Prevalence of *Listeria monocytogenes* in food samples from retail shops and street vendor stalls in Pretoria and the evaluation of bacterial probiotics as potential control measure

By

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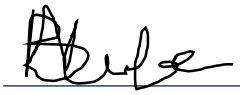
DECLARATION

I declare that the dissertation '**Prevalence of *Listeria monocytogenes* in food samples from retail shops and street vendor stalls in Pretoria and the evaluation of bacterial probiotics as potential control**' which I hereby submit for the degree of Magister Scientae at the University of Pretoria, is my own work and has not been previously submitted by me for a degree at this or any other tertiary institution.

Signature: 

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LIST OF ABBREVIATIONS

AAD	: Antibiotic associated diarrhoea
actA	: Actin A
ANOVA	: Analysis of Variance
avoS	: avocado isolate
BA-LA	: *Bifidobacterium animalis – Lactobacillus acidophilus* cocktail
BA-PA	: *Bifidobacterium animalis – Pediococcus acidilacti* cocktail
BHI	: Brain Heart Infusion
BLAST	: Basic Local Alignment Search Tool
BMEC	: Brain Macrovascular Endothelial Cells
Bp	: Base pairs
CBD	: Central Busines District
CDC	: Centre for Disease Control
CDAD	: *Clostridium difficile* antibiotic associated diarrhoea
CFS	: Cell Free supernatants
Cfu/g	: Colony forming units per gram
Cfu/ml	: Colony forming units per milliliter
cucS	: Cucumber isolate
Difco	: Detroit Infusion and Fermentation Company
Exo-Sap	: Exonuclease 1- Shrimp Alkaline Phosphatase
DNA	: Deoxy Ribonucleic acid
dNTP	: Deoxy Nucleotide Tri Phosphate
ECM	: Extra Cellular Matrix
FAO	: Food and Agricultural Organisation

FDA	: Food and Drug Administration
fsCFS	: filter sterilized Cell Free Supernatants
GABA	: gamma – Aminobutyric acid
GHP	: General Hygienic Practices
GMP	: General Manufacturing Practices
HCl	: Hydrochloric acid
HPP	: High Pressure Processing
HWF	: Hydrolyzed Whey Formula
IBD	: Inflamatory Bowel Disease
IBS	: Inriitable bowel syndrome
Inl	: Internalin
Kb	: Kilobase pairs
kDa	: Kilo Dalton
LAB	: Lactic acid Bacteria
LLO	: Listeriolysin O
MRS	: de Man, Rogosa and Sharpe
MRS-cys-HCl	: de Man, Rogosa and Sharpe supplemented with cysteine hydrochloric acid
MOX	: Modified Oxford Agar
MRSA	: Methicillin Resistant *Staphylococcus aureus*
MgSO4	: Magnesium Sulphate
NaOH	: Sodium Hydroxide
NCBI	: National Centre for Biotechnology Information
NEB	: New England Biolabs
NRF	: National Research Foundation

OD	: Optical Density
PCR	: Polymerase Chain Reaction
PI-PLC	: Phosphatidylcholine phospholipase
P.E.S.T	: Proline (P), Glutamic acid (E), Serine (S), Threonine (T)
rDNA	: ribosomal Deoxyribonuceic acid
rRNA	: ribosomal Ribonucleic acid
SCFA	: Short Chain Fatty acids
SO_2	: Sulphur dioxide
spp.	: species
RTE	: Ready To Eat meat
R. V	: Rapapport Vasilliadis
WHO	: World Health Organisation
w/v	: weight per volume
XLD	: Xylose deoxycholate

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Dedication

This dissertation is dedicated to Pretty Ncube, my mother, who has been constantly there for me in every way possible throughout this phase in my life. Your support and love has been colossal in me completing my studies, thank you.

Summary

Prevalence of *Listeria monocytogenes* in food samples from retail shops and street vendor stalls in Pretoria and the evaluation of bacterial probiotics as potential control measure

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Degree : **MSc (Microbiology)**

The capabilities of *Listeria monocytogenes* as a foodborne pathogen have been a topic of interest since it was linked to a coleslaw outbreak in the 1980s. The growth characteristics exhibited by this species increase its fitness as a pathogen, which has made it a challenge to the food and health industries. In South Africa specifically, this pathogen was responsible for the largest listeriosis outbreak to date as reported by the World Health Organization (WHO). During this outbreak, the limitations faced by the South African food and health industries in dealing with this pathogen were exposed. This then highlighted a need to reassess processes involved in the manufacturing, transportation, storage and retail of foods that support the growth of *L. monocytogenes*. An integral step to understanding the distribution of the pathogen is to determine its prevalence in the foods that support it. Subsequent to assessing its distribution, mechanisms to combat the pathogen in an effort to lessen the incidence of listeriosis have to be implemented. Antibiotics are generally used for the treatment of listeriosis while the use of probiotics as an alternative has recently been explored. Probiotics have been tested against this pathogen, with some showing potent anti-listerial activity. They employ various strategies in combating *L. monocytogenes,* ranging from nutrient competition to production of antagonistic substances.

The dissertation generated from the findings of the current study is organized into the four chapters. The first chapter (**Chapter 1- Literature Review**) gives an overview of characteristics of *L. monocytogenes* as a pathogenic species and the various methods

used to combat it. *L. monocytogenes*, one of twenty species within genus *Listeria*, is the most studied of all the species due to its pathogenic attributes. This pathogen is of interest because of its ability to thrive in environmental conditions such as extremes of pH, temperature and water activity, which would be considered challenging hurdles for many bacterial pathogens. This ability allows it to withstand harsh procedures involved in food manufacturing, processing, transportation and storage up until it reaches the consumer's plate. From then on, once ingested, the pathogen can weather the extreme pH of the gastric juices in the stomach, exiting to more conducive intestinal conditions. Once there where it causes severe gastrointestinal disturbances sometimes manifested as diarrhoea, before entering the circulatory system. It is in the blood where the pathogen causes septicaemia and through the blood, it reaches the brain where it causes meningitis, a common form of listeriosis.

The first chapter also outlines various alternatives in literature employed by the food processing industry to control *L. monocytogenes*. Use of temperature, pH and water extremes are common practices used against this pathogen. Also chemical treatments using nitrites, radicals, oxygen and its derivatives, have been explored. These methods, although numerous and diverse in anti-listerial mechanisms, are sometimes not feasible if not expensive for large-scale use. If all fails within the food processing environment and ingestion of *L. monocytogenes* occurs leading to listeriosis, the treatment of choice is antibiotics. The use of antibiotics was highly integral to overturning fatalities caused by infectious disease during the 20th century. Although highly beneficial, over usage and abuse of these drugs within the food and medical industries has led to the phenomenon of antibiotic resistance in multiple bacterial species and *L. monocytogenes* is no exception. This has prompted many to look into alternatives, of which probiotics have emerged as a viable option if managed correctly. Probiotics are "*live microorganisms which, when administered in adequate amounts, confer benefits to the host*". They belong to multiple genera and have a wide range of attributes that render them useful to human and animal health. Of the many properties possessed by probiotics, antimicrobial activity is the most useful when dealing with pathogens such as *L. monocytogenes.* Given the above information, the aim of this study was to determine the prevalence of *L. monocytogenes* in

foods acquired from retail stores and street vendor stalls in Pretoria and evaluate probiotics as a control measure against this pathogen.

In the first experimental chapter (**Chapter 2- Comparative analysis of the prevalence of *Listeria monocytogenes, Campylobacter spp.* and *Salmonella spp.* in retail stores and street vendor stalls in Pretoria)** the study followed a stepwise procedure to determine the prevalence of *L. monocytogenes* in different food samples acquired from retail stores and street vendors in and around the Pretoria CBD area. A total of 167 food samples were acquired based on availability and tested for the presence of *Listeria* spp. The prevalence of the common food pathogens, *Salmonella* spp. and *Campylobacter* spp. in those foods was also assessed for comparative purposes. Culture based identification techniques in the form of general and selective enrichment were employed to determine the presence of *Listeria* spp., *Salmonella* spp. and *Campylobacter* spp. in the collected food samples. A total of 37, 17 and 18 isolates were presumed to belong to *Listeria* spp., *Campylobacter* spp. and *Salmonella* spp. respectively. From the identified species, amplification of the 16S rRNA gene was performed to confirm the genera each isolate belonged to. Only 4 of the 37 presumptive *Listeria* isolates were confirmed to be *Listeria* spp. Of the 18 presumptive *Salmonella* isolates, only one was confirmed as belonging to the *Salmonella* genus. None were confirmed as *Campylobacter.* Thereafter, specie-specific *inlA* PCR was done to determine if any of the 4 *Listeria* isolates found were *L. monocytogenes* and only 2 were confirmed as *L. monocytogenes.* It is worth mentioning these confirmed *L. monocytogenes* isolates were found in samples acquired from street vendors. The multiple contamination sources and unhygienic practices associated with these vendor stalls were cited as possible reasons for presence of the pathogen in the foods acquired from those sites. Furthermore, antibiotic susceptibility testing was performed for the confirmed *L. monocytogenes* and *Salmonella* isolates. There was variation in the antibiotic susceptibility and resistance profiles between the two *L. monocytogenes* isolates. Antibiotic resistance in microorganisms is a growing concern in the health and food industries. Alternatives to antibiotics are currently a hot topic within the relevant sciences and an emerging option is the use of probiotics as a prophylactic or therapeutic agent, if not an adjunct to antibiotic use.

Having confirmed the presence of *L. monocytogenes* in food sold by the street vendors, this raises a need for controlling the pathogen. As mentioned previously, probiotics can substitute or assist antibiotics in an attempt to control pathogens. Taking that into consideration, in the second experimental chapter (**Chapter 3- Evaluation of probiotics as a control measure of *Listeria monocytogenes*)**, the study investigated the suitability of probiotics as a control measure against *L. monocytogenes*. Probiotics *Bifidobacterium animalis subsp. lactis* BB-12, *Lactobacillus acidophilus* L10, *Lactobacillus plantarum* 7.1E and *Pediococcus acidilacti* were used. Five *L. monocytogenes* strains, three controls previously isolated from foods and two that were isolated in this study (chapter 2) were used. Firstly, the *L. monocytogenes* strains were exposed to each of the suspended cultures of the above-mentioned probiotic strains in a spot inoculation assay and to their cell free supernatants (CFS) in a well diffusion assay. In the spot inoculation, *B. animalis subsp. lactis* and *L. acidophilus* inhibited the *L. monocytogenes* strains better than the other probiotics. The CFSs of all the probiotics used did not inhibit growth of any of the *L. monocytogenes* strains. The inhibitory activity of *Bifidobacterium* and *Lactobacillus* is attributed to nutrient competition. Secondly, the same *L. monocytogenes* strains which were used in the spot inoculation assay, were exposed to each of the abovementioned probiotics and their CFSs in two food matrixes, avocado and cucumber. *B. animalis subsp. lactis* was the only probiotic that significantly inhibited one of the *L. monocytogenes* strains. All the other probiotics; *L. acidophilus, L. plantarum* and *P. acidilacti* did not inhibit any of the *L. monocytogenes* strains. In addition to the individual probiotics, the study investigated the effects of a two species cocktail, one with *L. acidophilus* and *B. animalis* and the other with *P. acidilacti* and *B. animalis* on the growth of *L. monocytogenes* strains. None of the cocktails significantly inhibited the growth of any of the *L. monocytogenes* strains. The difference in inhibitory activity of high performing strains in the spot inoculation test compared to the food matrix tests was possibly due to the difference in properties of the growth matrixes used.

In the last chapter (**Chapter 4 – General Conclusions and Recommendations**), the general research findings and the recommendations for future work are given. Briefly, the study showed that *Listeria* was more prevalent than *Salmonella* and *Campylobacter* in all foods tested. Only 2 out of 167 food samples tested positive for *L. monocytogenes.* In the

spot inoculation test, the probiotics *B. animalis* and *L. acidophilus* inhibited most of the *L. monocytogenes* strains they were challenged with while *L. plantarum* and *P. acidilacti* were not as effective. Only *B. animalis* was capable of inhibiting *L. monocytogenes* strain growing on avocado during the food matrix tests. Variation in the inhibitory activity of probiotics and susceptibility of *L. monocytogenes* to antibiotics and probiotics was observed as a general trend throughout the study.

In future work, it would be beneficial to randomly repeat this study time and again to determine if the results found in this study are a norm in the retail stores and vendor stalls investigated or if they are a reflection of the reaction to the listeriosis outbreak. The study should also be extended to multiple locations throughout the country to give a better understanding of the pathogen's distribution. Focus on a single food group while increasing sample size might shed better light about *L. monocytogenes* prevalence within that food group. Environmental PCR techniques could enhance the discriminatory power of culture based techniques as well and allow for detection of non-culturable pathogen cells.

Introduction

Foodborne infectious diseases are a result of ingesting food contaminated by pathogens. Various genera and species of bacterial food pathogens have different mechanisms of infection and pathogenesis. Some such as *Bacillus cereus*, cause disease through production of toxins in the food ingested while others such as *Vibrio cholerae* produce the toxin in the intestinal region (Finkelstein 1996, Nelson et al 2011). It is the toxin which mediates disease progression in susceptible hosts (Bharati and Ganguly 2011). For other pathogens like *Neisseria meningitides* and *Listeria monocytogenes,* invasion of target tissues by the intact pathogen is necessary for development of disease (Southwick 1996, Spinosa 2007). There is a wide variety of bacterial food borne pathogens that have been implicated in multiple outbreaks. Assessing the distribution of such pathogens in food and environments that support their growth can allow for a more precise and informed reaction when they cause epidemics. Some of the more common and severe ones include but are not limited to *L. monocytogenes, Salmonella* and *Campylobacter*.

Campylobacter are Gram negative, non-spore forming, rod shaped, microaerophilic bacteria classified under the *Campylobacteriaceae* family (Janssen et al 2008). *Campylobacter jejuni* causes the majority of illnesses although *C. coli* and *C. lasi* have also been linked to some outbreaks (Hughes and Cornblath 2005). In 2012, pathogenic *Campylobacter* species were estimated to cause 850 000 annual illnesses in the USA alone (Pogreba - Brown et al 2018). *Campylobacter jejuni* is highly sensitive to dry conditions and atmospheric oxygen levels (Rodrigues et al 2016). It has a temperature optimum range of (37°C – 42 °C) and can survive in the intestinal region of birds, which has a temperature ranging between 41 and 42 °C (Duffy and Dykes 2006).

Salmonella are facultative anaerobic, non-spore forming, Gram negative bacilli which belong to the family *Enterobacteriaceae*. Pathogenic *Salmonella* species cause the largest number of gastroenteritis cases in the USA and were estimated to cause 1.2 million diseases per annum in 2012 (Bula-Rudas et al 2015). *Salmonella* mainly causes disease in children younger than the age of five although adults with debilitating disease and those over fifty are susceptible to infection (Bula – Rudas et al 2015). *Salmonella* spp. can survive a wide temperature range but optimally grow between 35 – 37 °C. The species within this

genera can tolerate a pH range of 3.8 - 9.5 though optimal growth is observed between 6.5 - 7.5 (Wirtanen and Salo 2016).

The genus *Listeria,* a member of the *Listeriaceae* family, is currently comprised of 20 species, which differ in host specificity and virulence (Leclerq et al 2019). It has strains that separate into multiple lineages, have diverse serotypes and differ in their virulence and distribution (Ward et al 2004*,* den Bakker et al 2008, Orsi et al 2008). *Listeria monocytogenes* is a Gram positive, non-spore forming, rod shaped member of this genus (Farber 1991). It can tolerate a pH range of (4 – 9), temperature range of (-0.5°C to 50°C) and low water activity (Walker 1987, Farber 1991). These traits increase its fitness as a pathogen and enable it to persist in various areas of a food manufacturing plant and to withstand food processing hurdles. They also promote its survival and proliferation during transportation, retail, consumer storage and preparation (Farber 1991).

Ingestion of food containing *L. monocytogenes* can lead to a disease known as listeriosis (Mclauchlin et al 2004). In humans, *L. monocytogenes* can infect both immune competent and compromised people; however, people with compromised immune systems, such as pregnant women, HIV-AIDS and cancer victims, are more susceptible (Castellazi et al 2013). Listeriosis has an elevated hospitalization rate (90.5%) and case fatality rate (21%) higher than food borne bacterial pathogens such as *Salmonella spp.* and *Campylobacter spp.* (Hernandez-Milian and Payeras-Cifre 2014). There has been an increase in non-perinatal listeriosis cases over the years (Rocourt 1996). This increment can be linked to a rise in immune-compromising diseases such as HIV/AIDS and cancer. The sub-Saharan region of Africa is responsible for 70% of HIV infections that occur globally (Amstrong-Mensah et al 2019). There were more than 9.5 million cancer fatalities in 2018 and 7.3% of those were from the African population (Dakubo 2019, Khazaei et al 2019). Taking these factors into consideration the South African population is a probable target for listeriosis.

The occurrence rate of listeriosis amongst human populations is increasing globally (Choi et al 2018). A study done in the Western Cape between 2012 and 2015 detected seventy-two cases of listeriosis (meningitis and bacteremia) in that specific time frame (NICD 2015). In the months between January 2017 and July 2018 in South Africa, more than 200 fatalities resulting from a listeriosis outbreak were recorded (Olanya et al 2019). The

outbreak was the biggest there has ever been globally (Kaptchouang Tchatchouang et al 2020). The exposure of the South African population to this pathogen is thus on the rise and if its distribution is not curtailed, it could pose a serious threat to the health of many, especially those with compromised immune systems. More focus should hence be directed towards studies that determine how extensive the listeriosis problem is in the country.

To treat listeriosis, antibiotics have proven to be very useful in a majority of cases (Hirsch 2008). Antibiotics usage though leads to emergence of antibiotic resistant strains due to misuse of these drugs by patients and health care professionals alike (Canton and Morosini 2011). Although other alternatives like phage therapy or quorum quenching are being pursued, probiotic therapy is on the forefront (Altamirano and Barr 2019). Probiotics are "*live microorganisms which, when administered in adequate amounts, confer health benefits to the host*" (Fijan 2016). Probiotics use different modes of action to inhibit pathogens including production of antimicrobial substances and nutrient competition (Denkova et al 2015). It is these attributes that can be manipulated when combating *L. monocytogenes* either *in-vitro*, *in planta* or *in-vivo.*

The aim of this study was to determine the prevalence of *L. monocytogenes, Salmonella spp.* and *Campylobacter spp.* in retail shops and street vendors around Pretoria and evaluate probiotics as a control measure against this pathogen. The specific objectives were:

1.) To determine the prevalence of *Listeria, Salmonella* and *Campylobacter* in foods acquired from retail stores and vendor stalls.
2.) To assess which of the *Listeria* strains, if any, are representatives of *L. monocytogenes.*
3.) To determine the antibiotic susceptibility profiles of confirmed *L. monocytogenes*, *Salmonella spp.* and *Campylobacter spp.* isolates.
4.) To analyse the effect probiotics have on the growth of *L. monocytogenes in vitro.*
5.) To analyse and compare the effect probiotics had on the growth of *L. monocytogenes* in the different food matrixes.

