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Abstract

This thesis describes a part of a larger project aimed at developing oral mucosal vaccines based on lactic acid bacteria (LAB). LAB are considered attractive candidates as vaccine delivery vectors because of their Generally Regarded As Safe (GRAS) status. Furthermore, many LAB are natural inhabitants of the gastrointestinal tract, where some are further thought to have probiotic effects on the host. *Lactobacillus plantarum* is of particular interest because of its ability to persist in the gastrointestinal tract of humans for up to seven days, and also because of its resistance to bile and low pH. Different delivery routes and targets may be considered to obtain efficient LAB-based mucosal vaccines, and this thesis describes the development of one such route, where *L. plantarum* is targeted to M-cells in the gastrointestinal tract through expression and surface-display of M-cell binding proteins. The idea behind this strategy is that M-cells may transcytose the bound bacteria to underlying Peyer's patches, where they are exposed to high concentrations of immune cells.

The proteins FimH from *Salmonella typhimurium* and enteropathogenic *Escherichia coli* and Invasin from *Yersinia enterocolitica* were selected for their ability to bind M-cell receptors. Plasmids were constructed for the expression, secretion and subsequent anchoring of FimH and Invasin in *L. plantarum*, using both cell wall- and lipoprotein anchors. Using western blotting and flow cytometry, it was shown that the proteins were produced and displayed on the surface of *L. plantarum*. Caco-2 cells were used as an *in vitro* model to investigate the ability of the proteins to promote internalization, and the recombinant *L. plantarum* strains all showed increased internalization compared to the strain harbouring an empty vector (pEV). However, the experiment did not distinguish between bound and internalized bacteria. Additional *in vitro* studies, using M-cell-like cells, showed signs of FimH- and Invasin-promoted transcytosis. In one of two individual experiments, promising results were obtained for several strains, in particular for the one expressing Invasin with an N-terminal lipoprotein anchor.

The work described in this thesis gives promising indications that FimH and Invasin are involved in promoting transcytosis of *L. plantarum* via M-cells. It thus seems reasonable to further pursue this strategy, starting with further analyses to evaluate the efficiency of the transcytosis process and to verify the possibility to deliver vaccine antigens to Peyer's patches.

Sammendrag

Denne oppgaven beskriver en del av et større prosjekt som har som mål å utvikle orale slimhinnevaksiner basert på melkesyrebakterier. Melkesyrebakterier er ansett som attraktive kandidater som leveringsvektorer av vaksineantigener ettersom de generelt er betraktet som trygge. Mange finnes dessuten naturlig i mage-tarmkanalen til mennesker, og enkelte er vist å ha probiotiske effekter på verten. *Lactobacillus plantarum* er spesielt interessant på grunn av sin evne til å vedvare i mage-tarmkanalen i opptil syv dager, og også på grunn av sin høye toleranse for gallesyre og lav pH. Ulike leveringsveier og mål er blitt vurdert for å oppnå en effektiv slimhinnevaksine, og denne masteroppgaven beskriver utviklingen av en slik alternativ leveringsvei, der *L. plantarum* er rettet mot M-celler i mage-tarmkanalen via ekspresjon og ankring av proteiner som binder reseptorer på M-celler. Idéen bak denne strategien er at M-celler kan frakte bundne bakterier til høye konsentrasjoner av immunceller i underliggende Peyerske flekker via en prosess som kalles transcytose.

Proteinene FimH fra *Salmonella typhimurium* og enteropatogen *Escherichia coli* og Invasin fra *Yersinia enterocolitica* ble valgt ut på grunn av sine egenskaper til å binde M-celle-reseptorer. Plasmider ble konstruert for ekspresjon, sekresjon og ankring av proteinene FimH og Invasin i *L. plantarum* ved bruk av cellevegg- og lipoprotein-ankere. Western blotting og flow cytometry viste at proteinene ble produsert og uttrykt på overflaten til *L. plantarum*. Caco-2 celler ble brukt som en *in vitro* modell for å undersøke proteinenes evne til å fremme opptak, og alle de rekombinante *L. plantarum* stammene viste økt opptak sammenliknet med stammen som ikke uttrykte protein. Dette forsøket kunne imidlertid ikke skille mellom bakterier som var tatt opp og bakterier som kun var bundet til overflaten av Caco-2 cellene. Ytterligere *in vitro* studier med M-celleliknende celler, viste tegn til at FimH- og Invasin fremmet transcytose. Ett av to individuelle forsøk viste lovende resultater for flere stammer, spesielt for den som uttrykte Invasin med et N-terminalt lipoprotein-anker.

Arbeidet beskrevet i denne oppgaven gir lovende indikasjoner om at FimH og Invasin er involvert i å fremme transcytose av *L. plantarum* via M-celler. Det synes derfor rimelig å forfølge denne strategien videre med ytterligere analyser for å vurdere effektiviteten av transcytoseprosessen og for å verifisere muligheten av å levere vaksineantigener til Peyerske flekker.

