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A comparative study of two *Lactobacillus fermentum* strains that show opposing effects on intestinal barrier integrity

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

Massey University

Manawatu, New Zealand

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2014

ABSTRACT

The *Lactobacillus* species can exert health promoting effects in the gastrointestinal tract (GIT) of humans through several mechanisms, which include pathogen inhibition, maintenance of microbial balance, immunomodulation and enhancement of the GIT barrier function. However, different strains of lactobacilli can evoke different responses in the host and not all strains of the same species can be considered health promoting. Two strains identified as *Lactobacillus fermentum*, namely AGR1485 and AGR1487, isolated from human oral cavities, exhibit opposing effects on intestinal barrier integrity. Studies have shown that AGR1485 maintains trans-epithelial electric resistance (TEER), a measure of GIT barrier integrity, across Caco-2 cell monolayers, while AGR1487 decreases TEER by 12 hours.

This work aimed to test the hypotheses that the varying effects shown by these two *L. fermentum* strains are related to phenotypic differences between the two strains and are mediated by the interaction of secreted and/or cell-associated bacterial components with the GIT epithelial layer. Differences in metabolic events that occur during the various phases of growth in bacteria can impact not only cellular structure and secreted molecules, but may also affect their interactions with the intestinal epithelial cells. TEER assays were conducted to investigate if variation in bacterial secreted molecules and cell wall components associated with various phases of microbial growth can affect Caco-2 cell TEER. The effect on Caco-2 cell TEER caused by both strains was independent of bacterial growth phase. To test the hypothesis that it is the bacterial structural and/or secreted components that influence

Caco-2 cell TEER, assays were conducted with live versus UV-killed bacteria on Caco-2 cells. Results showed that for both strains of *L. fermentum*, dead bacteria have similar effects on Caco-2 cell TEER as live bacteria, implying that direct bacterial contact with Caco-2 cells is necessary for the effects. Analogous to TEER assays, live AGR1487 increased mannitol permeability while UV-killed AGR1487 did not, implying that AGR1487 uses both cell surface structures and/or metabolites through distinct mechanisms to modulate host barrier properties. Subsequent experiments conducted using secreted metabolites from bacteria, Caco-2 cells and bacteria-Caco-2 cell interactions indicated that they have no effect on Caco-2 cell TEER, strengthening the assumption that bacterial cell surface-associated components are involved in mediating these effects.

The bacterial cells were subjected to ultrasonication followed by ultracentrifugation to isolate the bacterial cell wall extract. TEER assays conducted with the cell wall extracts from both strains resulted in decreasing Caco-2 cell TEER, although at high concentrations, further strengthening the role of bacterial cell surface components in influencing barrier integrity of the Caco-2 cells. To narrow down proteinaceous components of the cell wall extracts from both the strains that influence Caco-2 cell TEER, they were fractionated through size exclusion chromatography and the effects of these cell wall fractions on Caco-2 cell TEER were studied. One fraction of AGR1487 CW appeared to decrease Caco-2 cell TEER, although at a high concentration. However, the results could not be repeated when the same fraction was applied at concentrations that the proteins comprising this fraction would be found in

live AGR1487. Even the high concentration tested previously did not decrease Caco-2 cell TEER and the discrepancy in results remains unexplained.

The results reported in this dissertation have added to the knowledge that the two strains of *L. fermentum* AGR1485 and AGR1487 show differences in their genome size and in their phenotypic characteristics. In addition, these bacteria utilise both cell surface and/or secreted metabolites through multiple mechanisms to modulate host response. In the future, identification of specific bacterial effector molecules that influence host response will be a major step towards understanding strain-specific characteristics shown by *Lactobacilli*.

ACKNOWLEGEMENTS

I sincerely thank AgResearch and the Riddet Institute, a Centre of Research Excellence, for funding this research and my PhD fellowship and Massey University for honouring me with the Massey Doctoral Scholarship. It is with immense gratitude that I acknowledge and sincerely thank AgResearch for this project and allowing me the opportunity to work in the excellent research facilities and work environment that AgResearch provides. I want to specially thank the Riddet Institute for allowing me the opportunity to enter a PhD programme and to fulfil a personal dream.

I thank Professor Paul Moughan, whom I will always consider my mentor and cannot thank enough for having faith in me. I thank Professor Warren McNabb for his support and guidance, making me see the silver lining and providing every opportunity so I would not give up. My heartfelt gratitude to Dr Nicole Roy for her incredible compassion, her efficiency and patience and most of all, her always being available to listen and help. My sincerest gratitude to Dr Eric Altermann, whose vast knowledge has been a constant source of awe and inspiration to me and whose guidance helped me realise my own strengths and weaknesses. I wish to thank Dr Rachel Anderson, who guided me with her knowledge, helped me stay focussed and provided the most valuable support any student needs and that is encouragement. Thanks to all my excellent supervisors, for their valuable time, effort, kindness and faith in guiding me all the way through my PhD journey.

I wish to thank to Dr Bill Kelly for his valuable guidance and patience in teaching me laboratory techniques for conducting pulsed-field gel electrophoresis that

were new to me. I thank Dr Dongwen Luo, Dr Siva Ganesh and Dr John Koolaard for their invaluable help with the statistical analysis of my data. I wish to thank Dr Linley Scholfield for her excellent guidance, time and patience in teaching me all that she could about HPLC. I am extremely grateful to Kelly Armstrong for her excellent technical support and to both Kelly and Dr Mark McCann for their valuable advice in regards to tissue culture. Mannitol assays and TEER assays conducted with bacterial fractions reported in chapter 4 were done with Kelly Armstrong's expert technical assistance and I could not have completed those assays while writing this thesis without her help. I wish to extend my sincere gratitude to Bruce Sinclair for his expert advice and suggestions all through my PhD. Special thanks to Denise Martin for her support, advice and always being available for any help needed.

I am and will always be grateful to my friends and colleagues in the student room, who made this journey not only a learning experience but an adventure and a fun experience as well. Special thanks to Francisco, Dulantha, Sam and Quentin for providing me with invaluable help with my work and writing of my thesis and thanks to Nadja, Alicia, Milica, Carol, Haike, Filomena, Sonal, Tom, Sandeep and Eva for being such great office mates and always being there to help. The international environment that my fellow students provided is a memory I will treasure for life and each of them have helped me learn not just science, but so much more. I will cherish every moment I have spent with my colleagues and fellow students here at AgResearch.

I wish to thank my family for letting me embark on a really difficult journey and seeing me complete it with patience, love and a lot of sacrifices on their part. I wish to

thank Dr Abhimanyu Sarkar, for being a pillar of strength and always being there for me, I would not be here if it was not for him. My heart-felt gratitude to Dr. Abhik Ghosh for with his generosity and kindness of heart, providing my children and myself with fun escapades from reality during our toughest times. I wish to thank my parents and my sisters in India for making me realise the importance of being independent. My children, Avroneel Ghosh and Aishani Sarkar, no words can express what I feel for them. Their love and understanding, their patience and encouragement, have been the beacon that I have always needed. I dedicate this thesis to my children, Avroneel and Aishani, I did this for you. Last but the most important, I wish to thank The Almighty, for giving me the strength, courage and perseverance to complete this journey.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BHI	Brain-heart infusion
BLASTN	NCBI nucleotide Basic Local Alignment Search
BSA	Bovine serum albumin
cDNA	Complementary DNA
CFU	Colony-forming units
CM	Cell membrane
CnBP	Collagen binding protein
CLR	C-type lectin receptor
CPS	Capsular polysaccharides
CW	Cell wall
DC	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin

ddNTPs	Dideoxynucleotides
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
EDTA	Ethylenediamine tetra-acetic acid
EF-Tu	Elongation factor Tu
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
EPS	Exopolysaccharides
ERK	Extracellular signal regulated kinases
Fbp	Fibronectin binding protein
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G+C	Guanine plus cytosine
GlcNAc	N-acetyl-glucosamine

GIT	Gastrointestinal tract
H_2O_2	Hydrogen peroxide
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IFN	Interferon
IKK	IκB kinase
IL	Interleukin
IRAK	IL-1-receptor-asociated kinase
IRF	IFN-regulatory factor
IκB	Inhibitor of NF-κB
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LAB	Lactic acid bacteria
LACOG	*Lactobacillales*-specific clusters of orthologous genes
LTA	Lipoteichoic acids
LPS	Lipopolysaccharides
LSD	Least significant difference

M199	Medium 199
MAMPs	Microbe-associated molecular patterns
MAPK	Mitogen activated protein kinases
MBF	Mucus binding factor
MDCK	Madin Darby canine kidney
MLCK	Myosin light chain kinase
mRNA	Messenger RNA
MRS	Man, Rogosa and Sharpe
Msa	Mannose-specific adhesin
Mub	Mucus binding protein
MurNAc	β-1-4linked N-acetyl-muramic acid
MyD88	Myeloid differentiation primary response gene
NADH	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NEAA	Non-essential amino acids
NF-κB	Nuclear factor kappa B

NLR	NOD-like receptor
NOD	Nucleotide-binding and oligomerisation-domain
OD	Optical density
O_2	Superoxide anion radicals
$^{\cdot}OH$	Hydroxyl radicals
PAMPs	Pathogen-associated molecular patterns
PC	Polycarbonate (Transwell® cell culture inserts)
PCR	Polymerase chain reaction
PG	Peptidoglycan
PKC	Protein kinase C
PLA2	Group IIA phospholipase A2
PRRs	Pathogen recognition receptors
PFGE	Pulsed-field gel electrophoresis
REML	Restricted maximum likelihood
RNA	Ribonucleic acid
RNase	Ribonuclease A

ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
SDP	Sortase-dependent proteins
SEM	Standard error of the mean
Slp	S-layer proteins
SN	Supernatant
SOD	Superoxide dismutase
SrtA	Sortase enzyme
TA	Teichoic acids
TDCA	Taurodeoxycholic acid
TEER	Transepithelial electrical resistance
TGF	Transforming growth factor
TJ	Tight junction
TLR	Toll-like receptor
TNF	Tumour necrosis factor

UV	Ultraviolet
WPS	Wall polysaccharides
WTA	Wall teichoic acids
ZO	Zonula occludens

INTRODUCTION

"The doctor of the future will no longer treat the human frame with drugs, but rather will cure and prevent disease with nutrition."

-Thomas Edison

For centuries, humans have used lactic acid bacteria (LAB) in fermentation processes and in the last century the probiotic properties of LAB have gained them increasing attention in the scientific and industrial communities [1]. The genus *Lactobacillus*, the largest genus among LAB, is found in a wide variety of habitats, including vegetation, food resources and the gastrointestinal tract (GIT) of humans and animals, where some may reside as commensals [2]. The presence of lactobacilli is important for maintenance of the GIT microbial ecosystem and for providing protection against pathogen infection [3-5]. Some lactobacilli strains have been shown to reduce the incidence of diarrhoea, showing immune-modulatory activities, consequently leading to an improved GIT barrier function [6]. Several studies have suggested that natural anti-inflammatory molecules produced by probiotic bacteria can actively promote human health and have the potential to be used as therapeutic agents in human inflammatory disorders [7]. The beneficial properties exhibited by many lactobacilli on GIT health have resulted in their use in functional foods and our understanding of their significance to human health is growing.

Lactobacillus species can exert health promoting effects in the GIT through many mechanisms, which include pathogen inhibition, maintenance of microbial

balance, immunomodulation and enhancement of barrier function [6]. However, studies have shown that different strains of lactobacilli can evoke different responses in the host and therefore, results from one strain cannot be generalised to others and not all strains of the same species can be considered beneficial [5]. Strain variations may be related to variation and diversity of the cell surface architecture of lactobacilli and the bacterium's ability to express certain surface components or secrete specific compounds that may influence host-microbe interactions [8-10].

Lactobacilli show great diversity in cell surface architecture and are known to modify their surface properties in response to stress factors such as bile and low pH, and these adaptations may help their survival through the harsh environmental conditions encountered in the GIT [11, 12]. Different macromolecules constituting the cell wall of lactobacilli have been shown to contribute in maintaining bacterial cell integrity during environmental stress [13]. In recent years, multiple cell surface associated molecules have been implicated, either individually or collectively, in microbe-host interactions such as adherence of lactobacilli to the GIT lining, immunomodulation and protective effects on GIT epithelial barrier function [14, 15].

Considering the complexity of host-lactobacilli interactions involving host-cell signalling and regulation pathways, there may be several effector molecules in lactobacilli that regulate host response. Knowledge of the molecular mechanisms underlying the physiological characteristics of lactobacilli and identifying bacterial components that influence host-microbe interactions can strengthen the understanding of the impact of strain specificity and strain variation in lactobacilli.

1. CHAPTER 1: REVIEW OF LITERATURE

Part of the material presented in this chapter was published as a critical review in a special issue on "Mediators of inflammation & immune responses in the human gastrointestinal tract" in the journal Mediators of Inflammation: Sengupta, R, Altermann, E, Anderson, R C, McNabb, W C, Moughan, P J and Roy, N C. (2013). The role of cell surface architecture of lactobacilli in host-microbe interactions in the gastrointestinal tract. Mediators of Inflammation. Volume 2013, Article ID 237921, 16 pages.

1.1 THE HUMAN GASTROINTESTINAL BARRIER

The human gastrointestinal tract (GIT) is the body's largest interface with the environment and is host to a complex community of microorganisms. Humans and their symbiotic bacteria share an evolutionary fate and their mutual interactions are essential for human health and well-being [16]. The human GIT is dynamic and a complex ecosystem, an essential part of the digestive system that is constantly exposed to pathogenic and commensal bacteria as well as food and other luminal antigens. The functions of the GIT besides digestion and carbohydrate metabolism (which involves the uptake of nutrients, water and electrolytes), are to resist the passage of potentially detrimental pathogens while remaining tolerant to commensal bacteria. The barrier properties of the epithelium exclude pathogens and toxins and help in maintaining the balance between "protective" versus "harmful" intestinal bacteria [17]. The ability of the intestinal barrier to exhibit immunological tolerance towards commensal bacteria while inducing immunological responses restricting the penetration of harmful pathogens is crucial for the balance between health and disease. A breakdown in this balance may lead to chronic inflammation, as seen in the initiation and progression of intestinal diseases such as inflammatory bowel diseases [18], as well as diseases like colon carcinoma, the leading cause of death through cancer [19].

The human intestinal barrier has four different components that together provide defence against pathogens, while maintaining tolerance towards commensal bacteria (Figure 1.1) The four components are the epithelium, the mucus layer, the commensal bacteria community and the immune cells of the lamina propria [20, 21].

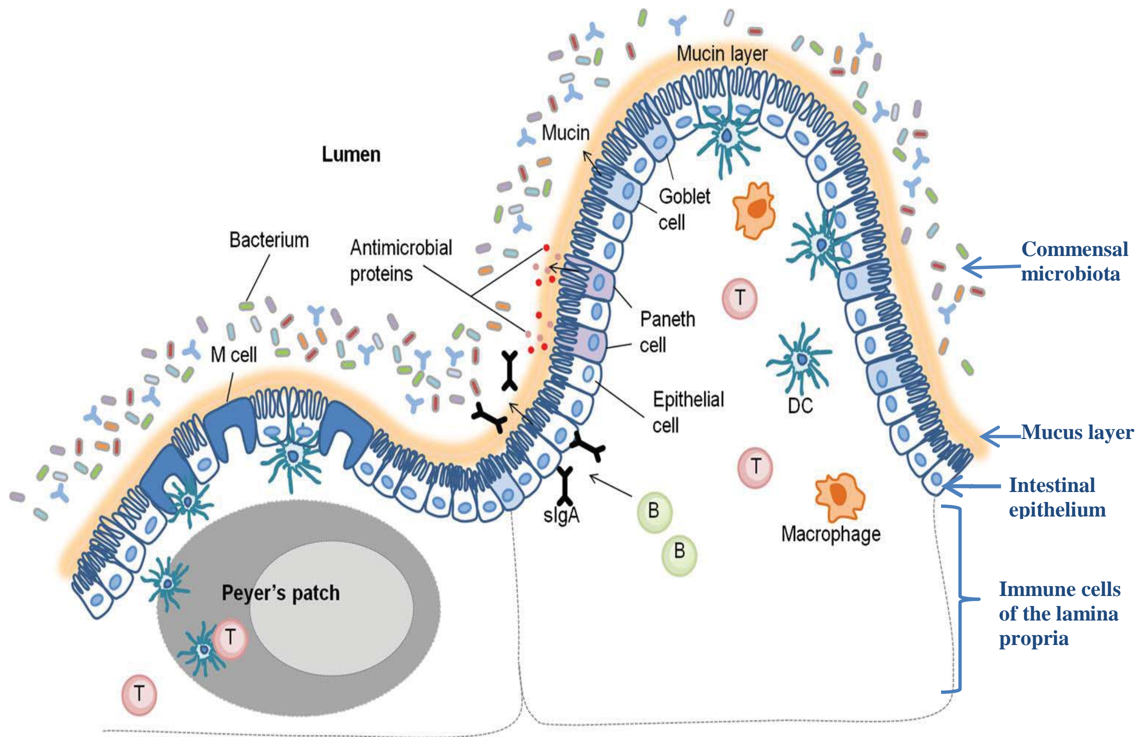


Figure 1.1 Components of the human intestinal barrier. The epithelium is composed of a one-cell-thick epithelial layer, separating the intestinal lumen from the internal lamina propria. The mucus layer protects the underlying epithelium while retaining antimicrobial compounds. The commensal microbiota protect against pathogens by competing for binding sites and by secreting antimicrobial compounds. The immune cells of the lamina propria help in defence against pathogens while tolerating commensals. Figure adapted from Lee *et al*. (2012) [10].

The epithelium provides a physical barrier between the highly colonised lumen and the internal milieu. The mucus layer is a chemical barrier that protects the underlying epithelium and acts as a medium for retaining, as well as transporting antimicrobial compounds between the luminal contents and intestinal epithelial cells (IEC). The commensal microbial community is composed mainly of hundreds of species of bacteria and one archaeal phyla (in mouth and colon), which protect against pathogens by competing for nutrients and binding sites and by secreting antimicrobial compounds [22]. The immune cells of the lamina propria provide the immunological barrier and help in defence against pathogens while tolerating commensal bacteria. These immune cells include macrophages, dendritic cells (DC), T-cells and antibody producing B-cells.

The intestinal epithelium, composed of a single layer of columnar epithelial cells, provides the physical barrier separating the lumen from the immunological barrier of the lamina propria. The IEC are joined together by tight junctions (TJ), adherence junctions, desmosomes and gap junctions [23]. Specialised IEC include enteroendocrine cells that secrete hormones, mucus secreting goblet cells and Paneth cells that provide defence by synthesising antimicrobial compounds [24]. Paracellular permeability is influenced by the TJ as they are the most apical connecting structures that seal the paracellular space between IEC. TJ provide a dynamic and selective barrier that regulates transport of nutrients, water and electrolytes while preventing pathogen entry. TJ play a crucial role in IEC proliferation, differentiation and polarisation, and together with adherens junctions (AJ), are involved in many intracellular signalling and transport pathways [25, 26]. Together TJ and AJ are known

as the ‘apical junctional complex’. While TJ and AJ are restricted to the apical domain, desmosomes are more widely distributed along the lateral membranes of neighbouring, polarised IEC. Gap junctions are interspersed among desmosomes and are involved in intracellular communication [27]. The intercellular junctional complexes determine paracellular permeability and play a crucial role in the functioning of the GIT.

The mucus layer is a dynamic and protective barrier overlying the IEC, composed predominantly of gel forming glyco-proteins called mucins that are synthesised and secreted by goblet cells [28]. Goblet cells are specialised columnar epithelial cells that secrete mucins by either simple exocytosis or compound exocytosis [29]. Simple exocytosis or baseline secretion involves release of newly synthesised mucin granules constitutively by the goblet cells. Exposure to bioactive factors such as hormones and inflammatory mediators (cytokines) however, can induce goblet cells to undergo compound exocytosis, resulting in the accelerated secretion of centrally stored mucin granules.

The mucus layer varies in thickness throughout the GIT and is thickest in the stomach, where it protects the underlying epithelium from highly acidic conditions. The layer is comparatively thin in the small intestine to aid bacterial sampling by the immune cells. In the colon, the mucus layer thickens and has two distinct layers with similar protein composition. The inner layer is dense and sterile, devoid of bacteria, while the outer layer is comparatively less dense and highly colonised by bacteria [30]. The mucus layer acts as a defensive barrier as it is the first anatomical site where the host encounters intestinal microbes. A protective function is suggested for the mucus layer due to consistent evidence showing enhanced mucus secretion in response to

intestinal microbes [31, 32] and structural alteration of the mucus layer in intestinal disorders such as Crohn's disease and ulcerative colitis [33].

The commensal microbial community is comprised of a vast, dynamic consortium of microorganisms that outnumber the cells of the human body tenfold [34]. The term "commensal" generally refers to microbes that co-exist without harming one another but without any obvious benefit, unlike pathogens that cause damage to the host. Bacteria can adhere to the mucosal surfaces of the GIT or remain unattached in the lumen of the intestine. The interaction of the microbiota with the intestinal cells plays a key role in the "cross-talk" between the epithelium and other cell types of the mucosa and influences the functional and developmental roles of the GIT [35]. The interactions between the bacteria and IEC varies from small to large intestine due to anatomical differences and is also influenced by the thickness of the mucus layer covering the epithelium. Several studies indicate that the composition and thickness of the mucus layer of the GIT are affected by the microbiota [32, 36]. The intestinal microbiota is discussed in more detail in the following section (Section 1.1.1).

The immune cells of the lamina propria form a part of the most complex immune systems of the human body, the intestinal immune system [37]. The lamina propria is the mucosal tissue, directly underlying the IEC, containing B cells, T cells, stromal cells, and anigen presenting cells such as macrophages and DC. The lamina propria contains organised lymphoid organs, including Peyer's patches in the ileum and mesenteric lymph nodes that are distributed throughout the small intestine and colon [38]. Lamina propria DC have been shown to penetrate epithelial tight junctions and directly sample the gut lumen for antigens [39]. Luminal antigens are also sampled by

specialised epithelial cells, the M cells, located in Peyer's patches and follicle associated epithelium [40]. The immune cells of lamina propria and the IEC recognise pathogens by means of pathogen recognition receptors (PRR), which leads to the activation of a pro-inflammatory response against the pathogens [41]. To maintain homeostasis, the intestinal immune system has to remain unresponsive to commensal bacteria and harmless food antigens, but respond robustly to harmful pathogens and a breakdown in the intestinal immune system leads to uncontrolled intestinal disorders.

1.1.1. THE INTESTINAL MICROBIOTA AND HOST-MICROBE INTERACTIONS

The human intestine is host to 10^{14} commensal bacteria with a biomass of 1.5-3 kg, comprising of 500-1000 different species of bacteria [42]. Although the composition of the microbiota differs between individuals and at each stage of life, adulthood is the most stable stage and is characterised by a distinct and differentiated microbiota [43]. The majority of these microbes reside in the human colon, where densities approach 10^{11}-10^{12} cells/mL [44]. The intestine has a remarkable exclusion function. The selection pressures presented by it result in the microbiota being dominated (greater than 90% of total) by just two divisions of bacteria (Bacteroidetes and Firmicutes) and one member of archaea (*Methanobrevibacter smithii*) [45, 46]. The survival of microbes and humans is interdependent and the human intestine can be seen as a "natural laboratory for studying the microevolution of humans" [34]. The gut microbiota, under normal physiological conditions, is composed mainly of commensal bacteria that benefit the host by mediating digestion of food, strengthening the immune system and preventing pathogen entry which could subsequently lead to disease in the

host. In addition to providing digestive benefits to the human host, these bacteria produce a variety of metabolites which have a potential impact on human health [47]. The role of the gut microbiota and its impact on human health and disease has become a major focus for scientific research in the past decade. It is now clearly evident that gut microbial imbalance (dysbiosis) can have a profound effect on the human immune system and has been linked to the development of autoimmune and inflammation related disorders [48]. The belief that a healthy GIT is essential to the general well-being of humans has led to investigations to find the appropriate balance of microbes that would promote health. Lactic acid bacteria (LAB) such as lactobacilli played a key role in this research due to their putative beneficial effects on intestinal health [49].

There is increasing support through experimental evidence for the role played by these bacteria in modulating the host immune system and the barrier properties of the intestinal epithelium [50]. The interaction between microbes and the epithelium in the GIT is crucial for regulation of intestinal immune homeostasis and for maintenance of host-microbe symbiosis. Several studies show that the breakdown of intestinal barrier integrity may be a result of altered intestinal microbial composition that is observed in several disorders [51, 52]. Dysbiosis occurs as a result of shifts in the composition of microbial communities in the human gut, which can affect host physiology thus compromising human health. Intestinal dysbiosis has been linked with the pathogenesis of inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), which results from a breakdown of the innate and adaptive immunity responses towards commensal microbiota [53]. Genetic susceptibility, defects in gut barrier function and an imbalance in the gut microbiota composition are considered to

be factors that impact the development of IBD [54]. Research has reported an abnormal GI microbiota in a subset of IBD patients, characterised by a decrease in *Firmicutes* and *Bacteroidetes* and a concomitant increase in *Proteobacteria* and *Actinobacteria* [51]. Among *Firmicutes*, IBD patients show a reduced presence of *Faecalibacterium prausnitzii* and IBD-associated dysbiosis has also been characterised by a decrease in the ratio between *F. prausnitzii* and *Escherichia coli* [55]. However, whether dysbiosis is a consequence of pathogenesis or the primary trigger that leads to inflammation subsequently causing IBD is still unclear. Faecal microbiota transplantation (FMT) has become an effective and increasingly popular procedure for treating patients with IBD [56]. It is considered the most direct way to change the patient's intestinal microbiota and FMT promptly restores normal donor-like intestinal microbiota composition dominated by the *Bacteroidetes* and *Firmicutes* with diminished proportions of members of *Enterobacteraceae* family [57]. However, the attempt at microbiota modification using FMT as treatment in IBD is in its infancy and future work should focus on refined and directed approach towards specific classes of organisms.

It is clear that the structure and composition of the commensal microbiota has an impact on human health. LAB are considered to be the most abundant group of bacteria that are linked to humans, associated particularly to the mucosal surfaces of the GIT [58]. Commensal LAB have drawn increased attention due to their suggested role in maintenance of health. Although lactobacilli make up a small portion of the gut microbiota, they and other LAB are predominant in the small intestine, where they provide protection against pathogens [59]. Several species of lactobacilli are

autochthonous (indigenous) and are able to colonise the mucosal surface of the GIT due to compatibility with the host immune system and specialised adhesion factors. However, there are allochthonous species of lactobacilli, which are transient and derived from food or from the oral cavity and these strains may inhabit the GIT for a limited time, probably even a few days [60]. Some transient species may not survive the harsh conditions of the GIT while others may resist, adapt and add to the commensal microbiota [61]. Over the past decade, the role played by lactobacilli and other commensal microbes in immunomodulation of the IEC has been the focus of considerable studies [41].

The GIT is a complex ecosystem that acts as a reservoir for both commensal and pathogenic bacteria. During the last few decades, it has become clear that the residential and ingested microbes that comprise the microbiota in the GIT exist in a continuum that ranges between mutualism and pathogenicity [62]. The microbiota plays an essential role in the general well-being of the host, including physiological functions such as digestion, assimilation of nutrients, protection against pathogens and immunomodulation. The microbiota is dynamic and there is a continuous interplay between commensal and pathogenic bacteria and the human host which makes it challenging to understand the mechanisms underlying how these bacteria contribute to human health. The complexity of the microbiota and the fact that it varies extensively between individuals adds to this challenge [63]. Although there are many factors that contribute to influencing the human microbiota, microbial interactions along with diet and host genotype have a major impact on the composition of individual microbial communities [64]. Interest in the beneficial

functions of the microbiota in human health has resulted in studies that were aimed at understanding the modes of action by which the microbiota confer their beneficial effects and contribute to human health.

Bacteria interact with the intestinal epithelium through various mechanisms that help maintain microbial homeostasis, modulate host response and preserve barrier integrity. These modes of action and some of the bacterial key components that influence host-microbe interactions are discussed in further detail in Section 1.4.2.

1.2 LACTOBACILLI

The LAB group comprises of Gram-positive, low guanine plus cytosine (G+C) content, microaerophilic, nonspore-forming, nutritionally fastidious rods and cocci that are functionally linked due to their common ability to primarily produce lactic acid from hexose sugars [58]. *Lactobacillus* is the largest genus within the LAB group, with a G+C content of less than 50 mol%, and belongs to the *Clostridium* branch [65]. Taxonomically, the *Lactobacillus* genus belongs to phylum *Firmicutes*, under the class *Bacilli*, order *Lactobacillale*s and family *Lactobacillaceae*. Their natural habitats encompass a variety of ecological niches, from foods such as dairy products, meats, vegetables, sourdough bread, and wine, to plants, sewage and to the mucosal surfaces such as the oral cavity, vagina and the GIT of humans and animals [66].

Traditionally lactobacilli have been known as starter cultures for food fermentations, but recent studies have suggested lactobacilli contribute to a variety of functions including intestinal barrier integrity, immunomodulation and pathogen resistance [3-5]. Recent studies involving animal models also suggest that lactobacilli

can modulate immune responses, possibly by activating macrophages, affecting the production of cytokines, increasing the activity of natural killer cells or by modulating immunoglobulin production [67-69]. These functions of lactobacilli as immunomodulators can be variable among different strains and the mechanisms involved are still not clearly understood.

Lactobacilli are present throughout the GIT in varying amounts. They are dominant in the proximal small intestine [70], a nutrient rich environment, while in the distal intestine they comprise only 1% of the microbes per gram of luminal content [71]. In the faecal microbiota, they present at most ~0.01%-0.6% and this proportion varies between individuals [72, 73]. Some lactobacilli have the ability to adhere to and interact with mucosal surfaces and the epithelium, while surviving the hostile conditions of the luminal environment and persisting against the competing microbiota [74]. These properties add to their potential to be used as probiotics that fit the parameters set by the Operating Standards in 2002 (FAO/WHO: Guidelines for the evaluation of probiotics in food). However, studies have shown that different strains of lactobacilli can evoke different responses in the host and therefore, results from one strain cannot be generalised to others [5, 75, 76].

Adherence of lactobacilli to the intestinal epithelium is an important characteristic as it promotes residence time and colonisation, stimulates microbe-host interactions through immunomodulation and provides protection to the intestinal barrier by various mechanisms including antagonistic activities against pathogens [77]. Bacterial cell surface components (adhesins, polysaccharides and proteins) play major roles in the adherence of lactobacilli to the intestinal epithelium, interactions that might

lead to pathogen exclusion and immunomodulation of host cells [78, 79]. Several studies implicate cell surface components, either individually or collectively, in microbe-host interactions [14, 15].

Lactobacilli show great diversity in cell surface architecture and are known to modify their surface properties in response to environmental stress factors such as bile and low pH, and these adaptations may help their survival through the harsh environmental conditions encountered in the GIT [11, 12]. Different macromolecules constituting the cell wall (CW) of lactobacilli have been shown to contribute to maintaining bacterial cell integrity during environmental stress [13]. The cell surface architecture of lactobacilli and their ability to express certain surface components or to secrete specific compounds, that act directly on the host cells, may thus influence the physicochemical properties of the bacterial cell and strain-specific properties.

1.2.1 CELL SURFACE STRUCTURES OF LACTOBACILLI

The cell envelope of lactobacilli, like that of all Gram positive bacteria, is composed of a bilipidic plasma membrane with embedded proteins encompassed by the CW. The bacterial CW consists of a thick multilayered sacculus made of peptidoglycan (PG), decorated with teichoic acids (TA) [wall teichoic acids (WTA) and/or lipoteichoic acids (LTA)], exopolysaccharides (EPS), proteinaceous filaments called pili and proteins that are anchored to the CW by different mechanisms (Figure 1.2). Some species of lactobacilli have an additional paracrystalline layer of proteins surrounding the PG layer, referred to as the S-layer.

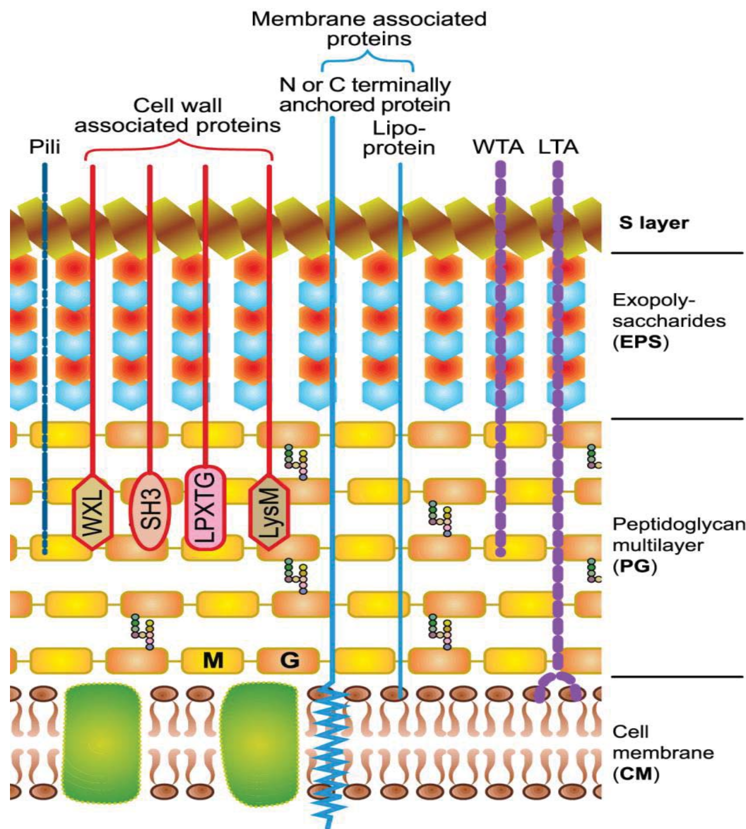


Figure 1.2 Cell envelope of lactobacilli. A schematic representation of a Gram-positive cell wall, and membrane-associated proteins found in the cell envelope of lactobacilli. The bilipidic cell membrane (CM) with embedded proteins is covered by a multilayered peptidoglycan (PG) shell decorated with lipoteichoic acids (LTA), wall teichoic acids (WTA), pili, proteins and lipoproteins. Exopolysaccharides (EPS) form a thick covering closely associated with PG and are surrounded by an outer envelope of S-layer proteins. The proteins are attached to the CW either covalently (LPXTG proteins) or non-covalently (exhibiting LysM, SH3 or WXL domains), lipid anchored to the CM (lipoproteins) or attached to the CM via N- or C-terminal transmembrane helix. M: N-acetyl-muramic acid; G: N-acetyl-glucosamine. Figure adapted from Kleerebezem *et al*. (2010) [80] and Bron *et al*. (2012) [81].

These macromolecules together may play crucial roles in determining species- and strain-specific characteristics of lactobacilli by influencing host-microbe interactions and microbial adaptations to the changing host environment.

1.2.1.1. Peptidoglycan

PG is the largest component of the bacterial CW and is an essential polymer in lactobacilli that determines the shape and preserves the integrity of the bacterial cell [82]. The PG layer has been described as a "fisherman's net" functioning both as a container and a sieve to the bacteria [82, 83]. The elastic nature of PG helps withstand stretching forces caused by bacterial turgor pressure, excludes large molecules from entering the bacterial cell and at the same time restricts the secretion of large proteins to the external milieu. Proteins with theoretical molecular masses as large as 49.4 kDa and 82.1 kDa have been reported to be secreted by *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* respectively [84, 85]. Some large proteins are unable to diffuse through the CW and are dependent on the CW expansion process to be dragged to the outer surface of the thick PG layer before being passively released into the external milieu [82, 83]. The threads of the PG net are polymers of covalently linked alternating residues of N-acetyl-glucosamine (GlcNAc) and β-1-4-linked N-acetyl-muramic acid (MurNAc). The glycan threads are held together by crosslinking pentapeptide side chains providing elasticity to the net. The pentapeptide side chain is made of alternating L- and D-amino acids and this attaches to the D-lactyl carboxyl group of MurNAc. Considerable variation occurs in the basic composition of the glycan strands and pentapeptides which impart strain-specific characteristics to the bacteria [86, 87]. For example, in several lactobacilli, resistance to vancomycin (a

glycopeptide antibiotic), was shown to be the result of a replacement of the C-terminal D-alanine residue of MurNAc-pentapeptide by D-lactate [88]. Following biosynthesis, assembly and incorporation of the PG subunits, modifications in the GlcNAc and MurNAc structures can occur and affect interactions between the host and lactobacilli [82]. These modifications include removal of acetyl groups from CW PG [89], 6-O-Acetylation of CW MurNAc residues [90] and the substitution of C6 MurNAc by teichoic and teichuronic acids [91]. These modifications can affect the physiology of the CW by increased sensitivity to autolysis, resistance to lysozyme and hydrophobicity of the cell envelope which in turn affects recognition by host receptors and bacterial adhesion [92, 93].

1.2.1.2. Teichoic acids

The TA are the second major component of CWs of lactobacilli and account for up to half of its dry weight [80]. They are anionic polymers consisting of repeating units of glycerol- or ribitol-phosphate, covalently linked to PG as WTA or attached to the cytoplasmic membrane through their lipid anchors as LTA [94-96]. A fraction of LTA can be found free in the CW or may be released into the extracellular medium through deacetylation of the lipid anchor, where they are recognised as ligands by receptors present on IEC [50]. LTA contribute to the anionic character of the CW and provide hydrophobicity, which in turn influences its adhesiveness [93].

The overall structure of TA is a chain made of phosphodiester-bound glycerol or ribitol residues hooked through a terminal 'linkage unit' on the C6 of the Mur*N*Ac residue of a growing PG chain. The structure of the linkage unit is well conserved and

comprises of a disaccharide N-acetylmannosaminyl β (1-4) glucosamine followed by glycerol phosphate. Differences in the nature of the sugars and number of phosphate residues can lead to the variation of TA structure [82]. There are considerable variations in structure and abundance between WTA and LTA molecules. Their size and physico-chemical properties depend on several factors such as species or strain, stage or rate of growth, availability of phosphate, acidity of medium, fermentable carbon sources, etc. [82]. Although all lactobacilli have TA in their CWs, not all *Lactobacillus* CWs contain WTA and some species appear to contain only LTA [97]. The LTA contributes to the anionic character of the CW and provide hydrophobicity, which in turn influences the adhesiveness of the cell envelope [93]. TA can function as a reservoir for phosphates and also as a scavenger of cations (Mg^{++} in particular) [98, 99]. The TA can also help in creating a pH gradient across the CW and are known to be involved in phage adsorption and autolysin activity [100]. For example, glycosylated TA have been reported to be essential for the adsorption of some *Lactobacillus plantarum* phages and studies with *Lactobacillus delbrueckii* subsp. *lactis* show the involvement of LTA in phage inactivation [101].

1.2.1.3. Cell wall polysaccharides

CW polysaccharides are omnipresent components of the bacterial cell envelope. They are generally neutral polysaccharides covalently bound to Mur*N*Ac of PG or loosely associated with it (wall polysaccharides, WPS) or released into the extracellular medium (EPS). When they form a thick capsule that is closely bound to the cell envelope they are referred to as capsular polysaccharide (CPS) [102, 103]. Distinction between these various classes of CW polysaccharides is often difficult. In

lactobacilli, EPS usually refers to extracellular polysaccharides that can be attached to the CW or released into the surrounding medium. The complex variations in the composition of EPS, which differ in the nature of the sugar monomers along with their linkages, distribution and substitution, add to the structural variety of the *Lactobacillus* cell envelope [104, 105]. EPS are generally composed of heteropolysaccharides consisting of different sugar moieties such as glucose, galactose, rhamnose, GlcNAc and N-acetylgalactosamine [106]. Residues of glucoronic acid, phosphate, acetyl and pyruvate groups may also be present in some strains of lactobacilli. Studies with *Lactobacillus rhamnosus* GG identified two different classes of EPS: long galactose rich molecules and short glucose/mannose rich EPS molecules [107]. Specific contributions of EPS to CW functionality are unclear, although their general role is to mediate interactions of lactobacilli with environmental components and promote bacterial adhesion and biofilm formation to inert or living surfaces [108, 109].

1.2.1.4. Pili and flagella

Pili are multi-subunit protein polymeric structures that have been identified at the genome level in some lactobacilli [110], although functionally analysed and characterised only in *Lactobacillus rhamnosus* GG [111, 112]. These non-flagellar appendages are an assembly of multiple pilin subunits that are covalently coupled to each other by the transpeptidase activity of the pilin-specific sortase [112, 113]. Resulting isopeptide bonds are formed between the threonine of an LPXTG-like motif and the lysine of YPKN pilin motif in the pilin subunits [114]. After assembly, the pilins are attached to the CW by a membrane bound transpeptidase, the housekeeping sortase [115]. The roles of pili in bacterial adhesion, invasion, aggregation, formation

of biofilms and modulation of immunity are well established [116, 117] but the receptors in the host that recognise these pili are still unknown and their function in signalling host response is unclear. The presence of flagella is an unusual feature found in lactobacilli and at present, at least twelve motile species of lactobacilli have been recognised [72]. The bacterial flagellum comprises of polymers of protein called flagellin, which is suggested to act as a ligand and mediate activation of signalling pathways and modulation of host immune cells [118].

1.2.1.5. Cell surface proteins

The cell surface proteins in lactobacilli are either anchored to the CW by various mechanisms or secreted from the bacterial cell into the surrounding medium, where they reassociate with the CW through electrostatic interactions [119]. Cell surface proteins include the S-layer proteins which, if present, constitute the major cellular proteins that surround the cell. A few examples of cell surface proteins include the 43 kDa collagen binding S-layer protein from *Lactobacillus crispatus*, two collagen-binding proteins of 45 and 58 kDa and a fibronectin-binding cell surface protein of 15kDa from *Lactobacillus acidophilus* CRL639 [120]. The cell surface proteins are categorised as CW-associated proteins, which are either attached covalently or non-covalently and membrane-associated proteins, which are either lipid anchored to the cell membrane (CM) or attached via an N- or C-terminal transmembrane helix (Figure 1.3).

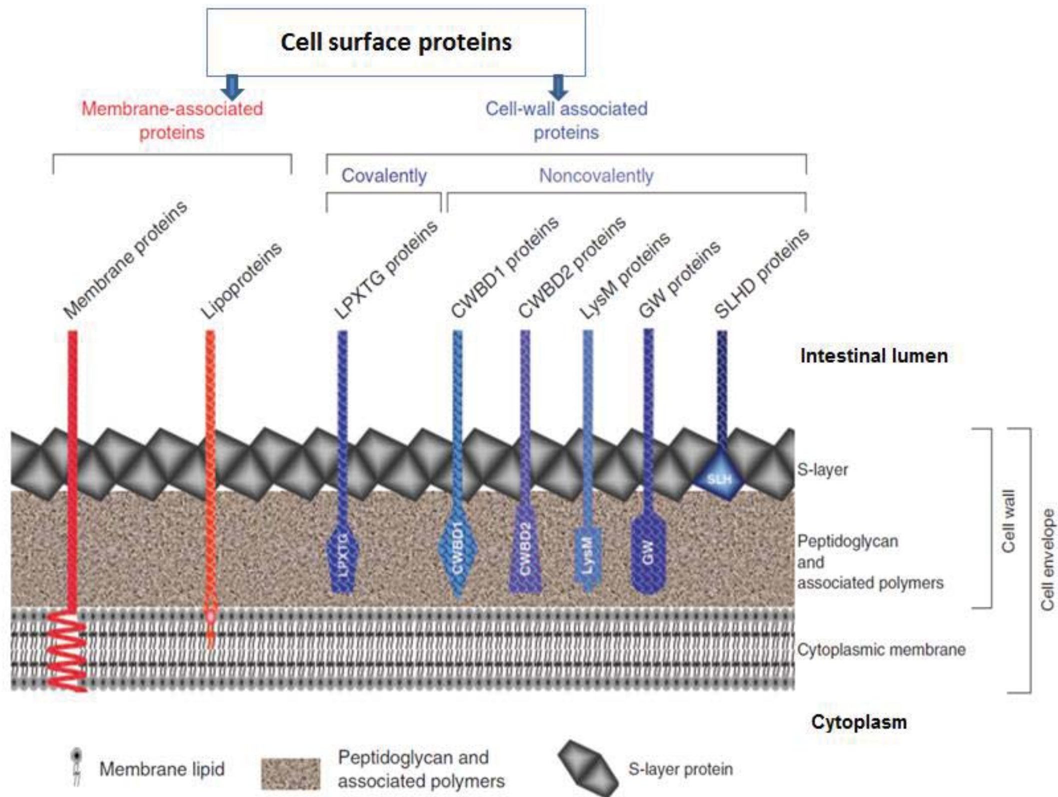


Figure 1.3 The different types of cell surface proteins found in Gram positive bacteria. The CW-associated proteins are attached either covalently (containing C-terminal pentapeptide LPXTG CW sorting motif) or non-covalently (exhibiting CW binding domains such as LysM, SH3 or WXL domains). The membrane-associated proteins are lipid anchored (lipoproteins) to the cell membrane (CM) or attached to the CM via N- or C-terminal transmembrane helix. M: N-acetyl-muramic acid; G: N-acetyl-glucosamine. Figure adapted from Desvaux et al. (2006) [121].

