Identification and functional characterisation of a novel surface protein complex of *Lactobacillus rhamnosus*

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Wesley Xingli Wen

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Abbreviations

Amp	Ampicillin
APF	Aggregation-promoting factor
Big-3	Bacterial immunoglobulin-like domain type-3
cfu	colony-forming unit
Cm	Chloramphenicol
COG	Cluster of orthologous genes
CWBD	Cell wall-binding domain
DC	Dendritic cell

DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epithelial growth factor
EGFR	Epithelial growth factor receptor
Em	Erythromycin
FRDC	Fonterra Research and Development Centre
GALT	Gut-associated lymphoid tissue
GIT	Gastrointestinal tract
GlcNAc	*N*-acetylglucosamine
h	hour(s)
HRP	Horse radish peroxidase
IEC	Intestinal epithelial cell
kbp	kilobase pair
LAB	Lactic acid bacteria
LTA	Lipoteichoic acid
MAMP	Microorganism-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MBP	Maltose-binding protein
min	minute(s)
MRS	Man-Rogosa-Sharpe
MurNAc	*N*-acetylmuramic acid
NF-κB	Nuclear factor κB
NLR	Nucleotide-binding oligomerisation domain-like receptor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pfu	plaque-forming unit
PG	Peptidoglycan
PPs	Phagemid particles
PR-1	Pathogenesis response domain 1
PRR	Pattern recognition receptor

PS	Polysaccharides
sec	seconds
Str	Streptomycin
TA	Teichoic acid
TBS	Tris-buffered saline
TBST	TBS-Tween
Tet	Tetracycline
TJ	Tight junction
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
v/v	volume/volume
w/v	weight/volume
WPS	Wall polysaccharide

Abstract

Proteins are the most diverse structures on bacterial surfaces; hence they are candidates for species- and strain-specific interactions of bacteria with the host, environment and other microorganisms. In probiotic bacteria, some surface and secreted proteins mediate interactions with the host and may consequently contribute to the health-promoting effects. However, a limited fraction of surface-associated proteins from probiotic bacteria have been functionally characterised to date. A secreted protein of *Lactobacillus rhamnosus* HN001, SpcA, containing two bacterial immunoglobulin-like domains type 3 (Big-3) and a domain distantly related to plant pathogen response domain 1 (PR-1-like), was previously shown to bind to HN001 cells, however the nature of its ligand on the surface of the cells was unknown. In this study, a series of binding assays first demonstrated that SpcA binds to a cell wall anchored protein of HN001. Next, the SpcA-"docking" protein, named SpcB, was identified using phage display. SpcB is a 3275-residue cell-surface protein that has all the features of large glycosylated serine-rich adhesins/fibrils from Gram-positive bacteria, including the hallmark glycoprotein signal sequence motif KxYKxGKxW and the cell wall anchor motif LPxTG. The *spcA* and *spcB* genes are located in a gene cluster, *spcBCDA,* which is present in 94 out of 100 strains of *L. rhamnosus* species and some strains of *L. casei* and *L. paracasei* whose genome sequences have been determined, but was absent from other *Lactobacillus* clades. To confirm the role of SpcB as the SpcA anchor and investigate the roles of these two proteins in surface properties of probiotic *L. rhamnosus* strains HN001 and GG, stable double-crossover mutations of these two genes were constructed. Binding assays to *L. rhamnosus* mutant cells confirmed dependence on SpcB in both GG and HN001 strains. Comparison of the wild-type and mutant surface properties suggested that SpcB in GG interferes with biofilm formation and aggregation, while it might contribute to the protective effect against TNFα-mediated disruption of the polarised Caco-2 cell monolayer integrity. Deletion of HN001 *spcB* or *spcA* had no effect on functions other than the SpcA binding. Our findings indicate that the roles of a surface protein can vary considerably among the strains of a species, requiring functional data to validate the bioinformatics-based hypotheses.

Chapter Ia: Lactobacilli as probiotics

1.1 Human health and probiotic bacteria

1.1.1 Anatomy and functions of the human intestinal epithelium

The human intestine plays important roles not only in food digestion and nutrient absorption but also in protection of human body from invasion by pathogens and absorption of inflammation- and allergy-causing food ingredients. Human intestinal epithelium is a mucus-covered cell monolayer and the majority of cells in gut epithelium are enterocytes. In addition, the gut epithelium also contains goblet and Paneth cells (Figure 1.1). Apart from their major structural role in the intestinal epithelium, the enterocytes carry out the key functions in nutrient absorption and processing. The goblet cells are responsible for mucus formation by producing the epithelium-protective mucin glycoproteins [1, 2] and for delivery of luminal antigens to the adjacent dendritic cells, generating regulated immune responses [3]. The Paneth cells have a dominant role in the innate immune response, producing antimicrobial factors that mediate defence against the intestinal pathogens [4]. Importantly, the human intestinal epithelium is also in close contact with the immune cells (Figure 1.1). Underneath the intestinal epithelium there is a second layer, called lamina propria, containing the lymphoid tissues such as the Peyer's patches. The Peyer's patch has a follicle centre and is covered by an "M cell"-containing epithelium. Luminal antigens can be taken up by an M cell and subsequently delivered to the immune cells (B cells, T cells, macrophage and dendritic cells) in the lamina propria. Activation of the immune cells ultimately results in the immune responses, such as modulated phagocytosis and production of specific antibodies and cytokines.

1.1.2 Human intestinal microbiota

The human gastrointestinal tract (GIT) has a dynamic and complex microbial community containing 10^{13}-10^{14} microorganisms which belong to several hundreds of species, consisting of bacteria, viruses, fungi, and archaea [5, 6]. This microbial ecosystem is established at birth and subsequently progresses towards a mature and stable microbial community. The composition and diversity of the human GIT microbiota is influenced

by both the host genetic factors and environmental factors [7]. Common environmental factors include age, diet, antibiotic usage and the intestinal health status [8].

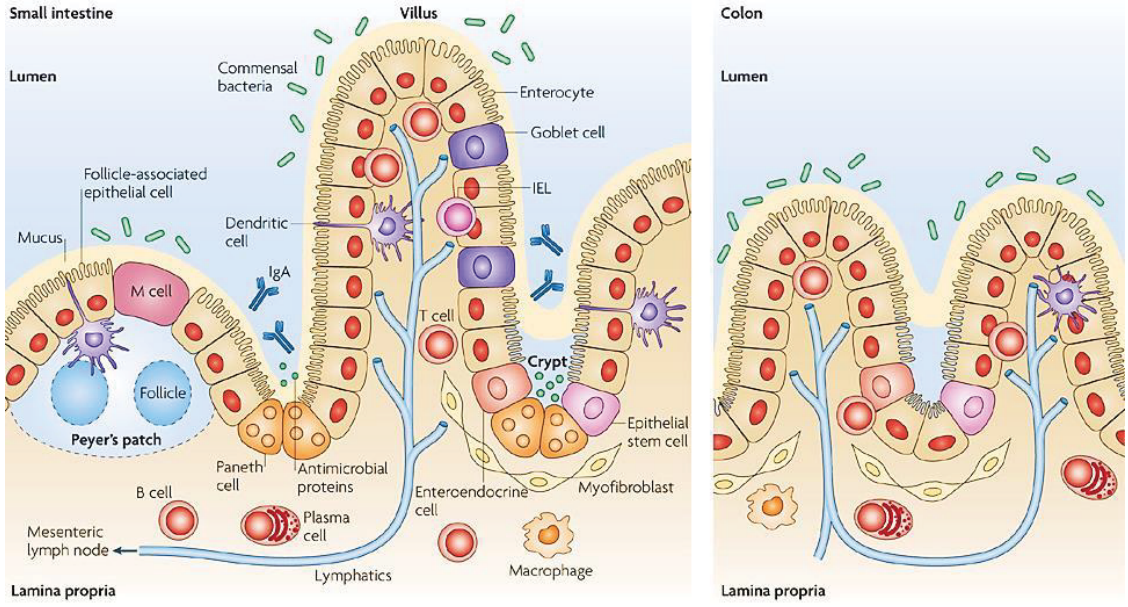

Figure 1.1. Anatomy of the intestinal immune system

A single layer of intestinal epithelial cells (IECs) provides a physical barrier that separates the trillions of commensal bacteria in the intestinal lumen from the underlying lamina propria. The IECs lining the lumen are bathed in nutrients, commensal bacteria, IgA and goblet cell-produced mucus. Epithelial stem cells proliferate and give rise to daughter cells with the potential to proliferate. These IECs then differentiate into villous or colonic enterocytes, which absorb nutrients (small intestine) and water (colon). In addition to differentiated enterocytes and goblet cells, progenitor IECs differentiate into both enteroendocrine cells, which secrete enteric hormones, and Paneth cells at the base of the small intestinal crypts. Beneath the IECs, the lamina propria is made up of stromal cells (myofibroblasts), B cells (especially IgA-producing plasma cells), T cells, macrophages and dendritic cells. Certain subsets of T cells and dendritic cells localise between the IECs. The small intestine has regions of specialized epithelium termed follicle-associated epithelium and microfold (M) cells that overlie the Peyer's patches and sample the intestinal lumen. IEL, intraepithelial lymphocyte. Adapted from [9] with permission.

The majority of gut microbe species are not cultivable using the current laboratory microbiological techniques, due to the complexity of their natural habitat. Bacteria, in particular the members of *Firmicutes* and *Bacteroidetes*, are thought to form the majority

of human GIT microbiota according to the recent studies that assessed the diversity and abundance of microbial species based on the 16S ribosomal DNA as a phylogenetic marker, using the next-generation sequencing technologies [5, 6, 10, 11]. Interestingly, a recent study suggested that statistically, according to the GIT bacterial composition, gut microbiomes in different individuals can be organised in clusters of similarities, designated enterotypes, which are represented by the composition of three dominant genera including *Bacterioides*, *Prevotella* and *Ruminococcus* [6]. Enterotypes are distinguishable between individuals through the significant variation of one of the three genera. The presence of each enterotype may reflect complex and specific interactions between the gut microbes and the human host. However, the validity of this method needs to be confirmed by further studies.

1.1.3 Implications of the GIT microbiota on human health

The homeostasis between resident microbiota and the host epithelial cells is important for maintaining human health. Firstly, the intestinal bacteria provide human host with absorbable nutrients. For example, some complex sugars directly consumed from the diet are relatively unabsorbable for the human host. With help from the gut microbes, those sugars can be degraded down to absorbable substances [12]. The members of intestinal microbiota can also synthesize various vitamins such as biotin and folate that are needed by the human host [6]. Secondly, the intestinal bacteria protect the gut epithelium from being attacked by the invading pathogens. Some gut bacteria secrete inhibitory substances such as bacteriocins, which directly inhibit the growth of the pathogens [13]. Commensals also indirectly restrict the colonisation of GIT by pathogens and their growth through competition for the epithelial adhesion sites and essential nutrients. In addition, the gut microbiota is thought to have significant effects on the maturation of the human immune system. The “microflora hypothesis” proposes that insufficient exposure to the gut microbes at early age of the host can lead to “abnormal maturation” of the intestinal microbiota and impair the development of the immune system, causing allergy and inflammation [14].

In a healthy individual, the epithelium senses the integrity of the intestinal microbiota and remains “silent” (physiologically homeostatic) to the commensal microorganisms,

maintaining the normal physiological functions of the human GIT. Compositional alteration of the intestinal microbiota can result in a significant change of the human immunity and health [15]. There is accumulating evidence showing that several diseases such as diabetes, obesity, neurological diseases, intestinal cancer and inflammatory bowel disease, could be related to the altered intestinal microbiota [10, 16-21]. In order to investigate the relationships between the intestinal microbiota and the host disease states, large-scale studies on the healthy human GIT microbiota are essential. Currently, two ongoing projects, MetaHIT (the European Union Project on Metagenomics of Human Intestinal Tract) [22] and the NIH Human Microbiome Project (USA) [23], are running towards this goal.

1.1.4 Probiotic microorganisms and their health-promoting effects

As the compositional shift of the human intestinal microbiota is significantly correlated with the disease and health states, it is hypothesized that artificial modification of the intestinal microbiota towards the health state may not only have a prophylactic effect, "promoting" health, but also reduce the severity of a disease or resolve diseases and therefore be considered as a stand-alone therapy. One of the suggested approaches to consumers is to eat "Good" bacteria, collectively designated "Probiotics", to improve their state of health or to protect themselves from disease. Probiotics are defined by the Food and Agriculture Organisation of the United Nations and the World Health Organisations as "live microorganisms which, when consumed in adequate amounts as part of food, confer a health benefit on the host" [24]. Actually, besides the live organisms, it has also been reported that the dead cells of some probiotic bacterial species can have similar beneficial effects as the live bacteria [25]. Scientific reports on this group of "good" bacteria can be even tracked back to 100 years ago [26], indicating their importance to human health.

Probiotic strain candidates are often isolates from GIT or sometimes urogenital system of healthy individuals (humans), or are of food origin; to ascertain that they are not pathogenic. The selection criteria for a probiotic bacterium generally include GRAS (Generally Recognised As Safe) status, the capacity of adhesion and colonisation, and the potential health-promoting effects. Most probiotic strains are lactic acid bacteria (LAB)

that produce lactic acid as the main carbon-based fermentation product. The representative genera of those bacteria are *Bifidobacterium* and *Lactobacillus,* which contain most extensively studied and utilised probiotic strains. In particular, acidophilic, non-sporulating and facultatively anaerobic Gram-positive bacteria belonging to *Lactobacillus* genus are well characterised and documented. Other species such as Gram-negative *Escherichia coli* Nissle 1917 [27, 28] and yeast *Saccharomyces boulardii* [29] are also suggested to promote human health.

Up to now, probiotic bacteria have been widely studied for their health-promoting effects in regard to the gastrointestinal disorders, enteric and urogenital diseases caused by pathogenic bacteria, cancers, serum cholesterol clearance and allergy [30-32]. Although overall it is still not well-understood how probiotic bacteria mediate these effects in most diseases, three general molecular mechanisms have been proposed [33]: 1) Direct and/or indirect clearance of pathogenic bacteria. For example, lactic acid produced by LAB lowers the pH of the intestinal tract generating a chemically harsh niche that inhibits the growth of most invading pathogens. Some probiotic bacteria can also synthesise and secrete proteinaceous bacteriocins [34, 35] to defend against the pathogens. The production of bacteriocins is influenced by the bacterial growth conditions such as the composition, pH and temperature of media [35], suggesting that bacteriocin-driven probiotic effects may involve very complex interactions between bacteria and the human intestinal epithelium. Indirect pathogen-inhibitory measures of probiotic bacteria may include competition for the adhesion sites or essential growth factors. 2) Enhancement of the epithelial barrier. The human intestinal epithelium with the mucus layer is considered as the first line of defence against the invading pathogens. Maintenance of the epithelial layer integrity is essential for human health. Several studies have shown that probiotic bacteria can protect the epithelial layer integrity via various mechanisms, such as promoting mucus secretion [36, 37], reducing intestinal cell death [38] and improving tight junction function [39, 40]. 3) Modulation of the human host immunity. Probiotic bacteria have been shown capable of stimulating the human immune cells, increasing the production of health-protecting cytokines and/or reducing the production of pro-inflammatory cytokines [41, 42].

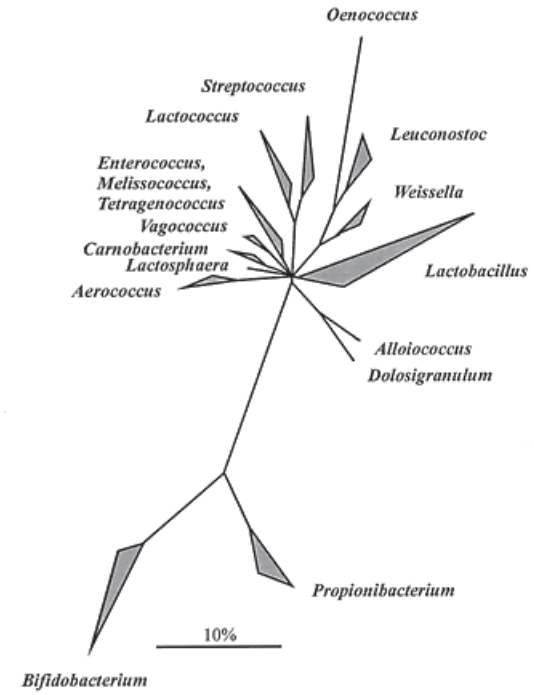

Figure 1.2. Phylogenetic tree of lactic acid bacteria

Consensus tree, based on comparative sequence analysis of 16S rRNA, showing the major phylogenetic groups of lactic acid bacteria (*Lactobacillus-Lactococcus* branch) with low mol% guanine plus cytosine in the DNA and the nonrelated Gram-positive genera *Bifidobacterium* and *Propionibacterium.* Adapted from [45].

1.1.5 Characteristics of lactic acid bacteria

LAB are metabolically similar Gram-positive bacteria belonging to about 20 genera, such as *Lactobacillus*, *Lactococcus*, *Aerococcus* and *Leuconostoc* (Figure 1.2) [43]. In addition to the Gram-staining feature, those bacteria are of rod or cocci morphology and non-sporulating. They ferment carbohydrates as energy source and produce lactic acid as main fermentation product so LAB strains have been broadly used in the food industry for centuries. The acid-tolerant feature of LAB is considered favourable in terms of their survival in the acidic regions of the human gut. Some LAB strains can also improve the texture, flavour and preservation of fermented food [44]. Even though *Bifidobacterium* species share the general features of LAB, they are phylogenetically distinguishable from the traditional LAB genera belonging to *Firmicutes* and have a high G+C content (55-67%) in their genomes, forming part of the *Actinomycetes* branch [43]. Furthermore, *Bifidobacterium* species grow poorly in the milk-based media and also ferment sugars in

a unique metabolic pathway that differs from the *Lactobacillus-Lactococcus* branch of LAB [45].

LAB are the main source from which probiotic bacteria are selected, mainly due to their commonly shared GRAS nature and ability to survive in the harsh human gut environment, which is anaerobic and acidic and contains high concentration of bile salts. The natural habitats of those LAB-origin probiotic bacteria are diverse, from various human body cavities to a broad range of food products such as dairy products, vegetables, poultry and fish. Among them, the members of *Lactobacillus* genus have been most widely studied as food fermenting bacteria and probiotics, in terms of the industrial applications and interactions with the human host.

1.2 Lactobacilli used as probiotics

1.2.1 General characteristics of lactobacilli

Lactobacilli share the common features of Gram-positive bacterial envelope and LAB metabolisms. In the laboratory, they grow well at 37℃ anaerobically, supplemented with 5% CO_2. The *Lactobacillus* genus belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and family *Lactobacillaceae*. More than 100 species have been discovered under this genus so far, forming the largest branch in the probiotics group. Lactobacilli are ubiquitous in various environments, from food to human body, where they can obtain sugars as energy sources. The composition and diversity of *Lactobacillus* species are often studied by the traditional bacterial cultivation method, DNA-DNA hybridisation and 16S rDNA-based analysis such as DGGE (Denaturing Gradient Gel Electrophoresis) and DNA sequencing. With the development and improvement of the next-generation sequencing methods, such as metagenomics and comparative genomic analysis, functional studies of *Lactobacillus* species have become possible. Therefore, in addition to the nucleic acid sequence-dependent analysis of specific phylogenetic markers such as 16S RNA, whole-genome-based methods focusing on the comparison of the gene content and conservation of core genes, COG (cluster of orthologous genes) greatly

improved our understanding of the diversity and function of *Lactobacillus* species in the human gut [46, 47].

Both cultivation- and molecular-biology-based studies suggest that lactobacilli such as *L. rhamnosus* and *L. casei* inhabit the infant intestinal tract within two months after birth [48, 49]. Diversity of lactobacilli in the human gut is very high and there are approximately 1,000 *Lactobacillus* phylotypes identified to date in this niche. The composition of *Lactobacillus* species in the gut can be influenced by the complex environmental factors. Although lactobacilli only represent a small proportion of the human intestinal microbiota, they play important roles in the human intestinal health and immunity.

1.2.2 Genomic features of *Lactobacillus*

At the time of writing (April 2015), more than 500 whole genome sequencing projects in regard to *Lactobacillus* genus have been carried out or are in progress according to the Bioproject Database of National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/bioproject) and the Gold Genomes Online Database (https://gold.jgi-psf.org/index). The published *Lactobacillus* genomes vary significantly in size, ranging from 1.29 Mbp (*L. iners* AB-1) to 3.35 Mbp (*L. plantarum* WCFS1) [50]. In some *Lactobacillus* species, one or more plasmids of various sizes exist, expanding the coding capacity of these *Lactobacillus* genomes. With the help of the sequenced and annotated *Lactobacillus* genomes, it has been shown that *Lactobacillus* genomes have extensive gene loss/decay and gene gain that are assumed to be due to the evolutionary adaption to their nutrient-rich niches [44]. For example, order *Lactobacillales* diverged from class *Bacilli* by the loss of 600-1,200 genes and gain of less than 100 genes [47, 51]. Many of these lost genes used to contribute to bacterial biosynthesis of cofactors and sporulation. These pathways are apparently not required by lactobacilli in their natural habitats such as dairy products and human gut that are rich in energy sources, cofactors and proteins.

Genetic acquisition is commonly observed in *Lactobacillus* genomes, such as gene duplication and horizontal gene transfer, in order to maximise the usage of nutrients from

the environment. Duplicated genes often encode carbohydrate-metabolic enzymes (e.g. enolase), proteolytic enzymes (e.g. peptidase) and transporters for carbohydrates and amino acids (e.g. phosphotransferase systems) [47]. Horizontal gene transfer systems also play important roles in moving genes between bacteria via mobile elements, such as plasmids, transposons, and prophages, which are widely found in *Lactobacillus* genomes [44, 51]. The genes on the mobile elements are often involved in transport and metabolism of nutrients, antibiotic resistance and bacteriocin production, which allow lactobacilli to adapt to a specific niche. For example, a megaplasmid of *L. salivarius*, pMP118, encodes 11% of total proteins contributing to bacteriocin production, resistance to bile salt and carbohydrate metabolism [52]. However, random DNA insertion into the genome (e.g via transposition) can sometimes cause the target chromosomal gene truncation that is observed in many lactobacilli such as *L. casei* ATCC393 [53], leading to further genomic and phenotypic diversity. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) as well as *cas* genes generate a prokaryotic immune system for bacteria from being attacked by bacteriophages or plasmids. The CRISPRs-related gene cluster was identified in some *Lactobacillus* genomes, such as *L. rhamnosus* ATCC53013 (GG), providing them with a potential to develop acquired phage resistance upon infection.

Recent comparative analyses of distantly and closely related *Lactobacillus* genomes have successfully demonstrated a method to identify niche-, species- or strain-specific genes or COGs. For example, the genes encoding mucus-adherent factors are more frequently found in the intestinal isolates (56%) compared to the dairy isolates (13%) [51, 54]. This will allow at least partial explanation of some specific probiotic traits of an individual strain. However, in comparison to the fast-pace development of evolutionary genomics, the progress in functional genomics is still unsatisfactory. Although many lactobacilli whose probiotic features have been demonstrated are widely used in the food fermentation products for their health benefit effects, the underlying molecular mechanisms are mostly unknown. The modern bioinformatics tools will allow functional prediction of genes, however the functions of significant proportion of genes are still unknown. Moreover, even for those genes that have bioinformatically assigned (proposed) functions, solid experimental evidence is still needed as the variability in the primary nucleic acid sequence of orthologous genes may lead to a novel or defective, rather than predicted function. For example, compared to *L. rhamnosus* GG, the percentage of genes shared by other *L. rhamnosus* strains is between 86.9% and 100%. However, 11 *L.*

rhamnosus human isolates demonstrated various metabolic phenotypes with respect to those shared metabolic pathways, suggesting mutations occurred in some of the genomes that inactivated or changed the pathways without the genes being physically eliminated from the genome [55].

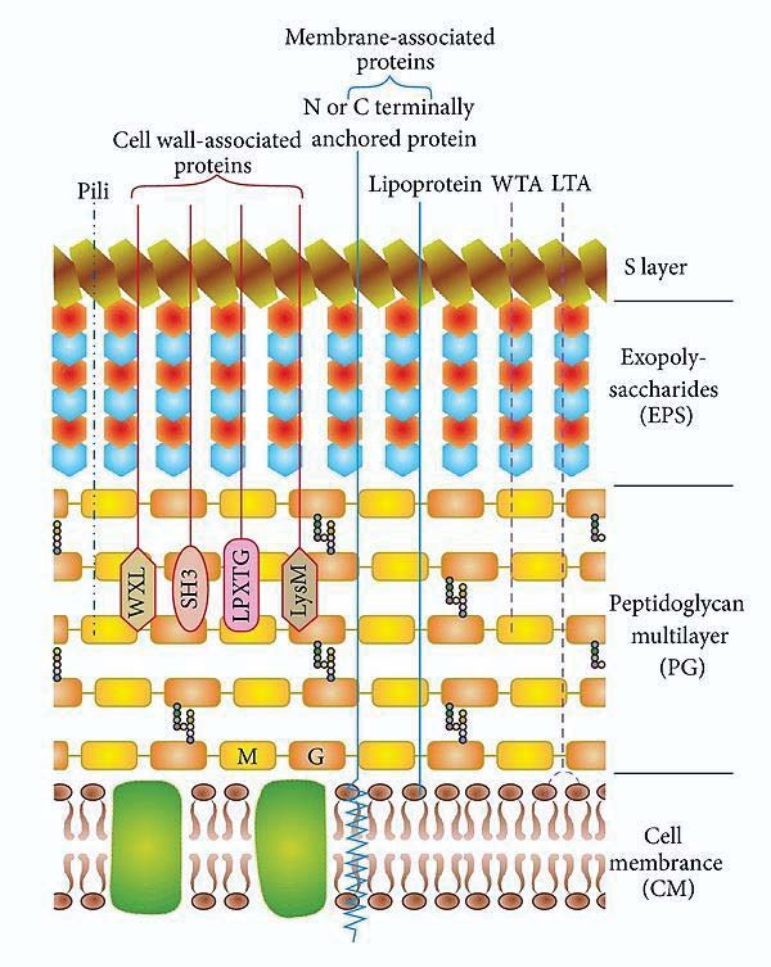

Figure 1.3. Cell envelope of lactobacilli with a schematic representation of cell-wall- and membrane-associated proteins

The bilipidic cell membrane (CM) with embedded proteins is covered by a multilayered peptidoglycan (PG) shell decorated with lipoteichoic acid (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 cell wall either covalently (LPXTG proteins) or noncovalently (exhibiting LysM, SH3, or WXL domains), lipid anchored in the CM (lipoproteins) or integrated into the CM via N- or C-terminal transmembrane helix. M: N-acetyl-muramic acid; G: N-acetyl-glucosamine. Adapted from [56] with permission.

1.2.3 General features of the cell surface of lactobacilli

Lactobacillus cell surfaces share common features of Gram-positive bacteria (Figure. 1.3), including a cytoplasmic membrane covered by multi-layer peptidoglycans (PGs) with associated teichoic acids (TAs), lipoteichoic acids (LTAs), cell wall polysaccharides (PSs) and proteins. In some species, a polysaccharide capsule or a protein layer (called S-layer) is present. Other specialised surface structures such as pili are also recently identified and characterised in lactobacilli [57, 58]. Like many Gram-positive bacteria, the surface properties of lactobacilli are quite dynamic and can be influenced by various environmental stresses [59, 60].

Gram-positive bacterial cell wall consists of 30-100 nm thick multiple peptidoglycan layers that maintain the cell shape and turgor pressure [61]. The overall chemical structure of peptidoglycan is similar between Gram-negative and Gram-positive bacteria, composed of peptide-cross-linked linear polysaccharide strands with alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) units that are linked with each other via glyosidic bonds, however the cell wall of Gram-negative bacteria is very thin, composed of only one to a few layers of peptidoglycan. There are variants of PGs among Gram-positive bacteria, mainly within the peptide-crosslinks between the adjacent polysaccharide strands [62]. Importantly, in addition to the structural role in protecting bacterial cells, PGs form a functional scaffold for anchorage of many extracellular molecules such as TAs, PSs and proteins. Furthermore, covalent modifications of PGs, such as *O*-acetylation, *N*-deacetylation and amidation of glycan strands, are important for various bacterial physiological functions [63].

TAs are cell-wall-associated polymers specific for Gram-positive bacteria. They are polymers of glycerol phosphate linked via phosphodiester bonds. They are commonly observed in Gram-positive bacteria. Given that they make up 60% of the Gram-positive bacterial cell wall mass, the multiple phosphates of TA result in an overall negative surface charge of bacteria [64]. Therefore, it is proposed that TAs can attract cationic ions and protons close to the cell surface to support the cell-surface enzymatic functions and maintain the pH gradient across the cell wall [65]. According to the localisation site, there are two types of TAs present, wall teichoic acid (WTA) and LTA. WTA is coupled with PGs through phosphodiester bond to the hydroxyl group of the associated MurNAc

residual, whereas LTA is anchored in the cytoplasmic membrane via a covalent link to the membrane lipids. Although the basic composition is similar between TA and LTA, they vary in various ways such as the repeat numbers and chirality of the core polymer units (e.g. glycerol phosphate) and modifications. In particular, TA or LTA modification with groups like *D*-alanine or carbohydrates is significantly related to the functions. Although neither TA nor LTA is essential for bacterial viability, the lack of either in *Bacillus subtilis* can lead to defective phenotypes in cell division, morphology and growth [66, 67]. However, lacking in both TA and LTA is lethal [68], suggesting certain common features shared by them are essential to maintain bacterial physiology. LTA also acts as a receptor for bacteriophage LL-H that infects *L. delbruekii* subsp. *Lactis* ATCC15808 [69]. Various LTA modifications can positively or negatively influence the binding efficiency of this bacteriophage [70].

PSs are also frequently present on the Gram-positive bacterial surface. Generally, based on the location, Gram-positive bacterial polysaccharides can be grouped as wall polysaccharide (WPS) and exopolysaccharide (EPS). The latter type of polysaccharide (EPS) is released from bacteria to the medium or loosely associated with the bacterial surface. In addition to the location, PSs vary significantly between bacteria in terms of composition, linkage and branching [63], suggesting their diverse functions. Heterogeneous PS structures can exist in different species, strains and even within a single cell under different growth conditions, as observed in *L. rhamnosus* GG that has a longer polysaccharide rich in galactose and also a shorter one rich in glucose [71]. The structural variation is most likely due to the diversity of gene clusters for PS production. The functions of PSs in Gram-positive bacteria are diverse. including maintenance of normal cell morphology and division [72], bacterial adhesion and biofilm formation [73], protection of bacteria from phagocytosis and immunomodulation [74].

In addition to the glycopolymers described above, Gram-positive bacterial surface contains numerous and diverse proteins, representing approximately 80% of all secreted proteins encoded by their genomes. After secretion, proteins that are covalently linked to a lipid moiety are anchored in the cytoplasmic membrane [75]. Those containing either a C-terminal LPxTG motif (sortase-dependent) [76] or cell wall binding domains are anchored to the cell wall. Some secreted proteins are just released to the surrounding environment [77]. Collectively, membrane- and cell wall-anchored proteins as well as

secreted proteins are called the secretome [78]. The secretome proteins play various roles in maintenance of bacterial physiology and interactions with other microbes and the environment, including human host. In particular, some bacteria contain "S-layer", an outmost coat covering the whole cell surface, which is made of a single protein unit [79, 80]. The S-layers are porous two-dimensional paracrystalline structures and consist of numerous repeated (glyco)protein subunits with molecular weight normally between 25 and 200 kDa. Compared to other Gram-positive bacteria, *Lactobacillus* S-layer proteins have relatively small size (25-71 kDa) and high p*I* (9.4-10.4) [80]. S-layers are assumed to assemble spontaneously and attach to the bacterial surface via non-covalent interactions. The conserved positively charged cell wall binding domains of S-layer proteins are proposed to bind to the negatively charged cell wall polymers such as TAs and PSs [80].

In addition, some Gram-positive bacteria have specialised proteinaceous structures that project from the surface, such as pili and flagella. In Gram-positive bacteria, pilus consists of one major pilin and two minor pilins. The large number of the major pilin subunits form the body of the pilus, whereas the small number of minor pilin subunits form the bottom and tip. Pilin subunits are connected to each other covalently through action of the pilus-specific sortase C. Assembled pilus is anchored to the PG via an LPxTG motif, recognised and processed by the housekeeping sortase A [81]. Pili are generally involved in adhesion, persistence and invasion [82]. Pili have been identified in *L. rhamnosus* and shown to play important roles in probiotic activities, such as autoaggregation, biofilm formation, adhesion to the intestinal epithelial cells, and immunomodulation of the host immune responses [41, 53, 83, 84]. Flagella are long surface appendages that function in bacterial motility, propelling bacteria through the liquid environment by rotation [85]. Although lactobacilli are generally considered non-motile, bacterial motility was observed in twelve *Lactobacillus* species [58]. Recently, the motility gene loci were analysed in *L. ruminis* and *L. mali*, showing that the motility gene loci in *Lactobacillus* genomes are closely related to those in carnobacteria and enterococci [58].