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Chapter 1

LITERATURE REVIEW

1.1 *L. monocytogenes* history and taxonomy

Though the first description of *L. monocytogenes* appeared in the 1920s, prior knowledge about its existence was already present (Seeliger 1988, FAO 2004). Isolates were not identified as belonging to any genus up until 1925 when Murray, Webb and Swann led an investigation into causes of sudden death in a rabbit population. Repeated isolation of the same gram positive bacilli from rabbits and guinea pigs in subsequent studies led to its first description as *Bacteria monocytogenes* (Murray et al 1926). They termed the isolates *B. monocytogenes* due to lack of similarities between isolates and any genera present at the time (Hof 2003).

Another researcher, Pirie, was working on a similar microorganism in South Africa (Schlech et al 2000). Pirie was conducting studies on a disease in veld rodents, specifically gerbils, in Johannesburg (Pirie 1927). He defined it as Tiger river disease describing the microorganism as a gram positive, rod shaped, catalase positive organism (Pirie 1927, Schlech and Achesson 2000). He first named the bacterium *Listerella hepatolytica* before changing it later on to *Listerella monocytogenes* (Hof 2003). The name was later changed to *Listeria* after *Listerella* was rejected because it had been used previously for a foraminifer species (Pirie 1940).

In the late 1940s to early 1950s, Reiss et al (1951) were working on isolates from an infant disease termed granulomatosis infantiseptica. This disease is characterized by presence of granulomas in the spleen, liver and lungs of affected newborns (Moore and Brogdon 1962). The isolates recovered were classified under *Corneybacterium* due to morphological similarities to the *Cornyebacteriaceae* family (Breed et al 1957, Wilkinson 1977). Seeliger then tested the isolates and observed a high resemblance to *Listeria* (Seeliger 1961). *Listeria* was a member of the *Cornyebacteriaceae* family up until 1974 when Bergeys Manual of Determinative Bacteriology placed it under *Lactobacillaceae* (Rocourt et al 2000). It was only in 2010 when *Listeria* was placed under *Listeriaceae* (Ludwig et al 2009).

The first human listeriosis case was reportedly a soldier in 1929 while the first perinatal case occurred in 1936 (FAO 2004). The first human outbreak was in infants in the form of

granulomatica infatisepticum (Reiss et al 1951, Hof 2003). *Listeria monocytogenes* only became a concern to food microbiologists in the 1980s when it was demonstrated that coleslaw could transmit the pathogen (Beuchat 1996). The importance of food in its transmission was further solidified by other outbreaks caused by cheese in USA and Switzerland (Conly and Johnston 2008). Currently, *L. monocytogenes* is considered a highly fatal pathogen by the CDC (CDC 2020).

The genus *Listeria* currently contains 20 species and 4 subspecies (LeClerq et al 2019, www.bacterio.net). The newly described species to the genus is *L. thailandensis* added in 2019 (LeClerq et al 2019). It has been proposed that all 20 species fall into four different clades. Three clades fall into one branch and the fourth clade which contains only one species, *L. grayi*, in another branch of the phylogenetic tree (den Bakker et al 2014). It is a diverse genus consisting of both pathogenic and non-pathogenic species (www.bacterio.net, Ludwig et al 2010). Only two species under *Listeria,* namely *L. monocytogenes* and *L. ivanovii,* are pathogenic (Alvarez-Ordóñez et al 2015). *L. ivanovii* infects animals while *L. monocytogenes* can infect both humans and animals (Guillet 2010).

L. monocytogenes is the type strain of the genus *Listeria*. It consists of multiple strains representing 13 serotypes namely 1/2a, 1/2b, 1/2c, 4b, 3a, 3b, 3c, 4a, 4b, 4c, IIIB4a, IIIB4b and IIIB4c (Pifaretti et al 1989, Roberts et al 2006, Orsi et al 2011, Braga et al 2017). These serotypes can all be classified under 4 lineages (Orsi et al 2011, den Bakker et al 2008). Lineage 1 consists of 1/2b, 3b, 3c and 4b while lineage 2 is represented by 1/2a, 1/2c and 3a (Pifaretti et al 1989). Lineage 3 first defined by Rasmussen et al 1995 is represented by 4a, 4b and 4c while lineage 4 consists of the rare strains IIIB 4a,IIIB 4b and IIIB 4c (Ward et al 2004, Roberts et al 2006). Lineage 1 has the highest pathogenicity potential containing the clinically relevant serotypes 1/2a and 4b followed by lineage 2 with 1/2b (Wiedmann 2002, Orsi et al 2011). The difference in pathogenic potential is most likely due to differential expression of genes between strains rather than absence of virulence factors (Liu et al 2004).

1.2. Sources of contamination

In a farm environment set up for either livestock or crop production, farm animals such as goats and chickens become reservoirs and transmitters of *L. monocytogenes* (Fenlon et al 1996). Wild birds and animals, interacting with farm produce often, can act as vectors (Hellstrom et al 2008, Alum et al 2016). The soil which harbors *L. monocytogenes* contaminates vegetables or livestock through direct and indirect contact (Locatelli et al 2013). Water in the farm environment has multiple uses ranging from watering plants, washing hands to irrigation purposes. If contaminated, the transmission capacity of farm water can be high and extensive (Giao and Keevil 2014, Guevremont et al 2017).

Silage, which is grass or fodder stored in airtight conditions without drying, is used to feed various farm animals such as cattle, goats and sheep (Merriam Webster dictionary, Grant and Ferraretto 2018). Spoiled silage contaminated with *L. monocytogenes* can lead to herd outbreaks when one silage lot is used to feed a whole herd (Vazquez-Boland et al 1992). After feeding on contaminated silage, cattle excrete manure harboring *L. monocytogenes.* This manure is used as fertilizer which introduces the pathogen to crops (Wiedmann et al 1996). These crops are harvested for sale to consumers further spreading the pathogen to potential hosts (Fenlon et al 1996).

Food can be contaminated on site in a processing plant where contamination is usually due to persistent strains (strains isolated from a site for multiple times over a given time period) or strains recently introduced into the manufacture site (Ferreira et al 2014). Tompkins et al (1999) broke down contamination into areas of high, indirect or low risk. High risk areas are sites where food manufacturing and processing occurs. Machines and personnel involved are implicated when contamination of a food product occurs due to their direct involvement (Tabit 2018). Indirect risk areas are sites adjacent to high risk areas, surfaces involved in processing of ready to eat foods and surfaces further from high risk areas. Low risk areas are characterized by infrequent sources of contamination such as raw food material or machines that move in and out of the production plant (Tompkins et al 1999, Ferreira et al 2014).

Contamination can also occur in a retail environment and consumer dwellings due to improper handling (Kaneko et al 1999, Sirsat et al 2014, Allum et al 2016). Some foods are stored well during transport only to be exposed to room temperature conditions in a retail store in which *L. monocytogenes* proliferates (Ferreira et al 2014). Fruits and vegetables which can harbor *L. monocytogenes* are sometimes left at room temperature allowing the pathogen to grow to infectious doses. Also, handling of ready to eat (RTE) foods, when done improperly, can lead to contamination (Endrikat et al 2010). Consumers sometimes do not have proper knowledge on food storage and handling before, during and after cooking hence they contaminate food in their homes (Mosupye and Holy 1999).

1.3 Foods implicated in *L. monocytogenes* outbreaks

Listeriosis is a food borne illness resulting from an infection by *L. monocytogenes* transmitted by various types of food. The food through which *L. monocytogenes* is transmitted must have intrinsic properties that allow for survival if not proliferation during manufacture, transport and storage (Farber and Peterkin 1991). *L. monocytogenes* can survive in a diverse range of foods from dairy products, different fruit and vegetable varieties, all types of meat and in RTE foods (El-Shamy et al 1993, Indrawattana et al 2011, Rothrock et al 2017, Tahoun et al 2017, Zhu et al 2017, Ziegler et al 2018).

1.3.1 Dairy

Cows can contract *L. monocytogenes* from the environment which is subsequently transmitted to milk (Bourry et al 1995). *L. monocytogenes* can survive in both natural and artificial milk (Xanthiakos et al 2006). It can also survive in yoghurt depending on the set of conditions present in yoghurt. Factors that influence proliferation of *L. monocytogenes* in yoghurt include the type of probiotic strain used, solids content and pH (Griffith and Deibel 1990). *L. monocytogenes* can also grow in cheese though factors such as pasteurization, type of starter culture and cheese play a role in its proliferation (Farber and Peterkin 1991, McAuliffe et al 1999, Cataldo et al 2007, Schvartzman et al 2011, Iulietto et al 2018). It has also been isolated from non-supporting foods such as ice cream albeit at a lower concentration (Poulliot et al 2015, Chen et al 2016).

1.3.2 Poultry

Contamination of poultry meat by *L. monocytogenes* at farm or processing sites has been extensively investigated (Berrang et al 2000, Goh et al 2012, Crespo et al 2013, Sasaki et al 2013, Rothrock et al 2017). Given it's ubiquitous nature; multiple opportunities for the pathogen to get in contact with poultry meat either at breeding, slaughter and processing sites are present (Vivant et al 2013) Within the farm environment, chicken eggs get in contact with contaminated chicken feces and soil can get contaminated with *L. monocytogenes* (Chemaly et al 2008, Rothrock et al 2017).

1.3.3 Meat and fish

Presence of *L. monocytogenes* in beef and pork meat has been established in many a study (Shen et al 2006, Hellstrom et al 2008, Gamboa-Marin et al 2012, Al-Mashhadany et al 2016). It mostly inhabits the surface though isolation from interior of muscles has been carried out (Johnson et al 1990, Farber and Peterkin 1991). Sea food like shrimp, crabmeat and white fish has been implicated as well (Weagant et al 1988, Jemmi and Guyer 1991). Factors that influence survival of *L. monocytogenes* in meat include type of tissue, its pH and temperature, the amount of background flora present, method of meat preparation and packaging (Grau and Vanderlinde 1988, Glass and Doyle 1989). Whether the meat is lean or fatty also plays a role since fatty foods can protect pathogens from acidity during gastric passage (Deshpande 2002).

1.3.4 Vegetables

A wide range of vegetables such as potatoes, radishes, cabbages, lettuces and green salads have been associated with *L. monocytogenes* (Sizmur and Walker 1988, Prazak et al 2002, Aytac et al 2010, Caggiano et al 2015, Ajayeoba et al 2016). Due to its expansive growth capabilities; it inhabits a diverse, wide range of habitats within the natural environment (Vivant et al 2013). During growth, vegetables are in contact with soil, water, insects and animals which contaminate plants with the pathogen (Weiss and Seeliger 1975, Kljujev et al 2018). Post-harvest, storage at room temperature or in non-freezing cold temperatures which is conducive for some vegetables might lead to proliferation of *L. monocytogenes* (Riquelme et al 1994). The absent or minimal processing required for most

vegetables before being palatable does not counter growth and increases risk of contamination (Nguyen et al 1994). However, some vegetables such as carrots have an anti listerial effect (Beuchat and Brackett 1990).

1.3.5 Ready to eat (RTE) foods

RTE foods can be eaten by the consumer without prior preparation or any thermal treatment (Rodriguez- Cavallini et al 2010). Examples are cold meats (polony, ham, and viennnas), vegetables (salads, cucumber, carrots) and certain desserts (WHO 2004, Tabit 2018). During processing of RTE foods; slicing, rolling and packaging is done by various machines and workers. It is during these processes were contamination is rife due to poor sanitary practices employed by those responsible (Grau and Vanderlinde 1993, Tabit 2018). Most if not all RTE foods are stored at cold temperatures then consumed straight after. Since *L. monocytogenes* can tolerate low temperatures, proliferation during storage is possible (Tasara and Stephan 2006).

1.4 Control of *Listeria monocytogenes*

L. monocytogenes can be controlled at various stages of contamination or occurrence. Control can be done at the manufacturing plant, retail site or consumer dwellings and lastly in the human host after ingesting foods containing *L. monocytogenes*. If *L. monocytogenes* is targeted and dealt with well in the processing plant, a lot of complications that occur downstream can be avoided (Ferreira et al 2014). Various bactericidal and bacteriostatic substances can be used to combat *L. monocytogenes* in industry (Chaitemwong et al 2014). These substances are most effective when applied not only to the correct site but also under a controlled strategy (Tompkins 2002).

Control of *L .monocytogenes* can be achieved by manipulating the intrinsic and extrinsic properties of food (FDA 2017). *L. monocytogenes* growth slows down at a pH of 4.4 and below hence acidic additives can lower pH inhibiting its proliferation (Shimojima et al 2016). Preservatives such as fumaric, acetic and benzoic acid can been used to maintain a low pH environment in food during storage (Gonzaloz-Fandos and Herrera 2014). Lowering of water activity to levels below 0.90 can deter growth of *L. monocytogenes* in food as

evidenced by low contamination levels in dried fish or hardened cheese with very low moisture content (Luber et al 2011, Shimojima et al 2016).

L. monocytogenes can withstand acidic pH ranges as low as 3 hence lowering the pH of the food to 4.4 may only give a bacteriostatic effect rather than a bacteriocidal effect (Ferreira et al 2003). It can also tolerate water activity levels as low as 0.7 hence lowering the moisture content to levels below 0.9 might not satisfactorily inhibit *L. monocytogenes* growth (Walker 1990, Farber and Peterkin 1991). Use of these control mechanisms in combination might enhance their anti-listeria effect (Leistner 2000). Maintaining sensorial properties is highly integral in food production and manipulating characteristics such as pH and water activity might affect the quality of food.

Control against *L. monocytogenes* can also be carried out using chemical alternatives. Inorganic acids like sulphite and nitrite have proven to have a high listericidal effect especially when used in combination with hydrostatic pressure (Brandt et al 2011). High temperature, short time (HTST) and low temperature, short time (LTST) pasteurization can also be used (Myer et al 2016, Escuder-Vieco et al 2018). Cooking can also expose *L. monocytogenes* to high temperatures though time taken to cook plays a role in the listericidal effect (Silva and Gibbs 2012, Khan et al 2016).

The temperature tolerance of *L. monocytogenes* is relatively high which increases its chances of surviving the temperature related hurdles (Farber and Peterkin 1991). Exposure of food to very high temperatures and chemical preservatives can affect the nutritional and sensorial properties of food (Khan et al 2016). We live in an age where consumers require food of high quality and safety while still maintaining its natural state and ensuring a long shelf life. The hurdles in food processing against bacterial pathogens have to be delicate enough for the consumers taste while being highly aggressive to pathogens (Rajkovic et al 2010).

New mechanisms of decontamination have been developed over the years that attempt to maintain all the qualities required of food by the public. Irradiation is one method which uses X–ray machines and gamma rays to kill of pathogenic bacteria (Solanki et al 2012). The disadvantages of irradiation methods such as X ray machines is that they are

expensive (Calado et al 2014). They also cannot be applied to all foods since they can compromise the sensorial quality in some fruits and dairy products (Stefanova et al 2010). There is also high pressure processing (HPP) also called cold pasteurization in which food, sealed off in a bag, is exposed to high pressures applied by water (www.hiperbaric.com). The drawback with HPP is that it is more efficient against Gram negative microbes than Gram positives like *L .monocytogenes* due to differences in cell membrane organization hence would not be suitable against the pathogen (Lucore et al 2000).

Use of ozone is another popular mechanism used to reduce the levels of bacterial pathogens while maintaining the shelf life of food especially in foods such as vegetables and fruits (Concha – Meyer et al 2015). It has been used in a highly effective combination with heat against *L. monocytogenes* (Sung et al 2014). Electrolysis of water which converts water into an acidic and alkaline form has also proven to be useful against *L .monocytogenes* strains that form biofilms (Khan et al 2016). The disadvantages of these methods is large scale disinfection which is very expensive hence will not be attempted by many food companies looking for cheaper but still effective methods.

All these methods mentioned above can be used against *L. monocytogenes* before consumption by consumer. If they fail, ingestion of food containing an infectious dose of *L. monocytogenes* by susceptible individuals leads to various health complications culminating in death.

1.5 *L. monocytogenes* infection

The success of a *L. monocytogenes* infection is based on its ability to safely transit through the stomach, cross epithelial barriers and move between cells without exiting into the intercellular space. *L. monocytogenes* can withstand gastric juices in the stomach using the acid adaptive tolerance system and glutamate acid decarboxylase amongst others facilitating a smooth transit to the intestinal epithelial cells, the site of infection (Smith et al 2012, Feehily et al 2014). It can also withstand bile secretions countering their effect using bile salt hydrolase or the bile exclusion system (Sleator et al 2005, Ridlon et al 2006). In the intestinal region, infection commences. The genes mainly responsible for pathogenicity

and virulence of *L. monocytogenes* are namely *inlA, inlB, prfA, hlyA, plcA, plcB, actA* and *mpl* (Figure 1) (Gouin et al 1994, Sheehan et al 1995):

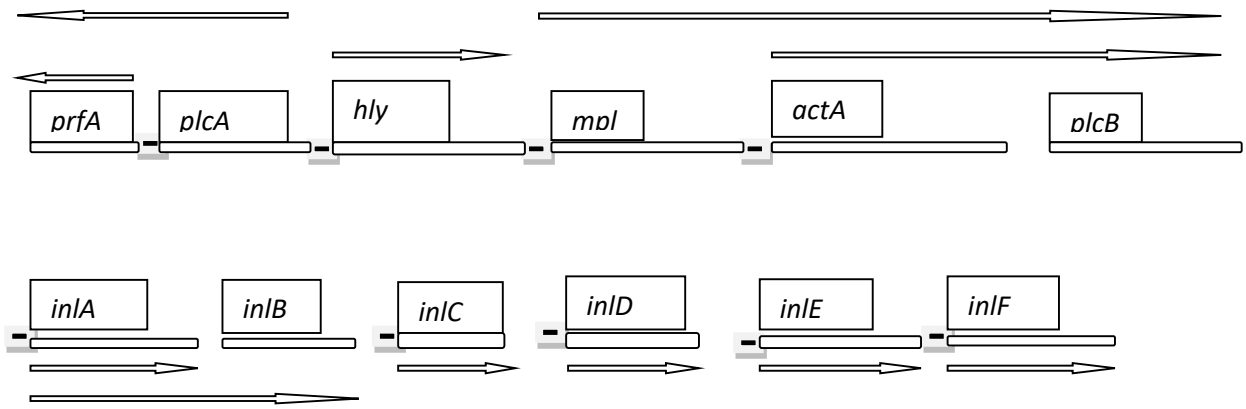

Figure 1: Structure of the *L. monocytogenes* virulence gene cluster. The boxes shaded black in between the genes show the motifs where *prfA* binds to activate expression of each gene. The arrows show the reading frame encoded and the direction (Gouin et al 1994, Sheehan et al 1995).

In the small intestine, molecular cross talk between *L. monocytogenes* pathogenic cells and intestinal cells begin. The *prfA* gene encodes PrfA proteins responsible for regulating expression of the *L. monocytogenes* virulence cluster (Wernars et al 1992). The first gene involved is *lmo1634* which encodes the Listeria Adhesion protein (LAP) (dos Santos et al 2018). LAP binds to the Hsp60 receptor present on surfaces of epithelial cells through its N2 domain (Burkholder and Bhunia 2010). Binding increases permeability of cell junctions and subsequently promotes invasion using the paracellular route (Kim and Bhunia 2013).

