Categorization of *Listeria monocytogenes* from food, environmental, and clinical origin in the Western Cape (South Africa)

by

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Human Research Ethics – (HREC) (NHLS/UCT - Clinical samples) – (HREC R020/2015)

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Summary

Listeria monocytogenes is a ubiquitous food pathogen responsible for the often-fatal infection, listeriosis. Foods that act as vectors of *L. monocytogenes* include meat products, seafood and fish products, dairy products, and ready-to-eat (RTE) foods. South Africa recently experienced the largest ever listeriosis outbreak during 2017-18, and despite this, information on *L. monocytogenes* is still lacking. Internationally, there is a high incidence of *L. monocytogenes* resistance towards antibiotics currently used as a treatment for listeriosis. However, in South Africa there is little information available on the resistance patterns of *L. monocytogenes* to these antibiotics. Additionally, due to the difficulty in controlling *L. monocytogenes* in the food processing environment, new methods such as bacteriophage treatment are being investigated to reduce *L. monocytogenes* numbers in these environments. Worldwide, studies have demonstrated the efficacy of bacteriophages against *L. monocytogenes*, but in South Africa, this technology is still unfamiliar and untested. Seeing as South Africa has a high burden of serious diseases, such as tuberculosis and HIV/AIDS, which often amplify the impacts of listeriosis, data on the efficacy of phage treatment, as well as currently used antibiotics, against *L. monocytogenes*, is greatly needed.

Using *L. monocytogenes* isolates from various origins (Clinical, Environmental, Raw meats, Raw seafood, and RTE) in the Western Cape, South Africa, the first objective was to classify *L. monocytogenes* isolates into lineage groups by using a recently developed PCR-RFLP method (based on SNPs within the *hly*A gene). The results showed an overrepresentation of Lineage I in Clinical environments and Raw seafood, while in the Environmental, Raw meats, and RTE categories, Lineages I and II were somewhat equally distributed. The prevalence of a high number of Lineage I isolates in categories other than Clinical contrasts with previous evidence that Lineage I is mostly associated with human listeriosis, while Lineage II is mostly associated with foods.

The second objective was to determine the susceptibility of *L. monocytogenes* isolates to a commercial bacteriophage (Listex™ P100), using spot tests. Additionally, the lineage group data was used to determine whether or not lineage classifications influenced bacteriophage susceptibility. The *L. monocytogenes* isolates from the Clinical, Environmental, Raw meats, and Raw Seafood categories were significantly susceptible to phage activity. However, a large fraction of isolates in the RTE category were tolerant to the phage, which disagrees with the findings of others. Additionally, both lineage groups were significantly susceptible to phage activity when considering all categories combined and lineage grouping did not significantly influence phage susceptibility.

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The final objective was to determine the antibiotic resistance of *L. monocytogenes* isolates to five antibiotics, namely ampicillin, chloramphenicol, erythromycin, gentamicin, and tetracycline, using the disc diffusion method (EUCAST). The results indicated that all isolates were significantly susceptible to ampicillin, and many isolates were resistant to chloramphenicol, erythromycin, and tetracycline. Clinical and Raw seafood isolates were significantly susceptible to all antibiotics, while Raw meats had the highest number of resistant strains.

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Opsomming

Listeria monocytogenes is 'n alomteenwoordige voedselpatogeen wat verantwoordelik is vir die dikwels noodlottige infeksie, listeriose. Voedselsoorte wat dien as vektore van *L. monocytogenes* sluit in vleisprodukte, seekos en visprodukte, suiwelprodukte, en gereed-om-te eet (RTE) voedsel. Suid-Afrika het onlangs die grootste listeriose-uitbraak gedurende 2017-18 ervaar, en ten spyte hiervan, ontbreek inligting oor *L. monocytogenes* nogsteeds in Suid-Afrika. Internasionale studies het hoë weerstandigheid gerapporteer teen antibiotika wat tans gebruik word as 'n behandeling vir listeriose. In Suid-Afrika is daar egter min inligting beskikbaar oor die weerstandigheid van *L. monocytogenes* aan hierdie antibiotika. Daarbenewens, as gevolg van die probleme in die beheer van *L. monocytogenes* in die voedselverwerkings-omgewing (voedselfabrieke), word nuwe metodes soos bakteriofaag-behandeling ondersoek om die teenwoordigheid van *L. monocytogenes* in hierdie omgewings te verminder. Wêreldwyd het studies die doeltreffendheid van bakteriofage teen *L. monocytogenes* gedemonstreer, maar in Suid-Afrika is hierdie tegnologie steeds onbekend en ongetoets. Aangesien Suid-Afrika 'n hoë las het van ernstige siektes soos tuberkulose en MIV/VIGS, wat dikwels die impak van listeriose vererger, is data oor die doeltreffendheid van bakteriofage, asook antibiotikum weerstandigheid, baie nodig.

Die eerste doel was om *L. monocytogenes* isolate van verskeie bronne (Kliniese, Omgewing, Rou vleis, Rou seekos, en RTE) in die Wes-Kaap, Suid-Afrika, te klassifiseer in een van drie linie groepe, deur die gebruik van 'n onlangs-ontwikkelde metode vir PCR-RFLP (gebaseer op enkel-nukleotied polimorfisme binne die *hly*A-geen). Die resultate het getoon dat daar ‘n oorverteenwoordigheid van Linie I was in isolate komende van listeriosis pasiënte (Klinies) en rou seekos, terwyl Linie I en Linie II iewat ewe versprei was in isolate komende van die Omgewing, Rou vleis, en RTE. Die voorkoms van 'n hoë aantal Linie I isolate in kategorieë anders as Kliniese, is in teenstelling met baie ander studies wat Linie I gevind het meestal van listeriose gevalle (d.w.s. kliniese), terwyl Linie II meer dikwels met voedsel geassosieer word.

Die tweede doel was om die vatbaarheid van *L. monocytogenes* isolate te bepaal vir 'n kommersiële bakteriofaag (Listex™ P100), deur middel van kol-toetse. Verder is die linie groepering gebruik om te bepaal of vatbaarheid vir die bakteriofaag daardeur beinvloed word. Die *L. monocytogenes* isolate van die Kliniese, Omgewings-, Rou vleis, en Rou seekos-kategorieë was aansienlik vatbaar vir die bakteriofaag. 'n Groot fraksie van isolate in die RTE-kategorie was egter onvatbaar vir die bakteriofaag, in teenstelling met ander se bevindings. Daarbenewens was beide linie groepe beduidend vatbaar vir bakteriofaag aktiwiteit by die oorweging van alle kategorieë gekombineer en linie groepering het nie beduidend hierdie vatbaarheid beïnvloed nie.

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Die finale doel was om die antibiotikum-weerstand van *L. monocytogenes* te bepaal teen vyf antibiotika, naamlik ampisillien, chloramphenicol, eritromisien, gentamisien en tetrasikliene, met behulp van die skyfdiffusie-metode (EUCAST). Die resultate het aangedui dat alle isolate beduidend vatbaar was vir ampisillien, en baie isolate was weerstandig teen chloramphenicol, eritromisien en tetrasikliene. Kliniese en Rou seekos-isolate was aansienlik vatbaar vir alle antibiotika, terwyl die Rou vleis kategorie die meeste weerstandige isolate bevat het.

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"Trust in the LORD with all thine heart; and lean not unto thine own understanding. In all thy ways acknowledge Him, and He shall direct thy paths." – *Proverbs 3:5-6*

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List of Conference-related Outputs

Keet, R & Rip, D. (2018) Establishing the genetic diversity of *Listeria monocytogenes* strains in the food processing environment and ready-to-eat foods in the Western Cape, South Africa and the associated public health risk; 26TH INTERNATIONAL ICFMH CONFERENCE, FOODMICRO 2018, BERLIN, GERMANY;

Keet, R & Rip, D. (2019) The activity of a commercial bacteriophage against strains of *Listeria monocytogenes* found in the South African food environment; ASM MICROBE 2018, SAN FRANCISCO, USA;

Keet, R & Rip, D. (2019) Susceptibility of *Listeria Monocytogenes* isolates from food, environmental, and clinical origin in South Africa against a commercial bacteriophage; IAFP ANNUAL MEETING 2019, LOUISVILLE, USA.

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List of Abbreviations

AMS	Antimicrobial stewardship
ATCC	American Type Culture Collection
BHI	Brain heart infusion
bp	base pairs
CDC	Centers for Disease Control and Prevention
CFU	Colony-forming units
DNA	Deoxyribonucleic acid
DoH	Department of Health
EFSA	European Food Safety Authority
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
GlcNAc	N-Acetylglucosamine
GRAS	Generally Recognized as Safe
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome
hly	hemolysin
HPP	High pressure processing
IPC	Prevention and Infection Control
LAB	Lactic acid bacteria
LLO	Listeriolysin O
MDR	Multidrug resistant
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
OD	Optical density
PBP	Penicillin-binding proteins
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field Gel Electrophoresis
PFU	Plaque-forming unit
RBP	Receptor-binding proteins
RFLP	Restriction Fragment Length Polymorphism
RTE	Ready-to-eat
SAASP	South African Stewardship Programme
SNP	Single nucleotide polymorphism
ST	Sequence type
TB	Tuberculosis

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TSA	Tryptic soy agar
WHO	World Health Organization
WTA	Wall teichoic acid

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Chapter 1
General Introduction

The global human population is ever expanding and shows no sign of abating in the near future. With it comes an increased need for food, and consequently an increase in food processing. Nowadays, convenience foods are increasingly being mass-produced, and food that require little preparation at home is progressively being favoured over more traditionally prepared meals that require long cooking times (Forsythe, 2010). As a result of increasing human dependence on different food producers to supply food, there has been a steady increase in foodborne related illnesses (Swaminathan & Gerner-Smidt, 2007; Forsythe, 2010). These illnesses can be caused by viruses (e.g. noroviruses, hepatitis A virus), parasites (e.g. *Toxoplasma gondii*), or bacteria (e.g. *Salmonella*, *Bacillus cereus* etc.), and their presence in food is usually a result of undercooking, cross contamination, or inappropriate storage temperatures (WHO, 2019). There are 600 million recorded cases of foodborne illnesses annually and about 420 000 people die as a result of eating food contaminated with pathogens (WHO, 2019). Thus, food pathogens represent one of the biggest challenges to food and medical industries alike.

One of the most important food pathogens is *Listeria monocytogenes*, a bacterium responsible for the serious, and often fatal, foodborne infection listeriosis (Forsythe, 2010). It is ubiquitous in nature, occurring in soil, water, and fertilizer (White *et al.*, 2002; de Noordhout *et al.*, 2014). It is quite resilient, being able to withstand high levels of nitrite and salt, and can grow at very low temperatures (0–4°C) (Forsythe, 2010; Lamont & Sobel, 2011). This means that *L. monocytogenes* can grow, and even thrive, in the refrigerator where other pathogens are often unable to grow (Montville *et al.*, 2012; de Noordhout *et al.*, 2014). Because of the bacterium's characteristics, foods often implicated as a risk for carrying *L. monocytogenes* include ready-to-eat meat products (such as deli meat and sausages), cold-smoked fish, dairy products (especially soft cheeses), and fresh produce (Hof, 2004; Manfreda *et al.*, 2005; Yücel *et al.*, 2005; Meloni *et al.*, 2009; Forsythe, 2010; Martins *et al.*, 2011; Montville *et al.*, 2012; Fallah *et al.*, 2013; Wang *et al.*, 2013; Ziegler *et al.*, 2019). The associated listeriosis risk for these foods arises because there is often no additional cooking step to kill bacteria before the food is consumed (Guenther *et al.*, 2009; Vasconcelos *et al.*, 2016; Henriques *et al.*, 2017). In the past few years, a steady increase in the presence of *L. monocytogenes* in various food types have led to a number of listeriosis outbreaks around the world (Aureli *et al.*, 2000; Buchanan *et al.*, 2017; Denise *et al.*, 2017; Gelbíčová *et al.*, 2018), and with a mortality rate as high as 30%, this increased presence of *L. monocytogenes* in food is a rising and important public health concern.

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There are two forms of listeriosis, namely non-invasive and invasive. The non-invasive form affects healthy-individuals and usually only leads to febrile gastroenteritis, which is self-limiting (Montero *et al.*, 2015). Non-invasive listeriosis will generally occur only when very high doses of *L. monocytogenes* are consumed (>10^5 CFU/mL) (Farber & Peterkin, 1991; Vázquez-boland *et al.*, 2001a). The invasive form, however, manifests in immunocompromised individuals, where it can lead to serious illnesses such as septicaemia, meningitis, and encephalitis (NICD, 2017a). Individuals that are particularly at risk are those with diseases such as cancer or HIV/AIDS (that weaken or compromise their immune system), babies or young children, the elderly, and pregnant women (Epstein *et al.*, 1996; de Noordhout *et al.*, 2014). Pregnant women specifically are 20 times more likely to be infected with *L. monocytogenes*, and while the women only experience mild symptoms, the infection is usually detrimental to the unborn babies and can lead to miscarriages (Lamont & Sobel, 2011). Tracing the source of a *L. monocytogenes* infection is often problematic, since the incubation period of the bacterium can be up to 90 days (Forsythe, 2010). Thus, by the time a patient is eventually diagnosed, the causal agent (i.e. the food source) can no longer be determined. Countries that are plagued by a high burden of serious diseases such as HIV/AIDS and tuberculosis, as in the case of South Africa, are particularly at risk, since the immunocompromised individuals are up to 1 000 times more likely to contract listeriosis than healthy individuals (Allerberger & Wagner, 2010; Bester & Essack, 2012; Nyasulu *et al.*, 2012; Moyane *et al.*, 2013). Infection with *L. monocytogenes* is usually treated with either one or a combination of various antibiotics. Currently the antibiotic of choice is ampicillin (a type of penicillin), alone or in combination with an aminoglycoside such as gentamicin (Charpentier and Courvalin, 1999; Hof, 2004). While *L. monocytogenes* is susceptible to a range of antibiotics, it is increasingly becoming antibiotic resistant, which is of great concern (Alonso-Hernando *et al.*, 2012; Fallah *et al.*, 2013; Carvalho *et al.*, 2019). Antibiotic resistant *L. monocytogenes* strains can have a particularly detrimental impact on immunocompromised individuals, and it is therefore necessary to continuously monitor *L. monocytogenes* resistance.

The food processing industry often exerts considerable effort to control *L. monocytogenes*, but it is generally accepted to be almost impossible to completely eradicate the bacterium from a processing facility (Todd & Notermans, 2011; Buchanan *et al.*, 2017; Chen *et al.*, 2017). Because of *L. monocytogenes*' close association with the environment, it can easily enter food processing facilities via incoming raw material (Todd & Notermans, 2011; Buchanan *et al.*, 2017). Once inside the food processing facility, the ability of *L. monocytogenes* to form biofilms then enables it to spread and persist within a facility even after cleaning and sanitation (Todd & Notermans, 2011; Buchanan *et al.*, 2017; Chen *et al.*, 2017). Moreover, several studies report increased resistance of *L. monocytogenes* towards sanitizers (Pan *et al.*, 2006; Strydom & Witthuhn, 2015; Kovacevic *et al.*, 2016). This has led food processors to explore alternative strategies to control

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L. monocytogenes within the food processing environment. One of these strategies involves the use of bacteriophages (a type of virus) as an "environmentally-friendly" biocontrol to eradicate these bacteria. Bacteriophages are very specific in their hosts targets; hence they can infect and kill *L. monocytogenes* without having an effect on other bacterial species (Guenther *et al.*, 2009; Moye *et al.*, 2018). Additionally, bacteriophages are also reported to be effective on biofilms (Soni & Nannapaneni, 2010a; Montañez-izquierdo *et al.*, 2012; Rodríguez-Melcón *et al.*, 2018), which is especially useful for food producers as there is always a risk of *L. monocytogenes* biofilm formation.

South Africa has recently experienced the largest ever global outbreak of listeriosis during 2017-18, which was traced back to a pork-meat processed product called polony (NICD, 2018a; Olanya *et al.*, 2019; Smith *et al.*, 2019b). More than 1 060 people were infected with *L. monocytogenes* and 216 died as a result (NICD, 2018b). Prior to the outbreak, listeriosis was a non-notifiable disease, i.e. health workers were not responsible for informing the authorities when dealing with cases of listeriosis (NICD, 2018c; WHO, 2018). Therefore, the same amount and quality of historic data that other countries (e.g. the United States) possess regarding listeriosis outbreaks and cases in general is not available in South Africa. Because South Africa does not have an active monitoring system for foodborne illnesses, the real impact of these infections may well be grossly underestimated. A better understanding of where the sources of *L. monocytogenes* are, and how the bacteria respond to antimicrobial treatments (e.g. bacteriophages or antibiotics), can help to improve control of the bacteria and could in turn potentially avoid another future listeriosis outbreak of such magnitude.

By making use of *L. monocytogenes* strains isolated from various origins and types of food in the Western Cape, South Africa, this dissertation aimed to answer three main questions, which are: 1) what are the main classification patterns in terms of lineage grouping of *L. monocytogenes*?; 2) can bacteriophage treatment be used as an effective alternative to antibiotic use in a South African context?; and 3) are currently used antibiotics still effective in controlling *L. monocytogenes* strains in South Africa? In other words, the flow of the dissertation was essentially concerned with what is present (i.e. *L. monocytogenes* classification), how effective are current methods at dealing with what is present, and finally is there a viable new alternative to deal with what is present. A detailed explanation follows.

The first objective was to classify *L. monocytogenes* into respective lineage groups by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Three common lineage groups are available for *L. monocytogenes* to be assigned to, which in turn can be subdivided into a total of 12 serotypes. However, the majority of strains are usually assigned to Lineage I (serotypes 1/2b and 4b) and Lineage II (serotype 1/2a). Serotype 1/2a and 1/2b are

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mostly isolated from food, while serotype 4b is characteristic of human listeriosis cases (Ward *et al.*, 2004; Swaminathan & Gerner-Smidt, 2007; Orsi *et al.*, 2010). Classifying *L. monocytogenes* from various origins in the Western Cape firstly sheds much needed light on lineage association patterns in South Africa, and secondly can be compared to patterns found elsewhere in the world.

The second objective was to determine whether these *L. monocytogenes* isolates are susceptible to a commercial bacteriophage, and whether lineage grouping differentially affects phage activity. The use of bacteriophage control is still relatively new, and not much is yet known about possible acquired bacterial resistance towards these phages. The few studies that have been done so far (outside of South Africa), determined phages to be effective against *L. monocytogenes* with no reported resistance. However, in 2015, local researchers tested the efficacy of a commercial bacteriophage, and found that in contrast to other studies, a large majority of *L. monocytogenes* strains were resistant to the bacteriophage (Strydom, 2015). Therefore, this part of the study was concerned not only with expanding the current knowledge base for potential bacteriophage usage in a South African context (since there is a great lack of information regarding this), but also to determine whether the findings here replicate or contradict what has been observed elsewhere. In other words, the aim was to establish whether the *L. monocytogenes* isolates in this study exhibit the same tolerance towards the mentioned commercial bacteriophage or not.

The third and final objective of this study was to assess the antibiotic susceptibility of *L. monocytogenes* isolates against five different antibiotics by using the disk diffusion method as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2012). Currently, there is a lack of information in South African literature on the possible antibiotic resistance of *L. monocytogenes* isolates from the food and clinical environment. This, together with the high levels of mortality associated with the recent outbreak and the fact that antibiotic-resistant strains of *L. monocytogenes* are generally on the rise, means that more in-depth studies into the efficacy of antibiotics currently used against *L. monocytogenes* in South Africa is desperately needed, since the outcomes of such studies can guide the management of *L. monocytogenes* in both food processing and clinical environments, potentially stemming the increased tide of *L. monocytogenes* resistance and thereby safeguarding current antibiotic treatments for future generations.

In conclusion, despite the significant effect that *L. monocytogenes* has on public health, very little research output has been generated on *L. monocytogenes* specific to the South African environment. Therefore, at the very least this study aimed to fill this gap by generating information on isolates from food, environmental, and clinical origin.

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Chapter 2
Literature Review

2.1. Introduction

The amount of food produced, processed, and distributed regionally and nationally increases annually, and with it so too does the need for the improved safety thereof (Forsythe, 2010). The potential therefore exists for the general public to get exposed to more pathogens every year as a result of this annual increase in food production, globalisation of the food chain, and changes in food processing methods (Swaminathan & Gerner-Smidt, 2007; Forsythe, 2010). However, consumers are continuously demanding that food be processed less and contain fewer additives, while simultaneously seeking an increasing variety of convenience foods and demanding longer shelf lives (Swaminathan & Gerner-Smidt, 2007; Montville *et al.*, 2012). Moreover, consumers want to spend less time on food preparation, resulting in an ever-increasing variety and production of ready-to-eat (RTE) foods. Such increased production of RTE foods have led to the emergence of psychrotrophic pathogenic bacteria, such as *Listeria monocytogenes* (Muñoz *et al.*, 2012), that are able to survive and rapidly multiply in raw and processed foods (Forsythe, 2010).

Listeria monocytogenes is a ubiquitous, Gram-positive, non-spore forming, motile bacterium responsible for the often fatal human disease listeriosis (Forsythe, 2010). *Listeria monocytogenes* is commonly associated with raw food products due to its presence in soil and water (White *et al.*, 2002; de Noordhout *et al.*, 2014) and tolerates a wide range of temperatures, being able to grow and multiply in the range of 0°C to 4°C, even though its optimum growth temperature is between 30°C and 37°C (Forsythe, 2010). It is thus very well adapted to refrigeration temperatures, which is in stark contrast to other pathogens that are mostly growth-inhibited at such low temperatures (de Noordhout *et al.*, 2014). Furthermore, *L. monocytogenes* has the ability to tolerate high salt concentrations (Lamont & Sobel, 2011) and is also resistant to levels of nitrite that inhibit the majority of other food pathogens (Forsythe, 2010). Spoilage bacteria cannot grow at refrigeration temperatures, which reduces microbial competition for *L. monocytogenes*, leading to its subsequent proliferation at such low temperatures (Montville *et al.*, 2012). In food production facilities, *L. monocytogenes* can survive by forming biofilms, thereby creating a steady supply of strains that are able to contaminate food (Todd & Notermans, 2011; Chen *et al.*, 2017). Foods that act as vectors of *L. monocytogenes* and which are also highly susceptible to contamination include meat products (especially deli meats), seafood and fish products, and pasteurized or raw milk products such as soft cheeses (Hof, 2004; Manfreda *et al.*, 2005; Yücel *et al.*, 2005; Meloni *et al.*, 2009; Forsythe, 2010; Martins *et al.*, 2011; Montville *et al.*,

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2012; Fallah *et al.*, 2013; Wang *et al.*, 2013; Ziegler *et al.*, 2019). Due to the lack of typical processing steps that reduce pathogens in RTE food, fresh fruit, and vegetables, these products can easily be contaminated with *L. monocytogenes* (Guenther *et al.*, 2009; Vasconcelos *et al.*, 2016; Henriques *et al.*, 2017). Thus, overall *L. monocytogenes* can tolerate a wide range of conditions that are necessary for the preservation and storage of food.

2.2. Growth and survival of *Listeria monocytogenes*

Listeria monocytogenes is a facultative anaerobic bacteria and commonly occurs in agricultural and food processing environments (Epstein *et al.*, 1996; Buchanan *et al.*, 2017). Soils with higher moisture content, or soil in close proximity to bodies of water, are associated with a higher prevalence of *L. monocytogenes*. The bacteria occurs within the intestines of various mammals, birds, and even crustaceans (Epstein *et al.*, 1996). As a result of its close association with animals and agricultural environments, food is considered to be the principal vehicle of contamination, with RTE foods being of particular significance, especially RTE foods which are kept at refrigeration temperatures for prolonged periods of time (Swaminathan & Gerner-Smidt, 2007; Buchanan *et al.*, 2017; Leong *et al.*, 2017). There are many ways in which *L. monocytogenes* can enter the food processing environment (Figure 2.1). Due to its ubiquitous presence in the agricultural environment, it can enter the food processing facility by means of raw material, movement of factory staff, improper hygiene practises, ineffective cleaning procedures, or even poor design of factory equipment (Todd & Notermans, 2011; Buchanan *et al.*, 2017).

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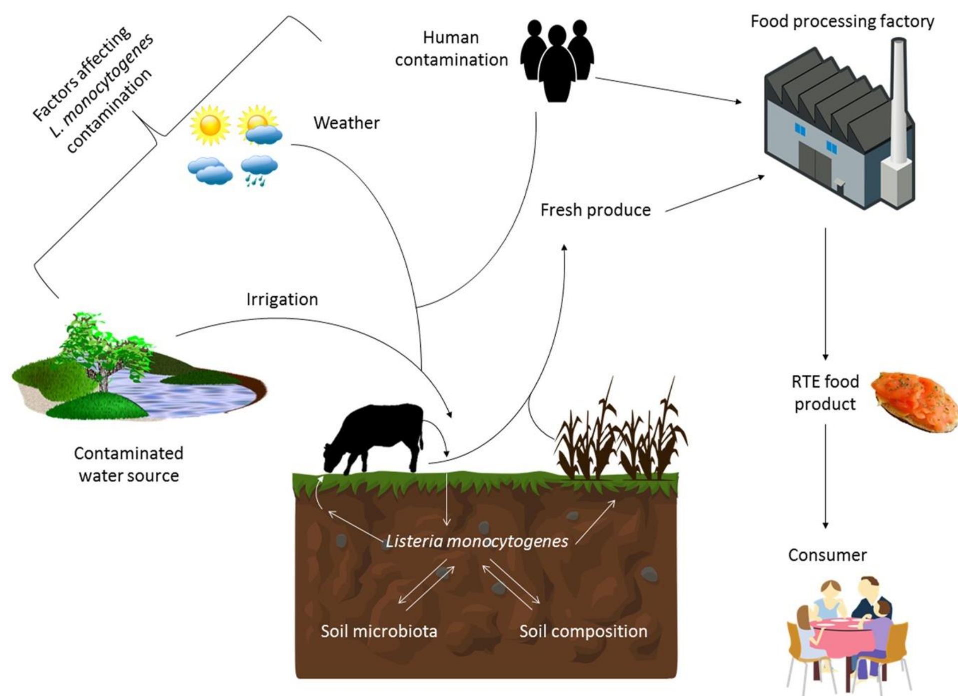

Figure 2.1 The various ways by which *L. monocytogenes* circulate in the environment and ultimately end up on the consumers plate: water sources, agricultural environments and associated livestock and/or fresh produce, and even employees and other humans associated with such food processing environments (NicAogáin & O'Byrne, 2016 used with permission).

Studies have shown that food processing equipment can convey contamination to uncontaminated food products. Using a slicer as an example, *L. monocytogenes* can be transferred from the blade to the food product, whilst simultaneously persisting on the blade even after numerous slices are made, thus acting as a semi-continuous source of contamination (Chaitiemwong *et al.*, 2014; Scollon *et al.*, 2016; Wang & Ryser, 2016). The frequency of *L. monocytogenes* in the food processing environment is not necessarily correlated to its frequency in food products (Buchanan *et al.*, 2017) and post-processing contamination is still thought to be the main cause of *L. monocytogenes* incidence (Jadhav *et al.*, 2012; Ferreira *et al.*, 2014). The ability of many strains of *L. monocytogenes* to endure adverse conditions such as high salt concentration, and low pH-, and low oxygen levels, often lead such strains to proliferate in food products (Montville *et al.*, 2012; Chen *et al.*, 2017). Lower temperatures also decrease the bacterium's metabolic rate and cause the cell membranes to become more rigid (NicAogáin & O'Byrne, 2016). This enables *L. monocytogenes* to better withstand salty environments, making cured meats a hospitable growth medium at such low temperatures (Montville *et al.*, 2012). Furthermore, due to its ubiquitous nature, complete eradication of *L. monocytogenes* from the food processing environment is nearly impossible. *Listeria monocytogenes* is able to survive in the food processing environment even after years of sanitation control, and there could be several possible explanations for this phenomenon (Buchanan *et al.*, 2017): strains can grow in small crevices which might be missed by disinfectants, leading to continuous re-introduction of the

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strains into the sanitized environment (Todd and Notermans, 2011; Allen *et al.*, 2016); persistent cells may be present; and the formation of biofilms and its protection of individual cells to sanitation (Buchanan *et al.*, 2017). Biofilms are immobile communities; a combination of bacterial cells and a self-produced mixture of polysaccharides, proteins, and extracellular DNA (Olivares *et al.*, 2013; Allen *et al.*, 2016; Friera *et al.*, 2017). Growth within a biofilm promotes interaction between cells and nutrients, possible harmful metabolites, and genetic material which may improve survival and growth (Buchanan *et al.*, 2017). Biofilm formation is possible on various surfaces such as glass, stainless steel, and polystyrene (Bonsaglia *et al.*, 2014). Several factors influence the extent of this biofilm formation, which include matrix composition, temperature, and even strain origin (Kadam *et al.*, 2013). It is unlikely that pure *L. monocytogenes* biofilms occur in the food processing environment, but rather that *L. monocytogenes* cells form part of a multispecies biofilm (Ferreira *et al.*, 2014). In the food processing environment, *L. monocytogenes* often coexists with *L. innocua*, and the presence of the latter species could be used as an indication that the environment is potentially contaminated with *L. monocytogenes* too (Costa *et al.*, 2018).

It is no surprise that in the last few decades there has been a steady increase in listeriosis outbreaks due to *L. monocytogenes* presence in foods (Aureli *et al.*, 2000; Buchanan *et al.*, 2017; Denise *et al.*, 2017; Gelbíčová *et al.*, 2018). In South Africa, listeriosis was non-notifiable prior to the 2017-18 outbreak. Thus, there are few records of listeriosis or the foods that could be implicated as a possible source prior to the SA 2017-18 outbreak. However, recent outbreaks in the USA (Figure 2.2) indicate that listeriosis outbreaks are mostly due to RTE food products. The South African listeriosis outbreak of 2017-18 was traced back to a local product called polony (Olanya *et al.*, 2019; Smith *et al.*, 2019b). Polony is a processed deli meat product similar to bologna sausage.

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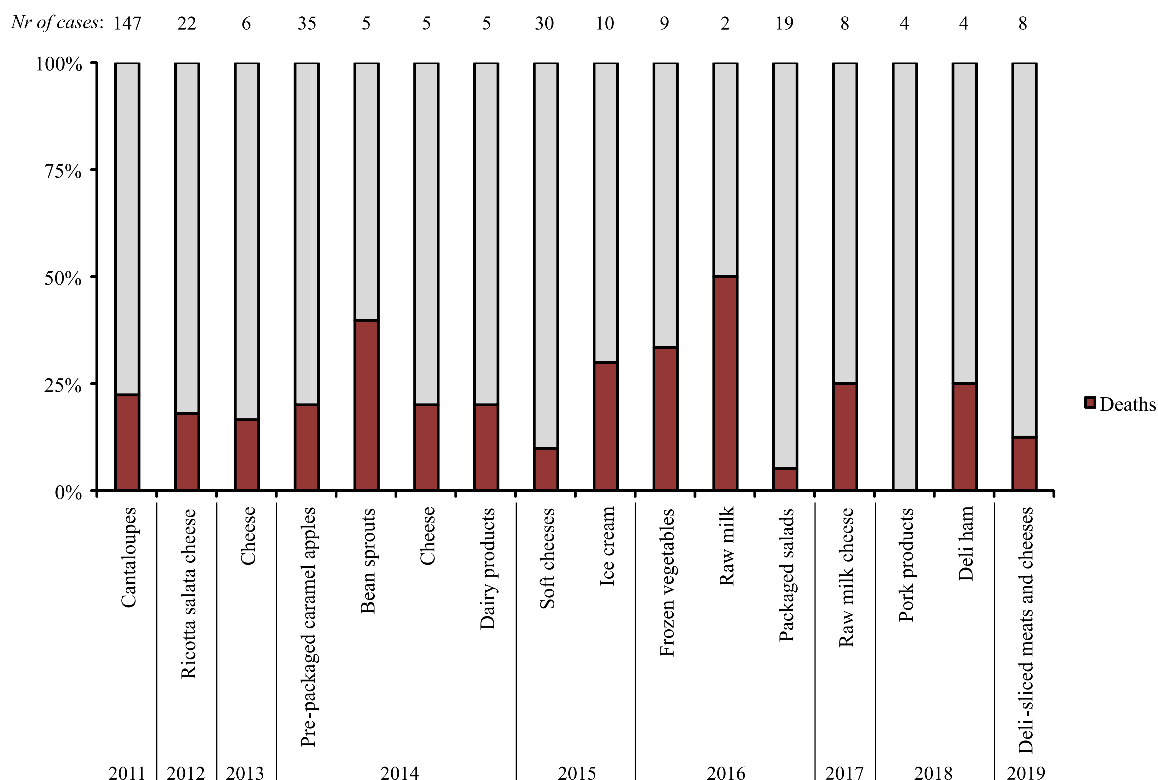

Figure 2.2 Multistate listeriosis outbreaks associated with various food products in the United States from 2011 to 2019 (data obtained from CDC, 2019).

A proactive approach needs to be taken in order to control *L. monocytogenes* and to prevent contamination of food (Buchanan *et al.*, 2017). The steps may include risk assessment, effective cleaning and sanitation procedures, control of personnel movement in and out of areas where food is prepared, and improved hygienic design of the processing facility (Buchanan *et al.*, 2017).

2.3. *Listeria monocytogenes* and its effect on humans

Listeriosis is a disease caused by infection with *L. monocytogenes*. It can lead to serious life-threatening conditions such as meningitis, encephalitis, and septicaemia. There are two forms in which listeriosis can occur, namely as non-invasive febrile gastroenteritis, which is non-lethal, or as a more invasive form, which has a mortality rate of 20 – 30% (Montero *et al.*, 2015). When people are infected with *L. monocytogenes,* the incubation period (i.e. the time until the physical manifestation of symptoms) can be up to 90 days, which makes it exceedingly difficult to establish the origin of the infection once a positive diagnosis has been made (Forsythe, 2010). Moreover, it is also believed that up to 10% of the human population are intestinal carriers of *L. monocytogenes* without ill effect (Grif *et al.*, 2003; Forsythe, 2010; Buchanan *et al.*, 2017), which further obscures the accurate identification of the bacterium's origin. In seemingly healthy individuals, an onset of febrile gastroenteritis may occur without warning, which is typically self-

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limiting (de Noordhout *et al.*, 2014), but such healthy individuals will only be affected after a large number of cells have been ingested (Dalton *et al.*, 1997; Aureli *et al.*, 2000; Montero *et al.*, 2015). In a one-year study focussing on the faecal carriage of *L. monocytogenes* in healthy individuals, the bacterium was isolated from volunteers even though none of them presented with febrile gastroenteritis symptoms (Grif *et al.*, 2003).

Listeriosis poses a much greater risk in individuals with weak cell-mediated immunities or compromised immune systems compared to healthy individuals (Forsythe, 2010). In immunocompromised individuals, listeriosis may lead to meningitis or septicaemia (NICD, 2017a). Jeopardized individuals include the elderly, pregnant women, unborn babies, neonates, and those with a compromised immunity as a result of illness, such as HIV/AIDS or cancer (Epstein *et al.*, 1996; de Noordhout *et al.*, 2014). In the outbreak that occurred in South Africa during 2017-18, it was confirmed that 78% of the adults diagnosed with listeriosis were HIV positive (NICD, 2017a). In fact, listeriosis is 100 – 1 000 times more frequently observed in HIV patients than in the other high-risk groups with comparable age (Allerberger & Wagner, 2010). Thus, the probability of individuals with compromised immune systems contracting listeriosis can be magnified depending on the reason for such weakened immune systems, and is not just dependent on whether individuals are immunocompromised *per se*.

Listeriosis is difficult to diagnose and the symptoms are often non-specific (Lamont and Sobel, 2011). In other words, symptoms vary according to which groups infected individuals belong to. For instance, unlike most other foodborne illnesses which mainly cause gastrointestinal symptoms, pregnant women with listeriosis might present with only mild flu-like symptoms (Lamont & Sobel, 2011), and can thus easily be misdiagnosed. In fact, a national survey conducted in the USA showed that only 18% of the pregnant women knew about listeriosis and less than 30% were aware that it was preventable by avoiding certain foods (Lamont & Sobel, 2011). Non-perinatal cases of listeriosis are often concurrent with diseases such as cancer and diabetes, and its presence is therefore masked by the magnitude and symptoms of these diseases, thus leading to non-diagnosis and subsequent non-reporting of listeriosis itself (de Noordhout *et al.*, 2014). Listerial meningitis symptoms presents similarly to acute bacterial meningitis and on occasion the central nervous system can be infected, presenting as encephalitis or rhombencephalitis (Allerberger & Wagner, 2010).

2.4. Genetic diversity of *Listeria monocytogenes*

Listeria monocytogenes can be divided into at least 12 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7) based on variation in the somatic (O) and flagellar (H) antigens (Seeliger & Langer, 1989; Rasmussen *et al.*, 1995; Borucki & Call, 2003; Doumith *et al.*, 2004; Liu *et al.*, 2006;

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Hyden *et al.*, 2016). Although recent literature suggests there might be more that 12 serotypes, there does not yet seem to be complete agreement on this (Liu, 2006; Chen *et al.*, 2014; Chen *et al.*, 2016) and thus only the well-known and established 12 serotypes are regarded here. Each serotype of *L. monocytogenes* has a unique wall teichoic acid (WTA). These distinctive WTA's are responsible for antigenic properties, making it possible to serotype *L. monocytogenes* (Shen *et al.*, 2017). There are three more common lineage groups identified for *L. monocytogenes*, namely Lineage I (serotypes 1/2b, 3b, 3c, and 4b), Lineage II (serotypes 1/2a, 1/2c, and 3a), and Lineage III (4a and 4c) (Figure 2.3). A fourth lineage group, which was previously thought to be a subgroup of Lineage III (IIIB), was recently reclassified as Lineage IV (Ward *et al.*, 2008; Orsi *et al.*, 2010; Lomonaco *et al.*, 2015). However, isolates from this lineage are mostly isolated from ruminants, and are thus not associated with human listeriosis cases (Gray *et al.*, 2004; Sauders *et al.*, 2004; Orsi *et al.*, 2010).