1.2.4 Adaptive and probiotic functions of lactobacilli cell surface structures in the human intestinal tract

The complex surface structures of probiotic bacteria are considered as the first line in contact with the physicochemical environmental factors, pathogens and human intestinal epithelial and immune cells. In lactobacilli, the presence and integrity of specific bacterial cell surface structures is responsible for the resistance to the environmental stress and are involved in colonisation as well as intra- and/or inter-kingdom signalling. For example, the components of *Lactobacillus* surface structure can be altered in response to bile salts, low pH and oxidative stress [56]. The compositional change such as fatty acid composition in cytoplasmic membrane is often seen in lactobacilli under these stressful conditions [86-88]. Low pH and bile salts can also induce the change in composition and relative amounts of proteins, PGs, LTAs and EPSs [60, 65, 89]. Besides their role in cell protection and adaptation to environmental conditions, LTAs, EPSs, specific surface proteins and structures, such as pili, are involved in adherence, persistence and colonisation in the human intestinal tract through binding to mucus, extracellular matrix components and/or specific receptors of the epithelial cells, respectively. Many lactobacilli can outcompete the intestinal pathogens for the adhesion sites available to bacteria in the human GIT. Furthermore, lactobacilli have evolved broad nutrient-processing machineries including extracellular proteolytic enzymes, carbohydrate hydrolases, and the dedicated transporters. Most importantly, many lactobacilli as probiotics possess diverse extracellular effector molecules that interact with the specific receptors on eukaryotic (human) cells, which can act as agonists or antagonists, triggering or blocking the cellular signalling pathways in the intestinal epithelial cells and/or immune cell [41, 90, 91]. This manipulation of host cell signalling was shown to result in programmed health-promoting responses, such as increased mucus secretion, enhanced function of tight junctions and anti-inflammatory immune responses [37, 39, 92]

1.3 Colonisation the human intestinal tract by lactobacilli

Many probiotic lactobacilli are not natural human GIT isolates. In order to exert an effect on the host, it is essential that probiotic lactobacilli can adhere to the epithelium and colonise, at least transiently, the GIT of humans. *In vitro* adhesion capacity to the human colorectal adenocarcinoma cell lines such as Caco-2 and HT-29 is generally considered as prerequisite for screening of probiotic strains [93]. However, the adhesion capacity of lactobacilli to these cells varies between different species and strains. It is believed that, besides adhesion to GIT epithelial cells, bacterial intra- and inter-species aggregation and biofilm formation are desirable traits that contribute to improved adhesion, colonisation and persistence of the probiotic strains in the host GIT. It is worth noting that all these traits involve complex bacteria-bacteria and bacteria-host surface interactions, most likely mediated by both non-specific and specific interactions and multiple extracellular factors. Although some factors that mediate these interactions were bioinformatically identified and experimentally supported, a deep insight is still needed to understand the molecular mechanisms of probiotic effects of probiotic bacteria and improve the strategies of their utilisation to promote human health. For example, given that probiotic effects are often species-/strain-specific, application of a mix of several probiotic stains can be used to presumably expand the spectrum of probiotic effects [37, 94, 95]. However, proper selection of probiotic strains will rely on the fundamental understanding of the traits of individual strains that should compensate the genetic limitations and result in a synergistic probiotic effect, rather than antagonism due to competition within the GIT.

1.3.1. Adhesion

Adhesion to the surface of the human intestinal epithelium is considered as the initial and essential step for probiotic bacteria to colonise in the human gut. Adherence of lactobacilli to the targets can competitively inhibit the binding by enteropathogens [96]. In pathogens, the disruption of adhesion normally leads to a diminished virulence [97, 98]. Compared to the well-characterised adhesion mechanisms utilised by Gram-positive pathogens, the

understanding of adhesion in probiotics lags far behind. The fast-pace development of next generation sequencing and advanced bioinformatics tools has offered opportunities to expedite discovery of adhesin-like molecules in *Lactobacillus* species. The identified and characterised adhesins in lactobacilli are summarised in Table 1.1, indicating the diverse nature of adhesin-target interactions. According to the nature and surface-anchoring modes, *Lactobacillus* adhesins can be grouped into sortase-dependent proteins [57, 99-105], S-layer proteins [99, 106], moon-lighting proteins [107-110] and non-proteinaceous factors (EPS and LTA) [100, 111].

Human intestinal epithelial cells are protected by a thick mucosal layer containing glycolipid and glycosylated mucins, which prevents adherence of most pathogens. However, mucus serves as a habitat for commensal as well as probiotic bacteria that can adhere to its components. The majority of *Lactobacillus* adhesins identified to date are sortase-dependent proteins that contain an N-terminal signal sequence for protein secretion and a C-terminal sortase-dependent cell wall anchoring motif (LPxTG), followed by hydrophobic residues and a positively charged tail. Depending on the particular adhesin domains contained within the extracellular (exposed) portion of these proteins, they have been demonstrated to bind to mucus and/or epithelium. Many of these proteins contain multiple mucus-binding (MUB) domains as observed in Mub of *L. reuteri* 1063 [112], LspA of *L. salivarius* UCC118 [104], Mub of *L. acidophilus* NCFM [99], MBF of *L. rhamnosus* GG [100] and Msa of *L. plantarum* WCFS1 [113]. MUB domains vary in size (from approximately 100 to 200 amino acids) and copy number (from 4 to 17 copies) in different mucus-binding proteins with similar organisation. These type of MUB-containing proteins are more frequently found in the human intestinal isolates of lactobacilli than in food-fermenting isolates [114], indicating their conserved function in colonisation of the intestinal mucosa. Another type of sortase-dependent mucus-binding surface structures are SpaCBA pili of *L. rhamnosus* GG [57, 83, 115-117], which are assembled by a pilus-specific sortase SrtC1 and anchored to the cell wall by the housekeeping sortase SrtA. The major pilin SpaA forms the pilus backbone. It is decorated by minor pilins SpaB and SpaC. In particular, SpaC, which is located at the tip as well as along the shaft of the pilus, has been shown to have a high affinity to mucin [116]. Intestinally, SpaC also mediates self-binding [84], which potentially increases the number of bacteria adhering to the mucus via aggregation.

Another group of *Lactobacillus* adhesins are the S-layer proteins. They not only form a surface shell protecting the bacteria, but also mediate adhesion of these bacteria to the host epithelium. Examples of this group of adhesins are SlpA of *L. acidophilus* NCFM, [99], CbsA of *L. crispatus* JCM 5810 [118], and SlpA of *L. brevis* ATCC8287 [119]. These S-layer proteins have also been demonstrated to bind to the human intestinal cells [99, 119] and extracellular matrix (ECM) components such as collagen and laminin [106].

Recently, some secreted and surface proteins that do not contain an obvious sequence for either secretion or surface-anchoring motifs have been identified in pathogenic and probiotic bacteria. These proteins are classically characterised as biosynthesis factors or cytoplasmic metabolic enzymes and were therefore named "moonlighting proteins" to signify a second function upon secretion. These anchorless proteins can be secreted into the spent growth media after culturing of lactobacilli and/or loosely associated with the bacterial surface. The examples of moonlighting proteins in lactobacilli are the translation factor Tu (EF-Tu) [108] and chaperone GroEL [107] of *L. johnsonii* La1 (NCC533), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *L. plantarum* LA318 [109] and *L. plantarum* 299v [120], enolase (ENO) of *L. plantarum* 299v [120], glutamine synthetase (GS) and glucose-6-phosphate isomerase (GPI) of *L. crispatus* ST1 [110]. The binding targets of these proteins include the human intestinal cell lines, mucin, collagen and laminin. The anchoring of the *Lactobacillus* moonlighting proteins to the bacterial surface and the adherence to the targets in human GIT and extracellular matrix are often pH-dependent [108, 120], suggesting that it is dependent on the protein charge or conformation. However, it is not known exactly why and how these moonlighting proteins are secreted.

In addition to proteinaceous molecules, surface polymers such as LTA and EPS have roles in adhesion of lactobacilli to the host surfaces. Anionic LTA significantly contributes to the surface charge and hydrophobicity that are considered to be important factors for non-specific interactions. In *L. johnsonii* La1, addition of purified LTA diminished the adherence capacity of this bacterium to Caco-2 cells [111]. However, insertional inactivation of *dltD*, gene of *L. rhamnosus* GG, encoding the enzyme responsible for esterification of LTA with *D*-alanine, did not reduce the bacterial adherence to the human intestinal cell lines, Caco-2 or HT-29 [121]. Therefore, the LTA-mediated bacterial adhesion is probably strain-specific. On the other hand, EPSs seem to

impair the *Lactobacillus* adhesion by shielding other surface adhesins as seen in *L. rhamnosus* GG [41, 100] and *L. acidophilus* CRL 639 [122].

Table 1.1. Examples of adhesins identified in lactobacilli

Strains	Adhesin name	Compartment	Surface localisation mechanism	Target for binding	Reference
L. acidophilus NCFM	Mub	Cell-wall-anchored	Sortase-dependent	Human intestinal epithelial cells and mucus	[99]
	FbpA	Cell-wall-anchored	Sortase-dependent	Human intestinal epithelial cells and fibronectin	[99]
	SlpA	Surface	S-layer	Human intestinal epithelial cells	[99]
L. brevis ATCC8287	SlpA	Surface	S-layer	Human intestinal epithelial cells, laminin, collagen, and fibronectin	[119]
L. casei BL23	p40	Secreted/surface-associated	No anchor	Human intestinal epithelial cells, mucin and collagen	[123]
	p75	Secreted/surface-associated	No anchor	Human intestinal epithelial cells, mucin and collagen	[123]
L. crispatus JCM 5810	CbsA	Surface	S-layer	Human intestinal epithelial cells, collagen I, collagen IV, laminin and bacterial LTA	[106]
L. crispatus ST1	GS	Secreted/surface-associated	Moonlighting	Collagen I and laminin	[110]
	GPI	Secreted/surface-associated	Moonlighting	Collagen I	
	LEA	Cell-wall-anchored	Sortase-dependent	Epithelial cells of human vagina	[101]
L. johnsonii La1	LTA	Membrane-anchored	LTA-specific targeting	Human intestinal epithelial cells	[111]
	EF-Tu	Secreted/surface-associated	Moonlighting	Human intestinal epithelial cells and mucus	[108]
	GroEL	Secreted/surface-associated	Moonlighting	Human intestinal epithelial cells and mucus	[107]
L. plantarum NCIMB 8826	D1	Secreted/surface-associated	No anchor	Mucin	[124]
L. plantarum LA318	GAPDH	Secreted/surface-associated	Moonlighting	Mucin	[109]
L. plantarum WCFS1	Msa	Cell-wall-anchored	Sortase-dependent	Mucin via mannose residue binding	[113]
L. plantarum 299v	GAPDH	Secreted/surface-associated	Moonlighting	Mucin, plasminogen and fibronectin	[120]
	ENO	Secreted/surface-associated	Moonlighting	Plasminogen and fibronectin	[120]
L. rhamnosus GG	SpaCBA pilus	Cell-wall-anchored	Sortase-dependent	Human intestinal epithelial cells and mucus	[57]
	MabA	Cell-wall-anchored	Sortase-dependent	Human intestinal epithelial cells	[102]
	MBF	Cell-wall-anchored	Sortase-dependent	Mucin	[100]
	EPS	Surface-associated	EPS-specific targeting	Mucin	[100]
L. reuteri JCM 1112^T	Lar_0958	Cell-wall-anchored	Sortase-dependent	Mucin	[105]
L. reuteri NCIB 104R	MapA	Secreted/surface-associated	No anchor	Human intestinal epithelial cells and mucin	[125]
L. reuteri NCIB 11951	CnBP	Secreted/surface-associated	No anchor	Collagen	[126]
L. reuteri 100-23	Lsp	Cell-wall-anchored	Sortase-dependent	Epithelium of the mouse forestomach	[127]
L. reuteri 1063	Mub	Cell-wall-anchored	Sortase-dependent	Mucin	[112]
L. salivarius UCC118	LspA	Cell-wall-anchored	Sortase-dependent	Human intestinal epithelial cells	[52, 104]

1.3.2. Aggregation

Many probiotic bacteria form multicellular aggregates when growing in the liquid media under laboratory conditions. This intra-species cell-cell aggregation is referred to autoaggregation. Some probiotic bacteria can even form inter-species aggregates with bacteria of other species, a phenomenon termed coaggregation. (Co)aggregation of bacteria is in general most frequently mediated by specific surface proteins [128], however the formation and properties of aggregates may be influenced by bacterial surface hydrophobicity [129] and environmental factors such as ions [130], oxygen [131] and carbohydrates [132].

Both autoaggregation and coaggregation are considered as desirable phenotypes of probiotic bacteria. Autoaggregation may help increase bacterial biomass to promote the persistence of probiotic bacteria in the human intestinal tract [133], adherence [129, 134, 135] and biofilm formation [128, 132, 136]. For example, an autoaggregation phenotype of *L. crispatus* M247 was found to be involved in reducing the severity of induced colitis in mice, modulating the expression of Toll-like receptors and generating anti-inflammatory responses [137]. This conclusion was made based on the loss of these functions in correlation with the loss of aggregation in a mutant of this strain (Mu5). Coaggregation of probiotic bacteria with the pathogens can potentially neutralise the epithelium-binding ligand(s) and toxins of the intestinal pathogenic microbes, thereby protecting the intestinal epithelial cell layers [138, 139]. For example, *L. kefir* strains (but not the non-coaggregating variants) coaggregate with pathogenic *Salmonella enteritidis* and significantly impair this pathogen's adhesion to and invasion of human intestinal epithelial cells [140]. Coaggregation is also considered to favour biofilm formation by multiple intestinal commensal species, forming a protective microbial layer [128].

Due to the importance of aggregation-promoting factors (APFs), they have been an object of intensive research. Currently known APFs of lactobacilli are shown in Table 1.2. *Lactobacillus* APFs are generally proteins, secreted and then surface-reassociated [141, 142] or surface-displayed (such as the S-layer proteins) [141] or cell-wall anchored (such as pili; [84]). Furthermore, there are examples of carbohydrates that reportedly have a role in aggregation [132]. APFs vary greatly in their nature, amount and growth

conditions/phase when they are expressed, hence the strategies and mechanisms of aggregation vary among lactobacilli. One of the first APFs discovered in lactobacilli, the Apf protein of the *Lactobacillus acidophilus* group (such as *L. gasseri* and *L. johnsonii*) is highly expressed in the stationary-phase of growth [143, 144]. Recently, knock-out mutants of genes encoding the *L. rhamnosus* GG SpaCBA pilus [84] and a *L. plantarum* BCIMB 8826 surface protein D1 [124] were constructed. Interestingly, both mutants not only lost the autoaggregating phenotype but also showed diminished mucus-binding capacity. In *L. rhamnosus* GG, SpaCBA pili mediate autoaggregation probably due to the self-binding property of SpaC subunits [84]. However the domains mediating autoaggregation remain to be determined in D1.

Table 1.2. Examples of aggregation-promoting factors identified in lactobacilli

Strains	Aggregation-promoting factors	Properties	Compartment	Reference
L. acidophilus M92	SlpA	45 kDa	S-layer	[134]
L. coryniformis DSM 20001^T	Cpf	19.9 kDa	Secreted/surface-associated	[141]
L. reuteri E10	Apf	32 kDa	Secreted/surface-associated	[143]
L. paracasei BGSJ2-8	Not characterised	200 kDa	Surface/Secreted	[145]
L. plantarum NCIMB 8826	D1	22 kDa	Surface/Secreted	[124]
L. rhamnosus GG	SpaCBA pilus	Covalently inter-linked multiple proteins	Cell-wall-anchored	[84]
L. reuteri 1063	Mub	353 kDa	Cell-wall-anchored	[135]
L. reuteri TMW1.106	EPS	Polysaccharide	Surface-associated	[132]

Furthermore, some of the APFs also mediate coaggregation with other bacterial species. For example, a 32 kDa protein isolated from some *L. reuteri* strains was shown to mediate coaggregation of *L. reuteri* with pathogenic *Escherichia coli* [143]. Another example is Cpf of *L. coryniformis* DSM 20001^T, that was shown to mediate coaggregation with *E. coli*, *Campylobacter coli*, and *Campylobacteria jejuni* [141]. Coaggregation interaction partners (cognate targets or ligands) on the surface of *E. coli* were shown to be the fimbriae and lipopolysaccharides [146].

1.3.3. Biofilm formation

In the natural habitat, many bacteria grow in biofilms, in contrast to the predominantly planktonic growth in the test-tubes. Biofilms are microbial communities embedded within a culture-produced matrix of extracellular polymeric substances. Formation of biofilm requires complex microbe-surface and microbe-microbe interactions, resulting in coordinated community behaviour, which is often regulated by quorum sensing [147]. Phenotypes of the microorganisms growing in biofilms are often important for coping with the environmental stress, such as low pH, bile salts, carbohydrates, antimicrobial factors and attack by the host immune responses [148-151]. Ability to form biofilms increases the virulence in many pathogenic microorganisms, whereas it is considered as a health-benefiting attribute in probiotic bacteria.

Biofilm growth can promote the persistence of probiotic lactobacilli in the GIT, increasing the time available to these beneficial bacteria to displace pathogens or to mask the pathogen binding sites on the GIT surface. The latter phenomenon is called “surface blanketing”. It is also thought that biofilm is conductive to the interactions with the human intestinal epithelium, which triggers specific beneficial signalling pathways.

In order to avoid the pathogenic microorganism adhesion to and integration into the *Lactobacillus* biofilms, some *Lactobacillus* strains produce biosurfactants [152, 153]. Furthermore, secreted substances by lactobacilli can downregulate the expression of biofilm-related genes in the incoming pathogens. For example, the EPS produced by *L. acidophilus* A4 can inhibit the biofilm growth of pathogenic *E. coli*, probably through downregulation of genes encoding for the adhesive pili called curli, and genes encoding the chemotaxis signalling molecules, preventing motility [154]. Biofilm growth in some lactobacilli induces production of antimicrobial and anti-inflammatory factors [155], which are also desirable traits of probiotic bacteria. Many *Lactobacillus* species can form biofilms, however very few biofilm factors have been reported to date (Table 1.3), indicating that biofilm-formation mechanisms are not well understood in *Lactobacillus* species.

Table 1.3. Examples of *Lactobacillus* factors involved in biofilm formation

Strain	Protein/factor name	Location	Reference
L. rhamnosus GG	MabA	Surface	[102]
	SpaCBA pilus	Cell wall	[41]
	LuxS	Cytosolic	[156]
	EPS	Surface	[41]
L. plantarum LM3	FlmA, FlmB,FlmC	Surface	[151]
	CcpA	cytosolic	[151]
L. reuteri 100-23	Lr70902	Surface/Secreted	[157]
	Lsp	Surface	[157]
	LuxS	Cytosolic	[158]
	TA	Surface	[159]
L. reuteri TMW1.106	GtfA	Cytosolic	[132]
	Inu	Cytosolic	[132]

Cytoplasmic central regulators, such as LuxS of *L. rhamnosus* GG [156] and *L. reuteri* 100-23 [158], and CcpA of *L. plantarum* LM3 [151], were shown to influence biofilm growth of these bacteria. LuxS is conserved in many bacteria, where in many cases it serves as a central factor for intra-/inter-species communication (quorum sensing). Knockout of *luxS* diminished the biofilm growth of *L. rhamnosus* GG, suggesting its involvement in this trait [41]. The *luxS* mutant of *L. reuteri* 100-23 was found to have increased the thickness of monospecies biofilm on the plastic surface and the epithelium of mice forestomach, suggesting a role in the biofilm structure [158]. Regulator CcpA is recognised as global regulator of gene expression in Gram-positive bacteria. In *L. plantarum*, the *ccpA* gene is essential for glucose-starvation-induced biofilm formation [151].

Surface proteins play important roles in biofilm formation of lactobacilli. Mutations in surface protein-encoding genes including *mabA* [41, 102] and *spaCBA* [41] of *L. rhamnosus* GG, *flmA*, *flmB* and *flmC* of *L. plantarum* LM3 [151] as well as *lsp* [157] and *lrh70902* [157] of *L. reuteri* 100-23, all impaired the biofilm formation capacity of the mutants relative to the corresponding wild-type parent strains. Non-proteinaceous polymers can also influence the biofilm growth of lactobacilli. For example, a *welE* knock-out mutant of *L. rhamnosus* GG, lacking the priming glycosyltransferase in the

synthesis of the EPS and completely devoid of this exopolysaccharide, promoted formation of biofilm on the plastic surface [41]. This finding suggests that the EPS may mask other surface biofilm factors (such as SpaCBA pili), decreasing the efficiency of the biofilm formation. Cytoplasmic glycan-synthesising enzymes are also involved in biofilm formation of lactobacilli, such as glucosyltransferase and inulosucrase of *L. reuteri* TMW1.106, which are responsible for synthesising gluco-oligosaccharides and fructo-oligosaccharides in this bacterium, respectively. Insertional inactivation of the corresponding genes (*gtf* and *inu*) eliminated the production of theses oligosaccharides, resulting in diminished biofilm formation [132].

On the other hand, *dltD* mutant of *L. reuteri* 100-23 whose TA lacks *D*-alanines along the polymer chain, was unable to form biofilm on epithelium of the murine forestomach, in contrast to the parental wild-type strain [159]. This finding suggested that *D*-alanylation is essential for the biofilm mode of growth for this strain. In contrast, *dltD* mutant of *L. rhamnosus* GG formed a biofilm on the plastic surface similar to that of the wild-type strain [121].

Overall, the effects of many identified biofilm factors, such as *D*-alanine on TA, can be strain-specific. In contrast, some factors, such as pilus are widely shown to either impair or facilitate the biofilm formation.

1.4 Molecular interactions between lactobacilli and the human intestinal epithelium

1.4.1 Crosstalk between probiotic bacteria and the human host

In addition to the pathogen-inhibitory traits, many probiotic bacteria have been shown to have capacities to improve the integrity of the intestinal epithelium, which helps avoid invasion of subepithelial tissues and systemic spread of the pathogens, promote the host immunity or reduce the severity of inflammation [160, 161]. Based on the body of immunological knowledge on the interactions between gut epithelium and the vast library of antigens to which it is exposed, host epithelial and immune cells invariably obtain environmental cues through the ligand-receptor interactions, for appropriate response that results in a gut homeostasis in healthy individuals. The effect of probiotic bacteria on this tissue should no doubt follow the same rules. The human intestinal epithelium consists of enterocytes, goblet cells, Paneth cells, enteroendocrine cells and M cells (Figure 1.1), which are responsible for nutrient absorption, mucus formation, secretion of antimicrobial factors, secretion of hormones, and probing of the microbial composition of gut lumen, respectively [9]. Importantly, the epithelial cells are also in close contact with the immune cells in the lamina propria. Together with dendritic cells and M cells, epithelial cells are responsible for interpreting the signals from the gut lumen and mounting appropriate types of immune responses (Figure 1.4). Microbial conserved surface structures, which are collectively called Microorganism-Associated Molecular Patterns (MAMPs), normally interact with the Pattern Recognition Receptors (PRRs) of host epithelial cells and immune cells, signalling the presence of microbes in the gut by triggering host cellular signalling pathways such as NFκB and other key pathways, resulting in expression of a range of innate defence molecules (e.g. antibacterial peptides such as defensins), as well as intercellular immune communication mediators such as cytokines and chemokines [9].

MAMPs are conserved surface structures. Some are non-protein molecules such as PG, lipopolysaccharides, WPS, LTA, TA in various microorganisms, however each of these molecules have strain-specific modifications, in particular in the oligosaccharide moieties.

Furthermore, MAMPs such as flagella and fimbriae are proteinaceous structures, which have quite diverse sequences among the strains and species. Receptors on eukaryotic cell surfaces that recognise MAMPs are PRRs. A dozen PRRs, each recognising a specific MAMP, combine to recognise diverse MAMPs. Toll-like receptors (TLRs), which are the best characterised PRRs, are a group of transmembrane proteins containing a characteristic extracellular leucine-rich-repeat-containing (LRR) domains that undergo homodimerisation or heterodimerisation upon ligand-binding, which in turn activate the cytoplasmic domains to initiate a signalling cascade [9]. These cytoplasmatic domains each contain a conserved Toll/Interleukin-1 Receptor (TIR) domain that recognises different cytoplasmic adaptor molecules. Moreover, other PRRs such as extracellular C-type lectin receptors (e.g. DC-SIGN) [162] and cytoplasmic nucleotide-binding oligomerisation domain-like receptors (NLRs) [163] are also involved in transmission of signals triggered by the intestinal microorganisms. In addition, membrane-bound or secreted coreceptors are often needed for primary recognition of MAMPs [9].

Stimulation of TLRs and NLRs in turn stimulate, via a variety of cytoplasmic adapter and signalling molecules, the mitogen-activated protein kinase (MAPK) pathway that ultimately results in transfer of the transcription factor called nuclear factor κB (NF-κB) from the cytoplasm into the nucleus [164]. This transcription factor, once in the nucleus, induces transcription of specific sets of genes involved in triggering innate and adaptive immune responses such as production of cytokines, antimicrobial substances and cytoprotective (anti-apoptotic) molecules (Figure 1.4). Cytokines further direct maturation and differentiation of dendritic cells that in turn activate and differentiate T-cells. Therefore, MAMPs of bacteria, including the probiotic bacteria, are bound to trigger sophisticated signalling system of human immune cells.

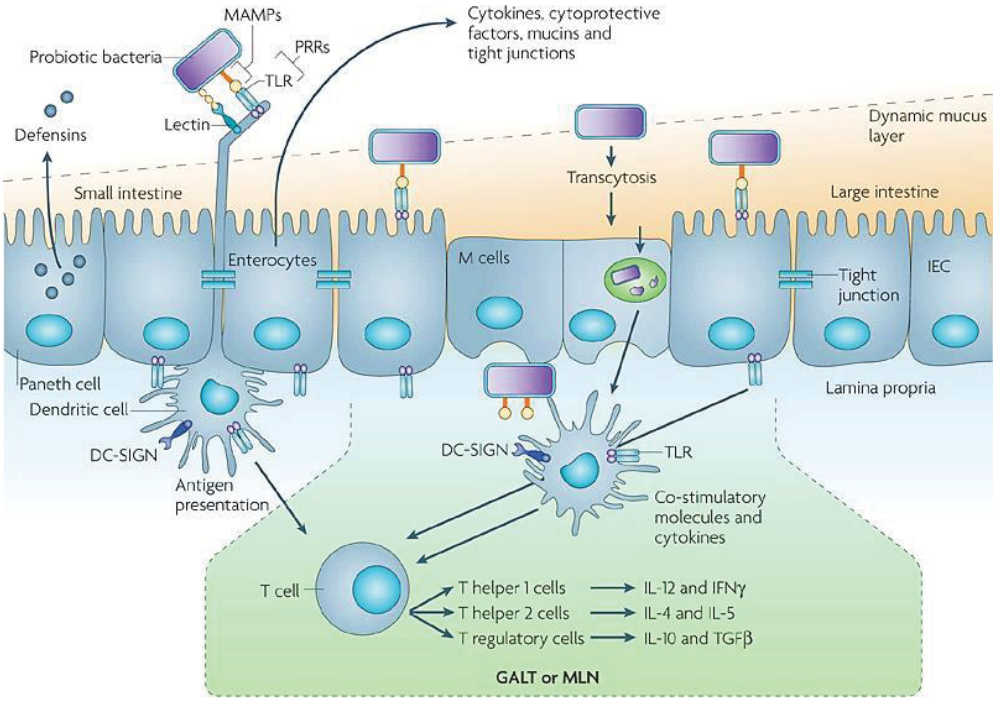

Figure 1.4. Interaction of probiotic bacteria with IECs and DCs from the GALT

A fraction of ingested probiotics are able to interact with intestinal epithelial cells (IECs) and dendritic cells (DCs), depending on the presence of a dynamic mucus layer. Probiotics can occasionally encounter DCs through two routes: DCs residing in the lamina propria sample luminal bacterial antigens by passing their dendrites between IECs into the gut lumen [165], and DCs can also interact directly with bacteria that have gained access to the dome region of the gut-associated lymphoid tissue (GALT) through specialized epithelial cells, termed microfold or M cells [166]. The interactions of the host cells with microorganism-associated molecular patterns (MAMPs) that are present on the surface macromolecules of probiotic bacteria induce a certain molecular response. The host pattern recognition receptors (PRRs) that can perceive probiotic signals include Toll-like receptors (TLRs) and the C type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN). Some molecular responses of IECs depend on the subtype of cell, for example, Paneth cells produce defensins and goblet cells produce mucus. Important responses of DCs against include the production of cytokines, major histocompatibility complex molecules for antigen presentation, and co-stimulatory molecules that polarise T cells into T helper or $CD4^{+}CD25^{+}$ regulatory T cells in the mesenteric lymph nodes (MLNs) or subepithelial dome of the GALT. IFNγ, interferon-γ; IL, interleukin; TGFβ; transforming growth factor-β. Adapted from [167] with permission.

1.4.2 Enhancement of the epithelial barrier function

Intestinal Epithelial Cells (IECs) are structurally and functionally polarised, containing an apical surface as well as a basolateral surface. The apical surface of IECs is normally covered by a protective mucus layer. The integrity of the human intestinal epithelial barrier is tremendously dependent on mucus secretion, proliferation of IECs, expression and modifications of cell skeletons and paracellular tight junctions (TJs).

Some probiotic lactobacilli can stimulate mucin production by IECs. For example, exposure of HT-29 cells to *L. rhamnosus* GG and *L. plantarum* 299v promoted mucin gene expression in an adhesion-dependent manner [36, 168]. The increased mucin production was also reported to impair the adherence capacity of enteropathogenic *E. coli* [168]. Other probiotics, such as those in a probiotic formula called VSL$^{\#}$3, could also stimulate the mucin gene expression and secretion in rat models [37].

TJs are proteinaceous structures, consisting of zonula occludens 1 (ZO1) proteins, occludins and claudins, which hold the adjacent IECs together tightly, blocking the paracellular spaces between IECs. This prevents the passage of inflammation-causing microorganisms and most MAMPs below the epithelial cell layer. In contrast to many pathogens that are capable of damaging TJs to translocate across the epithelium, some probiotic bacteria can enhance the integrity of TJs, which can be detected using various permeability assays, such as mannitol assay [160] and Transepithelial Electrical Resistance (TEER) assay [39]. However, molecular mechanisms by which probiotic bacteria modulate TJs are not completely revealed. Evidence suggests that lactobacilli help maintain posttranslational modifications and distribution of the TJ proteins that are often disrupted by the invasive intestinal pathogens [169, 170]. In the animal models, TJs were shown to be protected from enteroinvasive *E. coli* by *L. plantarum* through induction of elevated expression of TJ proteins [170, 171].

Besides an effect on TJs, probiotic lactobacilli can reduce cytokine- or chemical-induced apoptosis of IECs [38, 172]. Proliferation of IECs is regulated by the specific interactions between Epithelial Growth Factors (EGFs) and their receptors (EGFRs) on IECs. Upon stimulation, the EGFRs rapidly undergo dimerization and activate posttranslational modifications of the cytoplasmic domains, triggering a signalling cascade which

ultimately leads to cell proliferation, differentiation and viability [173]. Lactobacilli were shown to reduce the apoptosis by preventing EGFR inactivation by the enteroinvasive *E. coli* [123, 169, 170]. Interestingly, recent studies showed some evidence for molecular basis of this effect. For example, two characterised secreted *L. rhamnosus* proteins, p40 and p75, prevented the cytokine-induced apoptosis of different types of IECs [38, 172]. The p40 protein was also shown to transactivate the EGFR by stimulating the metalloproteinase (ADAM17)-dependent release of the heparin binding-EGFs from the young adult mouse colon cells and mouse colonic cells [90].

In addition, lactobacilli can help maintain the cytoskeletal organisation and function of IECs under the pathogen assault. For example, *Lactobacillus* species were shown to preserve the post-translational modifications and rearrangement of cytoskeletal proteins such as F-actin and actinin, which were blocked by virulence factors secreted from the pathogens [169, 170, 174, 175]. In another example, secreted factors from *L. rhamnosus* GG were shown to upregulate the expression of heat shock protein Hsp72, which in turn mediated protection of the IECs against cytoskeletal rearrangement via MAPK signalling pathway.

1.4.3 Immunomodulation

The intestinal microbiota has tremendous impact on the maturation of the human mucosal and central immune system homeostasis and responses, as described in Section 1.1.2. Immunomodulatory capacities are desirable traits of probiotic bacteria, which have been shown to promote both innate and adaptive host immune responses to eliminate the intestinal pathogens, while reducing the baseline inflammation and regulating the hypersensitivity to allergens. This is achieved in part through modulation of MAMP-PRR-dependent cellular signalling pathways (Figure 1.4).

Stimulation of the host innate immunity by probiotic bacteria usually involves MAMP-PRR-induced signalling, resulting in altered secretion of antimicrobial factors, secretory antibodies, chemokines and cytokines. For example, many lactobacilli can induce the expression and secretion of broad-spectrum antimicrobial peptides, including defensins, by IECs [176]. Some lactobacilli can also regulate secretion of other molecules such as

interleukins (IL) and interferons [177-180]. Furthermore, the intestinal mucosa contains secreted antibodies, which can be enhanced by lactobacilli as well [181, 182]. Alternatively, some lactobacilli can also upregulate the receptors such as TLRs on mononuclear cells, IECs, and DCs to promote the recognition of microbial surface structures generating amplified innate immune responses [183-185].

Unlike innate immunity, adaptive immunity in the gut mucosal tissues is regulated by the Gut-Associated Lymphoid Tissue (GALT) within the lamina propria, mediated by antigen-presenting cells. MAMPs of microorganisms can gain access to the immune cells of GALT through the specialised intestinal epithelial portal, called M cells (Figure 1.1 and 1.4), which take up microorganisms and their MAMPs to allow specific contact with the immune cells in lamina propria. Alternatively, microorganisms and their MAMPs can be sampled directly from the lumen of the intestinal tract by DCs. Macrophage and DCs play important roles in antigen presentation to stimulate adaptive immune responses, including maturation and differentiation of T and B cells, resulting in antigen-specific humoral and cellular responses. Immunomodulatory functions of probiotic bacteria often involve regulation of the balance between the populations of inflammatory helper T cells (T_{H17}) vs. regulatory T cells (T_{Reg}). T_{Reg} cells are responsible for producing the majority of IL-10 anti-inflammatory cytokines, which mediate suppression of T cell activities that results in inflammation and hypersensitivity to allergens [186].

Surface structures of probiotic bacteria have been shown to modulate the host immunity. For example, PG fragments of lactobacilli can mediate signalling through recognition by TLR-2 as well as NLRs. Variations in composition and structure of PG could trigger different signalling cascades. Besides peptidoglycans, LTA and TA can also induce signalling in the macrophage [187]. For example, it has been shown that *dltD* mutants of *L. rhamnosus* GG that lacked *D*-alanine modifications on LTA generated stronger anti-inflammatory cytokine response compared to the wild-type parental strain. In addition, surface proteins of lactobacilli were shown to play important roles in modulating the host immune responses. For example, SlpA, a well-known S-layer protein of *L. acidophilus* NCFM, was shown to interact with the DC-SIGN receptor of DCs to induce changes in cytokine production and T cell maturation [188]. Surface PSs of lactobacilli were also shown to be recognised by the multiple C-type lectins on DC and macrophage. For example, overproduction of the cell wall associated PSs of *L. casei* Shirota was shown to

suppress macrophage activation that normally results in pro-inflammatory responses [189].