1.2.1.5.1. Covalently anchored proteins

Covalently anchored proteins are further subcategorised into N- or C-terminally anchored proteins; lipid anchored proteins (lipoproteins) and sortase-dependent proteins. These proteins have a wide repertoire of functions, ranging from adhesion, transport, signal transduction, cell envelope metabolism, antibiotic resistance to microbe-host interactions. In *Lactobacillus* genomes, the N-terminally anchored proteins represent the largest group of cell surface anchored proteins and are mainly involved in cell envelope metabolism, extracellular transport and signal transduction, competence and protein turnover [80]. The C-terminal of signal peptides of proteins being transferred across the cytoplasmic membrane, contain a target sequence (cleavage site) which is cleaved for the mature protein to be released. However, many of the signal peptides of these proteins do not possess the cleavage motif in their C terminal and as such remain N-terminally linked to the cell membrane. In some cases where the cleavage site is present, the protein may still be anchored to the cell membrane by a C-terminal transmembrane domain. Many C-terminally anchored proteins are encoded by lactobacilli but the function of several of these proteins still remains unclear [80]. The lipid anchored proteins constitute the second largest group of predicted membrane-anchored proteins in lactobacilli and are involved in transport, adhesion, antibiotic resistance, sensory processes, homeostasis of the cell envelope and secretion, folding and translocation of proteins [80]. The C-terminal of the signal peptide of these lipoproteins contains the lipobox motif [L-(A/S)-(A/G)-C]. Lipidation followed by cleavage at the N-terminal of the Cys-residue in the lipobox results in the covalent binding of the lipoprotein to the cell membrane through thioether linkage [122].

Sortase-dependent proteins (SDP) or LPXTG-anchored proteins are cell surface proteins that reportedly play a crucial role in lactobacilli-host interactions [123]. These proteins are covalently attached to the PG and are characterised by the C-terminal pentapeptide (LPXTG) CW sorting motif. These proteins typically contain a cleavage site in their C terminal, with the LPXTG located in the C-terminal region of the mature domain followed by a stretch of hydrophobic residues and a positively charged tail [123]. The LPXTG motif is recognised by the sortase enzyme (SrtA), which cleaves between the T and G residues and then covalently links the threonine carboxyl group to an amino group of the PG cross-bridges [124]. Although SrtA recognises the sequence LPXTG, another sortase, called SrtB in *Staphylococcus aureus* has been reported to recognise and process proteins bearing the sequence NPQTN [125]. Recent studies involving cross-linked protein products of SrtA and SrtB indicate that different types of sortases may be able to attach proteins to distinct positions within the CW [126].

1.2.1.5.2. Non-covalently anchored proteins

These are proteins that are non-covalently bound to the bacterial cell surface through binding domains. Some proteins can also be found anchored to other CW proteins through protein-protein interactions. Some proteins are known to reassociate with the CW after being secreted through electrostatic interactions [127].

1.2.1.5.3. S-layer proteins

Many species of lactobacilli display a surface coating made of a crystalline, two dimensional array of protein or glycoprotein subunits assembled in lattices with different symmetries, also referred to as the S-layer. *Lactobacillus* S-layer proteins

represent 10 to 15% of total CW proteins. These proteins, ranging from 40 to 60 kDa, are highly basic, with stable tertiary structures [50]. S-layer proteins are the most prominent glycoproteins in prokaryotes. Although in lactobacilli, most S-layer proteins appear to be non-glycosylated, some lactobacilli have glycosylated S-layer proteins that have been identified [128]. S-layer proteins are non-covalently bound to the underlying PG CW, generally through secondary polymers such as LTA, WTA and neutral polysaccharides [127]. Properties such as adhesion, aggregation and pathogen inhibition have been related with the occurrence of particular types of S-layers. The functions of S-layer in lactobacilli are not just species- but are also strain-specific. Studies indicate that there may be a correlation between the different structural and chemical characteristics of the S-layer proteins with the surface properties of lactobacilli [107, 127]. There is ample evidence of S-layer proteins influencing the development of microbial communities as biofilms and therefore, it is likely that S-layer proteins have a role in the interaction of lactobacilli with other microorganisms [129].

1.2.1.5.4. Proteins displaying cell wall binding domains

Lactobacilli have enzymes with choline-binding, LysM, WXL and SH3b domains that help to keep the enzymes anchored to the bacterial cell surface. Choline-binding domains are mainly found in extracellular enzymes such as autolysins and are a stretch of 20 amino acids that have conserved multiple tandem repeats of aromatic residues and glycines. These proteins anchor to the cell surface by binding to the choline residues of WTA and LTA [130]. The LysM domain (lysine motif) is found in many extracellular enzymes that are suggested to have a PG binding function and are

involved in CW metabolism [131]. WXL domain containing proteins were identified in lactobacilli based on *in silico* analysis [132] and are suggested to interact with the PG layer through their protein C terminus. This domain has also been reported to mediate non-covalent binding between the bacterial CW of *Enterococcus faecalis* and other Gram positive bacteria [133]. SH3b domains have been identified in some lactobacilli and are involved in CW turnover. They have also been suggested to recognise specific sequences within the peptide cross-bridges of the PG, thus targeting and binding to the CW [134]. A putative domain composed of three α helices at the C- or N- terminal of an extracellular protein has been reported in some lactobacilli (*Lactobacillus plantarum, Lactobacillus johnsonii, Lactobacillus casei, Lactobacillus brevis, Lactobacillus helveticus* and *Lactobacillus gasseri*) [80]. This domain was found on cell-wall-degrading enzymes (Pfam PF01471) in these lactobacilli and is suggested to be involved in CW degradation through binding to the PG.

1.2.2 CELL SURFACE ADAPTATIONS OF LACTOBACILLI IN RESPONSE TO HOST ENVIRONMENT

The cell envelope is the first target of physico-chemical and environmental stress. Lactobacilli encounter several environmental stress factors during their transit through the GIT such as low pH, bile salts, oxidative and osmotic stress, along with starvation stress. Lactobacilli have developed sophisticated responses and adaptations to survive these stressors. Stress responses of lactobacilli rely on the coordinated expression or suppression of genes that act in concert to improve stress tolerance. These genes can alter cellular processes such as cell division, membrane composition, transport systems, housekeeping, DNA metabolism, etc. There are regulators that

control these genes and sometimes even other regulators. The integration of the bacterial response to various stress responses is provided by networks of regulators [135]. Lactobacilli respond to stress in specific ways dependent on the strain, species and the type of stress [136, 137].

1.2.2.1. Acid and bile stress

Survival under acidic conditions is achieved by adapting to low pH through a mechanism called acid tolerance response. Studies with acid- and bile-resistant variants of *L. acidophilus* suggest that an inducible pre-existing system co-exists with a *de novo* protein synthesis mechanism, which together protect against acid stress [138]. Bile acids are conjugated to glycine or taurine in the liver and enter the intestine where the amino acid may be deconjugated or hydrolysed by bile salt hydrolases (BSH) expressed by bacteria, including lactobacilli. Deconjugated bile acids are reabsorbed by passive diffusion, along with conjugated bile acids, and are efficiently conserved by enterohepatic recirculation [139]. In *L. plantarum*, the capacity to tolerate taurodeoxycholic acid (TDCA) has been attributed to the expression of TDCA hydrolase, but other studies have shown that BSH activity and resistance to bile are unrelated properties in lactobacilli [140, 141]. Many resistance mechanisms have been found to be common for bile and acid stress and result in alteration of lactobacilli cell surface structures [142]. Under bile and acid stress, the macromolecules composing the bacterial cell envelope (CW and cell membrane) contribute to maintaining the cell integrity. For instance, bile salts and cholesterol have been shown to induce changes in the lipid cell membrane of *L. reuteri* [11] while low pH causes alterations in the fatty acid composition of an oral strain of *L. casei* [12].

Screenings of acid and bile salt responses in lactobacilli have identified genes involved in PG biosynthesis and cell envelope functions. Gene expression analysis of *L. acidophilus* identified a high number of genes involved in PG and cell surface protein (e.g., SrtA) biosynthesis that are differentially expressed after bile exposure [143]. In *L. reuteri*, response to acidic conditions involves the ClpL chaperone, an ATPase with chaperone activity and a putative CW-altering esterase. These enzymes are also reported to be induced by bile exposure, further implying common resistance mechanisms for acid and bile stress [144, 145]. Other cell surface structures (LTA, WTA and EPS) have also been suggested to play roles in proper functioning of cell integrity in acidic conditions and in the presence of bile [146]. EPS biosynthesis reportedly involves suppression of genes after bile exposure as noted in *L. acidophilus* and *L. reuteri*, although the role of EPS in bile and acid resistance is still unclear [143, 145].

1.2.2.2. Oxidative and osmotic stress

In addition to acid and bile stress, the survival capacity of lactobacilli to oxidative and osmotic stress in the GIT is important. Lactobacilli are fermentative, aero-tolerant bacteria that possess oxidases, which utilise oxygen to oxidise substrates such as pyruvate to produce lactate or ethanol. These conversions produce reactive oxygen species (ROS) that cause oxidative stress in the cell and the ability to cope with this stress varies among different lactobacilli. Oxidative stress that can adversely affect cell fitness is caused by exposure to ROS resulting from partial oxygen reduction to superoxide anion radicals (O_2), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2).

ROS attacks proteins, lipids and nucleic acids and constitutes one of the major causes of ageing and cell death [137]. Polyunsaturated fatty acids are sensitive to ROS attack and the resulting peroxidation of membrane lipids and protein alteration affect cell membrane permeability and osmoregulation [147]. To minimise the damage caused by ROS, lactobacilli counteract ROS generation with the help of enzymes such as catalase, nicotinamide adenine dinucleotide (NADH) oxidase/peroxidise and superoxide dismutase (SOD) or non-enzymatic compounds such as ascorbate, glutathione and Mn^{2+}. Resistance to oxidative stress varies widely between species and strains. Stress handling mechanisms range from preventing formation of ROS, elimination of ROS and defence against oxidative damage to repair of oxidative damage [137].

The fatty acid composition of the cell membrane of *L. helveticus* has been shown to change under oxidative stress and this was reported to be due to an increased activity of the O_2–consuming fatty acid desaturase system, which reduces the free radical damage in the cell [13]. Interestingly, bile stress has also been shown to induce oxidative stress and studies indicate that the expression of glutathione reductase, the enzyme that reduces glutathione to glutathione sulfhydryl, is influenced by bile treatment [148]. Glutathione not only plays a key role in maintaining a proper oxidation state of proteins containing thiol groups, but also in protecting the bacteria from stress caused by low pH, chlorine compounds and osmotic stresses [142, 148]. Resistance to oxidative stress varies widely between species and strains and stress handling mechanisms range from preventing formation of ROS, elimination of ROS, defense against oxidative damage to repair of oxidative damage [137].

Lactobacilli are often exposed to changes in osmolarity of their environment, which can compromise essential cell functions. The bacterial cell membrane is permeable to water but is impermeable to most solutes. Changes in solute concentrations in the environment cause changes in cell turgor pressure which leads to changes in cell volume and adversely effects essential functions. To maintain turgor pressure in the cell and retain water, lactobacilli accumulate compatible solutes under hyper-osmotic conditions and release them under hypo-osmotic conditions [137]. In *L. acidophilus*, disruption of the cell division enzyme CdpA, caused an increased resistance to bile salts while showing reduced resistance to osmotic stress. Similar effects were shown by the SlpA mutant of *L. acidophilus*, which was more sensitive to osmotic stress while being more resistant to bile. According to these studies, certain components of the CW remain uncleaved or cross-linked resulting in immature structure of the CW in the mutant thus altering its phenotype [1, 149]. Studies with *L. alimentarius* showed that when grown under sublethal doses of NaCl, an increased tolerance was observed towards hyperosmotic conditions or an increased acid tolerance response against organic acids. Similar cross protection was observed when the cells were exposed to sublethal doses of these acids implying that common mechanisms were involved [150].

1.2.2.3. Starvation stress

The capacity to adapt to a specific nutritional environment is important to lactobacilli and ensures their residence time and survival in the GIT. Starvation is one of the most common stresses faced by lactobacilli. Bacterial growth itself contributes to nutrient exhaustion, accumulation of fermentation end products (e.g., lactic acid)

and subsequent starvation. Nutrient starvation in lactobacilli has been mainly studied by limiting the supply of carbohydrate, phosphate and nitrogen. Lactobacilli adapt to these nutritional limitations either by down-regulating nucleic acid and protein synthesis and/or protein degradation and amino acid synthesis [151]. Moreover, extreme environmental stress conditions, such as extreme acidic conditions, can indirectly provoke starvation by decreasing the activity of transporters resulting in reduced availability of essential nutrients that might be present in the extracellular environment [152]. Nutrient starvation leads to growth arrest and different lactobacilli have developed different strategies to survive starvation. Modification of cell morphology and cell division at the entry of the stationary phase, resulting in diminished cell size, has been reported in lactobacilli, some enterococci and lactococci, under these conditions [137, 153].

Starvation resistance mechanisms in lactobacilli are diverse as they occupy different niches and do not encounter the same starvation conditions. It is well established that bacteria become more resistant to various types of stresses and develop a general stress-resistant state on entering the stationary phase [136]. Carbohydrate starvation induces increased resistance to many stress conditions. Amino acid catabolism, in particular arginine degradation, plays a role in the enhanced survival of *Lactobacillus sakei* during stationary phase [154]. In *L. acidophilus*, 16 proteins were reported to be synthesised as a response to starvation, of which 7 were induced by stationary phase, while the others were induced in response to low pH [155]. In *Lactococcus lactis* subsp.*lactis* IL1403, glucose starvation was shown to induce resistance to many stresses (heat, low pH, oxidative and osmotic stress) [156].

Similarly in *L. bulgaricus*, lactose starvation increased resistance to heat, acid and bile stress [157]. The regulation of starvation-induced proteins in lactobacilli is still unclear. Although studies indicate a small overlap between stress-specific regulator genes and starvation regulator genes, many proteins can be commonly induced by more than one stress. However, only a few proteins are common to all stresses.

1.2.3 LACTOBACILLI-HOST INTERACTIONS INVOLVING BACTERIAL CELL SURFACE AND SECRETED FACTORS

Host-microbe interactions generally occur along mucosal surfaces and the human GIT provides the largest interface, harbouring a diverse bacterial community. The internal host environment is separated from the external milieu by only a layer of IEC that provide the first line of defence against bacteria, viruses, fungi and parasites that can act as pathogens. The indigenous commensal microorganisms that comprise the microbiota are closely associated with the GIT epithelium. Thus, the epithelium is important for the maintenance of GIT homeostasis in the presence of commensal microorganisms while preventing pathogen invasion [158]. Lactobacilli interact with the GIT through several mechanisms that help modulate the immune response of the host, preserve barrier integrity and maintain microbial balance through exclusion of pathogens by direct antimicrobial activity (production of bacteriocins or inhibitors), competitive exclusion (competing for binding sites) and/or stimulating anti-inflammatory immune responses (Table 1.1).

Table 1.1 Lactobacilli cell surface factors implicated in microbe-host interactions.

Lactobacillus species	Mechanism and effect	Cell surface factors	Target cells or host factors	References
L. reuteri	Adherence, pathogen inhibition, enhancement of epithelial barrier function	Mucus binding protein (Mub), collagen binding proteins (CnBP)	Epithelial cells and mucus, collagen	[145, 159, 160]
L. acidophilus	Adherence and aggregation, pathogen inhibition, maintenance of barrier function, immunomodulation.	Mucus binding protein (Mub), fibronectin binding protein (FbpA), S-layer proteins (SlpA), LTA and EPS	Epithelial cells and mucus, fibronectin, ECM components, Caco-2 cells	[14, 120, 161, 162]
L. plantarum	Adherence, enhancement of epithelial barrier function, immunomodulation	Mannose-specific adhesin (Msa), GAPDH	Epithelial cells and mucus, Caco-2 cells.	[163-166]
L. rhamnosus	Adherence, protection against pathogen, anti-apoptotic effects on intestinal epithelial cells	Fimbriae , mucus binding factor (MBF)	Mucus glycoproteins, intestinal epithelial cells	[112, 159, 167, 168]
L. salivarius	Adherence	Sortase dependent protein (LspA)	Intestinal epithelial cells and mucus	[169]
L. crispatus	Adherence, pathogen	S-layer proteins	HeLa cells	[170]

	inhibition, resistance to acid and bile			
L. brevis	Adherence, protection against stressors (low pH, bile, etc.), enhancement of barrier function	S-layer proteins (SlpA)	Intestinal epithelial cells	[171, 172]
L. kefir	Aggregation and protection against pathogens and stressors	S-layer proteins	Caco-2/TC-7 cells	[173, 174]
L. fermentum	Adherence	Mucus binding protein (32-mMubp)	Mucus	[175]
L. johnsonii	Adherence	LTA, elongation factor Tu (EF-Tu) and heat shock protein (GroEL)	Caco-2 cells, intestinal epithelial cells and mucus	[162, 176, 177]
L. ruminis	Motility, immunomodulation	Flagellin	Intestinal epithelial cells, HT29 and Caco-2 cells	[72]
L. casei	Maintenance of barrier function, increased mucus production, immunomodulation	EPS, Sortase dependent proteins (SrtA)	Caco-2 and HT29 cells, macrophages	[178-180]

1.2.3.1. Adherence factors of lactobacilli

Adherence of bacteria to the GIT mucosa is an important factor for colonisation and leads to direct interactions that can result in competitive exclusion of pathogens and the modulation of host responses. Adhesive mechanisms of human pathogenic bacteria have been studied extensively through the use of *in vitro* model systems. Human colorectal adenocarcinoma cell lines such as Caco-2 or HT-29 cells, immobilised intestinal mucus and extracellular matrices, quantitative measurements, microscopic enumeration and immunological detection methods have been used for assessing adhesive mechanisms [181, 182]. However, knowledge of the bacterial cell surface molecules mediating adhesion to the GIT mucosa is still limited. Genomics based approaches have revealed several bacterial cell surface associated proteins that bind to mucus and IEC [14]. *Lactobacillus* adhesins have been grouped into: mucus binding proteins; sortase-dependent proteins; S-layer proteins; proteins mediating adhesion to the extracellular matrix (ECM) components of IEC; and non-protein adhesins (LTA and EPS).

1.2.3.1.1. Mucus binding proteins

The IEC form a barrier between the host and the content of the lumen and are covered by a protective layer of mucus. The mucus layer exists in a dynamic equilibrium, balanced between production, degradation and physical erosion. It provides bacteria with only a short residence time in the GIT upon adhesion, thereby protecting the host against pathogens and undesirable bacterial colonisation [76]. However, the mucus layer also provides a habitat for commensal bacteria, such as

lactobacilli. Adherence of lactobacilli to mucus has been experimentally validated *in vitro* using adhesion assays with probiotic bacteria-pretreated intestinal mucus glycoproteins [183], as well as *in vivo* by microscopic analysis of biopsy samples [184]. *Lactobacillus* adhesion to mucus involves mucus binding proteins (Mub), which in addition to the same domain organisation typical of cell surface proteins (the N-terminal signal peptide and C- terminal LPXTG anchoring motif), share a mucus binding domain. Mub are encoded by protein coding *Lactobacillales*-specific clusters of orthologous genes (LaCOG) and contain one or more Mub repeats. Proteins containing Mub-repeats are abundant in lactobacilli that inhabit the GIT, suggesting that the Mub repeat is a functional unit that may be an evolutionary adaptation for survival in the GIT. Studies with *L. reuteri* 1063, implicated a cell surface protein, designated Mub, to be involved in interactions with mucin and colonisation of the GIT.

Another example of lactobacilli mucus adhesion, identified and functionally characterised, is the lectin-like mannose-specific adhesin (Msa) of *L. plantarum* WCSFS1 [166]. A database search using the sequence from the extracellular Mub domain of *L. reuteri* 1063 [185] and *L. acidophilus* NCFM [14], and Msa of *L. plantarum* WCFS1 [166] resulted in the identification of proteins containing multiple Mub domains in several species of LAB, suggesting that this is a LAB specific functional unit [186]. Studies with *L. fermentum* BCS87 have helped identify and characterise a 32 kDa surface-associated protein (32-mMubp) that is suggested to mediate adhesion to mucus [175]. The Mub domain consists of a series of amino acid residues, varying in size from 100 to 200 residues per domain [186]. Studies have shown that Mub and Mub-like proteins contribute to mucus binding and

autoaggregation, but high genetic heterogeneity among strains results in strain-specific diversity in adhesion to mucus [76].

Some lactobacilli (for example, *L. rhamnosus* GG) have fimbriae (also called pili) that reportedly enhance adhesion to mucus glycoproteins of the host cells with subsequent colonisation of the GIT [112]. Studies with *L. rhamnosus* GG have reported a mucus binding factor (MBF) with a presumed ancillary involvement in pilus-mediated mucosal adhesion [167]. However, fimbriae of some Gram positive pathogens, have been shown to induce pro-inflammatory responses [187] while the capsular polysaccharide of *L. rhamnosus* GG was found to shield fimbriae, possibly suppressing pro-inflammatory responses [159]. Such contradictory roles and possible positive effects of *L. rhamnosus* GG fimbriae still require clarification and need to be validated.

1.2.3.1.2. Sortase-dependent proteins

In lactobacilli, a subgroup of surface proteins that contains the LPXTG motif at their C terminal is recognised by SrtA. SrtA cleaves these proteins and anchors the resulting product to PG, thus incorporating these SrtA dependent proteins on the microbial surface. Although many sortase-dependent proteins are encoded by lactobacilli, the majority have no assigned function. Of the functionally characterised proteins belonging to this family, three correspond to the mucus adhesins of *L. reuteri* 1063 (Mub), *L. plantarum* WCFS1 (Msa) and *L. acidophilus* NCFM (Mub). LspA, of *L. salivarius* UCC118, is the fourth characterised sortase-dependent protein that also binds mucus and is known to mediate adhesion of this species to IEC [169, 188].

Recent studies with *L. casei* BL23 sortases and SrtA mutants suggest that SrtA might be involved in adhesion of this strain to Caco-2 and HT29 cells [179]. Although most sortase-dependent proteins of lactobacilli seem to have mucus binding capacity, not all of them have affinity to mucus components and the function of putative lactobacilli sortase-dependent proteins remains unclear [79]. Domain analysis of the extracellular proteins of *L. plantarum* WCFS1 involved in adhesion reported 10 out of the 12 identified proteins to contain the LPXTG motif. Their predicted role was adherence to collagen, fibronectin, chitin or mucus [132]. Of these 12 identified proteins, the role of Msa from *L. plantarum* in adhesion has been experimentally validated, but the roles of the other *in silico* identified putative adhesins are speculative and require *in vitro* and *in vivo* validation.

1.2.3.1.3. S-layer proteins

The S-layer proteins form the outermost interacting surface in different species of lactobacilli and have been shown to act as adhesins to IEC and components like mucus and extracellular matrix proteins. The role in adhesion of S-layer proteins of *L. acidophilus* (SlpA), *L. crispatus* (CbsA) and *L. brevis* (SlpA) has been experimentally validated [170, 171, 189]. Removal of the S-layers reduced bacterial aggregation in *L. acidophilus, Lactobacillus kefir* and *L. crispatus*, suggesting a functional involvement in this process [173, 174]. There is considerable evidence that aggregation directly influences the development of structured microbial communities as biofilms. Removal of the S-layer completely abolished co-aggregation, thus suggesting that it is mediated by S-layer proteins. Studies also suggest that S-layer proteins with lectin-like activity

interact with glycoproteins and polysaccharides and thus influence interactions of lactobacilli with other microorganisms [174].

Aggregation helps to form a physical barrier thus preventing colonisation by pathogens. Immunoblotting assays show direct interaction between *L. kefir* S-layer proteins and *Salmonella* surface adhesins. Pretreatment of *Salmonella* with purified S-layer proteins has been shown to protect two human IEC lines, parental Caco-2 and the TC-7 clone, from *Salmonella* invasion, but the protective effect was not observed when *Salmonella* were pretreated with non-aggregative strains [173]. These observations strengthen the theory that co-aggregation prevents invasion by *Salmonella* and protects epithelial cell damage. In *L. kefir*, the S-layer also influenced haemagglutination, but not adhesion to Caco-2 cells, unlike the S-layer of some strains of *L. acidophilus* that are involved in both Caco-2 adhesion and aggregation [160, 173, 174]. In *L. crispatus*, removal of the S-layer did not affect auto-aggregation or haemagglutination [170], suggesting that the S-layer may not be the only structure involved in these processes and that other covalently bound proteins or molecules such as LTA or lectin-like molecules can mediate adhesion to IEC.

1.2.3.1.4. Proteins mediating adhesion to extracellular matrix

The extracellular matrix (ECM) is a complex structure surrounding IEC and is composed of various proteins such as laminin, fibronectin and collagen. Some lactobacilli can bind to these proteins, thus competing with pathogens that have ligands for the same binding sites [120]. Examples of ECM binding adhesins are the fibronectin binding protein FbpA of *L. acidophilus* and the collagen binding protein

(CnBP) of *L. reuteri* [14, 190]. Pfam domain analysis of CnBP predicted a bacterial extracellular solute-binding domain (PF00497) that was also detected in mucus adhesion promoting protein (MapA), which was found to be a homologue for CnBP. Although MapA reportedly mediates the binding of *L. reuteri* to Caco-2 cells and mucus, database analyses detected no mucus binding domains, suggesting a role for the extracellular solute-binding domain of MapA in adhesion [79]. Other examples include the previously discussed S-layer proteins.

1.2.3.1.5. Non-protein adhesins

Lactobacillus adhesion to the GIT has also been shown to involve surface associated non-protein factors such as the LTA and EPS. LTA contribute to the anionic character of the CW and provide hydrophobicity, which in turn influences the adhesiveness of the cell envelope [93]. EPS may contribute to the physicochemical properties of the cell surface by shielding other cell surface adhesins, acting as ligands mediating adhesion and co-aggregation [191, 192]. In *L. acidophilu*s BG2FO4, carbohydrates on the bacterial CW were reported to be partly responsible for adhesion of this strain to Caco-2 cells and to mucus secreted by the mucus producing human adenocarcinoma cell line HT29-MTX cells [161]. In *L. johnsonii*, LTA has been reported to mediate adhesion to Caco-2 cells [15] and in *L. acidophilus*, different types of exopolysaccharides have been shown to influence adhesion to ECM components [120].

1.2.3.1.6. *Cytoplasmic and cell surface associated proteins as adhesins*

Two peculiar examples of cytoplasmic-localised proteins that act as surface-translocated adhesins in lactobacilli are elongation factor Tu (EF-Tu) and the heat shock protein GroEL of *L. johnsonii*. EF-Tu is involved in protein biosynthesis in the cytoplasm but has been reported as surface-translocated in many lactobacilli. In *L. johnsonii*, surface-translocated EF-Tu fulfils an alternative role of mediating adhesion to IEC and mucins. GroEL is a mediator of protein folding but when localised at the bacterial surface it mediates adhesion to human IEC and mucins [176, 177]. No domains or motifs have been found in either protein to account for their translocation across membranes. A cell surface associated enzyme GAPDH of *L. plantarum* LA318 has been found to mediate adherence to human colonic cells supposedly by recognising the sugar chains on the mucus and acting as a lectin-like protein [164]. GAPDH is surface localised although it lacks the conventional N-terminal signal sequence or a membrane anchoring motif.

Glycosylation of SlpA might be necessary for its interaction with dendritic cell-specific intercellular adhesion molecule-grabbing non-integrins (DC-SIGN) but needs to be validated as DC-SIGN is known to interact with glycosylated ligands of pathogens influencing host response to microorganisms [193]. EPS and other CW polysaccharides can be recognised by C-type lectin receptors (CLR) that are present on macrophages and DC. In *L. casei* Shirota, suppression of pro-inflammatory responses in macrophages is mediated by EPS thus indicating an immune suppressive role of CW polysaccharides [180]. Studies with *L. ruminis* show that some species of lactobacilli display flagella which act as microbe associated molecular patterns (MAMPs) that are

recognised by the toll-like receptors (TLR5) of the host and are suggested to activate the NF-κB pathway signalling in epithelial and immune cells of the host [72].

1.2.3.2. Preservation of epithelial barrier function

The modes of action by which bacteria are thought to contribute to human health include exclusion or inhibition of pathogens, preservation of epithelial barrier function and modulation of host immune responses [50]. Preservation and enhancement of the functioning of the intestinal epithelial barrier involves modulation of various signalling pathways that can result in enhancement of TJ expression and functioning [194, 195], induction and production of mucus [196] and antimicrobial compounds [197] and prevention of apoptosis [168].

Enhancement of intestinal epithelial barrier integrity by commensal bacteria has been observed in both *in vitro* and *in vivo* models. Some commensal bacteria have been shown to not only prevent but also reverse the effects of pathogens on intestinal barrier function [198]. For example, *L. plantarum* strain CGMCC 1258 protects against enteropathogenic *Escherichia coli* induced damage of the epithelial monolayer barrier (cultured Caco-2 cell monolayers) by preventing changes in the morphology. Another study has shown that *L. brevis* strengthens epithelial barrier function in healthy rats as assessed by mannitol permeability, with mannitol used as a probe to study colonic wall permeability [172]. Administration of *L. plantarum* DSM9843 and *L. reuteri* R2LC to rats with methotrexate-induced enterocolitis reduced the severity of enterocolitis [165]. *L. plantarum* DSM2648 has also been shown to increase epithelial barrier integrity when trans-epithelial electrical resistance (TEER) assays were used to measure of the

integrity of TJ between IEC [163]. Studies with interleukin-10 gene-deficient (IL-$10^{-/-}$) mice indicate that most of them develop chronic enterocolitis, as IL-10 has been suggested as an essential immunoregulator in the GIT and is a potent suppressor of macrophage and T cell functions [199]. Lactobacillus species have been shown to prevent chronic colitis in IL-$10^{-/-}$ mice [200].

Bacterial cell surface and secreted molecules play major roles in microbe-host “cross-talk” as they act as key ligands that interact with host receptors and influence signalling pathways that modulate host response. Studies with human IEC HT29 cells show that the lipid moiety of LTA from *L. johnsonii* strain La1 and *L. acidophilus* strain La10 inhibit *E.coli* and lipopolysaccharide (LPS) induced IL-8 production. IL-8, a chemokine, is a potent promoter of angiogenesis and is produced by IEC and these studies identified important bacterial cell surface factors that confer beneficial effects on the GIT [162]. Recent studies with *L. rhamnosus* GG using Caco-2 cells validate that interaction of LTA with TLR2/6 is needed for pro-inflammatory activities such as IL-8 induction and that the lipid chains of LTA and the D-alanine substituent are also important for IL-8 induction [201].

The IEC barrier is also affected by alterations in mucus and chloride secretion by epithelial cells. Mucin forms a physicochemical protective barrier for the underlying IEC and assists in the prevention of mechanical, chemical, enzymatic and microbial damage to the intestinal barrier and also restricts microbial invasion following adherence [196]. *In vitro* experiments with selected *Lactobacillus* strains have shown that adherence of enteropathogenic *E. coli* to human IEC is inhibited by induction of mucin gene expression [202]. Mucin is known to inhibit bacterial translocation and

studies with *L. rhamnosus* GG showed increased expression levels of mucin genes in a Caco-2 cell model [178]. Expression of mucin genes, induced by lactobacilli, has been shown to be dependent on direct cell contact between *L. plantarum* and IEC [202].

In addition to mucus production, modulation of TJ protein expression in IEC is an important factor in preserving epithelial barrier integrity. TJ proteins are dynamic structures that bind together IEC at their apical junctions and help maintain barrier integrity. Structural changes in TJ proteins influence their functionality. Claudins are transmembrane TJ proteins that determine the barrier properties of neighbouring IEC [203]. Zonula occludens-1 (ZO-1), a TJ protein, and F-actin, a structural component of the IEC cytoskeleton, are known to play important roles in maintaining cytoskeleton architecture of IEC thus preserving barrier integrity. *L. acidophilus* ATCC4356 has been shown to prevent disruption of the distribution of ZO-1 and occludin by enteroinvasive *E. coli* (EIEC) and enhance cytoskeletal and TJ protein structures such as occludin and actinin in IEC [204]. Several species of lactobacilli (For example *L. rhamnosus, L. acidophilus and L. plantarum*) have also been shown to improve barrier function in rats by increasing occludin expression and maintaining epithelial TJ [198, 205].

The adherence ability of commensal bacteria enables them to compete with pathogenic bacteria for receptors that are expressed on IEC, thus shielding them from damage caused by pathogenic bacteria and preserving barrier integrity [206, 207]. For example, *L. rhamnosus* R0011 and *L. acidophilus* R0052 inhibit infection of intestinal cells caused by exposure to enterohemorrhagic *E. coli* O157:H7 and enteropathogenic *E. coli* O127:H6 by reducing bacterial adhesion and cytoskeletal rearrangements [206].

Studies with specific *Lactobacillus* strains show that direct cell contact is needed to induce expression of opioid and cannabinoid receptors in intestinal epithelium mediating analgesic functions in the GIT implying involvement of cell surface related effector molecules [208]. The anti-apoptotic effect of *L. rhamnosus* GG on IEC is also dependent on direct cell contact [129]. The activation of the anti-apoptotic Akt/protein kinase B and inhibition of the activation of pro-apoptotic p38/mitogen-activated protein kinase by cytokines were suggested to prevent apoptosis in the IEC [168].

Beneficial effects of lactobacilli on IEC do not always require direct cell contact. For example, p40 and p75 are two secreted proteins of *L. rhamnosus* GG (LGG), that promote *in vitro* epithelial homeostasis [209]. These proteins are present in the spent supernatant of LGG and similar proteins were also identified in the supernatant of *L. casei* ATCC334 and ATCC393, which promoted IEC growth and inhibited TNF-α induced IEC apoptosis. The probiotic mixture VSL#3 made with *L. plantarum*, *L. acidophilus*, *L. casei* and *L. delbrueckii* subsp. *bulgaricus*, reportedly produces a soluble, proteinaceous factor that enhances IEC functioning by inducing mucins and stabilising TJ [210]. In addition to these proteins, low molecular weight, acid and heat stable peptides of LGG and probiotic mixtureVSL#3 are known to induce cytoprotective heat shock proteins in IEC [211, 212]. In the presence of pro-inflammatory stimuli, bacterial DNA from the VSL#3 mixture was shown to mediate anti-inflammatory effects by inhibiting IL-8 production, delaying NF-κB activation and reducing p38 MAPK activation [213].

1.2.3.3. Immunomodulation through interaction with host cell types

Bacteria are able to modulate immune responses of the host by interaction with the GIT mucosa. Bacterial surfaces exhibit characteristic features known as microbe associated molecular patterns (MAMP), which are usually CW components, such as LPS, PG, LTA and WTA, but can also be lipids, lipoproteins, proteins and nucleic acids [214, 215]. MAMPs are recognised by various pattern recognition receptors (PRR) that are expressed by many non-microbial cell types, for example, immune cells, IEC and hematopoietic cells. Recognition of these MAMPs by PRRs induces a signalling cascade that can result in the production of cytokines, chemokines and other effector molecules thus activating the innate immune response in the host (Figure 1.4). In the innate immune response, pathogens are detected by TLRs and other pattern recognition molecules leading to the production of antimicrobial peptides and recruitment of neutrophils resulting in antimicrobial response.

PRRs include toll-like receptors (TLR), nucleotide oligomerization domain (NOD)-like receptors (NLR) and C-type lectin receptors (CLR). Of these, TLRs and NLRs are well characterised receptors of the host immune system that are known to interact with bacterial cell surface components like the LTA and PG [41]. TLR signalling pathways involve the recruitment of adaptors such as myeloid differentiation primary response gene 88 (MyD88), which in turn activates the mitogen-activated protein kinase (MAPK) pathway and the nuclear factor κB (NF-κB) pathway signalling cascades [216].

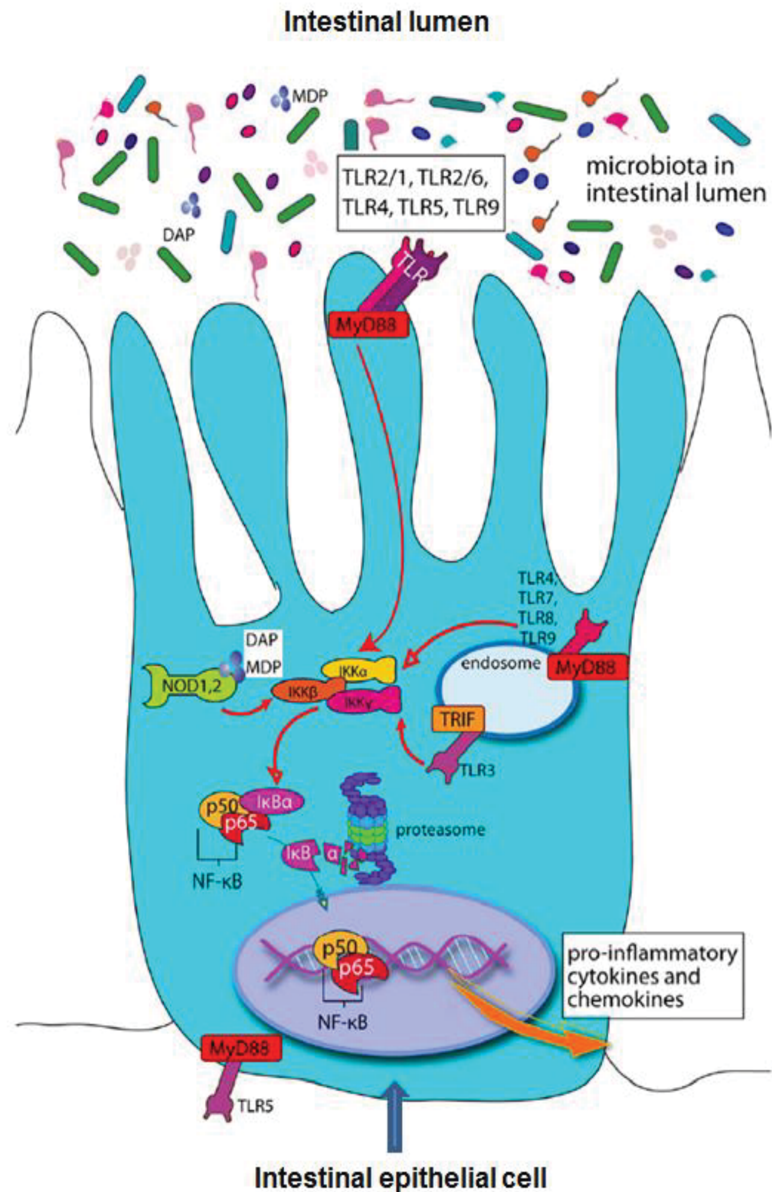


Figure 1.4 MAMP-PRR recognition and signalling in epithelial cells. A simplified representation of an intestinal epithelial cell showing the ligand receptors, adaptors and kinases, leading to the activation of MAPK and NF-κB pathways. Consequent translocation of NF-κB into the nucleus activates the expression of pro-inflammatory cytokines and chemokines. Figure adapted from Wells et al. (2010) [35].

Similarly, NOD receptors also activate the MAPK pathway and NF-κB pathway signalling cascades. Activation and translocation of NF-κB result in the transcription of numerous genes that regulate inflammatory responses. The transcription factor NF-κB is considered a master switch and genes regulated by NF-κB include those encoding cytokines such as interleukins (IL) and tumour necrosis factors (TNF). These changes in cytokine production can result in DC maturation and activation, which in turn modulates the activation and differentiation of T cells [217, 218]. The increased secretion of cytokines also attracts macrophages, neutrophils and other growth factors which help in proliferation of immune cells. The specific interactions of MAMPs with PRRs and the subsequent induction of signalling pathways depends on the microorganism and the reactivity of the host, which together play major roles in maintaining the functionality and homeostasis of the intestinal epithelial barrier.

Bacterial CW components such as LTA and lipoproteins are recognised by TLR2 in combination with TLR6, leading to activation of NF-κB. The two lipid chains of LTA have to be di-acylated and secreted as mature lipoprotein to mediate the interaction with the lipid-binding pocket of TLR2 [219]. WTA and LTA also bind to macrophage scavenger receptors such as SRA, a type I macrophage scavenger receptor that recognises LTAs, thus contributing to immune signalling [220]. LTA and SlpA interact with DC-SIGN on DC to induce cytokine release and T cell maturation. Activation of DC-SIGN by some strains of lactobacilli affects maturation of DCs, which reduces their capacity to induce IL-10 producing regulatory T cell responses against pathogens [68]. An additional line of defence is provided by the adaptive

immune system which actively provides antigen specific immune response and works in parallel with the innate immune system [221].

1.3 STRAIN SPECIFICITY AND CELL SURFACE FACTORS

Knowledge of roles played by bacterial MAMPs (LTA, WTA, PG and EPS) in strain-specific effects seen in lactobacilli is still in its infancy. Studies indicate that adherence characteristics, a major discriminating parameter in the colonising potential of commensal bacteria, show pronounced variation among strains, independent of species [5]. Strain specificity is undoubtedly linked to the wide variability and biochemical complexity of lactobacilli ligands and MAMPs as seen in the substitution levels of TA, the variable backbone alditol compositions of the WTA and the modifications of PG of the CW [81]. For example, *L. salivarius* str. Ls33 protects against chemically induced colitis in mice through the interaction of muramyl dipeptides present in its PG with NLR of the IEC. However, this protective effect is not observed for *L. acidophilus* str. NCFM, as variation in the PG composition of this strain blocks the NLR signalling pathway [222]. This illustrates the importance of the variable biochemistry of MAMPs such as PG to strain or species specificity. The ability of lactobacilli to induce host cytokine responses in immune cells can be strikingly different depending on both species and strain. Studies of DC responses to 42 *L. plantarum* strains indicate that the cytokines produced can vary from strain to strain, and different strains of the same species can have distinct pro-inflammatory and anti-inflammatory profiles, suggesting that multiple factors can influence immune phenotype [223]. Several studies suggest that cytokine responses of DC and

macrophages to lactobacilli can vary strikingly, depending on species and strain [75, 223, 224].

In addition, genetic variation and milieu-dependent switching between the multiple variants of CW polymers and/or TA adds to strain variation in lactobacilli. Studies with mutants of *Lactobacillus* strains that produce alternative LTA variants suggest that modifications to the LTA backbone can alter cytokine induction capacity thus increasing anti-inflammatory immune modulation [225, 226]. Chain length variation, subcellular localisation and interactions of these polymers most likely contribute to strain-specific characteristics and are still being experimentally validated [107, 227].

1.4 IN VITRO *MODELS OF THE INTESTINAL EPITHELIAL BARRIER*

In the mid-twentieth century, the development of *in vitro* human cell culture systems had been slow in progress due to limited viability of isolated IEC and their ability to retain the anatomical and biochemical features of *in vivo* differentiated cells [228]. In the 1970s, GIT tumours were used to establish several cell lines to study cancer mechanisms and therapies, but a decade later some of these tumour cell lines were being studied for their specific intestinal properties [229]. The discovery that human adenocarcinoma cell lines were able to express differentiation features that are characteristic of mature IEC, such as enterocytes and mucus cells, have made them valuable to be used as model systems for *in vitro* studies. These models have since been used for studies on a wide variety of intestinal functions, such as cellular differentiation, transport of nutrients and proteins, drug delivery and toxicity.

1.4.1 *In vitro epithelial cell model systems*

Many of the primary cell cultures, originating from the small intestine and colon, have proven to be unreliable due to their short life span, limited repeatability and their inability to express the differentiation characteristics of mature IEC [230, 231]. Therefore most of the established *in vitro* model systems of the intestinal epithelium are derived from immortalised cancer cell lines [232, 233]. There are several immortalised epithelial cell lines. For example, human colonic adenocarcinoma cell line (T84), Caco-2, HT29 and Madin-Darby canine kidney epithelial cell line (MDCK) are all used for transport and permeability studies [234-236]. Among these, the Caco-2 cell line has been used extensively as a model system to study interactions of pathogenic and commensal bacteria with intestinal surfaces.

