Antibiofilm properties of selected *Lactobacillus* species and their mechanism of action against *Listeria monocytogenes*

BY

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DECLARATION

I declare that the dissertation **Antibiofilm properties of selected *Lactobacillus* species and their mechanism of action against *Listeria monocytogenes*** which I hereby submit for the degree of Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other another tertiary institution.

Signature 

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List of Abbreviations

σ^A	: Sigma Factor A
σ^B	: Sigma Factor B
ActA	: Actin Assembly-Inducing Protein
ANOVA	: Analysis of Variance
ATCC	: American Type Culture
BapL	: Biofilm Associated Protein
BBB	: Blood-Brain Barrier
BHI	: Brain Heart Infusion
CDC	: Centers for Disease Control and Prevention
CDCs	: Cholesterol-Dependent Family of Cytolysins
CNS	: Central Nervous System
CFS	: Cell Free Supernatant
CFU/g	: Colony Forming Units per Gram
DC	: Dendritic Cell
cDNA	: Complementary Deoxyribonucleic Acid
DNA	: Deoxyribonucleic Acid
EPS	: Extracellular Polymeric Substances
FAO	: Food and Agriculture Organization
GAD	: Glutamate Decarboxylase
GC	: Guanine-Cytosine
GRS	: General Stress Response

GTI : Gastrointestinal Tract

HACCP : Hazard Analysis Critical Control Point

HMDS : Hexamethyldislazane

IgA : Immunoglobulin A

InlA : Internalin A

InlB : Internalin B

ISAPP : International Scientific Association of Probiotics and Prebiotics

LAB : Lactic Acid Bacteria

LAP : *Listeria* Adhesion Protein

LLO : Listeriolysin O

Met : Mesenchymal Epithelial Transition

MRS : De Man Rogosa and Sharpe

NaCl : Sodium Chloride

OD : Optical Density

ODC : Optical Density Cut-Off Value

PAMPs : Pathogen-Associated Molecular Patterns

PBS : Phosphate-buffered saline (PBS

PTFE : Polytetrafluoroethylene

PLC : Phospholipases C

PlcA : Phosphatidylinositol-Specific Phospholipase C

PlcB : Broad-Range Phospholipase C

ppm : Parts Per Million

PrfA : Positive Regulatory Factor A

PVC	: Polyvinyl Chloride
QACs	: Quaternary Ammonium Compounds
QS	: Quorum Sensing
RNA	: Ribonucleic Acid
RT qPCR	: Real-Time Quantitative Polymerase Chain Reaction
RTE	: Ready-To-Eat
spp.	: Species
SEM	: Scanning Electron Microscopy
TSB-YE	: Tryptone Soy Broth with 0.6 % Yeast Extract
Th	: T-Helper
WHO	: World Health Organization

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Dedication

This dissertation is dedicated to my late mother, the beautiful Motlagomang Deborah Masebe. You reside in my heart and I know your precious soul watched over me throughout this journey. Your last born daughter did it Mommy! I love you forever.

Summary

Antibiofilm properties of selected *Lactobacillus* species and their mechanisms of action against *Listeria monocytogenes*

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Degree : MSc (Microbiology)

The disastrous effects of the foodborne pathogen *Listeria monocytogenes* have been observed overtime by global listeriosis outbreaks claiming many lives. The spread of this pathogen is being reported at high levels with persistence spanning over several years in food-processing environments. The main source of contamination occurs in these very environments where *L. monocytogen*es present on surfaces comes into contact with food products and later infect consumers. The situation is exarcebated by the fact that *L. monocytogenes* forms biofilms, one very powerful virulence mechanism, that adhere to processing surfaces. The shortfall of commonly used antibiotics and sanitizers as treatment against *L. monocytogenes* biofilms due to antimicrobial resistance leads to a major safety crisis within the food industry. This limitation of antimicrobial agents sparked an interest in research of lactic acid bacteria (LAB) with certain probiotic properties as an alternative antibiofilm agent. Research has highlighted that the use of substances produced by LAB achieves inhibition and dispersal of *L. monocytogenes* biofilms. The antimicrobial substances of LAB have been revealed to showcase antilisterial activity, contributing to the combat of biofilm formation on surfaces. It is however known that the properties of probiotics cannot be generalized.

Taking these into consideration the current study aimed to determine whether selected Lactobacilli had the potential as an alternative control measure for biofilms formed by *L. monocytogenes* isolated from the food or food environments. The initial step of the current study was to screen *L. monocytogenes* strains for biofilm formation abilities. Then subsequently, the capabilities of cell

free supernatant (CFS) of selected LAB strains (*Lactobacillus acidophilus* La14 150B, *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* ATCC 4356) to inhibit as well as disperse the formation of biofilms of *L. monocytogenes* strains was investigated. *L. monocytogenes* ATCC 19115 and *L. monocytogenes* Cuc (originally isolated from cucumber) were classified as moderate biofilm formers, while *L. monocytogenes* 243 and *L. monocytogenes* Avo (isolated from avocado) were classified as strong biofilm formers. The strains isolated from the food processing environments were better biofilm formers that than the positive control *L. monocytogenes* ATCC 19115. After determining these categorizations, the *L. monocytogenes* strains were treated with CFS of LAB. All CFS managed to inhibit the formation of biofilms across all strains, significantly decreasing their biofilm former categories from either moderate or strong to weak ($p < 0.05$). *L. acidophilus* La14 150B performed better overall in inhibiting the biofilms. Cocktails of the CFS of LAB were prepared and additionally used as treatment. The cocktail ABC (*L. acidophilus* La14 150B + *L. plantarum* + *L. rhamnosus* ATCC 4356) had more efficient inhibition capabilities with both *L. monocytogenes* Avo and *L. monocytogenes* Cuc having the lowest recorded optical density values post treatment. The following step in this experiment explored the dispersal capabilities of the CFS of LAB. Though the preformed biofilms were not completely dispersed following treatment, the CFS were able to change the classification of all the *L. monocytogenes* strains into weaker biofilm former categories. *L. acidophilus* La14 150B was the most efficient of all LAB in removal of biofilms with significantly reduced optical density values ($p < 0.05$). Then, different cocktails of the CFS were prepared and assessed for their biofilm removal capabilities. It was deduced that all three CFS of LAB were able to significantly disperse the biofilms ($p < 0.05$). Collectively, the cocktail ABC (containing CFS of the three LAB test strains) had the greatest efficiency in dispersal abilities. From these results it was evident that cocktails were superior to individual CFS in the inhibition and dispersal of *L. monocytogenes* biofilms.

The observed inhibition and dispersal abilities of CFS of LAB (*L. acidophilus* La14 150B*, L. plantarum* and *L. rhamnosus* ATCC 4356) raised an interest in seeking to investigate their potency in removing *L. monocytogenes* biofilms adhered to different surfaces similar to those in contact with food in food processing environments. Scanning electron microscopy (SEM) revealed that both *L. monocytogenes* ATCC 19115 and *L. monocytogenes* 243 have a different attachment style on the hydrophilic stainless steel in contrast to the hydrophobic polyvinyl chloride (PVC). A two-layered biofilm structure with a honey comb complex was observed for both these *L.*

monocytogenes strains on stainless steel which was noticeably absent on PVC. Moreover, for the removal of *L. monocytogenes* biofilms on stainless steel coupons by CFS of LAB, all treatments managed to disperse the aggregated structures resulting in isolated cells. The antibiofilm properties of the different LAB was evidenced by a decline in the cell-clusters of *L. monocytogenes* ATCC 19115 and *L. monocytogenes* 243. On PVC, the three CFS exhibited the ability to interfere with and disrupt the aggregation of *L. monocytogenes* cells to each other as scattered cells were observed post treatment. Overall, *L. acidophilus* La14 150B dispersed the *L. monocytogenes* ATCC 19115 and *L. monocytogenes* 243 biofilms on both the stainless steel and PVC surfaces with the highest efficiency.

In order to determine the mechanism by which CFS of LAB interfered with the *L. monocytogenes* biofilm formation capabilities, the study further investigated how expression of the *L. monocytogenes prfA* gene is affected by the presence of CFS of LAB. The results demonstrated that the presence of all CFS of LAB (*L. acidophilus* La14 150B*, L. plantarum* and *L. rhamnosus* ATCC 4356) caused a significant downregulation in the expression of *prfA* ($p < 0.05$). This downregulation affects the coding of the PrfA regulator protein and disrupts *L. monocytogenes* biofilm formation. In correlation with the results observed for SEM analysis, the CFS of *L. acidophilus* La14 150B exhibited the highest antagonistic behavior, with expression of *prfA* recorded at a low 23% after treatment.

The results of the present study suggest that the CFS of LAB contain specific substances with antilisterial activity and antibiofilm properties. The presence of these substances contained within the CFS of LAB negatively affects the virulence gene *prfA*, decreasing the ability of *L. monocytogenes* to form biofilms. The study is of importance to the various food processing facilities and the food industry as it provides a potential safe alternative that can be used to limit any further outbreaks due to contamination of food products by *L. monocytogenes*. Cell free supernatants with demonstrated antibiofilm properties could be incorporated in industries and stipulated as the standard control measure for *L. monocytogenes* biofilms. Furthermore, for an enhanced effective treatment the cocktails of such CFS could be very beneficial. Thus cell free supernatants of lactic acid bacteria can be used to curb the formation of *L. monocytogenes* biofilms on food processing surfaces in the food industry and thereby contribute to improved food safety.

Introduction

Listeria monocytogenes is a gram positive, facultative intracellular bacterium that over the past century has progressed from an unclear zoonosis to a dynamic human foodborne pathogen (McMullen and Freitag, 2015). The initial discovery of *L. monocytogenes* dates back to 1924 where Murray and colleagues observed the pathogen presence in laboratory rabbits in Cambridge (Murray *et al.*, 1926). This ubiquitous pathogen shortly managed to infect humans and has since then exhibited excellent adaptability characteristics crucial in the mechanism of survival under extreme conditions. *L. monocytogenes* is able to survive in cold refrigerator temperatures (-1.5 to 4 °C) where food is regularly kept in storage. In addition, it can grow and multiply in a wide range of pH levels, high salt concentrations and low water activity (Jadhav *et al.*, 2012). This capability of *L. monocytogenes* to conquer multiple stresses presents an extensive challenge in the food chain.

Over the past decades *L. monocytogenes* has negatively affected food processing facilities as a result of soaring cases of contamination. Food products that are commonly contaminated include unpasteurised milks and cheeses, processed meats, ice cream, vegetables and fruits. Ready-To-Eat (RTE) foods – that do not require prior preparation - such hot dogs, deli meats and smoked fish are also included (Shamloo *et al.*, 2019). *L. monocytogenes* is most often transferred from processing surfaces, coming into contact with these foods, and later ingested by consumers. Following ingestion, this pathogen is able to cause the disease called listeriosis, which is especially severe for four groups of people: pregnant, elderly, young (particularly neonates) and the immunocompromised (Mateus *et al.*, 2013). *L. monocytogenes* has the highest mortality rate amongst all other foodborne pathogens with the worst ever global outbreak reported in South Africa in the years 2017-2018, where more than 1000 people were infected and over 200 of them lost their lives (Smith *et al.*, 2019). The prevalence of *L. monocytogenes* in food environments, leading to infection, is greatly attributed to its distinguishing ability to form biofilms (Martínez-Suárez *et al.*, 2016).

A biofilm is a consortium of cells that aggregate to each other, embedded in a slimy, extracellular matrix composed of extracellular polymeric substances (EPS). The EPS include nucleic acids, polysaccharides and proteins that bestow a protective layer to the complex of cells (Mann and Woznaik, 2012). The aggregation is orchestrated by mechanisms of quorum sensing and ensures efficient communication within cells, this strategy is also possible with other species creating a

'mixed/multi-species' biofilm (Van der Veen and Abee, 2011; Yan and Wu, 2019). The three-dimensional architectural biofilms of *L. monocytogenes* adhere to and grow on different types of surfaces in food processing facilities that encompass: stainless steel, polypropylene, wood, glass and rubber (Galié *et al.*, 2018). Additionally, biofilms attach to areas that are not regularly accessible such as floors, drains and pipes and can grow for years as they are not particularly cleaned as thoroughly as they should be (Colagiorgi *et al.*, 2017). Maximum and optimum attachment of a bacteria is due to high free surface energy and how wet the surface in question is, thus biofilms adhere differently on varied surfaces. Hydrophilic surfaces, in contrast to hydrophobic, with increased free energy are more favored by biofilms for attachment (Chmielewski and Frank, 2003). The concern indicated by Flemming *et al.* (2016) is that the *L. monocytogenes* biofilms have gained physical and mechanical resistance along with chemical protection which contribute to their overall persistence achieved via prominent virulence strategies.

It is crucial then to understand the virulence mechanisms of *L. monocytogenes*. An important major transcriptional activator is the regulon protein, Positive Regulatory Factor A (PrfA), that controls the expression of *L. monocytogenes* virulence genes. These genes drive the pathogenicity and include *prfA, plcA, hly, mpl, actA*, and *plcB* (Poimenidou *et al.*, 2018). Amongst those genes, *prfA* gene is responsible for encoding the PrfA protein that subsequently cues the lifestyle transition of *L. monocytogenes* from saprophyte to intracellular pathogen (de las Heras *et al.*, 2011). A study by Lemon *et al.* (2010) provided the first evidence that PrfA promotes biofilm formation and this has been of great interest to further investigate. Their report highlighted the importance of exploring a suitable treatment to disrupt *L. monocytogenes* virulence genes and ultimately eradicate biofilm formation.

Antibiotics and sanitizers have been used to inhibit and disperse biofilm growth of *L. monocytogenes* (Oloketuyi and Khan, 2017), however, overtime, resistance has deemed them particularly redundant. Gene transfer and exchange of other characteristics amongst pathogens has granted *L. monocytogenes* an advantage against most chemical treatments used commercially (Baquero *et al.*, 2020). An alternative biofilm control measure, lactic acid bacteria (LAB) with certain probiotic characteristics, was then introduced.

Probiotics are described as 'live microorganisms which when administered in adequate amounts confer a health benefit to the host' (FAO/WHO, 2001). The first recorded consumption of these microorganisms was reported by a Russian scientist, Elie Metchnikoff, who noticed that Bulgarian people who consumed fermented products containing LAB lived longer, indicating the probiotic properties (Culligan *et al.*, 2009). The principal functional properties of probiotics include adherence to epithelial surfaces, tolerance to acid and bile and antagonistic activity toward intestinal pathogens (Wan *et al.*, 2016). LAB, primarily falling under major the genus *Lactobacillus*, have the ability to secrete useful organic acids (lactic and acetic acid) and other antimicrobial substances such as bacteriocins, exopolysaccharides and biosurfactants (Kanmani *et al.*, 2013). A study by Gómez *et al.* (2012) showed that substances produced by Lactobacilli exhibit antilisterial activity against *L. monocytogenes* biofilm growth, proving the antagonistic effects. LAB are a safe and advantageous option that can be considered to reduce harmful pathogens. This study aimed to determine the ability of selected *Lactobacillus* spp. as an alternative biofilm control measure of *L. monocytogenes* on different surfaces, as well as elucidate its mechanism of action.

The specific objects were:

- To determine the biofilm formation capabilities of *L. monocytogenes* strains isolated from the food environment
- To determine the ability of cell free supernatants of selected *Lactobacillus* strains to inhibit the formation of, or disperse preformed *L. monocytogenes* biofilms
- To determine the potency of cell free supernatants of selected *Lactobacillus* strains for the removal of *L. monocytogenes* biofilms attached to various surfaces
- To quantify the expression levels of *L. monocytogenes prfA* gene in the absence and presence of cell free supernatants of selected *Lactobacillus* strains

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Chapter 1

Review of Literature

1.1 *Listeria monocytogenes*

1.1.1 History and characteristics

Listeria monocytogenes is a bacterium that was first discovered and studied in 1924 when E.G.D Murray and colleagues R. A Webb and M. B. R Swan observed cases of sudden death in six young laboratory rabbits in an animal breeding establishment in Cambridge. The first published description was given by them in 1926 (Murray *et al.*, 1926). Later on in 1927, J. Pirie investigated the unusual deaths of gerbils in Johannesburg, South Africa. Murray and J. Pirie worked independently of each other and both submitted their strains to the National Type Collection (Hof, 2003; Rocourt and Buchriese, 2007). Interestingly, the strains were clearly similar and proved to have the same identification. The strain was originally known as *Bacterium monocytogenes* but later renamed to *Listeria monocytogenes* in honour of Dr. Joseph Jackson Lister (Clarridge and Weissfeld, 1985). The first recorded culture isolated from humans dates back to just after the end of World War 1 in 1921 from a soldier suffering from meningitis, however, the first reported case of human *L. monocytogenes* infection was in 1929 in Denmark, (Nyfeldt, 1929; CDC, 1989).

Listeria monocytogenes is an intracellular, Gram-positive pathogen that is rod-shaped and a facultative aerobe (Chen *et al.*, 2019). It is a saprophytic organism, occupying natural ecological niches such as plants, soil, water, foodstuffs and animals (Fenlon, 1999; Vivant *et al.*, 2013). It is classified as an opportunistic foodborne pathogen due to properties that allow it to prevail in a wide range of conditions. For instance, the optimum temperature it survives at is 37°C, but it can still multiply within a range of -1.5 to 45°C (Meloni, 2014). This is of particular concern since refrigeration of food products usually occurs within those temperatures. *L. monocytogenes* has been shown to persist and survive in acidic conditions with pH levels lower than 4.0 and up to the value 9.6 (Lado and Yousef, 2007). It can tolerate salty environments with observed growth in levels of sodium chloride as high as 13 –14%. *L. monocytogenes* grows optimally at water activity (aw) levels of 0.97 but survival is still possible at a lower moisture content (Faber and Pagotto, 1992; Saraiva *et al.*, 2018). Through these characteristics it can contaminate different food products and then multiply and spread quite quickly within surroundings.

It is via ingestion of food, mainly contaminated during processing, that *L. monocytogenes* enters the human body. The foods mostly identified include unpasteurized milk, smoked meat, fruits,

vegetables, dairy, delicatessen and Ready-To-Eat (RTE) products. Ready-To-Eat foods are described as foods that are mostly pre-cleaned and precooked, ready for immediate consumption without prior preparation. They include salads, soft cheeses, cold cuts and luncheon meats (Huang and Hwang, 2012). The progression and multiplication of *L. monocytogenes* in the body causes the disease listeriosis. Listeriosis is extremely hazardous in high-risk individuals that include the elderly, pregnant women, neonates, and immunocompromised adults, with 20-30% of infections resulting in fatalities (Radoshevich and Cossart, 2018). The successful infection causes gastroenteritis, septicemia, meningitis, encephalitis, abortion or stillbirth of neonates (Vázquez-Boland *et al.*, 2001). A very alarming reality is *L. monocytogenes* associated outbreaks have been reported to cause the highest number of mortalities amongst other foodborne pathogens (Dewey-Mattia *et al.*, 2018).

A 2011 listeriosis outbreak across 28 states in the United States occurred as a result of contaminated cantaloupes linked to Jensen farms. A final report confirmed 147 total cases with 33 deaths (CDC, 2011). A recent major outbreak, worthy of noting as it was the largest globally to date, took place from 2017 to 2018 in South Africa. Over a 1000 people were infected and tragically more than 200 deaths recorded. The widespread outbreak of *L. monocytogenes* food poisoning was due to contaminated RTE processed meats. These meats included 'polony' manufactured by Enterprise Foods, a subsidiary of Tiger Brands in Polokwane (WHO, 2018; Thomas *et al.*, 2020).

1.1.2 Virulence and pathogenesis

1.1.2.1 Mechanism of action within the human body

Food contaminated with *L. monocytogenes* enters the body via ingestion and travels through the gastrointestinal tract (GIT) and during this time it encounters the low pH of the stomach and the duodenum (Gahan and Hill, 2014). In a healthy individual with a well-functioning immune system this acidic environment acts as a barrier and clears out the pathogen. For the immunocompromised, *L. monocytogenes* invades the intestinal cells and the host experiences flu-like symptoms. It overcomes and adapts to the acidity by the assistance of glutamate decarboxylase (GAD), which

works by mediating pH homeostasis (Smith *et al.*, 2013). Alternatively, *L. monocytogenes* can also be transmitted directly from a mother to a baby (fetus), otherwise known as vertical transmission. The source of this at most times being hospital acquired infections (Bell and Kyriakides, 2005).

The intestinal barrier is comprised of a monolayer of polarized epithelial cells called enterocytes, that are held together by tight and adherens junctions (Doran *et al.*, 2013). Adherens junctions are required for the integrity of tight junctions and are made of epithelial cadherins (E-cadherins) proteins connected to the cytoskeleton. Both junctions are located at the apical and luminal sides of the enterocytes (Rescigno and Di Sabatino, 2009). *L. monocytogenes* infects non-phagocytic cells including endothelial cells, fibroblasts and enterocytes (Ireton, 2007). In vivo, it enters almost all adherent cells, macrophages and similar cell lines, and is capable of efficiently internalizing 20 bacteria per cell (Portnoy *et al.*, 2002). The enterocytes are specifically infected via penetration of mucosal tissue or direct entry of the Peyer's Patches on the small intestines (Hof, 2001; Ribet and Cossart, 2015). The microfold cells are also infected as they are found on the surface of the Peyer's Patches. This occurs easier in people with a weakened immune system due the disruption of the T-cell-mediated defense system within their bodies having abnormal functionality (Rey *et al.*, 2020). To achieve pathogenicity in the different host cells and obtain full infection *L. monocytogenes* is assisted by a specific virulence mechanism.

1.1.2.2 Virulence genes

For any bacterial pathogen to progress within host cells it must first bind and then enter using some certain mechanisms. For *L. monocytogenes* the initiation of binding to cells is facilitated by the *Listeria* adhesion protein (LAP), which promotes translocation through epithelial cells using the paracellular route (Burkholder and Bhunia, 2010). LAP interacts with the cell receptor Heat shock protein (Hsp) 60, and initiates a complex signaling cascade stimulating secondary infection (Drolia and Bhunia, 2019). Binding by LAP is followed by internalization into the cells mediated by bacterial surface invasion proteins, Internalin A (InlA) and Internalin B (InlB). InlA binds to its cellular ligand E-cadherin, which is present at the adherens junction between epithelial cells (Drevetz and Bronze, 2008). InlB binds to the Met receptor, a tyrosine protein kinase which is a ligand for the hepatocyte growth factor, and the globular portion of the receptor for the first

component of complement (C1q) - ultimately mediating internalization via PI3-kinase activation (Cossart, 2001; Bleymüller *et al.*, 2016). The role of InlA in mediating cell entry has been confined to invasion across parts of the GIT, contrary to InlB which mediates entry into a wider variety of cells including hepatocytes, epithelial, and endothelial cells via the Met receptor (Jacquet *et al.*, 2004; Carvalho *et al.*, 2014). The binding of both these proteins, InlA and InlB, have detrimental effects of causing internalization of *L. monocytogenes* along with rearrangements to the cytoskeleton (Hamon *et al.*, 2006).

Internalization of *L. monocytogenes* is ensued through the process of phagocytosis by the host cells where it is encapsulated in a vacuole – forming a complete phagosome. The internalized bacterium is able to survive and can escape into the cytosol (Portnoy *et al.*, 2002). This escape, otherwise referred to as 'vacuolar lysis' (Figure 1.1), is mediated by pore-forming hemolysin protein listeriolysin O (LLO) (Vázquez-Boland *et al.*, 2001). LLO is a member of a cholesterol-dependent family of cytolysins (CDCs) and is active at the low phagosomal pH regulating bacterial escape from the phagosome (Wade *et al.*, 2015). Notably, it does not kill the host cell upon growth and replication within the cytosol because once the bacteria enter this environment the neutral pH inactivates LLO (Schnupf and Portnoy, 2007). In addition to LLO, *L. monocytogenes* secretes two phospholipases C (PLC) that also play a role in vacuolar escape. The first being a phosphatidylinositol-specific PLC (PI-PLC/plcA); and the other a broad-spectrum PLC (PC-PLC/plcB). PlcB is synthesized as a proenzyme activated by a secreted *L. monocytogenes* metalloprotease (Vazquez-Boland *et al.*, 2001; O'Riordan and Portnoy, 2002).

Entry into the cytosol granted by LLO, propels *L. monocytogenes* into adjacent cells allowing cell-to-cell spread with initial doubling times of approximately 40 minutes (Westcott *et al.*, 2007). Cell to cell spread (Figure 1.1) is facilitated by the ActA protein that works by F-actin nucleated by cytosolic bacteria and growing F-actin filaments (Lambrechts *et al.*, 2008). ActA forms a propelling-motion molecular motor as a transport protein that induces reorganization of the actin cytoskeleton (Pistor *et al.*, 1994; Jasnin *et al.*, 2013). This motion allows *L. monocytogenes* to have the ability to subsequently grow on carbohydrates and multiply intracellularly (Kuhn and Goebel,

2004).