Abbreviations

APC	Antigen Presenting Cell
BCG	Bacillus Calmette-Guérin
BSA	Bovine Serum Albumin
CLSM	Confocal Laser Scanning Microscopy
DC	Dendritic Cell
dsDNA	double stranded DNA
dNTP	deoxyriboNucleotide TriPhosphate
DTT	DiThioTreitol
EPEC	Enteropathogenic *Escherichia coli*
FITC	Fluorescein IsoThioCyanate
GRAS	Generally Regarded As Safe
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
IP	Inducer Peptide
LAB	Lactic Acid Bacteria
LDS	Lithium Dodecyl Sulphate
M-cell	Microfold Cell
MHC	Major Histocompatibility Complex
OD	Optical Density
PAGE	PolyAcrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
SEM	Scanning Electron Microscopy
SPase	Signal Peptidase
TEER	Trans Epithelial Electrical Resistance
TEM	Transmission Electron Microscopy

Contents

1   Introduction

Facilitated by the increasing globalization and traveling of people and animals, infectious diseases spread fast and easily all over the world. Infectious diseases account for one third of the deaths occurring worldwide each year, and the most cost-effective measure for preventing such diseases, is vaccination. Several effective vaccines already exist, but there is still an urgent need for developing new and improved vaccines for various diseases, like tuberculosis and HIV (Medina and Guzman, 2000). Considerable effort has been invested in the search for alternatives to existing injection vaccines, and mucosal vaccines have long been in the spotlight.

Since the early 1990s, scientists have investigated the potential of lactic acid bacteria (LAB) as delivery vectors of antigens in the development of mucosal vaccines (Wyszynska et al., 2015). Many LAB are natural inhabitants of the gastrointestinal tract, and they are Generally Regarded As Safe (GRAS). Some LAB are also thought to be probiotic, meaning that adequate amounts of the bacteria provide health beneficial effects on the host. This, together with their ability to survive the transit through the stomach, and to adhere to the surface of intestinal epithelium, make them promising candidates as delivery vectors for orally delivered mucosal vaccines (Wyszynska et al., 2015).

Oral delivery of LAB targeting antigens to mucosal dendritic cells has shown promising results to induce immunity (Mohamadzadeh et al., 2009). However, other delivery targets are also considered to enhance the efficacy of mucosal vaccines (Kim and Jang, 2014). This thesis describes the development of such an alternative delivery route, where *L. plantarum* is targeted to M-cells in the gastrointestinal tract through expression of M-cell binding proteins. Subsequent transcytosis by M-cells will then expose the bacterium to high concentrations of immune cells in the so-called Peyer's patches.

1.1   Lactic Acid Bacteria

Lactic acid bacteria (LAB) are Gram-positive bacteria with a low GC-content, and are characterized by their ability to ferment hexose sugars, forming lactic acid as the major product (Daniel et al., 2011). They are typically non-spore forming, acid tolerant and aerotolerant, though most prefer an anaerobic environment (Axelsson, 2004). LAB are also auxotrophic, meaning that they lack certain genes in various biosynthetic pathways, and as a consequence, they require environments rich in sugars, amino acids, nucleic acid derivatives, minerals and vitamins (Hayek and

Ibrahim, 2013). Due to their nutritional demands, LAB naturally grow in several food products, such as milk, meat and vegetables. Some are also found in the normal flora of mammals, inhabiting the gastrointestinal tract and the vagina (Daniel et al., 2011). For thousands of years, LAB have been used as a food preservative because of their ability to produce lactic acid. The low pH, together with antimicrobial compounds such as bacteriocins produced by the LAB, prevent growth of other microorganisms (Yang et al., 2012). Some strains, especially those that are able to colonize the intestinal mucus layer, are also considered to have probiotic effects when consumed, by maintaining homeostasis in the intestinal microflora. They are further found to modulate the immune system, affecting both mucosal and systemic immune responses, and they can reduce the risk of allergic reactions (Kim and Jang, 2014; Wyszynska et al., 2015). Their probiotic effects, tolerance to acidic environments and GRAS status have made LAB belonging to the *Lactobacillus* genus a promising tool for oral delivery of antigens (Wyszynska et al., 2015).

1.1.1   *Lactobacillus plantarum* WCFS1

Lactobacillus plantarum is found in a variety of environmental niches, and is one of few *Lactobacillus* species that are both involved in food and plant fermentations and are natural inhabitants of the human gastrointestinal tract. *L. plantarum* has been shown to survive the transit through the stomach, and once in the gastrointestinal tract, it can persist for up to seven days (Vries et al., 2006; Vesa et al., 2000). The complete genome of *L. plantarum* WCFS1 was sequenced in 2003 (Kleerebezem et al., 2003), and is today known as one of the largest among *Lactobacillus* spp. The versatility of *L. plantarum* can be explained from its large number of genes encoding sugar transport systems, which enables the bacterium to utilize a large variety of carbon sources. The genome sequence also shows that *L. plantarum* expresses a diverse collection of secreted and surface located proteins, which gives the bacterium potential to associate and exploit various surfaces and substrates. Many surface proteins resemble proteins that bind mucus and fibronectin as well as proteins that are involved in intercellular adhesion (Kleerebezem et al., 2003). Such adherence factors are generally desired for probiotic strains, as they are thought to promote the gut residence time of the bacteria, and also prevent colonization of pathogenic bacteria through competitive exclusion (Lebeer et al., 2008).

L. plantarum's characteristics and abilities have drawn considerable attention to this bacterial species as a possible delivery vector in the development of oral vaccines (Vesa et al., 2000; Diep et al., 2009). Several systems for gene expression of proteins in LAB have been developed, providing important tools for production, secretion

and anchoring of antigens or adjuvant proteins used to develop oral vaccines.

