Bioengineered *Lactobacillus casei* expressing internalin AB genes for control of *Listeria monocytogenes* infection

BY

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DECLARATION

I, Moloko Gloria Mathipa declare that this thesis entitled **'Bioengineered *Lactobacillus casei* expressing internalin AB genes for control of *Listeria monocytogenes* infection'** which I hereby submit for the degree **Philosophiae Doctor (Microbiology)** 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 tertiary institution.

Signature: ……..

Date: …….

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3. **Mathipa, M.G.,** Bhunia, A.K., Thantsha, M.S. (2019). *Lactobacillus casei* expressing internalin AB genes of *Listeria monocytogenes* protects Caco-2 cells from listeriosis-associated damages under simulated intestinal conditions. *PLoS ONE*. (**Accepted**)

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LIST OF ABBREVIATIONS

ActA	Actin assembly-inducing protein
AMPs	Antimicrobial peptides
BCA	Bicinchoninic Acid
BSA	Bovine serum albumin
BHI	Brain Heart Infused
CW	Cell wall
CDC	Center for Disease Control and Prevention
Ctx	Cholera toxin
CFU	Colony forming units
MRS	de Man Rogosa Sharpe
DMEM	Dulbecco's modified eagle's medium
ETEC	Enterotoxigenic *Escherichia coli*
FaSSIF	Fasted State simulated intestinal fluid
FeSSIF	Fed State simulated intestinal fluid
FBS	Fetal Bovine Serum
FbpA	Fibronectin binding protein
GI	Gastrointestinal
GIT	Gastrointestinal tract
GRAS	Generally regarded as safe
GMO	Genetically modified organisms

GAGs	Glycosaminoglycans
GFP	Green fluorescent protein
HBSS	Hanks' balanced salt solution
HGF	Hepatocyte growth factor
HIV	Human Immunedeficiency Virus
HUVEC	Human umbilical vein endothelial cells
IBD	Inflammatory bowel diseases
IFN	Interferons
IL-10	Interleukin 10
Inl	Internalin
KTCT	Korean Type Culture Collection
LDH	Lactate dehydrogenase
LAB	Lactic acid bacteria
LAP	*Listeria* adhesion protein
LLO	Listeriolysin O
LB	Luria-Bertani
MOX	Modified Oxford
MUCs	Mucins
MDR	Multi- drug resistant
MLST	Multilocus sequence typing
MOE	Multiplicity of exposure
MOI	Multiplicity of infection
MPO	Myeloperoxidase

MLCK	Myosin light chain kinase
OD	Optical density
PBS	Phosphate buffeed solution
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PPI	Proton-pump inhibitors
RBC	Ranitidine bismuth citrate
RTE	Ready to Eat
ST6	Sequence type 6
STEC	Shiga toxigenic *Escherichia coli*
SIF	Simulated gastrointestinal fluid
SDS	Sodium dodecyl sulfate
SD	Standard deviation
SN	Supernatant
TTFC	Tetanus toxin fragment C
TJ	Tight junctions
TEER	Transepithelial electrical resistance
TSB-YE	Tryptone Soy broth supplemented with 0.6% yeast extract
TNF- α	Tumor necrosis factor α
WT	Wild type
WHO	World Health Organization
ZO-1	Zonula occludens 1

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SUMMARY

Listeria monocytogenes is one of the common food pathogens implicated in different outbreaks. It has recently (2017-2018) been implicated in the South African listeriosis outbreak, ever reported, where 1060 people were infected resulting in 214 deaths. The ability of listeriosis to cause high case fatality rate (20 to 30%) when compared to most foodborne pathogens makes it an important pathogen and a substantial public health concern. *Listeria* is an intracellular pathogen that employs different virulence factors to cross the three significant barriers, namely, the intestinal epithelial, the blood-brain endothelial, and the feto-placental endothelial cell barrier, thereby causing listeriosis. As it is the case for most pathogenic infections, antibiotics have been the first line of defence against listeriosis, however, these undesirable effects in the gastrointestinal (GI) infections keep increasing and thus posing major clinical problems. Coupled with that is the increase in the number of bacteria referred to as "superbugs", those bacteria which have developed resistance against most of the commonly used antibiotics. The rise in these clinical problems, the increase in foodborne infections and the development of antibiotic resistance have led to a need for an alternative solution for these infections. There has been a growing interest in exploring probiotics as an alternative to antibiotics. Probiotics offer beneficial effects to the host and are able to inhibit pathogens through the use of different mechanisms including among others, competing for food and space with foodborne pathogens. They grow rapidly and colonize the gastrointestinal tract (GIT) either permanently or temporarily, consequently alleviate and prevent foodborne infections through mechanisms such as competitive exclusion. However, these probiotics are generic in their action, that is, they are non discriminatory in their action. Furthermore, they are not equally effective in all hosts nor against all pathogens. These limitations inspired the development or design of probiotics strains that will be targeted against specific pathogens. This can be achieved through a systematic understanding of the infection cycle of the pathogens, their virulence factors and disease mechanisms. Virulence genes from food-borne pathogens are cloned and expressed into probiotics through bioengineering in an effort to offer them direct competition for the same receptor sites to which pathogens attach, or for enhanced production of antimicrobial peptides and ultimately inhibition of the specific pathogen.

Listeria monocytogenes in its disease progression uses virulence factors such as *Listeria* adhesion protein (LAP), autolysin amidase (AmiA) for adhesion, while the bacterial surface proteins internalin A (InlA) and internalin B (InlB) are responsible for invasion through the host cells. Cloning and expression of these virulence factors into probiotics will potentially offer the recombinant probiotics an enhanced ability to compete and ultimately inhibit *L. monocytogenes*. Taking this into consideration, the current study intended to determine whether cloning and expressing the invasion proteins internalins A and B of *L. monocytogenes* into *Lactobacillus casei* using the expression vector pLP401-T would alleviate or prevent the *Listeria* associated damages *in vitro*.

The current study and its findings are organized into the five chapters of this thesis as follows. The first chapter of this thesis (**Chapter 1- Literature Review**) gives an overview of *L. monocytogenes* characteristics and pathogenesis, highlighting the virulence genes important for its infection. The various control measures used in clinical environment and food industry, their advantages and disadvantages are discussed. The limitations of these control measures and the need for an alternative measure are justified. Then probiotics as an alternative control for *L. monocytogenes* are described, taking into consideration their different modes of action. This gives a comprehensive explanation of the limitations of the wild type probiotics, including that they at times fail to inhibit pathogens, which emphasizes the demand for a robust strategy for their improvement. This is followed by discussion of the concept of probiotic engineering as an alternative strategy for improving the efficiency of probiotics for enhanced and targeted control of specific pathogens, explaining some applications where such recombinant strains have been explored. Recombinant probiotics are genetically modified organisms, therefore, due to the ethical reasons surrounding genetically modified organisms, safety concerns regarding recombinant probiotics were briefly addressed. This chapter ends by giving future perspectives regarding the use of recombinant probiotics.

In the first experimental chapter (**Chapter 2- Construction of recombinant *Lactobacillus casei* strain expressing the invasion proteins internalins A and B of *Listeria monocytogenes***), the research followed a stepwise procedure to clone and express the proteins. Firstly, the genomic DNA from *L. monocytogenes* F4244 (serotype 4b) was extracted and using the specific InlAB primers, the genes was amplified using PCR. The amplification of the InlAB genes was successful,

and the genes was subsequently purified for cloning. Using the specific restriction digestion enzymes, the genes and expression vector pLP401- T were digested and ligated using T4 DNA Ligase. Ligation of the two was successful and this was visualized by a band larger than that of the vector alone. The ligated pLP401- InlAB was transformed into *L. casei* through electroporation. A total of twenty-five transformants were obtained, which were subsequently tested for the presence of InlA, InlB and InlAB genes with their specific primers using PCR. The full length InlA, InlB and the genes InlAB were all amplified confirming their presence in the transformants (recombinant *L. casei*). The SDS-PAGE and Western blot were used to determine whether the internalins were expressed in the recombinants. The results showed that both InlA and InlB were expressed by the recombinant *L. casei* but not in its wild- type counterpart. The growth patterns of the wild-type *L. casei* strains (*L. casei* WT(Lbc^{WT})), *L. casei* with the vector without InlAB (Lbc^{V}) and *L. casei* with InlAB (Lbc^{InlAB})) were compared. Interestingly, there was no difference in the growth patterns of all the *L. casei* strains. The results from this chapter demonstrates that the cloning and expression of the proteins InlAB into the probiotic was successful and that expression of the foreign genes did not have observable negative effects on *L. casei* growth characteristics as growth curves of all the *L. casei* strains were comparable.

The successful cloning and expression of the invasion proteins InlAB allowed an opportunity to test if there were any differences in the effects that the recombinant *L. casei* would have on the inhibition of *L. monocytogenes in vitro.* In the second experimental chapter (**Chapter 3- Prevention of *Listeria monocytogenes* adhesion, invasion and translocation *in vitro* by the recombinant *Lactobacillus casei* expressing the internalin AB)**, the study investigated the ability of *L. casei* expressing the invasion genes internalin AB (InlAB) (Lbc^{InlAB}) to affect *L. monocytogenes* progression *in vitro* using the Caco-2 cells grown and maintained in the cell culture medium, Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (FBS). This construct was compared with a previously developed *L. casei* expressing *Listeria* adhesion protein (LAP) (Lbc^{LAP}). To achieve this aim, the ability of the *L. casei* strains to adhere to, invade and translocate through the Caco-2 cells were first investigated. The results showed a difference in all stages, with the recombinant *L. casei* showing enhanced activity then the wild-type counterpart. For microorganisms to be deemed a probiotic, they have to be able to competitively exclude pathogens. Taking that into consideration, the ability of all *L. casei* strains to inhibit *L. monocytogenes* adhesion using three different mechanisms, namely, Competitive

inhibition, inhibition and displacement of adhesion, was investigated. All *L. casei* strains competitively inhibited the adhesion of *L. monocytogenes*, however, none of them displaced the *L. monocytogenes* cells already adhered to the Caco-2 cells. It was worth noting that the recombinant strains expressing internalins showed enhanced inhibition than the Lbc^{WT} and Lbc^{V}. Subsequent to these positive results, it became intriguing to determine whether the observed *L. monocytogenes* inhibition would be influenced by duration of pre-exposure to the *L. casei* strains. The recombinant strains showed an enhanced ability to inhibit all the three infection stages, and this effect was increasing with the longer exposure times. In addition to the inhibition of the infection stages, the *Listeria* mediated cytotoxicity and effect of *L. monocytogenes* on the tight junction integrity of the Caco-2 cells were examined. Pre- exposure of Caco-2 to recombinant *L. casei* reduced *L. monocytogenes* mediated cell cytotoxicity and preserved the epithelial barrier. The results from this chapter showed that the recombinant strains enhanced effects against *L. monocytogenes* than the Lbc^{WT} and Lbc^{V}. When comparing the functional attributes of two bioengineered strains, the results revealed that Lbc^{InlAB} had a superior ability to prevent *L. monocytogenes* invasion and translocation, while Lbc^{LAP} strain was superior in preventing its adhesion.

Although the results of Chapter 3 summarized above were positive, they were still not appropriate for making inferences about how the recombinant *L. casei* would affect the *L. monocytogenes* intestinal infection phase as the media used did not sufficiently simulate the intestinal conditions. In the third experimental chapter (**Chapter 4- *Lactobacillus casei* expressing internalin AB genes of *Listeria monocytogenes* protects Caco-2 cells from listeriosis-associated damage under simulated intestinal conditions)**, the effects of the recombinant *L. casei* strain on *L. monocytogenes in vitro* under simulated intestinal conditions were investigated. As per the previous chapter, this part of the study determined the ability of the *L. casei* strains to adhere to, invade and translocate through the Caco-2 cells. The results were in agreement with the ones from Chapter 3, the recombinant strains had enhanced adhesion, invasion and translocation efficiencies. As expected, Lbc^{LAP} showed enhanced adhesion while Lbc^{InlAB} showed enhanced invasion and translocation. Furthermore, when tested for their ability to competitively inhibit *L. monocytogenes* under simulated intestinal conditions, the recombinant *L. casei* strains performed better than Lbc^{WT} and Lbc^{V}, with the inhibition efficiency improved by prolonged pretreatment of the Caco-2 cells

with the *L. casei* strains. The *Listeria* mediated cytotoxicity was reduced through pre- exposure to recombinant strains and additionally the epithelial tight junction was also maintained.

Then the last chapter (**Chapter 5 – General Conclusions and Recommendations**), the general research findings and the recommendation for future work, which will further advance the research in recombinant probiotics and their use for pathogen control, are given. Briefly, the study highlights are that recombinant probiotics expressing different virulence genes of *L. monocytogenes* can be targeted at different stages of its infection cycle, with the recombinant harbouring LAP and internalins targeting adhesion and invasion plus translocation, respectively. Thus, probiotic bioengineering could be used to target specific stages in the *L. monocytogenes* infection cycle to inhibit its colonization and infection progression. However, before these recombinant strains could be used in human applications, *in vivo* studies have to be conducted. Such studies will determine persistence of the recombinants and their expression of the foreign genes in the host, with discernible disease reduction. Additionally, as with all GMO, the safety issues pertaining application of the recombinant strains will have to be addressed. The effect of internalins on the beneficial properties of the *L. casei* strains will also need to be ascertained in order to confirm that they still qualify to be referred to as probiotics.

INTRODUCTION

Listeria monocytogenes is a Gram-positive bacterium that is responsible for a severe food-borne disease, listeriosis, characterized by meningitis, meningo-encephalitis, materno-fetal and perinatal infections (Cossart and Toledo- Arana, 2008). *Listeria* usually affects and causes diseases in immune-compromised individuals; therefore, it is termed an opportunistic pathogen. There are, however, instances in which normal healthy individuals were diagnosed with listeriosis (Schlech *et al*., 1983). The ability of *L. monocytogenes* to induce its own uptake by the phagocytic cells is very crucial in its pathogenicity (Cossart, 1997; Finlay and Cossart, 1997). This pathogen does not only induce its uptake into the host cells, it further has the ability to invade and reside in the mammalian cells (Lecuit *et al*., 1997). Most foodborne pathogens after ingestion cross the intestinal barrier then reach the lymph and the blood, the liver in which they replicate in hepatocytes, and also the spleen (Cossart and Toledo- Arana, 2008). *Listeria monocytogenes* is one of a few pathogens that can disseminate further to reach the brain and the placenta. Thus, *L. monocytogenes* as a pathogen can cross all the three host barriers: the intestinal, the blood-brain and the materno-fetal barriers (Hamon *et al*., 2006; Bonazzi *et al*., 2009; Ribet and Cossart, 2015).

The infection process of *L. monocytogenes* has been widely studied (Farber and Peterkin, 1991; Hamon *et al*., 2006; Cossart and Toledo- Arana, 2008). During infection, *Listeria* is internalized by the host cells using the "zipper mechanism", characterized by an intimate interaction between the bacterial cell and the host cell membrane, leading to the its progressive engulfment (Lecuit *et al*., 1997). This internalization is mediated by the direct interaction of the two surface proteins, internalin (inl) A and inlB. These are both host plasma membrane proteins that target E-cadherin and the hepatocyte growth factor receptor Met, respectively (Shen *et al*., 2000; Bonazzi and Cossart, 2011). After entry of the pathogen into cells: 1) the bacteria are entrapped into a vacuole, from which the membrane gets disrupted by the secretion of two phospholipases, PlcA and PlcB, and the pore-forming toxin listeriolysin O. 2) bacteria will then be released into the cytoplasm where they replicate and start to polymerize actin, thereby allowing themselves to pass into neighbouring cells by forming protrusions in the plasma membrane. 3) upon entry into the neighbouring cells, bacteria will be presented as a double- membrane vacuole allowing a new cycle of replication to take place in a second infected cell (Hamon *et al*., 2006). This phenomenon of

direct cell to cell spread allows dissemination of the bacteria in various infected tissues while being protected from host defences (Cossart and Toledo- Arana, 2008).

Listeria monocytogenes has a very wide host range; it has been isolated and identified in a minimal list of 27 species of animal from mammals and human, chicken, ticks, fish and crustaceans (Saha *et al*., 2015). It has been found in stream water, mud, sewage, slaughter house waste, silage, and sick room dust (Gray and Killinger, 1966). The ubiquitous nature of this bacterium makes its distribution easier. There has been a number of listeriosis outbreaks linked to contamination of food, including among others, fresh vegetables and/ or ready-to-eat meats. These outbreaks had then presented the need for an intervention strategy that can be used to prevent the infection, thereby protecting the susceptible people. As with the other disease caused by bacteria, antibiotics were and are still currently used to control the *L*. *monocytogenes* infection. Despite many therapeutic improvements made in the antibiotic field, their negative effects in the gastrointestinal (GI) infections (Rolfe, 2000) and microorganisms becoming resistant are increasing creating major clinical problems. Probiotics, are now an alternative in the treatment of intestinal disorders where medications, diet or surgery, disrupt the normal flora of the gastrointestinal tract (GIT), making the host animal even more susceptible to disease (Rolfe, 2000).

Probiotics are defined "*as live microorganisms which when administered in adequate amounts confer a health benefit on the host"* (Leahy *et al*., 2005). Among others, one of the criteria that probiotic cultures must meet is that they have to be able to inhibit pathogen(s) in the GIT. They have to be able to compete with the pathogen; either for nutrients or space; and successfully exclude them from the GIT (Ohashi and Ushida, 2009). One of the advantages of probiotics is that they allow for growth of beneficial microorganisms therefore displacing the harmful pathogens (Saarela *et al*., 2000). Therefore, providing the host with health benefits by altering their GIT microflora, reinforcement the mucosal barrier by, e.g. their adhering to the intestinal mucosa thus inhibiting pathogen adherence, pathogen inactivation, modification of bacterial enzyme activity, and influence on gut mucosal permeability, and regulation of the immune system (Betoret *et al*., 2003; Krasaekoopt *et al*., 2003).

Different enteric pathogens, the diseases they cause as well as symptoms of the disease caused have been studied intensively. One of those pathogens is *L. monocytogenes*, a widespread pathogen that can be found in unrelated hosts, in widely differing foods and living animals. It is a highly resistant bacterium that grows between 4 and 50°C, with optimum growth at 30- 37°C (Thévenot *et al*., 1994). Considering these characteristics, it is not surprising how difficult it will be to control this pathogen. *Listeria monocytogenes* is an opportunistic pathogen that persists mostly in immune- compromised people such as those with HIV, elderly, cancer and organ transplant patients and also in pregnant women. There have been rare cases reported where 'healthy' people were infected by this pathogen (Schlech *et al*., 1983) and these cases were attributed to exposure to high infective doses (Kaczmarski and Jones, 1989; McLauchlin *et al*., 1990; McLauchlin *et al.*, 1991).

Given the ability of the pathogen to grow in different environments such as water and soil, and in different temperatures, it is therefore noted that the pathogen can be found in fresh vegetables and freshly cut meat. It is ingested with the food product and then enters the GIT where it will interact with the epithelial cells and cause an infection. When left untreated, the bacteria spread out of the intestine to reach the liver, spleen, bladder, brain and the placenta (Hardy *et al*., 2004; Disson and Lecuit, 2012; Disson and Lecuit; 2013). The disease it causes, listeriosis, presents varying symptoms; they can mostly be flu- like, with co- symptoms such as fever, chills, muscle aches and diarrhoea or upset stomach. With regard to the presence of the pathogen in the nervous system, symptoms such as headache, stiff neck, confusion, loss of balance can occur. Infected pregnant women may experience only a mild, flu-like illness; however, infection can be transferred to the unborn baby through the placenta, leading to miscarriage, stillbirth, premature delivery, or infection of the newborn. The incidence of listeriosis is difficult to establish because it can easily be mistaken for a flu-like illness or gastroenteritis (Bortolussi, 2008). Misdiagnosis of listeriosis leads to mistreatment of this disease and therefore will end up with ineffective therapeutic intervention.

There are currently no preventative methods for the treatment of infection caused by *L. monocytogenes* except for those that have been outlined by the Centre for disease control and prevention (CDC) stating that safe food handling is very important, and that food should be thoroughly cooked. This on its own will not eliminate listeriosis, therefore, an alternative that can be used to protect the susceptible and other healthy people from listeriosis is needed. Antibiotics have been the first line of defence when coming to bacterial infections but there has been an escalation with the antibiotic resistance due to antibiotic overuse or misuse. Moreover, antibiotic resistance levels are also elevated among foodborne pathogens (Mache *et al*., 1997; Mache, 2002), including *L. monocytogenes*. The resistance of pathogens to antibiotics leads to inadequate infection control in health-care institutions, shortfalls in hygiene, sanitation, and public health and lack of surveillance and consequent late detection (Okeke *et al*., 2005). This therefore led to more research into probiotics.

Probiotics exert different beneficial effects on host species (Ouwehand *et al.,* 1999). Different studies on the enhancement of the activity and the viability of probiotics including multiple- stress adaptation (Mathipa and Thantsha, 2015) and microencapsulation (Amakiri *et al*., 2015) have been conducted. Bioengineering has also been used in the field of probiotics as a solution to broaden their efficacy (Bhunia, 2012) and also as a strategic approach to control enteric pathogens (Amalaradjou and Bhunia, 2013). Understanding the infection cycle of pathogens and then taking into consideration their virulence genes has introduced a new strategy to control these pathogens. The application of bioengineered probiotic strains that through their design harbour genes that can inhibit the pathogen from the GIT, represents an alternative strategy that can be used as a preventative method for enteric pathogens. This current study looked at the ability of the bioengineered probiotic *Lactobacillus casei* harbouring invasion genes internalin AB of *Listeria monocytogenes* in the prevention of listeriosis. A probiotic *Lactobacillus casei* was bioengineered to express internalin AB (Inl A and B), proteins essential for *L. monocytogenes*' entry into the non-phagocytic cells and the promotion of non- covalent binding to lipoteichoic acid on the host cell membranes, and then it was subsequently evaluated as an alternative method for control of *L. monocytogenes*

Aim of the study

The aim of the current study is to investigate the efficacy of the bioengineered *Lactobacillus casei* harbouring invasion genes internalin AB of *Listeria monocytogenes* in the prevention of listeriosis.

Objectives of the study

The specific objectives of the study are to:

1. Construct the recombinant *L. casei* strain carrying the invasion genes InlAB from *L. monocytogenes*

2. Investigate the ability of the bioengineered *L. casei* to prevent the adhesion, invasion and cytotoxicity of *L. monocytogenes* to the Caco- 2 cells *in vitro*

3. Investigate the ability of the bioengineered *L. casei* in the prevention of the adhesion, invasion and cytotoxicity of *L. monocytogenes* to the Caco- 2 cells in simulated gastrointestinal fluid (SIF) under anaerobic conditions

REFERENCES

Amakiri, A.C., Kalombo, L., Thantsha, M.S. (2015). Production and characterization of freeze-dried Vegetal BM 297 ATO-inulin lipid-based synbiotic microparticles containing *Bifidobacterium longum* LMG 13197. *Journal of Microencapsulation, 32(8)*:820-827.

Amalaradjou, M.A., Bhunia, A.K. (2013). Bioengineered probiotics, a strategic approach to control enteric infections. *Bioengineered, 4*:379- 387.

Betoret, N., Puente, L., Díaz, M.J., Pagán, M.J., García, M.J., Gras, M.L., Martínez-Monzó, J., Fito, P. (2003). Development of probiotic-enriched dried fruits by vacuum impregnation. *Journal of Food Engineering, 56*: 273-277.

Bhunia, A.K. (2012). Bioengineered probiotics- a solution to broaden probiotic efficacy! *Journal of Nutrition and Food Science, 2:e105*.doi:10.4172/2155-9600.1000e105.

Bonazzi, M., Cossart, P. (2011). Impenetrable barriers or entry portals? The role of cell-cell adhesion during infection. *Journal of Cell Biology, 195*: 349–358.

Bonazzi, M., Lecuit, M., Cossart, P. (2009). *Listeria monocytogenes* internalin and E-cadherin: From structure to pathogenesis. *Cell Microbiology, 11*:693–702.

Bortolussi, R. (2008). Listeriosis: a primer. *Canadian Medical Association Journal, 179:* 795-797.

Cossart, P. (1997). Subversion of the mammalian cell cytoskeleton by invasive bacteria. *Journal of Clinical Investigation, 99*:2307–2311.

Cossart, P., Toledo-Arana, A. (2008). *Listeria monocytogenes*, a unique model in infection biology: an overview. *Microbes and Infection, 10*: 1041–1050.

Farber, J. M., Peterkin, P. I. (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Reviews, 55*:476–511.

Finlay, B. B., Cossart, P. (1997). Exploitation of mammalian host cell functions by bacterial pathogens. *Science, 276*:718–725.

Gray, M.L., Killinger, A.H. (1966). *Listeria monocytogenes* and listeric infections. *Bacteriological Reviews, 30*:309-371.

Hamon, M., Bierne, H., Cossart, P. (2006). *Listeria monocytogenes*: A multifaceted model. *Nature Reviews: Microbiology, 4*:423–434.

Kaczmarski, E.B., Jones, D.M. (1989). Listeriosis and ready-cooked chicken. *Lancet, 1*:549.

Krasaekoopt, W., Bhandari, B., Deeth, H. (2003). Evaluation of encapsulation techniques of probiotics for yoghurt. *International Dairy Journal, 13*: 3-13.

Leahy, S.C., Higgins, D.G., Fitzgerald, G.F., van Sinderen, D. (2005). Getting better with bifidobacteria. *Journal of Applied Microbiology, 98*: 1303-1315.

Lecuit, M., Ohayon, H., Braun, L., Mengaud, J., Cossart, P. (1997). Internalin of *Listeria monocytogenes* with an intact leucine-rich repeat region is sufficient to promote internalization. *Infection and Immunity, 65*: 5309–5319.

Mache, A. (2002). *Salmonella* serogroups and their antibiotic resistance patterns isolated from diarrhea stools of pediatric outpatients in Jimma Hospital and Jimma Health center, southwest Ethiopia. *Ethiopian Journal of Health Science, 12:* 37-45.

Mache, A., Mengistu, Y., Cowley, S. (1997). *Salmonella* serogroups identified from adult diarrheal out-patients in Addis Ababa, Ethiopia: Antibiotic resistance and plasmid profile analysis. *East African Medical Journal, 74*:183-187.

Mathipa, M.G., Thantsha, M.S. (2015). Cocktails of probiotics pre-adapted to multiple stress factors are more robust under simulated gastrointestinal conditions than their parental counterparts and exhibit enhanced antagonistic capabilities against *Escherichia coli* and *Staphylococcus aureus*. *Gut Pathogens 7*:5.

McFarland, A.P., Savan, R., Wagage, S., Addison, A., Ramakrishnan, K., Karwan, M., Duong, T., Young, H.A. (2011). Localized delivery of interferon-β by *Lactobacillus* exacerbates experimental colitis. *PLoS ONE, 6*(2), E16967.

McFarland, L.V. (2005). Alternative treatments for *Clostridium difficile* disease: what really works? *Journal of Medical Microbiology, 54*:101-111.

Ohashi, Y., Ushida, K. (2009). Health-benefcial efects of probiotics: its mode of action. *Animal Science Journal, 80(4)*: 361–71.

Okeke, I.N., Klugman, K.P., Bhutta, Z.A., Duse, A.G., Jenkins, P., O'Brien, T.F., Pablos-Mendez, A. Laxminarayan, R. (2005). Antimicrobial resistance in developing countries. Part II: strategies for containment. *The Lancet Infectious Diseases, 5*: 568-580.

Ouwehand, A. C., Salminen, S., Isolauri, E. (2002). Probiotics: An overview of beneficial effects. *Antonie Leeuewenhoek 82*: 279–289.

Ribet, D., Cossart, P. (2015). How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes and Infection, 17:* 173- 183.

Rolfe, R.D. (2000). The role of probiotic cultures in the control of gastrointestinal health. *Journal of Nutrition 130:* 396- 402.

Saarela, M., Mogensen, G., Fondén, R., Mättö, J., Mattila-Sandholm, T. (2000). Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology, 84*: 197-215.

Saha, M., Bebnath, C., Pramanik, A.K., 2015. *Listeria monocytogenes*: An emerging food borne pathogen. *International Journal of Current Microbiology and Applied Sciences, 11*:52- 72.

Schlech III, W. F., Lavigne, P. M., Bortolussi, R. A., Alien, A. C., Haldane, E. V., Wort, A. J., Hightower, A. W., Johnson, S. E., King, S. H., Nicholls, E. S., Broome, C. V. (1983). Epidemic listeriosis-evidence for transmission by food. *The New England Journal of Medicine, 308*:203-206.

Shen, Y., Naujokas, M., Park, M., Ireton, K., 2000. InlB-dependent internalization of *Listeria* is mediated by the Met receptor tyrosine kinase. *Cell, 103*:501–510.

Thévenot, D., Dernburg, A., and Vernozy-Rozand, C. 2006. An Updated Review of *Listeria Monocytogenes* in the Pork Meat Industry and Its Products. *Journal of Applied Microbiology 10:* 7-17.

Chapter 1

Literature Review

Part of this chapter was published: **Mathipa, M.G.,** Thantsha, M.S. (2017). Probiotic engineering: Towards development of robust probiotic strains with enhanced functional properties and for targeted control of enteric pathogens. *Gut Pathogens, 9*(1), 28.

1.1. Foodborne pathogens

Poor hygiene and sanitation during food preparation can lead to the presence of different foodborne pathogens in food. Some of these pathogens or their toxins produced either before or after ingestion of such foods, can either act locally within the gastrointestinal tract (GIT), leading to development of illnesses, or disseminate to other parts of the body and damage cells/ tissues and ultimately the immune system (de Sousa, 2008). Incidences of foodborne illnesses are high in most developing countries as food control is a low priority issue due to limited funds. As a result of this, foodborne pathogens are the leading cause of illnesses and death in these countries (Fratamico *et al*., 2005). Most foodborne illnesses have diarrhoea as the primary symptom. Most societies consider diarrhoea a normal, natural condition; therefore, it usually goes unnoticed and/or untreated. The World Health Organization reported that in 1997, of the global total of 52.2 million deaths, 17.3 million were attributed to infectious and parasitic diseases, of which 2.5 million were due to diarrhoea (WHO, 1998). In 2007 the WHO reported that there were more than 1.5 billion cases of foodborne illnesses and more than 3 million annual fatality cases. These illnesses are not confined to developing countries. In the United States, foodborne pathogens cause an estimated 48 million illnesses annually (Scallan *et al*., 2011). Enteric pathogens account for high morbidity and mortality and are considered to be the fifth leading cause of death across all ages worldwide (Gupta *et al*., 2014). Amongst others, *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* are the most common food pathogens implicated (Manning *et al*., 2001, de Wit *et al*., 2001). When these pathogens are present in different food products, they can lead to different complications. Comparing the devastating effects caused by foodborne pathogens, listeriosis has been reported to have the highest hospitalization (90.5%) and case fatality rates (21%) (CDC, 2000).

1.2. *Listeria monocytogenes*: Background

The genus *Listeria* is composed of seventeen recognized species including *Listeria monocytogenes, Listeria seeligeri, Listeria ivanovii, Listeria welshimeri, Listeria marthii, Listeria innocua, Listeria grayi, Listeria fleischmannii, Listeria floridensis, Listeria aquatica, Listeria newyorkensis, Listeria cornellensis, Listeria rocourtiae, Listeria weihenstephanensis, Listeria grandensis, Listeria riparia, and Listeria booriae)* (Graves *et al*., 2010; Orsi and Wiedmann, 2016). Of all these, *L. monocytogenes* and *L. ivanovii* are reported to be the only species pathogenic to humans and animals, respectively (Farber and Peterkin, 1991). *Listeria monocytogenes* was first

isolated from infected rabbits and identified as *Bacterium monocytogenes* by Murray *et al*. (1926) and later renamed *L. monocytogenes* after Lord Lister by Pirie (1940). There are 13 serotypes of *L. monocytogenes* that have been identified, however, most of the human illnesses have been linked to serotypes 1/2a, 1/2b, and 4b (Silk *et al*., 2012, Cartwright *et al.*, 2013). Among the three serotypes, isolates of serotype 4b have reported to be responsible for the greatest proportion of listeriosis outbreaks and the largest number of cases per outbreak (Swaminathan and Gerner-Smidt, 2007). In 2011, however, serotypes 1/2a and 1/2b were implicated in the listeriosis outbreak in U.S. history. Previous studies have reported on the low rates of a sequence type 6 (ST6) *L. monocytogenes* among foodborne disease isolates. Koopmans *et al*., (2013) analysed clinical characteristics, treatment, genetic diversity, and outcome of 92 adults with *L. monocytogenes* meningitis. They concluded in their study that the emerging *L. monocytogenes* serotype 4b ST6 was identified as the main factor leading to a poorer prognosis. Althaus *et al.* (2017) reported that in 2016 there were five confirmed and two probable cases of listeriosis due to *L. monocytogenes* ST6. In 2018, *L. monocytogenes* ST6 has been implicated in the largest listeriosis outbreak in South Africa with a total of 1060 listeriosis cases and 214 deaths reported to date (CDC, 2018). Using the Multilocus sequence typing (MLST) Allam *et al.* (2018) reported that of the 521 clinical isolates that they sequenced using a whole genome sequencing approach, 443 (85%) of those isolates belonged to *L. monocytogenes* sequence type 6 (ST6).

1.2.1. Epidemiology

Listeria monocytogenes is a Gram-positive, facultative intracellular food-borne pathogen. It has the capacity to cause severe infections such as gastroenteritis, septicemia, abortion, meningitis, materno-fetal, perinatal infections and febrile gastroenteritis especially after ingestion of highly contaminated food products by humans and animals (Farber and Peterkin, 1991; Cossart and Toledo-Arana, 2008). The most common infection caused by *L. monocytogenes* is listeriosis. This pathogen is characterized by its low infection rate but a high mortality rate, as high as 20–30%, (Werbrouck *et al*., 2006). Groups of people most susceptible to this pathogen include the immunocompromised individuals (e.g. people with Human Immunodeficiency Virus (HIV)), elderly people, pregnant women, foetuses and neonates, those taking immunosuppressive medications following organ transplantation and those with cancer, autoimmune diseases, alcoholism and diabetes mellitus (Rubin *et al*., 1999; Khan and Wingard, 2001; Dropulic and

Lederman, 2016). During pregnancy, there is an escalated production of progesterone which leads to down-regulation of cellular (cell-mediated) immune functions (Smith, 1999), making pregnant women prone to infections. The consequences of foodborne illness can be particularly devastating during pregnancy as both the woman and her foetus are at risk. Listeriosis can also occur in people that are immune- competent (Schlech *et al*., 1983) however, the infection is usually asymptomatic or self-limiting (Koo *et al*., 2012).

The ability of *L. monocytogenes* to survive and multiply in a wide variety of conditions and environments presents a major concern for the food industry (Kathariou, 2002). Additionally, the incidence of listeriosis is difficult to establish and diagnose as it can easily be mistaken for influenza or gastroenteritis due to the similarity of symptoms (Bortolussi, 2008). This misdiagnosis of listeriosis can lead to its mistreatment and will end up with ineffective therapeutic interventions. The symptoms can vary from one person to another, including but not limited to influenza- like, with co- symptoms such as fever, chills, muscle aches and diarrhoea or upset stomach. Movement of the bacteria into the nervous system results in symptoms such as headache, stiff neck, confusion and loss of balance. Although infected pregnant women may experience only a mild, flu-like illness, infections during pregnancy can lead to miscarriage or can transfer the bacteria to the unborn baby through the placenta thereby causing spontaneous abortion, stillbirth, premature delivery, or infection of the newborn (Bortolussi, 2008).

1.2.2. *Listeria monocytogenes* outbreaks

The vehicle of infection in early *L. monocytogenes* outbreaks has been related to its direct transmission from infected animals to farm workers and veterinarians (Farber and Peterkin, 1991). Studies have reported on the different serotypes of *L. monocytogenes* that have been linked to clinical cases, most of those belonged to the subset of serotypes 1/2a, 1/2b, and 4b (Kathariou, 2002) with serotype 4b being linked to most outbreaks in the past (Kathariou, 2002; Knabel *et al*., 2012; Lomonaco *et al*., 2013) followed by serotype 1/2a and then 1/2b (Cartwright *et al*., 2013).

The first documented listeriosis outbreak affected 41 people (34 perinatal and 7 adults) was reported in Canada between March and September 1981, being linked to the epidemic strain serotype 4b. The implicated food was refrigerated coleslaw (Schlech, *et al*., 1983). Kaczmarski and Jones (1989) isolated serotype 1/2a from the stool of a patient who ate ready cooked chicken

nuggets. In 1990 Farber *et al*. isolated serotype 1/2b from the blood and synovial fluids of a patient who consumed large amounts of soft cheese. The first documented outbreak in the United States occurred in 1983 and it was linked to pasteurized milk (Cartwright *et al*., 2013). Later in 1985 from January to August, there was another outbreak in California, caused by Mexican-style soft cheese brand contaminated with strain 4b. It affected 142 people of which 93 were perinatal while 49 were adult cases (Linnan *et al*., 1988). In the perinatal cases, there were 48 deaths involving 30 fetuses plus newborn infants, and 18 non-pregnant adults while in the adult cases, 48 were immunosuppressed or elderly or had a severe chronic illness. Turkey frankfurters were also implicated in another outbreak that caused 54 adults and 12 perinatal cases and 8 deaths (CDC, 1989). Between 1998 and 2008, twenty-four listeriosis outbreaks were reported, which resulted in 359 illnesses, 215 hospitalizations and 38 deaths (Cartwright *et al*., 2013). There were even more outbreaks in the United States between 2009- 2017. Laksanalama *et al*. (2012) reported on the 2011 multistate listeriosis due to presence of serotype 1/2a and 1/2b in cantaloupe, which caused a total of 146 invasive illnesses, 30 deaths and one miscarriage. Angelo *et al*., (2017) reported the 2014- 2015 outbreak caused by pre-packaged caramel apples, which caused 35 cases, of which 34 were hospitalized and seven died. The most recent largest listeriosis outbreak was reported in South Africa. It began in January 2017 with around 1,060 known cases and over 214 laboratory confirmed deaths (CDC, 2018). This outbreak was caused by a widely consumed ready-to-eat processed meat product called "polony" and was also found in the processing environment (production facility) of the manufacturer of this implicated meat product (Allam *et al*., 2018). All these outbreaks were either due to environmental contamination or sanitation deficiencies, indicating a lack of attention to sanitation and hygiene (Jackson *et al*., 2018). This highlights the importance of sanitation during food production and preparation. Therefore, improved sanitary and hygienic practices by the food industry during food production, strict regulatory enforcements and continuous consumer education are necessary to prevent and control listeriosis.

1.2.3. Characteristics of *L. monocytogenes*

Listeria monocytogenes is widely present in nature, inhabiting both the soil and water. It is a hardy bacterium that can grow at extremely low temperatures as low as 3°C. It can therefore survive and multiply in food during and then cause illness later when the food is consumed without being

exposed to a listericidal process. This is evidently one reason why most food products, raw fruits and vegetables and animals, are contaminated with this pathogen. Despite contamination of most food products with this pathogen (Uyttendaele *et al*., 1999; Van Coillie *et al*., 2004), the incidence of it actually causing disease is very low. This is attributed to the different virulence potentials of each individual strain and the health status of the host (Brosch *et al*., 1993; Van Langendonck *et al.,* 1998; Norrung and Andersen, 2000; Werbrouck *et al*., 2006). Taking into consideration that immune competent people can contract listeriosis; this further explains that the different virulence potentials of the different *L. monocytogenes* strains could play a role in the susceptibility of listeriosis (Kelly *et al*., 1999; Farber *et al.,* 2000; Norrung and Andersen, 2000). This bacterium is not only taken up by professional phagocytes, but it can also invade non-phagocytic cells such as epithelial cells (Gaillard *et al*., 1987), hepatocytes (Dramsi *et al.,* 1995; Gaillard *et al*., 1996; Wood *et al*., 1993) and endothelial cells (Ireton *et al.,* 1999). It has the capacity to induce its own uptake into these non-phagocytic mammalian cells (Gaillard *et al*., 1987; Cossart, 1997; Finlay and Cossart, 1997; Ireton and Cossart. 1997).

1.2.4. *L. monocytogenes* pathogenesis

During the infection process, *L. monocytogenes* is able to cross three significant barriers, namely, the intestinal epithelial cell barrier, the blood-brain endothelial cell barrier, and the feto-placental endothelial cell barrier (Werbrouck *et al*., 2006). It can enter host cells either through active ingestion by phagocytic cells such as macrophages or through the interaction of specific proteins and receptors that control ingestion by normal non-phagocytic cells (da Silva *et al*., 2012). The interaction of a bacterial surface protein with a specific receptor on the plasma membrane of the host cell is known as the "zipper" mechanism (Isberg and Tran Van Nhieu, 1994; Mengaud *et al.,* 1996, Swanson and Baer, 1995). This interaction leads to the progressive engulfment of the bacterium (Mengaud *et al.,* 1996). The "zipper" mechanism involves a series of virulence factors that are responsible for attachment, invasion, growth and migration from cell to cell.

Listeria monocytogenes uses multiple virulence factors; however, they are not equally important in the infection process. Factors that have been reported to be more effective and critical to the infection process are PrfA, Internalin A (InlA), InlB, Actin assembly-inducing protein (ActA) and Listeriolysin O (LLO) (Koo *et al*., 2012). PrFA is a regulatory molecule that controls the

expression of the key virulence factors (Camejo *et al*., 2011; Kaur *et al*., 2007). Most of virulence genes responsible for the intracellular life cycle of *L. monocytogenes* are found on a region of the chromosome known as the PrfA-dependent gene cluster (Chakraborty *et al*., 1992). *Listeria* adhesion protein (LAP) is responsible for adhesion of the pathogen to epithelial cells (Jagadeesan *et al*., 2010). Internalin A mediates entry of *L. monocytogenes* into non-phagocytic cells while InlB promotes its non-covalent binding to lipoteichoic acid on the host cell membrane. ActA is involved at multiple stages: adhesion, invasion, evasion of host defences and cell-to-cell spread (Camejo *et al*., 2011; Lecuit, 2005; Southwick and Purich, 1996). Listeriolysin O is the main factor responsible for escape of *L. monocytogenes* from vacuoles (Southwick and Purich, 1996; Camejo *et al*., 2011).

1.2.5. Adhesion and Invasion of *L. monocytogenes*

1.2.5.1. *Listeria* adhesion

Being an intracellular foodborne pathogen, the initial interaction of *L. monocytogenes* with the intestinal epithelium is crucial in the establishment of its infection and also in promoting its spread to extraintestinal sites (Burkholder and Bhunia, 2010). The adhesion of *L. monocytogenes* has been well studied and the virulence proteins well reported. This stage involves numerous proteins including fibronectin binding protein (FbpA), Ami, CtaP, LAP and ActA. FbpA was reported to bind to fibronectin in the intestinal epithelium and on hepatocytes (Dramsi *et al*., 2004). Ami, an autolysin amidase, plays a role in the adhesion of the pathogen, however there aren't enough studies elucidating the mechanism and the host receptor to which it binds (Milohanic *et al*., 2001). CtaP, a cysteine transport-associated protein, has been reported to also contribute to adhesion of the pathogen to host cells (Xayarath *et al*., 2009). Reis *et al*. (2010) reported that LapB is involved in both adhesion to and invasion of host cells. Jaradat *et al*. (2003) and later Jagadeesan *et al*. (2010) identified the *Listeria* adhesion protein (LAP), a 104-kDa alcohol acetaldehyde dehydrogenase (lmo1634) and reported that it promotes adhesion of *Listeria* during the intestinal phase of infection. This protein interacts with the epithelial receptor, heat shock protein 60 (Hsp60), promoting adhesion to the host cells (Jagadeesan *et al*., 2011; Wampler *et al*., 2004). Once adhered, the pathogen then utilizes different proteins to invade the cells.

1.2.5.2. **The internalins**

Previous research has reported that the *L. monocytogenes* genome includes a large family (25 members) of proteins harbouring leucine-rich repeats known as internalins (Inl) (Bierne *et al*., 2007). They were all grouped in the same family as they all have the N-terminal signal sequence and an LRR domain in common, however, the presence of several other region helps differentiate amongst the internalin family. Of the internalins that were characterized, InlC, InlH, InlJ and Lmo2026 have been associated with the infection process, however, their functions are not yet clear (Lecuit, 2007; Seveau *et al*., 2007). The same applied to all the other internalins except InlA and InlB, which had been well characterized and reported to both being invasins necessary and sufficient to trigger internalization by normally nonphagocytic cells. InlA and InlB are the two surface proteins of *L. monocytogenes* that have been identified to promote host cell invasion and mediate host cell specific internalization (Gaillard *et al*., 1991; Lingnau *et al*., 1995; Dramsi *et al*., 1995; Lecuit *et al*., 1997). The internalins are not only responsible for the invasion of the pathogens to the cells of the host; they have also been reported as being responsible for the tranlsocation of the pathogen into the host cells (Braun *et al*., 1997; Lecuit *et al*., 1997; Pizarro-Cerda and Cossart, 2006). InlA and InlB have common structural features which are also shared by other proteins constituting the internalin multigene family (Farber and Peterkin, 1991; Dramsi *et al*., 1995). Their structure include an amino-terminal leucine-rich repeat domain (LRR) formed by tandem repeats of 20- 22 amino acids (Glaser *et al.,* 2001, Schubert *et al.,* 2001; Cabanes *et al.,* 2002) and the B-repeat region, separated by a highly conserved inter-repeat (IR) region (Dramsi *et al*., 1995; Farber and Peterkin, 1991).

1.2.5.2.1. **Internalin (InlA)**

Internalin A is an 800-amino-acid protein, containing a 15-LRR domain (Braun *et al*., 1997). Downstream of the LRR region it harbours an inter-repeat region that has been shown to be crucial for binding of the LRR domain to E-cadherin (Lecuit *et al*., 1997; Schubert *et al*., 2002). Mengaud *et al*. (1996) described E-cadherin as an adhesion molecule that is involved in the formation of adherent junctions at the three barriers; intestinal barrier, the blood–brain barrier, and the placenta, and has been identified as the cellular receptor for InlA. InlA was identified as enabling (Gaillard *et al.,* 1991; Seveau *et al*., 2007) and required by *Listeria* for its entry into human intestinal

epithelial cell line (Gaillard *et al*., 1991) and other cell lines expressing the E- cadherin receptor (Mengaud *et al*., 1996; Shen *et al*., 2000; Bonazzi *et al.*, 2009). This specific binding of InlA and the host cell receptor E-cadherin promotes invasion of enterocytes and crossing of the intestinal barrier (Lecuit *et al.*, 1999; Lecuit *et al*., 2001; Mengaud *et al.*, 1996; Pizarro-Cerdá *et al.*, 2004). InlA has also been reported to be responsible for the adhesion of *L. monocytogenes* that is initiated by the interaction of the protein InlA and the host receptor E-cadherin. It has been reported that the interaction between the LRRs expressed on the surface of *Listeria* is sufficient to promote the adhesion and entry into the host cells (Lecuit *et al*., 1997). InlA interacts specifically with the first ectodomain (EC1) of E- cadherin, the N-terminal EC repeat of E-cadherin also involved in the initial interaction between E-cadherin molecules on the surface of adjacent cells (Lecuit *et al*., 1999). This interaction then leads to the adherence of the bacteria resulting in the host cell invasion. It has already been established that InlA is responsible for the entry into the intestinal barrier, hence in studies on the *in vitro* assays of *Listeria* Caco-2 epithelial cells are used.

1.2.5.2.2. Internalin B (InlB)

Internalin B is a 630- amino-acid surface protein containing an 8-LRR domain (Domann *et al*., 1997), which interacts with three distinct host cell receptors, namely: 1) Met (Shen *et al.*, 2000), 2) gC1q-R (Braun *et al*., 2000), and 3) glycosaminoglycans (Jonquières *et al.*, 2001). It exists in two different forms, at the surface of the bacteria and also in the culture supernatants (Braun *et al*., 1997). The hepatocyte growth factor receptor, Met, is ubiquitous, allowing InlB to mediate internalization into a wider range of cell types (Bierne and Cossart, 2002; Cossart *et al*., 2000; Shen *et al*., 2000), including but not limited to hepatocytes, epithelial cells, fibroblasts and endothelial cells (Dramsi *et al.*, 1995; Gaillard *et al.*, 1996; Parida *et al.*, 1998; Lingnau *et al.*, 1995). This entry is responsible for the development of the systemic infection.

It has already been established that *Listeria* can cross all the three host barriers: the intestinal barrier, the blood-brain barrier and the materno-fetal barrier (Hamon *et al*., 2006; Bonazzi *et al*., 2009; Ribet and Cossart, 2015). There have been a number of incidences of meningitis and encephalitis that are associated with human *L. monocytogenes* infection; implying that this microorganism is able to breach the blood-brain barrier (Greiffenberg *et al.*, 1998). Entry into the blood- brain and the materno- fetal barrier has not been that well studied; however, InlB has been

implicated in this invasion. During *in vitro* studies cell lines such as HeLa, HEp-2, Henle 407, L929, Vero cells and hepatocytes are preferred when studying InlB (Braun *et al*., 1998; Dramsi *et al.*, 1995). Parida *et al*. (1998) reported that invasion of the human umbilical vein endothelial cells (HUVEC) is InlB dependent. This study was further supported by Greiffenberg *et al*. (1998) who reported that the invasion of *Listeria* into the human brain microvascular endothelial cells (HBMEC) depends only on InlB.