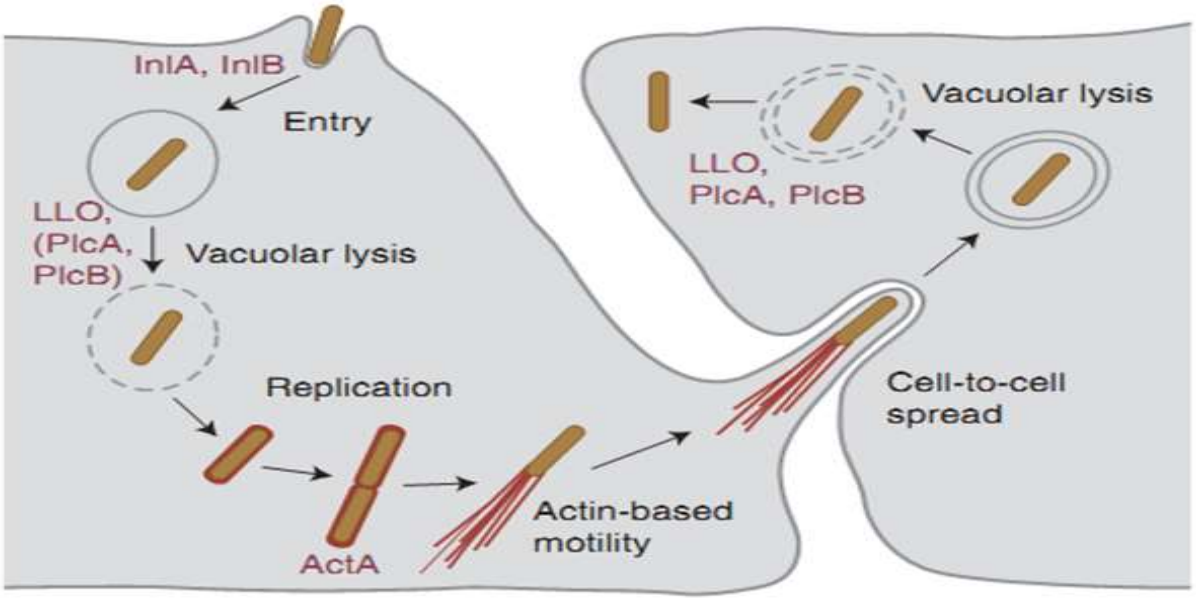

Figure 1.1: The intracellular cell cycle illustrating the host cell adhesion and invasion by *L. monocytogenes* and the different virulence genes involved (Pizarro-Cerdá *et al*., 2012)

1.1.2.3 Regulation by PrfA

The previously mentioned important virulence genes of *L. monocytogenes* that include *plcA, plcB, hly, mpl*, and *actA* are controlled by the key PrfA, which is the 'Positive regulatory factor A'. (Scortti *et al.*, 2007). PrfA is a 27-kDa site-specific DNA-binding protein regulon. It is the major transcriptional activator essential for the expression of most virulence genes and its activity, alongside the expression the *prfA* gene, is regulated by multiple mechanisms within *L. monocytogenes* (Wong and Freitag, 2004). A study by Poimenidou *et al*. (2018) highlighted that the main pathogenicity island of *L. monocytogenes* is the PrfA-virulence gene cluster that follow different evolutionary pathways affected by the strain origin and subtype. Both PrfA and its associated gene *prfA* are paramount in this process of pathogenesis. For the functionality of PrfA, two promoters $prfAp_1$ and $prfAp_2$ provide the initial levels required to direct the escape from the vacuoles of the host (Freitag and Portnoy, 1994). A third promoter, located upstream of the *plcA* gene, then participates in the high expression levels of *prfA* accelerates the spread of intracellular

bacteria to adjacent cells (Miner *et al.*, 2007). The expression and activation of PrfA is affected by the binding of a cofactor and posttranslational modifications (Wong and Freitag, 2004).

The expression and activity of PrfA is noticeably influenced by a wide range of physio-chemical signals which are present outside and inside the mammalian cell (Kuhn and Goebel, 2004; Gaballa *et al.*, 2021). Research shows that PrfA is temperature dependent and is primarily controlled by an RNA thermosensor formed by a temperature-sensitive region in the PrfA mRNA. This region inhibits any translation process occurring at low temperatures (Johansson *et al.*, 2002). PrfA encodes a protein that activates the transcription of LLO to maintain order in *L. monocytogenes* genes (Quereda *et al.*, 2018). PrfA binds specifically to a palindromic consensus sequence, otherwise known as the PrfA-box. The differences in the PrfA-box and the activation status of the PrfA protein itself is what ultimately determines the level of expression of the regulated virulence genes.

To assist *L. monocytogenes* PrfA in regulating virulence and withstanding several stress conditions is alternative sigma factor (σ^B). Stress tolerance mechanisms that promote pathogenesis are under the control of sigma B. The role it plays is to associate with RNA polymerase directing it to its promoter. The promoter re-programs the transcriptional profile of the cells to enable the expression of protective functions (van Schaik and Abee, 2005; Chaturongakul *et al.*, 2008). The genes under the control of σ^B are collectively known as the 'General Stress Response' (GRS) (NicAogáin and O'Byrne, 2016). According to Sleator and Hill (2002) the deletion of σ^B leads to the reduced survival in response to high salt concentrations.

1.1.2.4 Crossing over barriers in the human body

The ability of *L. monocytogenes* to colonize the gastrointestinal system is summarized by a pathway called transcytosis. It gains access to the adherens junction via the epithelial E-cadherins proteins present on cell surfaces in the body. Extruding cells from the tips of the intestinal microvilli detach and expose the E-cadherins to the luminal side of the intestine (Pentecost *et al.*, 2006). According to Nikitas *et al.* (2011) E-cadherins are not only luminally accessible around the tip of epithelial cells but also around mucus-expelling goblet cells and in villus epithelial folds. Essentially, *L. monocytogenes* adheres to accessible E-cadherins, is internalized and rapidly

transcytosed across the intestinal barrier to be released in the lamina propria via exocytosis. Once in the lamina propia, the pathogen crosses the intestinal barrier and then enters the bloodstream travelling to different bodily organs using actin-based mechanisms. The liver and spleen are the first and second target organs, respectively, following intestinal translocation (Vázquez-Boland *et al*., 2001; McDougal and Sauer, 2018). *L. monocytogenes* gain access to the liver through two ways: via Kupffer cells, by cell to cell spread, or by the direct invasion of hepatocytes from the Disse space after crossing the endothelial barrier lining the sinusoids (Demiroz *et al.*, 2021). The spleen is accessed through the splenic macrophages; and from those two target organs *L. monocytogenes* moves to different areas in the body (McElroy *et al.*, 2009).

The subsequent target organs are the brain and spinal cord –Central Nervous System (CNS)- and gravid uterus (Posfay-Barbe and Wald, 2009). The Blood-Brain Barrier (BBB) is responsible for maintaining homeostasis of the CNS microenvironment by restricting access of macromolecules, cells and pathogens. It is represented anatomically by the cerebral microvascular endothelium (Doran *et al.*, 2013). The brain microvascular endothelial cells are joint together by tight junctions and thus form a barrier, unfortunately this barrier may be surpassed by *L. monocytogenes* via parasitized leukocytes (Betz, 1992; Ireton, 2007). *L. monocytogenes* may also directly invade exposed sensory terminal results of the cranial nerve in the mouth and spread to the brain by centripetal migration (Vázquez-Boland *et al.*, 2001; Drevets and Bronze, 2008). For the intracarotid delivery, in contrast to the intravenous, *L. monocytogenes* is granted direct access to the vascular system of the brain. Ultimately CNS lesions involving the choroid plexus and ependymal of the cerebral ventricles result in meningitis in the brain and spinal cord and can prove fatal for the patient (Disson and Lecuit, 2012).

Listeria monocytogenes can all also travel to the female uterus. In cases where pregnant women are infected, the pathogen spreads via the gravid uterus to the unborn fetus and cause premature termination of pregnancy/ miscarriage (Posfay-Barbe and Wald, 2009). *L. monocytogenes* gains access to the fetus via hematogenous penetration of the placental barrier. Invasion begins in the decidua basalis and progresses to the placental villi where inflammatory infiltration and necrosis occur (Wolfe *et al.*, 2017). So collectively the pathogen is able to overcome three barriers – intestinal (gut), placental and blood brain barrier.

1.2 Biofilms

1.2.1 History and definitions

Biofilms are aggregated communities of microorganisms that attach to each other and to surfaces - a form of bacterial adherence. These adhered cells become fixed and embedded in a slimy matrix that is composed of extracellular polymeric substances (EPSs) such as polysaccharides, nucleic acids and proteins (Mann and Wozniak, 2012). The presence of cellulose in the biofilm matrix contributes to the resistance of cells to different types of mechanical forces and improves the adhesion to abiotic surfaces (Giaouris *et al.*, 2015). Flagella, pili, and membrane proteins initiate the adhesion on inanimate surfaces. These flagella are later lost after attachment and bacteria begin producing the EPS which provides resistance to disinfectants (Van Houdt and Michiels, 2005). The first description of biofilms was in the 17th century when Anton van Leeuwenhoek observed microbial aggregates on the scrapings of plaque that were present on his teeth (Chandki *et al.*, 2011). He used a simple microscope to view this assemblage of microbial cells and detailed that these surface associated cells exhibited a distinct phenotype with regards to gene transcription and growth rate (Donlan, 2002). It was only in 1978 that the term 'biofilm' was coined by Bill Costerton (Chandki *et al.*, 2011).

1.2.2 Functionality of biofilms

1.2.2.1 Formation stages and communication channels

The formation of biofilms generally follows four steps: 1) bacterial attachment to a surface; 2) micro-colony formation; 3) biofilm maturation and lastly 4) detachment of the bacteria (otherwise referred to as dispersal), which may potentially colonize new areas (Crouzet *et al.*, 2014). The bacterium, which are the primary colonizers of a given surface, form a biofilm by aggregation. Two types of aggregation exist: the first, auto-aggregation, is the attraction between the same species and the second, co-aggregation, is attraction between different species (Chandki *et al.*, 2011). The attached bacteria multiply overtime and are abl to secrete the extracellular matrix,

producing the mature mixed-population biofilms. The ubiquitous nature of biofilms allows them to form on a range of natural aqueous environments and surfaces (Bar-On and Milo, 2019).

The biofilm formation process influences and increases pathogen resistance and persistence. With the biofilm cluster acting like an army of soldiers attacking together on the surface, killing the entire unit becomes more challenging. This is due to the architectural characteristics of the biofilm such as thickness, density and spatial arrangement which define the functional properties of *L. monocytogenes* biofilms (dos Reis-Teixeiraa *et al.*, 2017). Biofilm formation, alongside other abilities such as antibiotic production, sporulation, conjugation, motility, competence, and bioluminescence regulated in response to signaling molecules of quorum sensing (QS) systems, embodies virulence factors (Rutherford and Bassler, 2012). QS is an important characteristic of biofilm associated bacteria and is also referred to as cell density mediated gene expression. The process entails expression of specific genes through the accumulation of certain signaling compounds mediating the intercellular communication (Processor, 1999; Chandki *et al.*, 2011). The bacterial cells ensue social interactions with each other using small diffusible signal molecules called autoinducers (Giaouris *et al.*, 2015). This type of communication can provide unique properties for the biofilm, such as antibiotic resistance genes at specific high level densities that contribute to protection (Processor, 1999; Chandki *et al.* 2011). Through QS the biofilm formation of *L. monocytogenes* enhance signaling triggers of the transcriptional activation of *actA* virulence gene regulated by PrfA. This results in the bacterial aggregating more effectively and forming biofilms (Giaouris *et al.*, 2015).

1.2.2.2 Mechanisms of resistance

Biofilms manage to efficiently resist most forms of combative treatment by delaying antimicrobial entry into the extracellular matrix via: physical restriction, slowing of growth rate of organisms inside the biofilm, or by expressing physiological changes through interaction of the organism and a surface (Donlan, 2000). The biofilm-associated resistance to different antimicrobial agents starts at the attachment stage and increases as the biofilm progressively ages. Other mechanisms that should be highlighted are the nutrient and oxygen depletion within the biofilm itself. The depletion leads to a stationary/non-growing state where the bacteria are less susceptible to growth-dependent

antimicrobial killing. Interestingly, some organisms have been shown to express biofilm-specific antimicrobial resistance genes (Patel, 2005).

Biofilms of the same bacteria may grow differently based on certain factors including the growth conditions, lineage, genotype and serotype. The growth conditions include salt content, nutrient availability and temperature and as a result challenge the efficiency of the biofilm (Marsden *et al.*, 2017). A sudden nutrient deprivation enhances the cellular adhesion of biofilms as it triggers a global cellular response to hypo-osmotic shock; while continuous nutrient deficiency hinders the maturation (Lee *et al.*, 2019). Bacteria have ways to avoid cell lysis caused by a great influx of water into the cytoplasm under sudden hypo-osmotic shock. One of these ways being mechanosensitive channels that mediate the influx of cytoplasmic solutes and water channels like aquaporins (Sleator and Hill, 2002). Addition of salt (0.85% w/v NaCl) significantly upregulates biofilm production and nutrient limitation (Lee *et al.*, 2019).

1.2.3 Biofilm formation in *L. monocytogenes*

For *L. monocytogenes*, the ability to form biofilms is a very important characteristic because it provides quick adaptability, resources and protection in harsh environments (Vogeleer *et al.*, 2014). The extracellular matrix of the biofilm is particularly responsible for the high prevalence and persistence of *L. monocytogenes* observed in different ecologic niches (Santos *et al.*, 2019). Exopolysaccharides, proteins, and eDNA are the main molecules within the biofilm matrix that serve as the protective material of bacteria against environmental stresses, antibiotics, antimicrobial agents and host immune responses (Donlan. 2000). A study by Franciosa *et al.* (2009) showed that both extracellular and surface proteins, Biofilm associated protein (BapL) and Internalin A (InlA) were found to be part of the matrix and play a role in the initial bacterial adhesion. All elements involved in the biofilm complex makes *L. monocytogenes* a resilient pathogen with a complex genomic system that enables it to react to different environmental conditions and antimicrobial agents (Chaturongakul *et al.*, 2008).

The key transcriptional activator PrfA, that positively regulates *L. monocytogenes* virulence genes, has been shown to have a great impact on extracellular formation of biofilms (Lemon *et al.*, 2010). In the study by Lemon *et al.* (2010) it was observed that mutants of *L. monocytogenes* that were

lacking PrfA were defective in surface-adhered biofilm formation. The biofilm defect occurred after initial surface adhesion, leading to the conclusion that PrfA plays a crucial role in promoting biofilm formation in *L. monocytogenes* and overall modulation of its lifestyle (Lemon *et al.*, 2010). The controlling factors of the PrfA transcript include housekeeping sigma factor A (σ^A) and the stress response sigma factor B (σ^B) (Nadon *et al.*, 2002). The σB has an effect on the phenotypic trait of biofilms and is positively regulated. Luo *et al.* (2013) and Price *et al.* (2018) in their studies highly suggested that PrfA is necessary only in the initial aggregation stages of biofilm formation and not so much in the colonization stage.

1.3 Biofilms in the food industry

1.3.1 Entry of *L. monocytogenes* into the food chain

Listeria monocytogenes often enter food processing environments through contaminated soil, water, plants and animal feces (Figure 1.2) (Vivant *et al.*, 2013). Overtime, these environments remain the primary source of contamination (Chmielewski and Frank, 2003; Giaouris *et al.*, 2014). *L. monocytogenes* biofilms attach to different surfaces present in processing facilities including stainless steel, plastic, glass, Teflon, wood, rubber, nylon and polystyrene, where cross-contamination is initiated (Ferreira, 2014). In addition, the biofilms are also found growing on non-contact surfaces such as floors, drains, pipes, sinks and walk-in cooler shelves and noticeably persist for several years in these areas (Hoelzer *et al.*, 2011).

Biofilms of *L. monocytogenes* prevail on surfaces in surroundings of cold refrigerator temperatures and high acidity and salt that are consider unbearable for other bacteria (Doyle *et al.*, 2001; Gardan *et al.*, 2003). When undergoing the presented stresses in processing environments biofilms form a monolayer or multilayers, where a significant change in physiology occurs inducing an increase in the level of tolerance and force of surface attachment (Beloin and Ghigo, 2005).

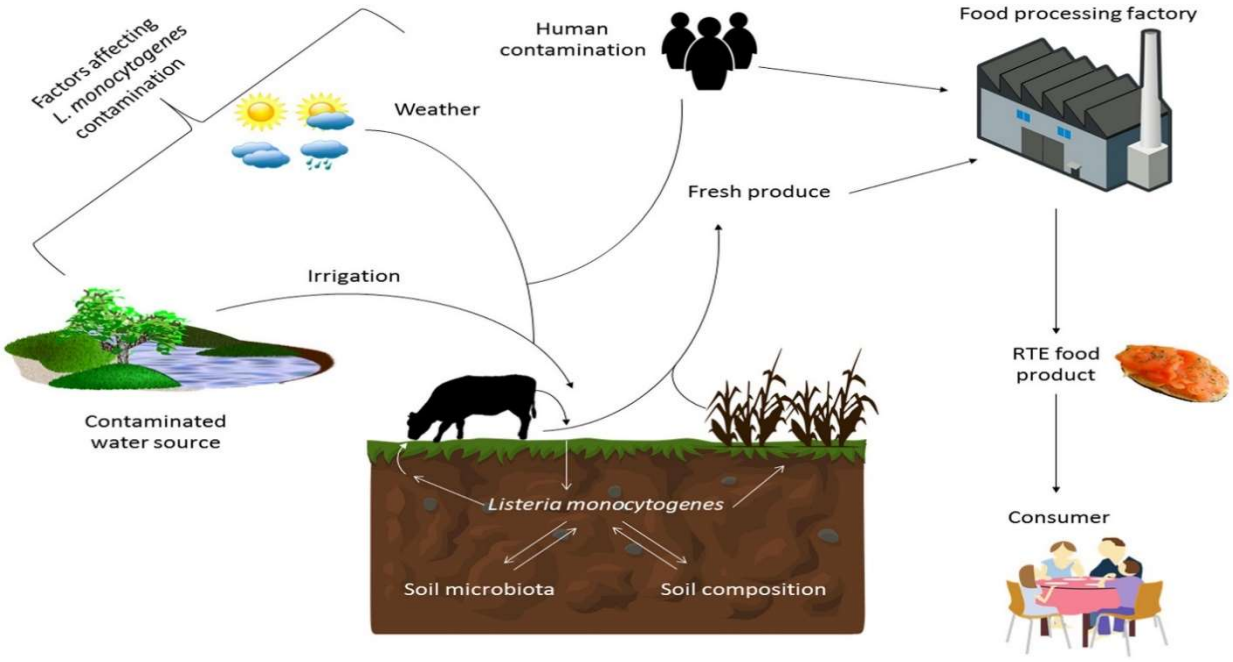

Figure 1.2: Flow-chart representing the source of and the factors affecting *L. monocytogenes* contamination, and channels it travels through to reach consumers (NicAogáin and O'Byrne, 2016*)*

1.3.2 Surface attachment

The adhesion of *L. monocytogenes* biofilms is influenced by various factors that affect the development, organization and microbial attachment of the biofilms to abiotic surfaces. The first being that physical properties of surfaces regulate cell attachment and physiology, that ultimately affect the early stages of biofilm formation. Secondly, the chemical properties influence the adhesion of cells to those surfaces and facilitate the progression to biofilm. Thirdly, the chemical communication between the cells gives way to the growth and organization (Renner and Weibel, 2011). Collectively, each step of biofilm formation is differently affected by environmental factors (Lee *et al.*, 2019).

The maximum attachment of bacterial cells depends upon high free cell energy or wettability of a surface (Chmielewski and Frank, 2003). For instance, stainless steel and glass have a high level of free surface energy and hence are hydrophilic. A hydrophilic nature allows the bacteria to attach far more greatly and form biofilms more easily, in contrast to hydrophobic surfaces like Teflon

and rubber that biofilms do not attach to as easily (Blackman and Frank, 1996). A study by Boulange-Petermann *et al.* (1993) displayed that the spreading pressure (βE) and balance of polar and Van der Waals forces of bacteria also influence the adhesion capabilities.

When cells undergo cold shock as a result of a rapid downshift in temperature, an environmental change induces modifications in bacterial cell surface proteins. The lipid composition is required to maintain membrane fluidity that facilitates adhesion and adaptation (Lee *et al.*, 2017). Lee *et al.* (2017) investigated *L. monocytogenes* biofilms that had been through sudden cold-stress and observed enhanced adhesion to surfaces. This adaption ability has an effect on the biofilm cells as they proliferate and multiply to hazardous levels during distribution and storage. The heighted strength of *L. monocytogenes* biofilm adherence to surfaces enhance their resistance to treatment and make their removal and control extremely difficult (Wang *et al.*, 2015).

1.3.3 Current methods of *L. monocytogenes* biofilm control

A multifaceted approach for the control of *L. monocytogenes* biofilm involves four different approaches 1) regularly monitoring high-risk and ready-to-eat food that are capable of growing the pathogen, 2) using the Hazard Analysis Critical Control Point (HACCP) to control spread to food supply, 3) using barriers and hurdles to inactivate *L. monocytogenes* biofilms in foods and lastly 4) educating high-risk individuals. (Farber, 1993; Ripolles-Avila *et al.*, 2019). Overtime the mentioned methods were implemented and coupled with the use of antibiotics and sanitizers to treat *L. monocytogenes* biofilms.

1.3.3.1 Antibiotics

Antibiotics, also known as antibacterials, are medications that destroy or slow the growth of bacteria (Aminov, 2010). The ‘accidental’ discovery of antibiotics dates back to 1928 when upon return from holiday British scientist, Alexandra Fleming, noticed something peculiar while working in his laboratory at St. Mary’s Hospital in London (Fleming, 1929). On a petri dish he accidently left open, Fleming observed colonies of *Staphylococcus* had been killed by a certain fungus growing on the same dish. The fungus, named *Penicillium*, from the substance penicillin,

proved how it could attack infectious bacteria. Penicillin was manufactured for commercial use and this catapulted research of other antibiotics that include cephalosporins, and fluoroquinolones (Aminov, 2010). The suggested mechanism of action of antibiotics used against *L. monocytogenes* biofilms was through disrupting essential processes and cell wall structure (Kapoor *et al.*, 2017).

Gradually the ability for antibiotics to inhibit and remove *L. monocytogenes* biofilms became ineffective due to resistance. Antibiotic resistance of the biofilm is attributed to gene transfer, through which bacteria communicate with each other. The resistance genes are acquired by a mating process with other bacteria called conjugation that provides *L. monocytogenes* with increased protection to the biofilm complex preventing degradation (Tatakis and Kumar, 2005; Lee *et al.,* 2010). This then led to the introduction of disinfectants described as sanitizers for the treatment of *L. monocytogenes* biofilms.

1.3.3.2 Sanitizers

Sanitizers are commercially used chemical detergents and cleaning agents that aim to degrade pathogenic bacteria and are applied in, on and around surfaces within food-processing environments. The most commonly used sanitizer in the food industry quaternary ammonium compounds (QACs) which work against bacteria, fungi, spores and viruses even at low level concentrations. QACs function by being active in the membrane of bacterial cells in the biofilm aggregate and disrupt the phospholipid bilayer. They cause cellular content leakage which results in bacterial death (Gerba, 2015). The advantage of QACs is that they are stable, present at low toxicity surface-active agents that are used up to a concentration level of 400 ppm. The disadvantage is that the misuse or overuse of QACs enhances the selection of new genetic elements that are horizontally transferred (Shapiro, 2015). The disadvantage is exploited by *L. monocytogenes* biofilms, coupled with active efflux pumps that extrude the QACs as a further tolerance mechanism (Rodríguez-López *et al.*, 2018). In a study by Nett *et al.* (2008) it was observed that cells embedded in the biofilm matrix, as opposed to planktonic cells, expressed different phenotypes which increased resistance to biocide treatment with QACs.

Chlorine-based compounds are another type of sanitizer that are cheap and easy to use against bacteria and fungi. Different compounds of this group are sodium hypochlorite, chlorine dioxide

gas or aqueous chlorine dioxide. The chlorine compounds function by using their fast-oxidizing nature to interact with the cellular membranes or penetrate directly into cell. They form N-chlorosaccharin groups that interfere with key enzymes and degrade cell (Wei *et al.*, 1985). The disadvantage of chlorine-based compounds is that if the cell wall thickness of *L. monocytogenes* biofilms is past a certain threshold, there is a struggle to penetrate through therefore protecting the cells (Rodríguez-López *et al.*, 2018).