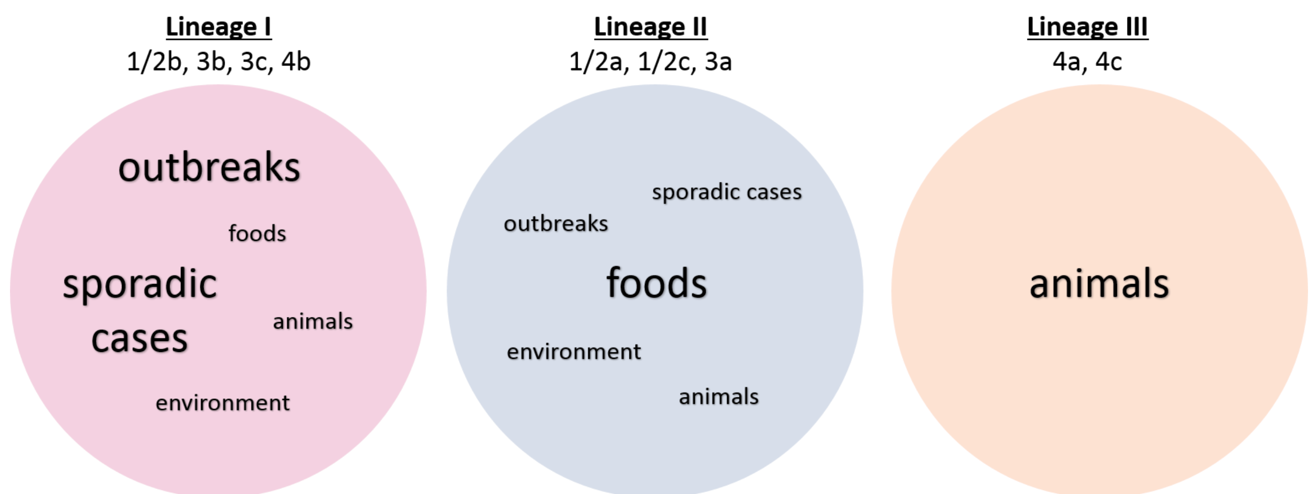

Figure 2.3 Association of *Listeria monocytogenes* lineage groups (and serotypes) with different ecological categories (word size represent the proportion of lineage groups obtained from each category) (information from Orsi *et al.*, 2010).

The most notable serotypes are 1/2b and 4b (Lineage I), which are most commonly associated with outbreaks of human listeriosis, and 1/2a and 1/2c (Lineage II), which are overrepresented among food isolates (Ward *et al.*, 2004; Swaminathan & Gerner-Smidt, 2007; Orsi *et al.*, 2010). Together they account for more than 95% of implicated foods and human listeriosis cases (Jadhav *et al.*, 2012; Hyden *et al.*, 2016).

It is suggested that the lineage groups of *L. monocytogenes* differ in terms of their pathogenicity and host specificity (Ward *et al.*, 2004). Differences in the prevalence of certain lineage groups in specific environments show that frequency of exposure to isolates from Lineage I does not necessarily correlate with the number of human listeriosis cases (Orsi *et al.*, 2010). This is due to the low occurrence of Lineage I serotypes in foods, even though such serotypes are

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responsible for most listeriosis cases. Instead, it is suggested that serotypes from Lineage I have increased pathogenicity when compared to those of Lineage II (Orsi *et al.*, 2010). A considerable number of *L. monocytogenes* strains isolated from foods have mutations that lead to premature stop codons in their *inl*A gene, leading to decreased virulence. This truncated and less virulent gene is especially common among isolates from Lineage II, specifically 1/2a and 1/2c serotypes (Nightingale *et al.*, 2008). This could offer an explanation as to why Lineage II isolates are not often associated with human listeriosis cases (Nightingale *et al.*, 2008). However, *L. monocytogenes* isolates display heterogeneity in virulence, so there is no clear association between virulence potential and strain origin (Montero *et al.*, 2015). It is, however, a possibility that variations in the structure of the virulence-associated genes can have an effect on the pathogenicity of the bacterium (Vines *et al.*, 1992). This idea is supported by epidemiological data that point to differences in the association of Lineages I and II with certain environments (Vines *et al.*, 1992). For example, the three lineages adapt differently to environmental stresses often encountered in the food processing environment (e.g. temperature fluctuations) which could explain why Lineage II is more frequently isolated from food products (Ward *et al.*, 2004; Orsi *et al.*, 2010). In other words, it is likely that Lineage II is more adapted to the environmental conditions found in food processing environments.

Isolates from Lineage III are seldom associated with human listeriosis cases. Researchers suggest that these isolates (e.g. 4a and 4c) are not virulent to humans and that their hosts are non-primate mammals (Wiedmann *et al.*, 1997; Jeffers *et al.*, 2001). Therefore, because of the infrequency of Lineage III isolates in foods (about 2%), there is a reduced chance of human exposure and subsequent infection (Ward *et al.*, 2004). It is also suggested that Lineage III is not well adapted to thermal inactivation processes (such as those encountered in a food production facility), making it more prevalent in animal production facilities (farms etc.) than in food manufacturing settings (Ward *et al.*, 2004).

2.5. Detection and subtyping of *Listeria monocytogenes*

Due to the high mortality rate associated with invasive listeriosis, it is essential that *L. monocytogenes* (from food origin) is detected and subtyped as early as possible, in order to reduce, or preferably, to prevent outbreaks. Various methods are used for the detection and subtyping of *L. monocytogenes,* each with its own advantages and disadvantages. In recent times researchers have started moving away from conventional methods which rely only on phenotypic characteristics, and are opting for genotypic methods which are more consistent and sensitive (Jadhav *et al.*, 2012). The incubation period for listeriosis can exceed 30 days, which means that

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it is necessary to develop molecular fingerprinting techniques that can be used to rapidly detect an outbreak where it could otherwise have been regarded as a sporadic case (Fox *et al.*, 2017).

Molecular fingerprinting techniques, such as restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE), and ribotyping, are becoming increasingly popular due to their ability to identify several strains of subtypes within a bacterial species (Gendel, 2004). This differentiation is crucial when investigating epidemiological outbreaks and is valuable for understanding the problems in the food industry caused by *L. monocytogenes* (Gendel, 2004; Jadhav *et al.*, 2012). With these methods one can recognize clusters that warrant further investigation. So although epidemiology is still necessary to establish a source for an outbreak, molecular subtyping techniques are superior for guiding studies in the right direction (Jackson *et al.*, 2016).

With conventional polymerase chain reaction (PCR), *L. monocytogenes* is detected by targeting a choice of genes including, but not limited to, *hly*A, internalin A (*inlA*), internalin B (*inlB*), and invasion associated protein *iap* (Jadhav *et al.*, 2012). It should be noted that since the technique analyses DNA, it cannot distinguish between living or dead organisms. When a negative signal is encountered, it is usually either due to the absence of the target DNA or the presence of inhibitory compounds used in enrichment broths (Jadhav *et al.*, 2012). Kaclíková *et al.* (2002) developed a detection method which takes only three days, and is faster than conventional methods, which usually take seven to ten days. It firstly makes use of a 48 h enrichment step, similar to that specified in EN ISO 11290-1 (using half Fraser and full Fraser broth), followed by a non-selective post enrichment in brain heart infusion (BHI) of five hours. This enrichment increases the sensitivity of the PCR method by reducing the risk of contamination by inhibitory compounds.

Restriction fragment length polymorphism (RFLP) is a sub-typing method whereby a virulent target gene (e.g. *hly*A) is first amplified by PCR, then subsequently digested with specific restriction enzymes (Rasmussen, O *et al*., 1992; Vines *et al*., 1992; Wiedmann *et al*., 1997; De Cesare *et al*., 2007; Rip & Gouws, in press; Meghdadi *et al*., 2019). The target gene contains polymorphisms which can be visualised as specific band sizes on agarose gel electrophoresis after digestion, making it possible to discriminate between serotypes within *L. monocytogenes* lineage groups (Rasmussen *et al.*, 1995; Wiedmann *et al.*, 1997). A single nucleotide polymorphism (SNP) is a mutation in a single base which is substituted by another nucleotide (Schork *et al.*, 2000). A study conducted by Vines *et al*. (1992) demonstrated the usefulness of RFLP to distinguish between not only lineage groups, but different serotypes of *L. monocytogenes.* They performed digests with different enzymes on four different virulence genes of the bacterium, namely *hly*A, *iap*, *Prf*A and *mpl*. When digesting the *hly*A gene with enzyme *Bst*UI (Bst1236I), they were able to

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distinguish serotypes 1/2b and 4b (Lineage I) from serotypes 1/2a and 1/2c (Lineage II), and also observed polymorphic differences between 1/2a and 1/2c (Vines *et al.*, 1992). This means that virulence-associated genes can be used for rapid detection and molecular characterization of *L. monocytogenes* (Vines *et al.*, 1992).

2.6. Epidemiology

2.6.1. Life cycle of *Listeria monocytogenes*

Once ingested by humans, infection with *L. monocytogenes* triggers a T-cell mediated immunity response, enabling macrophages to destroy the bacteria (Lamont & Sobel, 2011). Continuous exposure creates memory T-cells that are able to recognise the *L. monocytogenes* bacterium swiftly, providing necessary resistance against *L. monocytogenes* infection (Lamont & Sobel, 2011). T-cells are leucocytes (white blood cells) serving a core purpose in adaptive immunity, the system responsible for modifying the body's immune system to respond to specific pathogens (Lamont & Sobel, 2011). Individuals with underlying conditions that suppress their T-cell mediated immunity, such as HIV-patients, are more likely to succumb to listeriosis (Epstein *et al.*, 1996; Forsythe, 2010). This could explain why listeriosis has a higher association with HIV, therapies that suppress the immune system and pregnancy (Lamont & Sobel, 2011). For example, in Australia two cancer patients (i.e. compromised immunity as a result of illness) died of listeriosis after eating a hospital meal containing meat infected with *L. monocytogenes* (Todd & Notermans, 2011). Furthermore, in the elderly, susceptibility to listeriosis is related to reduced gastric acidity that occurs as a result of ageing (Forsythe, 2010). Thus, host factors increase the probability of infection with *L. monocytogenes* e (e.g. immune system suppression) and the consequent development of listeriosis can be fatal.

Once ingested in humans, *L. monocytogenes* is phagocytised and enters the epithelial cells. A transcriptional protein, protein regulatory factor A (*prf*A), is responsible for the switch between the extra- and intracellular lifecycle of *L. monocytogenes*. The bacterium possesses a surface protein, internalin, which exists in two forms, namely *Inl*A and *Inl*B. This protein together with its receptors (surface protein, E-cadherin) on the host epithelial cells facilitate adhesion (Epstein *et al.*, 1996; Bonazzi *et al.*, 2009; Lamont and Sobel, 2011). Because of this, *L. monocytogenes* does not disrupt or cause abrasions on the host cell's gastrointestinal tract (Epstein *et al.*, 1996). Once the macrophages, white blood, and plasma cells internalizes the bacterium, it is able to escape the vacuole and enter the cytoplasm, with subsequent proliferation (Epstein *et al.*, 1996; Doyle, 2001; Lamont & Sobel, 2011). The bacterium is able to escape due to the action of the pore-forming

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protein, listeriolysin O (LLO), encoded for by the *hly*A gene, which is innate to all *L. monocytogenes* species (Cossart *et al.*, 1989; Rasmussen, O *et al.*, 1991; Epstein *et al.*, 1996; Doyle, 2001).

One of the virulence factors, a surface protein called actin A (ActA), creates a propulsion system for the bacteria by inducing actin polymerization and forming filaments (Portnoy *et al.*, 1992). Once it is near the cell membrane it forms a protrusion, which is then engulfed by the adjacent cell (Doyle, 2001). This enables the bacterium to use the host cell's actin to provide the propulsion necessary for pathogenesis, allowing it to spread to adjacent cells (Epstein *et al.*, 1996). The intracellular cycle is able to continue since nearby cells are able to recognize pseudopodia-like structures, produced by the bacteria, which protrude from the host cell (Epstein *et al.*, 1996). By avoiding the extracellular environment, immunoglobulins cannot serve the major protective role as expected, explaining why immunocompromised individuals (due to reduced immunoglobulin activity) are more at risk for the disease (Epstein *et al.*, 1996). Therefore, effective treatment of listeriosis must take this intracellular life cycle into account and medication administered must be able to penetrate host cells.

2.6.2. Diagnosis of symptoms associated with *Listeria monocytogenes*

Clinically, listeriosis is diagnosed when the bacterium is isolated from body tissue or fluid, such as blood, cerebrospinal fluid (CSF), or in pregnancy-related cases, the placenta or foetus (Forsythe, 2010; Noll *et al.*, 2018). In patients with impaired immunity, severe illnesses can occur due to *L. monocytogenes* infections, namely sepsis, meningitis, or encephalitis, causing permanent damage or even death (de Noordhout *et al.*, 2014). In South Africa, *L. monocytogenes* is the second most common cause of acute bacterial meningitis (NICD, 2017a). Septicaemia and listerial meningitis cases have a mortality rate of 50% and 70% respectively, whereas perinatal-neonatal infections carry a mortality rate of greater than 80% (Forsythe, 2010). This is in stark contrast to other foodborne pathogens such as *Escherichia coli* and *Salmonella* that have a mortality rate of 11% and less than 1%, respectively (Laupland *et al.*, 2008).

The minimum infectious dose of *L. monocytogenes* is not yet known, but it is suggested that levels of 10^4 to 10^6 colony forming units (CFU)/g may be enough to cause disease, although for immunocompromised individuals this amount might be lower (Vázquez-boland *et al.*, 2001a; Swaminathan & Gerner-Smidt, 2007; Lamont & Sobel, 2011). Opinions regarding "safe" levels on RTE foods vary from absence in 25 g of food to 100 CFU/g of food, provided that *L. monocytogenes* is not able to multiply in the final product (Todd & Notermans, 2011). The 100 CFU/g limit is also not based upon strict dose-response formulas (European Commission, 1999). Several factors can have an influence on the minimum infectious dose of a foodborne pathogen such as *L. monocytogenes* (European Commission, 1999). These can include the status of the host's immune

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system (healthy vs. immunocompromised), strain virulence, the quantity of food consumed, and the number of organisms present on the food consumed (European Commission, 1999; Goulet *et al.*, 2013). It is suggested that, at a concentration as low as 1 CFU/g food, *L. monocytogenes* essentially occurs on all foods, and therefore cannot be responsible for causing listeriosis at such a low concentration, even in susceptible subjects, since it would be too common (European Commission, 1999). In any case, a zero incidence of *L. monocytogenes* in foods is likely not attainable (Chen *et al.*, 2003). The reason why levels necessary for infection is not yet determined is due to the high risks associated with listeriosis (i.e. death) and also because feeding studies on humans is unethical. (Chen *et al.*, 2003; Mclauchlin *et al.*, 2004). And although dose-response studies conducted on animals can give researchers some information, there are other factors that can also play a role, such as the food matrix, immunity of the consumer, and the pathogenicity and virulence of the *L. monocytogenes* strain in question (Vázquez-boland *et al.*, 2001a; Chen *et al.*, 2003; Mclauchlin *et al.*, 2004).

Gastroenteritis caused by *L. monocytogenes* is self-limiting with symptoms varying from fever, non-bloody diarrhoea, arthromyalgia, and headache (de Noordhout *et al.*, 2014; NICD, 2017a). Usually, the incubation period for gastroenteritis is less than 24 hours with the duration of the symptoms ranging from 1 – 3 days (NICD, 2017a). When listeriosis occurs during pregnancy, the incubation period can range from 17 – 67 days (Goulet *et al.*, 2013), presenting with mild flu-like symptoms, fever, backache, and headache (Mateus *et al.*, 2013). Maternal infections occur most often during the third trimester, this being the time where T-cell immunity is most suppressed (Allerberger & Wagner, 2010). Infection of pregnant women with *L. monocytogenes* can lead to premature birth, stillbirth, or spontaneous abortions (Committee Opinion No. 614, 2014; de Noordhout *et al.*, 2014). In pregnant women, sepsis often results in placental infection, which could lead to premature onset of labour and neonatal sepsis (NICD, 2017a). While infected mothers may be asymptomatic or only experience mild symptoms such as fever and diarrhoea, the neonatal illness is often severe and could lead to death of the child (Lamont & Sobel, 2011; Todd & Notermans, 2011; Committee Opinion No. 614, 2014). The two clinical forms of neonatal listeriosis are early onset (first seven days after birth) and late-onset (up to about four weeks after birth) (Allerberger & Wagner, 2010). Neonatal infection can occur by means of vertical transmission from mother to baby (e.g. via the vagina during natural birth), via inhalation of the infected amniotic fluid, or through transplacental infection from the mother's circulation (Becroft *et al.*, 1971; NICD, 2017a). Studies have shown that ampicillin administration at high doses, for prolonged use, can improve the chances of neonatal survival significantly (Lamont & Sobel, 2011). It is thus imperative that obstetricians are knowledgeable about the diagnosis and treatment of listeriosis, and moreover, how to prevent infection (Lamont & Sobel, 2011).

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Because listeriosis is not associated with the usual gastrointestinal symptoms often linked with foodborne illnesses, infection with *L. monocytogenes* is difficult to diagnose (Lamont & Sobel, 2011). Stool specimens are not routinely screened for *L. monocytogenes* due to the following reasons (Committee Opinion No. 614, 2014): gastroenteritis caused by *L. monocytogenes* is usually resolved within a few days in healthy individuals; the presence of *L. monocytogenes* in stool is short-lived, making it difficult to interpret positive or negative stool cultures; stool culture may present a false positive. Febrile gastroenteritis due to *L. monocytogenes* has a short incubation period (less than 48 hours), therefore the isolation of the bacterium from stool could assist in the identification of contaminated foodstuffs, making it particularly useful during listeriosis outbreaks (NICD, 2017a).

2.6.3. Treatment of listeriosis

In healthy individuals there is typically no treatment involved for listeriosis. *Listeria monocytogenes* usually concentrate in the liver, where they are removed from the circulatory system (Goulet *et al.*, 2013), making the disease self-limiting. Infection would thus be resolved by the time a diagnosis is made. In general, antibiotics that are able to target Gram-positive bacteria will be effective against *L. monocytogenes* (Maćkiw *et al.*, 2016). Bacteriostatic antibiotics are not effective due to *L. monocytogenes*' intracellular life cycle (Committee Opinion No. 614, 2014). Therefore, the preferred treatment for listeriosis is β-lactam antibiotics, such as penicillin or ampicillin, alone or in combination with other antibiotics such as aminoglycosides (Hof *et al.*, 1997; Charpentier & Courvalin, 1999; Allerberger & Wagner, 2010; Mateus *et al.*, 2013; Maćkiw *et al.*, 2016). Beta-lactam antibiotics are some of the most important and most used antibiotics (Jones *et al.*, 1997; Gullberg, 2014). They contain a nitrogen-containing beta-lactam ring in their molecular structure and work by binding a group of enzymes found anchored in the bacterial cell membrane, called penicillin-binding proteins (PBPs) (Papich & Papich, 2016). These proteins are involved in bacterial cell wall synthesis. Therefore, once these proteins are bound, they are unable to maintain the cellular wall structure. The inhibition of bacterial cell wall synthesis leads to autolysis and prevents infection in the host (Papich & Papich, 2016). The main target for β-lactams in *L. monocytogenes* is PBP 3 (Vicente *et al.*, 1990; Hof *et al.*, 1997). Cephalosporin antibiotics bind poorly to PBP 3 (Vicente *et al.*, 1990; Hof *et al.*, 1997), which could be the reason why *L. monocytogenes* is inherently resistant to cephalosporins. Although third-generation cephalosporins are used to treat other forms of meningitis, they are ineffective against listerial meningitis. The efficacy of ampicillin against *L. monocytogenes* is quite surprising due to poor penetration into the cerebrospinal fluid and low intracellular concentration, even at high dosages (Lutsar *et al.*, 1998). In spite of this, ampicillin inhibits the production of LLO and beta-galactosidase (both virulence factors), which may be the reason why it is still the

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recommended treatment for *L. monocytogenes* infection (Hof *et al.*, 1997). Although *L. monocytogenes* displays some resistance to ampicillin, it remains the preferred antibiotic due to its ability to penetrate the host cell efficiently (Lamont & Sobel, 2011).

Aminoglycosides (such as gentamicin) are bactericidal and work by inhibiting protein synthesis, specifically by binding the 30S subunit of ribosomes (Gullberg, 2014). Due to the similarity in structure between bacterial and human mitochondrial ribosomes, some antibiotics can often be damaging to the human mitochondria (Gullberg, 2014). Therefore the use of gentamicin is limited because of its toxicity and frequent side effects that include kidney damage and hearing loss (Lutsar *et al.*, 1998; Gullberg, 2014). They are often used together with β-lactams, but the effectiveness of this combination is still unclear (Bamford *et al.*, 2017). One study showed that gentamicin therapy will likely lead to earlier mortality (Mitjà *et al.*, 2009), whereas other studies showed combination therapy to be effective (Allerberger & Wagner, 2010; Thønnings *et al.*, 2016; Vasconcelos *et al.*, 2016).

Tetracyclines are broad-spectrum antibiotics, that are active against a wide range of bacteria (Gullberg, 2014). Due to the extensive use of tetracycline in the agricultural industry, and the subsequent increase in *L. monocytogenes* resistance towards tetracycline, it is no longer recommended for clinical use (MacGowan *et al.*, 1990; Gullberg, 2014). Chloramphenicol and macrolides (such as erythromycin) work by means of irreversible binding to the 50S subunit of the prokaryotic ribosomal subunits (Jelić & Antolović, 2016). Erythromycin is often used to treat listeriosis as a second-line treatment (Temple and Nahata, 2000).

In South Africa, listerial meningitis is currently the second most common cause of bacterial meningitis (Bamford *et al.*, 2017). In the past, patients that presented with meningitis symptoms were treated with ampicillin and gentamicin. However, this changed after the 2017-18 listeriosis outbreak, and third-generation cephalosporin treatment, together with ampicillin and gentamicin, is now recommended (NICD, 2017b; Schutte *et al.*, 2019). Immunocompromised individuals are usually treated with oral ampicillin or cotrimoxazole (NICD, 2017a). For patients that are allergic to β-lactams, treatment may be a combination of trimethoprim and a sulphonamide such as sulfamethoxazole (Hof *et al.*, 1997; Allerberger & Wagner, 2010; Maćkiw *et al.*, 2016). In South Africa, patients are treated with cotrimoxazole, or vancomycin together with gentamicin (NICD, 2017b). Clinicians also use trimethoprim-sulfamethoxazole or ampicillin in combination with gentamicin (Vasconcelos *et al.*, 2016). In order for antibiotic treatment against *L. monocytogenes* to be effective, the antibiotics should be able to penetrate and distribute within the host cell (Lamont & Sobel, 2011). Because of this, the inefficiency of antibiotics in listeriosis cases may be up to 70% (Hof, 2004). Currently, *L. monocytogenes* is treated successfully

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with ampicillin, penicillin, and amoxicillin (Temple & Nahata, 2000), but exhibits resistance towards cephalosporins, clindamycin, and chloramphenicol (Mylonakis *et al.*, 2002).

Although listeriosis in pregnant women could lead to adverse outcomes, early treatment may be effective at preventing such outcomes (Sisó *et al.*, 2012). It could be sensible to treat these patients pre-emptively with ampicillin when it is suspected that the patient could test positive for *L. monocytogenes* (Committee Opinion No. 614, 2014). Ampicillin is able to cross the placental barrier, bind to PBP3, and kill the bacteria (Lamont & Sobel, 2011). However, the importance of well-informed obstetricians should be stressed. It is crucial that they are knowledgeable about the diagnosis and treatment of *L. monocytogenes* infection and how to prevent infection so as to eliminate complications during pregnancy (Lamont & Sobel, 2011). When informing pregnant women about the risks of *L. monocytogenes* infection, emphasis should be placed on possible food sources, preventative measures, and the mortality rates associated with the disease (Mateus *et al.*, 2013).

2.6.4. Antibiotic resistance

The fact that *L. monocytogenes* is ubiquitous and able to grow in colder environments, is of great concern to the food processing environment (Olaimat *et al.*, 2018). This together with the emergence of resistant strains in the clinical environment has shifted focus towards the mechanisms by which food isolates are acquiring resistance to various antibiotics used to treat infections (Olaimat *et al.*, 2018). Despite *L. monocytogenes* being susceptible to a range of antibiotics, the emergence of antibiotic resistant strains are of great concern, as the mortality rate from listeriosis is up to 30% (Chen *et al.*, 2010a; Gómez *et al.*, 2014; Maćkiw *et al.*, 2016; Vasconcelos *et al.*, 2016; Escolar *et al.*, 2017; Noll *et al.*, 2018; Olaimat *et al.*, 2018).

Antibiotic resistance in bacteria (Figure 2.4) can either be phenotypic, intrinsic, or acquired (Olivares *et al.*, 2013; Gullberg, 2014; Munita & Arias, 2016; MacGowan & Macnaughton, 2017). Phenotypic resistance is not inherited and is not a result of genetic change. Rather, it is transient, and depends on the environmental conditions in which the bacterium lives (Olivares *et al.*, 2013). This type of resistance is usually associated with persistence, stationary growth cells, and biofilms. Persistent cells are cells that are able to avoid the effects of antibiotics, without undergoing actual genetic changes. If the selective pressure of antibiotics is removed, persistent cells can become antibiotic susceptible again (Corona & Martinez, 2013; Olivares *et al.*, 2013). Bacteria are able to communicate by means of quorum sensing (Friera *et al.*, 2017), and such communication can activate virulence genes and even lead to the formation of biofilms. The presence of biofilms could contribute to antibiotic resistance in different ways. Firstly, bacteria located in the bottom part of the biofilm have decreased metabolic activity due to oxygen and

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nutrient deficiency (Olivares *et al.*, 2013; Friera *et al.*, 2017). Such conditions make the bacteria dormant, leading to enhanced tolerance to antibiotics. This is especially the case with β-lactams which only work on actively growing bacteria (Gullberg, 2014). Secondly, by diffusing slowly through the matrix, the effect of the antibiotic is weakened. Biofilms in medical devices (such as catheters) are also at risk for re-colonization in a clinical setting (Olivares *et al.*, 2013; Friera *et al.*, 2017). Thirdly, biofilms are also associated with the presence of multidrug resistant (MDR) bacteria (Friera *et al.*, 2017). *Listeria monocytogenes* often forms part of a multispecies biofilm in the processing environment. This exposure to other *Listeria* spp. or even other species of bacteria could contribute to the horizontal gene transfer between strains of its own species or others (Allen *et al.*, 2016). Toomey *et al*. (2009) examined the ability of lactic acid bacteria (LAB) to transfer antimicrobial resistant genes to other species of bacteria. It was found that within a whole-milk matrix, genes conferring resistance to erythromycin and tetracycline were readily transferred from LAB to *L. monocytogenes.* Intrinsic resistance is defined as "resistance without any chromosomal mutation or acquisition of resistance genes" (Nikaido, 1994). This can either be because the bacteria lack a target site for the antibiotic, or because they have barriers in their cell wall that decreases permeability (Gullberg, 2014). An example of this is Gram-negative bacteria whose cell membrane is inherently impermeable to many antibiotic molecules (Cox & Wright, 2013). This can be seen in all *L. monocytogenes* strains that are intrinsically resistant to cephalosporins (Krawczyk-Balska & Markiewicz, 2016).

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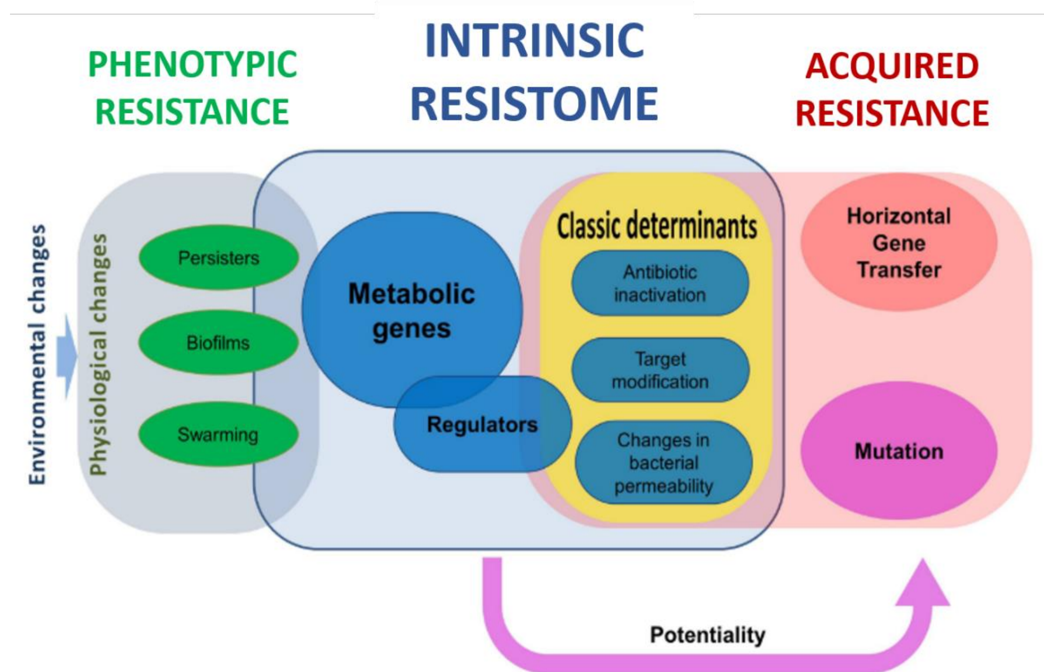

Figure 2.4 Bacteria can acquire resistance to antibiotics by means of horizontal gene transfer (from other bacteria) or by mutation of their own genes; phenotypic resistance can occur by means of biofilm growth, swarming adaptation or persistent cells (Olivares *et al.*, 2013 used with permission).

Acquired resistance occurs when otherwise susceptible bacteria are able to gain resistance due to mutations in their chromosomal genes or by gene transfer from other bacteria (Gullberg, 2014; Munita & Arias, 2016; MacGowan & Macnaughton, 2017). Antibiotic resistance can arise in several ways (Figure 2.5): alteration of antibiotic target sites, use of efflux pumps to eject antibiotics out of cells, reduced cell permeability, and enzymatic modification or inactivation (Wright, 2010; Gullberg, 2014; Allen *et al.*, 2016; Munita & Arias, 2016). Efflux pumps are prevalent features of all organisms and are used to force out toxic molecules that may harm host cells (Cox & Wright, 2013). But these efflux pumps could also expel antibiotics. *Listeria monocytogenes* makes use of an efflux pump, AnrAB, which is responsible for conferring resistance against nisin and β-lactam antibiotics (such as ampicillin, penicillin, and oxacillin) (Collins *et al.*, 2010). Genetic transfer can occur by three different ways: 1) conjugation – bacteria transfer plasmids (genetic material) by direct contact with each other; 2) transduction – the host bacterium's genes are incorporated into a bacteriophage, which then transfers this genetic material to another host bacterium; 3) transformation – bacteria take up naked DNA (foreign genetic material) from their environment and incorporate it into their own genome (Munita & Arias, 2016; MacGowan & Macnaughton, 2017).

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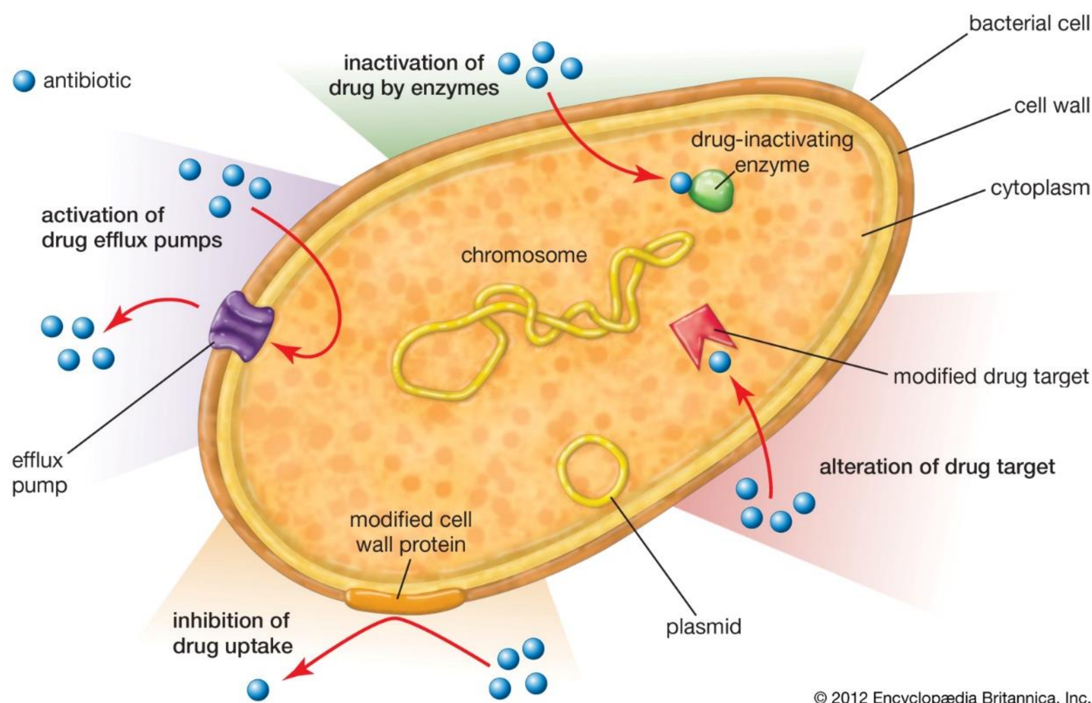

Figure 2.5 Mechanisms of antibiotic resistance can include inactivating enzymes, efflux pumps that pump antibiotics out of the cell, alteration of the bacterial cell wall, and the modification of the antibiotic target on the cell (by courtesy of Encyclopaedia Britannica Inc. © 2012, used with permission).

A few strategies have been proposed to reduce the risk of increased antibiotic resistance in recent years. Firstly, over-the-counter antibiotic prescriptions require stricter control and prescribers. Secondly, the general public consuming these antibiotics, could be better informed in order to prevent injudicious prescriptions and consumption of antibiotics (Davies & Davies, 2010; Wright, 2010; MacGowan & Macnaughton, 2017). It has been demonstrated recently that intrinsic resistance has existed long before the human use of antibiotics, but that injudicious use of antibiotics could exacerbate the resistance of bacteria as seen in clinical settings (Cox & Wright, 2013). The reduced use of antibiotics will reduce bacterial exposure to these chemicals, limiting the ability of the bacteria to acquire resistance (Wright, 2010). Intensifying surveillance of antibiotic use in clinical settings and in the farming environment will enable action to be taken early enough should resistance against existing antibiotics increase (Wright, 2010; MacGowan & Macnaughton, 2017).

2.7. Reduction of *Listeria monocytogenes* by use of bacteriophages

Bacteriophages (also known as viruses, and used interchangeably with the word "phage") are predatory organisms with the ability to infect bacterial cells (Figure 2.6). They are abundant in the environment and as a result are consumed unknowingly on a regular basis (García *et al.*, 2010). They are thus presumed to be safe for consumption with no adverse effects having ever been reported (Mahony *et al.*, 2011; Komora *et al.*, 2018). They are exceptionally host-specific

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which enables the phages to target one bacterial species without affecting another (Guenther *et al.*, 2009; Moye *et al.*, 2018).

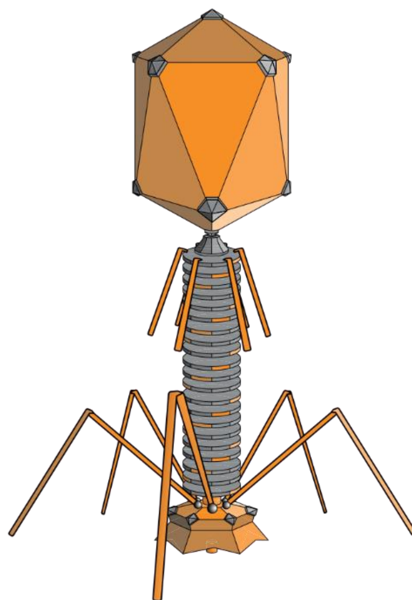

Figure 2.6 Anatomy of a bacteriophage typical of the *Myoviridae* family (Wikimedia, used with permission).

Bacteriophages were first discovered in 1915 when Frederick Twort noticed "glassy colonies" that had an antibacterial effect (Twort, 1915; El-Shibiny & El-Sahhar, 2017; Kakasis & Panitsa, 2019). Not long after the discovery, bacteriophages were successfully used to treat conditions such as staphylococcal skin disease, urinary tract infections, and surgical infections (Kakasis & Panitsa, 2019), just to name a few. However, the rise of the antibiotic era soon started and bacteriophage therapy shifted to the background (Kakasis & Panitsa, 2019). Unlike antibiotics, where the focus of treatment is on the infected patient, the use of bacteriophages is now mostly focused on eradicating spoilage and pathogenic bacteria from food products and the food processing environment. Several other approaches are already in use in the food processing environment. This includes treatments such as pasteurization, chemical sanitizers, irradiation, or high-pressure processing (HPP) (Moye *et al.*, 2018). There are some disadvantages associated with these approaches. Pasteurization can mostly be used only in liquid products, while HPP cannot be used on fresh food products (such as meat or produce). Irradiation could have a detrimental effect on the sensory qualities of food, and chemical sanitizers, although effective, have negative associations due to their harmful effect on the environment (Moye *et al.*, 2018). These methods also have a tendency to kill all (or most) of the microorganisms present, even potentially beneficial microorganisms (Moye *et al.*, 2018). This has led to an interest in the use of bacteriophages to reduce the microbial load not only in foods but also in the food processing

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environment, whilst simultaneously preserving the beneficial microflora (Gutiérrez *et al.*, 2016; Fister *et al.*, 2019).