1.2.6. *L. monocytogenes* control in the clinical environment and in the food industry

Listeria monocytogenes grows in soil, can be found in contaminated water, in the intestines of some animals and in fresh produce, thus increasing the probability of its presence in Ready-to-Eat (RTE) foods (Khan *et al*., 2016). The ability of *L. monocytogenes* to persist and survive in the different environments makes it a difficult pathogen to control. Its presence in food makes presents a high risk of infection in the susceptible group of people (Tompkin, 2002) therefore, industry must take stringent measures to control *L. monocytogenes* in ready-to-eat foods in which the organism can grow (Tompkin *et al*., 2015). Inhibition of this pathogen is a complex and difficult process, however there are regulations that have been set in place, particularly for RTE manufacturing and handling, to eliminate the chances of outbreaks. Cooperating with these regulations is the first step to inhibiting *Listeria* in food industries and the subsequent infections altogether. Of the regulations that have been set in place, when found in RTE food, the products are recalled from the market to ensure the safety of the public and to cross-contamination (from object to object, from food to object or from food to food) during manufacturing or in stores (Chmielewski & Frank 2003). Due to the different contamination points, it is crucial to focus at the different contamination points when preventing the spread and infection caused by *L. monocytogenes*. Pouillot *et al*. (2015) reported on the conditions that are considered when controlling *L. monocytogenes* and what the implications of those conditions were in retail. Furthermore, their report provided a scientific assessment of the risk of listeriosis associated with consumption of RTE foods in retail food stores. Most research that looks at the control of *L. monocytogenes* usually separates the strategies depending on the different contamination points. In the report released by the Risk Assessment Workgroup on the interagency retail *L. monocytogenes*, they reported that in order to control the spread of *L. monocytogenes* in retail, the following strategies should be followed. 1) Controlling the growth of *Listeria* by using growth

inhibitors and controlling the temperature. 2) Controlling contamination at its source (including other routes of contamination such as the slicer (deli meats and cheeses) or serving utensils (deli salad)) and inhibiting cross contamination. 3) Implementation of a sampling program, where sampling is done over a period of time to facilitate the detection of problems and trends and assess in a timely manner whether the environment in which RTE foods are exposed is under control (Gallagher *et al*., 2016).

There has been research on chemical antimicrobials being used as a control in the inhibition of *L. monocytogenes*. Bacteriophages has been reported to infect foodborne pathogens and therefore there is growing literature suggesting that they can be used to control these pathogens (Greer, 2005; Hudson *et al*., 2005). Listex^{TM} P100 is one of the commercial phages that have been shown to have listericidal activity on raw salmon fillets and channel catfish, however, during refrigerator conditions the phage activity was not as good (Soni and Nannapaneni, 2010; Soni, *et al.,* 2010). In addition to the fridge conditions, the phage was shown to show different results dependent on the food samples that were used in different studies (Guenther *et al*., 2009; Bigot *et al*., 2011). The same results were seen when a different phage, ListShield^{TM}, was used on the inhibition of *L. monocytogenes* on cut fruit. The results showed that the phage was not effective on apples but was effective on honeydew melon pieces which was attributed to the low pH on apples (Leverentz *et al*., 2003). Food preservatives have also been reported in the inhibition of *L. monocytogenes* in addition to the beneficial effects that they offer to the product itself (Zhu *et al.,* 2005). Salt of lactate has been used as an antimicrobial in meat products, its addition to food product with neutral pH offers good prospects for shelf- life prolongation (Houtsma *et al.*, 1993). Suitable amounts of salt lactate in combination with low pH has been reported to suppress the growth of *L. monocytogenes*. Previous study by Mbandi and Shelef (2001) investigated the effects of sodium lactate, sodium diacetate and sodium acetate on the inhibition of *L. monocytogenes* and *Salmonella enteritidis*. They reported that combinations of 2.5% sodium lactate and 0.2% sodium diacetate were bacteriostatic to *L. monocytogenes* at 10 °C, while at 5 °C, a combination of 1.8% sodium lactate and 0.1% sodium diacetate showed listeriostatic effect. Their study was supported by a later study by Zhu *et al*. (2005) that showed that during refrigerated storage *L. monocytogenes* increased by less than 1 log in RTE turkey hams containing 2% sodium lactate plus 0.1% sodium diacetate or 2% sodium lactate plus 0.1% potassium benzoate. In a different study, Janes *et al*. (2002) investigated the inhibitory effects of nisin against *L. monocytogenes* in RTE chicken, they reported

that treatment with nisin significantly suppressed the *L. monocytogenes* population by 1 to 3 log cycles. Martin *et al*. (2009) studied the inhibitory ability of a combination of lauric arginate with the antimicrobials potassium lactate and sodium diacetate formulated into the raw frankfurter batter. They reported that lauric arginate had an immediate effect in the reduction of *L. monocytogenes* growth, showing an almost 2 log reduction at time zero and maintained that decrease for 12 hours. As a different strategy to enhance the inhibition of *L. monocytogenes*¸ Jacobsen *et al*. (2003) reported that probiotics can be used as an alternative to chemical antimicrobials.

1.3. Probiotics

There have been different scientists studying the inhibition of *L. monocytogenes*, however the use of probiotics has been gaining more interest. They have been used to restore the balance of the gut microbial ecosystem and control pathogenic infections. Their administration assists in the prevention and control of food-borne illnesses, through a number of mechanisms including but not limited to, competitive exclusion of pathogens in the GIT, modulation of the host immune system and strengthening of the intestinal barrier (Wohlgemuth *et al*., 2010; Ceapa *et al*., 2013). There are different microorganisms that are used as probiotics, including strains from *Streptococcus, Enterococcus, Pediococcus, Weissella and Lactobacillus* (Ehrmann *et al.,* 2002) but most common ones are *Lactobacillus* and *Bifidobacteria* spp. These bacteria have met the criteria of probiotics and they also have nutritional and therapeutic effects (Rattanachaikunsopon and Phumkhachorn, 2010). Amongst others, one criterion that bacteria must meet in order for them to be regarded as probiotics is that they have to be able to survive and thrive throughout the GIT conditions and confer their beneficial effects. It is therefore important to understand their mechanisms of action in order for them to be used both prophylactic and as treatment options for the different food-borne diseases. The presence of food-borne pathogens in the human GIT affects the balance of the "good to bad" microorganisms. Apart from the presence of the pathogens in the GIT, there are different other factors that can affect the balance of the microorganisms in the host GIT. These different factors include stress, illness or antibiotic treatment, which changes the balance in the GIT in favour of harmful bacteria (Cremonini *et al*., 2002, Harish and Varghese, 2006). Another characteristic of probiotics is that they are able to protect the host from microbial imbalance. The

different mechanisms by which probiotics exclude pathogens from the human GIT are discussed next.

1.3.1. Probiotics' mechanisms of action against enteric pathogens

1.3.1.1. Competitive exclusion

Probiotics use this mechanism to exclude or reduce the growth of another microorganism in the GIT (Fuller, 1991). This can be achieved through different mechanisms (Rolfe, 1991), it could either be competition for nutrients or competition for space to adhere (Ohashi and Ushida, 2009). Microorganisms in any environment require nutrients to multiply and either cause or alleviate infections. The GIT is well known for its abundance in nutrients, therefore making it a great environment for microorganisms. The potential of probiotics to out-compete pathogens for these nutrients thus favours the growth of probiotics over that of the pathogens (Cumming and MacFarlane, 1997). During competition for nutrients, probiotics produce metabolites such as volatile fatty acids reducing the pH of the GIT. The reduction in the pH of the GIT makes it an unfriendly environment for pathogens and thus will lead to their inhibition because most of them cannot grow at low pH (Marteau *et al*., 1997; Chichlowski *et al.,* 2007).

On the other hand, competition for adherent space refers to the physical blocking of pathogenic bacteria colonization by probiotics from their favourite sites such as the intestinal villus, goblet cells and the colonic crypts (Chichlowski *et al.,* 2007). A key pathogenic factor of intestinal pathogens is their ability to attach to the surfaces of intestinal epithelial cells (Weinstein *et al*., 1998). A critical function of the microbiota is colonization resistance, to exclude pathogens from adhering and multiplying on the intestinal mucosal membrane. Adhesion and subsequent colonization of probiotics to the intestinal epithelium can competitively exclude the attachment of pathogens (Corr *et al*., 2009). Ability to adhere to the intestines is one of the pre- requisites for the colonization of probiotics and is also important for the interaction between the probiotic strains and the host (Juntunen *et al*., 2010). Probiotics can bind to intestinal cells via electrostatic interactions, steric forces or specific surface proteins. They have the ability to adhere to the intestinal cells in high quantities (Fuller, 1991; Collins and Gibson, 1999) thereby leaving no space for the pathogens to adhere and cause infection. Adherence of probiotics to the intestine and other

parts of the GIT becomes important as their physical presence at these sites blocks adhesion of the pathogens, thereby preventing infections (Bibiloni *et al*., 1995).

One strategy used by the lactic acid bacteria (LAB) probiotics is that they have a greater ability to adhere to the epithelial cells resulting in competitive exclusion of the pathogenic bacteria (Lee *et al*., 2003). Lactobacilli and bifidobacteria share carbohydrate-binding specificities with some enteropathogens (Nesser *et al*., 2000). *Bifidobacterium bifidum* and *Lactobacillus reuteri* have the ability to bind to glycolipids on the surface of the host cells to prevent attachment of certain pathogens that also bind to specific surface glycolipids (Wohlgemuth *et al*., 2010). Thirabunyanon and Thongwittaya (2012) reported in their study that they observed a reduction of *S. enteritidis* attachment to the surfaces of intestinal epithelial cells in the presence if probiotic *B. subtilis* NC11. This led to a complete exclusion of the pathogen in the GIT, which is the site where the infection process starts. Competitive exclusion of the pathogens by probiotics results in the inhibition of the pathogen from the GIT. This is corroborated with the fact that there is limited survival of the pathogen due to fewer nutrients available for their growth and proliferation and the unavailability of adherent space in the GIT will limit the growth of pathogens there. This exclusion of the pathogens from the GIT ultimately prevents diseases caused by the pathogen.

1.3.1.2. Production of inhibitory substances

In order to gain a competitive advantage when competing for space and nutrients, microorganisms release antimicrobial compounds. Antimicrobial compounds have a direct inhibition on several target pathogens (Volzing *et al.*, 2013). The mechanisms used by probiotics to inhibit pathogenic bacteria are interconnected. As already mentioned, exclusion of pathogens occurs due to the ability of probiotics to secrete organic acids such as acetic and lactic acids (Alakomi *et al*., 2000). The production of these organic acids leads to a decrease in the pH of the environment, making the microenvironment acidic therefore excluding pathogens that cannot survive acidic conditions (Wohlgemuth *et al*., 2010). The organic acids also have an effect on the pathogen metabolism and production of toxins, ultimately preventing disease.

The anti-pathogenic activity of probiotics is multifactorial (Servin, 2004). In addition to these acids, other probiotics produce other metabolites with antibacterial properties like H_2O_2, strain-

specific metabolites, bacteriocins, or non-lactic acid molecules (Oscáriz *et al*., 1999; Servin, 2004; Vilà *et al*., 2010; Dobson *et al*., 2012). Bacteriocins are small antimicrobial peptides produced for bacterial competition in a natural ecosystem (Volzing *et al.,* 2013). They may act as colonizing peptides by facilitating the introduction of probiotics into an already occupied niche on the intestinal epithelium. This allows for a competitive advantage to increase in probiotic density of the surface of the host intestines (Dobson *et al*., 2012). They can also act as killing peptides, by directly affecting pathogens. A study by Kim *et al*. (2003) evaluated the antimicrobial activity of the bacteriocins lacticin, pediocin and leucocin produced by LAB against *Helicobacter pylori.* These bacteriocins were able to significantly inhibit the growth of *H. pylori,* lacticin being the bacteriocin having the strongest effect against the gut pathogen.

Lactobacillus acidophilus has been reported to produce metabolites such as acidophilin, lactocidin and acidolin (Vilà *et al*., 2010) whereas *Bifidobacteria* produces bacteriocin like substances (Risoen *et al*., 2004) both inhibiting bacteria such as *Bacillus, Salmonella, Staphylococcus* and *E. coli, Clostridium perfringens, Listeria* species, among others (Schillinger and Lucke, 1989; Nielsen *et al*., 2010; Vilà *et al*., 2010). Fayol-Messaoudi *et al*. (2005) reported that the antibacterial effects of the probiotic *Lactobacillus* that inhibited the growth and killing pathogens was attributed to the synergistic action of lactic acid and the secreted non-lactic acid molecules. Certain probiotic strains can also stimulate the increase in the expression of host cell antimicrobial peptides. The intestinal cells of the host are able to produce defensins which can inhibit the functioning of pathogens thus aiding in the protection of the intestinal barrier (Dobson *et al*., 2012).

1.3.1.3. **Immune system modulation**

Probiotics displace pathogens through stimulation of host immunity (Meydani and Ha, 2000). There is considerable evidence to support the notion that probiotics displace pathogens in the GIT through stimulation of specific and nonspecific immunity to protect host against intestinal disease (Link-Amster *et al*., 1994; Malin *et al*., 1996). They modulate the host's immune system against pathogens' harmful antigens by activation of lymphocytes and production of antibodies (Ng *et al*., 2009). They can also stimulate the effects of different cells involved in innate and adaptive immunity; such as dendritic cells, macrophages, T cells and B cells, which enhances phagocytosis of gut pathogens (Viaşu-Bolocan *et al*., 2013). Probiotic strains such as *Lactobacillus rhamnosus*

and *Lactobacillus plantarum* adhere to gut associated lymphoid tissue enhancing both systemic and mucosal immunity (Behnsen *et al*., 2013). This enhancement stimulates the production of phagocytic cells more efficiently.

Probiotics can be recognized by the immune system through pattern recognition molecules such as Toll-like receptors. This recognition can lead to various intracellular signal transduction cascades and enhancement or reduction of pro- and anti-inflammatory cytokines. Link-Amster *et al*. (1994) fed 16 volunteers with fermented milk supplemented with probiotics *L. acidophilus, Bifidobacterium bifidum* Bb12 and *Streptococcus thermophilus,* at the same time they were injected with attenuated *Salmonella typhi* Ty21a vaccine. The results showed that there was an increase in the humoral immune response. Probiotics are able to stimulate the production of antibodies in the intestinal lumen, specifically immunoglobulin A. Immunoglobulin A represents the first line defence against infection and can inhibit the adhesion of pathogenic bacteria to the intestinal epithelia. It can interfere with adhesive cell receptors on the pathogens cell surface and cause bacterial agglutination. One study indicated that that oral administration of *Lactobacillus casei* enhanced the concentration of IgA in infants suffering from diarrhoea thereby shortening the duration of diarrhoea (Roberfroid, 2000; Viaşu-Bolocan *et al*., 2013). In a different study, Galdeano and Perdigon (2006) reported that when they fed BALB/c mice with *L.* casei, there was an increase in IgA^+ cells and IL-6-producing cells 7 days post administration. Ng *et al*. (2009) reported that administration of *L. rhamnosus* resulted in enhanced non- specific humoral responses reflected by an increase in the production of IgG, IgA and IgM from the circulating lymphocytes.

Furthermore, probiotics can stimulate an anti-inflammatory response, which can be used as an approach to reduce inflammation caused by gastroenteritis, enterocolitis and irritable bowel syndrome (Behnsen *et al*., 2013). An anti-inflammatory response is triggered when strains stimulate the activation of dendritic cells which secrete interleukin 10 (IL-10), a cytokine that plays a role in reducing inflammation. They also cause a decrease in the levels of pro-inflammatory cytokines during inflammation (Viaşu-Bolocan *et al*., 2013).

1.3.1.4. Improved barrier function

The integrity of the intestinal barrier needs to be maintained in order to prevent pathogens from reaching the intestinal cells leading to local and systemic infections. Gut pathogens have the ability

to disrupt the barrier when there is an imbalance in the microbial gut ecosystem (Culligan *et al*., 2009). Probiotics are able to maintain and repair this barrier after damage that may have been caused by gut pathogens. As an approach to repair the intestinal barrier probiotics can stimulate mucous secretion, chloride and water secretion and the binding together of submucosa cells by tight junctional proteins (Wohlgemuth *et al*., 2010).

Goblet cells express mucins (MUCs), which are either localized to the cell membrane or secreted into the lumen to form the mucous layer (McCool *et al*., 1994; Robbe-Masselot *et al*., 2008). There are 18 mucin-type glycoproteins that are expressed by humans (Culligan *et al*., 2009). In the human intestinal cell lines, *Lactobacillus* species increased mucin expression (MUC2 by Caco-2 cells; MUC2 and MUC3 by HT29), thus blocking cellular adhesion and invasion by pathogenic *E. coli* (Mattar *et al*., 2002; Mack *et al*., 2003). Kim *et al.* (2008) showed that the treatment of IL-10 gene-deficient mice with a combination probiotic VSL#3 (*L. casei*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii* subsp. bulgaricus, *Bifidobacterium longum*, *B. breve*, *B. infantis*, and *Streptococcus salivarius* subsp. thermophilus), resulted in normalization of colonic physiologic function and barrier integrity leading to a significant improvement in histologic disease (Madsen *et al*., 2001).

Tight junctions (TJ) form the continuous intercellular barrier between epithelial cells, which is required to separate tissue spaces and regulate selective movement of solutes across the epithelium (Anderson and Van Itallie, 2009). There are different proteins expressed on the TJ and the disruption of their expression leads to a dysfunctional epithelial barrier (Madsen *et al*., 2001). A study by Qin *et al*. (2005) reported that *L. acidophilus* increases the expression of occludin, a major component of TJ, in the gut mucosa of animals with cecal ligation and perforation, leading to a reduced bacterial translocation. A different study by Resta-Lenert and Barrett (2003) reported that probiotic bacteria, specifically *S. thermophilus* and *L. acidophilus*, prevented a reduction in the enteroinvasive *E. coli*-induced phosphorylation of the proteins occludin and zonula occludens 1 (ZO-1), thereby preserving the TJ structure. Furthermore, Parassol *et al*. (2005) showed that *L. casei* prevents the redistribution of the TJ protein ZO-1 away from the cell–cell contacts caused by infection with enteropathogenic *E. coli*.

1.4. The use of conventional probiotics for control of selected food pathogens

Due to the widespread use of antibiotics as therapeutic agents and the misuse of these antibiotics, there has been an increase in the antibiotic resistance of bacteria, an imbalance of normal microflora, and the presence of drug residues in food products (Fayol-Messaoudi *et al*., 2005). This brought upon a requirement for new intervention when coming to treating bacterial pathogens, leading to an escalation in the research field of the beneficial microorganisms, probiotics. Prevention and treatment of infections caused by the different pathogens are one of the effects that probiotics are extensively studied for (de Moreno de LeBlanc *et al*., 2010). When studying the prevention and treatment of pathogens, it is important to consider the complexity of the intestinal environment where a network of interactions among the microorganisms of the resident microbiota, epithelial and immune cells associated with the GIT, and nutrients exist (Hooper and Gordon, 2001; Bauer *et al*., 2006). The epithelial and the immune cells play a role in the modulation of the immune functions and they provide the first line of defence against the pathogenic bacteria. The resident microbiota has the ability to influence the composition and activity of the gut microbiota (de Moreno de LeBlanc *et al*., 2010). They also play a beneficial role in the treatment of disease caused by food-borne pathogens (Simon *et al*., 2005). Different microorganisms infect different parts of the host GIT, for example *Helicobacter pylori*, infects the gastric and duodenal mucosa, *Salmonella* spp. and *Clostridium difficile* cause inflammation in ileum and colon while *Shigella* sp. clearly prefers the colonic mucosa (Dupont, 1997).

Previous studies have shown the effects of probiotics, that when consumed as part of the daily diet they can maintain the immune system in an active state and prevent different intestinal disorders (de Moreno de LeBlanc *et al*., 2010). Valdez *et al*. (2001) reported that certain LAB probiotics inhibit apoptosis of macrophage infected with *Salmonella* preventing salmonellosis. Cano and Perdigón (2003) studied the preventative measure of *Lactobacillus casei* CRL 431 against *Salmonella* serovar Typhimurium, reporting that administrating probiotics for seven days had beneficial effects to the host. Findings of their study were confirmed by a different study (de Moreno de LeBlanc *et al*., 2010), in which they studied the preventative and continuous administration of probiotic *L. casei* CRL 431 against *S.* serovar Typhimurium and reported that the study group fed the probiotic for 7 days before the introduction of the pathogen and post infection experienced less severe infection compared to the control group without probiotics. They furthermore reported that 7-day administration of probiotics post infection resulted in better

protection against *Salmonella* infection. They concluded that the continuous administration of the probiotic improves the effects of the probiotics showed by the diminished counts of the pathogens in the intestine as well as their spread outside this organ.

More studies have been conducted on different pathogens to also show the efficacy of probiotic strains. *Helicobacter pylori* is a bacterium that plays a crucial role in the pathogenesis of chronic active gastritis and peptic ulcer disease in both adults and children with increasing amount of evidence supporting the hypothesis that it is an important co-factor in the development of gastric cancer (Uemura *et al*., 2001). *Helicobacter pylori* has been reported to be the only bacterium that has been linked to cancer to date and there is no vaccine that is currently licensed (Ruggiero, 2014). There are different therapeutic approaches that are used to treat *H. pylori*, however, there have been reports that suggest that some patients still remain infected after those approaches (Leung and Graham, 2002). Administration of alternative compounds that may increase the efficacy of the treatment and/or reduce side effects is of particular interest (Ruggiero, 2014), thus the proposed studying of administration of probiotics. There is growing evidence from different studies emphasizing the efficacy of probiotics in the management of *H. pylori* infection trickling different aspects of this infectious disease (Cats *et al*., 2003; Lionetti *et al*., 2011). Studies have reported that probiotics such as *Lactobacillus johnsonii* La1 (La1) or *Lactobacillus* GG exert bacteriostatic or bactericidal activities against a wide range of pathogens, including *H. pylori* (Bernet-Camard *et al*., 1997). Tursi *et al*. (2004) demonstrated that a 10-day quadruple anti-helicobacter therapy with ranitidine bismuth citrate (RBC) plus proton-pump inhibitors (PPI), amoxicillin and tinidazole obtains a high eradication rate, whereas supplementation with *Lactobacillus casei* significantly increased the eradication rate of *H. pylori* infection. This study concluded that the supplementation of the therapy with the administration of probiotics showed a slight improvement in the eradication of *H.* pylori. In a different study, Cruchet *et al*. (2003) have showed that *Lactobacillus johnsonii* La1 may interfere with *H. pylori* colonization in asymptomatic children and may be an effective alternative to modulate *H. pylori* infection. Probiotics can therefore be used as the first course of anti- *H. pylori* treatment or can be used in conjugation with the first line therapeutic approaches.

Shigella is one of the most antimicrobial-resistant bacteria (Opintan *et al*., 2007; Pazhani *et al*., 2008) and has been reported to cause gastroenteritis-induced deaths in 3-5 million children aged less than five years in developing countries (Sivapalasingam *et al.,* 2006; Mandomando *et al.,* 2009). The emergence of multiple drug resistance to cost-effective antimicrobials against *Shigella*

is a matter of concern in developing countries and resistance pattern of this bacterium is the cause of numerous clinical problems throughout the world (Mirnejad *et al*., 2013). Due to its resistance, the need for alternative treatment has therefore been deemed necessary. This raised an interest in the application of probiotics for the treatment of infections caused by *Shigella*. Zhang *et al*. (2011) studied the antimicrobial activity of the probiotics *Lactobacillus paracasei* subp. *paracasei* M5-L, *Lactobacillus rhamnosus* J10-L, *Lactobacillus casei* Q8-L and *L. rhamnosus* GG (LGG) against *Shigella sonnei*. They reported that the tested lactobacilii strains showed strong antimicrobial activity against *S. sonnei*. In a study to screen for the antimicrobial activity of probiotics against *S. sonnei*, Zhang *et al*. (2012) reported that *L. johnsonii* F0421 exhibited significant inhibitory activity and excluded, competed and displaced adhered *S. sonnei*. In a different study, Mirnejad *et al*. (2013) evaluated the nature of antimicrobial substances and properties of *L. casei* against multi-drug resistant clinical isolates of *S. flexneri* and *S. sonnei*. Their results indicated that *Lb. casei* showed strong antimicrobial activity against *S. flexneri* and *S. sonnei,* and they attributed pathogen inhibition to production of metabolites by the test *Lactobacillus*. In another study, Zou *et al*. (2013) studied the antimicrobial activity of nisin, a bacteriocin produced by *Lactococcus lactis* strains, against *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella Typhimurium* and *Shigella boydii*. They reported that there was a decline in pathogen populations, which was ascribed to the changes in the fatty acid profiles, cell viability, membrane permeability and depolarisation activity in response to nisin.

Listeria monocytogenes is a foodborne pathogen that causes devastating effects in human host, causing disease conditions ranging from stillbirth and premature delivery in perinatal cases (Mylonakis *et al*., 2002) to meningitis and septicemia in adults (Durand *et al*., 1993; Vázquez-Boland *et al*., 2001). There have been different studies using different probiotics to combat this food pathogen. In a study to demonstrate the activity of the antibacterial substances produced by bifidobacterial isolates, Touré *et al*. (2003) isolated six infant bifidobacterial strains from breast-fed infant faeces, with a potential antimicrobial activity against *L. monocytogenes*. These isolates actively inhibited *L. monocytogenes* by producing heat-stable proteinaceous substance. Their study indicated that the use of bifidobacterial strains capable of competing with pathogenic organisms following the probiotic approach would advantageously improve intestinal bacterial ecology and provides a useful alternative strategy for inhibiting intestinal pathogens. In 2007, Corr *et al*. studied the pretreatment of epithelial cells with strains of *Bifidobacterium* and *Lactobacillus* to

demonstrate that this can significantly interfere with subsequent invasion by *L. monocytogenes*. They reported that the pretreatment of intestinal epithelial cells with probiotic bacteria prior to infection with *L. monocytogenes* EGDe resulted in a significant decrease in listerial invasion (60–90%). In yet another study testing for the antagonistic effect of *Lactobacillus* strains against *E. coli* and *L. monocytogenes*, it was reported that *L. plantarum* WS4174 exhibited a stronger inhibitory effect against the Gram-Positive *L. monocytogenes* LMO26, possibly due to its higher sensitivity to low pH and the accumulation of lactic acid (Aguilar *et al*., 2011).

1.5. Limitations of conventional probiotics

Although probiotics provide numerous benefits to the host, they do have certain limitations. Certain studies have provided evidence where probiotic strains may be inefficient or ineffective in response to specific gut pathogens. Probiotics may release antimicrobial compounds that have a broad antimicrobial spectrum, however reports have suggested that there are limitations in the success of probiotics targeting specific pathogens. Therefore, a cocktail of various probiotic strains would need to be produced in order to enhance the effects against different pathogens within the gut (Kailasapathy and Chin, 2010). According to Koo *et al.* (2012), probiotics have a limited success in preventing the attachment of *L. monocytogenes* to intestinal monolayers. An experimental approach added *L. monocytogenes and Lactobacillus paracasei* to Caco-2 cells, results showed that the bacterial adhesion percentages of the pathogen with and without the probiotic strain were fairly similar. None of the lactobacilli were able to reduce the adhesion of the pathogen at significant levels. Furthermore, increasing the concentration of the probiotic strain also failed to displace the attached *L. monocytogenes.* The data from the study indicated that this probiotic strain as well as five other lactic acid bacterial strains could not prevent adhesion of this pathogen. Another report indicated that probiotics may also stimulate low levels of an immune response and low levels of an anti-inflammatory response (McCarthy *et al*., 2003). *Lactobacillus salivarius* and *Bifidobacterium infantis* were orally administered to mice suffering from colitis. Results indicated that TGF-β levels in mice treated and untreated with probiotics remained the same. TGF-β is an anti-inflammatory cytokine, the levels of this cytokine were not significantly increased but still maintained by *L. salivarius*, however, these were not maintained in the presence of *B. infantis*.

Most probiotics are administered as part of the food products or pharmaceutical preparations; therefore, they have to be able to withstand both the technological and gastrointestinal stress factors. The broad mode of action of probiotics and the differences from one probiotic to another is also an obstacle in their efficacy. It has been reported that the beneficial attributes of one strain or a cocktail of strains may not be reproducible and may vary from person to person (Karimi and Peña, 2008). In addition to that, the strain of the probiotic, the dosage, the route of administration, and the formulation of probiotic preparation can also affect the efficacy of a probiotic (Koo *et al*., 2012). Taking these into consideration, it can be realized that probiotics are still non-specific and discriminatory in their mode of action or ineffective in certain hosts (Bomba *et al*., 2002). The limitations discussed above introduces the need for more novel and innovative approaches in the use of probiotics for the prevention and treatment of foodborne pathogens. Previous literature has reported that the use of probiotics has been extended to deliver therapeutic and prophylactic molecules to the mucosal barrier of the host (Koo *et al*., 2012; Richter *et al*., 2009; Bhunia, 2012). Although probiotics have proven successful in control of enteric pathogens, they do have limitations. They are generic in nature and often fail to inhibit the attachment of certain pathogens at specific sites of infection and induce low levels of an immune response (McCarthy *et al*., 2003). A thorough understanding of the limitations of conventional probiotics, the behaviour of the pathogens and the mechanisms by which they cause disease (Amara and Shibi, 2015) provides possibilities to design new probiotic strains with desired characteristics and functionalities. Through genetic modification, novel bioengineered probiotic strains can be produced. Functioning of conventional probiotics in these novel strains can be strengthened to influence critical steps in the pathogenesis of disease. The strains can also be used to deliver drugs or vaccines, target a specific pathogen or toxin, mimic surface receptors and enhance an immune response within the host (Amalaradjou and Bhunia, 2013).

1.6. The concept of probiotic bioengineering or recombinant probiotics

The performance of the existing probiotic strains can be improved through the use of bioengineering. Bioengineering refers to the manipulation of a gene of a probiotic strain in order to improve the tolerance to the technological stress during food production and/or survival of the probiotic in the GIT to confer beneficial effects to the host (Upadrasta *et al*., 2011). This strategy

can be used in the design and construction of new probiotic strains harbouring genes of interest derived from the pathogens. It allows for production of proteins that were initially not present within the microorganism. Virulence factors of the pathogens can be cloned and expressed into the probiotics and subsequently administration of the recombinant probiotics will inhibit the development of infection. The inhibition of the pathogen by the recombinant probiotic will yield no clinical presentation of the symptoms. Furthermore, recombinant probiotics can be used to deliver drugs or vaccines, target specific pathogens or toxins, enhance an immune response and mimic cell surface receptors (Berg and Mertz, 2010). Most human receptors recognized by enteric pathogens or their toxins are well characterized. Also, by targeting a specific pathogen, this strategy deems the development of resistance to the vaccine or treatment unlikely. Bioengineering of probiotics is not entirely a new field, there has been research reporting the beneficial effects of this method. Culligan *et al*. (2009) reported on the main advantages of using recombinant probiotics in the treatment of enteric infection. The next section focuses on studies that were conducted on bioengineered probiotics aiming to improve different functional properties of the conventional strains.

1.6.1. Applications of probiotic bioengineering

1.6.1.1. Improvement of stress tolerance

There has been an increase in the use of probiotics due to their known effects to confer beneficial health to the host. However, there are still problems frequently associated with the incorporation of probiotic strains into food products. These problems include but are not limited to poor temperature, salt, and oxygen tolerance of some species or strains. Different approaches including pre- adaptation to stress, the use of oxygen-impermeable containers, microencapsulation (Desmond *et al*., 2004), incorporation of nutrients, and selection of stress resistant strains have been used in an attempt to address these problems (Shah, 2000). The use of bioengineering has been used in the field of stress adaptation, and there have been promising results.

The ability to confer additional stress tolerance in stress-sensitive cultures can lead to the development and delivery of novel probiotics with maximal therapeutic efficacy (Sleator and Hill, 2008). It has been reported that the two major heat shock proteins, GroES and GroEL are essential for the survival of bacteria at all temperatures (Fayet *et al*., 1989). In a study by Desmond *et al*.

(2004) the effect of overexpression of these heat-shock protein chaperones (GroES and GroEL) in the probiotic *L. paracasei* NFBC338 were investigated. Expression of these genes resulted in improved thermo-tolerance (heat tolerance) as well as increased solvent resistance by the probiotic strain. Furthermore, they compared the survival of the non- adapted parent strain, stress adapted and the recombinant probiotic during exposure to heat stress. They reported that the recombinant probiotic survived 10- and 54-fold better than the stress- adapted and non-adapted parent strains, respectively.

The survival of pathogens is usually dependent on the different systems that can help them overcome the different stress conditions present in the GIT. *Listeria monocytogenes* has to date three transport systems that have been linked to betaine and carnitine uptake (Sleator and Hill, 2002; Sleator *et al*., 2003). The first of these, BetL is a gene encoding the secondary glycine betaine transporter, which is linked to salt tolerance of *Listeria* (Sleator *et al*., 1999; Sleator *et al*., 2000). It has been reported that disrupting BetL results in reduced growth at 37°C in complex media of elevated osmolarity (Sleator *et al*., 1999). The reduction in the initial betaine uptake in the absence of BetL leads to diminished intracellular solute pools (Sleator *et al*., 2003), causing changes in the cell volume, intracellular solute concentration and the turgor pressure (Glaasker *et al*., 1996). Sheehan *et al*. (2006) studied the heterologous expression of the listerial betaine-uptake system (BetL) into the probiotic strain *L. salivarius* UCC118 using a nisin-controlled expression system. They reported that expression of this led to an increase in the resistance of the probiotic to several stresses (osmo-, cryo-, baro-, and chill), spray- and freeze-drying. Later in another study these researchers demonstrated that *B. breve* UCC2003 harboring the betaine-uptake (Betl) gene displayed an improved tolerance to gastric juice and elevated osmolarity (Sheehan *et al*., 2007).

Trehalose is a non-reducing disaccharide ubiquitously distributed in nature and is well known for its role in protecting cells against a variety of stresses (Jain and Roy, 2009). In *E. coli* it is synthesized in response to high osmolarity (Kempf and Bremer, 1998). Termont *et al*. (2006) cloned the trehalose synthesis gene (*ostAB*) from *E. coli* into *Lactococcus lactis* and reported that there was an enhanced probiotic's survival during freeze- drying, in high bile concentrations and its resistance to gastric acid. In a different study Carvalho *et al*. (2011) studied the expression of the trehalose synthesis into the same probiotic *Lactococcus lactis* and reported that trehalose plays a definite role in the protection of this bacterium against damage caused by acid, cold, or heat

shock. These studies provide evidence to proof that expression of genes from pathogenic species to improve stress tolerance of probiotics has been explored with promising results. However, further scientific assessment is still required to analyse the benefit of using these genes and interpretation by risk–benefit analysis (Sleator and Hill, 2008).

1.6.1.2. Production of antimicrobial peptides

The rise in development of antibiotic resistance of pathogens has led to a dire need for alternative methods to treat infections. Antimicrobial peptides (AMPs) have been explored as an alternative method for effective control of multi- drug resistant (MDR) pathogens (Mandal *et al,* 2014). As already mentioned, some probiotics produce several antimicrobial compounds and peptides as a defence mechanism against pathogens (Amalaradjou and Bhunia, 2013) but they are not specific. Probiotics can therefore be used as candidates for the production and delivery of therapeutic antimicrobial peptides within the host GIT targeting a specific action or pathogen. The current methods for production of AMPs has been reported to have several limitations, therefore an alternative strategy will be to use probiotic strains to express the different AMPs resulting in a combination strategy where hosts will get the probiotic effects with the production of the different AMPs (Mandal *et al*., 2014).

Volzing *et al*. (2013) chose *L. lactis* as an ideal vehicle for production and delivery of AMPs to the site of GI infection due to its ability to survive within the human gastrointestinal tract and its amenability to heterologous gene overexpression. In their study, they engineered a *L. lactis* strain to inducibly express and secrete AMPs with high activity against Gram- negative pathogens, specifically *E. coli* and *Salmonella* strains. The AMPs of interest, A3APO and alyteserin were selected and then cloned into *L. lactis* for the expression of the heterologous peptides. An expression cassette containing a codon-optimized sequence for alyteserin was fused with a Usp45 secretion signal sequence. This expression cassette was cloned under the control of a nisin inducible promoter and transformed into *L. lactis.* When the resulting recombinant strain was then tested against *E. coli* and *Salmonella* the results indicated that it successfully inhibited these pathogens while maintaining the host's viability. Inhibition of these pathogens by alyteserin was observed from concentrations ranging from 0.125- 1 mg/ml while the *L. lactis* strains remained

viable when exposed to the alyteserin supernatant at 1 mg/ml. This system showed potential as a therapeutic alternative to antibiotics in order to target and inhibit Gram-negative bacteria.

1.6.1.3. Enhancement of anti-inflammatory response

A group of chronic inflammatory disorders known as inflammatory bowel diseases (IBD) are responsible for the inflammation of the digestive tract. The two forms of the IBD are Crohn's disease and ulcerative colitis, both characterized by an uncontrolled inflammatory response to the luminal content (Khor *et al*., 2011). The treatment of IBDs poses a challenge as the current treatment options are either costly or cause severe side- effects in patients. There has been a number of studies on the treatment of IBDs and recent research has reported that probiotic bacteria may counteract the chronic inflammatory process (Bermúdez-Humarán *et al*., 2015). Elafin, is a protease inhibitor expressed in the intestinal epithelium, which contributes to reduction of inflammation. During inflammation there is an increase in elastase and myeloperoxidase (MPO) activity, elafin can inhibit the function of proteases thereby reducing inflammation (Sheil *et al*., 2008). Bermúdez-Humarán *et al*. (2015) bioengineered *Lactococcus lactis* to express elafin in mice suffering from colitis. The gene encoding for elafin was fused in frame with a gene encoding for a ribosome binding site and with a Usp45 secretion signal sequence and inserted into an expression vector. The recombinant plasmid was thereafter transformed into *L. lactis* and expression was induced under the control of a nisin induced promoter. Colonic inflammation was then induced in mice with dextran sodium sulphate and then the mice were subsequently orally treated with either wild type or recombinant *L. lactis.* Analysis of mice colons for inflammation parameters such as colonic thickness, elastase activities and granulocyte infiltration after 7 days, indicated that mice treated with recombinant *L. lactis* secreting the elafin showed a significant reduction in all inflammation parameters. However, mice treated with wild type probiotics did not show the same significant decrease in inflammation parameters their response was similar to that of the control untreated mice. Furthermore, comparison of efficiency of recombinant *L. lactis* secreting elafin to those expressing either anti- inflammatory cytokine IL-10 or TGF- β1 (to be discussed next) showed that elafin secreting strain was the most efficient. These results suggested that the protease inhibitor, elafin, was the most efficient anti-inflammatory molecule to be

delivered by a probiotic strain at the mucosal surface in order to treat inflammation (Bermúdez-Humarán *et al*., 2015).

Chronic inflammation of IBD patients can also be reduced through the administration of anti-inflammatory cytokines such as interleukin 10 (IL-10). IL- 10 plays a central role in down-regulation of inflammatory cascades and in the establishment of tolerance in the mucosa (Behnsen *et al*., 2013). Interferons (IFN), including IFN-α and IFN-β, are widely expressed cytokines involved in innate responses and additionally, these cytokines have an immunomodulatory role in the anti-inflammatory host response. The use of probiotic bioengineering to treat IBD has been studied, and it has been reported that this can indeed be used as an alternative. Several studies have been done with regard to probiotics expressing cytokines and other anti- inflammatory molecules such as IL-10 and TGF-β instead of elafin, using similar cloning procedures used for elafin. After transformation, recombinant probiotic strains were induced with nisin in order to either express IL-10 or TGF-β and orally administered to mice suffering from colitis. Recombinant *L. lactis* expressing TGF-β displayed beneficial effects by reducing MPO levels, overall reducing inflammation and colitis in 40% of the mice. However, the protective effects against colitis were higher in mice treated with recombinant probiotics expressing elafin than those treated with probiotics expressing IL-10 (Steidler *et al*., 2000). Another study reported that intra-gastric administration of *L. lactis* expressing recombinant IL-10, a cytokine used in clinical trials for treatment of IBD, could successfully prevent colitis in murine models (Kumar *et al*., 2016).

McFarland *et al*. (2011) investigated the effects of local administration of IFN-β on a murine model of colitis. They developed a transgenic *Lactobacillus acidophilus* strain that constitutively expresses IFN-β and reported that the resultant recombinant strain secreting IFN-β resulted in the exacerbation of colitis. Tumor necrosis factor α (TNF- α) is a cytokine that mediates the clinical symptoms of IBD (Behnsen *et al*., 2013). In a study by Vandenbroucke *et al*. (2010), they constructed a recombinant *L. lactis* to produce anti- TNF- α nanobodies and reported that daily administration of this strain reduced the colonic inflammation.

1.6.1.4. Enhancement of pathogen colonization exclusion

Enhancement of probiotic adhesion to the intestinal mucosal surface can be seen as a potential strategy in order to prevent adhesion and colonization of pathogenic bacteria. Strategies include

using gene products of target pathogens such as adhesins or secretory systems in probiotic bacteria to create a competitive environment for colonization (Koo *et al*., 2012). A number of researchers investigated the efficiency of this approach in improvement of competitive exclusion by enhancing binding or adhesion efficacy of the probiotics to host cells. When InlA from *L. monocytogenes* was cloned and expressed into the *L. lactis*, there was enhanced binding to human epithelial cells and bacterial internalization (Innocentin *et al*., 2009). Koo *et al*. (2012) developed a recombinant probiotic *L. paracasei* harbouring the *Listeria* adhesion protein (LAP) in order to control *L. monocytogenes* infection. LAP interacts with a heat shock protein 60 receptor in host cells and promotes adhesion of *Listeria* to host cells. Conventional and recombinant probiotic *L. paracasei* were added to Caco-2 cell monolayers separately, thereafter these monolayers were Giemsa-stained. Pre-exposure of Caco-2 cell monolayers to recombinant *L. paracasei* expressing LAP followed by the addition of *L. monocytogenes* led to a reduction of adhesion and translocation of the pathogen. The wild type probiotic strain had no significant reduction in the adhesion of the *L. monocytogenes* to the cell monolayer while the recombinant strain resulted in a 60% reduction of adhesion.

It has been shown that flagellins from *Bacillus cereus* are responsible for the adhesion of the bacterium to mucosal cells (Ramarao and Lereclus, 2006). Gut pathogens may also use fimbriae or flagella which are extended appendages on the surface of the cell wall, to adhere to host cell receptors. Therefore, expression of these specific appendages in probiotic strains would allow them to bind to the intestinal epithelium, excluding pathogenic binding. Taking that into consideration, Sánchez *et al*. (2011) cloned the surface- associated flagellin of *Bacillus cereus* CH and expressed it in the probiotic *Lactococcus lactis*. The recombinant strain adhered strongly to the mucin-coated polystyrene plates in an *in vitro* experiment and competitively inhibited the binding and adhesion of pathogenic *E. coli* and *S. enterica*.

Enterotoxigenic *Escherichia coli* (ETEC) K99 fimbriae have been reported to enhance the production of mucosal IgA and serum IgG1 fimbria-specific responses (Ascón *et al*., 2005), thereby increasing the immune responses at mucosal surfaces such as the gastrointestinal (GI) tract, the respiratory tract, and the vaginal tract (Blutt *et al*., 2012). Chu *et al*. (2005) cloned and expressed the K99 fimbriae from ETEC into the probiotic *L. acidophilus* and reported that the recombinant *L. acidophilus* was able to reduce the attachment of ETEC to porcine intestinal brush

border in a dose dependent manner. The reduction of the adherence of the pathogen by the recombinant probiotic prevents the binding of the pathogen, therefore inhibiting the infection.

1.6.1.5. Receptor mimicry system and toxin neutralization

One mechanism that pathogens use to invade the host cells and causes infection is through the production of toxins. These pathogens secrete toxins and sometimes express adhesins that bind to host cells via oligosaccharide receptors displayed on surface glycolipids or glycoproteins. The interaction between the released toxin and the specific oligosaccharide receptors on the surface of the human intestinal cells is an essential step during pathogenesis (Paton *et al*., 2010). Therefore, toxins or secretory systems of pathogens may also serve as potential targets in development of therapeutics (Rasko and Sperandio, 2010). Taking this into consideration, it thus becomes apparent that interfering with the toxin- receptor binding and adhesion can be used as a strategy to exclude the pathogen and subsequently minimize or control its infection (Paton *et al*., 2010). A therapeutic strategy would be to express toxin receptors on the cell surface of probiotic strains in order to mimic the receptor (Paton *et al*., 2006). This expression produces a lipopolysaccharide that mimics a host cell receptor, which, e.g. cholera toxin or ETEC heat-labile toxin could recognize and bind to. Therefore, upon infection, enterotoxins would bind to probiotic and become sequestered; protecting the host from a pathogenic infection (Paton *et al*., 2010).

There are a number of pathogens that secrete these toxins and amongst them, *Vibrio cholerae*, Shiga toxigenic *Escherichia coli* (STEC), enterotoxigenic *E. coli* (ETEC) and *Clostridium difficile*, just to name a few. Shiga toxigenic *E. coli* and ETEC both cause enteric infections, they cause gastrointestinal disease and diarrheal disease in humans, respectively. If left untreated, these pathogens can cause severe bloody diarrhoea associated with haemorrhagic colitis (Kitov *et al*., 2000). In an earlier study by Paton *et al*. (2000) the galactosyl-transferase genes from *Neisseria gonorrhoeae* were cloned and expressed into a non- pathogenic *E. coli*. The results showed that the recombinant *E. coli* was 100% effective in treating mice infected with the normally fatal shiga toxigenic *E. coli*. Then later in another study, these researchers cloned the glycosyltransferase gene, *Neisseria meningitidis* toxin specific receptor, into the probiotic *E. coli*, creating a competitive environment for toxin binding to the host cells. Expression of these genes created a cell surface mimic of a shiga toxin receptor. This led to competitive exclusion of the pathogen by

the probiotic and subsequently inhibiting its infection. This recombinant strain had a high binding capacity and efficacy in mouse models and was effective in neutralizing shiga toxin variants (Paton *et al*., 2006). Norton *et al*. (1995) cloned and expressed a tetanus toxin fragment C (TTFC) in *L. lactis.* They then reported that there were increased IgA levels in the host after oral administration of the recombinant probiotic, which led to protection of the host against the infections of the mucous membrane. These results were supported by other studies, where mice immunized with this recombinant probiotic showed more resistance to the lethal challenge with tetanus toxin than those that were not immunized (Robinson *et al*., 1997; Grangette *et al*., 2001).

Pathogens are able to control the expression of their virulence genes by sensing signals from their own species, other bacteria or their environment, a phenomenon termed quorum sensing (Amalaradjou and Bhunia, 2013). Interruption of quorum sensing of the pathogen can be used as an alternative strategy to control the pathogen. Cholera is a life- threatening gastrointestinal infection (Sack *et al*., 2004) that is caused by ingestion of water or food (usually undercooked shellfish) contaminated with *Vibrio cholera* (Paton *et al*., 2010). Following ingestion, *V. cholerae* passes through the stomach, colonizes the small intestine and then release cholera toxin (Ctx), which is responsible for its virulence. It has been hypothesized that neutralization of Ctx in the gut should prevent the disease from developing or at least speed up recovery from an established *V. cholerae* infection (Paton *et al*., 2010). The cloning and expression of Ctx into probiotics can therefore be used as an alternative strategy for the treatment of cholera. Focareta *et al*. (2006) constructed a probiotic *E. coli* encoding receptor GM1 to express the GM_1 ganglioside on its surface, which is capable of binding large amounts of Ctx and protecting infant mice from challenge with virulent *V. cholerae*. The resultant recombinant *E. coli* was capable of binding purified Ctx with high avidity and adsorbing >5% of its own weight of toxin *in vitro*. *Vibrio cholerae* releases cholera autoinducer-1 (CAI-1) and autoinducer-2 (AI-2) that accumulate when the population density increases at which point bacteria produce virulence factors (Amalaradjou and Bhunia, 2013). Duan and March (2010) constructed an AI-2 producing *E. coli* Nissle that co-expressed CAI-1. They reported an 80% reduction in Ctx binding to the intestines of mice pretreated with recombinant probiotic, which reduced the chances of infection. These results show that bioengineered probiotics can be administered for the prevention or treatment of enteric pathogens through receptor mimicry or toxin neutralization.

1.6.1.6. Vaccination

Probiotics may induce low levels of an immune response. Therefore, probiotics can be bioengineered to deliver immunogenic molecules to the intestinal mucosal surface to enhance the immune response. Recombinant probiotics can act as a vaccine arming the host immune system to deal with gut pathogens (Gardlik *et al*., 2012). In order to exploit a safe and effective vaccine for the prevention against K99 infections of ETEC, Wen *et al*. (2012) cloned and expressed ETEC adhesins K99 into the probiotic *L. casei*. They reported that there was an increase in the efficacy of the recombinant probiotic and that more than 80% of the vaccinated mice were protected after challenge with a lethal dose of standard strains.