The *L. monocytogenes* biofilm structure coupled with ineffective cleaning and disinfection procedures through use of sanitizers on surfaces and other 'hard-to-reach' areas contribute to continued contamination (Chmielewski and Frank, 2003). Since sanitizers applied as treatment in processing environments present disadvantages against the inhibition and dispersal of biofilm formation in *L. monocytogenes*, an alternative antagonistic method was introduced – probiotics.

1.4 Probiotics

1.4.1 History and definitions

The history of probiotics dates back to the start of human history in the Old Testament where Abraham was said to consume and offer people 'sour milk', or otherwise referred to as fermented milk, and overtime man then began to produce fermented food and beverages (Chavannavar and Unnikrishnan, 2004). The pioneer of probiotic studies is the Russian scientist, Elie Metchnikoff, who began his research in the 1900s of investigating the possible effects of microorganisms on human health. Metchnikoff associated the longevity of the rural people in Bulgaria to the regular consumption of fermented products such as yoghurt containing what would later be known as *Lactobacillus bulgaricus* (Gogineni *et al.*, 2013). He suggested that the lactobacilli might counteract the putrefactive effects of gastrointestinal metabolism contributing to aging and illness. He emphasized that certain probiotic foods make it possible for the intestinal microbe to modify the flora in the body and replace harmful microbes with beneficial ones (Gasbarrin *et al.*, 2016). Metchnikoff's research prompted him and other scientists to look further into probiotics and discovering many types (Ozen and Dinleyici, 2015).

The term probiotic is derived from Latin 'pro' and the Greek 'bios' which mean life (Hamilton-Miller *et al.*, 2003). The first description of probiotics was by German scientist Werner Kollath in 1953 to generally outline various organic and inorganic supplements that were able to restore the health of malnourished patients (Kollath, 1953). Later in 1965 a more expanded definition of probiotics in a different context was introduced and published by Lily and Stillwell. The definition by them was: 'the anaerobic bacteria that are able to produce lactic acid and stimulate the growth of other organisms'. In subsequent years Parker (1974) suggested that term should also include other substances that contribute to the intestinal microbial balance and not only microbial organisms. However, Fuller (1989) proposed an improved definition by deleting 'other substances' and narrowing it down to 'live microbial feed supplements which beneficially affects the host animal by improving its intestinal microbial balance'. The currently accepted definition of probiotics according to the World Health Organization (WHO) is: 'live microorganisms which when administered in adequate amounts confer a health benefit to the host' (FAO/WHO, 2001).

1.4.2 Properties of probiotic bacteria

Probiotics improve human and animal health as they contain live bacteria that build up the intestinal microbiota (Hati *et al.*, 2013). They are able to promote gut health in the gastrointestinal tract (GIT) and stimulate phagocytic activity through the ability to cross the intestinal mucous layer (La Fata *et al.*, 2018). For bacteria to be classified a probiotic it must have certain basic properties: it must be non-pathogenic, colonize the mucosal surface; be generally regarded as safe (GRAS), tolerate high concentrations of conjugated bile salts and low pH levels to allow movement through the GIT, not result in the formation of antibodies and possess extended protection against pathogens (Coombes and Maloy, 2007; Wells and Mercenier, 2008; Belicová *et al.*, 2013). At the beginning of 2014, Hill *et. al.* (2014) refined the FAO/WHO (2001) definition of probiotics for grammatical reasons to "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host". Hill *et al.* (2014) stated that probiotics must have "defined contents, appropriate viable count at end of shelf life and suitable evidence for health benefits," and further stated that all probiotics must be "safe for their intended use". The modification of the definition was followed by an updated version of probiotic properties. These properties were stipulated in a 2018 position statement by International Scientific Association of

Probiotics and Prebiotics (ISAPP) that lists the requirements an organism must meet to qualify it as a 'probiotic' for use in foods and dietary supplements (ISAPP, 2018). The four simple criteria are that microorganism must be: 1) sufficiently characterized, the strain must belong to an established genus and species/subspecies; 2) safe for intended use; 3) supported by at least one positive human clinical trial conducted according to generally accepted scientific standards or as per recommendations and provisions of authorities and lastly 4) alive in the product at efficacious doses throughout the entire shelf life; with a target of food products to have up to 10^7 CFU/ g at the end of the shelf life (Corcoran *et al.* 2006). Additionally, they must not exhibit the ability to confer antibiotic resistance genes to prospective horizontal genes through horizontal gene transfer (Mokoena, 2017).

Probiotics have the distinctive function of preventing infection. This function is mediated by increased defensins production and suppression of pro-inflammatory cytokines alongside improved epithelial tight junction and an increased level in the production of short-chain fatty acids during fermentation (Cook and Sellin, 1998; Delcenserie *et al.*, 2008; Liu *et al.*, 2011). Probiotics are able to adhere to the intestinal area by: lipoteichoic acids, specific surface proteins, steric surfaces or via electrostatic and hydrophobic interactions (Servin and Coconnier, 2003). The presence of some proteins on the surface like cell-wall anchored proteinases promotes hydrophobicity and adhesion in selected probiotic organisms (Zhang *et al.*, 2015). Additionally, secretory compounds from the cell wall are known to prevent colonization by pathogens and neutralize toxins (Banerjee *et al.*, 2009).

1.4.3 Health benefits of probiotics

Over the past two decades, different studies on probiotics have shown a great promise for benefiting human health. In infants this includes treatment of colic, periodontal disease and ulcerative colitis. It is also effective in treatment of gastrointestinal conditions in adults such as diarrhea, constipation, inflammatory bowel disease and irritable bowel syndrome (Hempel *et al.*, 2011; Rao *et al.*, 2016; Ford *et al.*, 2018). More general beneficial effects of probiotics are the overall improvement of intestinal health, reduction of serum cholesterol and enhancement of the

immune response (Kechagia *et al.*, 2013). The impacts of probiotics are strain specific, therefore, the impact observed for one strain can't be automatically applied for another (Abatenh *et al.*, 2018).

Probiotics are remarkable for their activity against uropathogens in humans such as *Candida albicans*, *Staphylococcus aureus* and *Escherichia coli* that infect the urinary tract that have gained antibiotic resistance. Several lactobacilli manage to colonize the uroepithelial cells. In a study by Tomás *et al.* (2003), *Lactobacillus acidophilus* CRL1259 of human origin inhibited the growth of uropathogenic *E. coli* and prompted the introduction and inclusion of this strain in products intended for vaginal application. Prominent metabolites in probiotics are responsible for the control of uropathogens are hydrogen peroxide and lactic acid (Ayeni *et al.*, 2011). These studies led to the study of a specific group of bacteria which often qualify as probiotics – lactic acid bacteria.

1.5 Lactic Acid Bacteria

1.5.1 Definitions and characteristics

Lactic acid bacteria (LAB) are a heterogeneous group of bacteria which play a very significant role in the various processes of fermentation. (Bintsis, 2018). They are Gram positive, aerotolerant non-spore forming, cocci or rod, catalase negative organisms. These bacteria are acid tolerant and immotile, possessing a low G + C content (Kaban and Kaya, 2008). LAB ferment carbohydrates to get energy and use endogenous carbon sources as the final receptor – lactic acid is the main fermentation product of the metabolism (Saeed and Salam, 2013). In addition to the organic acids lactic and acetic acid, LAB also produce several antimicrobial substances that include antimicrobial peptides (bacteriocins), reuterin, ethanol, diacetyl, carbon dioxide, and hydrogen peroxide (Liao and Nyachoti, 2017). Meanwhile, they can also produce a variety of products including short-chain fatty acids, amines, vitamins and exopolysaccharides during (Wang *et al.*, 2021). The habitats in which LAB occupy are usually where there is a rich nutrient supply such as decomposing plant material, fruit and other foods. In addition, they are found in cavities of humans and animals, specifically the ileum, colon and the oral cavity (König, and Fröhlich, 2009; Todorov, 2009). The mammalian intestine is colonized by 100 trillion microorganisms referred to as 'microbiota' that are vital for health and of which LAB are included (Hooper and Macpherson,

2010). LAB are found abundantly in the vaginal bacteria of women and inhibit the binding and growth of other bacteria to epithelial cells via production of lactic acid (Witkin and Linhares, 2017). LAB positively affects the innate and adaptive immune response within a host by binding to specific receptors on immune cells and other tissue (Tsai et *al.*, 2012). Those receptors prompt the production of regulatory T cells, chemokines, cytokines and activation of macrophages and dendritic cells (DC) (Wells and Mercenier, 2008). There is a core group of genera to which LAB belong, this consists of: *Lactobacillus, Pediococcus, Lactococcus, Enterococcus, Streptococcus* and *Leuconostoc* species. Nevertheless, to this day, the most studied one is *Lactobacillus* especially in the food industry due to the benefits of consumption (Martínez Cruz *et al.*, 2012).

1.5.2 Use and benefits in food industry

In fermented food processing, LAB degrade polysaccharides and produce monosaccharides or lactic acid which ultimately improve the quality of products (Wang *et al.*, 2021). LAB are commonly used as starter cultures in fermented dairy products such as traditional buttermilk, yoghurt, and cheese; but also in fermented meat and fish, cereals, beets, pickled vegetables, potatoes, sauerkraut, pickles and juices (Liu *et al.*, 2011). The degradation of proteins and the ability to produce aldehydes, alcohols, esters, acids and Sulphur compounds contribute to the development of specific flavors in fermented products (Bintsis, 2018). For application in foods the mainly used commercial LAB cultures include the strains: *L. acidophilus*, *Lacticaseibacillus casei*, *Lactobacillus curvatus, Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* (Champagne *et al.*, 2005).

By applying LAB, the food products are deemed as ‘functional foods’ as they not only provide nutrients and energy, but beneficially modulate targeted functions in the body. They enhance a certain physiological response via ingredients like bioactive compounds and dietary fiber that can aid with digestion (Nicolleti, 2012). As the gut epithelial barrier is strengthened, the digestive functionality within the body is overall enhanced by LAB proving the importance of this bacterial group for the food industry. The desirability also is due to their safe metabolic activity while growing in food, the long-lived uses and no record of clinical problems. All the mentioned reasons contribute to classifying the LAB as GRAS (Generally Recognized as Safe) and given a QPS

(qualified presumption of safety) status by the European Food Safety Authority (Leuschner *et al.*, 2010; Bourdichon *et al.*, 2012). It is important to remember that though LAB are beneficial not all of them are considered as probiotics, they have to meet the stipulated criteria first.

1.5.3 Mechanisms of action

Lactic acid bacteria have various mechanisms of action in which they exert their effects. The major mechanisms amongst other include inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms, production of antimicrobial substances and immunomodulation (Figure 1.3) (Bermudez-Brito *et al.*, 2012)

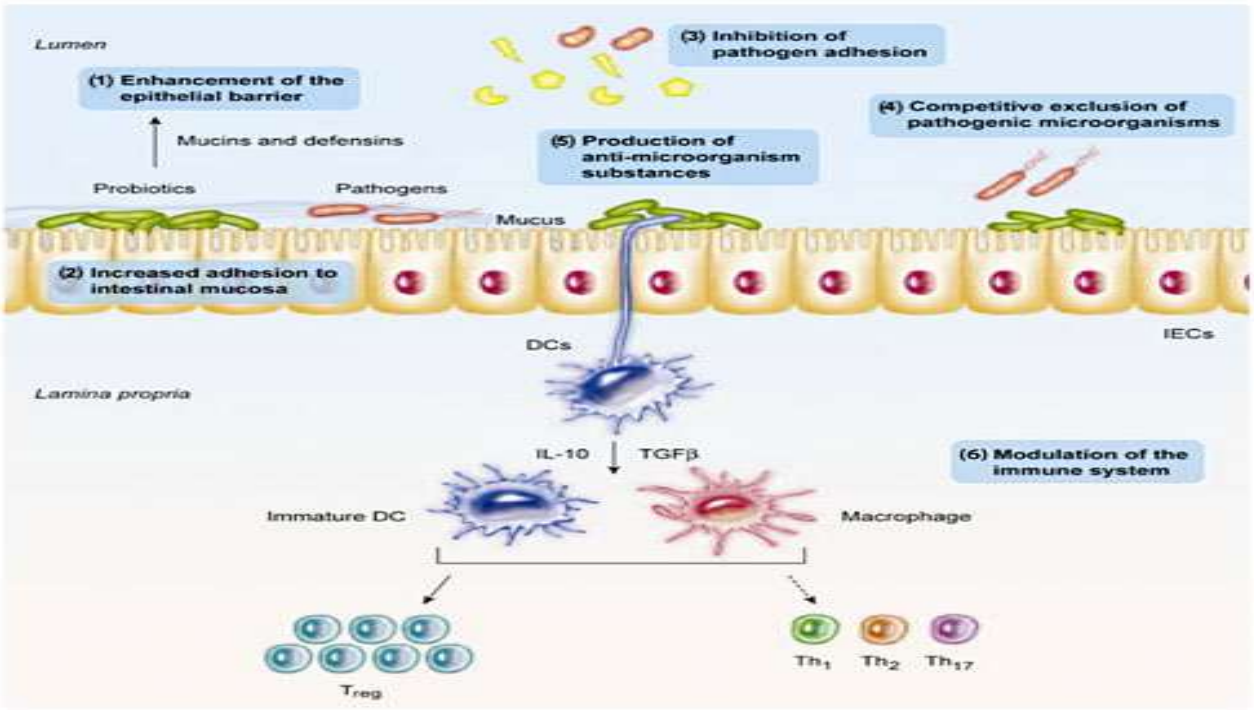

Figure 1.3: Mechanisms of action of lactic acid bacteria (Bermudez-Brito *et al.*, 2012)

1.5.3.1 Inhibition of pathogen adhesion

A wide range of pathogen growth are inhibited by LAB colonization through maintenance of health conditions. This is the main operational principle in infection prevention and treatment as well as restoration of microbial equilibrium in the gut (Abatenh *et al.*, 2018). The intestinal

epithelial cells are the first site targeted by pathogens and are also the first line of defense against invasion. LAB have the ability to block pathogens attachment through an increased adhesion to the intestinal mucosa. LAB induce secretion of complex glycoprotein mucin by epithelial cells to strengthen the barrier and cause an interference in pathogen adherence (Collado *et al.*, 2005). They use antibacterial mechanisms that increase the maintenance of intestinal microbial balance. This ensures the upregulation of epithelial cell functions, regulating intestinal immune cell responses and eliminates *L. monocytogenes* infection (Corr *et al.*, 2009). *Lactobacillus* proteins have been proven to promote mucous adhesion and surface adhesins present that mediate attachment to the mucosal layer (Buck et al., 2005). Inhibition of pathogenic bacteria adherence by LAB is achieved additionally achieved via means of steric hindrance at enterocyte pathogen receptors (Coconnier *et al.*, 1993). In a (2007) study by Corr *et al.* they observed that prior treatment of *L. acidophilus* strain NCDO 1748 on epithelial cells significantly ($p < 0.05$) reduced *L. monocytogenes* invasion by up to 90%. A study by Ndahetuye *et al.* (2012) proved that LAB inhibited the adhesion of *L. monocytogenes* by reducing the attachment level via bacteriostatic activity. With respect to *L. monocytogenes* biofilms a more recent study revealed that treating a surface with *Lactobacillus sakei* CRL1862 constituted a way of preventing the settlement of the biofilm (Pérez-Ibarreche *et al.*, 2016). This result provides an environmentally-friendly sanitation method to diminish contamination in food processing environments by use of LAB.

1.5.3.2 Competitive exclusion of pathogens

Lactic acid bacteria are able to vigorously fight and compete with pathogenic bacteria for benefits. This principle entails one species being the superior competitor for a single limiting resource, in order to not co-exist or occupy the same niche (Booth and Murray, 2008). The mechanism used by LAB to exclude and reduce the growth of another species include: production and secretion of antimicrobial substances lactic and acetic acid, eliminating the available bacterial receptor sites to form a hostile microecology, and competitive depletion of nutrients needed (Mukai *et al.*, 2002; Schiffrin and Blum, 2002). LAB gain a competitive advantage over *L. monocytogenes* through modifying the environment by inhibiting access to available nutrients. The prevention of *L. monocytogenes* growth maintained through saturation of its intended attachment receptor and colonizing the host cells so it is destroyed. Henderson *et al.* (2020) indicated that cell-to-cell spread

of LAB inhibited exchanging of information such as genetic material by *L. monocytogenes*, through conjugation, and therefore limited the extent of growth.

1.5.3.3 Immunomodulation

Another mechanism of action used by LAB is stimulation and modulation of specific and non-specific immune response. LAB have the ability to interact with dendritic and epithelial cells and with macrophages/monocytes and lymphocytes (Bermudez-Brito *et al.*, 2012). The innate immune system, that clears pathogens from the body with no long term immunity, responds to structures named pathogen-associated molecular patterns (PAMPs). This is in contrast to adaptive immune response, which does not provide long term immunity. This is all dependent on T and B lymphocytes specific for defined antigens (Gómez-Llorente *et al.*, 2010). The adaptive immune response pathway can be described in this way: the immune response is initiated by T-cell activation leading to cytokine production; followed by the induction of phagocytosis and IgA secretion; to modification of T-cell responses; enhancement Th1 responses and lastly attenuating Th2 responses (Soccol *et al.*, 2010). This process ultimately increases immunoglobulin-emitting cells in blood to accelerate antibody production proving beneficial in the prevention and therapy of infectious diseases.

1.5.3.4 Production of antimicrobial substances

There is a wide range of antimicrobial substances secreted by LAB that have been previously mentioned including organic acids, bacteriocins, reuterin, ethanol, diacetyl, carbon dioxide, and hydrogen peroxide; biosurfactants and several peptides that all degrade and kill pathogens (Ripert *et al.*, 2016). Meanwhile, they can also produce a variety of products including short-chain fatty acids, amines, vitamins, exopolysaccharides and biosurfactants during metabolism (Wang *et al.*, 2021). Organic acids that include acetic and lactic acid have exhibited strong inhibitory effects against Gram-positive bacteria and have been considered the main antimicrobial compounds responsible for inhibition of pathogenic growth (Alakomi *et al.*, 2000). Entry is gained into the bacteria by the un-dissociated form of the organic acid which later dissociates inside its cytoplasm.

The acid works by lowering the intracellular pH or intracellular accumulation of ionized form causing pathogen death (Russell and Diez-Gonzalez, 1998). For foodborne pathogens like *L. monocytogenes*, the antimicrobial substances can disrupt the growth and or other spoilage organisms in the GIT environment by inactivating toxins and creating an antagonistic environment (Abatenh *et al.*, 2018).

Many LAB produce bacteriocins and the common mechanism of bacteriocin-mediated killing is by destroying the target cells via pore formation and inhibition of cell wall synthesis. (Hassan *et al.*, 2012). These bacteriocins such as nisin, enterocin and sakacin are able prevent adhesion and biofilm formation on metallic and plastic surfaces (Rodríguez-López *et al.*, 2018). Nisin produced by *Lactococcus lactis* is one of the most common antimicrobials used within the food industry especially so for dairy products and acidic food. It has shown to be most effective in reducing the numbers of *L. monocytogenes* (Kaur *et al.*, 2013). In addition, LAB produce de-conjugated bile acids which are derivatives of bile salts. These acids possess a much high level of antimicrobial activity in comparison to bile salts synthesized by the host organism (Oelschlaeger, 2010).

1.5.3.4.1 Bacteriocins

Bacteriocins, are antimicrobial peptides that are ribosomally synthesized as primary metabolites, they are small cationic molecules of roughly 30-60 amino acids which form amphiphilic helices (Yang *et al.*, 2014). The molecules begin in an inactive stage and are later switched to an active state (Todorov, 2009; Perez *et al.*, 2014). These bacteriocins produced by LAB strains are used by the same strains to protect themselves by expressing a specific immunity protein, encoded in the bacteriocin operon. They primarily target energized bacterial cytoplasmic membrane vesicles to disrupt the proton motive force (Parada *et al*., 2007). Bacteriocins can be grouped and classified based on the following: molecular weights, post-translational modifications primary structures and the genetic characteristics (Mokoena, 2017). Three classes have been stipulated.

The first of the classes of bacteriocins is Class I lantibiotics, and are grouped based on their post-translational modification with nisin and lactocin as representatives; their modification result in formation of unusual amino acids such as lanthionine and methyllanthionine (Parada *et al*., 2007). Following those are the Class II bacteriocins that are small heat-stable, non-modified,

hydrophobic, cationic peptides. They are either chromosomally or plasmid-encoded, genes organizing the production of this class are grouped into operon clusters with the structural gene encoding prepeptide (Ennahar *et al.*, 2000). They are further divided into Class IIa and IIb, with Class IIa containing pediocin-like *Listeria* active peptides contributing to food preservation. For the class IIb bacteriocins to exert antimicrobial activity they require the synergistic activity of two complementary peptides. This subgroup has amphiphilic and hydrophobic regions which are mostly cationic (Zacharof and Lovitt, 2012; Perez *et al*., 2014). The final one is Class III bacteriocins which consists of large and heat-labile proteins with sizes exceeding 30 kDa, helveticin J is an example (Parada *et al*., 2007). In a study by Vijayakumar and Muriana (2017) the bacteriocin produced by *Lactobacillus curvatus* demonstrated effectiveness in the decrease 2>-log of *L. monocytogenes* decrease by attacking the cell envelope directly and destroying it. For the more recent study by Camargo *et al.*, 2018, it was deduced that bacteriocinogenic (their bacteriocins) LAB show an aptitude as agents to control *L. monocytogenes* biofilms – a major breakthrough for the food industry.

1.5.4 Cell free supernatant

The cell free supernatant (CFS) form is isolated by passing the LAB cultures through a nitrate cellulose filter, eliminating the cells. Antimicrobial substances mentioned (hydrogen peroxide, fatty acids, organic acids, ethanol, exopolysaccharides, bacteriocins, biocides) are released by the LAB and found in the CFS. A study by Mariam *et al.* (2014), CFS was isolated from LAB and co-cultured against the foodborne pathogens *L. monocytogenes*, *Salmonella* and *Staphylococcus aureus*. A strong growth inhibition ability by the CFS of LAB was observed. It is important to highlight the fact the inhibitory activities were stable following heat and protease treatment and some instances having a low pH indicated that CFS from LAB are suitable candidates for control alternatives (Poppi *et al.*, 2015). They are efficiently able to survive in a range of extreme conditions. CFS are effective not only against planktonic cells but biofilms too; the CFS of *Lactobacillus vurvatus* HH significantly reduced the biofilm formation of *Candida glabrata* ATCC 2001 by 79.4% and *C. albicans* ATCC 14053 by 61.1% and it can be used for prevention and treatment of candidiasis (Bulgasem *et al.*, 2015). In a study by Koo *et al.* (2012), CFS of LAB

were effective in reducing growth of *L. monocytogenes* biofilms after 8 weeks in cold refrigerator temperatures.

1.6 Conclusion

Listeria monocytogenes is a problematic foodborne pathogen that continuously manages to overcome adverse conditions. The ability *L. monocytogenes* has to form biofilms increases the burden of eradicating the strongly attached aggregates to various surfaces. Biofilms persistent on surfaces and areas in food processing facilities and ultimately come into contact with foods. The contamination of food products that are preferably consumed due to the convenience and ready-to-eat nature creates a major concern for the food industry. High mortality levels due to ingestion of this foodborne pathogen prompts the need of an alternative control measure. Commonly used antibiotics and sanitizers are no longer effective due to resistance of *L. monocytogenes* acquired through genetic exchange and transfer. An alternative, lactic acid bacteria (LAB) with certain probiotic properties has been introduced and investigated. These LAB have been proven to exhibit substances that have antilisterial activity to combat *L. monocytogenes* biofilm. The incorporation of LAB treatment commercially is a desirable option because of the antagonistic antimicrobial substances present in the cell free supernatant.