Thereafter, the internalin genes designated by the code *inl* are expressed synthesizing internalin proteins involved in adhsesion and invasion of *L. monocytogenes* (Hamon et al, 2006, Bonazzi et al 2009). Internalization occurs soon after LAP-Hsp60 binding mediated by the antigens internalin A (*inlA*) and internalin B (*inlB*) (Grundler et al 2013). InlA interacts with E-cadherin using its leucine rich repeats while InlB can interact with the receptors c-Met, gClq-R and glycosamines (Lecuit et al 2004, Niemann et al 2007). Internalization post binding is a complex process of which internalization by InlA is most understood. For InlA,

binding to E-cadherin leads to a complex array of processes all resulting in invasion of *L. monocytogenes* (Bonazzi et al 2009, Bonazzi 2012, Quereda et al 2016).

After entry into epithelial cells, *L. monocytogenes* is located inside a vacuole or phagosome (Portnoy et al 2002). Within the vacuole, *L. monocytogenes* produces superoxide dismutase which counters attacks by oxygen radicals (Welch et al 1979). It also produces peptidoglycan N-deacetylase (PgdA) enzyme which counters lysozyme in the phagosome through breaking down N-acetylglucosamine residues in the membrane (Boneca et al 2007). Escape occurs when pH increases in the vacuole signaling *L. monocytogenes* to produce substances such as listeriolysin (Burke et al 2014).

The *hlyA* gene encodes listeriolysin (LLO), a cholesterol dependent cytolysin that inserts into the vacuolar membrane. This leads to a disturbance in the pH gradient and creation of pores in the membrane through which proteins can exit (Gedde et al 2000). The PgdA enzyme then deacytelates N-acetyl glucosamine residues in the vacuolar membrane leading to dissolution of the membrane (Popowska et al 2004). The *plcA* and *plcB* genes encode for a phosphatidylcholine phospholipase C (PI-PLC) and phosphatidylinositol phospholipase C respectively (Pistor et al 1994, Kim and Bhunia 2008). These two phospholipases exit through pores formed and lead to dissolution from the exterior (Vazquez-Boland et al 2001).

LLO has a unique characteristic in that it is not toxic to the host cell after escape out of the cytosol (Glomski et al 2002). This lack of toxicity could be due to the presence of a so-called P.E.S.T sequence at its N terminal since its removal results in high toxicity and increased virulence (Lety et al 2001). It could also be due to a pH preference where LLO functions best at acidic ranges present in the vacuole but escape to the cytosol which functions at a higher pH range renders it inactivated (Portnoy et al 2002). Production of LLO does not seize due to its integral role of moving from cell to cell but its activity is limited by the above mechanisms (Anderluh and Gilbert 2014).

Within the cytosol, *L. monocytogenes* cells express a hexose phosphate transporter which enables it to use glucose-6-phosphate, a trait absent in other intracellular pathogens (Chico-Calero et al 2002). *L. monocytogenes* also express the *actA* gene producing ActA,

a surface actin polymerization protein capable of mediating actin nucleation and pathogenicity (Pantaloni et al 2001). ActA forms a binding site for Ena/Vasp proteins which in turn promote binding of profilin and F. actin capable of forming short, highly branched actin filaments responsible for actin polymerization (Geese et al 2002, Portnoy et al 2002). This branched actin system facilitates movement within the infected cell and also between adjacent cells (Dominguez et al 2010). Cell to cell spread then continues in this manner. From the epithelial cells, *L. monocytogenes* translocates to the liver or spleen (Rogers et al 1996, Aoshi et al 2009).

In the liver, *L monocytogenes* is exposed to an immune system attack (Melton-Witt et al 2012). Liver macrophages called Kupfer cells antagonize *L. monocytogenes* (Dramsi et al 1995). Also; hepatocytes release chemicals that attract neutrophils which combat bacterial infection (Witter et al 2016). Adhesion of neutrophils to infected hepatocytes is increased which results in formation of micro abscesses (Rogers et al 1996). Apoptosis is also induced in infected hepatocytes which helps in the reduction of *L. monocytogenes* (Miura et al 2000). In the spleen, dendritic cells, macrophages and neutrophils are in abundance in the organ hence antagonize it upon entry into the spleen (Aoshi et al 2009). The *L. monocytogenes* population left after an immune system attack in the liver or spleen can then move through the blood to the brain. *L. monocytogenes* can infect different endothelial cell types (Drevets et al 1995). In the brain, the target cell type is the brain macrovascular endothelial cells (BMEC) (Greiffnenberg et al 1998). In the brain, listeriosis manifests as either meningitis or encephalitis (Vazquez-Boland et al 2001).

In pregnant women, *L .monocytogenes* can disseminate to the placenta where it infects the foetus (Farber and Peterkin 1991). The foetus is separated from the mother by amniotic epithelium tissue, cytotrophoblastic and synctiotrophoblastic cell tissues (Robbins et al 2010). These cell types contain the surface receptors c-Met and glycoaminotransferases which bind internalin B (InlB) (Robbins et al 2010, Zhu et al 2017). Cytotrophoblastic cells and synctiotrophoblastic cells also express E-cadherin which *L. monocytogenes* can use to initiate invasion (Lecuit et al 2000). The pregnant mother is seldom affected by listeriosis except for exhibiting influenza like symptoms (FAO 2004).

1.6 Listeriosis outbreaks

There has been a number of listeriosis related outbreaks over the past years though most records are from the USA (Table 1.1). The most fatal outbreak was experienced by South Africa during the epidemic that occurred between January 2017 and December 2018. This shows how relevant listeriosis still is given the up to date medical and health practices in first world countries relative to third world countries. Tompkins (2002) postulated three different set-ups with respect to foods causing listeriosis outbreaks. In situation 1, isolated cases characterized by long incubation periods leads to information loss on the exact causal food. In situation 2, an epidemic occurs due to contamination of a single batch of food. Once the batch is finished, the epidemic stops. In situation 3, contamination of a processing plant or site were food production occurs leads to contamination of batches of food over multiple periods in time. Epidemics that result occur over long time periods at multiple locations (Tompkin 2002). All three of these scenarios occur before consumption hence prevention is better than cure in the case of listeriosis.

Table 1.1: Epidemics caused by *L. monocytogenes* in recent years.

Year	Contaminated food	Region	Hospitalization/ Fatalities	References
2013	Frozen vegetables	USA	9/3	CDC 2013
2014	Apples	USA	34/7	CDC 2014
2014	Bean sprouts	USA	5/2	CDC 2014
2014	Raw milk	USA	2/1	CDC 2014
2015	Soft cheese	USA	28/3	
2015	Vegetables, fruits, salads, RTE meat	South Africa	7/1	NCID
2015	Ice cream	USA	2/1	CDC 2015
2016	Mixed salads	USA	19/1	CDC 2016
2017	Cheese	USA	6/2	CDC 2017
2017	Ready to Eat polony	South Africa	1060/216	NCID
2020	Enoki mushrooms	USA	30/4	CDC 2020

Table 1.2 shows annual number of morbidities caused by pathogens in food (Scallan et al 2011). As shown, when comparing annual illnesses caused, *L. monocytogenes* ranks 22 out of 31 food pathogens. This does not give an indication of a dangerous pathogen until comparison of ratios between annual illnesses caused and number of fatalities is done. The result, *L. monocytogenes* causes an estimated 1 600 illnesses and 250 deaths

annually which implies 2 deaths for every 13 cases. This is the highest fatality rate of any of the pathogens on the table. When compared to the three top pathogens on the table, *Campylobacter*, *Salmonella* and *Norovirus*, *Campylobacter* causes a single death in 11 184 cases, *Salmonella* 1 in 2 631 cases and *Norovirus* 1 in 36 666 cases.

Table 1.2: Annual number of morbidities caused by pathogens in food (Scallan et al 2011).

Pathogen Type	Pathogen	Estimated annual illnesses*	Estimated annual hospitalizations*	Estimated annual deaths*
Bacteria	*Bacillus cereus*, foodborne	63,000	20	0
	Brucella spp.	840	55	1
	Campylobacter spp.	850,000	8,500	76
	Clostridium botulinum, foodborne	55	42	9
	Clostridium perfringens, foodborne	970,000	440	26
	E. coli (STEC) O157	63,000	2,100	20
	E. coli (STEC) non–O157	110,000	270	1
	Enterotoxigenic *E. coli* (ETEC)	18,000	12	0
	Diarrheagenic *E. coli* other than STEC and ETEC	12,000	8	0
	Listeria monocytogenes	1,600	1,500	250
	Mycobacterium bovis	60	31	3
	Salmonella spp., nontyphoidal	1,000,000	19,000	380
	S. enterica serotype Typhi	1,800	200	0
	Shigella spp.	130,000	1,500	10
	Streptococcus spp. group A, foodborne	240,000	1,100	6
	Streptococcus	11,000	1	0
	Vibrio cholerae, toxigenic	84	2	0
	V. vulnificus	96	93	36
	V. parahaemolyticus	35,000	100	4
	Vibrio spp., other	18,000	83	8
	Yersinia enterocolitica	98,000	530	29
Parasites	*Cryptosporidium* spp.	58,000	210	4
	Cyclospora cayetanensis	11,000	11	0
	Giardia intestinalis	77,000	230	2
	Toxoplasma gondii	87,000	4,400	330
	Trichinella spp.	160	6	0
Viruses	Astrovirus	15,000	87	0
	Hepatitis A virus	1,600	99	8
	Norovirus	5,500,000	15,000	150
	Rotavirus	15,000	350	0
	Sapovirus	15,000	87	0

1.7 Antibiotics and listeriosis

The introduction of antibiotics has been very beneficial to human health. Antibiotics significantly decreased fatalities related to infections caused by *S. pneumonia* and *S. aureus* in the early 1900s (Karchmer et al 1991, Bartlett and Mundy 1995). Approximately 70% of all amputations performed in WW1 were due to bacterial wound infections or done to prevent septicemia (Hirsch 2008). They have also greatly increased chances of patient survival in complex surgeries and medical procedures such as transplantation and chemotherapy (Friedman et al 2016).

Ampicillin and penicillin are the antibiotics of choice with respect to treatment of listeriosis while chloramphenicol is used in the instance of penicillin allergy (Allerberger and Wagne 2010, Olaimat et al 2018). Ampicillin and penicillin prevent transpeptidases from cross linking peptidoglycan residues in the cell membrane inhibiting biosynthesis of the cell wall leading to lysis (Chudobova et al 2014). Higher doses are usually required in pregnant women to increase the likelihood of the antibiotics crossing the placenta and umbilical cord (Janakiraman 2008). Gentamycin is an aminoglycoside which interrupts translation by inteferring with the function of 16 ribsomal RNA (Krause et al 2016). Combination with gentamycin is usually discouraged in pregnant women due to doubts concerning the toxicity of gentamycin especially at the high concentrations required (Temple and Nahata 2000, Janakiraman 2008). Other alternatives are trimethoprime, erythromycin, vancomycin and moxifloxacin (Temple and Nahata 2000, Sipahi et al 2008).

Though antibiotics have their benefits, they also have shortcomings that may result in limitations to their usage especially in combating bacterial infections (Friedman et al 2016). Abuse of antibiotics can lead to emergence of resistance in target species. *L. monocytogenes* strains over express methyl tranferases which ensures the stability of 23S rRNA, a target for erythromycin (Charpentier and Courvalin 1997, Granier et al 2011). Some strains can encode trimethoprim resistant dihydrofolate reductase mediated by the *dfrD* gene found in a plasmid (Charpentier and Courvalin 1997). Other strains have drug efflux pumps such as those belonging to the ATP-binding cassette transporter family which can actively rid the pathogenic cell of any antibiotics within (Granier et al 2011).

Genes that confer resistance are usually located in plasmids, for example, the tetracycline and ampicillin resistance genes (Bennet 2008, Leungtongkam et al 2018). Plasmid exchange can occur through horizontal gene transfer between pathogens and natural micro flora within a favourable environment resulting in the creation of antibiotic resistant pathogenic strains (San Millan et al 2018). The emergence of the multi-drug resistant *Clostridium difficile*, *Pseudomonas aeruginosa* and Methicillin Resistant *S. aureus* (MRSA) strains has been attributed to heavy antibiotic usage and is a testament to acquired antibiotic resistance (Deneve et al 2009, Harkins et al 2017, Miyoshi-Akiyama et al 2017).

Broad spectrum antibiotic action is not only specific to the pathogen but the natural host microbiota as the name suggests (Schjorring et al 2011, Spaulding et al 2018). The gut microbiome carries out multiple functions beneficial to both the microorganism and their human hosts. The amylolytic and proteolytic activity of bacteria within the gut is highly integral to the digestive process (Gibson et al 1989, Kaoutari et al 2013). Gut microbiota also break down non absorbed polysaccharides to short chain fatty acids (SCFA) essential for structure and function of colon cells (Scheppach 1994). Efficient SCFA production and function have also been recently linked to improved cardiovascular health (Chambers et al 2018).

A decrease in number and diversity of gut microbiota hence leads to a pleothora of complications including and not limited to reduced water and nutrient absorption and improper functioning of the intestines (Surawicz 2005, Guigoz et al 2008, Vecsei et al 2010). Depletion of beneficial microbes also disturbs the protective barrier thus increasing the chances of infection by intestinal pathogens (Hickson 2011). This leads to an imbalance in the ratio of beneficial to detrimental microbes. Dysbiosis results promoting the over growth of detrimental microbes like *C. difficile*, *Candida spp.* and *Klebsiella oxytoca* (Hogenauer et al 1998, Barbut 2002). The above factors singularly or combined can result in debilitating conditions inclusive of antibiotic associated diarrhoea (AAD) and Irritable bowel syndrome (IBS) amongst others (Hickson 2011).

The above mentioned disadvantages to antibiotic use are worsened by the recent decision by most pharmaceutical companies to slow down research and development of new antibiotics (Livermore 2004). This is due in part to financial returns and also regulations

that have been put in place to promote consumer safety. These regulations are hurdles which require effort and money to overcome (Kinch et al 2014). The continuous emergence of resistant strains and the health complications that result from antibiotics use coupled with limited research on new antibiotics has prompted some to look into alternatives and the use of probiotics has emerged as a viable option. Probiotics can be used as an adjunct or can completely replace antibiotic treatment depending on the ailment (Reid 2006).

1.8 Probiotics

1.8.1 What are they?

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer benefits to the host’’ (Fijan 2014). These “live microorganisms” are representative of a variety of fungi and bacteria (Fooks and Gibson 2002). One of the major bacterial representatives are the lactic acid bacteria composed of various genera namely *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Enterococci* and *Streptococci* (Fooks and Gibson 2000, Axelsson and Ahrne’ 2000, Soccol et al 2010). Lactic acid bacteria, as the name suggests, produce lactic acid as a by-product when they ferment carbohydrates (Walter 2008).

Bifidobacterium is another bacterial genera with strains commonly used as probiotics (Ciorba 2015). *Bifidobacterium* species form part of the first microbial population to colonise the infant gut post birth and their beneficial effects are well documented (Turroni 2009, O’Callaghan and van Sinderen 2016). *E. coli* species also have a probiotic representative; the *E. coli* Nissle strain (Oelschlaeger 2010). Members of the above genera offer certain advantages when used as probiotic strains. They are present in fermented foods where they naturally dominate the micro flora inhabiting the foods (Kim et al 2016). They are also part of the natural intestinal micro flora and are generally regarded as safe to use (Maragkoudakis et al 2009).

Fungi also have representatives in the form of the yeast and fungal strains such as *Saccharomyces cerevisae, S. boulardii*, *Kluyveromyces fragilis, K. marxianus, Aspergillus oryzae* and *A. niger* (Hudson et al 2016, Markowiak and Slizewska 2018). Their ability, as eukaryotes, to express and carry out post translational modifications of various types of

therapeutic proteins that cannot be processed by prokaryotes is unique amongst probiotics (Ciorba 2015). Fungi also exhibit exceptional survival properties within the harsh gut environment ensuring viability to carry out intended function (Hyde et al 2019).

1.8.2 History of probiotics

Microorganisms have been used, though unknowingly, for their beneficial effects since early in human history (McGovern 2007). Their use in fermentation of milk, bread, vegetables, wine and beer and the subsequent benefits of eating the fermented foods has been well documented by Greek, Egyptian and Roman civilizations (Chambers and Pretorius 2010, Gogineni et al 2013). Hippocrates, the father of medicine famously stated that “death sits in the bowels” hence the relation between gut health and homeostasis has been known since ancient times (Gasbarrini et al 2016). It was only in the late 1800s when Escherich formally described microbiota that colonize an infant gut and pointed out their assistance in digestion (Farre-Maduell and Cascals-Pascual 2019).

Tissier in the early 1900s found *Bifidobacterium* in the gut of infants and suggested that they were beneficial to the infant gut (Kechagia et al 2013). Round about the same time a pioneer in probiotic biology, Metchnikoff, found that Bulgarian citizens who consumed yoghurt containing high amounts of *Lactobacillus* were healthier than those who did not (Gogeneni et al 2013, Ozen and Dinleyici 2015). In the 1920s*, L. lactobacillus* was used in milk to improve digestion and in the 1930s; *L. casei* shirota strain was used in fermented milk owing to its ability to overcome hurdles in the gastrointestinal tract (Gogeneni et al 2013). The introduction of antibiotics resulted in probiotic research taking a back sit but as mentioned before, studies on probiotics are on the rise again (Belaga 2017).

1.8.3 Characteristics of probiotics

What qualifies a strain as a probiotic? Firstly, one has to consider that if the strain is incorporated into food, it will be exposed to processes involved in food manufacture. Post production, the product is stored under different conditions during transport, at the retail site and at the consumers dwelling. It has to be safe for consumption and remain viable as it transits through the gastro intestinal tract. When it reaches its destination, it has to be

as effective as intended. Thus a microorganism has to exhibit a vast array of capabilities to be considered as a probiotic strain.

Various mechanisms are involved in the manufacture of some food products. Processes such as freezing, drying, high pressure pasteurization and centrifugation amongst others are common in food production. Probiotic strains have to exhibit tolerance to these processes (Savini et al 2010, Roos and Livney 2016). Microorganisms are sometimes immobilized to protect the probiotic strain from such harsh conditions. Immobilization is entrapment or the attachment of microorganism cells to protective material (Mitropolou et al 2013). An example of an immobilization technique is micro encapsulation whereby microorganisms are entraped in a matrix which forms a core surrounded by encapsulation material such as alginate (Gbassi and Vandamme 2012).

The strain also has to be stable and maintain viability in the food matrix. This requires tolerance to various ingredients and properties specific to each food product (Kalliomaki et al 2007). Neutralizing the pH, prioritizing non-toxic ingredients, addition of growth factors and antioxidants during production can improve probiotic survival rates (Santo et al 2011). Whatever is done should not alter sensorial properties of the food product; taste, texture and appearance, so as to maintain the products' appeal to the consumer (Roos and Livney 2016). Additionally, the probiotic strain should be safe for consumption hence rigorous tests are required to ensure complications such as disruption of the normal immune system functioning and translocation to other parts of the body are avoided (Baken et al 2006).

After consumption, the probiotic strain must survive movement through the gastrointestinal tract. The first barrier is presented by the stomach's gastric acidic environment (Harzallah and Belhadj 2013). Also, the probiotic strain must tolerate bile and pancreatic juices which have a high alkaline pH and contain multiple digestive enzymes that inhibit bactericidal activity (Ruiz et al 2013). In the intestine, the probiotic strain needs to adhere to intestinal mucosa in order to colonize the intestinal region (Lahtinen 2012). After colonization, it has to exhibit anti-microbial activity ranging from preventing adhesion of pathogens, immune system modulation and nutrient competition to production of different types of antimicrobial substances. The various mechanisms of anti-pathogenic activity will be discussed in later sections.