Non-bactericidal infections can also be treated with bioengineered probiotics through an approach using vaccination delivery systems. Rotavirus is the most common cause of diarrhoea in children. It damages cells within the small intestine (enterocytes) and thereafter causes gastroenteritis. The viral proteins can disrupt the reabsorption of water within the human intestine and can also cause an inefficiency to digest lactose, resulting in milk intolerance for infants. Symptoms include nausea, vomiting, diarrhoea, fever and dehydration (Thirabunyanon, 2011). Gardlik *et al*. (2012) bioengineered *L. lactis* to express virus spike protein VP8, which induced anti-VP8 antibodies and IgA antibodies in mice. This induction occurred systemically and locally within the mouse intestine providing 100% protection against rotavirus. With oral vaccination being favoured above the other types of vaccination, using probiotics with their ability to withstand the GIT conditions can be used as an alternative mode of vaccination. There are several other advantages of delivery of vaccines using recombinant probiotics such as easy administration by consumers, a decreased risk in transmission of blood-borne diseases and the stimulation of both innate and adaptive immunity (Behnsen *et al*., 2013).

1.7. Safety concerns regarding bioengineered probiotics

Bioengineered probiotics are increasingly being studied as vehicles that can express and target delivery of specific genes targeting a specific foodborne pathogen. One of the main drawbacks of working with bioengineered probiotics is that they are classified as genetically modified organisms (GMO) (Kamada *et al*., 2005). The consumption of a bioengineered probiotic would thus evidently expose a GMO into a given environment. The nature of such probiotics regarded as GMO presents

a major limitation to their widely applications. It is well known that some consumers have ethical reasons for not consuming GMO for fear that such organisms may pose a danger to one's life (Snydman, 2008). However, these modified microorganisms have a great potential to address novel approaches for prevention and treatment of different human and animal pathological conditions. It is, therefore, important to establish criteria that can be used for the assessment of the environmental safety and tracing the fate of recombinant DNA *in vitro* and *in vivo*, which are both of great importance (Sorokulova, 2014). Hence, safety of these strains needs to be guaranteed in order not to possess antibiotic selection markers or to transfer genetically modified DNA to other bacteria (Kamada *et al*., 2005). Biological containment systems can be used to prevent dissemination of genetic material to other bacteria and to prevent a significant uncontrolled increase of probiotic cells within a given environment. An example of one method was to use a thymidilmate synthase gene in a recombinant *L. acidophilus* as a marker for plasmid maintenance which contained foreign inserted genes (Snydman, 2008).

When cloning and expressing the different virulent traits into probiotics, only traits that will not make the probiotics pathogenic should be used. It is also crucial that each bioengineered strain be carefully evaluated for virulence determinants and sensitivity to clinically relevant antibiotics before being deemed suitable as a probiotic (D'Silva, 2011). When cloning probiotics, therapeutic safety of recombinant probiotic carrier organisms is crucial, especially when the strain has to be used under diseased conditions. The risk exposure determination, risk assessment and safety assessment are essential to ensure protection for the population against any unintended consequences of the use of probiotics (Sanders *et al*., 2010).

1.8. Conclusions and Future Perspective

The rise in morbidity and mortality due to foodborne pathogens remains a serious concern world-wide and the need for an alternative strategy for the control and treatment of infections caused by pathogens is equally crucial. The application of probiotics in food for control of enteric pathogens has been explored and the probiotic market is growing in the world. The ability of probiotics to inhibit human enteric pathogen has been well researched and documented and this has led to their use as a therapeutic approach for treatment of enteric infections. These studies showed both their successes and limitations, mainly highlighting the generic nature of their mode of action and their

failure in controlling some specific pathogens. These limitations can be overcome, and functions of conventional probiotics enhanced to create a greater beneficial effect through the use of bioengineering. The modification of conventional probiotics by use of bioengineering technology has a great potential for design and development of novel therapeutic approaches for effective treatment of pathogens.

Thorough understanding the life cycle of pathogens post ingestion, and knowledge of the virulence factors they use to cause infections offers a strategy for development of bioengineered probiotics strains tailored to control targeted pathogens. By targeting a specific pathogen, the efficacy of the probiotics inhibiting both the pathogens and infection will be increased. Although still in the early stages, researchers have made impressive strides towards design of such probiotics, producing strains geared towards enhancement of various functional and/or technological probiotic properties. Results from most of such studies showed positive effects although in some few cases no benefits were reported. The bioengineered probiotics thus offer great potential to be used as novel therapeutic approach for the prevention and treatment of foodborne infections. More studies targeting different virulence genes and pathogens, including the less studied and emerging ones, are desired in order to establish the future of this field of research and how it will impact the food and health industries.

In addition to this, most bioengineered probiotics are designed to be orally administered, therefore, they must still be able to survive through both technological and gastrointestinal stresses. It is also crucial that these strains have scientifically validated health properties, demonstrated safety and good technological properties to be produced on a large scale (Paton *et al.,* 2006). They should remain viable in large numbers to confer the beneficial effects to the host and should not develop unpleasant flavours or textures upon their incorporation into foods (Norton *et al.,* 1995). These aspects should also be addressed in future studies on bioengineered probiotics.

1.9. REFERENCES

Aguilar, C., Vanegas, C., Klotz, B. (2011). Antagonistic effect of *Lactobacillus* strains against *Escherichia coli* and *Listeria monocytogenes* in milk. *Journal of Dairy Research, 78*(2), 136-143.

Alakomi, H.L., Skytta, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., Helander, I. M. (2000). Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane. *Applied and Environmental Microbiology, 66*(5), 2001-2005.

Allam, M. L., Tau, N. S., Smouse, S. T., Mtshali, P. M., Mnyameni, F., Khumalo, Z.T.H., Ismail, A., Govender, N., Thomas, J., Smith, A. (2018). Whole-genome sequences of *Listeria monocytogenes* sequence type 6 isolates associated with a large foodborne outbreak in South Africa, 2017 to 2018. *Genome Announcements, 6*(25)

Althaus, D., Jermini, M., Giannini, P., Martinetti, G., Reinholz, D., Nüesch-Inderbinen, M., Lehner, A., Stephan, R. (2017). Local Outbreak of *Listeria monocytogenes* Serotype 4b Sequence Type 6 Due to Contaminated Meat Pâté. *Foodborne Pathogens and Disease, 14*(4), 219-222.

Amalaradjou, M.A., Bhunia, A.K. (2013). Bioengineered probiotics, a strategic approach to control enteric infections. *Bioengineered, 4*(6), 379-87.

Amara, A.A., Shibi, A. (2015). Role of Probiotics in health improvement, infection control and disease treatment and management. *Saudi Pharmaceutical Journal, 23*(2), 107-114.

Anderson, J.M., Van Itallie, C.M. (2009). Physiology and function of the tight junction. *Cold Spring Harbor Perspectives in Biology* 1, no. 2 A002584.

Angelo, K.M., Conrad, A.R., Saupe, A., Dragoo, H., West, N., Sorenson, A., Barnes, A., Doyle, M., Beal, J., Jackson, K.A., Stroika, S., Tarr, C., Kucerova, Z., Lance, S., Gould, L.H., Wise, M., Jackson, B.R. (2017). Multistate outbreak of *Listeria monocytogenes* infections linked to whole apples used in commercially produced, prepackaged caramel apples: United States, 2014-2015. *Epidemiology and Infection, 154*(5), 848- 856

Ascón, M.A., Ochoa-Repáraz, J., Walters, N., Pascual, D.W. (2005). Partially assembled K99 fimbriae are required for protection. *Infection and Immunity, 73* (11), 7274- 7280.

Bauer, E., Williams, B.A., Smidt, H., Verstegen, M.W., Mosenthin, R. (2006). Influence of the gastrointestinal microbiota on development of the immune system in young animals. *Current Issues in Intestinal Microbiology, 7*(2), 35–51.

Behnsen, J., Deriu, E., Sassone-Corsi, M., Raffatellu, M. (2013). Probiotics: Properties, examples, and specific applications. *Cold Spring Harbor Perspectives in Medicine, 3*(3), A010074.

Berg, P., Mertz, J.E. (2010). Personal reflections on the origins and emergence of recombinant DNA technology. *Genetics; 184*(1), 9-17.

Bermudez-Humaran, L.G., Motta, J.P., Aubry, C., Kharrat, P., Rous-Martin, L., Sallenave, J.M., Deraison, C., Vergnolle, N., Langella, P. (2015). Serine protease inhibitors protect better than IL-10 and TGF-[beta] anti-inflammatory cytokines against mouse colitis when delivered by recombinant *Lactococci*. *Microbial Cell Factories, 14*(1), 26.

Bernet-Camard, M.F., Lievin, V., Brassart, D., Neeser, J.R., Servin, A.L., Hudault, S. (1997). The human *Lactobacillus acidophilus* strain La1 secretes a non-bacteriocin antibacterial substance(s) active *in vitro* and *in vivo*. *Applied and Environmental Microbiology, 63*(7), 27-47.

Bhunia, A.K. (2012). Bioengineered probiotics- a solution to broaden probiotic efficacy! *Nutrition and Food Sciences, 2*: e105.DOI:10.4172/2155-9600.1000e105.

Bibiloni, R., Pérez, P.F., de Antoni, G.L. (1999). Will a high adhering capacity in a probiotic strain guarantee exclusion of pathogens from intestinal epithelia? *Anaerobe, 5*(5), 519-524.

Bierne, H., Sabet, C., Personnic, N., Cossart, P. (2007). Internalins: A complex family of leucine-rich repeat-containing proteins in *Listeria monocytogenes*. *Microbes and Infection, 9*(10), 1156-1166.

Bierne, H., Cossart, P. (2002). InlB, a surface protein of *Listeria monocytogenes* that behaves as an invasin and a growth factor. *Journal of Cell Science, 115*, 3357–3367.

Bigot, B., Lee, W.J., McIntyre, L., Wilson, T., Hudson, J.A., Billington, C., Heinemann, J.A. (2011). Control of *Listeria monocytogenes* growth in a ready-to-eat poultry product using a bacteriophage. *Food Microbiology, 28*, 1448- 1452.

Blutt, S.E., Miller, A.D., Salmon, S.L., Metzger, D.W., Conner, M.E. (2012). IgA is important for clearance and critical for protection from rotavirus infection. *Nature, 5*(6), 712- 719.

Bomba, A., Nemcová, R., Mudronová, D., Guba, P. (2002). The possibilities of potentiating the efficacy of probiotics. *Trends in Food Science and Technology, 13*(4), 121-126.

Bonazzi, M., Lecuit, M., Cossart, P. (2009). *Listeria monocytogenes* internalin and E-cadherin: From structure to pathogenesis. *Cell Microbiology, 11*, 693–702.

Bortolussi, R. (2008). Listeriosis: a primer. *Canadian Medical Association Journal, 179*, 795- 797

Braun, L., Dramsi, S., Dehoux, P., Bierne, H., Lindahl, G., Cossart, P. (1997). InlB: an invasion protein of *Listeria monocytogenes* with a novel type of surface association. *Molecular Microbiology, 25*, 285–294.

Brosch, R., Catimel, B., Milon, G., Buchrieser, C., Vindel, E., Rocourt, J. (1993). Virulence heterogeneity of *Listeria monocytogenes* strains from various sources (food, human, animal) in immuno-competent mice and its association with typing characteristics. *Journal of Food Protection, 56*, 296–301.

Burkholder, K.M., Bhunia, A.K. (2010). *Listeria monocytogenes* Uses *Listeria* Adhesion Protein (LAP) to promote bacterial transepithelial translocation and induces expression of lap receptor Hsp60. *Infection and Immunity, 78*(12), 5062-5073.

Burkholder, K.M., Kim, K.P., Mishra, K.K., Medina, S., Hahm, B.K., Kim, H., Bhunia, A.K. (2009). Expression of LAP, a SecA2-dependent secretory protein, is induced under anaerobic environment. *Microbes and Infection, 11*(10), 859-867.

Cabanes, D., Dehoux, P., Dussurget, O., Frangeul, L., and Cossart, P. (2002). Surface proteins and the pathogenic potential of *Listeria monocytogenes*. *Trends in Microbiology, 10*, 238-245.

Camejo, A., Carvalho, F., Reis, O., Leittao, E., Sousa, S., Cabanes, D. (2011). The arsenal of virulence factors deployed by *Listeria monocytogenes* to promote its cell infection cycle. *Virulence, 2*, 379-394.

Cano, P.G., Perdigón, G. (2003). Probiotics induce resistance to enteropathogens in a re-nourished mouse model. *Journal of Dairy Research, 70*(4), 433-40.

Cartwright, E., Jackson, K., Johnson, S., Graves, L., Silk, B., Mahon, B. (2013). Listeriosis outbreaks and associated food vehicles, United States, 1998-2008. *Emerging Infectious Diseases, 19*(1), 1-9.

Carvalho, A.L., Cardoso, F.S., Bohn, A., Neves, A.R., Santos, H. (2011). Engineering trehalose synthesis in *Lactococcus lactis* for improved stress tolerance. *Applied and Environmental Microbiology, 77*(12), 4189- 4199.

Cats, A., Kuipers, E.J., Bosschaert, M.A., Pot, R.G., Vandenbroucke- Grauls, C.M., Kusters, J.G. (2003). Effect of frequent consumption of a *Lactobacillus casei*-containing milk drink in *Helicobacter pylori*-colonized subjects. *Alimentary Pharmacology and Therapeutics, 17* (3), 429-443.

Ceapa, C., Rezaiki, L., Kleerebezem, M., Knol, J., Ozeer, R. (2013). Influence of fermented milk products, probiotics and probiotics on microbiota composition and health. *Best Practice and Research Clinical Gastroenterology*, 27(1), 139-155.

Centers for Disease Control and Prevention, 2018. Listeriosis in South Africa. https://wwwnc.cdc.gov/travel/notices/alert/listeriosis-south-africa. Accessed April 2016.

Centers for Disease Control and Prevention. (2000). Preliminary Foodnet data on the incidence of foodborne illnesses- selected sites, United States, 1999. *Morbidity and Mortality Weekly Report, 50*, 241–246.

Centre for Disease Control and Prevention. (1989). Listeriosis associated with consumption of turkey franks. *MMWR Morbidity and Mortality Weekly report, 38*, 267- 268.

Chakraborty, T, Leimeister-Wachter, M, Domann, E, Hartl, M, Goebel, W, Nichterlein, T, Notermans, S. (1992). Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the prfA gene. *The Journal of Bacteriology, 174(2*), 568-574.

Chichlowski, M., Croom, J., McBrid, B.W., Havenstein, G.B., Koci, M.D. (2007). Metabolic and physiological impact of probiotics or direct-fed-microbials on poultry: A brief review of current knowledge. *International Journal of Poultry Science, 6*(10), 694-704.

Chmielewski, R., Frank, J. (2003). Biofilm formation and control in food processing facilities. *Comprehensive Reviews in Food Science and Food Safety*, *2*(1), 22-32.

Chu, H., Kang, S., Ha, S., Cho, K., Park, S.M., Han, K.H., Kang, S.K., Lee, H., Han, S.H., Yun, C.H., Choi, Y. (2005). *Lactobacillus acidophilus* expressing recombinant K99 adhesive fimbriae has an inhibitory effect on adhesion of enterotoxigenic *Escherichia coli*. *Microbiology and Immunology, 49*(11), 941-994.

Collins, M.D., Gibson, G.R. (1999). Probiotics, prebiotics and synbiotics: approaches for modulating the microbial ecology of the gut. *American Journal of Clinical Nutrition, 69*(5), 1025S–1027S.

Corr, S.C., Gahan, C.G.M., Hill, C. (2007). Impact of selected *Lactobacillus* and Bifidobacterium species on *Listeria monocytogenes* infection and the mucosal immune response. *FEMS Immunology & Medical Microbiology, 50*(3), 380-388.

Cossart, P. (1997). Subversion of the mammalian cell cytoskeleton by invasive bacteria. *Journal of Clinical Investigation, 99*, 2307–2311.

Cossart, P., Toledo-Arana, A. (2008). *Listeria monocytogenes*, a unique model in infection biology: an overview. *Microbes and Infection, 10*, 1041–1050.

Cossart, P., Pizarro-Cerda, J., Lecuit, M. (2000). Invasion of mammalian cells by *Listeria monocytogenes*: functional mimicry to subvert cellular functions. *Trends Cell Biology, 13,* 23–31.

Cremonini, F., Di Caro, S., Nista, E.C., Bartolozzi, F., Capelli, G., Gasbarrini, G. (2002). Meta-analysis: the effect of probiotic administration on antibiotic associated diarrhoea. *Alimentary Pharmacology and Therapeutics*, 16(8), 1461-1467.

Cruchet S, Obregon MC, Salazar G, Diaz E, Gotteland M. (2003). Effect of the ingestion of a dietary product containing *Lactobacillus johnsonii* La1 on *Helicobacter pylori* colonization in children. *Nutrition, 19,* 716- 721.

Culligan, E., Hill, C., Sleator, R. (2009). Probiotics and gastrointestinal disease: Successes, problems and future prospects. *Gut Pathogens, 1*(1), 1-19.

Cummings, J.H., Macfarlane, G.T. (1997). Role of intestinal bacteria in nutrient metabolism. *Clinical Nutrition, 16*(1), 3-11.

D'Silva, I. (2011). Recombinant technology and probiotics. *International Journal of Engineering and Technology, 3*(4), 288-293

da Silva, C.V., Cruz, L., da Silva- Araújo, N., Bodini Angeloni, M., Fonseca, B. B., de Oliveira Gomes, A., dos Reis Carvalho, F., Ribeiro Goncalves, A.L., de Freitas Barbosa, B. (2012). A glance at *Listeria* and *Salmonella* cell invasion: Different strategies to promote host actin polymerization. *International Journal of Medical Microbiology, 302,* 19- 32.

de Moreno de LeBlanc, A., Castillo, N.A., Perdigon, G. (2010). Anti-infective mechanisms induced by a probiotic *Lactobacillus* strain against *Salmonella enterica* serovar Typhimurium infection. *International Journal of Food Microbiology, 138*(3), 223-231.

De Sousa, C. P. (2008). The impact of food manufacturing practices on food borne diseases. *Brazilian Archives of Biology and Technology, 51*(4), 815-823.

De Wit, M.A., Kopmans, M.P., Kortbeek, L.M., van Leeuwen, N.J., Bartelds, A.J. (2001). Gastroenteritis in sentinel general practices. The Netherlands. *Emerging Infectious Disease 7,* 82-89.

Desmond, C., Fitzgerald, G. F., Stanton, C., Ross, R. P. (2004). Improved stress tolerance of GroESL-Overproducing *Lactococcus lactis* and probiotic *Lactobacillus paracasei* NFBC 338. *Applied and Environmental Microbiology, 70*(10), 5929-5936.

Disson, O., Lecuit, M. (2012). Targeting of the central nervous system by *Listeria monocytogenes*. *Virulance, 3(2),* 213- 221

Disson, O., Lecuit, M. (2013). *In vitro* and *in vivo* models to study human listeriosis: mind the gap. *Microbes and Infection, 15,* 971- 980.

Dobson, A., Cotter, P.D., Ross, R.P., Hill, C. (2012). Bacteriocin production: A probiotic trait? *Applied and Environmental Microbiology, 78*(1), 1-6.

Domann, E., Zechel, S., Lingnau, A., Hain, T., Darji, A., Nichterlein, T., Wehland, J., Chakraborty, T. (1997). Identification and characterization of a novel prfA-regulated gene in

Listeria monocytogenes whose product, IrpA, is highly homologous to internalin proteins, which contain leucine-rich repeats. *Infection and Immunity, 65*, 101–109.

Dramsi, S., Biswas, I., Maguin, E., Braun, L., Mastroeni, P., Cossart, P. (1995). Entry of *Listeria monocytogenes* into hepatocytes requires expression of InIB, a surface protein of the internalin multigene family. *Molecular Microbiology, 16*, 251–261.

Dramsi, S., Bourdichon, F., Cabanes, D., Lecuit, M., Fsihi, H., Cossart, P. (2004). FbpA, a novel multifunctional *Listeria monocytogenes* virulence factor. *Molecular Microbiology, 53*(2), 639-649.

Dropulic, L.K., Lederman, H.M. (2016). Overview of infections in the immunocompromised host. *Microbiology Spectrum, 4*(4) https://doi.org/10.2165/00003495-200161001-00001.

Duan, F., March, J. (2010). Engineered bacterial communication prevents *Vibrio cholerae* virulence in an infant mouse model. *Proceedings of the National Academy of Sciences of the United States of America, 107*(25), 11260-11264.

Dupont, H. (1997). *Lactobacillus* GG in prevention of traveler's diarrhea: An encouraging first step. *Journal of Travel Medicine, 4*(1), 1-2.

Durand, M., Calderwood, S., Weber, D., Miller, S., Southwick, F., Caviness, V., Swartz, M. (1993). Acute bacterial meningitis in adults- A review of 493 episodes. *The New England Journal of Medicine, 328*(1), 21-28.

Ehrmann, M.A., Kurzak, P., Bauer, J., Vogel, R.F. (2002). Characterization of *Lactobacilli* towards their use as probiotic adjuncts in poultry. *Journal of Applied Microbiology, 92*(5) 966-975.

FAO/WHO. Guidelines for the evaluation of probiotics in food. report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food; Ontario, Canada. April 30 and May 1, 2002. Accessed April 2019.

Farber, J. M., Daley, E. M., MacKie, M. T., Limerick, B. (2000). A small outbreak of listeriosis potentially linked to the consumption of imitation crab meat. *Letters in Applied Microbiology, 31*, 100–104.

Farber, J., Carter, A., Varughese, P., Ashton, F., Ewan, E. (1990). Listeriosis traced to the consumption of Alfalfa tablets and soft cheese. *New England Journal of Medicine, 322*(5), 338.

Farber, J.M., Peterkin, P.I. (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Reviews, 55*(3), 476-511.

Fayet, O., Ziegelhoffer, T., Georgopoulos, C. (1989). The groES and groEL heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. *Journal of Bacteriology, 171*(3), 1397-1385.

Fayol-Messaoudi, D., Berger, C.N., Coconnier-Polter, M.H., Liévin-Le Moal, V., Servin, A.L. (2005). pH-, Lactic acid-, and non-Lactic acid-dependent activities of probiotic Lactobacilli against *Salmonella enterica* Serovar Typhimurium. *Applied and Environmental Microbiology, 71*(10), 6008–6013.

Finlay, B.B., Cossart, P. (1997). Exploitation of mammalian host cell functions by bacterial pathogens. *Science, 276*, 718–725.

Fratamico, P., Bhunia, A., Smith, J. (2005). Foodborne pathogens: Microbiology and molecular biology. Wymondham, Norofolk, UK: Caister Academic Press.

Fuller, R. (1991). Probiotics in human medicine. *Gut, 32*(4), 439-442.

Gaillard, J. L., Berche, P., Mounier, J., Richard, S., Sansonetti, P. (1987). *In vitro* model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. *Infection and Immunity, 55*, 2822– 2829.

Gaillard, J. L., Jaubert, F., Berche, P. (1996). The inlAB locus mediates the entry of *Listeria monocytogenes* into hepatocytes *in vivo*. *Journal of Experimental Medicine, 183*,359–369.

Galdeano, C.M., Perdigon, C. (2006). The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. *Clinical and Vaccine Immunology, 13(2),* 219- 226

Gallagher, D., Pouillot, R., Hoelzer, K., Tang, J., Dennis, S., Kause, J. (2016). *Listeria monocytogenes* in retail delicatessens: an interagency risk assessment - risk mitigations. *Journal of Food Protection, 79*(7), 1076-1088.

Gardlik, R., Palffy, R., Celec, P. (2012). Recombinant probiotic therapy in experimental colitis in mice. *Folia Biologica, 58(6),* 238-245.

Glaasker, E., Konings, W.N., Poolman, B. (1996). Osmotic regulation of intracellular solute pools in *Lactobacillus plantarum*. *The Journal of Bacteriology, 178*(3), 575-582.

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., Berche, P., Bloecker, H., Brandt, P., Chakraborty, T., Charbit, A., Chetouani, F., Couvé, E., de Daruvar, A., Dehoux, P., Domann, E., Domínguez-Bernal, G., Duchaud, E., Durant, L., Dussurget, O., Entian, K.D., Fsihi, H., García-del Portillo, F., Garrido, P., Gautier, L., Goebel, W., Gómez-López, N., Hain, T., Hauf, J., Jackson, D., Jones, L.M., Kaerst, U., Kreft, J., Kuhn, M., Kunst, F., Kurapkat, G., Madueno, E., Maitournam, A., Vicente, J.M., Ng, E., Nedjari, H., Nordsiek, G., Novella, S., de Pablos, B., Pérez-Diaz, J.C., Purcell, R., Remmel, B., Rose, M., Schlueter, T., Simoes, N., Tierrez, A., Vázquez-Boland, J.A., Voss, H., Wehland, J., Cossart, P. (2001). Comparative genomics of *Listeria* species. *Science, 294*(5543), 849-852.

Grangette, C., Müller-Alouf, H., Goudercourt, D., Geoffroy, M.C., Turneer, M., Mercenier, A. (2001). Mucosal immune responses and protection against tetanus toxin after intranasal immunization with recombinant *Lactobacillus plantarum*. *Infection and Immunity, 69*(3), 1547-1553.

Graves, L.M., Helsel, L.O., Steigerwalt, A.G., Morey, R.E., Daneshvar, M.I., Roof, S.E., Orsi, R.H., Fortes, E.D., Milillo, S.R., den Bakker, H.C., Wiedmann, M., Swaminathan, B., Sauders, B.D. (2010). *Listeria marthii* sp. nov., isolated from the natural environment, Finger Lakes National Forest. *International Journal of Systematic and Evolutionary Microbiology, 60*(6), 1280-1288.

Greer, G.G. (2005). Bacteriophage control of foodborne bacteria. *Journal of Food Protection, 68*, 1102- 1111.

Greiffenberg, L., Goebel, W., Kim, K. S., Weiglein, I., Bubert, A., Engelbrecht, F., Stins, M., Kuhn, M. (1998). Interaction of *Listeria monocytogenes* with human brain microvascular endothelial cells: InlB-dependent invasion, long-term intracellular growth, and spread from macrophages to endothelial cells. *Infection and Immunity, 66*, 5260- 5267.

Guenther, S., Huwyler, D., Richard, S., Loessner, M.J. (2009). Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. *Applied and Environmental Microbiology, 75,* 93- 100.

Gupta, C., Prakash, D., Gupta, S. (2014). Genetically engineered probiotics. *African Journal of Basic and Applied Sciences, 6*(3), 57-64.

Hamon, M., Bierne, H., Cossart, P. (2006). *Listeria monocytogenes*: A multifaceted model. *Nature Reviews: Microbiology, 4,* 423–434.

Hardy, J., Francis, K.P., De Boer, M., Chu, P., Gibbs, K., Contag, C.H. (2004). Extracellular replication of *Listeria monocytogenes* in the murine gall bladder. *Science, 301,* 851-853

Harish, K., Varghese, T. (2006). Probiotics in humans-Evidence based review. Calicut *Medical Journal, 4(4):*e3.

Hooper, L.V., Gordon, J.I. (2001). Commensal host-bacterial relationships in the gut. *Science, 292*(5519), 1115-1118.

Houtsma, P.C., de Wit, J.C., Rombouts, F.M. (1993). Minimum inhibitory concentration (MIC) of sodium lactate for pathogens and spoilage organisms occurring in meat products. *International Journal of Food Microbiology, 20,* 247–257.

Hudson, J.A., Billington, C., Carey-Smith, G., Greening, G. (2005). Bacteriophages as biocontrol agents in food. *Journal of Food Protection,* 68, 426- 437.

Innocentin S, Guimaraes V, Miyoshi A, Azevedo V, Langella P, Chatel JM, Lefevre F. (2009). *Lactococcus lactis* expressing either *Staphylococcus aureus* Fibronectin-binding protein A or *Listeria monocytogenes* Internalin A can efficiently internalize and deliver dna in human epithelial cells. *Applied and Environmental Microbiology, 75(14),* 4870-4878.

Ireton, K., Cossart, P. (1997). Host pathogen interactions during entry and actin-based movement of *Listeria monocytogenes*. *Annual Reviews of Genetics, 31*,113–138.

Ireton, K., Payrastre, B., Cossart, P. (1999). The *Listeria monocytogenes* protein InlB is an agonist of mammalian phosphoinositide 3-kinase. *Journal of Biological Chemistry, 274*, 17025–17032.

Isberg, R. R., Tran Van Nhieu, G. (1994). Two mammalian cell internalization strategies used by pathogenic bacteria. *Annual Reviews of Genetics, 27,* 395– 422.

Jackson, K.A., Gould, L.H., Hunter, J.C., Kucerova, Z., Jackson, B. (2018). Listeriosis outbreaks associated with soft cheeses, United States, 1998-2014. *Emerging Infectious Diseases, 24*(6), 1116-1119.

Jacobsen, T., Budde, B.B., Koch, A.G., (2003). Application of *Leuconostoc carnosum* for biopreservation of cooked meat products. *Journal of Applied Microbiology, 95*, 242–249.

Jagadeesan, B., Fleishman Littlejohn, A., Singh, A., Mishra, K., La, D., Kihara, D., Bhunia, A.K. (2011). N-Terminal Gly224–Gly411 domain in *Listeria* adhesion protein interacts with host receptor Hsp60. *PLoS One, 6*(6), E20694.

Jagadeesan, B., Koo, O., Kim, K., Burkholder, K., Mishra, K., Aroonnual, A., Bhunia, A. (2010). LAP, an alcohol acetaldehyde dehydrogenase enzyme in *Listeria*, promotes bacterial adhesion to enterocyte-like Caco-2 cells only in pathogenic species. *Microbiology, 156*(Pt 9), 2782-2795.

Jain, N., Roy, I. (2009). Effect of trehalose on protein structure. *Protein Science, 18*(1), 24-36.

Janes, M.E., Kooshesh, S., Johnson, M.G. (2002). Control of *Listeria monocytogenes* on the surface of refrigerated, ready-to-eat chicken coated with edible zein film coatings containing nisin and/or calcium propionate. *Journal of Food Science, 67,* 2754- 2757.

Jaradat, Z.W., Wampler, J.W., Bhunia, A.W. (2003). A *Listeria* adhesion protein-deficient *Listeria monocytogenes* strain shows reduced adhesion primarily to intestinal cell lines. *Medical Microbiology and Immunology, 192*(2), 85-91.

Jonquières, R., Pizarro-Cerdá, J., Cossart, P. (2001). Synergy between the N- and C-terminal domains of InlB for efficient invasion of non-phagocytic cells by *Listeria monocytogenes*. *Molecular Microbiology, 42,* 955–965.

Juntunen, M., Kirjavainen, P. V., Ouwehand, A. C., Salminen, S. J., Isolauri, E. (2001). Adherence of Probiotic Bacteria to Human Intestinal Mucus in Healthy Infants and during Rotavirus Infection. *Clinical and Vaccine Immunology*, *8*(2), 293-6.

Kaczmarski, E., Jones, D. (1989). Listeriosis and ready-cooked chicken. *The Lancet*, *333*(8637), 549.

Kailasapathy, K., Chin, J. (2000). Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and Bifidobacterium spp. *Immunology and Cell Biology*, *78*(1), 80-88.

Kamada N, Inoue N, Hisamatsu T, Okamoto S, Matsuoka K, Sato T, Chinen H, Hong KS, Yamada T, Suzuki Y, Suzuki T, Watanabe N, Tsuchimoto K, Hibi T. (2005). Nonpathogenic *Escherichia coli* strain Nissle1917 prevents murine acute and chronic colitis. *Inflammatory Bowel Diseases, 11*(5), 455-463.

Karimi, O., Pena, A. (2008). Indications and challenges of probiotics, prebiotics, and synbiotics in the management of arthralgias and spondyloarthropathies in inflammatory bowel disease. *Journal of Clinical Gastroenterology, 42*(8), S136-S141.

Kathariou, S. (2002). *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *Journal of Food Protection, 65*(11), 1811-1829.

Kaur, S., Malik, S.V.S., Vaidya, V.M., Barbuddhe, S.B. (2007). *Listeria monocytogenes* in spontaneous abortions in humans and its detection by multiplex PCR. *Journal of Applied Microbiology, 103,* 1889-1896.

Kelly, J., Barnass, S., Sawicka, E., Dean, A. (1999). *Listeria* meningitis presenting in an immune-competent adult patient. *Hospital Medicine, 60,* 140–141.

Kempf, B., Bremer, E. (1998). Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Archives of Microbiology, 170*(5), 319-330

Khan, I, Khan, J, Miskeen, S, Tango, Cn, Park, Ys, Oh, Dh. (2016). Prevalence and Control of *Listeria monocytogenes* in the Food Industry - a Review. *Czech Journal of Food Sciences, 34*(6), 469-487.

Khan, S., Wingard, J. (2001). Infection and mucosal injury in cancer treatment. *Journal of the National Cancer Institute. Monographs, 2001*(29), 31-36.

Khor, B., Gardet, A., Xavier, R.J. (2011). Genetics and pathogenesis of inflammatory bowel disease. *Nature, 474*(7351), 307-17.

Kim, T.S., Hur, J.W., Yu, M.A., Cheigh, C.I., Kim, K.N., Hwang, J.K., Pyun, Y.R. (2003). Antagonism of *Helicobacter pylori* by bacteriocins of lactic acid bacteria. *Journal of Food Protection, 66*(1), 3-12.

Kim, Y., Kim, S.H., Whang, K.Y., Kim, Y.J., Oh, S. (2008). Inhibition of *Escherichia coli* O157:H7 attachment by interactions between lactic acid bacteria and intestinal epithelial cells. *Journal of Microbiology and Biotechnology, 18*(7), 1278- 1285

Kitov, P.I., Sadowska, J.M., Mulvey, G., Armstrong, G.D., Ling, H., Pannu, N.S., Read, R.J., Bundle, D.R. (2000). Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands. *Nature, 403* (6770), 669-673

Knabel, S.J., Reimer, A., Verghese, B., Lok, M., Ziegler, J., Farber, J., Pagotto, F., Graham, M., Nadon, C.A., Gilmour, M.W. (2012). Sequence typing confirms that a predominant *Listeria monocytogenes* clone caused human listeriosis cases and outbreaks in Canada from 1988 to 2010. *Journal of Clinical Microbiology, 50*(5), 1748-1751.

Koo, O.K., Amalaradjou, M.A.R., Bhunia, A.K. (2012). Recombinant probiotic expressing *Listeria* adhesion protein attenuates *Listeria monocytogenes* virulence *in vitro*. *PLoS ONE, 7*(1), 1-15

Koopmans, M.M., Brouwer, M.C., Bijlsma, M.W., Bovenkerk, S., Keijzers, W., Van der Ende, A., Van de Beek, D. (2013). *Listeria monocytogenes* sequence Type 6 and increased rate of unfavorable outcome in meningitis: Epidemiologic cohort study. (Report). *Clinical Infectious Diseases, 57*(2), 247-53.

Kumar, M., Yadav, A.K., Verma, V., Singh, B., Mal, G., Nagpal, R., Hemalatha, R. (2016). Bioengineered probiotics as a new hope for health and diseases: An overview of potential and prospects. *Future Microbiology, 11*(4), 585-600.

Laksanalamai, P., Joseph, L.A., Silk, B.J., Burall, L.S., Tarr, C.L., Gerner-Smidt, P., Datta, A.R. (2012). Genomic characterization of *Listeria monocytogenes* strains involved in a multistate listeriosis outbreak associated with cantaloupe in US. *PLoS ONE, 7*(7), 1-12.

Lecuit, M. (2005). Understanding how *Listeria monocytogenes* targets and crosses host barriers. *Clinical Microbiology and Infection, 11*, 430-463.

Lecuit, M. (2007). Human listeriosis and animal models. *Microbes and Infection, 9*(10), 1216-1225.

Lecuit, M., Dramsi, S., Gottardi, C., Fedor-Chaiken, M., Gumbiner, B., Cossart, P. (1999). A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*. *EMBO Journal, 18*, 3956–3963.

Lecuit, M., Ohayon, H., Braun, L., Mengaud, J., Cossart, P. (1997). Internalin of *Listeria monocytogenes* with an intact leucine-rich repeat region is sufficient to promote internalization. *Infection and Immunity, 65*, 5309–5319.

Lecuit, M., Vandormael-Pournin, S., Lefort, J., Huerre, M., Gounon, P., Dupuy, C., Babinet, C., Cossart, P. (2001). A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. *Science, 292*, 1722–1725.

Lee, Y. K., Puong, K. Y., Ouwehand, A. C., Salminen, S. (2003). Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. *Journal of Medical Microbiology, 52*(10), 925-930.

Leung, W., Graham, K. (2002). Rescue therapy for Helicobacter pylori. *Current Treatment Options in Gastroenterology*, *5*(2), 133-138.

Leverentz, B., Conway, W.S., Camp, M.J., Janisiewicz, W.J., Abuladze, T., Yang, M., Saftner, R., Sulakvelidze, A. (2003). Biocontrol of *Listeria monocytogenes* on freshcut produce by treatment with lytic bacteriophages and a bacteriocin. *Applied and Environmental Microbiology, 69*, 4519- 4526

Lingnau, A., Domann, E., Hudel, M., Bock, M., Nichterlein, T., Wehland, J., Chakraborty, T. (1995). Expression of the *Listeria monocytogenes* EGD inlA and inlB genes, whose products

mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and -independent mechanisms. *Infection and Immunity, 63*, 3896–3903.

Link-Amster, H., Rochat, F., Saudan, K., Mignot, O., Aeschlimann, J. (1994). Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunology and Medical Microbiology, 10*(1), 55-63.

Linnan, M.J., Mascola, L., Lou, X.D., Goulet, V., May, S. (1988). Epidemic listeriosis associated with Mexican-style cheese. *The New England Journal of Medicine, 319*(13), 823-828.

Lionetti, E., Principi, M., Scaccianoce, G., Maurogiovanni, G., La Rosa, M., Ierardi, E., Fontana, C., Sardaro, R., Cavallo, L., Francavilla, R. (2011). Probiotics and *Helicobacter pylori*. *European Journal of Gastroenterology and Hepatology, 7,* 121–128.

Lomonaco, S., Verghese, B., Gerner-Smidt, P., Tarr, C., Gladney, L., Joseph, L., Katz, L., Turnsek, M., Frace, M., Chen, Y., Brown, E., Meinersmann, R., Berrang, M., Knabel, S., (2013). Novel epidemic clones of *Listeria monocytogenes*, United States, 2011. *Emerging Infectious Diseases, 19*(1), 147-150.

Mack, D.R., Ahrne, S., Hyde, L., Wei, S., Hollingsworth, M.A. (2003). Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells *in vitro*. *Gut, 52*(6), 827–833.

Madsen, K., Cornish, A., Soper, P., McKaigney, C., Jijon, H., Yachimec, C., Doyle, J., Jewell, L., De Simone, C. (2001). Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology, 121*(3), 580-591.

Malin, M., Suomalainen, H., Saxelin, M., Isolauri, E. (1996). Promotion of IgA immune response in patients with Crohn's disease by oral bacteriotherapy with *Lactobacillus* GG. *Annals of Nutrition and Metabolism, 40(3),* 137- 145

Mandal, S.M., Silva, O.N., Franco, O.L. (2014). Recombinant probiotics with antimicrobial peptides: A dual strategy to improve immune response in immunocompromised patients. *Drug Discovery Today, 9*(8), 1045- 1050.

Mandomando, I., Jaintilal, D., Pons, M.J., Vallés, X., Espasa, M., Mensa, L., Sigaúque, B., Sanz, S., Sacarlal, J., Macete, E., Abacassamo, F., Alonso, P.L., Ruiz, J. (2009). Antimicrobial susceptibility and mechanisms of resistance in *Shigella* and *Salmonella* isolates from children under five years of age with diarrhea in rural Mozambique. *Antimicrobial Agents and Chemotherapy, 53*(6), 2450–2454.

Manning, G., Duim, B., Wassenaar, T., Wagenaar, J.A., Ridley, A., Newell, D.G. (2001). Evidence for genetically stable strain of *Campylobacter jejuni*. *Applied and Environmental Microbiology, 67*, 1185-1189.

Marteau, P., Minekus, M., Havenaar, R., Huis In't Veld, J.H.J. (1997). Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and the effects of bile. *Journal of Dairy Science, 80*(6), 1031-1037.

Martin, E.M., Griffis, C.L., Vaughn, K.L.S., O'Bryan, C.A., Friedly, E.C., Marcy, J.A., Ricke, S.C., Crandall, P.G., and Lary, Jr., R.Y. (2009). Control of *Listeria monocytogenes* by lauric arginate on frankfurters formulated with or without lactate/diacetate. *Journal of Food Science, 74*(6), M237-M241.

Mattar, A., Teitelbaum, D., Drongowski, H., Yongyi, R., Harmon, F., Coran, C. (2002). Probiotics up-regulate MUC-2 mucin gene expression in a Caco-2 cell-culture model. *Pediatric Surgery International, 18*(7), 586-590.

Mbandi, E., Shelef, L.A. (2001). Enhanced inhibition of *Listeria monocytogenes* and *Salmonella enteritidi*s in meat by combinations of sodium lactate and diacetate. *Journal of Food Protection, 64*, 640–644.

Mccarthy, J., O'mahony, L., O'callaghan, L., Sheil, B., Vaughan, E.E., Fitzsimons, N., Fitzgibbon, J., O' Sullivan, G.C.O., Kiely, B., Collins, J.K., Shanahan, F. (2003). Double blind, placebo-controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. *Gut, 52*(7), 975-980.

McCool, D.J., Forstner, J.F., Forstner, G.G. (1994). Synthesis and secretion of mucin by the human colonic tumor cell line Ls180. *Biochemical Journal, 302*(1), 111-118

McFarland, A.P., Savan, R., Wagage, S., Addison, A., Ramakrishnan, K., Karwan, M., Duong, T., Young, H.A. (2011). Localized delivery of interferon-[beta] by *Lactobacillus* exacerbates experimental colitis. *PLoS ONE, 6*(2), E16967.

Mengaud, J., Ohayon, H., Gounon, P., Mege, R. M., Cossart, P. (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. *Cell, 84,* 923–932.

Meydani, S.N., Ha, W.K. (2000). Immunological effects of yoghurt. *American Journal of Clinical Nutrition, 71(4),* 861-872

Milohanic, E., Jonquières, R., Cossart, P., Berche, P., Gaillard, J. (2001). The autolysin Ami contributes to the adhesion of *Listeria monocytogenes* to eukaryotic cells via its cell wall anchor. *Molecular Microbiology, 39(5),* 1212-1224.

Mirnejad R, Vahdati AR, Rashidiani J, Erfani M, Piranfar V. (2013). The antimicrobial effect of *Lactobacillus casei* culture supernatant against multiple drug resistant clinical isolates of *Shigella sonnei* and *Shigella flexneri in vitro*. *Iranian Red Crescent Medical Journal, 15(2),* 122-126

Murray, E., Webb, R., Swann, M. (1926). A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes*. *Journal of Pathology and Bacteriology, 29*(4), 407-439.

Mylonakis, E., Paliou, M., Hohmann, E., Calderwood, S., Wing, E. (2002). Listeriosis during pregnancy - A case series and review of 222 cases. *Medicine, 81*(4), 260-269.

Nesser, J.R., Granato, D., Rouvet, M., Servin, A., Teneberg, S., Karlsson, K.A. (2000). *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. *Glycobiology, 10*(11), 1193-1199.

Ng, S.C., Hart, A.L., Kamm, M.A., Stagg, A.J., Knight, S.C. (2009). Mechanisms of action of probiotics: Recent advances. *Inflammatory Bowel Diseases, 15*(2), 300-310.

Nielsen, D.S., Cho, G.S., Hanak, A., Huch, M., Franz, C.M., Arneborg, N. (2010). The effect of bacteriocin-producing *Lactobacillus plantarum* strains on the intracellular pH of sessile

and planktonic *Listeria monocytogenes* single cells. *International Journal of Food Microbiology, 141*, 53–59.

Norrung, B., Andersen, J. K. (2000). Variations in virulence between different electrophoretic types of *Listeria monocytogenes*. *Letters in Applied Microbiology, 30*, 228–232.

Norton, P.M., Le Page, R.W., Wells, J.M. (1995). Progress in the development of *Lactococcus lactis* as a recombinant mucosal vaccine delivery system. *Folia Microbiologica, 40*(3), 225-230.

Ohashi, Y., Ushida, K. (2007). Health-beneficial effects of probiotics: Its mode of action. *Animal Science Journal, 80*(4), 361-372

Opintan, J., Newman, M.J. (2007). Distribution of serogroups and serotypes of multiple drug resistant Shigella isolates. *Ghana Medical Journal, 41*(1), 8-29.

Orsi, R.H., Wiedmann, M. (2016). Characteristics and distribution of *Listeria* spp., including *Listeria* species newly described since 2009. *Applied Microbiology and Biotechnology, 100*, 5273- 5287.

Oscáriz, J., Lasa, I., Pisabarro, A. (1999). Detection and characterization of cerein 7, a new bacteriocin produced by *Bacillus cereus* with a broad spectrum of activity. *FEMS Microbiology Letters, 178*(2), 337-341.

Parassol N, Freitas M, Thoreux K, Dalmasso G, Bourdet-Sicard R, Rampal P. (2005). *Lactobacillus casei* DN-114 001 inhibits the increase in paracellular permeability of enteropathogenic *Escherichia coli*-infected T84 cells. *Research in Microbiology, 156*(2), 256-262.

Parida, S. K., Domann, E., Rohde, M., Muller, S., Darji, A., Hain, T., Wehland, J., Chakraborty, T. (1998). Internalin B is essential for adhesion and mediates the invasion of *Listeria monocytogenes* into human endothelial cells. *Molecular Microbiology, 28,* 81–93.

Paton, A., Morona, R., Paton, J.C. (2000). A new biological agent for treatment of Shiga toxigenic Escherichia coli infections and dysentery in humans. *Nature Medicine, 6*(3), 265-270.

Paton, A.W., Morona, R., Paton, J.C. (2006). Designer probiotics for prevention of enteric infections. *Nature Reviews Microbiology, 4*(3), 193-200.

Paton, A.W., Morona, R., Paton, J.C. (2010). Bioengineered bugs expressing oligosaccharide receptor mimics: Toxin-binding probiotics for treatment and prevention of enteric infections. *Bioengineered Bugs, 1*(3), 172-177.

Pazhani, G.P., Niyogi, S.K., Singh, A.K., Sen, B., Taneja, N., Kundu, M., Yamasak, S., Ramamurthy, T. (2008). Molecular characterization of multidrug-resistant *Shigella* species isolated from epidemic and endemic cases of shigellosis in India. *Journal of Medical Microbiology, 57*(7), 856-863.

Pirie, J. H.H. (1940). Genus listerella Pirie. *Science, 91*, 383-384.

Pizarro-Cerda, J., Cossart, P. (2006). Subversion of cellular functions by *Listeria monocytogenes*. *Journal of Pathology, 208*, 215–223.

Pizarro-Cerdá, J., Kühbacher, A., Cossart, P. (2012). Entry of *Listeria monocytogenes* in mammalian epithelial cells: An updated view. *Cold Spring Harbor Perspectives in Medicine, 2*(11), A010009-a010009.

Pouillot, R., Gallagher, D., Tang, J., Hoelzer, K., Kause, J., Dennis, S. (2015). *Listeria monocytogenes* in retail delicatessens: an interagency risk assessment-model and baseline results. *Journal of Food Protection, 78*(1), 134-145.

Qin, H.L., Shen, T.Y., Gao, Z.G., Fan, X.B., Hang, X.M., Jiang, Y.Q., Zhang, H.Z. (2005). Effect of lactobacillus on the gut microflora and barrier function of the rats with abdominal infection. *World Journal of Gastroenterology, 11*(17), 2591-2596.

Ramarao, N., Lereclus, D. (2006). Adhesion and cytotoxicity of *Bacillus cereus* and *Bacillus thuringiensis* to epithelial cells are FlhA and PlcR dependent, respectively. *Microbes and Infection, 8*(6), 1483-1491.

Rasko, D.A., Sperandio, V. (2010). Anti-virulence strategies to combat bacteria-mediated disease. *Nature Reviews Drug Discovery, 9*(2), 117-129.

Rattanachaikunsopon, P., Phumkhachorn, P. (2010). Antimicrobial activity of basil (ocimum basilicum) oil against *Salmonella Enteritidis in vitro* and in food. *Bioscience, Biotechnology, and Biochemistry, 74*(6), 1200-1204.

Reis, O., Sousa, S., Camejo, A., Villiers, V., Gouin, E., Cossart, P., Cabanes, D. (2010). LapB, a novel *Listeria monocytogenes* LPXTG surface adhesin, required for entry into eukaryotic cells and virulence. *The Journal of Infectious Diseases, 202*(4), 551-562.

