen interactions (38). Studies by Favaro et al. (12) and Rossi et al. (19) reported the nonpathogenic *Listeria* spp. Virulent strains, which caused infection in humans comprised of *inl*A, *inl*B, and incomplete *LIPI*-1 consisting of *prf*A, *hly*, and *plc*A genes. However, this was not the case in the present study as none of the genomes possess these genes. The present study conducted a complete genome analysis suggesting the presumed nonpathogenic *L. innocua* strains, especially ST132 and ST637, have more virulence factors and resistance genes than *L. welshimeri* strains isolated in SA. The presence of identical virulence and resistance genes could indicate horizontal gene transfer between the presumed nonpathogenic and pathogenic *Listeria* strains in SA.

Mobile genetic elements typically give rise to a diverse functional variation in the *Listeria* accessory genome (6, 39). Blasting of nucleotide sequences of the identified plasmids against the NCBI database revealed homology of these plasmids at more than 95% similarity with the virulence plasmid J1776 and *pLGUG1*. These plasmids are known to encode a number of genetic markers related to stress resistance (6). Some studies have shown *L. monocytogenes* can acquire resistance genes from the environment through plasmids and transposons, leading to the gradual increase in *L. Monocytogenes* resistance (6, 40). Wu et al. (28) showed *Listeria* carry multiple resistance genes *tetA*, *tetM*, *ermA*, *ermB*, *ermC*, and *aac(6')-Ib*. Generally, resistant genes *tetB* and *tetM* are frequently detected in mobile plasmids (41). This included features related to disinfectant resistance (*bcrABC* and *Tn6188_qac_1*), LGI3, and other stress resistance markers. The J1776 is a common virulent plasmid found in *L. monocytogenes* (26, 42). The *pLGUG1* specific genes encode a MATE family multidrug efflux pump in *listeria* spp. (40, 43). The present study revealed this was also common in *L. innocua* ST1085, ST132, and ST637 in the present study. Furthermore, the most common prophages detected were PHAGE_Lister_vB_LmoS_188 and PHAGE_Lister_LP_030_3 in *L. innocua* ST537. However, such prophages are primarily common in *L. monocytogenes* with adaptation and evolution roles in distant and closely related strains (23, 26, 44). Similarly, Palma et al. (23) and Orsi et al. (44) reported the differences in a prophage sequence differentiate four similar genome backbone *L. monocytogenes* isolates, indicating the importance of prophages in the differentiation of closely related *L. monocytogenes*. For example, the role of prophages in the adaptation and evolution of bacterial spp. has been reported to be associated with the acquisition of a prophage that contained a unique combination of virulence genes, which was probably generated through several recombination events (44).

Previous studies have successfully detected CRISPR repeats in *L. monocytogenes* genomes (3, 43, 45, 46). The CRISPR-Cas systems detected by previous studies were primarily type IB and IIA which is in agreement with the present work that primarily detected type IB and IIA in *Listeria* spp. Several pieces of evidence suggest at least some of the CRISPR-Cas systems detected in *L. monocytogenes* are functional with spacers that exactly match sequences of known *Listeria* phages and plasmids (45). The presence of CRISPR-Cas systems in the current *Listeria* genomes may suggest the lack of mobile genetic elements could be the result of a functional CRISPR-Cas systems in these isolates. The presence of CRISPR-Cas systems in many *Listeria* genomes suggests their suitability for biotechnological application against *L. monocytogenes*, although correct selection and adaptation of the systems will be crucial (45).

Conclusion. In order to gain an improved understanding of genome evolution in the genus *Listeria*, with particular attention to the evolution of virulence and stress tolerance, genome sequencing was conducted on isolates recovered from food and FPE. Analysis of 43 genome sequences representing two *Listeria* spp. (*L. innocua* and *L. welshimeri*) point to the fact that the presumed nonpathogenic *Listeria* spp. is unlikely to cause disease manifestation compared with pathogenic spp. due to the low occurrence of virulence factors such as *inl*A, *inl*B, and *LIPI*-1. The presence of genetic loci that have been previously associated with adaption/survival in stressful conditions was high in *L. innocua* especially *L. innocua* ST132 than in any of the *L. welshimeri* strains. The study highlights the low occurrence of important core genes could be the result of a functional CRISPR-cas system in the *Listeria* genomes.

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MATERIALS AND METHODS

Genome assembly and annotation of the bacterial strains. The isolates used in this study were collected between 2014 and 2019 and submitted at Onderstepoort Veterinary Research, SA, as part of research and/or routine diagnostics services. A total of 258 isolates from different geographical locations (processing facilities, butcheries, abattoirs, retail outlets, and cold stores) in SA were selected for sequencing. Of these isolates, 41 of these isolates were presumed to be nonpathogenic strains (*L. innocua*, 38 isolates and *L. welshimeri*, 3 isolates), and 217 isolates were pathogenic strains (*L. monocytogenes*). The *L. monocytogenes* results have been previously published by Mafina et al. (26) and Matle et al. (27). Here, we present the presumed nonpathogenic strains and three *L. monocytogenes* reference genomes which were used in this study for comparison purposes (Table 1). The presumed nonpathogenic isolates were preserved as lyophilized and subsequently revived by inoculation into brain heart infusion (BHI) broth and incubated at 37°C for 18 to 24 h. According to manufacture instructions, genomic DNA from BHI broth culture was extracted using the High Pure PCR Template Preparation Kit (Roche, Potsdam, Germany) and the quality of the DNA was assessed using Qubit flourimetric quantitation (Thermo Fischer Scientific, Waltham, MA, USA). The DNA libraries were prepared using Truseq DNA library preparation kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. The prepared libraries were loaded for 2×300 bp reads sequencing on Illumina HiSeq and Miseq Sequencing platform (Illumina, San Diego, CA, USA). The raw read quality was assessed with FastQC v.0.11.9 (47), and the adapters and low-quality reads were trimmed using Trimmomatic v.0.39 default settings (48). SPAdes v.3.13.1 program (49) was used for *de novo* assembly of each isolate. The resulting genome assembly was further quality assessed with QUAST v.5.0.2 (50) and annotated using Prokka v.1.13.7 (51).

Multilocus sequence type, pan-genome, and phylogenetic construction. Multilocus sequence type (MLST) profiles were obtained from the *Listeria* database hosted by the Pasteur Institute, France (http://bigsdb.pasteur.fr/listeria/). The MLST v.2.18.0 (52) was used to align reads against these profiles to determine the sequence types (STs) of each isolate.

The pangenome composition was extracted using Roary (53) and a core genome phylogenetic tree constructed with IQ-TREE v.1.6.6 (54). Pan-genome clusters were defined as core-genes: present in all isolates; soft-core genes: present in at least 95% of isolates; shell-genes: present between 15% and 95% of isolates; and cloud-genes: present in less than 15% of isolates. The core genome phylogenetic tree constructed using IQ-TREE was visualized using iTOL v.6.5 (55).

Genome screening for resistance markers and virulence factors. The resistance genes, *Listeria* genomic islands, and virulence factors were searched against a database created with genes retrieved from the *Listeria* database hosted by the Pasteur Institute, Paris, France (http://bigsdb.pasteur.fr/Listeria/). The plasmids database (PLSDB) (56) was searched for complete bacterial plasmid sequences. To identify putative prophages, genome assemblies were searched by the PHASTER (PHAge Search Tool – Enhanced Release) server (57). CRISPRCasFinder web server v1.1.2 (24) was used to search for CRISPR-Cas genes in the study genomes. All the genes of interest were interrogated using ABRicate v0.8.10 (https://github.com/tseemann/ABRicate) with minimum identity and coverage cut-offs values set by default settings.

Ethical statement. Ethical approval was obtained from the University of Pretoria, Faculty of Natural and Agricultural Sciences Research Ethics Committee (NAS324/2020). All methods in this study were approved by the University of Pretoria, Faculty of Natural and Agricultural Sciences Research Ethics Committee, and performed in accordance with the relevant guidelines and regulations.

Data availability. The data sets generated during and/or analyzed during the current study are available in the NCBI Sequence Read Archive (SRA) repository, BioProject ID accession number PRJNA804318 and the draft genomes are available at BioProject ID accession number PRJNA863749.

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We declare no conflict of interest.

The views presented in this article are those of the authors and do not represent an official position of the authors' affiliated institutions.

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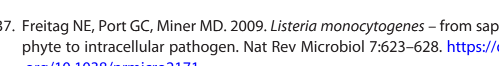

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CHAPTER FIVE

Chapter Five: mist: Multilocus In Silico Typing

Chapter Five: an online tool for the prediction of *Listeria* sequence types using next-generation sequencing raw data

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Short paper

To be submitted for Publication

mist: an online tool for the prediction of *Listeria* sequence types using Next Generation Sequencing raw data

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Keywords: Listeria, Sequence types, Online tools, Python, ST discovery, MLST

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INTRODUCTION

The *Listeria* species (spp.) belong to the *Listeriaceae* family and are widely distributed in nature (Buchanan *et al*, 2018). These species are problematic for both humans and animals, especially *L. monocytogenes* and *L. ivanovii,* respectively. *L. monocytogenes* is a food contaminant, and listeriosis is mainly associated with the consumption of these contaminated foods, mostly in immunocompetent individuals (Liu *et al*., 2013; Palaiodimou *et al*., 2021).

Molecular typing of bacterial pathogens is crucial to molecular epidemiology. The traditional methods such as multilocus sequence typing (MLST) are used for bacterial typing (Maiden *et al*., 1998). However, with the advances in next-generation sequencing (NGS) technologies, whole-genome sequencing (WGS) has recently emerged as a powerful tool for bacterial typing because it is faster and inexpensive (Kwong *et al*., 2015; Besser *et al*., 2018). WGS provides many important microbiological assays in silico, including core genome MLST (cgMLST) (Jolley *et al.*, 2012), and whole-genome MLST (wgMLST) (Cody *et al.*, 2013) and can allow for more acceptable typing resolution than the traditional MLST. Consequently, WGS data require end-users to possess a certain level of bioinformatic knowledge and skills to perform gene-based typing of bacterial isolates (quality control and genome assembly tools). Hence, accessible and easy-to-use platforms that remove these obstacles are required in clinical and public health microbiology.

Here, we describe "mist", a k-mer-based method for the rapid gene-based characterisation of bacterial isolates directly from genome sequence reads. "mist" has the advantage of being a web-based application. This application requires no installation, and the end-user just uploads their sequencing reads and submits the query. It runs on curated MLST schemes. "mist" was tested on a dataset of bacterial genome sequence reads with known ST information to validate its accuracy and performance.

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Materials and methods

Algorithm overview

The program, “mist”, uses stringMLST’s (Gupta *et al*., 2017) exact pattern matching of k-mers, a short DNA sequence of length k. This program characterises isolates by matching specific alleles for each locus to the typing scheme that shows k-mer hits. This algorithmic design allows “mist” to rapidly process sequencing read files with a small memory footprint.

Database

“mist” uses an “MLST” database file together with a profile definition file for the typing scheme along with allele sequences for each locus in the scheme retrieved from the PubMLST database (Jolley & Maiden, 2010). “mist” k-merizes each locus-specific allele sequence and records the corresponding allele and loci for each k-mer.

ST discovery

The ST discovery process run by stringMLST (Gupta *et al*., 2017) occurs in three stages. The first stage is filtering, then counting and reporting. In the filtering stage, “mist” removes a sequence read if the k-mer situated at the middle of the sequence read does not have a match in the “mist” database. If sequence reads have an exact match, they are k-merized during the counting stage. Each of these k-mers is then searched in the database, and all the matching allele and loci are recorded. “mist” processes all the input sequences and identifies the allele at each locus with the maximum counter value to generate an allelic profile and corresponding ST call (Figure 1).