1.4.2 Caco-2 monolayers as in vitro epithelial cell model system

The Caco-2 cell line has been well characterised, and is better suited than primary cell cultures for *in vitro* studies as it can express morphological and functional characteristics of mature IEC and has the ability to undertake trans-epithelial transport [131, 233]. A study comparing twenty human colon carcinoma cell lines revealed only Caco-2 cells to have the ability to undergo spontaneous enterocytic differentiation [237]. Under conventional culture conditions, Caco-2 cells differentiate structurally and functionally to form monolayers with polarisation, apical brush border microvilli and intercellular TJ [131, 238]. Caco-2 monolayers also exhibit electrical and ionic properties that closely resemble the epithelial cells of the small intestine [239]. Furthermore, electron microscopy has shown that some bacteria adhere to the apical

brush border present on the surface of the Caco-2 cells [240]. The reproducibility and long-term viability of Caco-2 cells along with their ability to be used in transport and toxicity studies confer significant advantages to this cell line over other *in vitro* models.

1.4.3 Cell and culture related factors influencing Caco-2 cell monolayers

Although, there are many advantages in using *in vitro* model systems, the limitations of using cancer-derived cell lines cannot be overlooked. As with all *in vitro* models, cells are grown in an artificial environment and there are several factors that make it difficult to extrapolate results to actual physiological conditions. For example, while Caco-2 cells possess the characteristic properties of cells in the distal ileum, electrical properties resemble those of the colonic origin [228]. Cell related factors such as number of passages influence different functions of this cell line. TEER was shown to increase in later passages in several studies [241, 242]. Culture related factors such as seeding density, time of culture and medium composition also influence the activity of the cell line. Seeding density is an important factor in the differentiation process, which starts only after the Caco-2 cells reach confluence. Factors such as seeding density and time of culture have to be strictly standardised to obtain comparable and reproducible results. The effects of seeding densities on Caco-2 cell differentiation have been shown to strongly affect monolayer structure and carrier mediated transport, while TEER and paracellular permeability showed no difference [243]. The morphological and biochemical characteristics of Caco-2 cells depend on the differentiation process. Studies have reported TEER values and mannitol

permeability to reach a plateau after 21 days in culture [244], while another study reported TEER to plateau after 15 days, while mannitol permeability declined after 8 days and levelled off after that [245]. The composition and physico-chemical characteristics of the cell culture medium also affect the biology of the Caco-2 cells, thus influencing the proliferation, differentiation, morphology and permeability of the cells. Investigation of Caco-2 cell lines have shown that the presence of serum in the medium did not generally affect TEER, but consistently lowered and stabilised the permeability of mannitol [245]. Thus, to ensure reproducibility of results, protocols have to be standardised for maintenance of the Caco-2 cell line and the bacterial cultures. In addition, the Caco-2 cell line is morphologically heterogenous and the properties of the monolayer are probably the sum of the characteristics of different cell types. There are several successful examples of the use of the Caco-2 cell monolayers as an *in vitro* human absorption surrogate [228, 246], but there have been reports of less efficient paracellular and active transport across Caco-2 monolayers than in the *in vivo* situation [247, 248].

1.4.4 In vitro *model system to study microbe-host interactions*

The development of *in vitro* epithelial models has facilitated microbe-host interaction studies on which the microenvironment of the human intestinal epithelium is mimicked. The polarity of IEC, especially those that line the mucosal surfaces, play a major role in microbe-host interactions. The luminal surface of the GIT is lined with a single layer of IEC with highly polarised apical and basolateral surfaces. The TJ which separate adjoining cells help maintain IEC surface polarity and prevent paracellular diffusion between cells. Caco-2 cells grow on permeable membranous

supports and obtain nutrients via basolateral feeding and can become fully polarised. This model mimics the *in vivo* setting wherein IEC obtain nutrients from the underlying blood vessels [249]. This model also makes it possible to study the interactions between bacteria and IEC by co-culturing. The epithelial TJ can be used to assess altered (or loss of) barrier function as a result of bacterial interactions with the epithelium using these *in vitro* models [250].

1.4.5 Trans-epithelial electrical resistance assay

The TEER assay has been widely used to study host-microbe interactions and the effects of bacterial strains on epithelial barrier function. It has also been used to test the effects of bacterial cell components and secreted molecules on IEC and provides reliable and reproducible results [163]. Caco-2 cells are seeded on microporous, permeable membranes on cell culture inserts (transwells) and allowed to differentiate into monolayers. The inserts allow the Caco-2 to become fully polarised and allows separation of the apical part, which is the cavity of the insert, from the basal compartment, which is the well in which the insert rests. The ability of the Caco-2 cells to form monolayers with TEER enables the measurement of changes in the paracellular ion flux which is linked to TJ integrity [251]. Disruption of TJ causes a reduction in TEER and increased paracellular permeability across the intestinal epithelium. The inserts allow free access of nutrients and ions to both the sides of the monolayer and TEER measurement. Bacteria can be added on the apical part of the insert, which provides convenient means to study the effects caused by either pathogenic bacteria or screening bacteria with putative health promoting properties on the TEER of Caco-2 cells.

1.4.6 Paracellular permeability of Caco-2 using mannitol assay

^{3}H mannitol, a non-metabolisable molecule with a molecular weight of 182 kDa, is an accepted marker for studying paracellular permeability in *in vitro* models such as the Caco-2 cell monolayers [245, 251, 252]. The Caco-2 cell monolayers mimic the colonic epithelia with a tighter and more discriminating paracellular pathway than that of the small intestine, which makes them suitable for permeability and absorption assays [251]. Studies on the structural-functional relationship of epithelial TJ have shown that ionic permeability may be regulated by different organisation of integral proteins, which do not affect solute passage [253]. Medium composition also evokes different responses in TEER and mannitol passage during the differentiation process of Caco-2 cells [245]. These studies suggest that TEER and mannitol flux, the two measures of monolayer permeability are not always correlated. Therefore, these assays should not be used as alternate methods of assessing TJ maturity, but as complementary methods.

Radiolabeled mannitol is widely used to determine monolayer integrity and IEC paracellular permeability in transepithelial models. ^{3}H-mannitol is added to the top of differentiated Caco-2 monolayers grown on permeable membranes (transwells placed in wells containing media). The amount of ^{3}H-mannitol that "leaks" through the monolayer to the basolateral side of the Caco-2 monolayers (into the media in the well) is measured at defined intervals. Permeability is calculated by measuring the fluorescence of the media in the wells taken at the defined time intervals.

TEER assays and permeability assays using marker molecules such as ^{3}H-mannitol have been extensively used to study intestinal barrier integrity and effects of host-microbe interactions [163, 251, 252]. However, it should be noted that the Caco-2 model has its limitations being a transformed cell line with properties representing different parts of the intestinal epithelium.

1.5 CONCLUSION AND FUTURE PERSPECTIVES

The cell envelope plays an essential role as the interacting surface with the GIT bacterial environment and in many aspects of the physiology and functioning of lactobacilli, including communication and adaptation to host-derived factors encountered in the GIT. Lactobacilli display considerable variation in their cell surface properties, which undoubtedly are important for the functioning of these bacteria. Database analyses and use of genetic tools has resulted in the identification of several effector molecules of lactobacilli that are proposed to be involved in direct interactions with host IEC or immune cells, many of these effector molecules are components of the CW itself [80]. Considering the complexity of host-lactobacilli interactions involving host-cell signalling and response-regulation pathways, it seems highly unlikely that single effector molecules regulate the entire host response. These molecules probably have an expanded repertoire of functions, in addition to playing crucial roles as building blocks of the bacterial CW [225]. Knowledge of the molecular mechanisms underlying the physiological characteristics of lactobacilli and identification of key bacterial structural or secreted components that influence host-microbe interactions can add to the understanding of the concept of strain specificity and variation.

1.6 AIM, HYPOTHESIS, APPROACH AND OUTLINE OF DISSERTATION

Lactobacillus fermentum is a major heterofermentative *Lactobacillus* species found in fermented foods, human oral cavities and in the human GIT [254, 255]. It is generally considered to be harmless and some studies have also indicated some *L. fermentum* strains to have beneficial effects, especially on GIT health [254, 256, 257]. Two strains of *L. fermentum*, isolated from the oral cavities of individuals, have been shown to exhibit different phenotypic properties and different effects on the hosts, especially on intestinal barrier function [163]. This presents an opportunity to study and understand the basis of the differences shown by the same species of bacteria and identify bacterial cell components, either structural or secreted, that impart strain-specific characteristics in lactobacilli. Recent studies with these two strains indicate that the negative effect on intestinal barrier integrity may be due to increased turnover of microtubule proteins in the IEC that result in TJ disassembly [258].

This research project aims to gain insight into the "cross-talk" that takes place between GIT bacteria and the host, into what makes strains of the same species act differently and have varying effects on barrier functionality. The hypothesis was that the genome size and phenotypic differences between the two *L. fermentum* strains are related to the different effects that these strains have on intestinal barrier integrity. The main questions to be answered, based on the purported differential effects shown by these two strains on intestinal barrier integrity were: (i) do phenotypic differences shown by the two strains influence the differential effects on IEC? (ii) does bacterial stage of growth have an impact on the effect of these strains on IEC? (iii) do viable and

non-viable (UV inactivated) bacteria have similar effects on IEC? and (iv) does the bacterial CW fraction have similar effect on Caco-2 cell TEER as whole bacteria?

The testing and validation of the hypotheses was achieved through the objectives outlined in Figure 1.5. The Caco-2 monolayer were used as a model system to understand the cause of these different effects, with TEER assays and ^{3}H-Mannitol assays being used as measures of paracellular permeability across Caco-2 monolayers.

The research described in Chapter 2 (Figure 1.5) investigated the differences and similarities in the phenotypic and genome sizes of the two strains of *L. fermentum*, AGR1485 and AGR1487. Lactobacilli encounter various harsh conditions as they transit through the GIT and in order to survive these stress challenges they evolve and adapt resistance mechanisms, which depend on the coordinated expression of genes (and also may involve loss, gain or mutation of genes) and regulators. Studying the differences shown in the stress responses constitutes a basis of comparison between strains. A high level of strain variation is seen not only in the ability to survive these challenges but may also be observed for sugar metabolism, which directly influences the strain's lifestyle and localisation in the GIT. To characterise the two strains of *L. fermentum*, they were subjected to genome size (16S rRNA sequencing, pulsed-field gel electrophoresis) and phenotypic (sugar utilisation, bile and pH tolerance assays) analyses.

In Chapter 3, experiments were designed and carried out to test the hypothesis that the differential behaviour of lactobacilli are related to their expression of cell surface molecules and/or their secretory capabilities while interacting directly with

their host cells. First, TEER assays were conducted with live versus UV-killed bacteria to test the hypothesis that the differential effects on Caco-2 cell TEER were the result of bacterial structural and/or secreted components and their interaction with IEC. Second, TEER assays were conducted using Caco-2 cells treated with bacteria taken from different phases of growth to study if bacterial growth phase affected host-microbe interactions. To determine the role of secreted molecules on Caco-2 cell TEER, further experiments were conducted using bacterial metabolites produced as a result of the interaction of bacteria with Caco-2 cells. Mannitol assays were conducted to determine the effect of UV-treated bacteria on the Caco-2 cells and as a verification of the effects on Caco-2 cell TEER.

In Chapter 4, experiments were conducted to test the hypothesis that the different effects that the strains have on Caco-2 cell TEER are caused by proteinaceous components present in the bacterial CW. Both the strains were treated to create bacterial CW extracts and these extracts were applied to Caco-2 cells to study their effects on Caco-2 cell TEER. Further fractionation of the bacterial CW extract was carried out using size exclusion chromatography and the resulting fractions were tested for their effects on Caco-2 cell TEER.

In Chapter 5, a summary and discussion of the main findings and further perspectives is given. An outline of the structure of the dissertation and the experimental chapters is presented in Figure 1.5.

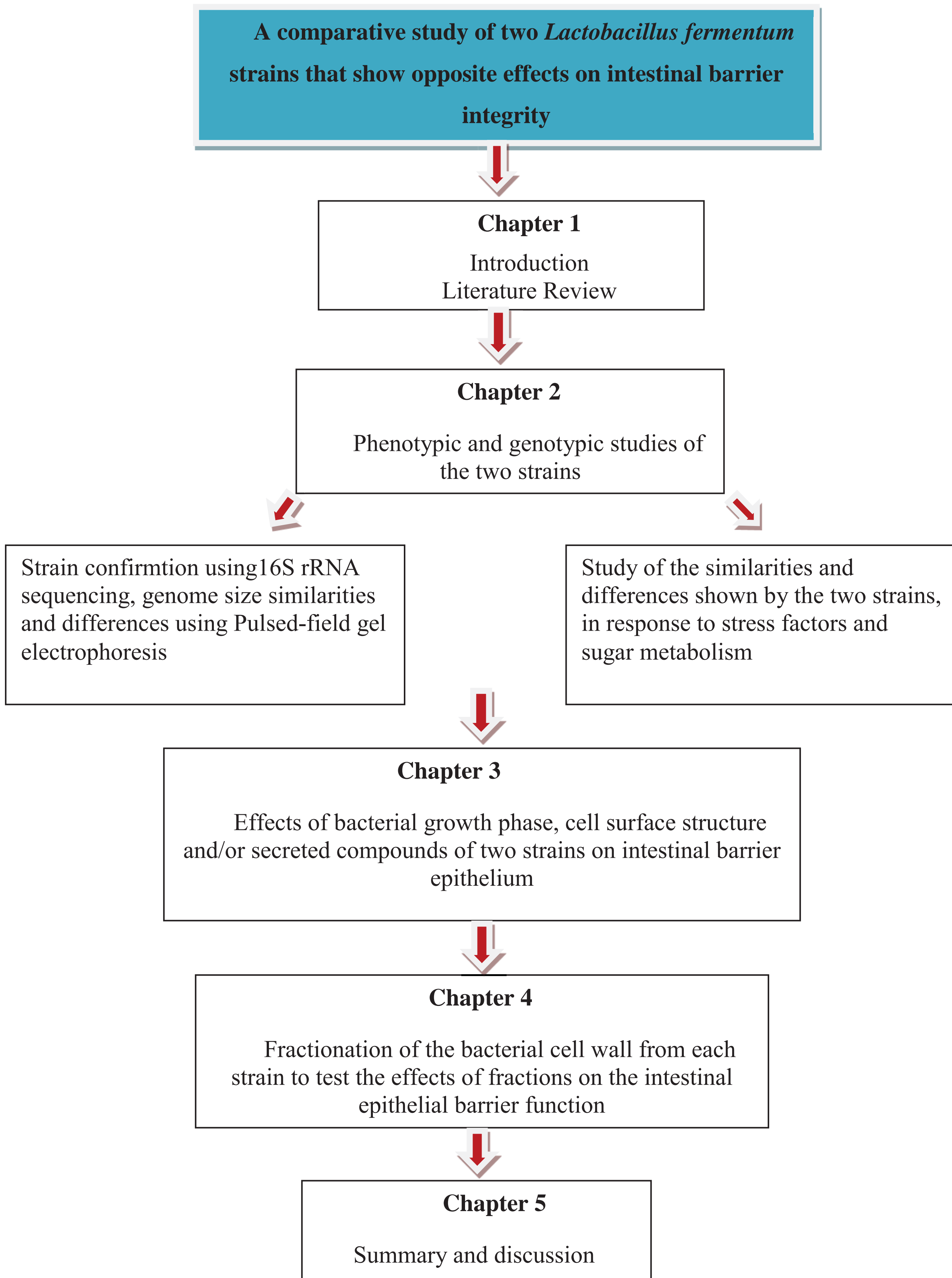


Figure 1.5 Structure of dissertation: Outline of thesis and research chapters.

2. CHAPTER 2: GENOME SIZE AND PHENOTYPIC DIFFERENCES BETWEEN THE TWO LACTOBACILLUS FERMENTUM STRAINS.

2.1 INTRODUCTION

The importance of the *Lactobacillus* species is well known in the food and fermentation industry, and use of these bacteria in industrial applications have been widely reported [3, 259]. The beneficial properties exhibited by many lactobacilli on gastrointestinal tract (GIT) health have resulted in their use as probiotics in foods, cultured milks and various pharmaceutical preparations [259-261]. In the GIT, the presence of lactobacilli is important for the maintenance and balance of the microbial ecosystem, as well as for providing protection against pathogens [3, 4, 262, 263]. However, studies have shown that different strains of lactobacilli, even from the same species, can evoke different responses in the host and therefore, results from one strain cannot be generalised to others [5].

Several factors are involved in the interaction between lactobacilli and intestinal epithelial cells (IEC) [264]. Studies have shown that lactobacilli benefit the host through a wide variety of mechanisms that seem to be strain-specific [265]. These mechanisms include protecting the host from pathogen invasion by competitive exclusion and/or production of organic acids and secretion of antimicrobial compounds, interacting with the epithelium that help modulate the immune response of the host and preserving barrier integrity of the GIT [9, 261, 266].

Lactobacillus fermentum is considered a “generally recognised as safe” (GRAS) organism by the US Food and Drug Administration. Clinical studies have reported that some strains of *L. fermentum* have beneficial effects on GIT health. For example, *L. fermentum* CECT5716, a naturally present probiotic strain in human breast milk,

reduced the incidence of upper respiratory tract and GIT infections in infants [267]. Research has also indicated that some strains of *L. fermentum* may have negative effects on intestinal barrier integrity, thus adversely affecting GIT health [163]. Two strains of *L. fermentum*, namely AGR1485 and AGR1487, isolated from human oral cavities, exhibit opposing effects on the intestinal barrier integrity [163, 258]. Studies have shown that AGR1485 increases or maintains barrier integrity across Caco-2 cells as compared to control medium. AGR1487, on the other hand, causes a decrease in intestinal barrier integrity [163, 258]. The effect of AGR1487 may be due to increased levels of microtubule proteins and a high turnover in tubulin gene expression in the Caco-2 cells that were treated with this bacterium [258]. Increased expression of tubulin genes are implicated in the disassembly of TJs between IEC [268].

2.2 AIM AND HYPOTHESIS

The aim of the research reported in this chapter was to investigate the differences and similarities in the genome sizes and phenotypic characteristics of the two strains of *L. fermentum*, AGR1485 and AGR1487. The two strains, isolated from human oral cavities, have different effects on the barrier integrity of confluent undifferentiated Caco-2 cells over 12 hours [163, 258]. These observations led to the hypothesis that the differences between the effects of these strains on Caco-2 cell barrier integrity are related to the genome size and phenotypic differences exhibited by the two strains. After confirming the previously noted impact of the two strains on intestinal barrier integrity using differentiated Caco-2 cell monolayers over a longer period of time, the characteristics of the two strains were further determined. The two strains of *L. fermentum*, were subjected to genetic (16S rRNA sequencing, pulsed-field gel

electrophoresis) and phenotypic (Gram staining, sugar utilisation, bile and pH tolerance assays) analyses.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains

L. fermentum strains AGR1485 and AGR1487 were previously obtained from the mouth lining, tongue and teeth of human volunteers [163]. Ethical approval from the New Zealand Health and Disability Committee was not required as the sampling was a non-invasive procedure and the volunteers were considered to be healthy individuals. The volunteers gave written consent for the collection and use of the samples for research purposes. AGR1485 was isolated from a healthy individual and AGR1487 was isolated from a then apparently healthy individual who was later diagnosed with ulcerative colitis.

2.3.1.1. Preparation of bacterial stocks and culture medium

The two strains were grown from frozen stocks (sample colonies stored previously as glycerol stocks at -80ºC) on de Man, Rogosa and Sharpe (MRS) agar (Acumedia manufacturers, Michigan, USA) plates for 48 hours at 37ºC in 5% CO_2. Fresh bacterial stocks were made by picking two to three fresh colonies and adding to a vial of freezing medium. Freezing medium was made by dissolving 3.7 g of Brain Heart Infusion powder (Becton Dickinson, Sparks, MD, USA) in 66 mL of distilled H_2O and the volume made up to 100 mL with 33 mL of glycerol (Fisher Scientific, Loughborough, UK) and mixed thoroughly. The mixture was dispensed in 1 mL

aliquots in cryogenic vials and autoclaved at 121ºC and 15 psi (0.1 MPa) for 20 minutes. Once the bacterial colonies were added to the vials, they were frozen at -80ºC.

Bacteria were grown from these frozen stocks by using sterile plastic loops to scoop approximately 1 μL of frozen stock from the vial and streaking on an MRS agar plate. The plates were incubated for 48 hours at 37ºC in 5% CO_2 and subcultured by inoculating a single colony from the plate into 10 mL of MRS broth and grown overnight at 37ºC in 5% CO_2 in Hungate tubes. MRS broth was prepared by mixing 55 g of lactobacilli MRS Broth powder (Acumedia manufacturers, Michigan, USA) in 1 L of distilled H_2O. The mixture was heated and stirred for dissolving and 10 mL aliquots were made in Hungate tubes and autoclaved at 121ºC and 15 psi for 20 minutes. MRS agar plates were made by adding 15 g of Bacteriological Agar (Oxoid, Hampshire, UK) to 1 L of MRS broth, autoclaving at 121ºC and 15 psi for 20 minutes, and then pouring onto Petri dishes inside a sterile flow hood. Once set, the plates were stored in plastic bags at 4ºC.

2.3.2 Gram staining

A single bacterial colony from each strain was picked from an agar plate and added to a drop of distilled H_2O on a glass slide. The droplet was spread and allowed to dry by gently flaming the slide. The slide was flooded with crystal violet to stain the bacteria and left for 30 to 60 seconds. The stain was poured off and the slide flooded with iodine solution and left soaking in the iodine solution for one to two minutes. The iodine solution was washed off with water and then decolourised with acetone alcohol until the decolouriser flowed colourless. Counterstaining was done with safranin for 30

to 60 seconds, followed by wash with water. Excess water was drained off, a drop of oil was placed on the stained smear and a cover slip was placed on it. The bacteria were visualised under bright field with 100X magnification and pictures taken, using a Leica DM2500 Microscope fitted with camera and LAS software to edit images.

2.3.3 Cell culture for transepithelial electrical resistance assay

Human colorectal adenocarcinoma cell line, Caco-2 cell ATCC HTB-37 (Manassas, Virginia, USA), stock cultures were grown in T175 flasks in 150 mL of M199 (GIBCO, Life Technologies) supplemented with 10% foetal bovine serum (GIBCO, Life Technologies), 1% non-essential amino acids (NEAA; MEM non-essential amino acids 100x solution, Life Technologies) and 1% penicillin-streptomycin (GIBCO, Life Technologies, 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin) at 37°C in 5% CO_2. The medium was replaced every 2 to 3 days and cells were sub-cultured weekly at a ratio of 1:3.

The transepithelial electrical resistance (TEER) assays were carried out using differentiated Caco-2 cells (16 to 20 days post seeding). Caco-2 cells (Passage number between 28 and 36) were seeded in 12 mm polyester Transwells with 0.4 µm membrane pore size (Transwell Polyester Products, Corning Incorporated) in 12-well plates at a concentration of $2x10^5$ cells/Transwell (500 µL/Transwell). Cells were grown in 1.5 mL/well of M199 with 10% foetal bovine serum, 1% NEAA and 1% penicillin-streptomycin at 37°C in 5% CO_2 for 15 to 19 days until they formed a differentiated monolayer. The day before the TEER experiment, medium from the Caco-2 cell monolayers was removed and M199 supplemented with only 1% NEAA

was added. Foetal bovine serum was not added as it causes the bacteria to grow rapidly. Penicillin and streptomycin were not added as those antibiotics would inhibit bacterial growth [163, 258].

2.3.3.1 Tight junction integrity

On the day of the TEER assay, the initial resistance values of the Caco-2 monolayers were recorded, which was considered to be the measurement for time 0 hour. The measurements were taken using an electrode chamber (ENDOHM-12 tissue culture chamber; World Precision Instruments, FL) using a voltohmeter (EVOM Epithelial Tissue Voltohmeter; World Precision Instruments). Caco-2 monolayers that had an initial TEER superior to 500 Ω/cm^2 were used for assays [245, 269]. The medium from the Transwells was then removed and replaced by 500 µL/Transwell of M199 with 1% NEAA (control), AGR1485 (0.9 OD_{600}) in M199 with 1% NEAA or AGR1487 (0.9 OD_{600}) in M199 with 1% NEAA (n=4 per treatment). The 24 hour TEER assay was carried out three times with 4 wells per treatment and resistance measured every 2 hours over 12 hours and then again at 24 hours.

AGR1485 and AGR1487 were grown from frozen stocks on MRS agar plates for 48 hours at 37ºC in 5% CO_2 and a single colony was sub-cultured into 10 mL MRS broth and grown overnight at 37°C in 5% CO_2. Bacterial cells in stationary phase (grown for 18 hours) were centrifuged at 5,000x*g* for 5 minutes and the bacterial cell pellet was re-suspended in M199 containing 1% NEAA to an optical density of 0.9, measured at 600 nm (OD_{600}) to keep the number of bacterial cells constant for each experiment.

The TEER was calculated from the resistance values (Equation 2.1), where background resistance was 12 Ω and membrane area 1.12 cm^2. To take into account variations in the initial TEER values between replicates, TEER values were normalised against the initial TEER values, and the change in TEER for each Transwell was calculated using Equation 2.2.

$$TEER = \left(resistance_{measured} - resistance_{background}\right) \times membrane\ area$$

Equation 2.1: Calculation of TEER [$\Omega.cm^2$]. Resistance [Ω], membrane area [cm^2]

$$\Delta TEER = \left(\frac{TEER - TEER_{initial}}{TEER_{initial}}\right) \times 100$$

Equation 2.2: Calculation of change in TEER (Δ TEER) [%]

A mixed effects repeated measures ANOVA model with subject (each Transwell) as the random effect, and treatment, time and their interaction as the fixed effects was applied for statistical analysis of the change in TEER. The correlation of change in TEER with time over the 12 hour period for each subject was assumed to be homogenous. Three separate experiments were carried out with four replicates per treatment for each experiment (total n=12 per treatment). Statistical analysis was carried out using meta-analysis with REML for a combined analysis of three related experiments, using package “lme4” and “predictmeans” in R, at probability values less than 0.05 considered as statistically significant.

2.3.4 16S rRNA sequencing

The two bacterial strains AGR1485 and AGR1487 were previously identified [163] and their identities reconfirmed as *L. fermentum* based on their 16S rRNA gene sequencing results. The genomic DNA from both the strains was isolated and subjected to PCR to amplify the 16S rRNA gene, which was then sequenced. The sequence generated was compared to known bacterial sequences in the National Centre for Biotechnology Information (NCBI) database to confirm their identification.

2.3.4.1 Genomic DNA Isolation

Genomic DNA was isolated from both the strains using DNeasy Blood & Tissue kit (Qiagen). 1.5 mL of an overnight bacterial culture was centrifuged for 5 minutes at 5,000x*g* and cell pellets washed with 0.85% NaCl. The resuspended pellet was washed again with distilled H_2O and centrifuged for 10 minutes at 5,000x*g*. The cell pellet was then resuspended in 180 µL enzymatic lysis buffer and incubated for 30 minutes at 37°C. 25 µL of Proteinase K and 200 µL of Buffer AL (lysis buffer from kit) was added and mixed thoroughly by vortexing. The mixture was incubated at 56°C for 30 minutes. 200 µL of ethanol (100%) was added to the sample and mixed thoroughly by vortexing. The mixture was pipetted into the DNeasy spin column placed in the provided 2 mL collection tube and centrifuged at 7,500xg for 1 minute. The flow-through was discarded. The spin column was placed in a fresh collection tube and 500 µL Buffer AW1 (wash buffer) was added, centrifuged at 7,500x*g* for 1 minute and flow-through was discarded. The spin column was then placed in a fresh collection tube and 500 µL Buffer AW2 (wash buffer) was added, centrifuged at 20,000x*g* for 3 minute and flow-

through discarded. The DNeasy spin column was placed in a clean microfuge tube and 200 µL of Buffer AE (elution buffer) was added to the membrane of the column. The column was incubated at room temperature for 1 minute and then centrifuged for 1 minute at 7,500x*g* to elute. The eluted DNA was quantified using a NanoDrop (Agilent 2100 Bioanalyzer) and stored at -20°C for subsequent use.

2.3.4.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was conducted in 25 µL reactions in an Eppendorf thermal cycler (Mastercycler pro S). PCR reactions were prepared in 0.2 mL PCR tubes by mixing 15.9 mL of autoclaved distilled H_2O, 2.5 µL of Taq buffer, 1 µL of $MgCl_2$ (25mM), 0.1 µL of Taq (5U/µL) and 2.5 µL of dNTP (2 mM) with 1 µL each of forward and reverse primers (Table 2.1). The primers used were at a concentration of 10 pmol/µL. 1 µL of DNA (30 ng) was added to each reaction. The PCR conditions were an initial denaturation step (94°C for 4 minutes) followed by 35 cycles of denaturation (94°C for 30 seconds), annealing (50°C for 30 seconds), extension (72°C for 1 minute) followed by a final step of elongation of 10 minutes at 72°C. The PCR products were purified using QIAquick PCR purification kit (Qiagen). Following the protocol, 5 volumes of Buffer PB (from kit) was added to 1 volume of the PCR products and mixed. The mixture was applied to a Qiaquick spin column, which was placed in 2 mL collection tube and centrifuged for 30 to 60 seconds at 17,900x*g* in a conventional bench-top microcentrifuge. The flow-through was discarded and the column placed back in the same collection tube. The bound DNA was then washed with 0.75 mL of Buffer PE (from the kit, with ethanol added) and re-centrifuged for 30 to 60 seconds. The flow-through was discarded, the column placed back in the same tube and

centrifuged for an additional minute. The column was then placed in a clean 1.5 mL microcentrifuge tube. The DNA was eluted by adding 50 µL of elution buffer (10mM Tris-Cl, pH 8.5) to the centre of the spin column, kept at room temperature for a minute and then centrifuged for 1 minute at same speed. The purified DNA was quantified before sequencing, using the NanoDrop (Agilent 2100 Bioanalyzer).

2.3.4.3 DNA sequencing and identification of strains

DNA sequencing service was provided by the Massey Genome Service (Massey University, Palmerston North, NZ). Reactions were prepared using 30 ng of purified PCR products (from 2.3.4.2) and mixing it with 3.2 pmol of forward or reverse primer (Table 2.1). The final volume was made to 15 µL with autoclaved distilled H_2O. Although the PCR was done using 1492R as the reverse primer, for the sequencing reaction, modified 514R (R2) was used to ensure an overlap of 320 bases for each strain. Full sequencing service for PCR products with fluorescent labelling using BigDye Terminator v3.1 was provided by Massey Genome Service. A Reaction Cycle Sequencing kit was used for sequencing PCR, followed by removal of unincorporated ddNTPs, precipitation of PCR products and capillary separation on an ABI3730 Genetic Analyser (Applied Biosystems) were included in the service provided. The sequencing data was returned as trace files, which were then analysed. The forward and reverse sequences were aligned, manually trimmed and edited using the Vector NTI ContigExpress software. The resulting consensus sequence was used for Basic Local Alignment Search (BLASTN) to compare with the nucleotide collection of the National Centre for Biotechnology Information (NCBI) database.

Table 2.1 Oligonucleotide sequences used for 16S rRNA sequence analysis.

Primer Name	***Sequence***	***Reference***
27F Forward primer (FD1 modified)	GAGTTTGATCCTGGCTCAG	Modified from Lane (1991) [270]
1492R Reverse primer (BAC1492R)	GGYTACCTTGTTACGACTT	Lane (1991) [270]
R2 Reverse (modified 514R Reverse primer for sequencing)	TACCGCGGCTGCTGGCAC	Modified from Jurgens (2000) [271]

2.3.5 Pulsed-field gel electrophoresis

The pulsed-field gel electrophoresis (PFGE) method used had been standardised for the identification of strains belonging to *L. fermentum* [272]. Standardisation involved selecting the appropriate restriction enzyme and establishing the parameters that are optimal for electrophoretic separation such as the duration of the electric pulse which can be most discriminative for strains within species [272, 273]. The restriction enzymes AscI and I-CeuI were selected as they had been previously shown (Kelly, unpublished data) to produce repeatable results and readable band profiles. These restriction enzymes and the optimised separation parameters obtained good quality restriction patterns suitable for specifying genetic relatedness as well as conclusive for genetic differentiation of the two *L. fermentum* strains.

For the preparation of plugs for PFGE, 10 mL of MRS broth was inoculated with a single colony of bacteria and incubated at 37°C for 18 hours. An aliquot of 1.5 mL of bacterial culture was centrifuged at 5,000x*g* for 5 min, the pellet washed in wash solution (1 M NaCl and 10 mM Trizma base in distilled H_2O, pH 7.6) and centrifuged at 5,000x*g* for another 5 minutes. The cell pellet was mixed 1:1 (v/v) with molten 2% pulsed-field certified low melt agarose (Bio-Rad, Hercules, CA, USA) in 125 mM EDTA equilibrated to 50ºC. The resulting mixture was dispensed into 10-well plug moulds (Bio-Rad, Hercules, CA, USA) and allowed to set at room temperature. Once solidified, plugs were transferred to Falcon tubes (up to 4 plugs per tube) in 4 mL of EC buffer (100 mM EDTA, 1 M NaCl, 35 mM N-Lauryl-sarcosine and 6 mM Trizma base in dH_2O, pH 7.6) containing 10 mg/mL lysozyme. Plugs were incubated with shaking for 18 to 24 hours at 37ºC to allow cell lysis to occur. The EC buffer/lysozyme

solution was replaced with 4 mL of 0.5 M EDTA-Sarkosyl solution containing 0.5 mg/mL of Proteinase K. Plugs were incubated for 16-24 hours at 37ºC with continuous agitation. Fresh EDTA-Sarkosyl/Proteinase K solution (4 mL) was added and the plugs were incubated for 16 to 24 hours at 37ºC with shaking. The EDTA-Sarkosyl/Proteinase K solution was replaced with TE (10 mM Trizma base and 1 mM EDTA) containing 1 mM phenylmethylsulfonylfluoride (PMSF) to eliminate residual Proteinase K activity, and incubated for 2 hours at 37ºC with shaking. Plugs were washed once in TE (10 mM Trizma base and 0.1 M EDTA) and stored at 4ºC in the same solution.

Slices of the plugs (1 to 3 mm) were digested with restriction enzymes (Asc I and I-Ceu I) and incubated for 20 hours at 37°C using respective incubation buffer provided by the manufacturer. The digested plugs were loaded onto an agarose gel (1% Seakem Gold Agarose). The gel was run for 20 hours, in 0.5x TBE at 14ºC using a CHEF-DR® III system powered by a Powerpac Basic (Bio-Rad, Hercules, CA, USA). The gel was subsequently stained with ethidium bromide for 30 minutes and bands were visualised and captured using a Kodak Gel Doc Box (KODAK Gel Logic 200 Imaging system) modified to accommodate a remotely controlled (Nikon Camera Control Pro 2) Nikon D700 digital camera (Nikon Corporation, Japan). The raw images were developed using Adobe Photoshop Lightroom 2.5.

2.3.6 Sugar utilisation

The API50CH kit (bioMérieux), containing 49 carbohydrates, is a ready-to-use kit used for the identification of the genus *Lactobacillus* and related genera and also to

study similarities and differences in sugar utilisation capabilities between strains. The strains were grown and sub-cultured twice under standard conditions. An aliquot of 1.5 mL from each strain was spun down and the pellet re-suspended in 1 mL of CHL medium (a ready-to-use medium which allows the fermentation of carbohydrates on the API50CH strip). This solution was then used to inoculate fresh CHL medium to an OD_{600} reading of 0.451 as per the protocol. The chambers in the strips of the API50CH kit were filled with the inoculated medium, the strips placed in trays, covered and incubated at 37°C for 48 hours according to the protocol. During the incubation, carbohydrates that were fermented to acids caused a decrease in pH, resulting in a change in colour of the indicator present in the medium. These carbohydrates were recorded as positive and the carbohydrates that did not cause a change in colour were recorded as negative for each strain tested. Results were recorded and the biochemical profiles obtained for the two strains were identified using the api*web*™ identification software with database (V5.1) (https://apiweb.biomerieux.com).

2.3.7 Bile and acid tolerance

For the bile tolerance assay the Walker and Gilliland procedure for measuring bile tolerance was followed [274]. Bacteria were grown from frozen stocks on MRS agar plates for 48 hours at 37°C for 18 hours in 5% CO_2. A single colony was picked from the plate and sub-cultured in 10 mL of MRS broth at 37°C for 18 hours in 5% CO_2. 10 mL MRS broth supplemented with 0.3% bile (Sigma) was inoculated with 100 µL of the bacterial culture. The control tubes contained MRS broth without any bile and were also inoculated with 100 µL of the bacterial culture. The experiment was carried out in triplicate. OD_{600} was measured hourly for 6 hours. A two way repeated

measures ANOVA test was applied to the absorbance (OD_{600}) to analyse the effects of strain, bile concentration and their interactions.

2.3.7.1 *Agar spot test*

To determine the effect of different bile concentrations on growth of bacteria, agar spot tests were conducted and colony forming units (CFU) were counted. For agar spot tests [275], bacteria were incubated in MRS broth at 37°C for 18 hours in 5% CO_2. A dilution series was made using 20 µl of a 10 mL overnight culture (sub-cultured twice) and added to 180 µL of MRS broth on a 96 well plate. A 10-fold dilution series was created with the dilution factor ranging from 10^1 to 10^8. From each dilution, 20 µl was taken and applied onto a quadrant on the agar plates with bile added (0.0%, 0.5%, 1%, 1.5% and 2%). The concentrations of bile were chosen to represent the range that might be found throughout the human GIT [163]. This spot test was conducted in triplicate. Colonies were counted after 48 hours of incubation at 37°C in 5% CO_2. The quadrants where the total number of colonies was lower than 100, were used to count number of colonies and the CFU were calculated based on dilution factor. For agar spot tests, quadrants containing 10-100 colonies per spot were chosen as counting was easy with less chances of error. A two way repeated measures ANOVA test was applied to the log10 transformed CFU count to analyse the effects of strain, bile concentration and their interactions. The probability-value of multiple comparisons between two strains at each level of bile concentration was adjusted by the Benjamini Hochberg method [276] for controlling the false discovery rate. Statistical analyses were done using R and package 'predictmeans'.

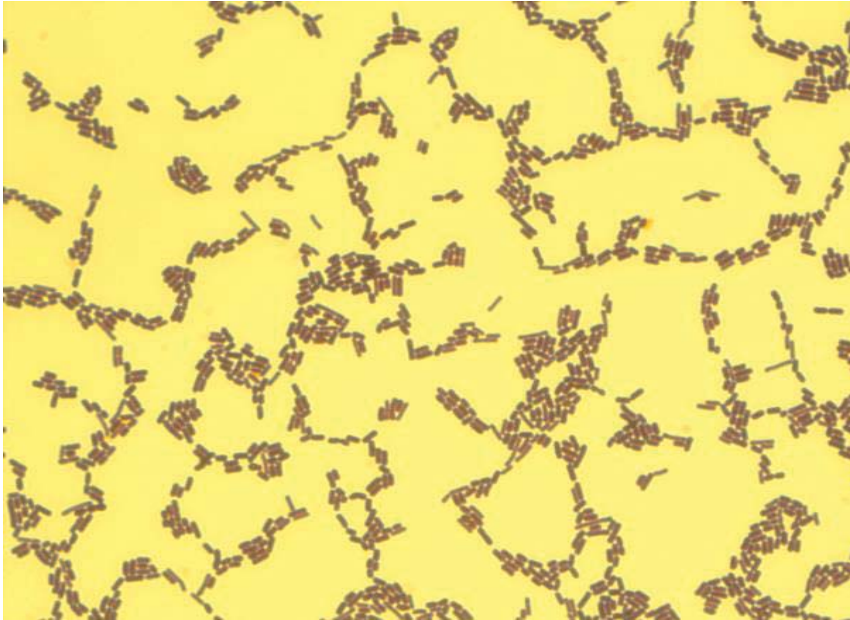
For acid tolerance tests, overnight bacterial cultures were vortexed, centrifuged (5,000x*g* for 5 minutes) and pellets re-suspended in MRS broth (control), and MRS broth acidified to pH 4 and pH 2 (pH adjusted with 6M HCl). The cultures were exposed for 2 and 4 hours, respectively, and spot tests conducted for each time point (0, 2 and 4 hours). The time points (2 and 4 hours) were chosen to represent the time it takes to pass through the stomach and the small intestine [163]. Bacterial viability was assessed using agar spot tests, following the same protocol described in the previous paragraph for the dilution series but using non-acidified standard MRS agar plates. Statistical analysis was done using three-way repeated measures ANOVA to the log10 transformed CFU response to detect effects of strain, pH, time and their interactions. All analyses were done using R and package 'predictmeans'. A pre-adaptation acid tolerance test was also conducted for both the strains by creating two treatment groups and each group was subjected to a pre-adaptation of 60 minutes to pH 5 (pH adjusted with HCl for one group and lactic acid for the second group). The pre-adaptation was followed by exposure to pH 3 (pH adjusted with 6M HCl) for 2 hours followed by spot tests. The control for both strains was not subjected to pre-adaptation but only the 2 hour exposure to pH 3 before spot test were conducted using non-acidified standard MRS agar plates.

2.4 RESULTS

2.4.1 Gram staining

Gram staining results showed that both AGR1485 and AGR1487, being lactobacilli, were Gram positive (Figure 2.1).

(A)



(B)

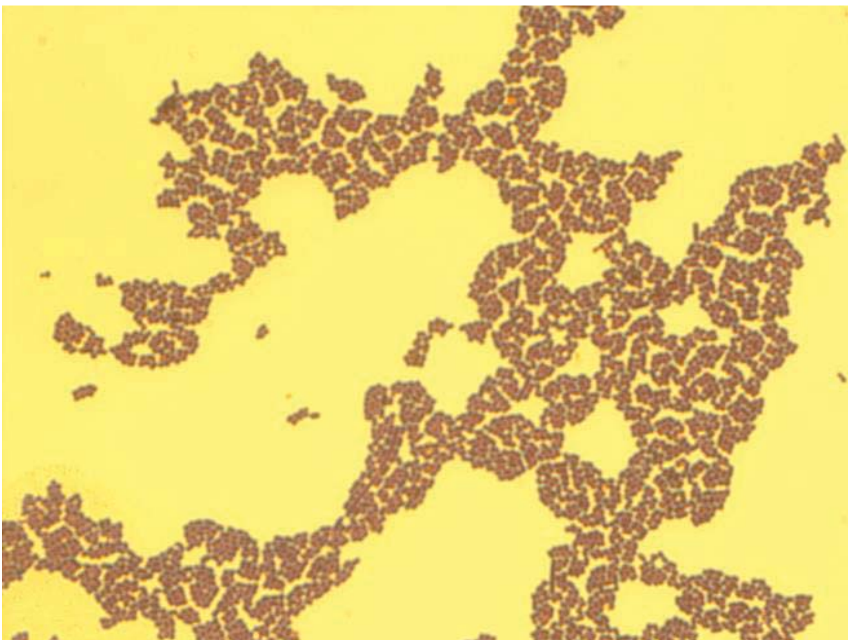


Figure 2.1 Gram stain of (A) AGR1485 and (B) AGR1487. Images captured under 100x magnification using a Leica DM2500 microcope fitted with camera.

AGR1485 appeared to be long, filamentous, rod shaped bacteria that were attached to each other in string-like manner. AGR1487 were shorter and had the appearance of round shaped cocci, clumped together forming clusters.

2.4.2 Effect of the two strains on transepithelial electrical resistance

The previously noted effects of the two strains on the integrity of tight junctions (TJ) between Caco-2 cell monolayers [163] were observed using undifferentiated Caco-2 cells (raw data in Appendix, Table 1). The TEER assays reported in this chapter were tested using differentiated Caco-2 cell monolayers over a longer time period (Figure 2.2). The TEER assay was conducted over 24 hours, using Caco-2 cell monolayers that were differentiated (16 to 20 days post seeding). AGR1485 maintained Caco-2 cell TEER for 12 hours, causing a 16% increase in TEER and had a positive effect but only after 24 hours, compared to the control medium (35% increase at 24 hours; $P<0.05$). AGR1487, on the other hand, decreased TEER across Caco-2 cell monolayers compared to the control medium (68% decrease at 12 hours and 76% decrease at 24 hours; $P<0.05$ after 10 hours and $P<0.005$ after 12 hours).