Resta-Lenert, S., Barrett, K.E. (2003). Live probiotics intestinal epithelial cells from the effects of infection with enteroinvasive *Escherichia coli*. *Gut, 52*(7), 988-998.

Ribet, D., Cossart, P. (2015). How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes and Infection, 17,* 173- 183.

Richter, J.F., Gitter, A.H., Gunzel, D., Weiss, S., Mohamed, W., Chakraborty, T., Fromm, M., Schulzke, J.D. (2009). Listeriolysin O affects barrier function and induces chloride secretion in HT-29/B6 colon epithelial cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 296*(6), G1350-G1359.

Riedo, F.X., Pinner, R.W., De Lourdes Tosca, M., Cartter, M.L., Graves, L.M., Reeves, M.W., Weaver, R.E., Plikaytis, B.D., Broome, C.V., Francis, X. R., Robert W.P., Maria De Lourdes, T., Matthew, L.C., Lewis, M.G., Michael, W.R., Robert, E.W., Brian, D.P., Claire, V.B. (1994). A point-source foodborne listeriosis outbreak: documented incubation period and possible mild illness. *The Journal of Infectious Diseases, 170*(3), 693-696.

Risoen, P.A., Ronning, P., Hegna, I.K., Kolsto, A.B. (2004). Characterization of a broad range antimicrobial substance from *Bacillus cereus*. *Journal of Applied Microbiology, 96*(4): 648-655.

Robbe-Masselot, C., Annkatrin, H., Ingemar, C., Michalski, J.C., Capon, C. (2008). Glycosylation of the two O-glycosylated domains of human MUC2 mucin in patients transposed with artificial urinary bladders constructed from proximal colonic tissue. *Glycoconjugate Journal*, *25*(3), 213-224.

Roberfroid, M.B. (2000). Prebiotics and Probiotics: are they functional goods? *The American Journal of Clinical Nutrition*, 71(6), 1682S–1687S,

Robinson, K., Chamberlain, L.M., Schofield, K.M., Wells, J.M., Le Page, R.W.F. (1997). Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. *Nature Biotechnology*, *15*(7), 653-657.

Rolfe, R.D. (1991). Population dynamics of the intestinal tract. In: Blankenship LC, editor. Colonization control of human bacterial enteropathogens in poultry. San Diego, Academic Press, 59- 75.

Rubin, R., Ikonen, T., Gummert, J., Morris, R. (1999). The therapeutic prescription for the organ transplant recipient: The linkage of immunosuppression and antimicrobial strategies. *Transplant Infectious Disease, 1*(1), 29-39.

Ruggiero, P. (2014). Use of probiotics in the fight against *Helicobacter pylori*. *World Journal of Gastrointestinal Pathophysiology, 5*(4), 384-391.

Sack, D.A., Sack, R.B., Nair, G.B., Siddique, A.K. (2004). Cholera. *Lancet, 363*(9404), 223-233.

Sánchez, B., López, P., González-Rodríguez, I., Suárez, A., Margolles, A., Urdaci, M. (2011). A flagellin-producing Lactococcus strain: Interactions with mucin and enteropathogens. *FEMS Microbiology Letters, 318*(2), 101-107.

Sanders, M., Akkermans, L., Haller, D., Hammerman, C., Heimbach, J., Hörmannsperger, G., Huys, G. (2010). Safety assessment of probiotics for human use. *Gut Microbes, 1*(3), 164-185.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A. (2011). Foodborne illness acquired in the United States-major pathogens. *Emerging Infectious Diseases, 17*(1), 1-15.

Schillinger, U, Lucke, F K. (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. *Applied and Environmental Microbiology, 55*(8), 1901-1906.

Schlech III, W. F., Lavigne, P. M., Bortolussi, R. A., Alien, A. C., Haldane, E. V., Wort, A. J., Hightower, A. W., Johnson, S. E., King, S. H., Nicholls, E. S., Broome, C. V. (1983). Epidemic listeriosis-evidence for transmission by food. *The New England Journal of Medicine, 308,*203-206.

Schubert, W.D., Göbel, G., Diepholz, M., Darji, A., Kloer, D., Hain, T., Chakraborty, T., Wehland, J., Domann, E., Heinz, D.W. (2001). Internalins from the human pathogen *Listeria*

monocytogenes combine three distinct folds into a contiguous internalin domain. *Journal of Molecular Biology, 312*(4), 783-794.

Servin, A. (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiology Reviews, 28*(4), 405-440.

Seveau, S., Pizarro-Cerda, J., Cossart, P. (2007). Molecular mechanisms exploited by *Listeria monocytogenes* during host cell invasion. *Microbes and Infection, 9*(10), 1167-1175.

Shah, N. (2000). Probiotic Bacteria: Selective Enumeration and Survival in Dairy Foods. *Journal of Dairy Science, 83*(4), 894-907.

Sheehan, V., Sleator, R., Hill, C., Fitzgerald, G. (2007). Improving gastric transit, gastrointestinal persistence and therapeutic efficacy of the probiotic strain *Bifidobacterium breve* UCC2003. *Microbiology, 153*(10), 3563-3571.

Sheehan, V.M., Sleator, R.D., Fitzgerald, G.F., Hill, C. (2006). Heterologous Expression of BetL, a Betaine Uptake System, Enhances the Stress Tolerance of *Lactobacillus salivarius* UCC118. *Applied and Environmental Microbiology, 72*(3), 2170-2177.

Sheil, B., MacSharry, J., O'Callagan, L., O'Riordan, A., Waters, A., Collins, J.K., Shanahan, E. (2006). Role of interleukin (IL-10) in probiotic-mediated immune modulation: An assessment in wild-type and IL-10 knock-out mice. *Clinical and Experimental Immunology, 144*(2), 273-280.

Shen, Y., Naujokas, M., Park, M., Ireton, K. (2000). InlB-dependent internalization of *Listeria* is mediated by the Met receptor tyrosine kinase. *Cell, 103,* 501–510.

Silk, B.J., Date, K.A., Jackson, K.A., Pouillot, R., Holt, K.G., Graves, L.M., Ong, K.L., Hurd, S., Meyer, R., Marcus, R., Shiferaw, B., Norton, D.M., Medus, C., Zansky, S.M., Cronquist, A.B., Henao, O.L., Jones, T.F., Vugia, D.J., Farley, M.M., Mahon, B.E. (2012). Invasive listeriosis in the foodborne diseases active surveillance network (FoodNet), 2004-2009: Further targeted prevention needed for higher-risk groups. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 54 Suppl 5*(5), S396-404.

Simon, O., Vahjen, W., Scharek, L. (2005). Microorganisms as feed additives - probiotics. *Advances in Pork Production, 16,* 161- 167

Sivapalasingam, S, Nelson, J.M., Joyce, K., Hoekstra, M., Angulo, F.J., Mintz, E.D. (2006). High prevalence of antimicrobial resistance among *Shigella* isolates in the United States tested by the national antimicrobial resistance monitoring system from 1999 to 2002. *Antimicrobial Agents and Chemotherapy*, *50*(1), 49-54.

Sleater, R.D., Gahan, C.G.M., Abee, T., Hill, C. (1999). Identification and disruption of BetL, a secondary glycine betaine transport system linked to the salt tolerance of *Listeria monocytogenes* LO28. *Applied and Environmental Microbiology, 65*, 2078-2083.

Sleator, R. D., Gahan, C. G., O'Driscoll, B., Hill, C. (2000). Analysis of the role of betL in contributing to the growth and survival of *Listeria monocytogenes* LO28. *International Journal of Food Microbiology, 60*(2-3), 261-268.

Sleator, R., Francis, G., O'Beirne, D., Gahan, C., Hill, C. (2003). Betaine and carnitine uptake systems in *Listeria monocytogenes* affect growth and survival in foods and during infection. *Journal of Applied Microbiology, 95*(4), 839-846.

Sleator, R.D., Hill, C. (2008). 'Bioengineered Bugs' – A patho-biotechnology approach to probiotic research and applications. *Medical Hypotheses, 70*(1), 167-169.

Smith, J. L. (1999). Foodborne infections during pregnancy. *Journal of Food protection, 62*(7): 818- 829.

Snydman, D. (2008). The safety of probiotics. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 46 Suppl 2*(S2), S104-111.

Soni, K.A., Nannapaneni, R. (2010). Bacteriophage significantly reduces *Listeria monocytogenes* on raw salmon fillet tissue. *Journal of Food Protection, 73*, 32- 38.

Soni, K.A., Nannapaneni, R., Hagens, S. (2010). Reduction of *Listeria monocytogenes* on the surface of fresh channel catfish fillets by bacteriophage Listex P100. *Foodborne Pathogens and Diseases, 7*, 427- 434.

Sorokulova, I. (2014). Recombinant probiotics: Future perspectives in disease treatment. *Journal of Probiotics and Health*, 2:e109. doi:10.4172/2329-8901.1000e109

Southwick, F.S., Purich, D.L. (1996). Intracellular pathogenesis of listeriosis. *The New England Journal of Medicine, 334(12),* 770-776.

Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., Fiers, W., Remaut, E. (2000). Treatment of Murine Colitis by *Lactococcus lactis s*ecreting Interleukin-10. *Science, 289* (5483), 1355- 1385.

Swaminathan, B., Gerner-Smidt, P. (2007). The epidemiology of human listeriosis. *Microbes and Infection, 9*(10), 1236-1243.

Swanson, J. A., Baer, S. C. (1995). Phagocytosis by zippers and triggers. *Trends in Cell Biology, 5*, 89–93.

Termont, S., Vandenbroucke, K., Iserentant, D., Neirynck, S., Steidler, L., Remaut, E., Rottiers, P. (2006). Intracellular accumulation of trehalose protects *Lactococcus lactis* from freeze-drying damage and bile toxicity and increases gastric acid resistance. *Applied and Environmental Microbiology, 72*(12), 7694.

Thirabunyanon, M. (2011). Biotherapy for and protection against gastrointestinal pathogenic infections via action of probiotic bacteria. *Maejo International Journal of Science and Technology, 5*(1), 108-128.

Thirabunyanon M, Thongwittaya N. (2012). Protection activity of a novel probiotic strain of *Bacillus subtilis* against *Salmonella Enteritidis* infection. *Research in Veterinary Science, 93*(1), 74-81.

Tompkin, R. (2002). Control of *Listeria monocytogenes* in the food-processing environment. *Journal of Food Protection, 65*(4), 709-725.

Tompkin, R.B., Swift-Eckrich, A., Downer's Grove, I.L. and Bernard, D.T. (2015). Industry guidelines to prevent contamination from *Listeria monocytogenes*. *National Pork Producers Council and the American Meat Association Fact-sheet http://www. extension. org/pages/27405/industry-guidelines-to-prevent-contamination-from-listeria-monocytogenes#. VTh1ndhFD1E.* Accessed, August 2016.

Touré, R., Kheadr, E., Lacroix, C., Moroni, O., Fliss, I. (2003). Production of antibacterial substances by bifidobacterial isolates from infant stool active against *Listeria monocytogenes*. *Journal of Applied Microbiology, 95,* 1058–1069.

Tursi, A., Brandimarte, G., Giorgetti, G.M., Modeo, M.E. (2004). Effect of *Lactobacillus casei* supplementation on the effectiveness and tolerability of a new second-line 10-day quadruple therapy after failure of a first attempt to cure *Helicobacter pylori* infection. *Medical Science Monitor, 10*(12), 662- 666.

Uemura, N., Okamoto, S., Yamamoto, S., Matsumura, N., Yamaguchi, S., Yamakido, M., Taniyama, K., Sasaki, N., Schlemper, R.J. (2001). *Helicobacter pylori* Infection and the Development of Gastric Cancer. *The New England Journal of Medicine, 345*(11), 784-789.

Upadrasta, A., Stanton, C., Hill, C., Fitzgerald, G.F., Ross, R.P. (2011). Improving the stress tolerance of probiotic cultures: recent trends and future directions. In: Tsakalidou E, Papadimitrou K, editors. Stress responses of lactic acid bacteria. *Springer, 395-438*.

Uyttendaele, M., De Troy, P., Debevere, J. (1999). Incidence of *Listeria monocytogenes* in different types of meat products on the Belgian retail market. *International Journal of Food Microbiology, 53,* 75–80

Valdez, J., Rachid, M., Gobbato, N., Perdigon, G. (2001). Lactic acid bacteria induce apoptosis inhibition in *Salmonella typhimurium* infected macrophages. *Food and Agricultural Immunology, 13*(3), 189-197.

Van Coillie, E., Werbrouck, H., Heyndrickx, M., Herman, L., Rijpens, N. (2004). Prevalence and typing of *Listeria monocytogenes* in ready-to-eat food products on the Belgian market. *Journal of Food Protection, 67,* 2480–2487.

Van Langendonck, N., Bottreau, E., Bailly, S., Tabouret, M., Marly, J., Pardon, P., Velge, P. (1998). Tissue culture assays using Caco-2 cell line differentiate virulent from non-virulent *Listeria monocytogenes* strains. *Journal of Applied Microbiology, 85,* 337–346.

Vandenbroucke, K., de Haard, H., Beirmaert, E., Dreier, T., Lauwerys, M., Huyck, L., Van Huysse, J., Demetter, P., Steidler, L., Remaut, E., Cuvelier, C., Rottiers, P. (2009). Orally

administered *L. lactis* secreting an anti-TNF Nanobody demonstrate efficacy in chronic colitis. *Mucosal Immunology, 3*(1), 49-56.

Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., Kreft, J. (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clinical Microbiology Reviews, 14*(3), 584-640.

Viaşu-Bolocan, L.V., Popescu, F., Bica, C. (2013). Probiotics and their Immunomodulatory Potential. *Current Health Sciences Journal, 39*(4), 204-210.

Vilà, B., Esteve-Garcia, E., Brufau, J. (2010). Probiotic micro-organisms: 100 years of innovation and efficacy; modes of action. *World's Poultry Science Journal, 66*(3), 369-380.

Volzing, K. N., Borrero, J. J., Kaznessis, Y., Sadowsky, M. (2013). Antimicrobial peptides targeting gram-negative pathogens, produced and delivered by lactic acid bacteria. *ACS Synthetic Biology*, *2*(11), 643-650.

Wampler, J.L., Kim, K.P., Jaradat, Z., Bhunia, A.K. (2004). Heat shock protein 60 acts as a receptor for the *Listeria* adhesion protein in Caco-2 Cells. *Infection and Immunity*, 72(2) 931-936.

Weinstein, D.L., O'Neill, B.L., Hone, D.M., MetCalf, E.S. (1998). Differential early interactions between *Salmonella enterica* serovar Typhi and two other pathogenic *Salmonella* serovars with intestinal epithelial cells. *Infection and Immunity, 66*(5), 2310.

Wen, L.J., Hou, X.L., Wang, G.H., Yu, L.Y., Wei, X.M., Liu, J.K., Wei, C.H. (2012). Immunization with recombinant *Lactobacillus casei* strains producing K99, K88 fimbrial protein protects mice against enterotoxigenic *Escherichia coli*. *Vaccine, 30*(22), 3339-3349.

Werbrouck, H., Grijspeerdt, K., Botteldoorn, N., van Pamel, E., Rijpens, N., van Damme, J., Uyttendaele, M., Herman, L., van Coillie, E. (2006). Differential InlA and InlB expression and interaction with human intestinal and liver cells by *Listeria monocytogenes* strains of different origins. *Applied and Environmental Microbiology, 72*, 3862–3871.

Wohlgemuth, S., Loh, G., Blaut, M. (2010). Recent developments and perspectives in the investigation of probiotic effects. *International Journal of Medical Microbiology, 300*(1), 3-10.

Wood, S., Maroushek, N., Czuprynski, C. J. (1993). Multiplication of *Listeria monocytogenes* in a murine hepatocyte cell line. *Infection and Immunity, 61*, 3068–3072.

World Health Organization. Life in the 21st century: A vision for all. 1998. http://www.who.int/whr/1998/en/whr98_en.pdf?ua=1 . Accessed 11 Apr 2016.

Xayarath, B., Marquis, H., Port, G., Freitag, N. (2009). *Listeria monocytogenes* CtaP is a multifunctional cysteine transport-associated protein required for bacterial pathogenesis. *Molecular Microbiology, 74*(4), 956-973.

Zhang, Y., Zhang, L., Du, M., Yi, H., Guo, C., Tuo, Y., Han, X., Li, J., Zhang, L., Yang, L. (2011). Antimicrobial activity against *Shigella sonnei* and probiotic properties of wild lactobacilli from fermented food. *Microbiological Research, 167*(1), 27-31.

Zhang, Y.C., Zhang, L.W., Ma, W., Yi, H.X., Yang, X., Du, M., Shan, Y.J., Han, X., Zhang, L.L. (2012). Screening of probiotic lactobacilli for inhibition of *Shigella sonnei* and the macromolecules involved in inhibition. *Anaerobe, 18*(5), 498-503.

Zhu, M., Du, M., Cordray, J., Ahn, D.U. (2005). Control of *Listeria monocytogenes* Contamination in Ready-to-Eat Meat Products. *Comprehensive Reviews in Food Science and Food Safety, 4*, 34- 42

Zhu, M.J., Mendonca, A., Ismail, H.A., Du, M., Lee, E.J., Ahn, D.U. (2005). Impact of antimicrobial ingredients and irradiation on the survival of *Listeria monocytogenes* and quality of ready-to-eat turkey ham. *Poultry Science, 84*, 613- 620.

Zou, Y., Jung, L., Lee, S., Kim, S., Cho, Y., Ahn, J. (2013). Enhanced antimicrobial activity of nisin in combination with allyl isothiocyanate against *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* Typhimurium and *Shigella boydii*. *International Journal of Food Science & Technology, 48*(2), 324-333.

Chapter 2

Construction of recombinant *Lactobacillus casei* strain expressing the invasion proteins Internalins A and B of *Listeria monocytogenes*

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2.1.ABSTRACT

Background: Probiotics when in present adequate numbers offer beneficial effects to the host and are able to inhibit pathogens. The use different mechanisms including competing for food and space with foodborne pathogens to alleviate infections. There has been an increase in foodborne infections, therefore, increasing the need for an alternative strategy. In order to enhance the inhibition of pathogens, probiotic engineering offers an alternative strategy. Virulence genes from foodborne pathogens are cloned and expressed into probiotics in an effort to offer them direct competition for receptor sites and ultimately inhibit the specific pathogen. **Aim:** In the current study the invasion proteins internalin A (InlA) and B (InlB) of *Listeria monocytogenes* were cloned and expressed into *Lactobacillus casei* using the expression vector pLP401-T. **Materials and Methods:** *L. monocytogenes* genomic DNA was extracted and then InlAB was amplified using Phusion High Fidelity polymerase. The expression vector and the amplified genes were purified and ligated. The resultant product was electroporated into *Escherichia coli* DH5α and subsequently into *L. casei*. Polymerase chain reaction was used to confirm the amplification of the genes in *L. casei*. Protein expression was confirmed by SDS-PAGE and Western blot. In preparation of comparison of the wild- type and recombinant *L. casei* strains, growth curves using OD 600 were constructed. **Results:** Agarose gel (1%) confirmed the amplification of the 4381 bp InlAB genes and the expression vector (9.8 kb). Subsequent to electroporation, transformants on the selective media showed amplification of InlA, InlB and InlAB in both *E. coli* and *L. casei*. SDS- PAGE showed the separation of the different proteins. Western blot confirmed expression of InlA and InlB by recombinant *L. casei* (Lbc^{InlAB}) and no expression in the wild- type *L. casei* (Lbc^{WT}) and the vector expression *L. casei* (Lbc^{V}). The growth curves showed that there were no significant differences in the growth profiles of all *L. casei* strains (Lbc^{WT}, Lbc^{V} and Lbc^{InlAB})**. Conclusion:** These results showed that InlAB was successfully cloned and expressed into *L. casei*.

2.2. INTRODUCTION

For most pathogenic infections, antibiotics have been the first line of defence, however, their negative effects in the gastrointestinal (GI) infections keep increasing and thus posing major clinical problems (Rolfe, 2000). The rise in these clinical problems has led to a need for an alternative solution for these infections. There has been a growing interest in studying probiotics as an alternative to antibiotics. In order to be deemed as probiotics, microorganisms have to meet criteria such as surviving in a low pH and high bile environment, adhering to intestinal epithelial cells and stabilizing intestinal microflora (Sleator and Hill, 2007) amongst others. Probiotics have been reported to multiply fast and colonize the gastrointestinal tract either permanently or temporarily (Culligan *et al*., 2009). Additionally, they are able to alleviate and prevent foodborne infections mechanisms such as competitive exclusion (Culligan *et al*., 2009).

Poor hygiene practices during food manufacturing and process can lead to the introduction of different foodborne pathogens in food. Amongst others, *Listeria monocytogenes* is one of the most common food pathogens implicated in infections (Manning *et al*., 2001, de Wit *et al*., 2001). It is a Gram-positive, facultative intracellular food-borne pathogen that has evolved to survive in diverse environments (Vazquez-Boland et al., 2001; Czuprynski, 2005; Gray and Bhunia, 2005). It is able to cross three significant barriers, namely, the intestinal epithelial cell barrier, the blood-brain endothelial cell barrier, and the feto-placental endothelial cell barrier (Werbrouck *et al*., 2006) to cause listeriosis. In its disease progression, it employs different virulence factors to attach, invade and move from one cell to another. Virulence factors responsible for its adhesion include but are not limited to *Listeria* adhesion protein (LAP), autolysin amidase (AmiA), and the Internalin (Inl) family of proteins (InlA, InlB, InlJ, and InlF) (Camejo *et al.*, 2011; Radoshevich and Cossart, 2018). For its invasion, it uses bacterial surface proteins internalin A (InlA) and internalin B (InlB) which bind to the host cell E-cadherin (Mengaud *et al.*, 1996) and/ or the Met receptor (also known as hepatocyte growth factor receptor) (Shen *et al*., 2000), respectively. E-cadherin is only expressed by a limited number of cell types, mostly cells of epithelial origin; therefore, InlA is necessary to promote *Listeria* entry into human epithelial cells (Bonazzi *et al.*, 2009). However, hepatocyte growth factor receptor, Met, is ubiquitous, allowing InlB to mediate internalization in a wider range of cell types, including but not limited to hepatocytes, epithelial cells, fibroblasts and endothelial cells (Dramsi *et al.*, 1995; Gaillard *et al.*, 1996; Parida *et al.*,

1998; Lingnau *et al.*, 1995). These virulence factors allow for the spread of the pathogen systemically causing listeriosis.

There is currently no treatment option for this food-borne pathogen except those stated by the Center for Disease Control (CDC) that summarizes the importance of hygiene when it comes to handling and preparation of food. Probiotics have been used to restore the balance of the gut microbial ecosystem and control pathogenic infections. They are defined as "*live microorganisms that when administered in adequate amounts confer a health benefit on the host*" (FAO/WHO, 2002). Their administration assists in the prevention and control of foodborne illnesses, through a number of mechanisms including but not limited to, competitive exclusion of pathogens in the gastrointestinal tract (GIT), modulation of the host immune system and strengthening of the intestinal barrier (Behnsen *et al*., 2013; Ceapa *et al.*, 2013). There have been different studies using different probiotics to combat *L. monocytogenes.* Touré *et al*. (2003) isolated six bifidobacterial strains from faeces of breast-fed infants and demonstrated that these isolates produced antibacterial substances with a potential antilisterial activity. Corr *et al*. (2007) reported that the pretreatment of intestinal epithelial cells with probiotic bacteria prior to infection with *L. monocytogenes* EGDe resulted in a significant decrease in listerial invasion (60–90%). In yet another study testing for the antagonistic effect of *Lactobacillus* strains against *Escherichia coli* and *L. monocytogenes*, it was reported that *L. plantarum* WS4174 exhibited a stronger inhibitory effect against *L. monocytogenes* LMO26, possibly due to its higher sensitivity to low pH and the accumulation of lactic acid (Aguilar *et al*., 2011).

Although probiotics have proven successful in control of enteric pathogens, they do have limitations. They are generic in nature and often fail to inhibit the attachment of certain pathogens at specific sites of infection and induce low levels of an immune response (McCarthy *et al*., 2003). A thorough understanding of the limitations of conventional probiotics, the behaviour of the pathogens and the mechanisms by which they cause disease (Amara and Shibi, 2015) provides possibilities to design new probiotic strains with desired characteristics and functionalities. Through genetic modification, novel bioengineered probiotic strains can be produced. Functioning of conventional probiotics in these novel strains can be strengthened to influence critical steps in the pathogenesis of disease. The novel bioengineered probiotic strains can also be used to deliver drugs or vaccines, target a specific pathogen or toxin, mimic surface receptors and enhance an

immune response within the host (Amalaradjou and Bhunia, 2013). Previously in the laboratory, a recombinant *L. paracasei* expressing the LAP of *L. monocytogenes* was designed as a control strategy in the *L. monocytogenes* infection *in vitro* (Koo *et al*., 2012). When comparing the inhibition ability of the wild type *L. paracasei* with the recombinant probiotic, the recombinant counterpart showed enhanced inhibition of *L. monocytogenes*. The same approach was followed to clone the LAP into the *L. casei* in our laboratory. In the current study the effects of recombinant probiotics in the inhibition of *L. monocytogenes*, by cloning and expressing the invasion genes InlAB of *L. monocytogenes* into probiotic *L. casei* was further elucidated.

2.3. MATERIALS AND METHODS

2.3.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 2.1. *Listeria monocytogenes* F4244 (serovar 4b) was cultured in tryptone soy broth supplement with 0.6% yeast extract (TSB-YE) or brain heart infused (BHI) at 37ºC for 18 h. The vector pLP401-T (Pouwels *et al.*, 2001) was used for the expression of InlAB in *Lactobacillus casei* ATCC344. *Escherichia coli* DH5α with vector was grown in Luria-Bertani (LB) broth supplemented with 50 µg/mL ampicillin. Wild-type *L. casei* (Lbc^{WT}) was grown in de Man Rogosa Sharpe (MRS) broth while the Lbc^{WT} (carrying pLP401T) vector control (Lbc^{V}) and recombinant Lbc^{InlAB} and Lbc^{LAP} (unpublished) strains were grown in MRS broth with 2 µg/ml erythromycin anaerobically at 37ºC for 16 h in an anaerobic jar. To induce expression of InlAB and LAP in recombinant *L. casei*, the bacteria were grown in modified MRS (1% w/v protease peptone, 0.5% w/v yeast extract, 0.2% w/v meat extract, and 0.1% v/v Tween 80, 37 mM $C_2H_3NaO_2$, 0.8 mM $MgSO_4$, 0.24 mM $MnSO_4$, 8.8 mM $C_6H_{14}N_2O_7$ in 0.1 M potassium phosphate buffer, pH 7.0) supplemented with mannitol (1% w/v) (Koo *et al.*, 2012).

Table 2.1: Bacterial strains and plasmids

Bacterial/ plasmids	Strains	Description	Source
Listeria monocytogenes	F4244	Wild type, serotype 4b	Our laboratory
Lactobacillus casei	ATCC344	Wild type	ATCC
Escherichia coli	DH5α	Wild type	Our laboratory
L. casei	LAP	*L. casei* expressing *Listeria* adhesion protein (Em^R 2µg/ ml)	Our laboratory
L. casei	$InlAB^+$	*L. casei* expressing InlAB of *L. monocytogenes* (Em^R 2µg/ ml)	This study
L. casei	$InlAB^-$	*L. casei* carrying control plasmid with no insert (Em^R 2µg/ ml)	This study
Plasmids			
pLP401T		Expression vector for *Lactobacillus*, (Am^R 50 µg /ml and Em^R 2µg/ ml)	Our laboratory

pLP401-InlAB		pLP401 carrying InlAB of *L. monocytogenes*	This study

* ATCC- American Type Culture Collection

2.3.2. Construction of *Lactobacillus casei* with Internalin AB (InlAB)

2.3.2.1. Genomic DNA extraction

L. monocytogenes F4244 was cultured in tryptone soy broth supplement with 0.6% yeast extract (TSB-YE) at 37^{o}C for 18 h. Genomic DNA was extracted using the boiling method. The DNA was harvested from overnight cultures by centrifugation at 10, 000 rpm for 10 minutes. The supernatant was discarded. The pellet was then resuspended in 100 µl of sterile water and heated at 95-100°C for 10 minutes. The mixture was then chilled on ice briefly afterwards and spun at 10,000 rpm for 10 minutes. The supernatant was saved and used for the subsequent experiments. The concentration of the DNA was measured using a Nanodrop (NanoDrop™ 2000, Fisher Scientific).

2.3.2.2. Polymerase chain reaction (PCR)

Primers were specifically designed for the genes with the restriction site inserted for specific restriction enzymes required for cloning (underlined), Table 2.2. They were obtained from Integrated DNA Technologies (Coralville, IA, USA) and annealing temperatures were calculated using the website http://www.thermoscientificbio.com/webtools/tmc/.

Table 2.2: Primers used for the amplification of the genes

Gene	Primer	Primer sequence (5'- 3')	Amplicon size (bp)
InlA	InlAStr	GTGAGAAGAAAACGATATGTATG	2394
	InlASto	CTATTTACTAGCACGTGCTTTTT	
InlB	InlBStr	GTGAAAGAAAAGCACAACCC	1893
	InlBSto	TCATTTCTGTGCCCTTAAATTAGC	

InlAB	InlABExp-F (NotI)	TAGCGGCCGCAACTATTGAAAAAGGAGTGTATATAGTG	4371
	InlABExp-R (XhoI)	GTCTCGAGTTTCTGTGCCCTTAAATTAGC	

Amplification of the DNA by the two different polymerases were performed as per the manufacturer's protocol. Amplification of DNA using Taq polymerase was performed with the reaction mix shown in Table 2.3. The thermocycling conditions were as follows: 3 min at 95°C for one cycle; then 15 s 95°C, 30 s annealing temperature (55°C for InlA and 49°C for InlB) and 1 min per kb at 72°C for 35 cycles; a final step of 10 min at 72°C for one cycle.

Table 2.3: Reaction composition for Taq polymerase.

Component	**50 µl Reaction**
DNA template	4 µl
5 x GoTaq Reaction Buffer	10 µl
$MgCl_2$ (50Mm)	4 µl
dNTP (10mM)	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Nuclease Free Water	28.75 µl
DNA Polymerase	0.25 µl

Amplification of DNA using Phusion polymerase (NEB) were performed with the reaction mix shown in Table 2.4. The PCR thermocycling conditions were: 2 min at 98°C one cycle, 20 s at 98°C, 20 s at 62°C (Annealing temperature) 2.5 min at 72°C for 35 cycles and a final step of 7 min at 72°C

Table 2.4: Reaction composition for Phusion polymerase

Component	50 µl Reaction
5X Phusion HF Buffer	10 µl
10 mM dNTPs	1 µl
10µM F- primer	2.5 µl
10µM R- primer	2.5 µl
Phusion DNA Polymerase	0.5 µl
$MgCl_2$ (50Mm)	1.5 µl
DMSO	1.5 µl
Nuclease Free Water	26.5 µl
Template DNA	4 µl

2.3.2.3. Gel electrophoresis of nucleic acids

Agarose gels made at a concentration of 1% (Fisher Scientific) were used for all the experiments with 1 mg/l ethidium bromide dissolved. For all the samples, 10 µl of DNA was loaded to the wells in agarose gels and electrophoresed in 1 x Tris-Acetate-EDTA (TAE, Fisher). GoTaq has a loading dye, in the case of Phusion polymerase 3 µl spots of loading buffer (Purple Dye, NEB) were aliquoted onto parafilm and mixed with samples prior to loading into the gel. Depending on the expected product size, either 100 bp or 1 kb molecular DNA ladder (Axygen Biosciences) were used in electrophoresis gel.

2.3.2.4. DNA purification

PCR products were purified with PureLink® PCR Purification Kit (Invitrogen) for the removal of the smaller unwanted DNA fragments, buffers, dNTPs and primer dimers. Briefly, 4 volumes of binding buffer (B2) with isopropanol were added to 1 volume of the PCR product (50–100 µl). The suspension was mixed well and added to the sample to the spin column. The spin column was centrifuged at room temperature at 10,000 × g for 1 min. The flow through was discarded and the spin column was placed into the collection tube. To the spin column, 650 µl of wash buffer was

added and then centrifuged at room temperature at 10,000 × g for 1 min. The flow through was discarded from the collection tube and the column was replaced into the tube. The column was then centrifuged at maximum speed for 2–3 min at room temperature to remove any residual wash buffer. The collection tube was then discarded, and then the spin column was placed in a clean 1.7 ml elution tube supplied with the kit. 50 µl nuclease free water was added to the centre of the column and then incubated at room temperature for 1 min. The column was then centrifuged at maximum speed for 2 min. The purified PCR product in the elution tube was stored to be used in subsequent experiments.

2.3.2.5. Plasmid extraction (*E. coli*)

To extract the vector pLP401-T that was initially maintained in *E. coli*, Qiagen Plasmid Miniprep kit was used for the extraction. All centrifugation was done at 10,000 x g. Briefly, *E. coli* DH5α with vector was grown in Luria-Bertani (LB) broth supplemented with 50 µg/mL ampicillin at 37^{o}C for 16 h. Then an overnight culture was centrifuged in a 1.5 ml tube to obtain a pellet (avoid oversized pellets). The supernatant was removed, and the pellet was then resuspend in 250 µl of the resuspension buffer. To the mixture, 250 µl alkaline lysis buffer was added, and the tube was gently inverted 4-5 times to mix (do not allow to incubate longer than 5 minutes to avoid risk of gDNA contamination). After incubation, 350 µl Neutralization buffer was added, and the tube was then inverted 4-6 times to mix well and centrifuge for 10 min. The supernatant was removed and loaded onto the column and the centrifuge the column for 1 min. The flow through was discarded and the column was washed by adding 750 µl wash buffer and centrifuged for 1 min. The flow-through was discarded, and the column was centrifuged at full speed for an additional 1 min to remove residual wash buffer. The column was transferred to a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl nuclease free water was added to the centre of the column and incubated the column at room temperature for 1 min, and then centrifuged for 1 min. The flow- through (plasmid) was stored at -20°C for subsequent experiments.

2.3.2.6. Restriction enzyme digestion and Ligation

2.3.2.6.1. Restriction enzyme digestion

The plasmid (pLP401-T) and the purified DNA were digested using the restriction enzymes NotI and XhoI (NEB). The enzymes were chosen for the restriction digest as they were incorporated in the primers. The restriction digestion reaction was done as the manufacture's protocol. Briefly, for a 50 µl reaction: 42 µl ddH2O, 5 µl 10X enzyme buffer, 1 µl DNA/ plasmid vector, 1 µl restriction enzyme A (5 units) and 1 µl restriction enzyme B (5 units) were added together. The suspension was the mixed up by pipetting up and down and incubated overnight at 37°C overnight (16 h). After the digestion, the enzymes were inactivated at 65 °C for 20 min for further use in ligation.

2.3.2.6.2. Ligation

For ligation, two experiment reactions were set up, one with the digested plasmid and DNA and the other one with the digested plasmid with no DNA. T4 DNA Ligase (NEB) was used and reaction was as per the manufacture's protocol. Briefly 2 µl T4 DNA ligase buffer, 1.5 µl digested vector, 3.5 µl digested PCR product, 12 µl nuclease free water and 1 µl T4 DNA ligase were added together. The mixture was carefully mixed by pipetting up and down and centrifuged briefly to have all reagent at bottom of the tube (to ensure that the reaction happens). The tubes were incubated overnight at 16 °C overnight (16 h). After the ligation, the reaction was stopped by heat inactivation at 65 °C for 10 min. The product of ligation was the designated pLP401T-InlABLm, which was used for electroporation.

2.3.2.7. Transformation of *E. coli*

2.3.2.7.1. Preparation of *E. coli* competent cells

Escherichia coli DH5α cells were prepared to make them electrocompetent for transformation purposes. Pre- warmed 250 ml LB broth was incubated with 2% overnight culture of *E. coli* DH5α and shaken (250 rpm) at 37°C until the optical density (OD) at 600 nm reached 0.5-0.6. The cultures were then chilled on ice for 15 min. The cells were harvested by centrifugation at 7,500 x g at 4°C for 10 min. The supernatant was decanted and followed with pipet to remove residual media. The cells were the washed four times in 250 ml, 100 ml, 50 ml and 25 ml of ice-cold distilled water, respectively. The cells were centrifuged at 7,500 xg at 4°C for 10 min between

every washing step. The cells were then resuspended in 1 ml of ice- cold 10% glycerol in water, aliquoted 60 µl in pre-chilled Eppendorf tubes and stored at -80°C.

2.3.2.7.2. Transformation by electroporation

Competent cells were thawed on ice and 50 µl of the cells was mixed with 1.5 µl of purified plasmid- DNA in an ice-cold cuvette with a 2 cm electrode gap. The electric pulse was then delivered by the Gene Pulser Xcell TM electroporation system (Bio-Rad, Richmond, CA) using the following parameter settings: 2.5 kV, 200Ω and 25 µF. Immediately after electroporation, competent cells were recovered in pre-warmed (37°C) 1 ml of super optimal broth with catabolite repression (SOC media). There were controls set up for this experiment: DNA control with no plasmid; plasmid control with no DNA, and electrocompetent cell control with no DNA or plasmid. The cells were then incubated at 37°C for 1 h. Transformants were selected using LB agar containing 50 µg/ml of ampicillin and the plates were incubated at 37°C overnight.

2.3.2.8. Transformation of *L. casei*

2.3.2.8.1. Preparation of *L. casei* competent cells

Overnight culture of *L. casei* was used, 2% of the overnight culture was inoculated into fresh MRS broth containing 0.5% sucrose and 0.5% glycine at 37°C until they reach a desired OD of between 0.5 and 0.8 at 600nm (OD_{600nm}~ 0.5 - 0.8). The cells were then harvested by centrifugation at 3,900 x g for 5 min at 4°C. The cells were then washed twice with ice- cold washing buffer (0.5 M sucrose, 10% glycerol) at 4°C and then collected. The cells were resuspended in 1 ml of the same washing buffer and stored at – 80°C.

2.3.2.8.2. Transformation by electroporation

Competent cells were thawed on ice and 50 µl was mixed with 1.5 µl of purified plasmid- DNA in an ice-cold cuvette with a 2-cm electrode gap. The electric pulse was then delivered by the Gene Pulser Xcell TM electroporation system (Bio-Rad, Richmond, CA) using the following parameter settings: 1.5 kV, 200Ω and 25 µF. There were controls set up for this experiment that were the same as the ones for *E. coli*: DNA control with no plasmid, plasmid control with no DNA, and

electrocompetent cell control with no DNA or plasmid. Immediately after electroporation, competent cells were recovered in pre-warmed (37°C) 1 ml of MRS containing 0.5 M sucrose, 20 mM $MgCl_2$, 2 mM $CaCl_2$. The cells were then incubated at 37°C for 1 h. Transformants were selected using MRS agar containing 2 µg/ml of erythromycin and the plates were incubated at 37°C 48 h anaerobically in anaerobic jars with Anaerocult A GasPacks and Anaerotest strips.

2.3.3. Plasmid extraction from *L. casei*

From the transformant colonies found on MRS-E plates, the colonies were then re- grown three times in MRS-E broth. Colonies that were still showing growth were then used for plasmid extraction to confirm presence of the genes. The plasmid extraction was done as per Mojtaba and Mehdi (2016) with minor modification. Briefly, cultures of the selected *L. casei* transformants were grown overnight at 37°C (anaerobically in MRS supplemented with 2µg/ ml Erythromycin). Post inoculation the cultures were centrifuged for 3 min at 12,000 rpm. These bacterial pellets were used for total DNA (genomic and plasmid) extraction. The pellet was washed thrice with 2 ml of NaCl-EDTA (30 mM NaCl, 2 mM EDTA, pH = 8.0) and resuspended in 100 µl of this buffer and then 100 µl of freshly prepared lysozyme solution (10 mg/ml in NaCl- EDTA) was added and mixed. To remove RNA, 1 µl of RNase A solution (20 mg/ml) was added to the mixture. This mixture was then incubated at 37°C for one hour with periodic shaking. The volume of the mixture was then made up to 500 µl with additional NaCl- EDTA, 50 µl of a 10% SDS solution and 10 µl of proteinase K solution (20 mg/ml). The contents were then thoroughly mixed and incubated at 55°C for one hour. After incubation, 200 µl protein precipitation solution (6 ml of 5 M potassium acetate, 1.15 ml of glacial acetic acid and 2.85 ml of distilled water) was added and vortexed at medium speed for 20 seconds and kept on ice for five minutes. The lysate was centrifuged at 12,000 rpm for three minutes and the supernatant was transferred to a clean 1.5 ml tube. DNA in the supernatant was precipitated with 600 µl of cold isopropanol and pelleted by centrifugation at 12,000 rpm at room temperature for 3 min. The supernatant was discarded, and the DNA pellet was washed once with freshly prepared 70% ethanol and air-dried. The final pellet obtained was then dissolved in 100 µl TE buffer (10 mM Tris HCl, 1 mM EDTA, pH = 8.0) and kept at 65°C for 15 min and the subsequently stored at −20°C till further analysis. To check for the presence of the genes InlA and InlB, Taq polymerase PCR was used as per 2.2.

2.3.4. Analysis of internalin expression by *L. casei*

2.3.4.1. Analysis of expression of the genes InlAB in the supernatant, the cell wall and in the intracellular fraction

Overnight cultures were used, 200 ml of the cultures was centrifuged (7000 x g for 10 min at 4° C) and supernatant (SN) removed for protein isolation. The cell pellet was retained for preparation of cell wall-associated protein. To isolate secreted proteins, the SN was filtered (0.22 µm filter), precipitated on ice with 10% trichloroacetic acid (v/v) for 40 min, and then centrifuged (14,000 x g for 10 min at 4° C). The resulting pellet was resuspended and washed twice with 1 ml ice-cold acetone, incubated on ice for 10 min and centrifuged (14, 000 x g for 5 min at 4°C). The supernatant was decanted, and the pellet was left to dry in the chemical hood (evaporate the residual acetone). The pellet was then resuspended in 250 µl alkaline rehydration buffer (100 mM Tris-base, 3% SDS, 3 mM DTT, pH 11), boiled for 10 min, and chilled on ice to cool down to room temperature then stored at -20°C for subsequent experiments. To isolate cell wall-associated protein, the bacterial pellet from above was washed twice in PBS. The resulting pellet was then resuspended in 500 µl protein extraction buffer (0.5% SDS, 10 mM Tris at pH 6.9), mixed by pipetting up and down to resuspend the pellet completely and then transferred the suspension into an Eppendorf tube. The resulting suspension was incubated at 37°C for 30 min with periodic shaking. The samples were then centrifuged (14,000 x g, 10 min, at 4 °C) and the resultant supernatant (containing cell wall-associated proteins) was retained and stored at -20 °C for use in subsequent experiments. The pellet from the cell wall fraction was resuspended in 200 µl of lysis solution B (100 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$) to lyse the cell. The suspension was sonicated on ice for 3 cycles of 20 sec each using a Sonifier 150D. The sample was centrifuged at 14, 000 x g for 5 min at 4 °C and the supernatant was collected and stored at -20 °C for use in subsequent experiments.

Protein concentrations were determined using the BCA (Bicinchoninic Acid) Protein Assay (Pierce, Rockford, IL), using bovine serum albumin (BSA) as a standard. Equal amounts of proteins (10µg of each fraction) was separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE) (7.5% - 12% acrylamide gel). Coomassie dye was used to visualize the proteins and imaged. For protein expression, the gel was transferred to an Immobilom-P membrane (Millipore, Billerica, MA) and immunoprobed with primary antibodies anti-InlA antibody mAb-2D12 (1.0 mg/mL) and anti- InlB pAb404 (1: 1000 in non- fat dry milk). The

membrane was incubated overnight on slow speed on a shaker at 4°C. Subsequent to that, horseradish peroxidase-coupled anti-mouse (InlA- Mab) and anti- rabbit (InlB- Pab) secondary antibodies (0.2 mg/mL; Jackson Immuno Research, West Grove, PA) (1: 2000 in no- fat dry milk) were incubated at room temperature on a shaker for 1 h. The membranes were developed with an enhanced chemiluminescence kit (Pierce). Furthermore, expression of InlA and InlB in the recombinant *L. casei* strains was determined by treatment with immunofluorescence staining. Overnight grown cultures were washed twice in PBS and treated with the primary antibodies, anti-InlA pAb 2D12 and anti- InlB pAb404 (diluted 1:500 in PBS). The suspension was then incubated at 37°C with constant shaking for 1 h. The cells were washed by centrifuging at 8,000 rpm for 3 min with PBS-Tween 20 (0.5%) at least 4 times. The pellet was resuspended in alexa- labelled anti- rabbit secondary antibody (diluted 1:500 in PBS) and the resultant mixture was incubated at 37°C for 1 h, away from light. The cells were then washed as above in PBS- Tween. The pellet was the resuspended in PBS and examined under a fluorescence microscope (Leica, model DMLB, Wetzlar, Germany) equipped with SPOT software (version 4.6.4.2, Diagnostic Instruments, Sterling Heights, MI, USA).

2.3.5. Growth curves of the *L. casei* strains

Lactobacillus casei strains: *L. casei* wild type (Lbc^{WT}), *L. casei* with the vector pLP401-T and no InlAB genes (Lbc^{V}) and *L. casei* expressing Internalins A and B (Lbc^{InlAB}) were grown under the conditions described above. The growth curve analysis of these strains was conducted for 24 h by measuring the cell density (OD_{600nm}) using the spectrophotometer (Beckman-DU80). At each time point, the culture was mixed by vortexing and the OD reading was taken, and 1 ml of the culture was used for plating. Lbc^{WT} was grown on deMan Rogosa Sharpe (MRS) agar while the Lbc^{V} and recombinant Lbc^{InlAB} were grown on MRS agar with 2 µg/ml erythromycin anaerobically at 37^{o}C for 48 h. This experiment was done twice in triplicates. Additionally, overnight cultures of the *L. casei* strains were used for phase contrast micrographs (Leica, model DMLB, Wetzlar, Germany) to show the morphology of the *L. casei* strains.

2.4. RESULTS

2.4.1. The amplification of the Internalins A and B

The specific primers that were used in the amplification of the genes were designed to start from the beginning of InlA and stop at the end InlB. Using gradient PCR, were able to optimize the amplification of the InlAB. The expected gene size of 4381 bp (InlA= 2403 bp, non- coding section= 85 bp and InlB= 1893 bp) was successfully amplified using the primers (**Fig. 2.1A**). The resultant PCR products were purified, and the products are showed on **Fig 2.1B.** Gene products from the PCR purification were used for the consequent experiments as they showed no primer dimers or any other products but the expected InlAB gene size.

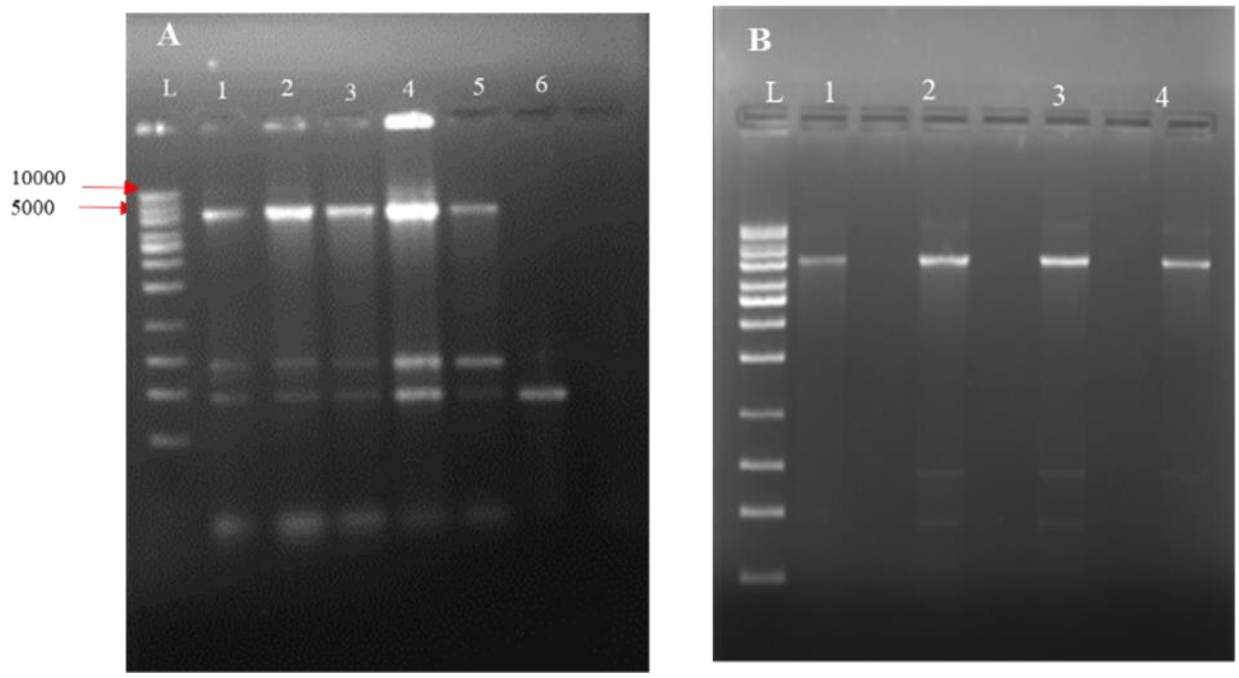


Figure 2.1: Agarose gel showing (**A**) gradient PCR (57- 61°C) amplified gene products for InlAB L- 1kb ladder, (Lane 1-5) and no amplification in the negative (Lane 6). (**B**): PCR Purification of the amplified products (58- 61°C), L- 1kb ladder (Lane 1-4)

2.4.2. Plasmid extraction

The *Lactobacillus* expression plasmid pLP401-T was used in this experiment. This was chosen as it has already been proven to express proteins in lactobacilli before (Koo *et al*., 2012). In our laboratory, the plasmid has been sub-cloned and maintained in *E. coli* DH5 α. Using the Qiagen plasmid extraction kit **Fig. 2.2** shows the successfully extracted plasmid pLP401T (9.8 kb) on lanes 2 and 4 using the 1kb Ladder DNA. For cloning purposes, on the same agarose gel, Lane 1 and 3 shows the purified amplified InlAB.