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Chapter 2

Screening of *Listeria monocytogenes* strains from foods for biofilm formation abilities and evaluation of selected *Lactobacillus* as antibiofilm control

2.1 Abstract

Listeria monocytogenes is a foodborne pathogen that constantly retains the ability to overcome all barriers used in the food industry to control pathogens. Its persistence in the food processing environments, accompanied by its successive growth forms, allows easier infection of humans. Formation of biofilms by *L. monocytogenes* expands its capabilities of persistence and resistance, making it even harder to eradicate. Lactic Acid Bacteria (LAB) in general, as well as those classified as probiotics, have been shown to be beneficial for control of different pathogens. Along with their antibacterial effects against various foodborne pathogens, they have also exhibited to production of substances with antilisterial properties. In this study, *L. monocytogenes* strains (*L. monocytogenes* strain 243, Avo and Cuc) isolated from foods and food environments were assessed for their ability to form biofilms and then classified based on their biofilm formation capabilities. *L. monocytogenes* ATCC 19115, was used as a positive control. Then the ability of *Lactobacillus acidophilus* La14 150B, *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* ATCC 4356 to prevent the formation of, or disperse preformed biofilms of the test *L. monocytogenes* strains was investigated. In the biofilm inhibition assays the cell free supernatants (CFS) of each of the LAB was used individually or in combination and were inoculated together with the *L. monocytogenes* strains into the microtiter plates. The biofilm dispersion assays were done by first allowing the *L. monocytogenes* strains to form biofilms in the wells of the microtiter plates and subsequently treating them with the CFS (individually or as a cocktail). All the microtiter well plates were incubated at 37°C for 48 hours and then biofilms were quantified used crystal violet staining. There was a significant decrease ($p < 0.05$) in the ability of *L. monocytogenes* strains to form biofilms because post-treatment with CFS of LAB, all the *L. monocytogenes* strains were categorized into weaker biofilm forming states. The CFS also dispersed preformed biofilms reflecting the strain as either weak or moderate biofilm formers, a reduction in biofilm-formation classification. The antibiofilm activity was enhanced when the CFS of different lactobacilli were used as a cocktail. All the tested lactobacilli exhibited anti-listerial properties, specifically the antibiofilm activity, thereby presenting as a safe alternative for containment of the spread of *L. monocytogenes* in food processing facilities.

2.2 Introduction

Listeria monocytgenes is a Gram positive, foodborne pathogen that is identified through cases of invasive disease, and linked to sporadic infections and outbreaks in certain food safety programs (Drevets and Bronze, 2008). *L. monocytogenes* poses a serious concern due to its adaptability features and prevalence under many stress conditions and different food storage areas. Refrigerators are the most common method of food storage and yet *L. monocytogenes* continues to overcome the surrounding low temperatures inducing persistence and dissemination in stored food products (Santos *et al*., 2019). This allows it contaminate various food products, including among others, ready-to-eat (RTE) foods such as luncheon meats and soft cheeses and *L. monocytogenes* contamination occurs (Rocourt *et al*., 2003; Ivanek *et al*., 2006). Once ingested it poses a greater risk of listeriosis disease infection for consumers in these susceptible populations groups: elderly, infants, pregnant women and immunocompromised patients - relative to the general population (Pizarro-Cerdá *et al.,* 2012). The detrimental effects of this pathogen has prompted awareness and much needed study due to the characteristics it possesses.

The presence of *L. monocytogenes* in food processing facilities and environments, is the main root and source of spread. The 'Farm-to-fork' continuum within the food industry is heavily impacted as a result of *L. monocytogenes* present on surfaces, drains and pipes coming into contact with food products. Other contamination channels are via poor, inadequate hygiene and improper post-processing procedures. The transfer of this pathogen happens from operator's hands and gloves to scales, cutting boards and slicing machines (Hoelzer *et al*., 2012). Additionally, *L. monocytogenes* can form mature biofilms, which are three-dimensional architectural structures made up of a matrix composed of extracellular polymeric substances (EPS). They can also form multispecies associations by establishing interspecies interactions with other biofilms (Liu *et al.,* 2016). Biofilms are categorized as the most widespread mode of growth in both natural and industrial realms and provide protection to harsh environments (Santos *et al.,* 2019). *L. monocytogenes* possess traits that make it a tri-factor having pathogenic power, the ability to form biofilms and ubiquity, qualifying it as a huge risk to the health sector (Esbelin *et al.,* 2018).

Over time the used treatment for *L. monocytogenes* was antibiotics, which fell short of their efficacy due to resistance. Through recombination and horizontal gene transfer, the exchange of antibiotic resistance genes from other pathogenic bacteria is allowed (Sun *et al.*, 2019). This led

to the use of commercially available sanitizers, also referred to as biocides. These collectively encompass the following: quaternary ammonium compounds (QACs), chlorine and acid compounds, but have unfortunately lost their absolute potency. The tolerance capacity of *L. monocytogenes* to antibiotics and sanitizers remains increasingly unsettling and a huge concern for the food industry (Rodríguez-López *et al.,* 2018). All these factors allow this mischievous microorganism to survive and persist within the food facilities and necessitated more research into alternative methods to combat its growth. This search for alternative methods for control of *L. monocytogenes* led to the introduction of lactic acid bacteria (LAB) with certain probiotic characteristics as potential control agents.

Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit to the host' (FAO/WHO, 2001). They can be found in yoghurt and other foods as well as dietary supplements. The two most common groups these bacteria belong to are: *Lactobacillus* and *Bifidobacterium* (Butel, 2014). Probiotics employ various mechanisms to fight against pathogens, including among others, the production of bacteriocins and organic acids; competition for nutrients and space and interference with pathogenic adhesion (Bermudez-Brito *et al*., 2012). Production of lactic acid by LAB affords them the antagonistic property against *L. monocytogenes* growth (Mangell *et al.,* 2002). It has been observed in the study by Gómez *et al.* (2016) that the cell-free supernatants (CFS) of probiotics are able to inhibit the formation of biofilms of pathogens over specified time periods. However, it is known that the properties of the probiotics are strain specific. Therefore, the aim of the current study was two-fold. Firstly, it aimed to screen the biofilm formation abilities of the *L. monocytogenes* strains isolated from the food environments. Secondly, the study aimed to evaluate abilities of CFS of *Lactobacillus acidophilus* La14 150B, *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* ATCC 4356 to inhibit formation of biofilms by these *L. monocytogenes* strains or to disperse their pre-formed biofilms.

2.3 Materials and Methods

2.3.1 Bacterial cultures

Listeria monocytogenes strains Avo and Cuc from Probiotics Research Group, Department of Biochemistry, Genetics and Microbiology, University of Pretoria were previously isolated from a store bought avocado and cucumber, respectively. *L. monocytogenes* 243 isolated from an avocado processing plant, was obtained from the Department of Food Science and Biotechnology, University of Free State, Bloemfontein, South Africa (Sibanda and Buys, 2017) and *L. monocytogenes* ATCC 19115 was purchased from the American Type Culture Collection (ATCC). These *L. monocytogenes* strains were all used as test strains and grown on *Listeria*-enrichment agar plates and then sub-cultured twice into Brain Heart Infusion (BHI) broth and incubated at 37°C for 20-24 hours.

Lactobacillus acidophilus La14 150B, *L. plantarum* and *L. rhamnosus* ATCC 4356 glycerol stocks, obtained from the Probiotics Research Group, University of Pretoria, were used as test LAB cultures. *Lactobacillus* spp. were sub-cultured twice in de Man Rogosa and Sharpe (MRS) broth (Merck, South Africa), followed by incubation at 37°C for 72 hours in anaerobic jars containing Anaerocult A gaspacks with Anaerotest strips (Merck, South Africa). Before use in the experiments, LAB cultures were standardized to an optical density of 0.2 at 600 nm.

2.3.2 Screening and categorization of *L. monocytogenes* strains for biofilm formation abilities

Overnight cultures of each *L. monocytogenes* strain (Avo, Cuc, 243 and ATCC 19115) were prepared by inoculating 200 µl of strain into 10 ml of BHI followed by incubation at 37°C for 18 hours. The optical density of the cultures was adjusted to 0.2-0.25 at 594 nm. Then 200 µl of each *L. monocytogenes* overnight culture was transferred to separate wells of the 24-well clear polyvinyl chloride (PVC) microtiter plates. The BHI medium was added to three wells to serve as the negative control. The plates were incubated for 48 hours aerobically at 37°C and subsequently quantified to determine biofilm formation within the wells according to methods of Djordjevic *et al.* (2002) and Gómez *et al.* (2016), with minor modifications. Briefly, broth solution in the bottom

of the wells was discarded and then treated with 2 ml of $\frac{1}{4}$ strength Ringer's solution to remove loosely attached cells. The remaining cells attached to the wells were gently washed thrice with sterile distilled water and thereafter the plates were emptied, inverted and allowed to dry for 30 minutes. Each well was treated with 150 µl of 1% crystal violet dye and left to stand for 45 minutes at room temperature. Then any unbound dye was washed off with sterilized water five times and the wells were treated with 200 µl of 95% ethanol and incubated at 4°C for 30 minutes to solubilize and destain them. After the 30 minutes, 200 µl of the contents of each well was transferred to a new sterile PVC microtiter plate. The absorbance of the wells was measured using a SpectraMax ® Paradigm ® Multi-Mode Detection Platform microtiter plate reader at 594 nm (OD_{595}). Then the *L. monocytogenes* strains were classified as either a non-biofilm, weak, moderate or a strong biofilm producer according to (Borges *et al.,* 2012) as follows: non-biofilm producers (OD ≤ ODC), weak biofilm producer (ODC < OD ≤ 2 × ODC), moderate biofilm producer (2 × ODC < OD ≤ 4 × ODC) or strong biofilm producer (4 × ODC < OD). The ODC was 0.05.

2.3.3 Preparation of cell free supernatants (CFS) of Lactobacilli

For each Lactobacilli, 200 µl was inoculated into 10 ml of MRS broth in a glass test tube and incubated at 37°C aerobically for 24 hours in anaerobic jars containing Anaerocult A gaspacks with Anaerotest strips. Then the cell free supernatant (CFS) were prepared using the method of Beristain-Bauza *et al.* (2016), without modifications. Briefly, following incubation, the culture was centrifuged at 4000 x g for 10 minutes at 20°C and the supernatant was filtered through a cellulose nitrate filter of 0.2 µm.

2.3.4 Biofilm formation by *L. monocytogenes* strains in the presence of CFS of lactic acid bacteria (LAB)

An overnight culture of each *L. monocytogenes* strain was prepared by inoculating 200 µl of the strain into 10 ml of BHI broth separately; this was vortexed for 5 seconds and incubated aerobically for18 hours at 37°C. From the overnight culture, 200 µl was transferred to each of the twelve wells of a 24 well microtiter plate. Then 2 ml of each Lactobacilli CFS was subsequently added to nine

out of the twelve wells containing the *L. monocytogenes* cultures to test for inhibition of biofilms. The three remaining wells to which no CFS was added served as the positive control. An additional three wells containing 200 µl BHI served as the negative control. The microtiter plate was incubated aerobically at 37°C for 48 hours. Then the plate was washed to remove excess media and unbound cells, and then the biofilm was quantified according to the method by Djordjevic *et al.* (2002) and Gómez *et al.* (2016) stipulated in **2.3.2**. Each experiment was repeated in three independent trials, with each treatment done in triplicate.

2.3.5 Biofilm formation by *L. monocytogenes* strains in the presence of CFS cocktails of LAB

Two hundred microliters of each *L. monocytogenes* strain was inoculated into 10 ml BHI broth and incubated for 18 hours at 37°C which formed the overnight culture. Cocktails containing different combinations of equal volumes of CFS from the different Lactobacilli strains were prepared (Table 1). From the overnight culture, 200µl was transferred to the wells of a 24 well PVC microtiter plate and immediately followed by treatment with 2 ml of each CFS cocktail. The microtiter plate was incubated for 48 hours at 37°C and was washed and quantified according to the method by Djordjevic *et al.* (2002) and Gómez *et al.*, (2016) stipulated in **2.3.2**.

Table 2.1: The CFS cocktails obtained by mixing equal volumes of the supernatants of LAB overnight cultures

CFS cocktail description	***L. acidophilus* La14 150B (A)**	***L. plantarum* (B)**	***L. rhamnosus* ATCC 4356 (C)**
AB	+	+	-
BC	-	+	+
AC	+	-	+
ABC	+	+	+

2.3.6 Dispersion of preformed *L. monocytogenes* biofilms by individual or cocktails of CFS of LAB

An overnight culture of each *L. monocytogenes* strain was prepared by inoculating 200 µl of the strain into 10 ml of BHI broth separately. *L. monocytogenes* biofilms for each strain were allowed to form by adding 200 µl of each overnight culture to a 24 well clear PVC microtiter plate and given an opportunity to aggregate and adhere for 48 hours at 37°C. After incubation, the non-adherent cells were removed by gentle pipetting while taking care not to remove the biofilm formed. Then 2 ml of each individual CFS of different LAB was added to each well with an existing biofilm and plates were then incubated at 37°C for a further 48 hours. This experimental set up was repeated with the cocktail treatment containing different combinations of equal volumes of CFS from the different Lactobacilli strains (Table 1). The microtiter plates were washed and biofilms quantified according to the method by Djordjevic *et al.* (2002) and Gómez *et al.* (2016) stipulated in **2.3.2**.

2.3.7 Statistical analysis of data

All the experiments were performed in triplicates in three independent trials. The values reported are averages and standard error of the means. The software GraphPad Prism 8.4.1 was used to analyze the results to perform the two-way ANOVA (Analysis of Variance) followed by the Tukey's multiple comparisons test ($p < 0.05$).

2.4 Results

2.4.1 Biofilm formation profiles of the test *L. monocytogenes* strains

The biofilm formation capabilities of the selected *L. monocytogenes* strains determined using the crystal violet biofilm staining assay are shown in Figure 1. All the strains were able to form biofilms within the microtiter wells, with the different strains displaying varied strengths of biofilm production. Based on the Borges *et al.* (2012) biofilm classification, *L. monocytogenes* Avo and

L. monocytogenes 243 strains were classified as strong biofilm producers while *L. monocytogenes* Cuc and *L. monocytogenes* ATCC 19115 were classified as moderate biofilm producers. Overall all the *L. monocytogenes* strains isolated from the food environment were stronger biofilm formers than the *L. monocytogenes* ATCC 19115 strain. The order of the *L. monocytogenes* strains from the strongest to the weakest biofilm former was as follows: *L. monocytogenes* 243 > *L. monocytogenes* Avo > *L. monocytogenes* Cuc > *L. monocytogenes* ATCC 19115. The four strains were used in subsequent experiments to observe how their biofilm producing abilities will be affected by CFS of LAB.

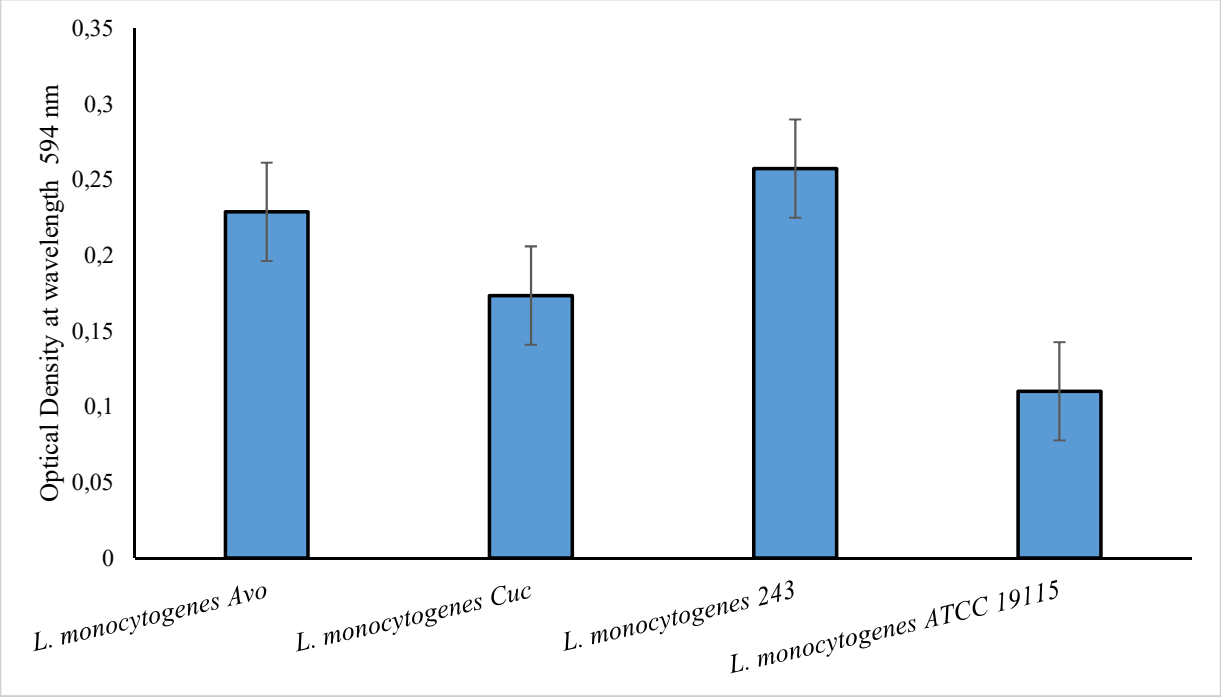

Figure 2.1: Biofilm formation profiles of the test *L. monocytogenes* strains.

2.4.2 Biofilm formation capabilities of *L. monocytogenes* strains in the presence of individual CFS of LAB

Figure 2 depicts the biofilm formation capabilities of *L. monocytogenes* strains when grown in the presence of CFS of individual LAB. The presence of CFS of LAB negatively affected biofilm formation of all *L. monocytogenes* strains, which was evident because in the absence of these treatments the strains formed dense/mature biofilms. All the *L. monocytogenes* strains post CFS

treatment were classified into weaker biofilm producer categories (Figure 2), indicating antagonistic abilities of the CFS. *L. monocytogenes* Avo and 243 were originally classified as strong biofilm formers but in the presence of all CFS they were categorized as weak biofilm formers. For *L. monocytogenes* Cuc, its moderate biofilm former status was demoted to a weaker category in the presence of all CFS treatments. Notably, *L. rhamnosus* ATCC 4356 CFS decreased the optical density measured at wavelength 594 nm (OD_{595}) of *L. monocytogenes* Cuc to below 0.05 meaning no biofilm formation occurred at all. *L. monocytogenes* ATCC 19115 was decreased from a moderate to a weak biofilm producer across all treatments too. Overall *L. acidophilus* La14 150B was the most effective with regards to inhibition of biofilm formation across all *L. monocytogenes* strains. This was concluded by the lower OD_{595} values recorded after treatment with *L. acidophilus* La14 150B and showed prominent inhibitory effects. *L. plantarum* CFS was the least effective of all the LAB in inhibiting biofilm formation, however, it still managed to change the classification of all the *L. monocytogenes* strains into a weaker category compared to the control (Figure 2). There were significant statistical differences ($p < 0.05$) between the OD_{595} values post-treatment with all three CFS in comparison to the control *L. monocytogenes* strains. However, there were no significant differences in inhibition of the biofilm formation by the CFS of the different LAB with each other ($p > 0.05$).

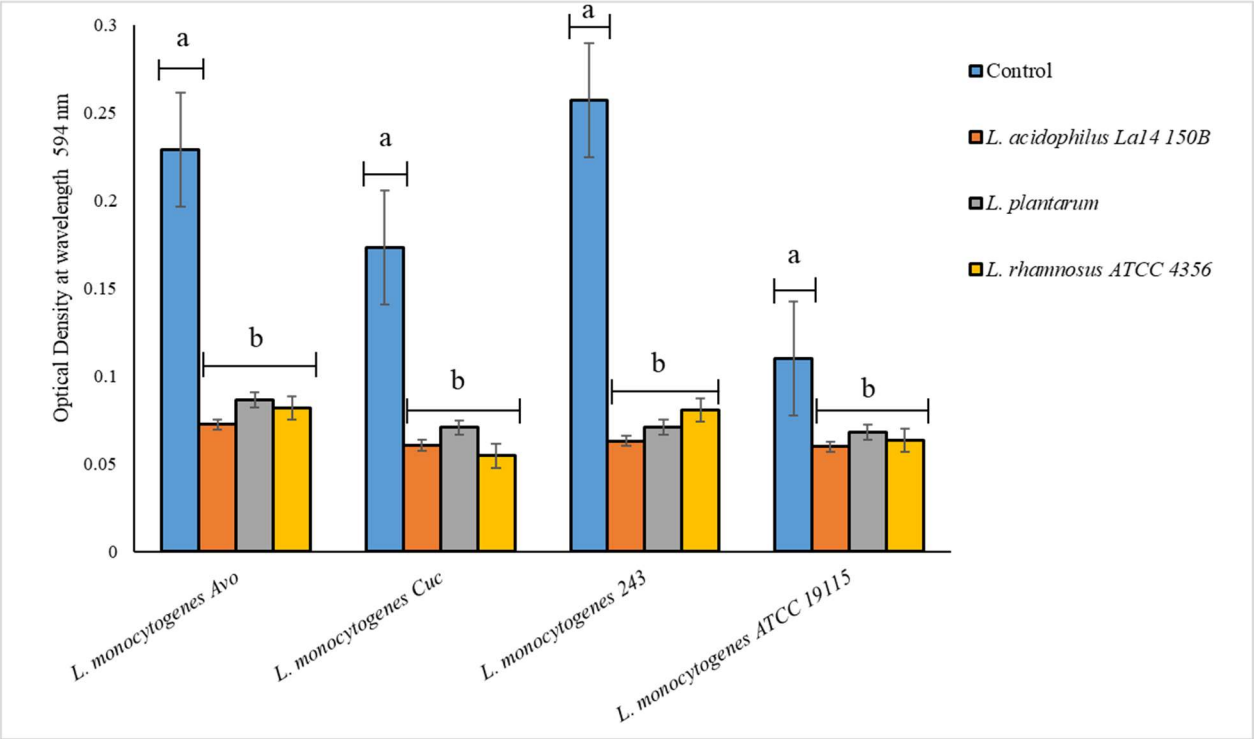

Figure 2.2: Biofilm formation capabilities of *L. monocytogenes* in the presence of individual cell free supernatants of lactic acid bacteria. Each bar represents the mean of triplicates from three separate trials and the error bars show the standard error. Bars represented with different letters are statistically different ($p < 0.05$), while those with the same letter have no statistical differences ($p > 0.05$)

2.4.3 Biofilm formation capabilities of *L. monocytogenes* strains in the presence of CFS cocktails of LAB

With the success of the CFS of individual LAB it was taken into consideration how their antagonistic activities could be further investigated. The cocktails created are outlined in (Table 1) and their effects on the *L. monocytogenes* are depicted in Figure 3. Similarly, to what was observed for CFS of individual LAB, the presence of the CFS cocktails classified the *L. monocytogenes* strains to weaker biofilm producing categories. Furthermore, the OD_{595} values post treatment with cocktails overall were lower than those obtained after treatment with individual CFS. Though the decrease was observed, statistically there were no significant differences in the biofilm inhibition

abilities of all the cocktails against the *L. monocytogenes* control strains. Of all the LAB CFS cocktails, cocktail BC (*L. plantarum* + *L. acidophilus* La14 150B) was the only cocktail to reduce a single strain (*L. monocytogenes* 243) from a strong to non-biofilm former. Interestingly and contrary to what was anticipated, in the presence of cocktail ABC (*L. acidophilus* La14 150B + *L. plantarum* + *L. rhamnosus* ATCC 4356), *L. monocytogenes* ATCC 19115 was promoted to a strong biofilm producer from a moderate category. Despite this alarming results obtained for *L. monocytogenes* ATCC 19115, for *L. monocytogenes* Avo and Cuc strains, the biofilm production was reduced, as indicated by the lowest OD_{595} values obtained, in the presence of cocktail ABC. The OD_{595} levels for *L. monocytogenes* Avo and Cuc strains in the presence of cocktail ABC were also the lowest in comparison to those in the presence of the other cocktails while for *L. monocytogenes* 243 the OD_{595} level was lowest in the presence of cocktail BC.