Based on their lifestyle, phages can be divided into two types, namely temperate and virulent phages (Figure 2.7). Virulent (lytic) phages are able to multiply within the host cell, and with subsequent bursting of the cell, a new generation of phage progeny is released (García *et al.*, 2010). Temperate phages are different in that they first enter the lysogenic phase when the phage DNA integrates with the host chromosome, becoming a prophage. They can enter the lytic phase when the prophages exit the host chromosome (García *et al.*, 2010). It is extremely important that temperate phages are not used in the food industry. Because of their ability to integrate their DNA into the host chromosome, they are capable of passing on virulent genes, leading to strains with new pathogenic potential (Moye *et al.*, 2018). This can be seen in pathogens such as *Escherichia coli* (with Shiga-toxin producing prophages) and *Vibrio cholera* (containing a phage that encodes for the cholera toxin). Thus prophages are vital in the adaptation of bacteria and their genetic diversity (Fortier & Sekulovic, 2013).

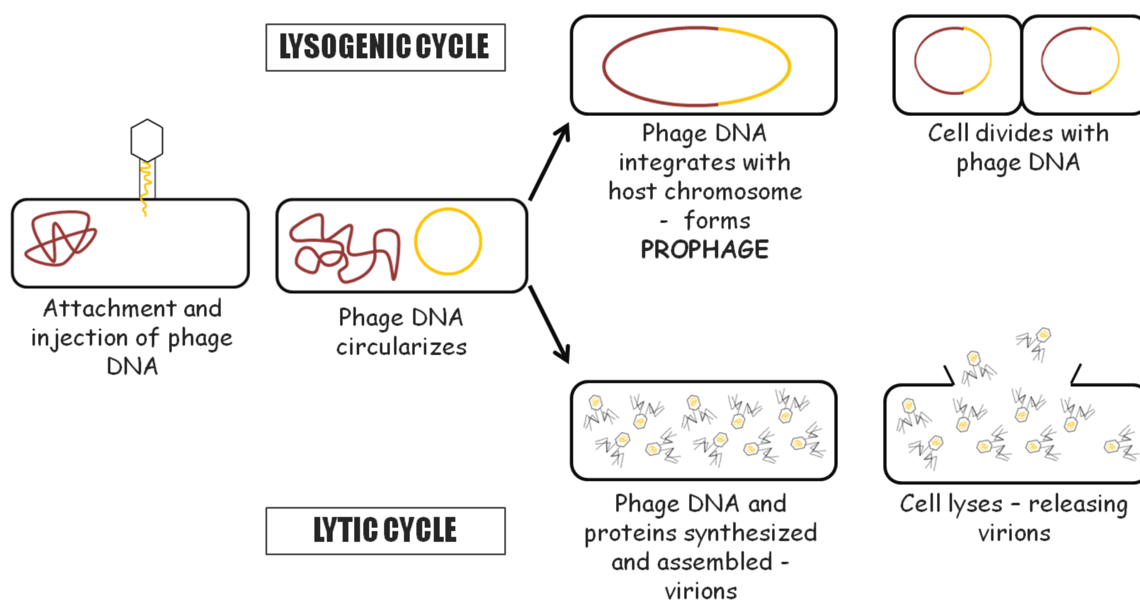

Figure 2.7 The life cycle of the bacteriophage can consist of two phases, namely the lysogenic phase, which leads to the formation of prophages, and the lytic phase which leads to cell lysis.

For the lytic phase to occur, the phage first needs to recognise and attach to the host, with subsequent insertion of nucleic acid. This attachment is mediated by receptor binding proteins (RPBs) on the phage tail fibres which recognize different receptors on the host cell. For phages from the *Myovidae* family (e.g. Myovirus A511 and P100), the two receptors that are utilized are wall teichoic acids (WTAs) and peptidoglycan (Figure 2.8). WTAs are the most abundant glycopolymers covalently bound to peptidoglycan on the bacterial cell wall (Shen *et al.*, 2017). The WTAs have several other biological roles in antibiotic resistance, virulence, and also phage

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attachment (Shen *et al.*, 2017; Dunne *et al.*, 2018). Different serotypes of *L. monocytogenes* have different sugar residues attached to the ribitol-phosphate backbone of the WTAs. The 1/2 serotype group's WTAs features L-rhamnose and *N*-acetylglucosamine (GlcNAc), while the WTAs of serotype 4b consists of GlcNAc with additional D-glucose and D-galactose (Bielmann *et al.*, 2015; Carvalho *et al.*, 2015). These wall decorations serve as receptors for phages, and if absent, could lead to non-attachment of a bacteriophage (Promadej *et al.*, 1999; Spears *et al.*, 2016). The cell wall teichoic acid glycosylation protein, *gtc*A, is associated with serotype 4b and is essential for the attachment of glucose and galactose to these WTAs. Mutations in this gene leads to a lack of galactose and reduced glucose, which in turn, reduces the probability of phage attachment (Promadej *et al.*, 1999; Spears *et al.*, 2016).

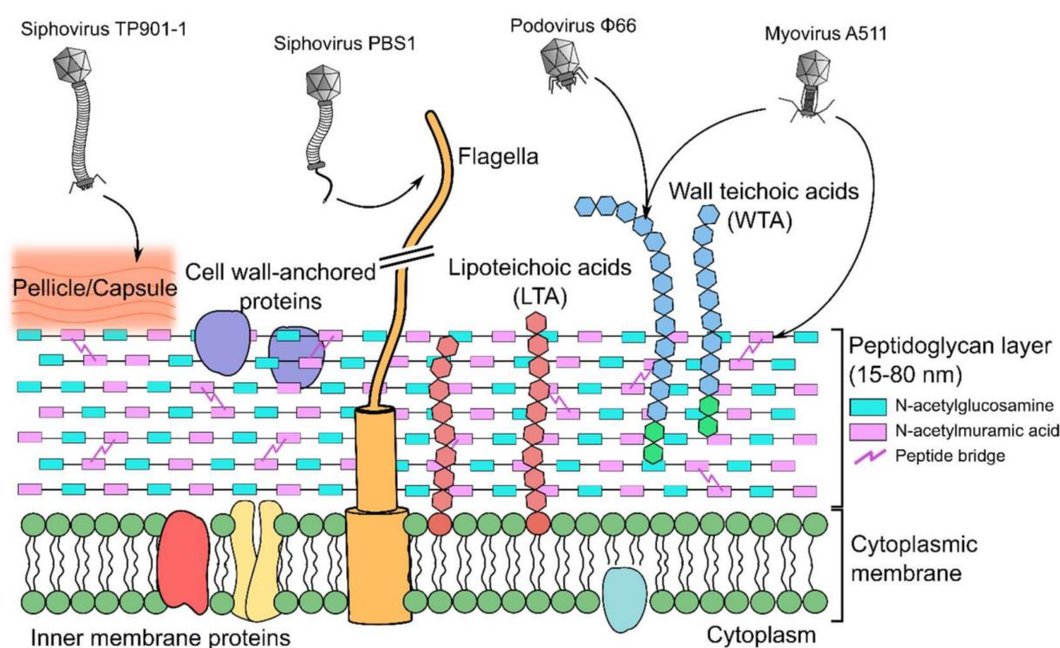

Figure 2.8 Different bacteriophages and their associated host receptors on the Gram-positive bacterial cell wall (Dunne *et al.*, 2018 used with permission).

After the phage genome is replicated, it takes over the host cell, forcing it to produce structural proteins solely for the phage. These proteins congregate to form new virions and lysis proteins, which causes the destruction of the host cell (García *et al.*, 2010). It is this destruction of the host bacterial cell which make phages useful for antibacterial activity. Phages which show lytic activity are particularly useful in the food processing environment since they don't incorporate their genome into the chromosome of the bacteria to form prophages but instead always eliminate the bacterial target cells (Guenther *et al.*, 2009). Previous studies have shown the application of bacteriophages to be useful against *L. monocytogenes* in the RTE food industry (Table 2.1) (Guenther *et al.*, 2009; García *et al.*, 2010; Moye *et al.*, 2018). The commercial bacteriophages used

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(PhageGuard Listex™, A511, Listshield™) were able to reduce bacterial counts on various food products by 1.8 to 5 log reductions (Carlton *et al.*, 2005; Guenther *et al.*, 2009; Soni & Nannapaneni, 2010b; Chibeu *et al.*, 2013; Oliveira *et al.*, 2014; Moye *et al.*, 2018). But even 1 to 2 log reductions in products such as deli meat could still reduce the mortality rate by 50% and 74%, respectively, for elderly people (Moye *et al.*, 2018). This indicates that although phage treatment is not able to completely eradicate *L. monocytogenes* from a food product, it is still beneficial in reducing the associated risk of certain foods.

Table 2.1 Results from the application of bacteriophage treatment on different foods

Food product	**Phage tested**	**Result**	**Reference**
Soft cheese	PhageGuard Listex™	A 3.5 log reduction after a single application, surviving colonies did not develop resistance	(Carlton *et al.*, 2005)
Chocolate milk, mozzarella cheese brine	A511, PhageGuard Listex™	*L. monocytogenes* completely eradicated	(Guenther *et al.*, 2009)
RTE solid foods	A511, PhageGuard Listex™	Up to 5 log reduction	(Guenther *et al.*, 2009)
Raw salmon fillets	PhageGuard Listex™	Up to 3.5 log reduction	(Soni & Nannapaneni, 2010b)
Raw catfish fillets	PhageGuard Listex™	Up to 2.3 log reduction, with no subsequent growth observed	(Soni & Nannapaneni, 2010b)
Cooked chicken	FWLLm1	Up to 2 log reduction, with subsequent growth observed	(Bigot *et al.*, 2011)
Melon and pear slices	PhageGuard Listex™	Up to 1.5 log reduction	(Oliveira *et al.*, 2014)
Apple slices	PhageGuard Listex™	No reduction	(Oliveira *et al.*, 2014)
RTE lettuce	ListShield™	Up to 1.1 log reduction	(Perera *et al.*, 2015)

Although relatively new, the application of phages in foods and the food processing environment may be more easily accepted by consumers than the use of preservatives. Since phages are isolated from the environment, they are considered "natural" and "environmentally-friendly". The idea that they target pathogenic bacteria without disturbing the natural microflora of the product means that consumers are potentially more likely to accept this new technology (Moye *et al.*, 2018). Nevertheless, convincing consumers that "viruses" can be beneficial for their food might be a challenge, although one study demonstrated that consumers are willing to pay more for a bacteriophage-treated product once they are informed about the food safety benefits (Naanwaab *et al.*, 2014). Therefore, it is imperative that consumers (and food manufacturers) be informed of possible benefits and safety of bacteriophages.

Bacteriophage control is not without disadvantages. Their specificity can also be seen as a limiting factor for use in the food industry, which is why it is not yet as widespread as other

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biopreservatives (García *et al.*, 2010). This high specificity means that in order to deal with foods that contain more than one food pathogen, a phage cocktail (a mixture containing more than one phage) needs to be used. There are different factors that influence the efficacy of a bacteriophage. The phage concentration, as well as the type of food sample and storage temperature, are all important (Guenther *et al.*, 2009). Furthermore, the phage and target bacteria need to be in contact with one another in order for bacterial eradication to occur (Hagens & Loessner, 2010). This can be challenging in a food processing environment, where there are numerous areas that can conceal bacteria. It is also suggested that the phage progeny might not be able to get into contact with pathogenic bacteria on the foodstuff, meaning increased phage concentration does not necessarily correlate with bacterial host reduction (Moye *et al.*, 2018). The food medium can also have an influence on the diffusion of the phage, making phage treatment potentially more effective on liquid foods in comparison to drier, solid foods (Moye *et al.*, 2018). Because the use of bacteriophages in the food industry is relatively new, not much is yet known about the bacterial host's resistance mechanisms or the possible occurrence of resistant bacteria in the food processing environment (Fister *et al.*, 2016). Phage resistance should thus not be excluded as a possibility (Guenther *et al.*, 2009; Fister *et al.*, 2016; Moye *et al.*, 2018). This could be due to bacteria possessing mechanisms that could either prohibit entry into the cell or by preventing phage replication (Fister *et al.*, 2016). It is proposed that phages with a broad host range be used in order to avoid potential resistance, or by applying phages with different host ranges in rotation (Guenther *et al.*, 2009). Extensive use of phage treatment in the food processing environment should also be avoided. This will reduce the long-term exposure of the bacterial host to the phage, thereby preventing the host bacterium from acquiring possible resistance (Moye *et al.*, 2018).

2.8. Surveillance data and the lack of information on listeriosis in South Africa

In developing countries, surveillance programs documenting foodborne illnesses are rarely put into practice (Todd & Notermans, 2011). In South Africa, there is currently no active monitoring being done on foodborne illnesses, and as a result these illnesses often remain underreported (Smith *et al.*, 2016). Although numerous studies have characterized *L. monocytogenes* from food processing facilities globally, not many outputs have been generated regarding *L. monocytogenes* in the South African setting (Rip & Gouws, in press).

In South Africa, a national listeriosis outbreak was declared in December 2017 and is believed to be the largest-ever global outbreak of listeriosis. As of 26 July 2018, there have been 1 060 laboratory-confirmed cases, where the cases with known outcome having a mortality rate of 27% (216/806) (NICD, 2018b). Prior to December 2017, listeriosis was not a notifiable disease

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with only a few sporadic cases reported (NICD, 2018a), hence published data relating to foodborne listeriosis is lacking in South Africa. In the Western Cape Province (South Africa) during September 2015 there was an increase in the number of human cases in which *L. monocytogenes* were isolated (Smith *et al.*, 2016). These isolates belonged to sequence type 6 (ST6), a sequence type often associated with listeriosis cases. In fact, 92% of clinical isolates obtained from patients during the 2017-18 outbreak belonged to ST6 (Smith *et al.*, 2016; NICD, 2018b; Schutte *et al.*, 2019). The 2017-18 SA listeriosis outbreak was officially declared to be over by the Minister of Health in September 2018, but the long incubation period of listeriosis means that new sporadic cases are still anticipated (DoH, 2018a). There is not much historical data on the economic burden of food pathogens in South Africa. However, a study by Olanya *et al.* (2019) estimated that the recall cost of the 2017-18 listeriosis outbreaks alone was $28.1 million. In the USA *L. monocytogenes* is the third most costly food pathogen, with an annual cost of between US$2.3 billion to $22 billion (de Noordhout *et al.*, 2014). In 2008, a listeriosis outbreak in Canada related to deli meat resulted in a $43 million loss for the company Maple Leaf (Fallatah, 2018). Additionally, the company had to settle $27 million in lawsuits. Thus, listeriosis is responsible for major economic impacts.

A challenge to epidemiological studies and exposure assessment of *L. monocytogenes* is the lack of data, with the infectious dose still unknown (Todd and Notermans, 2011). This leads to most companies in the food industry having a zero-tolerance policy requiring the absence of *Listeria* in a 25 g food sample. Even so, this stringent policy has not shown to be better for public health than more flexible criteria such as <100 *L. monocytogenes* CFU/g food (Forsythe, 2010; Montville *et al.*, 2012). In South Africa, there is no information on the microbiological limits of *L. monocytogenes* in food. The recently updated Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act No. 54 of 1972) includes strict regulations for bacteria such as *Salmonella*, *Escherichia coli*, and *Staphylococcus aureus*, but makes no mention of *L. monocytogenes.* The South African National Standard (SANS 885:2011) recommends a maximum allowable amount of 100 CFU/g at the end of shelf life in processed meat products. But this is merely a recommendation and not a rule enforceable by law. In order to ensure that exported foods are up to international standards, the microbial limits of *L. monocytogenes* set by different countries are used. In Canada (Bureau of Microbial Hazards, 2011), Europe (European Commission, 1999), Australia and New Zealand (Food Standards Australia New Zealand, 2014), a differentiation is made between foods that can support the growth of *L. monocytogenes* and those that cannot. According to the Compendium of Microbiological Criteria for Food (2016), foods that cannot support the growth of *L. monocytogenes* have the following criteria: a pH lower than 4.4, water activity less than 0.92, or a combination of pH less than 5.0 and water activity of less than 0.94. In RTE foods that support the growth of *L. monocytogenes,* the bacterium should not be detected in 5 x 25 g of sample. In

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foods that cannot support growth of the bacterium, the levels of *L. monocytogenes* should be less than 100 CFU/g.

In order to do better research into complex health challenges, a One Health approach (applied worldwide) has been proposed where multidisciplinary research comes together to give a better understanding of problems and potential solutions (Lebov *et al.*, 2017). In order to establish the origin and impact of *L. monocytogenes*, the food industry and its various processes can be linked with clinical aspects (e.g. hospital cases). The One Health approach focuses on three domains, namely environment, animal, and human (Lebov *et al.*, 2017). An example of this method would be to study soil and water where *L. monocytogenes* is found and the food processing industry (environment), the characteristics of the bacteria and the different strains (animal), and the listeriosis patients' behaviour and susceptibility factors (human) (Lebov *et al.*, 2017). This is especially important in a country such as South Africa, where it is estimated that 7.9 million South Africans are living with HIV, and about 330 000 people live with both HIV and tuberculosis (Molapo & Massy, 2019). Although the frequency of *L. monocytogenes* infection is low, the high degree of mortality, especially among the immunocompromised, validates the need to monitor epidemiological data (Montero *et al.*, 2015). By comparing *L. monocytogenes* isolates from both food samples and clinical cases, the probable source of contamination could be revealed, allowing the identification and regulation of foods that pose a health risk to the public (Montero *et al.*, 2015).

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Chapter 3

Lineage classification of *Listeria monocytogenes* isolates from food, environmental, and clinical origin by means of PCR-RFLP

3.1. Abstract

There is a paucity of South African literature investigating the relationships between *Listeria monocytogenes* lineages isolated from clinical and food processing environments. For this study, *L. monocytogenes* isolates from various origins (Clinical, Environmental, Raw meats, Raw seafood, and Ready-to-eat) were classified into one of three different lineage groups by using a recently developed method for PCR-RFLP (based on SNPs within the *hly*A gene of the bacterium). In addition, *L. monocytogenes* lineage groupings were scrutinised for patterns within the various categories of origin. The results showed an overrepresentation of Lineage I in the Clinical and Raw seafood, while in the Environmental, Raw meats, and Ready-to-eat categories, Lineages I and II were somewhat equally distributed. This finding was in contrast to other studies that found Lineage I to be mostly associated with human listeriosis, and Lineage II to be more frequently associated with foods. For *L. monocytogenes*, a clear association has not yet been made between different food types and lineage, so this study provides valuable insight into the distribution of the different lineages across a range of origins, especially in the South African context.

3.2. Introduction

Foodborne illnesses are a major public health concern, and it is estimated that about 420 000 people die annually as a result of consuming food contaminated with pathogenic bacteria (WHO, 2015). In recent years, consumers' lifestyles have changed such that they are consuming less 'homemade' meals (that undergo less processing) and more 'instant' foods (that undergo more processing), thus exposing them to a larger variety of possible foodborne pathogens (Allerberger & Wagner, 2010; Aparecida De Oliveira *et al.*, 2010). These pathogens can include bacteria, viruses, or parasites and their presence in food is often attributed to improper food handling, preparation, or storage (Martinović *et al.*, 2016). The world population is growing and the demography is shifting towards a higher number of elderly persons, which means an increased number of immunocompromised individuals. (Newell *et al.*, 2010). With increasing global trade, food ingredients are no longer restricted to local sources. There are several role players in an expansive food supply chain that do not all have the same food safety standards. Therefore, the

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risk of the consumer being exposed to pathogens are increasing. Subsequently more and more foodborne illnesses are emerging illustrating the need for ongoing research.

Listeria monocytogenes is a Gram-positive bacterium responsible for the foodborne infection, listeriosis. It is ubiquitous in the environment, can tolerate high salt concentrations and is also able to grow at temperatures as low as 0°C (Forsythe, 2010; Nowak *et al.*, 2015). Because of its presence in soil and water, it is often associated with raw food material (White *et al.*, 2002; de Noordhout *et al.*, 2014). This, together with its ability to survive long periods in food production facilities due to the formation of biofilms, makes the bacterium a major threat to the food industry (Swaminathan & Gerner-Smidt, 2007). Despite listeriosis being a rare disease (for example when compared to *Salmonella* infection), the mortality rate can be as high as 30% (Swaminathan & Gerner-Smidt, 2007), thus making *L. monocytogenes* a key pathogen in the food processing environment.

Listeria monocytogenes can be classified into three common lineages, namely I, II, and III, and these three lineages can together be further subdivided into 12 serotypes. Of the 12 serotypes, the three most notable are 1/2a, 1/2b, and 4b, which together account for more than 95% of listeriosis cases (Jadhav *et al.*, 2012; Hyden *et al.*, 2016). However, several studies agree that *L. monocytogenes* serotypes do not all have the same degree of virulence (Wiedmann *et al.*, 1997; Mclauchlin *et al.*, 2004; Ward *et al.*, 2004; Swaminathan and Gerner-Smidt, 2007; Manuel *et al.*, 2015). Isolates from Lineage I (1/2b and 4b) are mostly from human clinical cases of listeriosis, while isolates from Lineage II (1/2a and 1/2c) are mostly associated with foods and food processing facilities (Swaminathan & Gerner-Smidt, 2007; Nightingale *et al.*, 2008; Orsi *et al.*, 2010; Manuel *et al.*, 2015). Despite Lineage I isolates being overrepresented in human clinical cases, isolates from Lineage II can also cause listeriosis. Moreover, even though there are differences in the ratios of Lineages I and II and their respective associated environments, food is still thought to be the principal vehicle of *L. monocytogenes* contamination (Swaminathan & Gerner-Smidt, 2007; Leong *et al.*, 2016; Buchanan *et al.*, 2017). The reasons for Lineage II isolates being so common among food isolates are still unclear; however, several researchers propose the idea that these differences in frequency are due to mutations and variations in the bacterium's virulence genes (Vines *et al.*, 1992). Such mutations thus enable certain lineage groups to either adapt to the food processing facility (due to environmental pressures) or to establish easier within hosts by crossing the intestinal barriers (Manuel *et al.*, 2015). Due to these differences occurring between lineage groups, molecular characterization of *L. monocytogenes* can provide valuable insight as to how these different lineages associate with different origins.

The virulence gene *hly*A is innate to all *L. monocytogenes* bacteria and encodes for a pore-forming haemolysin (listeriolysin O) that allows the bacterium to escape the vacuole of the host's

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cells (Cossart *et al.*, 1989). Therefore, by using polymerase chain reaction (PCR), amplification of the *hly*A gene serves to confirm the presence of virulent *L. monocytogenes* strains. With PCR-restriction fragment length polymorphism (PCR-RFLP), *L. monocytogenes* isolates can successfully be differentiated into three different lineage groups, namely Lineage I (serotypes 1/2b, 3b, 3c and 4b), Lineage II (serotypes 1/2a, 1/2c and 3a), and Lineage III (serotypes 4a and 4c) (Rasmussen, O *et al.*, 1991; Vines *et al.*, 1992; Wiedmann *et al.*, 1997; De Cesare *et al.*, 2007; Bester, 2011; Meghdadi *et al.*, 2019). This classification ability is due to the presence of single nucleotide polymorphisms (SNPs) that are present in the virulence genes of *L. monocytogenes.* A SNP refers to a mutation in a DNA sequence, where a single nucleotide in a base pair can be substituted by another nucleotide (Schork *et al.*, 2000). These SNP's usually occur at specific points in the genome. Therefore, isolates with the same DNA sequences in the region of interest can be grouped together, and groups can be separated based on their differences in SNPs.

In South Africa, very few studies have yet attempted to investigate how lineage groupings of *L. monocytogenes* isolates relate to clinical and food processing environments (Ackermann, 2017; Rip & Gouws, in press). Considering that the largest ever recorded global outbreak of listeriosis recently occurred in South Africa (2017-18), and that different lineages can have different outcomes regarding listeriosis infection, an investigation into the relationships between lineage groups and how it disseminates in the food and the environment is warranted. In this study, *L. monocytogenes* isolates were obtained from various origins (clinical, food, and environmental) and, using a recently developed method for PCR-RFLP based on SNPs within the *hly*A gene of *L. monocytogenes* (Rip & Gouws, in press), classified isolates into one of three lineage groups. The main objectives of this study were thus: 1) to differentiate *L. monocytogenes* isolates from clinical, food, and environmental origin into their respective lineage groups, and 2) to determine whether certain lineage groups of *L. monocytogenes* are more prevalent in particular categories or not. Lineage I was expected to dominate in the Clinical Category, and Lineage II in isolates obtained from food origin.

3.3. Materials and methods

3.3.1. Sample collection and storage

A total of 192 isolates (presumed to be positive with *L. monocytogenes*) were collected from various origins. Twenty-two isolates from patients with listeriosis (Clinical) were received on 2% blood agar from the National Health Laboratory Service (NHLS Microbiology, Observatory) in the Western Cape region. Ethical clearance was obtained from NHLS *(HREC R020/2015)* and approved by the Research Ethics Committee: Biosafety and Environmental Ethics, Stellenbosch

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University *(Ethics #BEE-2018-1764)*. Presumed positive samples from various food origins (Environmental, Raw meats, Raw seafood, and Ready-to-eat) were received from an accredited food laboratory (Microchem) on either RAPID'L.Mono™ chromogenic agar (specific for *L. monocytogenes*) or PALCAM agar (specific for *Listeria* species). These plates were sealed and stored at -4°C until further examination. All isolates were also further divided into subcategories, where possible, within their respective categories (for example subdividing Environmental samples into equipment, drain, surfaces, etc.) (Addendum A). For the remainder of the chapter, "Ready-to-eat" refers specifically to one of the main categories in this study, whereas "RTE" refers to ready-to-eat foods in general.

3.3.2. Confirmation of *Listeria monocytogenes* presence

In order to confirm the presence of *L. monocytogenes* on acquired plates, colonies were streaked on RAPID'L.Mono™ agar and incubated for 24 h at 37°C. Once characteristic *L. monocytogenes* growth was observed (blue colonies with no halo), colonies from the RAPID'L.Mono™ were streaked on Tryptic Soy Agar (TSA) plates and incubated at 37°C for 20 – 24 h. The pure isolates were now ready to undergo a DNA isolation procedure and PCR. Glycerol stocks (25%) were also prepared from isolates grown on TSA for long term storage at -80°C.

3.3.3. DNA extraction and amplification

Isolates stored as glycerol stocks were grown overnight on Brain Heart Infusion (BHI) agar at 37°C. The genomic DNA was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (ZymoResearch) according to the manufacturer's instructions and subsequently stored at -20°C until further use. As a negative extraction control, DNase/RNase-free distilled water (UltraPure™, Thermo Fischer) was used instead of bacterial culture. In order to amplify the 730 bp region of the *hly*A gene, a forward primer with sequence 5'-CATTAGTGGAAAGATGGAATG-3' and reverse primer with sequence 5'-GTATCCTCCAGAGTGATCGA-3' were used (Blais *et al.*, 1995). PCR assays were performed in 25 µL reaction volumes, with a final concentration of the following compounds: 1X NH4 reaction buffer (Bioline), 0.2 mM of each of the four dNTPs (Thermo Scientific), 3 mM $MgCl_2$ (Bioline), 0.4 mM of each primer (IDT, Whitehead Scientific), 1.0 U of BIOTAQ Taq DNA polymerase (Bioline) (Rip and Gouws, in press) and 1 µL of undiluted template DNA. As a negative PCR control, DNase/RNase-free distilled water (UltraPure™, Thermo Fischer) was used instead of template DNA. As a positive control, *L. monocytogenes* ATCC 7644 was used. The amplification was performed in a T100™ Thermal Cycler (Bio-rad) with the following conditions: an initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, then a final extension at 72°C for 2 min.

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Bands were subsequently visualized by electrophoresis on 1.5% agarose gel (Lonza) stained with SmartGlow™ pre-stain (Whitehead Scientific). A 100 bp DNA ladder (GeneRuler, Thermo Scientific) was used as a reference marker. A running buffer of 1X TBE Buffer (Tris-borate-EDTA) was used and the gel viewed using Gel Doc™ XR+ System with Image Lab™ Software (Bio-Rad).

3.3.4. Restriction fragment length polymorphism (RFLP)

After PCR amplification of the *hly*A gene and visualisation by gel electrophoresis, the PCR amplicons were characterized by RFLPs (Rip and Gouws, in press). In a total volume of 15 µL, the restriction digestion mixture contained (final concentration): 3 µL PCR product, 1X FastDigest Green Buffer (Thermo Scientific), and Fast Digest restriction enzyme (0.5 unit/µL) (Thermo Scientific) and DNase/RNase-free distilled water (UltraPure™, Thermo Fischer). The digests were performed in a T100™ Thermal Cycler (Bio-rad) according to conditions as recommended by the supplier (Table 3.1). Products from the restriction digests were visualized by gel electrophoresis on a 1.5% agarose gel (Lonza, WhiteHead Scientific), stained with SmartGlow™ pre-stain (Whitehead Scientific). Undigested *L. monocytogenes* DNA was included as a negative control, and positive controls were included for Lineage I, II, and II, respectively (Table 3.1). A 100 bp DNA ladder (GeneRuler, Thermo Scientific) was used as a reference marker and gels were viewed using Gel Doc™ XR+ System with Image Lab™ Software (Bio-Rad). All isolates were subjected to digestion with each of the three restriction enzymes (Table 3.1), in three separate digests.

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Table 3.1 Enzymes and bacterial controls used to distinguish between lineage groups

Restriction enzyme*	Incubation conditions	Inactivation time	Cut site	Lineage	Expected band sizes (bp)*	Positive Controls
FastDigest *Nde* I	37°C for ≥ 60 min	65°C for 5 min	5'...CA↓TATG...3' 3'...GTAT↑AC...5'	I	320; 410	*Listeria monocytogenes* ATCC 23074 (serotype 4b)
FastDigest *Bfo*I	37°C for 5 min	65°C for 10 min	5'...RGCGC↓Y...3' 3'...Y↑CGCGR...5'	II	Cuts all serotypes: 274; 455 Lineage II: 178; 274; 278	*Listeria monocytogenes* ATCC 7644 (serotype 1/2c)
FastDigest *Bsh*12851	37°C for 15 min	80°C for 15 min	5'...CGRY↓CG...3' 3'...GC↑YRGC...5'	III	340; 390	*Listeria monocytogenes* ATCC 19114 (serotype 4a)

*Enzyme digest by means of restriction fragment length polymorphism (RFLP). See text for more details (Rip and Gouws, in press).

3.3.5. Statistical analysis

All statistical analyses were conducted in the R statistical environment (version 3.5.1) (R Core Team, 2017). In order to determine whether or not lineage groupings were significantly disproportionate, a series of binomial tests were conducted for each of the various categories. Binomial tests were chosen since in all cases experimental outcomes were binary, i.e. isolates were either classified as Lineage I or Lineage II according to the aforementioned methods. Expected probabilities of 0.5 were used in all instances in order to test the null hypothesis that lineage groupings were no different from random chance (i.e. proportions not significantly different from 50%). The binomial tests were performed with the function binom.test from the base package.

3.4. Results and Discussion

3.4.1. Positive *Listeria monocytogenes* samples

Of the 192 samples received, 180 presented with colonies that were presumed positive for *L. monocytogenes* on RAPID'L.Mono™ chromogenic plates (Oxoid) (Figure 3.1). These 180 isolates were selected for further study. Other types of growth observed was characteristic of either *L. innocua* and *L. welshimeri* (white colonies), or *L. ivanovii* (blue colonies with a halo). The phosphatidylinositol-phospholipase C (PIPLC) activity of *L. monocytogenes* makes it identifiable on RAPID'L.Mono™. It is unable to metabolise xylose, therefore it produces colonies without a

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halo. Only the samples that were positively identified as *L. monocytogenes* were used for further analyses.

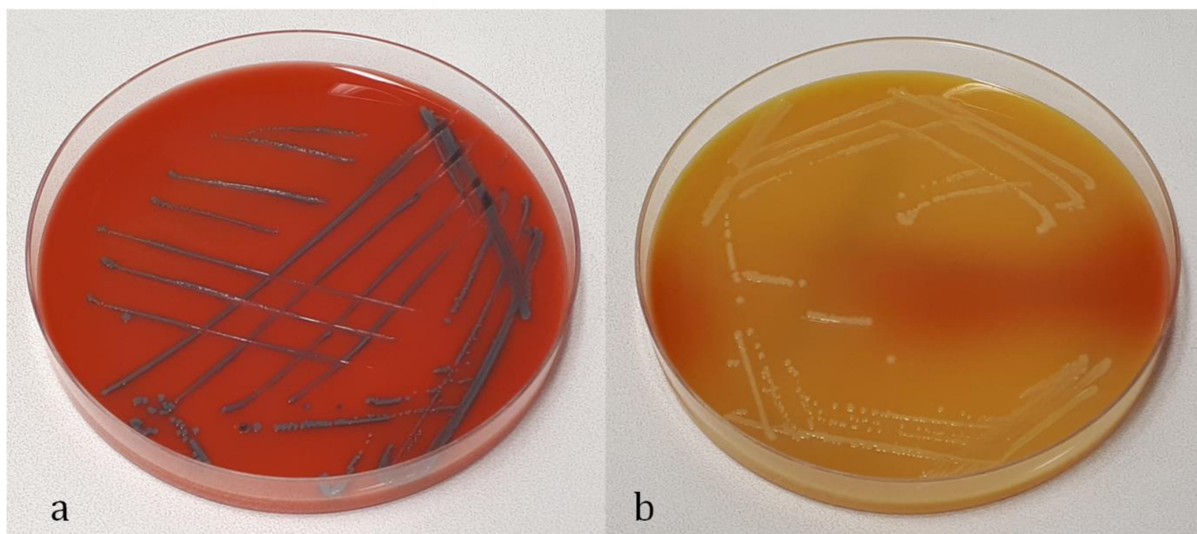

Figure 3.1 RAPID'L.Mono™ plates after 24 h incubation: light-blue colonies indicate positive *L. monocytogenes* growth (a) and white colonies indicate the growth of another *Listeria* species, namely *L. welshimeri* (b).

3.4.2. Polymerase Chain Reaction (PCR)

The *hly*A gene was amplified for 177 of the 180 isolates tested (Figure 3.2, only Ready-to-eat isolates shown as an example). The three isolates that tested negative for the *hly*A gene (thus not confirmed as *L. monocytogenes*) was not included for further study. There are bacterial species other than *L. monocytogenes* that are able to produce phospholipase C, which could make them appear similar on the RAPID'L.Mono™ plate (i.e. blueish colonies without a halo) (Gouws & Liedemann, 2005; Greenwood *et al.*, 2005). Thus, the three isolates which did not contain the *hly*A gene are suspected to have been other bacterial species (e.g. *Bacillus* or *Staphylococcus*) that resembled *L. monocytogenes* phenotypically.

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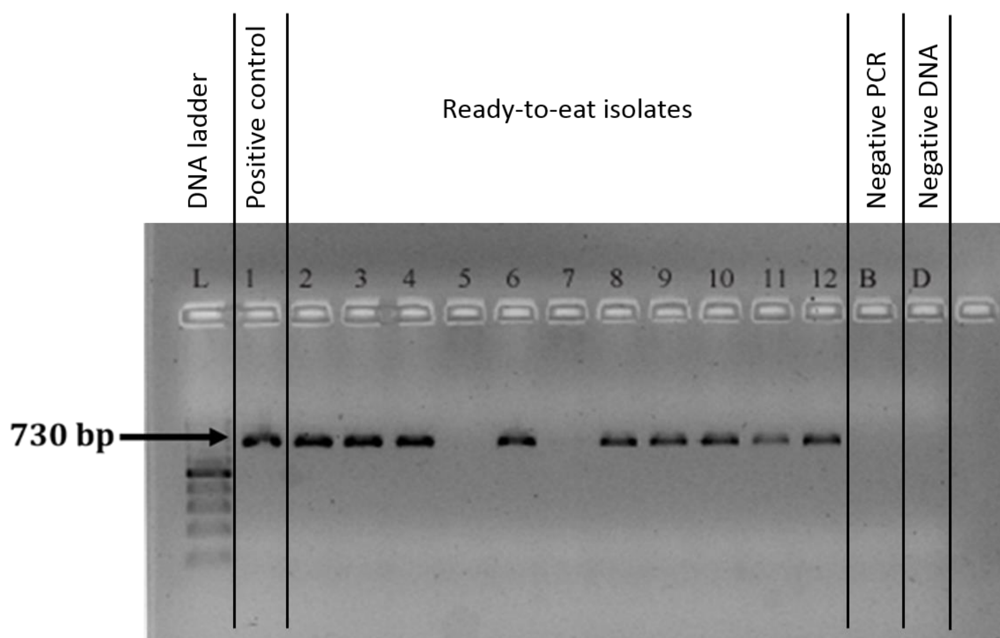

Figure 3.2 PCR amplification of *hly*A gene from *L. monocytogenes* isolates. L: 100 bp DNA ladder (GeneRuler, Thermo Scientific); **lane 1**: positive control (*L. monocytogenes* ATCC 7644); **lanes 2-4, 6, 8-12**: Ready-to-eat isolates; **lanes 5 and 7**: no *hly*A gene amplified; **lane B**: negative PCR control (no DNA); **lane D**: negative control from DNA extraction.