Overall, although some signalling responses induced by probiotic bacteria are immunostimulatory, they prevent inflammation and allergic response unlike those responses stimulated by pathogenic bacteria. The observed immunostimulatory and immunomodulatory effects are often strain-specific, suggesting the molecular immunomodulation mechanisms utilised by probiotic bacteria are diverse.

1.5 Secretion and surface display of the extracellular proteins of lactobacilli

1.5.1 Protein secretion

Approximately 25-30% of bacterial proteins are associated with the cell envelope or outside of bacterial cells [190]; they are collectively called "the secretome". Those extracellular proteins are initially synthesised in the bacterial cytoplasm and subsequently transported partially or fully across the cytoplasmic membrane to the cell envelope or secreted into the environment. Translocation across the bacterial cytoplasmic membrane is essential for functioning of extracellular proteins. In Gram-positive bacteria, there are various reported protein-transport mechanisms. The majority of the secretome proteins are translocated by the canonical SecYEG translocon and the corresponding accessory proteins. Other translocation pathways are non-canonical Sec translocons for glycosylated proteins, Tat (twin-arginine transport) for folded proteins containing prosthetic groups, or transporters for the components of surface organelles such as flagellar export apparatus (FEA), fimbrilin-protein exporter (FPE), or holins for export of phage lysins, peptide-efflux ABC transporters and WXG100 secretion system (Wss) [190-192]. Searching for the homologues of the key genes in the published *Lactobacillus* genomes showed that only genes involved in the canonical Sec pathway, non-canonical Sec pathway, FPE, peptide-efflux ABC and holin pathways are found *Lactobacillus* genomes [191].

1.5.1.1 The canonical Sec translocase

The canonical Sec translocase consists of an ATPase motor protein (SecA; also called SecA1) and a membrane protein-secretion channel (SecYEG), mediating translocation of the majority of unfolded proteins across the cytoplasmic membrane in Gram-positive bacteria (Figure 1.5a). The Sec translocase usually interacts with a heterotrimeric protein complex SecDF-Yajc that regulates the activity of SecA. In the cases of sorting the integral membrane proteins, YidC is also associated with the complex. Genes encoding this protein-secretion machinery are highly conserved in *Lactobacillus* species as observed in other Gram-positive bacteria.

Proteins transported via the canonical Sec pathway are synthesised as preproteins, which all contain a tripartite N-terminal signal sequence, consisting of a positively charged N-terminus, a hydrophobic core region (normally 15-25 residues) and a C-terminal polar terminus that often includes a cleavage site of the membrane-anchored signal peptidase [190]. While the precursor protein substrates are translocated across the cytoplasmic membrane, the signal peptides are cleaved off by the signal peptidases. Type I signal peptidase is dedicated to process the precursor proteins with canonical A-x-A-A cleavage site whereas Type II signal peptidase recognises the L-x-x-C cleavage site of the lipoproteins (also called the lipobox cleavage site).

1.5.1.2 The non-canonical Sec (SecA2) translocase

In addition to the conserved canonical SecYEG-SecA translocase, the genomes of many Gram-positive bacteria possess a SecA homologue, named SecA2, which was initially observed and characterised in *Mycobacterium*, *Listeria* and *Streptococcus* species [193-195]. Until recently, the fast-pace whole genome sequencing and metagenomic sequencing projects have revealed that SecA2 is also present in many other Gram-positive bacteria including several *Lactobacillus* species [196]. Although SecA2 homologues share variable sequence similarity with SecA, they are shown to be phylogenetically and functionally distinguishable membrane translocases [195, 196]. Unlike the canonical Sec translocase, the non-canonical Sec translocase in Gram-positive bacteria consists of either SecA2-SecY2-Asp4,5) (Figure 1.5b) or SecA2 in combination with SecYEG, also termed

"SecA2 only" (Figure 1.5c). The "SecA2 only" type of translocase is usually seen in the genomes of *Mycobacterium*, *Corynebacterium*, *Listeria*, *Bacillus* and *Clostridium* species.

The *secA2* sequence is generally shorter than *secA* within the same bacterial genome and is responsible for secretion of only narrow-spectrum substrates that are often required for virulence and/or colonisation [197]. Unlike SecA, SecA2 was reported to be non-essential for viability in Gram-positive bacteria such as *Listeria monocytogenes* [197]. In *Streptococcus* and *Staphylococcus* species, this type of non-canonical Sec system is dedicated to secreting a group of large glycosylated serine-rich adhesins, such as GspB of *Streptococcus gordonii*, which are important for virulence of those pathogenic bacteria [194]. These adhesins typically have an unusually long (~90 residues) N-terminal signal sequence, composed of an extended N-terminus containing a conserved KxYKxGKxW motif, a hydrophobic core region containing three conserved glycine residues and a C-terminal polar region containing the signal peptidase cleavage site. In GspB, the KxYKxGKxW motif and conserved glycine residues in the signal sequence and an adjacent AST (Ala, Ser and Thr-rich) motif direct the substrate protein towards the non-canonical Sec translocase, whereas the conserved glycine residues in the signal sequence and the the *O*-linked glycans in the glycosylated serine-rich region inhibit the sorting of the substrate proteins to the canonical Sec translocase. The genes encoding the substrate proteins are usually in the same gene cluster with the non-canonical Sec system components, together with two or more glycosyltransferases that *O*-glycosylate the substrate proteins. Compared to the well characterised SecA-mediated protein secretion mechanism, the knowledge on SecA2-based system is quite limited and is a subject of active research.

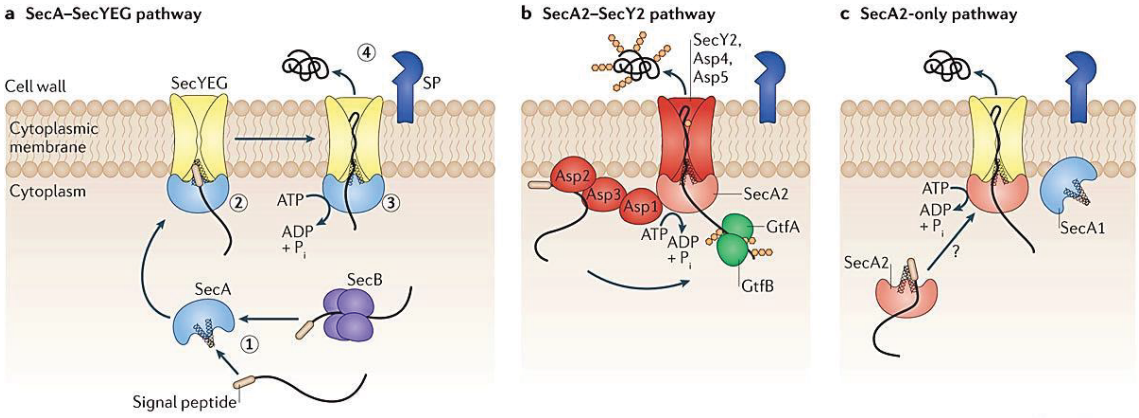

Figure 1.5. Models of Sec export

a) Post-translational Sec-mediated export is powered by the essential ATPase SecA. Preproteins synthesised with amino-terminal Sec signal peptides are bound by cytoplasmic SecA along a cleft between the two domains (step 1). Cytoplasmic chaperones, such as SecB, aid in keeping some preproteins unfolded before export and can directly deliver these preproteins to SecA. SecA delivers the preprotein to a membrane-spanning complex composed of SecY, SecE and SecG (step 2). SecA goes through rounds of conformational changes coupled to ATP hydrolysis to promote forward movement of the unfolded preprotein through the SecY channel (step 3). During or shortly after translocation, the signal peptide is removed by signal peptidases (SP) (step 4). b) The biogenesis of surface glycoproteins by SecA2–SecY2 systems involves both glycosylation factors (green) and export machinery (red) that are distinct from the canonical Sec machinery. Serine-rich proteins are synthesised with N-terminal signal peptides. The accessory Sec system proteins (Asps) promote SecA2–SecY2-mediated export by unknown mechanisms, but could target preproteins to the translocase and/or serve as a scaffold for the export complex. As glycosyl groups (orange hexagons) are added to the preprotein by cytoplasmic glycosylation factors, including the core GtfA and GtfB glycosyltransferases, SecA2 energizes transport of the unfolded preprotein through a channel formed by SecY2. c) SecA2-only systems lack a SecY2 channel; therefore, SecA2 probably uses the canonical SecYEG channel for export. The proteins that require SecA2-only systems for transport include proteins with N-terminal Sec signal peptides (pictured here) and also proteins lacking signal peptides. The ATPase activity of SecA2 probably drives the export of preproteins through the SecYEG channel, possibly in conjunction with SecA1. Adapted from [198].

1.5.1.3 Other protein translocation mechanisms

In addition to the SecA and SecA2, there are other genes found in *Lactobacillus* genomes encoding specialised types of protein secretion systems, including holins, FPE and ABC transporters.

Holins are small (60-150 residues) integral membrane proteins that are dedicated to secrete cytosolic fully folded muralytic enzymes [199]. Holins are often encoded by bacteriophage genomes and involved in the programmed host lysis by bacteriophage at the end of replication cycle. In *Lactobacillus* genomes, identified holin genes share low sequence homology to each other although they have predicted three-dimensional structural homology [191].

The FPE system is involved in the foreign DNA uptake by Gram-positive bacteria. In *Bacillus subtilis*, DNA uptake is mediated by pilus-like structure (pseudopilus). The pseudopilus components are proposed to be translocated to the cell surface through the FPE system consisting of several ComG proteins (ComGA-ComGG) and ComC that is a prepilin peptidase for cleavage of prepilin-like precursors at the cytoplasmic membrane [200]. The genes encoding the ComG homologues are widely present in *Lactobacillus* genomes, suggesting potential competency of lactobacilli.

Specialised ABC transporters are also encoded by the *Lactobacillus* genomes and dedicated for secretion of proteinaceous antimicrobial bacteriocins [191]. For example, many bacteriocins are synthesised in a prepeptide form, containing a leader peptide. The leader peptides share consensus sequences and contain two conserved glycine residues adjacent to the cleavage site [201]. However, these leader peptides are not processed by either the SecA translocation system or the type I signal peptidases. Instead, these prepeptides are recognised by cognate ABC transporters that remove the leader peptide through proteolytic cleavage and export the substrate across the cytoplasmic membrane [201].

1.5.2 Protein attachment to bacterial surface

The complex surface architecture of Gram-positive bacteria serves as a scaffold to display different extracellular proteins. After translocation across the cytoplasmic membrane of Gram-positive bacteria, proteins are either released into the medium or anchored on the surface of bacterial cells through various mechanisms, covalently or non-covalently.

1.5.2.1 Proteins containing transmembrane helices

Not all SecA-dependent extracellular proteins have a signal peptidase cleavage site in the "C" region of their signal peptides. Thus, after these preproteins are translocated across the cytoplasmic membrane, they are not released from the membrane. Instead, they are retained in the cytoplasmic membrane through the N-terminal signal peptide anchor. In lactobacilli, the N-terminally anchored proteins are involved in diverse extracellular functions such as transport, cell-wall metabolism and signalling [191]. Alternatively, some proteins with a C-region cleavage site can be anchored in the cytoplasmic membrane through their C-terminal end that contains a hydrophobic transmembrane helix as a membrane anchor, often called the "stop-transfer" signal or membrane anchor. The functions of the C-terminally anchored proteins in lactobacilli are largely unknown.

1.5.2.2 Covalently attached membrane proteins

Another group of cytoplasmic membrane anchored proteins are lipoproteins. These proteins carry a type II signal sequence that shares the similar tripartite organisation as type-I signal peptides except that the H region is shorter and C region has a signature lipobox motif (L-x-x-C). The cysteine residue in the lipobox motif is modified by the lipoprotein diacylglyceryl transferase. After the cleavage, the mature protein is covalently anchored in the cytoplasmic membrane through thioether linkage via the N-terminal Cys residue. *Lactobacillus* genomes encode various numbers of lipoproteins, which often play roles in substrate binding, antibiotic resistance and protein sorting [191].

1.5.2.3 Proteins containing a C-terminal cell-wall-anchor motif

In addition to the cytoplasmic membrane of Gram-positive bacteria, many extracellular proteins are covalently anchored to the PG layers. This type of proteins typically contains a cleavable N-terminal signal peptide as well as a C-terminal conserved peptidoglycan-anchoring motif, LPxTG, followed by a hydrophobic region and positively charged residues. The LPxTG motif is recognised by a transpeptidase, called sortase A (SrtA), which cleaves this motif between threonine and glycine residues and links the mature protein to the glycine residue of PG through the threonine residue [81]. Sortase-dependent peptidoglycan-anchored proteins are commonly present in lactobacilli and are involved in adhesion.

1.5.2.4 Cell wall-bound proteins

Many bacterial surface-attached proteins do not have a membrane anchor or LPxTG motif. Instead, these proteins contain one or more cell wall-binding domains (CWBDs) that non-covalently bind to the surface structures of Gram-positive bacteria. CWBDs include LysM, SLH, WxL, choline-binding domains and SH3.

LysM domain is found in many eukaryotic and prokaryotic proteins. In bacteria, LysM domain-containing proteins are often cell wall enzymes, such as hydrolases, peptidases, chitinases and esterases [202]. A LysM domain usually contains several LysM motifs (Pfam PF01476) each containing 44-65 residues. The optimal number of LysM motifs determines the function of these enzymes. Binding of LysM usually occurs at the specific site of peptidoglycan chains, most likely GlcNAc moiety[203]. Depending on the protein, the LysM motif can be located anywhere along a protein sequence, but is predominantly N- or C-terminal [191].

The SLH domains are mainly found in bacterial S-layer proteins that undergo self-assembly to form the tightly-knit S-layer on bacterial surface. Typically, Gram-positive S-layer proteins consist of three SLH domains that each contains 50-70 residues. These domains mediate the anchoring of the S-layer proteins to the bacterial surface [79, 80]. However, in *Lactobacillus* species, S-layer proteins do not have such SLH domains but instead contain two repeated amino acid motifs that share homology with the

carbohydrate-binding motifs of clostridial toxins and streptococcal glucosyltransferases [80]. It is proposed that the S-layer proteins of *Lactobacillus* species bind to the cell surface through secondary cell wall polymers, LTAs, TAs or neutral PSs [80, 204].

C-terminally located Wxl domain was initially discovered by *in silico* analysis of predicted proteins in genome sequences of lactobacilli, enterococci and listeria and located at the C-terminus of these proteins [205, 206]. The Wxl-domain-containing proteins were experimentally demonstrated to non-covalently bind to the cell wall of *E. faecalis*. Several *Lactobacillus* genomes, such as *L. plantarum*, *L. sakei*, *L. casei,* and *L. coryniformis*, are found to encode Wxl domain-containing proteins [63]. However, the functions of these proteins are unknown. In *L. plantarum*, a Wxl-containing protein is proposed to form a cell surface protein complex with other extracellular proteins, involved in carbon acquisition [205].

Bacterial SH3b domain (subfamilies SH3_3, SH3_4 and SH3_5) is the prokaryotic counterpart of the well-known eukaryotic SH3 domain and was shown to bind to the bacterial cell wall. The SH3b domain seems to bind to the various sites on the peptidoglycan in different bacteria. For example, in *Staphylococcus* species, the SH3_5 domain of lysostaphin ALE-1 binds to the cross-bridge of the cell wall [207, 208]. The binding is also dependent on the amino acid composition and length of the cross-bridge. On the other hand, SH3_5 domain from Acm2 protein of *L. plantarum* binds to the GlcNAc-containing glycan chains and targets this protein to the septum of dividing cells [209, 210]. In lactobacilli, the SH3b-containing proteins are proposed to function mainly in cell wall metabolism [191].

The choline-binding domain (Pfam PF01473) consists of multiple tandem copies of twenty-amino acid sequences that are rich in aromatic amino acids and glycines. Proteins carrying this domain are often enzymes, such as autolysins and cell wall hydrolases, which bind to the choline residues of LTA or TA of Gram-positive bacteria [191]. In lactobacilli, choline-binding domains are only found in few *Lactobacillus* species, such as *L. reuteri*, *L. salivarius* and *L. fermentum* [191].

1.5.3 Glycosylation of bacterial surface proteins

Glycosyl modification of proteins is widely observed in all domains of life. In particular, more than two-thirds of proteins are predicted to undergo glycosylation in eukaryotic cells [211]. In bacteria, the first identified glycosylated protein was the S-layer protein of hyperthermophilic C*lostridium* species [212]. According to the sites where glycans are covalently attached, there are two major protein glycosylation types, *N*-glycosylation (glycans are attached to the amide nitrogen of asparagine residues) and *O*-glycosylation (glycans are attached to the hydroxyl oxygen of serine or threonine residues). However, the glycosylation system is less characterised in bacteria compared to eukaryotic organisms. In bacteria, it has been reported that glycosylation has diverse impacts on the protein functions, such as solubility, stability, transport, assembly, adhesion, signalling and antigenic variation [213]. Therefore, glycoproteins are particularly important for both pathogenic and probiotic bacteria.

In pathogens, glycosylation can influence colonisation and virulence. For example, both flagella of *Campylobacter* species and pilin of *Neisseria* species are proposed to undergo phase-variable *O*-glycosylation that protects these bacteria from attack by the immune system and are potentially important for invasion and virulence [213, 214]. The glycans on proteins are also needed for adhesion of pathogens such as *Campylobacter* species to the host surfaces [215]. Disruption of *N*-glycosylation was demonstrated to significantly reduce capacity of some pathogens to invade and colonise their hosts [215, 216]. In *Streptococcus and Staphylococcus* species, glycans also help sorting of a group of adhesins through non-canonical Sec pathway [194]. In addition, *N*-glycosylation of protein VirB10 is essential for assembly of type IV secretion system required for transformation competence in *Campylobacter jejuni* [217].

In probiotic lactobacilli, glycoproteins are enzymes or immunomodulatory factors. S-layer proteins of various *Lactobacillus* species, including *L. reuteri*, *L. casei* and *L. acidophilus*, have been shown to interact with the DC-SIGN receptor of DC, to modulate the host immunity [218, 219]. For example, it has been demonstrated that in *L. acidophilus* NCFM, glycans on S-layer proteins are essential for recognition by DC-SIGN receptor and cytokine production in DC as well as T cell priming [219]. Another recently characterised glycoprotein is the major secreted protein of *L. rhamnosus* GG, called p75

or Msp1. This protein was shown to inhibit the cytokine-induced epithelial cell death and promote the cell growth [220]. Glycans on p75 enhance the stability of this protein and protect the protein from proteolytic degradation [221]. In *L. plantarum* WCFS1, the major autolysin, Acm2, is *O*-glycosylated in its AST domain in a species-specific manner [222]. In contrast, Acm2 isolated from *Lactococcus lactis* is not glycosylated [210].

Interestingly, it was shown that the removal of glycans from the AST domain of Acm2 of *L. plantarum* WCFS1, increased the peptidoglycan hydrolase activity of this enzyme [210]. It has been proposed by the authors that glycans maintain the proper conformation of AST domain which regulates the enzymatic activity of Acm2. This is the first example showing that *O*-glycosylation controls the enzymatic activity in bacteria.

1.6 Probiotic properties of *Lactobacillus rhamnosus*

In the past decades, *Lactobacillus* species have been broadly studied in terms of the health-promoting effects and their surface interactions with the host cells. Apart from well-studied *L. rhamnosus*, research publications using several other species such as *L. plantarum*, *L. acidophilus*, and *L. reuteri* have also demonstrated the diversity and specificity of their probiotic activities as reviewed in reference [56] and described in Section 1.3 and 1.4. For example, the work on *L. plantarum* species, which is a model probiotic microorganism, has not only revealed its clinical implications in intestinal disorders such as irritable bowel diseases [223] but also discovered (at least partially) the molecular mechanisms of adhesion (Table 1.1), MAMP-PRR interactions [224, 225] and NF-κB pathway-associated signalling [226]. However, these probiotic effects and their mediators are often strain-/species-specific. Therefore, it is often difficult to reveal specific function(s) of interest in one probiotic species based on the previous knowledge or bioinformatics prediction from other species. In this study, interactions between *L. rhamnosus* strains and their host were investigated.

L. rhamnosus species establishes in the intestinal tract of infants soon after birth, suggesting the importance of this species in the development of the human immunity [48]. Apart from the human intestinal tract [227], *L. rhamnosus* strains have been also widely

isolated from dairy products [57, 228] and human vaginal [229] and oral cavities [230]. Phylogenetically, the closest two species to *L. rhamnosus* are *L. casei* and *L. paracasei*, which together form the so-called "*Lactobacillus casei*" group.

L. rhamnosus has a relatively large genome (approximately 3.0-Mbp) in comparison to most lactic acid bacteria. Unlike *Bifidobacterium*, *Lactobacillus* genomes show higher genetic diversity [46]. Interestingly, in a recent report *L. rhamnosus* genomes were shown to encode the largest set of secretome proteins (more than 30% of the total proteins) among thirteen analysed *Lactobacillus* species [57, 231]. This suggests that *L. rhamnosus* might have excellent extracellular performance in communicating with the environment and human host. Even within the *Lactobacillus casei* group or *L. rhamnosus* species, strain-specific genetic and phenotypic features can be widely observed in surface properties (such as piliation), carbohydrate fermentation and TLR-2 activation [57, 232-235].

1.6.1 Clinical implications of *L. rhamnosus*

Probiotic *L. rhamnosus* strains, in particular GG, have been shown to have diverse health-promoting effects in clinical studies. Some results suggested that *L. rhamnosus* had potential therapeutic and/or preventive implications on the intestinal disorders. For example, various probiotic strains of this species have been shown to improve the intestinal barrier function in children with Crohn's disease [236], reduce the abdominal pain in children with irritable bowel syndrome [237], delay the first onset of pouchitis and maintain remission in patients with ulcerative colitis [238, 239], control the acute diarrhoea [33, 240], shorten the rotaviral diarrhoea and also significantly reduce the faecal rotavirus concentration in children [241-243] and prevent the antibiotic-associated diarrhoea [244-246].

Moreover, *L. rhamnosus* is capable of promoting human immunity and controlling eczema. For example, consumption of *L. rhamnosus* HN001 was shown to increase the activities of peripheral mononuclear cells and natural killer cells in healthy middle-aged and elderly people [247]. *L. rhamnosus* GG and HN001 could also prevent eczema in

infants [248-251]. Another probiotic strain *L. rhamnosus* 19070-2 could meliorate eczema in children when used in combination with *L. reuteri* DSM 122460 [252].

Interestingly, other studies demonstrated that *L. rhamnosus* strains GG has a potential to reduce the risk of dental caries [253, 254] and upper respiratory infections [255]. *L. rhamnosus* GR-1 was also able to control vaginal infections [256]. These findings suggest that *L. rhamnosus* can potentially have more diverse impacts on human health.

1.6.2 Molecular basis of the *L. rhamnosus*-mediated probiotic effects

One of the major bottlenecks for clinical use of probiotics is that the molecular basis underlying the probiotic functions is not completely known in most probiotic strains. However, numerous *in vitro* and *in vivo* studies have shown that *L. rhamnosus* has the potential to inhibit the intestinal pathogens, enhance the epithelial barrier functions and modulate the host immunity. For example, *L. rhamnosus* GG was shown to prevent the adhesion of pathogenic *E. coli* to the intestinal epithelial cells (HT-29) by stimulating mucus production [36, 168]. *L. rhamnosus* HN001 was also demonstrated to protect against the translocation of *Salmonella* Typhimurium in a mouse model [257]. Furthermore, the probiotic effects of *L. rhamnosus* on the inflammatory bowel disease have been at least partially attributed to the enhancement of barrier function. This could result from *L. rhamnosus*-induced expression and reorganisation of TJ proteins [258]. *L. rhamnosus* GG and HN001 were also shown to be capable of reversing the cytokine-altered TJ permeability [259, 260]. Alternatively, *L. rhamnosus* GG was shown to prevent the apoptosis and promote the proliferation of the intestinal cells to maintain the epithelial integrity [38, 172]. In addition, apart from blocking the pathogens' adhesion and post-adhesion inflammatory responses, *L. rhamnosus* strains such as GG and HN001 have been shown to interact with various immune cells resulting in specific immune responses, represented by the altered production of cytokines, chemokines and antibodies and modulated activities of immune cells, which might form the partial fundamental basis of probiotic effects on elimination of pathogens and relief of inflammation [41, 74, 90, 92, 261-263].

However, in contrast to common Gram-positive MAMPs such as PG, EPS and LTA, the information on the *L. rhamnosus*-specific proteinaceous effectors is basically obtained from SpaCBA pilus and the major secreted proteins, p75 and p40, of *L. rhamnosus* GG [38, 57]. SpaCBA pili have been demonstrated to play essential roles in adhesion, autoaggregation and biofilm formation [41, 84], which ensure the colonisation and persistence of this bacterium in the human gut. Interestingly, pili were also shown to regulate *L. rhamnosus* GG-induced host immune responses, such as IL-8 production and activation of ERK/MAPK in the intestinal cells [41, 115, 263]. However, whether these immune responses are direct (pilus-specific) or indirect (adhesion-dependent) is still to be revealed. Moreover, p75 and p40 are potent immune modulators that have been shown to prevent apoptosis and stimulate proliferation of the intestinal cells [38]. The subsequent studies revealed that p40 stimulated the release of HF-EGFs followed by activation of EGFRs, which in turn triggered the cellular signalling cascades and prevented the cytokine- and chemical-induced colitis [90, 264]. Other underlying molecular mechanisms are to be discovered.

1.6.3 *Lactobacillus rhamnosus* HN001

Approximately 20 years ago, in order to find strain(s) with typical characteristics of probiotics, an extensive screening of lactic acid bacteria was carried out at Fonterra Research and Development Centre (FRDC) to identify probiotic strains [228]. In the tests, a dairy isolate which was originally obtained from cheddar cheese, was identified as *Lactobacillus rhamnosus* and given the strain name HN001. This strain could adhere to various intestinal cell lines (Caco-2, HT-29 and HT29-MTX) and temporarily colonise the human gut [265].

In order to obtain an overview of the probiotic potential of HN001, a draft genome sequence (GeneBank accession # ABWJ00000000) of this bacterium has been generated, annotated and analysed. The estimated complete genome is about 3.0 Mbp in size and the average G+C content is around 46.4%, similar to those of other *Lactobacillus rhamnosus* genomes. Moreover, HN001 contains two plasmids, 8.75 Kbp (GeneBank accession # NC_011223) and 31.55 Kbp (GeneBank accession # NC_011225) in size. In particular, the larger plasmid carries an important microniche-specific lactose-utilisation gene

cluster, which allows HN001 to survive in dairy products (such as whey and milk, where lactose is the major carbon source). Through comparative analysis, many genes were identified and believed to be involved in contribution to flavour of food, survival in the host environment and health-promoting activities [266].

In the past years, the safety aspect and the probiotic effects of HN001 have been extensively studied [248, 257, 261, 265, 267-270] and several gene-deletion mutants have also been made (unpublished; FRDC). However, the molecular basis behind the probiotic effects observed in clinical and animal studies remains largely unknown. Besides the common Gram-positive structures such as LTA [271], understanding of the surface protein functions is limited to the bioinformatics prediction. In contrast to *L. rhamnosus* GG, HN001 genome does not carry SpaCBA pilus genes. Therefore, pilus could be an important surface structure responsible for variation in probiotic traits between GG and HN001.

Chapter Ib: Phage display as an efficient approach to study protein-protein interactions

Protein-protein interactions are important basis for both intracellular and extracellular biological activities. In the past decades, various biochemical and genetic approaches to study protein-protein interactions have been successfully developed and have been widely applied. Examples of the commonly used approaches are yeast two-hybrid assay [272], pull-down assay/mass spectrometry [273], far western blot [274], phage display [275] and other methods as reviewed in reference [276].

Yeast two-hybrid system is an *in vivo* system which include a bait protein fused to a DNA-binding domain and a target protein fused to a transcriptional activation domain of a transcriptional regulator. When a physical interaction is established between the bait protein and target protein, the activator and DNA-binding domains of transcriptional regulator are joined, resulting in a reporter gene activation. This allows the target protein to be subsequently identified. However, establishing a successful library of proteins fused to transcriptional activator domain in yeast *S. cerevisiae* is a prerequisite for the screening. In addition, only interaction between a protein pair can be identified at a time. This method is not suitable for surface and secreted proteins as the interaction takes part in the reducing environment of the yeast nucleus; furthermore the bait is limited to protein or DNA sequence and cannot be a complex entity such as cell or tissue.

Pull-down assay and far western blot are *in vitro* systems for capturing protein-protein interactions. Whereas the bait can be complex, the success is dependent on the assay conditions and protein properties. In pull-down assays, the bait protein is often fused to a tag such as glutathione-*S*-transferase and maltose-binding protein, which allow easy immobilisation and elution of the bait protein. The bait protein-binding proteins can be easily pulled down from the cell extract and subsequently analysed by protein gel electrophoresis and mass spectrometry. This method allows detection of protein complexes, however it is limited by the amount of target protein in an extract and affinity of particular protein-protein interaction(s). In the far western blot, proteins from the cell extract are firstly resolved on a two-dimensional protein gel and in turn blotted by the bait protein and bait protein-specific antibodies to detect the target. The target protein is typically identified by mass spectrometry.

Phage display is also an *in vitro* system, which utilises the potent virion-producing machinery of filamentous phage to establish a combinatorial genomic-proteomic library. Virions representing the phage display library display proteins or peptides on the surface, while containing corresponding coding sequence physically linked to the displayed protein by virture of packaging inside the virion. Therefore, isolation of a specific bait protein-binding virion will allow direct analysis of its coding sequence by simple DNA sequencing. In addition, screening of a phage display libraries can be carried out on baits of high complexity. Due to the advantages and technical feasibility of this method, phage display was used in this study.

1.7 An overview of Ff bacteriophage

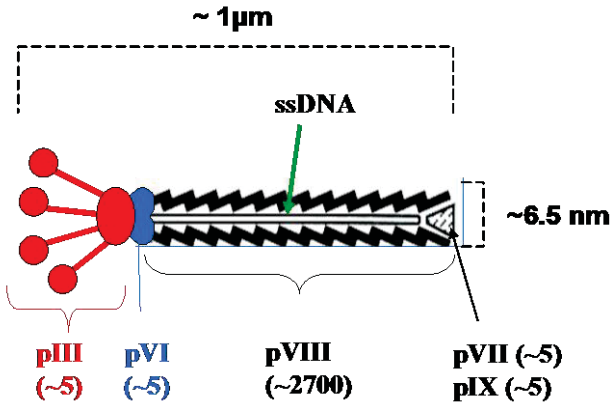

Figure 1.6. Schematic representation of Ff bacteriophage (not to scale)
Ff bacteriophage typically contains a circular single-stranded DNA (ssDNA) genome that is coated by about 2,700 copies of major capsid protein, pVIII, and 5 copies of each minor capsid protein, pIII, pVI, pVII and pIX. Adapted from [277].

Filamentous bacteriophages (or phages) are a group of long and thin bacterial viruses with a circular single-stranded DNA (ssDNA) genome. As only Ff (F-pilus specific) bacteriophages of *E. coli* are used in phage display technology, only these phages will be discussed. The Ff phage group includes f1, fd and M13, which share similar genomic sequences (98 % identity) and all use F-pilus as the primary receptor to infect their host [278, 279]. The length of a typical Ff phage is approximately 1 µm, but is not fixed and it depends on the size of the DNA genome. The Ff phage contains a simple and relatively

short DNA genome containing 6,407 nucleotides, encoding 11 proteins, pI to pXI. The protein coat of Ff phage consists of a few thousand copies of the major capsid protein, pVIII, and about five copies each of minor capsid proteins, pIII, pVI, pVII and pIX (Figure 1.6). Other phage-encoded proteins are involved in the different stages of Ff phage life cycles.

1.8 Ff bacteriophage life cycle

Ff phage life cycle (Figure 1.7) includes four steps: infection, genome replication, assembly and secretion [280]. The infection of Ff phage is initialised by the specific interactions between the capsid protein pIII and the surface primary F-pilus receptor and the periplasmic secondary TolQRA receptor of *E. coli* [281]. pIII is composed of three domains, named N1, N2 and C. N2 domain of pIII firstly binds to the tip of F-pilus and undergoes conformational changes after binding, causing the exposure of the binding sites for the secondary receptor on the N1 domain. This enables N1 domain to interact with periplasmic domain of the secondary receptor TolA to initialise virion uncoating and genome entry into the *E. coli* cytoplasm. However, the detailed mechanism is not quite clear. It was reported that covalent linkage between N1 and the C domain are essential for infection, as well as the TolQRA complex. Ff can infect *E. coli* that does not contain the F pilus at about 1% efficiency [282, 283].

After entry of the phage genome in the bacterial cytoplasm, the negative (-) strand is firstly synthesised by the host DNA-synthesis machinery using the phage genome (positive strand) as template, forming the double-stranded circular replicative form (RF). The replication protein of Ff phage, pII, binds to the RF at the (+) strand origin of replication and cleaves a specific sequence to generate free 3' end, which serves as a primer to allow synthesis of the positive strand [284]. In the early stage of infection, the newly synthesised positive strands of phage genomes are used as template to synthesise double-stranded DNA (about 50 copies per cell) in order to produce sufficient phage proteins. In the late stage of infection, the main body (except the packaging signal hairpin loop) of these positive strands are coated with ssDNA-binding protein, pV, and in turn subjected to assembly.