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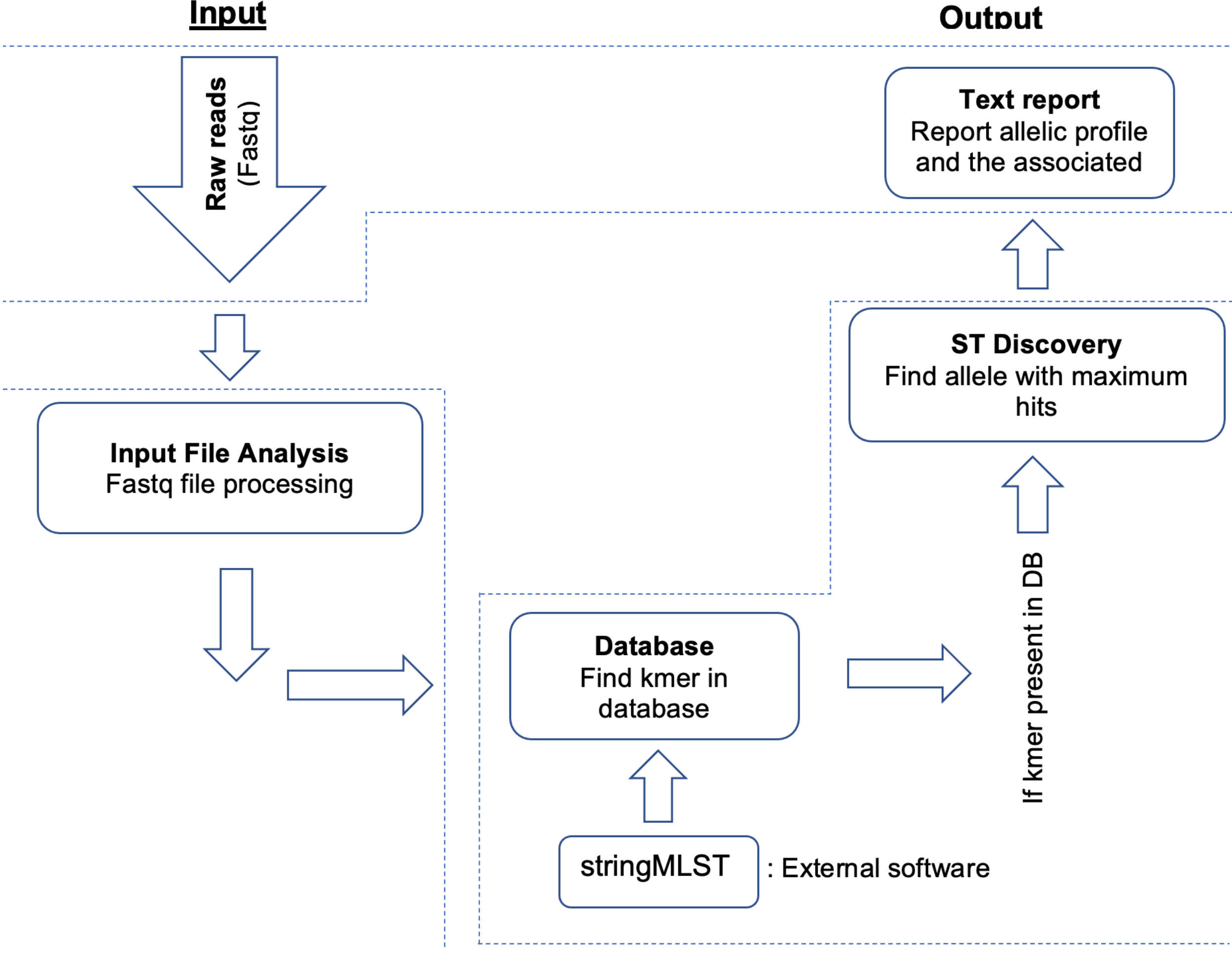

Figure 1. Schematic diagram of conceptual "mist" workflow for ST discovery routine.

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Implementation

The program "mist" was developed in Python 2.7 and implemented as an online tool at http://mist.bi.up.ac.za/. The accepted input is raw fastq paired-end read files. Results are printed in tab- or comma-separated text format.

Program Interface and Output visualization

Bioinformatics tools require users to have extensive knowledge and skills to use in research and clinical settings. In this study, we sought to develop a user-friendly platform that can be easily accessed online (http://mist.bi.up.ac.za/). The link directs users to the home page, which contains information about the tool's usage instructions (Figure 2). The user interacts with the system by simply clicking the browse button. This opens a dialog box that allows the user to select the desired input files from the local hard drives. Once selected, the files are uploaded and processed by clicking on the upload (Figure 2). If the upload was successful, the program interface displays that the upload was successful (Figure 3). The user interface provides information on the accepted input file formats. The interface also allows the user to provide their email address if they wish to be notified once the analysis has been completed. The fastq file upload menu allows the user to select paired-end read files as input. The input files must be paired-end, .gz compressed NGS read files (fastq format). "mist" outputs the results in a machine-readable tab- or comma-separated format (Figure 4B).

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Figure 2. "mist" web user interface showing the accepted input file types.

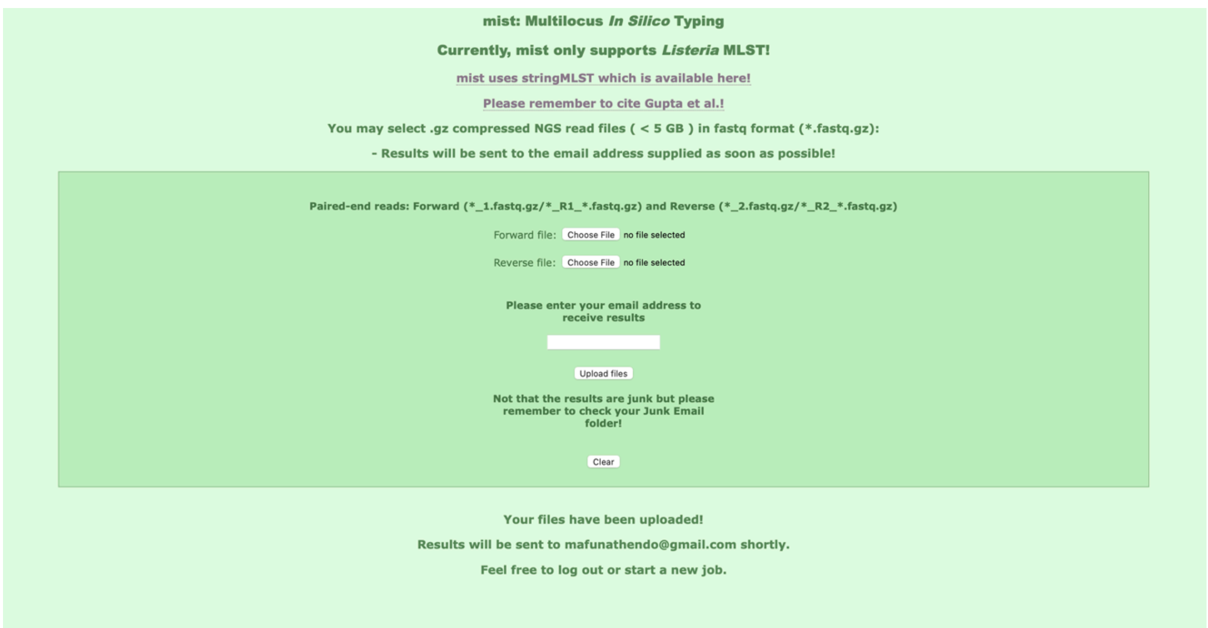

Figure 3. "mist" web user interface showing the successful upload message after the end-user has uploaded the correct input files.

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Performance evaluation

The accuracy and runtime of "mist" were evaluated in an accuracy test using a set of samples with known ST information. A total of 100 samples from *Listeria* spp. were used for these tests. "mist" correctly predicted the allelic profile and ST of all 100 samples tested with an average runtime of 30 s per sample, and 60 s upload time depending on the size of the sample. "mist" is a fast and reliable performance, together with its simple underlying algorithm, and easily accessible web platform, make it a suitable tool for genome-based *Listeria* typing by any user with or without bioinformatics knowledge and skills.

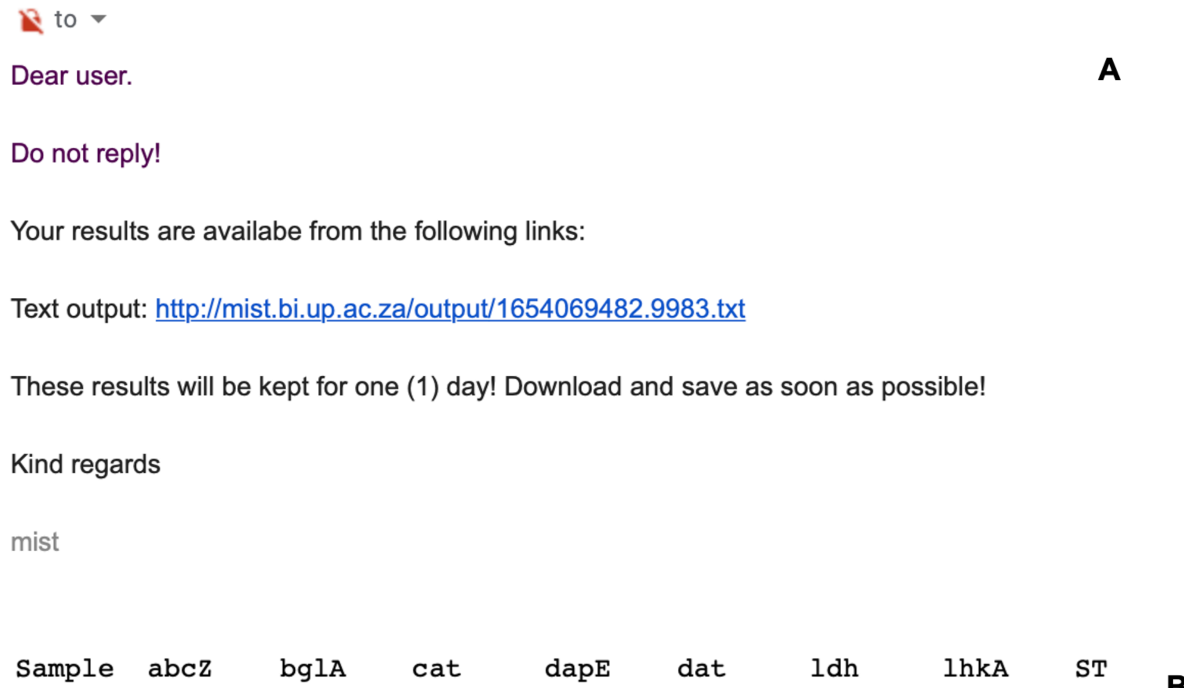

Figure 4. Screenshot of the email sent by "mist" to the end-user with the link to the MLST results (A). The email also shows how long the "mist" stores the results on the servers. Example of the results showing sample ID, seven housekeeping genes, and the corresponding ST, sent by "mist" to the end-user (B).

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Conclusion

We have developed a rapid online tool that uses .gz compressed NGS read files to predict *Listeria* sequence types. Although many alternative tools are currently available for *Listeria* ST prediction, “mist” predicts ST directly from raw DNA reads in fastq format generated by NGS sequencers. Other tools often require prior knowledge of the command line and the Linux operating system. This often deters non-specialist end-users from adopting NGS technologies and may require input files to be assembled from NGS raw reads to draft genomes. This process requires end-users to know about bioinformatics tools used to assemble such genomes. “mist” comes with a user-friendly graphical user interface and produces an easy-to-interpret output in human-readable format. This program can incorporate other common foodborne pathogens schemes for ST prediction in the future.

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CHAPTER SIX

Chapter Six: General discussion and conclusions

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6.1 Introduction

The research undertaken in this Ph.D. has revealed the importance of the implementation and use of WGS for pathogen surveillance and outbreak investigation. This chapter focuses on a discussion of the main findings of the different research chapters of this study to consolidate them with respect to the main aims/objectives of this study. To better understand the population structure and genomic diversity of *L. monocytogenes, Listeria innocua,* and *Listeria welshimeri* isolates in SA, a total of 258 isolates representing different meat and meat products were characterised using WGS. WGS is a very powerful tool for characterisation of *Listeria* as it allows an unprecedented subtyping resolution by using the entire genome of a bacterium to determine the strains diversity and virulence traits (Zeinali *et al*., 2009; Nielsen *et al*., 2017; Van Walle *et al*., 2018). The primary and universally accepted method for characterisation of *L. monocytogenes* isolates has been serotyping. This method has been used as a rapid tool for epidemiological investigations of listeriosis outbreaks and to understand the importance of certain serotypes in causing listeriosis in humans (Chenal-Francisque *et al*., 2013). The findings of the present study give a detailed overview of the genomic diversity and characteristics of *L. monocytogenes, L. innocua, L. welshimeri* in the meat value chain that can inform food safety risk-based decisions and risk assessment. WGS was used and provided a higher resolution compared to serotyping with various additional information available.