1.8.4 Probiotics in food production

The beneficial properties of probiotics to human health can be conferred directly through various mechanisms within the human body or indirectly through production and modification of food.

In food; they are involved in production of various types of foods as well as enhancing their sensorial properties (Roos and Livney 2016). Probiotic strains are highly beneficial as starter cultures to ferment milk in the production of cheese, yoghurt, kefir, koumiss amongst others (Stanton et al 1998, Elli et al 2015, Prado et al 2015). The strains are representative of various probiotic genera of which *L. acidophilus* and *B. lactis* are the most common (Parvez et al 2006, Cutting 2011). *L casei* is known to enhance the sensorial properties in parmesan cheese and kefir (Yerlikaya 2014). *Propioniobacterium* is required for maturation of Swiss cheese and through production of propionic acid and acetic acid enhances its flavor and aroma (Cousin et al 2011, Yerlikaya 2014).

Lactic acid bacteria are also integral to sausage production. Through production of lactic acid, they lower the pH promoting the coagulation of proteins (Thomas et al 2008). This results in firmness and cohesiveness of the meat product (Hugas and Monfort 1996). *Saccharomyces cerevisae* is the most common fungal probiotic. It is required for leavening which raises dough during baking of bread. It is also responsible for flavor and aroma in bread (Moyad 2008). *S. cerevisae* is also involved in brewing beer and wine making where it ferments carbon sources to ethanol (Lodolo et al 2008). It also produces sulphur dioxide (SO_2) and esters which improve the taste and aroma of alcoholic beverages (Lambrechts and Pretorius 2000).

1.8.5 Gastrointestinal activity

Probiotics can confer other non-antimicrobial benefits to the body. Their ability to digest lactose producing lactic acid is well documented (Kechagia et al 2013). *Lactobacillus* strains can reduce body cholesterol through uptake, use and conversion to other less detrimental compounds (Kobyliak et al 2016). LAB and *Bifidobacterium* are known to synthesize B-vitamins, folate and riboflavin, and K-vitamins which cannot be naturally synthesized by humans (Rossi et al 2011, Gu and Li 2016). They also produce short chain

fatty acids like butyric acid, an energy source for colon cells and can induce apoptosis in colon carcinoma cells (van Zanten et al 2012). Probiotic strains also bind mutagens and convert carcinogenic compounds into inactive compounds in the intestinal region (de Vrese et al 2001).

1.8.6 Antimicrobial activity

They can also ensure safety and preservation through limiting growth or reducing the population of pathogenic or spoilage microorganisms. Probiotics can act antagonistically against undesirable microorganisms in food in a variety of ways. Competitive exclusion through competition for space and nutrients can inhibit the proliferation of target pathogens (Moroni et al 2006). Bacterial growth requires acquisition of nutrients from a microorganism's immediate environment (Burrows 1936). The abundance of nutrients and acquisition rate of the microorganism determine the growth rate of a bacterial population. If the nutrient resource is shared, the microorganism which has a higher acquisition rate and is more efficient at nutrient usage will out-compete other microorganisms sharing the resource (Hibbing et al 2010). Lactic acid bacteria (LAB) produces lactic acid which chelates iron in the environment thus reducing its availability to other microorganisms (Presser et al 1997). Probiotic strains that are highly efficient in nutrient acquisition and use can thus limit the proliferation of spoilage and pathogenic in food.

To compete for space, microorganisms use structural factors that allow then to bind to the intestinal mucosa. This a structure composed of a top layer of mucus, forming a layer over epithelial cells and normal micro biota associated with the intestinal region (Ouwehand et al 2003). Mucus is a complex mixture of glycoproteins termed mucins, lipids, salts, digestive enzymes, antimicrobial peptides and antibodies (Johansson et al 2008, Derrien 2010). To bind to the mucus, bacteria express mucus binding proteins that enable them to attach (Sicard et al 2017). They also contain pilli or fimbriae, structures that allow them to adhere to mucus (Kankainen et al 2009). Binding to mucus might saturate binding sites preventing the attachment of pathogens to the mucus layer as they attempt to colonize the intestinal region (Hibbing et al 2010).

Binding to intestinal cells present within the intestinal mucosa is facilitated by S-layer proteins and fibronectin binding proteins (Hymes et al 2016) These proteins, expressed on the cell surface, allow them to bind to components of the extra cellular matrix (ECM) (Styriak et al 2003). Since probiotic strains cannot compete for specific receptor sites with pathogens, they more likely prevent adhesion of pathogens to cells through steric hindrance (Garcia-Cayuela et al 2014). Steric hindrance is the inhibition of a chemical reaction between two molecules due to arrangement of atoms (Merriam Webster). It can prevent the adhesion of bacteria to living or abiotic surfaces (Wiencek and Fletcher 1992).

Other proposed methods that may prevent adhesion are auto and co aggregation. Auto-aggregation is a process whereby bacteria from the same species aggregate together. Co –aggregation on the other hand is aggregation between different species (Rickard et al 2003). Auto aggregation between probiotic strains can result in the formation of a barrier that prevents binding of foreign microbes to the intestinal mucosa (Reid et al 1988). Co-aggregation, such as seen with *B. longum* and *L. monocytogenes*, can prevent the adhesion of a pathogen to mucus or to intestinal cells (Collado et al 2007).

Probiotics also produce different types of antimicrobial substances that can inhibit or reduce undesirable microorganisms. Lactic acid and other volatile acids synthesized by LAB reduce the pH of the surrounding environment which might not be tolerable to other inhabitants (Yildirim and Johnson 1998). It is also known to increase permeability of gram negative cells allowing entry of other substances (Calo-Mata et al 2008). Acetic acid, produced by LAB and *Bifidobacterium* is also known to exhibit bactericidal activity even at low concentrations owing to pH dependent effects (Moroni et al 2006). Probiotics can also produce hydrogen peroxide, carbon dioxide, bacterial enzymes and diacetyl (Hertzberger et al 2014, Azat et al 2016, Padmavathi et al 2018). Hydrogen peroxide inactivates important bio-molecules using the superoxide anion chain and can inactivate the lacto-peroxidase system (Mishra 1996). Carbon dioxide contributes to an anaerobic environment and inhibits the enzyme decarboxylase. It can also disturb normal functioning of the cell membrane (Erginkaya et al 2011, Leroy and DeVuyst 2004). Bacterial enzymes can antagonize other non-producing strains (Fooks and Gibson 2002, Schillinger 2014).

Diacetyl can disrupt arginine utilization and interfere with cytoplasmic membrane functioning (Maragkoudakis et al 2009).

In some cases, probiotic strains produce toxins known as bacteriocins. These are ribosomally synthesized substances of a proteinaceous nature (Cotter et al 2005). They are bactericidal or bacteriostatic against closely related species and do not harm the producer strain (Reeves 1965). Bacteriocins produced by gram positive microorganisms are divided into three classes. Class I peptides are smaller (<10kDa) and are represented by bacteriocins such as nisin and lactococcin (Parada et al 2007). Class II proteins are bigger in size than class I but are still less than 10kDa and exhibit unique properties such as heat tolerance (Sieiro-Alvarez et al 2016). Lastly, class III proteins, the largest in size, are heat unstable bacterial enzymes specific to certain species (Yang et al 2014). Bacteriocins produced by gram negative probiotics are separated into two classes, collicins and microcins. These classes differ in terms of size and mechanisms of action against target microorganisms (Yang et al 2014). Recently, two new group of bacteriocins have emerged namely circular and leaderless bacteriocins (Perez et al 2018).

Bacteriocin production has been observed in the genera *Lactococcus* specifically in species such as *L. lactis* which produces nisin and *L. lactis* spp. *cremoris* which produces diplococcin (Siegers and Entian 1995, Rea et al 2011). Bifidin, a bacteriocin, has also been detected in *Bifidobacterium* whose antimicrobial activity was attributed only to pH dependent effects (Yildirim and Johnson 1998). Bacteriocin production has also been reported in the lactobacillus strains *L. salivarus*, *L. plantarum* and *L. acidophilus* (Sanders et al 2001, van Hermet et al 2010, Flyn et al 2002). Studies implicating strains as bacteriocin producers have not been extensively done and more work is required in the field (Hergaty et al 2016).

Bacteriocins have various mode of action. Nisin, produced by *Lactococcus*, can interfere with cell wall formation and disrupt cell wall functioning (Breukink et al 1999). The active nisin peptide induces formation of voltage dependent pores in a target cell leading to effluxes in ions and low molecular weight substances resulting in a change and depletion of membrane potential and the proton motive force (Ruhr and Sahl 1985). Collicin type

toxins and lactococcins can inhibit proteins, interfere with replication and transcription and can disrupt septum formation (Balciunas et al 2013, Cavera et al 2015).

1.8.7 Strengthening of epithelial barrier function

As mentioned above, mucus forms a protective layer above intestinal cells. Some probiotic strains can induce high production of mucus antagonizing invading microbes. *Lactobacillus* species, specifically *Lb rhamnosus* and *Lb. plantarum*, have been used to induce muc2 and muc3 gene expression. These genes produce mucin which prevents adhesion of an attaching E. coli pathogenic strain to HT-29 cells (Mack et al 1999). Increasing barrier strength can also be achieved through enhancing tight junction function. Corr et al (2007) hypothesized that *Lactobacillus* and *Bfidobacterium* can produce a proteinaceous compound which can strengthen the barrier of epithelial cells. A study by Wang et al (2018) also showed the ability of *L. plantarum* to strengthen the epithelial barrier.

1.8.8 Modulating the immune system response

Probiotics can also modulate the immune system response. Adhesion to epithelial cells can lead to the production and activation of the antigen presenting dendritic cells which subsequently stimulates production of other immune cells (Moroni et al 2006, Koo et al 2012, Castellazi et al 2013). Expression of natural killer cells, macrophages, specific and non-specific cytotoxic cells subsequently follows (Ashraf and Shah 2014, Galdeano et al 2019). Through stimulation of pro and anti-inflammatory interleukins and cytokines, they can regulate the inflammatory response and normalize hypersensitivity reactions (Castellazi et al 2013). By improving normal gut mucosal function, probiotics can as a result, improve the immune functioning of the gut area (Isolauri et al 2001).

1.9 Probiotics and disease

1.9.1 Probiotics and food allergy

Food sensitivity can result in pathological manifestations in the body. It can be divided into two forms, food allergy and intolerance (Ortolani and Pastorello 2006). Food allergy is defined as “an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food” (Valenta et al 2015). It can be caused by the presence of small glycoproteins in food that act as epitopes that stimulate the immune system leading to allergic reactions such as inflammation (Castellazi et al 2013). Food intolerance is an inability to process or digest certain types of food due to lack of apparatus required such as lactose intolerance due to lack of lactase (Kretchmer et al 1972, Castellazi et al 2013).

Probiotics can prevent allergies in a couple of ways. A reduction in LAB and *Bifidobacterium* coupled with an increase in *Clostridium*, *coliforms* and *Staphylococcus* species is associated with a rise in allergic reactions (Ozdemir 2010). Since probiotics can improve intestinal microbial diversity, they can counter a reduction in beneficial microorganisms. Exposure of children to foreign antigens present on bacterial surfaces improves their immunotolerance preparing their immune systems for future exposure to food and pathogenic microbes. This is known as immune maturation and is usually mediated by *Bifidobacterium* species in infants (Sampson 2004). As mentioned, probiotics can modulate the immune system through stimulation of pro and anti-inflammatory substances. In this manner, they can ameliorate one of the main symptoms of allergic reactions, inflammation (Holt et al 1997).

Using probiotics to treat food allergies has been attempted successfully in various trials (Oak and Jha 2018, Tan-Lim and Esteba-Ipac 2018). Majama and Isolauri (2011) tested the hyper sensitivity of breast fed infants to hydrolyzed whey formula (HWF) after removal of cow’s milk from the diet. In the test group, the infants were fed HWF supplemented with *Lactobacillus* GG. In the control group, infants were fed HWF with no probiotic strain. In the test group, reduced incidences of atopic dermatitis were observed. Also, a reduction in

the α1 anti trypsin factor and TNF was linked to a reduction in inflammation (Majama and Isolauri 2011).

1.9.2 Antibiotic associated diarrhoea

As mentioned before, AAD results from multiple conditions that occur in the gut as a result of antibiotic usage. Probiotics can be used to combat some of the causes of AAD. Probiotics produce short chain fatty acids which subsequently leads to better absorption on carbohydrates and water in the intestines (van Zanten et al 2012). *L. fermentum* and *L. acidophilus* have been used for their amylolytic activity to improve digestion of raw starch in pigs further improving the absorption of carbohydrates (Lee et al 2001). LAB is also known to exhibit proteolytic activities which promote their use in milk products (O'Donkor et al 2007). The reduction of beneficial microbes associated with AAD is coupled with an increase in detrimental pathogens like *Clostridium difficile*. This pathogen causes a more severe form of AAD termed CDAD (Issa and Moucari 2014). It can produce a toxin that causes mild diarrhoea, excessive inflammation and can lead to death (Hickson 2011). *S. boulardii* can degrade the toxin produced by *C. difficile* while increasing levels of secretory IgA (Castagliuolo et al 1996, Qamar et al 2001).

1.9.3 Probiotics and necrotizing enterocolitis (NE)

Intestinal microbial imbalance in infants can lead to necrotizing enterocolitis (NE) (Silverman et al 2017). This disease is characterized by inflammation of parts of the intestinal region (Thompson and Bizarro 2008). In a study done by Lin et al (2008) the effect of *B. bifidum* and *L. acidophilus* on preventing NE in pre-term low weight infants was evaluated. They showed that the *Lactobacillus* and *Bifidobacterium* strains were highly effective in preventing necrotizing enterocolitis (Bells stage≥2) (Lin et al 2008).

1.9.4 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a range of gastrointestinal disorders characterized by diarrhoea and inflammation (Anbazhagan et al 2018). The two forms of the disease are Chrons' disease and ulcerative colitis (Floch 2011). IBD is characterized by a rise in *Bacteroides* and coliforms accompanied by lower numbers of LAB and *Bifidobacterium*

hence the ability of probiotics to increase intestinal diversity can reverse the effects of this condition (Bloom et al 2011). VSL3, a probiotic mixture containing *Lactobacillus, Bifidobacterium* and *Streptococcus* has been shown to maintain remission and prevent IBD (Veerappan 2012). Low butyrate production is also linked to IBD of which the ability of probiotic strains to produce butyrate can be handy in this respect (van Zanten et al 2012).

1.10 *L. monocytogenes* and probiotics

A lot of studies focusing on the antagonistic mechanisms of various probiotics strains on *L. monocytogenes* have been conducted. Preventing adhesion and production of bactericidal or bacteriostatic substances are the two main areas of focus with respect to anti-listerial activity. A few of these will be highlighted below.

1.10.1 Antimicrobial substances

Bacteriocin production by probiotics is well known. A wide range of bacteriocins from multiple genera are known to antagonize *L. monocytogenes. L. lactis*, when used as a starter culture in cheese production, produces lacticin 3147 which inhibits growth of *L. monocytogenes* (McAuliffe et al 1999). *E. faecium* K82 also exhibits the same effect in milk against *L. monocytogenes* owing to the enterocins A, B and P (Vandera 2016). *L. sakei* produces sakacin P which inhibits the proliferation of *L. monocytogenes* in cold salmon (Katla et al 2001). There are many other studies which implicate *B. bifidum, Carnobacterium* and *Leuconostoc* species in production of bacteriocins against *L. monocytogenes* (Yildrim et al 1999, Rickard et al 2003, Pilchova et al 2016). As mentioned above, probiotics strains also produce substances such as lactic acid and other volatile acids, carbon dioxide, hydrogen peroxide and diacetyl which antagonize *L. monocytogenes* in different ways (Kermanshahi and Qamsari 2015).

1.10.2 Inhibiting adhesion

To determine the effects of probiotic strains in-vitro on adherence of pathogens to intestinal cells, cell lines acquired from the intestinal region are used. The most common intestinal epithelial cell lines are the HT-29 and the Caco-2 cell lines. They are both human colon adenocarcinomas which exhibit various features of intestinal cells (Wang et al 2008,

Martinez- Marqueda et al 2015). The HT 29 cell line was used to test the capability of five probiotic strains in inhibiting adhesion of *L. monocytogenes* (Garriga et al 2014). The strains *L. rhamnosus CTC1679*, *L. rhamnosus GG, E. faecium* and *L. sakei* were used. The researchers concluded that all the probiotic strains could inhibit adhesion but the degree to which they inhibited *L. monocytogenes* adhesion and the mechanisms used were strain specific (Garriga et al 2014). Similar studies using CaCo2 or Jeg 3, the trophoblastic cell line, have reached the same conclusion (Bonazzi et al 2009, Koo et al 2012, Mathipa et al 2019).

1.10.3 Mouse models

Mouse models have also been used to understand the *in vivo* effects of probiotic strains against *L. monocytogenes*. Due to the genetic and physiological similarity of mice and also the genetic diversity within them, they have become invaluable to the biological and health sciences (Justice et al 2011, Perlman 2016). A study was done to determine the ability of *L. casei* to significantly reduce *L. monocytogenes* in germ free mice (de Waard et al 2003). The results showed a lower CFU in the organs of mice fed with *L. casei* than in the control group. The levels of alanine aminotransferase in the liver serum were also lower in the test group. The authors concluded that *L. casei* was effective in reducing *L. monocytogenes* infection and alleviating symptoms in mice (de Waard et al 2003). Similar studies have reached the same conclusion (Bambirra et al 2007, Vieira et al 2008, Archambaud et al 2012). All the above studies show a push towards bio control, a more natural approach to combating disease which has become popular amongst both the public and scientific communities.

1.11 Significance of study

There are a few *L. monocytogenes* prevalence studies that have been done in South Africa. Most studies have been done abroad. This makes the picture less clearer in South Africa with respect to *L. monocytogenes* and listeriosis hence limiting the effectiveness of any combat strategies against the pathogen.

The situation regarding the number of immune-compromised individuals in South Africa due to diseases such as AIDS and cancer is dire. *L. monocytogenes* infections are fatal in

such an environment hence more studies that attempt to determine the proportion of the population exposed to it especially in locations and in foods most associated with *L. monocytogenes* are required.

The constant search and manipulation of probiotics to find the most effective measure against food borne illness such as *L. monocytogenes* is still ensuing. Use of antibiotics is becoming ever more compromised and consumers are in demand of more natural methods to treat their ailments. Hence studies that pursue more natural remedies to pathogens with less adverse health effects should be a priority. The more studies are done, the higher the likelihood of finding more effective measures of dealing with these ailments and the root cause, pathogens.