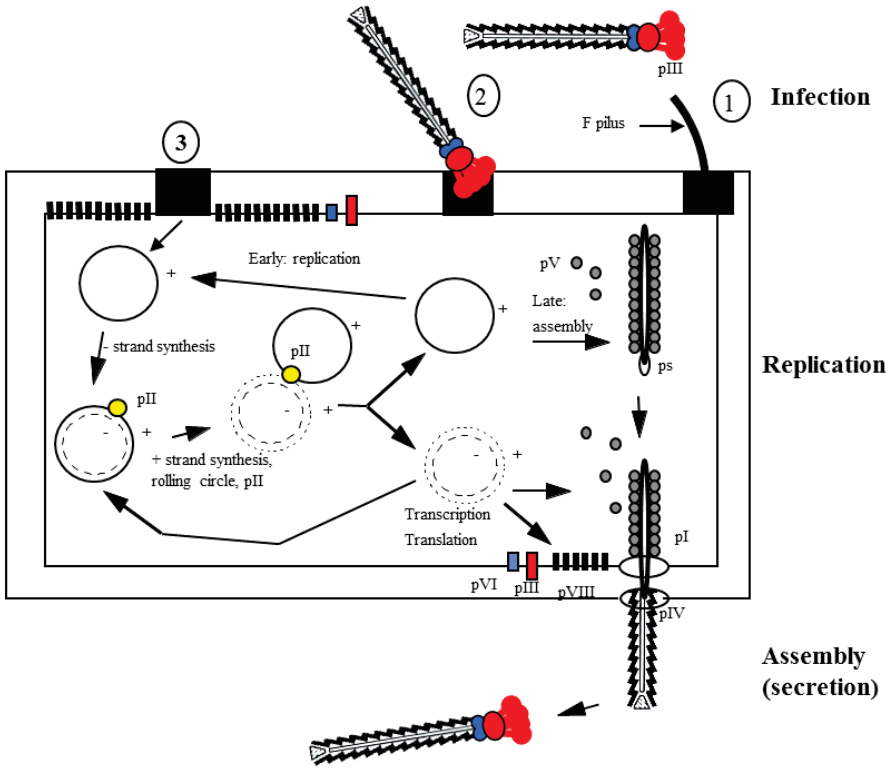

Figure 1.7. Schematic presentation of the life cycle of Ff bacteriophage
Infection is initiated by binding of the tip of the phage to the tip of the F pilus **(1)**. Secondary binding to the protein complex TolQRA embedded in the cytoplasmic membrane and spanning the periplasm **(2)**. Entry of ssDNA (+ strand) into the cell; coat protein integrates into the inner membrane as DNA enters the cytoplasm **(3)**. **Replication**: In the first stage negative (–) strand is synthesised by host proteins, starting from the (-) strand origin, primed by host RNA polymerase and carried out by the host DNA polymerase. At completion of negative (–) strand synthesis, the replicative form (RF) double-stranded DNA (dsDNA) is created. In the second stage phage protein II (pII) nicks the supercoiled dsDNA at the (+) strand origin, creating primer for the rolling circle synthesis of (+) strand. After one round, the product is cleaved off, again by pII, and the ends ligated. Early in the infection, (+) strand is recycled for replication; later, (+) ssDNA genome is coated by pV. This complex is the substrate for packaging into the viral particles. **Assembly/secretion**: pI/pXI phage proteins form multimers in the inner membrane while pIV multimer forms a large gated channel in the outer membrane; together they form the assembly machinery. An exposed hairpin loop at the tip of pV/ssDNA packaging substrate interacts with pI, and then distal phage tip proteins pVII and pIX, to initiate assembly. As ssDNA is extruded through the *E. coli* envelope, pVIII packs around it and in the end proteins pIII/pVI release phage from membranes. If pIII or pVI are non-functional, phage is not released from the cell membrane, forming long filaments which can be broken off by mechanical shearing. Adapted from [277] with permission.

Dimerization of DNA-coating protein pV shapes the DNA genome into a rod-like complex. The exposed packaging signal (a hairpin) interacts with cytoplasmic membrane assembly protein complex (ATPase) pI/pXI, and two capsid proteins, pVII and pIX, to initiate virion assembly. Protein pV is then displaced from the DNA and replaced by the major capsid protein pVIII. When the entire phage genome is coated with pVIII, another two capsid proteins, pIII and pVI, are added to the end of the virion, causing the release of phages from the inner membrane. It has been shown that addition of the C domain of pIII in combination with pVI is sufficient to trigger the release of phage [280, 285]. Protein pIV is the outer membrane component of phage assembly complex and its periplasmic domains interact with the inner membrane protein complex, pI/pXI [286]. Ff phage assembly does not kill *E. coli* host as it is assembled and released through a secretion-like process.

1.9 Ff phage display technology

The phage display systems are often based on either phage vectors (modified phage genomes) or phagemid vectors (specialised plasmids). In this study, the experiments were carried out using a phagemid vector-based phage display system. A typical phagemid vector contains the Ff phage origin of replication, an *E. coli* plasmid origin of replication, an Ff phage capsid protein-encoding gene, a multiple cloning site (for displaying a protein encoded by the insert through fusion with a coat protein) and an antibiotic-resistance marker. A helper phage is also required to supply all necessary phage proteins for the phagemid replication from the Ff origin in *E. coli,* assembly and secretion of complete virions, called phagemid particles (PPs). A helper phage itself is usually engineered to contain a defective Ff phage origin of replication and/or packaging sequence in order to allow phagemid genomes to be predominantly assembled and secreted. Desirably, the peptide encoded by a bacterial genomic fragment is expressed in the *E. coli* host and displayed (in fusion with the phage capsid protein) on PPs. The details of the phage display systems are reviewed elsewhere [280, 287, 288].

Phage display has been widely used to select, from large antibody, peptide, cDNA and microbial genomic libraries, those variants that bind to a ligand or “bait” of interest. This

is possible due to the physical link between the coding sequence packaged into the PPs or phage and protein displayed on the surface, which is the key principle of display technologies. Selection of a specific ligand from the phage display library against bait of interest is achieved from an affinity-purification method, called biopanning [289]. The high titres (up to 10^{13}/ml) of recombinant phages or PPs produced from *E. coli* [280] ensure that the representation of each individual recombinant library clone is high, thereby maximising the chance of affinity-selection of each library clone (variant), even those that bind to a "bait" of interest with a relatively low affinity. The selected variant is identified by DNA sequencing of the insert in the phage or phagemid. Phage display on Ff phage is ideal for surface and secreted proteins as the virion proteins themselves are integral membrane proteins and the virion is assembled at the cell envelope. Ff-based phage display has been used recently to identify functional extracellular proteins that bind to targets of interest from probiotic bacteria, such as the fibronectin-binding proteins of commensal bacterium *Lactobacillus casei* BL23 [290] and secretome proteins of *L. rhamnosus* HN001 and *L. reuteri* DSM20016T [291, 292].

1.10 SpcA, a *Lactobacillus rhamnosus* HN001-binding protein

In a preliminary study [277], a shot-gun HN001 phage display genomic library was constructed and screened using HN001 cell as bait, to identify potential aggregation proteins. In this screen, a partial ORF of a single gene (designated *spcA*) encoding an HN001-binding protein was identified [277]. As well as two bacterial immunoglobulin-like type 3 domains (Big-3; E-values $4e^{-04}$ and $3e^{-05}$) encoded by the inserts of recombinant phagemid clones selected for binding to HN001 cells, SpcA in all sequenced *L. rhamnosus* strains contained a third SCP-like extracellular protein domain (CD05379; E=$4e^{-3}$), which is C-terminal to the two Big-3 domains.

The Ig-like domains are commonly found in surface and secreted proteins in both prokaryotes and eukaryotes and are typically involved in extracellular adhesion and other binding activities [293, 294]. It was also shown, through deletion analysis, that, whereas

PPs displaying both Big-3 domains ($SpcA^A$ PPs) were able to bind to HN001, those displaying only N-terminal Big-3 domain ($SpcA^S$ PPs) were unable to bind [277].

The SCP protein family is present in diverse extracellular proteins of bacteria and eukaryotes. The initial identified protein in this family, was a murine extracellular glycoprotein [295]. Some members of this family are endopeptidases, some are transglycosylases and some have non-enzymatic roles. Analysis using the PHYRE protein structure prediction web server [296] selected a member of this family from plants, pathogen-recognition protein PR-1-like, as the top structural homologue of the SpcA C-terminal domain. The role of C-terminal domain of SpcA is, however, not possible to deduce, due to the lack of understanding of the exact function of the closest relative, PR-1, and the low homology to this or other proteins in the database.

Aims

One major bottleneck in clinical applications of probiotic bacteria is that the molecular mechanisms of their beneficial effects are mostly unclear. Until recently, some surface adhesins and immunomodulatory factors have been identified in *Lactobacillus* species; however the receptors on human intestinal cells are still to be discovered. Probiotic effects are also specific to individual species or strains, showing the complexity of specific interactions between probiotic bacteria and the human host.

SpcA is a recently identified *L. rhamnosus* HN001-binding protein, which is conserved in *L. rhamnosus* species. In *L. rhamnosus* GG and Lc705, SpcA contain 572 residues. However, as the partial ORF of *spcA* locates at the 3'-end of Contig00052 of HN001 draft genome, it was not certain whether the length and function of SpcA in HN001 was the same as in GG and Lc705. In addition, although described findings characterised the cell-binding domains of SpcA, the receptor of this protein on the surface of HN001 cell was unknown, nor was the role of the SpcA and its ligand in the HN001 interactions with the host and environment.

Therefore, this study aimed to identify the complete sequence of SpcA in HN001 and the

receptor of SpcA on HN001 surface and then to characterise the functions of SpcA and its receptor in the surface properties of *L. rhamnosus* and their interactions with the human host.

Chapter II: Materials and Methods

2.1 Materials and Culture Conditions

2.1.1 Bacterial strains, growth conditions and helper phage

The detailed list of bacterial strains and helper phage in this study is given in Tables 2.1 and 2.2, respectively. *Escherichia coli* TG1 was utilised to propagate phagemid vectors and for obtaining stocks of the helper phage VCSM13 (Stratagene). TG1 transformed with phagemid vectors or recombinant phagemids was infected with VCSM13 to allow packaging of phagemid DNA into phagemid particles (PPs) and display of *L. rhamnosus* proteins on the surface of the PPs. *E. coli* cells were grown in Yeast Extract Tryptone broth (Difco™ 2xYT) aerobically at 37 ºC with aeration. *Lactococcus lactis* MG1363 was used to construct and maintain the plasmids for replacement mutagenesis of genes in *L. rhamnosus* strains. MG1363 was cultured aerobically in M17 (Difco™) liquid or solid medium supplemented with 1% glucose at 30 °C. *Lactobacillus rhamnosus* strains HN001 and GG wild-type and their derived mutant strains (listed in Table 2.1) as well as other lactobacilli were propagated in the Man-Rogosa-Sharpe (MRS; Difco™) broth at 37°C anaerobically. Bacteriological Agar (Oxoid) was used at 1% (w/v) for solid medium. When required, antibiotics were added to the media at the following concentrations: 25 μg/ml chloramphenicol (Cm) and 100 μg/ml ampicillin (Amp) for *E. coli*; 5 μg/ml erythromycin (Em) for lactococci and lactobacilli; 5 μg/ml tetracycline (Tet) and 500 μg/ml streptomycin (Str) for lactobacilli.

Table 2.1. Bacterial strains used in this study

Strains	***Genotype***	***Reference***
Escherichia coli TG1	*supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5* (rK^- mK^-) [F′ *traD36 proAB lacI*^{q}Z*ΔM15*]	[297]
E. coli K1981	TG1//pYW01	[277]
E. coli K2090	TG1//pSpcA^A	[277]
E. coli K2208	TG1//pMBP-SpcA^{A1}	This study
E. coli K2212	TG1//pMal-c2X	This study
E. coli K2232	TG1//pSpcB^{N1}	This study
E. coli K2233	TG1//pSpcB^{N2}	This study

Lactococcus lactis MG1363	NCDO (prophage-cured)	[298]
Lactobacillus rhamnosus HN001	Wild-type	[228]
L. rhamnosus GG	ATCC53013; Wild-type	[57, 299]
L. rhamnosus L002	HN001 *rpsL1* (*str^R*)	FRDC
L. rhamnosus L003	HN001 *welE*::*em^R* (insertional mutant)	FRDC
L. rhamnosus L004	HN001 *rpsL1* (*str^R*) *ΔdltD*::*tet^R* (replacement mutant)	FRDC
L. rhamnosus L024	HN001 *rpsL1* (*str^R*) *ΔspcA*::*tet^R* (replacement mutant)	This study
L. rhamnosus L035	GG *rpsL1* (*str^R*)	This study
L. rhamnosus L036	GG *rpsL1* (*str^R*) *ΔspcA*::*tet^R* (replacement mutant)	This study
L. rhamnosus L041	HN001 *rpsL1* (*str^R*) *ΔspcB*::*tet^R* (replacement mutant)	This study
L. rhamnosus L042	GG *rpsL1* (*str^R*) *ΔspcB*::*tet^R* (replacement mutant)	This study
L. plantarum WCFS1	Wild-type	[300]

2.1.2 Plasmids, phagemids and phage

The plasmids, phagemids and phage used in this study are listed in Table 2.2.

Table 2.2. Phage, phagemids and plasmids

Name	***Description***	***Antibiotic resistance***	***Reference***
VCSM13	Helper phage	Kanamycin (Km)	Stratagene
pYW01	Phage display vector	Cm	[277]
pSpcA^A	pYW01::*spc*A (1-1128 nt)	Cm	[277]
pMal-c2X	Expression vector for construction of N-terminal MBP fusions	Amp	New England Biolabs
pMBP-SpcA^{A1}	pMal-c2X:: *spcA* (351-927 nt)	Amp	This study

pFRC027	A shuttle vector for marker-replacement mutagenesis in *L. rhamnosus*	Tet, Em	FRDC
pSpcA1^{KO}	pFRC027-derived knock-out construct for deletion of *spcA1*	Tet, Em	This study
pSpcB^{KO}	pFRC027-derived knock-out construct for deletion of *spcB*	Tet, Em	This study

2.1.3 Oligonucleotides

The primers and DNA probes used in this study are listed in Table 2.3.

Table 2.3. Oligonucleotides

Name	*Sequence*	*Restriction site*	*Application*
pWW18	AAGGAAAAAAGCGGCCGCGCTGC CGGTGTCCTCAAAGG	NotI	Cloning
pWW19	CAATTCCCATGGAGATCTCTCGAG CCATACCCTCCAAACTTCAGTG	NcoI	Cloning
pWW20	AATGCCCTAGGGTCGACCCCGGG TTTTACTGATGCCCCAACCGG	SalI	Cloning
pWW21	GGACTAGTGGACGGTAAAAACTC ACGCCC	SpeI	Cloning
pWW22	TTTACCGTCCCCCTGCAGGT		Sequencing
pWW23	TTCAACGTGCCGATCAAAGAC		Sequencing
pWW24	ACAACAAGTGAGAAAAGGGCTA		Sequencing
pWW25	CGATTAAAGATAAGGGAAGATAC		Sequencing
pWW26	ACTTTGTAAACAGCTACCGTCA		Sequencing
pWW30	TAGGCGCTTCAAAGGCATAATA		Sequencing
pWW31	TATTGCAGGTTGGTTGCTCCA		Sequencing
pWW37	CCGGAATTCAATAACCCAGAGCC GGAAGC	EcoRI	Cloning
pWW50	AAAACTGCAGCTATTATTTTGGAT CCATAAAACTAAAACG	PstI	Cloning

pWW59	AAGGAAAAAAGCGGCCGCCGTCG ATGTTTCAAGTGAGATC	NotI	Cloning
pWW60	CAATTCCCATGGGTGTTCACCCCA TTTAAAGAGAA	NcoI	Cloning
pWW61	AATGCCCTAGGGTCGACTTAACTG AAAGGAAGCCATTAATAT	SalI	Cloning
pWW62	GGACTAGTAGGTCTGGTTAACTTG ATTTTCTA	SpeI	Cloning
pWW69	CAAACAACGAGCATTCAGGG		Sequencing
pWW80	GGACGTGCTGACTGGGTTAAG		Sequencing
pWW81	CTCAGCTAGCAAGCCAAAGCG		Sequencing
pWW82	GTAGCAGCCCTCTAATTTGGC		Sequencing
pWW83	TCAAGCTTCGTAGCAGCCCTC		Sequencing
pWW86	CGCAATCCTGCCTTGATCAG		Sequencing

2.1.4 Solutions and buffers

Laboratory solutions and common buffers were prepared as described in Sambrook and Russell (2001) [301].

2.1.5 Chemicals, reagents, and enzymes

Common chemicals were purchased from Sigma-Aldrich, Merck Ltd, and BDH. All bacterial media (Difco^{TM}) were bought from Becton-Dickinson. Restriction endonucleases were obtained from Roche Applied Sciences and New England Biolabs Inc. DNA polymerases were purchased from Takara Bio (Japan). The DNA purification kits were purchased from Roche Applied Sciences, Qiagen and Life Technologies.

2.2 Methods

2.2.1 General molecular biology methods

General molecular biology techniques were implemented as instructed by Sambrook and Russell (2001) [301].

2.2.2 General DNA techniques

2.2.2.1 Genomic DNA purification from Lactobacillus rhamnosus

For PCR and Southern blot, genomic DNA was purified from *L. rhamnosus* strains using a modified method described by Jankovic *et al*. (2007) [291]. Briefly, bacteria were grown in 20 ml of MRS broth at 37℃ overnight. Cells were pelleted by centrifugation at 5,500xg for 10 minutes (min) at room temperature and subsequently resuspended in 20 ml of fresh MRS broth. The cultures were incubated at 37℃ for 2 hours (h). The cells were then harvested by centrifugation and washed twice in Buffer A (30 mM Tris pH 8.0, 5 mM EDTA and 50 mM NaCl) and the final cell pellet was resuspended in 0.5 ml of Buffer B [the lysis buffer; 50 mM Tris pH 8.0, 1 mM EDTA, 25% (w/v) sucrose, 20 mg/ml lysozyme and 20 µg/ml mutanolysin (Sigma-Aldrich)]. The resuspended cells were incubated in the Buffer B at 37℃ for 1 h to weaken the bacterial cell wall, followed by addition of 0.5 ml of 0.25 M EDTA and incubation at room temperature for 5 min. Next, 200 µl of 20% (w/v) SDS was added to solubilise the membranes and the mixture was incubated at 65℃ for 1 h. Proteinase K (10 µl of 20 mg/ml solution; Roche Applied Sciences) was added to the mixture and the reaction was incubated at 65℃ for 15 min to degrade the proteins. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) solution was added and mixed thoroughly. After centrifugation at 5000xg for 10 min, the DNA-containing aqueous phase was carefully transferred to a new tube and this purification step was repeated. DNase-free RNase was added to the aqueous fraction harvested after the second extraction at a final concentration of 100µg/ml and the reaction was incubated at 37℃ for 30 min followed by two phenol/chloroform/isoamyl alcohol extractions as described above until the white particulate protein layer at the interface was minimal. The DNA was precipitated by adding two volumes of cold 95% (v/v) ethanol

and incubation on ice for 30 min. The DNA pellet was obtained by centrifugation at 14,000xg at 4℃ for 10 min then washed in 70% (v/v) ethanol. Finally, the DNA pellet was air-dried and resuspended in 100 µl of 10 mM Tris-HCl pH 8.0.

2.2.2.2 Plasmid purification

Small scale (up to 5 ml of bacterial culture) plasmid purification from *E. coli* was carried out using a High Pure Plasmid Isolation Miniprep Kit (Roche) according to the manufacturer's instructions. For large scale plasmid purification from *E. coli* (25 ml of bacterial culture), a PureLink® HiPure Plasmid Filter Midiprep Kit (Life Technologies) was used according to the manufacturer's instructions.

Plasmid purification from *L. lactis* MG1363 was carried out using the same kits as described above, including additional steps to allow the lysis of these Gram-positive bacterial cell wall. Briefly, mid-log-phase ($OD_{600} \approx 0.6$) MG1363 culture (up to 5 ml for miniprep/100 ml for midiprep) was centrifuged at 5,500xg for 10 min to harvest the cells. The cell pellet was resuspended in Solution A [6.7% (w/v) sucrose, 50 mM Tris-HCl pH 8.0 and 1 mM EDTA] (379 µl for miniprep/6 ml for midiprep). Next, lysozyme (Sigma-Aldrich) solution (10 mg/ml in 25 mM Tris-HCl pH 8.0) was added to weaken the bacterial cell wall (97 µl for miniprep/1.5 ml for midiprep) followed by incubation at 37℃ for 10 min. The Solution B (50 mM Tris-HCl pH 8.0 and 0.25 M EDTA) was added (48.2 µl for miniprep/0.75 ml for midiprep) to stop the reaction. The cells were then collected by centrifugation at 1,500xg for 3 min and processed for plasmid purification by following the instructions described in the kit manuals.

2.2.2.3 Polymerase Chain Reaction (PCR)

The PCR reactions for cloning and sequencing were carried out using the PrimeSTAR GXL DNA polymerase (Takara Bio). For diagnostic PCR reactions used to confirm the presence of correct DNA fragments, SpeedSTAR HS DNA polymerase (Takara Bio) was used. The Whatman Biometra thermal cycler (UK) was used for all PCR reactions.

2.2.2.4 Southern blot

Amersham™ ECL Direct Labelling and Detection System (GE Health Life Sciences) and DIG Labelling and Detection Kit (Roche Applied Sciences) were used in the Southern blot experiments for confirmation of *L. rhamnosus* knock-out mutants, according to the manufacturers' instructions. Briefly, 3 µg purified genomic DNA of an *L. rhamnosus* strain was digested by either 30 unit HindIII (for confirmation of HN001*ΔspcA*) or PstI (for confirmation of HN001*ΔspcB,* GG*ΔspcA* and GG*ΔspcB*) at 37℃ overnight. Next, digested DNA fragments were resolved based on the size using 0.8% (w/v) agarose gel electrophoresis, transferred to positively charged nylon membrane, and hybridized with one of the labelled DNA probes (Probe-U1 and Probe-D for HN001*ΔspcA*; Probe-U2 and Probe-D for HN001*ΔspcB,* GG*ΔspcA* and GG*ΔspcB*). When using the ECL kit, horse radish peroxidase (HRP) was directly cross-linked to the probes using glutaraldehyde. After hybridization at 42℃ overnight and stringent washing, the membrane was incubated in the substrate solution provided in the kit. The chemiluminescence was detected by exposure of an X-ray film. When using the DIG kit, the probes were firstly labelled with digoxigenin using the random priming method. After hybridization at 42℃ overnight and stringent washing, the membrane was incubated in alkaline phosphatase conjugated anti-digoxigenin antibody solution. The excess unbound antibodies were removed by washing, followed by incubation in the chromogenic substrate solution provided in the kit to obtain visible bands on the membrane.

2.2.3 Preparation and transformation of *Escherichia coli* TG1 chemically competent cells

E. coli TG1 chemically competent cells were prepared and transformed as described by Sambrook and Russell (2001) [301] with slight modifications. Briefly, TG1 cells were grown in 100 ml of 2xYT broth until early-log-phase ($OD_{600nm} \approx 0.3$). The culture was chilled on ice and the cells were collected by centrifugation at 4,000xg at 4℃, washed in ice-cold 0.1 M $CaCl_2$ solution once and resuspended in 1 ml of ice-cold solution containing 0.1 M $CaCl_2$ and 10% (v/v) glycerol. Aliquots (100 µl) of competent cells were mixed with 10 µl of DNA solution of various concentrations (depending on the experiment) followed by incubation on ice for 30 min. Next, the cell-DNA mixture was

heated at 42℃ in a water bath for 2 min and subsequently chilled on ice for 2 min. SOC broth (1 ml) [301] was added to the transformed cells. The transformed cells were recovered by incubation at 37℃ for 1 h, followed by plating on 2xYT agar containing 100 µg/ml ampicillin and incubation at 37℃ overnight.

2.2.4 Preparation and transformation of *Lactococcus lactis* MG1363 electro-competent cells

L. lactis strain MG1363 was grown in 100 ml of M17 broth supplemented with 1% (w/v) glucose and 45 mM threonine [302] at 30℃ until OD_{600nm} reached 0.5. The culture was chilled on ice for 10 min and the cells were subsequently collected by centrifugation at 3,000xg at 4℃ for 10 min, followed by two washes in 20 ml of ice-cold EPB buffer (5 mM KPO4 buffer pH 7.4, 1 mM $MgCl_2$ and 0.5 M sucrose) and resuspension in 1 ml of chilled EPB buffer. A 100 µl aliquot of competent cells was mixed gently with 5 µl of desalted DNA (dialysed for 30 min against sterile distilled water on a floating MF-Millipore filter, type VS 0.025 µm). The cell-DNA mixture was transferred to a chilled 0.2 cm electroporation cuvette (Bio-Rad) and electroporated at (2.5 kV, 2.5 µF and 200 Ω) using a Bio-Rad Gene Pulser. The recovery broth [M17 broth supplemented with 1% (w/v) glucose and 0.5 M sucrose] was immediately added to the cells and the transformation mix was incubated at 30℃ for 2 h, followed by plating on M17 agar containing 1% (w/v) glucose, 50 mM sucrose and 5 µg/ml erythromycin. The agar plates were incubated at 30℃ for 3 days.

2.2.5 Preparation and transformation of *Lactobacillus rhamnosus* electro-competent cells

L. rhamnosus HN001 and GG electro-competent cells were prepared and transformed as described by Varmanen *et al*. (1998) [303] with modifications. Briefly, bacteria were grown in 100 ml of MRS broth supplemented with 2.5% (w/v) glycine and 300 ng/ml ampicillin at 37℃ until the OD_{600nm} reached 0.3. Cells were collected by centrifugation at 3,000xg at room temperature for 10 min, washed in 20 ml of V buffer$^{\delta}$ (7 mM KPO_4 buffer pH 7.4, 1 mM $MgCl_2$ and 0.5 M sucrose) at room temperature three times, and

resuspended in 1 ml of V buffer[δ]. A 100 µl aliquot of electro-competent cells was mixed with 5 µl of DNA of various concentrations and the mixture was transferred to a 0.2 cm electroporation cuvette (Bio-Rad). The electroporation was carried out at 1.5 kV, 25 µF and 200 Ω, followed immediately by addition of 1 ml of recovery broth [MRS supplemented with 20 mM $MgCl_2$, 2 mM $CaCl_2$ and 50 ng/ml (w/v) erythromycin]. The transformed cells were subsequently incubated at 37℃ for 3 h, followed by plating on MRS agar containing 3.5 µg/ml erythromycin and the plates were incubated at 37℃ for three days.

2.2.6 Basic phage and phagemid particle related protocols

The phage and phagemid particle methods used in this project were previously described by Jankovic (2008) [277] with slight modifications. Virions containing the helper phage genomes were termed phages, whereas virions containing phagemid ssDNA were termed phagemid particles (PPs).

2.2.6.1 Enumeration of phage and PPs

Enumeration of helper phage VCSM13 was carried out by titration on TG1 on 2xYT agar. Firstly, 0.2 ml of fresh stationary-phase culture of an indicator *E. coli* strain (TG1) was mixed with 2.5 ml of soft agar [0.5% (w/v)]. The mixture was poured on the top of a 2xYT agar plate and allowed to solidify. A serial phage stock solutions were prepared in 2xYT broth and 5 µl drops were placed on solidified soft agar layer. After incubation at 37℃ overnight, the plaques were counted and the titre of the phage stock was calculated expressed as plaque-forming units (pfu) per ml of the undiluted stock.

For enumeration of PPs, due to the presence of chloramphenicol-resistance marker, the titre of PPs was determined by counting the TG1 cells transduced to Cm-resistance. Firstly, 9 ml of melted antibiotic-free 2xYT agar was poured on the top of a 2xYT agar plate containing 25 µg/ml Cm and left for several min to solidify. The fresh overnight TG1 culture (0.2 ml) was added to 2.5 ml of soft agar and the mixture was poured on the top of this double-layer Cm-containing plate. The serial dilutions of PPs stock were placed onto the solidified soft agar as described above for the phage titration. After

overnight incubation at 37℃, the Cm^R colonies were counted and the titre of PPs stock was calculated and expressed as colony-forming units (cfu) per ml of the undiluted stock.

2.2.6.2 Production and purification of helper phage VCSM13 stock

A single plaque of the VCSM13 helper phage was picked, dissolved in 1 ml of 2xYT medium, incubated for 1 h at room temperature with slow agitation and filtered through a 0.45 μm filter to remove bacterial cells. The titre of VCSM13 in dissolved single plaque was estimated by titration on *E. coli* TG1 on 2xYT agar as described in Section 2.2.6.1 above. To make a plate stock, about 10^6 phages (pfu's) were mixed with 2.5 ml of 0.5% (w/v) soft agar in 2xYT and 200 μl of fresh overnight TG1 culture. The mixture was poured onto a 2xYT agar plate followed by incubation at 37℃ overnight. In order to obtain enough helper phages, multiple plates were prepared in parallel. Next day, 5 ml of 2xYT broth was added to each plate and subsequently incubated with slow agitation at room temperature for 1 h. Eluted phages were pooled together followed by centrifugation at 9,000xg for 20 min to remove most bacterial cells. The supernatant containing phages was further filtered (0.45 μm) to remove *E. coli* cells. The concentration of the phage stock (typically around 10^{12} pfu's per ml) was determined by titration as described above. The phage stock was routinely stored at 4℃.

2.2.6.3 Production and purification of PPs stocks

A fresh overnight culture of *E. coli* TG1 strain containing either a phagemid vector or a recombinant phagemid was diluted 100-fold into 100 ml of 2xYT broth supplemented with 25 μg/ml Cm and incubated with aeration at 37℃. When the OD_{600nm} of this culture reached 0.2 (approximately 10^8 bacteria per ml), *E. coli* was infected with helper phage VCSM13 at multiplicity of infection (m.o.i.) of 50 phages to 1 bacterium. Upon addition of the phage solution, the culture was incubated at 37℃ without agitation for 30 min, followed by incubation at 37℃ with aeration for 4 h. The culture was chilled on ice and centrifuged at 9,000xg at 4℃ for 20 min to remove bacterial cells. The supernatant was transferred to a centrifuge tube and mixed with 20 ml of ice-cold 6x polyethylene glycol 6000 (PEG)/NaCl solution [30% (w/v) PEG and 3 M NaCl] followed by incubation at 4℃ overnight to allow precipitation. Next day, the precipitated PPs were sedimented by

centrifugation at 9,000xg at 4℃ for 1 h, resuspended in 1 ml of 1xTris-buffered-saline (TBS) pH 7.4 and passed through a 0.45 μm syringe filter to remove the bacterial cells. The PPs stocks were supplemented with dimethyl sulfoxide (DMSO) at 7% and stored in aliquots at -80℃. The PPs were enumerated by titration as described in the Section 2.2.6.1.

2.2.7 Phagemid particle-lactobacilli binding assay and enzymatic treatment of *L. rhamnosus* HN001

2.2.7.1 PPs-lactobacilli binding assay

The PP-lactobacillus binding assay was performed according to a method described by Jankovic (2008) [277] with modifications. Briefly, about 10^9 cells from 1.5 ml of early-stationary-phase lactobacilli cultures were collected by centrifugation at 2,000xg at room temperature for 3 min. The cells were washed in TBS by centrifugation and resuspended in 0.6 ml of the same buffer. The PPs were mixed with the washed cells ($3x10^9$ PPs per assay; either the vector pYW01 or SpcA-displaying $pSpcA^A$ PPs). The cell-PPs mixture was incubated at room temperature for 3 h with slow rotation to allow binding. The unbound PPs were then removed by pelleting bacterial cells at 2000xg and resuspending in 1 ml of TBS (5 times). The cells containing bound PPs were resuspended in 50 μl of 2xYT broth and transferred to a new tube. The lactobacillus-bound PPs were enumerated by titration through direct addition of indicator *E. coli* strain TG1 to the lactobacilli suspension by adding 0.5 ml of the early-log-phase ($OD_{600nm} \approx 0.2$) TG1 culture followed by incubation at 37℃ for 30 min. The infected TG1 cells were diluted in 2xYT broth and enumerated by titration on a double-layer Cm-containing plate as described in Section 2.2.6.1. In order to identify the nature of the SpcA-binding partner, the enzymatically pre-treated HN001 cells were used in the assays (Sections 2.2.7.2 and 2.2.7.3).

2.2.7.2 Preparation of surface protein-free Lactobacillus rhamnosus HN001

HN001 cells from 1.5 ml of early stationary-phase culture were collected by centrifugation and washed once in TBS pH 7.4. The resulting cell pellet was then resuspended in 0.7 ml of Buffer P(+) (100 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$ and 5 mg/ml proteinase K) and Buffer P(-) (100 mM Tris-HCl pH 7.5 and 5 mM $CaCl_2$),

respectively. All samples were incubated at 37°C for 1 h. Cells were then washed four times in an equal volume of cell-wash buffer [TBS pH 7.4, 1 mM EDTA, protease inhibitor cocktail (Roche)], by pelleting and resuspension, followed by a 15-min incubation at 15 °C for all washes. After the final wash, cells were resuspended in 0.6 ml of the same buffer and used immediately in the binding assay as described above for untreated cells (Section 2.2.7.1). Buffer control for this experiment contained the cells treated in the same manner, except that proteinase K was omitted. To ensure that protease did not degrade pIII or displayed SpcA on the surface of PPs during binding assay (which would result in loss of infectivity and/or failure to bind HN001 cells due to degradation of pIII and SpcA, respectively), a mock protocol was set up using a mixture of cell-wash buffer (TBS pH7.4, 1 mM EDTA and protease inhibitor cocktail), and the proteinase K-containing buffer, allowing for expected protease dilution (achieved during the cell washing procedure after protease incubation) in the PPs-cell binding step. The titre remained the same, confirming no degradation of pIII and by deduction no degradation of displayed SpcA (data not shown).

2.2.7.3 Preparation of Lactobacillus rhamnosus HN001 protoplasts for binding assays

HN001 cells from 20 ml of late-exponential-phase culture were collected by centrifugation and resuspended in an equal volume of pre-warmed MRS broth. After the culture was incubated for another 2 h, cells from 1.5 ml of culture were collected and washed once in TBS pH 7.4 by centrifugation. The cell pellet was resuspended in 0.5 ml of L-M Buffer(+) [TBS pH 7.4, 25% (w/v) sucrose, 1 mM EDTA, 20 mg/ml lysozyme and 20 µg/ml mutanolysin] and L-M Buffer(-) [TBS pH 7.4, 25% (w/v) sucrose and 1 mM EDTA], respectively. After reaction was carried out at 37°C for 1 h, 0.5 ml of 0.25 M EDTA was added. The mixture was then incubated on ice for 5 min to stop the reaction. The resulting protoplasts were collected by gentle centrifugation (1000 x g), washed once with TBS and resuspended in 0.6 ml of the assay buffer (Section 2.2.7.1). Buffer control for this experiment contained the cells treated in the same manner, except that lysozyme and mutanolysin were omitted.