6.2 Main/Key Findings of the research

The main findings of this research study are:

1. Meat products are prone to contamination by diverse strains (multiple STs) of *L. monocytogenes* and there is a high level of diversity within each value chain control point in SA.
2. The distribution of the virulence genes, genomic islands, antimicrobial genes, plasmids, and prophages was not specific to a single ST. These virulence genes, genomic islands, antimicrobial genes, plasmids, and prophages were distributed

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across different STs and isolation sources in SA. This could present a major public health risk due to the ST's association with meat products and food processing environments.

3. The presumed non-pathogenic strains had less virulence factors and resistance genes due to restricted horizontal gene exchange caused by the presence of a functional CRISPR-cas system.
4. "mist": a rapid online tool that uses .gz compressed fastq NGS read files for sequence typing of *Listeria* spp. was successfully developed to aid in future research for end-users with or without prior knowledge about bioinformatics tools/skills.

Whole-genome sequencing as a surveillance tool for foodborne pathogens.

This research has shown that WGS provides the highest resolution for the analysis of bacterial isolates isolated from food and FPEs. The research studies undertaken as part of this PhD: "**Comparative genomics of *Listeria* species isolated from the meat processing chain in South Africa"** has shown that WGS is better for the characterisation *of L. monocytogenes* STs, serogroups, clonal complexes, and genomic determinants such as resistance genes, and virulence factors. WGS further revealed genetic relatedness of the *L. monocytogenes* strains isolated from SA using the Core-SNP Phylogenetic Clustering in Chapter 2. This study also detected non-pathogenic *Listeria* spp. including *L. welshimeri* and *L. innocua* from food and FPEs, during the national surveillance of *Listeria* spp. in SA was conducted through the WGS approach as reported in Chapter 3. Additionally, the tool listed in Chapter 4 and the frameworks outlined in this thesis can contribute to "real-time" applications of WGS.

The population structure of the Listeria spp. isolated food and food industries in SA.

The population structure and genomic diversity of *Listeria* isolate in SA were characterised by WGS. This method ensures the results are robust and reproducible. In Chapter 2 titled "**Whole genome-based characterisation of *Listeria monocytogenes* Isolates recovered from the Food Chain in South Africa**", WGS was employed to

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provide a detailed overview of the population structure of the pathogenic *Listeria* spp. (*L. monocytogenes*) which can cause listeriosis in humans. The findings of this chapter indicate that IIa and IVb serogroups and ST1, ST2, ST204, and ST321 were over-represented in our isolates. These isolates have been found to be the main cause of human listeriosis throughout the world. The findings in this study raise a lot of public health issues due to the detection of these isolates because they are associated with causing infection in humans and animals globally. This study also revealed the genomic diversity of the presumed non-pathogenic *Listeria* isolates in Chapter 3. A low occurrence of *L. welshimeri* was observed in the present study with only three isolates (ST1005, ST1084, and ST168) identified. These results indicate that *L. welshimeri* is not of immediate concern to public health and FPEs in SA. Of the presumed non-pathogenic isolates, *L. innocua* was the most dominant species isolated with ST537, ST1085, and ST132 being the most detected STs. These findings show a high genomic diversity of the *Listeria* isolates in SA with some of the strains having the potential to become virulent through the acquisition of new genetic materials because of horizontal gene transfer in the near future. However, the presence of a functional CRISPR-cas system in some isolates can prevent such acquisition of new genetic materials.

The mechanisms behind pathogenic and the presumed non-pathogenic isolate's tolerance and survival in food and food industries.

In order for both pathogenic and the presumed non-pathogenic *Listeria* isolates to survive and thrive in different environments, they need to acquire important genes responsible for biofilm formation, antimicrobial resistance, stress tolerance, and virulence. In Chapters 2 titled "**Whole genome-based characterization of *Listeria monocytogenes* Isolates recovered from the Food Chain in South Africa**" and Chapter 3 titled "**Comparative genomics of *Listeria* species recovered from meat and food processing facilities**", we investigated how these isolates manage to thrive in various niches. Our findings showed that both pathogenic and the presumed non-pathogenic *Listeria* isolates harbour genes for disinfectant resistance and stress survival which helps them survive in food industries. These isolates thrive where there is a wide range of suboptimal conditions, and accumulation of toxic heavy metals in the

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environment, as well as the disinfectants that are used as a control strategy. The ability of these isolates to develop resistance is of major concern in SA and throughout the world, as this ability makes these isolates difficult to control.

The virulence potential of pathogenic and the presumed non-pathogenic Listeria isolates.

In Chapters 2 and 3, we detected virulence factors that are expressed during the pathogenicity of *Listeria* isolates. These virulence factors are responsible for the bacterium's ability to cause disease. Important markers identified were *LIPI*-1 to *LIPI*-4 and internalins and other crucial markers indicated in Chapters 2 and 3. Our findings indicated that *L. monocytogenes* ST1, ST2, ST204, ST, 321 showed high virulence potential because most of these virulence markers were detected in their genomes. The presumed non-pathogenic isolates showed a very low occurrence of virulence factors. The *LIPI* cluster was not detected in any of the *L. welshimeri* isolates, which indicates that these isolates currently show no potential of being virulent or causing disease in humans. However, *L. innocua* harboured virulence factors, such as *LIPI,* that have been detected in virulent *L. monocytogenes* strains such as *LIPI*. This indicates that *L. innocua* has the potential to become virulent through the acquisition of virulence genes and other genetic materials through horizontal gene transfer.

Mobile genetic elements are found in Listeria isolates and what role do they play in these isolates?

Mobile genetic elements such as plasmids and prophages play a major role in providing diverse functional variation in the *Listeria* accessory genome. In the genomes of our isolates, plasmids and prophages were identified in both pathogenic and the presumed non-pathogenic isolates. In Chapter 3, the prevalence of these mobile genetic materials in the presumed non-pathogenic isolates was very poor. However, in pathogenic isolates, mobile genetic materials were detected in most of the isolates as shown in Chapter 2.

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The development of an online tool for the prediction of sequence types in Listeria.

A web-based program called "mist" was created for profiling *Listeria* STs from sequence data obtained directly from a sequencing platform. "mist" is user-friendly, easy to use, and requires no bioinformatics skills. The availability of this online application will aid future research and possible clinical detection of this pathogen. The disadvantage of mist is that you can only upload isolates individually not in bulk.

6.3 Future studies

Despite the work undertaken here, there are additional research objectives that can be investigated. Food is continuously being produced. Therefore, handle studies such as this need to become routine to stop possible outbreaks. Such studies that can be conducted in the future include:

1. "Phenotypic analysis of the presumed non-pathogenic *Listeria* spp. in the food industry".
2. "Identification and Characterisation of the CRISPR/Cas System in *Listeria* Strains from diverse sources".
3. "Comparative genomics of South African *Listeria* spp. with publicly available *Listeria* genomics from different parts of the world".
4. Expanding the current "mist" tool to incorporate other MLST schemes, serotyping, virulence profiling, and AMR.

These projects will add crucial information on the prevalence and genomic characteristics of the *Listeria* species in SA and how to control them.

6.4 Recommendations

1. The findings of this study have demonstrated that WGS offers tremendous potential for improving surveillance, source tracking, and outbreak investigations in the country. Therefore, it can be recommended that WGS should be considered the gold standard for typing of bacterial isolates from imported food products in the country.

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2. Future WGS-based studies investigating greater numbers of isolates from animal, food, and environmental sources are needed to better understand the evolution, population structure, virulence, and AMR dynamics of bacterial foodborne pathogens
3. Future work is still required to compare the WGS dataset produced from this study with clinical isolates from the same timeframe and geographic regions, to identify clusters and determine potential linkages to human foodborne cases and outbreaks, taking into consideration temporal, microbiological, and epidemiological evidence.

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6.5 Concluding remarks

Whole-genome sequencing can feasibly be implemented in public health microbiology to enhance the surveillance and control of bacterial pathogens in SA. The work in this thesis adds to the exponentially increasing body of evidence on the pathogen surveillance application of WGS in food industries. Undoubtedly, the detailed characterisation of microbial pathogens that WGS provides is far superior to traditional methods for comparing organisms and has contributed significantly to our understanding of foodborne pathogen disease transmission. Characterisation of *L. monocytogenes* isolates using WGS has provided valuable insights into strain diversity and virulence potential of isolates found in meat products consumed in SA. This study is to date, the largest to report baseline data on the presence of *Listeria* spp., including *L. monocytogenes, L. innocua,* and *L. welshimeri*'s serogroups, lineages, STs, and CCs across the meat value chain in SA. This study confirmed the heterogeneous distribution of *L. monocytogenes* CCs across different meat and meat products with evidence of over-representation of certain CCs. This study again illustrated meat products that are prone to contamination by diverse strains of *L. monocytogenes* within specific points in the value chain. This study further highlights the association of multiple STs of *Listeria* spp. to different meat products in SA and their resistance and virulence traits as well as genetic mutations of certain subgroups found in food products. This study also showed how these pathogens thrive in different environments and conditions by developing tolerance and resistance against such factors. This study also resulted in the development of a rapid online tool for sequence typing of *Listeria* spp. from NGS raw data. Therefore, the information generated here can be used in food safety risk assessment, management, and protection public health. However, these advances in sequencing and our understanding of foodborne pathogens have revealed a new set of questions to challenge conventional approaches to the pathogen surveillance and outbreak investigation. Future research directed in these areas will facilitate an even deeper knowledge of how to use the technology to prevent infectious disease transmission – a key objective in the endeavour for improved public health.

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APPENDIX 1

Population Structure of Non-ST6 *Listeria monocytogenes* Isolated in the Red Meat and Poultry Value Chain in South Africa

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Supplemental material

Article

Population Structure of Non-ST6 *Listeria monocytogenes* Isolated in the Red Meat and Poultry Value Chain in South Africa

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Keywords: *L. monocytogenes*, subtyping, serogroups, sequence types, clone complexes, pathogenic islands, lineages, inlA, sequencing

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Table S1: General and specific features of *Listeria* species genomes.