1.2   Inducible Gene Expression Using the pSIP-system

Gene expression systems can be used for cloning of target genes, and provides over-production of desired proteins. Such expression systems are mainly divided into constitutive- and inducible systems. Constitutive systems utilize strong promoters to obtain high production of the proteins of interest, and several such systems have been used to express proteins in *Lactococcus* and *Lactobacillus* (Brurberg et al., 1994; Kahala and Palva, 1999; Duong et al., 2011; Sasikumar et al., 2014). In cases where the protein of interest somehow interfere with the metabolism, or proves to be toxic to the host, inducible expression systems can be better suited. Ideally, these systems should have a low basal production, only producing proteins when induced. Several inducible systems exist, regulating gene expression by additives such as lactose or xylose, or by changing parameters like pH or temperature (Tauer et al., 2014). The NIsin Controlled Expression system (NICE) is the best known gene expression system in LAB (Mierau and Kleerebezem, 2005). Here, the bacteriocin nisin is used to activate a histidine-protein kinase, which is autophosphorylated, and further phosphorylates a response regulator. The response regulator initiates transcription by activating the nisA or nisF promoter. Genes of interest can be expressed by inserting the sequences downstream of one of these promoters (Kuipers et al., 1998). The system was originally constructed for expression in *Lactococcus lactis*, and both a two-plasmid- and a one-plasmid system were developed for other lactic acid bacteria (Mierau and Kleerebezem, 2005). The NICE system has many advantages, but considerable basal activity has been observed in *L. plantarum* and *Lactobacillus sakei* (Pavan et al., 2000; Sørvig et al., 2003). As a consequence, a strictly regulated expression system, called the pSIP vector expression system, was developed for *Lactobacillus* (Sørvig et al., 2003, 2005; Mathiesen et al., 2004).

The pSIP system is a one-plasmid expression system, which utilizes the regulatory promoters and genes involved in the production of the class II bacteriocins sakacin A and sakacin P. The bacteriocin promoter, e.g. the sakacin P promoter (P_{SppA}), controls the transcription of the gene of interest, and is regulated by an operon consisting of regulatory genes. These genes are under the control of the inducible P_{SppIP} promoter. In the natural bacteriocin-producing bacterium, the first gene in the regulatory operon encodes a peptide pheromone, also referred to as an induction peptide (IP). The peptide produced from this gene, activates the product of the following gene encoding a histidine-protein kinase (HK), which is located in the membrane. Through phosphorylations, the HK activates a response regulator (RR)

produced by the last gene in the operon. The activated RR binds and further activates the inducible promoters P_{SppA} and P_{SppIP}, initiating production of the proteins of interest and multiplying the strength of the induction signal, respectively. In the pSIP vectors (Figure 1.1), the IP-encoding gene has been deleted, and therefore only externally added IP can initiate transcription. In this way, the production of genes of interest can be strictly regulated (Sørvig et al., 2003), and the system does not need to contain the export machinery needed to secrete the IP.

The pSIP vectors are built up of cassettes, and all essential elements can easily be exchanged by restriction enzyme digestion (Figure 1.1) (Sørvig et al., 2005). The system also has replication determinants both for *E. coli* and lactobacilli, which makes it possible to construct the plasmids in *E. coli* before transforming them into *L. plantarum* (Sørvig et al., 2003). The plasmids constructed in this study are all derivatives of the pSIP401 vector shown in Figure 1.1.
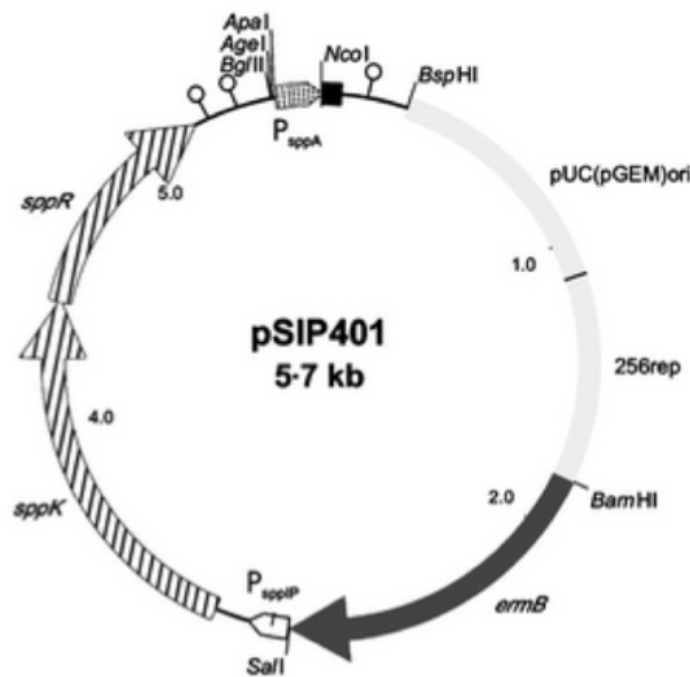
Figure 1.1. *The pSIP401 Expression Vector. Light-grey regions: Replication determinants. Dark-grey region: Erythromycin resistance marker. White region: Inducible P_{SppIP} promoter. Vertically hatched regions: Histidine protein kinase and response regulator genes. Dotted region: inducible P_{SppA} promoter. Black box: Multiple cloning site. Lollipop structures: Transcriptional terminator. Target genes are cloned downstream of the P_{SppA} promoter. The figure is taken from Sørvig et al. (2005)*

The pSIP vectors were first constructed for intracellular production in *Lactobacillus*, giving high expression levels when induced and low basal production when not induced (Sørvig et al., 2005). The system was then further developed for secretion of

proteins, by incorporating an exchangeable cassette consisting of signal peptides and target genes, into the pSIP401 vector, downstream of the P_{sppA} promoter (Mathiesen et al., 2008). Furthermore, a *SalI* restriction site was removed from a nonessential location (upstream of the P_{sppIP} promoter), so that a unique *SalI* site could be used in the secretion cassette. In 2009, Mathiesen et al. (2009) tested 76 signal peptides for secretion of NucA as a reporter protein through the Sec-pathway in *L. plantarum* (described in section 1.3 below). Of the 76 signal peptides analysed, 82% were functional, though the levels of extracellular protein detected varied by three orders of magnitude.