1.12 References

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Chapter 2

Comparative analysis of the prevalence of *Listeria monocytogenes*, *Campylobacter spp.* and *Salmonella spp.* in retail stores and street vendor stalls in Pretoria

2.1 Abstract

Listeria monocytogenes is a food borne pathogen that causes gastrointestinal complications, sepsis and can lead to death in immune-compromised individuals. Understanding the distribution of this pathogen within the formal and informal retail sector will assist relevant authorities in assessing the public's exposure to this pathogen and how to circumvent it. The current study investigates the prevalence of *L. monocytogenes* in food samples acquired from retail stores and street vendors around Pretoria. Its prevalence in the food samples was compared to that of *Salmonella* and *Campylobacter* spp. All pathogens were enriched in peptone water before subculturing onto their respective specific enrichment and then selective media. All presumptive colonies were confirmed by Gram staining, colony morphology, 16SrRNA gene sequencing and BLAST analysis. Amplification of the *inlA* gene was used to confirm the identity of any presumptive *L. monocytogenes* colonies. Out of 167 samples of vegetables, meat, ready-to eat (RTE) meats and fruit samples, 1.19 % tested positive for *L. monocytogenes*, 0.58% for *Salmonella* and 0% for *Campylobacter*. Avocado, cucumber and tripe meat tested positive for *Listeria* spp. while only avocado and cucumber tested positive for *L. monocytogenes.* It is worth mentioning that all *L. monocytogenes* were isolated from foods obtained from street vendor stalls and not retail stores. The poor hygiene practises of vendors probably contributed to this result. The prevalence of *L. monocytogenes* was higher than *Campylobacter* and *Salmonella* in all tested foods; however, it was prevalent in food from street vendors.

2.2 Introduction

Listeria monocytogenes is an intracellular foodborne pathogen with a host range inclusive of both human and animal species (Jemmi and Stephan 2006, Thakur et al 2018). It is ubiquitous and distributed across multiple diverse environments due to its expansive growth capabilities (Walker 1987, Farber and Peterkin 1991). The ability to tolerate cold temperatures, acidic pH and low water activity plays a role in its ubiquity and has led to its isolation from fruits, vegetables and meat from different animal and bird species (Jemmi and Guyer 1991, Sizmur and Walker 1988, Shen and Higgins 2006). Most infections result from eating the food raw in the form of ready-to-eat (RTE) salads, RTE meat and fruits (Tasara and Stephan 2006). The recent listeriosis epidemic in South Africa was attributed to consumption of ready to eat polony meat (Olanya et al 2019).

Ingestion of food containing *L. monocytogenes* can result in an illness known as listeriosis (Mclauchlin et al 2004). In healthy individuals, it is limited to febrile listeriosis characterized by gastrointestinal disturbances and flu-like symptoms (Ooi and Lorber 2005). In immune-compromised individuals however, it can manifest as sepsis, liver infections, meningitis and death (Ferreira et al 2014). In pregnant women, it causes complications that give rise to miscarriages, stillbirth and infant listeriosis (Janakiraman 2008). The current case fatality rate is at 16.25%, which went up to 27% during the listeriosis outbreak that occurred in South Africa between 2017 and 2018 (WHO 2020). It is hence of paramount importance to understand the nature and distribution of this pathogen as it might provide us with tools to limit its negative impact to human health.

In this study, the prevalence of two other foodborne pathogens, *Salmonella* and *Campylobacter* were investigated for comparative purposes. The genus *Salmonella* is composed of more than 2200 serotypes contained within two species, *S. enterica* and *S. bongori* (Akiba et al 2011, Fookes et al 2011). The *Campylobacter* genus also comprises of species such as *Campylobacter jejuni*, which are capable of causing a variety of complications to human health (Hughes and Cornblath 2005, CDC 2015). *Campylobacter* and *Salmonella* spp. are common foodborne pathogens. Their presence in food, especially in high numbers, along with *L. monocytogenes* would indicate a breakdown in parts the regulatory systems used to monitor foodborne pathogens. Their absence, especially in a

listeriosis outbreak, would point to faults within systems meant to regulate the proliferation and spread of *L. monocytogenes* only. The burden foodborne pathogens place on the health and economic industries is intolerable. The cost of treatment, a weakened, reduced labour force and stagnant investment can negatively impact a country, especially a developing one (Havelaar et al 2010, Hussain and Dawson 2013). In addition, food availability and security are highly compromised through contamination by food borne pathogens (Akeda 2015). In part the factors mentioned above relay the reasons why there is concern by the relevant industries.

Several studies have focused on the prevalence of *L. monocytogenes* in various foods from different regions in South Africa. Van Nierop et al (2005) investigated 99 frozen and fresh chicken carcases from various retailers in the North West province. A 19.2% prevalence rate of *L. monocytogenes* was reported. In Alice, a town from the Eastern Cape Province, Nyenje et al (2012) found *Listeria spp.* in 22% of food samples tested though presence of *L. monocytogenes* was not verified in any of the samples. The food samples were inclusive of vegetables, rice, pies, beef and chicken stews. Christison et al (2008) investigated 35 samples of ready to eat (RTE) meat from 4 retail delicatessens in Johannesburg and found 4% of the samples tested. To the best of our knowledge, no such study has been done in Pretoria. This provides a unique opportunity to assess the distribution of this pathogen in and around the Pretoria area. Taking all this into consideration, the current study aims to investigate the prevalence of *L. monocytogenes, Salmonella* spp. *and Campylobacter* spp. in food samples acquired from retail shops and street vendor stalls around Pretoria.

2.3 Materials and Methods

2.3.1 Collection of samples

Different food items grouped as meat, ready-to-eat cold meat, cheese, vegetables and fruits (Table 2.1) were bought from three major food grocery stores around Pretoria East and vendor stalls in the Pretoria CBD. The food products collected were picked as aseptically as possible, packaged into sterile bags and stored at temperatures equal to or

below 4°C. A total of 167 food samples were collected from all locations based on availability.

Table 2.1: Food items acquired from retail stores and street vendors around Pretoria.

Food item	**# of samples**	**Food item**	**# of samples**
Meat		**Fruits and Vegetables**	
Beef	9	Avocado	12
Chicken	9	Apples	9
Food item	**# of samples**	**Food item**	**# of samples**
Meat		**Fruits and Vegetables**	
Pork	9	Cabbage	14
Tripe	3	Cucumber	12
Ready-to eat cold meat		Green pepper	9
Chicken polony	9	Lettuce	9
French polony	9	Nectarines	3
Ham	9	Onions	9
Salami	9	Ready-made coleslaw	3
Cheese	12	Tomatoes	9

2.3.2 Microbiological analysis

2.3.2.1 Isolation of *Listeria*

Enrichment culture was done by mixing twenty five grams of the food product with 220ml of buffered peptone water in a stomacher bag and homogenized for 30 seconds in a

Stomacher Lab Blender 80 (Tekmar). The homogenate was incubated aerobically at 37°C for 24 hours. Subsequent to incubation, 100µl of the enrichment culture was used to inoculate 10 ml of *Listeria* enrichment broth (Difco) for selective enrichment of *Listeria*. The broth was incubated aerobically at 37°C for 48 hours. Thereafter, a 100µl volume of the broth was spread-plated onto MOX agar plates. The plates were incubated aerobically at 37°C for 48 hours. Black colonies surrounded by dark patches were identified as presumptive *Listeria* isolates. The isolates were stored at 4°C for further analysis.

2.3.2.2 Isolation of *Salmonella*

Enrichment was done as described for *Listeria.* Subsequent to incubation, a 100µl volume of the enrichment culture was inoculated into 10ml Rappaport Vassiliadis (RV) broth (Merck) and incubated aerobically at 37°C for 48 hours. Thereafter, a 100µl volume of broth culture was spread-plated onto Xylose Deoxycholate (XLD) agar (Merck) plates. The plates were incubated aerobically at 37°C for 48 hours. Pink colonies with a dark centre were identified as presumptive *Salmonella spp.* and were stored at 4°C for further analysis.

2.3.2.3 Isolation of *Campylobacter*

Enrichment was done as described for *Listeria*. The homogenate was incubated anaerobically in anaerobic jars with Anaerocult A gaspaks at 41°C for 24 hours. Subsequent to incubation, a 100µl volume of the homogenate was used to inoculate 10ml of Bolton broth (Merck). The broth culture was incubated anaerobically at 41°C for 48 hours. Thereafter, a 100µl volume of the broth culture was spread- plated onto Charcoal-Cefoperazone-Deoxycholate agar (Merck). The plates were incubated anaerobically at 41°C for 48 hours. Greyish colonies with a metallic green hue were identified as presumptive *Campylobacter spp.* The culture plates were stored at 4°C for further analysis.

2.3.2.4 Gram staining

All presumptive *Listeria, Salmonella* and *Campylobacter* isolates were prepared for Gram staining using standard procedures. The specimens were viewed under oil immersion lens of a light microscope and Gram status was assigned.

2.3.3 DNA extraction

Genomic DNA extraction from all presumptive *Listeria*, *Salmonella*, *Campylobacter* isolates was conducted using Prepman™ Ultra (Applied Biosystems) according to the manufacturer's protocol. Briefly, a single bacterial colony was suspended in 100µl Prepman™ Ultra reagent and incubated for 10 min at 100°C on a heating block. The suspension was then centrifuged at 14 000 x g for 30 min. The supernatant was transferred into a new Eppendorf tube. The concentration of DNA was evaluated on a Nanodrop at an absorbance ratio of 260/280 nm. A ratio of ~ 1.8 was indicative of presence of pure DNA. The quality and presence of DNA was also evaluated on a 1% agarose gel electrophoresis. The gel was stained with ethidium bromide in TAE buffer (Tris base, Acetic acid and EDTA) to a final concentration of 0.5µg/ml and then visualized using a gel Doc EZ imager (Bio-Rad). The DNA solution was stored at 4°C.

2.3.4 Amplification of 16S rRNA gene using PCR

The 16S rRNA gene of the bacterial isolates was amplified using the 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3') primers (Lane et al., 1991). The final concentration of each reagent in the final reaction was as follows: 0.3 µM of each respective primer, 2mM $MgSO_4$ (Qiagen), 2.5 mM of each dNTP, 0.06 U/µl Taq DNA polymerase and 5X reaction buffer (NEB) all added to a final volume of 20 µl. The PCR cycling conditions included an initial denaturation step at 94 °C for 3 minutes, followed by 30 cycles of denaturation at 94 °C for 1 minute, primer annealing at 55 °C for 1 minute, elongation at 72 °C for 30 seconds and a final elongation step at 72 °C for 5 minutes. The PCR products were stained with ethidium bromide and then visualized using gel electrophoresis as previously described. The gel was visualized on a gel Doc EZ imager (Bio-Rad). The PCR products were stored at 4°C.

2.3.5 Cleaning of PCR products

PCR products were purified using the Exo-Sap method according to the manufacturer's protocol (New England Biolabs). Briefly, 2 µl of Fast-AP (2U/µl) and 0.5 µl of Exo1 (1U/µl) was added to tubes containing amplicons. The tubes were incubated at 37°C for 15

minutes in a thermo cycler to activate the enzymes. Subsequently, the tubes were incubated at 85°C for 15 minutes to deactivate the enzymes.

2.3.6 Sequencing of amplicons

To confirm the genera of all presumptive *Listeria*, *Campylobacter* and *Salmonella* isolates, cycle sequencing was done. The amplicons were sequenced in both forward and reverse directions using the 27F/519R primer pair and labelled with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The components of the sequencing reaction mixture are listed in Table 2.2. Sequencing products were then analysed on an ABI 3100 Automated Capillary DNA Sequencer (Applied Biosystems, USA) in the DNA Sanger sequencing facility at the University of Pretoria. The sequences acquired were compared to sequences in the National Centre for Biotechnology Information (NCBI) database using BLAST analysis.

Table 2.2: Components of the sequencing reaction

Reagents	**Volume (µl)**
Nuclease free water	2.5
Sequencing buffer	2
Primer	1
Big dye	0.5
DNA sample	4

2.3.7 Amplification of *inlA* using PCR

In order to confirm the presumptive *Listeria* isolates as *L. monocytogenes*, the internalin A (*inlA)* gene of these isolates was amplified using the primers inlAF (5'-GTGAGAAGAAAACGATATGTATG-3') and inlAR (5'-CTATTTACTAGCACGTGCTTT-3') (Mathipa et al., 2019). The final concentration of each reagent in the final reaction was as

follows: 0.3 µM of each respective primer, 2mM $MgSO_4$, 2.5 mM of each dNTP, 0.06 U/µl Taq DNA polymerase and 5X reaction buffer (NEB) all added to a final volume of 20 µl. The PCR cycling conditions for the *inlA* gene included an initial denaturation step at 94 °C for 3 minutes followed by 34 cycles of denaturation at 94 °C for 1 minute, primer annealing at 45 °C for 45 seconds, elongation at 72 °C for 2 minutes 30 seconds and a final elongation step at 72 °C for 8 minutes. The presence of DNA was evaluated using gel electrophoresis. The gel was visualized on a gel Doc EZ imager (Bio-Rad).

2.3.8 Antibiotic susceptibility tests

The antibiotic susceptibility of positively identified *L. monocytogenes* and *Salmonella* isolates to the following 9 antibiotics: oxacillin (10 and 25 µg/ml), erythromycin (30 µg/ml), penicillin (10 µg/ml), gentamicin (10µg/ml), neomycin (10 µg/ml), streptomycin (10 and 25 µg/ml), chloramphenicol (30 and 50 µg/ml) and tetracycline (30 µg/ml) was assessed.

L. monocytogenes

The *L. monocytogenes* isolates were grown in brain heart infusion broth till they reached an O.D of 0.2-0.3. Then 100 µl of the broth culture was plated on brain heart infusion agar using the spread plate method. A Mast ring containing different antibiotics was then placed on the centre of the plate before incubation for 48 hours at 37°C. Susceptibility was indicated by presence of clearance zones around the discs. The radius of the clearance zones was measured in mm. Presence of a clearance zone equal to or larger than 6mm was considered positive *L. monocytogenes* inhibition (Fijan 2016).

Salmonella

The *Salmonella* isolates were grown in RV broth till they reached an O.D of 0.2-0.3. Then 100 µl of the broth culture was plated on XLD agar using the spread plate method. A Mast ring containing different antibiotics was then placed on the centre of the plate before incubation for 48 hours at 37°C. Susceptibility of the *Salmonella* isolates to any of the antibiotics was assessed as done for the *L. monocytogenes* isolates.

2.4 Results

2.4.1 Presumptive isolates using plating

A total of 72 presumptive *Listeria, Salmonella* and *Campylobacter* isolates were obtained from the 167 food samples. The selection of the presumptive isolates was based on colony morphological characteristics exhibited by each of the isolates on respective selective media and on their Gram staining properties as observed under a light microscope. Specifically; 37, 17 and 18 isolates were presumed to belong to the *Listeria, Salmonella* and *Campylobacter* genera, respectively.

2.4.2 DNA extraction

Genomic DNA was extracted from all the positive presumptive isolates. The representative extractions for the three bacterial species are shown in Figure 2.1. The concentrations of DNA as determined using Nanodrop ranged from 50 ng/µl to 300 ng/µl. DNA that was extracted was of good quality as it had a 280/260 nm ratio that ranged between 1.7 – 1.9.

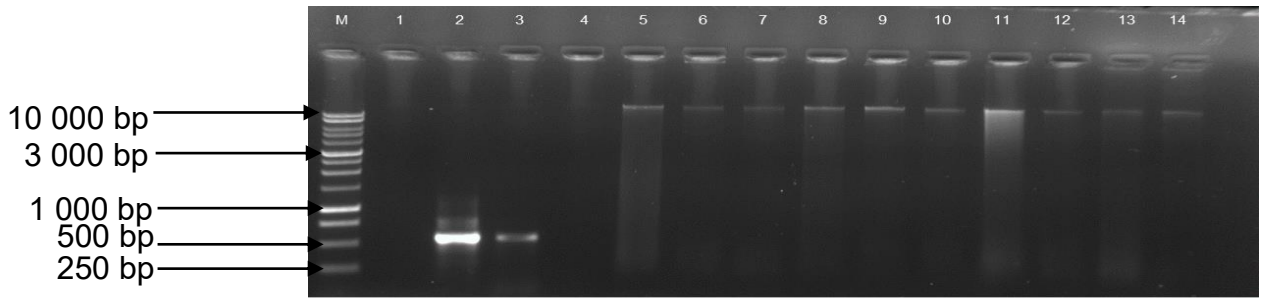

Figure 2.1: Genomic DNA (lanes 5-12) and 16S rRNA amplicons (lanes 2-3) from presumptive isolates. A 1kb DNA ladder was used. Lane M: DNA Ladder. Lanes 1 and 4: Empty. Lane 2: 16S rRNA band from presumptive Campylobacter isolate. Lane 3: Negative control. The band present is due to a contamination of the negative control. Lanes 5-6: *Campylobacter* ham isolate. Lanes 7-8: *Salmonella* salami isolate. Lane 9: *Listeria* French polony isolate. Lane 10: *Salmonella* French polony isolate. Lane 11: *Campylobacter* lettuce isolate. Lane 12: *Salmonella* lettuce isolate. Lane 13: *Listeria* cucumber isolate. Lane 14: *Salmonella* cucumber isolate.

2.4.3 16SrRNA gene amplification

2.4.3.1 *Listeria*

Amplification of the 16S rRNA gene in all presumptive *Listeria*, *Salmonella* and *Campylobacter* isolates was performed. Amplicon bands from a representative 16S rRNA PCR of presumptive *Listeria* isolates is shown in Figure 2.2. The expected gene size was 500bp.

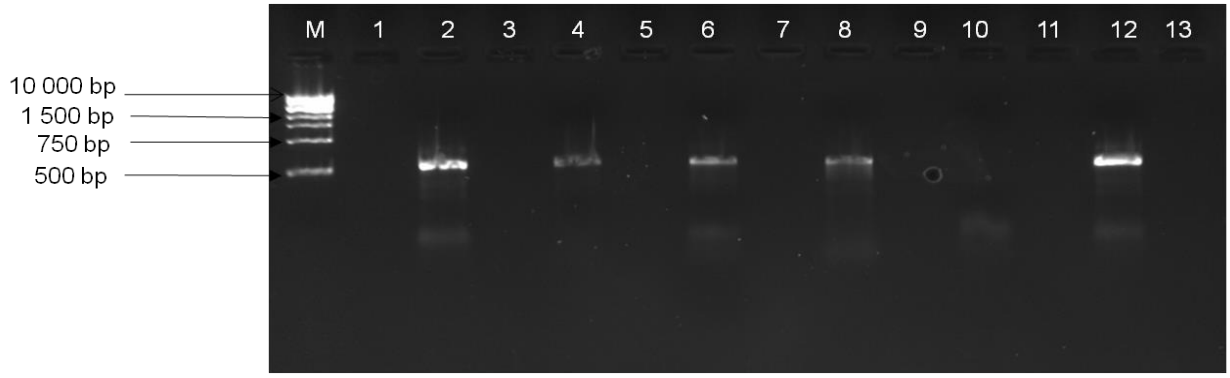

Figure 2.2: Amplicons of the 16S rRNA gene from presumptive *Listeria* isolates. A 1kb DNA ladder was used. Lane M: DNA ladder. Lane 2: Tripe meat isolate. Lane 4: Cucumber isolate. Lane 6: Avocado isolate. Lane 8: Tripe meat isolate. Lane 10: Negative. Lane 12: Tripe meat isolate. Lanes 1, 3, 5, 7, 9, 11, 13 and 14: No PCR products were loaded in respective wells.

2.4.3.2 *Salmonella*

Figure 2.3 shows the 16S rRNA amplicons from representative presumptive *Salmonella* isolates. The expected gene size was 500bp. The size of the bands on the DNA marker is indicated on the image.