LIPI	Sample ID	Sample	ST	CC	Lineage	Serogroup			Establishment		Sample origin
LIPI-1	1223_S20_L001_R1_001	1223	1	CC1	I	IVb	Raw	Poultry	Cold store	POE	Raw-Poultry
LIPI-1	1329_S45_L001_R1_001	1329	5	CC5	I	IIb	Raw	Poultry	Retail	North West	Raw-Poultry
LIPI-1	87_S22_L001_R1_001	87	2	CC2	I	IVb	Processed meat	Beef	Butchery	Gauteng	Processed meat-Beef
LIPI-1	1214_S19_L001_R1_001	1214	155	CC155	II	IIa	Raw	Poultry	Cold store	POE	Raw-Poultry
LIPI-1	230_S37_L001_R1_001	230	155	CC155	II	IIa	Processed meat	Beef	Butchery	Gauteng	Processed meat-Beef
LIPI-1	1746A_S29_L001_R1_001	1746A	5	CC5	I	IIb	Raw	Poultry	Retail	North West	Raw-Poultry
LIPI-1	1328_S13_L001_R1_001	1328	121	CC121	II	IIa	Raw	Poultry	Retail	North West	Raw-Poultry
LIPI-1	1745_S40_L001_R1_001	1745	5	CC5	I	IIb	Raw	Poultry	Butchery	North West	Raw-Poultry
LIPI-1	1854_S1_L001_R1_001	1854	2	CC2	I	IVb	Raw	Poultry	Cold store	POE	Raw-Poultry
LIPI-1	79_S31_L001_R1_001	79	2	CC2	I	IVb	Processed meat	Beef	Retail	Gauteng	Processed meat-Beef
LIPI-1	12_S27_L001_R1_001	12	121	CC121	II	IIa	Processed meat	Beef	Retail	Gauteng	Processed meat-Beef
LIPI-1	1680_S7_L001_R1_001	1680	321	CC321	II	IIa	Raw	Poultry	Retail	North West	Raw-Poultry
LIPI-1	67_S12_L001_R1_001	67	321	CC321	II	IIa	Processed meat	Beef	Butchery	Gauteng	Processed meat-Beef
LIPI-1	1824_S9_L001_R1_001	1824	9	CC9	II	IIc	Processed meat	Beef	Butchery	North West	Processed meat-Beef
LIPI-1	L1791_CTGAAGCT-TATAGCCT_L008_R1_001	L1791	5	CC5	I	IIb	Raw	Poultry	Cold store	POE	Raw-Poultry
LIPI-1	932_S10_L001_R1_001	932	321	CC321	II	IIa	Processed meat	Beef	Butchery	Free State	Processed meat-Beef

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LIPI-3	L346_CGCTCATT-GGCTCTGA_L007_R1_001	L346	876	CC1	I	IVb	Processed meat	Beef	Butchery	Limpopo	Processed meat-Beef
LIPI-3	L0812_CGTACG_L001_R1_001	L0812	288	CC288	I	IIb	Environmental	Pork	Abattoir	Mpumalanga	Surface sample
LIPI-3	L279_TCCGGAGA-CAGGACGT_L007_R1_001	L279	1	CC1	I	IVb	Processed meat	Beef	Retail	Gauteng	Processed meat-Beef
LIPI-3	L30_CGCTCATT-CAGGACGT_L006_R1_001	L30	2	CC2	I	IVb	Processed meat	Beef	Retail	Gauteng	Processed meat-Beef
LIPI-3	L337_CGCTCATT-CCTATCCT_L007_R1_001	L337	876	CC1	I	IVb	Processed meat	Beef	Butchery	Limpopo	Processed meat-Beef
LIPI-3	1223_S20_L001_R1_001	1223	1	CC1	I	IVb	Raw	Poultry	Cold store	POE	Raw-Poultry
LIPI-3	L16_TCCGGAGA-GGCTCTGA_L006_R1_001	L16	1	CC1	I	IVb	Processed meat	Beef	Retail	Gauteng	Processed meat-Beef
LIPI-3	1898_S36_L001_R1_001	1898	1	CC1	I	IVb	Raw	Beef	Retail	Eastern Cape	Raw-Beef
LIPI-3	L1022_TCCGCGAA-CCTATCCT_L007_R1_001	L1022	1	CC1	I	IVb	Processed meat	Beef	Retail	Limpopo	Processed meat-Beef
LIPI-3	L1027_TCTCGCGC-CCTATCCT_L007_R1_001	L1027	1	CC1	I	IVb	Processed meat	Beef	Retail	Limpopo	Processed meat-Beef
LIPI-3	L835_TAATGCGC-ATAGAGGC_L007_R1_001	L835	1	CC1	I	IVb	Processed meat	Beef	Butchery	Mpumalanga	Processed meat-Beef
LIPI-3	L889_CGGCTATG-ATAGAGGC_L007_R1_001	L889	1	CC1	I	IVb	Processed meat	Beef	Butchery	Gauteng	Processed meat-Beef
LIPI-3	l702_S17_L001_R1_001	l702	3	CC3	I	IIb	RTE	Poultry	Processing plant	Free State	RTE-Poultry
LIPI-3	L232_CGCTCATT-AGGCGAAG_L008_R1_001	L232	1	CC1	I	IVb	Processed meat	Beef	Butchery	Gauteng	Processed meat-Beef
LIPI-3	L1010_TCCGCGAA-ATAGAGGC_L007_R1_001	L1010	1	CC1	I	IVb	Processed meat	Beef	Retail	Limpopo	Processed meat-Beef

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LIPI-3	L586_ATTCAGAA-ATAGAGGC_L007_R1_001	L586	204	CC204	II	IIa	Processed meat	Mixed	Retail	North West	Processed meat-Mixed
LIPI-3	L825_TAATGCGC-TATAGCCT_L007_R1_001	L825	1	CC1	I	IVb	Processed meat	Beef	Butchery	Mpumalang a	Processed meat-Beef
LIPI-3	L995_CGGCTATG-GTACTGAC_L007_R1_001	L995	876	CC1	I	IVb	Processed meat	Beef	Butchery	Limpopo	Processed meat-Beef
LIPI-3	L584_ATTCAGAA-TATAGCCT_L007_R1_001	L584	1	CC1	I	IVb	Processed meat	Beef	Retail	North West	Processed meat-Beef
LIPI-3	L245_ATTACTCG-AGGCGAAG_L007_R1_001	L245	1	CC1	I	IVb	Processed meat	Beef	Retail	Gauteng	Processed meat-Beef
LIPI-3	L165_TCTCGCGC-AGGCGAAG_L006_R1_001	L165	876	CC1	I	IVb	RTE	Beef	Retail	Gauteng	RTE-Beef
LIPI-3	L1571_GAATTCGT-TAATCTTA_L008_R1_001	L1571	1	CC1	I	IVb	Raw	Poultry	Retail	North West	Raw-Poultry
LIPI-3	L362_CGCTCATT-GTACTGAC_L007_R1_001	L362	876	CC1	I	IVb	Processed meat	Pork	Processing plant	Limpopo	Processed meat-Pork
LIPI-3	L313_CGCTCATT-ATAGAGGC_L007_R1_001	L313	1	CC1	I	IVb	Processed meat	Beef	Retail	Gauteng	Processed meat-Beef
LIPI-3	L43_ATTCAGAA-CCTATCCT_L006_R1_001	L43	876	CC1	I	IVb	Processed meat	Beef	Retail	Gauteng	Processed meat-Beef
LIPI-3	L107_CGGCTATG-AGGCGAAG_L006_R1_001	L107	3	CC3	I	IIb	Processed meat	Beef	Butchery	Gauteng	Processed meat-Beef
LIPI-3	L29_CGCTCATT-TAATCTTA_L006_R1_001	L29	2	CC2	I	IVb	Processed meat	Beef	Retail	Gauteng	Processed meat-Beef
LIPI-3	L180_AGCGATAG-TATAGCCT_L006_R1_001	L180	1	CC1	I	IVb	Processed meat	Beef	Retail	Gauteng	Processed meat-Beef
LIPI-3	1848_S46_L001_R1_001	1848	1	CC1	I	IVb	Raw	Poultry	Cold store	POE	Raw-Poultry

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LIPI-3	L249_ATTACTCG-TAATCTTA_L007_R1_001	L249	876	CC1	I	IVb	Processed meat	Beef	Retail	Gauteng	Processed meat-Beef
LIPI-3	L0811_GTTTCG_L001_R1_001	L0811	288	CC288	I	IIb	Environmental	Pork	Abattoir	Mpumalanga	Surface sample
LIPI-3	185_S2_L001_R1_001	185	1	CC1	I	IVb	Processed meat	Beef	Retail	Free State	Processed meat-Beef
LIPI-3	L221_AGCGATAG-GGCTCTGA_L006_R1_001	L221	876	CC1	I	IVb	Processed meat	Beef	Retail	Free State	Processed meat-Beef
LIPI-3	L661_ATTCAGAA-GTACTGAC_L007_R1_001	L661	1	CC1	I	IVb	Processed meat	Beef	Butchery	Limpopo	Processed meat-Beef
LIPI-3	88_S26_L001_R1_001	88	1	CC1	I	IVb	Processed meat	Beef	Butchery	Gauteng	Processed meat-Beef
LIPI-3	L25_CGCTCATT-GGCTCTGA_L006_R1_001	L25	2	CC2	I	IVb	Processed meat	Beef	Processing plant	Gauteng	Processed meat-Beef
LIPI-3	165_S21_L001_R1_001	165	1	CC1	I	IVb	RTE	Beef	Retail	Gauteng	RTE-Beef
LIPI-3	L1028_TCTCGCGC-GGCTCTGA_L007_R1_001	L1028	204	CC204	II	IIa	Processed meat	Beef	Butchery	Limpopo	Processed meat-Beef
LIPI-3	L258_TCCGGAGA-GGCTCTGA_L007_R1_001	L258	1	CC1	I	IVb	Processed meat	Beef	Retail	Gauteng	Processed meat-Beef
LIPI-3	L732_GAATTCGT-GTACTGAC_L007_R1_001	L732	1421	CC1	I	IVb	Processed meat	Beef	Retail	Mpumalanga	Processed meat-Beef
LIPI-3	177_S20_L001_R1_001	177	876	CC1	I	IVb	Processed meat	Beef	Retail	Free State	Processed meat-Beef
LIPI-3	L253_ATTACTCG-GTACTGAC_L007_R1_001	L253	876	CC1	I	IVb	Processed meat	Beef	Retail	Gauteng	Processed meat-Beef
LIPI-3	L1216_TCTCGCGC-AGGCGAAG_L007_R1_001	L1216	1	CC1	I	IVb	Raw	Poultry	Cold store	POE	Raw-Poultry

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LIPI-3	L882_CGGCTATG-TATAGCCT_L007_R1_001	L882	1	CC1	I	IVb	Processed meat	Beef	Retail	Gauteng	Processed meat-Beef
LIPI-3	L359_CGCTCATT-CAGGACGT_L007_R1_001	L359	876	CC1	I	IVb	Processed meat	Beef	Butchery	Limpopo	Processed meat-Beef
LIPI-3	L35_CGCTCATT-CCTATCCT_L008_R1_001	L35	876	CC1	I	IVb	Processed meat	Beef	Butchery	Gauteng	Processed meat-Beef
LIPI-3	L636_ATTCAGAA-TAATCTTA_L007_R1_001	L636	1	CC1	I	IVb	Processed meat	Beef	Butchery	Limpopo	Processed meat-Beef
LIPI-4	L81_TAATGCGC-AGGCGAAG_L006_R1_001	L81	2	CC2	I	IVb	RTE	Beef	Butchery	Gauteng	RTE-Beef
LIPI-4	L120_TCCGCGAA-TATAGCCT_L006_R1_001	L120	87	CC87	I	IIb	Processed meat	Beef	Butchery	Gauteng	Processed meat-Beef
LIPI-4	L36_GAGATTCC-AGGCGAAG_L006_R1_001	L36	2	CC2	I	IVb	Raw	Pork	Processing plant	Gauteng	Raw-Pork
LIPI-4	L38_GAGATTCC-CAGGACGT_L006_R1_001	L38	2	CC2	I	IVb	Processed meat	Pork	Processing plant	Gauteng	Processed meat-Pork

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Table S2: Number of isolates for each category together with the different STs and serogroups found in each category.

Cluster	Size	STs	CCs	Lineage	Serogroup	Establishment	Sample Origin
1	100	'155', '204', '378', '122', '321', '7', '9'	'CC9', 'CC7', 'CC321', 'CC204', 'CC19', 'CC155'	'II'	'IIa', 'IIc'	'Butchery', 'Retail', 'Abattoir', 'Processing plant', 'Cold store'	'Raw-Pork', 'Processed meat-Beef', 'Raw-Poultry', 'Surface sample', 'Raw-Lamb', 'Processed meat-Poultry', 'RTE-Beef', 'Processed meat-Mixed', 'Raw-Beef'
2	93	'1421', '1430', '876', '1428', '1', '288', '2', '5', '820'	'CC5', 'CC288', 'CC1', 'CC2	'I'	'IVb', 'IIb'	'Butchery', 'Retail', 'Abattoir', 'Processing plant', 'Cold store'	'Raw-Pork', 'Surface sample', 'Raw-Poultry', 'RTE-Pork', 'Processed meat-Beef', 'RTE-Beef', 'Raw-Beef', 'Processed meat-Pork'
3	14	'121'	'CC121'	'II'	'IIa'	'Butchery', 'Retail', 'Cold store'	'Raw-Pork', 'Raw-Lamb', 'Raw-Poultry', 'Processed meat-Beef', 'Processed meat-Poultry', 'RTE-Beef'
4	4	'3', '5', '87'	'CC5', 'CC87', 'CC3'	'I'	'IIb'	'Processing plant', 'Butchery'	'RTE-Poultry', 'Processed meat-Beef'
5	2	'31'	'CC31'	'II'	'IIa'	'Abattoir'	'Surface sample'
6	2	'121'	'CC121'	'II'	'IIa'	'Processing plant', 'Retail'	'Raw-Poultry', 'RTE-Pork'

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7	1	'121'	'CC121'	'II'	'IIa'	'Retail'	'Processed meat-Beef'
8	1	'321'	'CC321'	'II'	'IIa'	'Butchery'	'Processed meat-Beef'

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APPENDIX 2

Whole genome-based characterization of *Listeria monocytogenes* Isolates recovered from the Food Chain in South Africa

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Supplementary Materials

Article

Whole genome-based characterization of *Listeria monocytogenes* Isolates recovered from the Food Chain in South Africa.