2.4.3 Identification of bacterial strains

The identities of the two strains AGR1485 and AGR1487 were confirmed as *L. fermentum* by comparing their 16S rRNA gene sequence to the nucleotide sequence collection of the NCBI database. The manual editing of sequences using Vector NTI generated 306 bases of clean read for AGR1485 and 302 bases for AGR1487.

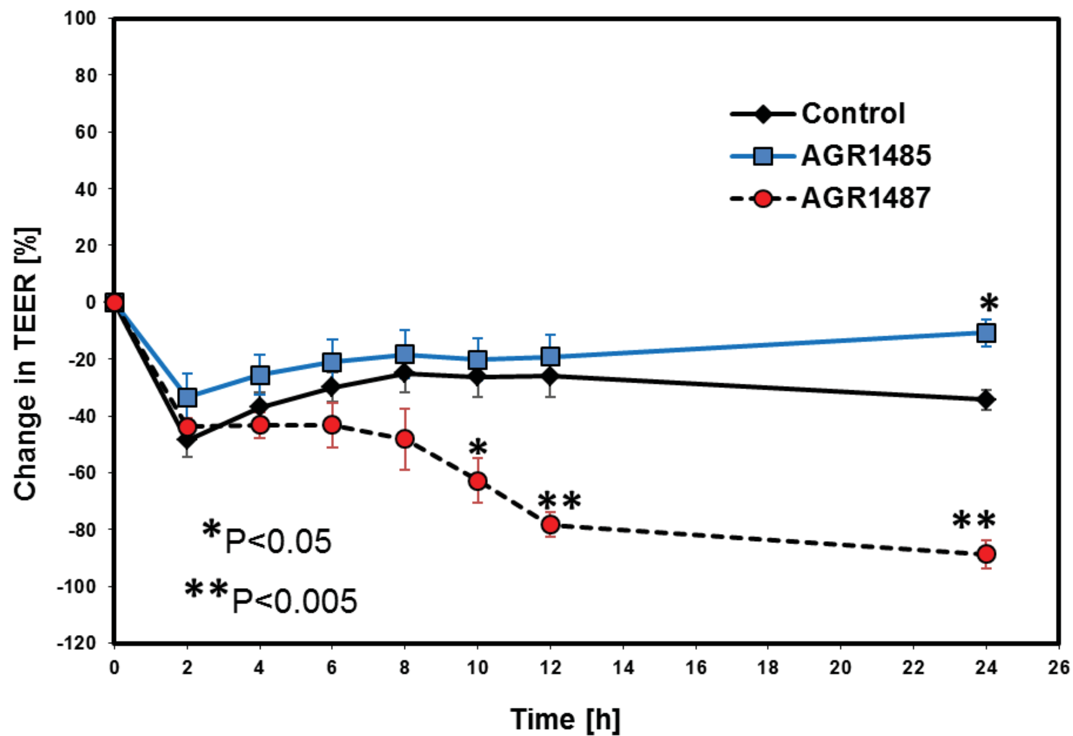


Figure 2.2 Change in trans-epithelial electrical resistance across Caco-2 monolayers over time. Change in transepithelial electrical resistance (TEER) across differentiated Caco-2 monolayers (day 16 to 20 post seeding) over time. The two strains AGR1485 and AGR1487 were taken from the late stationary phase of growth (18 hours). The change in TEER is the percentage change compared to the initial TEER for each monolayer. The values plotted are the mean values for twelve monolayers per treatment, data from three independent assays conducted and the error bars show the SEM.

The sequence of AGR1485 resulted in an alignment with highest sequence identity (99%) with the sequences of *L. fermentum* 28-3-CHN contig1.82 (whole genome shotgun sequence), along with *L. fermentum* ATCC 14931 contig00160 and *L. fermentum* IFO 3956 (whole genome). The trace of AGR1487 resulted in an alignment with 100% sequence identity with *L. fermentum* IFO 3956 (whole genome). Therefore, the identities of both the strains AGR1485 and AGR1487 were confirmed as *L. fermentum*.

2.4.4 Strain differentiation using pulsed field gel electrophoresis

The PFGE with genomic DNA from both strains gave clear band patterns with the restriction enzymes AscI (Figure 2.3A) and I-CeuI (Figure 2.3B). AGR1485 was more resistant to lysozyme compared to AGR1487 and therefore the bacterial pellets were treated to an additional wash with 0.85% NaCl to facilitate cell lysis during DNA extraction. There were differences noted between the band patterns of the two strains which could be used to distinguish the strains from each other. Comparison of the fragment sizes generated as a result of I-CeuI digests indicated that the genome size of AGR1485 was approximately 2,225 kb while that of AGR1487 was approximately 1,930 kb. The differences in the genome size and banding patterns suggest an insertion in the genome of AGR1485 and a deletion in the genome of AGR1487.

2.4.5 Sugar utilisation by the two strains

The API50CH (bioMérieux) test system was used to identify if there were any differences in sugar utilisation patterns between the strains. The lists of sugars and the fermentation results for the two strains are given in Table 2.2.

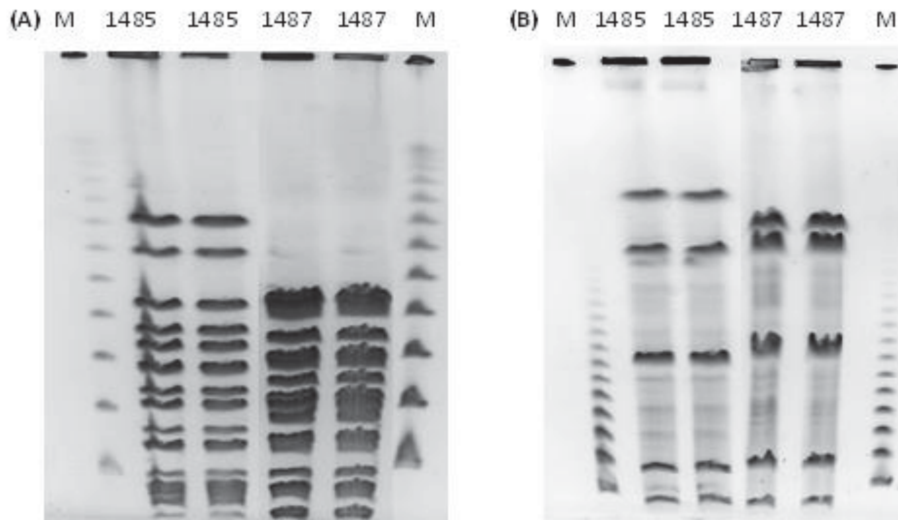


Figure 2.3 Pulsed field gel electrophoresis of genomic DNA from AGR1485 and AGR1487. (A) Genomic DNA from both strains AGR1485 and AGR1487 were digested with restriction enzyme AscI. The band patterns of PFGE of AscI digests show the similarities and differences between the strains. M is the λ marker used. (B) PFGE of I-CeuI digests of genomic DNA from both strains show similar but distinctive band patterns. M is the λ marker used.

Table 2.2 Comparison of sugar source utilisation between the two strains, AGR1485 and AGR1487, based on API50CH fermentation patterns. The table lists the sugars (control has no sugar) and the fermentation ability of the two strains.

Tube	Active Ingredients	AGR1485	AGR1487	Tube	Active Ingredients	AGR1485	AGR1487
0	Control	-	-	25	Esculin ferric citrate	+	+
1	Glycerol	-	-	26	Salicin	-	-
2	Erythritol	-	-	27	D-Cellobiose	-	-
3	D-Arabinose	-	-	28	D-Maltose	+	+
4	L-Arabinose	-	+	29	D-Lactose (bovine)	+	-
5	D-Ribose	+	+	30	D-Melibiose	+	+
6	D-Xylose	-	+	31	D-Saccharose (sucrose)	+	+
7	L-Xylose	-	-	32	D-Trehalose	-	+
8	D-Adonitol	-	-	33	Inulin	-	-
9	Methyl-bD-Xylopyranoside	-	-	34	D-Melezitose	-	-
10	D-Galactose	+	+	35	D-Raffinose	+	+
11	D-Glucose	+	+	36	Amidon(starch)	-	-
12	D-Fructose	+	+	37	Glycogen	-	-
13	D-Mannose	+	-	38	Xylitol	-	-
14	L-Sorbose	-	-	39	Genitiobiose	-	-

Tube	Active Ingredients	AGR1485	AGR1487	Tub e	Active Ingredients	AGR1485	AGR1487
15	L-Rhamnose	-	-	40	D-Turanose	-	-
16	Dulcitol	-	-	41	D-Lyxose	-	-
17	Inositol	-	-	42	D-Tagatose	-	-
18	D-Mannitol	-	-	43	D-Fucose	-	-
19	D-Sorbitol	-	-	44	L-Fucose	-	-
20	Methyl-aD-Mannopyranoside	-	-	45	D-Arabitol	-	-
21	Methyl-aD-Glucopyranoside	-	-	46	L-Arabitol	-	-
22	N-Acetylglocosamine	-	-	47	Potassium gluconate	+	-
23	Amygdalin	-	-	48	Potassium 2-ketogluconate	-	-
24	Arbutin	-	-	49	Potassium 5-ketogluconate	-	-

AGR1485 was able to utilise D-lactose, D-mannose and potassium gluconate and as such tested positive for these carbohydrates, while AGR1487 tested negative for the same sugars. AGR1485 was not able to utilise L-arabinose, D-xylose and D-trehalose and tested negative for these sugars in contrast to AGR1487 which gave positive results for the same sugars.

2.4.6 Ability of the two strains to tolerate gastrointestinal conditions

Bile tolerance was tested for both strains by comparing growth over 6 hours in MRS broth, with and without 0.3% bile (Figure 2.4A). AGR1485 failed to grow after 6 hours in 0.3% bile, and the OD_{600} was 87.6% lower compared to the control medium (growth in MRS broth in the absence of bile). AGR1487, however, continued to grow and at 6 hours, the OD_{600} was only 16.9% lower compared to the control medium. Pair-wise comparison of treatments (probability-values) showed that there was significant difference ($P>0.0001$) between AGR1485 and AGR1487 from 4 hours onwards when exposed to 0.3% bile. Spot tests were then conducted to study the effects of different concentrations of bile on the two strains. The results of the spot test indicated that the viability of AGR1485 decreased by 4.5 log units in the presence of 0.5% bile and 5-5.5 log units in the presence of 1% to 2% bile, whereas AGR1487 showed a decrease in viability of less than 1 log unit even in the presence of 2% bile (Figure 2.4B). Pair-wise comparison of the treatments showed significant difference in bile tolerance between AGR1485 and AGR1487 ($P>0.0001$) indicating that AGR1485 had lower tolerance to bile as compared to AGR1487.

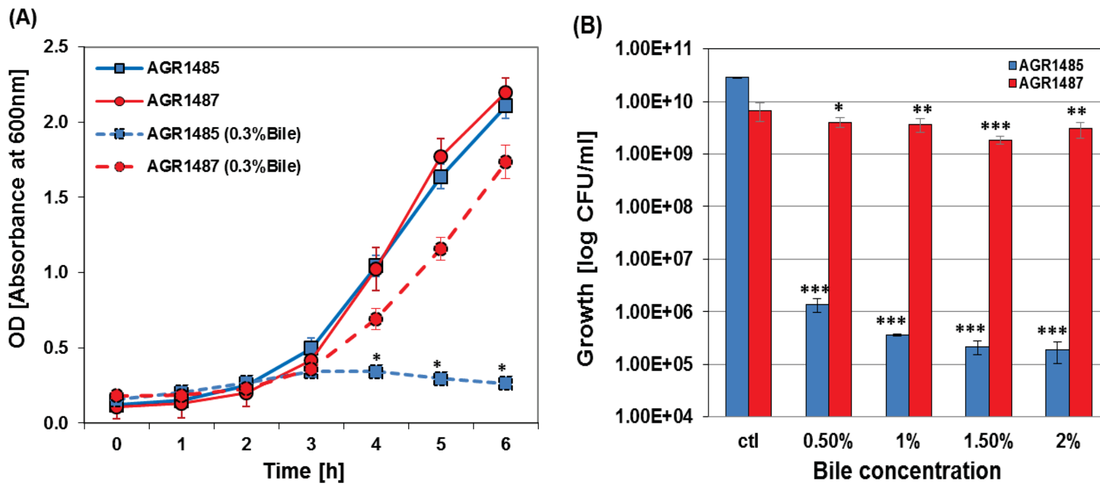


Figure 2.4 Growth of AGR1485 and AGR1487 in the presence of bile. (A) Growth of AGR1485 and AGR1487 in MRS medium with 0.3% bile and without bile (control) over 6 h. The values plotted are the means of three replicates and the error bars show SEM. *P<0.01 compared with control (without bile) values. (B) Agar spot test showing growth of AGR1485 and AGR1487 in different concentrations of bile (0.5%, 1%, 1.5% and 2%) as compared to each other. Control is bacterial growth in MRS broth without bile. The values plotted are the means of three replicates and the error bars show SEM. *P<0.1, **P<0.001 and ***P<0.0001 compared to their respective controls, the probability-values adjusted by Benjamini-Hochberg (BH) method for controlling the false discovery rate.

Initially, the spot tests conducted to study acid tolerance following pre-adaptation showed that both AGR1485 and AGR1487 tolerated pH 3 relatively better, compared to control (no pre-adaptation) when the bacteria were pre-adapted to pH 5 for 60 minutes with lactic acid. Pre-adaptation with HCl (pH 5) was not as effective as with lactic acid for either strain. But these spot tests resulted in too many colonies to be counted even at high dilutions. Acid tolerance was tested for both strains by exposing them to pH 4 and pH 2 for 2 and 4 hours before conducting spot tests to study viability, without pre-adaptation. Both strains were able to tolerate acidic conditions (pH 4 for 4 hours) without any significant loss of cell viability (Figure 2.5A & B). AGR1485 had a reduced viability of 3 to 3.5 log units at pH 2 for 2 hours and 4 hours respectively (Figure 2.5A). AGR1487 tolerated pH 2 for 2 hours with a reduced viability of 2 log units. However, for 4 hours, the viability of AGR1487 decreased by 4 log units (Figure 2.5B). Pair-wise comparison of probability-values showed significant difference in acid tolerance between AGR1485 and AGR1487 ($P>0.001$), indicating that AGR1485 had lower tolerance to acid as compared to AGR1487 at pH 2 at 2 hours.

2.5 DISCUSSION

The results of the TEER assays confirmed the previously noted differential effects of the two strains AGR1485 and AGR1487 on Caco-2 cell barrier integrity. AGR1485 maintained Caco-2 cell TEER similar to that of control and had a positive effect on TEER but only after 24 hours of exposure. AGR1487, on the other hand, reduced Caco-2 cell TEER as compared to control as early as 10 hours, thus compromising the barrier integrity of the Caco-2 cells.

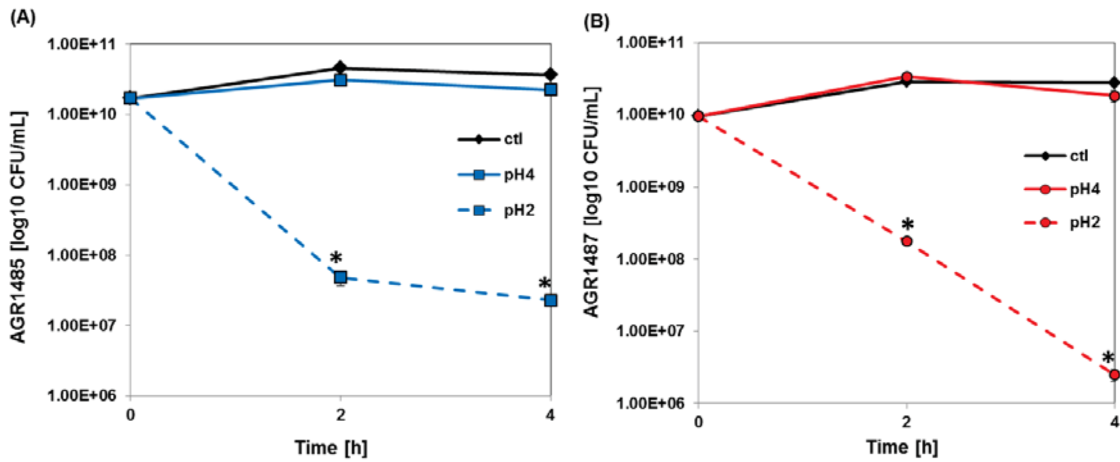


Figure 2.5 Agar spot test of (A) AGR1485 and (B) AGR1487 Both strains grown at pH 6 (growth in MRS broth used as control) compared to cultures exposed to pH 2 and pH 4 (MRS broth acidified with 6M HCl) for 0, 2 and 4 hours followed by spot test on MRS agar plates. The values plotted are the means of three replicates and the error bars show SEM. *$P<0.001$ compared to bacteria in control medium, the probability-values adjusted by Benjamini-Hochberg (BH) method for controlling the false discovery rate.

These results are similar to previously noted effects of AGR1485 and AGR1487 on undifferentiated Caco-2 cells [163]. In addition, the strains showed differences in their genome size, sugar utilisation capabilities and in their response to stress tolerance assays.

The identification and characterisation of *L. fermentum* and other members of this genus reported in studies were usually based on colony morphology, Gram stain reactions, 16 S rRNA sequencing [163] and sugar fermentation profiles in addition to studying enzymatic activities [5, 277]. For this study, PFGE of genomic DNA, after digestion with restriction enzymes AscI and I-CeuI, was used to differentiate the two bacterial strains from one another. Comparison of fragment sizes of the two strains resulting from the restriction digests with published data [278] indicate that the genome size of AGR1485 was approximately 2,225 kb and that of AGR1487 was 1,930 kb. This difference in genome size suggests that there was an insertion in the genome AGR1485 and a deletion in the genome AGR1487. The effect that AGR1487 has on the intestinal barrier integrity could be a result of the absence of genes and/or gene products that may play a role in the microbe-host interactions.

The compromising effect on barrier integrity may also be the result of morphological alteration caused mutations of genes that could be involved in structural biosynthesis and/or pathways that influence bacterial morphological characteristics. The phenotypic differences exhibited by these two strains could therefore be due to the presence, absence, or mutation of genes, resulting in strain-specific characteristics.

An observation made during genomic DNA extraction from both the strains, showed that AGR1485 was more resistant to lysozyme activity as compared to

AGR1487 and had to be treated with 0.85% NaCl (to remove EPS) in order to facilitate cell lysis, suggesting a difference in cell surface structure between the two strains. Many bacteria modify their PG in a variety of ways as a level of control over endogenous autolysins and other lytic enzymes [279]. One such modification is the *O*-acetylation of PG, that provides bacteria with resistance to endogenous autolysins as well as protection from lysozymes [280]. The resistance that AGR1485 exhibits towards lysozymes may be caused by a modification to the PG that contribute to its cell surface structure making this bacterium more resistant to lysozymes and autolysins.

The API50CH test highlighted the differences in sugar utilising capabilities between AGR1485 and AGR1487. Studies with *Lactobacillus delbrueckii* subsp. *lactis* 313 suggest that utilisation of different sugars stimulate the production of different cell surface proteins which has an effect on cell membrane associated proteinase activity [281]. Proteinase production is relative to CW biosynthesis and differences in CW biosynthesis may result in variation in its structural components. Variation in the peptidoglycan composition has been shown to impart strain-specific characteristics in lactobacilli and their interactions with host cells in activating metabolic pathways and eliciting immune responses [88, 222]. Therefore, variations in sugar utilisation capabilities of AGR1485 and AGR1487 may be indicative of differences in cell surface proteins and proteinase activity between the two strains, which in turn may influence host-microbe interactions and cause them to have differential effects on the host.

The possible genotypic differences of these strains may also be related to the difference in tolerance these bacteria show towards environmental stressors, such as bile and acidic conditions. Lactobacilli encounter various harsh conditions (e.g. bile, low pH, oxidative and osmotic stress) as they move through the GIT. To survive these conditions, they need to display many resistance mechanisms. These stress responses are dependent on the co-ordinated expression of genes that control different cellular processes [137, 282]. Many resistance mechanisms have been found to be common for bile and acid stress and result in alteration of lactobacilli cell surface structures [142]. For instance, bile salts and cholesterol have been shown to induce changes in the lipid cell membrane of *L. reuteri* [11] while low pH causes alterations in the fatty acid composition of an oral strain of *L. casei* [12]. Studies attribute differences in stress phenotypes to variation in stability or binding specificities of stress response regulatory proteins [283]. Screenings of acid and bile salt responses in lactobacilli have identified genes involved in PG biosynthesis and cell envelope functions. Gene expression analysis of *L. acidophilus* identified a high number of genes involved in PG and cell surface protein (e.g., SrtA) biosynthesis that are differentially expressed after bile exposure [143]. In *L. reuteri*, response to acidic conditions involves the ClpL chaperone, an ATPase with chaperone activity and a putative CW-altering esterase, which are also reported to be induced by bile exposure [144, 145]. AGR1485 was less tolerant to both bile and low pH as compared to AGR1487. This may be due to mutation in genes that make this strain less resistant to bile exposure as has been noted in studies with *L. reuteri* ATCC55730 [145]. The differences that AGR1485 showed in comparison to AGR1487 in bile and acid tolerance may be related to their cell surface proteins and/or secretory abilities. Structures of teichoic acids have been suggested to

affect proper functioning of cell integrity in acidic conditions and in the presence of bile [144, 145]. The cell surface structure of the strains can be an important indicator for the different stress responses that these strains exhibit [9, 136, 145]. AGR1487 may have a surface structure displaying proteins and/or secreting molecules that make it more tolerant to bile and low pH than AGR1485. AGR1485 may either lack or poorly express the proteins and/or components necessary to survive acidic conditions or to tolerate bile stress. There could also be alteration of expression of genes due to point mutations that influence the bacterial structure, thereby affecting its stress handling capabilities.

Many *Lactobacillus* species, including *L. fermentum*, are considered GRAS microorganisms. However, they have been implicated as opportunistic pathogens, under certain conditions, especially in immunocompromised individuals [284, 285]. The tolerance that AGR1487 exhibits towards high bile concentrations and low pH suggests that it may be better adapted to survive the passage through the GIT and that the GIT conditions of such immunosuppressed individuals may provide the ideal niche for AGR1487 to flourish and colonise. The detrimental effect that AGR1487 has on the intestinal barrier may play a role in the intestinal health of the genetically-susceptible individual and other such individuals, but based on current data such conclusions cannot be drawn [258]. AGR1485 however, has no such detrimental effects on intestinal barrier integrity thus stressing the strain-specific characteristics of lactobacilli.

3. CHAPTER 3: EFFECTS OF LACTOBACILLUS FERMENTUM CELL VIABILITY, GROWTH PHASE AND SECRETED COMPOUNDS ON INTESTINAL BARRIER EPITHELIUM.

3.1. INTRODUCTION

Lactobacilli are an important part of the human microbiota, considered to be protective organisms contributing to maintaining gastrointestinal tract (GIT) health. Many *Lactobacillus* species have been used as probiotics in foods and in various pharmaceutical preparations [259, 261]. There is accumulating evidence for the effectiveness of probiotics in the prevention and treatment of various diseases, including allergic diseases, diarrhoea and inflammatory disorders of the GIT [286, 287]. However, studies have shown that different strains of lactobacilli can evoke different responses in the host and therefore, results from one strain cannot be generalised to others [5, 75, 76].

Although lactobacilli seem to have few adverse effects on healthy individuals, some strains have been associated with serious infections in critically ill patients [288, 289]. There have been reports of infections caused by *Lactobacillus* species implicating them as opportunistic pathogens, under certain conditions, especially in immunocompromised individuals [284, 285]. For example, *Lactobacillus rhamnosus* GG (LGG) is a well characterised probiotic showing beneficial effects in patients with atopic dermatitis [290, 291]. However, there have been reports that supplementation with LGG during pregnancy and early infancy neither reduced the incidence, nor altered the severity of atopic dermatitis in affected children [292, 293]. However, supplementation with LGG was associated with an increased recurrence of wheezing bronchitis in children affected with atopic dermatitis and as such cannot be generally recommended as a primary preventive measure [294, 295]. In spite of these reports, the

resurgence of interest in the use of “all natural” products and the use of probiotics to treat and prevent illness, has increased the focus on *Lactobacillus* species [296].

Several mechanisms have been proposed to explain how lactobacilli exert their effects on hosts. These mechanisms include pathogen inhibition through exclusion, pathogen resistance, immunomodulation and maintenance of GIT homeostasis [3-5]. Experimentally, many of the effects of viable bacterial cells on the host can also be obtained using dead bacterial cells [297]. Inactivation of bacteria can be achieved by chemical and/or physical means. Most of these conventional techniques of inactivation cause some damage to the bacterial cellular structure, including denaturing surface proteins [298]. Short wave UV radiation treatment has been found to be the most suitable method of bacterial inactivation while maintaining the integrity of the bacterial structure [298, 299]. Some strains of UV-killed lactobacilli can be equally effective as live bacteria in eliciting responses in host cells [300]. Several studies suggest that the effects of lactobacilli on the host are related to the cell surface and/or secreted compounds of these bacteria interacting with the host cells [15, 74, 79]. However, the molecular mechanisms underlying the effects of lactobacilli on the host and the roles played by bacterial cell surface and/or secreted compounds in imparting these effects require elucidation [9, 79, 80].

Lactobacilli-host interactions are strain-specific and pronounced variation in effects on a respective host is observed among strains, even of the same species. Strain variation in lactobacilli influences characteristics such as adherence, a major parameter in the colonising potential of commensal bacteria [5], and the induction of pro- or anti-

inflammatory cytokines in host immune cells which can be either beneficial or detrimental to the host [75, 223, 224].

Bacterial cell surface components play major roles in the host-microbe ‘cross-talk’ as they act as the key ligands that interact with the host receptors and influence the host response [41]. The variability and biochemical complexity of the bacterial ligands as seen in the substitution levels of teichoic acids (TA), the variable backbone alditol compositions of the wall teichoic acids (WTA) and the modifications of peptidoglycan (PG) of the CW in lactobacilli are linked to strain-specificity and influence host-microbe interactions [81]. Differences in the expression of secreted and/or bacterial cell surface associated components are strain-specific and may also be related to the phase of bacterial growth. Transition from rapid growth (exponential/log phase) to a state of stress response (stationary phase) is associated with increased expression levels of genes that are involved in DNA repair, active metabolism and transport [301]. The changes in metabolic events that occur during the different phases of growth in bacteria impact not only their own cellular structure and secreted molecules, but may also affect their interactions with intestinal epithelial cells (IEC) [302, 303].

In the previous chapter, the phenotypic and genotypic similarities and differences of two strains of *Lactobacillus fermentum* (AGR1485 and AGR1487) were described. The results indicated that the strains showed differences, not only in their effect on Caco-2 cell barrier integrity, but also in the size of their genomes and in their response to stress tolerance assays.

3.2. AIM AND HYPOTHESIS

The aim of the research reported in this chapter was to test the hypothesis that the different effects on intestinal barrier integrity shown by the two strains of *L. fermentum* (AGR1485 and AGR1487) are mediated by the interaction of bacterial secreted and/or CW associated components with the epithelial layer. The effects of the bacterial growth phase on barrier integrity were determined by conducting TEER assays with bacteria taken from six different phases of growth (early log, mid log, late log, transition, early stationary and late stationary). Transepithelial electrical resistance (TEER) assays and ^{3}H-mannitol assays were conducted with live versus UV-inactivated bacteria interacting with Caco-2 cells monolayers to determine the effect of bacterial cell viability (implying possible roles of bacterial CW-associated and secreted components) on Caco-2 cell barrier integrity. To ascertain the role of bacterial secreted compounds in affecting intestinal barrier integrity, TEER assays were conducted using bacterial metabolites produced as a result of the interaction with Caco-2 cells.

3.3. MATERIALS AND METHODS

3.3.1 Cell culture for the intestinal barrier assays

Caco-2 cells (from passage numbers 28 to 36) were cultured in cell culture medium as previously described in Chapter 2, Section 2.3.3. For the mannitol assays, Caco-2 cells were cultured in M199 (GIBCO, Life Technologies) supplemented with 10% foetal bovine serum (GIBCO, Life Technologies), 1% non-essential amino acids (NEAA; MEM non-essential amino acids 100x solution, Life Technologies) and 1%

antibiotic-antimycotic (Gibco, 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone). The day before the TEER and mannitol assays, medium from the Caco-2 cell monolayers and the basal well was removed and replaced by M199 supplemented with 1% NEAA (500 µL/Transwell and 1.5 mL/well).

On the day of the assay, the initial resistance values of the Caco-2 cell monolayers were recorded, which was considered to be the baseline measurement (time = 0 hour). The measurements were taken as described in Chapter 2, Section 2.3.3.1.

3.3.2 Bacterial strains

The two strains of *L. fermentum*, AGR1485 and AGR1487, were grown from frozen stocks as previously described in Chapter 2, Section 2.3.1.1.

3.3.3 Bacterial growth curve

A 100 µl aliquot of each strain was inoculated into 10 mL MRS broth culture tubes (3 tubes/strain) and incubated at 37ºC in 5% CO_2. Optical density (OD_{600}) was measured every two hours for 24 hours. The growth curves for both strains were determined by plotting the OD_{600} against time. The anticipated timeframe for the logarithmic growth (log) phase of both strains was deduced from the respective half-logarithmic plots. Linear regressions were calculated using Sigma Plot 10 (Systat Software) to determine the specific OD_{600} that best represented early, mid and late log phases of growth. Mid log OD_{600} was estimated by evaluating data points that lay within the linear section of the growth curve, characteristic of the log phase of bacterial growth. The mid log OD_{600} was calculated using Equation 3.1.

$$OD_{600\ (mid\ log)} = \frac{OD_{600\ (max)} - OD_{600\ (min)}}{2}$$

Equation 3.1 Calculation of mid log OD_{600}. OD_{max}: maximum OD_{600} of linear section of growth curve, OD_{min}: minimum OD_{600} of linear section of growth curve.

The late stationary phase was chosen by time and not by OD_{600}, to be comparable to previous TEER assays conducted to study the effects of the two strains on the barrier integrity of the Caco-2 cells (bacteria taken from late stationary phase, grown for 18 hours after inoculation) [163].

3.3.4 Bacterial growth phase for transepithelial electrical resistance assays

Bacteria from the same culture harvested at six different phases of their growth were used as treatments to test the effects of growth phase on Caco-2 cell TEER. Bacterial cells were collected at the determined phases of their growth when the inoculated culture reached the corresponding OD_{600} or time point. Bacterial cells were centrifuged at 5,000x*g* for 5 minutes, the cell pellet was washed once in M199 containing 1% NEAA and resuspended in the same medium to an OD_{600} of 0.9 ensuring a constant number of bacterial cells from each growth phase. An aliquot of 500 μL of bacterial cells was then added to each Transwell of Caco-2 cells and the effect on TEER determined every 2 hours over a 12 hour period. For growth phase experiments, one 12-well plate was used per growth phase. Three separate experiments were carried out with four replicates per treatment for each experiment (total n=12 per treatment). Statistical analysis was carried out using meta-analysis with REML for a

combined analysis of three related experiments, using package “lme4” and “predictmeans” in R.[1]

3.3.5 Preparation of UV-killed bacteria

Use of short wave UV radiation has been reported to be a suitable method for inactivation of bacteria where it is important to maintain the integrity of the bacterial structure while rendering the bacteria non-viable. Therefore, methods were developed to test the viability of bacteria following UV radiation and to ascertain the effectiveness of UV-radiation in inactivating the two strains AGR1485 and AGR1487 by exposing them to UVC-radiation of 254 nm using a UV lamp (UVP, 3UV-38, 8 Watts, BIO-STRATEGY LTD).

3.3.5.1 Method development for UV inactivation of bacteria

3.3.5.1.1 UV inactivation of bacteria on agar plates

Preliminary experiments were conducted to confirm the effectiveness of UV-radiation in inactivating the two strains AGR1485 and AGR1487. Both strains were grown overnight in MRS broth, incubated at 37ºC in 5% CO_2. A 1 mL aliquot of bacterial cells from each strain was centrifuged at 5,000x*g* for 5 minutes, and resuspended in 1 mL of M199 containing 1% NEAA. Dilutions (1:1,000, 1:10,000) were prepared in M199 containing 1% NEAA and aliquots (100 µL) of these two dilutions of each strain were plated on MRS agar plates (3 agar plates per dilution). Half

[1] Statistical analysis was performed by Dr Dongwen Luo (AgResearch, Grasslands).

of the agar plate was covered with aluminium foil and the plate exposed to UVC-radiation (254 nm) for 3, 4 and 5 minutes (3 agar plates per time point). The UV treatment was carried out by placing the plates under a UV lamp (UVP, 3UV-38, 8 Watts, BIO-STRATEGY LTD). The plates were then incubated for 48 hours at 37ºC in 5% CO_2 and bacterial viability assessed by counting colonies and calculating CFU/mL based on dilution factor for both the UV-exposed and non-exposed (aluminium foil covered) sides.

3.3.5.1.2 UV inactivation of bacteria in M199

TEER assays on Caco-2 cell monolayers were conducted with bacteria resuspended to a concentration of 0.9 OD_{600}, therefore, it was necessary to determine the length of UV-exposure time to achieve 100% inactivation of both strains of bacteria at a concentration of 0.9 OD_{600}. Therefore bacterial cell pellets from both strains were re-suspended in M199 containing 1% NEAA to an optical density of 0.9 OD_{600} and subjected to UVC radiation. Aliquots (2 mL) of each strain were added to wells in a 6-well plate and exposed to UVC-radiation (254 nm) for 15 minutes, 20 minutes and 30 minutes, without ice and on ice. Ice was used to minimise damage and possible inactivation of bacterial cell surface components that might result from the heat generated due to UV radiation. The UV-inactivation was carried out for the control medium (M199 containing 1% NEAA) as well. The UV treatment was carried out by placing the 6 well plate (either on ice or without ice) containing the samples, under a UV lamp (UVP, 3UV-38, 8 Watts, BIO-STRATEGY LTD). The lamp was placed on a stable surface such that the plate was directly under the UV lamp to keep the distance (approximately 1.7 cm) from the plate constant. Bacterial viability was tested by plating

100 μL aliquots of the UV-treated bacterial cultures on agar plates, then incubating the plates for 48 hours at 37ºC in 5% CO_2 followed by counting colonies and calculating CFU/mL.

3.3.5.2 Determining the effect of UV-killed versus live bacteria on transepithelial electrical resistance

To determine the effect of UV-killed bacteria on Caco-2 cell TEER, both strains (AGR1485 and AGR1487) were subcultured under standard conditions (Section 3.4.2) and allowed to reach late stationary phase (grown for 18 hours). On the day of the assay, 1 mL of each culture was centrifuged at 5,000x*g* for 5 minutes, the supernatant discarded, and the bacterial cell pellets re-suspended in M199 containing 1% NEAA to an optical density of 0.9 (OD_{600}). Approximately 2 mL of the re-suspended bacterial culture was added to each well of a 6-well microtitre plate. The plate was placed on ice directly below a UV lamp (UVP, 3UV-38, BIO-STRATEGY LTD) and exposed to UVC radiation (254 nm) for 30 minutes. The length of UV exposure time was determined in Section 3.3.5.1.2 to be effective to achieve 100% inactivation for both strains. An aliquot of 500 μL of UV-killed bacterial cells of each strain was added to each Transwell of Caco-2 cells and the effect on TEER was determined every 2 hours over a 12 hour period. M199 media containing 1% NEAA, exposed to UV for 30 minutes on ice, was used as a control (500 μL/Transwell). Live (non-UV treated) bacteria from both strains were also used as treatments to compare their effects on Caco-2 cell monolayers. Three separate experiments were carried out with four technical replicates per treatment for each experiment (total n=12 per treatment). The three experiments were considered as biological replicates and the data within and

among the assays were averaged. Statistical analysis was carried out using repeated measures ANOVA (meta-analysis with REML) for a combined analysis of three related experiments, using package "lme4" and "predictmeans" in R. Statistical difference was declared between treatments for a given time point where the difference in means was greater than the least significant difference (LSD) at 5% as compared to control.[2]

3.3.5.3 Determing the effect of UV-killed bacteria on paracellular permeability (3H -mannitol assay)

Caco-2 cells were cultured as described in Section 3.3.1. Paracellular permeability of the monolayers was determined using ^{3}H-mannitol. A stock solution of ^{3}H-mannitol in 90% ethanol (American Radiolabelled Chemicals, St. Louis, Missouri, USA) was diluted in M199 (containing 1% NEAA). Overnight cultures of bacteria (stationary phase) were re-suspended in M199 containing 1% NEAA to an OD_{600} of 0.9, and UV treated before ^{3}H-mannitol was added.

On the day of the assay, the baseline TEER of the Caco-2 cell monolayers was recorded for each of the Transwells and treatments were prepared by mixing ^{3}H-mannitol solution with bacteria (untreated or UV-killed) or control medium (untreated or UV treated). For every 2 mL treatment (500 µL/Transwell), 80 µL of ^{3}H-mannitol was added (final concentration 9.25×10^4 Bq/mL). Caco-2 cell monolayers in Transwells were co-cultured with strains AGR1485, AGR1487, UV-killed AGR1485, UV-killed AGR1487, untreated and UV-treated control medium with no bacteria (M199 with 1% NEAA) over 12 hours, incubated at 37ºC in 5% CO_2. During the incubation, 75 µL of

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[2] Statistical analysis was performed by Dr Dongwen Luo (AgResearch, Grasslands).

cell culture medium (M199 containing 1% NEAA) was removed from each well (basal well in which the Transwell rests) every 2 hours for 12 hours. All samples were mixed with scintillation fluid (StarScint, Perkin Elmer, Waltham, Massachusets, USA) at a ratio of 1:1 and counted using a 1459 Microbeta Trilux scintillation counter (Perkin Elmer). The fluorescence detected from ^{3}H was used to calculate the amount of ^{3}H-mannitol in each well at each time point (T_n) using Equation 3.2.

$$Mannitol_{basal(T_n)} = \left(Mannitol_{sample(T_n)} \times \frac{(volume_{initial} - n \text{ x } volume_{sample})}{volume_{sample}} \right) + \sum_{k=0}^{n-1} Mannitol_{sample(T_k)}$$

Equation 3.2 Amount of ^{3}H-mannitol present in each basal well (Mannitol $_{basal}$) at each time point (***T_n*)**, n = 0, 1, …, 7, where these represent 0, 2, …, 12 hours. The Mannitol $_{sample}$ is the number recorded by the scintillation counter in disintegrations per minute (dpm) for each sample taken for a particular time point. The Mannitol $_{basal}$ is the number adjusted of the expected amount of ^{3}H-mannitol count for the entire basal well at time point ***T_n***, taking into consideration the initial volume of the basal well (volume $_{initial}$=1500 μL), volume of sample removed (volume $_{sample}$=75 μL), and the volumes removed for sampling at time points previous to ***T_n*** (***T_k=T_0*** to ***T_{n-1}***).

The percentage of ^{3}H-mannitol in the basal well that had passed from each Transwell was calculated for each time point using Equation 3.3.

$$Mannitol_{passed\ through}[\%] = \frac{Mannitol_{basal}}{Mannitol_{initial\ apical}} \times 100$$

Equation 3.3 Percentage ^{3}H-mannitol that passed through to the basal well [%] is the percentage of the amount of ^{3}H-mannitol that passed from the Transwell to the basal well taking into consideration the amount of ^{3}H-mannitol that was initially added to the Transwell.

Three separate experiments were carried out with four replicates per treatment for each experiment (total n=12 per treatment). Repeated measures ANOVA using “predict means” in R was used for statistical analysis. The data were log transformed to stabilise variance and back transformed to estimate the rate of ^{3}H-mannitol increase in the well (basal) assuming a straight line trend. The three experiments were used as biological replications and the data within the assays (for each treatment x time combination) had been averaged. A log transformation of ^{3}H-mannitol percentages gave satisfactory validation of ANOVA assumptions (assuming homogeneity of variance and normal distribution). Statistical difference was declared between treatments for a given time point where the difference in means was greater than the least significant difference (LSD) at 5% as compared to control.[3]

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[3] Statistical analysis was performed by Dr Siva Ganesh (AgResearch, Grasslands).

3.3.6 *Preparation of supernatants containing metabolites produced by bacterial-Caco-2 cell interactions*

Four treatment groups were prepared per strain (AGR1485 and AGR1487) with each treatment designed to test for molecules secreted either by Caco-2 cells, by bacteria or due to Caco-2-bacteria interactions that could affect Caco-2 cell TEER. The flow diagram (Figure 3.1) summarises the treatments that were made for each strain. Caco-2 cells were cultured and maintained as described in section 3.4.1.

Treatment 1 was designed to test if the interaction of metabolites secreted by Caco-2 cells (secreted into the media between days 15 and 16) with live bacteria for 12 hours could result in bacterial secretion of molecules that affect TEER when applied to Caco-2 cell monolayers. Treatment 1: On day 15 post seeding of the Caco-2 cells, the medium from four Transwells was removed and replaced by M199 containing 1% NEAA. On day 16, the supernatant from these Transwells was collected, centrifuged at 5,000x*g* for 5 minutes and filter sterilised using a 0.22 μm filter. The filter sterilised supernatant (SN) was used to re-suspend bacteria (grown to stationary phase in MRS medium, 18 hours) from each strain to an optical density of 0.9, measured at OD_{600}. The SN-bacteria mixture was aliquoted into four wells (500 μL/well) of a 12 well microtitre plate, incubated at 37ºC in 5% CO_2 for 12 hours. After 12 hours, the SN from each well was collected, centrifuged at 5,000x*g* for 5 minutes and sterile filtered using a 0.22 μm filter. The sterile filtered SN resulting from the Caco-2 SN-bacteria mixture was labelled as treatment 1.

Treatment 2 was designed to test if cellular components of dead Caco-2 cells interacting with live bacteria for 12 hours could result in secretion of bacterial molecules that affect Caco-2 cell TEER. Treatment 2: On day 16 post seeding of the Caco-2 cells, the medium from four Transwells was removed and the Caco-2 cell monolayers were trypsin treated to dislodge the cells from the Transwells and then centrifuged at 240x*g* for 3 minutes. The pelleted Caco-2 cells were re-suspended in M199 containing 1% NEAA and homogenised to kill the cells, using an OMNI-TH homogeniser (OMNI International) at the highest speed setting for 30 seconds. The lack of cell viability was verified under the microscope using trypan blue. The homogenised Caco-2 cells were mixed with bacteria (0.9 OD_{600}), aliquoted into four wells (500 µL/well) of a 12 well microtitre plate and incubated at 37ºC in 5% CO_2 for 12 hours. After 12 hours, the SN from each well was collected, centrifuged at 5,000x*g* for 5 minutes and sterile filtered using a 0.22 µm filter. The sterile filtered SN resulting from the homogenised Caco-2 cells-bacteria mixture was labelled as treatment 2.

Treatment 3 was designed to test if interaction of live Caco-2 cells with live bacteria for 12 hours resulted in secretion of molecules (either by bacteria or Caco-2 and/or both) that could affect Caco-2 cell TEER. Treatment 3: On day 16 post seeding of the Caco-2 cells, the medium from Caco-2 cell monolayers of four Transwells was removed and replaced by bacterial cells (500 µL/Transwell) that had been pelleted and re-suspended in M199 containing 1% NEAA (0.9 OD_{600}). These Transwells containing intact Caco-2 cells and live bacteria were incubated at 37ºC in 5% CO_2 for 12 hours. After 12 hours, the SN from each well was collected, centrifuged at 5,000x*g* for 5 minutes and sterile filtered using a 0.22 µm filter.