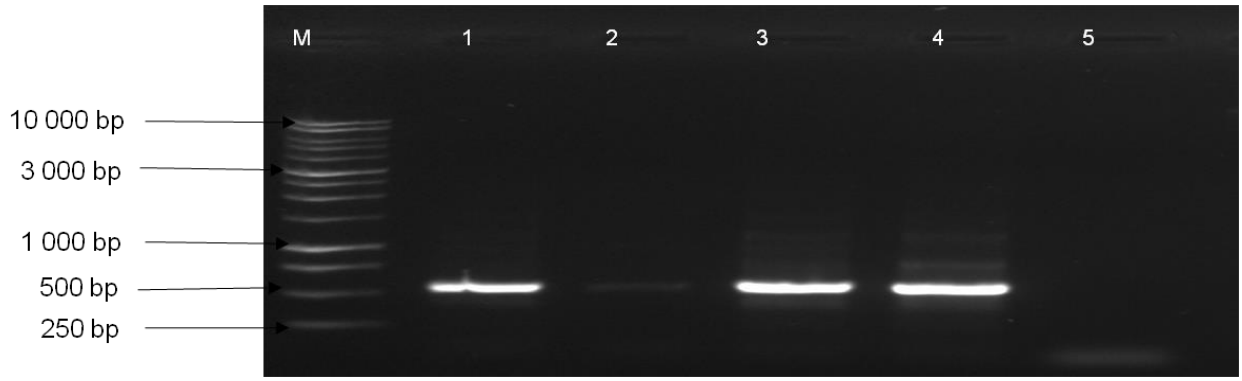

Figure 2.3: The 16S rRNA gene amplicons from presumptive *Salmonella* isolates. Ham (Lanes 1-4). A 1kb DNA ladder was used. Lane M: DNA ladder. Lane 1-4: Ham isolate. Lane 5: Negative control.

2.4.3.3 *Campylobacter*

Figure 2.4 shows the 16S rRNA amplicons from representative presumptive *Campylobacter* isolates. The expected gene size was 500bp. The size of the bands on the DNA marker is indicated on the image.

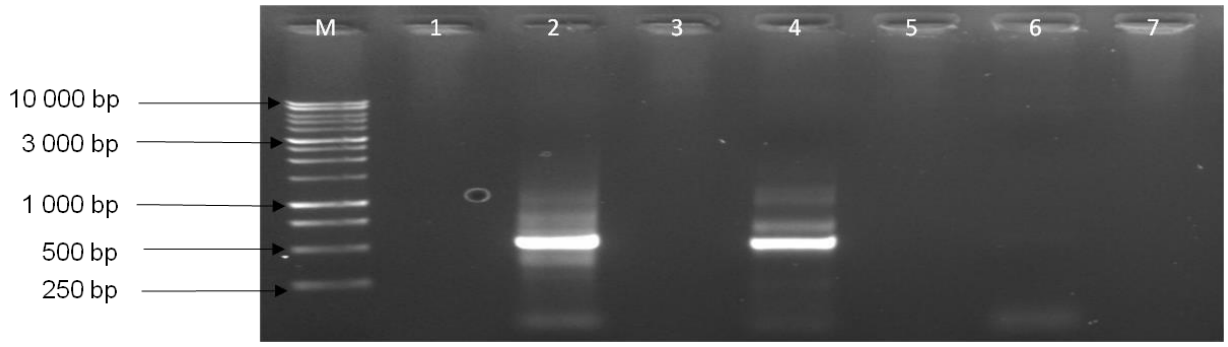

Figure 2.4: The 16S rRNA gene amplicons from presumptive *Campylobacter* isolates. A 1kb DNA ladder was used. Lane M: 1kb DNA ladder. Lane 2: Cabbage isolate. Lane 4: Lettuce isolate. Lane 6: Negative control. Lane 8: Tomato isolate. Lanes 1, 3, 5 and 7: Empty (No sample loaded).

2.4.4 Sequencing and BLAST analysis

Post amplification, the 16S rRNA gene amplicons from all presumptive isolates were sequenced and the sequences acquired analysed using BLAST. This was done to confirm the identity of each of the presumptive isolates as obtained using the culture dependent method. Of the 37, 17 and 18 presumptive *Listeria, Salmonella* and *Campylobacter* isolates respectively, only 4 were confirmed as *Listeria spp.* (10.8%) and 1 as *Salmonella* (5.9%). None were confirmed as *Campylobacter.* Two of the four *Listeria* isolates were isolated from avocado and cucumber while the other two were isolated from tripe meat. The single *Salmonella* isolate was from ham.

The other 67 remaining isolates represented multiple diverse genera. The genera isolated from each food group and the number of isolates representing that genus is shown in Figures 2.5 A - D. A total of 18 different bacterial genera were found in the 167 food samples tested. Each food group contained a diverse range of bacterial genera. The *Enterococcus* genus was the most represented in all food groups. The *Enterobacter* genus was also isolated from all the food groups tested. The *Staphylococcus*, *Proteus*, *Bacillus* and *Escherichia* genera were present in two of the food groups while the rest of the genera were represented only once in the four food groups tested.

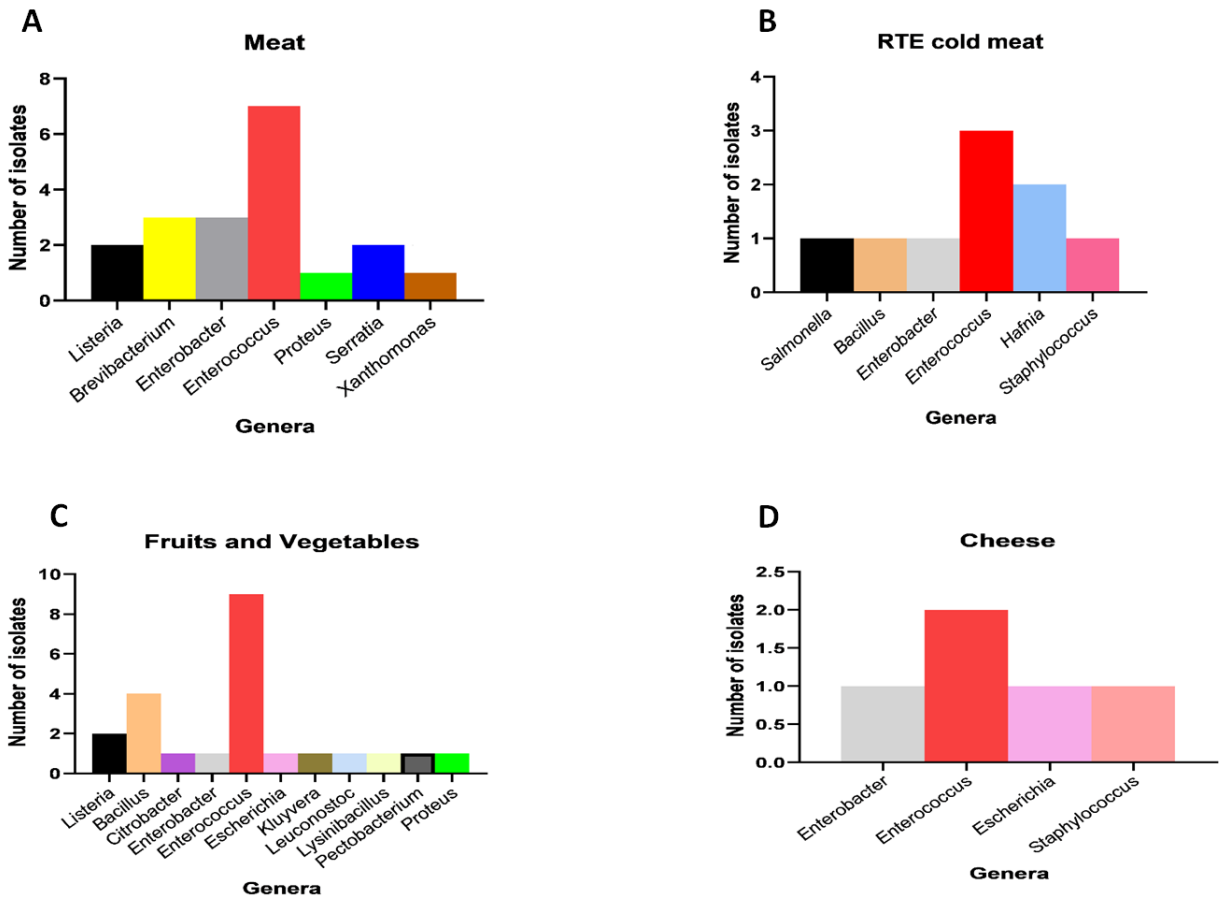

Figure 2.5: The number of isolates present in each genera isolated from the different food groups: A (Meat), B (RTE cold meat), C (Fruits and Vegetables) and D (Cheese) (Table 2.1).

2.4.5 Amplification of the *inlA* gene from suspected *L. monocytogenes* isolates

Amplification of the *inlA* gene in all *Listeria* isolates was performed. Amplicon bands from an *inlA* PCR of *Listeria* isolates is shown in Figure 2.6. Only 2 of the 4 *Listeria* isolates tested positive for presence of *inlA* bands. The isolates were acquired from avocado and cucumber. The expected gene size was 2.4kb. The size of the bands on the DNA marker is indicated on the image.

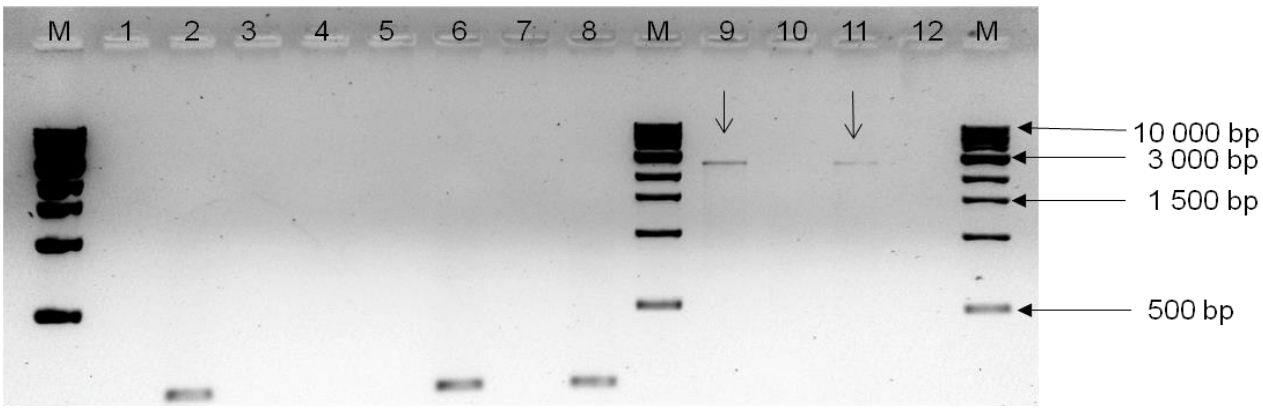

Figure 2.6: Amplification of *inlA* from suspected *L. monocytogenes* isolates. A 1kb DNA ladder was used. Lane M: DNA ladder. Lane 2: Negative control (primer dimers visible). Lane 6: tripe meat isolate. Lane 8: tripe meat isolate. Lane 9: isolate from avocado. Lane 11: isolate from cucumber. The bands in lanes 9 and 11 are indicated by a black vertical arrow. Lanes 1, 3, 4, 5, 7, 10 and 12: No PCR products were loaded in respective wells.

2.4.6 Prevalence of *L. monocytogenes*

Only 4 out of the 37 presumptive *Listeria* isolates were confirmed as *Listeria.* Of those 4, only 2 were confirmed as *L. monocytogenes* based on the presence of the *inlA* gene. Only 2 out of the 167 food samples were positive for *L. monocytogenes* (1.2%). The isolates from avocado and cucumber are referred to as *L. monocytogenes* avoS and *L. monocytogenes* cucS, respectively.

2.4.7 Antibiotic susceptibility

L. monocytogenes isolates

Both isolates from avocado and cucumber were sensitive to erythromycin, tetracycline, chloramphenicol and novobiocin, however, they showed resistance to oxacillin. The avocado isolate was resistant to fusidic acid and penicillin but sensitive to streptomycin while the cucumber isolate was resistant to streptomycin but sensitive to fusidic acid and penicillin.

Salmonella isolate

The *Salmonella* isolate was sensitive to erythromycin, chloramphenicol and fusidic acid but it was resistant to tetracycline, streptomycin, penicillin G, novobiocin and oxacillin.

2.5 Discussion

The 16S rRNA gene is highly conserved and is present in nearly all bacterial species (Janda and Abbot 2007). Hence it is highly useful in resolving bacterial identity in bioinformatics studies. In this study, 16S rRNA gene analysis was used to confirm the genus each of the presumed *Listeria* and *Salmonella* isolates. Amplification of species specific genes can then further corroborate the identity of isolate as belonging to a particular species. The *inlA* gene is specific to and is highly conserved within the *L. monocytogenes* species group (Poyart et al 1996). Amplification of this gene was used to confirm the identity of two *Listeria spp.* isolates as *L. monocytogenes.*

The use of phenotype based techniques and genotypic analysis in conjunction to confirm the identity of an isolate enhances the discriminatory power of either method (Tang et al 1998, Donelli 2013). A total number of 37 isolates were presumed to belong to *Listeria* while 18 were presumed to belong to *Salmonella* using culture dependent methods. After genotype based analysis of the same isolates, only 4 *Listeria* isolates and 1 *Salmonella* isolate were positively confirmed. The discrepancy between these results show how genotypic techniques can improve the resolving power of culture based phenotypic identification (Tang et al 1998). Culture based identification is based on phenotypic traits which are subject to change. This is because the genes that confer these phenotypic traits undergo processes such as mutations and horizontal gene transfer. These processes change the genetic makeup of an organism and subsequently the phenotypic characteristics which might lead to false positives and negatives (Dougherty et al 2014).

The prevalence of *L. monocytogenes* (1.2%) in this study was low (Table 2.6). This corroborates other previous studies by Little et al., 2007 who reported a 3.8% prevalence rate when testing 2686 samples of mixed salads. Van Pelt et al (2018) reported values as low as 0% while Ieren et al (2013) in Nigeria, reported a 3.9% prevalence rate out of 355 vegetable samples. The low prevalence in this study owes to the fact that all foods acquired

from retail shops, from which most samples were attained, tested negative for *L. monocytogenes* contamination. The retail store is the last line of defence against food pathogens before food is released to the public (Reimers 1994). It is hence of utmost importance that a zero tolerance to pathogens approach is taken within the retail environment. Absence of *L. monocytogenes* is a good indication that Hazard Analysis and Critical Control Point principles (HACCP) and Good Manufacturing Practices (GMP) are strictly adhered to by relevant parties (Little et al 2013). Major food retail shops benefit from up to date HACCP protocols since they inspire investor and consumer confidence while preventing potential economic losses.

The samples that tested positive for *L. monocytogenes* were avocado and cucumber samples acquired from street vendors. Microbial contamination due to non-hygienic practices by street vendors is possible (Bryan et al 1997, Feglo and Sakyi 2012). Fruit produce is exposed to flies which can carry bacteria from one stall to the next (Gupta et al 2014). Clustering of different fruits together and washing with the same water source can also be drivers of contamination (Ieren et al 2013). Consumers touching the fruits and resource sharing between vendors might also spread pathogens. Correlation between bacterial contamination and low hygiene has been investigated in several studies (Annor and Baiden 2011, Rosvoll et al 2015, Knight-Jones et al 2016). Hazards related to food contamination are further reinforced by the general lack of public knowledge on food safety hazards posed by vendor activity (Rane 2011). There is also a lack of or compromised intervention from responsible government structures due to absence of infrastructure and programs that can combat their temporary and transient nature (Adedeji and Ademuluyi 2009, Ajayeoba 2015). All the above mentioned factors play a vital role in the contamination of fruits and vegetables and exposure of consumers to foodborne pathogens (Gosh et al 2007).

Only a single ham sample (0.58%) was positive for *Salmonella*. This result is in accordance with studies by Sant'Ana et al (2011), Nyenje et al (2012) and Olobatoke and Mulugete (2015) who all found a low occurrence of *Salmonella* species in the food samples investigated. *Campylobacter* was not detected in any of the samples that were tested in this study. Our results were in agreement with previous studies by Kumar et al (2001),

Verhoeff-Brakennes et al (2011) and Reperant et al (2016) who all reported the low prevalence of *Campylobacter* isolates. *Salmonella* spp. and *Campylobacter* spp. are clinically relevant pathogens which cause a burden to the health industry on an annual basis (Scallan et al 2011, Devleesschauwer et al 2018). Their absence is a good indication of stringent and effective HACCP principles and GMPs in the retail stores investigated.

There was high bacterial genera diversity amongst the food samples investigated. The ubiquitous nature of bacteria on earth is not limited to any environment and the food production and processing environment is no exception. The presence of different genera in beef, pork and chicken meat has been reported (Ercolini et al 2011, Koo et al 2016, Pandit et al 2018, Poirier et al 2018). Dairy, vegetables and fruits have also proved to be efficient growth mediums for many bacterial species (Leff and Fierer 2013, Irlinger et al 2018). Our capability in eliminating food borne pathogens from the food supply chain is highly dependent on understanding the microorganisms that inhabit the different niches present from farm to consumer plates (Poirier et al 2018).

Enterococcus and *Enterobacter* were the most prevalent of all the bacterial genera isolated in this study. Both genera are highly abundant in the gastrointestinal tract thus unsanitary behaviour by food handlers can lead to contamination of food products (Dubin and Pamer 2017). *Enterococcus* and *Enterobacter* are responsible for a significant fraction of nosocomial infections (Giraffa 2002, Regli 2015). The contribution of these genera to the global antibiotic resistance crisis, especially within the hospital setting, is quite significant (Mehrad et al 2015, Prieto et al 2016). Members of the *Bacillus*, *Proteus* and *Staphylococcus* genera have also been implicated as food pathogens (Blackburn and McClure 2009, Tong et al 2015, Drzewiecka et al 2016). The latter genus consists of the devastating Methicilin Resistant *Staphylococcus aureus* which has become the poster strain for antibiotic resistance (Abdolmaleki et al 2019, Vetsergaard et al 2019).

There was variation in the resistance profile of *L. monocytogenes* avoS and *L. monocytogenes* cucS to various antibiotics was observed. Olaimat et al (2018) reported that resistance to oxacillin is intrinsic to all *L. monocytogenes* strains of which our results were in agreement. Penicillin and ampicillin are the antibiotics of choice with respect to treatment of listeriosis while chloramphenicol is used in the instance of penicillin allergy

(Conter et al 2009, Allerberger and Wagne 2010, Olaimat et al 2018). Both strains were sensitive to chloramphenicol. The *L. monocytogenes* avoS strain was resistant to penicillin while *L. monocytogenes* cucS was sensitive.

The inconsistency in susceptibility of target strains to antibiotics of choice can lead to compromised listeriosis treatment. Antibiotic resistance is a phenomenon spreading across various microbial pathogens (Ventola 2015). Mechanisms such as horizontal and vertical gene transfer between species and spontaneous mutations generate resistance in recipient strains (Viswanathan 2014). The rise in resistant strains has prompted pharmaceutical industries to either; improve the antibiotics already in existence or search for alternatives before most of the clinical isolates acquire resistance (Concia et al 2016, Edwards et al 2018, Singer et al 2019). Also, medical professionals need to assume responsibility and avoid over prescription and use of antibiotics (Read and Woods 2014).