The final grouping for isolates that were positive for the *hly*A gene according to origins were: Clinical (n=20); Environmental (n=31); Raw meats (n=31); Raw seafood (n=61); Ready-to-eat (n=34). Only three of the categories, for which additional information was available, were further divided into subcategories (Environmental, Raw meats, and Ready-to-eat).

3.4.3. Restriction fragment length polymorphism (RFLP)

Digests with the *Nde*I enzyme (Table 3.1; Figure 3.3; only selected examples shown) revealed 320 bp and 410 bp bands (Lineage I), although some lanes still had an additional 730 bp band that was slightly visible, suggesting that DNA was not completely digested. This was resolved by increasing the incubation time from 1 h to 3 h. Digests with *Bfo*I enzyme (Figure 3.4) and the resulting banding pattern confirmed that the isolates uncut with *Nde*I were from Lineage II. The enzyme digests all non-Lineage II isolates into band sizes of 274 bp and 455 bp and digests Lineage II isolates into band sizes of 178 bp, 274 bp, and 278 bp. The latter two bands (274 bp and 278 bp) are often seen as one band on the gel due to similar amplicon size, which is resolved by increasing gel electrophoresis running time and decreasing voltage.

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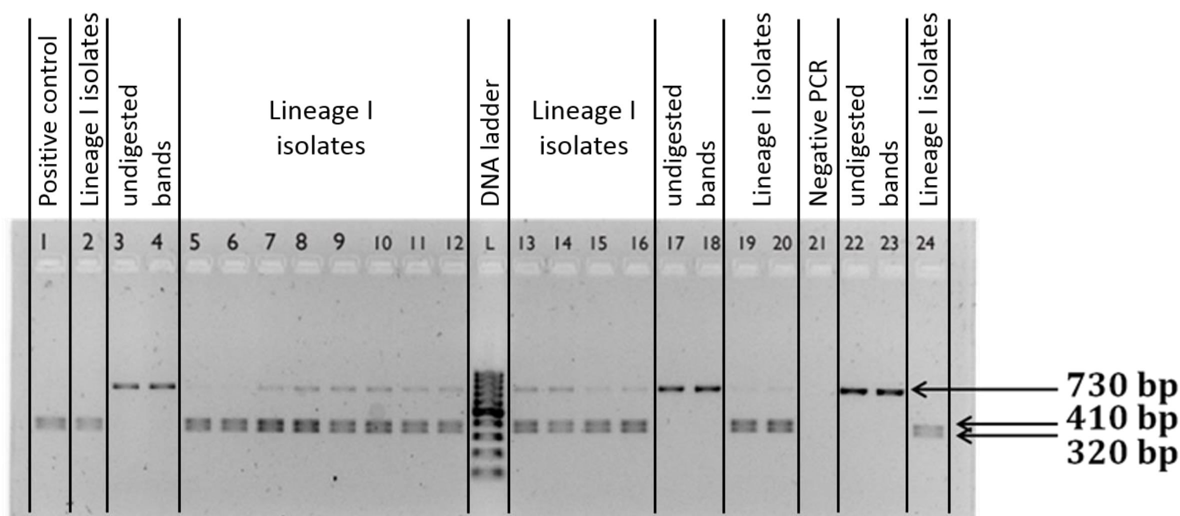

Figure 3.3 Digestion with *Nde*I enzyme revealed bands indicative of Lineage I (320 bp and 410 bp); single bands at 730 bp represent undigested DNA and indicates that such samples are not Lineage I *L. monocytogenes*. The positive control used is *L. monocytogenes* ATCC 23074 for Lineage I.

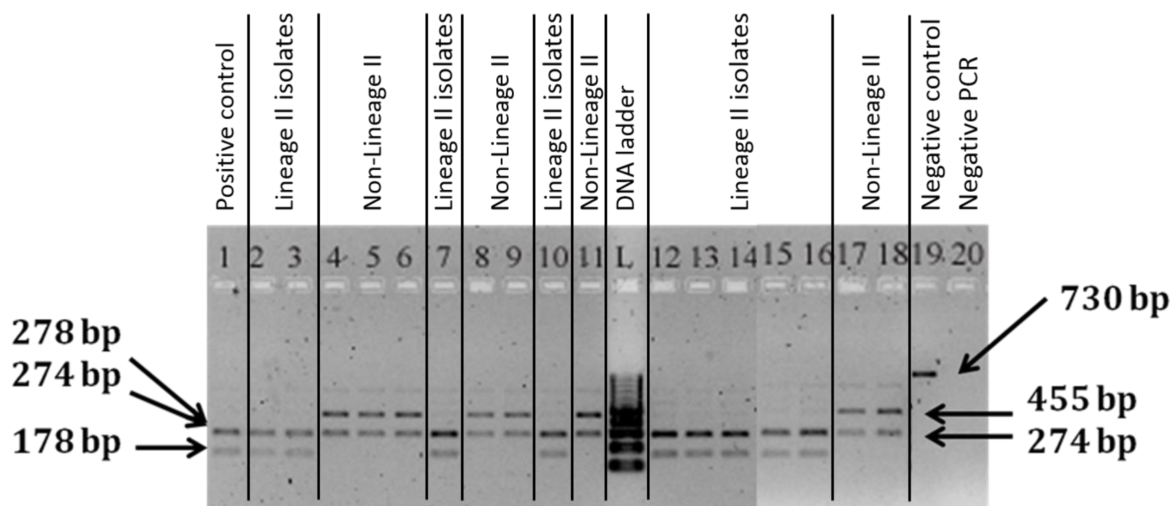

Figure 3.4 Digestion with *Bfo*I revealed bands indicative of Lineage II (178 bp, 274 bp and 278 bp); single band at 730 bp represent undigested DNA; bands at 455 bp and 274 bp represent non-Lineage II *L. monocytogenes* isolates.

The *Bsh*12851 enzyme digested only the reference isolate (*L. monocytogenes,* ATCC 19114, serotype 4a), indicating that Lineage III was not present in any of the sample isolates. This is a somewhat expected result since *L. monocytogenes* from Lineage III are mostly isolated from ruminants, and are rarely found in food or human clinical cases (Wiedmann *et al.*, 1997; Jeffers *et al.*, 2001; De Jesús & Whiting, 2003; Sauders *et al.*, 2004; Ward *et al.*, 2008; Orsi *et al.*, 2010; Tamburro *et al.*, 2010; Leong *et al.*, 2017). Prior studies have noted that Lineage III isolates have decreased virulence due to the lack of a virulent gene belonging to the internalin family (Liu *et al.*, 2006). These isolates are also more sensitive to thermal processing (Jeffers *et al.*, 2001; De

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Jesús & Whiting, 2003), providing an explanation on why this lineage is rarely isolated from food, the food processing environment, or human listeriosis cases.

All isolates were subjected to the PCR-RFLP method with results noted below. Of the five categories, Lineage I made up a significantly larger fraction of the Clinical (n=20, 97%, p<0.001) and Raw seafood (n=59, 95%, p<0.001) categories (Figure 3.5). The other three categories did not have significantly different fractions between Lineages I and II (Environmental: n=31, Lineage I=61%, Lineage II=39%, p=0.281; Raw meats: n=31, Lineage I=58%, Lineage II=42%, p=0.473; Ready-to-eat: n=34, Lineage I=53%, Lineage II=47%, p=0.864). In other words, Lineage I is highly characteristic of Raw seafood and Clinical samples, whereas no discernible pattern emerged for any of the other categories, and both lineages thus occur in similar proportions in these groups. Refer to Addendum A for a complete table with isolates, positive PCR results, and lineage grouping.

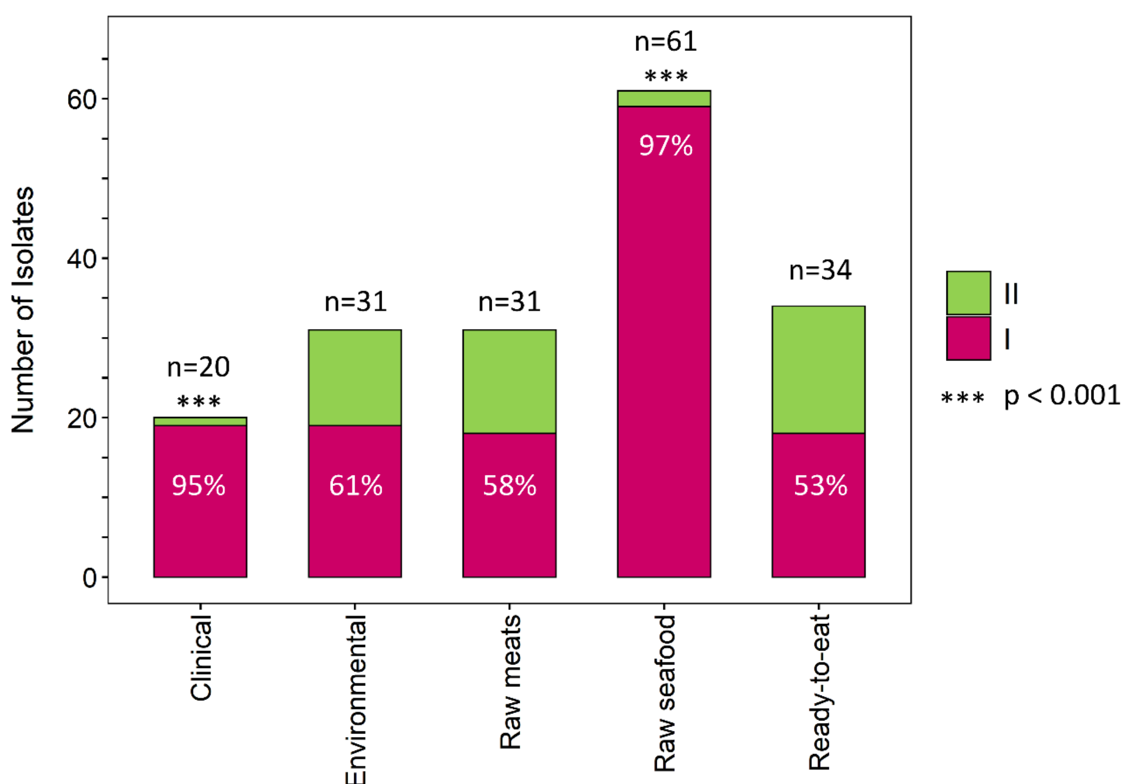

Figure 3.5 Distribution of lineage groups among isolates from different categories of origin indicated a significant association of Lineage I isolates with Clinical and Raw Seafood categories.

3.4.4. Lineage assemblage in subcategories

The categories Environmental, Ready-to-eat, and Raw foods, were further subdivided and investigated for patterns of lineage assemblage. In the Environmental category (Figure 3.6) it was found that isolates obtained from a factory worker's hand (n=1), floor (n=3), and surface (n=3)

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were exclusively made up of Lineage II (although caution should be used in the interpretation of these categories due to the low number of samples for each). However, the opposite was observed for drain (n=11) and equipment (n=9) samples where Lineage I made up a large fraction of samples (91% and 78%, respectively), which correlates with other studies (Hoelzer *et al.*, 2011; Ackermann, 2017; Smith *et al.*, 2019a). The results are somewhat similar to findings of Hoelzer *et al.* that Lineage I isolates associated significantly with surfaces such as floors and drains, whereas Lineage II isolates associated with surfaces that frequently come in contact with food.

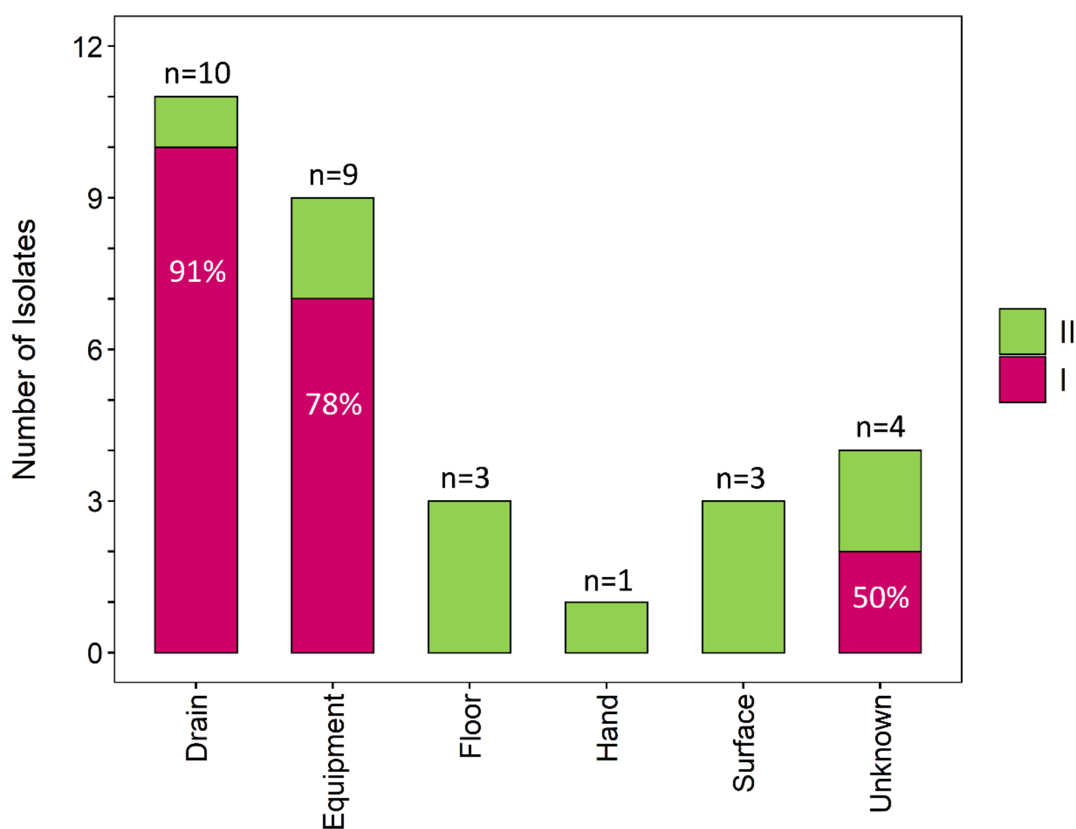

Figure 3.6 Distribution of lineage groups among different subcategories of Environmental isolates

For the Raw meats category it was found that Lineage II was exclusively associated with Pork (Figure 3.7). This is consistent with other reports finding Lineage II to be dominant among *L. monocytogenes* isolates from pork origin (Zoz *et al.*, 2017; Zuber *et al.*, 2019). However, as mentioned previously caution should be used when interpreting results of low sample size. Although the information is invaluable, the specific patterns might not necessarily be generalizable. Furthermore, opposite trends were observed between beef and chicken, where the former was primarily characterised by Lineage II (n=4, 75%) and the latter by Lineage I (n=10, 70%). The high occurrence of Lineage I in raw chicken isolates share some similarities with findings elsewhere (Zhang *et al.*, 2007; Fallah *et al.*, 2012). However, these findings do differ from others who found mostly Lineage II isolates from poultry (Fox *et al.*, 2012; Wang *et al.*, 2013;

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Oliveira *et al.*, 2018; Carvalho *et al.*, 2019). In agreement with our study, *L. monocytogenes* isolates from meat products have been previously associated with Lineage II (serotypes 1/2a and 1/2c) (Gilot & Genicot, 1996; Gianfranceschi *et al.*, 2009; Wang *et al.*, 2013). However Fox *et al.* (2012) found a higher incidence of serotype 4b (Lineage I) in beef isolates, while a study in France found an equal distribution of Lineage I and Lineage II isolates among *L. monocytogenes* in beef (Zoz *et al.*, 2017). Thus, there are inconsistent patterns in general for Raw meats isolates. The unknown category may possibly include a mixture of isolates from beef, pork, and chicken, thus explaining why there is a representation of both lineage groups as seen here.

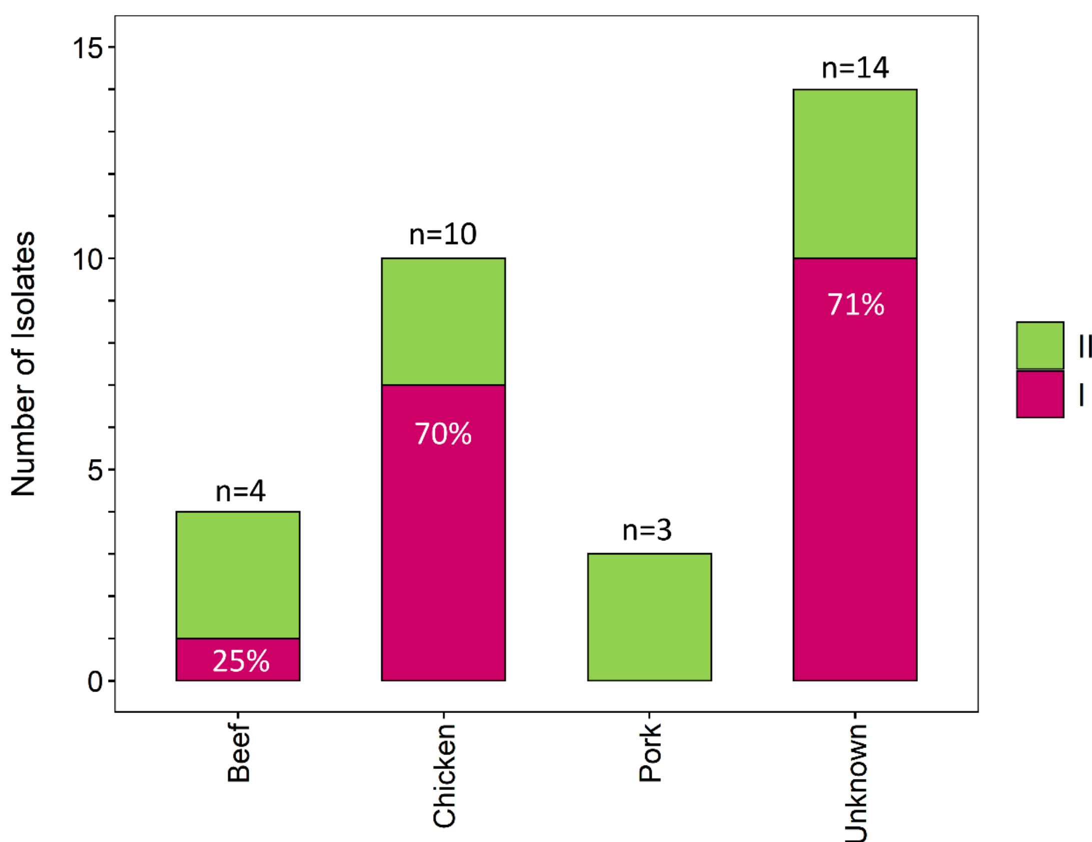

Figure 3.7 In the subcategories of Raw meats, it was found that Lineage II was exclusively associated with Pork.

Lineage II made up the larger portion for polony (n=5, 80%), and Lineage I for fresh produce (n=5, 80%). More or less equal lineage proportions were found for the deli meat (n=6, Lineage I=50%, Lineage II=50%) and hummus categories (n=15, Lineage I=47%, Lineage II=53%), while the single sample obtained for dairy was of Lineage I.

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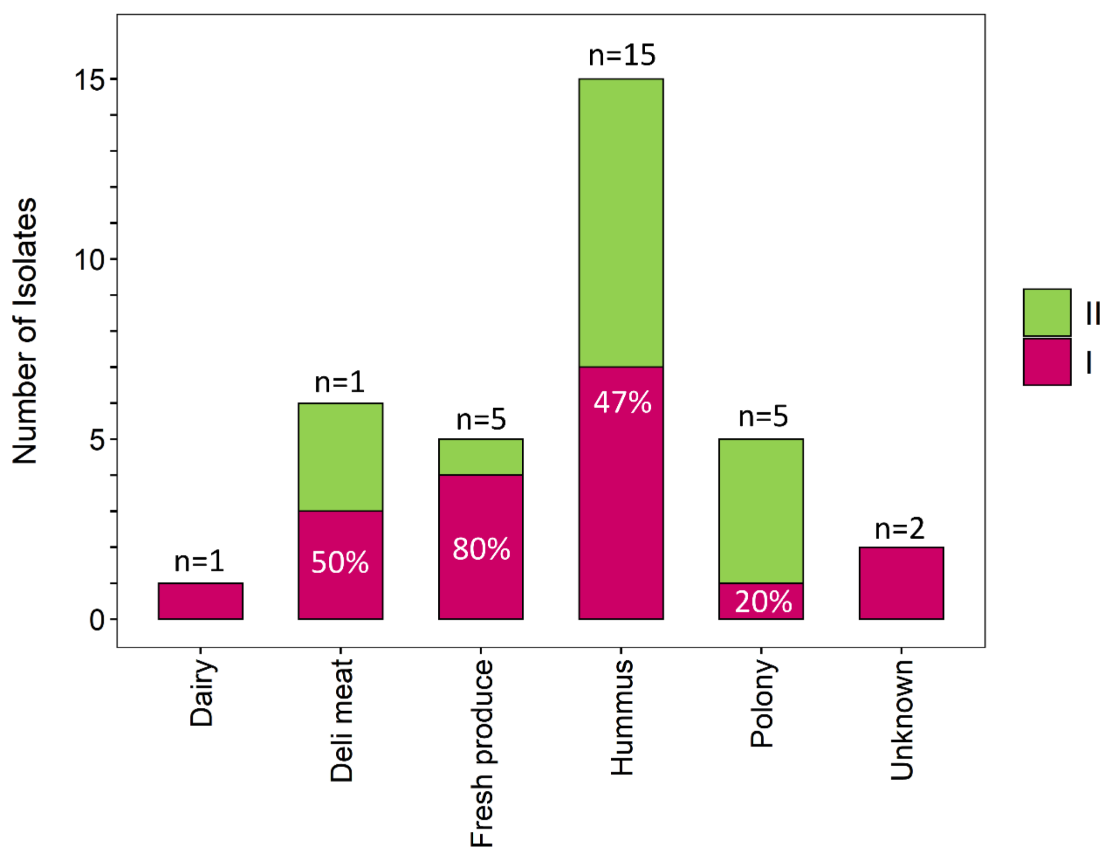

Figure 3.8 In the Ready-to-eat subcategories, Lineage II made up a larger portion for polony and Lineage I for fresh produce.

Lineage I and Lineage II isolates are usually more or less equally distributed among Ready-to-eat foods such as smoked fish, deli meats, and salads (Sauders *et al.*, 2004; Chen *et al.*, 2006; Shen *et al.*, 2006; Gianfranceschi *et al.*, 2009; Kramarenko *et al.*, 2013). However, contrasting trends have been observed where isolates from deli meat are either almost exclusively from Lineage II (Zuber *et al.*, 2019), or predominantly from Lineage I (Martins *et al.*, 2011). The overrepresentation of Lineage I isolates in fresh produce in this study agrees with other findings in South Africa, where isolates from carrot, coleslaw, and spring onions belonged to Lineage I (Rip & Gouws, in press). Additionally, studies outside the South African setting have also confirmed the presence of Lineage I isolates in fresh produce (Meloni *et al.*, 2009; Smith *et al.*, 2019a). It is unfortunate that there was only one isolate originating from dairy, but the fact that is was Lineage I is still useful. Past dairy-related outbreaks in the United States revealed that between 1985 and 2006, Lineage I isolates (either serotype 4b or 1/2b) were responsible for the majority of invasive and non-invasive listeriosis (Melo *et al.*, 2015), which is not surprising considering that Lineage I isolates have been dominant in diary plants (Miettinen *et al.*, 1999; Barancelli *et al.*, 2014).

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3.4.5. Prevalence of Lineage I isolates in human listeriosis cases

We expected that most, if not all, clinical isolates would be classified under Lineage I, since isolates from this lineage are usually associated with human listeriosis cases and outbreaks around the world (Aureli *et al.*, 2000; Jeffers *et al.*, 2001; Kathariou, 2002; Mclauchlin *et al.*, 2004; Orsi *et al.*, 2010), and since the clinical *L. monocytogenes* isolates in this study were obtained from patients that were infected during the SA listeriosis outbreak period (2017-18). About 91% of *L. monocytogenes* isolates from the SA 2017-18 listeriosis outbreak were of sequence type 6 (serotype 4b, Lineage I) (Smith *et al.*, 2019b), which corroborates the results of this study since all but one isolate belonged to Lineage I. This sequence type was also associated with two other outbreaks, namely one associated with cheese in the United States in 2013 (CDC, 2019) and the other with contaminated meat pâté in Switzerland in 2016 (Denise *et al.*, 2017). Serotype 4b (Lineage I) not only increases in virulence when exposed to colder temperatures (Orsi *et al.*, 2010), but can also rapidly adapt to grow and proliferate at human body temperature (Buncic *et al.*, 2001). This explains why they are better able to cause disease and are more frequently isolated from human listeriosis cases than *L. monocytogenes* isolates from Lineage II (Ribeiro & Destro, 2014). Only a single clinical isolate (CLM06) in this study belonged to Lineage II (Addendum A). However, the pattern is not always as clear. For example, clinical *L. monocytogenes* isolates collected in Belgium were mainly classified as Lineage I (64.2%) (Gilot and Genicot, 1996). This somewhat equal distribution of lineages among clinical isolates was also observed in Spain, where clinical isolates from invasive listeriosis were from both Lineage I (58.8%) and Lineage II (41.2%) (Ariza-Miguel *et al.*, 2006). In contrast, in Ireland, Lineage II was slightly more prevalent than Lineage I (65% vs. 35% respectively) (Fox *et al.*, 2012). Thus, unlike in the United States and South Africa where Lineage I appears to be dominant in clinical settings, both lineages seem to co-dominate in outbreaks in Europe and surrounds (Orsi *et al.*, 2010; Lukinmaa *et al.*, 2013), indicating a geographical lineage bias for *L. monocytogenes* in clinical settings.

3.4.6. Lineage I significantly associates with Raw seafood as opposed to Raw meats

The finding of dominant Lineage I isolates from raw seafood mirrors those of previous studies (Weagant *et al.*, 1988; Laciar & De Centorbi, 2002; Sauders *et al.*, 2004; Shen *et al.*, 2006; Chen *et al.*, 2010b; Momtaz & Yadollahi, 2013; Wang *et al.*, 2013; Montero *et al.*, 2015). Lineage I strains are better adapted to tolerate osmotic stress (Walecka-Zacharska *et al.*, 2013; Ribeiro & Destro, 2014) and in seafood, have increased ability to form biofilms (Djordjevic *et al.*, 2002; Takahasi *et al.*, 2009). The ability of *L. monocytogenes* to withstand moderately high salt levels and freezing

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temperatures, the fact that seafood is often stored at refrigeration temperatures and consumed with no prior cooking, and the high number of Lineage I isolates (higher mortality rate) associated with seafood, makes raw seafood a potential high risk food for the consumer (Harteminkand Georgsson, 1991; Embarek, 1994; Walecka-Zacharska *et al.*, 2013; Jami *et al.*, 2014).

3.4.7. *Listeria monocytogenes* lineage assemblage in the Ready-to-eat category

A high representation of Lineage I isolates in the Ready-to-eat category was expected since RTE foods are one of the main sources for infection with *L. monocytogenes* (Sauders *et al.*, 2004; Maćkiw *et al.*, 2016; Buchanan *et al.*, 2017; Gruyter *et al.*, 2018; Smith *et al.*, 2019a). There are conflicting views on the predominance of certain lineage groups in RTE foods. Some studies suggest a higher association of Lineage II (serotype 1/2a) with RTE foods and the meat processing industry (Zhang *et al.*, 2007; Gianfranceschi *et al.*, 2009). However, recent studies demonstrated the opposite, showing Lineage I serotypes (4b and 1/2b) to be the most prevalent in various RTE foods, namely cooked retail RTE foods (Chen *et al.*, 2014), milk and RTE milk products (Aurora *et al.*, 2009), and RTE meat products (Zhang *et al.*, 2007; Meloni *et al.*, 2009).

The prevalence of Lineage II isolates among the polony samples was unexpected. Polony (a locally made processed meat product) was implicated as the source of the 2017-18 listeriosis outbreak in South Africa, with the implicated strain belonging to Lineage I. This implicated strain was isolated from the polony producing facility, as well as from other food products in the facility. Our Ready-to-eat samples included five polony isolates, yet only one of them was classified as Lineage I. This result could be due the polony being contaminated with an isolate other than Lineage I, due to post-process handling.

The presence of Lineage I *L. monocytogenes* isolates from fresh produce is especially worrisome, as these products are often used in salads or other dishes that will be refrigerated for long periods of time, giving the bacteria opportunity to proliferate (Aparecida De Oliveira *et al.*, 2010). The exact source of *L. monocytogenes* on fresh produce is not yet known, i.e. whether contamination occurs on farm level or during post processing. However, it has been demonstrated that Lineage I isolates associated significantly more with natural environments such as pastures and uncultivated fields, compared to Lineage II isolates (Weis & Seeliger, 1975). For dairy products it is generally accepted that contamination occurs post-processing. This is because dairy products undergo pasteurization which effectively eliminates *L. monocytogenes.* The presence of *L. monocytogenes* on dairy products are due to post-processing contamination, often transferred from equipment, or even contamination by the end consumer (Autio *et al.*, 2000; Sauders *et al.*, 2004). This is a generally accepted theory for the contamination of most RTE foods (Tompkin, 2002). The prevalence of Lineage I isolates in RTE products is problematic, as serotype

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4b (Lineage I) is frequently associated with human listeriosis outbreaks (Borucki *et al.*, 2003; Jadhav *et al.*, 2012; Hyden *et al.*, 2016). Packaged RTE products are most likely to be consumed without further heating and are also stored at refrigeration temperatures, which increases the likelihood of *L. monocytogenes* growing to high numbers and thus leading to infection (Barancelli *et al.*, 2014).

3.4.8. *Listeria monocytogenes* in the food processing environment

Listeria monocytogenes is commonly associated with the food processing environment, particularly drains, floors, and processing equipment (El-shenawy, 1998). Contamination is often attributed to incoming raw material, movement of personnel, and ineffective cleaning procedures (El-shenawy, 1998; Tompkin, 2002; Carpentier & Cerf, 2011; Fallah *et al.*, 2013; Ferreira *et al.*, 2014). Moreover, complete eradication of *L. monocytogenes* from the food processing environment is difficult and the bacteria can persist due to the formation of biofilms, tolerance to chemical sanitizers, or even genes conferring a selective advantage for its survival (Jami *et al.*, 2014; NicAogáin & O'Byrne, 2016; Buchanan *et al.*, 2017; Muhterem-uyar *et al.*, 2018). Isolates from Lineage II possess certain genes that enable better adaptation and survival of the stressful conditions in the food processing environment (Chen *et al.*, 2006; Mullapudi *et al.*, 2008; Hoelzer *et al.*, 2011). This could possibly explain why Lineage II isolates are more frequently found on food contact surfaces, compared to Lineage I. In this study, the similar distribution of Lineage I and II isolates within the environment could be due to the diverse routes of contamination. For example, raw material entering the processing facility as well as the movement of personnel in and out of the facility (NicAogáin & O'Byrne, 2016). Improper staff hygiene and their movement within the food processing facility can consequently aid the spread of *L. monocytogenes* (El-shenawy, 1998; Fallah *et al.*, 2013; Gruyter *et al.*, 2018). Therefore, a proactive approach should be taken, with the focus on the hygienic design of the processing facility, educating personnel on food safety and associated risks, and effective monitoring and implementation of cleaning and sanitation procedures (Jami *et al.*, 2014). The presence of *L. monocytogenes* in factory drains is often seen as a consequence of contamination elsewhere in the factory (El-shenawy, 1998; Gruyter *et al.*, 2018)., The association of Lineage I with the drain isolates in this study is thus a risk factor for the consumer and the food processing staff to contract listeriosis.

3.4.9. General lineage associations

The distribution of *L. monocytogenes* lineages can be influenced by environmental stresses. For example, there are differences in how lineage groups of *L. monocytogenes* are associated with different origins and how they behave under stressful conditions (as experienced in the food

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processing environment), which in turn, influences their pathogenicity (Wiedmann *et al.*, 1997; Ward *et al.*, 2004; Hoelzer *et al.*, 2011; Manuel *et al.*, 2015; NicAogáin & O'Byrne, 2016). Various reasons have been proposed for this phenomenon. Strains of *L. monocytogenes* might enter the food processing facility by different routes (i.e. human versus raw material) which could explain a more common occurrence of one lineage over another (Hoelzer *et al.*, 2011). Temperature also seems to influence the prevalence of a specific lineage (Orsi *et al.*, 2010). For example, after cold storage at 4°C, *L. monocytogenes* serotype 4b isolates, as a group, tend to be more resistant to heat treatment at 60°C than 1/2a isolates (Buncic *et al.*, 2001). The genetic makeup of *L. monocytogenes* may also play a role in lineage distribution. The fact that 4b serotypes (Lineage I) are less likely to possess genes that confer resistance to sanitizers (Mullapudi *et al.*, 2008) is a potential reason why Lineage I isolates have less frequently been isolated from the food processing environment (Mullapudi *et al.*, 2008). Isolates from Lineage II are also more prone to recombination, enabling further adaptation to food processing environments (Lomonaco *et al.*, 2015), which could account for their frequency in these environments (Zuber *et al.*, 2019).

The dominant occurrence of one lineage group over another may also be influenced by the ability to form biofilms. While biofilm formation in seafood seems to be higher among Lineage I isolates (Takahasi *et al.*, 2009), Lineage II isolates produce biofilms with higher density than those of Lineage I (Pan *et al.*, 2009; Nilsson *et al.*, 2011; Combrouse *et al.*, 2013). The growth medium and nutrient availability has an influence on biofilm formation, and can thus likely influence the prevalence of one lineage over the other (Borucki *et al.*, 2003; Takahasi *et al.*, 2009; Reis-Teixeira *et al.*, 2017). Due to the overrepresentation of Lineage II isolates in food products and the processing environment, it has been suggested that a phenomenon known as "enrichment bias" exists. It proposes that Lineage I isolates are more sensitive to certain enrichment protocols than Lineage II isolates, making it possible to underestimate the presence of the former (Kathariou, 2002; Bruhn *et al.*, 2005). However this enrichment bias seems to be medium dependent, and is influenced by the nutrients in the medium rather than being lineage dependent (Gorski *et al.*, 2006). Lineage II isolates are able to better adapt to environmental conditions, thus the reason why they are more frequently isolated from food processing environments, as opposed to Lineage I (Orsi *et al.*, 2010). Nevertheless, due to the high number of Lineage I isolates, it is unlikely that enrichment bias played a role in this study.

The dominance of Lineage I in categories other than Clinical is surprising as it disagrees with the majority of studies involving the distribution of *L. monocytogenes* serotypes in food and the food processing environment. Most researchers agree that Lineage II serotypes (1/2a and 1/2c) are overrepresented in food isolates (Swaminathan & Gerner-Smidt, 2007; Nightingale *et al.*, 2008; Gianfranceschi *et al.*, 2009; Orsi *et al.*, 2010; Manuel *et al.*, 2015), so it is uncharacteristic

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that such a high number of Lineage I isolates were found in this study. It also contradicts findings in Italy (Gianfranceschi *et al.*, 2009) where the majority of isolates from food, environmental, and clinical origin belonged to Lineage II. Lineage I is known to carry a higher risk of human listeriosis (Rasmussen *et al.*, 1995; Wiedmann *et al.*, 1997; Orsi *et al.*, 2010) and it is estimated that Lineage I isolates are 100 times more likely to cause listeriosis than Lineage II isolates (Chen *et al.*, 2006). The collection of isolates during the outbreak period could be the reason for the dominance of Lineage I isolates across all categories; alternatively, it could be non-outbreak related strains, which can be confirmed using certain genotyping techniques, including but not limited to whole genome sequencing. The prevalence of Lineage I in all categories examined in this study is potentially worrisome, as stress factors encountered in the food environment could influence *L. monocytogenes*' ability to better survive within the human host and subsequently be more likely to cause disease.

3.5. Conclusion

The main aim of this chapter was to establish the lineage group assemblage among isolates from different origins in the Western Cape, South Africa. This study showed an overrepresentation of Lineage I in the Clinical samples (as expected), but even more concerning, a large fraction of Lineage I isolates in the Environmental, Raw meats, Raw seafood, and Ready-to-eat categories. Lineage I isolates are associated with a lower infectious dose (i.e. less bacteria are needed to cause listeriosis) and therefore pose a greater public health risk (Chen *et al.*, 2006), It is therefore imperative that future studies establish why Lineage I isolates are more prevalent in the South African food environment. However, due to the risk associated with RTE foods and the high number of Lineage II isolates on these products, the importance of isolates from this lineage should not be discounted.

The presence of *L. monocytogenes* in the food production environment and associated foods is of growing concern. Information on *L. monocytogenes* in South Africa is lacking, with most research articles published during 2018, i.e. after the SA 2017-18 listeriosis outbreak (Boatemaa *et al.*, 2019). Our findings differ from a majority of studies that find Lineage I to be mostly associated with human listeriosis and Lineage II to be more frequently associated with foods (Sauders *et al.*, 2004). Unlike other food pathogens (e.g. *Salmonella*), a clear association between different food types and *L. monocytogenes* lineage groups has not yet been found (Norrung & Skovgaard, 1993; Boerlin *et al.*, 1997; Sauders *et al.*, 2004; Ebner *et al.*, 2015). In order to establish whether there is an actual association between different categories and *L. monocytogenes* lineage groups, continued research is necessary within South Africa, with a specific focus on *L. monocytogenes* isolates from food, clinical, and environmental origin. This study provides insight

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into the distribution of the different lineages across a range of food categories, especially in the South African setting. A long-term study may offer valuable insight into whether the same proportion of *L. monocytogenes* lineages continue to exist in these categories. Additionally, it would be interesting to follow up this study with new post-outbreak isolates to determine if Lineage I still dominates in certain categories of origin. Furthermore, specific focus should be placed on resistance genes to elucidate whether, for example, resistance to sanitizers influence the prevalence of certain lineages (e.g. Lineage I). Finally, this study did not address the further subdivision of lineage groups into serotypes, and future studies should aim to resolve such finer classifications.