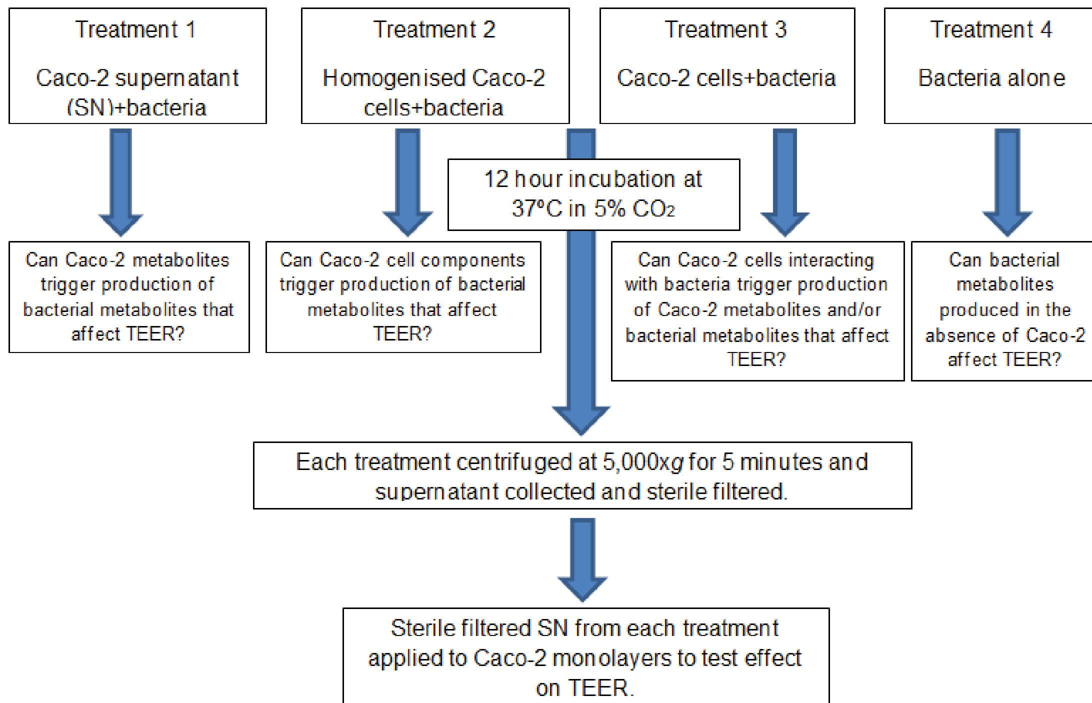

Figure 3.1 Flow diagram showing the treatments applied to the lactobacilli strains AGR1485 and AGR1487. Each treatment was designed to test for the activity of metabolites secreted by live Caco-2 cells, bacteria or as a result of Caco-2 (live or components) and bacterial interactions that might affect Caco-2 cell TEER.

The sterile filtered SN resulting from the Caco-2 cells-bacteria mixture was labelled as treatment 3.

Treatment 4 was designed to test if live bacteria secreted molecules that affect Caco-2 cell TEER without external stimuli. Treatment 4: On day 16, bacterial cells, that had been pelleted and re-suspended in M199 containing 1% NEAA (0.9 OD_{600}), were added to four wells of a 12 well microtitre plate (500 µL/well). These wells containing live bacteria were incubated at 37ºC in 5% CO_2 for 12 hours. After 12 hours, the SN from each well was collected, centrifuged at 5,000x*g* for 5 minutes and sterile filtered using a 0.22 µm filter. The sterile filtered SN containing metabolites secreted by live bacteria was labelled as treatment 4.

TEER assays were conducted using Caco-2 cell monolayers (16 to 20 days post seeding), using the sterile filtered SN collected from the previously co-cultured treatment groups (1-4) from each strain. The TEER was measured every 2 hours over a 12 hour period. Three separate experiments were carried out with four replicates per treatment for each experiment (total n=12 per treatment). Statistical analysis was carried out using repeated measures ANOVA (meta-analysis with REML) for a combined analysis of three related experiments, using package "lme4" and "predictmeans" in R. A statistical difference was declared between treatments for a given time point where the difference in means was greater than the least significant difference (LSD) at 5% as compared to control. [4]

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[4] Statistical analysis was performed by Dr Dongwen Luo (AgResearch, Grasslands).

3.4. RESULTS

3.4.1 Bacterial growth phases based on growth curves

The growth curves of both strains AGR1485 and AGR1487 were similar (Figure 3.2 A & B). Both strains grew exponentially for approximately 6 hours and then entered stationary phase which continued for the 18 hours studied. The ODs for the early, mid and late log phases were determined using linear regressions (Figure 3.2 C & D). The regression lines calculated for the log phase of the growth curves for both AGR1485 and AGR1487 indicated that by t=2 hours, both strains were in log phase and grew exponentially. An OD_{600} of 0.16 was chosen as early log phase for both strains. The growth curves also indicated that at t=6 hours, the growth pattern for both strains was no longer exponential, by which time both strains might have been in transition phase. Therefore, for AGR1485 (Figure 3.2 A), a late log OD_{600} of 1.8 was chosen and a mid log OD_{600} of 0.8 was calculated using Equation 3.1. For AGR1487 (Figure 3.2 B), a late log OD_{600} of 1.9 was chosen and a mid log OD_{600} of 0.9 was calculated. Based on the growth curves, an OD_{600} of 2.4 was chosen as the transition between log and stationary phase for both strains. The growth curves plateaued after reaching a maximum OD_{600} at t=8 hours for both strains, indicating that the bacteria were in stationary phase. An OD_{600} of 2.4 was chosen as early stationary for both the strains. The late stationary phase was selected on the basis of the time (18 hours after inoculation used in previous experiments [163]) instead of OD, to maintain consistency with previous experiments.

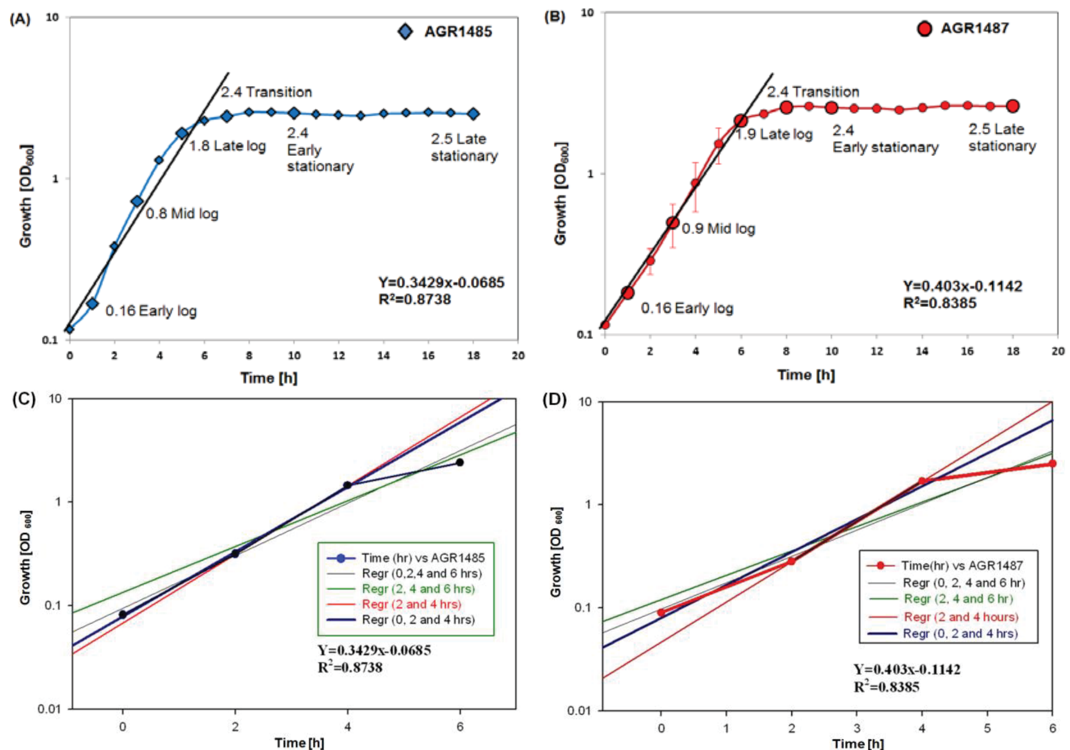

Figure 3.2 Growth curves for AGR1485 (A) and AGR1487 (B) and log phase of growth for AGR1485 (C) and AGR1487 (D). Both strains were grown in MRS broth at 37°C in 5% CO_2 over a 24 hour period with optical density measured at 600nm (OD_{600}) every 2 hours. The values plotted are the mean values (3 tubes/strain) and the error bars show the standard error of means (SEM). The OD measurement used for each growth phase is shown on each growth curve (enlarged) and the same OD was used for all three sets of growth phase experiments. The log phase of growth for AGR1485 and AGR1487 with linear regressions considering all 4 data points (0, 2, 4 and 6 hours) or excluding certain data points to find the best fit for calculating the mid log OD_{600}. The equation shown is for the regression line (0, 2 and 4 hours) that was the best fit for both the strains. Mid log OD was calculated as the mid value of early log OD and late log OD.

3.4.2 Effect of bacterial growth phase on transepithelial electrical resistance

The results of TEER assays conducted with bacteria taken from the six different phases of growth (early log, mid log, late log, transition, early stationary and late stationary) showed that AGR1485 (raw data in Appendix, Tables 2.1 and 2.3), taken from any phase of growth did not cause a significant change in Caco-2 cell TEER when compared to the control medium (Figure 3.3 A & B). However, a pair-wise comparison indicated that when taken from the transition phase, at 10 and 12 hours, AGR1485 decreased Caco-2 cell TEER by 19 and 22% respectively when compared to AGR1485 taken from the late stationary phase ($P<0.05$) (Figure 3.3 B). AGR1487 (raw data in Appendix, Tables 2.2 and 2.4), on the other hand, taken from all of the different growth phases decreased Caco-2 cell TEER ($P<0.05$) compared to the control medium from 8 hours onwards (Figure 3.3 C & D). All of the growth phases of AGR1487 caused a significant decrease in TEER as compared to control and pair-wise probability-value comparisons between the phases of AGR1487 showed no significant differences between any of the growth phases.

3.4.3 Effect of UV on bacterial growth on agar plates

Results of experiments exposing bacteria plated on agar plates to UV showed that UV-radiation (254 nm) was an effective method for inactivating both strains AGR1485 and AGR1487. Experiments conducted with both strains plated at different dilutions of bacteria in medium (1:1000, 1:10,000) on agar plates, showed that for AGR1485, after 3 minutes of UV-exposure, the dilution of 1:1000 dilution resulted in no colonies.

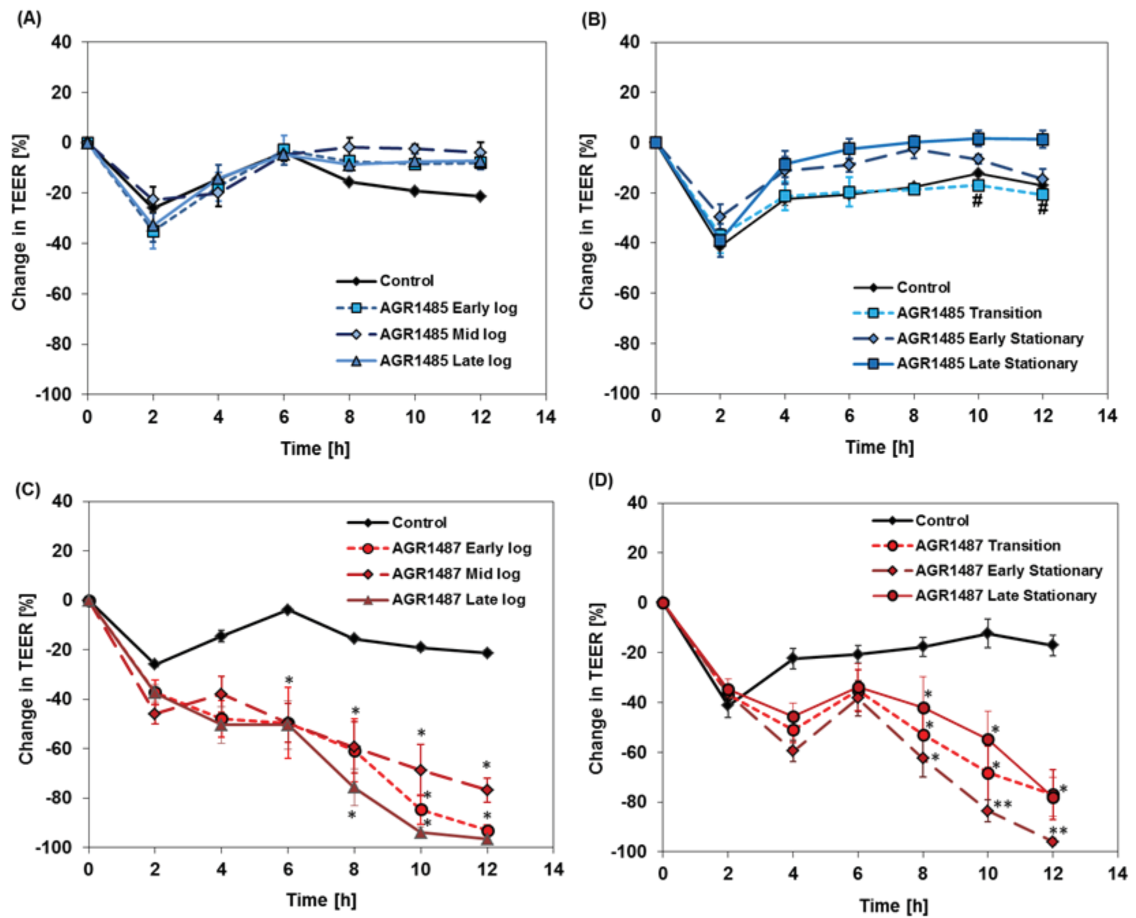

Figure 3.3 Effect of bacterial growth phase on trans-epithelial electrical resistance over time. The change in transepithelial electrical resistance (TEER) across Caco-2 cell monolayers is the percentage change compared to the initial TEER for each monolayer. The values plotted are the mean values for four monolayers per treatment for three separate experiments and the error bars show the standard error of means (SEM) (total n=12 per treatment). (A) Caco-2 cells exposed to AGR1485 taken from the early log, mid log and late log phases of growth. (B) Caco-2 cells exposed to AGR1485 taken from the transition between log and stationary phase, early stationary and late stationary phases of growth. (C) Caco-2 cells exposed to AGR1487 taken from the early log, mid log and late log phases of growth. (D) Caco-2 cells exposed to AGR1487 taken from the transition between log and stationary phase, early stationary and late stationary phases of growth. $^{\#}P < 0.05$ compared to late stationary, $^{*}P<0.05$ and $^{**}P < 0.001$ compared to the control medium.

However, for AGR1487, even after 4 minutes of UV-exposure, a few colonies were recorded. For both strains plated at a dilution of 1:10,000, a minimum of 4 minutes of UV-exposure was required to inactivate all bacteria (no bacterial colonies on UV-exposed side of the plate) (Table 3.1).

3.4.4 Effect of UV on bacterial growth in M199

To achieve complete inactivation of AGR1485 (resuspended to an optical density of 0.9 OD_{600} in a 2 mL volume), a UV exposure time of 15 minutes was needed, without ice or on ice (Table 3.2). For AGR1487, whether on ice or without ice, 15 minutes and 20 minutes of UV-exposure still resulted in a few colonies. An exposure time of 30 minutes, without ice or on ice, resulted in no colonies for AGR1487.

3.4.5 Effect of UV-killed bacteria on transepithelial electrical resistance

The results showed that treating AGR1485 for 30 minutes without ice or on ice, did not alter its effect on Caco-2 cell TEER as compared to 15 minutes and so both the strains could be treated to the same length of time of UV-exposure (Figure 3.4). Therefore, a UV-exposure time of 30 minutes on ice was determined to be an effective exposure time for inactivation of both AGR1485 and AGR1487. Live and UV-killed bacteria had similar effects on TEER across the Caco-2 cell monolayers.

AGR1485 (raw data in Appendix, Table 2.5), both live and UV-killed, did not cause a significant change in Caco-2 cell TEER as compared to the control medium at any time point (Figure 3.4 A).

Table 3.1 Bacterial viability after UV exposure on agar plates for varying periods of time. The number of colonies reported is the average of 3 agar plates per treatment for three sets of experiments (total n=9 per treatment). The dilution factor for each strain is shown in brackets.

Time period	*AGR1485 (1:1,000)*	*AGR1485 (1:10,000)*	*AGR1487 (1:1,000)*	*AGR1487 (1:10,000)*
2 minutes	>100	20	>100	>100
3 minutes	0	0	>50	>50
4 minutes	0	0	7	0

Table 3.2 Bacterial viability after UV exposure in M199 for varying periods of time. The number of colonies reported is the average of 3 agar plates per treatment for three sets of experiments (total n=9 per treatment).

Time period	*AGR1485*	*AGR1485 on ice*	*AGR1487*	*AGR1487 on ice*
15 minutes	0	0	4	41
20 minutes	0	0	0	2
30 minutes	0	0	0	0

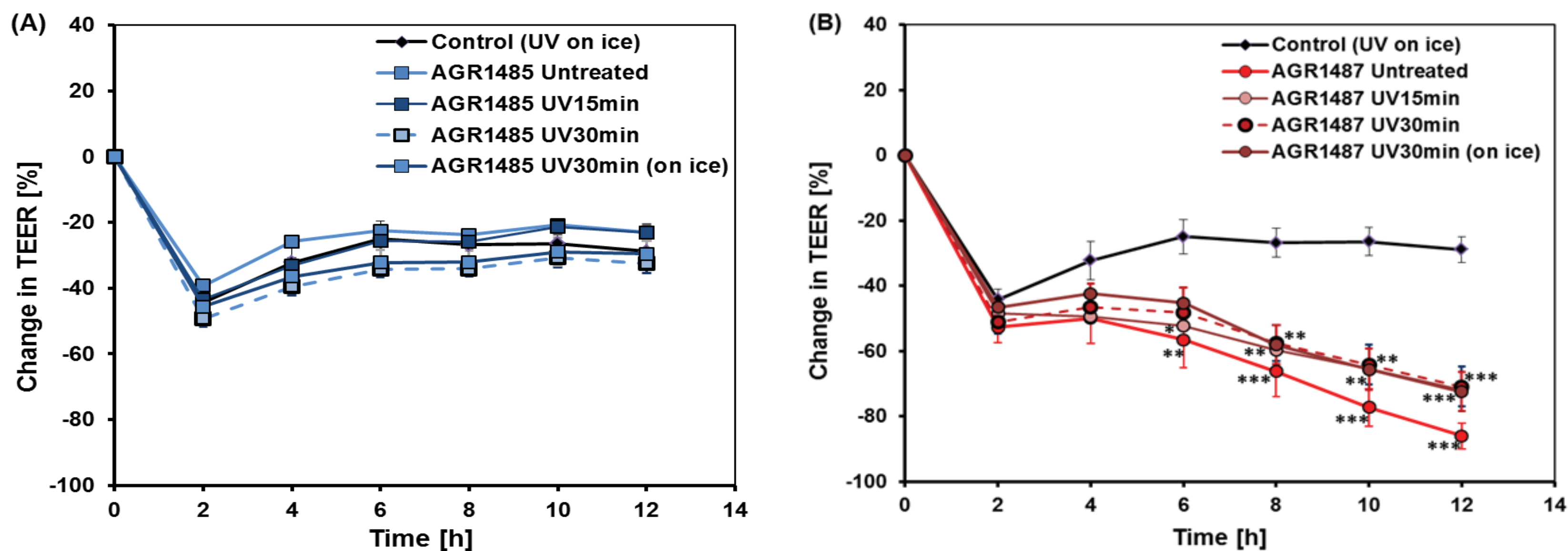

Figure 3.4 Effect of live versus UV-killed bacteria (AGR1485 and AGR1487) on Caco-2 cell monolayers over time. Change in transepithelial electrical resistance (TEER) across differentiated Caco-2 cell monolayers (days 16 to 20 post seeding) over time. The two strains AGR1485 and AGR1487, taken from the late stationary phase of growth (18 hours), live or UV-killed (15 minutes, 30 minutes and 30 minutes on ice) were used as treatments. UV-treated (30 minutes on ice) media (without bacteria) were used as control. The values plotted are the mean values for four monolayers per treatment for three sets of experiments and the error bars show the SEM (total n=12 per treatment), *P<0.05, **P<0.01 and ***P<0.001, compared to control.

On the other hand, both live and UV-killed AGR1487 (raw data in Appendix, Table 2.6) significantly decreased Caco-2 cell TEER ($P<0.01$ and $P<0.05$ respectively) compared to the control medium by six hours (Figure 3.4 B). At 12 hours, live AGR1487 significantly decreased Caco-2 cell TEER by 58% ($P<0.001$) as compared to the control, while UV-killed AGR1487 significantly decreased TEER by 44% ($P<0.001$). Pair-wise comparison showed no significant difference ($P>0.05$) between the effects of live AGR1487 and UV-killed AGR1487.

3.4.6 Effect of UV-killed bacteria on paracellular permeability

The results of the ^{3}H-mannitol assay showed that by 12 hours (raw data in Appendix, Table 2.9), there was no significant difference ($P>0.05$) in the amount of mannitol that had crossed over from the apical to the basal side of Caco-2 cell monolayers treated with non-UV-treated and UV-treated control medium (M199 with 1% NEAA). By 12 hours, live AGR1485 caused 6.6% mannitol to pass through to the basal well while UV-killed AGR1485 caused 4.7% mannitol to pass through to the basal well, both not significantly different ($P>0.05$) from the controls (Figure 3.5). On the other hand, by 12 hours, live AGR1487 caused 17.3% of mannitol to pass through to the basal well, which was significantly greater than the control ($P<0.001$) and AGR1485. However, UV-killed AGR1487 caused 7.3% mannitol to pass through to the basal well, which was not significantly different ($P>0.05$) from the control or AGR1485. Pair-wise comparison of probability-values showed that there was significant difference ($P<0.05$) between the effects of live AGR1487 and UV-killed AGR1487 on percentage mannitol passage at 10 and 12 hours.

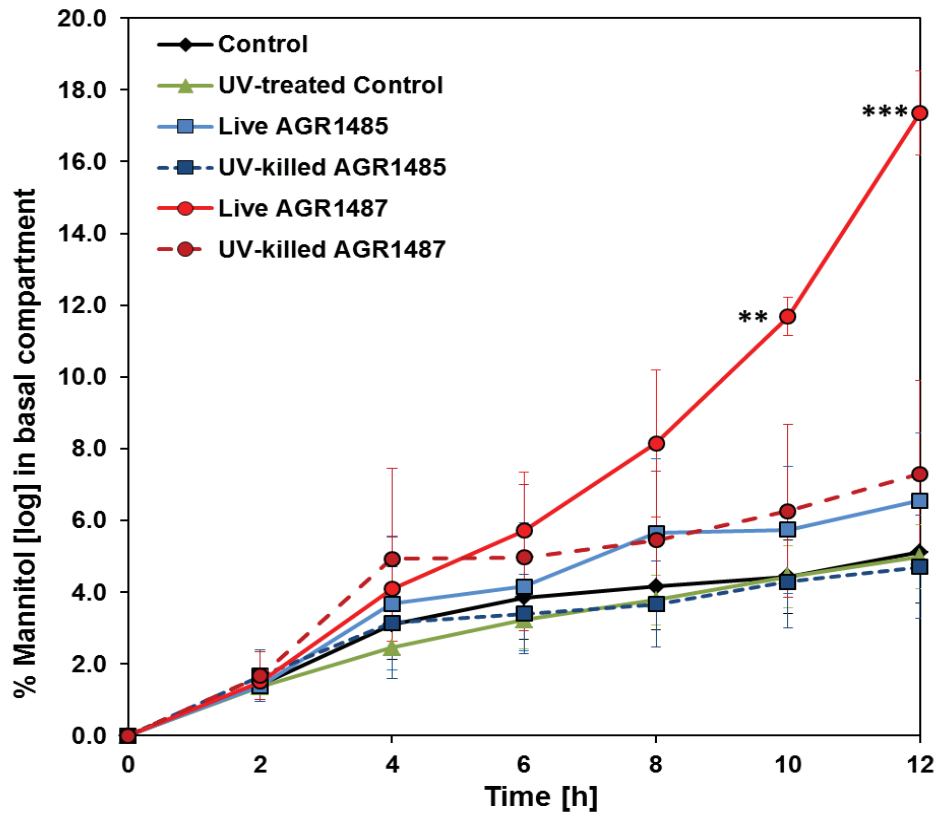

Figure 3.5 Effect of bacteria (live and UV-killed) on permeability of Caco-2 cell monolayers. Permeability of ^{3}H-mannitol across Caco-2 monolayers co-cultured with AGR1485 (live and UV-killed), AGR1487 (live and UV-killed) and non-UV-treated and UV-treated controls (no bacteria). The graph shows the mean (+/- SEM) % of ^{3}H-mannitol that crossed the Caco-2 monolayer from the cell culture Transwell to the basal well (n=12, 3 assays, 4 Transwells per treatment per assay), *P<0.05, **P<0.01 and ***P<0.001, compared to the control, based on the comparison of log (% mannitol) values.

3.4.8 Effect of metabolites produced by bacterial-Caco-2 interactions on transepithelial electrical resistance

Supernatants collected from 12 hour co-cultures of four treatment groups for each strain (treatments 1, 2, 3 and 4 designed to test the effect of metabolites resulting from bacteria, Caco-2 cells or bacterial-Caco-2 cell interactions) were applied to Caco-2 cell monolayers and the effects on TEER observed and compared with that of live bacteria.

At 12 hours, live AGR1485 (Figure 3.6A) did not cause any significant change ($P>0.05$) in Caco-2 cell TEER as compared to the control, and none of the four AGR1485 treatments caused any significant change ($P>0.05$) in Caco-2 cell TEER as compared to the control (raw data in Appendix, Tables 2.7). Live AGR1487 at 12 hours (Figure 3.6B) caused a significant decrease in Caco-2 cell TEER ($P<0.001$) as compared to the control (raw data in Appendix, Tables 2.8). In contrast, none of the four treatment groups of AGR1487 supernatant/metabolites caused any significant difference ($P>0.05$) in Caco-2 cell TEER as compared to the control.

3.5. DISCUSSION

The main results from the intestinal barrier integrity assays showed that both live and UV-inactivated AGR1487 decreased TEER across Caco-2 cell monolayers. The secreted metabolites produced by the bacteria, Caco-2 cells or as a result of bacteria-Caco-2 interactions did not affect Caco-2 cell TEER. However, only live AGR1487, and not UV-inactivated AGR1487, increased the rate of passage of mannitol between the Caco-2 cells.

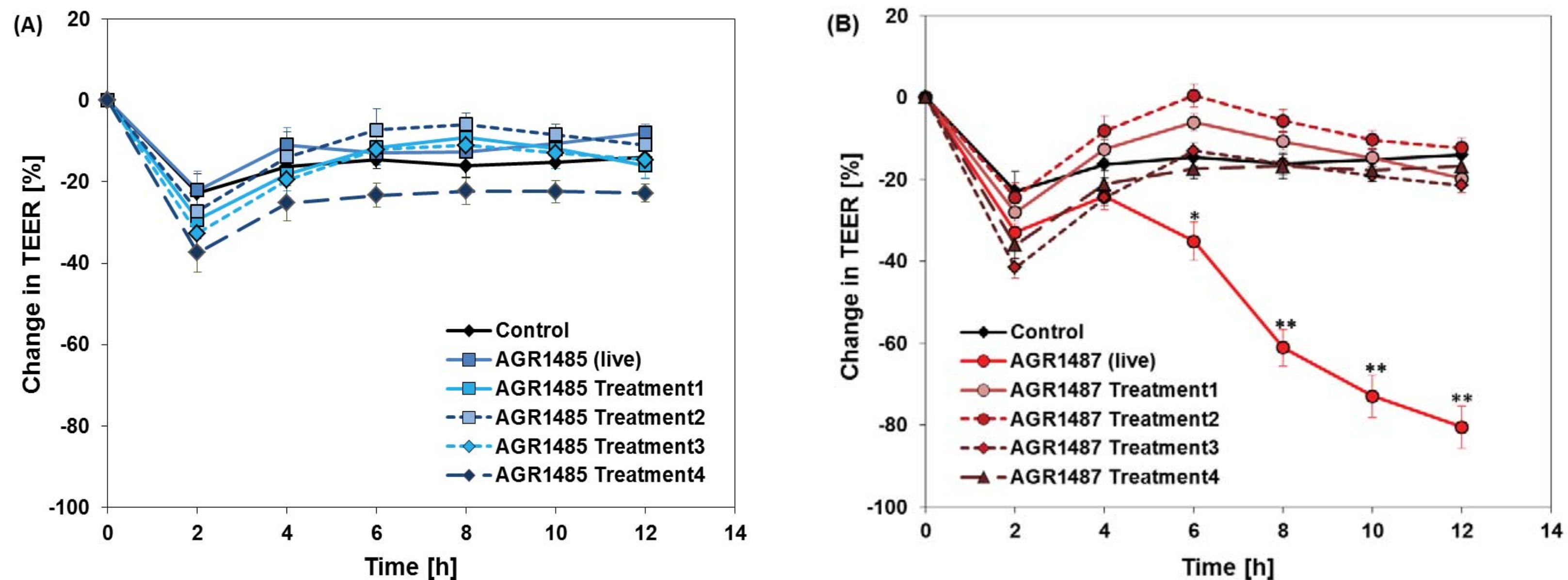

Figure 3.6 Effect of metabolites on trans-epithelial electrical resistance across Caco-2 cell monolayers. Change in transepithelial electrical resistance (TEER) across differentiated Caco-2 cell monolayers over time after treatment with live bacteria (0.9 OD_{600}) or supernatant (SN) collected from previously 12 hour co-cultured treatment groups of Caco-2 cells with (A) AGR1485 and (B) AGR1487. Supernatant collected from 12 hour treatment groups were filter-sterilised and applied to Caco-2 cell monolayers to study effects on TEER. Caco-2 cell monolayers were treated with Caco-2 cell monolayer SN + bacteria (Treatment 1); Homogenised Caco-2 cells + bacteria (Treatment 2); Caco-2 cell monolayer + bacteria (Treatment 3); bacteria alone (Treatment 4). *$P<0.05$ and ** $P < 0.001$ compared to control medium.

The seemingly conflicting results are likely related to the fact that the TEER and mannitol flux assays measure different characteristics of the epithelial barrier. It is possible that secreted molecules produced by AGR1487 while not involved in influencing TEER, may be involved in host-microbe interactions that influence paracellular permeability of the Caco-2 cells to non-ionic solutes such as mannitol. Collectively, these results imply that distinct mechanisms involving both cell surface-associated and/or secreted molecules might trigger different signaling pathways that result in the final response of Caco-2 cells to AGR1487. In terms of the effect on Caco-2 cell TEER, the data also showed that the effect of live AGR1487 was independent of the growth phase of AGR1487, and molecules secreted by AGR1487 alone and in combination with Caco-2 cells, did not cause the same detrimental effect on Caco-2 cell TEER. However, it is possible that the metabolites secreted were not present in sufficient quantities to affect Caco-2 cell TEER. In addition, the stability of the secreted molecules over time was also unknown and the experimental design did not allow for these effects to be detected. Keeping these limitations in mind, the results suggest that a direct bacteria-host interaction is required to affect TEER of the Caco-2 cells. It is possible that the bacterium's cell surface structural components may act as ligands for receptors present on the Caco-2 cells that trigger signaling pathways that influence TEER of the Caco-2 cells. The interaction of these bacterial ligands (bacterial cell surface proteins, TA, lipids and PG) with the epithelial toll-like receptors (TLR) may be required to elicit a host response. The observation that AGR1485, both live and UV-killed, did not affect the TEER across the Caco-2 cells, implies that the bacterial cell surface components required for these interactions may be absent or poorly expressed in this strain.

Literature reports provide supporting evidence for an interaction between bacterium's cell surface structural components and the TLRs. A study reported that both live and UV-inactivated LGG reduced flagellin-induced IL-8 production in Caco-2 cells, implying interaction of bacterial cell surface components with TLRs [300]. TLRs for various microbial components such as lipopolysaccharide (LPS), lipoteichoic acids (LTA) and flagellin play a role in signal transduction that result in the regulation of production of inflammatory mediators and several of the TLRs appear to act through common pathways [304]. Signaling pathways involve interactions between transmembrane proteins and several signaling proteins such as protein kinase C (PKC), myosin light chain kinase (MLCK) and mitogen-activated protein kinases (MAPK) and Rho GTPases. PKC expressed by IEC has been reported to be activated by stimulation of the TLR2 pathway (by bacterial ligands such as PG) leading to an increase in TEER and redistribution of TJ (TJ) protein zona occludens-1 (ZO-1) [305]. It would be important to determine if the cell surface components such as proteins, glycoproteins or LTA of AGR1487 may act as ligands for the TLR of Caco-2 cells and trigger a response that affects the barrier integrity of the Caco-2 cell monolayers.

In contrast, the observation that only live AGR1487 increased the mannitol flux across the Caco-2 cell monolayers indicates that this effect on mannitol flux may be caused by a metabolites produced by the bacterium, not the cell surface structure. A study reported the effects of colonic bacterial metabolites on Caco-2 cell paracellular permeability assessed by TEER and mannitol flux [306]. In this study, phenol, ammonia and bile acids increased permeability by reducing TEER and increasing mannitol flux whereas primary bile acids did not. The expression of occludin was

increased by bile acids but remained unaffected by phenol. The authors suggested that the increased permeability by phenol and ammonia was not related to the expression of occludin, one of the main TJ proteins responsible for TJ structure.

Several studies suggest that the paracellular channels, that influence ionic permeability (e.g. TEER assay) and flux of uncharged solutes (e.g. mannitol assay), discriminate on the basis of charge and not the size of the molecules [307, 308]. Epithelial TJ control the flow of ions and certain metabolites between IEC. Madin-Darby canine kidney (MDCK) cells, like Caco-2 cells, have been shown to differentiate into columnar epithelium and have been assessed as a tool for membrane permeability screening [309]. The TEER of MDCK cells has been shown to be altered by the differential expression of isoforms of TJ claudin; stable expression of claudin-2 is associated with decreased MDCK cell TEER while overexpression of claudin-4 is associated with increased MDCK cell TEER [307, 310]. Phosphorylation of the TJ protein ZO-1 has also been shown to decrease TEER in MDCK cells [311]. A study with Caco-2 cells expressing constitutively active MLCK mutant showed that inducing MLCK led to MLC phosphorylation which in turn decreased Caco-2 cell TEER and increased TJ permeability [312]. On the other hand, mannitol flux has been linked to the TJ occludin [313]. Expression of occludin in MDCK cells transfected with C-terminally truncated occludin resulted in increased MDCK cell TEER and paracellular flux of small molecular weight tracers [313]. In contrast, another study with MDCK cells reported that overexpression of TJ protein claudin-4 increased MDCK cell TEER without affecting mannitol flux suggesting that the two processes are distinct molecular mechanisms [307].

It is evident that paracellular permeability to ionic solutes (TEER) and non-ionic solutes (mannitol) is regulated by independent mechanisms, and different TJ proteins play a crucial role in these mechanisms. The seemingly conflicting results of UV-inactivated AGR1487 TEER and mannitol flux across the Caco-2 monolayers are likely due to UV-inactivation of production of metabolites that may trigger the cascade of signaling pathways that influence mannitol flux. However, the cell surface components of AGR1487, that may act as mediators of interaction with the TJ of the Caco-2 cells may remain unaffected by UV-treatment, and as such UV-inactivated AGR1487 caused a decrease in Caco-2 cell TEER similar to live AGR1487. The changes in TJ permeability as measured by the TEER and mannitol flux assays might also be related to the distribution of the TJ and AJ proteins and their interactions with the actin cytoskeleton that are crucial for the integrity of the epithelial barrier [314]. It has been reported that the probiotic *Escherichia coli* Nissle 1917 (EcN) restored enteropathogenic *E. coli*-induced barrier disruption in intestinal cells and this effect was associated with enhanced expression and redistribution of ZO-2 towards the cell boundaries [55]. There is also considerable evidence that indicates that transmembrane TJ proteins such as occludin and AJ E-cadherin and β-catenin interact with the actin cytoskeleton through plaque proteins such as ZO-1 and the distribution, alteration and assembly of these proteins affect barrier integrity [315, 316]. All these studies collectively imply that different TJ proteins interacting with the cellular cytoskeleton are involved in influencing TJ integrity and barrier functions.

Of particular relevance to the results presented here, a recent study reported that AGR1487 increases the expression levels of genes and the abundance of proteins that

have been implicated in the reduction of integrity of TJ between Caco-2 cells [258]. AGR1487 reportedly increased the expression levels of microtubule associated proteins [53], which have been linked to TJ disassembly [258, 317]. ZO-2 had an uneven distribution and was found to be localised in clusters on the Caco-2 cells treated with AGR1487, compared to uniform ZO-2 distribution for the control or AGR1485 treated Caco-2 cells [258]. It would be informative to determine if UV-inactivated AGR1487 affects the redistribution of ZO-2 of the Caco-2 cells and expression levels of tubulin genes similar to live AGR1487. This would imply a direct cell surface interaction of this bacterium with the Caco-2 cells.

Overall, the results of this study suggest that both AGR1487 cell surface components and/or metabolites can interact with Caco-2 cells through different pathways that control various characteristics and functions of the intestinal barrier integrity. The bacterial CW is a dynamic entity and lactobacilli display considerable variation in their cell surface architecture and properties. Considering the complexity of host-lactobacilli interactions involving host-cell signalling and regulation pathways, it is unlikely that single effector molecules regulate the IEC response to AGR1487. However, an effort to identify such bacterial components that modulate host response will strengthen and contribute to the understanding of the strain-specific characteristics of AGR1487.

4. CHAPTER 4: FRACTIONATION OF CRUDE BACTERIAL CELL WALL FROM AGR1485 AND AGR1487 TO DETERMINE EFFECTS OF FRACTIONS ON THE INTESTINAL BARRIER INTEGRITY.

4.1. INTRODUCTION

The cell wall (CW) of lactobacilli is a dynamic entity and plays an essential role in host-microbe communications as well as in adaptation to environmental and host-derived factors. Several studies implicate bacterial cell surface components, either individually or collectively, as mediators in host-microbe interactions [14, 15]. Lactobacilli show considerable variation in cell surface architecture and are known to modify their surface properties in response to the host environment. Endogenous stressors presented by the host, such as bile, cholesterol, acid and osmotic stress, have been shown to influence and alter lactobacilli CW composition, affecting peptidoglycan (PG) biosynthesis, expression of exopolysaccharides (EPS) and cell surface proteins, and substituting alanine-free esters with D-alanine residues in lipoteichoic acid (LTA) [1, 11, 143, 145].

Adherence is a major factor in the colonising potential of commensal bacteria. Adherence characteristics of lactobacilli to the host epithelium and mucosa are influenced by the bacterial CW components and show pronounced variation among different strains [5, 79]. Genomics-based approaches have revealed several bacterial cell-surface-associated proteins that are involved in adherence of the bacteria to host mucus and intestinal cells [14]. Cell surface proteins of lactobacilli are either anchored (covalently or non-covalently) to the CW or secreted into the external milieu, where they may re-associate with the CW through electrostatic interactions [119]. The CW-associated proteins have a wide repertoire of functions that range from adhesion, transport, signal transduction, cell envelope metabolism, and antibiotic resistance to host-microbe interactions [9, 80]. Bacterial surface-associated proteins interact with the

epithelial and immune cells of the host and trigger downstream responses in the host mucosa, inducing different signalling cascades that trigger changes in gene expression that result in physiological changes in host cells. Recognition and response of the host to microbes have been shown to involve bacterial surface components known as microbe-associated molecular patterns (MAMPs) that are recognised by the pattern recognition receptors (PRR) expressed by the host cells [9, 318].

Several effector molecules of lactobacilli that are proposed to be directly involved in host-microbe interactions have been identified [80, 318]. Recent studies have shown that a single gene mutation in *Lactobacillus acidophilus* NCFM results in the modification of the cell surface of this strain and confers improved anti-inflammatory properties by down-regulating the expression of pro-inflammatory cytokines such as interleukin-12 (IL-12) and tumour necrosis factor α (TNF-α) and enhancing the expression of anti-inflammatory cytokine IL-10 in dendritic cells (DC) when tested in mice [69]. Similar observations of enhanced anti-inflammatory capacity have been reported for a *Lactobacillus plantarum* mutant with modified teichoic acids [225] and a *Lactobacillus rhamnosus* GG *dlt*D mutant, that lacks D-alanine residues in its LTA [201]. Surface layer protein (Slp) extracts from *Lactobacillus helveticus* have been shown to inhibit the adhesion of enterohaemorrhagic *Escherichia coli* to epithelial cells (Hep-2 and T84 cell lines) thus providing protection against pathogens [319]. Some lactobacilli carry several *slp* genes and may express alternative S-layer protein genes that cause variation to the S-layer makeup and are most likely used in adaptation to various environmental stress situations [320, 321]. Studies with *Lactobacillus gallinarum* strains report the presence of two *slp genes*, although each isolated strain

expressed only one of the two genes. The expressed gene show a high sequence variation that resulted in different strains of the same species having different adherence targets for the encoded Slps [321]. The high degree of diversity of cell surface components found in lactobacilli not only imparts strain-specific characteristics to these bacteria but adds to the challenge of identifying effector molecules that may influence host-microbe interactions.

Findings reported in Chapters 2 and 3 and by Anderson *et al* [258] showed that the differential effects of the two strains of *Lactobacillus fermentum*, AGR1485 and AGR1487 on Caco-2 cell barrier integrity may be related to differences in their cell surface-associated components. Both live and UV-inactivated AGR1487 (but not culture supernatants) decreased the trans-epithelial electrical resistance (TEER) of Caco-2 cell monolayers, strengthening the assumption that bacterial cell surface components and not secreted molecules of AGR1487 affect the TEER of the Caco-2 cell monolayers.

4.2. AIM AND HYPOTHESIS

The aim of the research reported in this chapter was to test the hypothesis that the different effects that AGR1485 and AGR1487 have on Caco-2 barrier integrity are mediated by the interaction of bacterial CW-associated proteinaceous components with the epithelial layer. To test this hypothesis, methods were developed and optimised to obtain crude bacterial CW fractions from whole bacterial cell lysates, followed by sub-fractionation using size-exclusion high performance liquid chromatography (SE-HPLC), collecting fractions based on protein peaks and testing the effects of the

resulting fractions on TEER across Caco-2 cell monolayers. The assumption was that the components in an active fraction of AGR1487 CW can affect Caco-2 cell TEER similar to live AGR1487 when applied at the concentrations in which they are normally present in the live bacterial cells. To test this assumption, TEER assays were conducted with the bacterial CW fractions applied at different concentrations to determine their effects on Caco-2 cell barrier integrity.

4.3. MATERIALS AND METHODS

4.3.1. Bacterial strains

Two strains of *L. fermentum*, AGR1485 and AGR1487, were grown from frozen stocks and cultured under standard conditions (refer to Chapter 3, Section 3.4.2). Fresh frozen stocks were used for the TEER experiments described in Section 4.3.6.

4.3.2. Preparation of bacterial whole cell lysate and separation of crude cell wall from cell lysate supernatant

Two separate methods were used for the extraction of bacterial CW-associated components from both the strains AGR1485 and AGR1487. The first method used the chaotropic salt guanidine hydrochloride (GHCl). This method is effective for the extraction of non-covalently attached CW proteins (such as Slps and proteins exhibiting CW binding domains) and LTA (a fraction of which may occur free in the bacterial CW), from lactobacilli without causing cell lysis or disrupting covalently bound secondary CW polymers like carbohydrates and wall teichoic acids (WTA) [322]. Sonication followed by ultracentrifugation was used as the second method to obtain

bacterial whole cell lysate and for separation of bacterial CW-associated components from membrane-associated components.

4.3.2.1. Bacterial cell wall preparation using guanidine hydrochloride

The first method of extraction of bacterial CW components involved the removal of non-covalently bound components of the bacterial CW from both strains with the use of 4 M GHCl (pH 7.0). Both strains of bacteria (AGR1485 and AGR1487) were grown on MRS agar plates and subcultured in MRS broth as described in Chapter 2, Section 2.3.1.1. Bacteria were harvested by centrifugation at 8,000x*g* for 5 minutes at 4°C to obtain cell pellets. The bacterial cell pellets were weighed, a calculated amount of 4 M GHCl (1 mL of GHCl/10 mg of wet weight bacterial pellet) was added and mixed well. The mixture was incubated at 37°C for one hour. Incubation was followed by centrifugation at 18,000x*g* for 15 minutes at 4°C to separate solubilised surface components from the bacterial cells (Sigma3-18K centrifuge). A study with *L. rhamnosus* GG (LGG) reported that LGG filtrate with factors having molecular weights less than 5 kDa did not activate certain pathways [209]. In addition, surface-associated proteins of lactobacilli have reported to have molecular weights ranging from 15 kDa to 200 kDa, many of which are secreted into the external milieu [50, 323]. Keeping these molecular weights in mind, the supernatant was concentrated using a Vivaspin 2 concentrator, with a 10 kDa molecular weight cut-off (MWCO) (GE Healthcare). The supernatant was concentrated from an initial volume of 15 to 20 mL to a final volume of approximately 1.5 to 2 mL, and dialysed against ice-cold water. Dialysis was carried out twice for one hour each time, using 250 mL autoclaved milliQ water, with the dialysis apparatus placed in an ice bucket and with the water being stirred constantly. Dialysis

was done using Slide-A-Lyzer dialysis cassettes (10 kDa MWCO, Thermo Scientific). The sample in the dialysis cassette was then further dialysed against ice-cold M199 (250 mL). The final volume recovered from the dialysis cassette was approximately 2 mL, adjusted to 2 mL, an aliquot of which (20 μL) was then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to visualise the extracted proteins and compare the protein band patterns and molecular weights against a standard protein marker (SeeBlue Plus2 prestained marker, Invitrogen, Life Technologies).