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Keywords: sequence type, PCR serogroups, cgSNP, cgMLST, AMR, virulence profiles, Benzalkonium chloride resistance, stress tolerance, plasmids, prophages

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Figure S1. Distribution of *Listeria monocytogenes* serogroups, STs and CCs per study isolates. The coloured bar graph on lineage I indicate serogroups, sequence types and clonal complexes that belong to lineage I. The coloured bar graph on lineage II indicates serogroups, sequence types and clonal complexes that belong to lineage II.

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Figure S2. Core genome MLST phylogenetic analysis of *Listeria monocytogenes* isolates showing the association between MLST typing and isolation source isolates, (**A**) Meat category and (**B**) Establishment category isolates.

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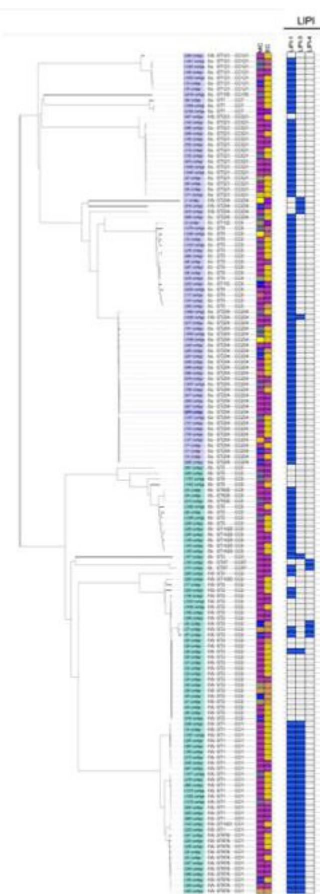

Figure S3. Core-genome MLST phylogenetic grouping of *Listeria* pathogenicity island across the *L. monocytogenes* isolates. The heat map shows the presence (blue) or absence (white) of genes involved in *L. monocytogenes* virulence. The isolation source MC and EC labelling on the heat map indicate the Meat category and Establishment category, respectively.

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Table S1: Number of isolates for each category together with the different STs and serogroups found in each category.

SampleID	Sequencetypes	ClonalComplex	Lineage	PCRSerogroup	Meat_category	Establisment_category	Number of Contigs	Largest contig	N50
L0150_contigs	ST7	CC7	II	IIa	Environmental-Pork	Abattoir	14	983105	426731
L1010_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail	18	618928	443868
L1022_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail	16	572342	418481
L1023_contigs	ST121	CC121	II	IIa	Processed_meat-Beef	Retail	25	816311	489509
L1024_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Butchery	23	592753	308200
L1026_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail	21	592753	321419
L1027_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail	22	557190	332251
L1028_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery	267	166053	49486
L104_contigs	ST1428	CC5	I	IIb	Processed_meat-Beef	Retail	14	1533492	2E+06
L1045_L007_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Butchery	18	896434	425806
L1045_L008_con	ST321	CC321	II	IIa	Processed_meat	Butchery	17	896434	4385

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tigs					-Beef				05
L105_contigs	ST1428	CC5	I	IIb	Processed_meat -Beef	Retail	12	829558	5027 09
L1059_contigs	ST7	CC7	II	IIa	Processed_meat -Beef	Retail	60	983105	4263 67
L107_contigs	ST3	CC3	I	IIb	Processed_meat -Beef	Butchery	12	1482268	5989 20
L117_contigs	ST9	CC9	II	IIc	Processed_meat -Mixed	Butchery	27	580100	4756 75
L120_contigs	ST87	CC87	I	IIb	Processed_meat -Beef	Butchery	17	724774	4818 77
L1201_contigs	ST121	CC121	II	IIa	Raw-Poultry	Coldstore	29	743095	5065 66
L1216_contigs	ST1	CC1	I	IVb	Raw-Poultry	Coldstore	15	572514	4184 81
L1225_contigs	ST121	CC121	II	IIa	Raw-Poultry	Coldstore	20	750506	5131 55
L1240_contigs	ST9	CC9	II	IIc	Raw-Beef	Coldstore	19	604796	4898 94
L1270_contigs	ST9	CC9	II	IIc	Raw-Poultry	Coldstore	339	154455	4293 8
L1331_contigs	ST204	CC204	II	IIa	Raw-Poultry	Retail	33	650260	4379 17
L1333_contigs	ST9	CC9	II	IIc	Raw-Poultry	Retail	29	1492333	5087

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									37
L1339_contigs	ST5	CC5	I	IIb	Raw-Poultry	Retail	19	1010255	5537 17
L142_contigs	ST321	CC321	II	IIa	Processed_meat -Beef	Butchery	15	945084	5446 34
L1426_contigs	ST5	CC5	I	IIb	Processed_meat -Beef	Retail	22	690051	4780 76
L1432_contigs	ST5	CC5	I	llb	Processed_meat -Beef	Retail	2387	79123	2792
L151_contigs	ST204	CC204	II	IIa	Processed_meat -Beef	Retail	371	174070	5012 5
L153_contigs	ST204	CC204	II	IIa	Processed_meat -Beef	Retail	21	531879	3966 54
L1538_contigs	ST204	CC204	II	IIa	Raw-Beef	Retail	21	541278	3909 88
L154_contigs	ST204	CC204	II	IIa	Processed_meat -Beef	Retail	23	531879	3966 54
L156_contigs	ST321	CC321	II	IIa	Processed_meat -Beef	Butchery	17	896434	4385 06
L157_contigs	ST204	CC204	II	IIa	Processed_meat -Poultry	Butchery	19	533231	3961 48
L1571_contigs	ST1	CC1	I	IVb	Raw-Poultry	Retail	15	573559	4188 49
L158_contigs	ST2	CC2	I	IVb	Processed_meat	Butchery	24	558217	3082

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					-Beef				00
L159_contigs	ST1428	CC5	I	IIb	Processed_meat -Beef	Butchery	18	1010256	5364 71
L16_contigs	ST1	CC1	I	IVb	Processed_meat -Beef	Retail	27	564721	3594 47
L160_contigs	ST121	CC121	II	IIa	Processed_meat -Beef	Butchery	24	814453	4804 98
L165_contigs	ST876	CC1	I	IVb	RTE-Beef	Retail	16	571859	5569 37
L1650_contigs	ST204	CC204	II	IIa	Raw-Poultry	Retail	25	541278	3987 08
L167_contigs	ST9	CC9	II	IIc	Processed_meat -Beef	Butchery	24	607507	4770 98
L1685_contigs	ST204	CC204	II	IIa	Raw-Poultry	Retail	24	541638	3975 11
L169_contigs	ST2	CC2	I	IVb	Processed_meat -Beef	Butchery	47	592753	3082 01
L17_contigs	ST204	CC204	II	IIa	Processed_meat -Beef	Retail	23	435626	3621 86
L1791_contigs	ST5	CC5	I	IIb	Raw-Poultry	Coldstore	2011	424567	9412
L180_contigs	ST1	CC1	I	IVb	Processed_meat -Beef	Retail	20	564597	4788 23
L1837_contigs	ST204	CC204	II	IIa	Raw-Poultry	Coldstore	15	698349	4379 18

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L2_contigs	ST122	CC9	II	IIc	Raw-Pork	Abattoir	14	908501	604955
L2018_contigs	ST155	CC155	II	IIa	Raw-Poultry	Retail	44	523705	363302
L221_contigs	ST876	CC1	I	IVb	Processed_meat-Beef	Retail	13	572059	556780
L228_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery	20	508973	390988
L229_contigs	ST1428	CC5	I	IIb	Processed_meat-Beef	Retail	15	1565556	2E+06
L230_contigs	ST1428	CC5	I	IIb	Processed_meat-Beef	Butchery	16	744186	503362
L232_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Butchery	15	572059	522281
L238_contigs	ST5	CC5	I	IIb	Processed_meat-Beef	Retail	17	536402	502857
L245_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail	22	564813	481532
L249_contigs	ST876	CC1	I	IVb	Processed_meat-Beef	Retail	16	571859	417225
L25_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Processing_plant	24	597900	321291
L250_contigs	ST204	CC204	II	IIa	RTE-Beef	Processing_plant	18	546847	390988

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L253_contigs	ST876	CC1	I	IVb	Processed_meat -Beef	Retail	15	572059	5567 80
L254_contigs	ST2	CC2	I	IVb	Processed_meat -Beef	Retail	20	600378	4250 69
L255_contigs	ST1430	CC2	I	IVb	Processed_meat -Beef	Retail	23	592799	3084 54
L257_contigs	ST204	CC204	II	IIa	RTE-Beef	Retail	21	508973	3909 88
L258_contigs	ST1	CC1	I	IVb	Processed_meat -Beef	Retail	20	564937	4810 49
L270_contigs	ST9	CC9	II	IIc	Processed_meat -Beef	Retail	17	604959	5071 59
L279_contigs	ST1	CC1	I	IVb	Processed_meat -Beef	Retail	13	683322	5644 56
L28_contigs	ST2	CC2	I	IVb	Processed_meat -Beef	Retail	27	597900	3082 00
L29_contigs	ST2	CC2	I	IVb	Processed_meat -Beef	Retail	110	475521	1316 74
L297_contigs	ST9	CC9	II	IIc	Processed_meat -Beef	Retail	22	843493	5061 02
L30_contigs	ST2	CC2	I	IVb	Processed_meat -Beef	Retail	25	475019	3214 19
L31_contigs	ST2	CC2	I	IVb	Processed_meat -Beef	Retail	25	558217	3212 95

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L313_contigs	ST1	CC1	I	IVb	Processed_meat -Beef	Retail	21	557190	332258
L32_contigs	ST2	CC2	I	IVb	Processed_meat -Beef	Retail	27	572084	308201
L33_contigs	ST2	CC2	I	IVb	Processed_meat -Beef	Retail	23	572084	415458
L337_contigs	ST876	CC1	I	IVb	Processed_meat -Beef	Butchery	22	933974	351334
L34_contigs	ST2	CC2	I	IVb	Processed_meat -Beef	Butchery	27	571629	308200
L346_contigs	ST876	CC1	I	IVb	Processed_meat -Beef	Butchery	23	573539	294252
L349_contigs	ST2	CC2	I	IVb	Processed_meat -Beef	Butchery	24	592410	321419
L35_contigs	ST876	CC1	I	IVb	Processed_meat -Beef	Butchery	27	571629	308200
L355_contigs	ST9	CC9	II	IIc	Processed_meat -Beef	Processing_plant	24	580087	475875
L359_contigs	ST876	CC1	I	IVb	Processed_meat -Beef	Butchery	22	572498	322665
L36_contigs	ST2	CC2	I	IVb	Raw-Pork	Processing_plant	25	571428	415458
L362_contigs	ST876	CC1	I	IVb	Processed_meat -Pork	Processing_plant	23	887237	359828