The pSIP system was further developed for C-terminal anchoring of a cancer antigen (OFA) through a cell wall anchor (described in section 1.3.3) (Fredriksen et al., 2010). The OFA sequence and a C-terminal *MluI* restriction site were ligated into a pSIP401 derivative with a signal sequence called Lp0373. Further, three versions of a cell wall anchor with a C-terminal *HindIII* restriction site were inserted (Figure 1.2a). The three anchors all consisted of an LPxTG-like motif and a proline-rich motif, but the sequence lengths differed. In 2012, pSIP401 derivatives with cassettes for N-terminal anchoring of proteins were constructed (Figure 1.2b) (Fredriksen et al., 2012). The two lipo-anchors (Lp1261 and Lp1452), the transmembrane anchor (Lp1568) and the LysM domain anchor (Lp3014) tested in the study, all led to surface display of Invasin from *Yersinia pseudotuberculosis*. Several studies around the world have successfully applied the pSIP system for recombinant expression of for example food relevant enzymes and enzymes or antigens of medical relevance in lactobacilli (Straume et al., 2006; Nguyen et al., 2011; Anbazhagan et al., 2013; Morais et al., 2013).
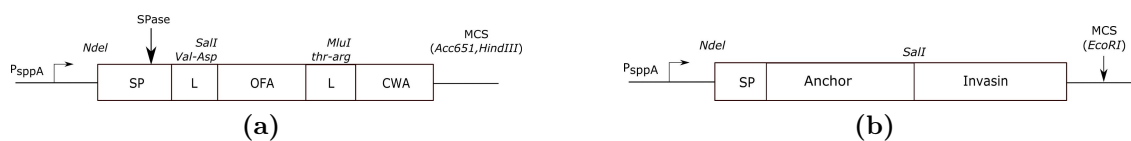
Figure 1.2. *Development of pSIP Expression Vectors for Surface Display of Proteins in L. plantarum. Panel (a) shows expression cassettes for secretion and C-terminal cell wall anchoring of OFA. Panel (b) shows expression cassettes for secretion and N-terminal anchoring of Invasin. The figures are taken from Fredriksen et al. (2010, 2012), respectively. The SalI and MluI restriction sites for digestion of OFA, and the SalI and EcoRI restriction sites for digestion of Invasin, are indicated.*

1.3   Anchoring of Proteins in Gram Positive Bacteria

Gram positive bacteria are well-suited host organisms for production and secretion of proteins, because they only have one cell membrane (Caspers et al., 2010). The proteins are translocated over the cytoplasmic membrane by seven different main secretion mechanisms (Kleerebezem et al., 2010). The major secretion pathway is the Sec pathway, which involves N-terminal signal peptides and the protein complex Sec-translocase, which translocates unfolded proteins (Tjalsma et al., 2000; Driessen and Nouwen, 2008). After secretion by the Sec-translocase, proteins can be anchored to the surface of Gram-positive bacteria by mainly four different types of anchors: Transmembrane anchors, lipo-anchors, LPxTG anchors and noncovalent cell wall anchors (Figure 1.3).
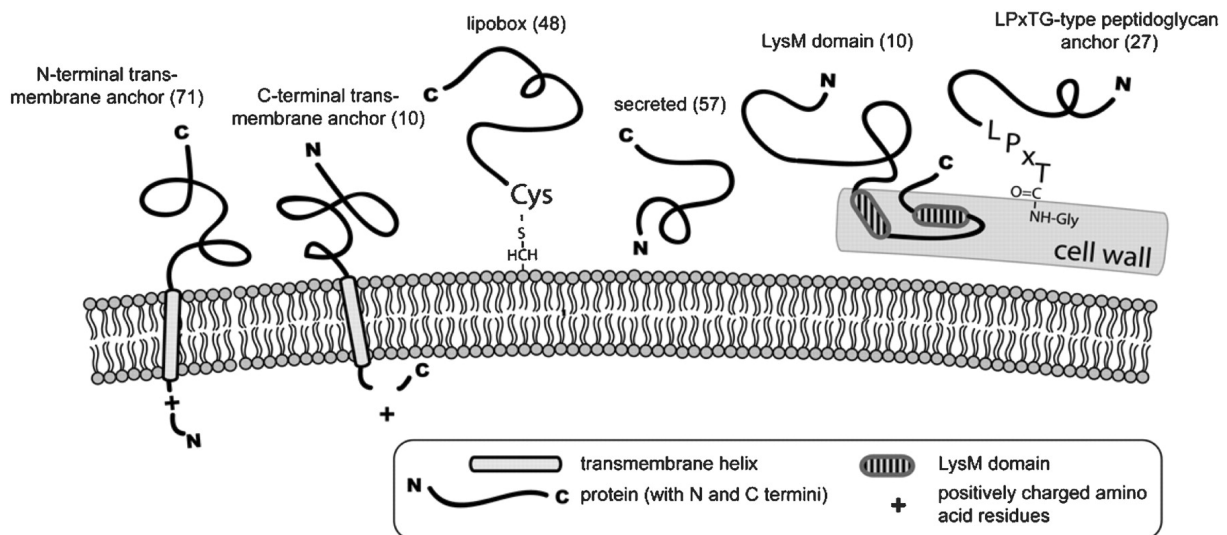
Figure 1.3. *Protein Anchors in L. plantarum. An N-terminal- and a C-terminal transmembrane anchor are shown, of which the N-terminal (which corresponds to a signal peptide lacking a cleavage site) is the most common. The third anchor from the left illustrates a lipoprotein anchor, which is anchored to the cell membrane through a lipobox motif. The two anchors shown to the right are both cell wall anchors. One anchors proteins non-covalently through domains with affinity to the cell wall, such as LysM-domains. The other anchors covalently through an LPxTG peptidoglycan anchor. The numbers of predicted proteins of the different types in L. plantarum are indicated in parentheses. See text for more details. The figure is taken from Boekhorst et al. (2006).*