4.3.2.2. Bacterial cell wall preparation using sonication and ultracentrifugation

The second method that was applied to extract bacterial CW components from AGR1485 and AGR1487 involved sonication of bacterial cells followed by centrifugation to remove intact bacterial cells from the sonicated mixture. The supernatant (SN) from the centrifuged mixture was then subjected to ultracentrifugation. The pellet from the ultracentrifugation was resuspended in M199 and used as bacterial crude CW extract and the SN was subjected to further ultracentrifugation (at higher speed and longer time) to separate traces of CW components that form a second pellet from any cytoplasmic or membrane components that remain in the SN. The SN from the second ultracentrifugation step was used as cell lysate SN. The CW extract and the cell lysate SN were then tested on Caco-2 cell monolayers to determine their effects on TEER. Bacteria taken from the late stationary phase (2.5 OD_{600}) (20 mL) were centrifuged at 8,000x*g* for 5 minutes to obtain cell pellets.

The bacterial cell pellets were resuspended in 2.5 mL of M199 (no supplements added) in a polypropylene tube and lysed using an ultrasonicator (Vibracell, Sonics & Materials Inc., Danbury, Connecticut, USA) fitted with a tapered microtip probe with a 3 mm tip diameter. Method optimisation was done by subjecting bacterial cultures to different numbers of bursts of sonication followed by checking samples under the microscope to determine the degree of cell lysis. For both the strains, samples were sonicated 10 times using 20 seconds bursts (Treatment 1) and 10 seconds bursts (Treatment 2), respectively, with intermittent two minute cooling periods between sonications. Cooling was achieved by keeping the polypropylene tube in an ice water bath during sonication and cooling periods.

The sonicated samples were then centrifuged (8,000x*g* for 15 minutes at 4°C) to remove residual intact cells in the lysates. The supernatant was centrifuged at 45,000x*g* for 20 minutes at 4°C (Sorvall RC 100 Ultracentrifuge) to pellet the CW. The CW pellet was resuspended in 1 mL of M199 (no supplements added). The supernatant was further centrifuged for 4 hours at 100,000x*g* at 4°C (Sorvall RC 100 Ultracentrifuge) to separate cytoplasmic and membrane components (forming the SN) from any remaining traces of CW components (forming the pellet). The second pellet was discarded and only the SN from the second ultracentrifugation step was collected and used as cell lysate SN to compare the effects of cytoplasmic and membrane components (SN) with the CW components on Caco-2 cell TEER. The volumes of the CW extract and the SN were adjusted to 2 mL with M199 and pH measured using a Beckman 340 pH Meter (Biolab Scientific Ltd). Aliquots of CW extract and the cell lysate SN were then analysed using SDS-PAGE.

4.3.2.3. SDS-polyacrylamide gel electrophoresis

SDS-PAGE of the bacterial CW preparations was carried out to determine the molecular weight distribution of proteins obtained by GHCl treatment and sonication. The samples were adjusted to 2 mL with M199 media and an aliquot of 20 µL of each sample was loaded onto a NuPAGE tris-acetate mini gel and run at 200 V for approximately 50 minutes in NuPAGE MES SDS running buffer (1X). A 5 µL aliquot of SeeBlue Plus2 Prestained Standard (1X) was used as a marker. The gel was stained in SimplyBlue Safe Stain for 1 hour followed by an overnight destaining procedure in milliQ water on a shaker. The gel was then vacuum-sealed in a plastic bag, and photographed using a scanner (RICOH, Aficio MP C5502A, Tustin, CA, USA).

4.3.2.4. Protein concentration determination of crude bacterial cell wall and cell lysate supernatant

The protein concentrations of the CW extract and cell lysate SN preparations were determined using Bradford assays [324], with bovine serum albumin (BSA) as the standard. Spectrophotometric measurements were performed using a Helios UV Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). BSA standards were made using BSA stock solution (2 mg/mL) diluted in M199 (no supplements) to yield standards with final concentrations ranging from 0 (Blank, M199) to 0.3 mg/mL. The range of absorbance was 0.2 to 0.9, and samples were diluted to ensure that the absorbance readings were within this range. Samples, including standards, were prepared by adding 100 µL of the sample to 1 mL of Bradford reagent. Disposable cuvettes with caps were used to measure absorbance at 595 nm. Absorbance for all

standards and samples was measured in duplicate. A linear standard curve was used to determine the protein concentrations (mg/mL) of the samples using the mean absorbance and corrected for the dilution factor. Based on the Bradford assays, the protein concentrations of the CW from both strains were standardised by adjusting volumes so that the amount of protein and the added volumes from the CW used as treatment could be kept constant for both the strains for all TEER assays. The amount of protein from the cell lysate SN from both the strains added to the Caco-2 cells was also kept constant for both the strains for all sets of experiments.

4.3.3. Fractionation of the crude bacterial cell wall

The CW extracts (resuspended in M199) from strains AGR1485 and AGR1487, (described in Section 4.3.2.2) were further fractionated using SE-HPLC to separate out the proteinaceous components present in the CW extract. SE-HPLC is reported to be the most suitable method for separation of macromolecules based on their molecular weights [325]. This analytical technique may be used to provide the "absolute" molecular weights and characterisation of polymers and biopolymers, in a non-destructive process from which the recovered samples can be used for subsequent studies [326].

4.3.3.1. Size-exclusion high performance liquid chromatography

For both bacterial CW extracts, SE-HPLC was carried out using a Biologic Duo Flow HPLC system (Bio-Rad, USA) with detection at 280 nm and 214 nm, using a Biologic Quad Tec UV-Vis detector and an automated fraction collector. The column used was a GE Healthcare Tricorn 10/600 high performance column packed with

Superdex 200 matrix (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The Superdex 200 column (dextran-agarose gel, bed height 600 mm, bed volume of approximately 45 mL and an internal diameter of 10 mm) has a separation range of 10 to 600 kDa for globular proteins. Standard proteins that result in peaks of known molecular weights, comprising of cytochrome c (12.4 kDa, Sigma-Aldrich, New Zealand), BSA (66 kDa, Sigma-Aldrich, New Zealand), and β-amylase (200 kDa, Sigma-Aldrich, New Zealand) were run and the elution times of the resulting peaks were then used to generate a standard curve. The equation from the standard curve was used to determine the approximate molecular weight range of the protein peaks that resulted from SE-HPLC of the AGR1485 and AGR1487 CW extracts. Each standard protein (1 mg) dissolved in 150 µL of M199. The three standard proteins were combined, filter sterilised using a 0.22 µm filter (Ultrafree Centrifugal filters, Durapore, Millipore) and applied to the Superdex 200 column using a 1 mL loop. The column was eluted with M199 (no supplements added) at a flow rate of 0.6 mL/min. A standard curve of elution time versus log molecular weight was plotted and used to determine the molecular weights of peaks resulting from SE-HPLC of the AGR1485 and AGR1487 CW extracts. Elution time was used as a parameter to calculate the corresponding molecular weights of the peaks.

The bacterial CW extracts were obtained from sonication and ultracentrifugation of bacterial culture (described in Section 4.3.2). Each strain was grown to late stationary phase (2.5 OD_{600}) and 40 mL of each bacterial culture was used to yield a bacterial CW pellet, which was resuspended in 500 µL of M199 and subjected to SE-HPLC fractionation. As sonication and ultracentrifugation were carried out in a non-sterile

environment, the CW extract from each strain was filtered sterilised using a 0.22 μm filter (Ultrafree Centrifugal filters, Durapore, Millipore) and applied to the Superdex 200 column. The Durapore microporous filter membranes display the lowest protein binding of any of the filter membranes and therefore a minimum loss of proteins due to filtration was assumed. Fractions were collected over the entire range of the elution volume, irrespective of a peak in absorbance, to ensure collection of non-protein (non-absorbing) compounds such as lipids and glycans. For both strains AGR1485 and AGR1487, the first fraction (Fraction 1) collected was determined by elution time rather than peak size, because this fraction was likely to contain non-proteinaceous bacterial CW components. The other three fractions were collected based on the generated peaks. The fractions collected were filter sterilised using 0.22 μm syringe filters (Millipore), concentrated using Vivaspin 20 concentrators (10 kDa MWCO for fractions 1 through 3, and 3 kDa MWCO for fraction 4) to a final volume of 2.5 to 3 mL for each fraction. The pH of each concentrated fraction was measured using a Beckman 340 pH Meter. The concentrated fractions collected from SE-HPLC, were filter sterilised using 0.22 μm syringe filters before being applied as treatments in TEER assays.

4.3.3.2. Protein concentration determination of bacterial cell wall fractions

Protein concentrations of the bacterial CW fractions resulting from SE-HPLC were determined by using the Bradford assay [324] as previously described in Section 4.3.4. To test the assumption that CW proteins present in normal concentrations can affect Caco-2 cell TEER, the CW protein concentration of live AGR1487 was determined. Live AGR1487 re-suspended in M199 containing 1% NEAA to an optical density of 0.9 OD_{600} was applied (500 μL/Transwell) to Caco-2 cell monolayers and

incubated for 12 hours at 37ºC in 5% CO_2. After 12 hours, bacteria and Caco-2 cells were harvested and subjected to sonication as described in Section 4.3.2.2. The protein concentration of the resulting CW extract of AGR1487 was determined using the Bradford assay as described in Section 4.3.4. The protein concentration of the Caco-2 cells from the control Transwells (without bacteria), harvested after the 12 hour incubation period, was used as background.

The proteins present in AGR1487 CW fraction 4 was calculated as a percentage of total protein present in all CW fractions. The latter was determined using the AGR1487 SE-HPLC chromatograms and by calculating the area under all the peaks (A=280 nm) resulting from the runs. The area under peaks 3 and 4 that were combined to prepare fraction 4 was then used to calculate the percentage of total CW proteins that were present in fraction 4. The calculations were done using R with function "auc" in package "MESS" to compute the area under the curves (AUC). The concentration of proteins of fraction 4 and the percentage of proteins present in fraction 4 compared to total proteins present in all CW fractions was used to calculate the concentration of proteins (fraction 4 proteins) that would be present in AGR1487 CW (0.9 OD_{600}) which has been shown to affect TEER of Caco-2 cell monolayers.

4.3.4. Transepithelial electrical resistance assay with crude bacterial cell wall and cell lysate supernatant

Caco-2 cells were cultured in M199 cell culture medium (supplemented with 10% foetal bovine serum, 1% NEAA) under conditions as previously described in Chapter 2, Section 2.3.3. Approximately 24 hours prior to the TEER assay, medium from the

Caco-2 cell monolayers and the basal well was replaced with M199 supplemented with 1% NEAA (500 μL/Transwell and 1.5 mL/well).

The bacterial CW preparations and the bacterial cell lysate SN preparations from both strains AGR1485 and AGR1487 were applied to Caco-2 cell monolayers to determine their effects on Caco-2 cell barrier integrity using TEER assays. M199 containing 1% NEAA was used as a control (500 μL/Transwell). Live bacteria (0.9 OD_{600}) from both strains AGR1485 and AGR1487 also used as treatments as a comparison to determine if the samples (containing calculated amounts of protein) elicited similar responses from the Caco-2 cell monolayers. Three separate experiments were carried out with four technical replicates per treatment for each experiment (total n=12 per treatment). On the day of the assay, the initial resistance values of Caco-2 cell monolayers were recorded, and considered to be the measurement for time 0 hour. Measurements were taken as described in Chapter 2, Section 2.3.3.1.

4.3.5. Transepithelial electrical resistance assay with bacterial cell wall fractions

Caco-2 cells were cultured in M199 cell culture medium as previously described in Section 4.3.4. Bacterial CW fractions resulting from SE-HPLC from both strains AGR1485 and AGR1487 were applied to Caco-2 cell monolayers to determine their effects on Caco-2 cell barrier integrity using TEER assays. TEER assays were conducted as described in Section 4.3.5.

4.3.6. *Transepithelial electrical resistance assay with different concentrations of AGR1487 cell wall fraction 4*

Caco-2 cells (from passage numbers 24 to 30) were cultured in M199 cell as previously described in Chapter 3, Section 3.3.1. Three different concentrations of AGR1487 CW fraction 4 were used as treatments on Caco-2 cells to determine their effects on Caco-2 cell TEER. The first concentration was the same concentration of fraction 4 that was used for TEER assays conducted for fractions 1 through 4 (Section 4.3.6.3). The second concentration was calculated to be the concentration of total CW proteins normally present in AGR1487 when applied at concentration of 0.9 OD_{600}. The third concentration was the calculated concentration of proteins that would be normally present in fraction 4 (% of total CW proteins) of AGR1487 applied at concentration of 0.9 OD_{600}. M199 media containing 1% NEAA was used as a control. Live AGR1485 and AGR1487 were also used as treatments to compare their effects on Caco-2 cell monolayers to those of the AGR1487 CW fraction 4. Three separate experiments were carried out with four technical replicates per treatment for each experiment (total n=12 per treatment).

4.3.7. *Statistical analysis*

Each of the treatments was tested in quadruplicate per TEER assay and each TEER assay was repeated three times. All the assays were treated as biological replicates and the data within each assay (for each treatment - time combination) were averaged. The statistical analysis was carried out using repeated measures ANOVA (meta-analysis with REML) for a combined analysis of all three related experiments,

using package “lme4” and “predictmeans” in R. All data were tested for normal distribution and homogeneity of variance and gave satisfactory validation of ANOVA assumptions. Statistical differences were declared between two treatments where the difference in means was greater than the LSD at 5% as compared to the control, and strength of difference shown as probability values.[5]

4.4. RESULTS

4.4.1. SDS-PAGE of cell wall preparations

Band patterns of proteins as visualised in the SDS-PAGE gels indicated that GHCl and sonication methods resulted in the extraction of proteins of varying sizes, ranging from over 100 kDa to approximately 10 kDa (lowest band close to the 14 kDa band of marker) (Figure 4.1). Both GHCl and sonication methods resulted in similar protein band patterns characteristic to each strain. However, the intensity of the protein bands resulting from sonication was greater and may have been due to the higher protein yield (Treatment 1 resulted in thicker protein bands as compared to the same protein bands from Treatment 2 and GHCl treatment).

The protein bands resulting from each strain showed that AGR1485 has two distinct high molecular weight bands (above 98 kDa) while AGR1487 shows just one band over 98 kDa. The SDS-PAGE gel results suggested that 10 bursts of 20 seconds sonication (Treatment 1) gave a higher protein yield compared to 10 bursts of 10 seconds sonication (Treatment 2) (Figure 4.1).

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[5] Statistical analysis was guided by Dr Siva Ganesh (AgResearch, Grasslands).

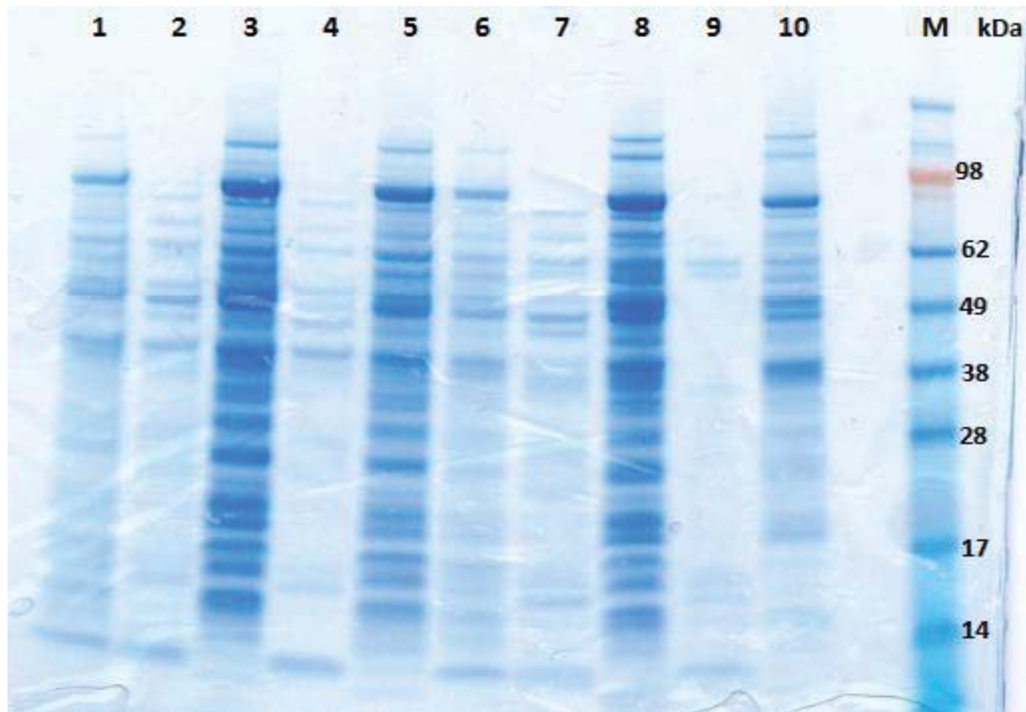


Figure 4.1 SDS-PAGE of bacterial cell wall and cell lysate supernatant preparations of AGR1487 and AGR1485. Protein bands visualised on gel showing cell wall (CW) and cell lysate supernatant (SN) fractions obtained using GHCl or sonication followed by ultracentrifugation. Lanes 1 through 5 are extracts from AGR1487 and are as follows: 1=CW (GHCl); 2=cell lysate SN and 3=CW (sonication, treatment 1); 4=cell lysate SN and 5=CW (sonication, treatment 2). Lanes 6 through 10 are extracts from AGR1485 and are as follows: 6=CW (GHCl); 7=cell lysate SN and 8=CW (sonication, Treatment 1); 9=cell lysate SN and 10=CW (sonication, Treatment 2). Treatment 1 involved 10 bursts of 20 second sonication treatment while Treatment 2 involved 10 bursts of 10 second sonication treatment to generate CW and cell lysate SN fractions. M=SeeBlue Plus2 prestained marker, with molecular weights shown in kDa.

In addition to all the protein bands resulting from GHCl treatment, sonication resulted in some additional protein bands with molecular weights between 14 kDa and 100 kDa. These additional protein bands observed from the sonication treatment (protein bands absent in the GHCl treatment) implied that sonication may have caused the release of covalently attached, CW-bound proteins, some membrane-bound and cytoplasmic components from the bacteria. The SDS-PAGE analysis also indicated that processing the cell lysate SN by additional ultracentrifugation at 100,000x*g* for 4 hours effectively removed most of the CW proteins.

4.4.2. Protein concentration of crude bacterial cell wall and cell lysate supernatant

The results for the concentrations of proteins from both the strains using the GHCl and sonication methods showed that, sonication followed by ultracentrifugation (Treatment 1) resulted in a higher protein yield (1.5 to 2.4 mg/mL) in the CW extract as compared to Treatment 2 (0.24 to 0.28 mg/mL) and the GHCl treatment (0.024 to 0.095 mg/mL). The protein yield of the AGR1485 CW resulting from sonication followed by ultracentrifugation was between 1.8-2.4 mg/mL (pH 7 to 7.2) and the protein concentration of the cell lysate SN was between 0.5-0.6 mg/mL (pH 6.5 to 7). For AGR1487 CW, the protein concentration was between 1.5-2 mg/mL (pH 7 to 7.2) and that of the cell lysate SN was between 0.5-0.6 mg/mL (pH 6.5 to 7.0). Based on the SDS-PAGE results and the protein yield from the various treatments, 10 bursts of 20 seconds sonication treatment followed by ultracentrifugation was accepted as the most suitable method for separating the bacterial CW and cell lysate SN components.

The protein yield of the CW from both the strains from the sonication method was consistent. As the minimum protein yield of AGR1487 CW was 1.5 mg/mL and that of AGR1485 CW was 1.8 mg/mL, a protein concentration of 1.42 mg/mL of both AGR1485 CW and AGR1487 CW was applied as treatment and was kept constant for all three TEER assays. Similarly for AGR1485 and AGR1487 cell lysate SN, the protein yield was consistently between 0.5 to 0.6 mg/mL. Therefore, a concentration of 0.5 mg/mL for both AGR1485 and AGR1487 cell lysate SN was applied as treatment for all three TEER assays.

The total protein yield of the CW extract of AGR1487 applied in concentrations that elicited a response from Caco-2 cells (bacterial concentration 0.9 OD_{600}) after 12 hour incubation was 0.127 mg/mL. The protein concentration of the control containing no bacteria was 0.06 mg/mL, which was used to correct for background protein.

4.4.3. Effect of bacterial cell wall extract and cell lysate supernatant on transepithelial electrical resistance

The results of TEER assays showed that AGR1485 CW (Figure 4.2A), caused an initial 67% drop in TEER at 2 hours, as compared to Caco-2 cell TEER at 0 hour. The Caco-2 cell TEER at 12 hours was still at negative 69% ($P<0.01$) as compared to Caco-2 cell TEER at 0 hour (raw data in Appendix, Table 3.1). For the control, however, after the initial 62% drop at 2 hours, the Caco-2 cell TEER by 12 hours was negative 27%, showing a 35% increase in cell TEER (Figure 4.2A). On the other hand, live AGR1485 and its cell lysate SN, caused no significant change ($P>0.05$) in Caco-2 cell TEER as compared to that of control over the 12 hours studied.

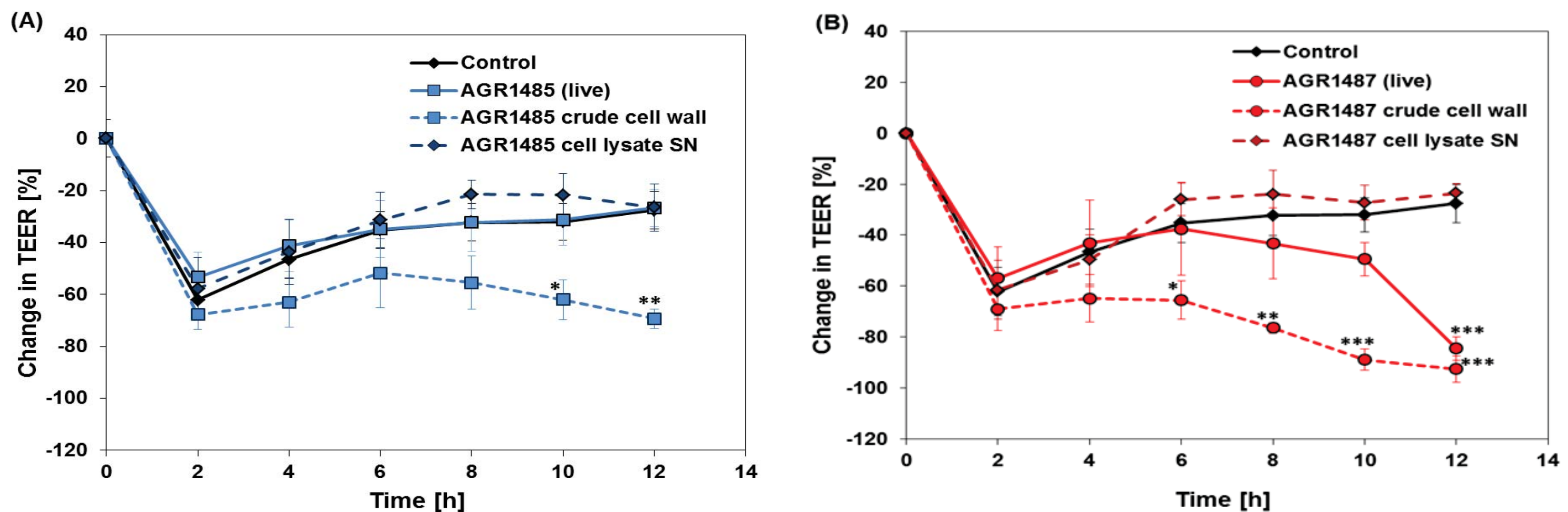
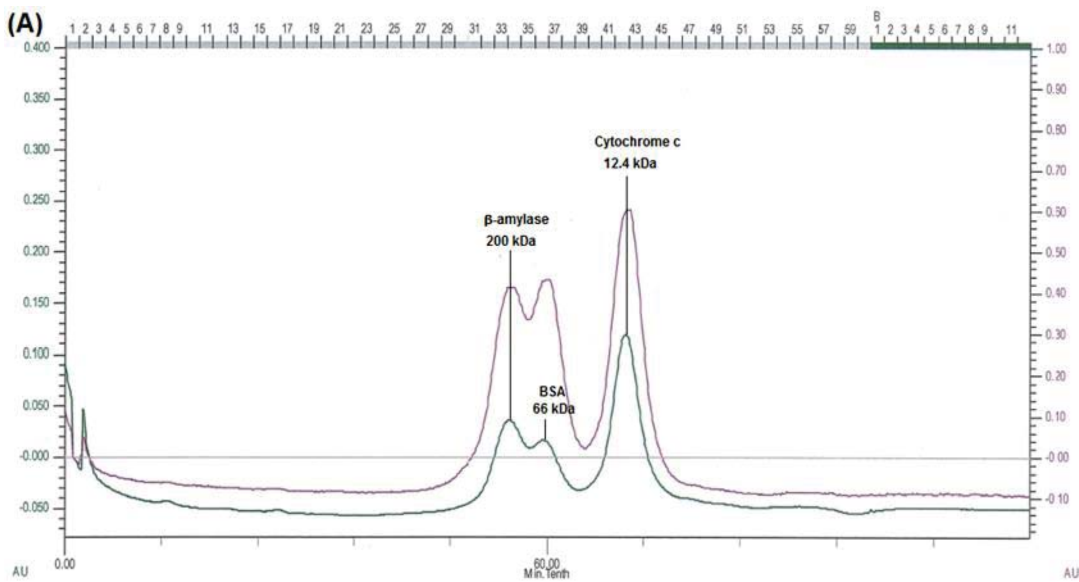


Figure 4.2 Effect of bacterial cell wall and cell lysate supernatant of (A) AGR1485 and (B) AGR1487 on Caco-2 monolayers over time. Change in TEER across differentiated Caco-2 cell monolayers (days 16 to 20 post seeding) over time. The two strains AGR1485 and AGR1487, taken from stationary phase (OD_{600} 2.5) were subjected to sonication followed by ultracentrifugation to yield bacterial CW (1.42 mg/mL) and cell lysate SN (0.5 mg/mL) fractions, which were used as treatments. Live bacteria (stationary phase, OD_{600} 0.9) were also used as treatments with M199 containing 1% NEAA as a control. The values plotted are the mean values for four monolayers per treatment for three sets of experiments and the error bars show the SEM (total n=12 per treatment), *$P<0.05$, **$P<0.01$ and ***$P<0.0001$, compared to control.

The results showed that AGR1487 CW caused an initial drop of 69% in TEER at 2 hours, as compared to Caco-2 cell TEER at 0 hour. The TEER decreased to 93% at 12 hours as compared to Caco-2 cell TEER at 0 hour, a significant change as compared to the control ($P<0.0001$) (Figure 4.2B). The cell lysate SN of AGR1487, on the other hand, did not significantly change ($P>0.05$) Caco-2 cell TEER as compared to that of the control over the 12 hours studied (raw data in Appendix, Table 3.2). Live AGR1487 caused a 56% initial drop in TEER at 2 hours, which decreased to 84% at 12 hours ($P<0.0001$). Pair-wise comparison of probability values showed that at 10 hours, AGR1487 CW significantly decreased ($P<0.05$) Caco-2 cell TEER (89%) as compared to AGR1485 CW (62%) At 12 hours, AGR1487 CW caused a 93% decrease in Caco-2 cell TEER while AGR1485 CW caused a 69% decrease and the difference was a trend.

4.4.4. Fractionation of bacterial crude cell wall

The bacterial CW pellets from the strains AGR1485 and AGR1487, resulting from sonication followed by ultracentrifugation were subjected to fractionation using SE-HPLC. The chromatogram shows the molecular weights and the elution times of the three standard proteins that were run as controls on the SE-HPLC column (Figure 4.3A) to generate a standard curve (Figure 4.3B). The chromatograms resulting from the SE-HPLC runs of the CW extracts from each strain show five main peaks of decreasing molecular weight range (Figure 4.4 and Figure 4.5). As no other peaks were observed during the last 30 minutes of each run, it was assumed that all the protein components in the sample had eluted during each run. The chromatographic pattern for each strain was reproducible every time (a prerequisite for an efficient fractionation procedure) [327, 328].



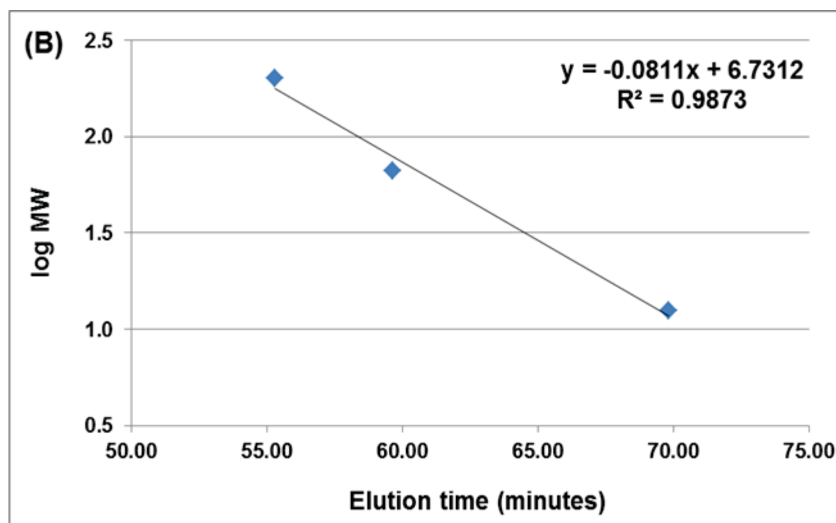


Figure 4.3 Chromatogram and standard curve of the standard proteins. Chromatogram showing the molecular weights of the standard proteins (absorbance at 280 nm). (A) The standard proteins used were β-amylase (200 kDa), bovine serum albumin (66 kDa) and cytochrome c (12.4 kDa). Green line=absorbance at 280 nm, purple line=absorbance at 214 nm. (B) The standard curve generated with the equation was used to calculate the approximate molecular weights of the peaks using elution time as a parameter.

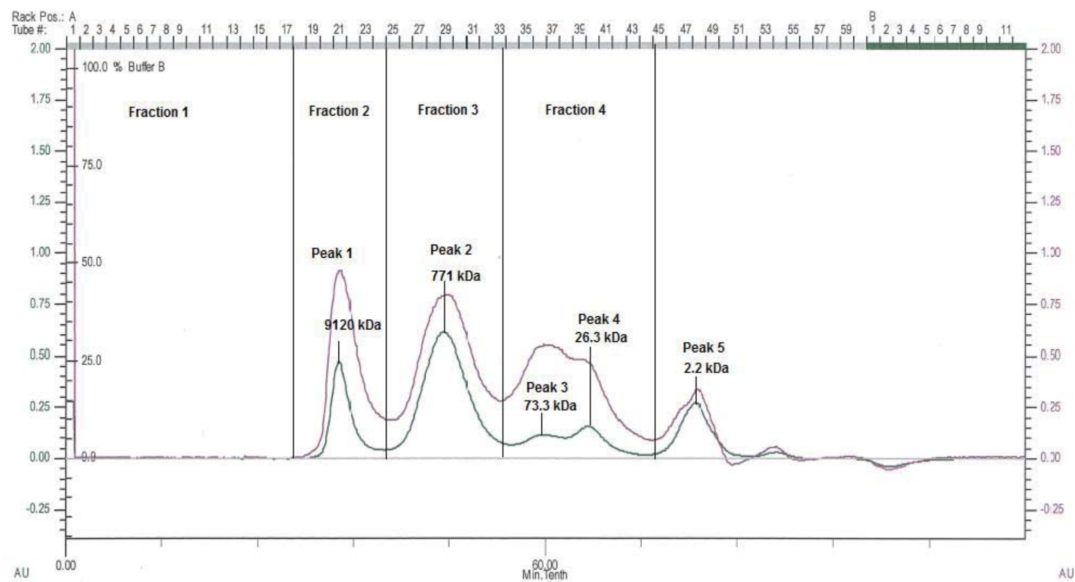


Figure 4.4 Chromatogram of the cell wall sample of AGR1485. Cell wall (CW) of AGR1485 was subjected to SE-HPLC. The molecular weights of the peaks shown were calculated using a standard curve, using elution time as a parameter. The chromatogram was used to prepare fractions by combining tubes and the fractions were then concentrated and used as treatments on Caco-2 cell monolayers. Green line=absorbance at 280 nm, purple line=absorbance at 214 nm.

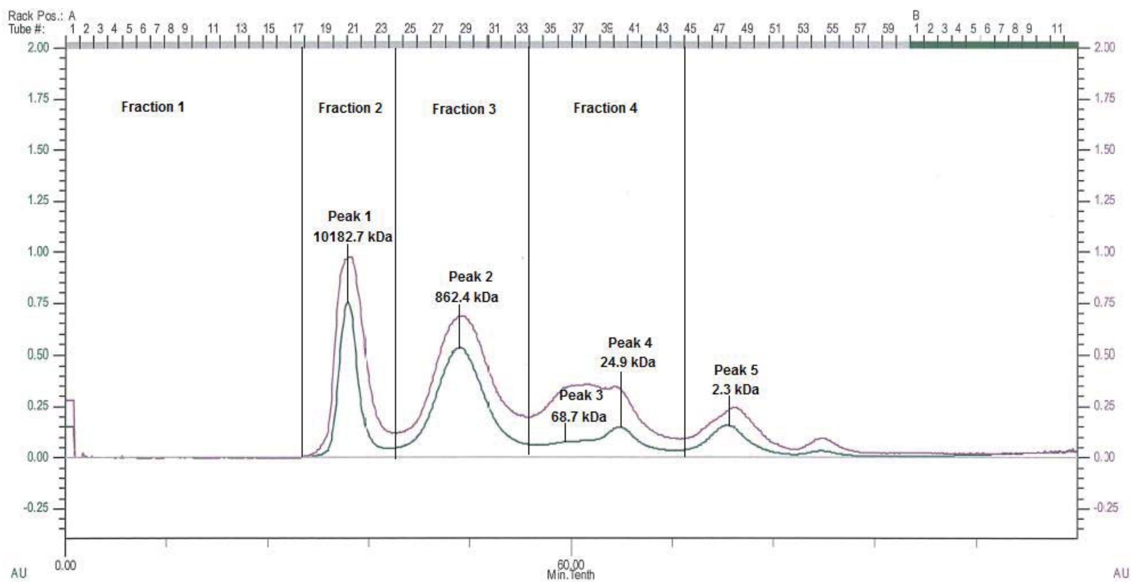


Figure 4.5 Chromatogram of the cell wall sample of AGR1487. Cell wall (CW) of AGR1487 was subjected to SE-HPLC. The molecular weights of the peaks shown were calculated using a standard curve, using elution time as a parameter. The chromatogram was used to prepare fractions by combining tubes and the fractions were then concentrated and used as treatments on Caco-2 cell monolayers. Green line=absorbance at 280 nm, purple line=absorbance at 214 nm.

The approximate molecular weight distributions of the fractions and individual peaks were determined by comparison with molecular weight standards (Figure 4.3). For both AGR1485 and AGR1487, peak 5 was determined to have a molecular weight of 2.2 kDa and 2.3 kDa respectively, but this fraction was not collected as studies have shown that lactobacilli Slps have apparent relative molecular weights of 40 kDa to 200 kDa [323, 329] and factors less than 5 kDa may not be effective in activation of certain pathways [209]. Therefore, peak 5, which may have comprised peptides or denatured protein fragments under 3 kDa, was considered to have a molecular weight too low to be relevant. The chromatogram generated for each individual run was used to determine the number of tubes (corresponding to the peaks) to be combined to prepare each fraction for the individual strains. Only peaks with molecular weights greater than 10 kDa (peaks 1 and 2) and greater than 3 kDa (peaks 3 and 4) were collected for further testing on Caco-2 cells.

4.4.5. Protein concentration of bacterial cell wall fractions

The pH of the fractions from both the strains was measured and was between 7.2 and 7.5. The protein concentrations of bacterial CW fractions resulting from SE-HPLC as determined by the Bradford assay are given in Table 4.1. All fractions were concentrated to a final volume of 2.5 to 3 mL. Based on the protein concentrations and the volumes of the concentrated fractions, the maximum amounts of proteins from each fraction were added to the Caco-2 cells to determine the effects of the fractions on Caco-2 cell TEER. As expected, fraction 1 of AGR1485 and AGR1487 CW had negligible amounts of protein. Therefore, 2 mL of the concentrated fraction 1 was used as treatment on the Caco-2 monolayers (500 μL/Transwell).

Table 4.1 Protein concentrations of fractions. Protein concentrations (mg/mL) of each of the concentrated fractions resulting from SE-HPLC of the CW of the strains AGR1485 and AGR1487 (used for TEER assays), as determined by Bradford assay.

Strain	HPLC	Fraction 1	Fraction 2	Fraction 3	Fraction 4
AGR1485	1	0.006	0.21	0.31	0.74
AGR1485	2	0.009	0.31	0.41	0.77
AGR1485	3	0.004	0.24	0.31	0.97
AGR1487	1	0.007	0.23	0.19	0.46
AGR1487	2	0.004	0.29	0.37	0.51
AGR1487	3	0.004	0.18	0.27	0.74
Conc. Used (TEER assay)	All 3 assays	2 mL*	0.20	0.20	0.45

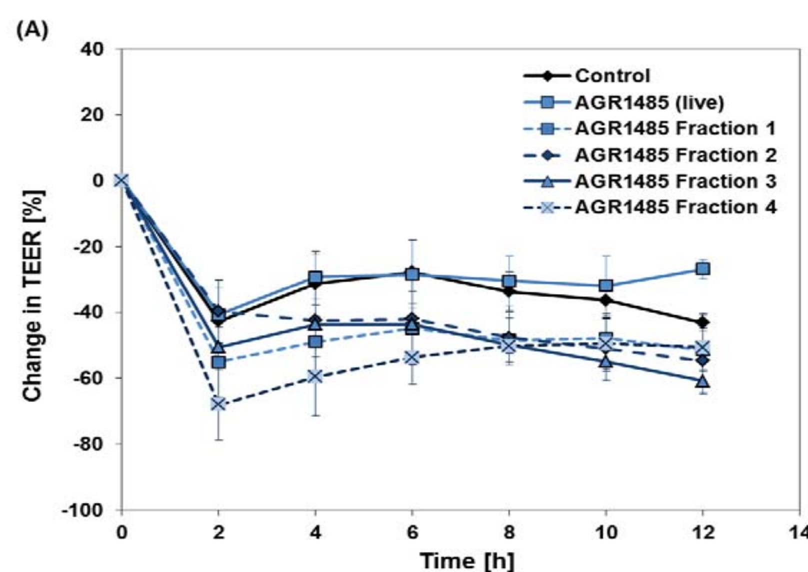
* Due to the low protein concentration, the concentrated volume was used as treatment.

For both AGR1485 and AGR1487, a protein concentration of 0.2 mg/mL was used as treatments for fractions 2 and 3 (Table 4.1). The CW fraction 4 was a combination of peaks 3 and 4 from the SE-HPLC runs and resulted in higher protein yields from both strains in the 2.5 to 3 mL concentrated volumes collected. Therefore, for CW fraction 4, a concentration of 0.45 mg/mL from both strains, AGR1485 and AGR1487, was used as treatment for all the TEER assays (500 µL/Transwell).

4.4.6. *Effect of bacterial cell wall fractions on Caco-2 cell transepithelial electrical resistance*

Results of the TEER assays using bacterial CW fractions prepared from SE-HPLC showed that for AGR1485, none of the fractions (fractions 1 through 4) caused any significant effect ($P>0.05$) on TEER of the Caco-2 cell monolayers as compared to that of the control, during the 12 hour assay (Figure 4.6A). Live AGR1485 also did not have any significant effect ($P>0.05$) on Caco-2 cell TEER as compared to that of the control (raw data in Appendix, Table 3.3). There was no significant treatment effect or treatment x time interaction effect. Therefore, a “pair-wise comparison of probability values” for “treatment” differences within each “time” point was not justified. Overall, there was no evidence for an effect of any of the AGR1485 CW fractions on Caco-2 cell TEER.

Live AGR1487 and the control decreased Caco-2 TEER by 43% at 2 hours (compared to Caco-2 cell TEER at 0 hour), but only AGR1487 significantly decreased TEER ($P<0.01$) to 90% by 12 hours (Figure 4.6B) (raw data in Appendix, Table 3.4).



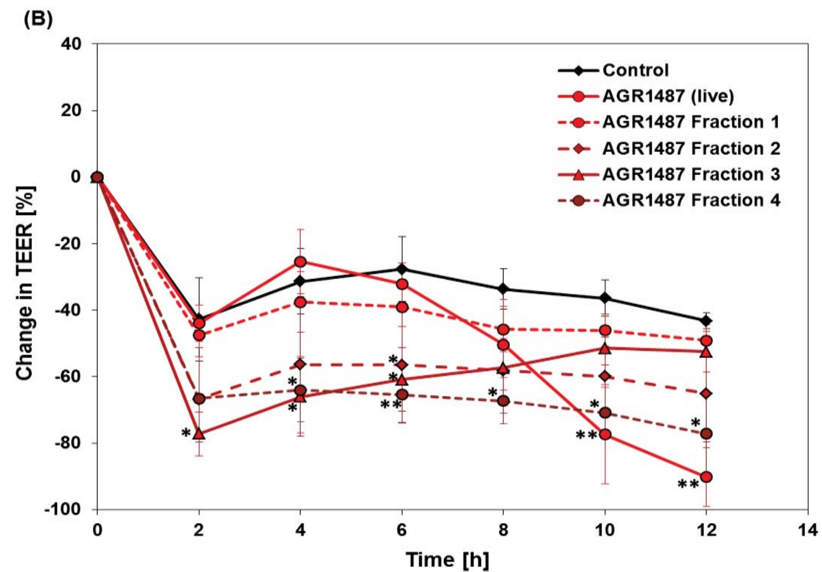


Figure 4.6 Effect of bacterial cell wall fractions of AGR1485 and AGR1487 on transepithelial electrical resistance. Change in transepithelial electrical resistance (TEER) across differentiated Caco-2 monolayers (days 16 to 20 post seeding) over time. Cell wall (CW) preparations from strains AGR1485 and AGR1487 were subjected to SE-HPLC and the resulting fractions were concentrated and used as treatments. Fraction 1 was speculated to comprise non-protein compounds of high molecular weights which may have eluted before the first peak of proteins detected. Fraction 2 comprised of the proteins within the first peak, fraction 3 contained proteins in the second peak and fraction 4 comprised of a combination of two peaks containing proteins of molecular weights between 200 kDa and 6 kDa. The values plotted are the mean values for four monolayers per treatment for three sets of experiments and the error bars show the SEM (total n=12 per treatment), *$P<0.05$ and **$P<0.01$, compared to control.

AGR1487 CW fraction 2 caused a 66% initial drop in Caco-2 cell TEER which remained at 65% over the period of the assay and at 12 hours was not significantly different ($P>0.05$) as compared to the control. AGR1487 fractions 3 caused a 77% initial drop, but by 10 hours the Caco-2 cell TEER had increased by 26%, and was similar to that of the control. However, fraction 4 of AGR1487 CW caused significant differences in Caco-2 cell TEER from 4 to 12 hours ($P<0.05$) as compared to control. After an initial 66% at 2 hours, fraction 4 of AGR1487 CW decreased Caco-2 cell TEER to 77% at 12 hours, (Figure 4.6B). Therefore, it appeared that AGR1487 CW fraction 4may have contained components that decreased TEER of the Caco-2 cell monolayers over the 12 hours studied.