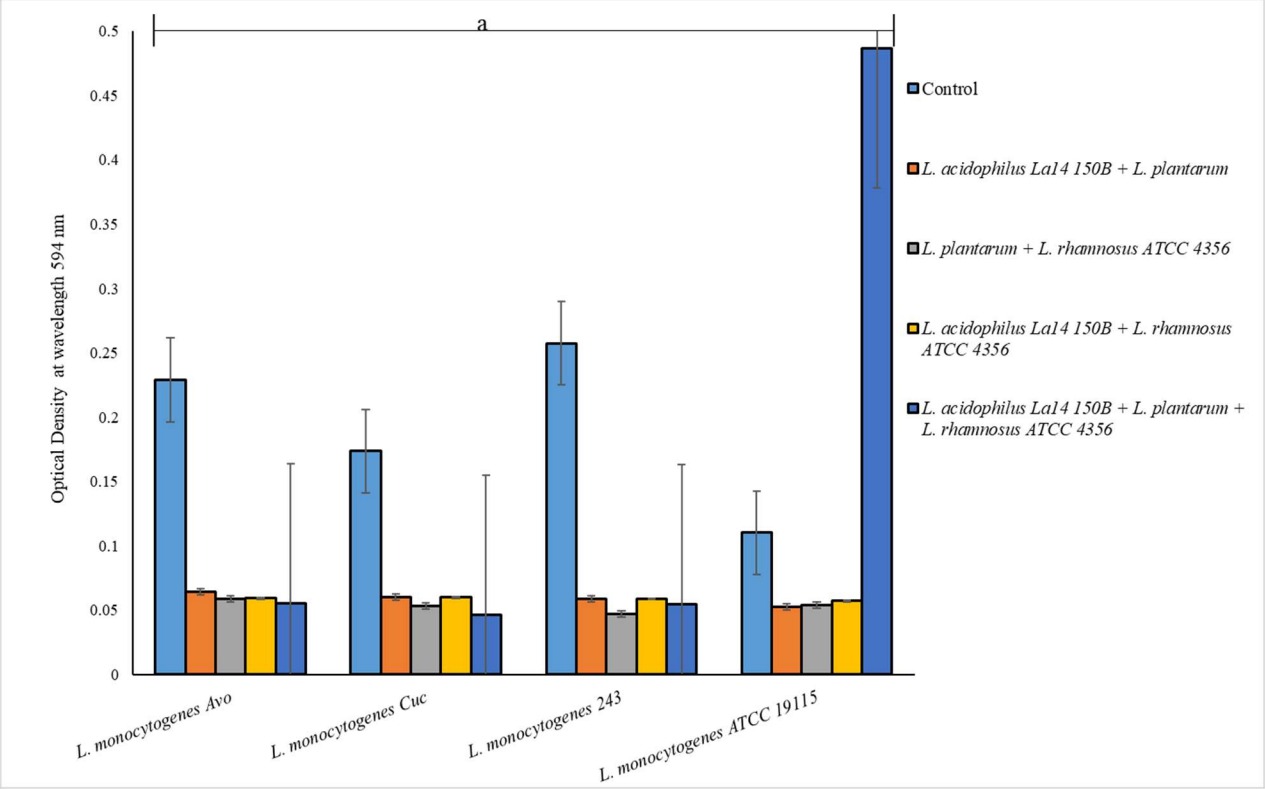

Figure 2.3: Biofilm formation capabilities of *L. monocytogenes* strains in the presence of cell free supernatants cocktails of lactic acid bacteria. Each bar represents the mean of triplicates from three separate trials and the error bars represent standard error. The 'a' represents statistical insignificance ($p > 0.05$).

2.4.4 Dispersion of preformed *L. monocytogenes* biofilms by CFS of individual LAB

We further investigated the ability of CFS to disperse *L. monocytogenes* biofilms already formed within the microtiter plates. Following the treatments with the CFS, the preformed *L. monocytogenes* biofilms were not completely dispersed but were classified into weaker biofilm forming categories. Individual CFS of *L. plantarum* and that of *L. rhamnosus* ATCC 4356 reduced the biofilm forming category of *L. monocytogenes* Avo from strong to moderate, while CFS *of L. acidophilus* La14 150B reduced it to a weak biofilm former (Figure 4). Both *L. monocytogenes* Cuc and ATCC 19115 strains were changed from the moderate biofilm former category to a weak biofilm former after treatment with individual CFS of all LAB.

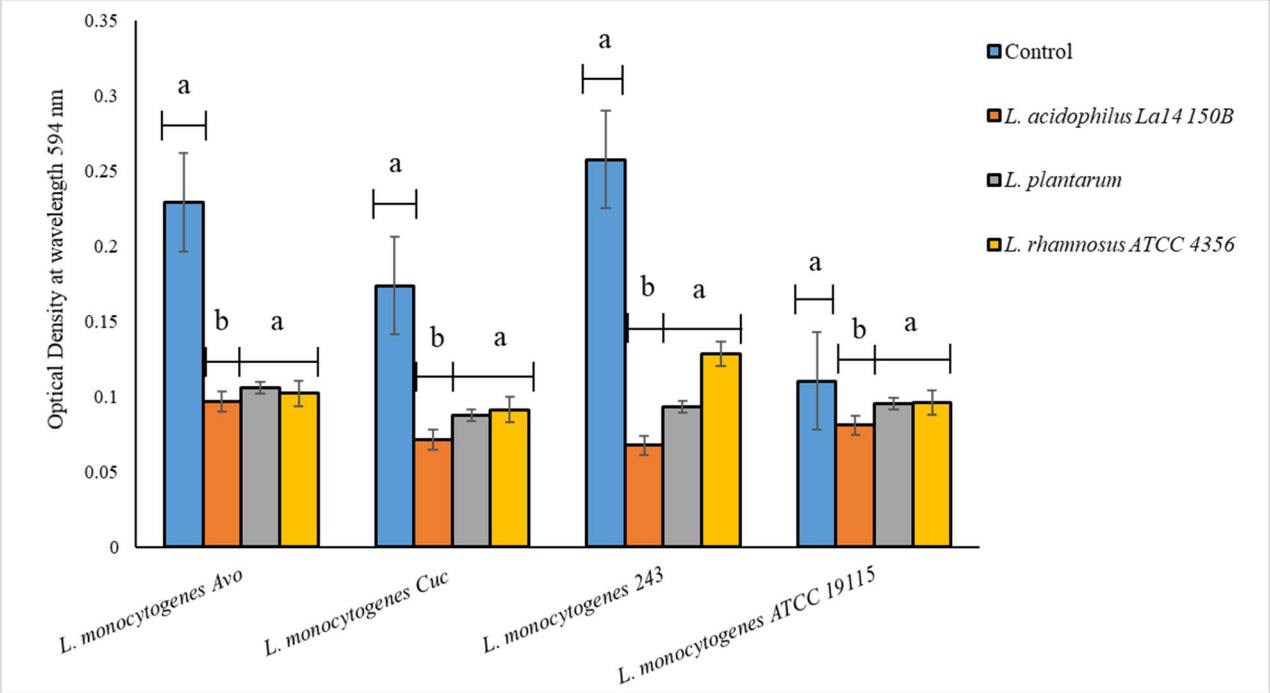

Figure 2.4: Dispersion of preformed *L. monocytogenes* biofilms by cell free supernatants of individual lactic acid bacteria. Each bar represents the mean of triplicates from three separate trials and the error bars represent standard error. Bars represented with different letters are statistically different ($p < 0.05$), while those with the same letter have no statistical differences ($p > 0.05$)

Overall, *L. acidophilus* La14 150B was the most efficient in dispersing preformed *L. monocytogenes* biofilms with significantly reduced OD_{595}values reflected ($p < 0.05$) when compared to OD_{595} values in absence of CFS treatment, as well as after treatment with CFS of *L. plantarum* and *L. rhamnosus* ATCC 4356. While *L. rhamnosus* ATCC 4356 was the least efficient. No significant differences were recorded for biofilm dispersion capabilities after treatment of pre-formed biofilms of all the *L. monocytogenes* strains with CFS of *L. plantarum* and *L. rhamnosus* ATCC 4356 when compared to the control ($p > 0.05$). None of the individual CFS of the tested LAB was able to completely disperse pre-formed biofilms of all the *L. monocytogenes* strains.

2.4.5 Dispersion of preformed *L. monocytogenes* biofilms by CFS cocktails of LAB

Figure 5 depicts the biofilm formation categories of the different *L. monocytogenes* strains as classified after their pre-formed biofilms were treated with CFS cocktails of the tested LAB. All the CFS cocktails significantly dispersed pre-formed biofilms ($p < 0.05$) when compared to the control and were more effective in dispersal than the individual CFS (Figure 4) indicative by lower OD_{595} values. All *L. monocytogenes* strains were classified as weak biofilm producers in the presence of the CFS cocktails, exhibiting their stellar dispersion abilities (Figure 5). For *L. monocytogenes* 243 and ATCC 19115 the cocktail treatments resulted in similar OD_{595} values reflecting consistency in CFS dispersal of the strains. Overall, the cocktail ABC (*L. acidophilus* La14 150B + *L. plantarum* + *L. rhamnosus* ATCC 4356) had the highest efficiency in dispersal abilities with the lowest recorded OD_{595} values across the *L. monocytogenes* strains. However, there were no significant differences in the disruption efficiency of the different CFS cocktails ($p > 0.05$) to each other. None of the cocktails changed the biofilm category of all the *L. monocytogenes* strains to non-biofilm formers.

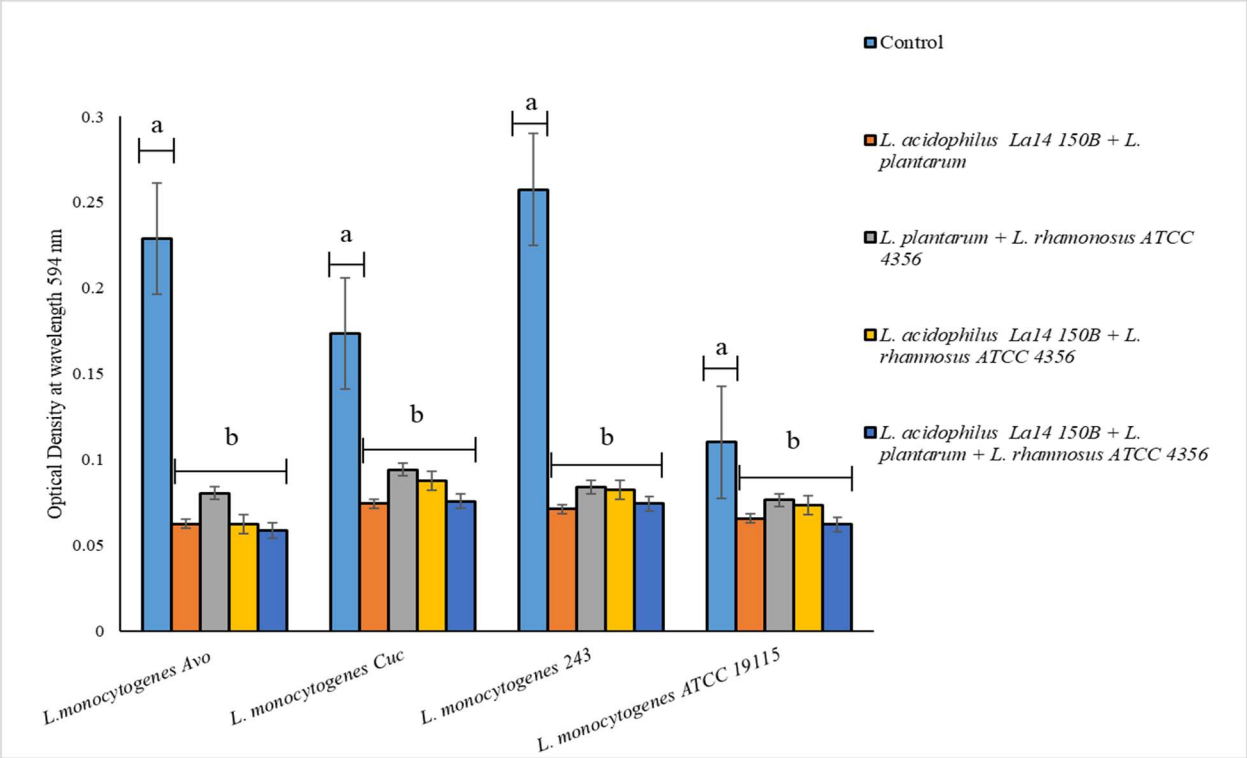

Figure 2.5: Dispersion of preformed *L. monocytogenes* biofilms by cell free supernatants cocktails of lactic acid bacteria. Each bar represents the mean of triplicates from three separate trials and the error bars represent standard error. Bars represented with different letters are statistically different ($p < 0.05$), while those with the same letter have no statistical differences ($p > 0.05$)

2.5 Discussion

The ever so adaptive *L. monocytogenes* continues to be a harmful pathogen with dire consequences for the population. The food industry relies on preservation methods including low pH, low water activity and low temperature and yet *L. monocytogenes* is able to survive these wide range of stress conditions resulting in a huge burden for producers (NicAogáin and O'Byrne, 2016). The key to the astounding persistence of this pathogen lies in the ability to exhibit strong virulence genes and exceptional transcriptional stress responses, and most importantly at the forefront the capacity to form biofilms (Colagiorgi *et al.*, 2016). Biofilms are the predominant mode of bacterial development in nature and are represented by the microbial cells that aggregate and ultimately attach to various surfaces. These sessile growing communities adhere to many areas in the food processing environments and can be present for years (Lee *et al.*, 2019). The *L. monocytogenes* biofilms come into contact with and contaminate food products leading to massive outbreaks with dangers of a large number of fatalities. Overtime antibiotics and sanitizers have been used for the inhibition and treatment of these biofilms, however, have been become ineffective due to resistance conferring a protective layer to the pathogen. This makes *L. monocytogenes* extremely hard to remove hence a new alternative had to be introduced. Since the discovery of lactic acid bacteria (LAB) and their safe use in fermentation processes, their application in bio-preservation made them a very attractive choice. Several studies began showcasing the antagonistic activities of LAB, De Martinis *et al*. (2001) reported that LAB from the family *Lactobacillaceae* inhibited the growth of *L. monocytogenes*. This propelled the indication that LAB and certain strains classified as probiotics, because of their therapeutic benefits, have impressive antilisterial activity.

In this study the *L. monocytogenes* strains from foods were first screened for biofilm formation abilities to assess their strength. All four strains were successful in forming biofilms but were classified differently. *L. monocytogenes* Avo and *L. monocytogenes* 243 strains were classified as strong biofilm producers. The classification ‘strong’ reveals that during the 48 hours’ time course an increased biofilm accumulation was observed. Whereas, *L. monocytogenes* Cuc and *L. monocytogenes* ATCC 19115 were categorized as moderate biofilm producers, indicative that during the incubation period only an adequate biofilm mass was observed with a slightly decreased number of viable cells aggregating together (Rodríguez-Lázaro *et al.,* 2019). Interestingly, all the *L. monocytogenes* strains isolated from the food and food processing facilities were stronger

biofilm formers than the *L. monocytogenes* ATCC 19115 strain; which concurs with the study by Rodríguez-Lázaro *et al.* (2019) that states isolates from food products have a significant capacity for forming biofilms with a possible higher protein content. These biofilms formed in food-processing facilities are often made up of a cluster of microbial communities for self-protection against unfavorable conditions and an exchange of information occurs mainly via quorum sensing using the *luxS* gene (Todorov *et al.,* 2018). The differences exhibited in the current study with regards to biofilm formation abilities of *L. monocytogenes* strains is dependent on multiple factors including the serotype of the strain which identifies based on cells surface antigens (Weiler *et al.*, 2013). Studies have observed how the phenotype of the biofilm is related to the clonal lineage due to specificities in the qualitative, quantitative and dynamic features expressed by the specific strain (Tasse *et al.*, 2018). In addition to those reasons, a study by Doijad *et al.* (2015) analyzed *L. monocytogenes* strains isolated from different sources and confirmed that the intensities of the biofilms formed correlated with the hydrophobicity, number of cells and amount of certain fatty acids of the strains. The fatty acid composition is increased in the aggregated form of the *L. monocytogenes* biofilm and suggested to play a role in the adhesion characteristics. The increase in fatty acid content causes a related increase in hydrophobicity and induces stronger biofilm formation (Gianotti *et al.*, 2008).

Additionally, in the study the evaluation of selected LAB: *Lactobacillus acidophilus* La14 150B, *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* ATCC 4356, as antibiofilm control for those *L. monocytogenes* strains was conducted. LAB has been considered an alternative for inhibition of biofilm formation of *L. monocytogenes* on account of the principal functional probiotic properties including tolerance to different stress conditions like acidic environments; and more importantly the antagonistic activity towards intestinal pathogens (Wan *et al.*, 2016). LAB also produce a wide range of metabolites such as a high level of organic acids – lactic and acetic acid (Tyler *et al.*, 2016). All three LAB in the current study pertain specific beneficial microflora. *L. acidophilus* is able to reduce gastrointestinal symptoms in lactose-intolerant individuals and relief symptoms of constipation (Gopal, 2011); *L. plantarum* has antioxidant and anti-inflammatory properties (Arasu *et al*., 2016); and *L. rhamnosus* strengthens the gut by treating diarrhea and also conferring protection against cavities (Han *et al.,* 2019). The mechanisms they use to promote gut health include nutrient competition, converting sugars to organic acids, competition for adhesion sites, alteration of pH and formation of a biological barrier that protects

the epithelial cells of the host (Emese *et al.,* 2011). The CFS of these LAB were deliberately used and purified to exclude the bacterial cell particles in the pellet and be left with the proteinaceous solution without any DNA.

In the current study *L. monocytogenes* Avo and 243 were both classified as strong biofilm producers and the remaining two *L. monocytogenes* Cuc and *L. monocytogenes* ATCC 19115 being categorized as moderate biofilm producers. Post treatment with CFS of the individual LAB inhibition of biofilm formation resulted as all four *L. monocytogenes* strains were no longer classified as moderate/strong biofilm producers but as weak biofilm producers. These results are consistent with Ibarreche *et al.* (2014) who found that lactobacilli strains *L. curvatus* CRL1532 and CRL705 and *L. sakei* CRL1862 were able to control *L. monocytogenes* biofilm growth. Another study that concurs with ours is by Gómez *et al.* (2016), they showed how three lactobacilli strains inhibited the pathogenic growth and adhesion of *L. monocytogenes*. The outcome Gómez *et al.* (2016) observed was suggested to perhaps be a combination of biosurfactants and bacteriocin production coupled with the mechanism of pathogen exclusion which can be considered for the current study. The OD_{595} values reported in the current study were significantly reduced ($p < 0.05$) in the presence of CFS in comparison to their absence. A recent similar experiment performed by Hossain *et al.* (2021) showed related results in which LAB including *L. plantarum* exhibited high inhibitory levels of *L. monocytogenes* biofilm formation. The inhibitory mechanism intriguingly also managed to suppress virulence gene expression for the *L. monocytogenes* strains.

Collectively LAB managing to prevent *L. monocytogenes* biofilm formation may be attributed to the production of various antimicrobial substances such as antimicrobial peptides, organic acids, hydrogen peroxide, bacteriocins and natural preservatives present in the CFS (Pelyuntha *et al.,* 2019). It was revealed by Li and Zhao (2016) that the CFS of *L. plantarum* showed antimicrobial activity against the food pathogen due to bacteriocin presence in the CFS. The more important organic acids, lactic, citric and acetic acid, also play a prominent role in being the efficient inhibitor of the biofilm formation process (Akbas, 2015). These antimicrobial substances of LAB kill the target organism by increasing the permeability of the cytoplasmic membrane and can initiate a mode of action in controlling the *L. monocytogenes* biofilms through inhibition, competition, exclusion, and displacement (Camargo *et al.,* 2018). Though statistically there was no difference amongst the three LAB themselves post treatment ($p > 0.05$), *L. acidophilus* La14 150B was the

best in inhibiting the formation of the biofilm with regards to lower OD_{595} values observed. Second was *L. rhamnosus* ATCC 4356 and lastly was *L. plantarum*. This might be as result of *L. acidophilus* not being affected by environmental stress factors promoting the antagonistic properties as concluded by Pereira and Gómez (2007). Interestingly Kim *et al.* (2006) discovered that the *L. acidophilus* can interfere with the exopolysaccharide and influence biofilm formation in *L. monocytogenes*. LAB have continued to show their excellent properties and capabilities in being able to disrupt the growth of pathogenic bacteria.

Cocktails of the CFS of the LAB were produced to assess the net result and compare to the prior individual treatment for the inhibition of *L. monocytogenes* biofilm formation. The choice to incorporate cocktails was prompted by the significant success seen previously with the single CFS of LAB, reflecting exceptional significant differences. In addition, two studies by Vinderola *et al.* (2014) and Gómez *et al.* (2012) showcased how potentially probiotic LAB combined can act synergistically or even have an additive effective in the antimicrobial activity against *L. monocytogenes*. Statistically there was no significant differences ($p > 0.05$), most likely as a result of the outlier of the combination ABC treatment (*L. acidophilus* La14 150B + *L. plantarum* + *L. rhamnosus* ATCC 4356) on *L. monocytogenes* ATCC 19115 (Figure 3), which surprisingly promoted it from a moderate biofilm former to a stronger biofilm contrary to the anticipated weaker characterization. Despite that, the cocktail treatments yielded lower OD_{595} values across other strains in comparison to individual CFS treatment and classified all the strains to weak biofilm formers. This indicated that the different *Lactobacillus* spp. strains combined contributed to a greater net effect in the inhibition of biofilm formation; seen by the lower ranks *L. monocytogenes* placed within the weak biofilm-forming category. Mixture of all LAB expressed a synergistic effect and this could have been due to all the antimicrobial metabolites present in the CFS. Our study concurs with Koo *et al.* (2012), where CFS of three LAB strains were combined and an increased activity in antilisteria was observed - the growth of *L. monocytogenes* was reduced by a log value of 0.6. The mechanism suggested to explain these results is that the combination of LAB leads to a decrease in pH and the enhancement of organic acid production including other metabolites hydrogen peroxide and bacteriocins. These promoted levels of antimicrobial substances assist greatly in the heighted ability to disrupt the formation of *L. monocytogenes* biofilms (Lim *et al.*, 2011). A mixture of LAB induces a significant synergistic

stimulatory effect and raises its antibiofilm mechanism of action (Chorostowska-Wynimko *et al.*, 2001).

In the final part of the current study the effect of CFS of individual and cocktails LAB on dispersion of preformed *L. monocytogenes* biofilms was investigated. The strains were given a period of 48 hours to form in their optimum growth temperature of 37□ thus giving them time to adhere to the surface before being treated with the individual or cocktails of the CFS. All three CFS of the individual *Lactobacillus* spp. dispersed the preformed biofilms. The *L. monocytogenes* strains were classified into lower categories either of weak or moderate biofilm formers. CFS managed to dismantle the biofilm structure and remove some of the cells aggregated together. This was in agreement with a study by (Zhao *et al.*, 2013) which found the treatment with LAB eliminated detectable levels of preformed *L. monocytogenes* biofilms. This substantial reduction was attributed to the colonization of the pathogen allowing mitigation of its persistence. Another study by Jara *et al.* (2020) gave us further insight as how LAB possibly achieves this, their study indicated how Lactobacilli microcolonies are able to trap *L. monocytogenes* cells and prohibit them from assembling together. *L. acidophilus* La14 150B was the most efficient in diminishing the biofilms fixed on the PVC microtiter plate and was significantly different ($p < 0.05$) in comparison to the other two treatments by *L. plantarum* and *L. rhamnosus* ATCC 4356

The categorization of all the *L. monocytogenes* strains as weak biofilm producers after treatment (Figure 5) with CFS cocktails is consistent with the knowledge that a combination of the LAB can create a synergistic effect that is more powerful against a pathogen (Lim *et al.,* 2011). As expected the CFS cocktails dispersed the *L. monocytogenes* significantly ($p < 0.05$) better than the individual treatment with lower OD_{595} values being reflected. In two related studies by Qiao *et al.* (2008) and Wang *et al.* (2015), accumulated lactic acid present in the cocktail CFS of LAB may be responsible for the dispersal of pathogenic biofilms such as of *L. monocytogenes.* This may be achieved by the lactic acid destroying the biofilm structure causing cell membrane damage that results in protein leakage. Most often it is extremely difficult to disperse a biofilm when attached to a surface, but the CFS cocktails in the currently managed to do so. The high biofilm removal activity of CFS can be related to certain released anti-biofilm compounds that include exopolysaccharide and biosurfactants (Kim and Kim, 2009). The production of biosurfactants, which are amphiphilic compounds that reduce surface tension, by LAB, may explain the success of dispersing the *L.*

monocytogenes biofilms. Biosurfactants are important in the food industry because they reduce adhesion of *L. monocytogenes* to glass and silicon rubber, therefore, limiting the spread of this pathogen (Gómez *et al.,* 2016).

In addition to biofilm inhibition, the food industry may explore CFS of LAB as a bio preservation methods using the antimicrobial metabolites present for the improvement of food safety. This may be an intriguing alternative because the food products will be able to retain their sensory qualities such as flavor, colour, nutritional value and texture with no associated risk (Reis *et al.,* 2012; Camargo *et al.,* 2018).