1.3.1   Transmembrane Anchors

Proteins secreted by the Sec-translocase pathway have an N-terminal signal peptide consisting of three different domains, called N, H and C. After or during the translocation by the Sec-translocase, the signal peptide is cleaved off. This however,

only happens if a cleavage motif targeted by an SPase (Type-I or Type II) in the C region is present. These cleavage sites are not always present, and in such cases, the protein will remain N-terminally anchored to the cell membrane. In *Lactobacillus*, proteins anchored in this way constitute the largest fraction of membrane anchored proteins, and these proteins are mainly involved in extracellular processes. Some of the proteins that do contain a cleavage site leading to secretion, can still be anchored to the cytoplasmic membrane through a C-terminal transmembrane domain. The functions of C-terminally anchored proteins are unknown (Kleerebezem et al., 2010).

1.3.2   Lipoprotein-Anchors

Lipoproteins, predicted to be the second most common class of membrane anchored proteins in *Lactobacillus*, are also translocated by the Sec pathway (Kleerebezem et al., 2010). The signal peptides however, differ from those for the transmembrane proteins, as they contain a shorter hydrophobic H-domain and a C-domain with a lipobox motif [L-(A/S)-(A/G)-C]. The lipobox motif contains a conserved Cys-residue, which is modified by a diacylglyceryl transferase, which transfers a diacylglyceryl moiety to the Cys-residue. N-terminally of the Cys-residue, a Type II SPase cleaves the protein, and the mature protein becomes covalently anchored to the cell membrane through a thioether linkage, with its C-terminal part protruding into the environment. Most lipoproteins predicted to be encoded by *Lactobacillus*, are involved in ATP-binding cassette (ABC) transporters, where they function as substrate-binding proteins. Other tasks include adhesion and antibiotic resistance, as well as protein secretion, folding and translocation (Hutchings et al., 2009; Kleerebezem et al., 2010).

1.3.3   LPxTG Anchors

As the name implies, LPxTG-anchored proteins have a conserved LPxTG- or LPxTG-like motif. This motif is located C-terminally, and anchors the protein covalently to the cell wall (Boekhorst et al., 2005). The LPxTG-like proteins have an N-terminal signal peptide sequence that directs the secretion, and contains a cleavage motif for Type I SPase, which cleaves the signal peptide after translocation through the Sec pathway. Although the protein is cleaved, it stays arrested in the membrane due to a C-terminal stretch of hydrophobic residues and a positively charged tail, following the LPxTG motif. At this point, the LPxTG motif is recognized by a membrane-associated transpeptidase called sortase, which cleaves the motif between the T and G residues. Further, the sortase covalently attaches the resulting threonine carboxyl group to the peptidoglycan (Kleerebezem et al., 2010). LPxTG-like motifs anchor

proteins C-terminally, meaning that the N-terminal part of the protein protrudes into the extracellular environment. These anchors have been used in several studies of surface display of different proteins (Kajikawa et al., 2011; Liu et al., 2008; Fredriksen et al., 2010). For example, in a study by Bermúdez-Humarán et al. (2004), LPxTG anchored E7 oncoprotein in *Lactococcus lactis* proved to elicit an immune response in mice. According to Fredriksen et al. (2012), sortase-mediated anchoring of proteins has been extensively explored compared to other types of anchoring.

1.3.4   Non-Covalent Cell Wall Anchors

Non-covalent cell wall anchors are anchors that bind through various binding domains, such as lysine motif (LysM) domains, choline-binding domains, SLH domains and SH3 domains. The LysM domain is a widely distributed domain for anchoring of proteins to peptidoglycan and chitin, and is assumed to recognize N-acetylglucosamine moieties (Buist et al., 2008). The majority of proteins anchored through a LysM domain in *Lactobacillus* are predicted to perform enzymatic functions related to the cell wall (Kleerebezem et al., 2010).