4.4.7. Concentration of proteins in AGR1487 cell wall fraction 4

The AGR1487 chromatograms used for calculating the AUC for total bacterial CW proteins (covering all the protein peaks in the runs) showed that it was 962.75 cm^2 and AUC for AGR1487 CW fraction 4 (covering protein peaks 3 and 4) was 196.63 cm^2. Therefore, the percentage of protein present in fraction 4 was calculated to be approximately 20% of the total CW proteins. As reported in Section 4.4.2, the total protein yield of the CW extract of AGR1487 applied in concentrations that elicited response from Caco-2 cells (bacterial concentration 0.9 OD_{600}) after 12 hour incubation was 0.127 mg/mL. Based on that concentration, the concentration of proteins normally present in AGR1487 CW fraction 4 was determined to be 0.025 mg/mL (20% of the 0.127 mg/mL total protein present in the CW extract of AGR1487 at a concentration of 0.9 OD_{600}).

4.4.8. Effect of different concentrations of AGR1487 CW fraction 4 on Caco-2 cell transepithelial electrical resistance

The results of TEER assays of AGR1487 CW fraction 4 applied to Caco-2 cell monolayers at varying concentrations are shown in Figure 4.7 (raw data in Appendix, Table 3.5). The concentrations of AGR1487 fraction 4, used were 0.45 mg/mL (same concentration as previous TEER assays reported in Section 4.4.6), 0.127 mg/mL (normal concentration of total CW proteins) and 0.025 mg/mL (normal concentration of proteins in AGR1487 fraction 4). AGR1487 CW fraction 4 applied at a concentration of 0.45 mg/mL significantly increased Caco-2 cell TEER during the 12 hour assay. There was no initial drop of cell TEER at 2 hours observed for AGR1487 CW fraction 4 (0.45 and 0.025 mg/mL), but an increase in Caco-2 cell TEER by 32% and 6% respectively was noted. AGR1487 CW fraction 4 (0.45 mg/mL) continued to maintain an increased Caco-2 cell TEER throughout the 12 hour assay while AGR1487 (0.025 mg/mL) increased Caco-2 cell TEER for the first 8 hours of the assay before dropping to the same level as control at 12 hours. AGR1487 CW fraction 4 applied at 0.127 mg/mL, caused an initial drop of 3% at 2 hours and maintained cell TEER similar to control over the 12 hours studied. After the initial 47% drop at 2 hours, live AGR1487 had caused a further 53% drop in Caco-2 cell TEER at 12 hours. Live AGR1485 did not have any significant effect on Caco-2 cell TEER as compared to control throughout the 12 hours studied.

Overall, none of the AGR1487 CW fraction 4 concentrations decreased Caco-2 cell TEER similar to live AGR1487 over the 12 hours studied, including the concentration (0.45 mg/mL) that had previously shown to decrease Caco-2 cell TEER.

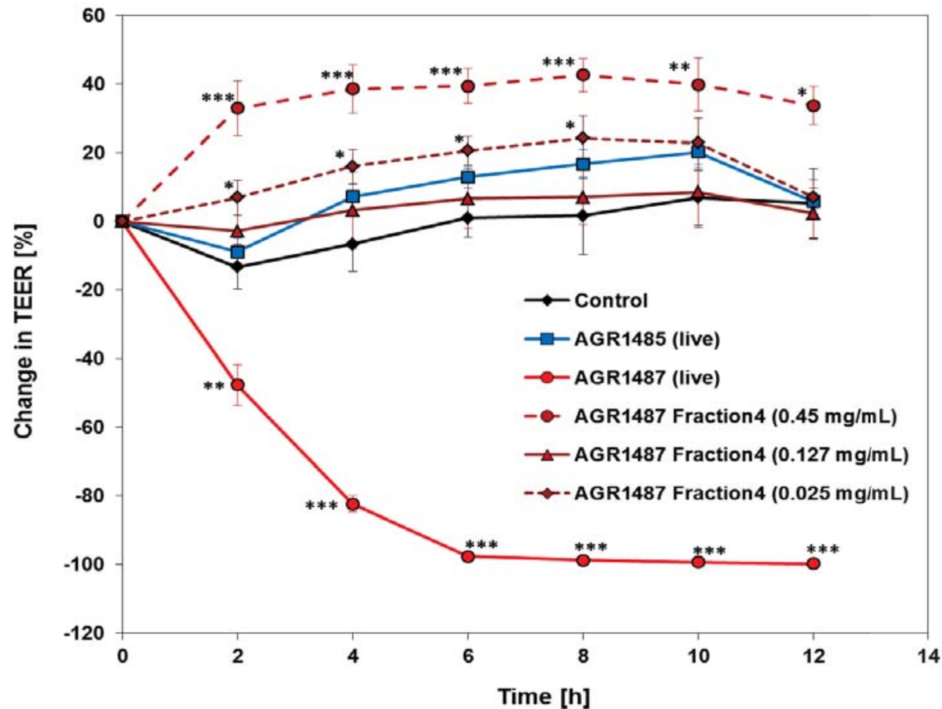


Figure 4.7 Effect of AGR1487 cell wall fraction 4 applied at different concentrations on transepithelial electrical resistance. Change in transepithelial electrical resistance (TEER) across differentiated Caco-2 cell monolayers (days 16 to 20 post seeding) over time. The bacterial cell wall (CW) preparation from AGR1487 was subjected to SE-HPLC and the resulting fraction 4 was concentrated and used at three different concentrations as treatments: 0.45 mg/mL (concentration used for previous TEER assays, Figure 4.6); 0.127 mg/mL (normal concentration of total CW proteins) and 0.025 mg/mL (normal concentration of proteins in AGR1487 fraction 4). Live bacteria, AGR1485 and AGR1487 (stationary phase, 0.9 OD_{600}) were also used as treatments and M199 containing 1% NEAA was used as control. The values plotted are the mean values for four monolayers per treatment for three sets of experiments and the error bars show the SEM (total n=12 per treatment), *P<0.05, **P<0.01 and ***P<0.0001 as compared to control.

4.5. DISCUSSION

The main results from the TEER assays indicated that the CW components of AGR1487 reduced the barrier integrity of the Caco-2 cell monolayers over 12 hours in a similar pattern to that of live AGR1487. This implies that all the bacterial components that are required to influence the physiological changes in the Caco-2 cells related to the TEER of the monolayers are present in AGR1487 CW. The TEER assays conducted with fractions resulting from SE-HPLC of AGR1487 CW identified one CW fraction (fraction 4) that decreased TEER over the 12 hours studied.

However, it must be noted that in the first set of TEER assays, the concentrations of the proteins used to test each of the fractions from the bacterial CW were several folds higher (0.45 mg/mL) than the concentrations at which these CW proteins would be normally present in live bacteria (0.025 mg/mL). The assumption was that the components comprising AGR1487 CW fraction 4 can affect Caco-2 cell TEER similar to live AGR1487 at normal concentrations. However, in the second set of TEER assays, none of the concentrations of AGR1487 CW fraction 4 (0.025 to 0.45 mg/mL) decreased Caco-2 cell TEER similar to that of live AGR1487, including the same high dose of AGR1487 CW fraction 4(0.45 mg/mL) that had decreased cell TEER as compared to control during the first series of TEER assays. On the contrary, the high dose of AGR1487 CW fraction 4 increased Caco-2 cell TEER in the second set of TEER assays. The non-reproducible effects of AGR1487 CW fraction 4 on Caco-2 cell TEER could be due to limitations of the SE-HPLC fractionation procedure and variations that may affect the two different biological systems, the Caco-2 cells and AGR1487 (live or CW fractions), used in this study.

SE-HPLC using Superdex columns have been used for purification and fractionation of proteins and natural organic matter and the stability and properties of the resulting fractions have been well studied [328, 330]. However, non-specific interactions of components being fractionated with the column matrix can result from adsorption mechanisms and ionic interactions, which can affect the fractionation procedure and efficiency [328]. The chemical-physical properties of the components play a role in their ionic and adsorptive interactions and can cause selective retention of solutes resulting in retarded elution. The high concentration of CW extracts (as used in these studies) can overload the column and during fractionation, solutes with a high molecular weight, which are more susceptible to van der Waals forces, can get adsorbed reversibly onto the gel phase and thus retarded [328]. One of the limitations of using Superdex 200 columns used in this study is that in some applications, substances such as denatured or precipitated proteins and non-specifically bound lipoproteins or lipids do not elute within one column volume, thus rendering the fractionation procedure inefficient. It is possible that even if the sonication and ultracentrifugation procedures resulted in CW extracts with all the bacterial CW components present, the fractionation step with SE-HPLC may not have yielded the active components, which might not have eluted due to the limitations of the column.

Another limitation could be linked to the fact that each TEER assay was conducted using fractions resulting from independent SE-HPLC runs. It is possible that LTA and/or PG fragments, which are bacterial CW components that can act as ligands for Caco-2 cell receptors, could have eluted along with the protein comprising AGR1487 CW fraction 4. LTA and PG fragments are non-protein components that

would not result in a peak and hence their presence would not be detected by the UV-detector of the SE-HPLC system. The Bradford assays also would not measure LTA or PG concentration in any of the CW fractions. Although the conditions of the SE-HPLC were kept the same for all the runs, it is possible that due to limitations of the SE-HPLC column [328], some LTA and/or PG fragments may have eluted in variable quantities along with AGR1487 CW fraction 4. The possible variation of presence of undetected LTA and PG components in the AGR1487 CW fraction 4 prepared by independent SE-HPLC runs may have resulted in the discrepancy in the results between the first and second series of TEER assays. The observation that none of the fractions of AGR1487 CW decreased Caco-2 cell TEER similar to live AGR1487 may also indicate that the fractionation procedure may have physically damaged the components of AGR1487 CW or separated out the cell surface components or ligands present in AGR1487 CW that are important for the CW fractions to affect Caco-2 cell TEER.

The fractionation procedure using SE-HPLC might have affected the structure, composition and activity of bacterial ligands (through denaturation of proteins, structural damage to the lipid chains that might act as ligands, or separation of these ligands that work in conjunction). Studies have reported the importance of LTA and peptidoglycan in host-microbe interactions [8, 331] and showed that non-protein bacterial ligands (PG, LTA, and DNA) via their recognition by receptors (TLR, NLR and CLR) present on the Caco-2 cells, can affect the response of Caco-2 cells. This ligand-receptor interaction induces a signalling cascade that involves recruitment of adaptors such as the myeloid differentiation primary response gene 88 (MyD88) and

activation of several pathways such as the mitogen-activated protein kinase (MAPK) and the nuclear factor κB (NF-κB) pathways [35, 216].

It is also important to note that the Caco-2 cells used to conduct the TEER assays to determine the effects of the different concentrations of AGR1487 CW fraction 4 were from a different batch from lower passage numbers, with an extra antifungal-antimycotic added to the culture medium. Two of the three TEER assays were conducted by an experienced technical staff and is reflected in the minimal initial drop in TEER resulting from efficient handling of the cells. Cell related factors such as number of passages and medium composition can influence different functions of this cell line [241, 242, 245]. In addition, the AGR1487 CW used to prepare fraction 4 for the second set of TEER assays, compared to the first set of TEER assays, was from a different batch of frozen stock. Both the Caco-2 cells and AGR1487 are two different biological systems and variations in either system may have impacted the response of the Caco-2 cells to the AGR1487 CW fraction 4. However, this is unlikely as the effects of live bacteria on these Caco-2 cells were similar to those observed in previous studies [163, 258].

In conclusion, AGR1487 CW had a similar effect on TEER of Caco-2 cell monolayers to live AGR1487. This effect of CW proteins was noted at high concentrations and remains to be confirmed at concentrations normally found in live AGR1487. Nonetheless, this might be an indication that CW may contain all the components that affect TEER across the Caco-2 cell monolayers. AGR1487 CW fraction 4, at a higher concentration than that normally found in live AGR1487, decreased Caco-2 cell TEER, however, the results could not be repeated at the same or

lower (including normal) concentrations. The discrepancy in the results of TEER assays conducted with AGR1487 CW fraction 4 suggests the need for further method development. The method for isolating bacterial CW fractions using SE-HPLC may need further modifications and optimisation for the differential elution of denatured proteins or non-specifically bound lipoproteins and for the detection and isolation of LTA and PG ligands, to remove potential confounding factors that might have limited the assessment of AGR1487 CW fraction 4 on Caco-2 cell barrier integrity. Modified and optimised methodologies can lead to the identification and characterisation of specific molecules present in the AGR1487 CW that are potential ligands for host receptors. Identification of these effector molecules that influence host-microbe interactions and modulate intestinal barrier integrity will be a significant step towards understanding the strain-specific characteristics of AGR1487.

5 CHAPTER 5: SUMMARY AND GENERAL DISCUSSION

5.1 BACKGROUND

The research reported in this dissertation was part of a wider research programme which aims to understand how foods interact with the resident microbes, and epithelial and immune cells of the gastrointestinal tract (GIT) to maintain intestinal barrier function. The resulting knowledge can be used to develop new ingredients and foods that may promote human health and wellness.

The GIT microbial communities and their composition are a major driving force for individual well-being and health. The human GIT, consisting of a complex array of cell types, is the largest interface between man and his environment, and acts as a barrier to protect the body from the entry of microbes and antigens. The intestinal barrier can be divided into four components: a physical, a chemical, an immunological and a microbial barrier. The microbial barrier comprises 10^{14} commensal bacteria, comprising of 500-1000 different species [42], which are involved in a molecular "cross-talk" with the other intestinal barrier components. The host-microbe interactions play functional roles in the development of the GIT, in modulating the host immune system and the barrier properties of the intestinal epithelium [50]. In healthy individuals, the GIT promotes the uptake of nutrients and provides protection from pathogenic microbes, while remaining tolerant to commensal bacteria. These host-microbe interactions are crucial for the regulation of intestinal immune homeostasis and for maintenance of host-microbe symbiosis. A disruption of the symbiotic balance may lead to dysfunction and disease, and may affect human well-being [51, 52]. Ultimately, this balance is maintained by complex interactions of gene-based networks both within

the host, and between the host and its microbial community whereby marker molecules can trigger individual responses and cascades [35, 50, 80].

Due to the interest in the use of "all natural" products to reduce the risks of GIT disorders, there has been an increased focus on the use of probiotics, such as the *Lactobacillus* species. Lactobacilli form an integral part of the microbiota and are generally considered to be protective organisms that are required to maintain a healthy GIT. *Lactobacillus fermentum*, one of the most common species of lactobacilli to be isolated from the GIT [296], is considered a "generally recognised as safe" (GRAS) organism, with several studies indicating some *L. fermentum* strains to have beneficial effects on GIT health [254, 256, 257].

However, different species of lactobacilli can evoke different responses in the host and not all strains of the same species are necessarily beneficial [9, 50, 258]. The work described in this dissertation highlights the differential effects of the two strains of *L. fermentum*, AGR1485 and AGR1487, on intestinal barrier integrity.

5.2 SUMMARY OF RESULTS

The overall aim of the research reported here was to describe phenotypic similarities and differences between the two strains of *L. fermentum* (AGR1485 and AGR1487), and then determine if these differences were related to the known differential effects that these two strains have on intestinal barrier integrity. These investigations were carried out in an attempt to identify bacterial cell surface components that may impart strain-specific characteristics and influence host-microbe interactions. Caco-2 cell monolayers were used as a model system to study the effects of

the two strains of bacteria and bacterial components on intestinal barrier integrity. Transepithelial electrical resistance (TEER) assays and ^{3}H-mannitol assays were used as measures of tight junction (TJ) integrity and paracellular permeability of the Caco-2 cell monolayers, respectively.

In Chapter 2 of this dissertation, the results of the phenotypic analyses and genome size comparison showed that the strains AGR1485 and AGR1487 exhibit differences not only in their effect on intestinal barrier integrity, but also in their genome sizes, sugar utilisation capabilities and responses to stressors (acid and bile stress tolerance assays). AGR1487 decreased TEER across the Caco-2 cell monolayers, while AGR1485 did not influence TEER. The API50CH test highlighted differences in sugar utilising capabilities between the two strains, which may be indicative of differences in cell surface components as carbohydrate utilisation has been shown to affect cell-envelope-associated proteinase activity that may in turn influence host-microbe interactions [281]. The difference in genome size between AGR1485 and AGR1487 as indicated by pulsed-field gel electrophoresis may be indicative of the presence or absence of genes causing morphological alterations that may impart strain-specific characteristics that influence host-microbe interactions. These genotypic differences may also be related to the differences in stress tolerance shown by the two strains. AGR1485 showed a lower tolerance to bile and low pH compared to AGR1487.

In Chapter 3, TEER assays conducted using Caco-2 cell monolayers with live versus UV-killed bacteria indicated that for both the strains, dead bacteria had similar effects on the intestinal barrier integrity to the live bacteria. AGR1487 (both live and UV-inactivated), decreased TEER across Caco-2 cell monolayers by 60 to 80%, and this

effect was independent of the bacterial growth phase. Experiments conducted with metabolites produced by AGR1485 and AGR1487, Caco-2 cells or as a result of interactions between the Caco-2 cells and either strain, did not affect Caco-2 cell TEER as compared to control. These results strengthened the assumption that bacterial surface components, and not secreted molecules, are involved in the host-microbe interactions that affect TEER across the Caco-2 cells. Analogous to TEER assays, live AGR1487 increased mannitol-flux across Caco-2 cell monolayers indicating a destabilisation of the Caco-2 cell TJ integrity. However, UV-inactivated AGR1487 did not affect mannitol flux, indicating that secreted molecules of AGR1487 might influence the paracellular permeability of mannitol. Results of the TEER assays and mannitol flux assays collectively, suggest that AGR1487 may use both bacterial cell surface components as well as secreted molecules through distinct mechanisms to influence the Caco-2 cell barrier properties.

In Chapter 4, it was shown using TEER assays conducted with Caco-2 cell monolayers that crude bacterial cell wall extract (CW) from AGR1487 caused a decrease in TEER across the Caco-2 cell monolayers similar to that found with live AGR1487. On the other hand, the bacterial cell lysate supernatant (SN) from both the strains (AGR1485 and AGR1487) did not change Caco-2 cell TEER as compared to the control medium. These results indicated that the CW contained components that affected Caco-2 cell TEER, although the CW was ten times more concentrated than normally found in live bacteria. Therefore, the CW was subsequently fractionated using SE-HPLC in an attempt to narrow down possible candidates (proteins and lipoproteins) that may act as ligands that trigger responses in Caco-2 cell monolayers thereby

affecting TEER. For AGR1487, a CW fraction (namely fraction 4) was identified which decreased TEER of the Caco-2 cell monolayers, as compared to the control, but the fraction was tested at non-physiological concentrations. To test the assumption that the components comprising AGR1487 CW fraction 4 could affect Caco-2 cell TEER when applied at concentrations at which they are normally found in live AGR1487, the TEER assays were repeated with varying concentrations (above and within a physiological range) of the AGR1487 CW fraction 4. None of the concentrations of the AGR1487 CW fraction 4, including the same high concentration tested before, however, affected TEER of the Caco-2 cell monolayers in the second set of TEER assays. This discrepancy in results remains unexplained. Overall, the results imply that although the AGR1487 CW may have contained the cell surface components required to affect Caco-2 cell TEER, the effect might require synergy of several CW molecules to modulate TEER of the Caco-2 cell monolayers. The fractionation process may have separated out these molecules thus negating the synergistic effect and as such none of the fractions could affect TEER. These results highlight the need for further research studying the combined and separate effects of these bacterial cell surface components in affecting the barrier integrity of the intestinal epithelium.

5.3 GENERAL DISCUSSION

5.3.1 Advancement of knowledge

The work described in this dissertation focuses on the differential effects of two strains of *L. fermentum* (AGR1485 and AGR1487) on intestinal barrier integrity. It also sought to identify bacterial components that may be responsible for these effects. The

overall results indicate that the barrier integrity of Caco-2 cell monolayers was compromised by live AGR1487, UV-inactivated AGR1487 and AGR1487 CW, but not by secreted molecules produced by AGR1487 or AGR1487-Caco-2 interactions. These results collectively suggest that the components necessary to affect Caco-2 cell TEER are CW components of AGR1487. However, UV-inactivated AGR1487 did not have the same effect as live AGR1487 on mannitol flux assays implying, that not all of the barrier properties of Caco-2 cells are influenced by bacterial cell surface-associated components. It is possible that distinct mechanisms involving both surface-associated and secreted molecules might trigger different pathways to elicit a final response from Caco-2 cells to AGR1487.

A recent study reports that physical interaction of AGR1487 cells with Caco-2 cells is required for the decrease in TEER caused by this strain as AGR1487 supernatant did not decrease the TEER of Caco-2 cell monolayers but increased it [258]. The study also reported that AGR1487 had greater adherence to Caco-2 cells (as compared to AGR1485) that may facilitate the host-microbe interactions necessary to trigger certain signalling pathways that influence host response. The authors suggest that the compromising effects of AGR1487 on the Caco-2 cells may be caused by the altered distribution of zona occludens (ZO-2, a junctional plaque protein) around each Caco-2 cell along with an increased abundance of microtubule-associated proteins (suggesting disassembly of TJ proteins) that was observed in Caco-2 cells treated with AGR1487. The observation reported here that UV-inactivated AGR1487 caused a decrease in TEER similar to live AGR1487 clearly indicates the cell surface components of

AGR1487 as possible ligands that induce host signalling pathways involved in maintenance of TJ integrity.

5.3.2 Lactobacilli cell surface components affecting host-microbe interactions

Many studies have reported that direct cell contact between lactobacilli and intestinal epithelial cells (IEC) is necessary for some microbe-host interactions. Some of these studies have also identified bacterial cell surface components that play crucial roles in these interactions thereby modulating some host pathways [196, 204, 332]. For example, adherence of *L. plantarum* 299v to HT-29 epithelial cells and induction of mucin expression was dependent on direct cell contact between lactobacilli and IEC [196]. The *adh* mutant of *L. plantarum* 299v (strain unable to produce mannose sensistive binding ligand required for adherence to HT-29 cells) had lost the ability to induce mucin expression and resulted in 10-times less adherence to IEC. Studies with *L. acidophilus* ATCC 4356 report that direct cell contact was required for inducing anti-inflammatory and anti-apoptotic effects in the IEC through the activation of mitogen-activated protein kinases (MAPK), prevention of inactivation of epidermal growth factor (EGF) receptor, and the prevention of inhibitor of NF-κB (IκB) degradation [204, 209, 332]. The specific anti-apoptotic effect exerted by *L. rhamnosus* GG (LGG) on IEC through the inhibition of tumour-necrosis factor alpha (TNF-α)-stimulatory activation of MAPK p38, also required direct cell contact [168]. Several of these *in vitro* studies identify multiple signalling pathways involving MAPKs, EGF receptor, TLRs and IκB (linked to NF-κB pathway) that have important functions in the regulation of host cell cytoskeleton, stabilisation of TJ and immune responses of IEC. It

is evident that multiple cell surface-associated factors of lactobacilli can influence a complex network of interacting signalling pathways that modulate the host response.

5.3.3 Lactobacilli secreted molecules affecting host-microbe interactions

The results of the studies reported in this dissertation showed that the secreted metabolites resulting from bacteria, Caco-2 cells or bacteria-Caco-2 cell interactions did not affect the TEER of the Caco-2 cells. The mannitol flux assays showed that only live AGR1487 caused an increase in mannitol flux (UV-inactivated AGR1487 did not increase mannitol flux), suggesting the possibility of the involvement of bacterial secreted molecules in influencing the selective properties of paracellular permeability in the Caco-2 cells.

The roles played by secreted molecules in lactobacilli-host interactions have been well documented. For example, the two secreted proteins, p40 and p75 (CW-associated hydrolases) of LGG have been reported to protect TJ integrity (involving protein kinase C and MAPK) as well as inhibit TNF-induced apoptosis (involving phosphatidylinositol 3-kinase (PI3-K) and activation of EGF receptor) in IEC [168, 194]. These effects are brought about by activation of separate kinases involving different co-receptors in the host, indicating that these bacterial proteins can influence host response through more than one pathway. Similar sized proteins (recognised by p40 and p75 antisera) identified in the supernatant of *L. casei* ATCC334, stimulated the activation of Akt (or protein kinase B) and inhibited TNF-induced apoptosis in IEC [209]. Soluble, low molecular weight heat and acid-stable peptides of LGG have also been shown to induce heat shock

proteins in IEC through the activation of the MAPK signalling pathway [211]. TJ stability and induction of mucin production in IEC have been reported to be influenced by soluble proteinaceous factors present in the VSL#3 probiotic mixture comprising eight probiotic bacterial strains [210].

These studies taken together suggest that multiple cell surface and secreted components of lactobacilli exert an effect on IEC through mediation of multiple signalling pathways. However, the exact mechanisms of action involving specific *Lactobacillus* effector molecules and their corresponding host receptors, and the downstream signalling events still remain unclear and need to be characterised in most cases.

5.3.4 The AGR1487 cell wall

The research reported in this dissertation showed that the AGR1487 CW affected TEER across Caco-2 cell monolayers in a similar manner to live and UV-inactivated AGR1487, which suggests that the components required to affect TEER of the Caco-2 cells are present in the AGR1487 CW. The effect found with AGR1487 CW (at ten times concentration) was not found for its fractions. The fractionation procedure, may have separated out the cell surface components that possibly have a synergistic effect on the Caco-2 cells and may be the reason that none of the AGR1487 CW fractions resulting from SE-HPLC affected the TEER of Caco-2 cell monolayers similar to live AGR1487. Recognition of AGR1487 and the resulting response of Caco-2 cells probably involve multiple cell surface components and/or secreted metabolites of AGR1487 that may act as ligands (MAMPs) for the Caco-2 cells (PRRs). The MAMP-

PRR interactions result in further activation of various adaptor molecules (such as myeloid differentiation primary response protein (MyD88), EGF receptors, peptide transporters) to initiate signalling cascades that involve the consecutive actions of several kinase systems (NF-κB, MAPK, PKC and PI3K) [35, 216].

It is important to note that the host response to a microorganism is dependent on several factors, such as the concentration of the MAMPs along with their accessibility for their specific PRRs, specific physical features of the MAMPs to ensure recognition by their respective PRRs, presence of other microbial effector molecules that may affect host response (for example, production of acids and inoles) and most importantly, the combination of distinct MAMPs that may act in conjunction with their respective PRRs and associated co-receptors. For example, flagellins (MAMP) from *Lactobacillus ruminis* interact with TLR5 (PRR) that can induce pro-inflammatory signalling [72]. Indeed, various factors of the host and microbes modulate the flagellin-TLR5 interactions and only flagellin delivered as a monomer can interact with the TLR-5 MAMP [333]. Similarly, fimbriae/pili of bacteria are also dedicated to adherence to host cells through binding to TLR4, involving the engagement of specific co-receptors and adaptor proteins in host lipid rafts to modulate signalling pathways and the response of the host cells [334]. Some lactobacilli, for example LGG, mediate adherence of fimbriae to host mucus glycoproteins which may enhance the interaction of these bacteria with host PRR inducing the associated signalling pathways [112]. In contrast, fimbriae of some pathogens have been shown to induce pro-inflammatory responses [187]. Thus it is not only the amount of the MAMP but the specific structural features of the MAMP that are important in the MAMP-PRR interactions. It is possible that unlike AGR1485,

AGR1487 expresses pro-inflammatory flagellin proteins, which result in a negative effect on TEER of the Caco-2 cell monolayers.

Lactobacilli cell surface-associated proteins have been implicated in mucus adhesion and modulation of immune response of the host [79]. A study reported that the putative glycoprotein S-layer protein A (SlpA) of *Lactobacillus acidophilus* NCFM interacts with dendritic cells (DC) through the C type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), but this interaction may be dependent on the glycosylation of SlpA. DC-SIGN is a phagocytic receptor which promotes phagocytic uptake of bacteria in phagolysosomes and can also induce signalling pathways that involve Raf-1 kinase-dependent acetylation of transcription factor NF-κB and MAPK [335]. The protein band patterns from the SDS-PAGE showed differences between the CW protein composition of AGR1485 and AGR147. The CW of AGR1487 may contain proteins required to trigger response in the Caco-2 cells that are absent in AGR1485. In contrast, the CW of AGR1485 may contain proteins that lack the potential to act as ligands required to trigger signalling pathways.

In addition to cell surface-associated and secreted proteins, lipoteichoic acid (LTA) molecules (present on almost all lactobacilli) may also act as ligands for TLR2. However, the two lipid chains of LTA must be exposed for the interaction with the lipid-binding pockets of TLR2 to induce signalling. The digestion of these bacteria in phagolysosomes of the host cells enables the two acyl chains of LTA (MAMP) to become accessible for the PRR. A mutational approach in *Lactobacillus plantarum* suggests that D-alanylation of LTA mediates pro-inflammatory interactions through TLR2, an effect that could not be confirmed in LGG by the same experimental approach

[97, 225]. However, the mutations of LTA can affect several MAMP-PRR interactions by having a pleiotropic effect on other bacterial cell surface components thus indicating the importance of specific structural features of the MAMP in host-microbe interactions. The LTA present in the CW of AGR1487 may be a variant of that present in AGR185 CW which may result in the differential effects that these strains show on the barrier integrity of the Caco-2 cells.

Peptidoglycan (PG) can also act as a ligand for TLR2, but before PG can be detected by host PRR, PG needs to be hydrolysed through autolysis or remodelling. PG can interact with NOD2 only after its peptide ligand muramyl dipeptide (MDP) is taken up by the apical peptide transporter (PEPT1), subsequently activating the NF-κB pathway [336]. All these reports emphasise the variety of possible bacterial ligands with their specific physical features and their involvement with several, sometimes common, host pathways that modulate the response from the host [337]. Therefore, the recognition of AGR1487 and the response of the Caco-2 cells can involve multiple bacterial ligands interacting with several host receptors, and surface-located PRR often co-operate with co-receptors and may not function in isolation.

5.3.5 Strengths/limitations of methodologies used

The strengths and limitations of the experimental designs and the techniques used are important to discuss. A limitation of the experimental design for studying the effects of secreted metabolites on TEER of the Caco-2 cell monolayers was that it did not account for the stability or the concentration of the secreted metabolites over time. However, similar to the present report, a previous study has also indicated that for

AGR1487 to decrease Caco-2 cell TEER, direct bacteria to host cell interaction was required [258]. It is possible that the metabolites secreted that affect properties of the Caco-2 cell monolayers (for example paracellular permeability of non-ionic solutes) are produced as a direct interaction between bacteria and Caco-2 cells. It is possible that these metabolites are stable for only a short period of time, need to be at a certain concentration and may require other bacterial CW components to act as mediators to trigger a response from the Caco-2 cells. Therefore, investigation of the effects of secreted metabolites from both the strains (AGR1485 and AGR1487) need to be modified to establish the involvement of bacterial secreted metabolites and mixtures of these metabolites in influencing the barrier properties of Caco-2 cells.

Another potential limitation of the studies reported here was the use of the concentrations of AGR1487 CW and its fractions applied on the Caco-2 cells. The concentration of AGR1487 CW and its fractions in the initial study were ten times higher than their concentrations in live AGR1487. Results of studies with the collagen-binding protein CbsA protein of *L. crispatus* in *L. casei* support the assumption that the binding capacity of entire S-layers (as found in live bacteria) is far more efficient than its protein subunits [338]. Moreover, lactobacilli contain a diverse group of adhesive factors, both proteinaceous and non-proteinaceous, that interact with the host to modulate host response [79]. The expression and activity of these adhesins are most likely influenced by conditions presented by the host. Thus, the application of the CW extracts and the resulting fractions at high concentrations was made in an attempt to ensure that an effect could be observed.

The experimental design using SE-HPLC column focused on the separation of bacterial cell surface proteins and glycoproteins and studying their effects on TEER of the Caco-2 cell monolayers. One of the limitations of SE-HPLC using Superdex 200 columns is that substances such as denatured or precipitated proteins and non-specifically bound lipoproteins or lipids do not elute within one column volume, thus resulting in an inefficient fractionation procedure. It is possible that during the fractionation step with SE-HPLC, some of the components remained bound to the column, and might not have eluted due to the limitations of the column. In addition, LTA and PG fragments are non-protein components that would not result in a peak and hence their presence or absence in the eluted fractions would not be detected by the UV-detector of the SE-HPLC system. Given the importance of LTA and PG as possible ligands that affect host response, the fractionation procedure for the AGR1487 CW requires modifications and optimisation to separate bacterial cell surface proteins, and to separate LTA and PG fragments. The effects of each of these potential ligands could then be tested independently and in combination with each other to ascertain their roles in affecting the barrier integrity of the host.

5.3.6 Next steps and suggested improvements in methodologies

The limitations of the fractionation procedure of the CW of AGR1487 emphasises the need for development and optimisation of separation techniques of the bacterial components due the importance of the physical features of these potential ligands. However, to gain an understanding of the molecular mechanisms involved in host-microbe interactions, it is important to consider the limitations of using *in vitro* models

and how these limitations may affect the host-microbe interactions that occur in the host.

The two strains of *L. fermentum*, AGR1485 and AGR1487, are facultative anaerobes that can survive under aerobic conditions as well as under anaerobic conditions in the human GIT. The physiology of the facultative anaerobic bacteria can undergo considerable alteration depending on its environment. For example, a study reported that in enterohaemorrhagic *Escherichia coli* (EHEC) O157:NM, exposure to anaerobic conditions led to the expression of fimbriae although fimbriae are absent in this strain under aerobic conditions [339]. Genes encoding for pili/fimbriae have been identified at the genome level in some lactobacilli, but they have been functionally analysed and characterised only in LGG [110, 111]. The importance of fimbriae as a possible ligand and in adherence has been discussed in Section 5.3 and as such it is clear that the effects of facultative anaerobes such as AGR1485 and AGR1487 are likely to differ based on their environment. All experiments reported here were carried out under aerobic conditions. Therefore, to better mimic the effects of these facultative anaerobic bacteria, it would be appropriate to use *in vitro* models that are more similar to the *in vivo* conditions.

The research reported in this dissertation used the Caco-2 cell monolayer as a model for the GIT epithelial barrier. As previously described in Chapter 1, despite the advantages of the use of differentiated Caco-2 cells as an *in vitro* model, it is a cancer-derived cell line and as such may not always be truly representative of the *normal* human intestinal cells. Although the *in vitro* models can never fully represent the dynamic and complex nature of the *in vivo* situation, recent studies have shown the

development of several *in vitro* models that better mimic the human GIT and are more suited for studying microbe-host interactions as they would take place in the GIT. Complex three dimensional models using hydrogels to represent microvilli on which Caco-2 cells are seeded have been developed [340]. A microfluidic model was used to study host-microbe interactions which allowed co-culture of both commensal and pathogenic bacteria with IEC [341]. An apical anaerobic model has been recently developed using the Caco-2 cell line and the Transwell co-culture system and has been used to co-culture commensal obligate anaerobes with Caco-2 cells [342]. Such models provide the advantages of the *in vitro* models while better representing the complexity and dynamic qualities of the *in vivo* models.

A major limitation that may affect the host-microbe interactions studied using the Caco-2 cell model is the absence of mucus. As described previously in Chapter 1, the mucus layer plays an important role in bacterial adherence and is the first barrier that the bacteria encounter in the human GIT. While the Caco-2 cells represent enterocyte-like cells, the human GIT is composed of various different types of cells (such as goblet cells, endocrine cells and M cells) that interact with the bacteria to determine the host response. Models using co-cultures of two or more cell lines have been developed to overcome these limitations and may be a better representation of the *in vivo* physiological conditions. For example, a triple co-culture of Caco-2 cells, HT29-MTX cells and Raji B cells was developed to mimic the human GIT epithelium representing the absorptive enterocyte-like cells, mucus producing cells and cells that can induce M-cell phenotype in Caco-2 cells [343].

The combined use of multiple cell lines co-cultured to establish an *in vitro* model closely representing the human GIT and the use of co-culture chambers such as the apical anaerobic model would be a closer representation of the *in vivo* conditions of the GIT. Such a model would be ideal to study the effects of bacterial components, food components, drugs and toxins in a physiological intestinal environment that can include both commensal as well as pathogenic bacteria. These studies using dual environments that are suitable for co-culturing both bacteria and host cells can lead to a better understanding of the host-microbe interactions, the mechanisms involved in these interactions and in the identification of key components of bacterial and/or host origin that are involved in regulating the functions of the GIT barrier.

The next step would be to study the effects of UV-inactivated AGR1487 compared to those caused by live AGR1487 on the adherence to Caco-2 cells. Additionally, the effects of UV-inactivated AGR1487 on the distribution of ZO-2 and the expression levels of microtubule-associated proteins in Caco-2 cells, as has been studied for live AGR1487 [258], could also indicate possible roles played by the cell surface-associated components of AGR1487 in influencing TJ integrity. Mannitol flux assays studying the effects of secreted molecules produced by AGR1487 can verify if bacterial metabolites influence paracellular permeability of mannitol across Caco-2 cell monolayers. Although genes for pili have been identified at the genome level in some lactobacilli [110], their presence in AGR1487 would be a novel finding and it would be informative to investigate if their presence is dependent on the environmental conditions (anaerobic versus aerobic). Further studies could also investigate if the presence or

absence of pili/fimbriae influences the adherence properties of *L. fermentum* strains and the pro- or anti-inflammatory responses they may elicit from IEC.

The strain AGR1485 was isolated from a healthy individual and AGR1487 was isolated from an individual subsequently diagnosed with IBD. It may be of interest to study in detail different effects these two strains (live and UV-inactivated) and their culture supernatants have on biomarkers such as pro-inflammatory cytokines. A recent study with *L. rhamnosus* and its cell-free culture supernatant (SN) applied on *E. coli*-challenged human dendritic cells (DC) showed that the levels of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8 and IL12p70 were higher in DC treated with live probiotic bacteria than DC treated with the probiotic SN [344]. In presence of *E. coli*, however, the SN was more effective in reducing the production of pro-inflammatory cytokines than the probiotic bacteria. The study with Caco-2 cells treated with AGR1485 and AGR1487 has reported the differences in the gene expression and protein abundance profiles through whole genome expression analysis and LC-MS/MS [258]. A similar approach could be taken to compare the effects of AGR1487 and AGR1487 SN on Caco-2 cells to compare differentially expressed proteins and cytokines.

Other biomarkers such as faecal calprotectin and lactoferrin, considered to be "hallmarks of neutrophilic intestinal inflammation" and invariably present in IBD [345] could be used to study the effects of AGR1487 in order to find a possible correlation between IBD and AGR1487. Serum markers such as immunoglobulin (Ig)A and IgG class antibodies anti-*Saccharomyces cerevisiae* (ASCA) and IgG class anti-neutrophil cytoplasmic antibodies (ANCA) are used in diagnosing IBD [345]. The presence of

these serum markers, which can be determined through enzyme immunoassays and by indirect fluorescent antibody assays, can be used to study and compare the effects of AGR1487 with other strains. Generally, health promoting bacteria increase the production of anti-inflammatory cytokines such as IL-10 and TGF-β, while reducing pro-inflammatory cytokines such as TNF-α, interferon-γ and IL-8 [200, 346]. The effect of AGR1487 on the production of these cytokines could be informative and indicative of its possible link to IBD.

The genomes of AGR1485 and AGR1487 have been sequenced, assembled and are currently being annotated for comparative genomic analyses to identify functional genes and genetic differences between the two strains. Standardisation of a separation protocol to obtain bacterial proteinaceous compounds that influence the phenotypic characteristics of the strains could be followed by proteomic analysis. The proteomics data would supply a list of proteins that are expressed by the bacteria. Comparison of the proteomics data from the two strains AGR1485 and AGR1487 could indicate differentially expressed proteins. These differentially expressed proteins and their activity could then be further analysed and be used in conjunction with the information obtained from the *in silico* analyses of the genome sequences of the two strains. Proteomic data, genomic information and gene function distribution analysis could be used for designing future *in vitro* and *in vivo* experiments that could clarify the activities of possible novel proteins that may explain the differential behaviours of the two strains AGR1485 and AGR1487.

5.4 CONCLUSIONS

The two strains of *L. fermentum*, AGR1485 and AGR1487, are closely related genetically but are different phenotypically. These two strains, although belonging to the same species, exhibit different effects on the GIT barrier integrity, presumably due to the differential expressions of certain bacterial components such as proteins, lipoproteins and other complex molecules. The lactobacilli cell surface is a dynamic entity and plays an important role in the MAMP-PRR interaction inducing signalling cascades that ultimately trigger the host cell response to the bacteria. The surface molecules of lactobacilli that impart health-promoting rather than pathogenic characteristics to these bacteria are not easy to delineate [9].

The effects on intestinal barrier integrity caused by these strains and detailed herein were studied in isolation, and may not have positively or negatively impact the host-microbe balance in the *in vivo* situation. As the research in this dissertation suggests, the bacterial CW components play an important role in modulating host response. However, it is likely that the host-microbe interactions are a result of complex interactions involving various bacterial surface and secreted molecules with different host receptors and associated co-receptors. The final outcome whereby a symbiotic balance is achieved is likely due to the interactions of many stimulatory and inhibitory effector molecules, resulting from the "cross-talk" among many species, strains and cell types. These are only preliminary insights into what must be a complex, multifactorial, finely tuned process.

Currently, the knowledge of the host-microbe interactions and the participating components is restricted to *in vitro* and animal studies, with a few human clinical studies. However, the progress of functional genomics has resulted in the analysis of available *Lactobacillus* genomes, identifying putative effector molecules that can target host receptors [14, 186]. However, experimental validation following *in silico* analysis is important for proper understanding of the molecular mechanisms by which lactobacilli interact with the GIT. Knowledge of the molecular mechanisms that influence host-microbe interactions and the effects of the resident microbiota functions in the human GIT is vital to allow transition from *in vitro* studies to whole animal and human studies. This knowledge may lead to a more focused approach to the use of bacterial species as "natural forms" of preventive measures for reducing the risks of GIT diseases.

Thus, further research is needed to expand an understanding of the molecular mechanisms of action involving each of the microbial cell surface-associated molecules and the corresponding host molecules that regulate signalling pathways and subsequently modulate host response. The identification and characterisation of bacterial ligands and their specific host receptors can ultimately lead to the development of strains with enhanced health benefits.

APPENDIX

Raw Data from Chapter 2 TEER Experiments:

Table 1. Effect of AGR1485 and AGR1487 on TEER across Caco-2 monolayers over time.

Time	Assay 1			Assay 2			Assay 3		
	Resistance (ohms)								
	Control	AGR1485	AGR1487	Control	AGR1485	AGR1487	Control	AGR1485	AGR1487
0	1092	1205	755	3790	3710	3530	4820	4920	967
	353	630	1119	3550	3970	3590	4200	4350	828
	1159	1424	1283	3520	3830	3810	4170	4400	1240
	975	688	1265	3780	3940	3790	3760	4760	1064
2	429	1080	480	2370	2800	2190	1270	1870	497
	275	652	720	2550	3290	2010	1370	1440	515
	727	1480	850	1980	2450	2320	1490	1330	484
	510	627	493	3100	2410	1960	850	1230	679
4	455	906	557	2560	2990	1710	1925	2700	548
	290	755	894	2880	3190	1570	2260	2260	587
	868	1554	881	2570	2730	1550	2370	2040	590
	685	608	540	3230	2970	1360	1250	1890	815
6	497	788	530	2680	3020	1310	2300	3000	485
	348	853	1310	2990	2910	1300	2690	2700	554
	948	1844	1195	2830	2730	1290	2980	2390	569
	738	607	418	3100	3040	1150	1540	2360	760
8	675	1414	451	2430	2340	970	2470	3110	354
	343	812	1268	2660	2110	1010	2890	2990	437
	1380	1767	1765	2410	2320	1060	2930	2730	453
	1135	755	282	2490	2610	940	1620	2930	628
10	694	1338	285	2320	2420	740	2590	3050	199
	350	754	807	2430	1980	770	2880	3040	301
	1398	1662	1364	2180	2400	880	2840	2780	313
	1156	742	243	2240	2240	790	1630	2820	523
12	701	1484	194	2250	2675	630	2550	3020	93
	359	721	396	2460	1760	650	2890	3180	158
	1395	1700	829	2180	2433	790	2860	2840	158
	1163	750	120	2250	2514	690	1620	2860	186
24	615	1257	-3	2200	3067	1326	2630	3340	16
	291	763	9	2520	3365	800	2890	3610	19
	985	1543	14	2100	3125	1607	2920	3280	16
	756	730	6	2360	3316	1315	1680	3470	19

Raw Data from Chapter 3 TEER Experiments:

Effect of bacterial growth phase on TEER across Caco-2 monolayers (blank cells represent damaged Transwells).