2.2.8 Electron microscopy

Electron microscopy was carried out to confirm the binding of $SpcA^{A}$-displaying PPs to HN001 cells using a previously reported method [304] with slight modification. Briefly, after the binding step as described in Section 2.2.7.1 [except that the assay buffer was PBS pH 7.4 (phosphate-buffered-saline) instead of TBS pH 7.4], equal volume (about 630 µl) of 8% (v/v) paraformaldehyde was added to the assay buffer followed by incubation at room temperature for 15 min. The reaction was stopped by adding 32 µl of 1 M $NaBH_4$ and the mixture was incubated at room temperature for 5 min. The cells were then washed once in PBS and subsequently resuspended in 0.2 ml of anti-fd-phage rabbit polyclonal antibodies (AB6188; Abcam®; 1:100 dilution in PBS) followed by incubation at room temperature for 1 h. The cells were subsequently collected by centrifugation and resuspended in gold-conjugated anti-rabbit polyclonal antibodies (1:10 dilution in PBS). The mixture was incubated at room temperature for 1 h. The cells were then collected by centrifugation and resuspended in 0.2 ml of H_2O. Cells were further diluted until their density was suitable for microscopy and examined under an electron microscope directly.

2.2.9 Expression and purification of MBP-SpcA fusion

DNA sequence encoding the N-terminal portion of SpcA, containing both Big-3 domains, but lacking the signal sequence ($SpcA^{A1}$, residues 117 to 309) was amplified by PCR, using genomic DNA as template and primers pWW37 and pWW50 (Table 2.3). The primers introduced EcoRI and PstI sites at the ends of the amplicon. The PCR product and the expression vector pMal-c2X (New England Biolabs) were digested with EcoRI and PstI restriction enzymes and ligated to EcoRI-PstI-cut pMAL-c2X vector which encodes maltose-binding protein N-terminal tag (MBP), to obtain recombinant plasmid pMBP-$SpcA^{A1}$. The MBP-$SpcA^{A1}$ fusion was then expressed in TG1 cells and affinity-purified using amylose resin (New England Biolabs) according to the manufacturer's instructions. Purity of the protein was analysed by SDS-PAGE [305].

2.2.10 Screening of *Lactobacillus rhamnosus* HN001 phage display genomic library using MBP-SpcA as bait

Purified MBP-SpcA^{A1} fusion was used as a bait to screen the HN001 shot-gun phage display library [277] to identify SpcA docking protein on the surface of HN001. Four rounds of panning were carried out. In the first round of panning, amylose resin which binds MBP tag of the MBP-SpcA^{A1} fusion with a high affinity was used to capture the MBP-SpcA^{A1}-recombinant PPs complexes in the enrichment step of the library panning. Briefly, 4 µg MBP-SpcA^{A1} fusion was mixed with 10^{11} PPs from the library in 1 ml of TBS in a microcentrifuge tube. After incubation at room temperature for 3 h, 50 µl of amylose resin in TBS was added and the mixture was incubated for 1 h. The amylose resin with bound proteins and phage particles was then collected by centrifugation and washed five times with 1 ml of TBS to eliminate unbound proteins and PPs. After the last wash, the bound PPs and MBP-SpcA^{A1} protein were eluted by resuspending the amylose resin in 500 µl of TBS containing 10 mM maltose at room temperature for 15 min. The PPs co-eluted with MBP-SpcA^{A1} fusion were amplified by infecting *E. coli* TG1 to transduce the phagemid DNA; the transfected (Cm^{R}) cells were then superinfected with VCSM13 helper phage to produce the PPs as described in Section 2.2.6.3. In the three subsequent rounds of panning, the affinity-enrichment procedure was carried out in the microtitre plates (MaxiSorp^{TM}, Nunc). The bait protein (MBP-SpcA^{A1}) and controls (MBP and BSA) were immobilised on the surface of microtitre plate wells at 4℃ overnight and the wells were subsequently blocked by 2% (w/v) BSA in TBS. About 3 x 10^{9} phage particles from the previous panning round on MBP-SpcA^{A1} bait were added. After incubation and washing, MBP-SpcA^{A1}-bound phage particles were eluted with 100 µl of low-pH elution buffer [100 mM Glycine-HCl, 0.1% (w/v) BSA, pH 2.2] at room temperature for 15 min and neutralized by 6 µl of 2 M unbuffered Tris. Eluted PPs were amplified by infecting TG1 cells and concentrated as described above. The plasmid profiles after each round of panning were examined. The profile of plasmids eluted from parallel controls, panned on BSA and MBP, was used as a comparison, to distinguish the plasmid bands selected on the MBP-SpcA^{A1} fusion relative to those selected by panning on the controls. One plasmid band that was identified in the plasmid profile after the fourth round of panning on MBP-SpcA^{A1}, but absent from the profiles of plasmids eluted from the same round of panning on negative controls (BSA and MBP) was excised from the gel, purified and transformed into TG1. Inserts of ten plasmids isolated from

individual transformants were analysed by DNA sequencing to identify open reading frames (ORFs) from which the inserts were derived.

2.2.11 Phagemid particle-based western blot

To confirm binding of PPs displaying fragments of protein SpcB ($SpcB^{N1}$ and $SpcB^{N2}$) to the $SpcA^{A1}$, a new method of phagemid particle-western blots (or PP-western blots) was developed. The MBP-$SpcA^{A1}$ fusion protein was partially cleaved by a sequence-specific protease, Factor Xa (Novagen), whose recognition site is between the MBP tag and the SpcA protein in pMBP-$SpcA^{A1}$. The Factor Xa digestion reaction was prepared according to the product manual (New England Biolabs) and incubated at 37°C for 2 h. Two sets of digested proteins and undigested controls were analysed by SDS-PAGE; gel portion containing one set of samples was stained by Coomassie brilliant blue and the portion containing a duplicate set was transferred to the nitrocellulose membrane for blotting with $SpcB^{N1}$- and $SpcB^{N2}$-displaying PPs. The membranes were blocked in TBST [TBS; 0.5% (v/v) Tween-20] containing 5% (w/v) skim milk at 4°C overnight. The membranes were subsequently exposed to 1 x 10^9 PPs in TBST at room temperature for 2 h and the unbound phage particles were washed away with TBS. The bound PPs were in turn detected by primary antibodies against M13 phage coat protein pVIII and alkaline phosphatase-conjugated secondary antibodies using the standard western blot protocols [301]. The $SpcB^{N1}$ PPs and PP-$SpcB^{N2}$ PPs-bound bands on the membrane were detected by an alkaline phosphatase assay [301].

2.2.12 Construction of *Lactobacillus rhamnosus* knock-out mutants

To study the function of *spcA* and *spcB* cluster, those genes were deleted (knocked out) from the genomes of HN001 and GG, respectively, through a marker-replacement-mutagenesis (double-crossover) method. However, as the 3' end of *spcA* (3' 180 nucleotides) and the downstream DNA had not been sequenced at the time when the *spcA* knock-out mutant was made, *spcA1* [5' end (0.6 kbp) of *spcA*] was replaced by the marker, which disrupted the transcription and translation of the downstream gene sequence and resulted in a null mutant. Briefly, the gene-flanking upstream (U) and downstream (D) homologous sequences ($spcA^U$ and $spcA^D$ for *spcA1* or $spcB^U$ and $spcB^D$ for *spcB*) were

PCR-amplified using HN001 genomic DNA as template and the specific primers [pWW18 and pWW19 for spcA^{U}; pWW20 and pWW21 for spcA^{D}; pWW59 and pWW60 for spcB^{U}; pWW61 and pWW62 for spcB^{D} (Section 2.1.3)]. Next, the two PCR products, upstream and downstream, were double-cleaved with NotI-NcoI and SalI-SpeI enzyme combinations, respectively. These inserts were sequentially cloned into NotI-NcoI and SalI-SpeI sites flanking the tetracycline-resistance marker (*tet*^{R}) of the replacement vector pFRC027, to obtain a replacement-mutagenesis plasmid (pSpcA1^{KO} or pSpcB^{KO}). The cloning experiments were carried out in *Lactococcus lactis* MG1363 as described in Section 2.2.4. Those plasmids were subsequently transformed into a recessively streptomycin-resistant (Str^{R}) *rpsL1* (*str*^{R}) *L. rhamnosus* strain (HN001 *rpsL1* or GG *rpsL1*), as described in Section 2.2.5. The erythromycin-resistant (Em^{R}) transformants were grown in MRS liquid medium containing 5 μg/ml Em overnight to select for the recombinant plasmid acquisition. These cultures were used to inoculate the MRS broth containing 5 μg/ml Tet and 500 μg/ml Str, to select for the double-crossover recombinants. Tet^{R} and Str^{R} bacteria were further plated on Em-containing MRS agar plates to identify the true double cross-over recombinants that have lost the vector backbone, including the *em*^{R} marker, in contrast to the potential ectopic recombinants or single cross-over mutants whose *rpsL* wild-type allele has mutated to *rpsL1*. Bacteria from the Em^{S} colonies were further amplified and their genomic DNA was analysed by diagnostic PCR and Southern blot (Section 2.2.2.4) to confirm the correct double cross-over gene replacement.

2.2.13 Autoaggregation assay

Cells from 5 ml of early-stationary-phase (~18 h) culture of *L. rhamnosus* strains were collected by centrifugation at 1,500xg at room temperature for 3 min, washed once in PBS pH 7.4, resuspended and diluted in the same buffer and immediately transferred to a 1-2 ml cuvette. The volume of the buffer was adjusted to obtain the density of OD_{600nm}, 0.25 ± 0.05 (A_0). For each strain, the sample was prepared in triplicate. The cell suspension was left undisturbed at room temperature for 24 h. After that, the absorbance (A_{24}) at OD_{600nm} was measured. As the spectrophotometer (Thermo Fisher Scientific; USA) detects the OD above the layer of sedimented (autoaggregated) cell layer in the cuvette, decrease in the absorbance is a measure of aggregation. The autoaggregation percentage was determined as $(A_0 - A_{24})/A_0 \times 100$.

2.2.14 *In vitro* biofilm assay

This assay was performed as described by Lebeer *et al*. (2007) [156, 306]. Briefly, approximately $3x10^7$ *L. rhamnosus* cells in 0.2 ml of AOAC (Difco™) medium were added to each well of a 96-well microtitre plate that was subsequently closed by a matching lid (Nunc no. 269789) with a hanging peg in each well. After the plate was incubated at 37℃ in an anaerobic jar for 24 h, the lid was transferred to a new plate containing 0.2 ml of pre-warmed AOAC medium in each well and the incubation was continued under the same conditions. The medium change was repeated again after 48 h. In total, the lid with biofilm-forming bacteria was incubated in AOAC medium at 37℃ for 72 h. The lid containing the pegs with the attached bacteria were briefly rinsed in 0.2 ml of PBS pH 7.4 and subsequently stained in 0.2 ml of 0.1% (w/v) crystal violet (Sigma-Aldrich) in isopropanol-methanol-PBS [1:1:18 (v/v)] with slow agitation at room temperature for 30 min. The pegs were rinsed in 0.2 ml of H_2O and air-dried at room temperature for 30 min. The stain from the attached bacteria was extracted by incubating the pegs in 0.2 ml of ethanol-acetone (4:1) solution with slow agitation for 20 min. The extracted stain (135 µl from each peg) was transferred to a microtitre plate and the absorbance at OD_{570nm} was measured with a spectrophotometer (Thermo Fisher Scientific).

2.2.15 Hydrophobicity assay

To study the potential change on the hydrophobicity property of the mutants, this assay was carried out according to a method described by Palmer (2008) [307] with modification. Briefly, cells were collected by centrifugation as described in Section 2.2.13, washed once in PBS pH 7.4 and resuspended in PBS containing 3 M NaCl to adjust cells to OD_{600nm}=0.7 ± 0.05 (A_0). This cell suspension (2 ml) was mixed with hexadecane by vortexing for 30 seconds (sec), followed by incubation at room temperature for 20 min. After that, 1 ml of aqueous layer was transferred to a cuvette and the absorbance at OD_{600nm} was measured (A_{20}). The hydrophobicity was expressed as the percentage of cells that fractionated to the organic phase and calculated using equation: $(A_0 - A_{20})/A_0$ x 100.

2.2.16 Mammalian cell culture

Caco-2 (HTB-37; ATCC) cells were grown and maintained at 37℃ in 5% CO_2 in T175 flasks containing 25 ml of M199 standard medium [M199 tissue culture medium (GIBCO, Life Technologies), supplemented with 10% (v/v) foetal bovine serum (FBS; GIBCO, Life Technologies), 1% (v/v) non-essential amino acids [MEM non-essential amino acids (NEAA) 100× solution, Sigma-Aldrich] and 1% penicillin-streptomycin (P/S; 10,000 units penicillin G sodium salt and 10000 g streptomycin sulphate in 0.85% saline; GIBCO, Life Technologies)]. Caco-2 cells growing in the flasks were routinely subcultured once a week at the ratio of ~1:3 [39]. Briefly, when the confluence of Caco-2 cells reached 70-80%, the medium was discarded followed by addition of 15 ml of TrypLE™ Express (Life Technologies) and incubation at 37℃ for 15 min, followed by adding of the medium (15 ml) to neutralise the reaction. The Caco-2 cells were subsequently collected by centrifugation at room temperature at 1,200xg for 3 min and gently resuspended in 4 ml of fresh medium. The cell concentration was determined using a Countess® Cell Counting Chamber Slide (Life Technologies) and 1.5 ml of concentrated Caco-2 cells were used to seed a new T175 flask containing 25 ml of fresh medium and incubated under the conditions described above, until the next split. The medium was changed every 3-4 days. In parallel, a proportion of the collected Caco-2 cells were diluted in the fresh medium to $4x10^5$/ml to seed the Cornings® Transwell polyester membrane cell culture inserts (0.4 µm; CLS3470) for polarised growth. Briefly, each insert was seeded with 200 µl of diluted Caco-2 and 810 µl of M199 standard medium was added to the bottom compartment underneath the inserts. The Caco-2 cells growing in the inserts were incubated for 18 days under the same conditions as those growing in flasks prior to the assays. During that time, the medium was changed twice a week. The passage numbers of Caco-2 cells used in the assays described below were between 30 and 35.

2.2.17 Bacterial adhesion assay

For all adhesion assays, Caco-2 cells were grown in the inserts as described in Section 2.2.16. On day 18 (one day prior to addition of the treatments), the M199 standard medium in both inserts and well compartments below the inserts were replaced with the M199 assay medium [M199 supplemented with NEAA only (no FBS or P/S)] and the

cultures were incubated for 24 h. In parallel, *L. rhamnosus* strains were grow in MRS broth anaerobically at 37℃ overnight until early stationary phase. Next day, cells from 1 ml of lactobacilli culture were collected by centrifugation at 13,000g for 1 min, resuspended and diluted in the M199 assay medium to $5x10^6$/ml. The apical M199 assay medium over the Caco-2 polarised monolayer in the inserts was replaced with 200 µl of medium containing the diluted bacteria. After incubation at 37℃ for 3 h, the Caco-2 cell layer was washed three times in PBS pH 7.4. After the last wash, the inserts were transferred to an empty plate. TrypLE™ Express (Life Technologies) was then added, 100 µl and 600 µl to the inserts and the basal compartment below the inserts, respectively, followed by incubation at 37℃ for 15 min. The detached Caco-2 cells with associated lactobacilli (~100 µl) were transferred to a new tube and mixed with 900 µl of PBS by vigorous pipetting, which lyses the treated Caco-2 cells. The cell suspension in PBS was diluted in MRS broth, plated on MRS agar and incubated at 37℃ overnight to titre the released bacterial cells based on the number of the colony-forming units.

2.2.18 Transepithelial electric resistance (TEER) assay

TEER assays were implemented by following a previously reported protocol with modifications [39]. Briefly, as for the adhesion assay, Caco-2 cells were grown in M199 standard medium in the transwell inserts as described in Section 2.2.16. Formation of polarised cell monolayer was monitored by measuring the TEER values. On day 18, the inserts were transferred to the CellZscope (Nanoanalytics, Germany) units and the medium was replaced by M199 assay medium (260 µl and 810 µl for apical and basal compartments, respectively). The Caco-2 cultures were incubated for 24 h and in parallel the TEER values were recorded every hour. *L. rhamnosus* strain cultures were prepared as described above, except that the cells were resuspended and diluted in either M199 assay medium or M199 assay medium supplemented with TNFα (100 µg/ml), at a density corresponding to OD_{600nm} of 0.9±0.03. The wells (basal compartment) were refilled with 810 µl of M199 assay medium with or without TNFα, whereas the apical compartment was refilled with 260 µl of bacterial suspensions, with or without TNFα. During incubation at 37℃ for 24 h, the TEER values were recorded every hour.

Chapter III: Discovery of SpcA-binding ligand

3.1 SpcA binds to a cell-wall associated protein, specific to the tested *L. rhamnosus* strains

Given that the SCP-like or PR-1-like domains in other organisms are versatile and have been reported to perform a variety of functions, from enzymatic to host-defence, it is likely that the SpcA Big-3 domains may bind to the bacterial cell surface ligand in order to immobilise the PR-1-like domain on the surface of the cell. In that case, it is expected that SpcA is anchored through a cognate species-specific docking molecule. In order to determine the SpcA tropism or species-specificity, the binding profile of $SpcA^A$ PPs, which were produced from a previously identified HN001-binding phage display clone carrying sequence encoding $SpcA^A$ (Section 1.9.2), to various lactobacilli was tested with empty vector PPs as a negative control. In the binding assays (Section 2.2.7), lactobacilli cells were incubated with either vector PPs or $SpcA^A$ PPs to allow specific binding. After washing off the unbound PPs, lactobacilli containing the bound PPs mixed with *E. coli* TG1 indicator strain in order to enumerate the bound PPs. Each internalised phagemid genome conferred the Cm^R phenotype to the infected TG1 cell; hence the titration was carried out by counting the TG1 colonies at appropriate dilution on plates containing chloramphenicol. The number of recovered PPs was expressed as the number of cfu's of TG1. The relative binding efficiency of SpcA to lactobacilli was determined by the difference between the recovered $SpcA^A$ PPs and vector PPs.

Firstly, the binding affinity of selected $SpcA^A$ PPs to various *Lactobacillus* species was tested. According to the phylogenetic relationships with HN001 as described in [308], a closely related strain *L. rhamnosus* GG as well as a distantly related strain *L. plantarum* WCFS1 were initially tested. This assay showed that the ratios of $SpcA^A$ PPs/vector PPs were 327, 35 and 1.6 in *L. rhamnosus* HN001, *L. rhamnosus* GG and *L. plantarum* WCFS1, respectively (Figure 3.1). The results suggested that SpcA could bind to both HN001 and GG of *L. rhamnosus* species at various efficiencies but not *L. plantarum* WCFS1. The binding affinity of SpcA to a few other *Lactobacillus* species/strains was also tested, including *L. plantarum* ATCC14197, *L. sakei* Lb790 and *L. acidophilus* NCFM. However, no significant binding was observed (data not shown). Thus, these findings suggested that SpcA might bind to a *L. rhamnosus*-specific ligand.

As described in Section 1.5.2, bacterial surface proteins can bind the cell surface through non-covalent attachment to the cell wall polymers, such as LTA or carbohydrate, or to a specific surface protein. Therefore, two *L. rhamnosus* HN001 mutants were subsequently tested: HN001*ΔwelE*, which contains an insertional mutation in the *welE* gene resulting in failure of synthesizing a long galactose-rich exopolysaccharide, and HN001*ΔdltD*, which has a deleted *dltD* gene which is responsible for *D*-alanylation of LTA. Compared to the wild-type HN001strain, $SpcA^A$ PPs could bind to both mutants at relatively high efficiencies (Figure 3.2), suggesting *welE*-dependent EPS and alanylated LTA were not required for binding of SpcA to HN001 cells.

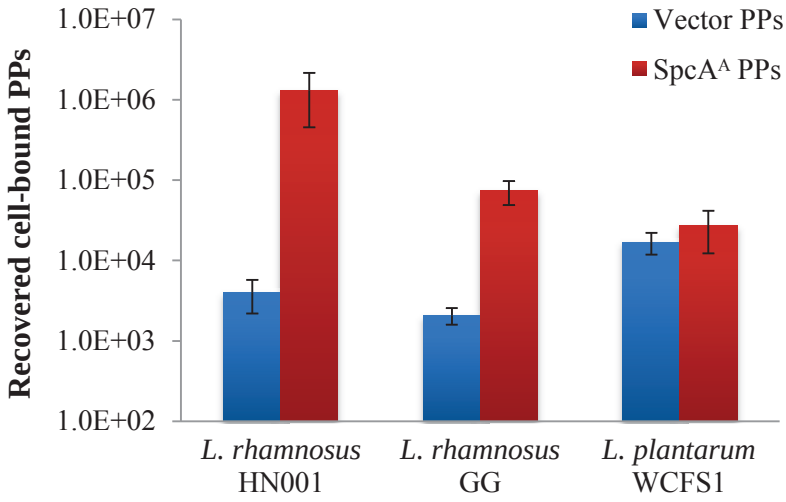

Figure 3.1. PPs-lactobacilli binding assays with various *Lactobacillus* species or strains

Binding of $SpcA^A$ PPs (HN001-binding PPs displaying $SpcA^A$) was tested on *L. rhamnosus* HN001, *L. rhamnosus* GG and *L. plantarum* WCFS1. Vector PPs (empty PPs displaying only phage proteins) were used as negative control. After binding, washing, elution and infection in *E. coli* TG1, the total number of the recovered cell-bound PPs were TG1 determined. The values represent the mean of three independent experiments and the error bars represent standard deviation.

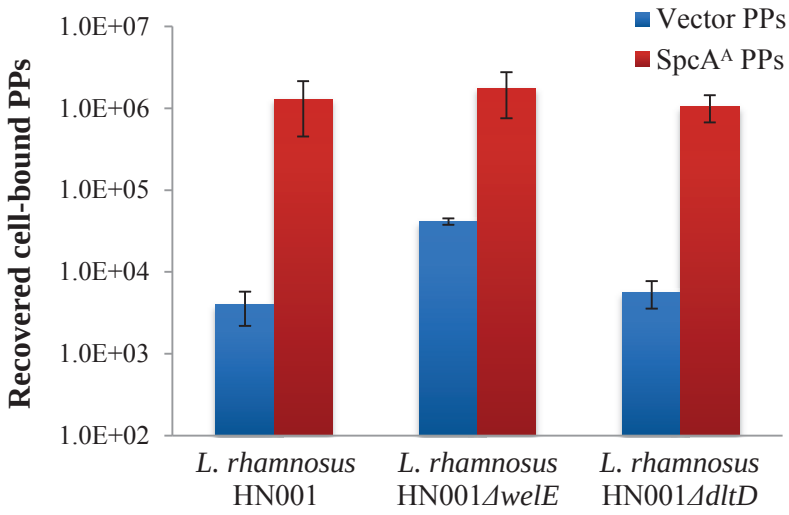

Figure 3.2. PPs-lactobacilli binding assays with *L. rhamnosus* HN001 mutants
Binding of SpcA^{A} PPs was tested on *L. rhamnosus* HN001 wild-type, HN001*ΔwelE* (a *welE* mutant unable to synthesize a surface polysaccharide) and HN001*ΔdltD* (a *dltD* mutant without *D*-alanylation modification of LTA). Vector PPs were used as a negative control. The values represent the mean of three independent experiments and the error bars represent standard deviation.

Next, to test whether SpcA^{A} PPs interact with the surface proteins and/or peptidoglycans, the cell surface of HN001 was pretreated by either proteinase K, which non-specifically degraded the exposed portions of surface proteins, or a mutanolysin-lysozyme mix, which removed the peptidoglycans including the attached cell-wall-associated proteins and other polymers. Compared to the control group (untreated cells), the enzymatic reactions involved a few more centrifugation-washing steps which could potentially cause extra changes to the bacterial surface properties in addition to the enzymatic modification. Therefore, an additional negative control group (cells treated in the same manner as enzymatic treatment group except the enzymes were omitted) was included for each assay. The result showed that both proteinase K and lysozyme-mutanolysin mix significantly decreased the binding efficiency of SpcA^{A} PPs to the enzymatically treated HN001 cells (Figure 3.3). In contrast, the buffer controls showed similar binding efficiencies as the untreated cells, suggesting the additional washes did not affect the binding. Therefore, the observed impaired binding affinity of SpcA^{A} PPs to the enzymatically treated HN001 cells was due to the loss of surface proteins in the proteinase K-treated cells and bacterial cell wall in the lysozyme-mutanolysin-treated cells.

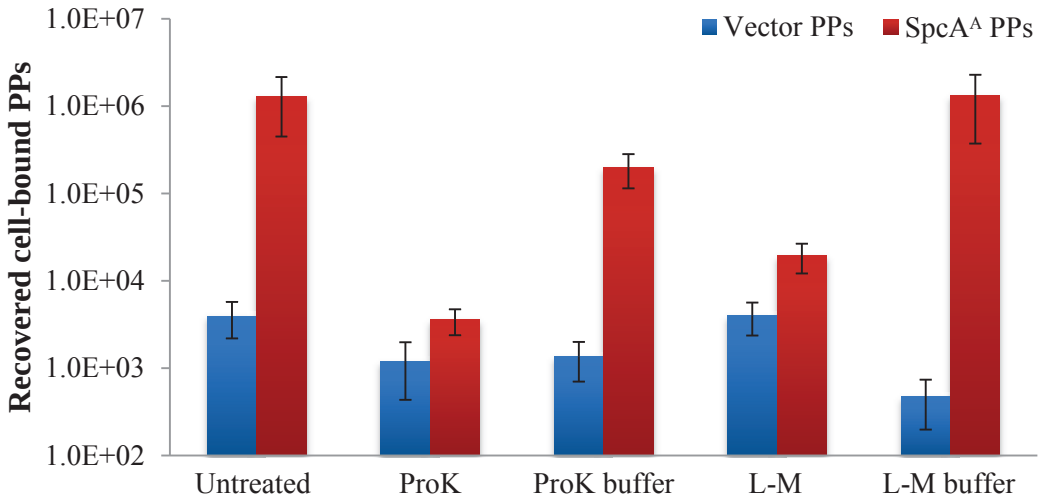

Figure 3.3. PPs-lactobacilli binding assays with enzymatically treated *L. rhamnosus* HN001

Binding of SpcA^{A} PPs was tested on enzymatically treated *L. rhamnosus* HN001. The vector PPs were used as negative control. ProK, proteinase K-treated cells; ProK buffer, cells processed in the same manner as in the ProK assay, except the proteinase K was omitted; L-M, lysozyme-mutanolysin-treated cells (spheroplasts); L-M buffer, cells processed in the same manner as in the L-M assay, except the lysozyme and mutanolysin were omitted. The number of cell-bound PPs was compared between untreated and treated cells. The values represent the mean of three independent experiments and the error bars represent standard deviation.

In conclusion, the lack of binding to protease-treated cells and protoplasts, as opposed to binding to cells lacking an EPS or with an altered LTA suggests binding of SpcA^{A} PPs to a cell wall-anchored protein.

3.2 SpcB, the largest surface protein in *L. rhamnosus*, is the SpcA-docking protein

As described above, SpcA appeared to bind to a cell wall protein of *L. rhamnosus* HN001, therefore this protein ligand could potentially exist in a previously constructed HN001 phage display library [277]. To identify the SpcA-binding ligand(s) from this phage display library, an affinity-screening (biopanning) experiment was carried out (Section 2.2.10). Firstly, the DNA sequence encoding both Big-3 domains without signal sequence (residues 117-309; named MBP-SpcA^{A1}) was cloned in a commercial *E. coli* expression vector, pMal-c2X, in fusion with a *malE* gene encoding maltose-binding protein (MBP) tag (data not shown). Both the fusion, MBP-SpcA^{A1}, and the protein tag, MBP, were separately produced in *E. coli* TG1 and affinity-purified using amylose columns. The MBP-SpcA^{A1} fusion was used as bait to screen the HN001 phage display genomic library.

The first round of panning was carried out by immobilising the bait (MBP-SpcA^{A1}) pre-incubated with the HN001 shot-gun phage display library, to the amylose resin. Binding to amylose was done to increase the amount of bait and the volume of the library, in order to maximise the number of library clones in the initial biopanning round. This method usually allows panning of ~10^{11} PPs compared to the microtitre plate method ~10^{10}. The subsequent three rounds (2-4) of panning experiments were performed using MBP-SpcA^{A1} fusion immobilised on the microtitre plates. In the rounds 2-4, the library was first depleted from MBP-binding clones by first binding to immobilised MBP tag; the remaining unbound PPs were then used for binding to the MBP-SpcA^{A1} fusion. Binding, washing and elution steps were performed for both MBP and MBP-SpcA^{A1} and plasmid profiles were analysed for both eluates, to distinguish between SpcA^{A1}- and potential MBP-binding PPs. Analysis of the plasmid profiles after the fourth round of panning indicated enrichment of discrete plasmid bands (Figure 3.4). The bands that were present in the eluate from the immobilised MBP-SpcA^{A1}, but absent from the MBP control eluate were excised from an agarose gel, purified and subsequently transformed into *E. coli* TG1 strain to obtain individual clones. Ten random transformants were analysed by DNA sequencing, which revealed that the inserts in five clones encoded the N-terminal domain of SpcB, the largest protein in the HN001 genome (3275 residues in length). This protein

is encoded by *spcB* gene, located upstream of *spcA* in a gene cluster, named *spcBCDA*. These five transformants represent two clones containing distinct but overlapping inserts, named $SpcB^{N1}$ (residues 32-391) and $SpcB^{N2}$ (residues 211-586). Both inserts were in frame with upstream vector-encoded PelB signal sequence and downstream c-Myc tag and C-terminal domain of pIII, confirming that they were able to be displayed on the surface of PPs.

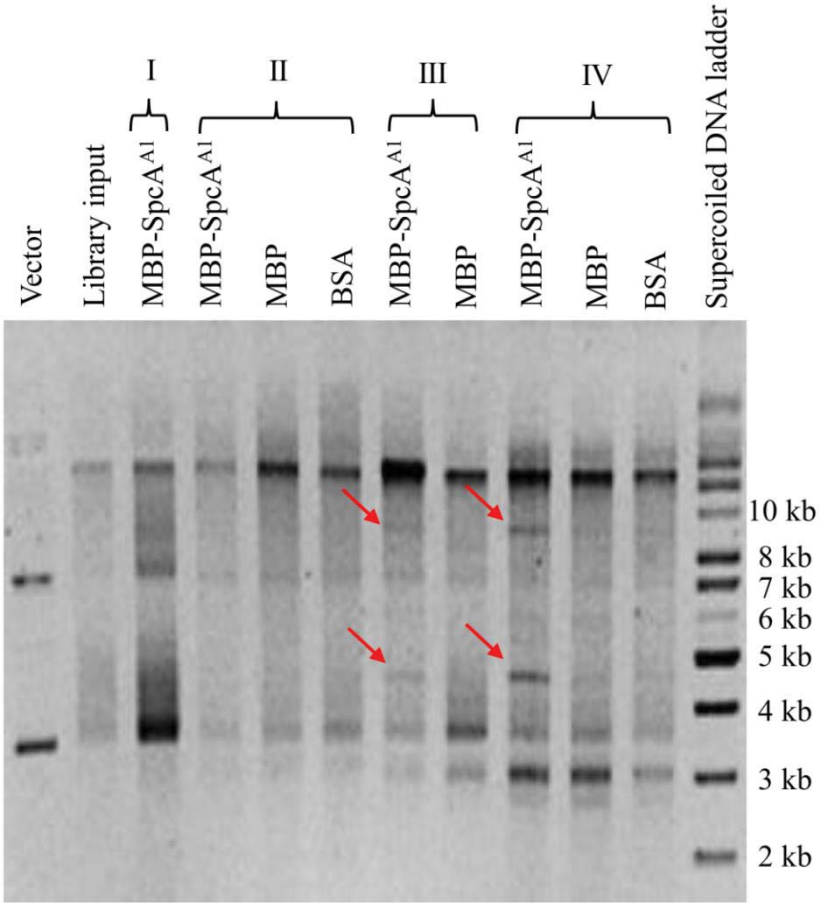

Figure 3.4. The plasmid profiles over four rounds of affinity-screening (biopanning) of the HN001 phage display library using purified MBP-$SpcA^{A1}$ fusion protein as bait

The panning was carried out over four rounds of affinity selection and amplification (I to IV) to enrich the specific binders. From the second to the fourth rounds of panning, MBP and BSA were also included as control baits. Vector represents the phagemid vector used to construct the phage display library. The plasmid bands representing the enriched phagemid genomes are indicated by the red arrows.

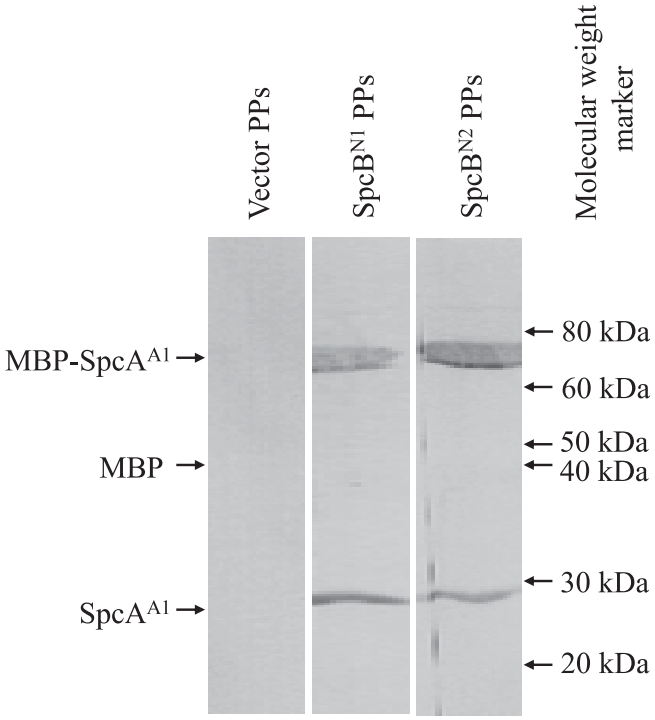

Figure 3.5. Confirmation of the physical interaction between SpcA and SpcB by phagemid particles (PPs) western blot

Affinity-purified MBP-SpcA^{A1} was partially cleaved by a specific protease, Factor Xa, which cuts between the MBP and SpcA^{A1}. Uncut fusion protein and proteolysis products, MBP and SpcA^{A1}, were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were cut along the lanes in the gel, and each strip was blotted with one of the three PP samples: Vector PPs, PPs containing empty vector (negative control); SpcB^{N1} PPs, PPs displaying SpcB^{N1} (residues 59-391); SpcB^{N2} PPs, PPs displaying SpcB^{N2} (residues 211-595); MBP, maltose-binding protein (N-terminal tag). The bound PPs were detected using primary rabbit anti-M13 phage antibodies, followed by anti-rabbit IgG secondary antibodies conjugated with alkaline phosphatase, and visualized by alkaline-phosphatase chromogenic assays.