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Chapter 4

Susceptibility of *Listeria monocytogenes* isolates from food, environmental, and clinical origin in South Africa to a commercial bacteriophage P100

4.1. Abstract

Controlling *L. monocytogenes* in the food processing environment is difficult at best and an ongoing struggle. Therefore, novel methods are continuously being sought to control the bacterium and to reduce, or preferably avoid, the risk of food contamination. One such novel control method concerns the use of bacteriophages, a group of microorganisms that have long been known, but which have only recently started to generate interest. The interest in bacteriophage treatment as an alternative control method is in part due to *L. monocytogenes* becoming increasingly resistant towards currently implemented control methods. Thus, the aim of this study was to determine the susceptibility of *L. monocytogenes* isolates from various origins, such as food, environmental, and clinical against a commercial bacteriophage (Listex™ P100). Firstly, bacteriophage susceptibility tests were conducted on the *L. monocytogenes* isolates by means of spot tests. Secondly, lineage group data was compared with susceptibility results to determine whether or not lineage classifications influenced the susceptibly of the *L. monocytogenes* towards the bacteriophage. The *L. monocytogenes* isolates obtained from Clinical, Environmental, Raw meats, and Raw Seafood were significantly susceptible to phage activity. However, a large fraction of isolates in the Ready-to-eat category were tolerant to the phage, which is in disagreement with the finding of others. Additionally, both lineage groups were significantly susceptible to phage activity when considering all categories combined, and lineage groups did not significantly influence phage susceptibility.

4.2. Introduction

Listeria monocytogenes is one of the biggest foodborne pathogens in the food industry. Being a psychrotrophic bacterium, it has the ability to actively grow at refrigeration temperatures (Forsythe, 2010; Montville *et al.*, 2012), and together with this also has the ability to tolerate high salt concentrations (Lamont & Sobel, 2011). Its ubiquitous nature in the environment and tendency to form biofilms on various surfaces in the food processing industry (e.g. stainless steel workbenches) makes it nearly impossible to completely eradicate from the food processing environment (Todd & Notermans, 2011; Buchanan *et al.*, 2017; Chen *et al.*, 2017). This is a

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challenging aspect for food industries in terms of keeping food products free from contamination by *L. monocytogenes*, seeing that virtually all such products come into contact with such contaminated surfaces at some stage during production. This can be especially problematic in ready-to-eat foods (RTE), since these products do not undergo further sterilization treatment once they are packaged, thus leaving them highly vulnerable to contamination by *L. monocytogenes* derived from workplace surfaces (Forsythe, 2010). Furthermore, not only are consumers demanding a larger variety of RTE foods, they also want increased shelf lives for these products (Swaminathan & Gerner-Smidt, 2007; Montville *et al.*, 2012). Therefore, the steady increase in the production of RTE foods that are distributed regionally and nationally, has led to a higher prevalence of food pathogens such as *L. monocytogenes* (Muñoz *et al.*, 2012).

Foods that have often been implicated with the presence of *L. monocytogenes* are meat products (especially delicatessen meats), fish and seafood products, and dairy products (such as soft cheeses) (Vázquez-boland *et al.*, 2001b; Swaminathan and Gerner-Smidt, 2007; Forsythe, 2010; Montville *et al.*, 2012; Buchanan *et al.*, 2017). Likewise, RTE fruits and vegetables, similar to many other RTE products, can easily be contaminated with *L. monocytogenes* due to the minimal processing that these products undergo (e.g. no heat treatment) (Vasconcelos *et al.*, 2016). Due to the difficulty in controlling *L. monocytogenes* in food processing environments, a proactive approach needs to be taken in preventing food contamination, as well as generally reducing the overall microbial load in these environments (Buchanan *et al.*, 2017). Such proactive approaches include the use of disinfectants and standard sterilization procedures. However, the fact that these biocides are becoming increasingly ineffective at controlling *L. monocytogenes* has led researchers to start investigating other control measures, such as bacteriophage biocontrol.

Bacteriophages are viruses with the ability to infect and lyse bacterial cells. They are ubiquitous in nature and are consumed unknowingly on a regular basis (EFSA, 2009; García *et al.*, 2010; Mahony *et al.*, 2011). Bacteriophages are therefore assumed to be safe for consumption, with no adverse effects having ever been reported (Carlton *et al.*, 2005; Hagens & Offerhaus, 2008; EFSA, 2009; Mahony *et al.*, 2011; Komora *et al.*, 2018). Bacteriophages are highly host-specific, meaning that they can infect and lyse only one species of bacteria, while having no effect on others (Hagens & Offerhaus, 2008; Guenther *et al.*, 2009; Moye *et al.*, 2018). This specificity is especially valuable for the treatment of fermented foods where the natural microflora should not be destroyed in order to maintain product quality (Carlton *et al.*, 2005; Hagens & Offerhaus, 2008; Guenther *et al.*, 2009). Although this host specificity can be very useful for the food industry, the application of such bacteriophages for targeting pathogenic bacteria in food processing facilities is still relatively new, even though the concept of using bacteriophages to treat or prevent bacterial infections has been around for a long time, having even preceded the discovery of

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antibiotics (Sulakvelidze & Alavidze, 2001). However, the high usage levels of antibiotics in the United States and Western Europe for controlling pathogenic microorganisms long overshadowed the usefulness of bacteriophage therapy as a treatment, almost causing it to be forgotten. But the threat of increasing antibiotic resistance in many microorganisms has recently forced researchers to start investigating alternative methods to control pathogenic microorganisms, which has led to a revived interest in the use of bacteriophage therapy for controlling such pathogenic microorganisms (Mylonakis *et al.*, 2002; Chen *et al.*, 2010a; Rahimi *et al.*, 2010; Lamont & Sobel, 2011; Mahony *et al.*, 2011; Maćkiw *et al.*, 2016; Noll *et al.*, 2018).

Bacteriophages can have either a lytic or lysogenic lifecycle (Figure 4.1), with the former leading to lysis of bacterial cells. In the lysogenic cycle, phages incorporate their own DNA into a host's chromosome, forming prophages (García *et al.*, 2010). This enables the phages to pass on virulent genes, leading to bacterial strains with higher pathogenic potential (García *et al.*, 2010; Fortier and Sekulovic, 2013; Moye *et al.*, 2018). Lysogenic (or temperate) phages are thus not used in the food industry (Hagens & Offerhaus, 2008; Hagens and Loessner, 2010; Klumpp *et al.*, 2013), and will not be discussed further in this chapter. In the lytic (or virulent) cycle, the bacteriophage recognizes and attaches to the bacterial host cell wall, penetrates the cell wall by means of tail associated proteins, and secretes its nucleic acid into the cytoplasm of the host (Hagens & Offerhaus, 2008; EFSA, 2009; Shen *et al.*, 2017). Phages from the *Myrovidae* family (e.g. P100) make use of two different receptors on the host cell, namely wall teichoic acids (WTAs), and sugars in the peptidoglycan layer.

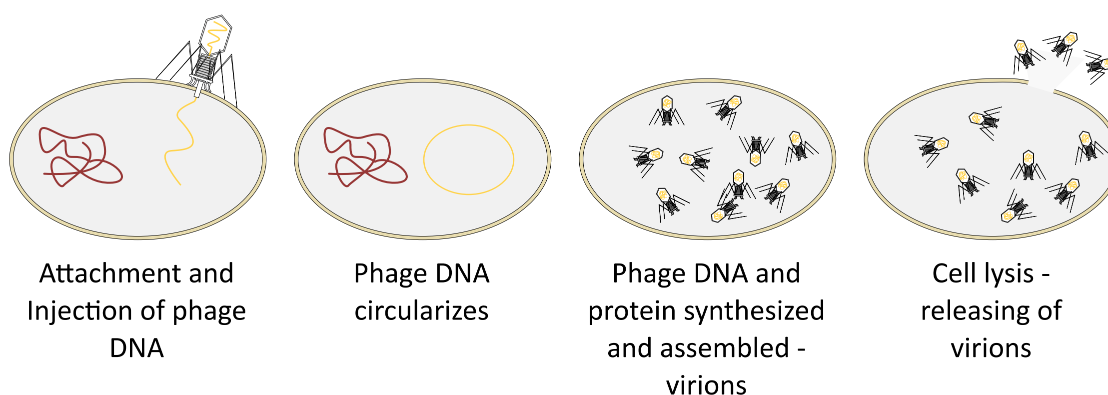

Figure 4.1 The simplified life cycle of a lytic (virulent) bacteriophage leads to lysis of the host cell**.**

Several studies on the efficacy of bacteriophages have shown them to be useful at controlling *L. monocytogenes* in the food production industry (Guenther *et al.*, 2009; García *et al.*, 2010; Moye *et al.*, 2018). For example, commercial bacteriophages (such as A511 and Listex™ P100) are effective on an assortment of food products, which include: dairy products, such as cheese and chocolate milk (Carlton *et al.*, 2005; Guenther *et al.*, 2009); RTE foods (Guenther *et al.*, 2009;

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Cláudia *et al.*, 2017); raw seafood (Soni & Nannapaneni, 2010b; Soni *et al.*, 2010); poultry and fresh produce (Leverentz *et al.*, 2003; Oliveira *et al.*, 2014; Perera *et al.*, 2015). The commercial P100 phage preparation, Listex™ P100, has been approved by the Food and Drug Administration and is also currently used in the United States, Canada, and Switzerland (Aprea *et al.*, 2018). Phage P100 has a broad host range within the *Listeria* genus, being able to target and successfully infect approximately 95% of *Listeria* species, including *L. ivanovii*, *L. innocua*, and *L. monocytogenes* (specifically serogroups 1/2 and 4) (Carlton *et al.*, 2005; Hagens & Loessner, 2007; Guenther *et al.*, 2009). Serotypes often differ in how they respond to sanitizers or in their ability to form biofilms, but there has been little research on whether bacteriophages have better efficacy against strains of a certain serotype or lineage group.

Because bacteriophage treatment in the food industry is still relatively new, little is still known about the bacterial host's resistance mechanisms or the possible occurrence of phage resistant bacteria in the processing environment (Fister *et al.*, 2016). Phage resistance should thus not be excluded as a possibility (Guenther *et al.*, 2009; Fister *et al.*, 2016; Moye *et al.*, 2018). It should also be noted that, concerning disinfectants and sanitizers used in the food industry, the term "resistance" is somewhat loosely defined. Resistance can either be phenotypic (e.g. colony growth) or genotypic (expression of certain genes that facilitate resistance) (Cerf *et al.*, 2010). It is therefore suggested that the term "susceptibility" be used when studying bacteriophages in order to distinguish between resistance types (Cerf *et al.*, 2010), and such terminology is followed throughout this text. Unlike antibiotic susceptibility testing, there is great variation in tests within bacteriophage research. A soft agar overlay method is typically employed for the isolation and quantification of phages after reduction of the bacterial host (Soni *et al.*, 2010). The spot tests method (also known as spot-on-lawn or spot assay) is recommended to test whether a phage mixture of known concentration shows activity against the bacterial host (Hyman & Abedon, 2010; Hyman, 2019).

To the author's best knowledge, not much information has yet been generated on the use of bacteriophages in South Africa. Only one study specifically investigated the susceptibility of *L. monocytogenes* isolates from foods and the food processing environment (Strydom, 2015). Therefore, the main aim of this study was to determine the susceptibility of *L. monocytogenes* isolates from various origins, such as food, environmental, and clinical (all from the Western Cape, South Africa), against a commercial bacteriophage (Listex™ P100). The first objective was to determine whether or not isolates from specific origins varied in their susceptibility towards the commercial bacteriophage, and secondly to determine whether or not lineage classifications influenced the susceptibly of the *L. monocytogenes* towards the bacteriophage.

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4.3. Materials and Methods

4.3.1. Sample collection and storage

Refer to Chapter 3 for detailed information regarding sample numbers, collection, and exact methods followed for lineage classification, and Addendum A for additional sample information and classification results. Briefly, all isolates were cultured and only those that were confirmed positive for *hly*A gene amplification by means of polymerase chain reaction (PCR) and classified into lineage groups by restriction fragment length polymorphism (RFLP), were subsequently included for bacteriophage susceptibility tests. For the remainder of the chapter, "Ready-to-eat" refers specifically to one of the main categories in this study, whereas "RTE" refers to ready-to-eat foods in general.

4.3.2. Method optimization

As phage P100 is already a purified phage mixture, the decision was made to proceed with the spot test method. With this technique, a bacterial lawn is first grown on a petri dish, with subsequent addition of small drops of phage dilutions. If phages are able to adsorb to and kill the bacteria, a zone of clearance will appear after incubation.

Prior to conducting phage susceptibility tests by means of a spot test, a series of optimization tests were performed. This was due to the variation of test methods found in literature. Firstly, two liquid media (used to dilute bacteriophage solution) were tested, namely distilled water and 0.85% saline solution (Oxoid, ThermoFischer Scientific) (Soni *et al.*, 2010; Rossi *et al.*, 2011; Chibeu *et al.*, 2013; Nóbrega *et al.*, 2014; Strydom & Witthuhn, 2015). Different concentrations of phage solution (original concentration 10^{11} PFU/mL) were also tested, that is 10^9 PFU/mL, 10^8 PFU/mL, 10^7 PFU/mL, and 10^6 PFU/mL (Leverentz *et al.*, 2004; Carlton *et al.*, 2005; Rossi *et al.*, 2011; Strydom & Witthuhn, 2015). These concentrations were chosen since the recommended phage concentration for application on a food product is 10^8 PFU/cm^2 (Leverentz *et al.*, 2004; Guenther *et al.*, 2009; Guenther & Loessner, 2011), not exceeding 10^9 PFU/cm^2 which is the maximum allowable amount to be used on foods (Moye *et al.*, 2018). Log-phase *L. monocytogenes* cultures were used (OD600=0.6) and diluted to a final concentration of 10^7 CFU/mL. Following the optimization test results (Table 4.1), a 0.85% saline solution was used to dilute the phage stock to a final concentration of 10^9 PFU/mL, since this combination was optimal for visualizing phage susceptibility.

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Table 4.1 Optimizations tests showed a 0.85% saline solution used to dilute the phage to a concentration of 10^9 PFU/mL to be the most likely combination to confirm phage susceptibility. "Y" indicates zone of lysis; "-" indicates no zones of lysis

	Distilled water				Saline solution (0.85%)			
	10^9	10^8	10^7	10^6	10^9	10^8	10^7	10^6
Sample 1	Y	-	-	-	Y	-	-	-
Sample 2	-	-	-	-	-	-	-	-
Sample 3	Y	Y	-	-	Y	Y	Y	Y
Sample 4	Y	Y	-	-	Y	Y	-	-
Sample 5	Y	-	-	-	Y	-	-	-
Sample 6	Y	Y	Y	Y	Y	Y	Y	Y
Negative control	-	-	-	-	-	-	-	-

4.3.3. Bacteriophage susceptibility tests

Bacteriophage activity was tested by using the spot test method (Figure 4.2) (Loessner & Busse, 1990; Hyman & Abedon, 2010; Denes *et al.*, 2015; Khan Mirzaei & Nilsson, 2015; Estela *et al.*, 2016). Bacterial colonies (grown overnight at 37°C) were resuspended from tryptic soy agar (TSA) plates into 5 mL brain heart infusion (BHI) broth (Oxoid, ThermoFischer Scientific) and grown for 3 h at 37°C, to obtain log-phase growth (OD600=0.6) and was further diluted with BHI to obtain 10^7 CFU/mL. A log-phase culture was used as it is more favourable for phage attachment than stationary growth (Krueger & Fong, 1937; Braun *et al.*, 2006). Cells in stationary growth are often smaller in size, decreasing the likelihood of being encountered by the bacteriophage. Longer incubations can also lead to possible gene mutations that confer a competitive advantage to the bacterial cell, leading to false negatives (Braun *et al.*, 2006). A lawn of bacteria was grown by aliquoting 100 µL of the diluted, log-phase bacterial culture on a petri dish and employing the spread plate technique. The bacterial lawn was dried for approximately 30 min at room temperature before the bacteriophage solution (Listex™ P100) was applied. Ten µL of phage (diluted with 0.85% saline solution to obtain a final phage concentration of 10^9 PFU/mL) was dropped on each plate. The negative control was 10 µL of phage-free 0.85% saline solution. The plates were left to dry completely at room temperature before they were incubated for 18 – 20 h at 30°C. The plates were incubated at this temperature as susceptibility is most likely to occur at 30°C as opposed to 37°C (Tokman *et al.*, 2016). After incubation, the plates were inspected to determine whether or not a lysis zone (zone of inhibition) had formed.

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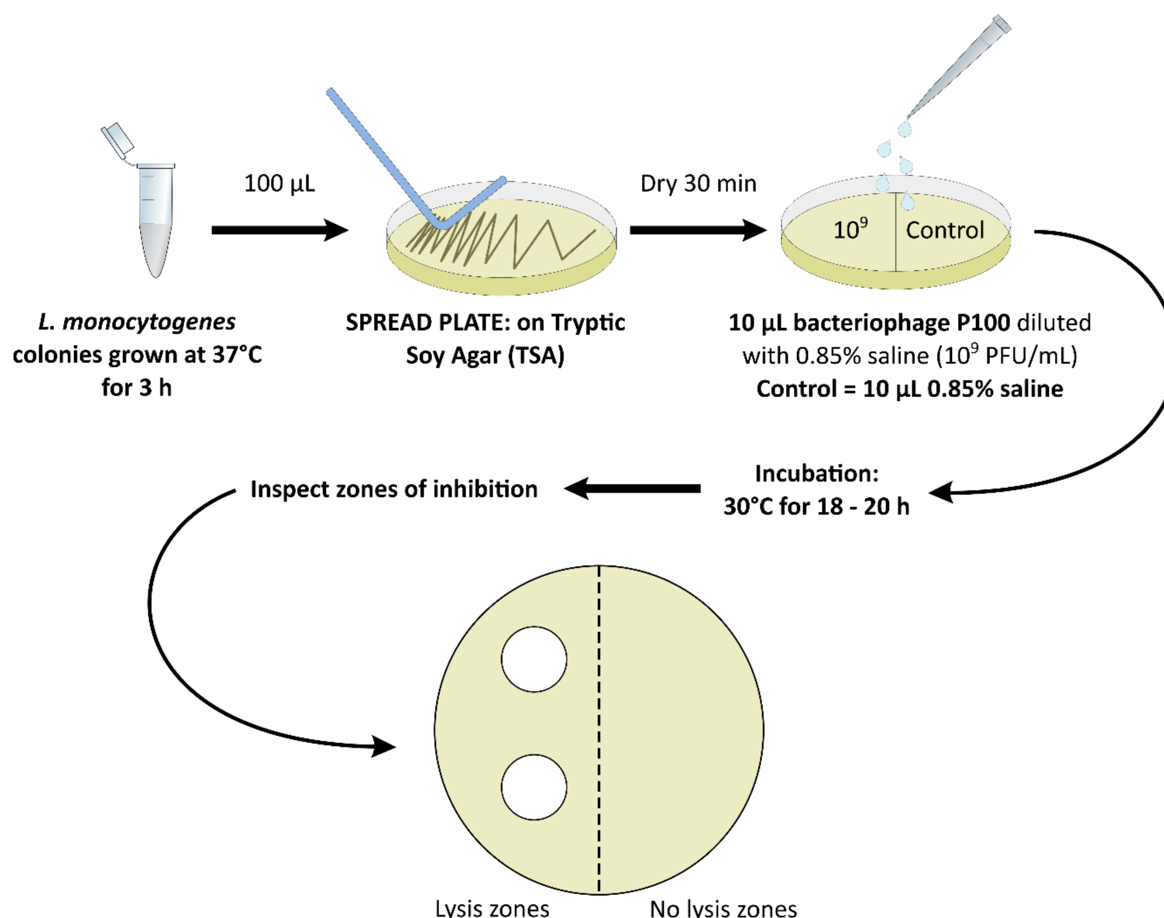

Figure 4.2 Outline of the spot test method used to test susceptibility of *L. monocytogenes* isolates against the bacteriophage P100. Lysis zones indicate isolate is susceptible to phage; if no lysis zones are observed, it implies that the isolate is tolerant to the phage.

For the purpose of the study, the terms "tolerant/tolerance" were used instead of "resistant" and refers to the phenotypic "resistance" observed on the bacterial lawn, when no zone of lysis/inhibition was present. The terms bacteriophage and phage are used interchangeably.

4.3.4. Statistical analysis

All statistical analyses were conducted in the R statistical environment (version 3.5.1) (R Core Team, 2017). In order to determine whether or not susceptibility patterns were statistically significant, binomial tests for the various categories were conducted. Binomial tests were chosen since experimental outcomes were all binary, i.e. isolates were either classified as susceptible or tolerant according to the aforementioned methods. Expected probabilities of 0.5 were used in all instances, in order to test the null hypothesis that phage effectiveness was equal to 50% (i.e. no better than random chance). The binomial tests were performed with the function binom.test

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from the base package. Furthermore, it was determined whether *L. monocytogenes* lineage groups (Chapter 3) influenced phage susceptibility. For this, a Chi-square test was conducted using the function chisq.test and the factors phage susceptibility (susceptible or tolerant) and Lineage (I or II).

4.4. Results and Discussion

After observing growth on RAPID'L.Mono™ agar (Oxoid), 180 isolates (from food, environment, and clinical origin) presented with colonies that were phenotypically positive for *L. monocytogenes* on RAPID'L.Mono™ agar (i.e. light-blue colonies). Of these 180 isolates that were subjected to PCR, the *hly*A gene was amplified in 177 isolates, thus confirming the identity of *L. monocytogenes.* The three samples for which the *hly*A gene could not be amplified (thus confirming an identity other than *L. monocytogenes*) were discarded and not used for further analyses. The remaining 177 *hly*A-positive isolates were screened for susceptibility against a commercial bacteriophage.

From the bacteriophage susceptibility tests, zones of lysis (Figure 4.3) were observed in 151 of 177 *L. monocytogenes* isolates (85%), meaning that 15% of the isolates exhibited tolerance.

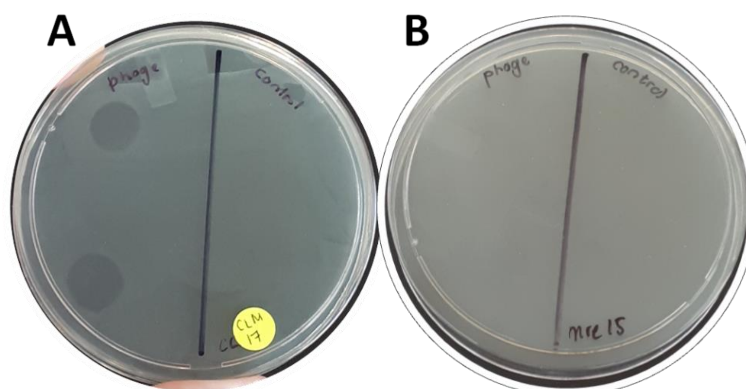

Figure 4.3 Zones of lysis (A) indicate susceptibility to phage; lack of lysis zones on the bacterial lawn (B) indicates tolerance to phage.

Listeria monocytogenes was significantly susceptible to phage activity in four of the five categories (Figure 4.4): Clinical (n=20, 95% susceptibility, p<0.001), Environmental (n=31, 84% susceptibility, p<0.001), Raw meats (n=31, 81% susceptibility, p<0.01), and Raw seafood (n=61, 97% susceptibility, p<0.001). The results from the Raw seafood isolates is similar to the findings of several researchers demonstrating the efficacy of phage P100 on raw seafood (Soni and Nannapaneni, 2010b; Soni *et al.*, 2010; Baños *et al.*, 2016). It should be noted however that these studies were conducted on the food product itself and not on the pure isolates as in our study. No

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significant results were observed in susceptibility from the Ready-to-eat category (n=34, 65% susceptibility, p=0.121).

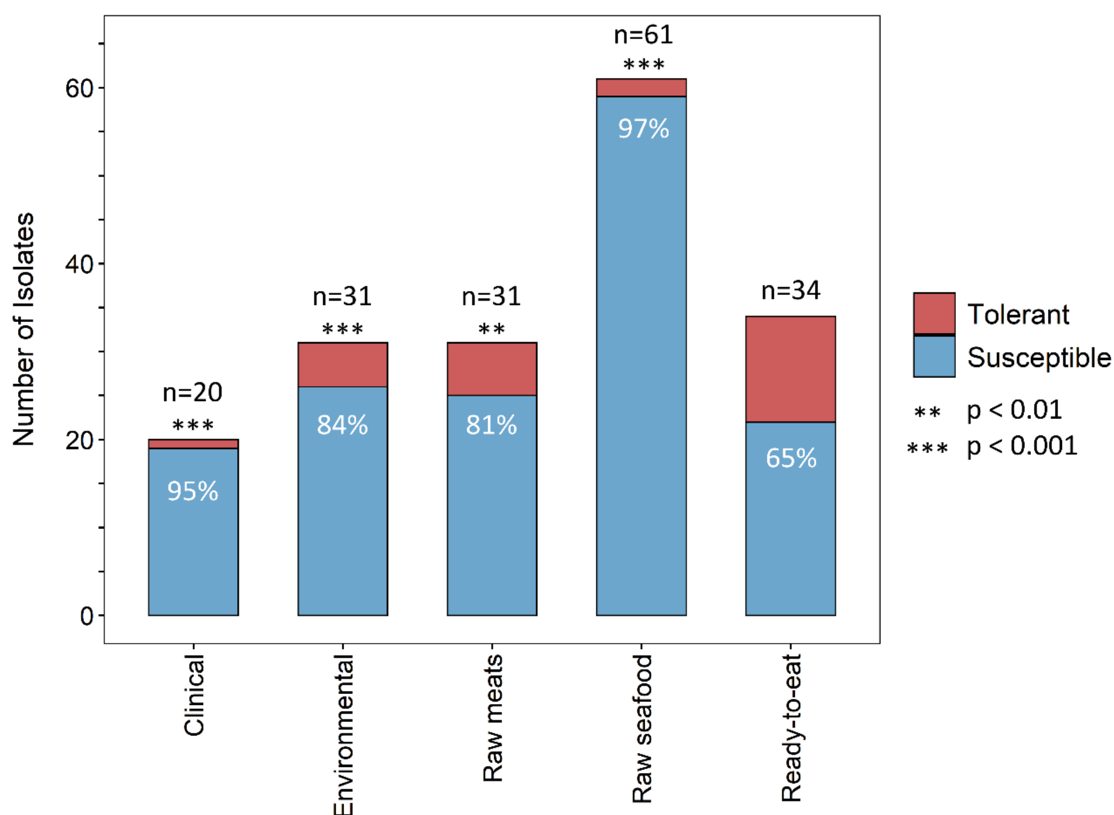

Figure 4.4 Susceptibility of *L. monocytogenes* isolates from different categories to bacteriophage. Isolates from all categories except Ready-to-eat were significantly susceptible to the bacteriophage. Significance values for phage susceptibility must be interpreted only on a per-category basis.

The categories Environmental, Ready-to-eat, and Raw foods, were further subdivided and investigated for patterns of bacteriophage susceptibility. Subdivision of the Environmental category (Figure 4.5) revealed that isolates from factory drains (n=11, 91% susceptibility), equipment (n=9, 78% susceptibility), floor (n=3, 67% susceptibility), hand (n=1), and surface (n=3, 67% susceptibility) showed susceptibility to phage activity. Although the low sample count in the subcategories makes the generalization of patterns difficult, it is possible that differences in drain cleaning procedures versus that of food processing equipment may influence *L. monocytogenes*' ability to adapt its cell wall and thus withstand or be susceptible to phage attachment (Cheng *et al.*, 2007; Bielmann *et al.*, 2015).

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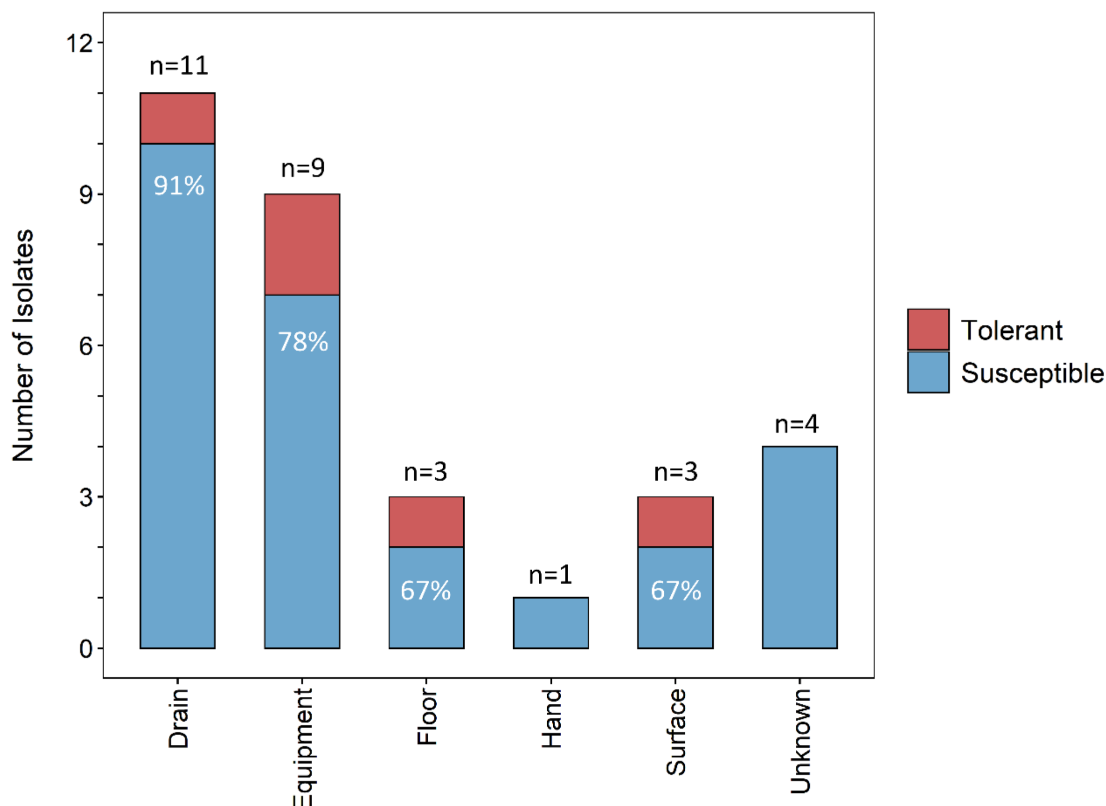

Figure 4.5 Susceptibility of *L. monocytogenes* isolates from Environmental origin to bacteriophage.

In the subcategories of the Raw meats isolates, there was a higher rate of tolerance from beef isolates (n=4, 50% susceptibility), in comparison to chicken (n=10, 70% susceptibility) and pork (n=4, 100% susceptibility). Isolates that were classified as "Unknown" were mostly susceptible, however, this is of little value since a finer classification cannot be assigned to the isolates. The lack of studies conducted specifically on raw meats such as poultry and beef make it difficult to explain the patterns of phage susceptibility observed here. Previous work showed that phages did not significantly reduce counts of *L. monocytogenes* on raw beef stored at 4°C (Dykes & Moorhead, 2002), but this was attributed to the low amount of phage used (Hagens & Loessner, 2010).

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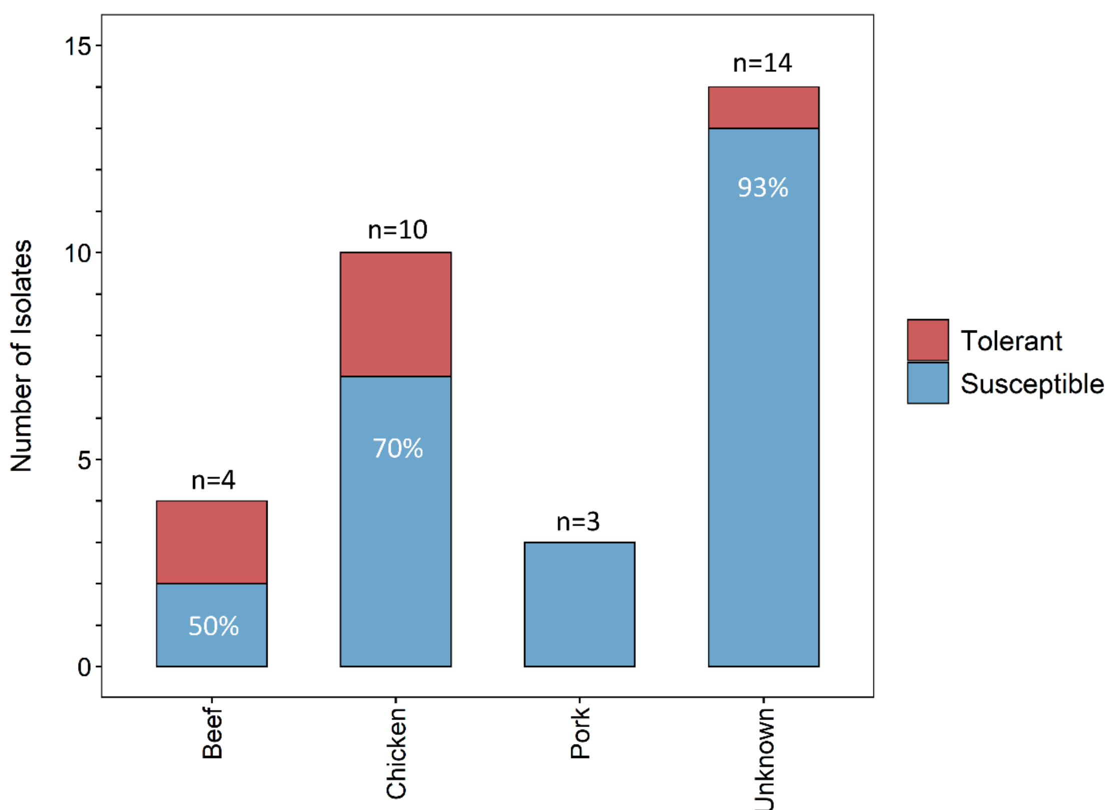

Figure 4.6 Susceptibility of *L. monocytogenes* isolates from Raw meats to bacteriophage.

In the Ready-to-eat category (Figure 4.7), isolates from deli meat were all susceptible to the bacteriophage (n=6, 100% susceptibility), which is consistent with findings of phage efficacy studies conducted on processed meat products (Guenther *et al.*, 2009; Rossi *et al.*, 2011; Gutiérrez *et al.*, 2017). Tolerance was observed in isolates obtained from fresh produce (n=5, 40% susceptibility), hummus (n=15, 60% susceptibility), and polony (n=5, 40% susceptibility). Although only one dairy isolate was analysed in this study, the use of phages has previously found to be effective on cheeses (Carlton *et al.*, 2005; Guenther & Loessner, 2011). Studies on fresh produce, however, showed that phage activity differs among fruits types (Leverentz *et al.*, 2003, 2004; Aparecida De Oliveira *et al.*, 2010), possibly due to differences in pH levels associated with the various types. The Ready-to-eat category in this study included *L. monocytogenes* isolates from an assortment of products (hummus, delicatessen meats, dairy, etc.), and such diversity could lead to varying results. Spot tests are reported to overestimate the efficacy of a bacteriophage due to the presence of produced bacteriocins that can lyse bacteria (Khan Mirzaei & Nilsson, 2015). Even with this overestimation, a large number of isolates from the Ready-to-eat category were tolerant to the phage and the results from this category were not significant. This means that given an isolate from such a category, chances that the phage will be able to destroy

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the host bacteria is no better than random (about 50%). Given that Listex™ (a commercial phage P100 product) has recently been approved for use on RTE foods (Mehmet, 2019), this finding is rather worrisome.

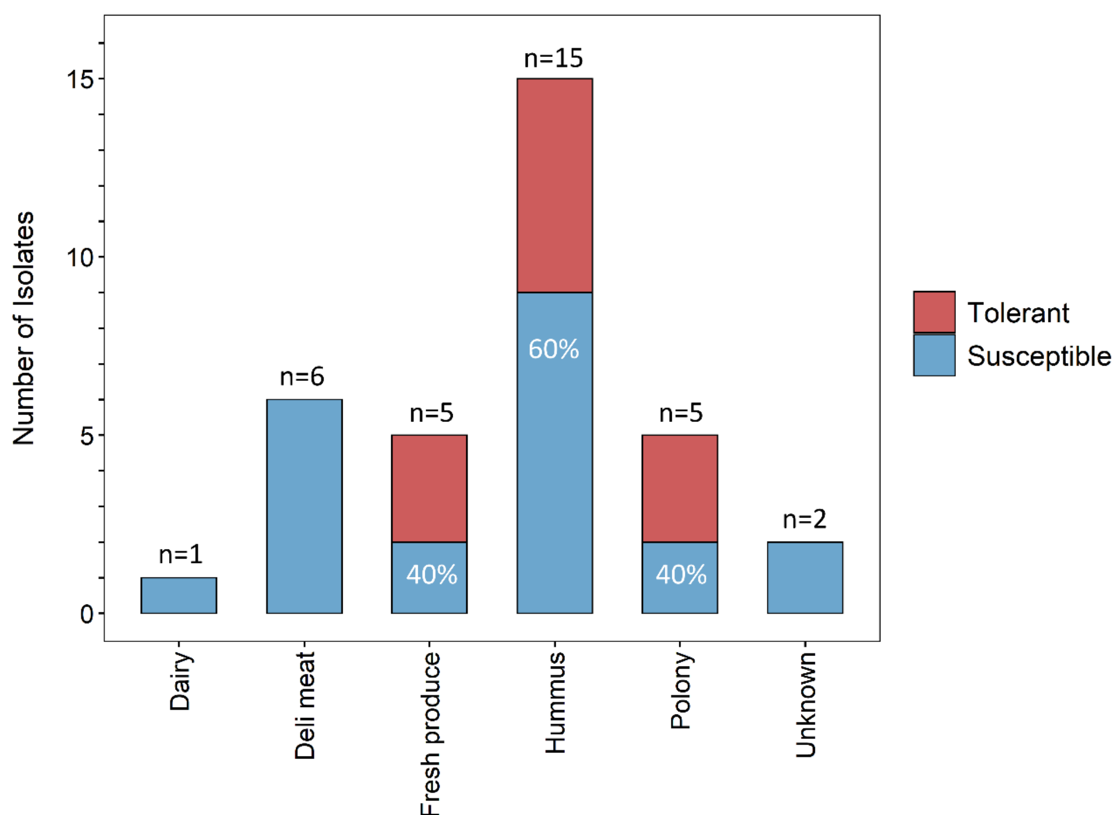

Figure 4.7 Susceptibility of *L. monocytogenes* isolates from Ready-to-eat category to bacteriophage.