2.6 Conclusion

The use of cell-free supernatants (CFS) of LAB is a promising viable alternative for the control of *L. monocytogenes* biofilms in food processing facilities. The study showed how effective using *Lactobacillus* species is for the prevention and dispersal of biofilms. Crucial antagonistic behavior was exhibited by the LAB and the results found are instrumental to the food industry and provides a means to ensure food security. The LAB used in their CFS form were able to significantly ($p < 0.05$) reduce the formation of biofilms. We can conclude that *L. acidophilus* La14 150B was the most efficient at inhibiting the formation of biofilms with regards to individual treatment. The cocktails performed better in comparison to the individual treatment proving much more potent as an antagonist. The cocktail ABC (*L. acidophilus* La14 150B, *L. plantarum* and *L. rhamnosus* ATCC 4356) was the most potent of the three against the biofilms. With this information it would be advantageous when developing other future cocktails to consider the inclusion of *L. acidophilus* La14 150B knowing g its great antilisterial levels shown in the current study. Further knowledge has been provided on how LAB with probiotic properties can combat persistence of extremely detrimental biofilms that contaminate food products during processing. This can assist the environments and facilities that handle food products to relieve the pressure and economic burden introduced with the presence of *L. monocytogenes* biofilms.

2.7 References

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Chapter 3

Efficacy of cell free supernatants of different Lactobacilli against *Listeria monocytogenes* biofilms formed on various surfaces

3.1 Abstract

Listeria monocytogenes adheres differently to surfaces, which in food-processing facilities affects its transmission to food products. Many such surfaces that food products come into contact with exist in these food-processing environments, ranging from rubber to glass and Teflon. These surfaces are classified as either hydrophilic or hydrophobic, measuring the level of free surface energy determining how loose or tightly the bacteria will attach. Successful cleaning of biofilms off contact surfaces differs, being influenced by how vigorous the biofilm has adhered to the surface. It is therefore important to test the efficacy of control agents for removing *L. monocytogenes* biofilms formed on various surfaces. This study investigated the potency of cell free supernatants (CFS) of selected lactic acid bacteria (LAB) (*Lactobacillus acidophilus* La14 150B, *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* ATCC 4356) for removal of *L. monocytogenes* ATCC 19115 and 243 biofilms attached to different surfaces. The biofilms were allowed to form for 48 hours on two different surfaces; polyvinyl chloride (PVC) (hydrophobic) and stainless steel (hydrophilic), and then treated with the CFS to assess biofilm dispersal abilities. The integrity of the biofilm was viewed using Scanning Electron Microscopy (SEM) after treatment with CFS. A stronger adhesion to the stainless steel surfaces by the biofilm pre-treatment was observed in comparison to the PVC. Cell free supernatant of all three LAB were able to disperse biofilms of both *L. monocytogenes* strains, indicated by a change from the initial clustered, thread-like formed biofilms to isolated, scattered cells. *L. acidophilus* La14 150B exerted the greatest dispersal capabilities overall being able to disintegrate the assembled biofilm structures. Cell free supernatants of the tested LAB have the potential as alternative safer agents for control of *L. monocytogenes* on different surfaces used in the food-processing environments.

3.2 Introduction

Listeria monocytogenes is a pathogen that contaminates a variety of food products consumed by the public, resulting in its detrimental disease, listeriosis, in consumers. Though its outbreaks are not very common, the numbers of hospitalizations and fatalities occurring from its infections are very high. These are detected from reported cases of abortion in pregnant women and complications for the elderly and young children (Galié *et al.*, 2018). *L. monocytogenes* is able to persist within various food-processing facilities with a wide range of stresses such as low pH, temperature and water activity; making it excessively problematic for the food industry which relies on the mentioned stresses for food preservation (NicAogáin and O'Byrne, 2016). The ability of this intracellular pathogen to replicate in adverse conditions promotes the risks associated with its presence.

A few examples of food products that *L. monocytogenes* may be found in include: fruits, unpasteurized milk, ready-to-eat (RTE) products, frozen vegetables, meat and dairy products. Noticeably, the most commonly reported contaminated foods have been narrowed down to fresh cheese, smoked fish and cold-cut meats which come into contact with the pathogen during processing (Rothrock Jr *et al.*, 2017). The source of entry into the food facilities is mostly via water, soil, personnel clothing and equipment (NicAogáin and O'Byrne, 2016). *L. monocytogenes* then spread over to locker rooms, hallways and toilets. Inadequate cleaning methods escalate its multiplication in most areas, particularly on surfaces.

L. monocytogenes growth gives rise to biofilms, a consortium of cells attached to each other, which form and adhere to industrial surfaces. This happens more frequently where food residues accumulate and *L. monocytogenes* gains an easier access to nutrients enhancing cell aggregation (Colagiorgi *et al.,* 2017). The biofilms form quickly in a three-step process that first involves conditioning the surface and binding the cells to that surface, a step that irreversible. Secondly, irreversible micro-colonies develop and form on the surface and thirdly; the biofilm's tridimensional structure is built creating a complex ecosystem ready for dispersion (Srey *et al.*, 2013; Coughlan *et al.*, 2016). The surfaces to which biofilms adhere include polytetrafluoroethylene (PTFE) used in conveyor belts; polyester used as a floor sealer; stainless steel used for the majority of the equipment, polystyrene as a material for the drains; rubber used in joints, wood and also glass (Abdallah *et al.*, 2014; Ripolles-Avila *et al*., 2019). All mentioned

surfaces are either hydrophobic or hydrophilic, determined by the surface energy. When the surface energy is low, the molecules in the water droplets are more tightly attracted to each other versus to the surface (Chieng *et al.*, 2019). Therefore, the selection of surface materials used in food processing environments is crucial as it has an effect on pathogenic biofilm adherence. Nevertheless, many different materials are used in the food processing facilities.

The control of *L. monocytogenes* biofilms on surfaces is a major challenge as it demands sufficient cleaning and the application of adequate sanitization procedures, which at times is not completely achieved. This hurdle is primarily due to the resilience biofilms confer via the prominent extracellular matrix. Biofilms allow for the transfer of cell molecules and offer the embedded cells protection against toxic compounds (Galié *et al.*, 2018). The *L. monocytogenes* biofilm matrix can sense and respond to the physicochemical stresses it encounters by a transcriptional response resulting in homeostatic functionality (O'Byrne and Karatzas, 2008).

The commonly used treatment to control the spread of *L. monocytogenes* biofilms in food processing facilities are sanitizers or otherwise referred to as biocides. These sanitizers include: quaternary ammonium compounds (QACs), peracetic acid, hydrogen peroxide and sodium hypochlorite. The sanitizers operate by acting as oxidizing agents creating reactive oxygen species (ROS) to damage the cell membrane and cellular components. These positively charged water soluble compounds of sanitizers ultimately cause bacterial lysis (Jennings *et al.*, 2015). Unfortunately, overtime *L. monocytogenes* biofilms have gained physical, chemical and mechanical resistance to sanitizers, a problem that inspired a search for alternative biofilm control agents, which led to the introduction of lactic acid bacteria (LAB) with certain probiotic properties as a possible alternative control (Flemming *et al.*, 2016). Studies by Lobos *et al.* (2009) and Gómez *et al.* (2016) revealed that the bactericidal effect of sanitizers on bacterial biofilms were improved by LAB, reports which further encouraged research into the use of probiotics as an advantageous treatment. The current study aimed to investigate the potency of CFS of selected LAB (*Lactobacillus acidophilus* La14 150B, *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* ATCC 4356) against *L. monocytogenes* biofilms attached to PVC (a hydrophobic surface) - and stainless steel (a hydrophilic surface).

3.3 Materials and Methods

3.3.1 Bacterial cultures

The preparation of the bacterial cultures *Listeria monocytogenes* ATCC 19115 and 243; and LAB *Lactobacillus acidophilus* La14 150B*, Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* ATCC 4356 were conducted as described in Chapter 2, section **2.3.1**.

3.3.2 Preparation of *L. monocytogenes* bacterial suspensions

The preparation of the bacterial suspension of the *L. monocytogenes* strains and methods of inoculation on food surfaces were done according to Milanov *et al*. (2009) with modifications. Briefly, overnight cultures of both *L. monocytogenes* strains (ATCC 19115 and 243) were prepared separately by inoculating 200 µl of the culture into 10 ml of BHI broth, followed by incubation at 37°C for 18 hours. Each culture was serially diluted up to 10^{-6} dilution using ¼ strength Ringer's solution. Then 100 µl of the10^{-4}, 10^{-5} and 10^{-6} dilutions were plated onto BHI agar plates, and then and the plates were incubated at 37°C for 24 hours. Subsequently, three to four isolated colonies grown on the BHI agar were inoculated into 3 ml of Tryptone soy broth with 0.6 % yeast extract (TSB-YE) in a glass test tube. The test tubes were incubated for 24 hours at 25°C. The optical density of the inoculum was adjusted to 0.2-0.25 at 594 nm by spectrophotometry before use in experiments.

3.3.3 Preparation of cell free supernatants (CFS) of the selected lactic acid bacteria (LAB)

The preparation of the CFS was conducted as described in Chapter 2, section **2.3.3**.

3.3.4 Preparation of various surfaces to be used for biofilm growth

The stainless steel was cut out into 2 cm x 2.5 cm rectangular coupons while PVC was cut out into circular coupons with a radius of 2 cm and total circumference of 12.57 cm. The coupons were boiled for 5 minutes in a detergent solution and then rinsed with distilled water five times to

remove any residual detergent. The coupons were then stored in 100% ethanol and passed through a flame prior to their use in the experiments.

3.3.5 Biofilm formation

The coupons of stainless steel were placed into separate wells of a sterile polystyrene 6-well plate where 100 µl of *L. monocytogenes* ATCC 19115 and 243 prepared bacterial suspensions were transferred onto each coupon surface separately. The same was done for the PVC coupons. All coupons were incubated at 25°C for 3 hours to allow adhesion to occur. Following the 3-hour incubation period, the non-adherent bacteria were removed by pipetting and washing with 3 ml of sterile Phosphate-buffered saline (PBS). Immediately after that, 300 µl of CFS of each LAB (*L. acidophilus* La14 150B, *L. plantarum* and *L. rhamnosus* ATCC 4356) was transferred as treatment onto each coupon surface and incubated for 1 hour at 25°C. The coupons were immersed in 5 ml of sterile TSB-YE within the wells and incubated for 7 days at 25°C. Every second day of the incubation the old medium from the wells was replaced with 5 ml of fresh TSB-YE.

3.3.6 Scanning electron microscopic (SEM) analysis of biofilms on stainless steel and PVC

Following 7 days of incubation the stainless steel and PVC coupons were removed from the wells and washed by mild pipetting with 3 ml of sterile PBS to remove the medium and non-adherent cells. The preparation of the samples for microscopy was done according to Booyens *et al.* (2014) with minor modifications. Briefly, the coupons were fixed using 2.5% glutaraldehyde in 0.075 mol-1 phosphate buffer (pH 7.4) for 30 minutes. They were subsequently washed three times in 0.15 mol-1 PBS before being dehydrated in a series of graded alcohol concentrations (30%, 50%, 70%, 90% and 100% ethanol) for 15 minutes each. They were further left in 100% ethanol for 30 minutes as the final dehydration step. All the coupons were left covered for 1 hour with a 50:50 hexamethyldislazane (HMDS) and 100% ethanol mixture. The mixture was removed and then HMDS alone was added covering the sample for 1 hour. This was removed and fresh HMDS was again added but this time the container was left open for the container to dry. The cells were critically dried for 24 hours before being coated with carbon. The stainless steel coupons were

coated directly. The PVC circular coupons were first mounted onto aluminum stubs and then coated with carbon. The resulting cells were viewed using a Zeiss Crossbeam 540 FEG and Zeiss 540 Ultra scanning electron microscope (SEM).

3.4 Results

3.4.1 Removal of *L. monocytogenes* biofilms on stainless steel coupons by CFS of LAB

Listeria monocytogenes ATCC 19115 formed clusters of mature biofilm groups with an adjoined honey-comb structure on the stainless steel surfaces (Figure 3.1 a). The number of cells bound to each other decreased after the treatment with the CFS of LAB with an overall difference in structural appearance observed. The decline in aggregation reflects anti-biofilm properties of the different LAB.

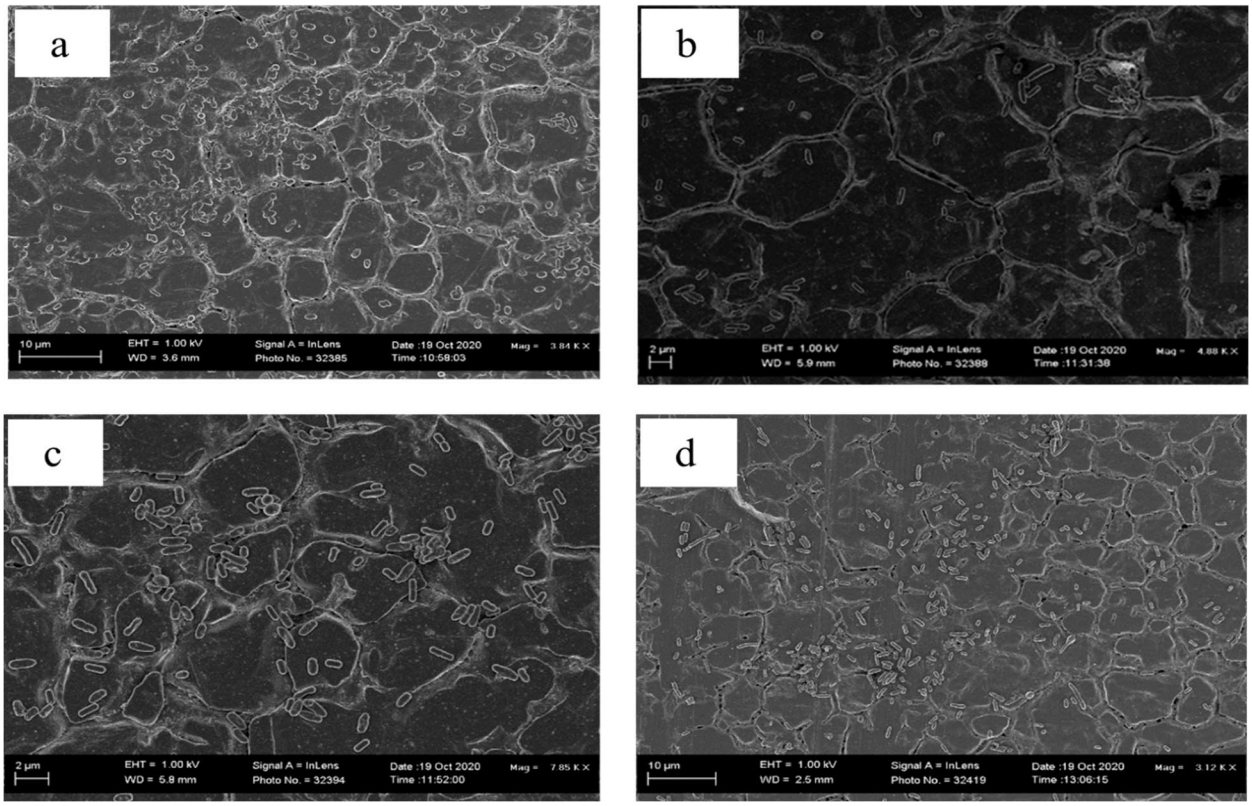

Figure 3.1: *L. monocytogenes* ATCC 19115 biofilms on stainless steel coupons after 7 days of incubation in TSB at 25°C (a) control, and after treatment with CFS of (b) *L. acidophilus* La14 150B, (c) *L. plantarum,* (d) *L. rhamnosus* ATCC 4356

The extent of biofilm disruption varied for the CFS of the different LAB (Figure 3.1 b, c & d). The CFS of *L. acidophilus* La14 150B was the most efficient in disrupting the biofilm structure of *L. monocytogenes* ATCC 19115, breaking the cell to cell attachments (Figure 3.1 b). *L. rhamnosus* ATCC 4356 was the least effective of the three CFS tested (Figure 3.1 d).

Listeria monocytogenes 243 also formed mature biofilms on a bed of honey-comb structures on stainless steel; with the biofilm composing of larger group of cells bound together (Figure 3.2 a) than those observed for *L. monocytogenes* ATCC 19115. Following the treatment with the all three CFS of the LAB, the *L. monocytogenes* 243 cells were more isolated and fewer counts of the larger groups were detected (Figure 3.2 b, c & d).

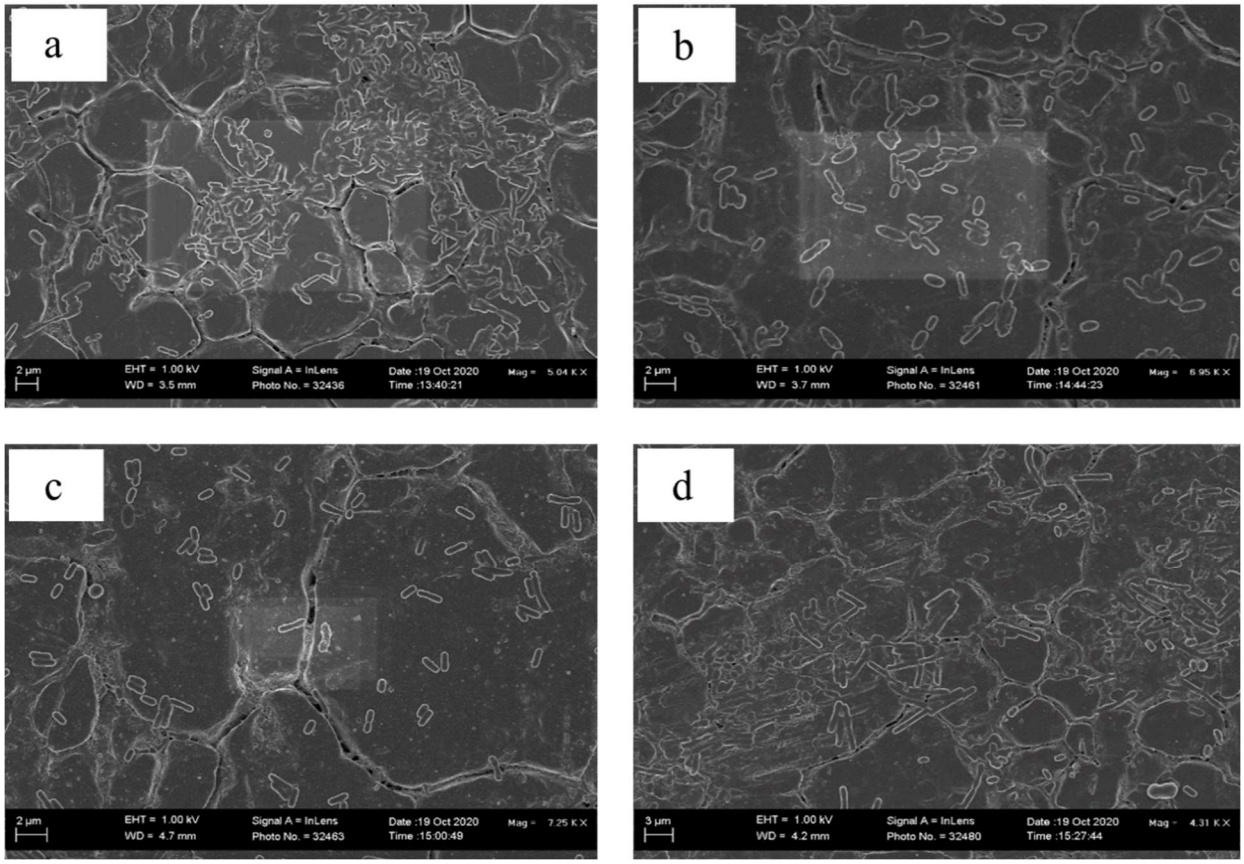

Figure 3.2: *L. monocytogenes* 243 biofilms on stainless steel coupons after 7 days of incubation in TSB at 25°C (a) control, and after treatment with CFS of (b) *L. acidophilus* La14 150B, (c) *L. plantarum,* (d) *L. rhamnosus* ATCC 4356

Interestingly, the CFS of *L. plantarum* exhibited the most effective anti-biofilm properties against *L. monocytogenes* 243 where the growth and attachment of cells to each other was dispersed (Figure 3.2 c). Similar to what was observed for *L. monocytogenes* ATCC 19115, the supernatant of *L. rhamnosus* ATCC 4356 was the most inefficient.

3.4.2 Removal of *L. monocytogenes* biofilms on PVC coupons by CFS of LAB

Figure 3.3 depicts the scanning electron images of *L. monocytogenes* ATCC 19115 on polyvinyl chloride (PVC). The PVC illustrates a difference in the way the *L. monocytogenes* strain grows and adheres in comparison to stainless steel with more swollen, dense structures observed. The untreated *L. monocytogenes* ATCC 19115 formed long bundle-shaped, three dimensional complexes with no honey-comb structures. The edges of the rod-shaped cells in the biofilm were not straight-line defined (flat), but were bulged (Figure 3.3 a). After treatment with the CFS of the LAB (Figure 3.3 b, c & d) the scattering of the textured and bulged aggregated cells was observed. The cell mechanism of joining together in a thread-like manner was disrupted in the presence of CFS of LAB, leading to the disappearance of the bundle structure that was observed for the untreated biofilm. Overall, as was observed for the stainless steel coupons, the biofilm of *L. monocytogenes* ATCC 19115 were increasingly more isolated post-treatment with CFS of *L. acidophilus* La14 150B (Figure 3.3 b) while the CFS of *L. rhamnosus* ATCC 4356 was less effective, depicted by presence of some clusters of cells after treatment (Figure 3.3 d).

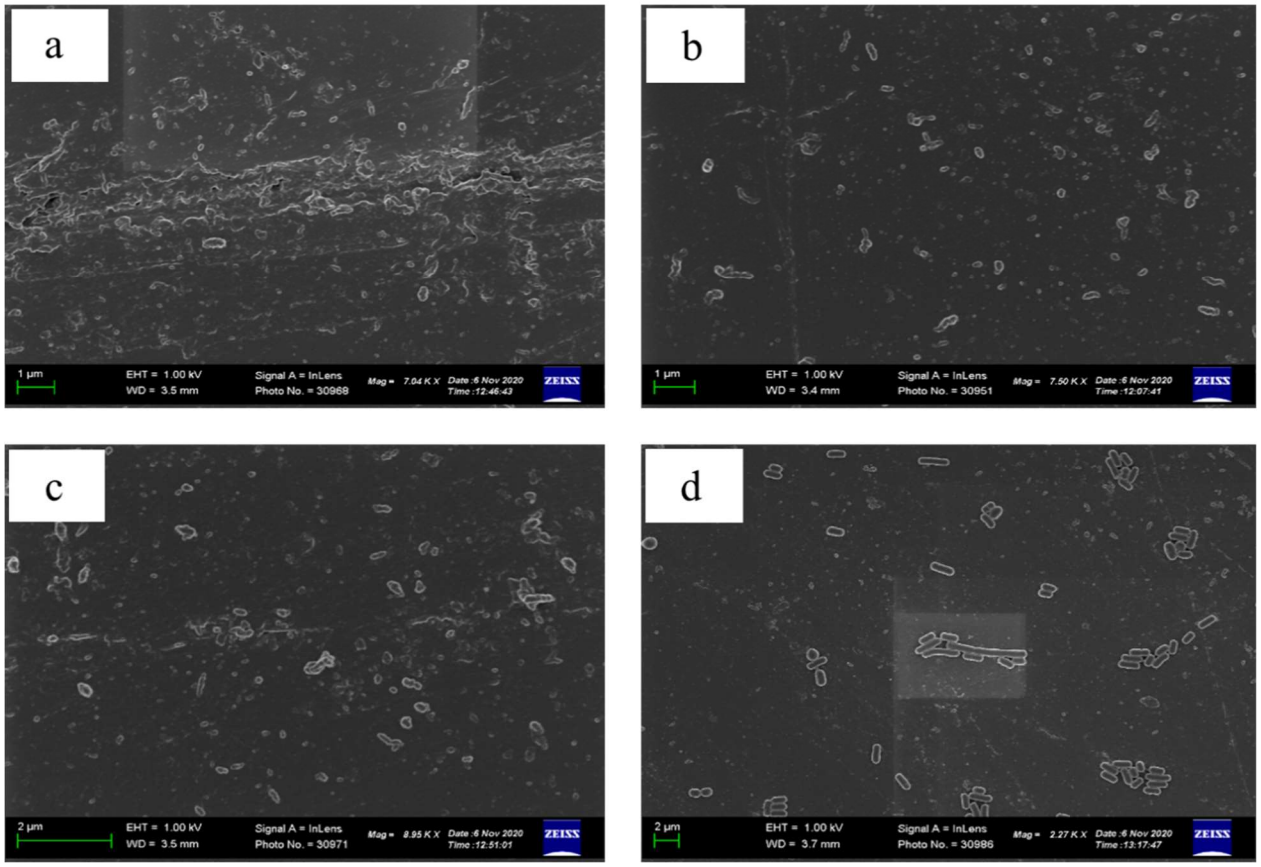

Figure 3.3: *L. monocytogenes* ATCC 19115 biofilms on PVC coupons after 7 days of incubation in TSB at 25°C (a) control, and after treatment with CFS of (b) *L. acidophilus* La14 150B, (c) *L. plantarum* (d) *L. rhamnosus* ATCC 4356

In Figure 3.4, the bulged rod-shaped *L. monocytogenes* 243 adhered to each other, creating a dense structure (Figure 3.4 a). All CFS of LAB exhibited the ability to interfere with and disrupt the aggregation of cells to each other (Figure 3.4 b, c & d). Treatment with CFS of LAB resulted in appearance of isolated rod-shaped cells with minimum thread like connection, a change from the untreated control micrographs. Overall *L. acidophilus* La14 150B dispersed the cells with the highest efficiency (Figure 3.4 b).