Table 2.1 Effect of AGR1485 taken from early, mid and late log phases of growth on TEER across Caco2 monolayers over time.

Time	Assay 1				Assay 2				Assay 3			
	Resistance (ohms)											
	AGR1485				AGR1485				AGR1485			
	Control	Early log	Mid log	Late log	Control	Early log	Mid log	Late log	Control	Early log	Mid log	Late log
0	3839	3604	4657	3996	5200	6050	5640	4150	3580	3600	2910	3340
	3492	4713	5004	5687	5040	5000	4780	4060	3660	3420	2260	3470
	3649	3806	4433	5699	5240	5400	4750	4170	3560	3890	2980	2820
	3716	3985	4646	4847		5030	5120	4960	3810	3900	3140	3060
2	3291	3055	3627	2059	2500	2720	4440	1820	2400	2450	2200	2450
	3089	3727	3324	2025	3270	3380	3210	2850	2390	2220	2130	2890
	3134	3593	2585	1207	2440	2030	3750	3220	2430	2500	2290	2740
	3145	3649	1790	2787		3300	3940	3470	2400	700	3050	2040
4	3548	3828	4041	3223	4080	2970	3900	2060	2770	3060	2180	2740
	3638	4668	4209	3660	3720	3420	2780	3660	2790	2680	2560	3250
	3627	3772	3425	2518	3540	2620	3530	3740	2780	3130	2380	3150
	3739	4175	3212	4075		3220	3500	4260	2930	1840	1690	2790
6	4086	4646	3951	3526	4760	3220	4380	2730	3220	3410	2930	3180
	3895	5497	4198	4511	4220	3840	3740	3850	3150	2990	2310	3300
	4063	4556	3649	3806	4300	2980	4120	4070	3080	3350	2950	3260
	4075	4903	3772	4209		3460	4130	4610	3350	2730	3240	3220
8	3223	3481	3851	3503	5050	3450	4100	2990	3040	3720	3050	2850
	2966	4209	4041	4713	4420	4050	3430	3600	2930	3230	2450	3060
	3123	3638	3470	4444	4490	3220	3640	3750	2900	3560	3170	3010
	3111	3593	3772	4377		3690	3700	3940	3200	3130	3390	3000
10	2988	3459	4164	3313	5120	3570	3900	3200	2830	3610	2990	2920
	2899	4097	4411	4579	4540	4190	3230	3680	2950	3130	2380	3150
	2988	3447	3974	4478	4500	3520	3500	3920	2770	3490	2950	3110
	2943	3571	4299	4265		3850	3500	4260	3060	3360	3080	3130
12	2854	3380	3739	3257	4320	3420	4060	2970	2900	3560	2960	3060
	2708	4108	3638	4489	3970	3930	3380	3170	2860	3170	2430	3180
	2820	3391	3324	4534	4070	3460	3710	3260	2750	3420	3130	3050
	2719	3481	3761	3750		3630	3650	3610	2990	3660	3360	3260

Table 2.2 Effect of AGR1487 taken from early, mid and late log phases of growth on TEER across Caco2 monolayers over time.

Time	Assay 1				Assay 2				Assay 3			
	Resistance (ohms)											
	AGR1487				AGR1487				AGR1487			
	Control	Early log	Mid log	Late log	Control	Early log	Mid log	Late log	Control	Early log	Mid log	Late log
0	3440	3480	4160	3930	5200	4920	4350	4280	3580	3870	3290	3180
	3130	3650	4120	3980	5040	5400	5150	4520	3660	3870	3130	3330
	3270	3810	4010	4000	5240	5610	5650	3800	3560	3480	3190	2990
	3330	3190	3630	4910		6020	5880	4650	3810	3980	2740	2860
2	2950	2680	800	2650	2500	2150	2550	1490	2400	3050	2530	1800
	2770	1340	1280	2720	3270	740	3140	3120	2390	2900	2730	2580
	2810	2220	2730	1840	2440	2390	3520	1600	2430	2600	2430	1660
	2820	2380	820	2120		2940	1900	2740	2400	3330	1670	2190
4	3180	3170	1390	2970	4080	1100	1830	1190	2770	3290	1630	2310
	3260	1980	2110	3140	3720	390	2550	3060	2790	3280	2390	2890
	3250	2830	2900	2690	3540	1640	2810	1310	2780	2850	1410	2200
	3350	2850	1450	2820		2430	1250	1340	2930	3370	2020	2680
6	3660	3570	2030	2900	4760	500	1480	710	3220	3230	3040	2760
	3490	2090	2690	3210	4220	180	2170	2670	3150	3100	2890	3280
	3640	3230	3200	2940	4300	990	2490	740	3080	2610	2900	2630
	3650	3080	2180	2750		1910	790	850	3350	3160	2490	3050
8	2890	2800	1880	1410	5050	270	650	280	3040	2850	2990	2080
	2660	1720	2260	2210	4420	70	650	640	2930	3380	3030	2990
	2800	2620	3060	2180	4490	420	1470	230	2900	2680	2950	2230
	2790	2770	2400	700		1070	200	430	3200	2590	2460	1960
10	2680	210	1380	270	5120	180	190	70	2830	2070	2600	940
	2600	820	2100	640	4540	40	130	170	2950	2980	2550	1820
	2680	1160	2560	620	4500	200	450	60	2770	1790	2780	860
	2640	1530	2190	110		570	70	130	3060	850	1960	740
12	2560	100	590	110	4320	140	80	10	2900	860	2400	410
	2430	330	1250	160	3970	30	70	30	2860	2290	2670	840
	2530	370	1030	160	4070	150	100	10	2750	1010	2690	270
	2440	620	1400	40		360	30	50	2990	310	1360	240

Table 2.3 Effect of AGR1485 taken from transition, early and late stationary phases of growth on TEER over time.

	Assay 1				Assay 2				Assay 3			
	Resistance (ohms)											
		AGR1485				AGR1485				AGR1485		
Time	Control	Transition	Early stationary	Late stationary	Control	Transition	Early stationary	Late stationary	Control	Transition	Early stationary	Late stationary
0	3320	4010	3080	2980	4780	4370	4150	4310	3120	2910	3250	2960
	3120	4780	2830	3440	4750	5300	4500	4080	2970	2950	3130	3220
	3740	3880	3640	2890	4320	5260	3980	2960	2980	2800	3050	3260
	3730	5340	3110	3100	4250	5360	3900	4500	2800	2980	3370	2950
2	2280	2760	2900	2180	3250	2520	3020	2820	2380	2170	1880	2480
	2730	1710	2610	1720	2520	3930	3540	2250	1850	2150	2030	2070
	2820	2230	2890	2030	2020	3760	1910	1840	2260	2200	1940	1440
	2450	3510		1450	2560	2450	2490	2250	1210	1650	2050	2050
4	2740	3180	3230	2850	4410	3080	3920	4020	2840	2660	2480	3070
	2830	2470	2880	3140	4270	4410	4640	3310	2560	2680	2550	3060
	3100	2870	3430	2700	3640	4380	2750	2680	2900	2540	2530	2630
	2910	4880		2710	3830	3240	3430	3940	2090	2340	2750	2890
6	2720	3070	3120	2950	4780	3010	4140	4050	2890	2630	2550	3000
	2750	2780	2880	3470	4640	4150	4590	3580	2630	2840	2490	3290
	2930	2920	3540	2760	4330	4200	3080	2970	2890	2690	2570	3050
	2770	4840		2920	4240	3420	3680	4270	2420	2700	2880	3150
8	2590	3270	3100	2810	4720	2670	4230	3860	2940	2880	3060	3130
	2600	3260	2850	3530	4660	3650	4850	3670	2850	2890	2850	3460
	2750	3080	3510	2760	4540	3680	3400	3020	2950	2760	2850	3400
	2700	5390		3130	4420	3180	3900	4420	2550	2720	3370	3290
10	2650	3030	3110	3040	4520	2820	3710	3700	2890	2930	2830	3260
	2650	3330	2820	3700	3440	3950	4440	3510	2760	2880	2800	3580
	2870	2970	3590	2990	4310	4120	3190	2940	2910	2780	2900	3430
	2750	5240		3240	4120	3600	3580	3850	2490	2810	3260	3480
12	2660	3020	3020	2840	4490	1580	2200	3820	3000	2980	2530	3390
	2750	3370	2780	3670	4200	3650	4590	3560	2890	2960	2420	3640
	2950	2860	3420	2830	4330	3650	3390	2930	3030	2850	2420	3470
	2740	5050		3040	4280	3260	3710	4000	2630	2910	2660	3520

Table 2.4 Effect of AGR1487 taken from transition, early stationary and late stationary phases of growth on TEER over time.

	Assay 1				Assay 2				Assay 3			
	Resistance (ohms)											
		AGR1487				AGR1487				AGR1487		
Time	Control	Transition	Early stationary	Late stationary	Control	Transition	Early stationary	Late stationary	Control	Transition	Early stationary	Late stationary
0	3320	5050	3110	3210	4780	5250	5200	4630	3120	2870	3140	3040
	3120	4520	3130	3240	4750	5050	4940	4370	2970	2500	3300	3150
	3740	3570	3560	3080	4320	4550	4510	4490	2980	2970	3170	2850
	3730	4450	2850	2800	4250	4300	4900	4500	2800	2520	3350	2730
2	2280	1950	1580	890	3250	3250	2740	1980	2380	1420	1890	1760
	2730	1700	3050	2800	2520	2340	2600	1420	1850	2260	2160	1600
	2820	2300	2130	1870	2020	2820	2580	1520	2260	2100	1930	1720
	2450	2020	1580	1550	2560	2830	1870	1840	1210	2160	1290	1740
4	2740	2320	1680	1770	4410	3280	3320	2550	2840	1890	2450	2540
	2830	2600	3050	3300	4270	2580	3310	1780	2560	2510	2730	2620
	3100	2450	2320	2740	3640	3250	3150	1990	2900	2540	2490	2420
	2910	2440	1670	2740	3830	2280	1990	2170	2090	2570	2060	2400
6	2720	2390	1640	1890	4780	1450	1980	1190	2890	2170	2380	2430
	2750	2830	3100	3200	4640	2290	2370	1090	2630	2600	2760	2620
	2930	2510	2310	2760	4330	3500	2910	1600	2890	2790	2670	2590
	2770	2380	1660	2810	4240	630	330	710	2420	2660	2170	2500
8	2590	1420	1080	1630	4720	250	250	140	2940	2090	1540	2400
	2600	2500	1490	2660	4660	480	300	130	2850	2630	2420	3340
	2750	2360	1680	2400	4540	1430	760	350	2950	2650	2640	2900
	2700	1300	1150	2860	4420	20	40	60	2550	1870	1630	2240
10	2650	200	490	780	4520	60	90	40	2890	1820	450	1530
	2650	760	280	2090	3440	140	80	40	2760	2630	1560	3070
	2870	1850	880	1480	4310	210	230	60	2910	2660	1300	2440
	2750	170	530	2800	4120	20	20	30	2490	990	680	1910
12	2660	90	110	340	4490	20	40	30	3000	1090	140	800
	2750	260	50	680	4200	40	30	20	2890	2460	540	2560
	2950	1010	140	380	4330	20	60	20	3030	2550	340	1650
	2740	70	120	210	4280	10	10	10	2630	450	170	1350

Table 2.5 Effect of live versus UV-killed AGR1485 on TEER across Caco2 monolayers over time.

	Assay 1					Assay 2					Assay 3				
	Resistance (ohms)														
Time	Control (UV30min on ice)	AGR1485 (no UV)	AGR1485 (UV15 min)	AGR1485 (UV30 min)	AGR1485 (UV30 min on ice)	Control (UV30min on ice)	AGR1485 (no UV)	AGR1485 (UV15 min)	AGR1485 (UV30 min)	AGR1485 (UV30 min on ice)	Control (UV30min on ice)	AGR1485 (no UV)	AGR1485 (UV15 min)	AGR1485 (UV30 min)	AGR1485 (UV30 min on ice)
0	1460	1460	1430	1730	1800	2060	2110	2380	2030	1810	1569	1400	1351	1650	1855
	1300	1640	1360	1500	1480	1800	2550	2150	2460	1740	1974	1746	2460	1626	2600
	1490	1600	1640	1320	1430	1710	2460	1690	1651	1680	1591	1871	1852	1777	1569
	1160	1850	1950	1410	1560	1882	2120	1880	2400	2540	1299		1499	2170	1654
2	747	756	808	1008	793	837	1124	977	973	597	774	1051	826	789	1046
	682	883	677	750	687	994	1154	893	1322	1211	836	955	1507	701	1215
	721	930	716	647	632	901	915	1161	701	669	852	1164	1172	801	947
	684	978	929	624	653	841	793	1056	1049	1028	538		773	835	655
4	766	881	956	1147	919	1229	1596	1392	1299	759	885	1225	899	946	1146
	741	1078	816	870	825	1348	1734	1345	1831	1383	967	1190	1710	913	1484
	847	1105	870	740	753	1325	1431	1533	940	846	956	1387	1375	994	1068
	755	1197	1143	767	817	1162	1116	1445	1420	1406	592		931	1005	845
6	789	969	1065	1225	980	1408	1599	1472	1265	748	1104	1211	958	1027	1196
	842	1164	934	968	898	1478	1853	1478	1852	1218	1243	1214	1852	1067	1609
	951	1183	989	797	880	1427	1473	1551	943	823	1155	1365	1593	1077	1131
	835	1285	1296	853	837	1278	1190	1356	1375	1359	717		1051	1103	969
8	795	1007	1094	1269	1007	1372	1725	1852	1319	767	1092	1154	997	1010	1232
	851	1227	968	985	898	1436	2040	1719	1895	1245	1225	1160	1740	1091	1572
	988	1207	1031	833	934	1419	1636	1689	974	827	1125	1323	1529	1054	1118
	851	1323	1339	900	847	1287	1323	1410	1442	1433	703		1109	1082	1012
10	891	1016	1172	1341	988	1482	1669	1814	1117	638	1056	1155	989	1019	1210
	879	1255	1046	1054	917	1481	1911	1690	1792	962	1278	1187	1815	1103	1572
	1057	1271	1090	865	957	1550	1674	1664	875	726	1131	1360	1544	1084	1164
	885	1346	1432	932	878	1366	1334	1376	1292	1207	705		1162	1069	1003
12	866	981	1127	1264	950	1277	1651	1919	1073	606	985	1137	991	1026	1275
	909	1198	1004	1020	890	1302	1848	1753	1670	885	1156	1183	1842	1128	1618
	1053	1228	951	820	938	1367	1628	1614	796	704	1026	1334	1534	1076	1199
	894	1286	1368	896	852	1254	1357	1414	1186	1091	662		1167	1003	1037

Table 2.6 Effect of live versus UV-killed AGR1487 on TEER across Caco2 monolayers over time.

Time	Assay 1					Assay 2					Assay 3				
	Resistance (ohms)														
	Control (UV30min on ice)	AGR1487 (no UV)	AGR1487 (UV15 min)	AGR1487 (UV30 min)	AGR1487 (UV30 min on ice)	Control (UV30min on ice)	AGR1487 (no UV)	AGR1487 (UV15 min)	AGR1487 (UV30 min)	AGR1487 (UV30 min on ice)	Control (UV30min on ice)	AGR1487 (no UV)	AGR1487 (UV15 min)	AGR1487 (UV30 min)	AGR1487 (UV30 min on ice)
0	1460	1690	1290	1430	1400	2060	2200	2620	2480	2320	1569	1657	1766	1685	2720
	1300	1850	1300	1200	1730	1800	2500	2740	2840	2790	1974	1586	2360	1085	1437
	1490	1750	1380	1390	1550	1710	2530	2780	2900	2070	1591	1709	2400	1840	1438
	1160	1460	1440	1550	1900	1882	2130	2180	2540	2030	1299	1446	1980	2400	1437
2	747	406	701	686	729	837	879	917	635	471	774	834	445	743	899
	682	460	583	787	985	994	832	926	606	672	836	655	1144	415	553
	721	577	646	801	912	901	857	687	855	443	852	891	920	598	726
	684	407	535	783	1273	841	630	721	668	683	538	939	587	603	605
4	766	184	505	676	669	1229	1100	1226	803	600	885	1018	530	853	1025
	741	311	477	777	1038	1348	1056	1222	812	848	967	715	1430	503	674
	847	405	554	861	959	1325	1135	974	1171	641	956	967	1206	773	834
	755	284	425	768	1392	1162	734	976	943	845	592	1109	737	858	718
6	789	88	159	467	316	1408	1111	1458	851	777	1104	831	550	829	1039
	842	150	265	653	822	1478	1137	1520	911	1147	1243	596	1488	513	746
	951	172	307	745	685	1427	1247	1201	1166	887	1155	749	1317	807	810
	835	119	250	536	1195	1278	787	1094	940	935	717	1000	744	906	702
8	795	38	43	182	116	1372	860	1343	778	632	1092	591	502	738	934
	851	80	109	360	425	1436	898	1383	833	1058	1225	396	1366	473	661
	988	75	117	459	282	1419	970	1134	977	847	1125	535	1194	753	717
	851	64	109	245	529	1287	623	977	705	792	703	797	677	843	583
10	891	22	22	75	67	1482	699	1369	737	587	1056	340	462	669	792
	879	48	61	202	221	1481	791	1311	793	1021	1278	202	1190	424	553
	1057	33	63	249	116	1550	858	1126	828	854	1131	313	1043	686	628
	885	31	63	116	226	1366	500	836	557	753	705	555	563	778	504
12	866	17	15	32	33	1277	650	1191	732	561	985	172	347	590	597
	909	29	30	73	83	1302	666	985	787	889	1156	90	1017	385	434
	1053	22	32	60	47	1367	852	936	796	803	1026	137	842	619	552
	894	21	31	36	73	1254	421	717	542	646	662	264	406	715	442

Table 2.7 Effect of metabolites (AGR1485) on TEER across Caco2 monolayers over time.

	Assay 1						Assay 2						Assay 3					
	Resistance (ohms)																	
Time	Control	Treatment1	Treatment2	Treatment3	Treatment4	AGR1485	Control	Treatment1	Treatment2	Treatment3	Treatment4	AGR1485	Control	Treatment1	Treatment2	Treatment3	Treatment4	AGR1485
0	675	701	776	794	750	602	725	540	776	611	865	598	1356	1440	1266	1308	1317	1258
	695	750	690	717	667	717	775	771	598	584	751	750	1145	1500	1256	1570	1172	1440
	710	740	779	744	879	647	696	624	679	596	799	615	1270	1361	1322	1336	1501	1364
	615	696	735	662	754	778	750	765	704	662	647	725	1358	1351	1541	1266	1094	1260
2	488	528	438	664	473	540	635	443	505	395	443	491	912	928	1275	943	1031	965
	661	558	543	618	422	573	555	536	409	311	535	616	825	976	1098	975	745	934
	601	330	661	554	455	558	425	430	323	316	481	507	1021	1114	1170	1048	914	998
	520	507	619	483	485	535	604	548	393	316	362	545	987	1111	940	823	800	973
4	483	609	560	740	542	567	696	438	584	460	549	566	1046	1080	1384	1122	1265	1106
	605	682	624	676	464	629	660	596	462	370	599	695	1006	1189	1284	1251	964	1209
	587	484	749	630	560	628	508	506	416	389	536	575	1172	1282	1455	1318	1106	1227
	495	575	725	555	553	606	680	586	504	406	410	603	1142	1292	1111	1067	1068	1114
6	540	610	612	836	564	545	650	484	609	499	565	512	1080	1183	1491	1207	1195	1199
	623	696	640	729	490	609	639	666	511	416	634	626	1071	1307	1417	1368	935	1310
	599	559	777	667	592	593	494	527	475	441	599	528	1192	1399	1586	1392	1049	1292
	545	619	740	616	572	613	645	620	566	454	453	549	1211	1424	1196	1130	1064	1134
8	528	622	658	776	605	550	601	504	642	533	567	518	1015	1167	1483	1212	1187	1216
	628	716	626	683	498	616	602	704	542	475	618	604	1075	1309	1393	1403	938	1309
	619	572	732	630	620	597	484	555	516	484	571	534	1182	1449	1581	1401	1099	1302
	540	616	719	578	595	609	627	687	610	498	458	551	1231	1407	1227	1179	1078	1138
10	558	607	628	787	589	554	609	511	637	527	591	537	981	1027	1344	1164	1092	1217
	625	728	606	656	491	618	629	716	576	487	654	645	1072	1129	1294	1331	929	1336
	618	603	710	611	624	602	504	585	565	498	616	557	1197	1314	1462	1269	1097	1316
	546	594	683	559	560	633	625	679	647	518	466	574	1228	1299	1144	1134	1075	1139
12	515	554	625	769	571	545	577	464	607	489	589	542	1187	952	1325	1199	1121	1349
	627	703	580	627	466	620	599	651	552	478	627	626	1141	1056	1318	1352	952	1460
	623	597	675	569	608	603	492	566	549	476	592	545	1287	1255	1420	1282	1141	1433
	535	571	665	548	545	650	595	621	615	507	468	560	1338	1414	1122	1159	1123	1249

Table 2.8 Effect of metabolites (AGR1487) on TEER across Caco2 monolayers over time.

	Assay 1						Assay 2						Assay 3					
	Resistance (ohms)																	
Time	Control	Treatment1	Treatment2	Treatment3	Treatment4	AGR1487	Control	Treatment1	Treatment2	Treatment3	Treatment4	AGR1487	Control	Treatment1	Treatment2	Treatment3	Treatment4	AGR1487
0	675	719	694	794	727	730	725	715	618	775	731	675	1356	1600	1632	1815	1327	1191
	695	947	583	856	806	820	775	830	641	867	823	659	1145	1606	1622	1573	1378	1261
	710	794	905	761	798	672	696	667	762	681	760	672	1270	1572	1533	1467	1483	1275
	615	779	744	672	740	659	750	699	818	656	693	597	1358	1483	1733	1230	1723	1277
2	488	565	612	600	564	487	635	562	379	394	425	469	912	1007	1284	870	999	796
	661	659	516	525	594	653	555	487	544	425	468	475	825	1113	1120		963	843
	601	595	715	505	538	492	425	485	438	396	333	448	1021	1071	1306	920	1182	623
	520	626	656	420	482	451	604	490	483	395	359	419	987	1245	1262	673	937	784
4	483	650	660	702	673	562	696	679	448	545	500	547	1046	1361	1651	1213	1204	854
	605	759	578	675	697	724	660	592	650	574	590	572	1006	1430	1509		1164	971
	587	679	822	606	630	560	508	603	552	508	454	549	1172	1325	1670	1210	1400	645
	495	712	743	516	619	462	680	574	602	480	440	505	1142	1566	1661	957	1289	799
6	540	697	747	784	683	462	650	735	533	663	562	507	1080	1484	1693	1384	1248	552
	623	866	614	796	708	582	639	654	742	704	670	571	1071	1482	1604		1131	829
	599	773	916	691	651	501	494	630	651	604	523	552	1192	1350	1626	1271	1364	471
	545	807	805	613	630	368	645	629	732	555	511	489	1211	1514	1697	1016	1320	579
8	528	687	673	720	727	348	601	686	484	638	519	337	1015	1426	1592	1355	1247	177
	628	828	566	757	717	365	602	618	709	702	620	337	1075	1408	1578		1154	452
	619	712	869	663	683	401	484	622	654	592	524	335	1182	1252	1591	1219	1368	185
	540	797	735	587	660	253	627	589	682	541	502	311	1231	1387	1596	981	1372	236
10	558	695	655	706	708	213	609	654	455	625	514	298	981	1396	1516	1288	1135	71
	625	798	524	733	722	188	629	601	663	669	626	316	1072	1286	1375		1109	176
	618	696	853	649	669	273	504	584	647	574	528	324	1197	1173	1457	1160	1349	63
	546	756	698	568	675	152	625	589	680	545	524	297	1228	1231	1487	907	1365	77
12	515	649	628	709	702	109	577	550	443	570	525	266	1187	1440	1546	1318	1225	35
	627	753	508	724	712	93	599	564	618	620	625	295	1141	1226	1458		1146	67
	623	678	829	608	665	145	492	516	596	539	544	295	1287	1116	1507	1181	1304	35
	535	727	668	558	679	84	595	530	621	507	529	272	1338	1220	1572	928	1416	32

Table 2.9 Effect of live and UV-killed bacteria on ^{3}H-mannitol (% mannitol crossed from culture Transwell to basal compartment) permeability across Caco-2 monolayers over time.

	Assay 1						
Time	0	2	4	6	8	10	12
Live AGR1485	0	1.3941	3.6857	4.1547	5.6537	5.7387	6.5586
UV-killed AGR1485	0	1.6745	3.151	3.3979	3.6711	4.2977	4.7023
Live AGR1487	0	1.5073	4.0926	5.7124	8.1492	11.6763	17.3649
UV-killed AGR1487	0	1.6744	4.9334	4.9719	5.4476	6.2663	7.3064
Control	0	1.4173	3.1059	3.8514	4.1619	4.4326	5.1182
UV-treated Control	0	1.3652	2.455	3.2292	3.7861	4.4332	4.9847
	Assay 2						
Time	0	2	4	6	8	10	12
AGR1485	0	0.3067	3.1946	3.0851	3.5955	3.0617	3.2541
AGR1485UV	0	1.2428	2.6912	1.9129	2.0777	2.2417	2.4892
AGR1487	0	0.4805	2.5311	2.8273	3.5606	0.926	2.0236
AGR1487UV	0	1.1511	4.3815	3.5223	3.3368	4.1891	4.4753
Control	0	0.1989	1.7016	1.999	2.1134	1.7643	2.4566
ControlUV	0	0.4055	1.4755	1.4122	1.1929	1.4965	1.5457
	Assay 3						
Time	0	2	4	6	8	10	12
AGR1485	0	0.177073	1.844403	1.781183	2.075863	1.767673	1.878756
AGR1485UV	0	0.717531	1.553765	1.104413	1.199561	1.294246	1.43714
AGR1487	0	0.277417	1.461331	1.632342	2.055713	0.534626	1.168326
AGR1487UV	0	0.664588	2.52966	2.033601	1.926502	2.418578	2.583816
Control	0	0.114835	0.982419	1.154123	1.220172	1.018619	1.418319
ControlUV	0	0.234116	0.85188	0.815334	0.688721	0.864005	0.89241

Raw Data from Chapter 4 TEER Experiments:

Table 3.1 Effect of AGR1485 cell lysate supernatant (CL SN) and cell wall on TEER across Caco-2 monolayers over time.

	Assay 1				Assay 2				Assay 3			
	Resistance (ohms)											
Time	Control	AGR1485	AGR1485 CL SN	AGR1485 Cell wall	Control	AGR1485	AGR1485 CL SN	AGR1485 Cell wall	Control	AGR1485	AGR1485 CL SN	AGR1485 Cell wall
0	1410	1540	1670	2790	4820	4920	4380	3540	3230	3160	938	3060
	2300	1570	2090	3000	4200	4350	4580	4600	2810	3010	863	2540
	1540	1580	1810	2960	4170	4400	4340	4340	2920	3080	986	2900
	1810	2600	1670	3050	3760	4760	2890	3420	3310	3190	879	2560
2	709	795	813	770	1270	1870	774	656	1395	2680	508	1550
	1003	699	580	870	1370	1440	1248	620	1590	1780	630	1140
	744	616	601	590	1490	1330	1409	593	1750	1700	632	1280
	836	592	632	680	850	1230	458	484	1165	1850	592	880
4	866	997	1113	1810	1925	2700	1329	850	1930	2780	591	1290
	1369	974	873	1430	2260	2260	2170	860	2090	2290	790	1300
	994	932	859	1590	2370	2040	2470	720	2180	2250	776	830
	1100	1199	885	1270	1250	1890	727	620	1790	2490	812	1010
6	1087	1179	1321	2720	2300	3000	1940	1060	2230	2840	639	1740
	1643	1232	1238	1810	2690	2700	2880	1130	2300	2650	870	1680
	1107	1186	1149	2010	2980	2390	3140	900	2340	2560	854	1080
	1332	1645	1103	1590	1540	2360	982	720	2300	2780	890	1200
8	1137	1283	1385	2100	2470	3110	2370	1140	2130	2810	707	1535
	1653	1342	1492	1770	2890	2990	3140	1320	2170	2750	952	1468
	1115	1298	1372	2000	2930	2730	3290	1050	2230	2640	907	1040
	1342	1886	1258	1400	1620	2930	1080	740	2290	2860	963	970
10	1060	1290	1362	1802	2590	3050	2620	1180	1970	2740	704	1312
	1661	1345	1556	1655	2880	3040	3150	1310	2020	2710	903	1055
	1129	1216	1415	1811	2840	2780	3230	1080	2020	2650	882	876
	1387	1908	1294	899	1630	2820	1130	720	2170	2830	953	650
12	1076	1264	1271	1168	2550	3020	2520	1160	2080	2700	662	1133
	1704	1381	1545	1440	2890	3180	2910	1310	1950	2720	843	713
	1097	1236	1419	1478	2860	2840	2900	1090	1930	2650	834	758
	1298	1879	1263	398	1620	2860	1110	700	2120	2840	870	488

Table 3.2 Effect of AGR1487 cell lysate supernatant (CL SN) and cell wall on TEER across Caco-2 monolayers over time.

	Assay 1				Assay 2				Assay 3			
	Resistance (ohms)											
Time	Control	AGR1487	AGR1487 CL SN	AGR1487 Cell wall	Control	AGR1487	AGR1487 CL SN	AGR1487 Cell wall	Control	AGR1487	AGR1487 CL SN	AGR1487 Cell wall
0	1410	2180	1540	4250	4820	5100	5930	5430	2120	2010	5930	2370
	2300	1390	1580	4200	4200	4670	5190	4650	2200	2220	5190	2220
	1540	1950	2210	4010	4170	2980	5680	4730	2360	2640	5680	2860
	1810	1800	1810	5390	3760	4920	5820	5510	2180	2010	5820	2490
2	709	1064	675	620	1270	2420	779	612	972	1517	779	967
	1003	528	741	593	1370	2540	1082	464	1520	1607	1082	1081
	744	508	859	687	1490	970	856	700	1093	1120	856	700
	836	676	1000	638	850	660	880	472	874	1546	880	1126
4	866	1404	966	820	1925	2980	1540	850	1450	2060	1540	1084
	1369	755	1075	650	2260	2870	2000	580	2050	2080	2000	1298
	994	858	1317	800	2370	1330	1690	940	1660	1660	1690	796
	1100	938	1321	700	1250	860	2120	690	1170	2030	2120	1290
6	1087	1582	1197	1100	2300	3150	2750	1240	1541	2260	2750	1028
	1643	951	1329	730	2690	2900	3340	720	1996	2280	3340	1265
	1107	1192	1633	890	2980	1660	3360	1250	1833	1850	3360	824
	1332	1189	1557	800	1540	1040	4370	980	1296	2160	4370	1219
8	1137	1640	1217	1200	2470	3000	2690	1470	1759	1690	2690	631
	1653	927	1400	710	2890	2750	3090	800	2060	2530	3090	849
	1115	1367	1679	890	2930	1560	3350	1440	1970	1850	3350	652
	1342	1223	1587	810	1620	1000	3910	1160	1420	1390	3910	599
10	1060	1500	1133	1150	2590	2830	2840	1410	1780	3090	2840	168
	1661	806	1322	670	2880	2560	3230	830	1920	1482	3230	248
	1129	1268	1585	850	2840	1630	3440	1470	1950	666	3440	279
	1387	1053	1504	790	1630	1060	3870	1240	1450	180	3870	129
12	1076	797	1198	1050	2550	1530	3540	1710	1760	91	3540	66
	1704	540	1396	600	2890	920	3750	780	1770	390	3750	90
	1097	852	1658	770	2860	680	4020	1380	1840	136	4020	111
	1298	641	1580	760	1620	790	4480	1180	1400	46	4480	45

Table 3.3 Effect of AGR1485 cell wall fractions on TEER across Caco-2 cell monolayers over time.

	Assay 1						Assay 2						Assay 3					
	Resistance (ohms)																	
Time	Control	AGR1485	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Control	AGR1485	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Control	AGR1485	Fraction 1	Fraction 2	Fraction 3	Fraction 4
0	3820	4020	4720	4660	4400	4200	2560	3380	3550	3160	3320	4310	3630	3870	3600	3580	3600	4010
	3600	4210	4140	4580	5090	4230	2870	2940	3580	3670	3780	3630	3870	4170	4020	4120	4140	3460
	4250	4660	4360	4400	4440	4030	3050	2980	3290	2970	3620	4110	4030	4170	3600	3760	3800	3440
	3990	3530	4240	4510	4520	4400	2730	3550	3510	3530	3470	3960	3800	3850	3970	4050	4200	3990
2	1630	2140	786	1950	1800	2050	2180	2160	2450	2430	2380	1090	1890	3000	1270	2030	1580	680
	1700	2350	2040	2820	2570	2260	2590	2550	2510	2560	2450	1060	2380	2330	1370	2140	1100	540
	1110	1510	2010	1900	1910	2430	1610	2000	2090	2580	2370	1640	1090	2090	1200	2670	1890	600
	1750	1680	1190	1420	1980	2020	2680	2940	2200	2750	2240	780	2370	1510	1330	2270	1250	530
4	2150	2750	1000	2680	2460	2530	2300	2560	2620	2370	2390	1810	2500	2980	1280	1400	1850	940
	2320	3120	2660	3050	3000	2530	2750	2800	2680	2700	2540	1480	2810	2900	1340	1520	1380	610
	1760	2190	2660	2580	2580	2760	2110	2410	2260	2470	2490	2320	1860	2440	1320	1540	1890	670
	2130	2510	1770	1970	2560	2420	2610	3070	2500	2690	2340	1070	2720	1810	1310	1620	1620	710
6	2240	2620	1040	2660	2290	2450	2390	2860	2650	2590	2470	2370	2460	2530	1480	1340	2070	1520
	2450	2800	2610	2620	1570	2400	2760	3030	2850	2970	2680	1680	2640	2660	1570	1370	1710	940
	2050	2580	2600	2620	2420	2590	2370	2660	2430	2610	2720	2620	2170	2290	1570	1390	1950	1050
	2700	2700	1990	2120	2390	2480	2710	3150	2820	2890	2480	1260	2660	1790	1530	1480	1930	1190
8	2160	2650	860	2380	1940	2070	2030	2540	2100	2170	2130	2330	2330	2500	1690	1560	2160	2410
	2200	3080	2230	1930	410	1960	2280	2700	2340	2530	2300	1510	2430	2610	1760	1540	2100	1750
	2130	2750	2270	2270	1720	2150	2080	2500	2150	2310	2360	2330	2320	2280	1890	1460	2260	1950
	2640	2780	1960	1970	1570	2220	2370	2900	2420	2420	2200	1230	2460	1750	1880	1610	2280	1980
10	2070	2560	760	2230	1690	1820	1960	2620	2240	2140	2080	2320	2540	2300	1720	1570	2210	2780
	1990	2860	2150	1460	110	1520	2190	2750	2420	2410	2080	1340	2370	2450	1800	1410	2110	2300
	2030	2730	2130	1980	1070	1770	1860	2470	2180	2240	2090	2230	2320	2190	2020	1410	2230	2420
	2470	2850	1980	1830	890	1960	2210	2940	2450	2380	2010	1280	2350	1550	1920	1540	2300	2280
12	2040	2520	650	2060	1500	1840	1680	2280	2020	1940	1890	2130	2140	3370	1780	1660	2040	2910
	1910	2890	1940	1090	50	1240	1800	2370	2130	2000	1710	1130	2130	3130	1850	1400	2060	2530
	1990	2790	1940	1690	630	1520	1460	2280	1960	2010	1700	1980	2160	3140	1940	1300	2100	2560
	2400	2830	1880	1640	690	1850	1900	2570	2190	2140	1720	1290	2140	2830	1880	1460	2030	2380

Table 3.4 Effect of AGR1487 cell wall fractions on TEER across Caco-2 cell monolayers over time

	Assay 1						Assay 2						Assay 3					
	Resistance (ohms)																	
Time	Control	AGR1487	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Control	AGR1487	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Control	AGR1487	Fraction 1	Fraction 2	Fraction 3	Fraction 4
0	2560	2620	3910	4020	3420	3900	3630	4460	3570	3830	4580	5030	4260	4680	3950	4120	4490	3760
	2870	2380	4070	3970	4260	4540	3870	3880	3770	3920	4510	4600	3950	4100	4510	4290	4590	4440
	3050	2380	3680	4350	3980	4030	4030	4380	3750	3660	5030	5350	3860	4640	4370	4150	4350	4260
	2730	2780	4220	4080	3800	3950	3800	4260	3830	4170	4650	4320	3940	4490	4580	4580	4990	4010
2	2180	1000	1740	1280	1220	1210	1890	2650	1960	2470	1650	1360	2500	2700	2000	522	503	930
	2590	1260	2600	2180	720	1360	2380	2180	2450	1870	1880	2090	2310	3590	2080	402	557	1610
	1610	990	2280	1460	820	1320	1090	2350	2480	2160	1330	1600	1150	2520	2160	379	452	1450
	2680	1470	1780	880	1000	1380	2370	2490	1870	1950	1330	1800	2080	2760	1720	464	486	1440
4	2300	1270	1630	1620	1600	830	2500	2820	2980	3310	2730	2240	3690	4040	2450	458	684	1030
	2750	1720	2170	2800	960	920	2810	2980	3180	2780	2810	2890	3720	4400	2590	215	616	1490
	2110	1350	1950	1820	1120	780	1860	3030	3360	2970	2360	2580	1580	4050	2670	251	572	1420
	2610	1920	2130	1310	1540	960	2720	2900	2420	3030	2330	2620	3650	4170	2320	305	572	1370
6	2390	1170	1720	1710	2090	820	2460	2840	2600	2820	2460	2200	2850	3320	2610	729	1028	1150
	2760	1820	2010	2870	1350	890	2640	2980	2850	2640	2490	2540	2920	3560	2690	331	940	1490
	2370	1480	1990	1940	1530	680	2170	3060	2940	2640	2330	2520	1390	3510	2850	393	907	1480
	2710	1960	2260	1640	2060	1000	2660	2890	2190	2800	2290	2390	2850	3520	2600	433	860	1340
8	2030	322	1500	1440	2420	1010	2330	620	2500	2690	2240	2110	2640	3100	2280	1025	1399	1140
	2280	1336	1730	2380	1640	1070	2430	1580	2620	2550	2270	2260	2620	3530	2320	262	1155	1120
	2080	1258	1720	1860	1840	760	2320	2360	2670	2480	2210	2350	1320	3490	2400	304	1089	1310
	2370	923	1880	1670	2440	1180	2460	1510	2110	2620	2200	2190	2620	3590	2320	431	1119	960
10	1960	92	1630	1440	2490	1100	2540	150	2360	2410	2020	2060	2340	2750	2070	1960	2280	1180
	2190	476	1920	2340	1770	1150	2370	240	2490	2260	2060	2090	2330	3190	2120	194	1940	990
	1860	599	1910	1840	2050	790	2320	610	2540	2200	2000	2190	1250	3230	2320	237	1980	1210
	2210	215	2220	1690	2670	1220	2350	330	2100	2310	2040	2060	2350	2970	2220	457	1820	790
12	1680	32	1680	1410	2560	1310	2140	30	2320	2270	1850	2020	2230	360	1940	2350	1090	14
	1800	107	1850	2060	1820	1080	2130	40	2350	2100	1930	2000	2190	2070	1860	109	2540	14
	1460	94	1920	1690	2050	720	2160	70	2430	2040	1880	2050	1240	2120	2080	151	2320	16
	1900	45	1940	1690	2650	1260	2140	80	1950	2080	1900	2000	2230	650	2020	465	1940	15

Table 3.5 Effect of AGR1487 fraction 4 (applied at different concentrations) on TEER across Caco-2 cell monolayers over time (blank cells represent damaged Transwells).

	Assay 1						Assay 2						Assay 3					
	Resistance (ohms)																	
Time	Control	AGR1485	AGR1487	Fr4 (0.45mg/mL)	Fr4 (0.127 mg/mL)	Fr4 (0.025 mg/mL)	Control	AGR1485	AGR1487	Fr4 (0.45mg/mL)	Fr4 (0.127 mg/mL)	Fr4 (0.025 mg/mL)	Control	AGR1485	AGR1487	Fr4 (0.45mg/mL)	Fr4 (0.127 mg/mL)	Fr4 (0.025 mg/mL)
0	1831		1973	2150		2300	1347	1382	815	1324	1541	1429	1473	1743	1502	1551	1471	1560
	1984	2130	870	2230	2500	2370	1498	1554	1501	1295	1644	1453	570	1410	1733	1238	1777	1812
	2260	2110	1866	2050	2310	2090	1346	1391	1613	1383	1465	1398	1503	715	1532	1808		1758
	2100	2020	2090	2120	2430	2310	1393	1583	1557	1140	1576	1492	1675	1747	895	1870	1564	1576
2	1544		1165	2640		2470	1356	1179	311	1864	1789	1664	1356	1670	1029	2200	693	1659
	1674	1953	549	2520	2560	2320	1526	1337	784	2050	1817	1687	548	1465	1008	1648	1816	1884
	1859	1865	737	2590	2390	2210	1363	1163	822	1952	1686	1603	1405	690	814			1658
	1769	1759	826	2430	2360	2300	1301	1387	539	1647	1705	1782	463	1732	679	2300	1326	1579
4	1697	2250	496	2830		2760	1446	1549	142	1948	1882	1775	1382	1749	236	2130	740	1652
	1902	2220	242	2780	2730	2670	1663	1801	223	2060	1911	1804	552	1553	306	1720	1860	1930
	1965	1994	327	2690	2600	2420	1465	1672	194	2070	1751	1713	1543	715	220			1776
	1936		325	2660	2660	2600	1451	1738	180	1722	1789	1848	479	1814	279	2410	1396	1649
6	1808	2350	79	2850		2810	1553	1586	47	1895	1907	1808	1804	1907	38	2240	779	1791
	2070	2310	45	2870	2820	2830	1666	1899	21	1988	1862	1769	612	1644	49	1830	2120	2060
	2080	2090	53	2670	2690	2570	1501	1692	23	1993	1689	1743	1702	754	42			1920
	2010		56	2680	2780	2700	1532	1813	32	1636	1747	1821	490	2050	43	2480	1502	1800
8	1927		44	3070		2970	1717	1706	45	1929	2090	1973	1754	1904	27	2120	801	1739
	2180	2660	26	2770	2750	3040	1816	1995	22	2070	1389	1891	388	1644	30	1800	2200	2040
	2150	2380	31	2680	2720	2730	1578	1757	22	2140	1776	1862	1670	739	28			1970
	2080	2190	33	2680	2850	2830	1617	1886	6	1756	1844	1867	504	1930	28	2440	1496	1780
10	1968		28	3320		2870	1749	1662	21	1924	2100	2100	1835	2210	19	2100	790	1678
	2170	2590	20	2750	2640	3010	1847	1813	19	2060		1966	606	1840	27	1840	2080	1986
	2060	2350	21	2700	2620	2620	1572	1776	19	2080	1772	1924	1833	790	25			1934
	2120	2160	22	2670	2740	2810	1653	1980	6	1815	1843	1879	528	2130	25	2380	1553	1720
12	1939		23	2790		2830	1814	1872	5	1912	1739	1979	1707	1762	13	2050	819	1660
	2100	2550	17	2620	2300	2840	1902	1944	4	1974		1931	596	1649	23	1830	2080	1990
	2000	2240	18	2580	2430	2080	1579	1733	0	2050	1737	1760	1743	737	22			1860
	2010	1970	19	2600	2610	2630	1668	1891	13	1783	1813	1785	514	1947	23	2340	1510	1570

DRC 16

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Ranjita Sengupta

Name/Title of Principal Supervisor: Warren McNabb

Name of Published Research Output and full reference:

The role of cell surface architecture of lactobacilli in host-microbe interactions in the gastrointestinal tract. Mediators of Inflammation. Volume2013, Article ID 237921, 16 pages.

In which Chapter is the Published Work: Chapter 1, Review of literature

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The article was published as a critical review in a special issue on "Mediators of inflammation & immune responses in the human gastrointestinal tract".

Ranjita Sengupta (digitally signed)

Candidate's Signature

27th June, 2014

Date

Warren McNabb (digitally signed)

Principal Supervisor's signature

30th June, 2014

Date

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