Finally, both lineage groups were significantly susceptible to phage activity when considering all categories combined (Lineage I: n=116, 87% susceptibility, $p<0.001$; Lineage II: n=35, 80% susceptibility, $p<0.001$), and lineage groups did not significantly influence phage susceptibility (Figure 4.8) ($\chi^2=1.001$, $p=0.317$). The same pattern of susceptibility was seen when looking at the subcategories; in other words, whether or not isolates were susceptible to the phage was not dependent on belonging to a specific lineage group.

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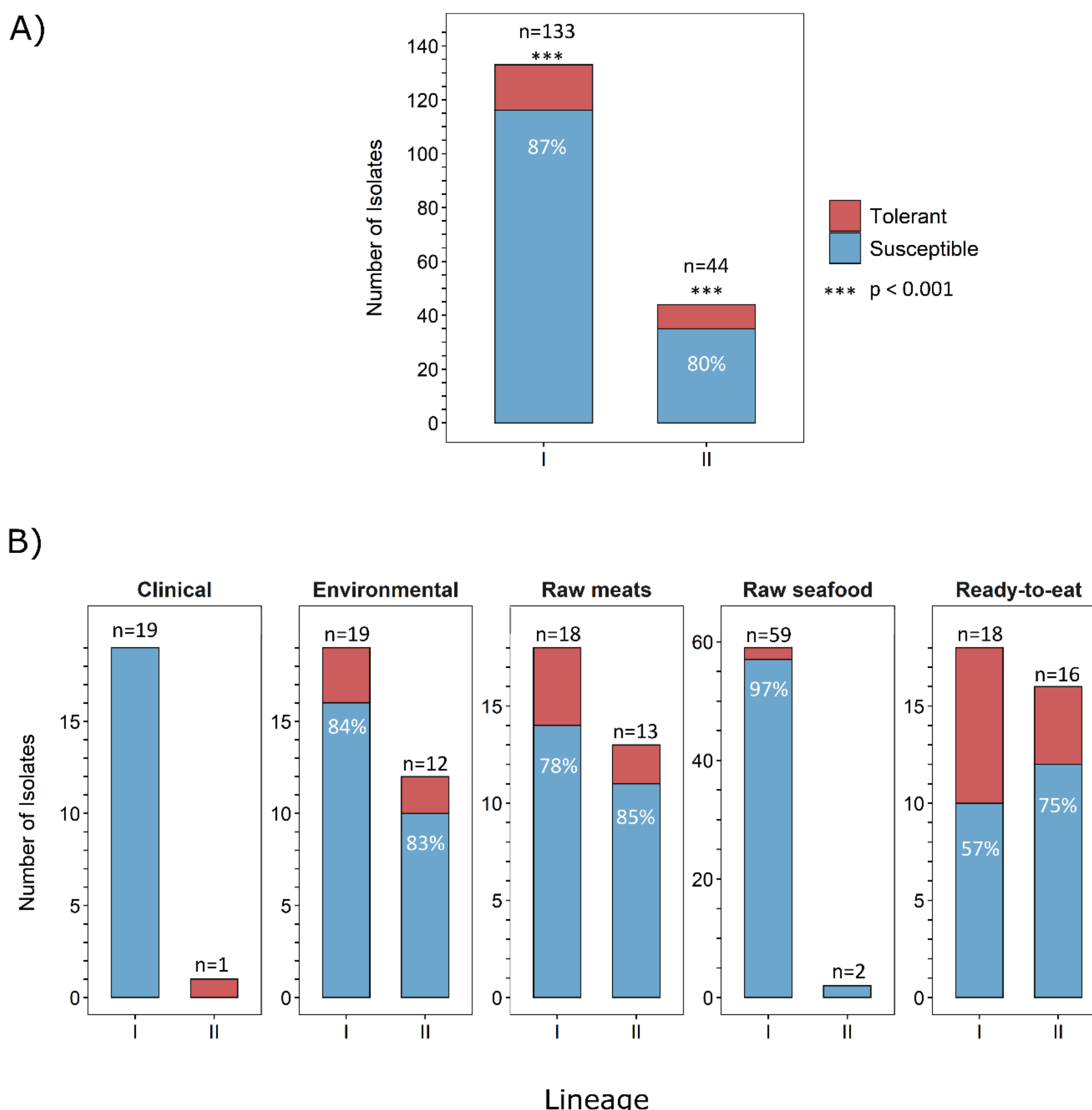

Figure 4.8 Both lineages were equally susceptible to phage activity, when considering main categories (A) and subcategories (B) of origin.

4.4.1. Phage susceptibility of isolates in Clinical category

All but one isolate in the Clinical category were susceptible to the bacteriophage. These isolates were all from Lineage I, while the one isolate that showed tolerance was from Lineage II (Addendum A). As there were no associations between lineage groups and susceptibility, this result is not necessarily generalizable. However, a number of studies showed serotype 4b (Lineage I) to be more susceptible to phages (Kim *et al.*, 2008; Vongkamjan *et al.*, 2012). Currently, listeriosis is not treated with bacteriophages; however, bacteriophages are used to treat diseases such as bacterial dysentery, salmonellosis, and cerebrospinal meningitis. Studies have also shown that oral administration of phages are effective in reducing *Escherichia coli* (Bruttin & Bru, 2005) and *L. monocytogenes* intestinally (Mai *et al.*, 2010). It should be emphasised that testing phage

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efficacy as therapeutic treatment against *L. monocytogenes* was not an aim in this study. However, although phage P100 was developed for use in the food processing industry and thus direct food application, it is nevertheless useful to investigate phage efficacy on isolates from clinical origin. This could give researchers insight as to how the bacterium adapts, and if different environmental pressures (e.g. the human host versus the food processing factory) causes *L. monocytogenes* to develop different resistant mechanisms. Thus, phage therapy as a treatment against *L. monocytogenes* in clinical settings remains a useful avenue for future research.

4.4.2. Association of *Listeria monocytogenes* lineage groups with phage susceptibility

We found no association between lineage groups and phage susceptibility. Phages need to recognize certain sugars on the WTAs of bacteria for successful attachment (Wendlinger *et al.*, 1996). A bacterial strain can become resistant if cell wall modifications (caused by environmental stresses) cause the wall sugar conformations to change, making phage attachment impossible, and thus prohibiting phage entry (Denes *et al.*, 2015; Fister *et al.*, 2016). Host tolerance can thus be gained via gene mutations controlling the addition of WTAs sugars, which lead to the non-attachment of the phage. Because WTAs determine the antigenic properties of *L. monocytogenes* (i.e. serotype classification) (Seeliger & Langer, 1989), and because sugar additions on WTAs determine phage attachment to *L. monocytogenes* (Eugster *et al.*, 2015; Trudelle *et al.*, 2019), it is more likely that an association exists between phage susceptibility and serotype, instead of lineage grouping.

Different *L. monocytogenes* serotypes differ in their cell wall structures. Phages subsequently differentially target serotypes due to such cell wall differences (Denes *et al.*, 2015), with serogroup 4 (4a, 4b and 4c) being the most sensitive to phage activity (Hagens *et al*. (2007). Furthermore, serogroup 3 strains mostly display phage resistance (Hagens & Loessner, 2007). Although phage P100 targets 95% of *Listeria* species and specifically serotypes 1/2 and 4 of *L. monocytogenes* (Loessner & Busse, 1990; Carlton *et al.*, 2005), it does not target *L. monocytogenes* serogroup 3. Thus, the possibility of tolerant isolates observed here being serotype 3b or 3c (Lineage I) or serotype 3a (Lineage II) cannot be excluded since serotype 3a has been linked to an outbreak associated with butter (Lyytikäinen *et al.*, 2002), even though serotypes 3a and 3c are generally rare in the food environment and human listeriosis cases (Nho *et al.*, 2015). Therefore, additional serotyping on these isolates will be beneficial to better determine the reasons for tolerance observed here.

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4.4.3. Key factors that play a role in phage susceptibility

Bacteria are continuously adapting to environmental stresses for better survival. As mentioned, mutations causing a change in cell wall structure is one such mechanism (Strydom & Witthuhn, 2015). Even a small structural change can result in non-attachment of the phage (Hyman & Abedon, 2010). Furthermore, it is likely that the processing of food products have different effects on the growth and adaptation of *L. monocytogenes*, which in turn influences phage adsorption to the cell wall (Cheng *et al.*, 2007; Bielmann *et al.*, 2015). However, phage resistance might not occur often due to the exertion experienced by bacteria from cell wall structure modification (Klumpp *et al.*, 2013).

True bacterial resistance to a bacteriophage can be due to mutations within the bacterial genome (consequently avoiding phage attachment), whereas transient resistance (or “tolerance") can be due to external factors (Hyman & Abedon, 2010). These factors can influence phage susceptibility, namely, phage and bacterial host concentration (Leverentz *et al.*, 2004; EFSA, 2009), physiological state of the bacterial host, temperature (Tokman *et al.*, 2016), and food matrix and composition (EFSA, 2009; Guenther *et al.*, 2009). There are thus numerous ways in which bacteria can acquire phage tolerance (Guenther *et al.*, 2009; Fister *et al.*, 2016; Aprea *et al.*, 2018)

The efficacy of bacteriophage control depends on the interaction between the phage and the target bacterial host. However, each of these microorganisms are influenced by different factors that could make efficient removal of the bacterial host less likely. It is important to consider the initial bacterial host count and phage concentration (Leverentz *et al.*, 2004; EFSA, 2009). A phage needs to diffuse into the bacterial cell for successful activity, which therefore requires the phage and the host bacteria to be in close proximity for bacterial eradication to occur (Hagens & Loessner, 2010). This can pose a challenge in food processing environments and food products, where complex surface structures can reduce phage accessibility towards bacteria which means bacteria can remain inaccessible. For the initial application of bacteriophages to successfully replicate and eradicate bacteria, the phage concentration needs to be high enough (Leverentz *et al.*, 2004; Hagens & Offerhaus, 2008; Hagens & Loessner, 2010; Strydom & Witthuhn, 2015). In the food industry *L. monocytogenes* are often only present in low numbers, and it is therefore recommended that sufficient amount of phage be used in order to ensure this contact between phage and host cell (Klumpp *et al.*, 2013; Strydom & Witthuhn, 2015). Biofilms may influence the probability of contact between phage and host. Phage P100 is effective in reducing biofilms on stainless steel (Soni & Nannapaneni, 2010a; Montañez-izquierdo *et al.*, 2012) and polystyrene (Rodríguez-Melcón *et al.*, 2018) surfaces, and although the load of bacterial cells in a biofilm might seem easily accessible for the phage, the polymeric substances

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within a biofilm might impede access to the bacterial host (Hyman & Abedon, 2010). When used specifically on foods, phage progeny (phage offspring) might not be able to make effective contact with the pathogens contaminating the product, and therefore a higher phage dosage would not necessarily lead to improved bacterial reduction (Moye *et al.*, 2018). This is because the food matrix influences phage movement (Klumpp *et al.*, 2013). The movement will be hampered in solid foods versus that of liquid foods, decreasing the likelihood of interaction between phage and host (Guenther *et al.*, 2009; Guenther & Loessner, 2011). Additionally, foods with irregular surfaces provide additional spaces that shelter *L. monocytogenes*, and as a result phage treatment has been more successful when used in liquid foods as to when compared to solid foods (EFSA, 2009; Guenther *et al.*, 2009; Moye *et al.*, 2018). This study was conducted on bacterial isolates recovered from food, as opposed to tests conducted on the food product itself (as in many other studies), thus the issue with diffusion on the food matrix is eliminated. However, the food product type to be treated must be carefully noted to ensure optimal treatment, since different food types can significantly impact on treatment efficacy, even when environmental conditions are identical (Guenther *et al.*, 2009). This is because the characteristics of the food type, such as the presence of acid or microflora (e.g. within cheese), will influence the stability and activity of the phage, while the state of the food product to which phage is applied (solid or liquid), will influence phage movement. The processing of these food products is also likely to have different effects on the growth and adaptation of the host bacterium, which in turn influences phage adsorption to the cell wall. The tolerance observed in some of the isolates in this study was likely a result of actual genetic difference between strains, since phage efficacy could not have been influenced by food types and thus food matrices.

Phenotypic or transient resistance can be influenced by temperature (temperature-based tolerance) (Leverentz *et al.*, 2003; Kim *et al.*, 2008; Kim & Kathariou, 2009; Mahony *et al.*, 2011; Tokman *et al.*, 2016). At the bacterium's optimum growth temperature (37°C), the cell wall has less rhamnose (a type of sugar needed for phage attachment) available (Tokman *et al.*, 2016). However, there are also instances where strains of *L. monocytogenes* demonstrate resistance at colder temperatures (Kim & Kathariou, 2009; Kim *et al.*, 2012). This could be an issue in a practical application in the food processing facility, where temperatures are usually much colder than the optimum growth temperature for a food pathogen (10-12°C). Moreover, even though phage P100 is reportedly effective at refrigeration temperatures, this efficacy is highly influenced by host status (physiological/nutritional) and environmental conditions (Miguéis *et al.*, 2017). For example, the efficacy of phages on *Salmonella* and *Campylobacter* spp. at cooler temperatures (~4°C) is conditional on a phage concentration higher than what would normally be used for other bacteria. This is also the case for *Listeria* phages, where a higher than normal concentration is needed for improved efficacy (Carlton *et al.*, 2005; Nóbrega *et al.*, 2014; Miguéis *et al.*, 2017),

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thus increasing control costs and making regulation problematic (Mahony *et al.*, 2011; Moye *et al.*, 2018). This higher concentration is required for sufficient phage replication, which in turn is needed for complete eradication of bacteria (Leverentz *et al.*, 2003; Carlton *et al.*, 2005; Klumpp *et al.*, 2013). Furthermore, applying phage to a surface of food product with a high concentration of bacterial host could eventually lead to subsequent emergence of resistant *L. monocytogenes* strains (Guenther & Loessner, 2011; Vongkamjan *et al.*, 2013; Fister *et al.*, 2016). For example, phage tolerant *L. monocytogenes* isolates emerged in an Austrian dairy plant only after a phage product was used in the facility (Fister *et al.*, 2016), which confirms that phage tolerance can arise as a result of phage application (Fister *et al.*, 2016). Such tolerance can take place at the adsorption step, when the phage attaches to the bacterial host. In order to avoid this potential tolerance, it has been suggested that phages with a broad host range be used or by rotationally applying phages with different host ranges (Guenther *et al.*, 2009; Klumpp *et al.*, 2013). Alternatively, the use of phage treatment should be reserved as a last resort only when *L. monocytogenes* persists within the niche of a food factory (Moye *et al.*, 2018), which can potentially prevent the phage from gaining tolerance through longtime exposure.

Correct timing of phage application is important, i.e. application of phage at critical points where contamination with *L. monocytogenes* is most likely (Hagens & Offerhaus, 2008; Guenther & Loessner, 2011). In order to avoid the emergence of phage resistance within the food processing environment, products should be removed from the production area after phage treatment, and surfaces coming in contact with phages should be sufficiently cleaned (Guenther & Loessner, 2011; Aprea *et al.*, 2018; Moye *et al.*, 2018). This could pose as a challenge for production facilities that already have a set production flow in place, making it difficult to change the movement of products. Food products are therefore treated just before packaging (Hagens & Loessner, 2010; Klumpp & Loessner, 2013). However, there is a risk that phages may persist in the food processing facility even long after treatment has stopped (Fister *et al.*, 2016). The persistence of phages in the environment could lead to phage resistant bacteria as a result of bacterial genome mutations (Fister *et al.*, 2019). It is therefore imperative to ensure the complete removal of phages after treatment to avoid further distribution of the phage within the facility (Sommer *et al.*, 2019). The likelihood of phage resistance is higher when treatment is used in the environment as opposed to foods (Hagens & Loessner, 2010). It is therefore suggested that phage treatment be reserved for use on foods, whereas biocides and sanitizers should be used in the food processing environments (Hagens & Loessner, 2010).

The classification of the host strain can affect phage efficacy, for example the lineage grouping (or serotypes) of the bacterial host (in this case *L. monocytogenes*). A potential challenge in the food industry may thus arise from not knowing which lineage group dominates at the time

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of phage application. Furthermore, the condition of the bacterial host also plays a role in whether or not phage adsorption will take place (EFSA, 2009). Bacteriophages are more likely to attach to bacterial cells in log-phase growth than those that are in stationary phase (Krueger & Fong, 1937; Braun *et al.*, 2006; EFSA, 2009). Stressed or injured cells can shift to the stationary phase and may have altered cell wall conformations, which would impede phage attachment to the host (Denes & Wiedmann, 2014). Subsequent mutations in *L. monocytogenes* genes can confer a growth advantage to the bacterium during this stationary phase, which could lead to increased virulence (Bruno & Freitag, 2011) and possibly affect the attachment of phage to the cells of *L. monocytogenes,* leading to phage tolerance. This presents a technical problem since bacteriophages used in the food processing environment are more likely to encounter stressed or injured cells in a stationary phase, as opposed to healthy, log-phase cells (Denes & Wiedmann, 2014; Strydom & Witthuhn, 2015). Thus, although this study was conducted under "best case scenario" conditions, i.e. log phase culture, a temperature optimal for phage attachment, and known lineage groupings, it is unlikely that these conditions will be encountered in the food processing environment.

Interestingly, the only other South African study (to the author's knowledge) conducted on phage P100 showed a very high number of *L. monocytogenes* isolates to be 'resistant' (Strydom, 2015). The authors attributed this resistance to the presence of prophages. This is probably due to prophages already being present in the *L. monocytogenes* strains (Klumpp & Loessner, 2013), as phage P100 is a strictly virulent phage, meaning it is unable to become a prophage (Carlton *et al.*, 2005; Guenther *et al.*, 2009).

Finally, it is important to note that tolerance, as observed in this study, was based purely on phenotypic observations and does not necessarily imply that *L. monocytogenes* isolates were resistant. It is possible that the isolates could revert back to susceptibility once they are exposed to a different set of environmental conditions (Guenther & Loessner, 2011). If phage treatment is to be used effectively in industry, tests will have to be conducted at the same food processing and storage temperatures to ensure phage activity is optimized for specific environmental conditions. Thus, although the results presented here contribute significantly to our understanding of the potential efficacy and applicability of phage treatment in the South African food processing environment, the lab experiments are still mostly preliminary and do not necessarily match the environmental conditions of food processing facilities. Future studies should subsequently build on the work presented here by focussing on industrial conditions, for example determining the optimal phage concentration needed specific to the food or environment to which it will be applied (Guenther & Loessner, 2011). Such studies will establish whether phage control is a viable option for implementation in the food industry.

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4.5. Conclusion

This study indicated that while a large majority of *L. monocytogenes* isolates were susceptible to phage P100, numerous isolates were still tolerant. Bacteriophage control offers a valuable tool, especially in the light of *L. monocytogenes* increasingly gaining resistance to antibiotics in clinical settings, and to sanitizers used in the food processing environment (Strydom & Witthuhn, 2015), in addition to consumers wanting more eco-friendly biocontrol options (in the processing facility and in foods). It is important to note that observed susceptibility patterns may change should phage treatment be applied directly to food products, as opposed to application to strains isolated from food. Firstly, the phage tests in this study were conducted on bacterial isolates recovered from food products instead of on the food products. In industry, the phage mixture will be applied directly to the solid or liquid food medium. This medium will influence the diffusion of the phage and subsequently influence whether or not the phage encounters the bacterial host present in the food. Secondly, temperature also plays a major role in phage attachment, and consequently the possible phage-resistance of the host. The tests in this study were conducted at 30°C to fit optimal phage-adsorption conditions (as described in literature), but in the food processing industry, these temperatures will be well below 25°C. This means that an isolate that presents as susceptible here, might present as tolerant once the phage is applied at cooler temperatures. It is therefore recommended that a similar study be repeated with food samples from various origins in South Africa, where phage is applied to different food surfaces and incubated under a variety of temperatures, as encountered in the food industry. In fact, incubation of phage treated isolates at temperatures more characteristic of food processing environments (i.e. colder), instead of optimum incubation temperatures for the pathogen, could well be the most informative way forward to establish the viability of phage control for the food industry (Vongkamjan *et al.*, 2013). Another interesting avenue to explore concerns the effectiveness of the phages against stationary phase cultures since this study only involved log-phase isolates (Bryan *et al.*, 2016; Abedon *et al.*, 2017). With so many variables influencing phage attachment, and thus its efficacy, it is imperative that future bacteriophage efficacy studies be tailored to suit the environmental conditions in which phage treatment will be applied, as bacteriophage efficacy tests on an industrial scale are yet to be conducted (Aprea *et al.*, 2018).

Despite the increasing interest in the use of bacteriophages as a means to control *L. monocytogenes*, there is still a gap in knowledge on how these phages will act in the food processing environment, and in turn how *L. monocytogenes* will adapt to treatment by these phages. Unlike antibiotic susceptibility tests, there is no single standardized protocol yet to test whether isolates are susceptible to bacteriophages. Plaque assays and spot tests all have varying results (Hyman and Abedon, 2010; Khan Mirzaei & Nilsson, 2015), making the determination on

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whether an isolate is susceptible or resistant very difficult. It is therefore imperative that future studies not only focus on standardizing susceptibility tests but that such studies also be aligned towards industrial-scale application since that is ultimately the end goal of bacteriophage treatment.

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Chapter 5

Antibiotic susceptibility of *Listeria monocytogenes* isolates from food, environmental, and clinical origin in the Western Cape, South Africa

5.1. Abstract

Listeria monocytogenes is one of the top foodborne pathogens responsible for food related fatalities. Despite South Africa experiencing the largest ever global listeriosis outbreak, information on the bacterium and its resistance towards antibiotics is severely lacking. Furthermore, until now it remained to be discovered whether or not *L. monocytogenes* antibiotic resistance patterns in South Africa mirror resistance patterns elsewhere in the world. The aim of this study was therefore to determine antibiotic resistance of *L. monocytogenes* isolates from diverse origins in the Western Cape, South Africa (clinical, food, and environment) to five different antibiotics, namely ampicillin, chloramphenicol, erythromycin, gentamicin and tetracycline, using the disc diffusion method as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). This study indicated that all the isolates were significantly susceptible to ampicillin, while a large number of isolates were resistant to chloramphenicol, erythromycin, and tetracycline. Clinical and Raw seafood isolates were significantly susceptible to all antibiotics, while Raw meats had the highest number of resistant strains. This study makes a valuable contribution to the lack of data on antibiotic resistance and multidrug resistant strains of *L. monocytogenes* in the South African environment, and how it compares to resistance patterns found in other countries.

5.2. Introduction

Foodborne illnesses, caused by food pathogens, are a major threat to public health the world over and are responsible for hundreds of thousands of human deaths every year (WHO, 2019). The most important pathogens responsible for such a high number of deaths include *Salmonella*, *Toxoplasma gondii,* and *Listeria monocytogenes*, causing salmonellosis, toxoplasmosis, and listeriosis, respectively (White *et al.*, 2002; WHO, 2015; EUFIC, 2016). Moreover, the deaths resulting from these illnesses are increasing annually (Bari & Yeasmin, 2018). Specifically, *L. monocytogenes* is a major food contaminant and is ever-present in the food processing environment. As such, it is a particularly challenging microorganism to control.

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Listeria monocytogenes is a Gram-positive, facultatively anaerobic, non-spore forming bacterium (Forsythe, 2010) that is ubiquitous in the environment and thus commonly associated with raw food material, where it acts as a food pathogen (White *et al.*, 2002; de Noordhout *et al.*, 2014). Despite its optimum growth temperature of between 30°C and 37°C, it is able to grow and proliferate at refrigeration temperatures (Forsythe, 2010), which means that refrigeration is not an effective method of control against it. In recent years, there have been several listeriosis outbreaks around the world, with the foods mostly implicated being dairy, meat and seafood, and fresh produce (Fallah *et al.*, 2012; CDC, 2019). It therefore seems the presence of this pathogen in the food processing environment is on the rise.

Listeria monocytogenes is responsible for causing a serious infection, called listeriosis. The source of infection is mainly by eating contaminated foods; however, the infection can also be transmitted to an unborn baby from its mother via the placenta (Allerberger & Wagner, 2010). Listeriosis rarely affects healthy individuals and only manifests if high numbers of *L. monocytogenes* cells are ingested, which could at most lead to an onset of febrile gastroenteritis, and as such, the illness is self-limiting in these individuals (Dalton *et al.*, 1997; Aureli *et al.*, 2000; de Noordhout *et al.*, 2014; Montero *et al.*, 2015). Listeriosis is however, detrimental in immunocompromised individuals, i.e. individuals that have a suboptimal immune system such as pregnant women, neonates, the elderly, or patients with cancer, TB or HIV (Epstein *et al.*, 1996; Forsythe, 2010; de Noordhout *et al.*, 2014). In these individuals, listeriosis can often develop into life-threatening illnesses such as septicaemia and meningitis, leading to a high number of fatalities annually (Allerberger & Wagner, 2010). Because listeriosis is such an important and potentially fatal disease, it is imperative to effectively and properly control *L. monocytogenes* not only in the food processing industry, but particularly in environments that are frequented by immunocompromised individuals, such as hospitals and clinics. In food processing environments, *L. monocytogenes* is usually controlled by various sanitizers that contain quaternary ammonium compounds, peroxyacetic acid, or chlorine (Food and Drug Administration, 2017), whereas in listeriosis patients control is achieved with antibiotic treatment (Hof *et al.*, 1997; Allerberger & Wagner, 2010).

The current preferred antibiotic treatment for listeriosis in immunocompromised individuals is ampicillin, used as a standalone treatment or in combination with an aminoglycoside such as gentamicin (Charpentier & Courvalin, 1999; Hof, 2004). For treating listeriosis in pregnant women, erythromycin is usually the antibiotic of choice (Alonso-Hernando *et al.*, 2012). Although *L. monocytogenes* is known to be susceptible to a wide variety of antibiotics, it is intrinsically resistant to cephalosporins, as such antibiotics are unable to bind to PBP 3 (penicillin-binding protein), which is the main target for β-lactams in *L. monocytogenes* (Vicente

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et al., 1990; Hof *et al.*, 1997). However, there are various ways by which *L. monocytogenes* (and bacteria in general) can acquire resistance to antibiotics, which include horizontal gene transfer, genetic mutations, and the use of efflux pumps (Cox & Wright, 2013; Gullberg, 2014; Allen *et al.*, 2016; Noll *et al.*, 2017; Wright, 2019).

Due to the high mortality rate of individuals that have contracted listeriosis, which can be as high as 40% among immunocompromised individuals, the emergence of antibiotic-resistant *L. monocytogenes* strains has been of great concern (Todd & Notermans, 2011). Furthermore, an even bigger cause for concern is the increase of such antibiotic-resistant *L. monocytogenes* strains in recent years and several studies have reported the emergence of such strains in the food processing environment (Addendum B). Fortunately, some antibiotics are still highly effective treatments against *L. monocytogenes*. For example, β-lactams (such as ampicillin and penicillin) are still the most effective antibiotics against *L. monocytogenes*, with aminoglycosides (e.g. gentamicin) being only slightly less effective (Vitas *et al.*, 2007). At present, there are several general uses for antibiotics beyond the treatment of infectious diseases, some of which include: 1) prevention of disease and growth promotion in animal production industry (e.g. tetracycline); 2) as biocides in household and toiletry products (e.g. triclosan); and 3) therapeutic use in clinical settings (e.g. ampicillin) (Cerf *et al.*, 2010; Davies & Davies, 2010; Christensen *et al.*, 2011; Karmi, 2014). It should be noted that different geographical environments, as well as the different applications of antibiotics (e.g. clinical or veterinary use), can have an influence on the resistance or susceptibility patterns of *L. monocytogenes* isolates (Wang *et al.*, 2013; Allen *et al.*, 2016).

In South Africa, a listeriosis outbreak in 2017-18 was thought to be the largest global outbreak on record, with 1 060 cases and 216 deaths reported (NICD, 2018b). Thus, there is a great need to establish which antibiotics are still effective treatments against *L. monocytogenes*. Currently, there is a general paucity of information in South African literature on the possible antibiotic resistance of *L. monocytogenes* isolates from the food and clinical environment. This, together with the high levels of mortality associated with the recent outbreak and the fact that antibiotic-resistant strains of *L. monocytogenes* are generally on the rise, is, therefore, an urgent call for a more in-depth investigation into the efficacy of antibiotics currently used against *L. monocytogenes* in South Africa. The objective of this study was thus to assess the antibiotic susceptibility of *L. monocytogenes* isolates from clinical, food, and environmental origin against five different antibiotics used in clinical treatment. By comparing *L. monocytogenes* isolates from the environment, food samples, and clinical cases, it could be determined whether certain patterns of resistance exist.

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5.3. Materials and Methods

5.3.1. Sample collection and storage

Refer to Chapter 3 for detailed information regarding sample numbers, collection, and exact methods followed for lineage classification, and Addendum A for additional sample information and classification results. Briefly, all isolates were cultured and only those that were confirmed positive for *hly*A gene amplification by means of polymerase chain reaction (PCR) and classified into lineage groups by restriction fragment length polymorphism (RFLP) and were subsequently included for antibiotic testing. For the remainder of the chapter, "Ready-to-eat" refers specifically to one of the main categories in this study, whereas "RTE" refers to ready-to-eat foods in general.

5.3.2. Antibiotic susceptibility tests

Listeria monocytogenes isolates stored as glycerol stocks were streaked onto tryptic soy agar (TSA) and grown overnight at 37°C. Antibiotic susceptibility testing of the different *L. monocytogenes* isolates was performed by the disc diffusion method as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2012). The overnight cultures of *L. monocytogenes* were suspended in 0.85% saline solution to obtain a McFarland standard of 0.5 (Oxoid™, ThermoFischer Scientific). The suspensions were applied on Mueller-Hinton agar supplemented with 5% defibrinated horse blood and 20 mg/L β-NAD (MH-F agar) (Oxoid™) with a cotton swab in three different directions (each rotating 60°) and antibiotic discs were applied, with a sterilized tweezer, within 15 min of swabbing. The supplemented agar (MH-F) is recommended for fastidious bacteria such as *L. monocytogenes* (EUCAST, 2012). Five different antibiotics (Oxoid™) were tested, namely ampicillin, chloramphenicol, erythromycin, gentamicin, and tetracycline (Table 5.1). After 20-24 h incubation at 37°C, the zones of inhibition were measured with a calliper and the results were interpreted according to EUCAST recommendations (EUCAST, 2012). Currently, there are no set breakpoints for *L. monocytogenes* except for ampicillin and erythromycin, therefore the breakpoints of *Staphylococcus aureus* was used to interpret the zones of inhibition for the remaining antibiotics (Chen *et al.*, 2010a; Maćkiw *et al.*, 2016; Noll *et al.*, 2017). *Staphylococcus aureus* ATCC 25923 was used as a control strain (Wiggins *et al.*, 1978; Chen *et al.*, 2010a; Wang *et al.*, 2015a; Maćkiw *et al.*, 2016). The inhibition zones were classified as susceptible or resistant. Isolates that were resistant to at least one antibiotic from three or more different antimicrobial categories were classified as multi-drug resistant (MDR) (Magiorakos *et al.*, 2012) (Table 5.1). The choice of antibiotics tested was based on the use of these antibiotics in a clinical setting, as well as the emergence of resistance against certain antibiotics used for listeriosis (Alonso-Hernando *et al.*, 2012; Fallah *et al.*, 2013).

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Table 5.1 Isolates were classified as susceptible (S) or resistant (R) based on the inhibition zones (mm) observed; isolates that were resistant to at least one antimicrobial from three or more antimicrobial category were classified as multidrug resistant (MDR) (EUCAST, 2012)

Antibiotic	Antimicrobial category	Mode of action	Disc content (μg)	Zone diameter breakpoint (mm) (EUCAST, 2012)	
				S	R
Ampicillin	Beta-lactams	Bacterial cell wall synthesis inhibitor	10	≥16	<16
Chloramphenicol	Phenicols	Inhibits protein synthesis (prevents growth)	30	≥18	<18
Erythromycin	Macrolides	Inhibits protein synthesis (bacteriostatic)	15	≥25	<25
Gentamicin	Aminoglycoside	Inhibits protein synthesis (leads to cell death)	10	≥18	<18
Tetracycline	Tetracycline	Inhibits protein synthesis (ribosomal inhibitor)	30	≥22	<19

5.3.3. Statistical analysis

All statistical analyses were conducted in the R statistical environment (version 3.5.1) (R Core Team, 2017). In order to determine whether or not antibiotic resistance patterns were statistically significant, binomial tests for the various categories were conducted. Tests for each antibiotic were performed separately. Binomial tests were chosen since experimental outcomes were all binary, i.e. isolates were either classified as susceptible or resistant according to the aforementioned methods. Expected probabilities of 0.5 were used in all instances, in order to test the hypothesis that antibiotic effectiveness was equal to 50% (i.e. no better than random chance). The binomial tests were performed with the function binom.test from the base package.

5.4. Results and Discussion

After observing growth on RAPID'L.Mono™ agar (Oxoid), 180 isolates (from food, environment, and clinical origin) presented with colonies that were phenotypically positive for *L. monocytogenes* on RAPID'L.Mono™ agar (i.e. light-blue colonies). Of these 180 isolates that

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were subjected to PCR, the *hly*A gene was amplified in 177 isolates, thus confirming the identity of *L. monocytogenes.* The three samples for which the *hly*A gene could not be amplified (thus confirming an identity other than *L. monocytogenes*) were discarded and not used for further analyses. The remaining *hly*A-positive 177 isolates were screened for their antibiotic sensitivity patterns.

Antibiotic susceptibility tests indicated that all the isolates (n=177) were susceptible to ampicillin (Addendum A). Isolates from the Clinical category (n=20) were significantly susceptible (p<0.001) to all five antibiotics tested (Figure 5.1). Interestingly, one isolate (classified as Lineage I, see Chapter 3) from the Clinical category showed resistance to four antibiotics, namely chloramphenicol, erythromycin, gentamicin, and tetracycline. Eight of all the isolates included in this study exhibited multidrug resistance, i.e. these isolates were resistant to three or more antibiotics.

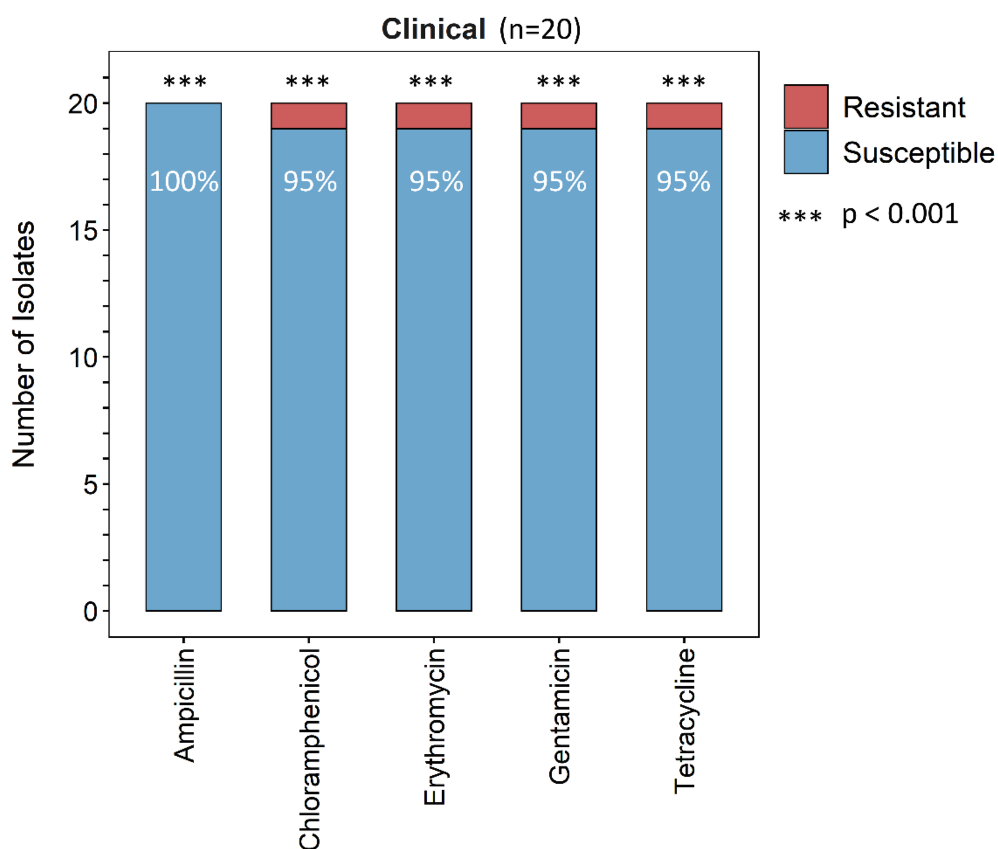

Figure 5.1 Susceptibility of *Listeria monocytogenes* isolates from Clinical category to respective antibiotics.