To confirm binding to SpcA^{A1}, rather than the MBP portion of the bait, the MBP-SpcA^{A1} fusion was digested with the sequence-specific protease Factor Xa, whose cleavage site is engineered between the MBP and SpcA^{A1}. Binding of SpcB^{N1}- and SpcB^{N2}-displaying PPs to either of the proteolytic products was determined by a phagemid particle-western blot. This is a blot in which the partially digested MBP-SpcA^{A1} fusion, separated by SDS-PAGE and transferred onto a nitrocellulose membrane, was probed individually with the SpcB^{N1}- or SpcB^{N2}- displaying PPs (or the empty vector control PPs), followed by

detection of the bound PPs using anti-phage antibodies. This experiment showed binding of the $SpcB^{N1}$ PPs and $SpcB^{N2}$ PPs to $SpcA^{A1}$ portion and also to the uncut MBP-$SpcA^{A1}$ fusion, but not to the MBP band, proving that this is specific $SpcA^{A1}$-$SpcB^{N}$ interaction (Figure 3.5). The sequence overlap between the two inserts, $SpcB^{N1}$ and $SpcB^{N2}$, determines an $SpcA^{A1}$-binding domain, from residue 211 to 391, named "A domain".

3.3 Features of SpcB protein sequence and domain organisation resemble those of a group of large Ser-rich Gram-positive bacterial adhesins

The SpcB primary sequence organisation (Figure 3.6a; Appendix I) resembles that of large glycosylated Ser-rich surface adhesins in Gram-positive bacteria [309], including the characteristic signal sequence that contains an extended N-terminal positively charged sequence, and a glycine-rich hydrophobic region [194].

The high-complexity SpcA-binding domain (A region) is preceded by a relatively short Ala-Ser-Thr-rich (AST) low-complexity region (L1; residues 67-213) at the N-terminus of the mature protein and is followed by a nearly 3000-residue long low-complexity Ala-Ser-rich domain (L2; residues 389-3178) in *L. rhamnosus* HN001 and GG [57, 299]. As a difference from other Gram-positive Ser-rich proteins, SpcB does not have a short highly conserved Ser-rich motif; instead, it is organised as imperfect repeats of about 175 residues in length between residues 427 and 2028. In addition, four almost identical 111-residue repeats are located between residues 2592 and 3035; these repeats are more hydrophobic than the upstream low-complexity region. Besides the predominant Ala (44) and Ser (20) residues, these repeats contain a large number of charged residues, mainly Lys (14) and Asp (13), with other residues having low representation and were therefore named ASKD repeats (Figure 3.6b). Relatively high frequency of these four residues is maintained along the whole low-complexity region which makes up most of the protein (Figure 3.7).

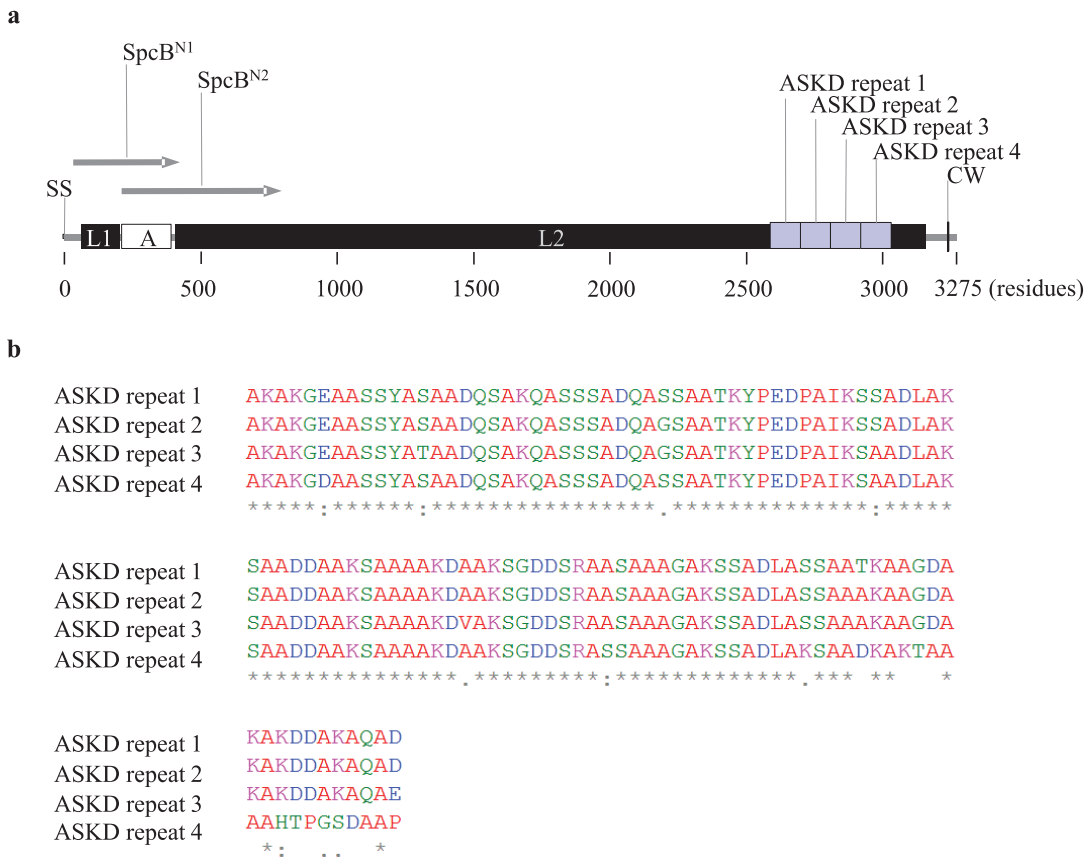

Figure 3.6. Schematic representation of SpcB protein organisation and mapping of the SpcA-binding region

a) SpcB protein organisation. SS, the KxYKxGKxW signal sequence; L1, low-complexity region I; A, high-complexity region containing SpcA-binding sequence; L2, low-complexity region II; ASKD, repeats rich in Ala, Ser, Lys and Asp residues; CW, cell-wall anchor, including LPxTG motif. The grey block arrows above the scheme represent the two N-terminal SpcB fragments that were affinity selected from the HN001 phage display library using purified MBP-SpcA^{A1} protein as bait. b) Alignment of the protein sequences of four ASKD repeats.

Along the whole length of the low-complexity region the PHYRE server predicts both the disordered structure (with high probability) and α-helical secondary structure (with variable probability). It is therefore possible that this part of the molecule forms a helical fibril. The C-terminal LPxTG motif [310] and transmembrane helix of SpcB correspond to a typical cell wall anchor. Finding that the ligand is a *L. rhamnosus*-unique cell-wall anchored protein is consistent with the SpcA^{A} PP binding assays described in Section 3.1.

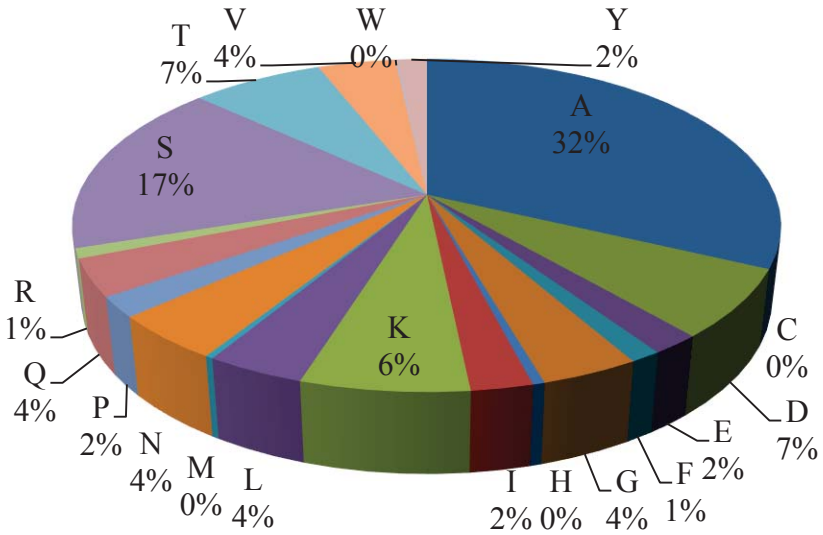

Figure 3.7. Amino acid composition of SpcB

The abundance of each amino acid is expressed as the percentage of the total number of amino acid residues.

Whereas no homologues were identified for the Ala-Ser-rich sequence which represents the majority of the protein (when the BLASTP search was carried out with the low-complexity filter turned on), in the species other than *L. rhamnosus L. casei* and *L. paracasei* belonging to "*Lactobacillus* casei group", the high-complexity SpcA-binding domain had low similarity to one protein in the NCBI database, a hypothetical KxYKxGKxW-signal sequence protein of the human gut bacterium *Lactobacillus parafarraginis* F0439 (ZP_09393738.1). This hypothetic protein has 32% identity (BLASTP); $E=3e^{-12}$) to SpcB between residues 233 and 449, corresponding roughly to the N-terminal high complexity domain that mediates binding to SpcA Big-3 domains; furthermore, the two proteins have a conserved signal sequences (residues 1-60; 57% identity; $E=2e^{-10}$ by BLAST analysis). The SpcA-binding domain is not annotated in the pfam database and did not give significant hits in the PHYRE 3D alignment; hence it may be considered a unique domain. The remaining portion of *L. parafarraginis* ZP_09393738.1 protein also has a low-complexity Ala-Ser rich region arranged in imperfect repeats.

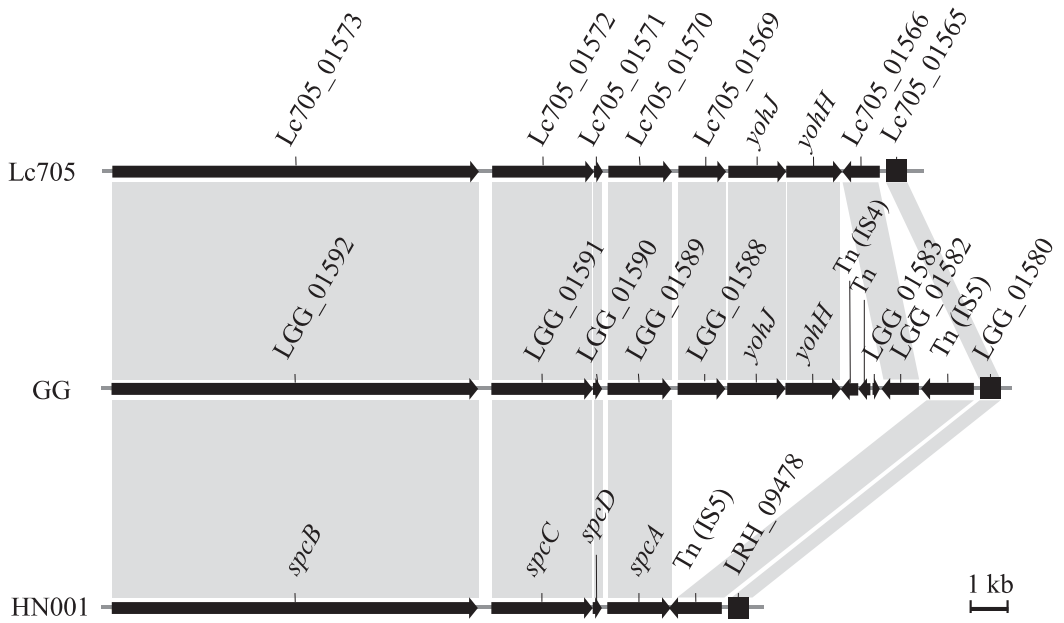

Figure 3.8. Schematic representation *spcBCDA* gene cluster organisation in *L. rhamnosus* strains, Lc705, GG and HN001

The homologous DNA sequences between the three strains are indicated by the grey shadows. Tn, transposase gene; *yohJ* and *yohH*, predicted glycosyltransferase genes.

3.4 Comparative analysis of *spcA*-downstream region in *Lactobacillus rhamnosus* genomes

This study started with only an incomplete draft HN001 genome where *spcA* locates at the end of Contig00052 (Accession ABWJ01000002) without a known 3'-terminal sequence. Given that the downstream regions of the genetic loci encoding KxYKxGKxW signal sequence-containing adhesins (such as GspB) usually contain several genes essential for secretion and post-translational modification of these proteins [194], it was worth to identify the missing sequence in HN001. Although the available primary sequences in *spcBCDA* are highly conserved between HN001 and GG (Figure 3.8), all PCR reactions failed to amplify this region in the HN001 genome with the reverse primers designed based on the GG sequence downstream of *spcA*. This suggested that the regions downstream of *spcBCDA* were not identical between HN001 and GG. A HindIII restriction site-based Southern blot experiment (see Figure 4.2 in Chapter IV) which was

carried out in this study using a probe corresponding to *spcA* further supported this hypothesis by showing bands of different size in HN001 from those predicted for GG. Using GG genome as a scaffold, contigs of the HN001 draft genome were then aligned. The genome alignment identified that the next closest downstream contig to Contig00052 is Contig00069 (Accession ABWJ01000036) and the gap region, whose sequence information is missing from HN001, is 8467 nt in length in GG. A subsequent PCR experiment containing a forward primer complementary to Contig00052 and a reverse primer complementary to Contig00069 was used to amplify this unknown gap region using HN001 genome as template. Surprisingly, a band representing the size of this gap region in HN001 was only about 1.5 kbp. This confirmed the noticed genomic variation in the region downstream of *spcA* between GG and HN001 as described above. Next, this PCR product was sequenced to close this genomic gap in HN001. Figure 3.8 illustrated the genetic conservation in *spcBCDA* cluster and genetic variation in the downstream region of this cluster between HN001 and another two well annotated *L. rhamnosus* genomes, Lc705 and GG [311].

Interestingly, a conserved downstream transposase gene (Tn; IS5 family) was noted in the reverse direction relative to the *spcBCDA* cluster in both HN001 and GG genomes. However, in GG genome, there are several ORFs (LGG_01588-LGG_01582) between *spcBCDA* and IS5 (LGG_01581), whereas in HN001 genome, IS5 is found immediately downstream of truncated *spcA*, causing the loss of these ORFs including two predicted glycosyltransferase-encoding genes (*yohJ* and *yohH*). This possibly resulted from an early recombination-deletion event in the HN001 genome due to the presence of transposases IS5.

Based on gene organisation in clusters that contain a gene encoding a KxYKxGKxW large Ser-rich adhesins, which are typically glycosylated, glycosyltransferases are expected to be present in the cluster and to play essential roles in glycosylation and secretion of these proteins. In the Lc705 genome, *yohJ* and *yohH* are conserved whereas the IS5-type transposase gene found in GG and HN001 genomes is missing. The variable sequence downstream of *spcBCDA* might therefore potentially affect the functions of *spcBCDA* components.

Summary

In this chapter, the receptor of HN001-binding protein SpcA was characterised through a series of PPs-lactobacilli binding assays with either different *Lactobacillus* species/strains or HN001 with defective surface structures. The findings suggested that SpcA bound to a cell-wall anchored protein, specific to the tested *L. rhamnosus* species. The following affinity-screening of the HN001 phage display library with MBP-SpcA^{A1} as bait identified a large alanine/serine-rich protein, SpcB, as the docking protein of SpcA.

The overall protein organisation of SpcB resembles a group of large and glycosylated Gram-positive adhesins. Genes encoding *spcB* and *spcA* are located within the same gene cluster, *spcBCDA*, which is conserved in the genomes of *L. rhamnosus* strains, HN001, GG and Lc705. However, in the HN001 genome, this cluster is incomplete, missing three genes downstream of *spcA*. Additionally, three genes from a different gene cluster are also missing from HN001. The functions of the missing cluster genes may be involved in glycosylation of SpcB, hence this modification, if present, could be strain-specific.

Chapter IV: Analysis of the SpcB and SpcA roles in L. rhamnosus HN001 and GG

4.1 Construction of *L. rhamnosus spcA* and *spcB* knock-out mutants

SpcA-SpcB is a novel type of surface protein complex identified in *Lactobacillus* genus. Although from the sequence information and demonstrated interaction it can be proposed that SpcB and SpcA correspond to a cell wall-bound adhesin-type fibrillary and possibly glycosylated protein, and a cognate associated protein, respectively, their biological function cannot be deduced from the their primary amino acid sequences. It can be hypothesised that the SpcB adhesin could be involved in microbe-microbe or microbe-host interactions, and that SpcA could add to the functionality through immobilisation of the PR-1 domain to the surface of bacterium. Given that the SpcA-SpcB interaction has been demonstrated in two probiotic, but biologically distinct strains in the terms of the surface properties, HN001 and GG, and that the two strains also differed in potential to glycosylate SpcB, it was of interest to compare the roles of the two proteins in these two strains.

To study the roles of SpcA and SpcB in microbe-microbe and microbe-host interactions, *spcA* and *spcB* genes were each deleted separately from the genomes of *L. rhamnosus* HN001 and GG using a double-crossover marker-replacement method. The corresponding mutants were named HN001*ΔspcA*, HN001*ΔspcB*, GG*ΔspcA* and GG*ΔspcB*. Due to the double cross-over these mutations were irreversible and hence could be maintained in the antibiotic-free media and their phenotypes could be compared directly to the wild-type strains under the same growth conditions. It is worth noting that unlike *ΔspcB* mutants whose full-length *spcB* sequence was replaced by the marker, only ~0.6 kbp 5'-end sequence (named *spcA1*) was replaced in *ΔspcA* mutants of HN001 and GG. The reason for this was that the sequence downstream of the *spcA* ORF in HN001 genome was unknown at the time of the mutant construction; hence the 3' region of the gene had to be placed downstream of the tet^R (tetracycline-resistance marker encoding gene) marker in order to allow double-crossover recombination. The deletion removed the translation start of the *spcA*, and the replacement marker, tet^R, which was inserted in the same orientation as the rest of *spcA* sequence, contained a translational stop codon

followed by a transcription terminator, hence the 3' portion of *spcA* that remained in the knock-out construct was not transcribed and translated, making this a null mutant.

Due to the conserved homologous sequences in HN001 and GG, the same recombinant mutagenic plasmids were used for deletion of *spcA* or *spcB* in both strains. The mutagenic plasmids (Figure 4.1a) for double-crossover recombination deletion of *spcA* and *spcB* were constructed in the same vector pFRC027 (unpublished; FRDC) by sequentially cloning two gene-flanking homologous fragments (Flanking-U and Flanking-D), using *Lactococcus lactis* MG1363 as the primary cloning host (Section 2.2.4). In addition to the cassette aimed for gene replacement (tet^R marker flanked by two cloned homologous fragments flanking the deleted/replaced genomic sequence), the mutagenic plasmid contains an em^R marker (erythromycin-resistance marker-encoding gene), and a dominant wild-type allele of *rpsL* gene that renders the recipient strain sensitive to antibiotic streptomycin. After the correct constructs were identified in *Lactococcus lactis* MG1363 and confirmed by sequencing, they were individually transformed into mutants of *L. rhamnosus* strains, HN001 *rpsL1* and GG *rpsL1,* which each contained a recessive mutation conferring streptomycin-resistance phenotype to the *rpsL1* mutants (Figure 4.1b).

After internalisation of the mutagenic plasmid, the transformed cells showed Tet^R, Em^R and Str^S phenotypes prior to recombination with the chromosome (Figure 4.1c). The transformants were subjected to Tet and Str selection for double-crossover recombinants (see the Figure 4.1 legend for explanation). During recombination, the target gene (*spcA* or *spcB*) was replaced by the tet^R marker. In bacterial cells that contained double-crossover homologous recombination, the plasmid backbone (except Flanking-U, tet^R and Flanking-D) would have been lost, therefore their antibiotic-resistance phenotypes were expected to become Tet^R, Str^R and Em^S (Figure 4.1d). The positively selected to Tet^R and Str^R recombinants were replica-plated on plates also containing Em, to distinguish between the true recombinants that have lost the vector backbone and the single-crossover integrants or non-homologous recombinants whose *rpsL* (Str^S) allele in the vector backbone mutated to the *rpsL1* (Str^R) allele. This type of spontaneous mutation is frequent when lactobacilli are grown on Str-containing plates.

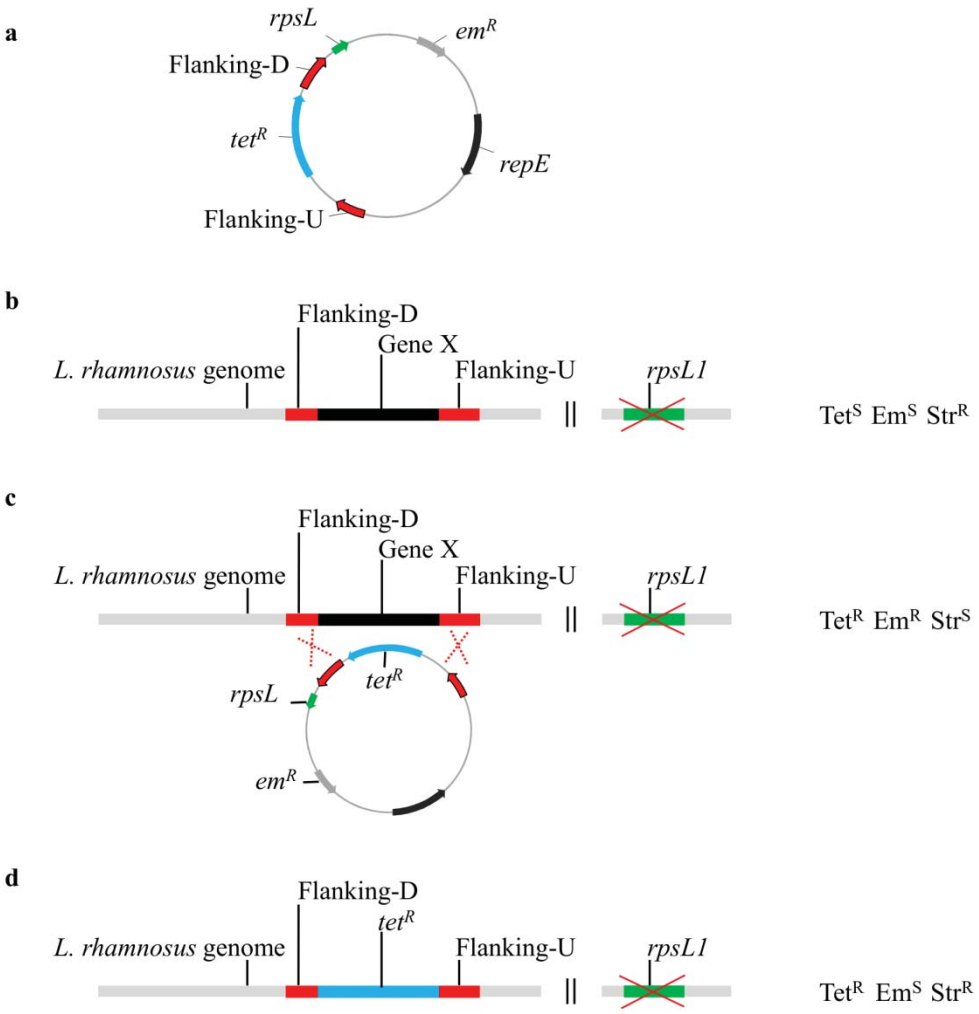

Figure 4.1. Construction of *L. rhamnosus* knockout mutants

a) Mutagenic plasmid. Flanking-U and Flanking-D, *L. rhamnosus* sequences flanking the genomic sequence planned to be deleted; tet^R (blue), a gene encoding tetracycline-resistance marker; em^R (light grey), a gene erythromycin-resistance marker; *rpsL* (green), a dominant wild-type gene responsible for bacterial streptomycin sensitive phenotype; *repE* (dark grey), a replication protein-encoding gene. b) The parent strains used to construct the gene-knockout mutants were *rpsL1* point mutants of *L. rhamnosus* HN001 and GG, respectively. This mutation rendered 30S ribosomal protein S12 unable to bind to streptomycin, resulting in streptomycin resistance. Gene X (black) represents the target gene to be deleted, flanked by the upstream and downstream homologous sequences (red), Flanking-U and Flanking-D. c) After transformation and before recombination, the internalized mutagenic plasmid rendered the parent strain resistant to Tet and Em but sensitive to Str (due to the presence of a dominant wild-type *rpsL* allele in the plasmid backbone). The transformants were then subjected to selection against Tet and Sm. Tet was the selection for retention of the tet^R marker, whereas Str was selection for loss of the plasmid backbone carrying *rpsL*, which forced the double-crossover recombination to occur between the homologous regions flanking the tet^R marker. d) After recombination, the tet^R marker replaced the target gene, whereas the plasmid backbone containing the em^R marker and *rpsL* was lost. The obtained mutant was resistant to Tet and Str but sensitive to Em.

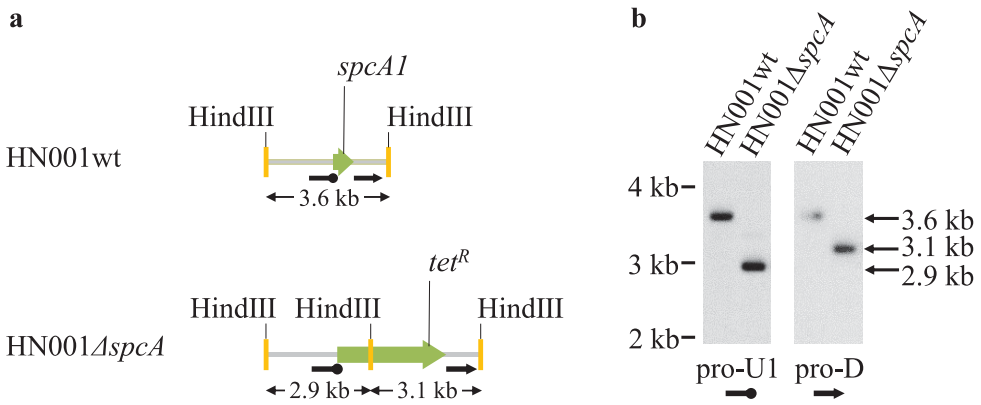

Figure 4.2. Confirmation of the putative HN001*ΔspcA* mutant by Southern blot
a) Schematic representation of the HindIII restriction maps (yellow vertical lines) around the *spcA* loci in *L. rhamnosus* HN001 genome. The black lines with a dot or an arrow represent the probes, pro-U1 or pro-D, respectively, used in Southern blot. *spcA1*, 5' terminus of *spcA* to be replaced; *tet*^{R}, tetracycline-resistance marker-encoding gene. b) Southern blot. Blot contains HindIII-digested genomic DNA of the candidate Tet^R, Str^R and Em^S recombinants and the wild-type controls resolved by agarose gel electrophoresis. The blot was sequentially hybridized with probes pro-U1, and pro-D.

The recombinants that were Tet^R, Str^R and Em^S were further screened by PCR with diagnostic primers and then confirmed by Southern blotting using suitable probes from the upstream and downstream homologous sequences that flanked the replaced segments of genomic DNA (Figure 4.2 and 4.3).Firstly, two separate diagnostic PCR reactions, for upstream and downstream crossover, were used to confirm both recombination endpoints. In each PCR reaction, one primer was designed to anneal only to the bacterial chromosome, but not the vector, and the other one was complementary to the *tet*^{R} gene inserted into the chromosome. Only genomic DNA isolated from the true mutants, but not the wild-type strain or recombinants containing insertion of the *tet*^{R} gene into a different locus was expected to generate a PCR product in each of these reactions. The PCR assays confirmed expected products in all tested Tet^R, Str^R and Em^S candidates (data not shown). The recombinants that were positive in PCR were further tested by Southern blotting to confirm the recombination (Figure 4.2 and 4.3). This experiment, in which appropriate restriction digests and probes corresponding to the sequences upstream and downstream from the *tet*^{R} gene were used, confirmed the correct gene-replacement recombination in these mutants.

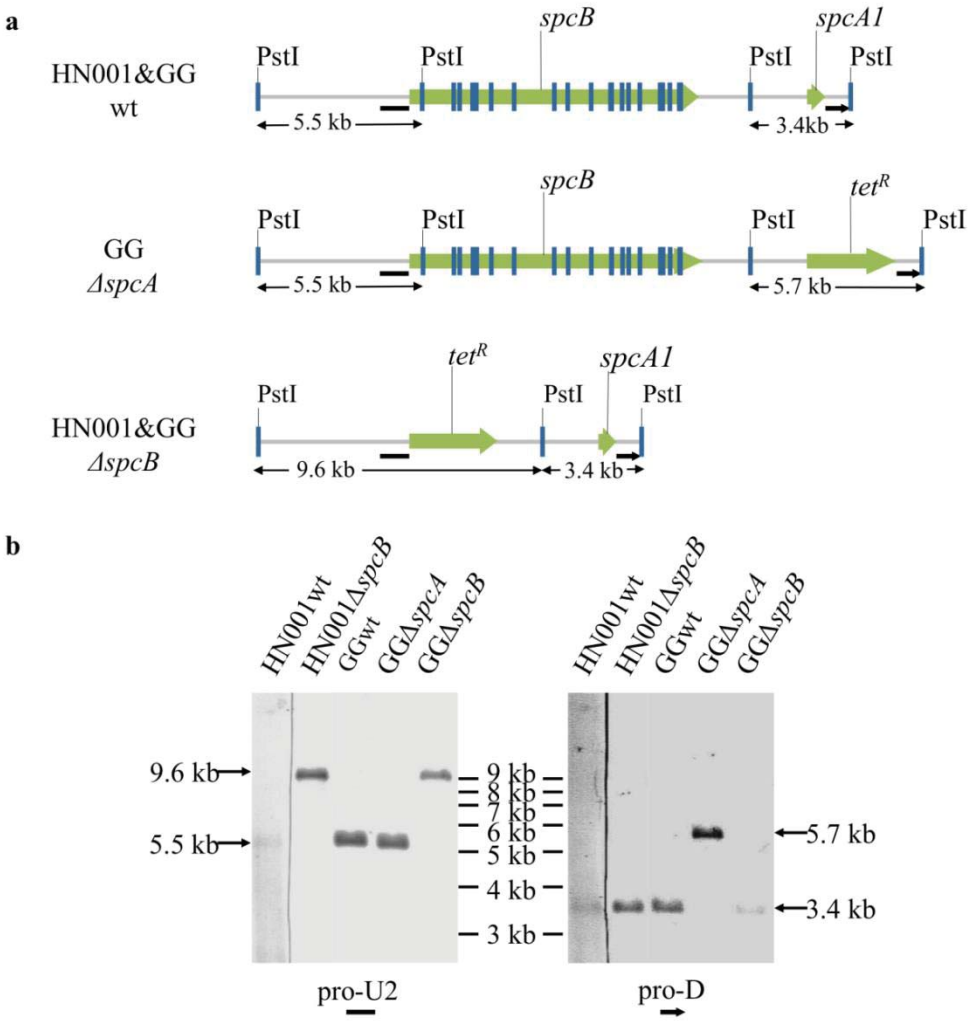

Figure 4.3. Confirmation of the constructed *L. rhamnosus* knockout mutants, HN001*ΔspcB*, GG*ΔspcA* and GG*ΔspcB* mutants, by Southern blot

a) Schematic representation of the PstI restriction maps (blue lines) of the *spcA* and *spcB* loci in *L. rhamnosus* HN001 and GG genomes. The black lines without and with an arrow correspond to probes pro-U1 or pro-D, used in Southern blot. *spcA1*, 5' terminus of *spcA* to be replaced; *tet*^{R}, tetracycline-resistance marker-encoding gene. b) Southern blot. Blot contains PstI-digested genomic DNA of candidate Tet^R, Str^R and Em^S recombinants and the wild-type controls resolved by agarose gel electrophoresis. The blot was sequentially hybridized with probes pro-U2 and pro-D, respectively.

In summary, the knock-out mutants of *spcA* and *spcB* genes were successfully constructed, allowing for the testing of the role of these two genes in properties dependent on the surface structures in *L. rhamnosus* strains HN001 and GG.

4.2 SpcA binding to *Lactobacillus rhamnosus* is SpcB-dependent

As previously described in Section 3.1 & 3.2, SpcB is a surface ligand of SpcA, which was selected by screening of phage display library using the SpcA as bait. Direct interaction of the two proteins was confirmed by phage-western blot. However, bacterial surfaces are complex, containing a large number of cell-wall-anchored proteins. Given that screening of the phage display library often discovers the ligand(s) with highest binding affinity to the bait, it was possible that SpcA binds to other redundant ligands on the surface of *L. rhamnosus*. To test whether SpcB is the exclusive SpcA-binding partner or there was redundancy of the cognate ligands, PPs-lactobacilli binding assays were carried out with the *L. rhamnosus* knockout mutants, HN001*ΔspcB* and GG*ΔspcB*. Another two mutants, HN001*ΔspcA* and GG*ΔspcA*, were also used as control to exclude the possibility that the failure of binding of SpcA^{A} PPs to the *spcB*-defective *L. rhamnosus* mutants was due to the presence of the chromosomally integrated *tet*^{R} marker.