1.4   The Human Mucosal Immune System

Mucosal layers line organ cavities such as the respiratory-, urogenital- and gastrointestinal tracts of the human body. The layers are made up of a single layer of epithelial cells, which is renewed every 2-3 days. Among the epithelial cells there are goblet cells that produce mucin, the major component in the viscous mucus covering the epithelial layer (Figure 1.4). The mucosal layer of the gastrointestinal tract in humans has a surface area of $\sim$200 m^2, enabling absorption of large amounts of nutrients (Hooper et al., 2012). The intestinal mucosae functions as a selectively permeable barrier, protecting the internal environment from the sometimes hostile external environment (Turner, 2009).
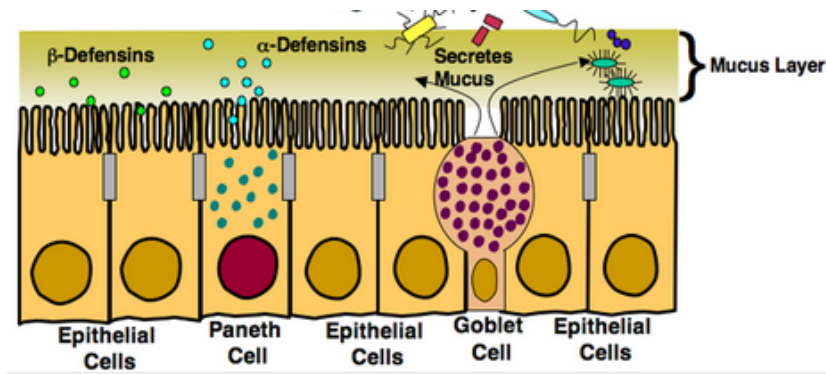
Figure 1.4. *Intestinal Epithelium Cells. The figure illustrates cells in the mucosal layer of the gastrointestinal tract, such as epithelial cells like enterocytes, paneth cells, which produce anti-microbial peptides called defensins, and mucin-producing goblet cells. The figure is taken from McGhee and Fujihashi (2012).*

The external environment on the luminal side of the mucosal layer, consists of complex communities of 100 trillions of microorganisms from more than 1000 different species, which are commonly called the intestinal microbiota. The microbiota is dominated by bacteria, and a certain set of colonizers is conserved in all humans. Apart from this so-called core gut microbiota, the composition of bacteria and archaea varies highly between individuals (Tremaroli and Backhed, 2012), and research claims that diet plays an important role in affecting this composition in humans and other mammals (Wu et al., 2011; Russell et al., 2011; De Filippo et al., 2010). One example is a study by Walker et al. (2011), showing a strong increase in the relative abundance of the starch degrading bacteria *Ruminococcus bromii* and *Eubacterium rectale* in 14 overweight men following a diet rich in resistant starch. The microbiota's ability to degrade otherwise indigestible food, provides the body with important nutrients, such as short-chain fatty acids, vitamins and amino acids, that it cannot produce itself (Gerritsen et al., 2011). In addition to the symbiotic microorganisms present, various pathogens can also enter into the lumen, and utilize the mucosal layers to invade the internal tissues. The need to protect the host from harmful pathogens, and at the same time contain the beneficial microbiota, has driven the evolution of the immune system to elicit immune responses and induce tolerance as necessary (Hooper et al., 2012).

The mucus coating of the epithelial cells limits the interaction between microbes in the intestinal lumen and the mucosal epithelial cells. In addition, some specialized epithelial cells called Paneth cells (Figure 1.4), express antimicrobial proteins, mostly α-defensins, which kill bacteria, fungi and some viruses by permeabilizing the cell membrane (Hooper et al., 2012; Ganz, 2003; Schroeder et al., 2014). Underneath the epithelial layer, the mucosal tissue known as the lamina propria (Figure

1.5), hosts B cells, T cells, and so-called antigen presenting cells (APCs) such as dendritic cells and macrophages (Wells et al., 2011).

Dendritic cells (DC) located in the lamina propria, sample microbes and antigens by extending dendrites through the epithelial cell layer, into the lumen. Derived peptides from the phagocytosed antigens are then presented by DCs to naive T cells in the mesenteric lymph nodes via major histocompatibility complex (MHC) Class I or II. Microbes and antigens can also be sampled by specialized atypical epithelial cells called microfold cells (M-cells) (Figure 1.5) (Mabbott et al., 2013). In contrast to other epithelial cells in the mucosal membrane, M-cells are not covered with mucus; they have flattened apical membrane surfaces and express specific markers. The specific markers or receptors on the apical side (facing the lumen, see Figure 1.5) of M-cells bind antigens and microorganisms from the intestinal lumen and deliver them to the underlying Peyer's patches by a process called transcytosis (Azizi et al., 2010). The Peyer's patches are organized lymphoid structures containing a large number of immune cells, including B cells, DCs, macrophages and T-cells. The Peyer's patches are further linked to mesenteric lymph nodes through lymphatic vessels (Jung et al., 2010). When antigens reach Peyer's patches, DCs phagocytose them and directly present derived peptides to naive T cells.