Isolates from Raw seafood category (n=61) were significantly susceptible (p<0.001) to all five antibiotics tested (Figure 5.2, ampicillin not shown). There were a few isolates from this category resistant to chloramphenicol, erythromycin, and tetracycline, which is in agreement with other

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studies conducted on *L. monocytogenes* isolated from seafood (Fallah *et al.*, 2013; Jamali *et al.*, 2015).

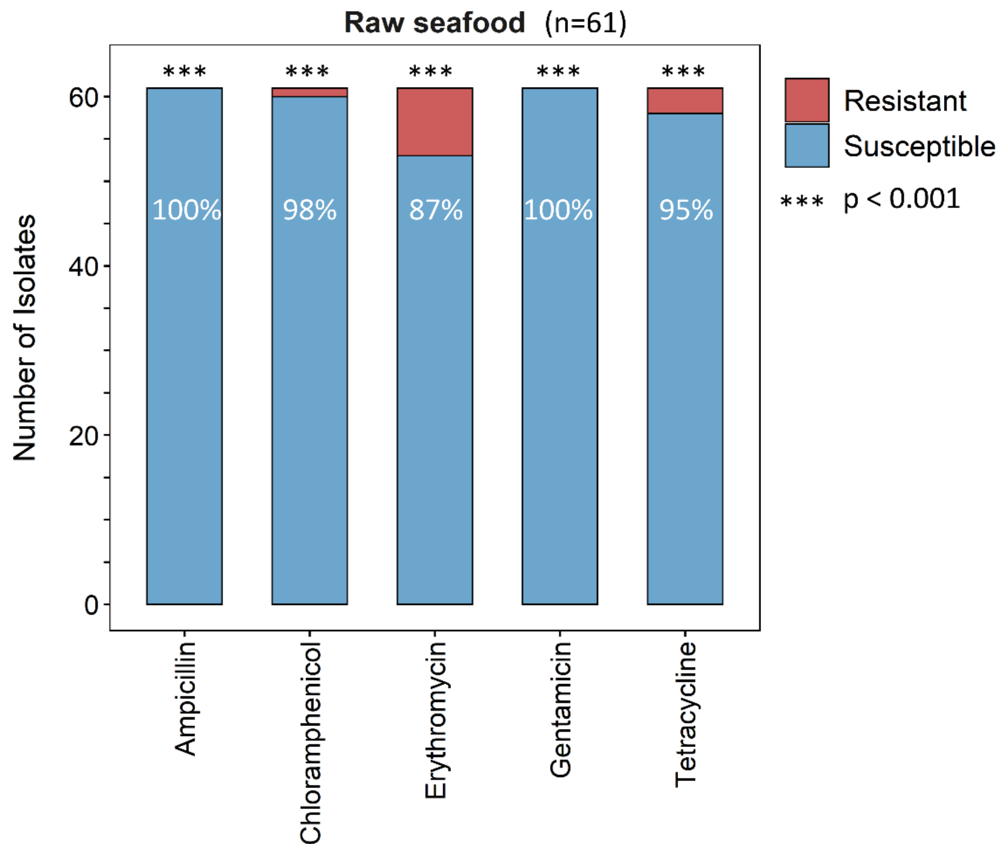

Figure 5.2 Susceptibility of *Listeria monocytogenes* isolates from Raw seafood category to respective antibiotics.

Isolates from the Ready-to-eat category (n=34) were also significantly susceptible (all p<0.001) to all antibiotics tested (Figure 5.3).

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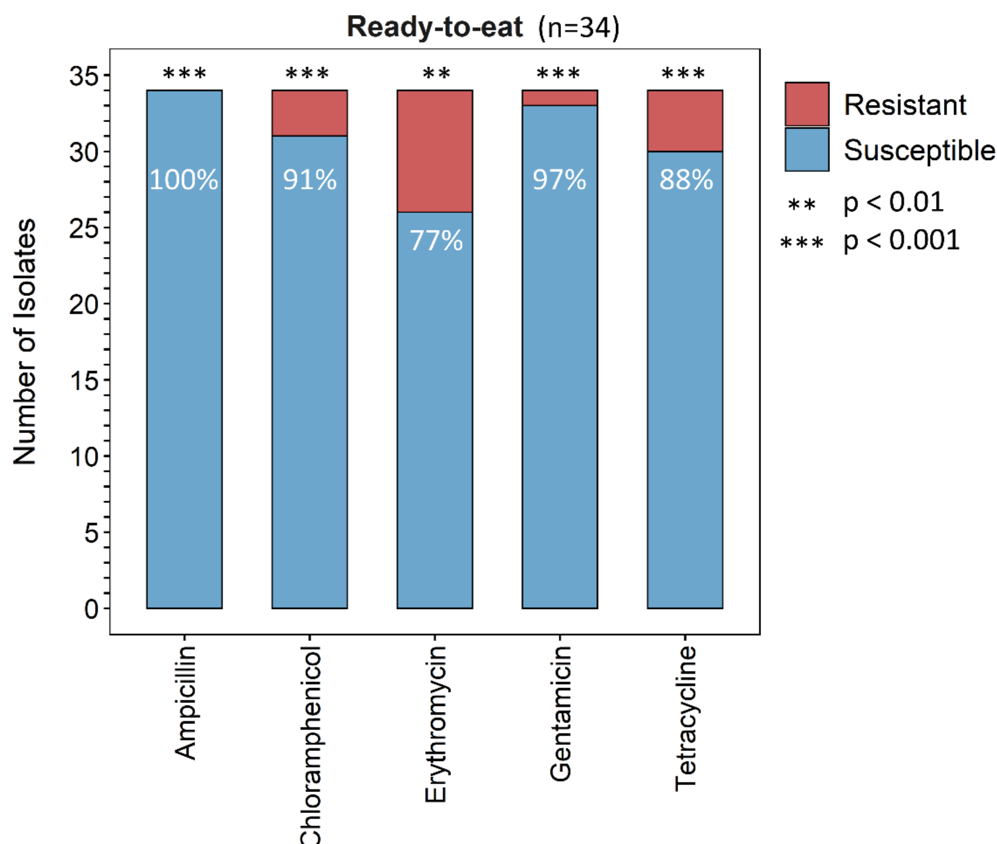

Figure 5.3 Susceptibility of *Listeria monocytogenes* isolates from Ready-to-eat category to respective antibiotics.

Subdivision of the Ready-to-eat category revealed that hummus (n=15) isolates were all susceptible to gentamicin and tetracycline (Figure 5.4), however, there were isolates resistant to chloramphenicol (n=15, 93% susceptibility) and erythromycin (n=15, 87% susceptibility). Isolates from deli meat (n=6) were all susceptible to chloramphenicol and gentamicin, but there were isolates resistant to erythromycin (n=6, 50% susceptibility) and tetracycline (n=6, 93% susceptibility). There were five isolates originating from polony (n=5) and although all of these isolates were susceptible to gentamicin, there were a few that showed resistance to chloramphenicol (n=5, 60% susceptibility), erythromycin (n=5, 80% susceptibility), and tetracycline (n=5, 80% susceptibility). Chloramphenicol- and tetracycline resistance have been reported previously among *L. monocytogenes* isolates from RTE meat products (Wang *et al.*, 2015b; Escolar *et al.*, 2017). The fresh produce (n=5) isolates were all susceptible to chloramphenicol, however there were isolates displaying resistance to erythromycin (n=5, 60% susceptibility), gentamicin (n=5, 80% susceptibility) and tetracycline (n=5, 60% susceptibility). The one dairy isolate was susceptible to all antibiotics.

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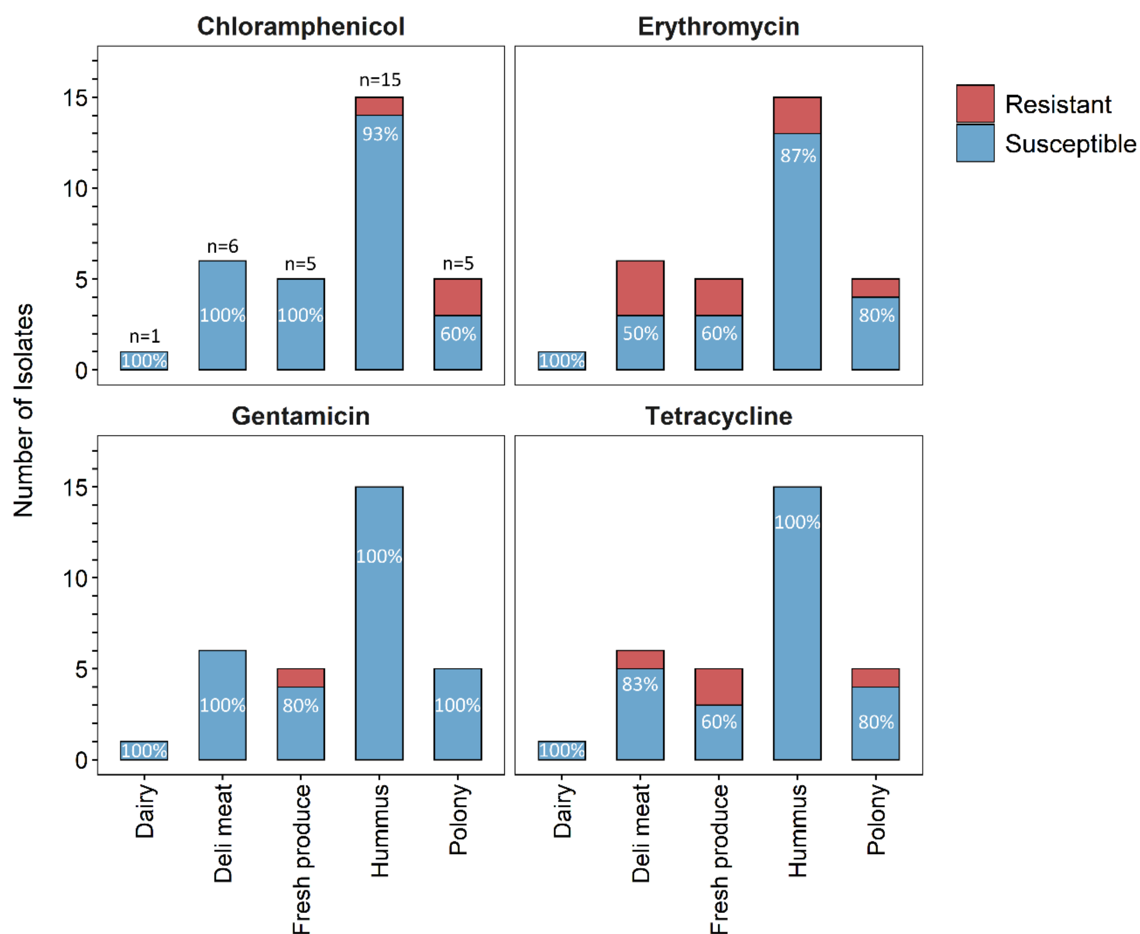

Figure 5.4 Susceptibility of *Listeria monocytogenes* isolates from Ready-to-eat subcategories to respective antibiotics.

In the Environmental category (Figure 5.5), isolates were significantly susceptible to ampicillin (not shown), chloramphenicol ($p<0.01$), gentamicin and tetracycline (both $p<0.001$). A large fraction of Environmental isolates were resistant to erythromycin (n=31, 61% susceptibility), however, these results were not significant ($p=0.281$).

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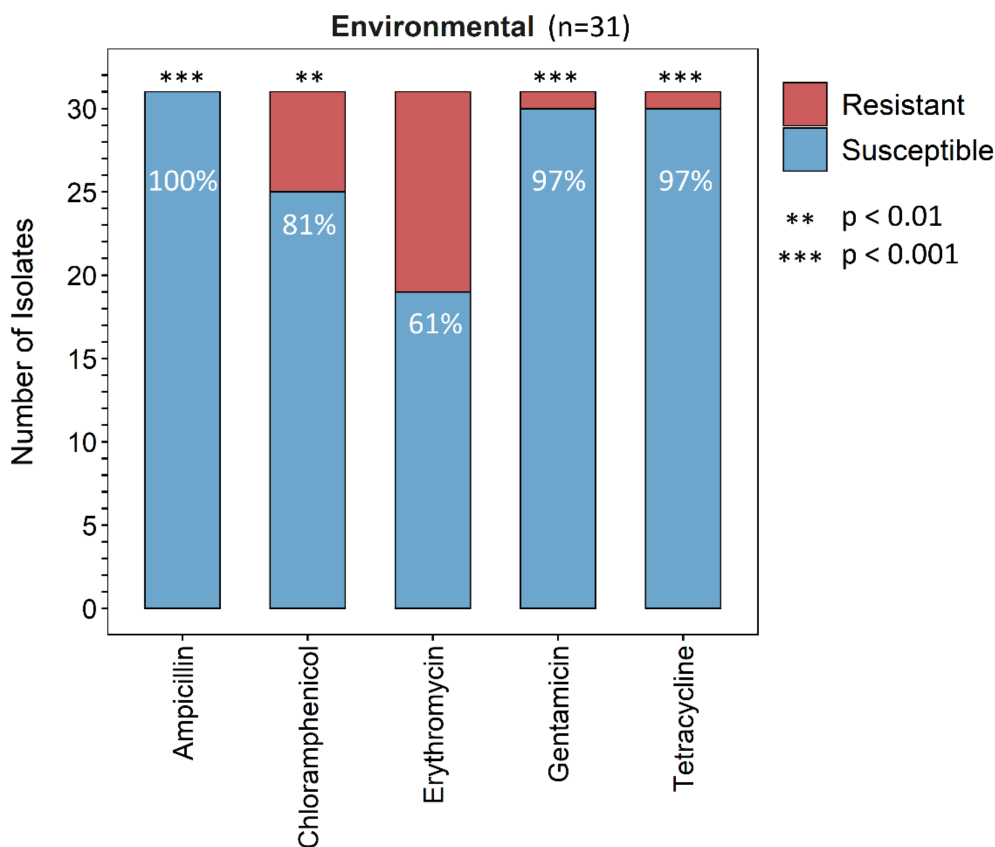

Figure 5.5 Susceptibility of *Listeria monocytogenes* isolates from Environmental category to respective antibiotics.

Further subdivision of the Environmental category (n=31) (Figure 5.6) indicated that the isolates originating from food processing equipment (n=9) were all susceptible to chloramphenicol, gentamicin, and tetracycline. However, there were isolates resistant to erythromycin (n=9, 78% susceptibility). The isolates from factory drains (n=11) were susceptible to tetracycline (100% susceptibility) and gentamicin (n=11, 91% susceptibility), however a smaller fraction showed susceptibility to chloramphenicol (n=11, 64% susceptibility) and erythromycin (n=11, 55% susceptibility). The "hand" isolate (n=1) was resistant to chloramphenicol, erythromycin, and tetracycline. The surface isolates (n=3) were all resistant to gentamicin and tetracycline, while a greater fraction of these samples were resistant to erythromycin (n=3, 67% susceptibility).

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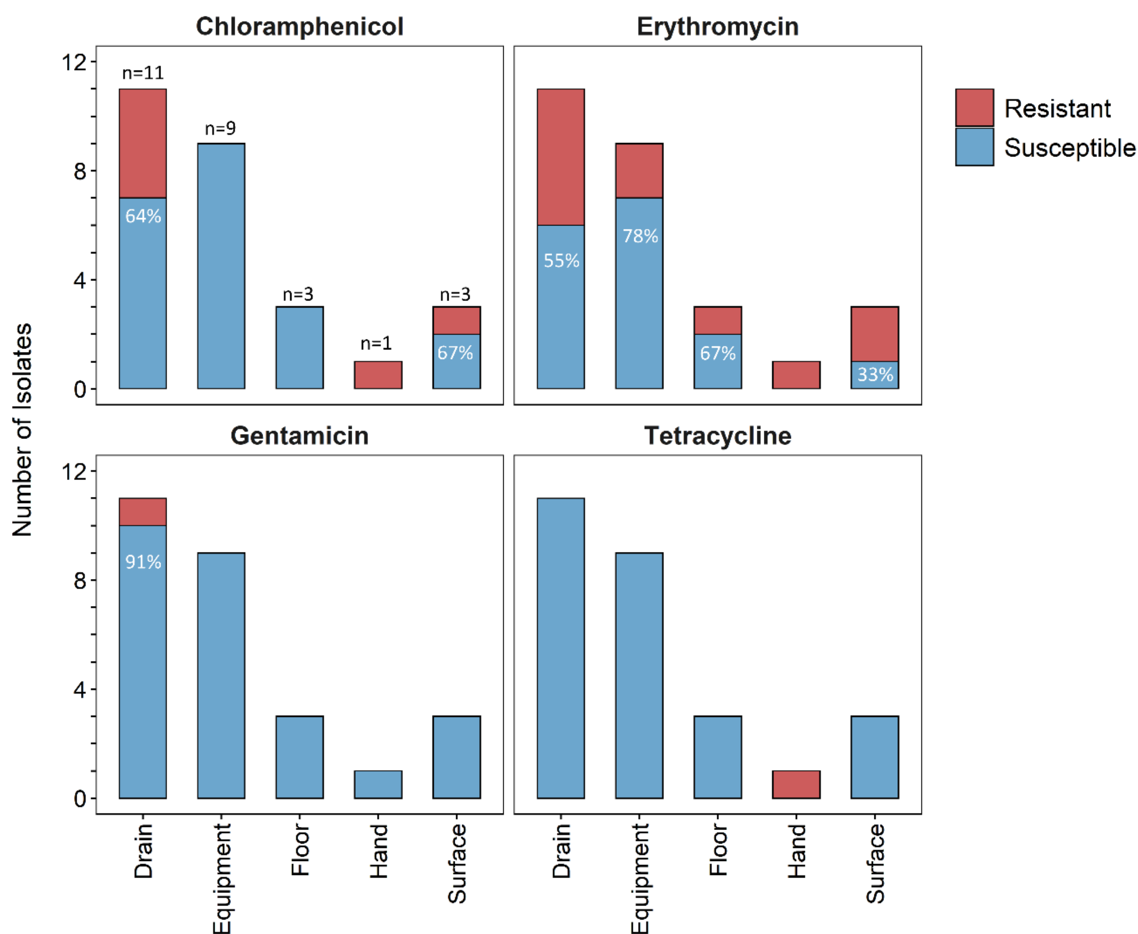

Figure 5.6 Susceptibility of *Listeria monocytogenes* isolates from Environmental subcategories to respective antibiotics.

The Raw meats isolates (n=31) (Figure 5.7) showed significant susceptibility to ampicillin ($p<0.001$, not shown), chloramphenicol ($p<0.01$) and gentamicin ($p<0.001$), and while a large fraction of isolates were susceptible to tetracycline (n=31, 65% susceptibility), it was not significant ($p=0.15$). Slightly more than half of the isolates showed resistance to erythromycin (n=31, 45% susceptibility), however, these results were also not significant ($p=0.72$). Isolates from this category also exhibited the highest level of multidrug resistance (13%).

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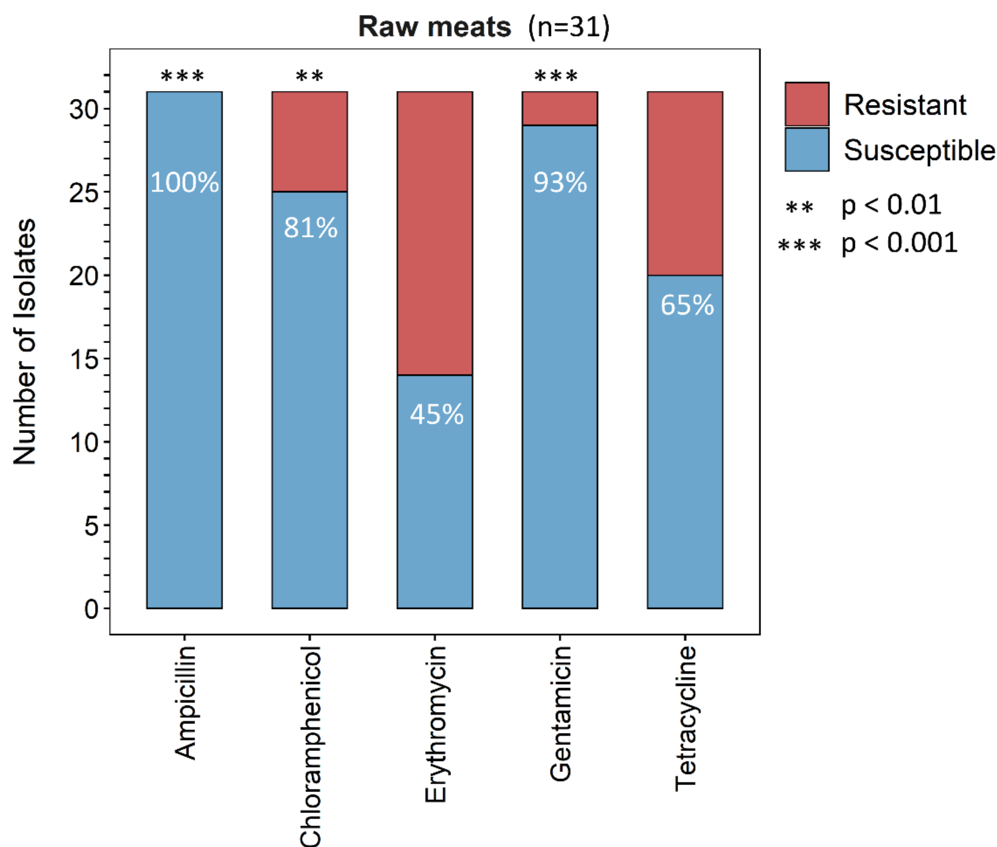

Figure 5.7 Susceptibility of *Listeria monocytogenes* isolates from Raw meats subcategories to respective antibiotics.

Subdivision of the Raw meats category (n=31) (Figure 5.8) revealed that the chicken isolates (n=10) were all susceptible to chloramphenicol and gentamicin (p<0.01). While there were a few isolates resistant to erythromycin (n=10, 80% susceptibility), a large fraction was resistant to tetracycline (30% susceptibility). All the beef isolates (n=4) were resistant to erythromycin, with a few isolates resistant to chloramphenicol and gentamicin (n=4, 75% susceptibility). There was an equal distribution of beef isolates susceptible and resistant to tetracycline (n=4, 50% susceptibility). Pork isolates (n=3) were all resistant to gentamicin, while a few of the isolates were resistant to chloramphenicol, erythromycin and tetracycline (n=3, 67% susceptibility).

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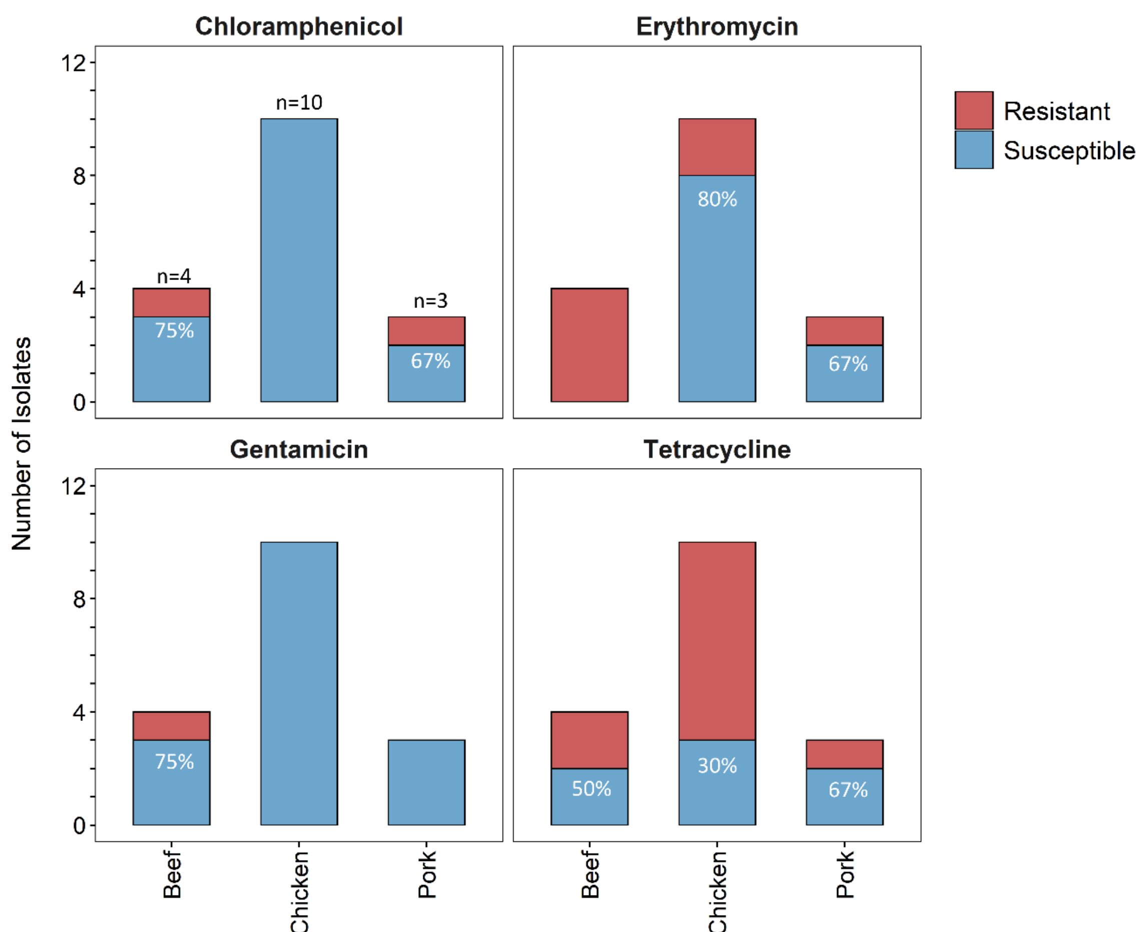

Figure 5.8 Susceptibility of *Listeria monocytogenes* isolates from Raw meats subcategories to respective antibiotics.

Observing statistically significant susceptibility in the main categories of origin was reassuring, since it indicates that antibiotics are performing better than random chance. However, it should be kept in mind that the presence of even a single resistant strain is a cause for concern, irrespective of the effectiveness of the antibiotic in question, since such a strain could eventually transfer resistance to other strains, and could itself establish and proliferate. In other words, in the light of the impacts of *L. monocytogenes* on human health (i.e. potential death) every single sample that shows antibiotic resistance is a cause for concern. Thus, antibiotic resistant bacteria are, without exception, unwanted in any setting at any time and the existence of antibiotic resistant *L. monocytogenes* strains is therefore never trivial, irrespective of the amount of strains found.

The eight isolates that exhibited multidrug resistance (resistance to at least one antibiotic in three or more classes), were all resistant to erythromycin and tetracycline. Most of the MDR isolates (Figure 5.9) came from Raw meats, followed by Ready-to-eat, Environmental and Clinical. There were no MDR isolates in the Raw seafood category.

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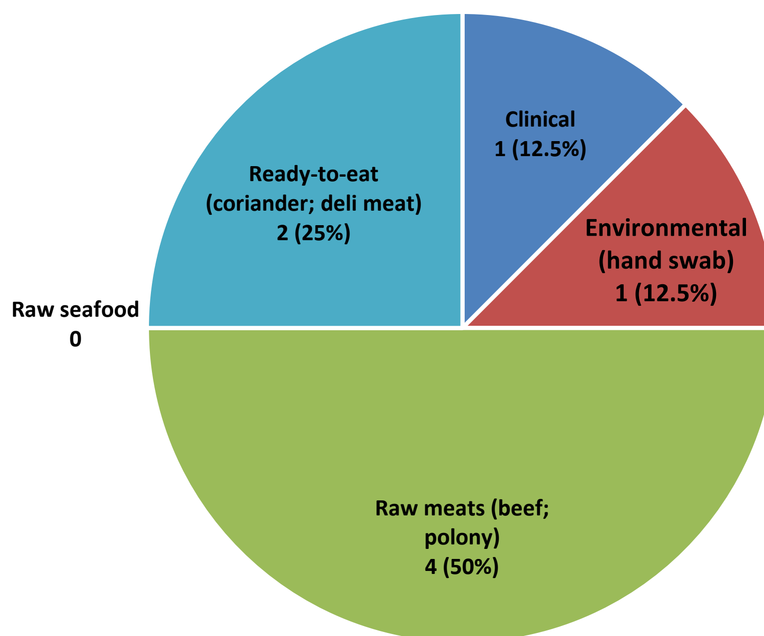

Figure 5.9 MDR isolates (n=8) was mostly observed in samples originating from Raw meats, while the rest originated from Ready-to-eat, Clinical and Environmental. No MDR isolates originated from Raw seafood.

5.4.1. No resistance to the current antibiotic of choice, ampicillin

The fact that none of the isolates were resistant to ampicillin was encouraging, as this is the current antibiotic of choice against *L. monocytogenes* in South African clinics and hospitals (Bamford *et al.*, 2017; NICD, 2017b), and ironically it is the most ineffective antibiotic (out of the ones studied here) elsewhere in the world (Addendum B). However, for the effective treatment of *L. monocytogenes* infections, several antibiotics are recommended, and not just ampicillin alone. This is because the effect of ampicillin on *L. monocytogenes* is mostly bacteriostatic (inhibiting growth and reproduction), even though it is a bactericidal antibiotic (Moellering *et al.*, 1972; Allerberger & Wagner, 2010). Therefore, it is often used together with an aminoglycoside such as gentamicin (Moellering *et al.*, 1972). As with our study, other researchers have also reported 100% susceptibility to ampicillin (Alonso-Hernando *et al.*, 2009; Davis & Jackson, 2009; Haubert *et al.*, 2016). However in stark contrast to this, a number of international studies have recently demonstrated a high presence of ampicillin resistant strains in raw meats (Yücel *et al.*, 2005; Pesavento *et al.*, 2010; Fallah *et al.*, 2012; Wang *et al.*, 2015a; Abdollahzadeh *et al.*, 2016), dairy (Rahimi *et al.*, 2010), RTE foods (Maćkiw *et al.*, 2016; Escolar *et al.*, 2017), seafood (Fallah *et al.*, 2013; Jamali *et al.*, 2015), and clinical isolates (Safdar & Armstrong, 2003). Specifically, poultry products in Turkey, Brazil, and Iran, revealed a high number of strains resistant to ampicillin (Ayaz & Erol, 2010; Fallah *et al.*, 2012; Carvalho *et al.*, 2019), while isolates from clinical and seafood origin, also exhibited resistance to ampicillin (Safdar & Armstrong, 2003;

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Abdollahzadeh *et al.*, 2016). Thus, while the situation in South Africa is still favourable for the use of ampicillin as a treatment against *L. monocytogenes*, the numerous aforementioned studies should serve as a warning that previously effective antibiotics are currently losing their efficacy. The situation in South Africa should, therefore, be carefully monitored since resistant strains of *L. monocytogenes* might arise or be introduced from elsewhere in the near future, which could have devastating consequences.

5.4.2. Clinical isolates show a high level of susceptibility

It was interesting to note that although the Clinical isolates were highly susceptible, one isolate was resistant to four antibiotics, namely chloramphenicol, erythromycin, gentamicin, and tetracycline (Addendum A). Resistance to these antibiotics among clinical strains have been reported (Safdar & Armstrong, 2003; Abdollahzadeh *et al.*, 2016). Noll *et al*. (2017) also reported the occurrence of multidrug resistance among strains from human listeriosis cases (56%, n=259), obtained during an outbreak in Austria and Germany, but found no resistance against the two antibiotics of choice, namely ampicillin and gentamicin. However, the one clinical resistant isolate in this study was resistant to gentamicin as well. It is concerning that such a strain exists, and its origins should ideally be investigated further.

In South Africa, antibiotic resistance among other diseases (such as tuberculosis) is on the rise (Nyasulu *et al.*, 2012). From our study, it was evident that antibiotic resistance was much more prevalent in environmental and food categories as compared to the clinical category. This is also confirmed by other studies which demonstrate a higher prevalence of *L. monocytogenes* resistance among environmental isolates compared to clinical (Charpentier & Courvalin, 1999; Duffy *et al.*, 2001; Safdar & Armstrong, 2003; Li *et al.*, 2007; Morvan *et al.*, 2010; Magiorakos *et al.*, 2012). There are several factors contributing to bacteria acquiring resistance. Firstly, medical patients that have been prescribed antibiotics as treatment often neglect to complete the full course, thus leading to the incomplete eradication of all bacteria which in turn give rise to resistant strains (Olaimat *et al.*, 2018). Secondly, it is also suspected that long term exposure to triclosan (found in many household products), at sub-lethal levels, could increase resistance in non-pathogenic bacteria, which could then in turn transfer resistance, via horizontal gene transfer, to *L. monocytogenes* against various aminoglycosides, such as gentamicin, kanamycin, and streptomycin (Davies & Davies, 2010; Christensen *et al.*, 2011; Meyer *et al.*, 2013; Allen *et al.*, 2016).

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5.4.3. Multidrug resistance among *Listeria monocytogenes* isolates

Multidrug resistant (MDR) *L. monocytogenes* isolates have been previously reported (Morvan *et al.*, 2010; Cetinkaya *et al.*, 2014; Kevenk & Terzi Gulel, 2016). MDR bacteria are especially problematic since they are associated with a higher mortality rate than susceptible bacteria, and also higher cost of hospitalization, as antibiotic therapy often needs to be extended (Tanwar *et al.*, 2014; Medina & Pieper, 2016; Munita & Arias, 2016). MDR strains circulating in the environment can be transferred to food processing plants via incoming raw material or factory personnel. It is concerning that the isolate obtained from a factory worker (Figure 5.6) was resistant to three different antibiotics. This is a potential threat to the consumer, should it spread to food that will be consumed without further cooking. Such strains are destroyed upon cooking of raw foods, however, by introducing MDR strains into a processing facility, there is a risk of strains circulating and spreading into the immediate environment. It could be assumed that non-pathogenic MDR bacteria are not problematic, however horizontal gene transfer between pathogenic and non-pathogenic bacteria can lead to the former acquiring resistance genes from the latter (Davies & Davies, 2010; Wright, 2010; Haubert *et al.*, 2016). Soil studies have already revealed that the vast majority of non-pathogenic, soil-dwelling bacteria found in the environment are intrinsically resistant to multiple antibiotics (Cox & Wright, 2013). This is a result of the high amounts of antibiotic compounds that have accumulated in these environments over time, thereby selectively pressurising such soil bacteria to adapt and develop resistance genes ("resistome") (Wright, 2010, 2019). For example, animal waste from agricultural systems where antibiotics are used spreads to agricultural soils and leads to increased antibiotic residues in soil and water (Meyer *et al.*, 2013). The resistance genes of non-pathogenic bacteria are homologous to those found in pathogenic bacteria and could easily result in the latter acquiring resistance genes from the former (Wright, 2010; Meyer *et al.*, 2013). Thus, environmental reservoirs offer a constant flow of genes that are able to confer resistance to susceptible bacteria (Cox & Wright, 2013; Wright, 2019). An example of this is where *tet*(M) genes (conferring tetracycline resistance by protecting a bacterium's ribosomes from interacting with the antibiotic) are transferred from other non-pathogenic Gram-positive bacteria to *L. monocytogenes* (Bertrand *et al.*, 2005; Morvan *et al.*, 2010; Munita & Arias, 2016). In fact, *tet*(M) genes are readily transferable from the commensal bacterium, *Enterococcus faecium*, to *L. monocytogenes* (Haubert *et al.*, 2016). Additionally, it is also known that *L. monocytogenes* can acquire erythromycin and tetracycline resistance from lactic acid bacteria, but this transfer is however influenced by the food medium (Allen *et al.*, 2016). This could explain the increased resistance observed among environmental and food isolates as when compared to clinical isolates in our study.

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5.4.4. The use of antibiotics in the agricultural industry

This study's results tie in well with previous studies wherein *L. monocytogenes* isolates originating from poultry, pork, and other meats exhibited resistance to erythromycin and tetracycline (Pesavento *et al.*, 2010; Fallah *et al.*, 2012; Wang *et al.*, 2015c). However, contrary to our findings, the isolates from these studies also exhibited resistance to gentamicin and chloramphenicol. Tetracycline resistance is the most frequently reported antibiotic resistance among *L. monocytogenes* species (Charpentier & Courvalin, 1999; Li *et al.*, 2007; Chen *et al.*, 2010a; Wang *et al.*, 2013). Our study aligns with this observation since levels of tetracycline resistance were high among the raw meat isolates tested here. This is not surprising since antibiotics are often used as growth promoters in sub-therapeutic levels in the poultry and meat industries, leading to reduced antibiotic effectiveness (Fallah *et al.*, 2012). Reasons for the use of antibiotics (especially tetracycline and erythromycin) in the animal industry (poultry, pork, and cattle) include controlling infections (i.e. disease control), optimizing feed efficiency, and acting as growth promoters (Warris, 2010; Moyane *et al.*, 2013; Gómez *et al.*, 2014; Allen *et al.*, 2016; Paridah *et al.*, 2016; Ferri *et al.*, 2017; Mund *et al.*, 2017). Furthermore, tetracycline-resistant strains of *L. monocytogenes* have already been isolated from the meat processing environment (Gómez *et al.*, 2014), as well as raw meat products (Pesavento *et al.*, 2010; Fallah *et al.*, 2012; Wang *et al.*, 2015a). In fact, the resistance of *L. monocytogenes* to tetracycline can be up to 23% (Chen *et al.*, 2010a; Noll *et al.*, 2018). In South Africa, tetracycline contributes to 17% of the total amount of antibiotics used in the agricultural industry (DoH, 2019). The overuse of tetracycline also affects other bacteria. For instance, there is a higher prevalence of tetracycline resistance among *Campylobacter* bacteria from commercially produced chicken in South Africa, as opposed to those that are produced on a small scale (Bester &, 2012), which is attributed to frequent sub-therapeutic antibiotic use. There have been several reports outside of South Africa of accumulated tetracycline residues detected in chicken meat (Amjad *et al.*, 2005; Hakem *et al.*, 2013; Karmi, 2014; Sattar *et al.*, 2014), while in South Africa residues of both tetracycline and erythromycin have been found in meat samples (DoH, 2019).