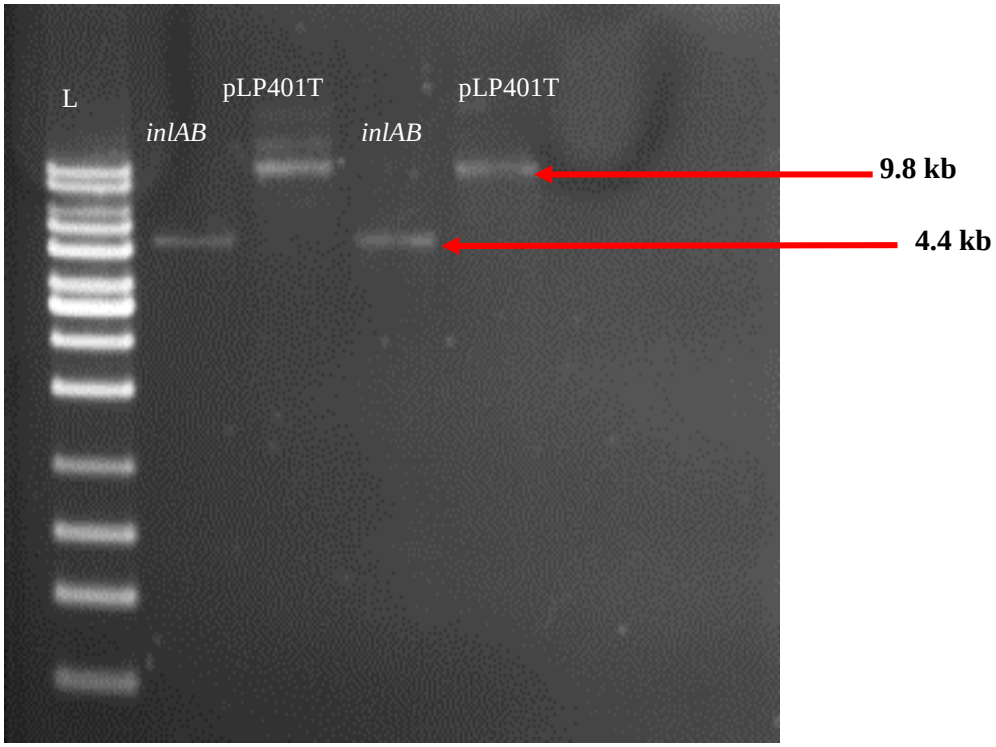


Figure 2.2: Agarose gel showing the difference in sizes for amplified InlAB (Lane 1 and 3) and pLP401T (Lane 2 and 4).

2.4.3. Cloning of InlAB genes into *L. casei*

2.4.3.1. Restriction enzyme digestion and Ligation

To produce the designated pLP401-InlAB, the purified PCR product and the extracted plasmid pLP401T were digested with the restriction enzymes NotI and XhoI. The resultant digested products were then ligated using the T4 DNA Ligase. **Fig. 2.3** shows that when the purified PCR products were digested with the restriction enzymes, it was not different to the undigested counterpart. However, when pLP401T was digested with the same digestion enzymes, there were two resultant bands both with sizes smaller than 9.8kb (the size of undigested pLP401T). The two digested products (PCR product and pLP401-T) were ligated, and the resulting products are shown on **Fig. 2.3.** There are still two products visible on the gel, however the band size of one of them was larger than that of pLP401T. These final products were then stored at -20 °C to be used for the next experiments.

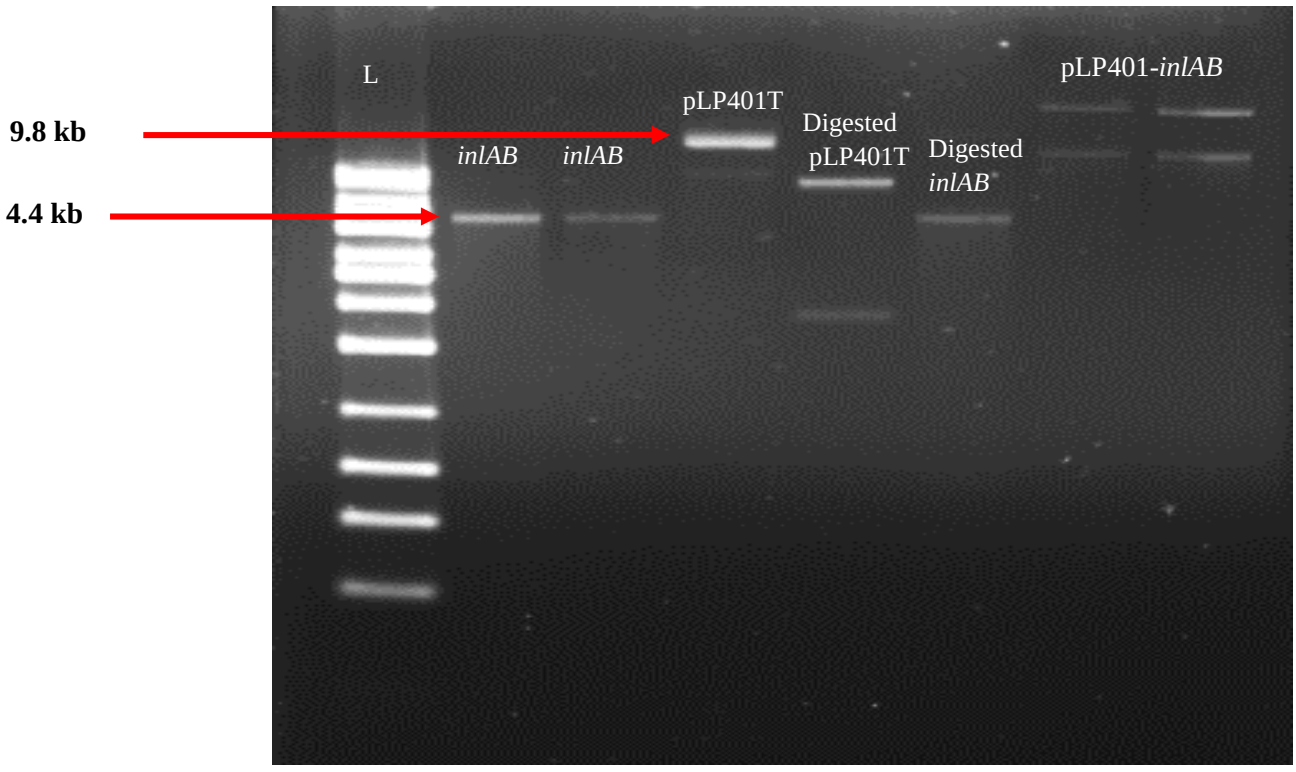


Figure 2.3: Agarose gel showing the amplified *inlAB*, plasmid pLP401T, restriction enzyme digested *inlAB* and pLP401-T and ligation products of the *inlAB* and pLP401-T. Lane L: 1kb Ladder DNA Marker.

2.4.3.1.1. Transformation into *E. coli* by electroporation

Following ligation of the PCR product (InlAB) and pLP401-T, the resultant pLP401-*inlAB* was used for the subsequent experiments. **Fig. 2.4** shows the plasmid map (14.2 kb) of ligated *Lactobacilli* expression vector pLP401T (9.8 kb) with InlAB (4.4 kb) that was used for transformation. The pLP401-InlAB was transformed into electrocompetent *E. coli* DH5α cells. The electroporated product was grown on BHI agar supplemented ampicillin (50 µg/ ml). There were three colonies growing on the BHI plates and those colonies were selected by sub-culturing twice in BHI with ampicillin (50 µg/ ml). Plasmids from the transformed *E. coli* DH5α were extracted and the presence of InlAB was confirmed using PCR. **Fig 2.5** shows the extracted plasmids from the transformants and the amplified InlAB. The InlAB genes were amplified in two of the transformants and in the positive control, *L. monocytogenes* while there was no amplification in the third transformant and Ec^{WT}. Following the confirmation for the presence of InlAB in the two resultant plasmids, the plasmid from $Ec^{InlAB-1}$ was then subsequently transformed into electrocompetent *L. casei*. The electroporated product was grown on MRS agar with erythromycin (2 µg/ ml). The positive colonies were selected by sub-culturing twice in MRS broth with erythromycin (2 µg/ ml).

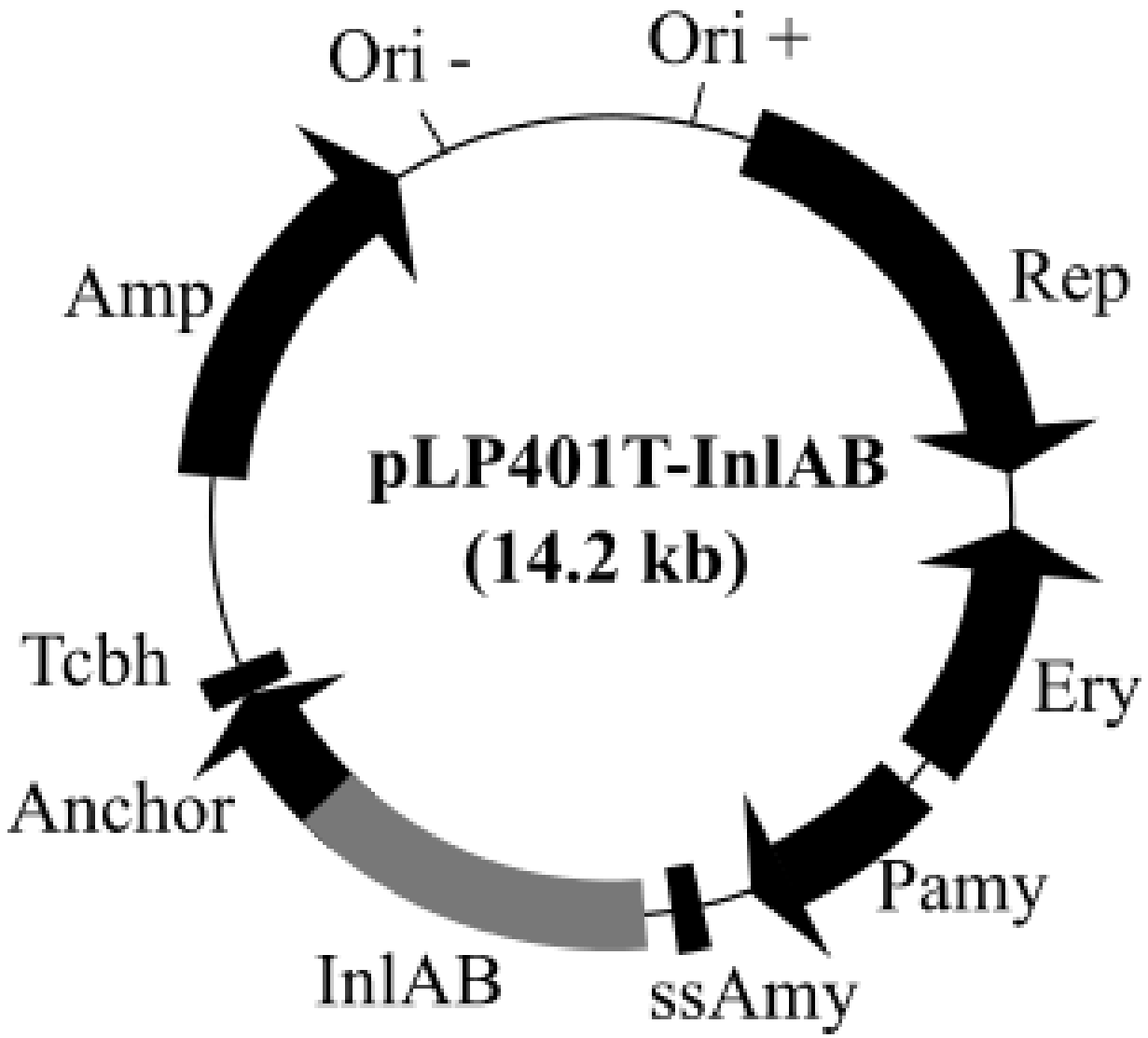


Figure 2.4. Plasmid map (14.2 kb) of InlAB expression vector pLP401T (9.8 kb)-InlAB (4.4 kb) (Pouwels *et al*., 2001). **Ery**, erythromycin resistance gene; **Amp**, ampicillin resistance gene; **Ori+** = origin of replication of *E. coli*, **Ori-** = origin of replication of *Lactobacillus*; **InlAB**, Internalin A and B; **Pamy**, a-amylase promoter gene; **ssAmy**, secretion signal (36 aa) and the N-terminus (26 aa) of a-amylase gene; **Anchor**, anchor peptide (117 aa) gene of *L. casei*; **Tcbh**, transcription terminator of the cbh (conjugated bile acid hydrolase) gene; **Rep**, repA gene.

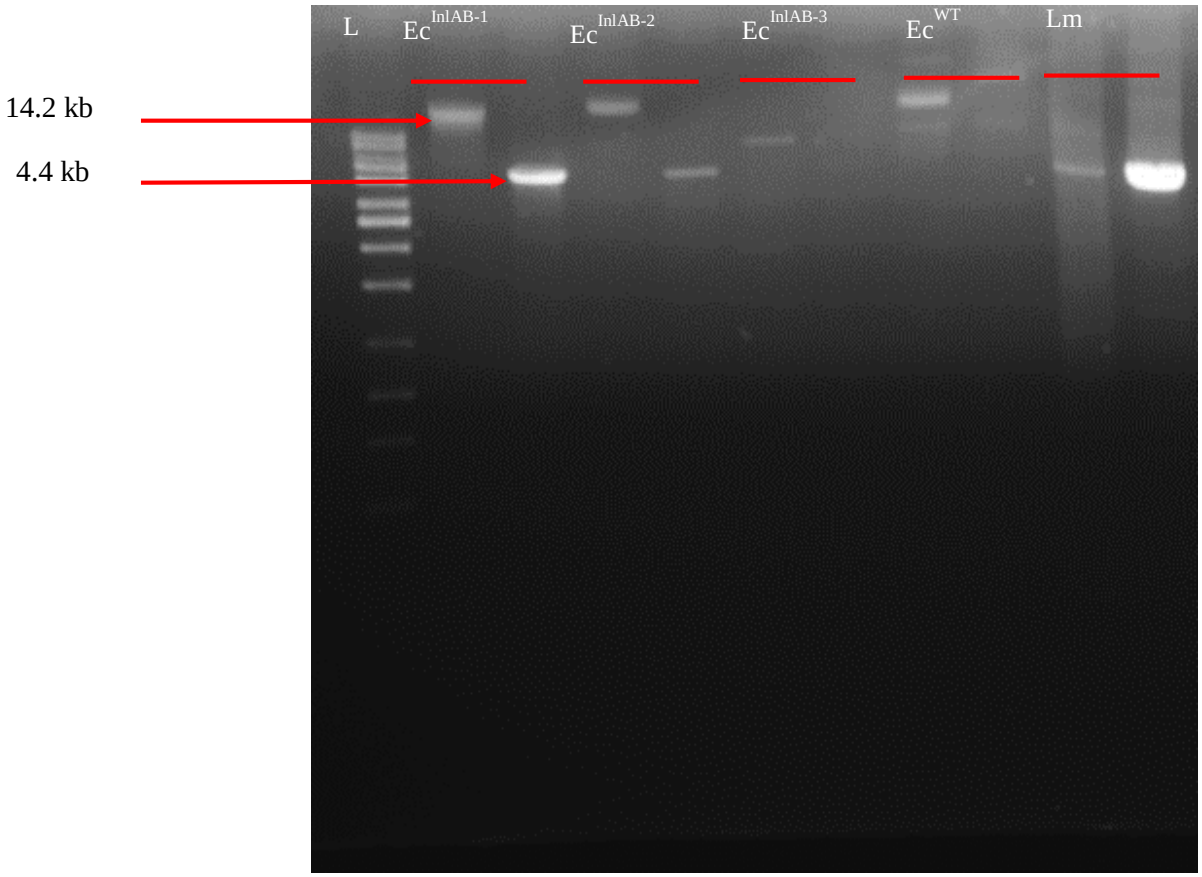


Figure 2.5. Agarose gel showing PCR amplified gene products for *inlAB*, *inlA*, and *inlB* of InlAB-expressing 3 recombinant *E. coli* DH5α strains ($Ec^{InlAB-1}$, $Ec^{InlAB-2}$, $Ec^{InlAB-3}$) and *L. monocytogenes* (Lm) and Ec^{WT}. Lm: *L. monocytogenes* F4244 (Positive control) and Ec^{WT} (Negative control).

2.4.3.1.2. Transformation into *L. casei* by electroporation

Following the confirmation for the presence of InlA and B in the resultant *E. coli* DH5α plasmid, the plasmid (Ec^{InlAB1}) was then subsequently transformed into electrocompetent *L. casei*. There were 25 transformant colonies on the MRS-E plates. The colonies were subcultured three times in MRS broth with erythromycin (2 µg/ ml). **Fig. 2.6** the results from the recombinant *L. casei* transformants. From the plasmid extraction, the 25 colonies all showed the presence of the pLP401-InlAB (**Fig. 2.6A)**. To confirm the presence of the genes in the resultant pLP401-InlAB, PCR for both InlA and InlB was performed. Both InlA (**Fig. 2.6B**) and InlB (**Fig. 2.6C**) were present in all the 25 *L. casei* transformants. Additionally, the presence of the two genes in all the 25 transformants served as a good control. From the 25 colonies, 3 were randomly chosen to be used for the subsequent experiments. To check for the presence of the full length InlAB, PCR using the specific primers was performed in the 3 chosen cultures (Lbc^{InlAB1}, Lbc^{InlAB2} and Lbc^{InlAB3}). For the 3 chosen cultures, **Fig. 2.6D** shows that there was an amplification of the genes *inlA, inlB* and InlAB, confirming that transformation into *L. casei* was successful.

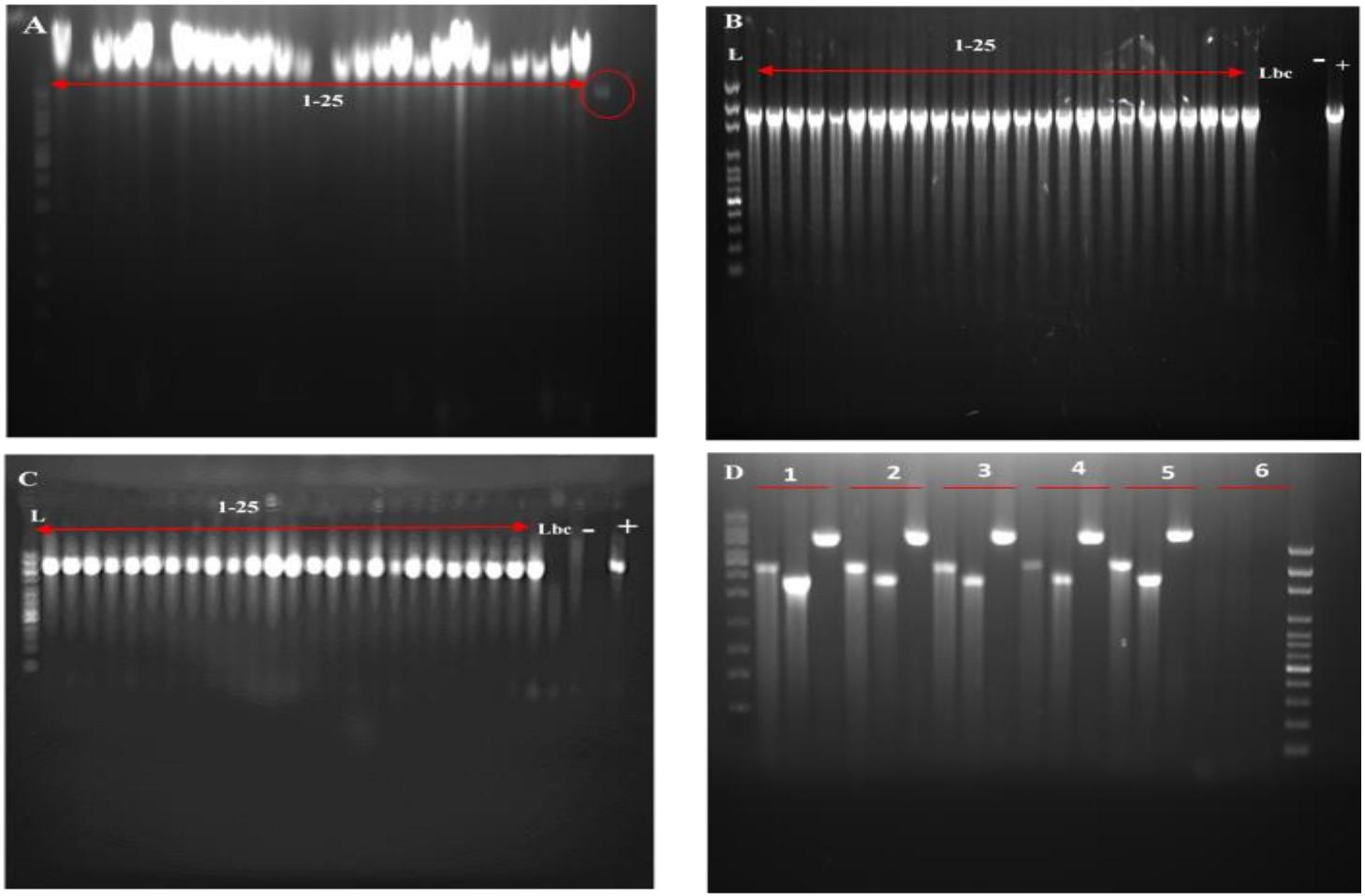
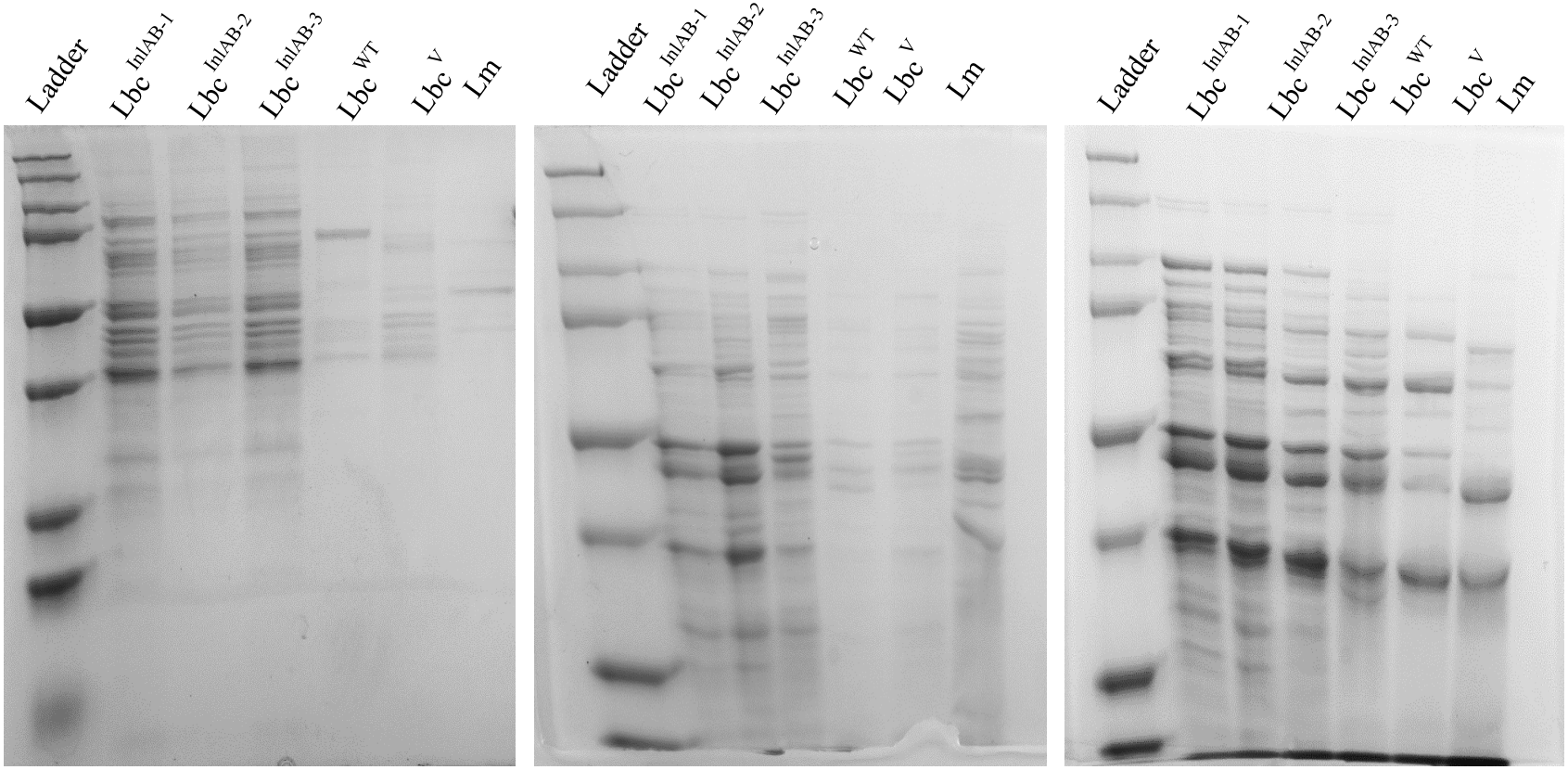


Figure 2.6. Agarose gel (1 % w/v) showing: **A:** Plasmids extracted from the positive transformants of *L. casei*. Lane 1-25- Plasmids isolated from 25 different colonies of *L. casei*. Lane 26- pLP401-T. **B:** Polymerase chain reaction products from *L. casei* with the primer set InlA (2403 bp) and **C:** InlB (1893 bp). Lane L- 1 kb Ladder DNA marker, Lanes 1-25, Lbc (Lbc^{WT}) and – (No DNA) are Negative controls and + (Positive control). **D:** PCR for InlA, InlB and InlAB. 1-4 positive transformants of *L. casei* (Lbc-^{InlAB} 1, 2, 3 and 4), 5: *L. monocytogenes* F4244 (Positive control) and 6: Lbc^{WT} (Negative control)

2.4.4. Confirmation of protein expression by SDS PAGE, Western blot and Immunofluorescence staining

The separation of the proteins was visualized using SDS-PAGE (**Fig. 2.7A**), showing the separation of the proteins in the supernatant, cell wall and the intracellular fraction of all the *L. casei* strains and *L. monocytogenes*. Western blot assay confirmed the expression of both InlA and InlB proteins in the different cellular fractions (supernatant, cell wall and intracellular) of Lbc^{InlAB} and *L. monocytogenes* while absent in Lbc^{WT} or Lbc^{V} (Lbc carrying only empty pLP401-T vector) cell fractions (**Fig. 2.7B**). Immunofluorescence staining also confirmed the surface expression of InlA and InlB in Lbc^{InlAB} strain and *L. monocytogenes* (**Fig. 2.7C**). These data indicate that both InlA and InlB were successfully expressed in Lbc^{InlAB} strain and were associated with the cell wall. Transformant 1 ($Lbc^{InlAB-1}$) was used for the rest of the experiments.

A



Supernatant Cell Wall Intracellular

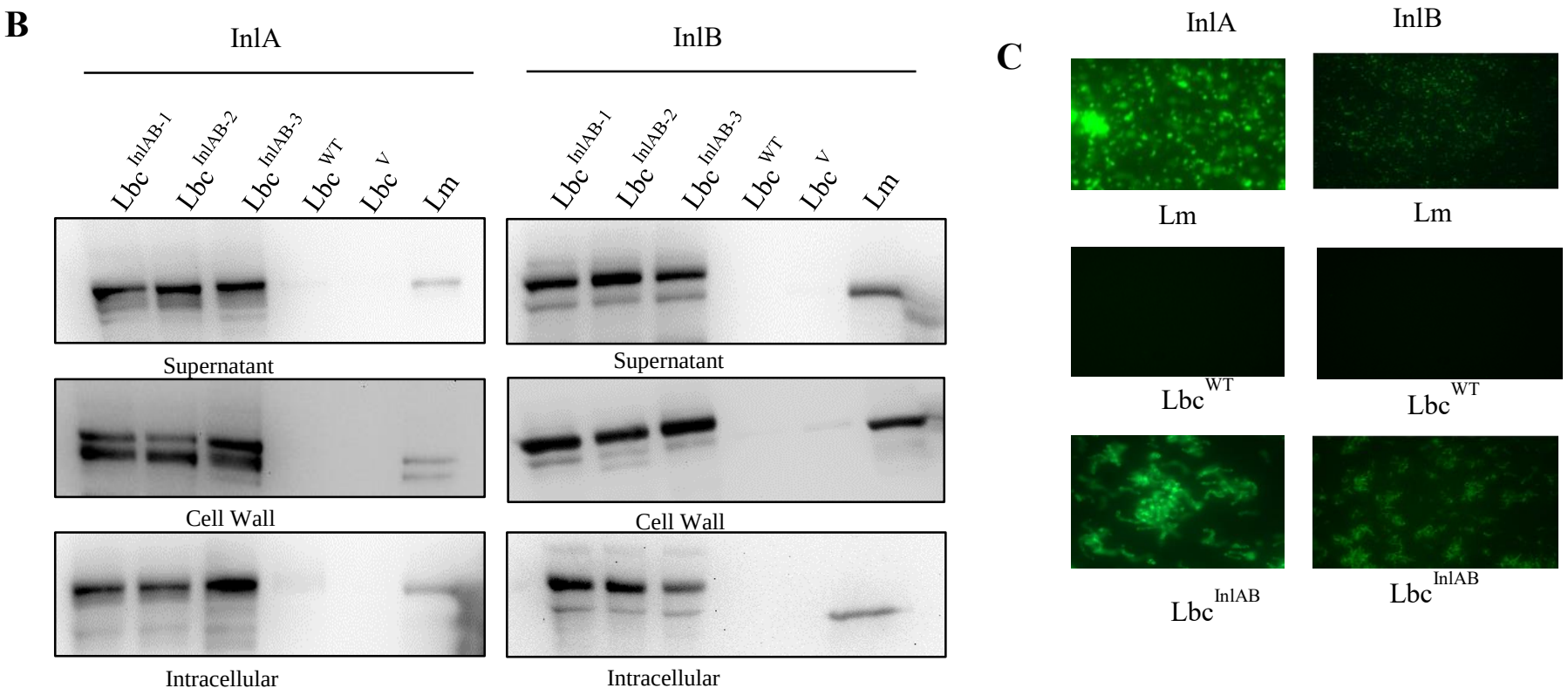


Figure 2.7. Expression of Internalin (InlA) and InlB in the recombinant *Lactobacillus casei* (Lbc^{InlAB}). (**A**) SDS PAGE showing the protein separation in the different cellular fractions of the *L. casei* strains and *L. monocytogenes*, (**B**) Western blot showing expression of Internalin (InlA) and InlB in the recombinant *L. casei* strains ($Lbc^{InlAB-1}$, $Lbc^{InlAB-2}$, $Lbc^{InlAB-3}$, Lbc^{WT} and Lbc^{V} in the different cellular fractions (supernatant, cell wall and intracellular) and *L. monocytogenes* F4244 (Lm). (**C**) Immunofluorescence staining of bacteria (magnification 1000×) with anti-InlA mAb-2D12 and anti-InlB pAb404. Lbc^{InlAB} and Lm (control) cells indicated the presence of InlA and InlB (green) and no expression in Lbc^{WT}.

2.4.5. Growth curves and cell morphologies of *L. casei* strains

In order to determine whether the expression of InlAB affects the growth of *L. casei*, growth curves of the Lbc^{WT}, $Lbc^{V,}$ and Lbc^{InlAB} were compared. Both optical density (**Fig. 2.8A**) and the viable cell counts (log CFU/ ml) (**Fig. 2.8B**) data showed similar growth profiles for all three strains over time. Furthermore, in phase contrast micrographs (**Fig. 2.8C**), all three strains Lbc^{WT}, Lbc^{V} and Lbc^{InlAB} maintained a typical elongated curve-shaped morphology; however, Lbc^{V} and Lbc^{InlAB} formed slightly longer chains.

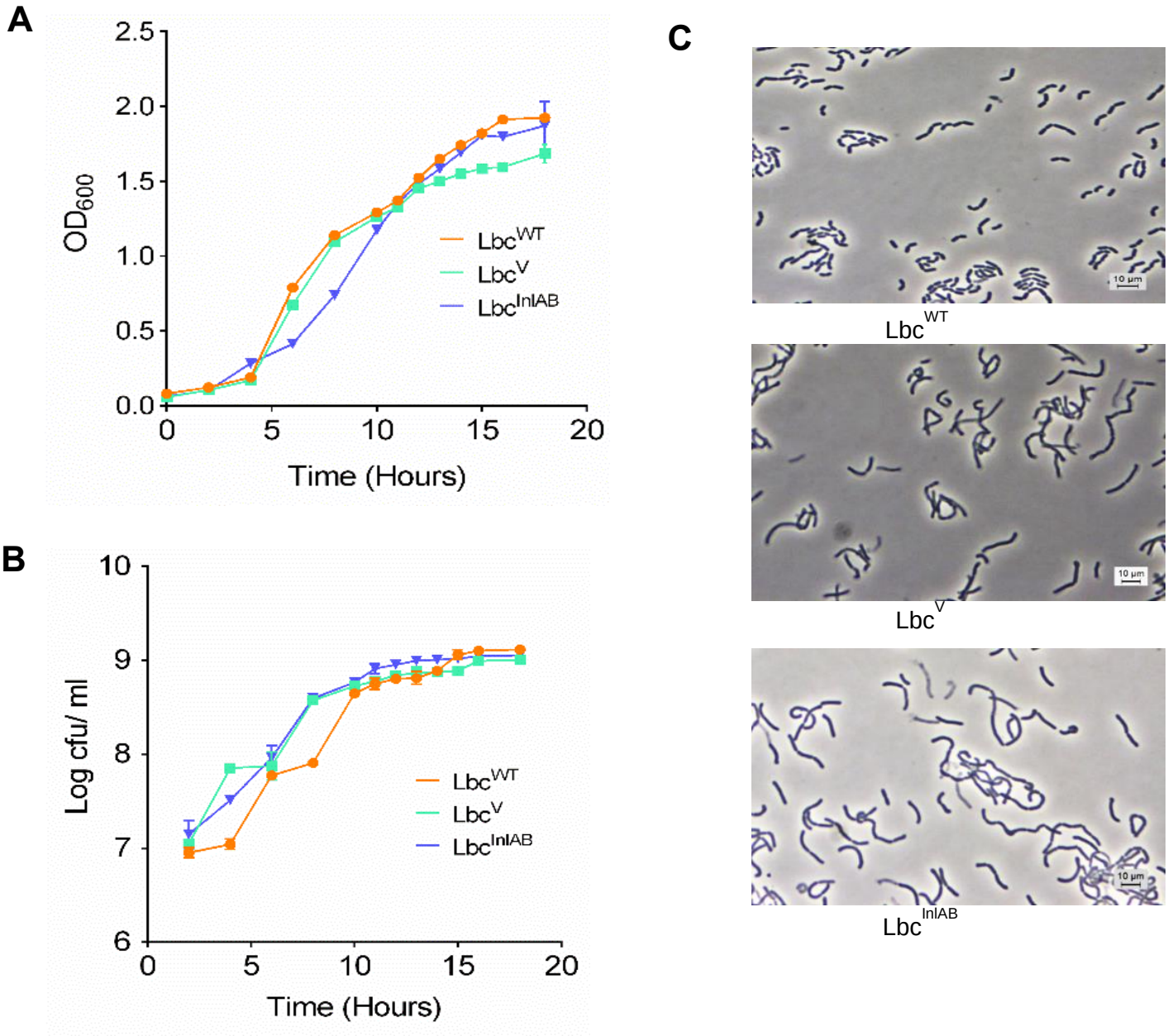


Figure 2.8. Panel showing *L. casei* growth curves (**A**) optical density measurement (OD at 600 nm), (**B**) bacterial counts, and (**C**) phase contrast microscopic images of Lbc^{WT}, $Lbc^{V,}$ and Lbc^{InlAB}. This experiment was performed twice in triplicates.

2.5. DISCUSSION

Adhesion of beneficial bacteria termed probiotics to host surfaces is a crucial aspect as it confers subsequent mechanical clearing of pathogens (Jayashree *et al.*, 2018), at the same time for pathogens, it is essential for progression of the infection. Most commensal and pathogenic bacteria interact with hosts and then express adhesive molecules on their surfaces that promote such interactions (Toumola *et al*., 1999; Kline *et al*., 2009). Several studies have evaluated the ability of probiotics for control of *L. monocytogenes* infection. Puertollano *et al*. (2008) evaluated the immunomodulatory effects of *Lactobacillus plantarum* against *L. monocytogenes* infections in mice and reported that administration of this probiotic resulted in a reduction in the production of proinflammatory cytokines, which circumvented *Listeria*-mediated cytotoxicity. In a different study, dos Santos *et al*. (2011) studied how the administration of *L. delbrueckii* influenced the clearance of *L. monocytogenes* from various mice organs such as the liver, spleen and peritoneal cavity. They reported that the *L. delbreuckii* administration yielded a faster clearance of the bacteria from these organs and ultimately protected the mice against death caused by *L. monocytogenes*. However, the use of probiotics to inhibit foodborne pathogens has always mostly been generic, with few reports on a specific probiotic for a specific pathogen.

In an effort to offer probiotics an enhanced ability to compete with a specific pathogen, an advanced understanding of the infection cycle of the foodborne pathogen in question and consideration of its virulence genes has presented a new strategy for its control. For most pathogens to cause infections there is a specific recognition of receptors for toxins or adhesion factors, which is essential for the initiation of the infection process. Thorough understanding of the infection cycle, in this case the specific recognition of gene and receptor recognition can be adapted to develop infection intervention strategies. This can be achieved through expression of the virulence genes coding for molecules that bind to host cell receptors in probiotic bacteria (Paton *et al.*, 2010). Expression of the genes from the enteric pathogen by the probiotic that allows it to competitively inhibit binding of the pathogen to its receptor.

L. monocytogenes is a facultative intracellular pathogen able to invade, survive and multiply inside epithelial cells. Adhesion and invasion of the host cell are crucial steps in its life infection as they allow it to replicate in host cell's cytoplasm (Camejo *et al*. 2011). *L. monocytogenes* employs LAP which interacts with the Hsp60, its host cell receptor, promoting bacterial adhesion to intestinal

cells (Wampler *et al*. 2004; Burkholder and Bhunia, 2010). Once adhered, it employs amongst others, the two major invasion proteins InlA and InlB, which mediate its entry into different non-phagocytic cell types (Gaillard *et* al., 1991; Dramsi *et al*. 1995). The host cell receptor for InlA is E-cadherin (Mengaud *et al*., 1996), a transmembrane glycoprotein involved in cell-cell adhesion while InlB has various host receptors: gC1qR, c-Met and glycosaminoglycans (GAGs) (Bleymuller *et al.*, 2016). The interaction of both InlA and InlB and their receptors have been reported to be necessary and sufficient to promote invasiveness (Braun *et al.* 1998). The crucial role of InlA and InlB for *L. monocytogenes* pathogenesis has been confirmed by researchers elsewhere. Gründler *et al*. (2013) compared invasion of different cell lines by wild type *L. monocytogenes* in comparison with that of mutants whose InlA or InlB gene were deleted. They reported strong diminished invasion of cells by these mutants, confirming the fundamental role of both surface proteins during cellular entry. The interaction of these proteins and the host cell receptor can be exploited as a strategy for inhibition of adhesion, which can then prevent colonization of the intestine by *L. monocytogenes*, and thereby prevent its infection (Tuomola *et al*., 1999). Taking that into consideration, it was hypothesized that the expression of InlA and InlB into a probiotic strain will result in its enhanced ability to invade the cells depending on the cell type and the receptors. Hence, in the current study, the invasion proteins InlA and InlB were expressed in a probiotic *L. casei* for enhanced targeted control of *L. monocytogenes* infection.

Successful cloning and expression of the genes in the different cellular fractions of the probiotic was confirmed by Western blot and immunofluorescence staining. There was expression of both InlA and InlB in the three fractions; supernatant, cell wall and intracellular fractions, while there was no expression when the anti- InlA and anti- InlB was reacted with the same fractions of Lbc^{WT}. Expression of these proteins in cellular fractions of the recombinant probiotic *L. casei* strain shows its potential to attach to their specific receptors on the mammalian cells usually recognized and used by *L. monocytogenes* for its attachment to these cells to cause or advance an infection. Koo *et al.* (2012) reported that the ability of probiotics to express recombinant proteins (LAP) in the different fractions makes them available for interaction with mammalian cells, allowing them to directly compete with those pathogens that attach to the same receptors.

It has previously been reported that expression of new genes resulted in changes in growth of the recombinants when compared to their wild-type counterparts (Ludwig *et al.* 2001; Ramos *et al.*,

2004; Li *et al.* 2016). The effects of the resultant Lbc^{InlAB} on the growth of *L. casei* were investigated. Similar growth curves obtained for the *L. casei* wild type and recombinant Lbc^{InlAB} suggest that expression of the extra genes by the recombinant *L. casei* did not have any effect on its growth rate. This is a desirable result as it indicates that growth and consequent colonization potential of the recombinant strain will be comparable to that of the parental strain from which it was derived.

2.6. CONCLUSION

Expression of different virulence genes offers an alternative strategy with potential for targeted control of *L. monocytogenes* infection. The invasion genes InlAB was successfully cloned and expressed into *L. casei.* The expression of the genes had no effects on the growth of the *L. casei* strains, however, the recombinants maintained a typical elongated curve-shape as opposed to the rod- shaped morphology of the wild- type counterparts.

2.7. REFERENCES

Aguilar, C., Vanegas, C., Klotz, B. (2011). Antagonistic effect of *Lactobacillus* strains against *Escherichia coli* and *Listeria monocytogenes* in milk. *J Dairy Res., 78*(2), 136-143.

Amalaradjou, M.A.R., Bhunia, A.K. (2013) Bioengineered probiotics, a strategic approach to control enteric infections, *Bioengineered, 4*, 291-299.

Amara, A.A., Shibl, A. (2015). Role of probiotics in health improvement, infection control and disease treatment and management. *Saudi Pharmaceutical Journal, 23*(2), 107-114.

Behnsen, J., Deriu, E., Sassone-Corsi, M., Raffatellu, M. (2013) Probiotics: properties, examples, and specific applications, *Cold Spring Harbor Perspective Biology, 3*: a010074.

Bleymuller, W., Lammermann, N., Ebbes, M., Maynard, D., Geerds, C., Niemann, H. (2016). Met-activating residues in the b-repeat of the *Listeria monocytogenes* invasion protein InlB. *Journal of Biological Chemistry, 291*(49), 25567-25577.

Bonazzi, M., Lecuit, M., Cossart, P., (2009). *Listeria monocytogenes* internalin and E-cadherin: From structure to pathogenesis. *Cell Microbiology, 11*, 693–702.

Braun, L., Dramsi, S., Dehoux, P., Bierne, H., Lindahl, G., Cossart, P. (1997) InIB: An invasion protein of *Listeria monocytogenes* with a novel type of surface association, *Molecular Microbiology, 25,* 285-294.

Burkholder, K.M., Bhunia, A.K. (2010) *Listeria monocytogenes* uses *Listeria* adhesion protein (LAP) to promote bacterial transepithelial translocation, and induces expression of LAP receptor Hsp60, *Infection and Immunity, 78,* 5062-5073.

Camejo, A., Carvalho, F., Reis, O., Leitao, E., Sousa, S., Cabanes, D. (2011) The arsenal of virulence factors deployed by *Listeria monocytogenes* to promote its cell infection cycle, *Virulence, 2*, 379-394.

Ceapa, C., Rezaiki, L., Kleerebezem, M., Knol, J., Ozeer, R. (2013). Influence of fermented milk products, probiotics and probiotics on microbiota composition and health. *Best Practice and Research Clinical Gastroenterology*, *27(1),* 139-155

Corr, S., Li, Y., Riedel, C.U., O'Toole, P.W., Hill, C., Gahan, C.G.M. (2007). Bacteriocin production as a mechanism for the anti-infective activity of *Lactobacillus salivarius* UCC118, *Proceeding of the National Academy of Sciences (USA), 104*, 7617-7621.

Culligan, E.P., Hill, C., Sleator, R.D. (2009). Probiotics and gastrointestinal disease: successes, problems and future prospects. *Gut Pathogens, 1*, 19.

Czuprynski, C. (2005). *Listeria monocytogenes*: Silage, sandwiches and science. *Animal Health Research Reviews, 6*(2), 211-217.

de Wit, M.A., Kopmans, M.P., Kortbeek, L.M., van Leeuwen, N.J., Bartelds, A.J. (2001). Gastroenteritis in sentinel general practices. The Netherlands. *Emerging Infectious Disease, 7*, 82-89.

dos Santos, L., Santos, M., de Souza Silva, H., Arantes, R., Nicoli, J., Vieira, L. (2011). Monoassociation with probiotic *Lactobacillus delbrueckii* UFV-H2b20 stimulates the immune system and protects germfree mice against *Listeria monocytogenes* infection. *Medical Microbiology and Immunology*, *200*(1), 29–38.

Dramsi, S., Biswas, I., Maguin, E., Braun, L., Mastroeni, P., Cossart, P. (1995). Entry of *Listeria monocytogenes* into hepatocytes requires expression of InIB, a surface protein of the internalin multigene family. *Molecular Microbiology, 16*, 251–261.

FAO/WHO (2002). Guidelines for the evaluation of probiotics in food. In Food and Agriculture Organization of United Nations and World Health Organization Working Group Report (London, Ontario).

Gaillard, J.L., Berche, P., Frehel, C., Gouin, E., Cossart, P. (1991). Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. *Cell, 65,* 1127–1141.

Gray, K.M., Bhunia, A.K. (2005). Specific detection of cytopathogenic *Listeria monocytogenes* using a two-step method of immunoseparation and cytotoxicity analysis. *Journal of Microbiological Methods, 60*(2), 259-268.

Gründler T, Quednau N, Stump C, Orian-Rousseau V, Ishikawa H, Wolburg H, Schoten H, Tenenbaum T, Schwerk C. (2013). The surface proteins In1A and In1B are interdependently required for polar basolateral invasion by *Listeria monocytogenes* in a human model of the blood-cerebrospinal fluid barrier. *Microbes and Infection, 15*(4), 291-301.

Jayashree, S., Karthikeyan, R., Nithyalakshmi, S., Ranjani, J., Gunasekaran, P., Rajendhran, J. (2018) Anti-adhesion property of the potential probiotic strain *Lactobacillus fermentum* 8711 against methicillin-resistant *Staphylococcus aureus* (MRSA), *Frontiers in Microbiology, 9,* 411. doi: 10.3389/fmicb.2018.00411

Kline, K.A., Falker, St., Dahlberg, S., Normark, S., Henriques-Normark, B. (2009). Bacterial Adhesins in Host-Microbe Interactions. *Cell Host & Microbe, 5*(6), 580-592.

Koo, O.K., Amalaradjou, M.A.R., Bhunia, A.K. (2012). Recombinant probiotic expressing *Listeria* adhesion protein attenuates *Listeria monocytogenes* virulence *in vitro*. *PLosONE, 7*(1):e29277. doi:10.1371/journal.pone.0029277.

Li, C., Sun, J.W., Zhang, G.F., Liu, L.B. (2016). Effect of the absence of the CcpA gene on growth, metabolic production, and stress tolerance in *Lactobacillus delbrueckii* ssp. bulgaricus. *Journal of Dairy Science, 99*(1), 104-111.

Lingnau, A., Domann, E., Hudel, M., Bock, M., Nichterlein, T., Wehland, J., Chakraborty, T. (1995). Expression of the *Listeria monocytogenes* EGD inlA and inlB genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and -independent mechanisms. *Infection and Immunity, 63*, 3896–3903.

Ludwig, H., Homuth, G., Schmalisch, M., Dyka, F., Hecker, M., Stülke, J. (2001). Transcription of glycolytic genes and operons in *Bacillus subtilis*: Evidence for the presence of multiple levels of control of the gapA operon. *Molecular Microbiology, 41*(2), 409-422.