Antigens presented by MHC class I, stimulate activation of cytotoxic CD8+ T cells which kill infected host cells, and antigens presented by MHC class II stimulate activation of CD4+ T cells. CD4+ T cells, also known as T helper (T_H) cells, can differentiate into either T_H1 or T_H2 cells which produce different kinds of signal molecules, called cytokines. T_H1 cells mediate cellular immunity and inflammation, and T_H2 cells prime naive B cells and help regulating the antibody-mediated immune response (Lea, 2006). Activated B and T cells migrate through the lymph and are transported through the bloodstream to specific mucosal sites. Here, they differentiate into effector cells and memory cells with specific receptors for the antigen, ensuring that a new infection with the same antigen will induce a rapid immune response (Holmgren and Czerkinsky, 2005).
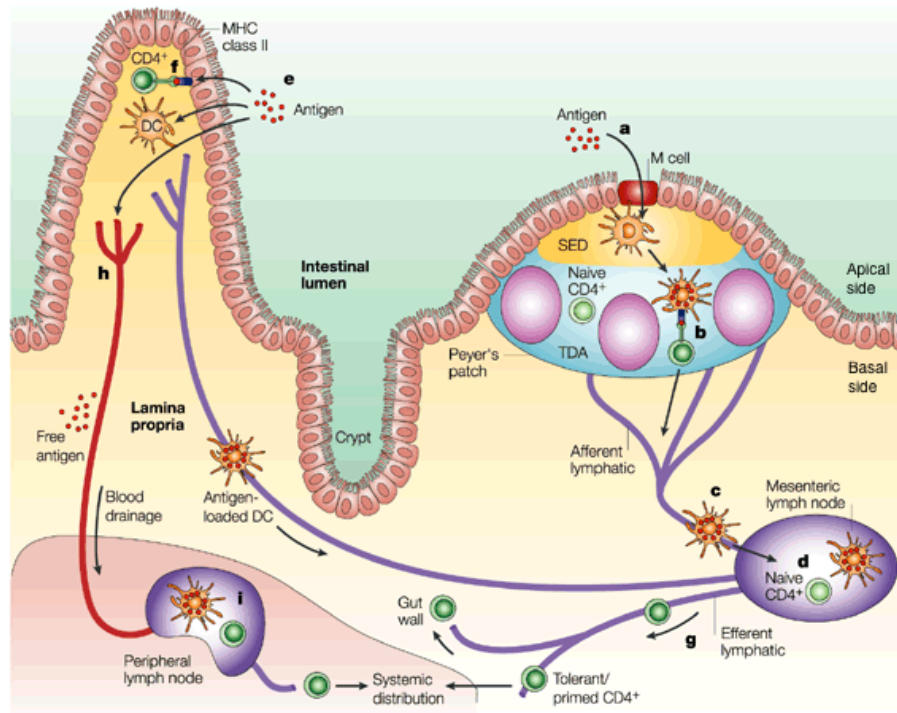
Figure 1.5. *The Mucosal Surface of the Gastrointestinal Tract. Antigens enter through M-cells (a), and are transcytosed to dendritic cells in the Peyer's patches (b). Antigen-loaded dendritic cells from Peyer's patches can be transported through lymphatic vessels (c) to mesenteric lymph nodes, where they can stimulate T-cells (d). Dendritic cells can also sample antigens from the lamina propria (e), and these antigens are transported directly to mesenteric lymph nodes. Antigen-responsive CD4+ T cells leave mesenteric lymph nodes (g) and end up at different mucosal sites. The apical- and basal sides of the cells are indicated to the right. The figure is taken from Mowat (2003).*

Many infections are caused by pathogens entering the body through mucosal layers, and in recent years, an interest in developing mucosal vaccines to prevent these infections has emerged (Holmgren and Czerkinsky, 2005). Different routes to administer such vaccines, including genital-, rectal-, inhalation-, sublingual-, nasal- and oral delivery, have been investigated. Nasal- and oral delivery have shown the most promising results when it comes to initiating immune responses. As many pathogens enter the body through the epithelial layer of the gastrointestinal tract, oral delivery of vaccine antigens through the same route is considered a favourable option (Azizi et al., 2010; Kim and Jang, 2014).

Various pathogens exploit M-cells and Payer's patches to enter the host organism, by expressing certain proteins that bind specific M-cell receptors. Examples of such pathogenic bacteria are the diarrhea causing enteropathogenic *Escherichia coli*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* which both can cause ileocolitis, the most common type of Crohn's disease, and *Salmonella typhimurium* which

causes typhoid fever in humans. (Jung et al., 2010; Kidgell et al., 2002). Using the receptor-specific proteins from such pathogens to target M-cells, has gained increasing attention in the development of efficient oral mucosal vaccines (Azizi et al., 2010; Kim and Jang, 2014; Wang et al., 2014; Davitt and Lavelle, 2015) and is also addressed in this study.

1.4.1   M-Cell Receptor Specific Proteins

The protein that causes transcytosis of enteropathogenic *E. coli* and *S. typhimurium* across M-cells, is called FimH, which is a subunit located on the fimbrial tip of Type I fimbria (Yu and Lowe, 2009). FimH does not specifically bind M-cells, but generally glycoproteins containing mannose, and a typical type 1 fimbriated bacterium has 200-500 fimbriae on its surface, enhancing the bacteria's chance of colonizing the mucosae (Schembri et al., 2001). Although enteropathogenic *E. coli* FimH and *S. typhimurium* FimH only share 15% primary structure identity, the tertiary structures are highly similar, and they both bind glycoprotein 2 (GP2) receptors located on M-cells (Hase et al., 2009; Kisiela et al., 2011; Kim and Jang, 2014). Hase et al. (2009) showed that *S. typhimurium* expressing a fragment of tetanus toxoid, was transcytosed to Peyer's patches where it induced a significant antigen-specific antibody production in mice, while a FimH-deficient mutant of the same strain induced a lower response, as it was probably not transcytosed to the Peyer's patches. GP2-deficient mice also gave a lower response, indicating that FimH binding to GP2-receptors mediate transcytosis to Peyer's patches, a property that can be utilized in the delivery of mucosal vaccines. The adhesive domain of FimH is located N-terminally (Schembri et al., 2001).

Invasin is the primary invasion factor of *Y. enterocolitica*, and the protein has high affinity to a subset of β_1-integrin receptors found on the apical side of M-cells. On epithelial cells, these receptors are only located on the basal side (see Figure 1.5), which means that only β_1-integrin on M-cells are available for binding on the luminal side. Because of this distribution of β_1-integrin receptors, Invasin can specifically target M-cells in the gastrointestinal tract (Uliczka et al., 2011). Invasin binds the receptors with a hundred-fold higher affinity than the natural ligand of β_1-integrins, fibronectin. Invasin is a non-fimbrial adhesin, consisting of four domains in a rod-like structure located in the outer membrane, and it binds β_1-integrins through its C-terminal domain (Dersch and Isberg, 2000; Souza dos Reis, 2010).