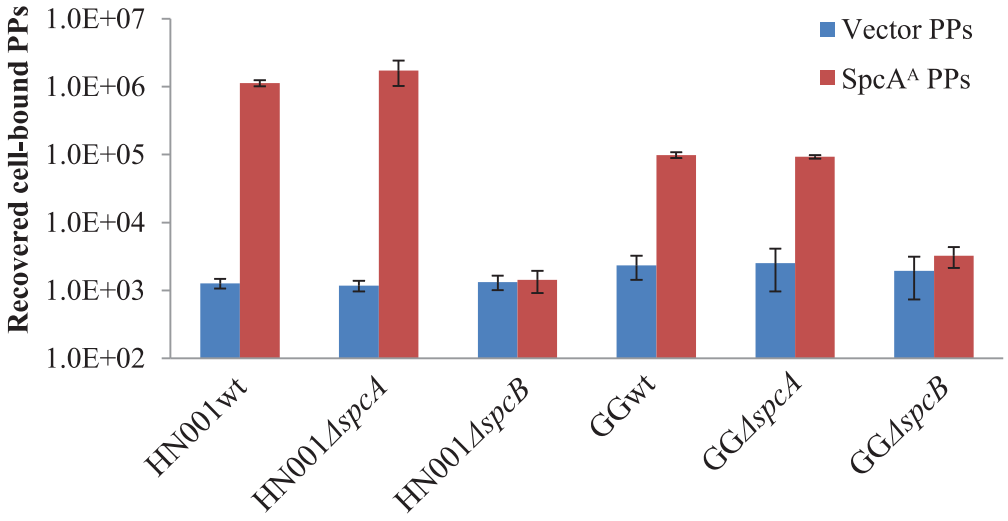

Figure 4.4. PPs binding assay to *L. rhamnosus* knockout mutants

Binding of SpcA^{A} PPs (HN001-binding PPs displaying SpcA^{A}) was tested on *L. rhamnosus* mutants, HN001*ΔspcA*, HN001*ΔspcB*, GG*ΔspcA* and GG*ΔspcB* and the corresponding wild-type parent strains as controls. The total number of the recovered cell-bound PPs was determined by TG1 titration. The values plotted represent the means of three independent experiments. The error bars represent standard deviation.

As illustrated in Figure 4.4, compared to the binding of SpcA to wild-type HN001 and GG (the difference between the recovered $SpcA^A$ PPs and vector PPs), binding to the HN001*ΔspcB* and GG*ΔspcB* mutants was almost completely eliminated, suggesting that SpcB is the sole binding partner of SpcA on the surface of *L. rhamnosus*. On the other hand, SpcA binding to the HN001*ΔspcA* and GG*ΔspcA* mutants remained the same as to the wild-type strains demonstrating that SpcA binding was neither affected by the loss of *spcA* nor the presence of the integrated tet^R marker, supporting the previous finding that SpcB is the cognate "docking" ligand of SpcA. This also suggested that endogenously produced SpcA did not compete for binding sites on SpcB with phage-displayed SpcA.

4.3 SpcB may affect autoaggregation of *Lactobacillus rhamnosus* GG

Like many other lactic acid bacteria, *L. rhamnosus* HN001 and GG form multi-cell aggregate (autoaggregation) in the liquid media under laboratory conditions. This phenotype is generally considered to be favourable for biofilm formation, adhesion and colonization in the human gut as discussed in Section 1.3.2. Although both surface and secreted autoaggregation factors have been reported in *Lactobacillus*, except the SpaCBA pilus of *L. rhamnosus* GG (absent from HN001) [84], the autoaggregation factor has not been reported in other *L. rhamnosus* strains. Due to the adhesin-like and hydrophobic nature, SpcB was hypothesised to contribute to autoaggregation. Furthermore, the cell-binding property of SpcA was hypothesised to potentially mediate aggregation by cross-linking the cells to each other. To test this phenotype in the mutants, autoaggregation assays were carried out.

Given that the *Lactobacillus* cultures form characteristic flake-like aggregates that sediment in the bottom of the culture tubes, aggregation of the overnight stationary cultures was compared visually between wild-type HN001 and GG, and corresponding mutants. However, no obvious difference was observed. Microscopic examination of these cultures also showed similar autoaggregation levels.

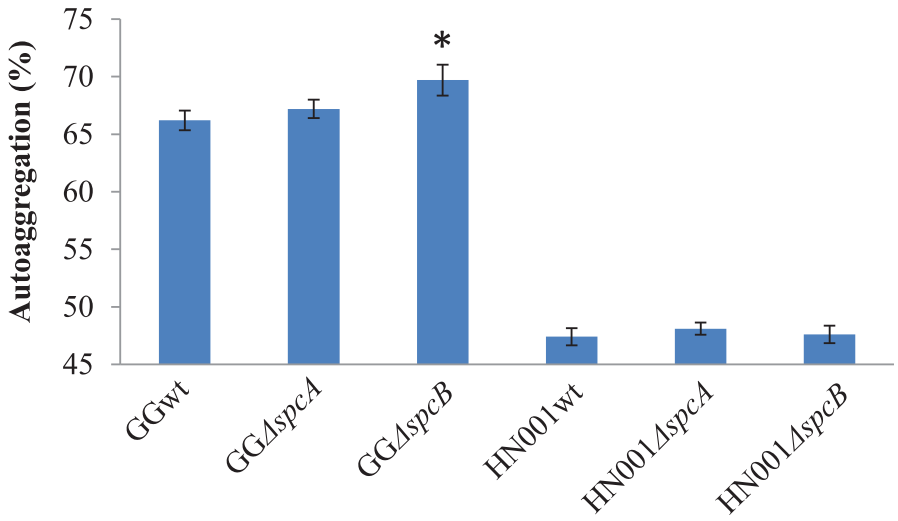

Figure 4.5. Comparison of the autoaggregation properties between the wild-type strains and knockout mutants of *L. rhamnosus*

Aggregation of the wild-type strains (HN001wt and GGwt) of *L. rhamnosus* and mutants (HN001*ΔspcA*, HN001*ΔspcB*, GG*ΔspcA* and GG*ΔspcB*) was measured using a spectrophotometric autoaggregation assay. Cells from the stationary-phase *Lactobacillus* cultures were collected by centrifugation and diluted in PBS pH 7.4 until OD_{600nm} reached 0.25±0.05. The absorbance of the cell suspension transferred into the spectrophotometer cuvettes and measured immediately (A_0) and after 24 hours (A_{24}). The percentage of autoaggregation was calculated s (A_0 - A_{24})/ A_0 x 100. Each value is the mean of three independent experiments, each carried out in triplicate. The error bars represent standard deviation. Significant difference (*p*-value < 0.05) is indicated with an asterisk.

As autoaggregation can be mediated by complex cell-cell surface interactions, it is possible that this phenotype results from the presence of more than one autoaggregation factors. If SpcA or SpcB are minor autoaggregation factors, the change in the corresponding mutants might be not easily observed. Aggregation was further quantitatively measured using a spectrophotometric assay (Section 2.2.13) based on the fact that the extent of aggregation correlates with faster sedimentation rate in the liquid, resulting in a lower absorbance in the upper layer of the cell suspension. The absorbance at OD_{600nm} of the upper layer was measured prior- and post-incubation, respectively, to determine the percentage of aggregation.

The assay data (Figure 4.5) showed that the autoaggregation level of GG was approximately 40% higher than that of HN001. No significant difference in autoaggregation was observed among HN001wt, HN001*ΔspcA* and HN001*ΔspcB* (47.4, 48.1 and 47.6%, respectively). Interestingly, on the other hand, GG*ΔspcB* demonstrated a slightly but consistently higher autoaggregation level (69.7%) than the wild-type GG (66.2%) (p-value < 0.05; Wilcoxon-Mann-Whitney test). The GG*ΔspcA* mutant showed similar autoaggregation level (67.2%) as the GG wild-type. Therefore, the results suggested that SpcB might negatively influence autoaggregation in *L. rhamnosus* GG but not HN001 and SpcA was not involved in autoaggregation of either HN001 or GG under the test conditions.

4.4 SpcA and SpcB may negatively regulate biofilm formation in *Lactobacillus rhamnosus* GG

As discussed in Section 1.3.3, biofilm helps bacterial colonisation and survival against selective pressures in the harsh environmental conditions. In the case of probiotics, biofilm formation may also protect the human intestinal epithelium from colonization by pathogens. Pilus was previously reported as a major biofilm-formation factor in *L. rhamnosus* GG [41]. However, biofilm-formation capacity was also shown to be influenced in the GG *luxS* [156] and *mabA* [102], knockout mutants and also by medium components [306]. This suggests that biofilm formation is a complex phenomenon that involves the expression of multiple genes.

In particular, of genes involved in biofilm formation in GG, HN001 lacks the pilus-encoding *spaCBA* genes, thus the biofilm-formation capacities could be strain-specific between these two *L. rhamnosus* strains. SpcB has a typical protein domain organisation feature like those fibril-like adhesins suggesting its potential in binding, and an N-terminal high-complexity binding domain, hence it may have a role in biofilm formation. To test whether SpcA or SpcB has a role in biofilm formation, an *in vitro* biofilm assay was implemented with HN001 and GG wild-type and mutants.

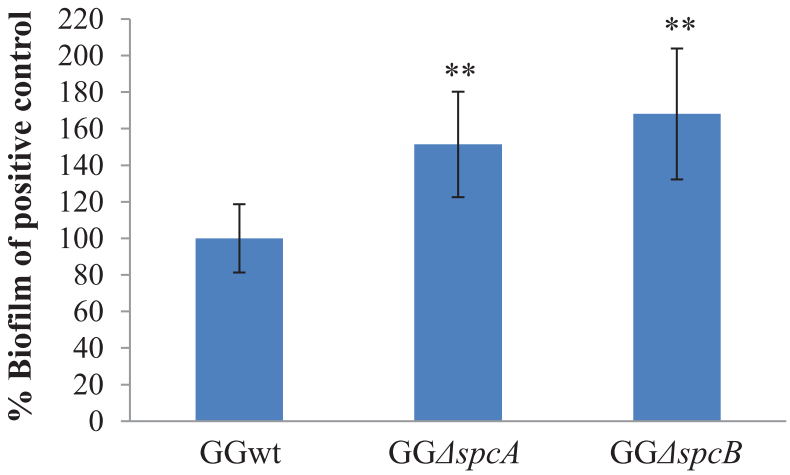

Figure 4.6. Comparison of the *in vitro* biofilm-formation capacities of the *L. rhamnosus* GG knockout mutants with their parent strain

The wild-type strain (GGwt) and knockout mutants (GG*ΔspcA* and GG*ΔspcB*) of *L. rhamnosus* GG were assessed for the biofilm formation on polystyrene. Biofilm formation was measured relative to the wild-type (100%). The values plotted represent the mean of N=24 experimental values (three independent experiments, each performed in 8 replicates). The error bars represent standard deviation. Significant differences (*p*-value < 0.01) are indicated with double asterisks.

Given that *L. rhamnosus* strains form very little biofilm in MRS medium [306], the tested lactobacilli were grown in AOAC medium in a 96-well microtitre plate covered by a polystyrene lid with a hanging peg in each well. Over the incubation time, biofilm-forming bacteria attached to the surface of the hanging pegs and started biofilm growth. Peg-attached bacteria were stained by crystal violet, which was subsequently extracted with ethanol-acetone and quantified spectrophotometrically. All tested *L. rhamnosus* HN001 strains (HN001wt, HN001*ΔspcA*, and HN001*ΔspcB*) failed to attach to the pegs over the medium-only background, showing no biofilm formed under the given growth and assay conditions (data not shown).

On the other hand, *L. rhamnosus* GG strains formed biofilm (Figure 4.6), consistent with previously reported findings [306]. Using the amount of biofilm formed by GGwt as standard (100%), The GG*ΔspcA* and GG*ΔspcB* mutants, interestingly, showed an increased biofilm formation ability relative to the wild-type GG strain, 151.4% and 168.1%, respectively (*p*-value < 0.01, Wilcoxon-Mann-Whitney Test). Therefore, the

assay data suggested that both SpcA and SpcB might have a direct or indirect negative influence on biofilm growth of *L. rhamnosus* GG.

4.5 SpcA and SpcB do not affect the overall hydrophobicity of *Lactobacillus rhamnosus* HN001 and GG

Bacterial cell-cell or cell-surface interactions often involve primary non-specific interactions, mediated by the various surface properties such as hydrophobicity and electrostatic charge [312]. For probiotics, the net surface hydrophobicity may potentially influence their important probiotic features, such as aggregation or coaggregation with other bacteria as well as adhesion to the host intestinal epithelial cells or inorganic surfaces [129, 313, 314].

Given that SpcB is an alanine-rich protein containing 1360 hydrophobic residues, it was possible that it could influence the overall hydrophobicity of *L. rhamnosus* cells. In this hydrophobicity assay, lactobacilli in an aqueous buffer, at a standardized density as determined by absorbance (A_0) were mixed with a hydrophobic solvent, hexadecane, which does not mix with water. After separation of the mixture into the aqueous and solvent layers over 20 min, the absorbance (A_{20}) of the cells remaining in the aqueous layer was measured. The amount of bacteria partitioned into the organic solvent was obtained by subtracting the bacteria remaining in the aqueous phase from the input bacteria. The hydrophobicity was expressed as the percentage of the hexadecane-partitioned bacteria relative to the input bacteria.

The hydrophobicity assay data (Figure 4.7) indicated that *L. rhamnosus* GGwt (74.5%) showed approximately 2.3-fold higher value than HN001wt (32.6%), showing the variation in surface hydrophobicity between the HN001 and GG strains. However, no significant difference in hydrophobicity was observed in the tested *L. rhamnosus* mutants (HN001*ΔspcA* and HN001*ΔspcB*, GG*ΔspcA* and GG*ΔspcB*) in comparison to the

corresponding parental wild-type strains. Therefore, SpcA and SpcB were not responsible for the hydrophobicity observed for *L. rhamnosus* strains under the assay conditions.

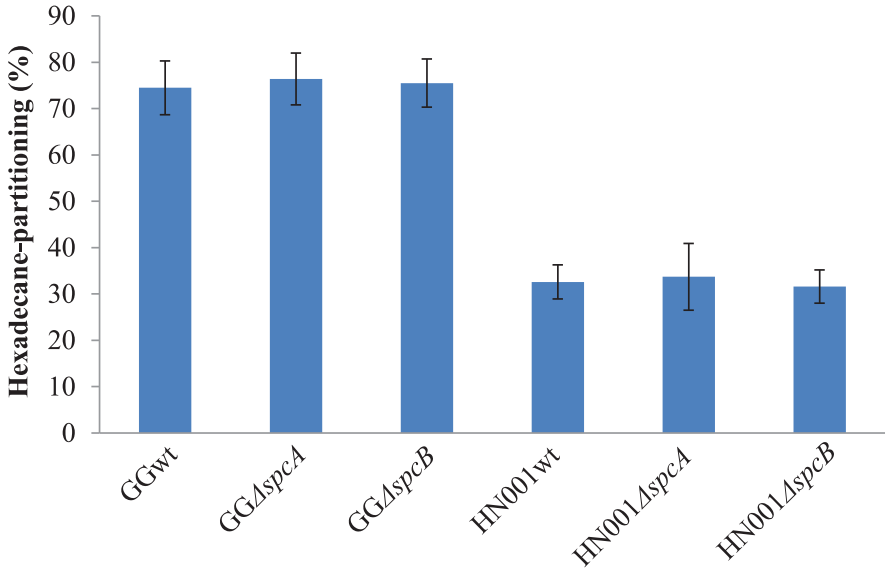

Figure 4.7. Comparison of the surface hydrophobicity between the *L. rhamnosus* wild-type strains and their mutants

The surface hydrophobicity of the *L. rhamnosus* wild-type strains (GGwt and HN001wt) and knockout mutants (GG*ΔspcA,* GG*ΔspcB,* HN001*ΔspcA* and HN001*ΔspcB*) was assayed for their affinity to a hydrophobic solvent, hexadecane, as a measure of overall hydrophobicity. The relative hydrophobicity is expressed as the percentage of solvent-partitioned bacteria relative to the input bacteria, as determined by optical density at OD_{600nm}. The plotted values represent the mean of 6 measurements, obtained from three independent experiments, each in duplicate. The error bars represent standard deviation.

4.6 SpcA and SpcB do not affect *in vitro* adhesion of *Lactobacillus rhamnosus* HN001 and GG to Caco-2 cells

Ability of probiotics to adhere to the intestinal cell lines is often one of the definition criteria for probiotics. Both *L. rhamnosus* HN001 and GG have been reported capable of binding to Caco-2 cell monolayers [41, 315]. A few adhesion-associated proteins of *L. rhamnosus* GG, SpaCBA pilus [41], MabA [102] and MBF [100] have been experimentally demonstrated to promote binding of this bacterium to the components of the intestinal cells. Therefore, it seems that adhesion to the intestinal cells is mediated by multiple strain-specific factors.

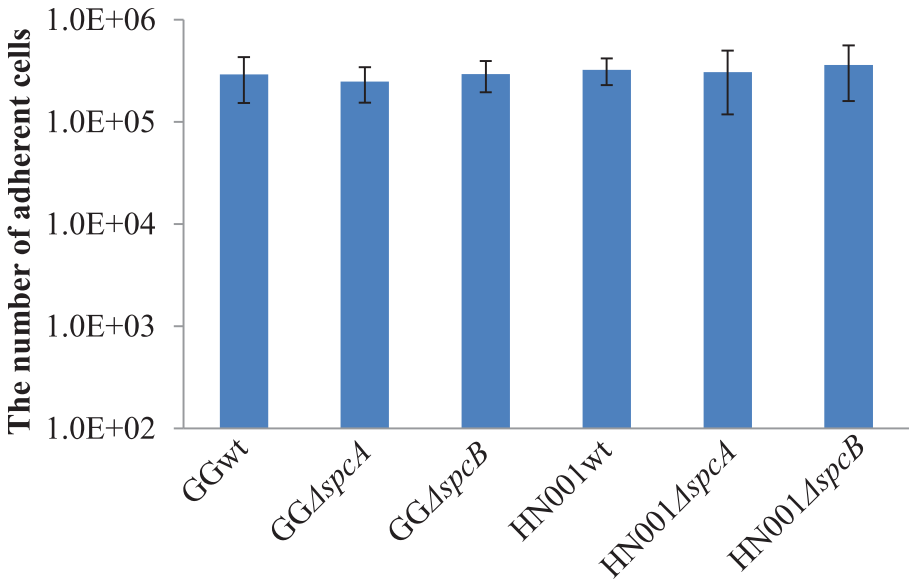

Figure 4.8. Comparison of the adhesion to the Caco-2 cells between the wild-type strains and knockout mutants of *L. rhamnosus*

Stationary-phase lactobacilli cells of the wild-type strains (GGwt and HN001wt) and their knockout mutants (GG*ΔspcA*, GG*ΔspcB,* HN001*ΔspcA* and HN001*ΔspcB)* were tested for adhesion to the fully differentiated Caco-2 cell monolayers. The values represent the mean of nine measurements obtained from three independent experiments (each done in triplicate). The error bars represent standard deviation.

As described in Section 3.3, SpcB shares common features of the signal sequence and protein organisation features of the adhesin/fibril-like proteins in streptococci and

staphylococci. Therefore, *in vitro* adhesion assays were undertaken to compare the adhesion capacities between the wild-type strains and their mutants. Cells from the *L. rhamnosus* wild-type strains and knockout mutants were incubated with the fully differentiated Caco-2 cell monolayers and then Caco-2-attached lactobacilli were eluted, lysed and quantified by counting cfu's on MRS agar. The number of recovered Caco-2-attached bacteria was compared between the tested strains. The assay data (Figure 4.8) showed that the adhesion efficiency was similar between HN001 and GG. However, no significant difference was observed between any mutants and corresponding parental wild-type strains. This result suggested that SpcA and SpcB did not have a detectable impact on the adhesion capacities of the tested *L. rhamnosus* strains under the assay conditions.

4.7 SpcB of *Lactobacillus rhamnosus* GG may improve the barrier function of the TNFα-treated polarised Caco-2 cell layer

Some probiotic bacteria have capacities to promote human health through maintaining the integrity of the intestinal epithelium [316]. *Lactobacillus* strains, including GG and HN001, were previously shown to promote the TJ functions in the permeability assays [259, 260] and also to ameliorate the inflammatory effects of cytokine- and chemical-induced colitis on the TJ structures. Apart from the characterised secreted proteins, p40 and p75, surface-anchored *L. rhamnosus* proteins may potentially modulate the composition and/or distribution of the TJ proteins. In particular, in HN001 the molecules involved in the tight junction health has not been revealed.

In this study, a TEER assay was used to investigate the roles of SpcA and SpcB of *L. rhamnosus* strains in maintenance of the TJ integrity of Caco-2 cell layers. In the time-course of TEER assays, the electrical resistance values across the polarised Caco-2 cell monolayers grown in the inserts of the transwell plates were examined in the presence of either a wild-type strain or a corresponding knockout mutant. In another set of TEER assays, TNFα (a proinflammatory cytokine, which has been shown to impair the TJ

function by increasing the permeability of the Caco-2 cell monolayers leading to a decrease in the TEER values) was included in the bacterial treatments to test whether SpcA and/or SpcB have a role in the protective effects against the inflammatory damage to the TJ integrity.

The TEER data for each treatment was plotted over time as shown in Figure 4.9, which showed a typical three-phase changes, including an initial drop (adaption to the treatment), a subsequent increase (promoted production of the TJ proteins) followed by a decrease as a result of the medium acidification by lactobacilli). In the assays containing bacterial treatments without TNFα, both GGwt and HN001wt treatments were shown to generate strain-specific increases in TEER change, approximately 40% and 60% more than their corresponding initial values, whereas the controls did not have significant difference over time (Figure 4.9a and Figure 4.9c). However, no significant difference in change of TEER was observed between the wild-type strains and their mutants of both GG and HN001, thus SpcA and SpcB are not molecules responsible for promoting the TJ integrity of the Caco-2 cell layers (in the absence of TNFα).

In the assays with TNFα challenge (Figure 4.9b and 4.9d), there was a 10-20% drop in the TEER change in the controls, indicating that the TJ integrity was compromised by this proinflammatory cytokine. Both GG and HN001 wild-type strains compensated for the negative influence of TNFα on the Caco-2 cell layers and eventually increased the TEER change by approximately 40% and 20%. The effect of GGwt was more prominent than that of HN001wt, suggesting that GG has a relatively greater ability to protect the TJ integrity than HN001. The HN001 *ΔspcA* and *ΔspcB* mutants showed no significant difference in change of TEER (Figure 4.9d). However, GG*ΔspcB* mutant showed a significant loss of the protective effects on TJs against TNFα challenge, by only increasing the TEER value to the corresponding initial level, without improving them as observed in the control (p-value < 0.05) (Figure 4.9b). In contrast, the GG*ΔspcA* mutant behaved similarly to the wild-type strain. Therefore, the overall TEER assay data suggested that SpcB in *L. rhamnosus* GG might be involved in protection of the TJ integrity against the inflammatory damage in a strain-specific manner.

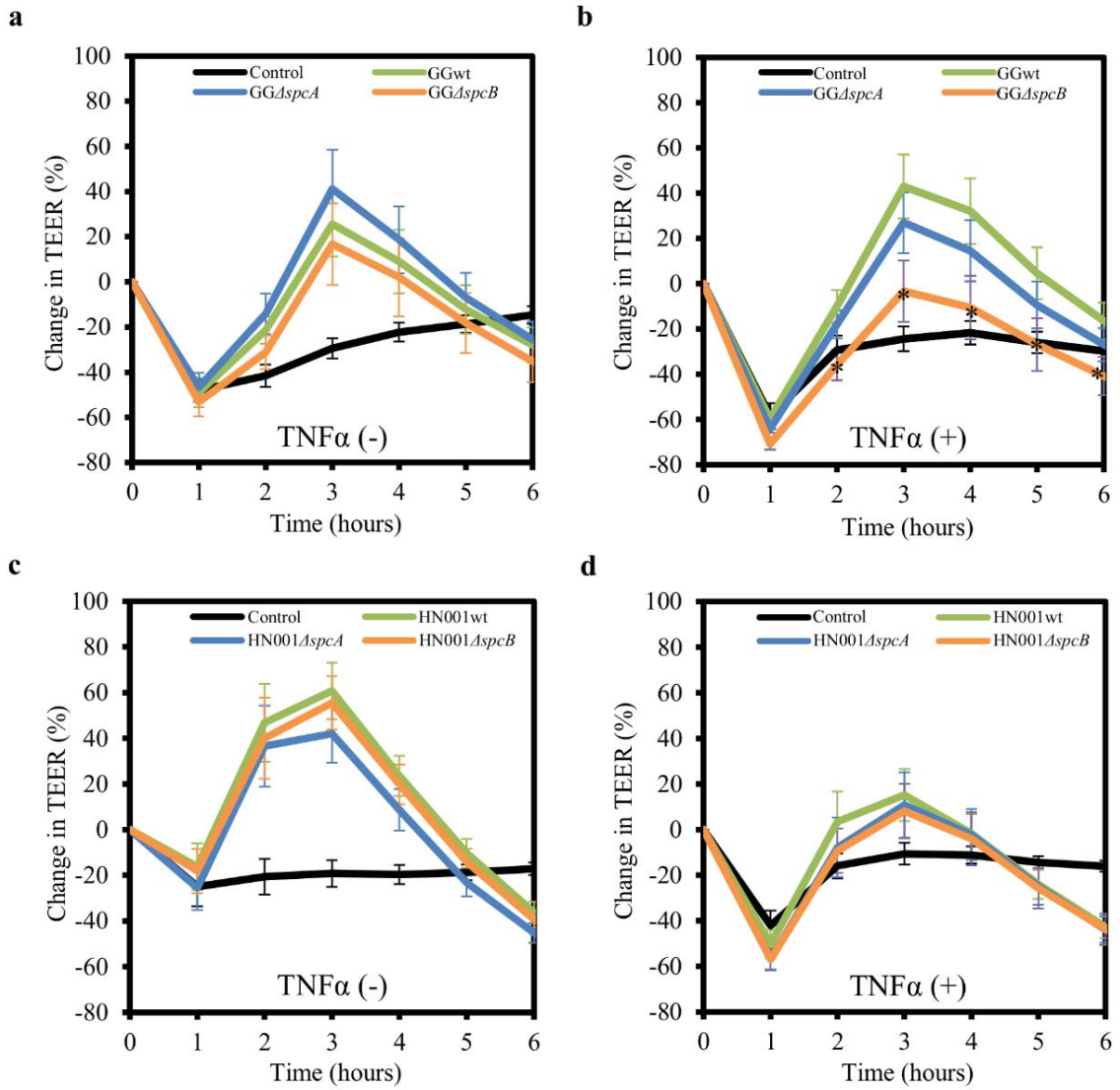

Figure 4.9. Impact of the *L. rhamnosus* GG and HN001 wild-type strains and knockout mutants on the integrity of Caco-2 cell monolayers

The electrical resistance data across the fully differentiated Caco-2 cell monolayers were measured in the presence of a particular bacterial treatment, containing either a wild-type strain or a knockout mutant of *L. rhamnosus* (a and c). The assay medium was used as a control. In another set of treatments, TNFα was also added (b and d). The change in TEER for each insert was calculated using the following formula: change in TEER (%) = (current TEER/initial TEER – 1) x 100. The values plotted represent the mean of 12 measurements (three independent experiments, each containing four replicates). The error bars represent standard error of the mean. The data was analysed using repeated measures ANOVA in the software package RStudio version 0.98.1049. Compared to the control, treatments with a difference greater than the least significant difference at 5% at a given time point were considered statistically different, as indicated by an asterisk.

Summary

As described in Chapter III, SpcA-SpcB is a novel type of bacterial surface protein complex. To investigate the roles of SpcA and SpcB, in some properties of *L. rhamnosus* relevant to its probiotic traits, knock-out mutants of the genes encoding these two proteins were constructed in *L. rhamnosus* HN001 and GG using a marker-exchange method.

The subsequent PPs-lactobacilli binding assays demonstrated that SpcA binding to HN001 and GG was SpcB-dependent, confirming the SpcA-SpcB interaction was specific. In analyses of several properties of the constructed knockout mutants, both SpcA and SpcB were shown to affect biofilm formation (only tested in GG as HN001 does not form biofilms). SpcB, but not SpcA, may be involved in autoaggregation in GG. However, effects of SpcA and SpcB on aggregation were not observed in HN001. Interestingly, while GG, but not HN001, formed biofilms on the plastic surfaces, GG also aggregated faster in the planktonic state of growth in comparison to HN001. Assays testing hydrophobicity did not indicate roles of SpcA and SpcB in this property, however it showed that the GG strain was much more hydrophobic than HN001.

Although SpcA and SpcB were shown not to affect the ability of HN001 or GG to adhere to the Caco-2 cells *in vitro*, SpcB in *L. rhamnosus* GG, but not HN001, was interestingly found to protect the tight junction integrity when the cells were subjected to TNFα challenge. Overall, while SpcB is present on both tested strains, as determined through binding of its interaction partner SpcA, only in GG was this protein involved in other assayed properties relevant to bacteria-bacteria and bacteria-host interactions, suggesting that the role of this protein in some probiotic properties is strain-dependent and this may correlate with differences in the genetic locus, which is incomplete in HN001, missing the associated glycosyltransferases.

Chapter V: Discussion

5.1 SpcA and SpcB of *L. rhamnosus* strains form a novel type of interacting surface protein complex.

Probiotic bacteria are believed to promote human health; however the molecular mechanisms underlying the probiotic effects are not clear in most strains. *L. rhamnosus* is one well characterised species, represented by strains such as GG and HN001. Within *L. rhamnosus* species, variations in bacterial surface properties and probiotic traits have been broadly observed [57, 232, 260, 317]. These differences indicate that, despite the presence of detailed genome sequence and other omics-type of information, a gap of understanding the cellular and molecular biology differences exists, in particular those that pertain to the cell envelopes and interactions with the host. Recently, some proteinaceous effectors have been shown to be important in maintaining the probiotic traits of *L. rhamnosus* species, represented by pilus and the major secreted proteins, p75 and p40 [227]. In HN001, information on these effector molecules is not reported.

In this study, the surface binding site of a previously discovered *L. rhamnosus* HN001-binding protein, SpcA, was successfully characterised by an initial series of $SpcA^{A}$ PPs-lactobacilli binding assays. Failure in binding to other tested *Lactobacillus* species and protein-/peptidoglycan-free HN001 suggested that SpcA was anchored on the cell surface through binding to a cell wall protein specific to *L. rhamnosus* species. Thus, this SpcA-binding protein was proposed to be contained within an existing *L. rhamnosus* HN001 phage display library. Subsequent screening of this phage display library, using MBP-$SpcA^{A1}$ as a bait, revealed peptides derived from the N-terminal "A domain" of a large LPxTG-containing protein, SpcB, which was further confirmed as the SpcA-binding partner (Figure 5.1). Interestingly, although binding of SpcA to *L. rhamnosus* HN001 was confirmed in various ways as discussed above, in an early electron microscopy experiment (method described in Section 2.2.8), in which HN001-bound $SpcA^{A}$ PPs were fixed using paraformaldehyde and labelled sequentially by the primary anti-phage antibodies and secondary gold-conjugated antibodies, only a small proportion (less than 1 in 100) of HN001cells were seen to have attached PPs (Appendix II). This suggested that either SpcB expression might not be uniform among the cells in the bacterial culture or the binding efficiency of SpcA to HN001 was restricted by other unknown factors.

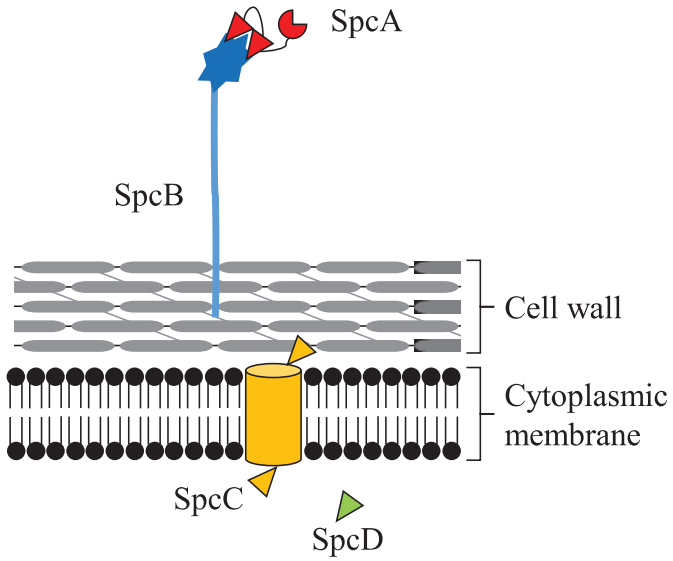

Figure 5.1. Model of the SpcB-SpcA complex and position of SpcC and SpcD
SpcA (red), a secreted protein containing two Big-3 domains (triangle shape) and a third domain (PR1); SpcB (blue), a cell wall-anchored fibril-like protein; SpcC (yellow), a putative membrane channel-like protein containing two Big-3 domains (one on each side of the cytoplasmic membrane); SpcD (green), a putative cytosolic protein only containing a Big-3 domain.

The *spcA* and *spcB* genes were found to belong to a cluster of ORFs (*spcB-spcA*) unique to the "*Lactobacillus casei* group" (*L. rhamnosus*, *L. casei* and *L. paracasei*) and highly conserved in *L. rhamnosus* species. The complete or incomplete cluster was found to be almost ubiquitous among the *L. rhamnosus* strains in a recent comparative genomics study (*spcB* exists in 94 out of 100 *L. rhamnosus* sequenced genomes) [232]. However, the frequency of the *spcB* homologue is much lower in the other species apart from *L. rhamnosus* in this particular *Lactobacillus* group. A study has shown that the *spcB* homologue is present in only 10 out of 37 *L. paracasei* genomes [234]. Although there has not been a comparative *L. casei* genomic analysis reported so far, according to the genome sequences deposited in the National Centre for Biotechnology Information (NCBI) database, *spcB* is found in only 9 out 29 complete and draft *L. casei* genomes. Compared to *L. rhamnosus*, the overall SpcB homology is higher in *L. casei* than that in *L. paracasei*. Interestingly, "A domain" of SpcB is conserved among the SpcB sequences in *L. casei* and *L. paracasei*, suggesting that SpcA and SpcB in these species very likely form a protein complex as seen in *L. rhamnosus*.