2.6 Conclusion

From all the food samples that were collected from both the retail stores and the street vendors, *L. monocytogenes* was more prevalent than *Salmonella* and *Campylobacter*. The strains that identified as *L. monocytogenes* were from samples collected from the street vendors and not the retail stores. There was a significantly low to no presence of the food borne pathogens *Salmonella*, *L. monocytogenes* and *Campylobacter* from samples collected from retail shops; owing to GMP, GHP and HACCP principles that are followed by every member of the food supply chain from the farm or production site to the consumer's plate. Constant monitoring and implementation of HACCP by retail and the education of vendors on hygiene and their role in disease spread can help reduce and finally eradicate foodborne pathogens. It will be interesting, in the future, to perform a similar study to determine whether the results obtained will be sustainable, that is to check whether the GMPs are practised throughout or whether it was by chance just due to the listeriosis outbreak scare.

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Chapter 3

Evaluation of bacterial probiotics as a potential control measure against *Listeria monocytogenes*

3.1 Abstract

The use of probiotics against *Listeria monocytogenes* in the food industry is well documented. Their potential as an alternative or adjunct to antibiotics could ameliorate antibiotic resistance. The aim of this study was to evaluate the suitability of various probiotics as a control measure in the food processing environment against *L. monocytogenes* strains acquired from food samples. The probiotic species used were *Bifidobacterium animalis*, *Lactobacillus acidophilus, Lactobacillus plantarum* and *Pediococcus acidilacti.* Five *L. monocytogenes* strains were each challenged with each of the four probiotics in a spot inoculation test. Thereafter, the Cell Free Supernatants (CFS) of each of the probiotics were employed in a well diffusion assay against each of the *L. monocytogenes* strains. In addition, the antagonistic anti-listerial activity of the probiotic strains or their CFS in avocado and cucumber samples spiked with each of the *L. monocytogenes* strains was evaluated. The sensitivity or resistance of the *L. monocytogenes* strains to probiotic cocktails (*B. animalis* + *L. acidophilus* and *B. animalis* + *P. acidilacti*) within the food matrix was analyzed. *B. animalis* inhibited growth of four out of the five *L. monocytogenes* strains while *L. acidophilus* was bacteriocidal to three of the five strains. *L. plantarum* inhibited only one of the five strains while *P. acidilacti* inhibited none. Inhibition of *L. monocytogenes* strains by each of the probiotic strains or their CFSs in both food matrixes was not statistically significant, except for inhibition of *L. monocytogenes* T62 in avocado by *B. animalis*. Properties of *L. monocytogenes,* the food medium and conditions during storage used in the study could have had an impact on the inhibitory effects of the tested probiotic strains.

3.2 Introduction

The use of probiotics against *Listeria monocytogenes* has been researched in depth (Koo et al 2012, Million et al 2013, Iulietto et al 2018). *Bifidobacteria, Enterococcus, Pediococcus, Lactococcus* and *Lactobacillus* species have all shown, to some degree, bacteriostatic and listericidal activity against *L. monocytogenes in vitro* (Jacobsen et al 1999, Corr et al 2007, Koo et al 2012, Hassanzadazar et al 2014).There are multiple anti-listerial mechanisms employed by various species in these genera. Understanding the antagonistic nature of these anti-listerial species will allow more precise and effective combat of *L. monocytogenes.*

The benefits of using probiotics are not limited to anti-pathogenic activity but extend to healthy bodily function as well. They promote gastrointestinal health through growth suppression of undesirable microbes and also strengthen the epithelial barrier, which subsequently boosters the immune system (Xue et al 2011, Koo et al 2012, Galdeano et al 2019). They have also been linked to improved treatment of intestinal complications such as inflammatory bowel disease, Crohns' disease and ulcerative colitis (Jonker and Stockbrugger 2003, Bibiloni et al 2005, Fedorak 2010). Probiotics can synthesize short chain fatty acids and the essential B vitamins amongst other beneficial substances (Sheridan et al 2014). Contribution to mental health through production of compounds such as serotonin and GABA has also been reported in some studies (Forsthye and Bienenstock 2009, Hemarajata and Versalovic 2013).

Antibiotic resistance is another reasonable cause for further investigation into probiotic efficacy. Prescription of antibiotics for each and every ailment and subsequent abuse by patients has resulted in the emergence of antibiotic resistant pathogen strains (Langford and Morris 2017, Llewelyn 2017). Also, the heavy usage of antibiotics during intensive poultry and cattle farming has contributed massively to the dilemma (Phillips et al 2004, Landers et al 2012). Abuse or misuse of antibiotics can have dire consequences, from the emergence of Methicillin Resistant *Staphylococcus aureus* resistant to multiple different antibiotics to the antibiotic resistant *Clostridium difficile* (Tenover et al 2012, Tracey et al 2015). Combined this with reluctance from pharmaceutical industries to develop new

antibiotics or improve those already on the market, this catastrophe can only worsen (Singer et al 2019).

The emergence of antibiotic resistant food borne *L. monocytogenes* strains is a developing concern in the food industry (Olaimat et al 2018). Some strains show resistance to erythromycin while some can encode trimethoprim resistant dihydrofolate reductase (Charpentier and Courvalin 1999, Granier et al 2011). Resistance to ampicillin, which is the antibiotic of choice in listeriosis treatment, has also been reported (Yucel et al 2005, Arslan and Ozdemir 2008). Hence an alternative is urgently required, of which the use of antibiotics in conjunction with probiotics can reduce over usage of the former. Also, biocontrol is encouraged in a time where many different chemicals interact, under different environmental conditions, with other substances whose synergistic or potentiating capacity is unknown (Mastroni 2008).

Probiotics combat pathogenic species in a variety of ways. Competitive exclusion, employed by probiotics, is a common occurrence between bacterial species occupying the same habitat or niche (Hibbing et al 2010). This competition can be classified as a scramble or a contest. In a scramble competition, the competitors fight for nutrients present in the surrounding environment (Nicholson 1954). In a contest, more direct antagonism against specific species is observed (Nicholson 1954). Alongside competition for nutrients, strains produce bacteriocins, lactic acid and hydrogen peroxide amongst many other substances in an attempt to negatively impact the growth of target strains (Reeves 1965, Raccach et al 1989, Mishra and Lambert 1996). The aim of this part of the study was hence to evaluate various probiotics against specifically *L. monocytogenes* isolates from food samples in an effort to determine their suitability for control of *L. monocytogenes* in the food-processing environment.

3.3 Materials and Methods

3.3.1 Bacterial cultures

Four different probiotic strains: *Bifidobacterium animalis susp. lactis* BB-12, *Lactobacillus acidophilus* L10, *Lactobacillus plantarum* 7.1E and *Pediococcus acidilacti* were used in antagonistic tests against five *L. monocytogenes* strains. *B. animalis* and *L. acidophilus* freeze-dried cultures were acquired from the ChR-Hansen culture collection and DSM nutrition, respectively. The *L. plantarum* and *P. acidilacti* strains were both obtained from glycerol stock cultures in the Probiotics Research Group laboratory, University of Pretoria. *L. acidophilus, L. plantarum* and *P. acidilacti* were subcultured thrice in De Man, Rogosa and Sharpe (MRS) broth (Oxoid) while *B. animalis* was subcultured in MRS broth supplemented with 0.05% v/v hydrochloride cysteine (MRS-cys-HCL). The broth cultures were subsequently spread plated onto MRS agar (Oxoid) plates and incubated anaerobically in anaerobic jars with Anaerocult A gaspaks at 37 ˚C for 72 hours and thereafter maintained on the same medium at 4 ˚C.

A total of five *Listeria monocytogenes* strains were used in the study. Three *L. monocytogenes* strains, namely, *L. monocytogenes* T62, *L. monocytogenes* 159 and *L. monocytogenes* 243 were acquired from the Food Science Department at the University of Pretoria. These strains were used as controls.The other two strains, *L. monocytogenes* avoS and *L. monocytogenes* cucS, were isolated from the avocado and cucumber fruits, respectively (Chapter 2 of this study). *L. monocytogenes* avoS and *L. monocytogenes* cucS had already exhibited resistance to multiple antibiotics including those used in current treatment regimens. The strains were grown in brain heart infusion agar (BHI) (Oxoid) at 37 ˚C for 48 hours and then maintained on the same medium at 4 ˚C.

3.3.2 Spot inoculation test

The probiotic cultures (*L. acidophilus, L. plantarum, P. acidilacti*) were grown in MRS broth and *B. animalis* in MRS-cys-HCl until they reached an optical density at 600nm (OD_{600}) of 0.2 which equates to a concentration of approximately 10^8 cfu/ml. Each probiotic culture was serially diluted in ¼ strength Ringer's solution and each dilution was spotted on MRS

agar. The spots were allowed to develop over 24 hours through incubation anaerobically at 37 ˚C.

Listeria monocytogenes strains were grown in BHI broth at 37˚C until they reached an OD_{600} of 0.2. A 100µl volume of the broth culture from each of the strains was used to inoculate 3 ml of 0.7% (w/v) solution of soft water agar. Each of the *L. monocytogenes* cultures was poured over each of the four respective probiotics spotted on an MRS agar plate, and the plates were incubated anaerobically at 37˚C for 48 hours. A zone of inhibition with a diameter of 6mm or more was considered positive *L. monocytogenes* growth inhibition (Fijan 2016). All tests were done in triplicate in three independent trials.

3.3.3 Preparation of cell free supernatants (CFS) of probiotics

Probiotic strains were grown until they reached an OD_{600} of 0.2. The broth cultures were then centrifuged at 4 000rpm for 10 minutes at 4°C. The supernatants were filter sterilized through a 0.22µm cellulose acetate syringe filter and thereafter autoclaved to remove any planktonic cells possibly present. The CFSs were spread plated on MRS agar post autoclaving to rule out presence of viable probiotic cells. To ensure that none of the antagonistic activity was acidic pH dependent, aliquots of autoclaved filter sterilized CFS were neutralized to a pH of 7 using 1M NaOH.

To check for heat stability of potential antagonistic substances, aliquots of CFSs were boiled at 100˚C for 2 hours. To eliminate anti-listerial activity from proteinaceous molecules, portions of the CFS were treated with Proteinase K (Qiagen) at 37˚C for 2 hours. The enzyme was subsequently inactivated by incubating the CFS at 100˚C for 20 minutes. The supernatants were stored at 4˚C before use.

3.3.4 Well diffusion assay

The five *L. monocytogenes* strains (OD600=0.2) were each spread plated onto BHI agar plates for preparation of the bacterial lawn. Five millimeter holes were punched onto the inoculated BHI agar plates using the back of a sterile glass pipette. Each of the *L. monocytogenes* strains was treated with the CFS of each probiotic strain as follows: *L. monocytogenes* and filter sterilized CFS (fs CFS); *L. monocytogenes* and pH neutralized

fs CFS; *L. monocytogenes* and heat treated fs CFS and *L. monocytogenes* and Proteinase K treated fs CFS. A 2 fold dilution series of each of the probiotic CFS was made. Fifty microliters of each of the dilutions was pipetted into the holes. The plates were then incubated anaerobically at 37˚C for 48h hours. A zone of inhibition with a diameter of 6mm was considered positive *L. monocytogenes* growth inhibition. All tests were done in triplicate in three independent trials.

3.3.5 *L. monocytogenes* growth in avocado and cucumber

The *L. monocytogenes* strains avoS, cucS and T62 were used in the food inoculation tests described in 3.3.5 to 3.3.8. The tests were set up to determine if the probiotic strains inhibited the growth of the *L. monocytogenes* strains or were of no effect in the food matrix used. The bacterial strains were grown in BHI broth until they reached an OD_{600} of 0.2. Ten grams of each of the food samples was ground and placed in a sterile Petri dish. Two milliliter aliquots of the *L. monocytogenes* broth cultures were mixed with each of the ground food samples. Then 1g of the inoculated food sample was transferred to 9 ml of BHI broth in a stomacher bag and was homogenized for 30 seconds in a Stomacher Lab Blender 80 (Tekmar). Thereafter, to quantify the numbers of *L. monocytogenes* present in the food sample prior to incubation, a 10 fold serial dilution of the resultant suspension was conducted. The dilutions were spread plated onto BHI agar plates and then the plates were incubated at 37 ˚C for 48 hours.

To determine how *L. monocytogenes* grows in the inoculated food samples, the samples were incubated anaerobically at 4˚C for 7 days. Then 1g of the inoculated food sample was taken and prepared for enumeration of *L. monocytogenes* as was done for the initial (day 1) samples. All experiments were done in triplicate and in three independent trials. The growth of *L. monocytogenes* in the absence of probiotics was used as a control to assess the growth inhibitory capacity of each of the probiotic strains used.

3.3.6 Growth of *L. monocytogenes* in food samples in presence of probiotics

The *L. monocytogenes* and probiotic strains were grown until they reached an OD_{600} of 0.2. A mass of 10g of the food samples was ground and placed in sterile Petri dishes. Two milliliter aliquots of each of the *L. monocytogenes* and probiotic strain cultures were mixed

in a test tube and used to inoculate each of the ground food samples. The inoculated food samples were incubated anaerobically at 4˚C for 7 days. Then 1g of the samples was prepared for enumeration of *L. monocytogenes* as described in section 3.3.5. All experiments were performed in triplicate and in three independent trials.

3.3.7 Growth of *L. monocytogenes* in food samples in presence of probiotic CFS

The *L. monocytogenes* and probiotic strains were grown until they reached an OD_{600} of 0.2. Cell free supernatants of each of the probiotic strains were prepared as previously described in section 3.3.3. A volume of 2ml of each probiotic CFS was mixed with a 2ml aliquot of each *L. monocytogenes* broth culture in a test tube. Then 10g of the food sample was ground and placed in sterile Petri dishes. The mixtures were then used to inoculate each of the food samples. The inoculated food samples were incubated anaerobically at 4˚C for 7 days. After 7 days; quantification of *L. monocytogenes* was carried out as detailed in 3.3.5. All experiments were performed in triplicate and in three independent trials.

3.3.8 Growth of *L. monocytogenes* on food sample in presence of probiotic cocktails

Three of the four probiotic strains were selected for use as part of the probiotic cocktails employed against the *L. monocytogenes* strains avoS, cucS and T62. Two probiotic cocktails, *B. animalis* + *L. acidophilus* (BA-LA) and *B. animalis* + *P. acidilacti* (BA-PA), were prepared. The *L. monocytogenes* and probiotic strains were grown until they reached an OD_{600} of 0.2. A volume of 2ml of each of the probiotic culture broths were then mixed together to form a cocktail. Then 10g of the food samples was ground and placed in sterile Petri dishes. Two milliliters of each probiotic cocktail was then mixed with 2ml aliquots of *L. monocytogenes* broth culture in a test tube and used to inoculate each of the food samples. The inoculated food samples were incubated anaerobically at 4˚C for 7 days. After 7 days; quantification of *L. monocytogenes* was carried out as detailed in section 3.3.5. All experiments were performed in triplicate and in three independent trials.

3.3.9 Statistical analysis

The experiments were performed in triplicate for each *L. monocytogenes* strain - probiotic strain combination. After means and standard deviation were determined, experimental data was analyzed using GraphPad Prism 8.01 (Graphpad Software, Inc., La Jolla, USA). Statistical analysis was performed by one way ANOVA (Analysis of Variance). P values ≤ 0.05 were considered statistically significant while p values > 0.05 were taken as statistically non- significant. The difference between growth of *L. monocytogenes* in a food matrix in the absence and presence of various probiotic strains was compared. Statistically significant inhibition ($p \leq 0.05$) was regarded as less growth in the presence of a particular probiotic strain.

3.4 Results

3.4.1 Spot inoculation test

Figure 3.1 depicts the anti-listerial activity of each of the probiotic strains against the different *L. monocytogenes* strains *in-vitro.* The anti-listerial activity as assayed using the spot inoculation test was scored according to Fijan (2016), where a 6mm zone of inhibition was regarded as a positive result. *B. animalis* was effective against all *L. monocytogenes* strains except *L. monocytogenes* strain 243. *L. acidophilus* inhibited growth of all *L. monocytogenes* strains tested with the exception of *L. monocytogenes* avoS and *L. monocytogenes* 243. *L. plantarum* and *P .acidilacti* were the least effective, with *L. plantarum* showing inhibitory activity against *L. monocytogenes* cucS only while *P. acidilacti* did not inhibit growth of any of the strains tested.

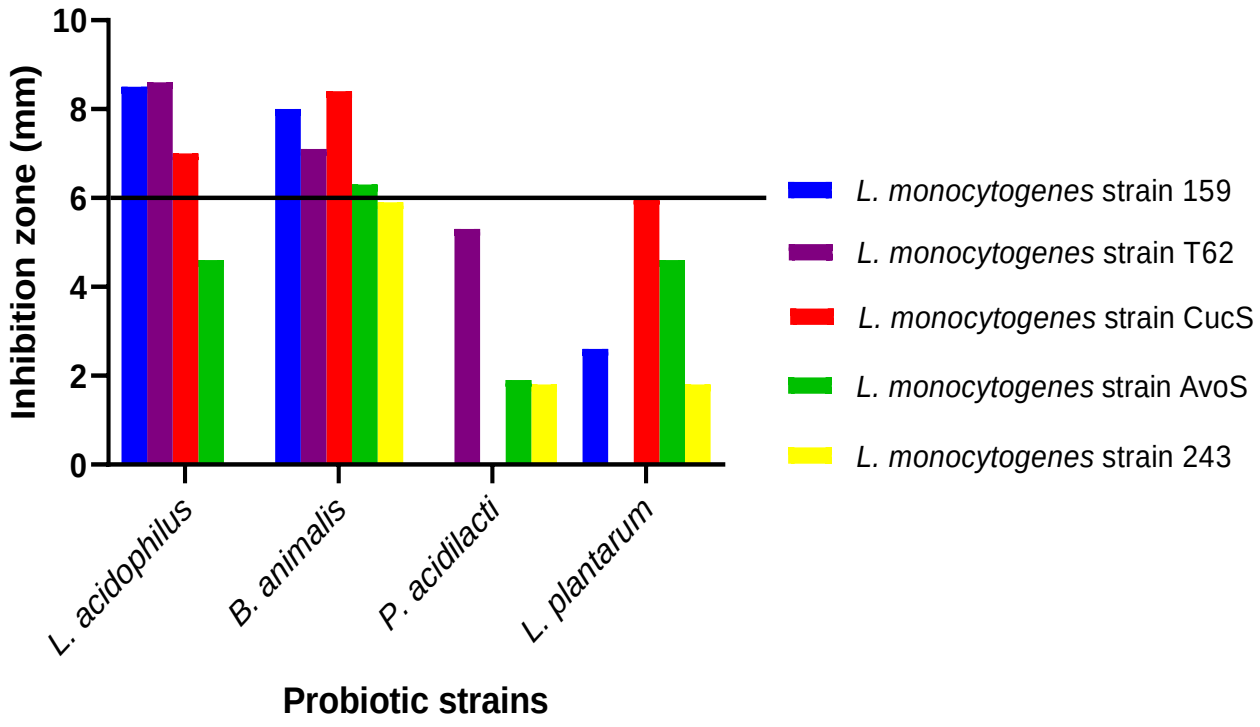

Figure 3.1*:* Inhibition of *L. monocytogenes* strains by probiotic strains *in-vitro*. The line on the graph represents 6mm; any result below was considered insignificant while any result on or above the line was considered effective inhibition.