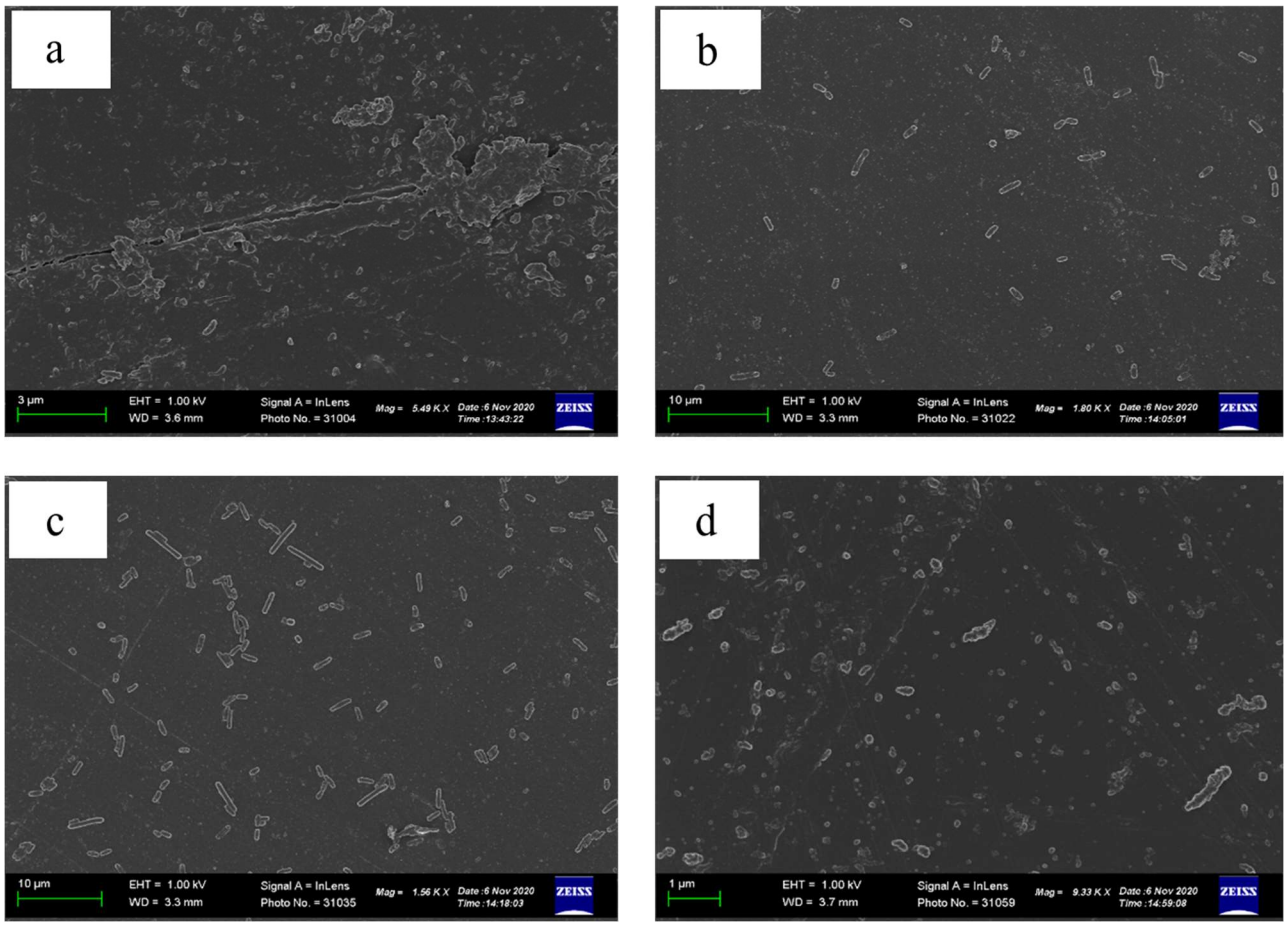

Figure 3.4: *L. monocytogenes* 243 biofilms on PVC coupons after 7 days of incubation in TSB at 25°C (a) control, and after treatment with CFS of (b) *L. acidophilus* La14 150B, (c) *L. plantarum,* (d) *L. rhamnosus* ATCC 4356

3.5 Discussion

It has been reported that *L. monocytogenes* can persist and adapt for extended periods of time in food processing facilities because of its ability to form biofilms. The biofilms formed on surfaces and food matrices have various composition and attachment styles and use certain genes in the production of the actual biofilm structure (Gurgu *et al.,* 2019). Prevalent strains of *L. monocytogenes* have a good adhesion ability due to the presence of flagella, pili and membrane proteins (Lemon *et al.,* 2007). The formation of biofilms happens in stages and the stage to be highlighted here is the fourth stage, maturation. During this stage, the biofilms develop into a rigid structure by means of the cellular production of extra-cellular polymeric substances (EPS) (Bogino *et al.,* 2013). The biofilms reach the point of maturation when the structures are crossed by channels or pores of water, ensuring an exchange of nutrients and metabolites (González-Rivas *et al.,* 2018). Even before reaching full maturity *L. monocytogenes* biofilms adapt and mitigate in harsh surroundings exhibiting strong stress survival mechanisms (Tasara and Stephan, 2006).

These mature formed biofilms pose a threat and have a tremendous direct negative effect in food processing environments. They affect: the functioning of mechanical parts that may be congested or blocked; energy consumption becoming higher as a result of decreased heat transfer levels and accelerated rate of corrosion of surfaces underneath the biofilms (Téllez, 2010). In food plants the types of surfaces that biofilms form on range from plastic, cement, metal, glass to wood (Trachoo, 2003). In the current study the stainless steel and polyvinyl chloride (PVC) were used as surfaces for growth of *L. monocytogenes* biofilms and then microscopy assessment was performed to determine how these biofilms were influenced by treatment with CFS of three different LAB strains. Stainless steel was selected to represent the hydrophilic group because the majority of surfaces and equipment in food processing facilities are made from it (Skåra and Rosnes, 2016). For the opposite group of surfaces, hydrophobic, PVC was the desirable choice as it is a versatile polymer widely used for the construction of door and window profiles and more significantly pipes and cable insulation. It is ranked the world's third largest thermoplastic material and more manufacturers are gravitating towards its utilization (Khomchu *et al.*, 2017).

Both *L. monocytogenes* ATCC 19115 and 243 were able to efficiently colonize the surfaces and form biofilms, but the way in which the biofilms formed on each surface was noticeably different. On the PVC the biofilms were densely compacted and the three-dimensional rod shapes had

irregular bulges on the edges. These differed from the biofilms on the stainless steel coupons. The *L. monocytogenes* rod cells were more clearly shaped and formed regular firm structures on stainless steel and were less clumped although the cells still managed to strongly and efficiently aggregate together onto the surface in a biofilm nature, forming a honey-comb like layer. The different characteristic with regards to growth patterns and attachment can be attributed to the respective wettability. Like glass, stainless steel is a hydrophilic surface and therefore has a higher wettability and level of free surface cell energy. This enables greater attachment of the bacteria and forms defined biofilms more easily (González-Carrasco *et al.*, 2019). The level of free surface energy influences the degree of biofilm contact with the physiological environment and affects the protein adsorption and adhesion (Puliyalil *et al*., 2019). For metals closely similar to stainless steel, bacteria growing on the surface can use iron and magnesium of the metal as terminal electron acceptors in respiration (Nealson and Finkel, 2011). The similarity between the biofilms of the test strains (*L. monocytogenes* ATCC 19115 and 243), was that the group of cells forming the biofilms were all binding in a tight adjacent manner, one cell to another and to the surface. The results of the current study were consistent with those found by Milanov *et al*. (2009) who investigated the ability of several *L. monocytogenes* strains to form biofilms on stainless steel. The structures of the biofilms produced in their study had various appearances from a confluent, uniform monolayer of bacterial cells to large, individual, three dimensional cell aggregates which were similar with the morphological biofilm structure range observed in our study.

The two *L. monocytogenes* selected strains adhered to and differently formed biofilms on the two different surfaces. This is not unusual as the ability to form biofilms generally differs between strains of *L. monocytogenes*. These differences are related to the individual features of the strains, their persistence and phylogenetic origin (Norwood and Gilmour, 2009; Djordjevic *et al*., 2002). Furthermore, the experimental conditions such as temperature, incubation period, composition of nutritive medium, the type and characteristics of the surface (substrate) are also crucial factors affecting biofilm formation (Milanov *et al*., 2009). Thus, the differences in biofilms formed by the different *L. monocytogenes* strains in the current study could be attributed to different strain features, as well as characteristics of the substrates. Kalmokoff *et al.* (2001) reported that *L. monocytogenes* Scott A strain did not form biofilms on stainless steel surface at room temperature in brain heart infusion broth. However, later on Marsh *et al.* (2003) demonstrated that at 35°C in Tryptone Soy broth the exact same strain, Scott A, formed a biofilm with a three-dimensional

'honey-comb' structure. The honey-comb structures observed by Marsh *et al.* (2003) were consistent with the biofilm features observed on stainless steel coupons in the current study. Furthermore, findings of this study correlated with those of a recent study by Kıran *et al*. (2021), who investigated co-incubation of strain *L. monocytogenes* 32 and CFS of *L. plantarum* on three different surfaces, namely, stainless steel, polypropylene and PVC. They reported that the dense three-dimensional structures were formed in the absence of CFS being in agreement with Renier *et al.* (2011) who previously confirmed the three-dimensional biofilm formation by untreated *L. monocytogenes*. However, post treatment with the CFS of *L. plantarum*, cells within the biofilm were greatly reduced and dispersed.

Chae and Schraft (2000) mentioned the significance surface type has on the adherence ability of *L. monocytogenes*. In their study they observed the EPS of biofilms formed by *L. monocytogenes* strains on glass surfaces. These biofilms had a two-layer structure with the cells binding in a thread-like manner to one another and to the surface. They attributed this structure formed on the hydrophilic surface, to static conditions used in the experiment, whereby the bacteria attached to the surface and produced daughter cells. This is insightful for the current study and suggests a possible explanation for the two-layer structure observed - (honey-comb layer plus thread-like bound cell layer) – on the hydrophilic stainless steel, as these coupons were was also incubated under static conditions. Oh and Marshall (1996) confirmed the rapid adherence capacity *L. monocytogenes* has on stainless steel than other surfaces and being able to reach irreversible stages within a few hours. Stainless steel surfaces absorb metabolites that LAB produce, such as bacteriocin nisin, and reduce the adhesion ability of food-isolated *L. monocytogenes* strains (Guerra *et al.,* 2009). Arevalos-Sánchez *et al*. (2012) corroborated this and indicated the efficacy of nisin to reduce adhesion of the foodborne pathogen on both glass and stainless steel surfaces.

Following the treatment with the different LAB, the biofilm structures were dispersed and less aggregation of the cells was observed. The LAB are able to engage in a diverse range of active competitive strategies to achieve dispersal including, among others, production of antimicrobial compounds and metabolites (released into culture media, hence present in CFS), interfering with the competitors signaling and motility; and finally by directly forcing the dispersal of the competitor (Falagas and Makris, 2009; Hibbing *et al.* 2010). Overall the CFS of LAB *L. acidophilus* La14 150B had the highest anti-listerial ability and more dispersal of preformed

biofilms was achieved in its presence in comparison to those of *L. plantarum* and *L. rhamnosus* ATCC 4356. *L. acidophilus* is able to combat pathogenic biofilms via its antimicrobial, antibiofilm and antiadhesive capabilities through production of biosurfactants; it may be able to do so more efficiently in comparison to the other LAB due to accelerated dispersal traits (Walencka *et al.*, 2008). These biosurfactants are structurally diverse surface-active amphipathic molecules in CFS of LAB. They reduce interfacial and surface tension and interfere with the contact angle (Mukherjee and Das, 2010; Satpute *et al.*, 2018). They operate through various antagonistic mechanisms, specifically: directly interfering with membrane functions and energy generating structures, decreasing the cell surface hydrophobicity, which reduces the level by which microbes can adhere to the surface, and lastly, they also enhance the cell permeability resulting in a leakage of metabolites (Surekha *et al.*, 2016). All these explain how microbial biofilm colonization can be inhibited but more importantly, for the current study, how dispersal may be achieved by all three LAB. When biosurfactant derived from *L. acidophilus* NCIM 2903 were investigated by Satpute *et al.* (2018), antibiofilm abilities were observed proving how LAB can destroy the integrity of pre-existing biofilms.

Dispersal of a pathogenic biofilm usually concurs with alteration of the biofilm EPS components, then the modulation of these components are influenced by the transduction of dispersal signals (Yang *et al.*, 2012). LAB can directly attack physical membrane, disfigure the biofilm structure and interrupt the protein confirmations of the pathogen (Surekha *et al.,* 2016). The current study concurs with theirs in that, differences in the conformation and shape of cell aggregates were evident post treatment with the selected CFS of LAB strains. In a study by Jara *et al.* (2020), the interaction of *Lactobacillus* seemed to interfere with the synthesis of EPS and species inside the biofilms of *L. monocytogenes*. This is another possible mechanism by which CFS of LAB were able to control the *L. monocytogenes* biofilm, however this mechanisms of natural immobilization for CFS needs to be further investigated. The antagonistic activity of LAB can be attributed to a collective range of powerful vehicles: competitive exclusion, immune modulation, stimulation of host defense systems, production of organic acids or hydrogen peroxide that lower pH, production of antimicrobials such as bacteriocins, antioxidants, and production of signaling molecules that trigger changes in gene expression (Ratsep *et al.*, 2014; Saxelin *et al.*, 2005). All these factors have an effect on biofilm construction and the disruption thereof. This information can assist in prolonging the life of biomaterials and limiting opportunistic infections.

3.6 Conclusion

The presence of *L. monocytogenes* biofilms within the food-processing environments remains a major concern for the entire food industry. Biofilms continue to persist and be transferred from different surfaces during processing to food products, giving rise to major health problems. This study investigated the potency of cell free supernatants of selected LAB, *L. acidophilus* La14 150B, *L. plantarum* and *L. rhamnosus* ATCC 4356, to disperse *L. monocytogenes* ATCC 19115 and 243 biofilms attached to different surfaces. *L. monocytogenes* ATCC 19115 and 243 strains form biofilms on both the hydrophilic stainless steel and hydrophobic PVC surfaces, with the mature biofilms characterized by the formation of a honey-comb structure evident on stainless steel but not on the PVC. These CFS induce a change in the structural complex of the *L. monocytogenes* biofilms resulting in isolated, individual cells, an indication of their efficacy for removal of biofilms. *L. acidophilus* La14 150B exhibits the best anti-listerial and anti-biofilm activity. A spray containing individual or a cocktail form of the LAB selected in this study could be suggested in food-processing facilities as an alternative control measure for pre-formed *L. monocytogenes* biofilms.

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Chapter 4

Quantitative analysis of *Listeria monocytogenes prfA* gene expression in the presence and absence of cell free supernatants of selected Lactobacilli: Towards elucidation of the anti-biofilm mechanism of action

4.1 Abstract

Foodborne pathogens continue to pose a threat to the food industry. The presence of certain virulence genes within these pathogens has an overall effect on the way they conduct normal functionalities. In *Listeria monocytogenes* many different genes work together in contributing to its pathogenicity and making it resilient. *L. monocytogenes* has a virulence gene cluster that is positively regulated by a transcriptional activator, the PrfA. The pathogenicity island of *L. monocytogenes* is further stimulated by the formation of biofilms and their persistence on surfaces. Several studies including our current study, reported that lactic acid bacteria (LAB) have antilisterial activity which negatively impacts the ability of *L. monocytogenes* to form biofilms in food-processing environments. This part of the study aimed to investigate the possible mechanism by which cell free supernatants (CFS) of selected LAB affect biofilm formation by comparing the expression of *prfA* gene of *L. monocytogenes* ATCC 19115 in the presence and absence of CFS. Real-time quantitative PCR (RT qPCR) was used to measure the levels of *L. monocytogenes* ATCC 19115 *prfA* expressed in the presence and absence of CFS of selected LAB (*Lactobacillus acidophilus* La14 150B, *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* ATCC 4356). The results indicated a significant decrease in the expression levels of *prfA* gene in the presence of all three LAB treatments ($p < 0.05$). The gene expression values were measured relative to the control strain, which gives a 100 % expression under normal untreated conditions. In the presence of CFS of LAB, downregulation was observed for *prfA* gene expression, with levels of 23 %, 36 % and 59 % recorded in presence of CFS of *L. acidophilus* La14 150B, *L. plantarum* and *L. rhamnosus* ATCC 4356, respectively. Thus, the CFS interrupt the expression of *prfA* which ultimately disturbs the functionality of the main regulator PrfA. This causes a downregulation of the *L. monocytogenes* ATCC 19115 virulence genes and promotes the inhibition of biofilm formation.

4.2 Introduction

The manner in which *Listeria monocytogenes* inhabits a wide range of reservoirs and adheres to abiotic surfaces while managing to withstand environmental stresses is astounding (Poimenidou *et al.*, 2016). Following its transmission to humans via the ingestion of contaminated food, *L. monocytogenes* may overcome barriers within the body and cause illnesses such as gastroenteritis after intestinal translocation. Alternatively, it may be carried by lymph or blood fluid making its way to the mesenteric lymph nodes, spleen and liver; leading to meningoencephalitis, placentitis and neonatal septicemia or abortion (Bhunia, 2018). With its advanced virulence mechanisms that contribute to enhanced movement and persistence, *L. monocytogenes* facilitates its own internalization by non-phagocytic cells and replicates within phagocytes (Carvalho *et al.,* 2014). The weaponry it presents constructs a major hurdle that remains difficult to jump.

The severity of the effects of *L. monocytogenes* prompted the research of this harmful pathogen on a molecular level. The main pathogenicity island in *L. monocytogenes* is the PrfA-virulence gene cluster (pVGC) that comprises of *prfA, plcA, plcB, hly, inlAB, mpl* and *actA* genes (Dussurget *et al.,* 2002). These diverse sets of genes play a role in carbohydrate metabolism, transport and cell envelope processes which promote survival (Kazmierczak *et al*., 2006). The *prfA* gene is responsible for encoding the Positive Regulatory Factor A (PrfA) protein, required for transcription of pVGC that includes *prfA* itself (Poimenidou *et al.*, 2016). PrfA is the major transcriptional gene regulator that positively regulates and promotes expression of most the known listerial virulence genes. The expression is modulated by an RNA thermosensor mechanism that enables translation of the *prfA* mRNA at temperatures strictly at or close to 37°C. It is additionally controlled by a trans-acting riboswitch in the 5' UTR region (Johansson *et al*., 2002). The *L. monocytogenes* core PrfA regulon is an important transcription factor in that it controls the bacterial passage from extracellular to the intracellular stages of infection (Pieta *et al.*, 2014). Finding a way in which another bacterium can be used as an antagonist to alter the functionality of PrfA has continuously been strongly sought after to hinder the progression of virulence and limit the pathogenicity.

It has been highlighted how *L. monocytogenes* has the ability to form biofilms that are surrounded by a slimy extracellular matrix, increasing the difficulty of their removal (López *et al.*, 2010). The biofilm structure allows survival within a wide range of harsh conditions and therefore inhibition

of biofilm formation is paramount. Understanding the relationship between virulence genes and biofilm formation can help identifying inhibition strategies. A study by Zhou *et al.* (2010) explored the role of PrfA on *L. monocytogenes* biofilm development. Their results indicated a reduction in biofilm production for strains that lacked a functional PrfA; which ultimately concluded that PrfA facilitates a major role in biofilm formation in *L. monocytogenes*. Interestingly, it was observed in another study by Tirumalai and Prakash (2012) that PrfA is most necessary in the initial stages of biofilm formation and aggregation.

In the previous chapters, LAB were successful in inhibiting and dispersing *L. monocytogenes* biofilms on respective surfaces however their mechanism of action is not known. It was hypothesized that the antilisterial capabilities of LAB could be possible due to the change(s) they may impose on the expression of virulence genes, specifically those involved in biofilm formation. Therefore, the current study aimed to investigate the expression of *L. monocytogenes* ATCC 19115 *prfA* gene in the presence and absence of cell free supernatants (CFS) of selected Lactobacilli (*Lactobacillus acidophilus* La14 150B, *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* ATCC 4356) as a step towards understanding how these CFS affect biofilm formation of *L. monocytogenes*.

4.3 Materials and Methods

4.3.1 Bacterial cultures

The preparation of the bacterial cultures *Listeria monocytogenes* ATCC 19115; and LAB cultures (*Lactobacillus acidophilus* La14 150B, *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* ATCC 4356) were conducted as described in Chapter 2, section **2.3.1**.

4.3.2 Preparation of cell free supernatants (CFS) of selected lactic acid bacteria (LAB)

The preparation of the CFS was conducted as described in Chapter 2, section **2.3.3**.

4.3.3 Preparation and treatment of *L. monocytogenes* test culture

An overnight culture of *L. monocytogenes* ATCC 19115 strain was prepared by inoculating 200 µl of the bacterial culture into 10 ml of BHI broth; this was vortexed for 5 seconds and incubated aerobically for 18 hours at 37°C and served as the control sample. For the three treatment samples, 200 µl of the previously prepared *L. monocytogenes* ATCC 19115 culture was transferred to three separate glass test tubes containing 10 ml of BHI broth. The cultures were vortexed for 5 seconds and incubated for 18 hours at 37°C aerobically. Immediately following the incubation, 2 ml of the CFS of each LAB (*L. acidophilus* La14 150B, *L. plantarum* and *L. rhamnosus* ATCC 4356) was added to each of the respective test tubes already containing the *L. monocytogenes* ATCC 19115 culture. These were incubated for 24 hours at 37°C and served as the treated samples.

4.3.4 RNA extraction

Total RNA was extracted from overnight cultures of *L. monocytogenes* ATCC 19115 control and treated samples using the PureLink ® RNA Mini Kit with Trizol ® reagent (Thermo Scientific) according to the manufacturer's instructions. The RNA was eluted with RNase-Free water and

quantified using the NanoDrop™ 2000 spectrophotometer, Thermo Scientific, Wilmington, DE, USA. It was then stored at -80°C.

4.3.5 cDNA synthesis using Reverse Transcriptase

The cDNA was synthesized with 2 µl total RNA of control and treated samples using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). This was done following the manufacturer's instructions, which was optimized to generate first strand cDNA for use in two-step RT qPCR. The quality was assessed using the NanoDrop™ 2000 spectrophotometer, Thermo Scientific, Wilmington, DE, USA.

4.3.6 Real Time quantitative PCR

4.3.6.1 Primer design

The primers (Table 4.1) were developed and designed using Basic Local Alignment Search Tool (BLAST) in combination with the Primer Design 4.1.

Table 4.1: Data of the *L. monocytogenes* genes used for real-time qPCR

Gene	**GenBank® accession number**	**Primers sequences**		**Length (bp)**
		Forward	**Reverse**	
*prfA (*Gene of interest)	JN703898.1	tagcgagaacgggaccatca	aacgtatgcggtagcctgct	136
GAPDH (Reference gene)	FJ890134.1	aggtgacttccgtcgtgcac	gaacacgttgagcagctccg	128
bgla (Reference gene)	FM180366.1	cggtcacattactgacggtcc	ggaagatacgggaccaagcga	146

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; bgla: beta-glucosidase

4.3.6.2 Gene expression quantification

Real-time quantitative PCR (RT qPCR) was conducted using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA) according to the protocol's reaction set-up. Reactions were carried out in QuantStudio™ 5 Real-Time PCR System 384-well block (Applied Biosystems, USA). The RT qPCR was set at 10 µL with 5 µL of SYBR Green reference dye, 1.5 µL nuclease-free water, 0.5 µL of each primer (forward and reverse) and 2.5 µL of cDNA template. The standard cycling parameters consisted of: 50°C for 2 minutes and 95°C for 2 minutes of holding cycles for UDG activation and Dual-Lock™ DNA polymerase respectively. This was followed by 40 cycles of 95°C for 15 seconds of denaturing and 56°C for 1 minute anneal/extend stage with the fluorescent signal collected at the extension step. The experiment was performed with biological triplicates and technical quadruplicates.