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L38_contigs	ST2	CC2	I	IVb	Processed_meat-Pork	Processing_plant	26	558217	308201
L40_contigs	ST2	CC2	I	IVb	RTE-Pork	Processing_plant	26	572091	308200
L41_contigs	ST2	CC2	I	IVb	Raw-Pork	Processing_plant	22	572973	415458
L43_contigs	ST876	CC1	I	IVb	Processed_meat-Beef	Retail	15	564661	480007
L44_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail	26	571429	308200
L46_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail	23	571436	415458
L47_contigs	ST321	CC321	II	IIa	Raw-Pork	Retail	18	896434	425800
L48_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Retail	17	896434	438506
L50_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Retail	18	896434	425046
L53_contigs	ST820	CC5	I	IIb	Processed_meat-Beef	Butchery	21	741144	433655
L54_contigs	ST820	CC5	I	IIb	Processed_meat-Beef	Butchery	23	741144	266903
L540_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Retail	20	508137	390988

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L549_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Retail	21	509489	390988
L552_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Butchery	17	896642	438506
L58_contigs	ST9	CC9	II	IIc	Processed_meat-Beef	Retail	22	604791	509107
L584_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail	20	564809	481048
L586_contigs	ST204	CC204	II	IIa	Processed_meat-Mixed	Retail	30	507621	390988
L612_contigs	ST820	CC5	I	IIb	Raw-Poultry	Butchery	21	741144	475542
L617_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery	19	650260	437917
L636_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Butchery	19	564597	479675
L661_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Butchery	22	994802	371848
L678_contigs	ST9	CC9	II	IIc	Raw-Pork	Retail	21	1001696	509416
L68_contigs	ST9	CC9	II	IIc	Processed_meat-Beef	Butchery	18	604953	506801
L682_contigs	ST9	CC9	II	IIc	Processed_meat-Beef	Retail	22	604797	506849

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L71_contigs	ST204	CC204	II	IIa	RTE-Beef	Butchery	25	541278	397611
L711_contigs	ST321	CC321	II	IIa	Raw-Lamb	Retail	16	896434	425700
L712_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Retail	18	896434	425050
L72_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Retail	23	541469	398710
L723_contigs	ST321	CC321	II	IIa	Raw-Poultry	Retail	16	896396	425050
L732_contigs	ST1421	CC1	I	IVb	Processed_meat-Beef	Retail	16	619834	564654
L74_contigs	ST121	CC121	II	IIa	RTE-Beef	Retail	24	816311	489571
L75_contigs	ST121	CC121	II	IIa	Processed_meat-Beef	Retail	24	775552	506943
L759_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Retail	29	698289	437918
L791_contigs	ST204	CC204	II	IIa	Raw-Poultry	Retail	22	541437	397015
L81_contigs	ST2	CC2	I	IVb	RTE-Beef	Butchery	21	558217	321419
L817_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery	16	698286	437922

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L818_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery	19	541277	397600
L825_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Butchery	13	941022	418481
L83_contigs	ST5	CC5	I	IIb	Processed_meat-Beef	Butchery	14	751209	514555
L835_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Butchery	14	683322	564667
L836_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery	19	698983	437917
L837_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery	19	698286	437918
L838_contigs	ST204	CC204	II	IIa	RTE-Beef	Butchery	24	541092	390988
L870_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery	15	698276	437917
L872_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Butchery	21	896437	425426
L882_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail	13	597495	564667
L889_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Butchery	13	941022	418481
L916_contigs	ST2	CC2	I	IVb	RTE-Beef	Retail	21	558217	321419

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L938_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Retail	13	945084	548420
L96_contigs	ST7	CC7	II	IIa	Processed_meat-Beef	Retail	14	569308	462385
L995_contigs	ST876	CC1	I	IVb	Processed_meat-Beef	Butchery	22	781766	556739
L996_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Butchery	16	945084	549460
L1_contigs	ST204	CC204	II	IVb	Raw-Beef	Abattoir	765	945084	18853
L37_contigs	ST2	CC2	I	IVb	Processed_meat-Pork	Processing_plant	463	163041	50040
L70_contigs	ST9	CC9	II	IIc	Processed_meat-Beef	Retail	22	604957	489790
L141_contigs	ST87	CC87	I	IIb	Processed_meat-Beef	Butchery	371	174070	50125
L0230_contigs	ST9	CC9	II	IIc	Processed_meat-Beef	Butchery	56	604791	475675
L369_contigs	ST121	CC121	II	IVb	Processed_meat-Beef	Retail	2398	61066	2242
L555_contigs	ST5	CC5	I	IIb	Raw-Poultry	Butchery	2046	77108	4246
L608_contigs	ST204	CC204	II	IIc	Raw-Poultry	Retail	1028	144807	8405
L815_contigs	ST204	CC204	II	IVb	Processed_meat-Beef	Retail	1472	66058	8616

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L819_contigs	ST204	CC204	II	IVb	RTE-Beef	Butchery	650	91604	19853
L937_contigs	ST321	CC321	II	IVb	Processed_meat-Beef	Retail	2485	61056	2671
L942_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Butchery	2097	61056	4152
L961_contigs	ST5	CC5	I	IIb	Processed_meat-Beef	Butchery	1963	77863	4985
L77_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail	40	475791	308194
L223_contigs	ST110	CC9	II	IIc	RTE-Pork	Butchery	49	995993	475455
L42_contigs	ST2	CC2	I	IVb	RTE-Pork	Processing_plant	541	157093	37490

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Table S2: Number of SNP per each isolate.

SampleID	Total SNPs	Num of SNPs inside recombinations	Num of SNPs outside recombinations	Num of Recombination Blocks	Bases in Recombinations	r/m	rho/theta	Genome Length	Bases in Clonal Frame
L0150_contigs	50	41	9	3	1443955	4,555555	0,333333	2750252	1422576
L0230_contigs	45	0	45	0	985510	0	0	2838591	1912593
L1010_contigs	276	229	47	24	2168503	4,87234	0,510638	2538039	665099
L1022_contigs	110	103	7	9	2160600	14,714286	1,285714	2537303	664934
L1023_contigs	16	0	16	0	1516237	0	0	2750859	1346345
L1024_contigs	19	5	14	1	2178984	0,357143	0,071429	2536964	645005
L1026_contigs	56	48	8	5	2178984	6	0,625	2537430	645039
L1027_contigs	24	8	16	1	2163745	0,5	0,0625	2547851	665640
L1028_contigs	148	136	12	8	500421	11,333333	0,666667	2831559	2355082
L1045_L007_contigs	12	12	0	2	1322574	0	0	2755759	1540718
L1045_L008_contigs	0	0	0	0	1322574	0	0	2755569	1540524
L104_contigs	0	0	0	0	2069525	0	0	2603947	761737

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L1059_contigs	0	0	0	0	1443955	0	0	2770041	1425687
L105_contigs	196	174	22	19	2069478	7,909091	0,863636	2603481	761470
L107_contigs	4616	821	3795	21	2146015	0,216337	0,005534	2595411	693049
L117_contigs	0	0	0	0	985510	0	0	2838502	1912680
L1201_contigs	0	0	0	0	1516227	0	0	2749965	1346216
L120_contigs	0	0	0	0	2153820	0	0	2583448	679323
L1216_contigs	102	41	61	7	2167542	0,672131	0,114754	2538171	660725
L1225_contigs	86	56	30	7	1516237	1,866667	0,233333	2751216	1347295
L1240_contigs	130	90	40	1	985510	2,25	0,025	2847827	1915302
L1270_contigs	0	0	0	0	985510	0	0	2836004	1904744
L1331_contigs	0	0	0	0	506704	0	0	2818753	2353530
L1333_contigs	159	90	69	1	985510	1,304348	0,014493	2851620	1917358
L1339_contigs	129	79	50	8	2060473	1,58	0,16	2603667	770289
L141_contigs	1280	920	360	32	2186208	2,555556	0,088889	2452542	650202
L1426_contigs	237	148	89	12	2069436	1,662921	0,134831	2606332	762140

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L142_contigs	0	0	0	0	1322574	0	0	2754607	1540066
L1432_contigs	0	0	0	0	1791638	0	0	2512790	976928
L151_contigs	0	0	0	0	500190	0	0	2823439	2364227
L1538_contigs	31	14	17	1	506704	0,823529	0,058824	2820747	2355105
L153_contigs	0	0	0	0	500190	0	0	2823805	2364535
L154_contigs	0	0	0	0	500190	0	0	2821719	2362542
L156_contigs	7	5	2	1	1322574	2,5	0,5	2755982	1540945
L1571_contigs	47	33	14	4	2160619	2,357143	0,285714	2537922	665214
L157_contigs	0	0	0	0	500190	0	0	2822931	2363755
L158_contigs	0	0	0	0	2174267	0	0	2528411	645902
L159_contigs	118	85	33	8	2069470	2,575758	0,242424	2603245	761331
L160_contigs	0	0	0	0	1516229	0	0	2751278	1346809
L1650_contigs	0	0	0	0	509599	0	0	2822007	2353563
L165_contigs	150	138	12	13	2165092	11,5	1,083333	2545976	666686
L167_contigs	0	0	0	0	985510	0	0	2839954	191412

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									5
L1685_contigs	41	23	18	1	506959	1,277778	0,055556	2821389	2355756
L169_contigs	14	14	0	1	2178984	0	0	2537060	645048
L16_contigs	242	204	38	19	2170543	5,368421	0,5	2546324	665197
L1791_contigs	0	0	0	0	1925942	0	0	2504683	861932
L17_contigs	0	0	0	0	509599	0	0	2821452	2352169
L180_contigs	60	49	11	4	2166989	4,454545	0,363636	2549037	665637
L1837_contigs	15	14	1	1	507603	14	1	2819392	2353307
L1_contigs	0	0	0	0	312250	0	0	2668031	2388594
L2018_contigs	14748	9279	5469	177	1454400	1,696654	0,032364	2746480	1412106
L221_contigs	0	0	0	0	2169803	0	0	2543260	658388
L223_contigs	81	40	41	2	985510	0,97561	0,04878	2851839	1917445
L228_contigs	0	0	0	0	509599	0	0	2830993	2353686
L229_contigs	0	0	0	0	2069447	0	0	2603466	761652
L230_contigs	187	149	38	16	2071121	3,921053	0,421053	2602032	761070
L232_contigs	456	375	81	20	2166758	4,62963	0,246914	2537698	661790

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L238_contigs	271	166	105	13	2069460	1,580952	0,12381	2605929	762135
L245_contigs	136	118	18	13	2163744	6,555555	0,722222	2547812	665323
L249_contigs	0	0	0	0	2161923	0	0	2542980	666073
L250_contigs	18	10	8	1	509599	1,25	0,125	2830989	2353545
L253_contigs	103	64	39	4	2172812	1,641026	0,102564	2534862	655910
L254_contigs	142	13	129	2	2161855	0,100775	0,015504	2528864	657088
L255_contigs	273	110	163	11	2163423	0,674847	0,067485	2538427	660543
L257_contigs	0	0	0	0	509599	0	0	2831087	2353515
L258_contigs	150	108	42	14	2169265	2,571429	0,333333	2546670	665537
L25_contigs	91	74	17	7	2170935	4,352941	0,411765	2528374	647805
L270_contigs	0	0	0	0	985510	0	0	2840546	1912541
L279_contigs	311	278	33	27	2167661	8,424242	0,818182	2537228	662112
L28_contigs	52	31	21	3	2161672	1,47619	0,142857	2528663	656914
L297_contigs	0	0	0	0	985510	0	0	2839943	1913242
L29_contigs	283	236	47	20	2168542	5,021276	0,425532	2541687	657789
L2_contigs	79	0	79	0	985510	0	0	2838439	1914136
L30_contigs	28	18	10	3	2163165	1,8	0,3	2527000	655331
L313_contigs	0	0	0	0	2169228	0	0	2548101	664511