3.4.2 Well diffusion assay

There were no zones of inhibition observed for all the probiotic CFS. Thus all the CFS failed to inhibit growth of *L. monocytogenes in-vitro*.

3.4.3 Probiotics against *L. monocytogenes* in food samples

Figure 3.2 depicts the effect each probiotic species had on the growth of *L. monocytogenes* avoS, cucS and T62 strains in avocado and cucumber. The inhibition of *L. monocytogenes* avoS growing on avocado by *B. animalis* (p = 0.7572), *L. acidophilus* (p = 0.999), *L. plantarum* (p = 0.9999) and *P. acidilacti* (p = 0.5586) was statistically insignificant. There was no significant difference in inhibition of *L. monocytogenes* cucS strain by *B. animalis, L. acidophilus*, *L. plantarum* and *P. acidilacti*, with p values of 0.7572, 0.999, 0.9999, and 0.5586 respectively.

Similarly, the inhibitory effects of *B. animalis* (p = 0. 999), *L. acidophilus* (p = 0.0616), *L. plantarum* (p = 0.8677) and *P. acidilacti* (p = 0.9999) on *L. monocytogenes* T62 growing on cucumber were not statistically significant. Only one of the probiotic strains, *B. animalis* was capable of significantly inhibiting growth of *L. monocytogenes* T62 on avocado (p= 0.0013). The other probiotic strains *L. acidophilus* (p = 0.5454), *L. plantarum* (p = 0.9355) and *P. acidilacti* (p = 0.4057) did not significantly impede *L. monocytogenes* T62 growth in the same food matrix (avocado).

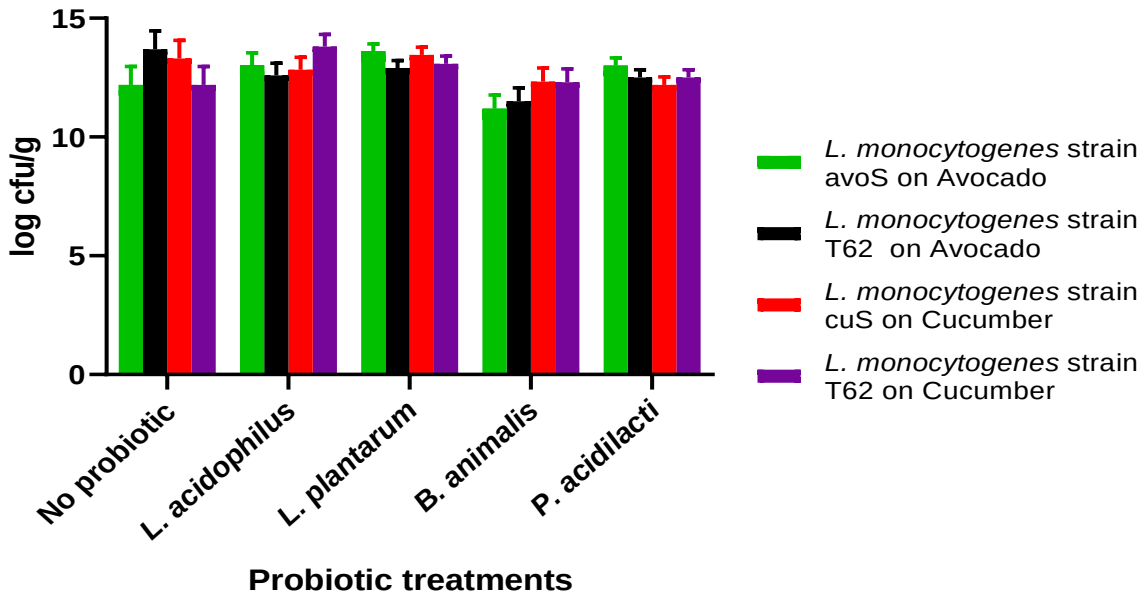

Figure 3.2: Growth of *L. monocytogenes* strains in the avocado and cucumber food matrixes in the absence and presence of probiotic strains.

3.4.4 Probiotic cell free supernatants against *L. monocytogenes* in food sample

Figure 3.3 illustrates the effect cell free supernatants (CFSs) of each of the probiotic strains had on the growth of *L. monocytogenes* strains avoS, cucS and T62 in avocado and cucumber fruits. The exposure of *L. monocytogenes* avoS to the CFSs of *B. animalis, L. acidophilus* and *L. plantarum* did not significantly impede its growth in the avocado fruit, with p values of 0.8872, 0.2863 and 0.2863, respectively. Interestingly, the growth of *L. monocytogenes* avoS on avocado was significantly enhanced in the presence of the CFS of *P. acidilacti* (p = 0.0123). No statistically significant inhibition was observed when the *L.*

monocytogenes cucS strain growing on cucumber was treated with the CFSs of *B. animalis* (p= 0.8872), *L. acidophilus* (p = 0.8872), *L. plantarum* (p = 0.8872) and *P. acidilacti* (p = 0.9999).

Growth of *L. monocytogenes* T62 in avocado in the presence of the CFSs of *B. animalis, L. acidophilus*, *L. plantarum* and *P. acidilacti* did not result in statistically significant inhibition of the pathogen with p values of 0.9999, 0.1415, 0.4849 and 0.9999, for the different probiotics respectively. Similar results were observed when *L. monocytogenes* T62 was grown in cucumber in presence of the CFSs of *B. animalis* (p = 0.9999), *L. acidophilus* (p = 0.2863) and *L. plantarum* (p = 0.4849). As observed for *L. monocytogenes* avoS, exposure of *L. monocytogenes* T62 growing on cucumber to the CFS of *P. acidilacti* (p = 0.003) significantly enhanced its growth.

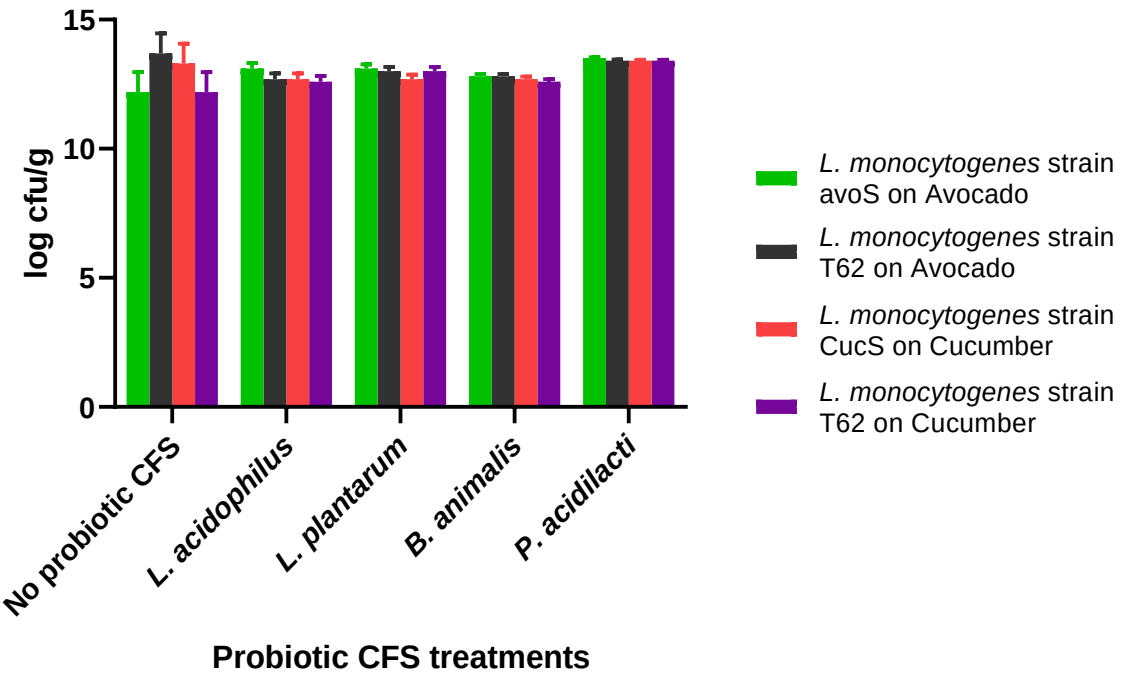

Figure 3.3*:* Growth of different *L. monocytogenes* strains in the presence or absence of cell free supernatants of the various probiotics.

3.4.5 Probiotic cocktails against *L. monocytogenes* in food sample

Figure 3.4 shows the impact cocktails of *B. animalis* - *L. acidophilus* (BA-LA) and *B. animalis* – *P. acidilacti* (BA-PA) had on the growth of *L. monocytogenes* strains avoS, cucS and T62 when grown in the avocado and cucumber matrixes. The BA-LA (p = 0.5514) and BA-PA (p = 0.6511) cocktails did not significantly inhibit the growth of *L. monocytogenes* avoS in avocado. The growth of *L. monocytogenes* cucS on cucumber was also not significantly hindered by either the BA-LA (p = 0.2189) or BA-PA (p = 0.999) cocktail. Challenging *L. monocytogenes* T62 growing in avocado with either BA-LA (p = 0. 2189) or BA-PA (p = 0.999) did not significantly impede its growth. Also, no significant inhibition of *L. monocytogenes* T62 growing on cucumber by either the BA-LA (p = 0.6511) or BA-PA (p = 0.4541) cocktails was observed.

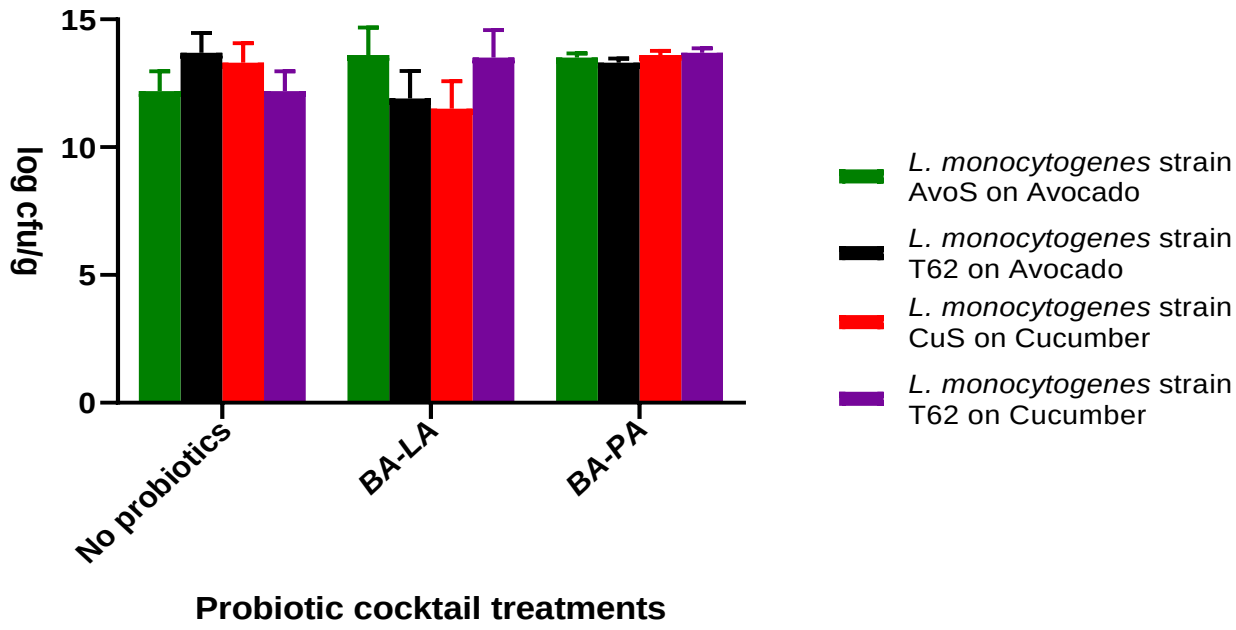

Figure 3.4*:* Growth of different *L. monocytogenes* strains in the presence and absence of probiotic cocktails.

3.5 Discussion

B. animalis and *L. acidophilus* strains inhibited growth of multiple *L. monocytogenes* strains while *L. plantarum* and *P. acidilacti* inhibited a single strain and none, respectively in the spot inoculation test. Several studies have shown the anti-listerial potential of *Bifidobacterium spp.* (Toure et al 2003, Kheadr et al 2004, Jesus et al 2016). *Lactobacillus spp.* have also been shown to inhibit growth of *L. monocytogenes in vitro* (Wilson et al 2005, Corr et al 2007). Antagonistic activity in these species is attributed to production of antimicrobial substances, nutrient and space competition (Yildrim et al 1999, Toure et al 2003, Georgieva et al 2015, Chen et al 2019). The antagonistic substances produced include bacteriocins, hydrogen peroxide, lactic and acetic acids (Klaenhammer 1993, Vandenbergh 1993, Yildrim et al 1998, Aroutcheva et al 2001).

There was variation in inhibitory capability between the two probiotic *Lactobacillus* strains used. This indicates interspecies variation within the *Lactobacillus* genera with respect to anti-listerial activity. The anti-pathogenic capabilities of *Lactobacillus* species are specific to each representative strain (Lievin-Le-Moal and Servin 2014). Inter genera variation in inhibitory activity towards *L. monocytogenes* growth was also observed. This indicates specie-specific anti- listerial activity within the various genera and this should be taken in to consideration when targeting the pathogen (McFarland et al 2018, Ansari et al 2019).

Multiple *L. monocytogenes* strains showed resistance to probiotic inhibition in the spot test. None of the probiotics inhibited all the strains. *L. monocytogenes* can proliferate in conditions of low pH and water activity and contains catalase for breaking down hydrogen peroxide. The ability to generate resistance to bacteriocins has been observed and discussed in various studies (Rekhif et al 1994, Vadyvaloo et al 2004, Kjos et al 2011, Macwana and Muriana 2012, Slozilova et al 2014). These factors can afford *L. monocytogenes* strains a competitive advantage when challenged with probiotics.

None of the probiotics, their CFSs and cocktails had an effect on the growth of *L. monocytogenes* on either the avocado or cucumber matrix. The only exception was the inhibition of *L. monocytogenes* T62 growing on avocado by *B. animalis.* The inhibitory ability of *B. animalis* towards *L. monocytogenes* T62 was already demonstrated in the spot

test. Interestingly, exposure of *L. monocytogenes* T62 and *L. monocytogenes* avoS to the CFS of *P. acidilacti* enhanced growth of both pathogenic strains. The growth promoting effect of *Bifidobacteria* on *L. monocytogenes* was previously reported by Yang et al 2017. The authors attributed the effect to proteins produced by the probiotic species.

The difference in inhibitory capacity of the probiotics used between the spot test and the food inoculation test indicates that properties of the food matrices might have had an influence on probiotic activity. The predominant micro flora in fruit possibly competes with probiotics for nutrients and niches (Janisiewicz and Korsten 2002). Nutrient composition and bioavailability determines which micro flora dominate the plant surface (Zgadzaj et al 2016). Presence of oxygen and the water activity of fruit can also impact the growth of probiotics given their anaerobic nature. The impact intrinsic factors of foods have on probiotics during storage should be thoroughly investigated before incorporation to ensure optimum activity of the probiotic strain.

The absence of anti-listerial activity could also be due to shortcomings resulting from characteristics of the probiotics used. The minimum growth temperature of *Bifidobacteria*, *Lactobacillus* and *Pediococcus* ranges between 8°C and 22°C (Gunther and White 1961, Papagianni and Anastasiadou 2009, Modest et al 2014). At this temperature, optimal production of bacteriocin and other antagonistic substance in these probiotics is compromised (Tomas et al 2003, Zamfir and Grosu-Tudor 2009). In this study, they were used at a temperature of 4°C which would further compromise their activity. The cold temperature can act as selective pressure in favour of *L. monocytogenes* as it can survive and proliferate at 4°C (Walker et al 1990). This should be taken into account if probiotics are to be applied to food that supports *L. monocytogenes.*

3.6 Conclusion

When evaluating the general effect *in vitro* of all probiotic strains used, *L. acidophilus* and *B. animalis* were the most effective, inhibiting four of the five *L. monocytogenes* strains challenged. More studies assessing the antagonistic mechanisms of these two species should be pursued to better understand how they can be of benefit to the food industry. In an age of biotechnology, molecular manipulation of these strains can be and has been done to insure the combat against *L. monocytogenes* is with ever increasing efficiency, considering its re-emergence as observed in the past year in South Africa.

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Chapter 4

General conclusions and recommendations

General conclusions

- *Listeria* species were more prevalent than *Salmonella* or *Campylobacter* species in all food samples tested. Four food samples, namely, avocado, cucumber, cow intestines and rumen tested positive for *Listeria* species. Only a single food item, ham, was positive for *Salmonella* while there was no presence of *Campylobacter* in any of the food items.

- Only two (avocado and cucumber) out of 167 food products tested positive for *L. monocytogenes*. Both food items were acquired from street vendors whose practices were non hygienic and surroundings rife with contamination sources. None of the food samples acquired from retail stores tested positive for the pathogen.

- There were variations between the antibiotic resistance and susceptibility profiles of the two *L. monocytogenes* isolates and between the *L. monocytogenes* and *Salmonella* species.

- The *Lactobacillus acidophilus* L10 and *Bifidobacterium animalis* subsp. *lactis* BB-12 strain best inhibited most of *L. monocytogenes* strains in the spot inoculation tests. The other probiotics used, namely *Pediococcus acidilacti* and *Lactobacillus plantarum* 7E1, did not prove to be as effective although the latter did exhibit inhibitory action towards a single *L. monocytogenes* strain.

- None of the cell free supernatants of the probiotics inhibited the growth of any of the *L. monocytogenes* isolates in the well diffusion assay.

- *B. animalis subsp. lactis* BB-12 inhibited the growth of a control *L. monocytogenes* strain on an avocado fruit. In all the other antagonistic food inoculation tests, none of the probiotics exhibited any significant inhibition of *L. monocytogenes* growth in any of the food matrixes used.

- Variation in susceptibility of *L. monocytogenes* strains to various antibiotics and probiotics was observed as a general trend during the antagonistic tests. The medium in which the tests were carried also seems to play a role.

Recommendations for future work

- The prevalence of *L. monocytogenes* in food samples acquired from retail stores in Pretoria should be assessed again in the future. This should be done to determine if the low prevalence found in this study is a usual occurrence due to stringent hygienic practices or if it was a fluke result based on reaction to the listeriosis outbreak.

- Focus on a single food group that supports *L. monocytogenes* growth such as RTE food and increasing the sample size might shed better light on the prevalence of the pathogen in that food group. Also, the time spent on this project should be increased to include all four seasons over a period of 2 years. This would allow for seasonal comparisons and hence a better picture of when the pathogen prevails more.

- Given the growing population in the inner cities and rapid increase in street vendors, more studies on prevalence of various foodborne pathogens should be conducted to determine if safety practices are being followed. The prevalence of different pathogens in multiple locations within the country should be investigated in order to give a framework through which food safety policies can be established.

- Employing environmental PCR techniques using *L. monocytogenes* specific genes to determine presence of *L. monocytogenes* can enhance the discriminatory power of culture based techniques allowing for rapid detection of the pathogen in suspected samples.