4.3.7 Statistical analysis of data

Relative gene expression was determined using the Pfaffl method (Pfaffl, 2001) with a slight modification of incorporating the geometric average of all relative quantities of the multiple reference genes used. The software GraphPad Prism 8.4.1 was used to analyze the results to perform the two-way ANOVA (Analysis of Variance) followed by the Tukey's multiple comparisons test ($p < 0.05$).

4.4 Results

4.4.1 NanoDrop™ 2000 spectrophotometer quality assessment

RNA previously isolated was used as the template for synthesizing cDNA and an assessment of the purity and quality was conducted. The NanoDrop™ 2000 spectrophotometer results also indicated that high concentrations of cDNA of good quality, free from protein and salt contamination - observed from the 260/280 and 260/230 ratios, respectively were obtained for both control *L. monocytogenes* ATCC 19115 and that cultured in presence of CFS of different LAB (Table 4.2) Thus, all cDNA was suitable for use in downstream RT qPCR.

Table 4.2: Purity and quality parameters of synthesized cDNA of *L. monocytogenes* ATCC 19115 grown in presence of CFS of different LAB as measured using the NanoDrop™ 2000 spectrophotometer

Treatment	**[cDNA] (ng/µl)**	**A260 (Abs)**	**A230 (Abs)**	**260/280 ratio**	**260/230 ratio**
Control	1325.90	26.51	16.23	1.73	1.95
CFS of *L. acidophilus* La14 150B	1407.60	28.15	16.61	1.69	2.00
CFS of *L. plantarum*	1177.10	23.54	14.05	1.77	2.03
CFS of *L. rhamnosus* ATCC 4356	1312.20	29.27	16.56	1.85	2.11

4.4.2 Melt curve plot analysis of RT qPCR

Following the RT qPCR, a melt curve plot was constructed to visualize the integrity of the run (Figure 4.1). Solid, defined peaks were successfully obtained for the control and all three treatments. Most importantly, no amplification was observed in the non-template control (NTC) indicating no contamination in the experiment and the strong efficiency of the primers used.

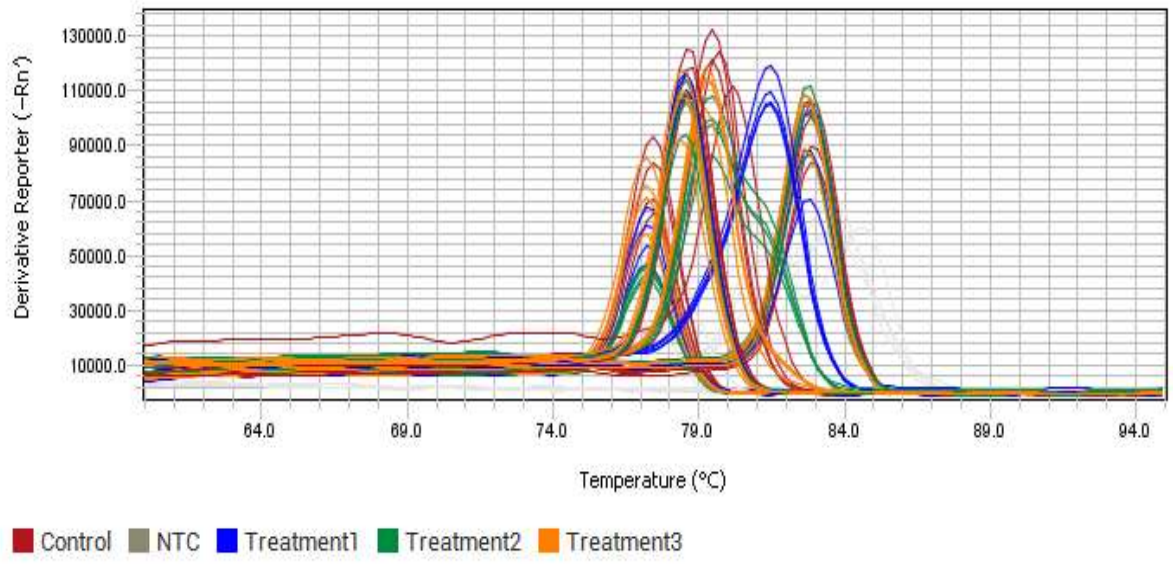

Figure 4.1: Melt Curve Plots. The different peaks are the control *L. monocytogenes* ATCC 19115, and those treated with CFS of different LAB: *L. acidophilus* La14 150B (Treatment 1), *L. plantarum (*Treatment 2) and *L. rhamnosus* ATCC 4356 (Treatment 3). The NTC represents the non-template control.

4.4.3 *prfA* gene expression quantification

The investigation conducted was to determine the effect LAB presence had on the expression of the *prfA* gene coding for the regulatory factor PrfA. The results (Figure 4.2) are indicative that in the presence of CFS of all LAB strains there was a statistically significant decrease in the *prfA* gene expression ($p < 0.05$). The values shown are gene expression fold changes measured via a relative quantification qPCR experiment using a comparative cycle threshold (Ct), with reference genes GAPDH and bgla as an internal control. Fold change is interpreted as a percentage where a fold change of 1 means 100% expression under normal conditions. Hence, the value of 1 observed for the untreated control *L. monocytogenes* ATCC 19115. A fold change value above 1 shows upregulation of the gene of interest relative to the control and values below 1 are indicative of gene downregulation relative to the control. For Treatment 1 (CFS of *L. acidophilus* La14 150B) a downregulation was observed with a value of 0.23 indicating a 23 % gene expression relative to control. This is nearly only a quarter of the normal expression of *prfA*. In the second treatment

(CFS of *L. plantarum*) a value of 0.36 was measured which is a 36 % gene expression relative to the control, a downregulation. Finally, Treatment 3, (CFS of *L. rhamnosus* ATCC 4356) yielded the value 0.59, a 59 % gene expression relative to the control. Thus, in the presence of all three LAB a downregulation in the expression of *prfA* was observed, the *L. monocytogenes* ATCC 19155 virulence functionality was affected. *L. acidophilus* La14 150B had the greatest antilisterial abilities in comparison to the other two treatments.

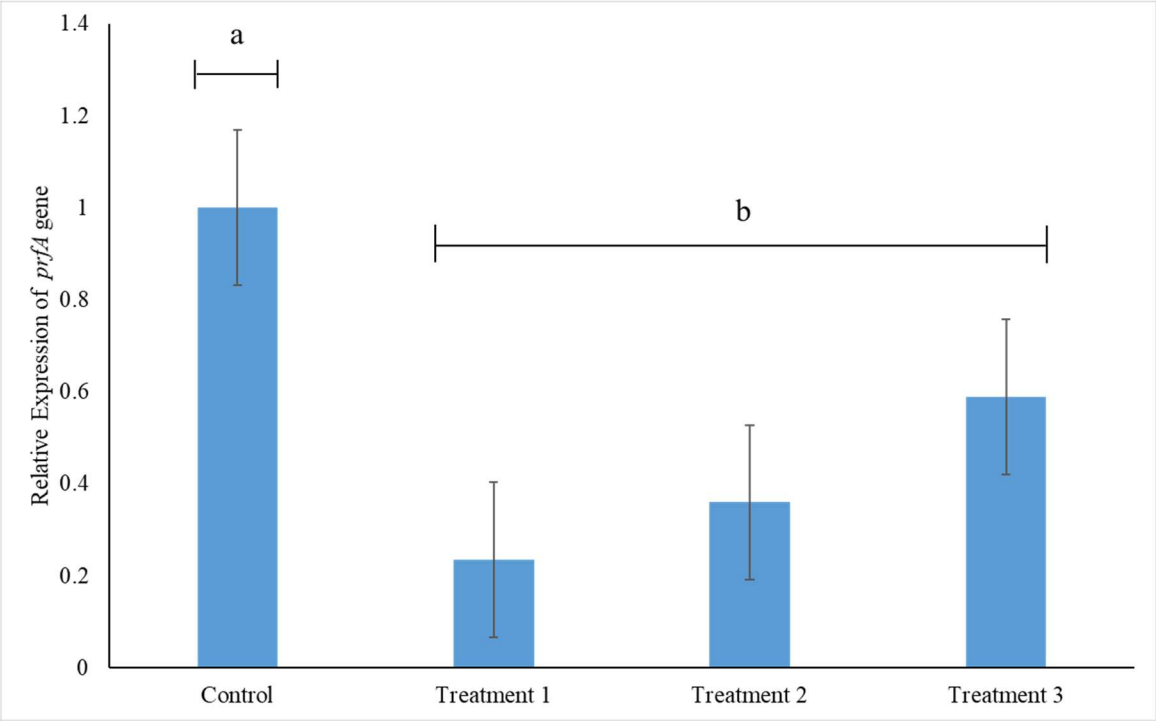

Figure 4.2: Fold change expression of *L. monocytogenes* prfA gene expression relative to a pair of reference genes (Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-glucosidase (bgla)). The control is untreated *L. monocytogenes* ATCC 19115; Treatments 1, 2, 3 are *L. monocytogenes* ATCC 19115 treated with CFS of *L. acidophilus* La14 150B, *L. plantarum* and *L. rhamnosus* ATCC 4356, respectively. Bar heights indicate mean expression of the gene in triplicate samples while error bars indicate standard error. Bars with different letters are significantly different ($p < 0.05$) while those with same letter have no significant differences ($p > 0.05$).

4.5 Discussion

In past and recent times an interest has sparked into the virulence functionality of *L. monocytogenes* that enable it to replicate and persevere for prolonged periods. The calculated cycle of pathogenesis it follows introduces several ways of adaptability in different conditions. One of ways is the *L. monocytogenes* proteosurfaceome, these proteins on the surface create a link between the bacteria and the environment. This link assumes a significant role in communication, stress resistance, chemical sensing and a balance of nutrients and toxins within the cell (Cordwell, 2006). Another crucial adaptability mechanism is biofilm formation by aggregating *L. monocytogenes* microbial cells creating a complex surrounded by an extracellular polymeric substance. This complex provides increased fitness and protection and becomes extremely challenging to eradicate (Flemming *et al.*, 2016). Biofilm formation occurs when the once planktonic cells cluster together via quorum sensing and synchronize the expression of certain regulation genes and adhere to various surfaces (Rémy *et al.*, 2018). The biofilm structure enhances the transfer of nutrients and increases the chance to acquire new traits (Colagiorgi *et al.*, 2017; Galie *et al.*, 2018). Although the entire process of biofilm formation and attachment in *L. monocytogenes* is due to a combination of multiple mechanisms, PrfA. as the key transcriptional activator coded by the *prfA*, is often highlighted amongst the rest due to its important role in virulence.

The current study looked at the *prfA* gene expression in the presence and absence of cell free supernatants (CFS) of selected lactic acid bacteria (LAB), *L. acidophilus* La14 150B, *L. plantarum* and *L. rhamnosus* ATCC 4356, using RT qPCR. A decrease in the expression of *prfA* was observed for *L. monocytogenes* ATCC 19115 influenced by treatment with individual CFS of the three LAB. *L. acidophilus* La14 150B had the highest antilisterial activity in comparison to the other LAB, with the lowest gene expression level of 23%, a significant downregulation of the *prfA*. This was in contrast to the lower 36% and 59% of *L. plantarum* and *L. rhamnosus* ATCC 4356 respectively. This may strongly be due to the fact that *L. acidophilus*, as previously shown by Liguori *et al.* (2015), yields the highest level of lactic acid production among other *Lactobacillus* strains. El-Mokhtar *et al.* (2020) further confirmed that *L. acidophilus* has stronger antibacterial and antibiofilm activities. This approach of utilizing LAB to decrease the virulence of *L. monocytogenes* has been reported to operate through modulation of gene or protein expression by

bacterial signaling mechanisms conferred by the LAB (Loh *et al.*, 2012). LAB are then able to interfere with the process of switching *L. monocytogenes* from inactive to active form and break the chain of pathogenesis. The way CFS of LAB achieves these disruptions can be attributed to a wide range of properties that include but are not limited to: competitive exclusion, competition for nutrients and niche competition; the production of microbial inhibitory compounds mostly organic acids: lactic and acetic and bacteriocins (Yap *et al.* 2021).

LAB have the potential to control the development of *L. monocytogenes* through significant antlisterial activities and antagonistic metabolites. In addition, several studies have identified nutrient competition as the prominent mechanism used by LAB behind the inhibition of *L. monocytogenes* in presence of multispecies. In competitive exclusion the LAB compete for available nutrients and cause depletion of supply to *L. monocytogenes*, the opponent, resulting in cell growth disruption or death. The opponent struggles to survive due to insufficient nutrients in the microenvironment and the faster uptake by LAB (Holt, 2017). It is observed that in the presence of co-incubation with LAB, *L. monocytogenes* often only represent a small part within the bacterial population (Chorianopoulos *et al.*, 2008; Guillier *et al.*, 2008). These studies possibly give a strong suggestion as to why the *prfA* levels in the current study decreased, due to the posed competition evoked by *L. acidophilus* La14 150B, *L. plantarum* and *L. rhamnosus* ATCC 4356. Another study conducted by Saraoui *et al.* (2016) showcased the inhibition of *L. monocytogenes* via competitive exclusion by LAB after being co-cultured. The LAB operate by communication through quorum sensing where production and release of auto-inducers occurs to achieve exclusion (Tannock *et al.*, 2005).

LAB produce a wide variety of antimicrobial compounds apart from main organic acids. The compounds include diacethyl, acetoin, ethanol, carbon dioxide, hydrogen peroxide, and exopolysaccharides (Capozzi *et al.*, 2021). In a recent review Vieco-Saiz *et al.* (2019) concluded that organic acids generate a selective barrier that leads to the alteration of cell metabolism and virulence progression, the damage of enzymes and the destruction of genetic material. According to Ricke (2003) lactic acid reduce the intracellular pH and prevent the active transport of excess internal protons that require cellular adenosine triphosphate consumption resulting in energy depletion within the cell. Studies by Wang *et al.* (2015) and Surendran Nair *et al.* (2017) reported that virulence of *L. monocytogenes* was inhibited by organic acids produced by LAB that mainly

targeted specific metabolic functions including replication and aggregation of cells, leading to premature death. This is consistent with our study yielding a reduction in the virulent *prfA* levels after treatment with organic-acid producing LAB. In previous studies by researchers Sun and O'Riordan (2013) and Bermudez-Brito (2014), their findings showcased the antilisterial activity of LAB against *L. monocytogenes*. They found that short chain fatty acids such as butyrate present in the CFS of LAB enhances barrier integrity and induces epithelial cell differentiation; and most importantly indirectly inhibit the virulence gene expression at the transcriptional level. The findings of the current study concur with these and the reasons why downregulation of the *prfA* in the presence of LAB was observed can be closely correlated.

Bacteriocins are ribosomally-synthesized bacterial antimicrobial peptides that possess direct antagonistic activity towards closely related Gram positive bacteria including *L. monocytogenes* (Kumariya *et al.* 2019). Upadhyay *et al.* (2016) reported that five LAB of which one was *L. plantarum* B-4456 bacteriocin significantly reduced the virulence gene expression of *L. monocytogenes* ($p < 0.05$). This was attained by pore formation inducing the inhibition of cell-wall and nucleic acid synthesis and disrupting protein synthesis. The antilisterial activity of LAB bacteriocins was also reported by Jeong and Moon (2015) and Trinetta *et al.* (2012) where *L. rhamnosus* CJNU 0519 derived rhamocin and *L. sakei* derived sakacin, both respectively decreased the virulence expression of *L. monocytogenes*. These results concur with the current study where LAB caused a decline in virulence.

A study by Lemon *et al.* (2010) reported the first evidence that PrfA of *L. monocytogenes* has a significant impact on extracellular biofilm formation, with mutants lacking the PrfA being defective in surface-adhered biofilm formation. The mutant PrfA showed a defect in the biofilm after initial adhesion and had wild-type flagella motility. This pivoted upcoming studies to investigate the important role PrfA has on biofilm formation and critically analyze its functionality. Following that study, Zhou *et al*. (2011) also confirmed that PrfA does have a strong impact and influence on biofilm formation in *L. monocytogenes,* the strain that lacked the functional PrfA had reduced production of biofilm. In a later study by Luo *et al.* (2013) it was observed that a loss of PrfA dramatically altered gene expression in *L. monocytogenes* biofilms and therefore resulted in reduced biofilm formation abilities. They identified that 185 genes are associated with PrfA and biofilm formation. Of these 185 genes, 175 were observed to alternate in the regulation pattern

when comparing the wild-type and the PrfA mutant. The previous findings mentioned are indicative of how the same can be suggested for the current study; that a decrease in *prfA* gene expression post treatment with the CFS of LAB led to a decrease in the biofilm formation abilities of *L. monocytogenes* ATCC 19115 strain. The normal expression of PrfA promotes the aggregation and formation of biofilms whereas the mutated form lacking optimum expression decreases biofilm formation. This information allowed us to elude a possible mechanism by which LAB may use to both inhibit and disperse biofilm formation of *L. monocytogenes*. Since the expression of *prfA* plays a role in the initial stages of biofilm formation, the results obtained in the current study are crucial and may serve as the prevention of the subsequent pathogenic steps. LAB application can assist in making *L. monocytogenes* less virulent and prove beneficial in the long run to decrease the overall pathogenicity by controlling the regulator PrfA– a consistent means of ensuring downregulation

4.6 Conclusion

The effects of LAB treatment on *prfA* expression in *L. monocytogenes* has not been extensively reported which propelled the current study analyzing the expression in the presence and absence of cell free supernatants of *L. acidophilus* La14 150B, *L. plantarum* and *L. rhamnosus* ATCC 4356. The presence of CFS of each of the three LAB resulted in significant downregulation of the expression of *prfA*. Thus, the results suggest that the CFS of LAB not only disrupts the preformed biofilms of *L. monocytogenes*, but also influences the key regulator for virulence genes, which includes genes for biofilm formation. The downregulation of *prfA* negatively affect the PrfA and its associated virulence gene expression and can deem the pathogenicity of *L. monocytogenes* redundant.

With the knowledge of how these specific LAB strains can decrease *prfA* gene expression in *L. monocytogenes*, treatment of the surfaces in the food environments with the LAB could be implemented to prevent biofilm formation. This viable approach for the reduction of *L. monocytogenes* virulence using antagonistic mechanisms of LAB could furthermore prevent the prominent persistence.

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Chapter 5

General conclusions and recommendations

5.1 General conclusions

- All the test *Listeria monocytogenes* strains were able to form biofilms, although they differed with regards to the degree to which they form these structures, whereby some were classified as moderate biofilm formers while others were categorized as strong biofilm formers. Worth noting is that all the *L. monocytogenes* strains isolated from food processing facilities were stronger biofilm formers than the test *L. monocytogenes* ATCC 19115 strain. This finding further highlights that the challenges faced by the food industry with regards to the safety concerns caused by proliferation of *L. monocytogenes* cannot be underestimated, and continues to need serious attention if the food industry is determined to produce food that will not cause disease in consumers.

- Antilisterial activity, specifically antibiofilm properties of *Lactobacillus acidophilus* La14 150B, *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* ATCC 4356, was confirmed as indicated by the change in the biofilm former category level of all the *L. monocytogenes* strains when they were grown in the presence of cell free supernatant (CFS) of the lactic acid bacteria (LAB). This indicated that the LAB released certain antimicrobial substances into the culture medium some which negatively impacted the ability of *L. monocytogenes* to form mature biofilms.

- The cocktail containing CFS of *L. acidophilus* La14 150B + *L. plantarum* + *L. rhamnosus* ATCC 4356 (ABC) exhibited the highest level of potency, the best inhibitor of L. monocytogenes biofilms. This results suggest that the CFS of the LAB had a synergistic effect which boosted the antibiofilm activity as opposed to when individual CFS were used.

- All LAB were efficient in the dispersal of preformed *L. monocytogenes* biofilms with *L. acidophilus* La14 150B showcasing the best biofilm removal abilities. Once again a cocktail of *L. acidophilus* La14 150B + *L. plantarum* + *L. rhamnosus* ATCC 4356 (ABC) was the most powerful in disintegrating the preformed biofilms of all the *L. monocytogenes* strains. The adherence style and attachment mechanisms of these existing biofilms were

significantly impacted by the presence of the individual and cocktail CFS. The disruption of the aggregated structures was observed, evidenced by overall decrease to weaker biofilm former categories.

- Scanning electron microscopy revealed changes in the structural-biofilm complex following treatment with cell free supernatants (CFS) of the different lactic acid bacteria (LAB). The initial biofilm characteristics of aggregated cells joined in a cell-to-cell manner were scattered and isolated subsequent to exposure to CFS. These results pointed out the biofilm dispersal abilities of the antagonistic LAB and their interference with the adhesion of *L. monocytogenes* biofilm to both the stainless steel and polyvinyl chloride (PVC) surfaces. The potency of the CFS of the different test LAB differed, with CFS of *L. acidophilus* La14 150B being the most efficient while that of *L. rhamnosus* ATCC 4356 being the least efficient in disassembling the preformed biofilms.

- The *Listeria monocytogenes* strains attached to and differently formed the biofilms on the hydrophobic and hydrophilic surfaces. On the hydrophilic stainless steel, *L. monocytogenes* biofilms formed two-layer structures versus the single layered biofilms formed on the hydrophobic PVC.

- The expression of the major transcriptional regulator, *prfA,* which is associated with the ability of *L. monocytogenes* to efficiently form biofilms, was significantly decreased in the presence of CFS of all the testes LAB. The downregulation of this gene negatively impacts the major transcriptional regulator, PrfA, and deconstructs the virulence functionality. Thus, the results suggest that one of the mechanisms by which CFS of LAB interferes with biofilm formation by *L. monocytogenes*, is through their negative impact on PrfA. Consistently with observations from SEM, downregulation of this regulator was mostly pronounced for the *L. monocytogenes* grown in the presence of *L. acidophilus* La14 150B and the least affected in presence of *L. rhamnosus* ATCC 4356.

5.2 Recommendations for future work

- As the cell free supernatant (CFS) of lactic acid bacteria (LAB) were efficient in the inhibition and dispersal of *Listeria monocytogenes* biofilms of the selected strains, investigating which specific antimicrobial substances are responsible for this result would be a desirable option. This way a more accurate alternative for the control of *L. monocytogenes* can be provided.

- The efficiency of the cell free supernatant of *Bifidobacterium*, as opposed to Lactobacilli, for the control of biofilms in the food industry can be explored, not only for biofilms formed by *L. monocytogenes* but other foodborne pathogens such as *Escherichia coli*, *Campylobacter jejuni*, *Staphylococcus aureus*, *Salmonella enterica* and *Bacillus cereus*. It is envisaged that the antimicrobial metabolites *Bifidobacterium* produce, which include organic acids and hydrogen peroxide, can be potent against other pathogenic biofilms as previously observed with the successful inhibition of oral biofilm-forming bacteria.

- The efficiency of CFS of different LAB of biofilms under different stress conditions for example, incubation temperatures ranging from freezing to warm temperatures, different relative humidity and oxygen levels can also be explored, providing a new perspective. In addition, assessing the biofilm formation capabilities of *L. monocytogenes* strains in the presence of CFS at various time intervals might give a sufficient overview of when and for how long during the food production process their application are likely to have a significant impact on food safety and quality.

- With the great success of CFS cocktails in comparison to individual treatment, a wider inclusion of more LAB strains combined together could be investigated and might create an improved synergistic effect.

- Increasing the number of surfaces and aligning them to those used currently in most commercial food-processing environments such as glass, wood and rubber, will be advantageous. Contrasting the effects CFS of LAB on these surfaces will give a better understanding of the role of hydrophobicity in biofilm formation of *L. monocytogenes*.

- Recombinant forms of *L. monocytogenes* strains with altered virulence genes such as *inlB*, *hlyA* and *actA* could be explored in how their biofilm formation would be influenced by CFS of LAB. The mutated nature may demote the pathogenicity and enhance the antilisterial activity of LAB.

- It is not known how LAB specifically affect the *prfA* gene expression and ultimately the PrfA regulator, therefore extensive assessment on the molecular level may prove beneficial in eventually pinpointing the mechanism of action (MOA) of CFS. However, the MOA is envisaged to be complex since our results already show impact on *prfA* which is involved in regulation of many genes. Quantifying the effect of CFS on other important virulence genes is an avenue that should also be explored.