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L31_contigs	0	0	0	0	2170952	0	0	2528373	647704
L32_contigs	0	0	0	0	2194500	0	0	2528631	625777
L337_contigs	300	251	49	25	2183303	5,122449	0,510204	2545626	655471
L33_contigs	33	22	11	2	2194201	2	0,181818	2528138	625529
L346_contigs	343	295	48	27	2172282	6,145833	0,5625	2547012	659270
L349_contigs	158	128	30	13	2179000	4,266667	0,433333	2536228	644502
L34_contigs	149	132	17	12	2194091	7,764706	0,705882	2528130	626578
L355_contigs	0	0	0	0	985510	0	0	2837697	1912004
L359_contigs	0	0	0	0	2175959	0	0	2547001	655557
L35_contigs	374	315	59	32	2168755	5,338983	0,542373	2543915	666231
L362_contigs	0	0	0	0	2166677	0	0	2545417	661991
L369_contigs	0	0	0	0	1491385	0	0	2466697	1235128
L36_contigs	82	80	2	5	2178542	40	2,5	2527704	643983
L37_contigs	668	521	147	34	2263179	3,544218	0,231293	2393783	536074
L38_contigs	0	0	0	0	2170926	0	0	2528035	647496
L40_contigs	0	0	0	0	2198643	0	0	2528033	625049
L41_contigs	135	114	21	8	2173828	5,428571	0,380952	2527069	647242
L42_contigs	0	0	0	0	2261521	0	0	2391017	536082
L43_contigs	127	77	50	9	2162047	1,54	0,18	2535098	663608
L44_contigs	0	0	0	0	2176123	0	0	2528288	643994

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L46_contigs	0	0	0	0	2170937	0	0	2527507	647754
L47_contigs	0	0	0	0	1322574	0	0	2755743	1540697
L48_contigs	7	7	0	1	1322574	0	0	2755821	1540715
L50_contigs	0	0	0	0	1322574	0	0	2755190	1539528
L53_contigs	144	124	20	14	2072010	6,2	0,7	2601212	761295
L540_contigs	2	0	2	0	509599	0	0	2832125	2354723
L549_contigs	5	0	5	0	509599	0	0	2830682	2353096
L54_contigs	0	0	0	0	2069609	0	0	2601268	761121
L552_contigs	1	0	1	0	1322586	0	0	2755223	1539856
L555_contigs	0	0	0	0	1454987	0	0	2525286	1282252
L584_contigs	0	0	0	0	2163747	0	0	2547247	665173
L586_contigs	33	31	2	2	509599	15,5	1	2837772	2354396
L58_contigs	0	0	0	0	985510	0	0	2840278	1913631
L608_contigs	0	0	0	0	840303	0	0	2679046	1929237

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L612_contigs	53	42	11	5	2069693	3,818182	0,454545	2600430	761363
L617_contigs	30	16	14	1	507603	1,142857	0,071429	2845218	2357837
L636_contigs	193	169	24	20	2169182	7,041667	0,833333	2548950	664921
L661_contigs	56	8	48	1	2163744	0,166667	0,020833	2547103	665428
L678_contigs	1	0	1	0	985510	0	0	2839409	1913303
L682_contigs	0	0	0	0	985510	0	0	2840831	1912966
L68_contigs	0	0	0	0	985510	0	0	2841762	1913995
L70_contigs	0	0	0	0	985510	0	0	2839822	1913398
L711_contigs	0	0	0	0	1322574	0	0	2756782	1541616
L712_contigs	15	15	0	2	1322586	0	0	2755129	1539756
L71_contigs	0	0	0	0	509599	0	0	2821984	2352746
L723_contigs	9	8	1	1	1322586	8	1	2755666	1540469
L72_contigs	18	14	4	1	509599	3,5	0,25	2822697	2353429
L732_contigs	0	0	0	0	2163684	0	0	2537828	661969

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L74_contigs	35	30	5	4	1516239	6	0,8	2750188	1345574
L759_contigs	17	14	3	1	507603	4,666667	0,333333	2818736	2352502
L75_contigs	0	0	0	0	1516229	0	0	2751893	1346836
L77_contigs	116	5	111	1	2161752	0,045045	0,009009	2538675	660296
L791_contigs	26	16	10	2	509599	1,6	0,2	2821164	2352501
L815_contigs	3279	3078	201	77	821512	15,313433	0,383085	2606990	2169886
L817_contigs	0	0	0	0	507603	0	0	2820444	2354277
L818_contigs	20	14	6	1	507603	2,333333	0,166667	2820023	2353856
L819_contigs	0	0	0	0	323942	0	0	2667924	2378980
L81_contigs	0	0	0	0	2240198	0	0	2392817	551103
L825_contigs	0	0	0	0	2160692	0	0	2536460	665179
L835_contigs	0	0	0	0	2166131	0	0	2538962	662639
L836_contigs	0	0	0	0	507603	0	0	2820444	2353112
L837_contigs	0	0	0	0	507603	0	0	2817835	2351613

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L838_contigs	21	6	15	1	500312	0,4	0,066667	2818766	2359918
L83_contigs	202	149	53	14	2064237	2,811321	0,264151	2606667	770360
L870_contigs	1	0	1	0	507603	0	0	2819481	2353400
L872_contigs	0	0	0	0	1322586	0	0	2755561	1540391
L882_contigs	235	191	44	22	2167511	4,340909	0,5	2537460	661049
L889_contigs	0	0	0	0	2160692	0	0	2536455	665186
L916_contigs	75	65	10	7	2161675	6,5	0,7	2528721	656730
L937_contig	0	0	0	0	1321348	0	0	2497939	1394376
L938_contigs	0	0	0	0	1322574	0	0	2755630	1540658
L942_contigs	16153	13981	2172	186	2311602	6,436924	0,085635	2475207	619219
L961_contigs	0	0	0	0	924317	0	0	2535699	1745907
L96_contigs	110	19	91	3	1443955	0,208791	0,032967	2745187	1421746
L995_contigs	0	0	0	0	2161965	0	0	2545830	666473
L996_contigs	11	11	0	2	1322574	0	0	2755043	1540457

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Table S3: Biofilm formation associated genes.

	Listeria monocytogenes MLST				**isolation sources**			**Biofilm formation associated genes**							
SampleID	**Sequencetypes**	**Clonal Complex**	**Lineage**	**PCRSerogroup**	**Meat_category**	**Establisment_category**		**recO**	**inlL**	**prfA**	**actA**	**lmo0673**	**bapL**	**lmo2504**	**luxS**
L0150_contigs	ST7	CC7	II	IIa	Environmental-Pork	Abattoir		+	+	+	+	-	-	+	+
L1010_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L1022_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L1023_contigs	ST121	CC121	II	IIa	Processed_meat-Beef	Retail		+	-	+	-	-	+	+	+
L1024_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L1026_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L1027_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L1028_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	-
L104_con	ST142	CC5	I	IIb	Processed_meat-	Retail		-	-	+	-	+	-	+	+

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tigs	8				Beef										
L1045_L 007_conti gs	ST321	CC321	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	+
L1045_L 008_conti gs	ST321	CC321	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	+
L105_con tigs	ST142 8	CC5	I	IIb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L1059_co ntigs	ST7	CC7	II	IIa	Processed_meat-Beef	Retail		+	+	+	+	-	-	+	+
L107_con tigs	ST3	CC3	I	IIb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L117_con tigs	ST9	CC9	II	IIc	Processed_meat-Mixed	Butchery		+	+	+	+	-	-	-	+
L120_con tigs	ST87	CC87	I	IIb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L1201_co ntigs	ST121	CC121	II	IIa	Raw-Poultry	Coldstore		+	-	+	-	-	+	+	+
L1216_co ntigs	ST1	CC1	I	IVb	Raw-Poultry	Coldstore		-	-	+	-	+	-	+	+
L1225_co ntigs	ST121	CC121	II	IIa	Raw-Poultry	Coldstore		+	+	+	+	-	-	+	+
L1240_co ntigs	ST9	CC9	II	IIc	Raw-Beef	Coldstore		+	+	+	+	-	-	-	+

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L1270_contigs	ST9	CC9	II	IIc	Raw-Poultry	Coldstore		+	+	+	+	-	-	-	+
L1331_contigs	ST204	CC204	II	IIa	Raw-Poultry	Retail		+	+	+	+	-	-	+	+
L1333_contigs	ST9	CC9	II	IIc	Raw-Poultry	Retail		+	+	+	+	-	-	-	+
L1339_contigs	ST5	CC5	I	IIb	Raw-Poultry	Retail		-	-	+	-	+	-	-	+
L142_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	+
L1426_contigs	ST5	CC5	I	IIb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L151_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Retail		+	+	+	+	-	-	+	+
L153_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Retail		+	+	+	+	-	-	+	+
L1538_contigs	ST204	CC204	II	IIa	Raw-Beef	Retail		+	+	+	+	-	-	+	+
L154_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Retail		+	+	+	+	-	-	+	+
L156_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	+
L157_contigs	ST204	CC204	II	IIa	Processed_meat-Poultry	Butchery		+	+	+	+	-	-	+	+

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L1571_contigs	ST1	CC1	I	IVb	Raw-Poultry	Retail		-	-	+	-	+	-	+	+
L158_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L159_contigs	ST1428	CC5	I	IIb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L16_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L160_contigs	ST121	CC121	II	IIa	Processed_meat-Beef	Butchery		+	-	+	-	-	+	+	+
L165_contigs	ST876	CC1	I	IVb	RTE-Beef	Retail		-	-	+	-	+	-	+	+
L1650_contigs	ST204	CC204	II	IIa	Raw-Poultry	Retail		+	+	+	+	-	-	+	+
L167_contigs	ST9	CC9	II	IIc	Processed_meat-Beef	Butchery		+	+	+	+	-	-	-	+
L1685_contigs	ST204	CC204	II	IIa	Raw-Poultry	Retail		+	+	+	+	-	-	+	+
L169_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L17_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Retail		+	+	+	+	-	-	+	+
L180_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+

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L1837_contigs	ST204	CC204	II	IIa	Raw-Poultry	Coldstore		+	+	+	+	-	-	+	+
L2_contigs	ST122	CC9	II	IIc	Raw-Pork	Abattoir		+	+	+	+	-	-	-	+
L2018_contigs	ST155	CC155	II	IIa	Raw-Poultry	Retail		-	+	+	+	-	-	+	+
L221_contigs	ST876	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L228_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	+
L229_contigs	ST1428	CC5	I	IIb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L230_contigs	ST1428	CC5	I	IIb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L232_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L238_contigs	ST5	CC5	I	IIb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L245_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L249_contigs	ST876	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L25_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Processing plant		-	-	+	-	+	-	+	+

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L250_contigs	ST204	CC204	II	IIa	RTE-Beef	Processing_plant		+	+	+	+	-	-	+	+
L253_contigs	ST876	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L254_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L255_contigs	ST1430	CC2	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L257_contigs	ST204	CC204	II	IIa	RTE-Beef	Retail		+	+	+	+	+	-	+	+
L258_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L270_contigs	ST9	CC9	II	IIc	Processed_meat-Beef	Retail		+	+	+	+	-	-	-	+
L279_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L28_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L29_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L297_contigs	ST9	CC9	II	IIc	Processed_meat-Beef	Retail		+	+	+	+	-	-	-	+
L30_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+

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L31_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L313_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L32_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L33_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L337_contigs	ST876	CC1	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L34_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L346_contigs	ST876	CC1	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L349_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L35_contigs	ST876	CC1	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L355_contigs	ST9	CC9	II	IIc	Processed_meat-Beef	Processing_plant		+	+	+	+	-	-	-	+
L359_contigs	ST876	CC1	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L36_contigs	ST2	CC2	I	IVb	Raw-Pork	Processing_plant		-	-	+	-	+	-	+	+

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L362_contigs	ST876	CC1	I	IVb	Processed_meat-Pork	Processing_plant		-	-	+	-	+	-	+	+
L38_contigs	ST2	CC2	I	IVb	Processed_meat-Pork	Processing_plant		-	-	+	-	+	-	+	+
L40_contigs	ST2	CC2	I	IVb	RTE-Pork	Processing_plant		-	-	+	-	+	-	+	+
L41_contigs	ST2	CC2	I	IVb	Raw-Pork	Processing_plant		-	-	+	-	+	-	+	+
L43_contigs	ST876	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L44_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L46_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L47_contigs	ST321	CC321	II	IIa	Raw-Pork	Retail		+	+	+	+	-	-	+	+
L48_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Retail		+	+	+	+	-	-	+	+
L50_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Retail		+	+	+	+	-	-	+	+
L53_contigs	ST820	CC5	I	IIb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L54_contigs	ST820	CC5	I	IIb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+