In GG, as in HN001, SpcB is 3275 residues in length, whereas in Lc705 it has 3390 residues, due to an extra 111-residue ASKD repeat, and several other short insertions/duplications, suggesting that the function of SpcB might be strain-specific. SpcB has a signal sequence motif, KxYKxGKxW, and organisation typical of Gram-positive glycosylated adhesins, exemplified by the GspB adhesin of *Streptococcus gordonii* that binds to human platelets, and a mucus-binding fibril-like adhesin of *Streptococcus parasanguinis*, Fap1 [194, 318]. However, the signal sequence of SpcB (51 residues) is shorter than that of GspB (90 residues) as it contains less residues in the charged N-terminus. Furthermore, the gene organisation surrounding the *spcB* ORF is very different from that of *gspB* and *fap1*. The *gspB* and *fap1* genes are in the same cluster/operon with the glycosyltransferase-encoding genes and genes encoding a secondary/alternative Sec translocon complex (SecA2, SecY2 and in some cases SecE2 and SecG2) [191, 319]. The secondary translocon, either in combination with or independently of the standard translocon (SecAF, SecYEG), is required for export of GspB and Fap1 from their respective organisms. The same gene cluster also typically encodes two or more transmembrane or cytosolic accessory proteins that are required for, or facilitate, the secretion of glycosylated precursors [191, 319].

In contrast, the conserved *spcB-spcA* cluster in *L. rhamnosus* includes only two other genes: *spcC* and *spcD*. Neither *secA2* nor *secY2* has been annotated in the sequenced *L. rhamnosus* genomes. This suggests that transport of SpcB in *L. rhamnosus* could be mediated by the canonical SecA/YEG translocase, possibly with the help of the cluster-encoded accessory proteins. The candidates for accessory proteins for secretion of SpcB are SpcC and SpcD (the former of which is a multiple-transmembrane-helix-containing integral membrane protein containing two Big-3 domains, presumably one on each side of the cytoplasmic membrane, and the latter is a cytosolic protein containing a single Big-3 domain) (Figure 5.1). Genes encoding these two proteins are located between *spcB* and *spcA*. Unlike the GG genome where this cluster is followed by seven ORFs including two glycosyltransferase genes (*yohJ* and *yohH*), in HN001 genome, downstream region of *spcBCDA* cluster is truncated by apparent insertion of an IS5-family transposase gene near the 3'end of *spcA*, resulting in the loss of the downstream cluster sequence, including the predicted glycosyltransferase genes. Glycosylation is known to be important for export of the KxYKxGKxW motif-containing adhesin/fibril-like proteins [194]. Even though HN001 lacks the two GtfA family glycosyltransferases closely linked to the

spcBCDA cluster, it contains four genes encoding additional GtfA homologues elsewhere in the genome that are highly conserved with the missing glycosyltransferases. Therefore, it is possible that the SpcB polypeptide is still glycosylated in HN001, despite the deletion of adjacent glycosyltransferases.

A few recent proteomic studies have separately shown that the SpcB homologues (LGG_01592 and LC705_01573) were produced in *L. rhamnosus* GG and Lc705 [75] and also the expression level of this gene in *L. rhamnosus* GG is probably independent (at least under the test conditions) on the growth conditions (such as growth phase and media) and environmental stress (acid and bile) [60, 89, 320, 321]. Interestingly, using mass spectroscopy, 105 different peptides from LGG_01592 were identified in *L. rhamnosus* GG cell wall fraction [75]. This protein is therefore produced in *L. rhamnosus* GG and targeted to the cell wall. The mass fingerprinting approach used in Savijoki et al. (2011) can not assign peptides to a protein if they are glycosylated, therefore many proteolytic peptides derived from SpcB in *L. rhamnosus* GG must be unmodified [91]. Whatever possible differences in modification between HN001 and GG, they do not affect binding of SpcA, as shown in the PPs-lactobacilli binding assays.

Eight different antisera to predicted immunogenic peptides were custom-raised in this thesis, four against SpcB and the other four against SpcA; however they all failed to result in antibodies that can recognise either SpcB-pIII or SpcA-pIII fusions within phagemid particles, or those proteins in GG or HN001 (total, cell wall or trichloroacetic acid-concentrated supernatant protein extracts), suggesting that the antibody titres were very low (data not shown). Other than extremely bad fortune, this failure could indicate intrinsic low immunogenicity of SpcB and SpcA.

A series of lectin screening experiments carried out in this study, using various lectins (Lectin screening kit I; Vector Labs.) specific to mannose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine and fucose, failed to distinguish the wild-type strain and the corresponding GG and HN001 mutants of *spcA* and *spcB* in the glycosylated protein profiles (data not shown), suggesting that SpcB is possibly modified by other type of sugars. Modifications with ConA-reactive sugars reported in the major secreted proteins of GG, p75 and p40, were confirmed in these experiments.

5.2 Roles of SpcA and SpcB in surface properties of *Lactobacillus rhamnosus*

Extracellular proteins are known to mediate both intra- and inter-species interactions, such as autoaggregation, coaggregation and biofilm formation. In probiotic bacteria, some of these proteins are responsible for their probiotic traits, such as exclusion of pathogens, adhesion capacity, biofilm formation and immunomodulation [41, 80]. Unlike the Gram-positive pathogens, whose molecular mechanisms are well characterised, including large number of proteins mediating interactions with other bacteria and a variety of host cell surface molecules, extracellular matrix and signalling molecules, comparatively little is known about surface proteins of Gram-positive probiotic species, including *Lactobacillus* species. Although *L. rhamnosus* is a well-known species in terms of the probiotic functions, the published cell biology and molecular biology experimental data mostly come from a single strain, GG. However, a significant variation in the probiotic traits has been observed in a large-scale comparative analysis of *L. rhamnosus* strains, such as the variation in the genes encoding pili and their roles in binding to mucus, as well as TLR-2 responses [232]. This suggests that the probiotic traits could be mediated by multiple surface factors, which may have synergistic or antagonistic effects. Overall, more biological information is required in order to gain a deeper insight into the strain-specific probiotic traits.

In this study, SpcA-SpcB was identified as a novel cell surface protein complex from probiotic *L. rhamnosus*. Apart from the general adhesin organisation for SpcB, the lack of homology with known surface proteins prevented any prediction as to the biological function of this complex based on the primary amino acid sequences of the two proteins. Out of the three SpcA domains, the two Big-3 domains of SpcA have been shown to be required for interaction with A-domain of SpcB, however the function of the third (PR-1-like) domain cannot be predicted using bioinformatics tools, although PR-1 is, interestingly, involved in plant-pathogen interactions in an unknown manner. Similarly, even though SpcB shares the typical signal sequence and protein organisation features of Gram-positive adhesin/fibril-like proteins, such as GspB and Fap1, the amino acids sequences and compositions are different, including the N-terminal high-complexity

domain that is, based on the position, expected to be mediating adhesion to extracellular targets. The closest prediction for the binding targets is that they are proteins containing immunoglobulin-like domains, by analogy with its binding partners, Big-3 domains of SpcA.

To functionally characterise SpcA and SpcB, knockout mutants of the respective genes were constructed in both GG and HN001 background using a marker-exchange method. Unlike insertional mutagenesis, this method generated genetically stable mutants even in the absence of the antibiotics, allowing direct phenotypic comparisons between the wild-type strains and the derived mutants in the same antibiotic-free medium. The antibiotic-resistance profiles, diagnostic PCR and Southern blot data all confirmed the correct replacement deletion of genes in the putative mutants. However, several parallel attempts of construction of the complementing or overexpressing strains were unsuccessful using two expression plasmid vectors carrying a promoter from either SlpA- or EF-Tu-encoding gene, respectively (unpublished; FRDC). Although an SpcA-expressing plasmid was constructed, SpcA was not detected in any fraction of HN001 by western blot (data not shown), suggesting the heterologous expression of proteins is very difficult in *L. rhamnosus* species. Attempts to construct of SpcB-expression plasmid in lactococci were unsuccessful as well, probably due to the large size of *spcB* gene (9828 nt), which lowered the transformation efficiency.

The knock-out constructs were utilised to determine the roles of SpcB and SpcA in various properties of *L. rhamnosus* GG and HN001. In the binding assays of phagemid particles (PPs) displaying $SpcA^{A}$, binding to *L. rhamnosus* GG and HN001 was shown to be SpcB-dependent. Binding of $SpcA^{A}$ PPs to *ΔspcB* mutants was equal to that of the negative control; therefore this supported the previous hypothesis that SpcB was a cognate "docking" protein of SpcA. On the other hand, binding of $SpcA^{A}$ PPs to *ΔspcA* mutants was not affected, suggesting that SpcA (or at least its Big-3 domains) did not self-bind or compete with PPs for binding to SpcB. The Ala-Ser-rich low complexity regions of SpcB were predicted by the PHYRE server to have either disordered or helical secondary structure. If the latter is correct, the low-complexity sequences along the 95% of the SpcB length could make a cell-associated fibril, as found for the similarly organised Ser-Ala-rich adhesins in Gram-positive pathogens. Alternatively, SpcB as a fibrillar protein might serve as a scaffolding protein for anchoring of other proteins (such as SpcA)

to the cell surface, as seen in the anaerobic bacterial cellulosome complex, which contains a fibrillar scaffoldin and attached enzymes, functioning in degradation of plant cell walls [322]. Interestingly, in addition to the four nearly perfect ASKD repeats annotated in this study (Figure 3.6), along L2 Ala-Ser-rich low complexity region that forms the central portion of the SpcB sequence (Appendix I), nine imperfect repeats containing approximately 175 amino acids in length were identified, already annotated as Ser-Ala-175 repeats in the Conserved Domain database (NCBI). Therefore, these repeats in SpcB may potentially serve as docking sites for anchoring various molecules.

With respect to other surface properties, the GG*ΔspcB* mutant was shown to have a slightly increased autoaggregation; however *ΔspcB* mutant HN001 and *ΔspcA* mutants of GG and HN001 did not show any significant changes in this phenotype. Even though the observed difference in GG*ΔspcB* mutant was small, it was consistently detected and statistically significant. This suggested that SpcB might have a minor regulatory impact on autoaggregation in GG but not HN001. It is not too surprising that SpcB is not an major autoaggregation factor in GG as the SpaCBA pilus has been shown to be essential for autoaggregation in this strain, probably through homophilic binding of SpaC [84]. However, as discussed earlier, a single probiotic trait can be regulated by multiple bacterial factors. SpcB could be a negative regulator for autoaggregation in GG. In contrast to GG, strain HN001 does not contain the *spaCBA* pilus genes but still shows an autoaggregation phenotype. This indicates that factor(s) other than pilus must be involved in autoaggregation. Interestingly, autoaggregation of HN001 was much lower relative to that measured in GG, (47.4% vs. 6.2%, respectively), and this correlates with absence of pili in HN001. The absence of pili correlates with the lack of SpcB effect on aggregation in HN001, hence SpcB may counteract the pili in autoaggregation in GG, but this function may be biologically superfluous due to already low aggregation in HN001 and therefore not taken up by any protein. This possibility remains to be explored in the future.

With respect to biofilm formation, both GG*ΔspcA* and GG*ΔspcB* mutants were shown to form increased amount of biofilm over 72 h on polystyrene surface compared to the wild-type strain, suggesting that SpcA and SpcB may affect the biofilm growth of GG or stimulate biofilm attrition. Biofilm formation in bacteria is influenced by both central regulatory factors and variable surface molecules [323]. In *L. rhamnosus* GG, apart from *luxS*, which has a regulatory role, other genes implicated in the biofilm growth mode

encode surface structures. Among these biofilm factors, the SpaCBA pilus is found to be essential for biofilm formation. Loss of SpaCBA pilus or minor pilin SpaC or treatment with anti-SpaC antibodies was shown to eliminate biofilm formation in GG [41]. MabA is a minor biofilm-promoting factor, whose disruption was reported to reduce the biofilm formed by about 50% by GG [102]. In contrast, disruption of gene *welE*, encoding a glycosyltransferase catalysing the initial step of EPS synthesis resulted in significantly increased biofilm formation [100]. This could be due to partial masking of one or more biofilm-promoting factors (proteins) by the galactose-rich EPS. Therefore, the SpcA-SpcB complex discovered in this thesis may play a similar role as EPS in biofilm formation in GG, by interfering with the interactions involved in biofilm formation. In contrast to GG, HN001 did not form detectable biofilm under the same conditions (data not shown). This is not too surprising as the HN001 genome does not contain the SpaCBA pilus-encoding genes, which are known to be essential for biofilm formation in GG.

As SpcB is a large Ala-Ser rich protein, hydrophobic alanine residues of this protein could potentially influence the bacterial surface hydrophobicity, which is responsible for nonspecific bacteria-bacteria interactions involved in the probiotic straits, such as autoaggregation and biofilm formation. However, in this study, no difference in surface hydrophobicity was observed between any tested mutant and its parent strain. This indicated that neither SpcA nor SpcB significantly contributes to the net surface hydrophobicity of GG and HN001 under the test conditions. Interestingly, the assay data showed that GG is much more hydrophobic than HN001, at least when using hexadecane as hydrophobic agent in the partitioning assay. Therefore, besides the pili, increased hydrophobicity of GG may favour this bacterium in binding to a hydrophobic surface of various sources, such as polystyrene.

5.3 Roles of SpcA and SpcB in bacteria-host interactions

The integrity of human intestinal epithelium is maintained by the intact TJ structures between the adjacent intestinal epithelial cells and essential for exclusion of the luminal pathogens and their toxins, as well as allergens [324]. Strains of some probiotic bacterial species, such as *L. rhamnosus* and *L. plantarum*, have been shown to have abilities of not

only improving on the TJ integrity but also can compensate or even reverse the negative effects of the cytokine- or chemically-induced inflammation on the tight junction integrity [38-40, 260, 325]. However, apart from p40 and p75 characterised in *L. rhamnosus* GG [38, 264], other effectors and the corresponding molecular mechanisms involved in the intestinal epithelial integrity are still unknown.

In this study, both GG and HN001 wild-type strains were shown in the TEER assays to be capable of positively impacting and also reversing the negative effects of TNFα on the TJ integrity. However, compared to the wild-type strain, GG*ΔspcB* mutant interestingly demonstrated a much lower ability to reverse the TNFα–caused drop in TEER change across the polarised Caco-2 monolayers. This finding suggested that SpcB of GG might have a role in reversing the cytokine-induced destabilisation of the TJs in the Caco-2 monolayer.

The TNFα-mediated increase in Caco-2 cell layer's permeability is proposed to result from impaired production and distribution of the TJ protein components (such as ZO1), in an NF-κB dependent manner [326]. The NF-κB p50/p65 has been shown to bind to the promoter of myosin light-chain kinase (MLCK)-encoding gene causing an increase in transcription, translation and enzymatic activity of MLCK, as well as the downstream signalling that results in the increased TJ permeability [327, 328]. Another report indicates that TNFα also induces phosphorylation of extracellular signal regulated kinases, ERK1 and ERK2 (MAPK group), in Caco-2. Apart from TNFα, the signalling pathways involved in maitenance of the TJ integrity between the intestinal cells also include a series of signalling molecules, including the Rho family GTPases, protein kinase C (PKC) and MAPK kinases [40, 325].

Lactobacillus strains, such as *L. rhamnosus* GG and *L. plantarum* ATCC8014 have been shown to reverse one or more TNFα-dependent signalling pathways as described above to recover the intestinal epithelial integrity [258, 259, 325]. For example, *L. rhamnosus* GG was shown to prevent the TNFα-induced nuclear translocation of NF-κB, blocking the downstream signalling pathways [259]. The recent studies on secreted proteins of *L. rhamnosus* GG, p40 and p75, have provided a putative molecular basis for probiotic effects on the intestinal epithelial integrity. Both p40 and p75 have been shown to inhibit the TNFα-induced intestinal cell apoptosis and stimulate the intestinal cell growth,

coupled to the PI3K/Akt-activation pathways [38]. Further studies also indicate that the previously observed protective effects of p40 on the intestinal cells are EGFR-dependent [90, 264].

However, not all signalling events mediated by *L. rhamnosus* GG are attributed to p40 and p75. For example, *L. rhamnosus* GG cell, but not p75 or p40, inhibit the TNFα-stimulated p38/MAPK activation and induce ERK1/2 [38]. However, these effectors on *L. rhamnosus* GG cell have not been identified so far. As the absence or presence of SpcB in GG does not affect the TEER change in the absence of TNFα, it could specifically interfere with the TNFα-dependent signalling pathways. SpcB might be such a cell-surface-anchored immunomodulatory protein, which can reverse one or more TNFα-induced signalling cascades to protect the intestinal epithelial integrity. The SpcB interference could be direct or indirect, depending on whether the signalling cascades in Caco-2 are triggered by SpcB itself or a SpcB-dependent molecule.

In the strain HN001, no matter whether TNFα was present or absent, no difference in TEER change was observed between the *ΔspcA* or *ΔspcB* mutant and the wild-type, showing that the function of SpcB was strain-specific, and that in HN001 SpcB is redundant with respect to stabilising the TJs in the presence of TNFα. Different effects of SpcB from GG and HN001 on the stabilising the TJs could stem from potential differences in glycosylation. As the two glycosyltransferase genes are missing in the HN001 genome, SpcB in this strain could be nonglycosylated. Alternatively, SpcB is glycosylated in HN001 by other nondedicated glycosyltransferases, however compositionally or structurally the modification is different from the one in GG, resulting in different effects on cell signalling that controls the stability of the TJs.

Another research question relevant to *L. rhamnosus*-host interactions is whether SpcB and/or SpcA promote adhesion of *L. rhamnosus* to Caco-2 cell layers. A few surface proteins in GG have been demonstrated to mediate adhesion to Caco-2 cells, in particular SpaCBA pilus [41, 100, 102]. However, less information is available for HN001, a *L. rhamnosus* strain that does not have a pilus. In the *in vitro* adhesion assays, both GG and HN001 strains adhere equally efficiently to the Caco-2 cells. Furthermore, no significant difference was observed between the wild-type strains and *ΔspcA* or *ΔspcB* mutant of GG and HN001, suggesting neither SpcA nor SpcB was a major adhesin in either of the two

strains under the test conditions. As SpcB of GG has been shown to interfere with TNFα-induced destabilisation of the Caco-2 polarised monolayer integrity, this protein possibly has a much weaker interaction with the Caco-2 cells than that of the putative major adhesins of *L. rhamnosus*.

Chapter VI: Conclusions and further directions

6.1 Conclusions

Using phage display, a previously identified *L. rhamnosus* HN001-binding protein, SpcA, was experimentally demonstrated to interact with a large cell wall-anchored protein, SpcB. SpcA-SpcB was shown to be a novel bacterial surface protein complex and unique to the *Lactobacillus casei* group. The *spcB* and *spcA* genes are located in the same gene cluster, *spcBCDA*, which is highly conserved among *L. rhamnosus* strains. However, the downstream regions of this gene cluster are strain-dependent. In particular, two glycosyltransferase-encoding genes located downstream of the cluster are missing in HN001 genome due to a transposase-mediated truncation.

Through phenotypic analysis of the GG and HN001 knockout mutants of single genes, SpcA and SpcB were showed to have various strain-specific roles in both microbe-microbe and microbe-host interactions, correlating with the presence of the cluster-dedicated glycosyltransferases. In GG, SpcB was found to slightly affect autoaggregation and both SpcA and SpcB were also shown to negatively influence biofilm formation. In addition, SpcB of GG supported recovering the TNFα-compromised TJ integrity. Although SpcB amino acid sequences are almost identical in GG and HN001, the dependence of some surface properties on SpcA and SpcB were only observed in the strain GG, but not in HN001, suggesting the strain-specific roles for these proteins.

As a single probiotic trait might be regulated by multiple factors, the findings from this study will allow a deeper insight into some previously well characterised probiotic traits of *L. rhamnosus*.

6.2 Further directions

This study has shown that SpcA binds to its cognate surface "docking" ligand, SpcB, in *L. rhamnosus* GG and HN001. As *spcB* gene is conserved in 94 out of 100 analysed *L. rhamnosus* strains and a few *L. casei* and *L. paracasei* strains, it would be interesting to do a comparative study of whether this protein is expressed in all those strains, using the easy $SpcA^{A}$ PPs-lactobacilli binding assay as described in this study.

Many proteins can be potentially noncovalently attached to Gram-positive bacteria, hence most likely SpcA is not the only cell-surface-binding protein of *L. rhamnosus*. SpcA was, however, repeatedly isolated as a sole CDS in library screens for binding to wild-type *L. rhamnosus*. As mutants of *spcB* do not bind SpcA, yet aggregate efficiently, use of this mutant as bait offers an opportunity to discover novel *L. rhamnosus*-binding proteins, potentially involved in aggregation, by screening the HN001 phage display library.

To study the physiochemical properties, export, posttranslational modification and structural function, detection of SpcB is essential. The SpcB-overexpressing strains were not constructed in this study despite repeated attempts, using purpose-constructed vectors for *L. rhamnosus* HN001. However, the list of potential vectors was not exhausted, hence it is possible to test other *L. rhamnosus*-specific expression plasmids (unpublished; FRDC) or a broad host-range plasmid pTRKH2, which has been shown to successfully express a peptidase of *L. rhamnosus* HN001 [329]. Moreover, due to the large size of *spcB* gene, which is often a restricting factor for transformation into Gram-positive bacteria, individual regions of SpcB (such as some of the Ala-Ser-175 repeats) can be attempted. This could also provide an opportunity to functionally map the SpcB domains for the SpcB roles observed in GG. Given that heterologous expression of proteins is technically difficult in *L. rhamnosus* species, alternatively, *Lactococcus lactis* can be used an expression host. *L. lactis* has been successfully demonstrated to be functional for heterologous expression of *L. rhamnosus* GG SpaFED pili that are naturally not expressed above the detectable level in GG [330]. The *spcBCDA* cluster also contains another two genes, *spcC* and *spcD*, which are proposed to be dedicated for export and stability of SpcB or other accessary components. To confirm their roles, knockout mutants of the respective genes are needed using the methods described in this study.

In *L. rhamnosus* GG, SpcB was found to have negative impact on both autoaggregation and biofilm formation, which are considered as important probiotic traits. However, the intestinal lactobacilli are normally not model microorganisms for studying biofilm formation. The molecular mechanisms and dynamics of biofilms in probiotic bacteria are therefore not well-understood. Although polystyrene-dependent *in vitro* biofilm-formation capacities of GG mutants were analysed in this study, further exploration of the biofilm properties would give more insight into the role of SpcB in the biofilm formation. For example, biofilm-formation capacity of GG can be tested in a flow-cell reactor that is used for other biofilm-forming species such as *Pseudomonas* [331] or other more complex setups with the attachment substrates other than plastic. In addition to the *in vitro* biofilm formation, animal models could be used to examine the roles of SpcB of GG in the colonisation of the gastrointestinal tract *in vivo*, which depends on the ability to form biofilms [332]. The GG cells would be monitored by specific detection using the fluorescent *in situ* hybridisation (FISH) technique, as described by Lebeer *et al.* (2011) [333]. Despite the presence of other minor factors, overall evidence to date pinpoints SpaCBA pilus as the key or essential factor for maintaining both phenotypes of *L. rhamnosus* GG [41, 84]. Therefore, SpcB might specifically or nonspecifically interact with the SpaCBA pilus to weaken these two phenotypes. It is worth investigating further whether purified single pilin subunits or the assembled pili interact with SpcB.

In the TEER assays, GG SpcB was demonstrated to support recovering of the TJ integrity of TNFα-compromised Caco-2 cell monolayers. This might involve complex signalling pathways, mediated by molecules such as NF-κB, MLCK, ERK/MAPK and PKC [259, 328, 334, 335]. Thefore, it is worth testing which signalling molecules are the targets of GG SpcB-mediated improvement of the TJ integrity. Moreover, *L. rhamnosus* GG has been shown to prevent cytokine-induced cell death in both human and mouse intestinal cells, through activation of EGFR and Akt and inhibition of p38/MAPK. The former has been attributed to a secreted protein, p40 [90, 264], whereas the latter is only known to be mediated by the *L. rhamnosus* GG cells (not p40) but the corresponding surface molecule has not been revealed. Therefore, SpcB may potentially be involved in inhibition of p38/MAPK, however this needs to be tested. To further characterise this protective role of SpcB in the TJ integrity, expression and distribution of the TJ proteins, such as ZO1, have to be examined in the TNFα-dependent intestinal epithelial inflammation models in the presence of the GG wild-type vs. *ΔspcB* mutant, or in the

presence of purified recombinant SpcB. Importantly, a more general protective role of SpcB should also be investigated by testing other human polarised intestinal cell lines (such as HT-29 and T84), or mouse colonic epithelial cells (MCE), or mouse models, as previously described [90, 264].

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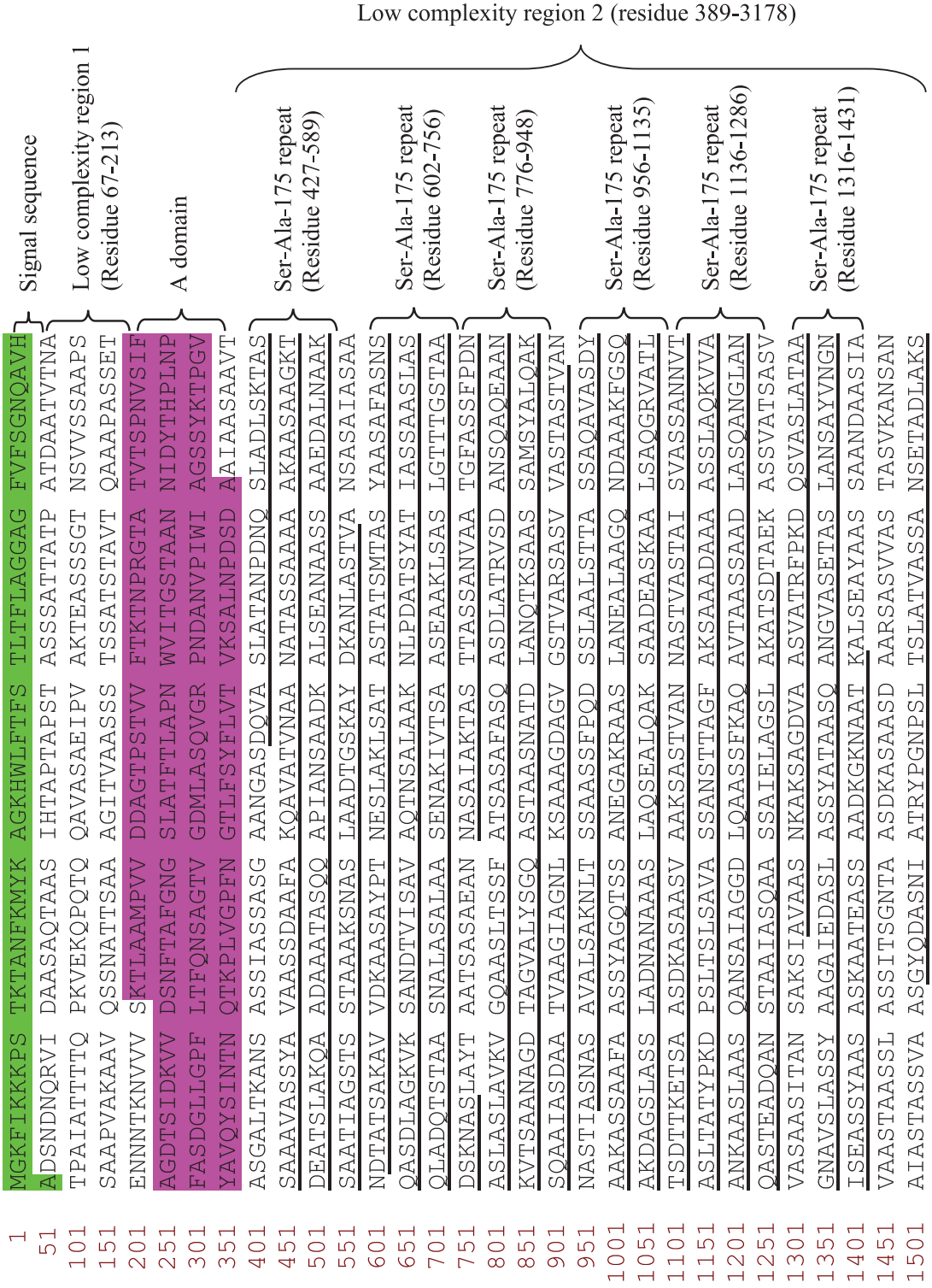
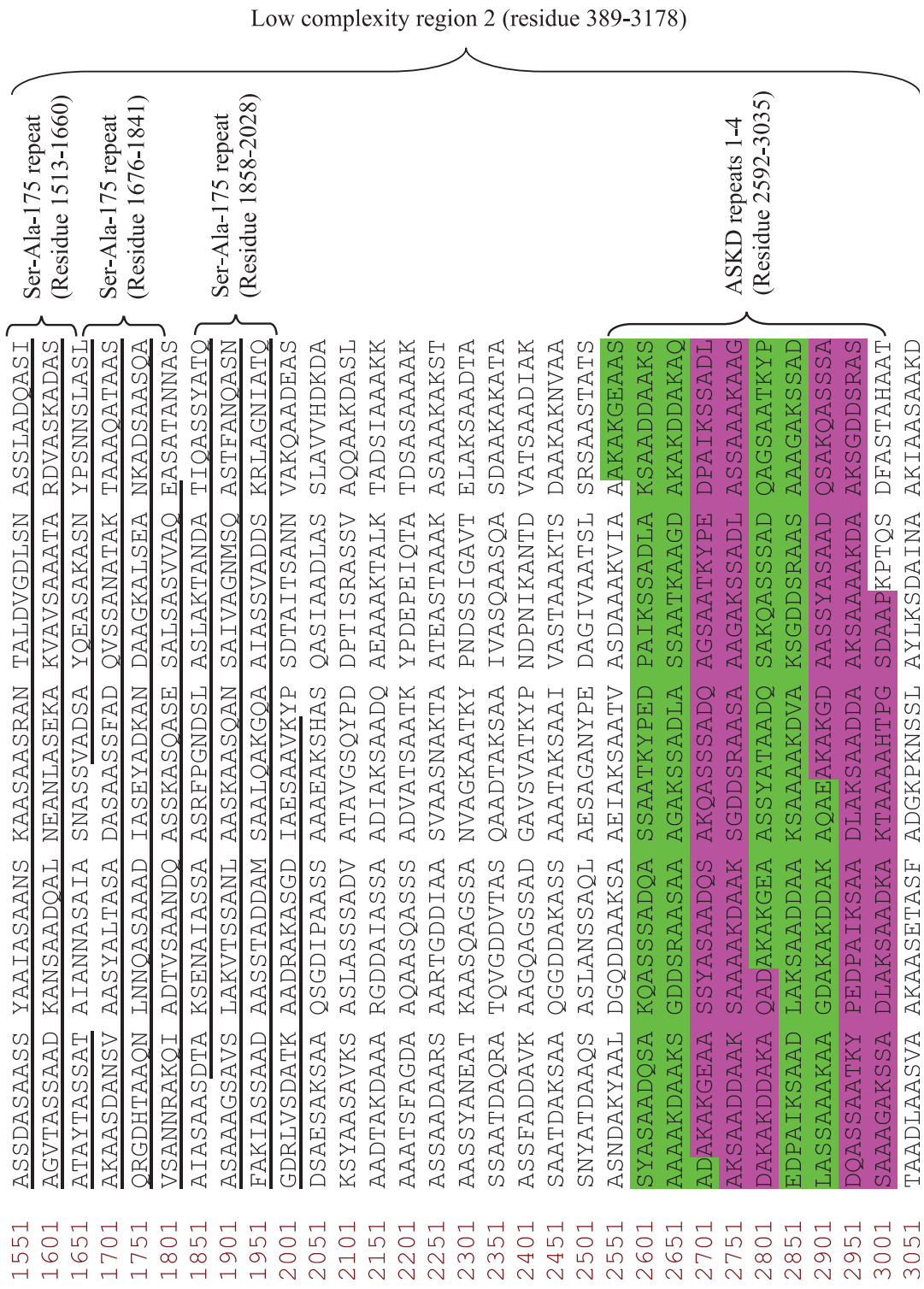
Appendix I. Protein sequence and annotation of SpcB



Appendix I. Protein sequence and annotation of SpcB

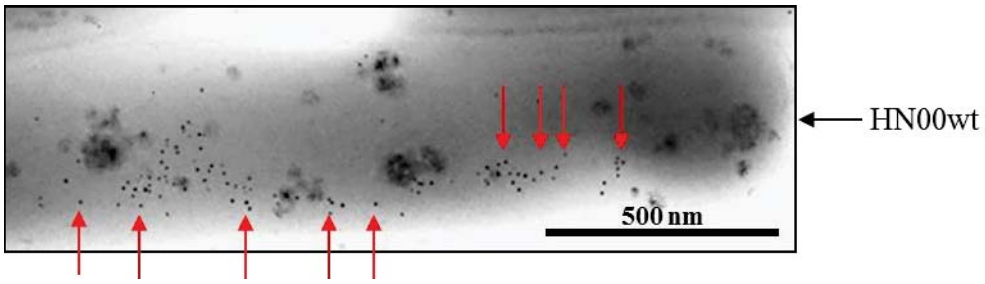


Appendix I. Protein sequence and annotation of SpcB

```
3101  AASFAALSKA AQELAHDYVD PNKAAQALSY AAQYASSATA AADKAKSAAT
3151  RAQTDRDLAE QIASHDVDTK GVKDVDGTSK NPKSQDVGHS ELGANSGSQD
3201  NTVHTVATDT SVGDKTNPGT TADIIAKNGS GQTKDGSGHT LPQTGETEEA
3251  ALLALAGIVL MGTLAAMGKK KHRED
```

LPxTG anchor

Appendix II. Electron microscopy



Electron microscopy. SpcA^{A} PPs were firstly incubated with wild-type HN001. After washing off the unbound PPs, the PPs-HN001 complex was fixed by paraformaldehyde. Then cell-bound PPs were in turn detected by anti-phage antibodies and gold-labelled secondary antibodies. The sample was subsequently examined under an electron microscope. HN001wt (background) is indicated by the black arrow and the cell-bound PPs (black dot) is indicated by the red arrows.