Antibiotic resistance also occurs among *L. monocytogenes* isolates from fresh produce (e.g. coriander). Such isolates in our study were resistant to erythromycin, gentamicin, and tetracycline. Similarly, erythromycin and tetracycline resistant *L. monocytogenes* have been previously isolated from vegetables (David & Odeyemi, 2007; Vasconcelos *et al.*, 2016). Aminoglycosides (which include gentamicin) are used in plant crop industries to control fire blight (Gullberg, 2014). The sub-therapeutic use of these antibiotics is highly concerning since some of these antibiotics are not completely broken down during cooking processes, leaving residues behind that are ingested by the consumer (Javadi, 2011). This can lead to a disturbance

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in the consumer's microflora, due to continuous exposure to small amounts of antibiotic residues (Gullberg, 2014; Mund *et al.*, 2017). Tetracycline is not intrinsically biodegradable, therefore residues may persist in the environment (soil and surface water) for long periods of time (Wellington *et al.*, 2013). It is theorized that antibiotic resistance existed long before the first human use of antibiotics (Cox & Wright, 2013; Meyer *et al.*, 2013; Wright, 2019). However, increased use of and reliance on antibiotics have intensified the selective pressures among non-pathogenic bacteria, causing pathogenic bacteria to acquire resistance genes. RTE foods do not undergo heat treatment prior to consumption, meaning that individuals are at greater risk for listeriosis if they consume these types of foods.

The use of antibiotics in the South African agricultural industry is governed by two acts, namely the "The Fertilisers, Farm, Feeds, Agricultural Remedies and Stock Remedies Act" (Act 36 of 1947) (Fertilizers Farm Feeds Agricultural Remedies and Stock Remedies, 2008) and the "Medicines and Related Substances Control Act" (Act 101 of 1965) (Medicines and Related Substances Act, 2005). Although the Department of Agriculture, Fisheries, and Forestation is responsible for monitoring the use of these antibiotics, it is presented with a difficult task, as under these acts, antibiotics can be purchased by farmers over the counter without a prescription, making monitoring a complicated undertaking (DoH, 2016). Older antibiotics, such as tetracycline, can be registered as either stock or veterinary medicine, and for the former, no record of use is needed, which makes monitoring its use especially difficult (Henton *et al.*, 2011).

5.4.5. The use of disinfectants in food processing environments and its effect on antibiotic resistance

In addition to antibiotic use, biocides and disinfectants are also used in the processing of raw meat. Because of its association with foodborne pathogens, such as *Salmonella*, *Campylobacter* and *L. monocytogenes*, raw meat and chicken often undergo decontamination procedures in order to reduce potential foodborne outbreaks (Alonso-Hernando *et al.*, 2010). However, it has been suggested that the sub-inhibitory use of these decontaminants may lead to bacteria acquiring antibiotics resistance (Walsh *et al.*, 2003; Alonso-Hernando *et al.*, 2009). It is suggested that exposure of *L. monocytogenes* to stressful conditions (e.g. osmotic stress, temperature fluctuations etc.) within the food processing environment can have an effect on its resistance to clinical antimicrobials (Allen *et al.*, 2016). This could explain why environmental *L. monocytogenes* isolates show a high number of antibiotic resistance. It could be protected by a biofilm in the food processing environment, and share the space with other bacteria that transfer resistance to the *L. monocytogenes* isolates.

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A recent study on the antibiotic resistance of *L. monocytogenes* in the meat processing environment recovered a tetracycline-resistant strain, while other strains showed decreased susceptibility to penicillin (Gómez *et al.*, 2014). Furthermore, exposure to sub-inhibitory concentrations of poultry-washing chemicals leads *L. monocytogenes* and *Salmonella enterica* to acquire resistance to multiple antibiotics, including erythromycin and chloramphenicol (Alonso-Hernando *et al.*, 2009). This is particularly worrisome since the European Food Safety Authority (EFSA) reports contaminated red meat and poultry to be responsible for 25% of foodborne related outbreaks (EFSA, 2007). This is consistent with our results, namely: *L. monocytogenes* exhibited high levels of resistance to multiple antibiotics, specifically erythromycin, chloramphenicol, and tetracycline. Although few studies have attempted to investigate the correlation between decontamination procedures and possible antibiotic resistance, the high levels of *L. monocytogenes* resistance found in raw meat and chicken samples in our study suggests that such a correlation potentially exists. In fact, EFSA recommends that chemical decontaminants (or "biocides") should be evaluated prior to their use for carcass washing, in order to assess the potential development of antibiotic resistance, especially in instances where *L. monocytogenes* is the target bacterium (EFSA, 2010). Because resistant bacteria are already present in the environment, the continued sub-therapeutic use of antibiotics will put even more selective pressure on these bacteria, which will lead to an increase of resistance genes (Davies & Davies, 2010; Meyer *et al.*, 2013). Thus, current regulations should be scrutinized and updated so that the effects of such therapeutic use can be minimised.

5.4.6. *Listeria monocytogenes* antibiotic resistance patterns are geographically biased

The patterns of antibiotic resistance of *L. monocytogenes* are not the same across different countries (Figure 5.10). In other words, patterns of *L. monocytogenes* antibiotic resistance seem to be geographically biased, in that they are not consistent across various regions of origin. When comparing results of other researchers (Addendum B) with the results from this study, remarkably different resistance patterns emerged, especially for ampicillin and erythromycin. While the exact drivers and mechanisms underlying such differences are still unclear, additional studies on the resistance of *L. monocytogenes* isolates from the South African environment would be of great benefit to determine firstly whether the patterns in this study remain consistent if replicated in a similar fashion, and secondly why these patterns differ so greatly from other countries. Finally, an investigation into whether differences in antibiotic use influences resistances patterns in different countries would be invaluable.

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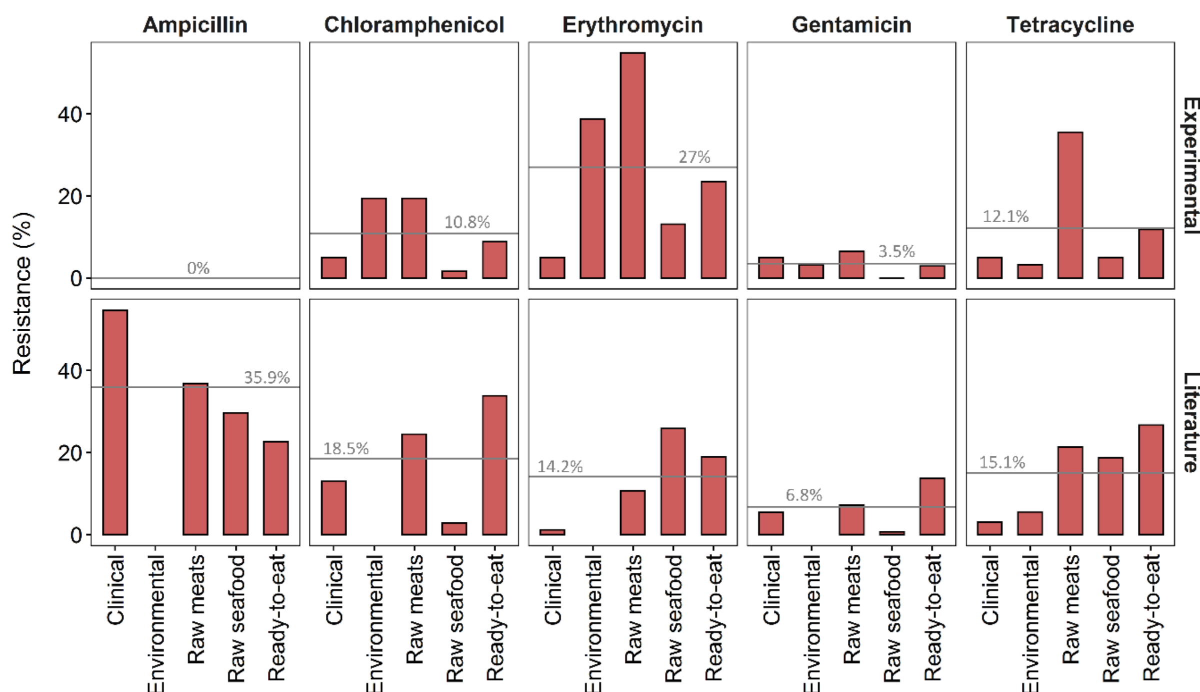

Figure 5.10 Comparison between experimental (n=177 isolates in this study) versus literature (n=48 studies; see Addendum B for references) antibiotic resistances of *L. monocytogenes*; grey lines indicate mean values of resistance across all categories for each antibiotic and source (literature or experimental) combination.

5.5. Conclusion

To the best of the authors' knowledge, this is the first study in South Africa in which antibiotic resistance for *L. monocytogenes* was determined simultaneously for isolates from both clinical and various food origins. We determined that ampicillin was still the most effective treatment against *L. monocytogenes* irrespective of origin. We also showed that a large portion of isolates were resistant to chloramphenicol, erythromycin, and tetracycline. From literature, there is a definite correlation between the use of antibiotics in the agricultural and clinical industry and the appearance of antibiotic resistance (Meyer *et al.*, 2013). Information on the agricultural use of antibiotics in South Africa is scarce, with only a handful of studies attempting to quantify this amount (Henton *et al.*, 2011; Eagar *et al.*, 2012). In contrast to other countries, such as the United States and China, the majority of antibiotics acquired in South Africa is for human consumption (DoH, 2019). In a country such as South Africa where a high burden of diseases such as tuberculosis and HIV/AIDS exists, the increase in resistant pathogenic *L. monocytogenes* strains could increase the number of annual fatalities of such immunocompromised individuals (Bester & Essack, 2012; Nyasulu *et al.*, 2012; Moyane *et al.*, 2013). Therefore, it is vital to continue surveillance studies on the increase of antibiotic resistant *L. monocytogenes* strains. Understanding the degree of antibiotic resistance of bacteria could lead to better administration

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of antibiotics, and in the case of *L. monocytogenes,* could help reduce mortality rates from listeriosis (Nyasulu *et al.*, 2012). Finally, antibiotic resistance patterns differ from country to country (Ayaz & Erol, 2010; Fallah *et al.*, 2012). Thus, resistance patterns in South Africa might not be relatable to resistance patterns of other countries.

The National Department of Health gives several challenges hindering comprehensive infection prevention and control (IPC) (DoH, 2016), some of which include: 1) lack of accountability; 2) lack of trained IPC practitioners; 3) lack of basic resources; and 4) lack of research. In other words, research specifically focussed on antibiotic resistance in South Africa is lacking. It is imperative to study the possible increase in antibiotic resistance among *L. monocytogenes* isolates in South Africa. This will provide researchers with a better understanding of resistance development, and in doing so will minimise the detrimental effect of resistant *L. monocytogenes* in clinical settings. Since the administration of antibiotics is governed by two different acts, approximating the actual amount of antibiotics use in South African agriculture is very difficult (Henton *et al.*, 2011). The South African Stewardship Programme (SAASP) aims to promote the development of antimicrobial stewardship (AMS) in the public and private sector. The South African government has now implemented a strategic framework for 2018-2024 to increase surveillance of administered antibiotics and strengthen antibiotic stewardship, while taking a One Health approach (DoH, 2016, 2018b). Doing research with a One Health approach is extremely important and useful. Many factors influence the emergence of antibiotic resistance, including but not limited to: injudicious use of antibiotics by patients, overuse of antibiotics in the agricultural industry, and even the use of disinfectants in processing facilities. With a topic as multidimensional as antibiotic resistance, it is essential to take a collaborative approach and include isolates from different origins, since resistance is often transferred between different sectors (i.e. environmental, agricultural, clinical) (McEwen & Collignon, 2018). This study was regionally limited in its nature, and it is, therefore, crucial that future studies include isolates from different geographical regions and not only different origins, which, in conjunction with the latter, will enable a broader set of generalizations to be made regarding antibiotic resistance in *L. monocytogenes*. This work sets a foundation for future researchers to build on with more information on *L. monocytogenes* in the South African environment and its associated antibiotic resistance patterns.

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Chapter 6

General Discussion and Conclusions

South Africa experienced the largest ever recorded global outbreak of listeriosis during 2017-18, motivating for the characterization of *Listeria monocytogenes* from diverse origins and investigating resistance patterns.

The first objective of this study was to classify *L. monocytogenes* isolates into lineage groups by PCR-RFLP. There was an overrepresentation of Lineage I isolates in all of the categories tested. This is of great concern since Lineage I is most often associated with human listeriosis cases, and not so much with food. A lower number of *L. monocytogenes* from Lineage I is needed to cause listeriosis (i.e. lower infectious dose). Therefore, in a country with a high burden of disease such as South Africa (i.e. more immunocompromised individuals), the high prevalence of Lineage I isolates in food that is destined for human consumption is a great public health risk.

The second objective was to determine the susceptibility of the *L. monocytogenes* isolates to a commercial bacteriophage (Listex™ P100) and to determine whether susceptibility was linked to lineage grouping. While a large number of isolates were generally found to be susceptible to the phage, the distribution of susceptible and tolerant isolates was somewhat equal in the Ready-to-eat category. This has important implications especially in light of the recent approval of Listex™ P100 use on ready-to-eat (RTE) foods. Thus, this part of the study demonstrates that phage treatment alone would likely not yet be viable in the food processing environment and will still have to be used in conjunction with conventional methods, instead of as a standalone alternative. These results are valuable, especially since not much is yet known about the resistance of the *L. monocytogenes* to these phages.

The final objective was to conduct antibiotic susceptibility tests on the *L. monocytogenes* isolates. All isolates were susceptible to ampicillin (the current antibiotic of choice for treatment of listeriosis), which is reassuring; however, a large fraction of isolates were resistant to erythromycin, chloramphenicol, and tetracycline. This is of particular concern especially considering the high number of listeriosis cases in South Africa and emphasises the need to continuously monitor antibiotic efficacy and to search for alternative antibiotics. An unexpected finding was how not only antibiotic resistance patterns, but also lineage associations, differed from one country to another. Finally, a few multidrug resistant strains were observed, and such strains specifically are of great concern due to their potential impact on human health, especially if such strains become dominant in the food processing environment. These results again emphasise why undertaking research on *L. monocytogenes* and antibiotic resistance with specific

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focus on the South African environment is so important, instead of relying only on international *L. monocytogenes* data.

In South Africa, the 2017-18 outbreak was declared over on the 3rd of September 2018. Subsequently, there has been sporadic reports of listeriosis, which the authorities have noted as normal, stating that it is "below the expected range" (NICD, 2019). This is because the expected range of listeriosis infections (in countries such as the USA that have routine surveillance) is about 2 to 5 cases per 1 million individuals. However, when the incidences of diseases such as TB or HIV/AIDS in South Africa is compared to a country such as the USA (Figure 6.1), it becomes evident that South Africa has a much higher number of immunocompromised individuals, meaning a higher number of individuals vulnerable to contracting listeriosis (Vanleeuw & Loveday, 2015; CDC, 2017; STATS SA, 2018; Talwar *et al.*, 2019). Because listeriosis only recently became notifiable in South Africa, it is possible that there is a lack of awareness of *L. monocytogenes* among the general public, which leads to a lack of reporting or failure to detect the bacteria in time. The long incubation period and varied symptoms could mean that not all patients may be diagnosed in time for antibiotic treatment. This again emphasizes the need for regular monitoring of antibiotic efficacy against *L. monocytogenes* and highlights why the results of this study are so valuable.

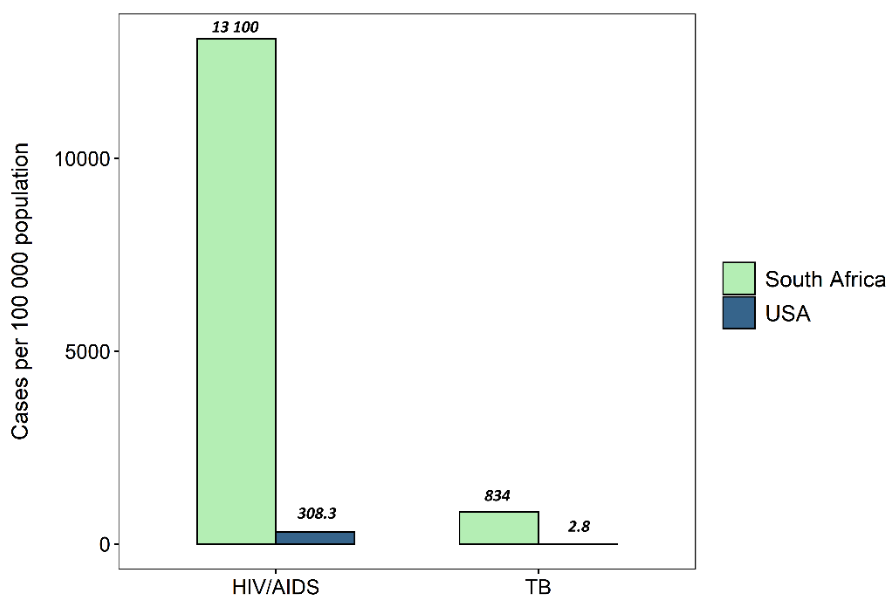

Figure 6.1 The prevalence HIV/AIDS and TB in South Africa in comparison with its incidence in the United States.

The results of this study have addressed some fundamental questions about *L. monocytogenes* in the Western Cape, but knowledge gaps still remain and there is great potential to expand on this work. Specifically, some recommendations for future studies are:

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- Increasing the resolution of *L. monocytogenes* classification. A clear association between *L. monocytogenes* lineage groups and food types was not found in this study and seems generally to be the case elsewhere. Thus, for example, serotyping and whole-genome sequencing will increase the genetic resolution and would provide valuable information not only on the source of the *L. monocytogenes* strains, but could shed light on whether instead a link exists between serotype groups and food types.
- Investigating how bacteriophages perform when exposed to diverse environmental conditions (such as found in the food processing environment, for example colder temperatures). Such experiments would be invaluable for determining the efficacy of bacteriophages during commercial food processing, since various factors can influence phage efficacy. Additionally, confirming whether phage susceptibility remains the same after inoculating different RTE foods (similar to that tested before by other researchers: salmon, deli meat etc.), to determine whether food medium has an effect on susceptibility. Since the present study investigated the susceptibility of pure *L. monocytogenes* isolates obtained from different origins within the Western Cape, future studies that build on this by using the same *L. monocytogenes* isolates and determining whether phage susceptibility is consistent or not, would be invaluable for the management of *L. monocytogenes*.
- Continuing with observational studies on antibiotic resistance of *L. monocytogenes* in South Africa by specifically broadening the scope so as to include other provinces. By determining antibiotic resistance of *L. monocytogenes* in other provinces, comparisons can be made with the results of this study to determine whether resistance patterns remain consistent or not, the result of which will have important management implications.

In conclusion, this study provides an invaluable baseline of the classification and resistance patterns of *L. monocytogenes* in the Western Cape, and overall it contributes significantly to filling the knowledge gap currently existing in South Africa regarding *L. monocytogenes*. One of the strengths of this study is the diverse nature and large number of isolates that were examined, and emphasizes the need for research to be conducted with a One Health approach, whereby samples originating from various sectors are examined simultaneously. This study also highlights the need for further, and more in-depth research, on *L. monocytogenes* with specific focus on the South African context. Research with a One Health approach is important and bacterial resistance is multidimensional, with resistance often spread between different sectors (environmental, agricultural, and clinical). This study therefore serves as a steppingstone to future collaborative efforts that aim to combat the ever-present threat of *L. monocytogenes.*

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Addendum A

Listeria monocytogenes isolates categorized into lineage groups, antibiotic- and phage susceptibility

Sample	Category	Subcategory (additional description, as provided by supplier)	Lineage	Ampicillin	Chloramphenicol	Erythromycin	Gentamicin	Tetracycline	Phage
CLM01	Clinical	Unknown	I	-	-	-	-	-	-
CLM06	Clinical	Unknown	II	-	-	-	-	-	T
CLM04	Clinical	Unknown	I	-	-	-	-	-	-
CLM15	Clinical	Unknown	I	-	-	-	-	-	-
CLM02	Clinical	Unknown	I	-	-	-	-	-	-
CLM05	Clinical	Unknown	I	-	-	-	-	-	-
CLM07	Clinical	Unknown	I	-	-	-	-	-	-
CLM08	Clinical	Unknown	I	-	-	-	-	-	-
CLM09	Clinical	Unknown	I	-	-	-	-	-	-
CLM10	Clinical	Unknown	I	-	-	-	-	-	-
CLM11	Clinical	Unknown	I	-	-	-	-	-	-
CLM12	Clinical	Unknown	I	-	-	-	-	-	-
CLM14	Clinical	Unknown	I	-	-	-	-	-	-
CLM16	Clinical	Unknown	I	-	-	-	-	-	-
CLM17	Clinical	Unknown	I	-	R	R	R	R	-
CLM18	Clinical	Unknown	I	-	-	-	-	-	-
CLM19	Clinical	Unknown	I	-	-	-	-	-	-
CLM20	Clinical	Unknown	I	-	-	-	-	-	-
CLM21	Clinical	Unknown	I	-	-	-	-	-	-

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Sample	Category	Subcategory (additional description, as provided by supplier)	Lineage	Ampicillin	Chloramphenicol	Erythromycin	Gentamicin	Tetracycline	Phage
CLM22	Clinical	Unknown	I	-	-	-	-	-	-
MEN42	Environmental	Drain (jam unit)	I	-	-	-	-	-	-
MEN30	Environmental	Drain (prep area)	I	-	R	R	-	-	-
MEN34	Environmental	Drain (production)	I	-	-	R	-	-	-
MEN21	Environmental	Drain (production)	I	-	-	-	-	-	-
MEN26	Environmental	Drain (prep area)	I	-	R	-	-	-	-
MEN23	Environmental	Drain (production)	I	-	-	-	-	-	-
MEN31	Environmental	Drain (cooking area)	II	-	R	R	-	-	-
MEN47	Environmental	Drain	I	-	R	R	-	-	-
MEN01	Environmental	Drain (mixer)	I	-	-	-	R	-	-
MEN03	Environmental	Drain (LR peeler)	I	-	-	-	-	-	-
MEN04	Environmental	Drain (mixer)	I	-	-	R	-	-	T
MEN24	Environmental	Equipment (veg room)	I	-	-	-	-	-	-
MEN49	Environmental	Equipment (small bizerba)	II	-	-	-	-	-	-
MEN46	Environmental	Equipment	I	-	-	-	-	-	-
MEN48	Environmental	Equipment	I	-	-	R	-	-	-
MEN05	Environmental	Equipment (major slicer)	I	-	-	-	-	-	T
MEN07	Environmental	Equipment (blender)	II	-	-	-	-	-	-
MEN09	Environmental	Equipment (glass machine motor cover)	I	-	-	-	-	-	T
MEN11	Environmental	Equipment	I	-	-	R	-	-	-
MEN12	Environmental	Equipment	I	-	-	-	-	-	-
MEN36	Environmental	Floor	II	-	-	R	-	-	-
MEN27	Environmental	Floor (despatch)	II	-	-	-	-	-	-
MEN08	Environmental	Floor (chiller)	II	-	-	-	-	-	T

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Sample	Category	Subcategory (additional description, as provided by supplier)	Lineage	Ampicillin	Chloramphenicol	Erythromycin	Gentamicin	Tetracycline	Phage
MEN38	Environmental	Hand (during production)	II	-	R	R	-	R	-
MEN25	Environmental	Surface (chiller door handles)	II	-	-	-	-	-	-
MEN14	Environmental	Surface (red cutting boards)	II	-	R	R	-	-	T
MEN15	Environmental	Surface (production tables)	II	-	-	R	-	-	-
MEN16	Environmental	Unknown	I	-	-	-	-	-	-
MEN17	Environmental	Unknown	I	-	-	-	-	-	-
MVA03	Environmental	Unknown	II	-	-	R	-	-	-
MVA52	Environmental	Unknown	II	-	-	-	-	-	-
MRA32	Raw meats	Beef (mince)	II	-	-	R	R	R	T
MRA32a	Raw meats	Beef (mince)	II	-	-	R	-	-	-
MRA18	Raw meats	Beef	I	-	R	R	-	R	-
MRA03	Raw meats	Beef (mince)	II	-	-	R	-	-	T
MRA31	Raw meats	Chicken (whole birds)	I	-	-	-	-	R	T
MRA34	Raw meats	Chicken (fresh breasts)	I	-	-	R	-	R	T
MRA40	Raw meats	Chicken (mixed portions)	I	-	-	-	-	R	-
MRA41	Raw meats	Chicken (skins)	I	-	-	-	-	-	-
MRA42	Raw meats	Chicken (frozen thighs)	I	-	-	-	-	R	-
MRA37	Raw meats	Chicken (fillets)	II	-	-	-	-	-	-
MRA43	Raw meats	Chicken (pieces)	II	-	-	-	-	R	-
MRA46	Raw meats	Chicken (mixed portions)	I	-	-	-	-	R	T
MRA17	Raw meats	Chicken (drumsticks)	I	-	-	R	-	R	-
MVA15	Raw meats	Chicken	II	-	-	-	-	-	-
MRA36	Raw meats	Pork (BBQ portions)	II	-	R	R	-	R	-

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Sample	Category	Subcategory (additional description, as provided by supplier)	Lineage	Ampicillin	Chloramphenicol	Erythromycin	Gentamicin	Tetracycline	Phage
MRA47	Raw meats	Pork (pork belly)	II	-	-	-	-	-	-
MDM	Raw meats	Pork (mechanically deboned pork meat)	II	-	-	-	-	-	-
MRA23	Raw meats	Unknown	I	-	-	R	-	-	-
MRA26a	Raw meats	Unknown	II	-	R	R	-	R	-
MRA26	Raw meats	Unknown	II	-	R	R	-	-	-
MRA20	Raw meats	Unknown	I	-	R	R	-	-	-
MRA35	Raw meats	Unknown	I	-	-	-	-	-	-
MRA45	Raw meats	Unknown	I	-	-	-	-	-	-
MRA39	Raw meats	Unknown	II	-	-	-	-	-	-
MRA11	Raw meats	Unknown	I	-	-	R	-	-	T
MRA13	Raw meats	Unknown	I	-	-	-	R	-	-
MRA14	Raw meats	Unknown	I	-	-	R	-	-	-
MRA20a	Raw meats	Unknown	I	-	-	R	-	-	-
MRA21	Raw meats	Unknown	I	-	R	R	-	-	-
MRA33	Raw meats	Unknown	II	-	-	R	-	-	-
MRA30	Raw meats	Unknown	I	-	-	R	-	-	-
MRA02	Raw seafood	Seafood	I	-	-	-	-	R	T
MRA09	Raw seafood	Seafood	I	-	-	-	-	-	-
MRA10	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA01	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA02	Raw seafood	Seafood	I	-	-	R	-	R	-
MVA04	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA05	Raw seafood	Seafood	I	-	-	R	-	-	-
MVA06	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA07	Raw seafood	Seafood	I	-	-	-	-	-	-

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Sample	Category	Subcategory (additional description, as provided by supplier)	Lineage	Ampicillin	Chloramphenicol	Erythromycin	Gentamicin	Tetracycline	Phage
MVA08	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA09	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA10	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA11	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA13	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA14	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA16	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA17	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA18	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA19	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA20	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA21	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA22	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA25	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA26	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA27	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA28	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA29	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA30	Raw seafood	Seafood	I	-	-	R	-	-	-
MVA31	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA32	Raw seafood	Seafood	I	-	-	-	-	-	T
MVA33	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA34	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA35	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA36	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA37	Raw seafood	Seafood	I	-	-	-	-	-	-

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Sample	Category	Subcategory (additional description, as provided by supplier)	Lineage	Ampicillin	Chloramphenicol	Erythromycin	Gentamicin	Tetracycline	Phage
MVA38	Raw seafood	Seafood	I	-	R	-	-	-	-
MVA39	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA40	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA41	Raw seafood	Seafood	I	-	-	R	-	-	-
MVA42	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA43	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA44	Raw seafood	Seafood	I	-	-	R	-	-	-
MVA45	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA46	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA47	Raw seafood	Seafood	I	-	-	R	-	-	-
MVA48	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA49	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA50	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA51	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA55	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA56	Raw seafood	Seafood	I	-	-	R	-	-	-
MVA57	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA58	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA59	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA60	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA61	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA62	Raw seafood	Seafood	II	-	-	-	-	R	-
MVA63	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA64	Raw seafood	Seafood	I	-	-	-	-	-	-
MVA65	Raw seafood	Seafood	I	-	-	R	-	-	-
NPD4A	Raw seafood	Seafood	II	-	-	-	-	-	-

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Sample	Category	Subcategory (additional description, as provided by supplier)	Lineage	Ampicillin	Chloramphenicol	Erythromycin	Gentamicin	Tetracycline	Phage
MRE39	Ready-to-eat	Dairy	I	-	-	-	-	-	-
MRE21	Ready-to-eat	Deli meat (bacon breakfast griller)	II	-	-	R	-	-	-
MRE29	Ready-to-eat	Deli meat	II	-	-	-	-	-	-
MRE40	Ready-to-eat	Deli meat (cheese griller)	I	-	-	R	-	R	-
MRE10	Ready-to-eat	Deli meat (pastrami)	I	-	-	-	-	-	-
MRE11	Ready-to-eat	Deli meat (pressed beef)	I	-	-	-	-	-	-
MRE17	Ready-to-eat	Deli meat (pepper beef)	II	-	-	R	-	-	-
MRA04	Ready-to-eat	Fresh produce	I	-	-	R	-	R	T
MRE37	Ready-to-eat	Fresh produce (cucumber after dipping)	I	-	-	-	-	-	-
MRE31	Ready-to-eat	Fresh produce (cucumber after dipping)	II	-	-	-	-	-	T
MRE32	Ready-to-eat	Fresh produce (coriander after dipping)	I	-	-	R	R	R	T
MRE34	Ready-to-eat	Fresh produce (cling peach)	I	-	-	-	-	-	-
MRE28	Ready-to-eat	Hummus	I	-	-	-	-	-	T
MRE38	Ready-to-eat	Hummus	II	-	R	-	-	-	-
MRE35	Ready-to-eat	Hummus	II	-	-	-	-	-	-
MRE01	Ready-to-eat	Hummus	I	-	-	-	-	-	T
MRE02	Ready-to-eat	Hummus (tarama salata)	II	-	-	-	-	-	-
MRE03	Ready-to-eat	Hummus (jalapeno)	II	-	-	-	-	-	-
MRE04	Ready-to-eat	Hummus (zaatar)	II	-	-	-	-	-	-
MRE05	Ready-to-eat	Hummus (orange)	I	-	-	-	-	-	T

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Sample	Category	Subcategory (additional description, as provided by supplier)	Lineage	Ampicillin	Chloramphenicol	Erythromycin	Gentamicin	Tetracycline	Phage
MRE06	Ready-to-eat	Hummus (peri-peri)	I	-	-	-	-	-	-
MRE07	Ready-to-eat	Hummus	II	-	-	R	-	-	-
MRE08	Ready-to-eat	Hummus (red pepper)	II	-	-	R	-	-	-
MRE09	Ready-to-eat	Hummus	I	-	-	-	-	-	T
MRE13	Ready-to-eat	Hummus (chipotle)	I	-	-	-	-	-	T
MRE14	Ready-to-eat	Hummus	I	-	-	-	-	-	T
MRE15	Ready-to-eat	Hummus (jalapeno)	II	-	-	-	-	-	-
Hb2	Ready-to-eat	Polony	I	-	-	-	-	-	-
Hb3	Ready-to-eat	Polony	II	-	-	-	-	-	-
Hb5	Ready-to-eat	Polony	II	-	-	-	-	-	T
Hb6	Ready-to-eat	Polony	II	-	R	-	-	-	T
Hb4	Ready-to-eat	Polony	II	-	R	R	-	R	T
MRE16	Ready-to-eat	Unknown	I	-	-	-	-	-	-
MRE18	Ready-to-eat	Unknown	I	-	-	-	-	-	-

R = resistant
T = tolerant
'-' = susceptible/sensitive
Isolates from the same category are not necessarily from the same retail outlet, or environmental origin. Isolates with similar descriptions are not referring to duplicates.

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Addendum B

Studies indicating the prevalence of antibiotic resistance of *Listeria monocytogenes* to ampicillin, chloramphenicol, erythromycin, gentamicin, and tetracycline

Antibiotic	Origin	Reference	*L. monocytogenes* isolates resistant/isolates tested (%)
Ampicillin	Clinical, USA	(Safdar and Armstrong, 2003)	6/65 (9.2%)
	Clinical, Iran	(Abdollahzadeh *et al.*, 2016)	14/14 (100%)
	Seafood, Iran	(Fallah *et al.*, 2013)	107/278 (38.5%)
	Seafood (open-air fish markets), Iran	(Jamali *et al.*, 2015)	9/43 (20.9%)
	Chicken, Iran	(Abdollahzadeh *et al.*, 2016)	14/14 (100%)
	Poultry, Iran	(Fallah *et al.*, 2012)	44/98 (44.9%)
	Raw meat, Italy	(Pesavento *et al.*, 2010)	8/40 (20%)
	Pork, China	(Wang *et al.*, 2015c)	3/26 (11.5%)
	Meat products, Turkey	(Yücel *et al.*, 2005)	5/66 (7.5%)
	Dairy products, Iran	(Rahimi *et al.*, 2010)	5/19 (26.3%)
	RTE products, Spain	(Escolar *et al.*, 2017)	8/25 (32%)
	RTE products, Poland	(Maćkiw *et al.*, 2016)	20/210 (9.5%)
Chloramphenicol	Clinical, USA	(Safdar and Armstrong, 2003)	7/54 (12.9%)
	Seafood, Iran	(Fallah *et al.*, 2013)	9/278 (3.2%)
	Seafood (open-air fish markets), Iran	(Jamali *et al.*, 2015)	1/43 (2.3%)
	Poultry, Iran	(Fallah *et al.*, 2012)	24/98 (24.5%)
	Dairy products, Iran	(Rahimi *et al.*, 2010)	2/19 (10.5%)
	Vegetables, Nigeria	(David and Odeyemi, 2007)	60/104 (57.7%)
	RTE meat products, China	(Wang *et al.*, 2015a)	11/33 (33.3%)
Erythromycin	Clinical, USA	(Safdar and Armstrong, 2003)	1/84 (1.9%)
	Seafood, Iran	(Fallah *et al.*, 2013)	7/278 (2.5%)
	Seafood (open-air fish markets), Iran	(Jamali *et al.*, 2015)	12/43 (27.9%)
	Seafood, Poland	(Skowron *et al.*, 2018)	33/70 (47.1%)
	Poultry, Iran	(Fallah *et al.*, 2012)	15/98 (15.3%)
	Raw meat, Italy	(Pesavento *et al.*, 2010)	2/40 (5%)
	Pork, China	(Wang *et al.*, 2015c)	3/26 (11.5%)
	Dairy products, Iran	(Rahimi *et al.*, 2010)	3/19 (15.8%)
	Vegetables, Nigeria	(David and Odeyemi, 2007)	23/104 (22.1%)

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Antibiotic	**Origin**	**Reference**	***L. monocytogenes* isolates resistant/isolates tested (%)**
Gentamycin	Clinical, USA	(Safdar and Armstrong, 2003)	2/52 (2%)
	Clinical, Iran	(Abdollahzadeh *et al.*, 2016)	1/14 (7.1%)
	Seafood, Iran	(Fallah *et al.*, 2013)	2/278 (0.72%)
	Chicken, Iran	(Abdollahzadeh *et al.*, 2016)	1/14 (7.1%)
	Poultry, Iran	(Fallah *et al.*, 2012)	10/98 (10.2%)
	Raw meat, Italy	(Pesavento *et al.*, 2010)	3/40 (7.5%)
	Pork, China	(Wang *et al.*, 2015c)	1/26 (3.9%)
	Dairy products, Iran	(Rahimi *et al.*, 2010)	1/19 (5.3%)
	Vegetables, Nigeria	(David and Odeyemi, 2007)	33/104 (31.7%)
	RTE products, Spain	(Escolar *et al.*, 2017)	1/25 (4%)
Tetracycline	Clinical, USA	(Safdar and Armstrong, 2003)	2/66 (3%)
	Seafood, Iran	(Fallah *et al.*, 2013)	52/278 (18.7%)
	Poultry, Iran	(Fallah *et al.*, 2012)	34/98 (34.7%)
	Raw meat, Italy	(Pesavento *et al.*, 2010)	1/40 (2.5%)
	Chilled pork, China	(Wang *et al.*, 2015c)	7/26 (26.9%)
	Dairy products, Iran	(Rahimi *et al.*, 2010)	3/19 (15.8%)
	Vegetables, Nigeria	(David and Odeyemi, 2007)	32/104 (30.8%)
	RTE products, Spain	(Escolar *et al.*, 2017)	12/25 (48%)
	RTE meat products, China	(Wang *et al.*, 2015a)	4/33 (12.1%)
	Food processing environment, Canada	(Kovacevic *et al.*, 2013)	3/54 (5.6%)