Manning, G., Duim, B., Wassenaar, T., Wagenaar, J.A., Ridley, A., Newell, D.G. (2001). Evidence for genetically stable strain of *Campylobacter jejuni*. *Applied and Environmental Microbiology, 67*, 1185-1189.

Mengaud, J., Ohayon, H., Gounon, P., Mege, R. M., Cossart, P. (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. *Cell, 84*, 923–932.

Parida, S. K., Domann, E., Rohde, M., Muller, S., Darji, A., Hain, T., Wehland, J., Chakraborty, T. (1998). Internalin B is essential for adhesion and mediates the invasion of *Listeria monocytogenes* into human endothelial cells. *Molecular Microbiology, 28*, 81–93.

Paton, A.W., Morona, R., Paton, J.C. (2010) Bioengineered bugs expressing oligosaccharide receptor mimics: toxin-binding probiotics for treatment and prevention of enteric infections, *Bioengineered Bugs, 1*, 172-177.

Pouwels, P.H., Vriesema, A., Martinez, B., Tielen, F.J., Seegers, J.F., Leer, R.J., Jore, J., Smit, E. (2001) Lactobacilli as vehicles for targeting antigens to mucosal tissues by surface exposition of foreign antigens. *Methods in Enzymology, 336*, 369-389.

Puertollano, E., Puertollano, M.A., Cruz-Chamorro, L., Alvarez de Cienfuegos, G., Ruiz-Bravo, A., de Pablo, M.A. (2008). Orally administered *Lactobacillus plantarum* reduces pro-inflammatory interleukin secretion in sera from *Listeria monocytogenes* infected mice. *British Journal of Nutrition, 99(4)*, 819- 825

Radoshevich, L., Cossart, P. (2018). *Listeria monocytogenes*: towards a complete picture of its physiology and pathogenesis, *Nature Review: Microbiology, 16*, 32-46.

Ramos, A., Neves, A.R., Ventura, R., Maycock, C., Lopez, P., Santos, H. (2004). Effect of pyruvate kinase overproduction on glucose metabolism of *Lactococcus lactis*. *Microbiology, 150*(4), 1103-1111.

Rolfe, R.D. (2000). The role of probiotic cultures in the control of gastrointestinal health. *Journal of Nutrition 130*, 396- 402.

Shen, Y., Naujokas, M., Park, M., Ireton, K. (2000). InlB-dependent internalization of *Listeria* is mediated by the Met receptor tyrosine kinase. *Cell, 103*, 501–510.

Sleator, R.D., Hill, C. (2008). "Bioengineered bugs"—a patho-biotechnology approach to probiotic research and applications. *Medical Hypotheses, 70*, 167–169.

Tuomola, E., Ouwehand, A., Salminen, S. (1999). The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. *FEMS Immunology & Medical Microbiology, 26*(2), 137-142.

Vàzquez-Boland, J. A., Dominguez-Bernal, G., Gonzàlez-Zorn, B., Kreft, J., Goebel, W. (2001). Pathogenicity islands and virulence evolution in *Listeria*. *Microbes and Infection, 3*, 571–584.

Wampler, J.L., Kim, K.P., Jaradat, Z., Bhunia, A.K. (2004) Heat shock protein 60 acts as a receptor for the *Listeria* adhesion protein in Caco-2 cells. *Infection and Immunity, 72*, 931-936.

Werbrouck, H., Grijspeerdt, K., Botteldoorn, N., van Pamel, E., Rijpens, N., van Damme, J., Uyttendaele, M., Herman, L., van Coillie, E., (2006). Differential InlA and InlB expression and interaction with human intestinal and liver cells by *Listeria monocytogenes* strains of different origins. *Applied and Environmental Microbiology, 72*, 3862–3871.

Chapter 3

Prevention of *Listeria monocytogenes* adhesion, invasion and translocation *in vitro* by the recombinant *Lactobacillus casei* expressing the Internalins AB

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3. ABSTRACT

Background: *Listeria monocytogenes* is an intracellular foodborne pathogen that has been implicated in a number of outbreaks including the recent largest listeriosis outbreak in South Africa. There are currently no preventative methods for the treatment of foodborne infections, therefore, raising a need for an alternative strategy. Advanced understanding the pathogens' infection cycle and consideration of their virulence genes offers this alternate strategy for its control. Probiotic bioengineering is a strategic approach to broaden efficacy for control of enteric pathogens such as *L. monocytogenes*. The current study investigates the ability of a *Lactobacillus casei* expressing the invasion genes internalin AB (InlAB) (Lbc^{InlAB}) of *L. monocytogenes* for its control. This construct was compared with a previously developed *Lb. casei* expressing listeria adhesion protein (LAP) (Lbc^{LAP}). **Materials and Methods:** The adhesion, invasion and translocation profiles of *L. casei* strains as well as their ability to competitively exclude *L. monocytogenes in vitro* were investigated. Inhibition of *L. monocytogenes* adhesion, invasion and translocation through Caco-2 cells, pre- exposed to the *L. casei* strains for 1, 4, 16, or 24 h (MOI =10) was investigated. **Results:** There was a significant increase ($p<0.0001$) in the ability of Lbc^{InlAB} to adhere, invade and translocate through Caco-2 cells compared to Lbc^{WT}. The recombinant *L. casei* strains were able to competitively exclude *L. monocytogenes* and inhibited adhesion, invasion and translocation over time. Comparison of functional attributes of two bioengineered strains revealed that Lbc^{InlAB} had a superior ability to prevent *L. monocytogenes* invasion and translocation, than the Lbc^{LAP}. However, Lbc^{LAP} strain exhibited prevented *L. monocytogenes* adhesion than the Lbc^{InlAB}. Pre- exposure of Caco-2 to recombinant *L. casei* strains showed a reduction in *L. monocytogenes* mediated cell cytotoxicity and epithelial barrier dysfunction. **Conclusion:** The results suggest that recombinant strains expressing different virulence genes of *L. monocytogenes* can be targeted at different stages of its infection cycle, with the recombinant harbouring LAP and internalins targeting adhesion and invasion plus translocation, respectively. Thus, probiotic bioengineering could be used to target specific stages in the *L. monocytogenes* infection cycle to inhibit its colonization and infection progression.

3.2. INTRODUCTION

Listeria monocytogenes is a Gram-positive, facultative intracellular foodborne pathogen that has evolved to survive in different environments both inside and outside mammalian hosts (Vazquez-Boland *et al*., 2001; Czuprynski, 2005; Gray *et al*., 2006). The most common infection caused by this bacterium, listeriosis, is acquired by ingesting contaminated food products (Hamon *et al.*, 2006). The intensity of infection is dependent on the host. In healthy individuals, the infection is usually a self-limiting gastroenteritis; however, in immunocompromised individuals and pregnant women, the bacterium is capable of causing systemic infections that lead to meningitis, encephalitis and, in the case of pregnant women, infection of the developing foetus, which can lead to abortion, stillbirth or neonatal infections (Drevets and Bronze, 2008; Freitag *et al*., 2009). When compared to most foodborne pathogens the incidence of listeriosis is low, however, its high case fatality rate (20 to 30%) has potentially made it a considerable public health concern (Mead *et al*., 1999). Most recently (2017-2018), the largest outbreak of listeriosis documented occurred in South Africa where 1060 people were infected resulting in 214 deaths due to the consumption of ready-to-meat sausage products, Polony (Allam *et al.* 2018).

As an intracellular pathogen, *L. monocytogenes* invades mammalian cells, escapes from host cell phagosomes, replicates within the cytosol, and spreads into neighbouring cells (Hamon *et al*., 2006; Freitag *et al*., 2009). A number of bacterial factors are required for its intracellular replication and cell-to-cell spread, to facilitate its escape from vacuoles, its survival, proliferation and motility within the host cells (Vazquez-Boland *et al*., 2001). There are several virulence factors involved in the *L. monocytogenes* infection process, however not equally important, that have been identified, and their roles well established (Cossart and Lecuit, 1998; Cossart, 2001; Cossart and Vàzquez-Boland *et al*., 2001). Amongst the virulence factors, *L. monocytogenes* employs the *Listeria* adhesion protein (LAP), in its adhesion during the intestinal phase of infection. For the subsequent step, invasion, the pathogen uses among other factors, the internalins (InlA and InlB) (Robbins *et al*., 2010: Camejo *et al.*, 2011).

The ability of *L. monocytogenes* to invade a number of non-phagocytic cells and to cross the intestinal (Nikitas *et al.*, 2011; Drolia *et al.*, 2018), blood–brain (Ghosh *et al*., 2018) and feto-placental barriers (Hamon *et al.*, 2006; Le Monnier *et al*., 2007; Wolfe *et al*., 2017) makes it a

burden. In model cell culture systems, adherence and invasion of host cells require several determinants (Kathariou, 2002), however, the adhesion and invasion efficiencies vary with the cell line or the type of cells used (Cossart, 2011). A number of bacterial surface proteins, including the InlA and InlB, have been shown to contribute to bacterial invasion of host cells (Seveau *et al*., 2007; Stavru *et al*., 2011). Studies on these proteins have reported that they are both necessary and sufficient for bacterial entry into cell types such as enterocytes, hepatocytes, fibroblasts, epithelial and endothelial cells (Hamon *et al*., 2006). For entry into cells, InlA binds E-cadherin, a host cell adhesion molecule, whereas InlB binds to the hepatocyte growth factor (HGF) receptor, Met (Pizarro-Cerda *et al.*, 2006). The difference in the receptors yields each protein working individually to gain entry into certain cells although sometimes interdependent. With regards to the different barriers that the pathogen is able to cross, it has been reported that InlA facilitates entry into human intestinal epithelial cells (Lecuit *et al*., 2001) and crosses the gut epithelial barrier by transcytosis (Nikitas *et al*., 2011), while InlB contributes to invasion of human hepatic and M cells (Lecuit, 2007, Chiba *et al*., 2011). *L. monocytogenes* also can cross the gut epithelial barrier by disrupting epithelial tight junction by using LAP through a paracellular route (Burkholder and Bhunia, 2010; Drolia *et al*., 2018). LAP interaction with epithelial receptor, Hsp60 results in the activation of NF-kB and myosin light chain kinase (MLCK) and mislocalization of tight junction proteins resulting in the opening of tight junction for bacterial passage to lamina propria (Drolia *et al*., 2018). Once the pathogen invades the cells, it multiplies and using cell-to-cell spread causing systemic infection that can proliferate in immune- compromised individuals as stated before.

Probiotic engineering is a strategy that has been used to design probiotics to offer enhanced functions and probiotics. Additionally, the recombinant probiotics have been reported to better compete with pathogens when compared to their wild- type counterparts. Sheehan *et al.* (2006) cloned and expressed BetL from *L. monocytogenes* into *Lactobacillus salivarus*, they reported that recombinant *L. salivarius* strain showed a significant increase in betaine accumulation compared to the wild type. In a study by Volzing *et al*. (2013), they reported that *Lactococcus lactis* expressing the peptides A3APO and alyteserin showed activity against pathogenic bacteria *Escherichia coli* and *Salmonella enterica* better than the wild- type *L. lactis.* In a different study Borrero *et al.* (2015) genetically engineered *L. lactis* to detect *Enterobacter faecalis* in the environment and then produce a set of antimicrobial peptides, the wild- type strain did not have those functions. These studies propose that microbial bioengineering offers an alternative strategy

to enhance the functions of probiotics. Taking that into consideration, the current study investigates the ability of the recombinant *L. casei* expressing internalin AB (Lbc^{InlAB}) to inhibit the adhesion, invasion and translocation of *L. monocytogenes in vitro*. This construct was compared to the previously cloned *L. casei* expressing LAP (Lbc^{LAP}).

3.3. MATERIALS AND METHODS

3.3.1. Bacterial strains and growth conditions

L. monocytogenes F4244 (serovar 4b) was cultured in tryptone soy broth supplement with 0.6% yeast extract (TSB-YE) or brain heart infusion (BHI) at 37ºC for 18 h. Wild-type *L. casei* (Lbc^{WT}) was grown in de Man Rogosa and Sharpe (MRS) broth while the *L. casei* carrying the pLP401T empty vector (Lbc^{V}) and recombinant Lbc^{InlAB} and Lbc^{LAP} (unpublished) strains were grown anaerobically at 37ºC for 16 h in MRS broth containing 2 µg/ml erythromycin.

3.3.2. Recombinant *L. casei* strains' adhesion and invasion into Caco-2 cells

3.3.2.1. Caco-2 cell culturing

Human colon carcinoma cell line Caco-2 (HTB37; American Type Culture Collection) was cultured in Dulbecco's modified eagle's medium (DMEM) with high glucose (HyClone™, GE, Logan, UT) supplemented with 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals, GA) (D10F). The cells were grown in flasks (Greiner- Bio-One) for up to 10-12 days or until differentiated and then trypsinized (Gaillard and Finlay, 1996). They were then seeded in 12-well plates at a density of 1×10^5 cells/ well and incubated at 37°C in the presence of 7% CO_2 for 10–12 days until they reached confluence (10^6 cells/well).

3.3.2.2. Adhesion and invasion assays

Overnight grown (18 h) bacterial cultures were washed twice with PBS, adjusted to OD 600 = 1 and were suspended in D10F to a final concentration of 1×10^7 CFU/ml to achieve a multiplicity of infection (MOI) or multiplicity of exposure (MOE), 10. The Caco-2 cell monolayer was washed three times using DMEM, and then exposed separately to the *L. casei* strains (Lbc^{WT}, Lbc^{V}, Lbc^{InlAB} or Lbc^{LAP}) and *L. monocytogenes* and incubated for 1 h at 37ºC in a gas atmosphere with 5% CO_2 (Koo *et al.*, 2012). To enumerate bacterial adhesion, the Caco-2 cell monolayer was first washed thrice using DMEM and then treated with 0.1% Triton X-100 (37ºC, 10 min). For the invasion assay, the monolayers were exposed to *L. monocytogenes* and *L. casei* and then washed as performed in the adhesion assay, treated with gentamycin (50 µg/ml, 1 h) and with 0.1% Triton X-100 (37ºC, 10 min). The lysed cell suspensions from both adhesion and invasion experiments

were serially diluted in PBS before plating on MRS, MRS supplemented with erythromycin (2 µg/ml) and Modified Oxford (MOX) agar for Lbc^{WT}, recombinant *L. casei*, and *L. monocytogenes*, respectively. All the plates were incubated at 37ºC for 24-48 h before bacterial enumeration.

3.3.3. Determination of *L. monocytogenes* exclusion mode by *L. casei* strains

The competitive exclusion assay was performed as before (Koo *et al.*, 2012) with minor modifications. Bacterial cultures were prepared as above and were suspended in D10F to a final concentration of 1×10^7 CFU/ml. For competitive adhesion, *L. monocytogenes* was co-inoculated with each of the *L. casei* strains (Lbc^{WT}, Lbc^{V}, Lbc^{InlAB} or Lbc^{LAP}) to Caco-2 cell monolayer (MOI, 10) and incubated for 1 h. Adherent bacteria were enumerated as above.

In the inhibition of adhesion assay, the Caco-2 monolayers were first inoculated with each *L. casei* strain (MOE, 10) and incubated for 1 h, and washed to remove unbound bacteria using DMEM. *L. monocytogenes* was then added to the wells and plates were incubated for 1 h, followed by an enumeration of adherent bacteria by plating. For displacement of adhesion, Caco-2 cells were first inoculated with *L. monocytogenes* (MOI, 10) and incubated for 1 h, and washed to remove unbound bacteria. *L. casei* strains were then added to the wells and plates were incubated for 1 h. Adhered bacteria were released by treatment with 0.1% Triton X-100 (37ºC, 10 min) and plated on MRS, MRS supplemented with 2 µg/ml of erythromycin and MOX agar plates for enumeration of Lbc^{WT}, recombinant *L. casei* and *L. monocytogenes*, respectively.

3.3.4. Inhibition of *L. monocytogenes* adhesion and invasion by *L. casei* strains

The Caco-2 cell monolayers were washed and then exposed to the *L. casei* strains (MOE, 10) for 1, 4, 16 and 24 h at 37°C in the humidified incubator with 5% CO_2. Excess medium in the wells containing unbound *L. casei* was removed and replaced with 500 µl of *L. monocytogenes* suspended in D10F (MOI, 10), and the plates were incubated for 1 h at 37°C with 5% CO_2. The adherent bacteria were enumerated by plating as above.

For inhibition of *L. monocytogenes* invasion, the Caco-2 cell monolayers were exposed to each *L. casei* strain (MOE, 10) for 1, 4, 16 and 24 h at 37°C with 5% CO_2. Excess *L. casei* cells were removed and replaced with 500 µl of *L. monocytogenes* suspended in D10F (MOI, 10) and

then incubated at 37°C with 5% CO_2 for 1 h. The cell monolayers were washed, treated with gentamycin (50 µg/ml) for 1 h, and determined for invading bacteria by plating.

3.3.4.1. Caco-2 cells cytotoxicity assay

To determine Caco-2 cell cytotoxicity induced by *L. monocytogenes* after pre-exposure to *L. casei* over time, the LDH assay was performed. The supernatants after infection with *L. monocytogenes* for 1 h were collected and used to analyse for lactate dehydrogenase (LDH) enzyme release. Caco-2 cells that were treated with 500 µl of 0.1% Triton X-100 per well were used as a positive control while those treated with DMEM were used as the negative control. From the supernatants collected, 100 µl were transferred to the 96-well flat bottom plate in triplicates and was analysed using Pierce LDH cytotoxicity assay kit (Thermo Scientific, USA) following the protocol from the manufacturer.

3.3.5. Transcellular translocation of *L. casei* strains and subsequent inhibition of *L. monocytogenes* transepithelial translocation by recombinant *L. casei*

The Caco-2 cells were grown in 12 well trans-well filter inserts (3-µm pore size) for 20-25 days to reach confluence (Burkholder and Bhunia, 2010; Drolia *et al.*, 2018). TEER of Caco-2 cells was quantified using the Millicell ERS (Millipore, Billerica, MA) and a TEER value of more than 200 Ω/cm^2 was used for all the experiments. For determining baseline translocation by *L. casei* strains or *L. monocytogenes*, the Caco-2 cells were washed and then the bacteria were added (MOI, 10) separately to the apical side of the trans-well at 37°C with 5% CO_2 for 2 h. The liquid from the basal well was collected, serially diluted in PBS and then plated for the enumeration of bacterial cells (CFU/ ml).

For the inhibition of *L. monocytogenes* translocation, *L. casei* cells were first added to the apical wells (MOE, 10) and incubated for 1, 4, 16 and 24 h at 37°C with 5% CO_2 and *L. casei* counts in the basal wells were determined by plating on MRS agar. Subsequently, excess *L. casei* cells were removed from the apical well, and replaced with 500 µl of *L. monocytogenes* (MOI, 10) and then incubated at 37°C with 5% CO_2 for 2 h. *L. monocytogenes* counts in the basal wells were determined by plating on MOX agar plates.

3.3.5.1. Epithelial Tight Junction Integrity Analysis

Quantification of (TEER) of Caco-2 cells was measured before and after the exposure to the bacteria was performed using Millicell ERS system (Millipore, Billerica, MA) as described before (Burkholder and Bhunia, 2010). Furthermore, the integrity of the tight junctions between Caco-2 cells was determined by measuring FD4 permeability in a spectrofluorometer. After exposure to *L. monocytogenes*, the tight junction permeability using Dextran^{FITC} (Mr 3–5 kDa; Sigma) permeability through the transwell filter inserts was analysed. Dextran^{FITC} (1 mg/ ml) was added to the transwell and incubated at 37°C for 1 h. Samples from the apical and basolateral chambers was collected and read in a SpectraMax Gemini EM fluorescent plate reader (Molecular Devices; Sunnyvale, CA).

3.3.6. Statistical analysis

All data were analysed using Prism 7 software (Graphpad software Inc., United States), and significance was assigned at $p < 0.05$. Where appropriate, Turkey's multiple comparisons, with $p<0.005$ as a significant difference was used to identify statistically significant differences.

3.4. RESULTS

3.4.1. Adhesion, invasion and translocation characteristics of recombinant Lbc^{InlAB}

The abilities of the different *L. casei* strains (Lbc^{WT}, Lbc^{V}, Lbc^{InlAB} and Lbc^{LAP}) to adhere to, invade and translocate through or across the Caco-2 cells were compared to those of *L. monocytogenes*. This served to determine how these functionalities are influenced by expression of the different *L. monocytogenes* genes (InlAB and LAP) and establish which of the recombinants performed better at these stages or processes. **Figure 3.1A** shows the adhesion profiles of the *L. casei* strains when compared to *L. monocytogenes*. There were no statistically significant differences in the adhesion of *L. monocytogenes* F4244 (10%) and that of Lbc^{WT} ($p=0.8466$) and Lbc^{V} ($p=0.9964$) to the Caco-2 cells, which both showed adhesion rate of 11% under the condition employed. However, there were significant differences in adhesion between these strains and Lbc^{InlAB} as well as between them and Lbc^{LAP}. There was an increase in the adhesion of the recombinant *L. casei* strains, Lbc^{InlAB} adhered at higher levels (13%), which was statistically different to that of *L. monocytogenes* ($p=0.0015$), Lbc^{WT} ($p=0.0153$) and Lbc^{V} ($p=0.0042$). Similarly, recombinant Lbc^{LAP} showed increased adhesion to the Caco-2 cells, statistically different ($p<0.0001$) to those recorded for *L. monocytogenes,* Lbc^{WT} and Lbc^{V}. When comparing the two recombinant *L. casei* strains, it was observed that Lbc^{LAP} showed significantly higher ($p<0.0001$) adhesion (17.8%) than levels recorded for Lbc^{InlAB}.

Figures 3.1B and **3.1C** show respectively, invasion and translocation profiles for *L. monocytogenes* and the *L. casei* strains. *L. monocytogenes* is well known for its ability to invade and translocate through the Caco-2 cell monolayer. We recorded 10.7% and 7.5% for its invasion and translocation, respectively. On the contrary, Lbc^{WT} and Lbc^{V} did not invade nor translocate through the Caco-2 cells, both showing 0.18% and 0.13% for invasion and translocation, respectively. There was an increase in both the invasion and translocation efficiencies due to the presence of InlAB and LAP in *L. casei*. Lbc^{InlAB} invaded and translocated through the Caco-2 cells by 8.0% and 5.3%, respectively, levels which were significantly higher compared to those of Lbc^{WT} and Lbc^{V}. Invasion (1.8%) and translocation (0.83%) levels for Lbc^{LAP} were significantly higher than Lbc^{WT} and Lbc^{V} ($p<0.0001$), but significantly lower than for Lbc^{InlAB} ($p<0.0001$). As expected, *L. monocytogenes* was able to invade and translocate the monolayer at significantly

higher levels than obtained for all the *L. casei* strains. What was worth noting is that invasion and translocation of Lbc^{InlAB} through the Caco-2 cells was at significantly higher levels than all the other *L. casei* strains.

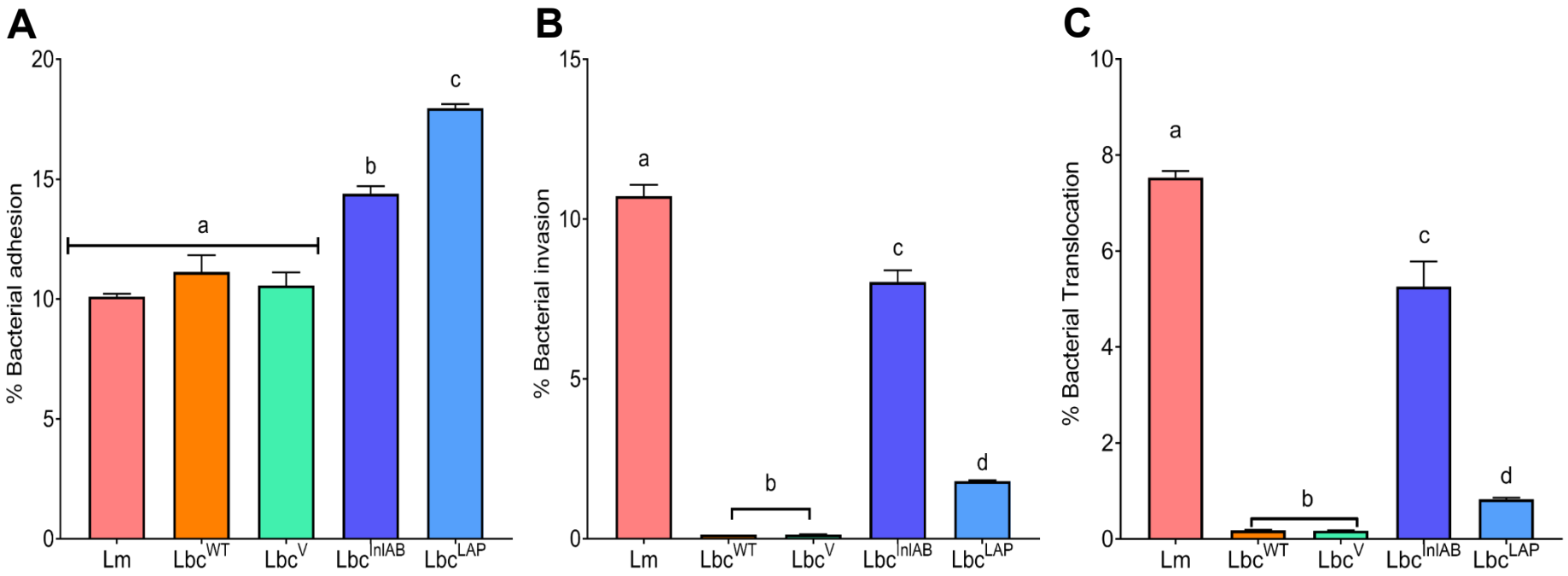

Figure 3.1. Adhesion, invasion and translocation profiles of *Listeria monocytogenes* (Lm) and *Lactobacillus casei* (Lbc) to Caco-2 cells. (**A**) Adhesion, (**B**) Invasion, and (**C**) Translocation of the Caco-2 cells by *L. monocytogenes* and *L. casei* strains (Lbc^{WT}, Lbc^{V}, Lbc^{InlAB} and Lbc^{LAP}). Percentages were calculated relative to the inoculums that were added to the Caco-2 cells. Data are average (SD) of three independent experiments performed in duplicate (n=6). For each time point, bars marked with different letters (a, b, c, d) indicate a significant difference at $P<0.05$.

3.4.2. Competitive exclusion of *L. monocytogenes* by recombinant Lbc^{InlAB}

Probiotics utilize the mechanism of competition, either for adhesion space or for food, to inhibit pathogens. There are different ways by which probiotics can competitively inhibit pathogen adhesion and subsequently the infection. Three competitive exclusion mechanisms, namely competitive adhesion, inhibition and displacement of adhesion were evaluated, to determine the most effective for control of *L. monocytogenes* by the recombinant *L. casei* strains (**Fig. 3.2**). The adhesion of *L. monocytogenes* to Caco-2 cells in the absence of *L. casei* strains was recorded as 100% in all the assays and was used to calculate the relative adhesion in the presence of the *L. casei* strains. For the competitive adhesion assay, **Fig. 3.2A** shows that adhesion of *L. monocytogenes* was only slightly reduced when it was added simultaneously with either Lbc^{WT} or Lbc^{V}, with no statistical differences in adhesion ($p=0.9136$ and $p=0.9986$ for Lbc^{WT} and Lbc^{V}, respectively). Adhesion of *L. monocytogenes* was reduced by 24% and 29% when co-inoculated with Lbc^{InlAB} and Lbc^{LAP}, respectively. Both recombinant strains significantly ($p<0.0001$) decreased adhesion of *L. monocytogenes* by competitive adhesion, however there was no statistical difference in reduction of its adhesion by Lbc^{InlAB} and Lbc^{LAP} ($p=0.4138$). In the inhibition of adhesion (**Fig. 3.2B**), the results were similar with what was observed for competitive adhesion assay. There was no significant reduction in adhesion of *L. monocytogenes* when the Caco-2 cells were pre-exposed to Lbc^{WT} ($p=0.9993$) and Lbc^{V} ($p=0.9536$). Pretreatment of the monolayer with either of the recombinant strains (Lbc^{InlAB} and Lbc^{LAP}) significantly reduced ($p<0.0001$) subsequent adhesion of *L. monocytogenes*. Interestingly, there was a statistical difference ($p=0.0074$) in the reduction of adhesion between the two recombinant *L. casei* strains, with Lbc^{LAP} (26%) showing better reduction in adhesion than the Lbc^{InlAB} (19%). For both the competitive adhesion and inhibition of adhesion assays, there were significant differences ($p<0.0001$) between Lbc^{WT} and Lbc^{V} and the recombinant *L. casei* strains (Lbc^{InlAB} and Lbc^{LAP}). All the *L. casei* strains failed to dislodge the *L. monocytogenes* cells already bound to the Caco-2 cell monolayer (**Fig. 5C**). There were no significant differences in displacement of *L. monocytogenes* by Lbc^{WT} ($p=0.8323$), Lbc^{V} ($p=0.6518$), Lbc^{InlAB} ($p=0.6570$) and Lbc^{LAP} ($p=0.2173$). Furthermore, there were no statistical differences among all the *L. casei* strains in the displacement of *L. monocytogenes.*

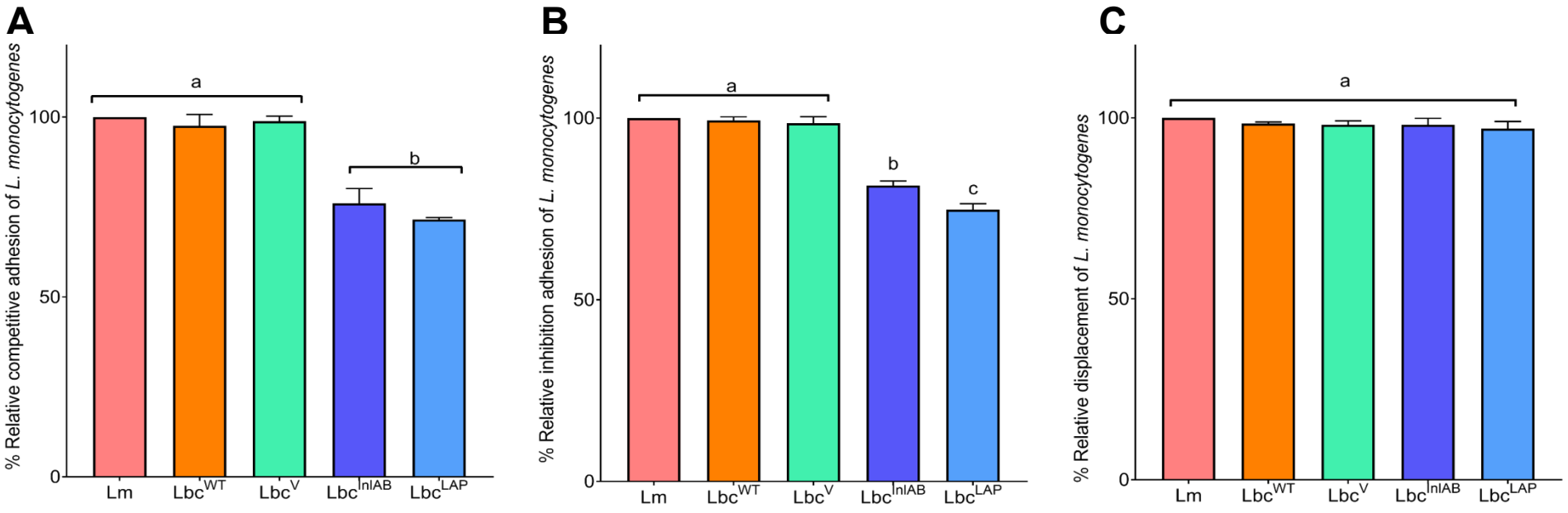

Figure 3.2. Competitive exclusion of *Listeria monocytogenes* (Lm) adhesion to Caco-2 cells by *L. casei* strains (Lbc^{WT}, Lbc^{V}, Lbc^{InlAB} and Lbc^{LAP}), analyzed by three different exclusion mechanisms. (**A**) Competitive adhesion: Caco-2 cells were exposed to *L. casei* strains with Lm simultaneously, (**B**) inhibition of adhesion: Caco-2 cells were pre-exposed to *L. casei* strains for 1 h before infection with Lm, and (**C**) Displacement of adhesion: Caco-2 cells were infected with Lm for 1 h before treatment with *L. casei* strains (1 h). Adhesion of Lm alone to Caco-2 cells was presented as 100% and percent adhesion was calculated relative to that. Data are averages of three experiments ran in duplicates (n=6). For each time point bars marked with different letters (a, b, c) indicate a significant difference at $P<0.05$.

3.4.3. Inhibition of *L. monocytogenes* adhesion, invasion and transcellular migration over time

Considering that the results presented above indicated that inhibition of adhesion yielded pronounced inhibition of *L. monocytogenes* by the *L. casei* strains, it was further explored how this mechanism will be influenced by time. In addition to adhesion, the effect on other stages of *L. monocytogenes* intestinal infection stages, namely, invasion and translocation was examined. Inhibition of *L. monocytogenes* when the Caco-2 cells were pre- exposed to the *L. casei* strains for 1 h was already shown (**Fig. 3.2B**). Therefore, it was necessary to investigated whether prolonged exposure of Caco-2 cells to the *L. casei* strains will yield equal or more pathogen inhibition. The Caco-2 cells were pre- exposed to the *L. casei* strains (Lbc^{WT}, Lbc^{V}, Lbc^{InlAB} and Lbc^{LAP}) for 1, 4, 16 and 24 h. The results (**Fig. 3.3A**) showed that the longer the exposure to *L. casei* strains the more the inhibition. When the Caco-2 cells were exposed to Lbc^{WT} or Lbc^{V} for 1 h, there was no statistical difference in the inhibition when compared to the pathogen control. Over time, exposure to Lbc^{WT} showed a significant reduction ($p<0.0001$) in adhesion of *L. monocytogenes* with 8.2%, 8.8% and 11.83% recorded for 4, 16 and 24 h, respectively. There were no statistically significant differences between reductions of *L. monocytogenes* adhesion by Lbc^{WT} and Lbc^{V} for all the exposure periods. On the contrary, there was a statistical difference ($p<0.0001$) in the adhesion of the pathogen control compared to exposure the recombinant Lbc^{InlAB} and Lbc^{LAP} even at 1 h, with 22.81% and 21.79% reductions obtained for Lbc^{InlAB} and Lbc^{LAP}, respectively. Worth noting, pre-exposure of the Caco-2 cells to the recombinant Lbc^{InlAB} and Lbc^{LAP} for 1 h showed better inhibition compared to that obtained for 24 h pre-exposure to either Lbc^{WT} or Lbc^{V}. Although there was a statistical difference at 1 h for the recombinant *L. casei* strains, prolonged exposure resulted in even more increased inhibition. Reductions of 53.59% and 64.43% were recorded for Lbc^{InlAB} and Lbc^{LAP}, respectively, after 24 h. When comparing inhibition reductions for Lbc^{InlAB} and Lbc^{LAP}, they were not statistically different when pre- exposed for 1 h ($p>0.9999$) and 4 h ($p= 0.9711$) but were significantly different ($p<0.0001$) after 16-24 h, with Lbc^{LAP} showing a more enhanced reduction than Lbc^{InlAB}.

Using the same competitive exclusion mechanism, the inhibition of invasion of *L. monocytogenes* by the *L. casei* strains was investigated. Results obtained were similar to those observed for inhibition of adhesion. Pre-exposure of the Caco-2 cells to Lbc^{WT} and Lbc^{V} for up to 16 h did not

yield any significant reduction ($p<0.9999$) of *L. monocytogenes* invasion when compared to the control (**Fig. 3.3B**). The longest exposure (24 h) to these strains decreased invasion of the Caco-2 cells by *L. monocytogenes* by 15% ($p<0.0001$). Pre- exposure of the monolayer to Lbc^{InlAB} and Lbc^{LAP} for 1 h statistically reduced ($p<0.0001$) the invasion of the cells by *L. monocytogenes*. Longer exposure to Lbc^{InlAB} and Lbc^{LAP} showed an even enhanced decrease of invasion (**Fig. 3.3B**), with Lbc^{InlAB} showing higher reduction in invasion than Lbc^{LAP}. After 24 h pre-exposure, Lbc^{InlAB} and Lbc^{LAP} showed significantly different ($p<0.0001$) reductions of invasion of the Caco-2 cells by *L. monocytogenes,* with 51.71% and 31.93% decreases obtained due to these recombinants, respectively.

The ability of the pathogen to enter the cells is always crucial in the infection cycle of intracellular pathogens. The inhibition of translocation of the pathogen into the cells over time was therefore studied. Pre- exposure of the Caco-2 cells to Lbc^{WT} and Lbc^{V} did not show any significant reduction in *L. monocytogenes* translocation ($p<0.9999$), with a decrease of only 2% recorded after 24 h (**Fig. 3.3C**). Similarly, to what was observed for inhibition of adhesion and invasion, pre-exposure of the cells to Lbc^{InlAB} and Lbc^{LAP} for 1 h significantly reduced translocation of *L. monocytogenes* through the Caco-2 cells ($p<0.0001$). Longer exposure to Lbc^{InlAB} and Lbc^{LAP} showed an enhanced decrease of translocation, with Lbc^{InlAB} being more effective than Lbc^{LAP}. Reductions of 57.14% and 52.46% were obtained due to Lbc^{InlAB} and Lbc^{LAP}, respectively. These levels were significantly higher than those due to the presence of Lbc^{WT} and Lbc^{V}. Furthermore, reductions by Lbc^{InlAB} were significantly higher than those by Lbc^{LAP} for 4 - 24 h pre-exposure periods ($p<0.0001$).

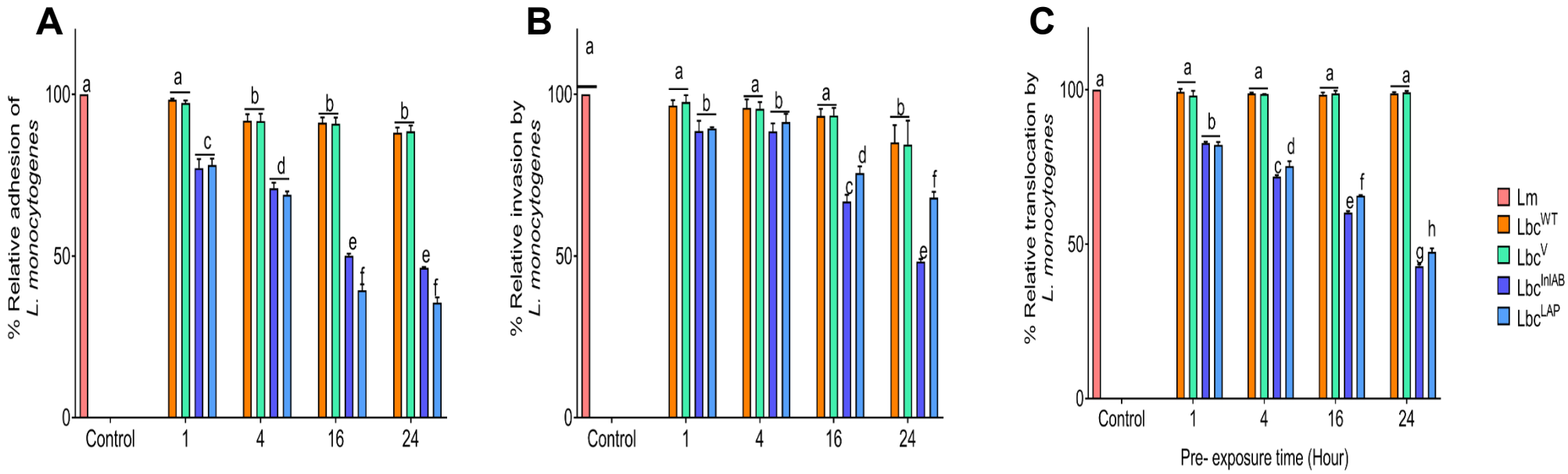

Figure 3.3. Inhibition of *Listeria monocytogenes* (Lm) adhesion (**A**), invasion (**B**) and transcellular translocation (**C**) by the *L. casei* strains (Lbc^{WT}, Lbc^{V}, $Lbc^{InlAB,}$ and Lbc^{LAP}). Caco-2 cells were pre-exposed to the *L. casei* strains for 1, 4, 16 and 24 h before infection with Lm for 1 h for adhesion and invasion and 2 h for translocation. Data are averages of three experiments ran in duplicates (n=6). For each time point bars marked with different letters (a, b, c, d, e, f, g, h) indicate a significant difference at $P<0.05$.

3.4.4. Inhibition of cytotoxic effects of *L. monocytogenes* on Caco-2 cells by *L. casei*

In addition to studying the inhibition of adhesion the cytotoxicity induced by *L. monocytogenes* to Caco-2 cells in the presence and absence of the *L. casei* strains was investigated (**Fig. 3.4**). The lactate dehydrogenase assay showed that *L. monocytogenes* caused 64.38% cytotoxicity to Caco-2 cells in the absence of *L. casei* strains. When the cells were pre- exposed to the *L. casei* strains, a reduction in cytotoxicity was observed. After 1 h pre-exposure to the *L. casei* strains 11%, 90% and 98% cytotoxicity reductions were recorded for Lbc^{WT}, Lbc^{InlAB} and Lbc^{LAP}, respectively. Pre-exposure for 24 h resulted in 3.2%, 55% and 79% reductions by Lbc^{WT}, Lbc^{InlAB} and Lbc^{LAP}, respectively. Although the *L. casei* strains protected the cells, the level of protection offered decreased over time. After 24 h, there were no differences in cytotoxicity in the presence of Lbc^{WT} when compared to control. For the recombinant *L. casei* strains a significant difference in the protection of the cells was observed, with Lbc^{InlAB} showing less protection when compared to Lbc^{LAP}.

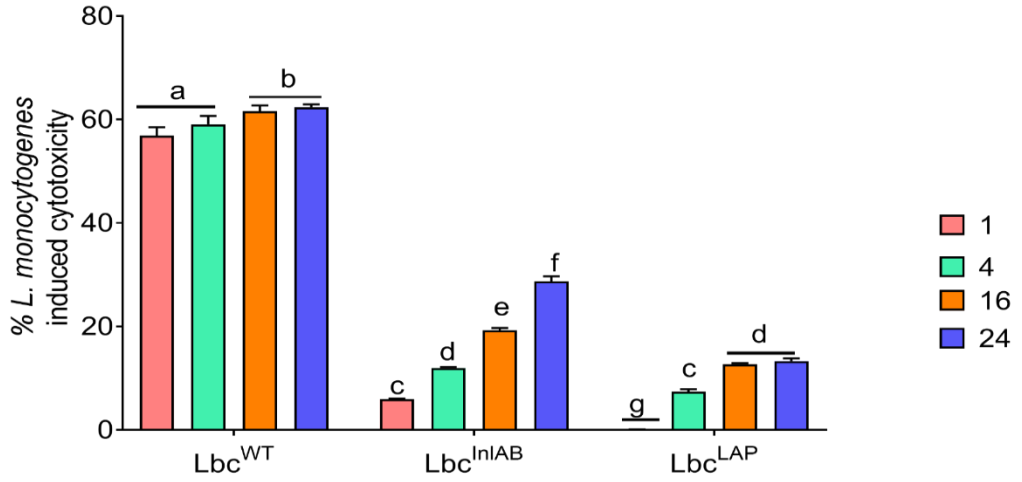

Figure 3.4. Cytotoxicity of *Listeria monocytogenes* (Lm) in Caco-2 cells pre-exposed with *Lactobacillus casei* over time (1, 4, 16, 24 h). Cytotoxicity value for *L. monocytogenes* treatment (1 h) in the absence of *L. casei* strains was 64.38%. Data are averages of three experiments ran in duplicates (n=6). For each time point bars marked with different letters (a, b, c, d, e, f, g) indicate a significant difference at $P<0.05$.

3.4.5. Recombinant Lbc^{InlAB} protects epithelial tight junction barrier integrity

This study further monitored the effect of *L. monocytogenes* on the tight junction integrity of Caco-2 cells in presence and absence of *L. casei* strains using the TEER and Dextran^{FITC} permeability analyses. For TEER analysis, electrical resistance of cells before their exposure to the *L. casei* strains and after their subsequent infection with *L. monocytogenes* for 2 h were measured. Cells pre- exposed only to *L. monocytogenes* for 2 h were used as the control. **Figure 3.5A** shows the percent TEER changes measured and calculated as per Koo *et al*. (2012). The TEER reduction when cells were exposed to *L. monocytogenes* without treatment with *L. casei* strains was 16.9%. In the presence of the different *L. casei* strains TEER reductions were lower than that recorded for the cells treated with *L. monocytogenes* alone, ranging from 9.50%- 16.7%, 2.6%- 8.53% and 1.67%- 6.52%, for Lbc^{WT}, Lbc^{InlAB} and Lbc^{LAP}, respectively. Significant protection was evident for Lbc^{InlAB} and Lbc^{LAP} than for Lbc^{WT}. However, as was observed for cytotoxicity assay, prolonged exposure to all *L. casei* strains resulted in a decrease in level of protection. There were no statistically significant differences ($p>0.9999$) between the integrity of cells treated with *L. monocytogenes* alone and those treated with Lbc^{WT}. Nevertheless, significant differences were still attained for cells treated with Lbc^{InlAB} or Lbc^{LAP} and *L. monocytogenes* alone. Although both recombinant *L. casei* strains protected the Caco-2 cells from the damaging effects of *L. monocytogenes*, Lbc^{LAP} showed better protection than Lbc^{InlAB}. However, when comparing Lbc^{InlAB} and Lbc^{LAP} at 24 h, there was no significant difference in TEER reductions ($p=0.5755$).

Figure 3.5B shows results of Caco-2 tight junction permeability in the presence and absence of *L. casei* strains. When the cells were only infected with *L. monocytogenes* for 2 h, 2.76% of the Dextran^{FITC} was recovered at the basal side. The level of Dextran^{FITC} recovered at the basal side decreased when the trans-well membranes were pre-exposed to the *L. casei* strains, Lbc^{WT}, Lbc^{InlAB} or Lbc^{LAP}. Differences of 23%, 50% and 95% for Lbc^{WT}, Lbc^{InlAB} and Lbc^{LAP}, respectively, were obtained when compared to the control membrane that was not pre-exposed to *L. casei* strains for 2 h. The levels of recovered Dextran^{FITC} increased with an increase with exposure time, with levels getting closer to those of membrane not exposed to any probiotic. After 24 h pre- exposure to *L. casei* strains, there were differences of 6.88%, 27% and 82% for Lbc^{WT}, Lbc^{InlAB} and Lbc^{LAP}, respectively when compared to the control membrane. Significant differences in recovered

Dextran^{FITC} levels were obtained due to the presence of the different *L. casei* strains. However, it was evident that Lbc^{LAP} showed recovery of less Dextran^{FITC}, which means it was better at protecting the integrity of the Caco-2 cells, followed by Lbc^{InlAB} and then Lbc^{WT}. These results further elucidated those obtained for inhibition of translocation and the TEER reduction experiments.

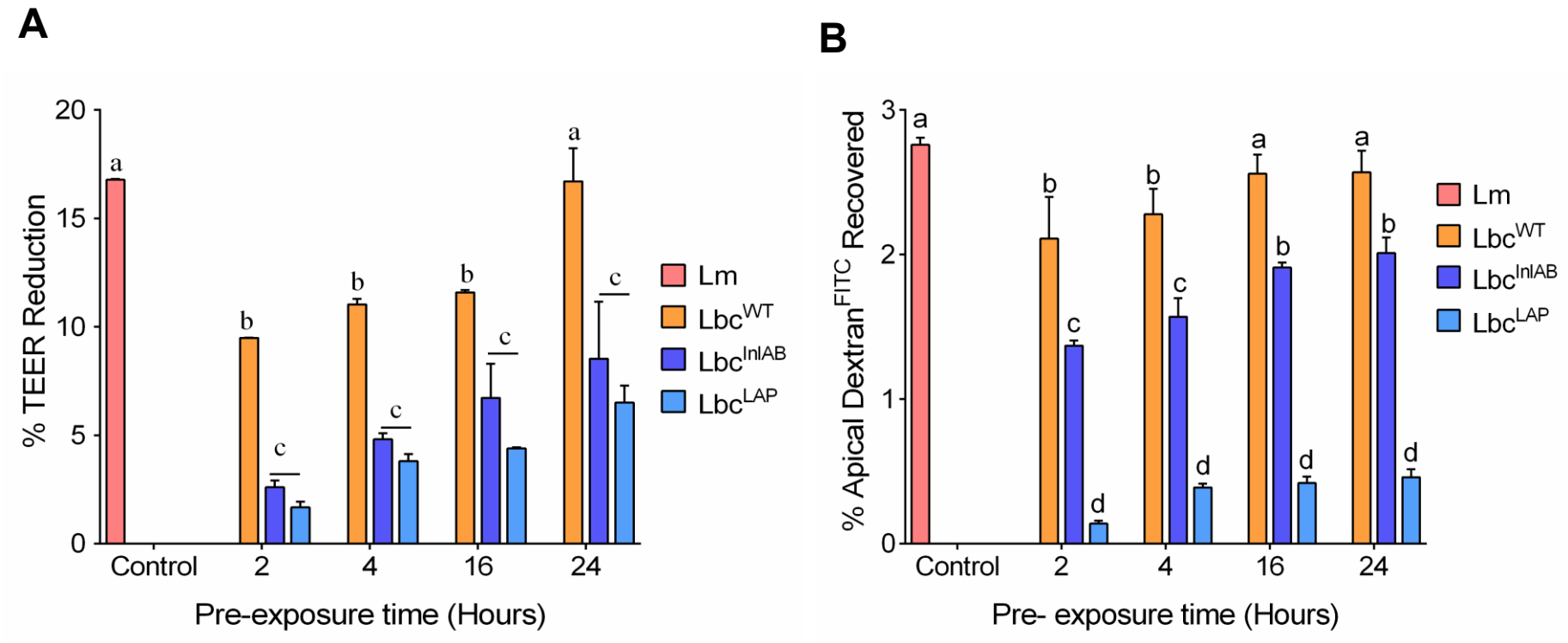

Figure 3.5. Caco-2 cell permeability analysis using (**A**) transepithelial electrical resistance (TEER) and (B) 4-kDa Dextran^{FITC} (FD4) permeability assay. Caco-2 cells monolayers were grown in trans-well inserts and treated with *L. casei* strains (Lbc^{WT}, Lbc^{V}, Lbc^{InlAB} or Lbc^{LAP}) for 2, 4, 16, and 24 h, before their infection with *Listeria monocytogenes* (Lm) for 2 h. TEER measurements before and after exposure to *L. monocytogenes* treatment alone were 268.9±2.3 and 224.5±4.7 respectively, with a 16.5% change. Values are averages of two experiments analysed in triplicate. % TEER reduction was calculated as per Koo *et al*. (Koo *et al.*, 2012) as 1 – $TEER_{after}$ / $TEER_{before}$ x100. (B) FD4 recovery after Lm was 2.76± 0.03%. Values are averages of three independent experiments performed in duplicates (n=6). For each time point, bars marked with different letters (a, b, c, d) indicate a significant difference at $P<0.05$.