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L540_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Retail		+	+	+	+	-	-	+	+
L549_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Retail		+	+	+	+	-	-	+	+
L552_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	+
L58_contigs	ST9	CC9	II	IIc	Processed_meat-Beef	Retail		+	+	+	+	-	-	-	+
L584_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L586_contigs	ST204	CC204	II	IIa	Processed_meat-Mixed	Retail		+	+	+	+	-	-	+	+
L612_contigs	ST820	CC5	I	IIb	Raw-Poultry	Butchery		-	-	+	-	+	-	+	+
L617_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	+
L636_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L661_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L678_contigs	ST9	CC9	II	IIc	Raw-Pork	Retail		+	+	+	+	-	-	-	+
L68_contigs	ST9	CC9	II	IIc	Processed_meat-Beef	Butchery		+	+	+	+	-	-	-	+

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L682_contigs	ST9	CC9	II	IIc	Processed_meat-Beef	Retail		+	+	+	+	-	-	-	+
L71_contigs	ST204	CC204	II	IIa	RTE-Beef	Butchery		+	+	+	+	-	-	+	+
L711_contigs	ST321	CC321	II	IIa	Raw-Lamb	Retail		+	+	+	+	-	-	+	+
L712_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Retail		+	+	+	+	-	-	+	+
L72_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Retail		+	+	+	+	-	-	+	+
L723_contigs	ST321	CC321	II	IIa	Raw-Poultry	Retail		+	+	+	+	-	-	+	+
L732_contigs	ST1421	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L74_contigs	ST121	CC121	II	IIa	RTE-Beef	Retail		+	-	+	-	-	+	+	+
L75_contigs	ST121	CC121	II	IIa	Processed_meat-Beef	Retail		+	-	+	-	-	+	+	+
L759_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Retail		+	+	+	+	-	-	+	+
L791_contigs	ST204	CC204	II	IIa	Raw-Poultry	Retail		+	+	+	+	-	-	+	+
L81_contigs	ST2	CC2	I	IVb	RTE-Beef	Butchery		+	-	+	+	+	-	+	+

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L817_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	+
L818_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	+
L825_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L83_contigs	ST5	CC5	I	IIb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L835_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L836_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	+
L837_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	+
L838_contigs	ST204	CC204	II	IIa	RTE-Beef	Butchery		+	+	+	+	-	-	+	+
L870_contigs	ST204	CC204	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	+
L872_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	+
L882_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L889_contigs	ST1	CC1	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+

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L916_contigs	ST2	CC2	I	IVb	RTE-Beef	Retail		-	-	+	-	+	-	+	+
L938_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Retail		+	+	+	+	-	-	+	+
L96_contigs	ST7	CC7	II	IIa	Processed_meat-Beef	Retail		+	+	+	+	-	-	+	+
L995_contigs	ST876	CC1	I	IVb	Processed_meat-Beef	Butchery		-	-	+	-	+	-	+	+
L996_contigs	ST321	CC321	II	IIa	Processed_meat-Beef	Butchery		+	+	+	+	-	-	+	+
L1_contigs	ST204	CC204	II	IVb	Raw-Beef	Abattoir		+	+	+	+	-	-	+	+
L37_contigs	ST2	CC2	I	IVb	Processed_meat-Pork	Processing_plant		+	-	+	+	+	-	+	+
L70_contigs	ST9	CC9	II	IIc	Processed_meat-Beef	Retail		+	+	+	+	-	-	-	+
L141_contigs	ST87	CC87	I	IIb	Processed_meat-Beef	Butchery		+	-	+	+	+	-	+	+
L0230_contigs	ST9	CC9	II	IIc	Processed_meat-Beef	Butchery		+	+	+	+	-	-	-	+
L608_contigs	ST204	CC204	II	IIc	Raw-Poultry	Retail		+	+	+	+	-	-	+	+
L819_contigs	ST204	CC204	II	IVb	RTE-Beef	Butchery		+	+	+	+	-	-	+	+

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L77_contigs	ST2	CC2	I	IVb	Processed_meat-Beef	Retail		-	-	+	-	+	-	+	+
L223_contigs	ST110	CC9	II	IIc	RTE-Pork	Butchery		+	+	+	+	-	-	-	+
L42_contigs	ST2	CC2	I	IVb	RTE-Pork	Processing_plant		+	-	-	-	+	-	+	+

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APPENDIX 3

Comparative genomics of *Listeria* species recovered from meat and food processing environment

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Supplementary Materials

Article

Comparative genomics of *Listeria* species recovered from meat and food processing environment

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Keywords: sequence type, AMR, virulence profiles, Benzalkonium chloride resistance (BC), stress tolerance, plasmids, prophages

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Table S1: Table 1: general and specific features of *Listeria* spp. genomes.

Sample Number	***Sequence types***	***Species***	**Sample Type**	**Meat category**	**Food Animal spp**	**Establisment category**	**Province**
L3	ST537	*L. innocua*	Patties (after processing)	Processed meat	Pork	Retail	Gauteng
L4	ST537	*L. innocua*	Vienna (after processing)	RTE	Pork	Processing plant	Gauteng
L6	ST537	*L. innocua*	Polony (after processing)	RTE	Pork	Processing plant	Gauteng
L7	ST537	*L. innocua*	Patties (after processing)	Processed meat	Beef	Processing plant	Gauteng
L8	ST537	*L. innocua*	Mince	Processed meat	Beef	Retail	Gauteng
L11	ST537	*L. innocua*	Vienna (after processing)	RTE	Pork	Abattoir	Gauteng
L13	ST537	*L. innocua*	Vienna (after processing)	RTE	Poultry	Processing plant	Gauteng
L14	ST537	*L. innocua*	Polony (after processing)	RTE	Poultry	Processing plant	Gauteng
L15	ST537	*L. innocua*	Polony	RTE	Poultry	Processing plant	Gauteng
L18	ST537	*L. innocua*	Wors	Processed meat	Beef	Retail	Gauteng
L19	ST537	*L. innocua*	Mince	Processed meat	Beef	Retail	Gauteng
L21	ST537	*L. innocua*	Wors	Processed meat	Beef	Retail	Gauteng
L22	ST537	*L. innocua*	Wors	Processed meat	Beef	Retail	Gauteng
L23	ST1084	*L. welshimeri*	Mince	Processed meat	Beef	Retail	Gauteng
L24	ST537	*L. innocua*	Beef minced meat	Processed meat	Beef	Butchery	Gauteng
L52	ST448	*L. innocua*	Wors	Processed meat	Beef	Butchery	Gauteng
L57	ST537	*L. innocua*	Mince	Processed meat	Beef	Retail	Gauteng
L59	ST537	*L. innocua*	Patties	Processed meat	Beef	Retail	Gauteng
L60	ST537	*L. innocua*	Beef biltong	RTE	Beef	Retail	Gauteng
L61	ST537	*L. innocua*	Biltong	RTE	Beef	Retail	Gauteng
L62	ST168	*L. welshimeri*	Wors	Processed meat	Beef	Retail	Gauteng

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L63	ST537	*L. innocua*	Mince	Processed meat	Beef	Retail	Gauteng
L64	ST537	*L. innocua*	Beef biltong	RTE	Beef	Retail	Gauteng
L69	ST537	*L. innocua*	Patties	Processed meat	Beef	Retail	Gauteng
L80	ST537	*L. innocua*	Biltong	RTE	Beef	Butchery	Gauteng
L84	ST132	*L. innocua*	Beef patties	Processed meat	Beef	Butchery	Gauteng
L85	ST132	*L. innocua*	Minced meat	Processed meat	Beef	Butchery	Gauteng
L145	ST637	*L. innocua*	Minced meat	Processed meat	Beef	Butchery	Gauteng
L166	ST1085	*L. innocua*	Beef patties	Processed meat	Beef	Butchery	Gauteng
L171	ST132	*L. innocua*	Beef patties	Processed meat	Beef	Retail	Gauteng
L181	ST1085	*L. innocua*	Beef wors	Processed meat	Beef	Retail	Free State
L186	ST1085	*L. innocua*	Beef mince meat	Processed meat	Beef	Retail	Free State
L241	ST599	*L. innocua*	Pork russian	Processed meat	Pork	Butchery	Gauteng
L505	ST1085	*L. innocua*	Beef wors	Processed meat	Beef	Retail	North west
L519	ST1085	*L. innocua*	Beef mince	Processed meat	Beef	Retail	North west
L735	ST599	*L. innocua*	Beef mince	Processed meat	Beef	Retail	Mpumalanga
L755	ST637	*L. innocua*	Beef mince	Processed meat	Beef	Butchery	Mpumalanga
L1034	ST1005	*L. welshimeri*	Russian wors chicken	Processed meat	Poultry	Retail	Limpopo
L1036	ST1085	*L. innocua*	beef wors	Processed meat	Beef	Retail	Limpopo
L1221	ST1610	*L. innocua*	Chicken leg Quarter	Raw	Poultry	Cold store	POE
L1335	ST1480	*L. innocua*	Chicken wing	Raw	Poultry	Retail	North west

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APPENDIX 4

List of some of the commands and parameters used in this study

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```
# QUALITY CHECK
# FastQC:

$ fastqc listeria/*.fastq.gz
```

```
# RAW SEQUENCE TRIMMING AND ADAPTER CLIPPING

#For paired-end reads:
#Trimmomatic

$ for R1 in listeria/*1.fastq.gz
do
        R2=${R1/1.fastq.gz/2.fastq.gz}
        java -jar /apps/trimmomatic-0.36/trimmomatic-0.36.jar PE -threads 12 -phred33 \
        $R1 $R2 trim_paired_${R1##listeria/} trim_unpaired_${R1##listeria/} \
        trim_paired_${R2##listeria/} trim_unpaired_${R2##listeria/} \
        ILLUMINACLIP:/apps/trimmomatic-0.36/adapters/TruSeq2-PE.fa:2:30:10 \
        LEADING:15 TRAILING:15 SLIDINGWINDOW:4:15 MINLEN:35 >> trimmomatic.cmds
done
```

```
# In silico MLST

$ mlst --scheme Listeria monocytogenes listeria_output/contigs.fasta > mlst.tab

$ stringMLST.py --predict -d ./listeria -k 35 -P LM > mlst.tab
```

```
# GENOME ASSEMBLY with SPAdes:

$ for R1 in listeria/*1.fastq.gz
do
        R2=${R1/1.fastq.gz/2.fastq.gz}
        out=${R1##listeria/}
        spades.py --careful -1 $R1 -2 $R2 -o listeria_output/${out%%1.fastq.gz}
done
```

```
# Basic assembly quality check with Quast

for R1 in listeria_output/*.fna
do
        out=${R1##listeria_output/}
        quast.py $R1 -o quast_output/${out%%.fna}
done
```


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```
# REMAPPING READS BACK TO DRAFT ASSEMBLY with snippy
(see https://github.com/tseemann/snippy)

# Read alignment with Snippy
$ snippy --cpus --force --outdir sample1/sample1 --ref
ref.gbk --R1 sample1/R1.fq.gz --R2 sample1/R2.fq.gz

#Generate core genome SNP alignment and pseudo-whole-genome alignment

$ snippy-core sample1/sample1 sample2/sample2 ... sampleN/sampleN
```

```
# Draft genome auto-annotation with Prokka

$ for R1 in listeria_output/*.fasta
do
        out=${R1##listeria_output/}
        prokka ${R1} --outdir listeriat_annotated/${out%%_.fasta} --prefix ${out%%_.fasta}
done
```

```
# Pan-genome analysis with Roary

$ roary -f roary -v -p */prokka/*.gff
```

```
# Approximate maximum likelihood tree estimation with FastTree

$ FastTree -nt -gtr < core.aln > tree.newick
```

```
# In silico detection of acquired resistance mechanisms with abricate
#database used:
% abricate --list

DATABASE       SEQUENCES  DBTYPE  DATE
argannot       1749       nucl    2022-Jun-10
card           2241       nucl    2022-Jun-10
megares        6635       nucl    2022-Jun-10
ncbi           4324       nucl    2022-Jun-10
plasmidfinder  263        nucl    2022-Jun-10
resfinder      2434       nucl    2022-Jun-10
vfdb           2597       nucl    2022-Jun-10

$ abricate --db vfdb --quiet listeria.fa >
```


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