3.5. DISCUSSION

One of the important properties of probiotic bacteria is their ability to adhere to the target sites for their colonization in the gut for expression of optimal functionality **(**Duary *et al*., 2011). Attachment to intestinal epithelial cells also represents one of the essential steps in establishment of pathogen infection (Candela *et al*., 2008). Subsequent to pathogen adhesion, invasion and translocation through the cells are also important for infection progression. Certain lactobacilli reportedly share the same binding sites with pathogens (Neeser *et al*., 2000), an attribute which is acclaimed to lead to inhibition of the pathogen (Lee and Puong, 2002). This statement suggests that the better the adhesion ability of the probiotic or the pathogen, the better its effects, whether in conferring beneficial effects or causing an infection. Therefore, the recombinant probiotic has to compete with, and preferably out compete *L. monocytogenes*, for adhesion sites or receptors on the epithelial cells. The expression of the invasion genes InlAB afforded the probiotic the ability to adhere to the Caco-2 cells more than *L. monocytogenes*, Lbc^{WT} and Lbc^{V}. What was even more intriguing was that Lbc^{LAP} adhered to Caco-2 cells at levels significantly higher than Lbc^{InlAB}. This observation highlights or confirms the critical role played by LAP as opposed to internalins (Drolia *et al*., 2018), at this stage of *L. monocytogenes* infection process.

Invasion and translocation of bacteria through the host cells may result in their transfer to other organs, thereby potentially causing bacteraemia, septicaemia and multiple organ failure (Berg, 1995). Pavan *et al*. (2003) reported that some lactobacilli do translocate into the cells however, they are rapidly eliminated by the host immune system and thus may not be found even when administered in higher doses. Liong (2008) further reported that in most of the studies where probiotics were administered at high dosages to healthy subjects, there was usually no probiotic translocation. Therefore, it was imperative that the assays were performed to ascertain whether the recombinant Lbc^{InlAB} was able to translocate through the Caco-2 cells. The absence of the required factors or molecules, in this case internalins, hindered Lbc^{WT} and Lbc^{V} from invading and translocating the Caco-2 cells. On the contrary, their presence promoted better invasion and translocation of Caco-2 cells by Lbc^{InlAB}. Invasion and translocation were also observed for Lbc^{LAP} albeit less pronounced than by Lbc^{InlAB}. Converse to the argument for adhesion, the differences displayed between the recombinant strains harbouring the different virulence genes indicate that it is the presence of internalins that is required for invasion and translocation. Although LAP has

been reported to be necessary for adhesion there has been studies showing that it also plays a role in the translocation of *L. monocytogenes.* Burkoholder and Bhunia (2010) compared the invasion and translocation of wild type *Listeria* with that of *lap*-deficient mutant, they reported that the invasion and translocation of the WT was greater than that of the mutant suggesting that LAP might also serve as an invasion. However, when they manipulated the expression of Hsp60, they found no influence in the invasion while there was a change in the translocation, suggesting that LAP plays a role in the translocation and not the invasion of *L. monocytogenes* into host cells. Previous studies reported that adhesion and invasion of *L. monocytogenes* occur by separate mechanisms that involve different proteins located on the bacterial cell wall (Gilot *et al*., 1999; Jacquet *et al*., 2002; Jaradat and Bhunia, 2003). Improved invasion and translocation of the recombinant Lbc^{InlAB} was envisaged as both InlA and InlB are employed by *L. monocytogenes* to invade cells. Results of this study were in correlation with reports of researchers elsewhere. Lebrun *et al.* (1996) reported that expression of InlA gene from *L. monocytogenes* in non-invasive *Enterococcus faecalis* strains allowed their efficient entry into Caco-2 cells. Furthermore, Guimaraes *et al*. (2005) reported enhanced internalization of *Lactococcus lactis* expressing InlA and attributed it to specific binding of InlA to E-cadherin expressed on epithelial/endothelial cells, which was absent in the wild type *L. lactis*. In a recent study by Yano *et al.* (2018), they cloned and expressed murinized InlA into non-invasive *L. lactis* for DNA delivery. In their results they showed the recombinant *L. lactis* significantly increased DNA delivery, even to the polarized epithelial cells, although the efficiency of DNA delivery to polarized cells was lower than that to unpolarized cells. Their results suggest that DNA transfer from *L. lactis* occurs predominantly in the peyer's patch, but not in the intestinal epithelial cells, in an invasin-independent manner.

Probiotics can employ three different anti-infective strategies against pathogens: competitive inhibition, inhibition and displacement of adhesion to prevent or reduce adhesion of the pathogens to the host cells. Previous studies (Tuomola *et al*., 1999; Gueimonde *et al*., 2006 and Collado *et al*. 2007) mentioned that the degree of adhesion of probiotic strains is not proportional to its degree of competitive inhibition, inhibition and/or displacement of pathogen adhesion. Therefore, the adhesion of the probiotics should always be investigated simultaneously with its ability to reduce the adhesion of the pathogen to the same cells. Lee *et al*. (2003) reported that when incubated together, lactobacilli were able to compete with eight pathogens for adhesion to Caco-2 cells. However, in the current study, adhesion of *L. monocytogenes* to Caco-2 cells was the same in the

presence or absence of wild type *L. casei*, indicating the limitation of this conventional probiotic strain to compete with this pathogen for the adhesion site on the cells. Co- incubation of probiotics and pathogens has been previously reported to result in an increase in the adhesion of the pathogen (Collado *et al*. 2007). Averse to findings of their study, in the current study both co- incubation with and pre-exposure to the recombinant *L. casei* strains significantly decreased *L. monocytogenes* adhesion. Reduction by competitive adhesion and inhibition of adhesion mechanisms was higher for Lbc^{LAP} than Lbc^{InlAB}, once again highlighting the requirement for different virulence factors at these stages of *L. monocytogenes* infection cycle. Results reported here were in agreement with those by Jankowska *et al*. (2008) who reported that probiotics decreased adherence of pathogens to cells in competitive inhibition and inhibition of adhesion studies. Lee and Puong (2002) deduced that the degree of competition was probably determined by the affinity of adhesins on respective bacterial surfaces for the stero-specific receptors they are competing for, or their relative positions in the case of steric hindrance. This reasoning further elucidates the enhanced inhibition of *L. monocytogenes* by the recombinant *L. casei* expressing the invasion genes InlAB of *L. monocytogenes.* The ability of the recombinant Lbc^{InlAB} to inhibit adhesion of *L. monocytogenes* better than the wild type probiotic is thus attributed to its expression of the receptor genes as the pathogen. This expression means that they will compete for attachment to the same receptor which results in direct competition for the adhesion sites and ultimately inhibition of pathogen adhesion. Previous studies have reported the ability of probiotics to exclude or displace pathogens from mucus and intestinal cells (Lee *et al*., 2003; Sherman *et al*., 2005). In the current study, none of the *L. casei* strains (Lbc^{WT} and Lbc^{V}, Lbc^{InlAB} and Lbc^{LAP}), could displace *L. monocytogenes* already attached to Caco-2 cell monolayer. This observation was in correlation with previously published literature (Lee *et al.*, 2003; Candela *et al.,* 2008). In the competitive exclusion of pathogens, degrees of displacement were generally much lower than those of inhibition achieved by competition and inhibition (Lee *et al.*, 2003). They argued that *L. rhamnosus* LGG would not be able to competitively displace an adhered pathogen unless the pathogen detaches from the receptor and then binding of LGG to the same receptor hinders reattachment of the pathogen to the receptor. Most foodborne pathogens with high affinity for the receptor would not detach and would reattach readily (Lee *et al*., 2003). Other researchers however argue that the displacement activity exerted by probiotics towards enteropathogens is related to mechanisms other than mere competition for common adhesion sites (Candela *et al*., 2008). These

above-mentioned reasons could be an explanation for the observed failure of probiotics to remove the *L. monocytogenes* cells already attached to the monolayer. Thus the results presented here then propose that the recombinant *L. casei* will be effective as a prophylactic rather than a therapeutic intervention.

Once it was established that the recombinant *L. casei* strains best inhibit *L. monocytogenes* by inhibition of adhesion, the efficiency of this mechanism at adhesion, invasion and translocation stages influenced by probiotic pretreatment period was determined. Prolonged exposure to recombinant *L. casei* strains enhanced their ability to reduce *L. monocytogenes* at all these stages. Interestingly, even after this protracted exposure period, Lbc^{LAP} still outperformed Lbc^{InlAB} for inhibition of *L. monocytogenes* adhesion while the latter surpassed Lbc^{LAP} in invasion and translocation studies. Tight junctions hold epithelial cells together, forming a branch of network sealing strands that provide a physical intercellular barrier that restricts paracellular transport (Giepmans and Van Ijzendoorn, 2009). They are responsible for preventing diffusion of microorganisms and other antigens across the epithelium (Ulluwishewa *et al*., 2011). Maintenance of the tight junction integrity is thus crucial for infection control. Pre- exposure of mammalian cells to probiotics before infection with pathogen can maintain tight junction integrity and thereby inhibit pathogen infection (Helmy *et al*., 2017). In correlation with results observed for adhesion, invasion and translocation assays, the *L. casei* strains protected and maintained the tight junction integrity as indicated by results of TEER reduction, $Dextran^{FITC}$ and cytotoxicity assays. However, the longer pre-exposure of Caco-2 cells to *L. casei* strains before infection with *L. monocytogenes* compromised their ability to protect tight junction integrity. Therefore, an appropriate time has to be determined that will not only allow reduction of *L. monocytogenes* adhesion, invasion and translocation, but that will achieve that without sacrificing or compromising the tight junction integrity. So far, it appears as though exposure period between 4-16 h will ensure both these desirable effects are attained.

3.6. CONCLUSION

Recombinant *L. casei* strains expressing different virulence genes of *L. monocytogenes* can be targeted at different stages of its infection cycle, with the recombinant harbouring LAP and internalins targeting adhesion and invasion plus translocation, respectively. These recombinant *L. casei* strains will be effective as a prophylactic rather than therapeutic intervention. The recombinant strains will potentially offer dual effects, the ability to confer general beneficial effects attributed to conventional probiotics, and enhanced specific control of a targeted pathogen, specifically *L. monocytogenes*.

3.7. REFERENCES

Allam, M. L., Tau, N. S., Smouse, S. T., Mtshali, P. M., Mnyameni, F., Khumalo, Z.T.H., Ismail, A., Govender, N., Thomas, J., Smith, A. (2018). Whole-genome sequences of Listeria monocytogenes sequence type 6 isolates associated with a large foodborne outbreak in South Africa, 2017 to 2018. *Genome Announcement, 6*(25).

Berg, R. (1995). Bacterial translocation from the gastrointestinal tract. *Trends in Microbiology, 3(4),* 149-154.

Borrero, J., Chen, Y., Dunny, G.M., Kaznessis, Y.N. (2015). Modified lactic acid bacteria detect and inhibit multiresistant enterococci. *ACS Synthetic Biology, 4(3),* 299- 306.

Burkholder, K.M., Bhunia, A.K. (2010). *Listeria monocytogenes* uses *Listeria* adhesion protein (lap) to promote bacterial transepithelial translocation and induces expression of LAP receptor Hsp60. *Infection and Immunity, 78(12),* 5062-5073.

Camejo, A., Carvalho, F., Reis, O., Leitão, E., Sousa, S., Cabanes, D. (2011). The arsenal of virulence factors deployed by *Listeria monocytogenes* to promote its cell infection cycle. *Virulence, 2*(5), 379-394.

Candela, M., Perna, F., Carnevali, P., Vitali, B., Ciati, R., Gionchetti, P., Rizzello, F., Campieri, M., and Brigidi, P. (2008). Interaction of probiotic *Lactobacillus* and *Bifidobacterium* strains with human intestinal epithelial cells: Adhesion properties, competition against

enteropathogens and modulation of IL-8 production. *International Journal of Food Microbiology, 125*(3), 286-292.

Chiba, S., Nagai, T., Hayashi, T., Baba, Y., Nagai, S., Koyasu, S. (2011). Listerial invasion protein internalin B promotes entry into ileal Peyer's patches *in vivo*. *Microbiology and Immunity, 55*(2), 123-129.

Collado, M.C., Meriluoto, J., Salminen, S. (2007). Measurement of aggregation properties between probiotics and pathogens: *In vitro* evaluation of different methods. *Journal Microbiological Methods, 71*(1), 71-74.

Cossart, P. (2001). Molecular and cellular basis of the infection by *Listeria monocytogenes*: An overview. *International Journal of Medical Microbiology, 291*(6), 401-409.

Cossart, P. (2011). Illuminating the landscape of host-pathogen interactions with the bacterium *Listeria monocytogenes*. *Procedings of National Academy of Science USA, 108*(49), 19484-19491.

Cossart, P., Lecuit, M. (1998). Interactions of *Listeria monocytogenes* with mammalian cells during entry and actin-based movement: Bacterial factors, cellular ligands and signaling. *EMBO Journal, 17*(14), 3797-3806.

Czuprynski, C. (2005). *Listeria monocytogenes*: Silage, sandwiches and science. *Animal Health Research Reviews, 6*(2), 211-217.

Drevets, D.A., Bronze, M.S. (2008). *Listeria monocytogenes*: Epidemiology, human disease, and mechanisms of brain invasion. *FEMS Immunology and Medical Microbiology, 53*(2), 151-165.

Drolia, R., Tenguria, S., Durkes, A.C., Turner, J.R., Bhunia, A.K. (2018). *Listeria* adhesion protein induces intestinal epithelial barrier dysfunction for bacterial translocation. *Cell Host & Microbe, 23*(4), 470-484.e7.

Duary, R., Rajput, Y., Batish, V., Grover, S. (2011). Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. *Indian Journal of Medical Research, 134*(5), 664-671.

Freitag, N.E., Port, G.C., Miner, M.D. (2009). *Listeria monocytogenes* -from saprophyte to intracellular pathogen. *Nature Reviews in Microbiology, 7*(9), 623.

Ghosh, P., Halvorsen, E.M., Ammendolia, D.A., Mor-Vaknin, N., O'riordan, M.X.D., Brumell, J.H., Markovitz, D.M., Higgins, D.E. (2018). Invasion of the brain by *Listeria monocytogenes* is mediated by inlf and host cell vimentin. *MBio, 9*(1).

Giepmans, B.N.G., Van Ijzendoorn, S.C.D. (2009). Epithelial cell–cell junctions and plasma membrane domains. *Biomembranes, 1788*(4), 820-831.

Gilot, P., André, P., Content, J. (1999). *Listeria monocytogenes* possesses adhesions for fibronectin. *Infection and Immunity, 67*(12), 6698–6701.

Gray, M.J., Freitag, N.E., Boor, K.J. (2006). How the Bacterial Pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. *Infection and Immunity, 74*(5), 2505-2512.

Gueimonde, M., Jalonen, L., He, F., Hiramatsu, M., Salminen, S. (2006). Adhesion and competitive inhibition and displacement of human enteropathogens by selected lactobacilli. *Food Research Internationaal, 39*, 467-471.

Guimarães V.D., Gabriel, J.E., Lefèvre, F., Cabanes, D., Gruss, A., Cossart, P., Azevedo, V., Langella, P. (2005). Internalin-expressing *Lactococcus* lactis is able to invade small intestine of guinea pigs and deliver DNA into mammalian epithelial cells. *Microbes and Infection, 7*(5), 836–844.

Hamon, M., Bierne, H., Cossart, P. (2006). *Listeria monocytogenes*: A multifaceted model. *Nature Review. Microbiology, 4*(6), 423-34.

Helmy, Y. A., Kassem, I. I., Kumar, A., Rajashekara, G. (2017). *In vitro* evaluation of the impact of the probiotic *E. coli* Nissle 1917 on *Campylobacter jejuni*'s invasion and intracellular survival in human colonic cells. *Frontiers in Microbiology, 8*, 1588

Jacquet, C., Gouin, E., Jeannel, D., Cossart, P., Rocourt, J. (2002). Expression of ActA, Ami, InlB, and Listeriolysin O in *Listeria monocytogenes* of human and food origin. *Applied and Environmental Microbiology, 68*(2), 616-622.

Jankowska, A., Laubitz, D., Antushevich, H., Zabielski, R., Grzesiuk, E. (2008). Competition of *Lactobacillus paracasei* with *Salmonella enterica* for adhesion to Caco-2 cells. *Journal of Biomedical Biotechnology*, 357964.

Jaradat, Z.W., Bhunia, A.K. (2003). Adhesion, invasion, and translocation characteristics of *Listeria monocytogenes* serotypes in Caco-2 cell and mouse models. *Applied and Environmental Microbiology, 69*(6), 3640-3645.

Kathariou, S. (2002). *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *Journal of Food Protection, 65*(11), 1811-29.

Koo, O., Amalaradjou, M., Bhunia, A. (2012). Recombinant probiotic expressing *listeria* adhesion protein attenuates *Listeria monocytogenes* virulence *in vitro* (Recombinant Probiotic Attenuates Infection). *PLoSONE, 7*(1), E29277.

Le Monnier, A., Autret, N., Join-Lambert, O.F., Jaubert, F., Charbit, A., Berche, P., and Kayal, S. (2007). ActA is required for crossing of the fetoplacental barrier by *Listeria monocytogenes*. *Infection and Immunology, 75*(2), 950-957.

Lebrun, M., Mengaud, J., Ohayon, H., Nato, F., Cossart, P. (1996). Internalin must be on the bacterial surface to mediate entry of *Listeria monocytogenes* into epithelial cells. *Molecular Microbiology, 21*(3), 579-592.

Lecuit, M. (2007). Human listeriosis and animal models. *Microbial Infection, 9*(10), 1216-1225.

Lecuit, M., Vandormael-Pournin, S., Lefort, J., Huerre, P., Gounon, C., Dupuy, C., Babinet, C., Cossart, P. (2001). A transgenic model for listeriosis: Role of internalin in crossing the intestinal barrier. *Science, 292*(5522), 1722-1725.

Lee, Y. K., Puong, K. Y., Ouwehand, A. C., Salminen, S. (2003). Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. *Journal of Medical Microbiology, 52*(10), 925-930.

Lee, Y., Puong, K. (2002). Competition for adhesion between probiotics and human gastrointestinal pathogens in the presence of carbohydrate. *British Journal of Nutrition, 88* (S1), S101-S108.

Liong, M. (2008). Safety of probiotics: Translocation and infection. *Nutrition Reviews, 66*(4), 192-202.

Mead, P. S., Slutsker, L., Dietz, V., McCraig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M., Tauxe, R.V. (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases, 5*(5), 607-625.

Neeser, J., Granato, D., Rouvet, M., Servin, A., Teneberg, S., Karlsson, E. (2000). *Lactobacillus johnsonii* Lal shares carbohydrate-binding specificities with several enteropathogenic bacteria. *Glycobiology, 10*(11), 1193-1199.

Nikitas, G., Deschamps, C., Disson, O., Niault, T., Cossart, P., Lecuit, M. (2011). Transcytosis of *Listeria monocytogenes* across the intestinal barrier upon specific targeting of goblet cell accessible E-cadherin. *Journal of Experimental Medicine, 208*(11), 2263-2277.

Pavan, S., Mercenier, A., and Desreumaux, P. (2003). Use of mouse models to evaluate the persistence, safety, and immune modulation capacities of lactic acid bacteria. *Clinical and Diagnostic Laboratory Immunology, 10*(4), 696-701.

Pizarro-Cerdá, J., Cossart, P., Herrington, C.S., Douek, D.C. (2006). Subversion of cellular functions by *Listeria monocytogenes*. *Journal of Pathology, 208*(2), 215-223.

Robbins, J., Skrzypczynska, K., Zeldovich, V., Kapidzic, M., Bakardjiev, A., Schneider, D. (2010). Placental syncytiotrophoblast constitutes a major barrier to vertical transmission of *Listeria monocytogenes* (*Listeria* Infection of the Placenta). *PLoS Pathogens, 6*(1), E1000732.

Seveau, S., Pizarro-Cerda, J., Cossart, P. (2007). Molecular mechanisms exploited by *Listeria monocytogenes* during host cell invasion. *Microbes and Infection, 9*(10), 1167-1175.

Sheehan, V.M., Sleator, R.D., Fitzgerald, G.F., Hill, C. (2006). Heterologous expression of BetL, a betaine uptake system, enhances the stress tolerance of *Lactobacillus salivarius* UCC118. *Applied and Environmental Microbiology, 72*(3), 2170-2177.

Sherman, P.M., Johnson-Henry, K.C., Yeung, H.., Ngo, P.S. C., Goulet, J., Tompkins, T. A. (2005). Probiotics reduce enterohemorrhagic *Escherichia coli* O157:H7- and enteropathogenic *E. coli* O127:H6-induced changes in polarized T84 epithelial cell monolayers by reducing bacterial adhesion and cytoskeletal rearrangements. *Infection and Immunology, 73*(8), 5183-5188.

Stavru, F., Bouillaud, F., Sartori, A., Ricquier, D., Cossart, P. (2011). *Listeria monocytogenes* transiently alters mitochondrial dynamics during infection. *Proceedings of National Academy of Science USA, 108*(9), 3612-3617.

Tuomola, E., Ouwehand, A., Salminen, S. (1999). The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. *FEMS Immunology and Medical Microbiology, 26* (2), 137-142.

Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J., Roy, N.C. (2011). Regulation of tight junction permeability by intestinal bacteria and dietary components. *Journal of Nutrition 141*(5), 769–776.

Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J., Kreft, J. (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clinical Microbiology Reviews*, 14(3), 584–640.

Volzing. K., Borrero, J., Sadowsky, M.J., Kaznessis, Y.N. (2013), Antimicrobial peptides targeting Gram-negative pathogens, produced and delivered by lactic acid bacteria. *ACS Synthetic Biology., 2*(11), 643- 650.

Wolfe, B., Wiepz, G.J., Schotzko, M., Bondarenko, G.I., Durning, M., Simmons, H.A., Mejia, A., Faith, N.G., Sampene, E., Suresh, M., Kathariou, S., Czuprynski, C.J., Golos, T.G. (2017). Acute fetal demise with first trimester maternal infection resulting from *Listeria monocytogenes* in a nonhuman primate model. *MBio, 8*(1).

Yano, A., Takahashi, K., Mori, Y., Watanabe, S., Hanamura, Y., Sugiyama, T., Inoue, N. (2018). Peyer's patches as a portal for DNA delivery by *Lactococcus lactis in vivo*. *Biological and Pharmaceutical Bulletin, 41*(2), 190-197.

Chapter 4

Lactobacillus casei expressing internalin AB genes of *Listeria monocytogenes* protects Caco-2 cells from listeriosis-associated damages under simulated intestinal conditions

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4. ABSTRACT

Background: *Listeria monocytogenes* is an intracellular pathogen that survives ingestion and then employ a number of strategies to survive challenging gastrointestinal conditions. It then proliferates in the gut and subsequently cause listeriosis. Therefore, inhibition of its adherence to receptors on the intestine is crucial in controlling its infection. In this study, we investigated the effects of the recombinant *L. casei* expressing InlAB on *L. monocytogenes* epithelial infection processes under simulated intestinal conditions. The effect of the resultant recombinant *L. casei* (Lbc^{InlAB}) on *L. monocytogenes* was compared to that of the previously cloned *L. casei* expressing *Listeria* adhesion protein (Lbc^{LAP}). **Materials and Methods:** The confluent Caco-2 cell monolayer was pre-exposed to different *L. casei* strains at a multiplicity of exposure ((MOE) =10) for various exposure times before infection with *L. monocytogenes* at a multiplicity of infection ((MOI) =10) under simulated intestinal conditions. Subsequently, adhesion, invasion and translocation of the Caco-2 cells by *L. monocytogenes* under these conditions were investigated. Furthermore, its mediated cytotoxicity on Caco-2 cells and impact on tight junction integrity were analysed. **Results:** There was a significant increase ($p<0.0001$) in adherence to, invasion and translocation through the Caco-2 cells by the recombinant *L. casei* strains when compared to the wild type. Although the recombinant strains exhibited enhanced inhibition of *L. monocytogenes*, none of them was able to displace *L. monocytogenes* cells already attached to the monolayer. Lbc^{InlAB} displayed a considerably pronounced inhibition of invasion and translocation while Lbc^{LAP} better reduced adhesion. Additionally, pre-exposure to recombinant strains reduced cell mediated toxicity and protected the tight junction integrity with Lbc^{LAP} showing better effects than Lbc^{InlAB}. **Conclusion:** Thus, the recombinant *L. casei* expressing internalin AB shows potential for use as a prophylactic intervention strategy for targeted control of *L. monocytogenes* intestinal infection phase.

4.2. INTRODUCTION

Listeria monocytogenes is abundant in nature and has proven to proliferate under various environments conditions (Smith *et al*., 2013) including temperatures between -1.5 to 45°C and broad pH range of 4.0 to 9.6. It is well suited to survive in foods, transit through the gastrointestinal tract to ultimately cause the disease generally referred to as listeriosis. This is dependent on different strategies that are employed by the microorganisms, affording them the ability to survive the challenging microenvironments of the gastrointestinal tract (Hamon *et al*., 2006; Schuppler and Loessner 2010). It enters the body through gastrointestinal mucosal surfaces to cause infections (Drolia and Bhunia 2019). In its disease progression, *L. monocytogenes* employs the *Listeria* adhesion protein (LAP), reported promoting its adhesion and transmigration across the epithelial barrier during the intestinal phase of infection (Jagadeesan *et al*., 2010; Drolia *et al*. 2018). It then uses a number of proteins including among others the surface protein internalin A (InlA) (Mengaud *et al*., 1996; Lecuit *et al*., 1997) and internalin B (Chiba *et al*., 2011) to attach to and gain entry into host cells. This invasion has also been shown to be associated with murine M cells both *in vivo* and *in vitro* (Jensen *et al*., 1998).

Attenuated strains of foodborne pathogens such as *L. monocytogenes* have been used as vaccines for their control. However, such strains present two most important risks. Firstly, the attenuated strain has a potential for reversion to its virulent phenotype post administration. Secondly, they can be virulent in partially immunocompetent (young infants; elderly) or immunocompromised individuals as they retain residual virulence (Mercenier *et al*., 2000). These risks prompted an interest into a search for alternative strategies for pathogen control. Non-pathogenic transient bacteria in the digestive tract were then suggested as an alternative that can be used to substitute the pathogenic bacteria (Marelli *et al*. 2011; Tarahomjoo, 2012). Probiotics are candidates of choice for such bacteria as they have been reported to confer a health benefit on the host (FAO/WHO, 2002). They have been shown to competitively inhibit foodborne pathogens thereby ultimately controlling infections (Amalaradjou and Bhunia 2013; Behnsen *et al*. 2013). Their advantages include but are not limited to colonization of the mucosal surface, acid and bile salts tolerance allowing for survival and transition through the gastrointestinal tract (GIT) and their continued colonization at this site (Amalaradjou and Bhunia, 2013). However, several studies

reported that probiotics are generic in their action and that they sometimes fail to inhibit some pathogens.

In the effort to enhance the effectiveness or antipathogenic effects of probiotics, bioengineering has been used as an alternative strategy. This strategy is used for protection against pathogens by cloning and expression of pathogens' virulence genes into probiotics and subsequently used to competitively exclude pathogens. These genetically engineered probiotics, depending on the virulence genes they are expressing, can be used either as a prophylactic or as treatment alternatives of the specific pathogens. This mechanism has been reported by different researchers and showed enhanced inhibition of the pathogens by bioengineered probiotics when compared to their wild- type counterparts (Chu *et al.* 2005; Sánchez *et al*. 2011; Koo *et al.* 2012). Due to promising results reported in these studies for control of different enteric pathogens, the invasion genes internalin AB (InlAB) of *L. monocytogenes* was cloned and expressed into probiotic *Lactobacillus casei* for the inhibition of *L. monocytogenes* adhesion, invasion and translocation *in vitro*, using the Caco-2 cells grown and maintained in the cell culture media, Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (FBS) (Mathipa *et al*., 2019). The resultant recombinant probiotic showed enhanced inhibition on all the infection stages compared to the wild-type *L. casei*. Although the results were positive, they were still not appropriate for making inferences about how the recombinant *L. casei* would affect the *L. monocytogenes* intestinal infection phase as the media used did not sufficiently simulate the intestinal conditions.

The Caco-2 cell culture model is widely used as a well-established method to assess potential intestinal permeability (Ingels *et al*., 2004; Fossati *et al*., 2008). Different media such as the buffered salt solution, Hanks' balanced salt solution (HBSS) buffered with HEPES (10 mM) at pH 7.4, and the DMEM containing amino acids and vitamins both supplemented with glucose have been commonly used in permeability studies. However, these media have been criticised for supporting cell growth, which render them inappropriate models for the epithelial infection process (Kapitza *et al*., 2007; Lind *et al*., 2007). This raised the need for alternative media that can address this shortcoming and thereby give a better simulation of the intestinal conditions. In order to address this, simulated intestinal fluids (SIF), namely, the Fed State SIF (FeSSIF) originally proposed for evaluation of drug dissolution kinetics (Galia *et al*., 1998), and Fasted State SIF (FaSSIF), were checked for their compatibility with Caco-2 monolayer. It was found that FaSSIF

exhibited cytotoxicity to Caco-2 while FaSSIF was compatible with the Caco-2 monolayer (Ingels *et al*., 2002, Ingels *et al*., 2004; Fossati *et al*., 2008). Brouwers *et al*., (2006) reported that data generated using FaSSIF were similar to that obtained with actual human intestinal aspirates collected in the fasted state. Hence, in order to determine the effect of the recombinant *L*. *casei* expressing InlAB on the epithelial infection process of *L*. *monocytogenes in vitro*, Caco-2 cell culture model and FaSSIF as the medium was used.

4.3. MATERIALS AND METHODS

4.3.1. Bacterial strains, plasmids and growth conditions

Listeria monocytogenes F4244 (serovar 4b) was grown in Tryptone Soy broth supplement with 0.6% yeast extract (TSB-YE) or Brain Heart Infused (BHI) at 37^{o}C for 18 h. *Lactobacillus casei* WT (Lbc^{WT}) was grown in de Man Rogosa Sharpe (MRS) broth while the Lbc^{WT} (carrying pLP401T) vector control (Lbc^{V}) and recombinant *L. casei* expressing InlAB (Lbc^{InlAB}) were grown in MRS broth with 2 µg/ml erythromycin anaerobically at 37^{o}C for 16 h. Recombinant *L. casei* expressing LAP (Lbc^{LAP}) (Our laboratory) was also grown in MRS broth containing 2 µg/ml erythromycin anaerobically at 37^{o}C for 16 h.

4.3.2. Preparation of the Fasted state simulated intestinal fluids (SIF)

The fasted state simulated intestinal fluid was prepared as per Dressman *et al*., (1998). Briefly, 0.78 g potassium hydrogen phosphate (KH_2PO_4), 3.28 g of potassium chloride (KCl), 5 mM sodium taurocholate (representative bile salt) and 1.5 mM lecithin were suspended in 150 ml distilled water. The pH of the solution was adjusted to pH 6.8 with 1M NaOH or 1M HCl, and then its volume was made up to 200 ml with distilled water. The SIF was sterilized by filtering through 0.2 µm filter to avoid thermal denaturation of the media components. The SIF was stored in the fridge, at 4°C, and used within 24 h post preparation.

4.3.3. Recombinant *L. casei* adhesion and invasion of Caco-2

4.3.3.1. Caco-2 cell culture

Human colon carcinoma cell line Caco-2 (HTB37; American Type Culture Collection) was cultured in Dulbecco's modified eagle's medium (DMEM with high glucose, HyClone^{TM}, GE, Logan, UT) supplemented with 10% Fetal Bovine Serum (FBS Atlanta Biologicals, GA) (D10F). The cells were grown in flasks (Greiner- Bio-One) for up to 10- 12 days. The cells were then trypsinized as per Malik and Yadav (2013). Briefly, 3 ml trypsin solution was added to the confluent cells and then incubated at 37°C for 7 minutes to remove the cells from the surface. To inactivate trypsin, 7 ml of D10F was added to the solution and thoroughly pipetted up and down to break the cell clumps. The cells were then seeded in 12-well plates at a density of 1 x 10^5 cells/

well. The plates were incubated at 37°C in the presence of 7% CO_2 in a cell culture incubator for 10–12 days until they reached confluency (10^6 cells/ well) or until monolayers are formed.

4.3.3.2. Adhesion and invasion assays

The Caco-2 cells were grown in the 12-well plates until they reached confluency (10^6 cells/ well). Overnight (18 h) bacteria pre-cultivated in their respective broths were used. The bacterial cultures were washed twice with PBS, their absorbance adjusted to OD 600 = 1 and then they were suspended in FaSSIF to a final concentration of 1×10^7 CFU/ml (MOE = 10 or MOI =10). The Caco-2 cell monolayer was washed three times with DMEM. The monolayer was then exposed separately to the *L. casei* strains (Lbc^{WT}, Lbc^{V}, Lbc^{InlAB} or Lbc^{LAP}) and *L. monocytogenes* and incubated at 37ºC with 5% CO_2 for 1 h. Excess media was removed, and the cell monolayer was washed three times with DMEM. To enumerate the adhered bacterial cells, cell monolayers were treated with 0.1% Triton X-100, incubated at 37ºC for 10 min. For the invasion assay, the monolayers were exposed to *L. monocytogenes* and *L. casei* and then washed as was done in the adhesion assay, treated with gentamycin (50 μg/ml, 1 h) and with 0.1% Triton X-100 (37ºC, 10 min). The lysed cell suspensions from both adhesion and invasion experiments were serially diluted in PBS before plating on MRS, MRS supplemented with erythromycin (2 μg/ml) and Modified Oxford (MOX) agar for Lbc^{WT}, recombinant *L. casei*, and *L. monocytogenes*, respectively. All the plates were incubated at 37ºC for 24- 48 h before bacterial enumeration.

4.3.4. Determination of *L. monocytogenes* exclusion mode by the *L. casei* strains

The competitive exclusion assay was done as per Koo *et al.* (2012) with minor modifications. The absorbance of the bacterial cultures was adjusted to OD 600 = 1 after they were washed twice with PBS, and then they were suspended in FaSSIF to a final concentration of $\times 10^7$ CFU/ml (MOI = 10). For competitive adhesion, *L. monocytogenes* was co-inoculated with each of the *L. casei* strains (Lbc^{WT}, Lbc^{V}, Lbc^{InlAB} or Lbc^{LAP}) to Caco-2 cell monolayer and incubated for 1 h. Adherent bacteria were enumerated as before.

In the inhibition of adhesion assay, the Caco-2 cell monolayers were first inoculated with each *L. casei* strain and incubated for 1 h. After incubation, bacteria not bound to the monolayer were removed by washing of the wells four times using DMEM. *L. monocytogenes* was then added to

the wells and plates were incubated for one more hour. Adhered bacteria were released and plated as above. For displacement of adhesion, Caco-2 cells were first inoculated with *L. monocytogenes* and incubated for 1 h. Then unbound bacteria were washed off as in the inhibition of adhesion assay. *L. casei* strains were then added to the wells and plates incubated for another 1 h. Adhered bacteria were released and plated on MRS, MRS supplemented with 2 µg/ml of erythromycin and MOX agar plates for enumeration of Lbc^{WT}, recombinant *L. casei* and *L. monocytogenes*, respectively (Koo *et al*., 2012).

4.3.5. Inhibition of adhesion and invasion by the *L. casei* strains

Bacteria were pre-cultivated in their respective broths for 18 h. The bacterial cultures were washed twice with PBS after adjusting their absorbance to OD 600 = to 1, followed by their resuspension in FaSSIF to the final concentration of 1×10^7 CFU/ml (MOE/MOI = 10). The Caco-2 cell monolayer was washed and then exposed to the *L. casei* strains for 1, 4, 16 and 24 h at 37°C in the humidified incubator with 5% CO_2. Excess medium in the wells containing unbound *L. casei* was removed and replaced with 500 µl of *L. monocytogenes* suspended in FaSSIF, and the plates incubated for 1 h at 37°C with 5% CO_2. The cells were then washed thrice using DMEM. To enumerate the adhered bacterial cells, cell monolayers were treated with 0.1% Triton X-100, incubated at 37ºC for 10 min before plating onto the respective microbiological media as already mentioned.

For inhibition of *L. monocytogenes* invasion, the Caco-2 cell monolayers were washed three times with DMEM and then exposed to each *L. casei* strain for 1, 4, 16 and 24 h at 37°C with 5% CO_2. Excess *L. casei* strains were removed and replaced with 500 µl of *L. monocytogenes* suspended in SIF and then incubated for 1 h at 37°C with 5% CO_2. To remove the non-adhered bacteria, the cell monolayers were washed three times with DMEM and then treated for 1 h with gentamycin (50 µg/ml). The invading bacterial counts were determined by plating as above.

4.3.5.1. Caco-2 cells cytotoxicity assay

To determine Caco-2 cell cytotoxicity induced by *L. monocytogenes* after pre-exposure to *L. casei* over time, we performed the LDH assay (Koo *et al*. 2012). The supernatants after infection with

L. monocytogenes for 1 h were collected and used to analyse for lactate dehydrogenase (LDH) enzyme release. Caco-2 cells that were treated with 500 µl of 0.1% Triton X-100 per well were used as a positive control while those treated with DMEM were used as the negative control. From the supernatants collected, 100 µl were transferred to the 96-well flat bottom plate in triplicates and was analysed using Pierce LDH cytotoxicity assay kit (Thermo Scientific, USA) following the protocol from the manufacturer.

4.3.6. Transcellular translocation of *L. casei* strains and subsequent inhibition of *L. monocytogenes* transepithelial translocation by recombinant *L. casei*

The Caco-2 cells were grown in 12 well trans-well filter inserts (3-µm pore size) for 20-25 days to reach confluence. TEER of Caco-2 cells was quantified using Millicell ERS system (Millipore, Billerica, MA) and a TEER value of more than 200 was used for all the experiments. Overnight (18 h) bacteria pre-cultivated in their respective broths were used. The bacterial cultures were washed twice with PBS and then resuspended in FaSSIF (MOE = 10). For determining baseline translocation by *L. casei* strains or *L. monocytogenes*, the cell monolayer was washed three times with DMEM and then the bacteria were added separately to the apical wells, followed by incubation of microwell plates at 37°C with 5% CO_2 for 2 h. The liquid from the basal well was collected, serially diluted in PBS and then plated for the enumeration of viable cells (CFU/ ml).

For the inhibition of *L. monocytogenes* translocation, *L. casei* strains were first added to the apical wells and incubated for 1, 4, 16 and 24 h at 37°C with 5% CO_2. The liquid from the basal wells was collected, serially diluted in PBS and then plated on MRS plate for enumeration of *L. casei* as described. Subsequently, excess *L. casei* were removed and replaced with 500 µl of *L. monocytogenes* suspended in FaSSIF (MOI = 10) and then incubated for 2 h at 37°C with 5% CO_2. The liquid from the basal wells was removed and serially diluted in PBS and then plated on MOX plates for the enumeration *L. monocytogenes*.

4.3.6.1. Epithelial Tight Junction Integrity Analysis

Transepithelial electrical resistance (TEER) of Caco-2 cells was measured before and after the exposure to the bacteria using Millicell ERS system (Millipore, Billerica, MA). Furthermore, we

analysed the epithelial tight junction integrity as per Koo *et al*. (2012). After exposure to *L. monocytogenes*, the tight junction permeability using Dextran^{FITC} (Mr 3–5 kDa; Sigma) permeability through the transwell filter inserts was analysed. Fluorescence of the samples collected from the apical and basolateral chambers was read in a SpectraMax Gemini EM fluorescent plate reader (Molecular Devices; Sunnyvale, CA).

4.3.7. Statistical analysis

All data were analysed using Prism 7 software (GraphPad software Inc., United States), and significance was assigned at $p < 0.05$. Where appropriate, Turkey's multiple comparisons, with $p<0.005$ as a significant difference was used to identify statistically significant differences.

4.4. RESULTS

4.4.1. Adhesion, invasion and translocation profiles of *L. monocytogenes*, *Lb. casei* (WT) and recombinant *L. casei*

Probiotics and foodborne pathogens transit through the gastrointestinal tract (GIT) in order to offer beneficial effects or cause infection, respectively. It was therefore imperative that to determine how expression of invasion genes by *L. casei* would impact its ability to adhere to, invade and translocate the Caco-2 cells under simulated intestinal conditions and compare it to *L. monocytogenes*. **Figure 4.1A** depicts the adhesion profiles of the *L. casei* strains and *L. monocytogenes* to Caco-2 cells in simulated intestinal fluid. There were no statistically significant differences in the adhesion of *L. monocytogenes* F4244 versus Lbc^{WT} ($p= 0.4436$) or Lbc^{V} ($p= 0.9914$) to the Caco-2 cells, which showed adhesion percentages of 7%, 8% and 7.8%, respectively. Conversely, recombinant *L. casei* strains expressing the different genes of *L. monocytogenes* adhered to Caco-2 cells at levels significantly higher than those recorded for *L. monocytogenes* ($p= 0.0002$ for Lbc^{InlAB} vs *L. monocytogenes* and $p <0.0001$ for Lbc^{LAP} vs *L. monocytogenes*). Worth noting, adhesion of Lbc^{LAP} was significantly higher than that of Lbc^{InlAB} ($p= 0.0229$).

Invasion (**Fig. 4.1B**) and translocation (**Fig. 4.1C**) profiles of the *L. casei* strains and *L. monocytogenes* in simulated intestinal conditions were investigated. The strains Lbc^{WT} and Lbc^{V} displayed similar trends in invasion and translocation through the Caco-2 cells, both showing 0.08% and 0.13% for invasion and translocation, respectively. There was an increase in both the invasion and translocation of the recombinant *L. casei* (Lbc^{InlAB} and Lbc^{LAP}). Lbc^{InlAB} invaded and translocated through the Caco-2 cells at levels significantly higher compared to those of Lbc^{WT} and Lbc^{V}. Invasion and translocation levels for Lbc^{LAP} were not significantly different to Lbc^{WT} and Lbc^{V} ($p<0.79$), but significantly lower than for Lbc^{InlAB} ($p<0.0001$). *L. monocytogenes* was able to invade and translocate the Caco-2 cell monolayer at significantly higher levels than obtained for all the *L. casei* strains. What was worth noting is that invasion and translocation of Lbc^{InlAB} through the Caco-2 cells was at significantly higher levels than all the other *L. casei* strains.

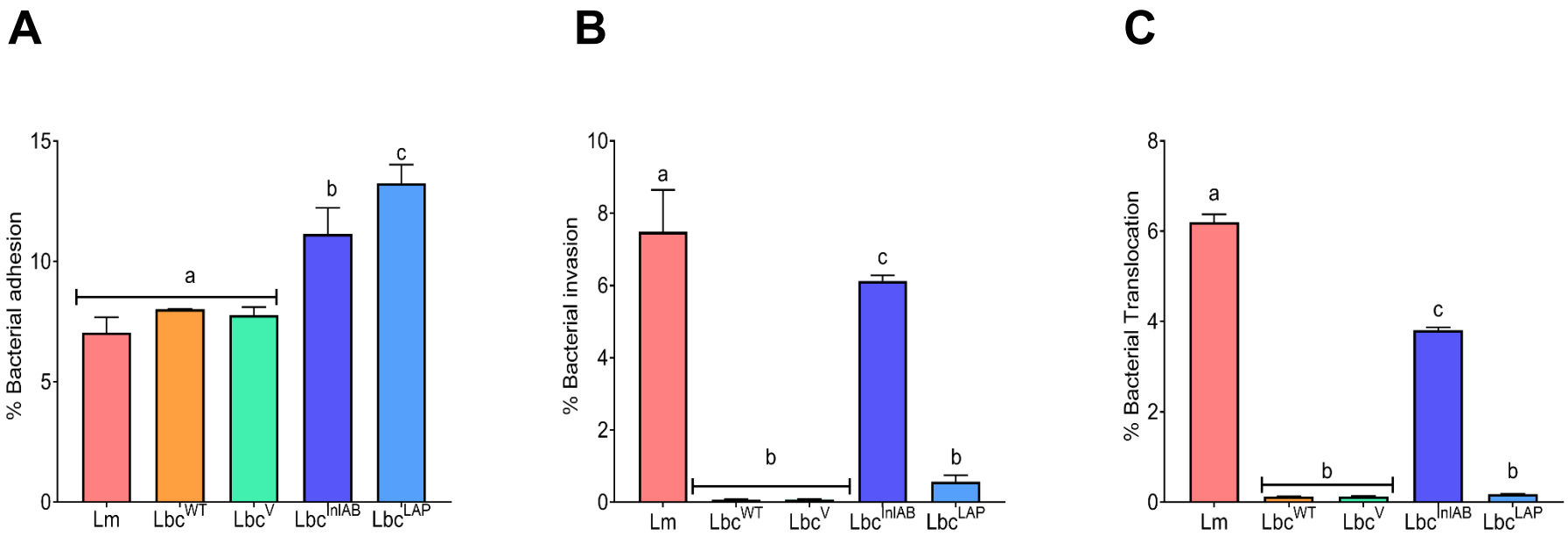

Figure 4.1: Adhesion **(A)**, Invasion **(B)** and Translocation **(C)** of *Listeria monocytogenes* (Lm) and *L. casei* strains (Lbc^{WT}, Lbc^{V}, Lbc^{InlAB} and Lbc^{LAP}) to Caco-2 cells. Percentages were calculated relative to the inoculums that were added to the Caco-2 cells. Data are average (SD) of three independent experiments performed in duplicate (n=6). For each time point bars marked with different letters (a, b, c) indicate a significant difference at $P<0.05$.

4.4.2. Mechanisms of exclusion of *L. monocytogenes* by the *L. casei* strains

Probiotics employ various mechanisms of competition to inhibit or reduce adhesion of pathogens to the intestinal cells. In order to determine which mechanism is employed by recombinant *L. casei* against *L. monocytogenes*, competitive, inhibition and displacement were evaluated as possible mechanisms. Competitive adhesion, inhibition and displacement of adhesion of *L. monocytogenes* by the *L. casei* strains (Lbc^{WT}, Lbc^{V}, Lbc^{InlAB} and Lbc^{LAP}) was evaluated under simulated intestinal conditions (**Fig. 4.2**). The adhesion of *L. monocytogenes* to Caco-2 cells in absence of the *L. casei* strains was recorded as 100% in all the assays and was used to calculate the relative adhesion in the presence of the *L. casei* strains.

Figure 4.2A shows that adhesion of *L. monocytogenes* to Caco-2 cells was insignificantly reduced when it was co-inoculated with Lbc^{WT} and Lbc^{V} ($p= 0.9941$). Reductions of 5.67% and 6% in adhesion of *L. monocytogenes* were recorded for LbcWT and Lbc^{V}, respectively. When co-inoculated with the recombinant strains (Lbc^{InlAB} and Lbc^{LAP}), there was a significant reduction ($p< 0.0001$) in the adhesion of *L. monocytogenes.* There was a 20.48% and 22.34% adhesion reduction by Lbc^{InlAB} and Lbc^{LAP}, respectively. Although both Lbc^{InlAB} and Lbc^{LAP} reduced the adhesion of *L. monocytogenes*, there was no statistical difference in their reduction levels ($p= 0.2620$).

In the inhibition the adhesion (**Fig. 4.2B),** adhesion of *L. monocytogenes* to the Caco-2 cells was reduced by 2.92% and 3.05% due to their pre-exposure to Lbc^{WT} and Lbc^{V}, respectively. The reductions recorded were significant for both Lbc^{WT} ($p= 0.0494$) and Lbc^{V} ($p= 0.0391$), however as expected, there was no significant difference when comparing the inhibition of *L. monocytogenes* adhesion by Lbc^{WT} vs. Lbc^{V} ($p=0.4588$). Adhesion of *L. monocytogenes* was reduced by 18.88% and 14.38% due to pre-exposure of the Caco-2 cells to the recombinant strains Lbc^{InlAB} and Lbc^{LAP}, respectively. Interestingly, these recorded reduction levels were significantly higher when compared to adhesion of *L. monocytogenes* alone ($p<0.0001$). Furthermore, there was a significant difference ($p< 0.0033$) in the reduction of adhesion between the two recombinant strains, with Lbc^{LAP} better decreasing adhesion than Lbc^{InlAB}. Significant differences ($p<0.0001$) were also obtained when comparing inhibition of adhesion by Lbc^{WT} or Lbc^{V} versus Lbc^{InlAB} or Lbc^{LAP}. When looking at the results for displacement of adhesion (**Fig 4.2C**), there were no

significant differences in the adhesion of *L. monocytogenes* alone when compared to in the presence of any of the *L. casei* strains. Furthermore, there were no statistical differences among all the *L. casei* strains in the displacement of *L. monocytogenes*. Thus, the results show that inhibition of adhesion is the mechanism of competition used by the recombinant *L. casei* to reduce interaction of *L. monocytogenes* with the intestinal cells.

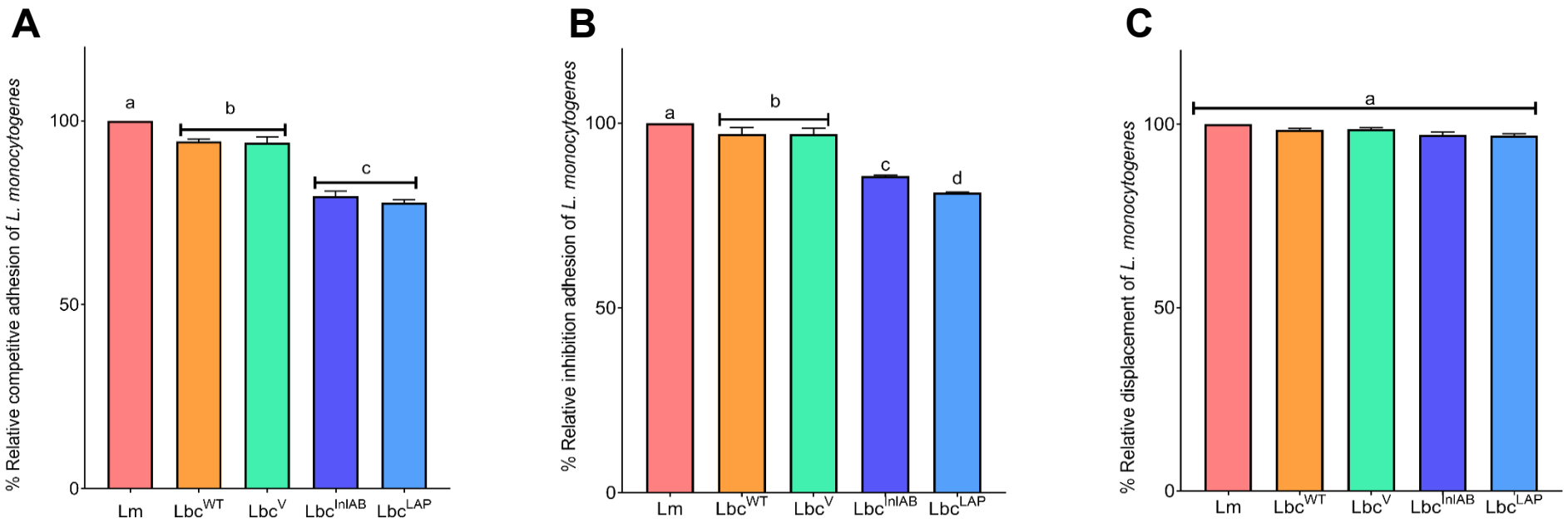

Figure 4.2. Competitive exclusion of *Listeria monocytogenes* (Lm) adhesion to Caco-2 cells by *L. casei* strains (Lbc^{WT}, Lbc^{V}, Lbc^{InlAB} and Lbc^{LAP}), analysed by three different exclusion mechanisms. (**A**) Competitive adhesion: Caco-2 cells were exposed to *L. casei* strains with Lm simultaneously, (**B**) inhibition of adhesion: Caco-2 cells were pre-exposed to *L. casei* strains for 1 h before infection with Lm, and (**C**) Displacement of adhesion: Caco-2 cells were infected with Lm for 1 h before *L. casei* strains (1 h). Adhesion of Lm alone to Caco-2 cells was presented as 100% and percent adhesion was calculated relative to that. Data are averages of three experiments ran in duplicates (n=6). For each time point bars marked with different letters (a, b, c, d) indicate a significant difference at $P<0.05$.

4.4.3. Inhibition of *Listeria monocytogenes* (Lm) adhesion, invasion and translocation over time

In order to determine how inhibition of *L. monocytogenes* adhesion to Caco-2 in SIF will be influenced by duration of pre-exposure of the cell monolayer to *L. casei* strains, adhesion, invasion and translocation of Caco-2 cells under simulated intestinal conditions by *L. monocytogenes* over a 24 h period were evaluated. The effect of different exposure periods to *L. casei* strains on adhesion of *L. monocytogenes* to Caco-2 cells is presented in **Fig. 4.3A**. Adhesion of *L. monocytogenes* was more reduced the longer the Caco-2 cells were pre-treated with Lbc^{WT}, with reductions of 3.29%, 4.51 and 12.96% recorded for 4, 6 and 24 h pre-exposure times, respectively. Significant reductions due to Lbc^{WT} were recorded after 4 h ($p=0.0007$) and 16-24 h ($p<0.0001$). Improved reductions were obtained due to pre-exposure to recombinant *L. casei* strains, with reduction levels of 14.36% and 18.58% after 1 h as well as 57.66% and 61.52% recorded for pre-exposure to Lbc^{InlAB} and Lbc^{LAP}, respectively. Contrary to what was observed for Lbc^{WT}, significant reductions in adhesion ($p<0.0001$) were obtained for Lbc^{InlAB} and Lbc^{LAP} for all exposure periods. Furthermore, even though prolonged exposure to either of the recombinant *L. casei* strains enhanced inhibition of adhesion, it was interesting to observe that pre-exposure to Lbc^{LAP} maintained significantly higher reductions than Lbc^{InlAB} throughout the 24 h ($p<0.0001$). Similar trends were observed for invasion (**Fig. 4.3B)** and translocation (**Fig. 4.3C**) of Caco-2 cells by *L. monocytogenes* subsequent to their prolonged pre-exposure to *L. casei* strains. Pre- exposure of the Caco-2 cells to Lbc^{WT} for 1 to 16 h showed no significant reduction of invasion ($p= 0.3088$), however the 24 h exposure time resulted in a significant reduction ($p<0.0001$) (**Fig. 4.3B**). However, translocation of *L. monocytogenes* was significantly reduced ($p<0.0001$) by this strain from 1 h up to 24 h pre-exposure times (**Fig. 4.3C**). No significant reduction in invasion were obtained due to pre-exposure of the Caco-2 cells to Lbc^{InlAB} or Lbc^{LAP} for 1-4 h, while the significant reduction ($p<0.0001$) was evident for 16 h to 24 h pre-exposure period to these strains. Similarly, when comparing the recombinant *L. casei* strains, there were no significant differences at 1 and 4 h pre-exposure, however, they exhibited significant differences after 16 and 24 h pre-exposure ($p<0.0001$). Pre- exposure to Lbc^{InlAB} and Lbc^{LAP} for 24 h showed a significant ($p<0.0001$) reduction of *L. monocytogenes* invasion with 48.96% and 32.22% reductions recorded for Lbc^{InlAB} and Lbc^{LAP}, respectively (**Fig. 4.3B**).

For translocation assays (**Fig. 4.3C**), after 24 h of pre-exposure we recorded reductions of 17.81% and 15.67% for Lbc^{InlAB} and Lbc^{LAP}, respectively. Prolonged exposure of the Caco-2 cells to the recombinants showed an even significantly ($p<0.0001$) enhanced reduction of translocation. Intriguingly, Lbc^{InlAB} was always significantly better than Lbc^{LAP} ($p<0.0001$) at reducing invasion and translocation of Caco-2 cells by *L*. *monocytogenes*. Overall, the results indicate that the longer the Caco-2 cells were pre-exposed to *L*. *casei* strains before their infection with *L*. *monocytogenes*, the more the adhesion, invasion and translocation of *L*. *monocytogenes* was reduced. Furthermore, the presence of listeria adhesion protein enhanced inhibition of adhesion while internalins enhanced inhibition of invasion and translocation.

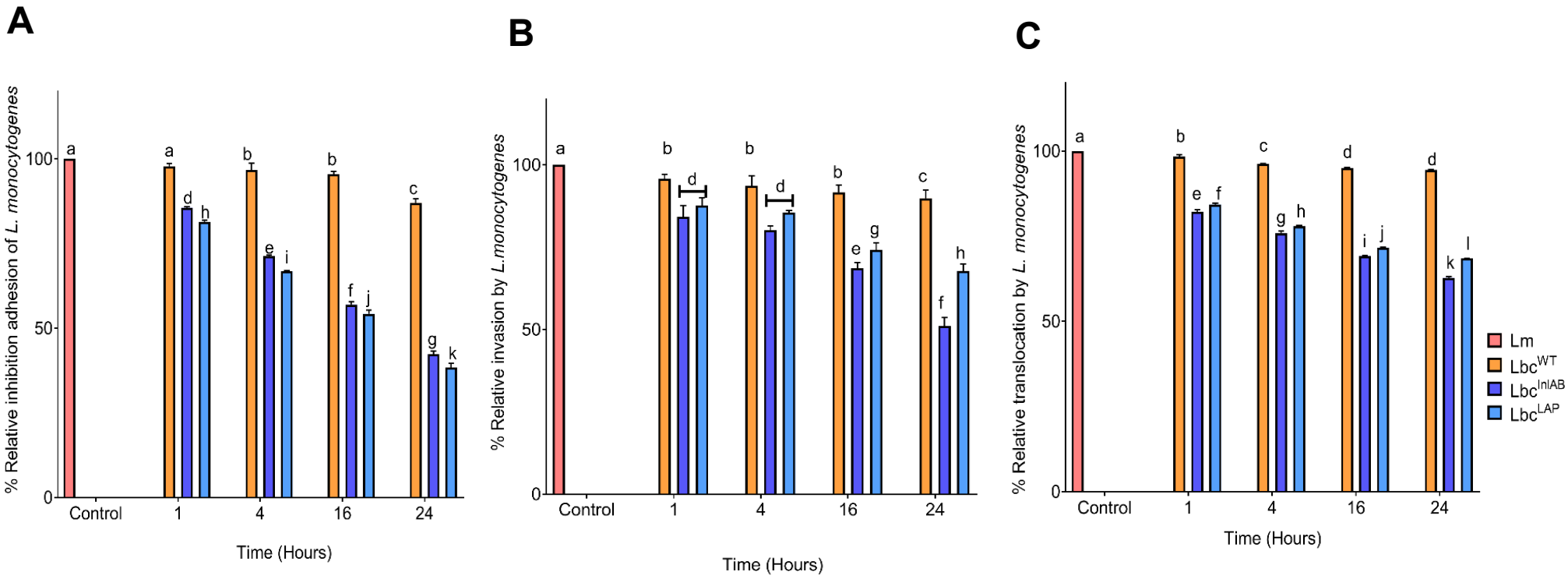

Figure 4.3. Overtime inhibition of *Listeria monocytogenes* (Lm) adhesion (**A**), invasion (**B**) and translocation (**C**) by *L. casei* strains (Lbc^{WT}, Lbc^{InlAB} and Lbc^{LAP}). Caco-2 cells were pre-exposed to the *L. casei* strains for 1, 4, 16 and 24 h before infection with Lm for 1 h for adhesion and invasion and 2 h for translocation. Data are averages of three experiments ran in duplicates (n=6). For each time point bars marked with different letters (a, b, c, d, e, f, g, h, I, j, k, l) indicate a significant difference at $P<0.05$.

4.4.4. Cytotoxicity of *L. monocytogenes* to Caco-2 cells in presence of *L. casei* strains

L. monocytogenes mediated cytotoxicity to the Caco-2 was investigated (**Fig. 3.4**) using the lactate dehydrogenase (LDH) assay. In the absence of *L. casei* strains, *L. monocytogenes* treatment for 1 h induced 70.25% cytotoxicity to Caco-2 cells. Pre- exposure of the cells to *L. casei* strains showed a reduction in the cell cytotoxicity. *L. monocytogenes* induced only 63.7% and 65.42% cytotoxicity after 1 h and 24 h when pre-exposure to Lbc^{WT}, respectively, while there was 8.45% and 30.45% when pre-exposure to Lbc^{InlAB} for 1 and 24 h, respectively (**Fig. 3.4**). When pre- exposed to Lbc^{LAP} for 1 and 24 h, *L. monocytogenes* induced only 0.34% and 18.25% cytotoxicity, respectively. These data indicate pretreatment with recombinant *L. casei* strains provide a significant protection ($p<0.0001$) against the cytotoxic effect of *L. monocytogenes* than the Lbc^{WT}.

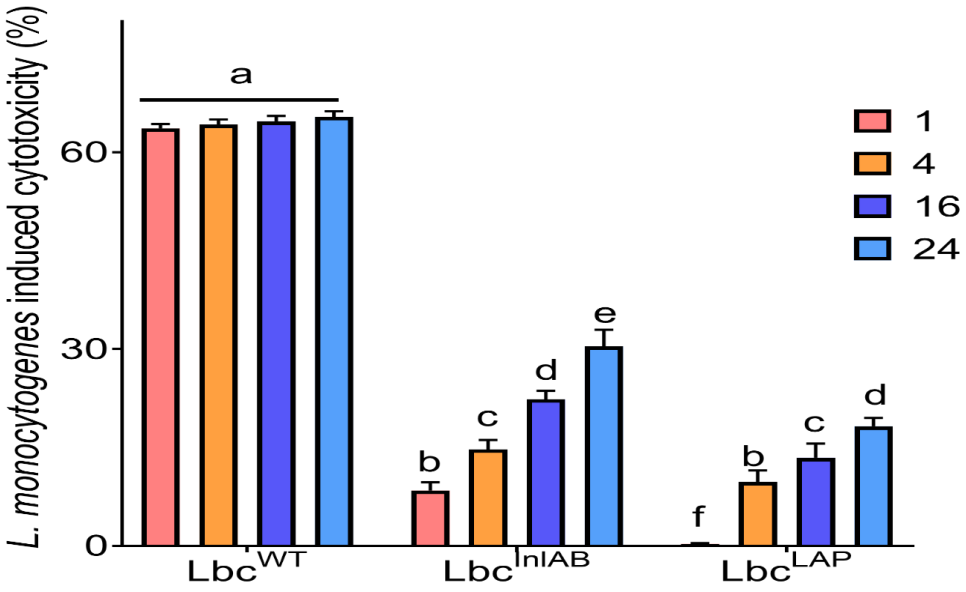

Figure 4.4: Cytotoxicity of *Listeria monocytogenes* (Lm) in Caco-2 cells pre-exposed with *L. casei* over time. Cytotoxicity value for *L. monocytogenes* treatment in the absence of *L. casei* strains was 70.25%. Data are averages of three experiments ran in duplicates (n=6). For each time point bars marked with different letters (a, b, c, d, e, f) indicate a significant difference at $P<0.05$.

4.4.5. Epithelial tight junction integrity analysis

The integrity of the Caco-2 cells infected with *L. monocytogenes* alone or after their exposure to *L. casei* strains in simulated intestinal fluid was measured using the TEER (**Fig. 4.5A**) and Dextran^{FITC} (**Fig. 4.5B**) analyses. The results obtained for both analyses complemented those for cytotoxicity analysis. When the Caco-2 cells were pre- treated with *L. casei* strains for all exposure periods tested, there were lower TEER reduction changes than that of cells treated with *L. monocytogenes* alone (**Fig. 4.5A**). TEER reduction changes were also lower due to pre-exposure to recombinant *L. casei* strains than that due to their wild-type counterpart. When comparing recombinant strains, TEER reductions were lower for Lbc^{LAP} than Lbc^{InlAB}. These results showed that under simulated intestinal conditions, recombinant *L. casei* strains protected the integrity of tight junctions between the Caco-2 cells, with Lbc^{LAP} showing better protection than Lbc^{InlAB}. However, as was also observed for cytotoxicity analysis, prolonged exposure of Caco-2 cells to the *L. casei* strains in SIF had negative effects on Caco-2 cells as it resulted in higher TEER reductions when compared to shorter exposure periods. Nevertheless, even after 24 h, the TEER reductions for Caco-2 cells pre-exposed to *L. casei* strains were still lower than those of cells that were treated with *L. monocytogenes* alone.

The results of the Dextran^{FITC} analysis indicated that pre-treatment of the Caco-2 cells with *L. casei* strains reduced their permeability induced by *L. monocytogenes* infection as the amount of Dextran^{FITC} stain recovered from the basal chamber of the transwell plate was always higher for cells infected with *L. monocytogenes* alone than that for those pre-exposed to *L. casei* strains (**Fig. 4.5B**). Comparing the amount of dye recovered in the basal chamber for cells pre-exposed to *L. casei* strains, recombinant *L. casei* strains (Lbc^{InlAB} and Lbc^{LAP}) showed better protection than Lbc^{WT}, however, Lbc^{LAP} showed better protection than Lbc^{InlAB}. These differences are in agreement with those found in the inhibition of translocation and the TEER reduction, meaning that Lbc^{LAP} was better at protecting the integrity of Caco-2 cells under simulated intestinal conditions. The amount of dye recovered increased with an increase in exposure to *L. casei* strains, with levels higher after 24 h than after 2 h for all the probiotic strains. This results confirmed observations from the cytotoxicity and TEER reduction assays, which showed that prolonged exposure of Caco-2 cells to the *L. casei* strains in SIF had negative effects them.

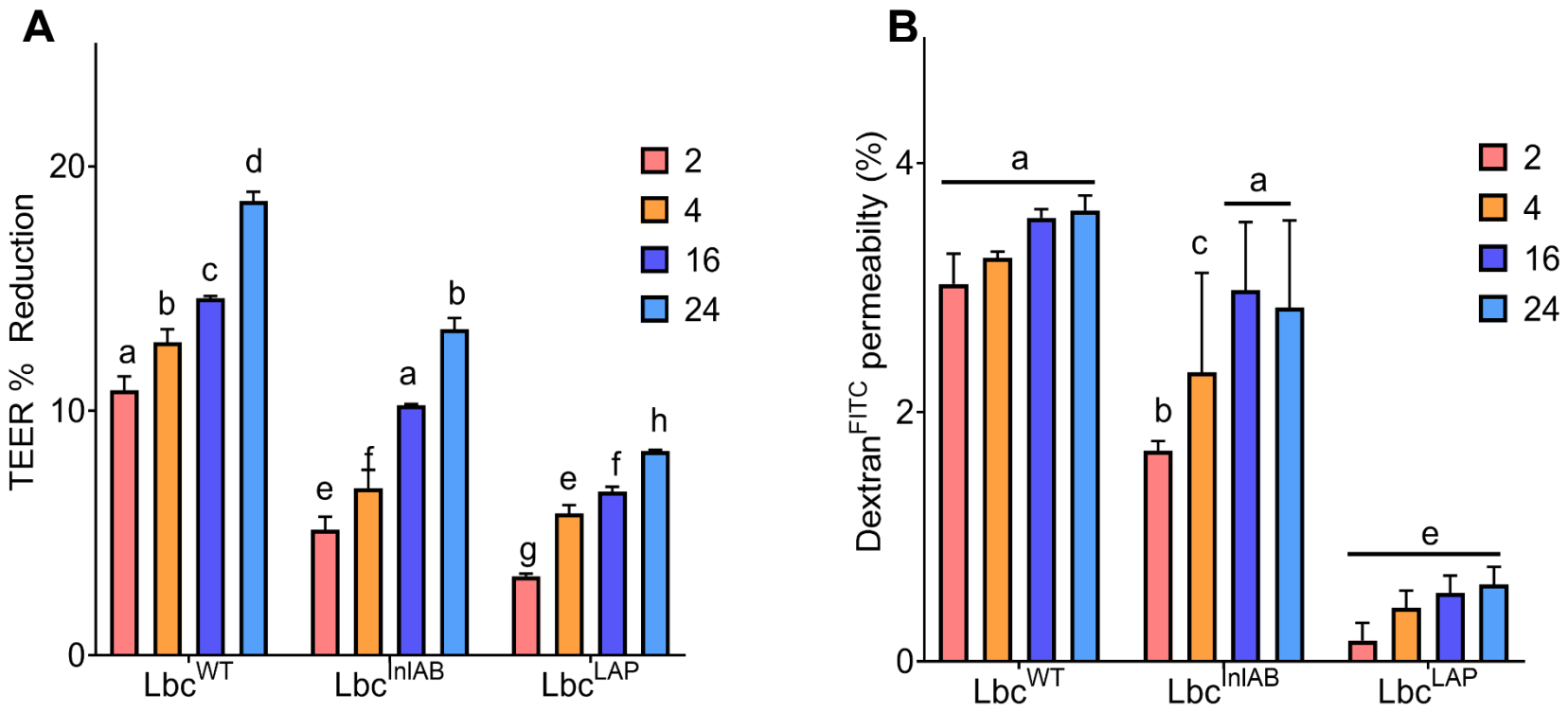

Figure 4.5: Caco-2 cell permeability analysis using transepithelial electrical resistance (TEER) and Dextran^{FITC} permeability assay. Caco-2 cells monolayers were grown in transwell inserts and treated with *L. casei* (Lbc^{WT}, Lbc^{V}, Lbc^{InlAB} or Lbc^{LAP}) for 2, 4, 16, and 24 h, before their infection with *Listeria monocytogenes* (Lm) for 2 h. **A:** TEER measurements before and after exposure to *L. monocytogenes* treatment alone were 268.9±2.3 and 224.5±4.7 respectively, with a 20.5% change. % TEER reduction was calculated as per Koo *et al.* (2012) as $1 - TEER_{after} / TEER_{before} *100$. **B:** Tight junction integrity of Caco-2 cells was also monitored with Dextran^{FITC} translocation across the membrane. Dextran^{FITC} recovery after *L. monocytogenes* was 3.72± 0.03%. Data are averages of three experiments ran in duplicates (n=6). For each time point bars marked with different letters (a, b, c, d, e, f, g, h) indicate a significant difference at $P<0.05$.

4.5. DISCUSSION

With the increase in microbial resistance to antibiotics there has been a need to study alternative methods in the prevention or treatment of foodborne diseases. Initially, inactivated or attenuated pathogens were used as vaccines; however, with the risks associated with the use of these there has been an increased interest in using safe non-pathogenic bacteria as a substitute (Tarahomjoo, 2012). Probiotics' have been reported to offer beneficial health-promoting effects on host and are generally regarded- as-safe (GRAS), therefore, making them an attractive alternative (Wyszynska *et al*., 2015). They are more preferred over inactivated or attenuated pathogens as vaccines, which come with the risk of possible reversion to virulent phenotypes in hosts as well as the possibility for becoming virulent, especially in immunocompromised individuals (Nabel, 2013; Tarahomjoo, 2012). The use of probiotics in the inhibition of pathogens has been reported in literature, however, they have been generic in their application and have been reported to be less effective against some pathogens. In the effort to enhance the effectiveness of probiotics, bioengineering has been used as an alternative strategy. This strategy is used for protection against pathogens by cloning and expression of pathogens' virulence genes into probiotics and subsequently used to competitively exclude pathogens. These genetically engineered probiotics, depending on the virulence genes they are expressing can be used either as a prophylactic (vaccination) or as treatment alternatives of the specific pathogens. The current study reports of the use of probiotic engineering for targeted control of *L. monocytogenes*. Although there have been concerns regarding probiotic bioengineering, it was reported that that probiotics retain their GRAS status even after expression of heterologous genes (Kumar *et al*., 2016).

During the infection process, *L. monocytogenes* employs the *Listeria* adhesion protein (LAP) for its adhesion to epithelial cells (Jagadeesan *et al*., 2010; Drolia *et al*. 2018; Drolia and Bhunia, 2019) and invasion genes internalin A (InlA) and InlB to invade a wider range of mammalian cells (Dietrich *et al*., 1998). Therefore, in order to construct a probiotic strain with enhanced ability for targeted control of *L. monocytogenes*, we previously cloned and expressed the invasion proteins InlA and InlB into a probiotic *L. casei* (Mathipa *et al*., 2019). In this study it was observed that the recombinant probiotic showed an enhanced ability to adhere to, invade and translocate through Caco-2 cells *in vitro*. Furthermore, in the previous results there was a significant difference in the inhibition of *L. monocytogenes* by the recombinant *L. casei* strains as opposed to the wild-type

counterparts (Mathipa *et al*., 2019). These experiments were performed *in vitro* using the Caco-2 cells grown and maintained in the cell culture media, DMEM supplemented with fetal bovine serum (FBS). In its disease progression, *L. monocytogenes* has to overcome diverse suboptimal microenvironments that usually constitute the host's defence system (Gahan and Hill, 2005) in order to colonize the host GI tract and cause infection. These conditions include but not limited to low acid in the stomach and high bile concentration in the small intestine. In an effort to better understand the epithelial infection processes of *L. monocytogenes* as influenced by the recombinant *L. casei* strains, in the current study, we investigated the effect of InlAB expressing *L. casei* on the inhibition of *L. monocytogenes* in FaSSIF. Gamboa and Leong (2013) reported that SIF has osmolality that is similar to that of human cells thus making this fluid a better medium to be used in *in vitro* intestinal model. The results in **Fig. 4.1** show that the expression of InlAB and LAP by the recombinant Lbc^{InlAB} and Lbc^{LAP} exhibited an enhanced adhesion, invasion and translocation as opposed to Lbc^{WT}. However, it was worth noting that Lbc^{LAP} showed a better adhesion as opposed to Lbc^{InlAB} that showed better invasion and translocation. Guimarães *et al.* (2005) cloned and expressed the invasion gene internalin A of *L. monocytogenes* into *Lactococcus lactis* and reported that the probiotic showed an enhanced ability to invade epithelial cells. In a different study, Koo *et al.* (2012) cloned and expressed the LAP into probiotic *Lactobacillus paracasei* and reported that the probiotic exhibited enhanced adhesion to the Caco-2 cells. Although these studies reported on the enhancement of probiotics through genetic engineering, the cells that they used were maintained in media that was supporting the growth of the epithelial cells. Taking this into consideration, the results of the current study, suggests that the recombinant Lbc^{InlAB} would show the same results *in vivo*.

Researchers elsewhere have investigated the intestinal phase of *L. monocytogenes* infection process in artificial gastrointestinal fluid systems (Begley *et al*., 2002; King *et al*., 2003; Formato *et al*., 2007). These studies reported on the behaviour of *L. monocytogenes* when it was introduced on its own to these conditions. However, the current study investigated the intestinal infection phase of *L. monocytogenes*. The ability of the *L. casei* strains to competitively exclude *L. monocytogenes* using three different mechanisms: competitive adhesion, inhibition and displacement of adhesion, under simulated intestinal conditions was evaluated (**Fig. 4.2)**. The results revealed that during competitive adhesion and inhibition of adhesion, adhesion of *L. monocytogenes* to Caco-2 cells was reduced by the *L. casei* strains, with recombinant Lbc^{InlAB} and

Lbc^{LAP} exhibiting an enhanced reduction compared to Lbc^{WT}. These results were in agreement with previous studies that reported on the competitive exclusion of pathogens. It has been shown that some probiotics share binding specificities with some pathogens (Fujiwara, *et al.,* 2001; Neeser and Granato, 2000), making it possible for direct competition between the probiotics with specific pathogens for receptor sites on the host cell (Collado *et al*, 2010). Lee and Puong (2002) reported that the inhibition of pathogens by probiotics could be due to interaction of specific adhesins and receptors present in both probiotic and pathogen, affording ability to compete for attachment to the same receptors. Converse to the results for competitive adhesion and inhibition of adhesion, all the *L. casei* strains failed to displace *L. monocytogenes* cells that had already adhered to Caco-2 cells. Failure of these strains to displace the pathogens already attached to the epithelial cells was in correlation with reports by other researchers. Lee *et al*. (2003) reported that in the competitive exclusion of pathogens, rates of their displaced were generally lower than those achieved by competition and inhibition. Gueimonde *et al.* (2006) also reported that the displacement profiles of pathogens by probiotics were different from those observed for the competitive adhesion and inhibition of pathogen adhesion.

Previously Barmpalia-Davis *et al*. (2008) reported that artificial gastrointestinal conditions closely simulate the dynamics of GIT, therefore, giving an indication of the pathogenesis. Bernbom *et al.* (2006) reported that in order to eliminate the influence of the indigenous microflora during pathogenesis studies and thereby simplify results interpretation, *in vitro* models of the intestinal system can be used. Taking these studies into consideration, the inhibition of the consequent stages in the infection cycle, adhesion, invasion and translocation of *L. monocytogenes* under simulated intestinal conditions over various exposure times was studied (**Fig. 4.3)**. Prolonged exposure of the Caco-2 cells to *L. casei* strains showed an enhanced inhibition of *L. monocytogenes*. Furthermore, it was worth noting that in all the stages the recombinant *L. casei* strains were better at inhibiting *L. monocytogenes* than Lbc^{WT}. Similar to the results from the adhesion, invasion and translocation profiles of *L. casei* strains (**Fig. 4.1)**, when comparing the inhibition of *L. monocytogenes* by the recombinant Lbc^{InlAB} and Lbc^{LAP} under simulated intestinal conditions, Lbc^{LAP} showed better inhibition of *L. monocytogenes* adhesion while Lbc^{InlAB} showed better inhibition of invasion and translocation. This was attributed to the expression of the LAP in Lbc^{LAP} and invasion genes InlAB in Lbc^{InlAB}. Koo *et al*. (2012) also reported that prolonged exposure of

the Caco-2 cells to recombinant *Lb. paracasei* expressing LAP showed an enhanced inhibition of *L. monocytogenes* adhesion, invasion and translocation.

Consequent to studying the inhibition of translocation, the tight junction integrity using electrical resistance (**Fig. 4.5A**), Dextran^{FITC} (**Fig. 4.5B)** assays in the presence of the *L. casei* strains were also monitored. In agreement to the results observed for the inhibition of adhesion, invasion and translocation, there was an enhanced protection of tight junction integrity through pre- exposure to *L. casei* strains under simulated conditions. *L. monocytogenes* translocation has been reported to potentially occur in the stomach (Conlan, 1997), the small intestine (MacDonald *et al*., 1980; Marco *et al*., 1992; Pron *et al.,* 1998) or the lower intestine (Nishikawa *et al*., 1996) in murine models. The enhanced protection of the tight junction by the *L. casei* strains in simulated intestinal conditions will result in reduction of *L. monocytogenes* translocation, therefore, inhibiting *Listeria* infection.

4.6. CONCLUSION

The current study shows that probiotics engineering can be used as an alternative strategy in the inhibition of individual pathogens and additionally target different stages on infection depending on the virulence genes cloned and expressed under simulated intestinal conditions. Presumably, this is the first report to be documented on the inhibition of *L. monocytogenes* by the recombinant *L. casei* strains under simulated intestinal conditions, an indication of *in vivo* conditions. Thus, recombinant *L. casei* strains show potential for use as a prophylactic intervention strategy for control of *L. monocytogenes* infection, targeting different stages of its intestinal infection phase.

4.7. REFERENCES

Amalaradjou M, Bhunia A. (2013). Bioengineered probiotics, a strategic approach to control enteric infections. *Bioengineered, 4(6)*: 379-387.

Barmpalia-Davis, I.M., Geornaras, I., Kendall, P.A., Sofos, J.N. (2008). Differences in survival among 13 *Listeria monocytogenes* strains in a dynamic model of the stomach and small intestine. *Applied and Environmental Microbiology, 74(17)*: 5563-5567.

Begley, M., Gahan, C.G.M., Hill. C. (2002). Bile stress response in *Listeria monocytogenes* LO28: adaptation, cross-protection and identification of genetic loci involved in bile resistance. *Applied and Environmental Microbiology, 68,* 6005–6012.

Behnsen, J., Deriu, E., Sassone-Corsi, M., Raffatellu, M. (2013). Probiotics: Properties, examples, and specific applications. *Cold Spring Harbour Perspectives in Medicine, 3(3),* A010074.

Bernbom, N., Licht, T.R., Saadbye, P., Vogensen, F.K., Nørrung, B. (2006). *Lactobacillus plantarum* inhibits growth of *Listeria monocytogenes* in an *in vitro* continuous flow gut model but promotes invasion of *L. monocytogenes* in the gut of gnotobiotic rats. *International Journal of Food Microbiology, 108(1),* 10-14.

Brouwers, J., Tack, J., Lammert, F., Augustijns, P. (2006). Intraluminal drug and formulation behavior and integration in *in vitro* permeability estimation: A case study with amprenavir. *Journal of Pharmaceutical Science,* 95(2), 372-383.

Chiba, S., Nagai, T., Hayashi, T., Baba, Y., Nagai, S., Koyasu, S. (2011). Listerial invasion protein internalin B promotes entry into ileal Peyer's patches *in vivo*. *Microbiology and Immunology, 55(2),* 123-129.

Chu, H., Kang, S., Ha, S., Cho, K., Park, S.M., Han, K.H., Kang, S.K., Lee, H., Han, S.H., Yun, C.H., Choi, Y. (2005). *Lactobacillus acidophilus* expressing recombinant K99 adhesive fimbriae has an inhibitory effect on adhesion of enterotoxigenic *Escherichia coli*. *Microbiology and Immunology, 49(11),* 941-948.

Collado, M.C., Gueimonde, M., Salminen, S. (2010). Probiotics in Adhesion of Pathogens: Mechanisms of Action. *Bioactive Foods in Promoting Health,* 353-370.

Conlan, J. (1997). Neutrophils and tumour necrosis factor-α are important for controlling early gastrointestinal stages of experimental murine listeriosis. *Journal of Medical Microbiology, 46(3),* 239-250.

Dietrich, G., Bubert, A., Gentschev, I., Sokolovic, Z., Simm, A., Catic, A., Kaufmann, S.H., Hess, J., Szalay, A.A., Goebel, W. (1998). Delivery of antigen-encoding plasmid DNA into the

cytosol of macrophages by attenuated suicide *Listeria monocytogenes*. *Nature Biotechnology, 16(2),* 181-185.

Drolia, R., Bhunia, A.K. (2019). Crossing the epithelial barrier via *Listeria* adhesion protein and Internalin A. *Trends in Microbiology,* doi: 10.1016/j.tim.2018.12.007 (Published online, Jan 17, 2019)

Drolia, R., Tenguria, S., Durkes, A.C., Turner, J.R., Bhunia, A.K. (2018). *Listeria* adhesion protein induces intestinal epithelial barrier dysfunction for bacterial translocation. *Cell Host Microbe, 23(4),* 470-484.

FAO/WHO (2002). Guidelines for the evaluation of probiotics in food. Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food; Ontario, Canada.

Formato, G., Geornaras, I., Barmpalia, I.M., Skandamis, P.N., Belk, K.E., Scanga, J.A., Kendall, P.A., Smith, G.C., Sofos, J.N. (2003). Effect of acid adaptation on growth during storage at 10°C and resistance to stimulated gastric fluid of *Listeria monocytogenes* inoculated onto bologna formulated with or without antimicrobials. *International Journal of Food Microbiology, 86(3),* 283-292.

Fossati, L., Dechaume, R., Hardillier, E., Chevillon, D., Prevost, C., Bolze, S., Maubon, N. (2008). Use of simulated intestinal fluid for Caco-2 permeability assay of lipophilic drugs. *International Journal of Pharmaceutics, 360(1-2),* 148-155.

Fujiwara, S., Hashiba, H., Hirota, T., Forstner, J.F. (2001). Inhibition of the binding of enterotoxigenic *Escherichia coli* Pb176 to human intestinal epithelial cell line HCT-8 by an extracellular protein fraction containing BIF of *Bifidobacterium longum* SBT2928: Suggestive evidence of blocking of the binding receptor gangliotetraosylceramide on the cell surface. *International Journal of Food Microbiology, 67,* 97–106

Gahan, C., Hill, C. (2005). Gastrointestinal phase of *Listeria monocytogenes* infection. *Journal of Applied Microbiology, 98(6*), 1345-53.

Galia, E., Nicolaides, E., Hörter, D., Löbenberg, R., Reppas, C., Dressman, J.B. (1998). Evaluation of various dissolution media for predicting *in vivo* performance of class I and II drugs. *Pharmaceutical Research, 15(5),* 698-705.

Gamboa, J.M., Leong, K.W. (2013). *In vitro* and *in vivo* models for the study of oral delivery of nanoparticles. *Advance Drug Delivery Reviews, 65(6),* 800-810.

Gueimonde, M., Jalonen, L., He, F., Hiramatsu, M., Salminen, S. (2006). Adhesion and competitive inhibition and displacement of human enteropathogens by selected lactobacilli. *Food Research International, 39,* 467-471.

Guimarães, V.D., Gabriel, J.E., Lefèvre, F., Cabanes, D., Gruss, A., Cossart, P., Azevedo, V., Langella, P. (2005). Internalin-expressing *Lactococcus lactis* is able to invade small intestine of guinea pigs and deliver DNA into mammalian epithelial cells. *Microbes and Infection, 7(5-6),* 836-844.

Hamon, M., Bierne, H., Cossart, P. (2006). *Listeria monocytogenes*: A multifaceted model. *Nature Review Microbiology, 4(6),* 423-434.

Ingels, F., Beck, B., Oth, M., Augustijns, P. (2004). Effect of simulated intestinal fluid on drug permeability estimation across Caco-2 monolayers. *International Journal of Pharmaceutics, 274(1-2),* 221-322.

Ingels, F., Deferme, S., Destexhe, E., Oth, M., Van den Mooter, G., Augustijns, P. (2002). Simulated intestinal fluid as transport medium in the Caco-2 cell culture model. *International Journal of Pharmaceutics, 232,* 183–192.

Jagadeesan, B.K., Koo, O.P., Kim, K.M., Burkholder, K., Mishra, K., Aroonnual, A., Bhunia, A. (2010). LAP, an alcohol acetaldehyde dehydrogenase enzyme in *Listeria,* promotes bacterial adhesion to enterocyte-like Caco-2 cells only in pathogenic species. *Microbiology, 156(Pt 9),* 2782-2795.

Jensen, V.B., Harty, J.T., Jones, B.D. (1998). Interactions of the invasive pathogens *Salmonella typhimurium, Listeria monocytogenes,* and *Shigella flexneri* with M cells and murine peyer's patches. *Infection and Immunity, 66(8),* 3758-3766.

Kapitza, S.B., Michel, B.R., Van Hoogevest, P., Leigh, M.L.S., Imanidis, G. (2007). Absorption of poorly watersoluble drugs subject to apical efflux using phospholipids as solubilizers in the Caco-2 cell model. *European Journal of Pharmaceutics and Biopharmaceutics, 66(1),* 146-158.

King, T., Ferenci, T., Szab, E.A. (2003). The effect of growth atmosphere on the ability of *Listeria monocytogenes* to survive exposure to acid, proteolytic enzymes and bile salts. *International Journal of Food Microbiology, 84*(2), 133-143.

Koo, O.K., Amalaradjou, M.A.R., Bhunia, A.K. (2012). Recombinant probiotic expressing *Listeria* adhesion protein attenuates *Listeria monocytogenes* virulence *in vitro*. *PLoS ONE, 7(1)*: e29277.

Lecuit, M., Ohayon, H., Braun, L., Mengaud, J., Cossart, P. (1997). Internalin of *Listeria monocytogenes* with an intact leucine-rich repeat region is sufficient to promote internalization. *Infection and Immunity, 65(12),* 5309–5319.

Lee, Y., Puong, K. (2002). Competition for adhesion between probiotics and human gastrointestinal pathogens in the presence of carbohydrate. *British Journal of Nutrition, 88(Suppl 1),* S101-S108.

Lee, Y.K., Puong, K.Y., Ouwehand, A.C., Salminen, S. (2003). Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. *Journal of Medical Microbiology, 52(Pt 10)*: 925-930.

Lind, M., Jacobsen, J., Holm, R., Müllertz, A. (2007). Development of simulated intestinal fluids containing nutrients as transport media in the Caco-2 cell culture model: Assessment of cell viability, monolayer integrity and transport of a poorly aqueous soluble drug and a substrate of efflux mechanisms. *European Journal of Pharmaceutical Science, 32(4-5),* 261-270.

MacDonald, T.T., Carter, P.B., Carter, P.B. (1980). Cell-mediated immunity to intestinal infection. *Infection and Immunity, 28(2),* 516-523.

Marco, A.J., Prats, N., Ramos, J.A., Briones, V., Blanco, M., Dominguez, L., Domingo, M. (1992). A microbiological, histopathological and immunohistological study of the intragastric inoculation of *Listeria monocytogenes* in mice. *Journal of Comparative Pathology, 107(1),* 1-9.

Marelli, B., Perez, A.R., Banchio, C., De Mendoza, D., Magni, C. (2011). Oral immunization with live *Lactococcus lactis* expressing rotavirus VP8* subunit induces specific immune response in mice. *Journal of Virological Methods, 175(1),* 28-37.

Mathipa, M.G., Bhunia, A.K., Thantsha, M.S. (2019). *Lactobacillus casei* expressing internalins A and B reduces *Listeria monocytogenes* interaction with Caco-2 cell*s in vitro*. *Microbial Biotechnology*, *12(4),* 715-729.

Mengaud, J., Ohayon, H., Gounon, P., Mege, R.M., Cossart, P. (1996). E-Cadherin Is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into Epithelial Cells. *Cell, 84(6),* 923-932.

Mercenier, A., Muller-Alouf, H., Grangette, C. (2000). Lactic acid bacteria as live vaccines. *Current Issues of Molecular Biology, 2(1),* 17-25.

Nabel, G. (2013). Designing Tomorrow's Vaccines. *New England Journal of Medicine, 368(6),* 551-560.

Neeser, J., Granato, D. (2000). *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. *Glycobiology*, *10(11*), 1193- 1199.

Nishikawa, S., Miura, T., Sasaki, S., Nakane, A. (1996). The protective role of endogenous cytokines in host resistance against an intragastric infection with *Listeria monocytogenes* in mice. *FEMS Immunology and Medical Microbiology, 16(3-4),* 291-298.

Pron, B., Boumaila, C., Jaubert, F., Sarnacki, S., Monnet, J.P., Berche, P., Gaillard, J.L. (1998). Comprehensive study of the intestinal stage of listeriosis in a rat ligated ileal loop system. *Infection and Immunology, 66(2),* 747-755.

Sánchez, B., López, P., González-Rodríguez, I., Suárez, A., Margolles, A., Urdaci, M. (2011). A flagellin-producing *Lactococcus* strain: Interactions with mucin and enteropathogens. *FEMS Microbiological Letters, 318(2),* 101-117.

Schuppler, M., Loessner, M.J. (2010). The opportunistic pathogen *Listeria monocytogenes*: Pathogenicity and interaction with the mucosal immune system. *International Journal of Inflammation,* doi: 10.4061/2010/704321.

Smith, J.L., Liu, Y., Paoli, G.C. (2013). How does *Listeria monocytogenes* combat acid conditions? *Canadian Journal of Microbiology, 59(3)*, 141-152.

Tarahomjoo, S. (2012). Development of Vaccine Delivery Vehicles Based on Lactic Acid Bacteria. *Molecular Biotechnology, 51(2),*183-199.

Wyszynska, A., Kobierecka, P., Bardowski, J., Jagusztyn-Krynicka, E.K. (2015). Lactic acid bacteria-20 years exploring their potential as live vectors for mucosal vaccination. *Applied Microbiology and Biotechnology, 99(7)*, 2967-2977.

Chapter 5

General Conclusions and Recommendations

General conclusions

- The recombinant *Lactobacillus casei* strain expressing the internalins A and B, the virulence proteins used by *L. monocytogenes* for invasion of and translocation into host cells, as well as for mediation of host cell specific internalization, was successfully developed.
- The expression of the internalins changed the morphology of *L. casei* from the usual rod-shaped to elongated curve shaped cells. However, expression of these genes did not produce any negative effects on growth of *L. casei* as the growth profiles of the wild-type and recombinant strains were similar. This is an esteemed outcome which indicates that growth and potential consequent colonization of the recombinant *L. casei* is more likely to be equivalent to and not inferior to that of the conventional wild-type strain.
- In terms of the interaction of lactobacilli with the Caco-2 cells, the recombinant *L. casei* strains adhered to, invaded as well as translocated the Caco-2 cells better than the wild-type strain. This indicated improved colonization potential of the recombinant strain. Furthermore, this result was evidence illustrating that the elongated curve shape of the recombinant did not compromise the ability of *L. casei* to adhere to the intestinal cells. Adhesion is a critical step for colonization of the gastrointestinal tract by probiotics, hence it is desirable that it be maintained in the recombinant strain.
- The recombinant *L. casei* strains competitively excluded *L. monocytogenes* and inhibited adhesion, invasion and translocation more than their wild-type counterpart both in tissue culture media and in simulated intestinal conditions, with efficiencies more remarkable the longer the duration of the interaction of the probiotics with the Caco-2 cells before introduction of *L. monocytogenes*. This result indicates that time is a critical factor that has to be taken into consideration in real-life applications of the recombinant strain for *L. monocytogenes* control.
- Recombinant *L. casei* strains weakened the *L. monocytogenes* mediated cell cytotoxicity and protected the integrity of the epithelial barrier junctions. This could possibly minimize dissemination of *L. monocytogenes* from the gastrointestinal tract to remote sites.
- Although the recombinant *L. casei* strains exhibited enhanced competitive and inhibition adhesion of *L. monocytogenes*, none of these strains was able to displace *L. monocytogenes* cells already attached to the Caco-2 cells. Thus, the recombinant *L. casei* strains will be

effective as a prophylactic rather than therapeutic intervention for listeriosis. Comparison of functional attributes of two bioengineered strains revealed that recombinant *L. casei* strain expressing internalins was superior in prevention or reduction of *L. monocytogenes* invasion and translocation while the strain expressing *Listeria* adhesion protein (LAP) better prevented *L. monocytogenes* adhesion. Thus, recombinant *L. casei* strains expressing different virulence genes of *L. monocytogenes* can be targeted at different stages of this pathogen's infection cycle, with the recombinants harbouring LAP and internalins directed towards adhesion and invasion plus translocation, respectively.

- Overall, the current study shows that probiotics engineering is a promising alternative strategy that can be used in the control of specific foodborne pathogens. These recombinant probiotics will potentially offer dual effects; the ability to confer general beneficial effects attributed to conventional probiotics, and enhanced specific control of a targeted pathogen, in this case, *L. monocytogenes*.

Recommendations for Future work

The findings of the current study demonstrate potential for the recombinant strains to be used as an alternative method for targeted and enhanced control of listeriosis. However, before the strains can be endorsed for direct application in humans, additional studies are required. These studies should include but not limited to the following:

- *In vivo* studies that will determine the persistence of the recombinant strains, their ability to express the foreign genes, specifically in the absence of antibiotic pressure present in *in vitro* trials, with associated noticeable disease reduction.

- Due to the role some internalins play in the trasplacental transmission of *L. monocytogenes* and evidence supporting their role in vertical transmission of this pathogen from the mother to the foetus, a study of the effect of the recombinant strain on *L. monocytogenes* progression in pregnant guinea pig model will also be valuable, with this model chosen based due to close resemblance of guinea pig placenta to that of humans.

- One of the main limitations regarding the use of GMO is safety. Therefore, safety issues relating to application of the recombinant strains have to be addressed.

- The use of recombinant *L. casei* strains in the study showed an enhanced inhibition of target specific stages of infection of *L. monocytogenes* in the current study. It would be interesting to investigate the efficacy of a cocktail of the two recombinant strains, to determine if that would have better effects when compared to the individual strains.

- In order to be deemed as probiotics, microorganism have to meet criteria such as survival in a low pH and high bile environment, adhering to intestinal epithelial cells and stabilizing intestinal microflora. There were no studies to check for whether the *L. casei* strain retained the functional properties that qualifies it as a probiotic. Therefore future studies investigating if the recombinant *L. casei* still conform to those criteria even with the expression of the foreign genes, will have to be conducted.

- Microencapsulation is a method used in the probiotic field for production of probiotics with high viability, with the encapsulants selected based on their ability to protect the probiotic cultures in products during storage and gastrointestinal transit. The recombinant strains will also have to be formulated into a delivery vehicle that will allow high viability, long shelf life and easy administration. Therefore, different encapsulation methods will be compared to determine the encapsulation method best suited for delivery of the bioengineered *L. casei* strains.