1.5   *In Vitro* Models of M-Cells

The human intestinal Caco-2 cell line is commonly used as an *in vitro* model of the intestinal epithelium to, for example, screen for drug candidates. Caco-2 cells are continuous lines of heterogeneous epithelial colorectal adenocarcinoma cells, and when grown over time on semi permeable filters, they polarize and spontaneously differentiate into monolayers with characteristics very similar to the small intestinal epithelium, such as the formation of microvilli (Lakshmana Rao and Sankar, 2009). This spontaneous differentiation occurs after the cell layer has reached confluence (5-7 days), and can be assessed by measuring the levels of a well characterized marker for the functional differentiation of intestinal epithelial cells; the hydrolase sucrase-isomaltase (Coconnier et al., 1994). When the Caco-2 cells differentiate, tight junctions between the polarized cells are formed, resulting in two clearly distinguishable apical- (upper) and basal-(lower) domains. Research has shown that the receptors for Invasin (β_1-integrins) are present only on the basal side of both polarized, differentiated Caco-2 cells and epithelial cells in the intestine (Liévin-Le Moal and Servin, 2013; Uliczka et al., 2011). Non-polarized, undifferentiated Caco-2 cells that have not had time to polarize and obtain distinct apical and basal domains, express the receptors in a different pattern where the receptors are available for binding. Non-polarized, undifferentiated Caco-2 cells can therefore be used as a model for internalization of bacteria expressing Invasin. This was demonstrated in a study by Critchley et al. (2004), where recombinant *E. coli*, expressing Invasin from *Y. pseudotuberculosis*, was incubated with such non-polarized, undifferentiated Caco-2 cells. After 48 hours, *E. coli* was internalized into 80% of the Caco-2 cells. In contrast to the β_1-integrins, the GP2 receptor, which binds FimH proteins, occur only in modest amounts on Caco-2 cells (Werner et al., 2012).

As M-cells differ from regular epithelial cells in the intestinal barrier, Caco-2 cells alone do not provide an optimal model. Therefore, a different model needs to be used to analyse *in vitro* transcytosis of bacteria across M-cells. Such a model was developed by Kernéis et al. (1997) who found a method to grow cells with M-cell characteristics by co-culturing polarized and differentiated Caco-2 cells with lymphocytes from Peyer's patches (PP lymphocytes). When the lymphocytes were present, the organized apical microvilli of the Caco-2 cells, which are typical of epithelial cells, became disorganized, and the cells obtained a more flattened surface characteristic of M-cells. These changes applied for all the Caco-2 cells in the monolayer, and not only for the ones that were in physical contact with the PP lymphocytes, implying that soluble factors were involved (Kernéis et al., 1997). The M-cell-like character of the cells was demonstrated by showing that they were able to transcytose *Vibrio*

cholerae from the apical surface to the basal compartment with a 100-fold increase compared to Caco-2 cell monocultures. The translocation was also shown to depend on temperature, as the translocation only occurred at 37°C, and not at 4°C. The PP lymphocytes that induced the conversion of Caco-2 cells comprised both B- and T cells. To investigate whether B- and T cells contributed equally to the conversion of Caco-2 cells into M-like-cells, further experiments using one lymphocyte type at time in the co-cultures were performed. Raji cells exhibiting B cell markers were found to significantly trigger the transcytotic activity, while Jurkat cells with T cell markers, only did so to a very low extent (Kernéis et al., 1997). The mechanism for the formation of M-cell-like cells is not fully understood (Kernéis et al., 1997). However, Lügering et al. (2004) suggested that a specific microenvironment, including B cells, T cell receptor $(TCR)_{\alpha\beta}$- and CD4 positive T cells and interleukin-4, is likely to induce M-cell differentiation from epithelial cells.

A simplified version of this *in vitro* M-cell model was developed by Gullberg et al. (2000). This model (Figure 1.6) includes a co-culture of polarized and differentiated Caco-2 cells with Raji B cells, but in this case the Raji B cells were physically separated from the Caco-2 cells. In this approach, the alteration of microvilli was found to be nonuniform, which indicated that only a subpopulation of Caco-2 cells was converted into M-cell-like cells. Still, latex microparticles were translocated with a 40-fold increase compared to Caco-2 monocultures (Gullberg et al., 2000). Gullberg et al. (2000) also identified some M-cell markers on the M-cell-like cells, like upregulation of Sialyl Lewis A antigen and downregulation of alkaline phosphatase. The expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule was also altered. The model developed by Gullberg et al. (2000) has been used in several studies investigating transcytosis of various bacteria through M-cells, and all studies showed increased transcytosis through the M-cell-like cells compared to Caco-2 monocultures (Maresca et al., 2007; Martinez-Argudo and Jepson, 2008; Paixão et al., 2009; Finn et al., 2014). Martinez-Argudo and Jepson (2008) however, observed significant variability in the extent of transcytosis of *Salmonella enterica*, which may be taken to confirm the observation by Gullberg et al. (2000) of nonuniform conversion of Caco-2 cells. According to Des Rieux et al. (2007), the M-cell-like cell model can be further improved by inverting the Caco-2 cells prior to the co-incubation with Raji B cells (Figure 1.7). The study showed that transcytosis of nanoparticles was 50-fold higher in inverted co-cultures compared to Caco-2 monocultures, while only a 3-fold increase was seen in the normal co-cultures compared to Caco-2 monocultures. Des Rieux et al. (2007) also found that close contact between the Caco-2 cells and the Raji B cells seemed to induce a more functional and more reproducible *in vitro* model.

Importantly, M-cell-like cells obtained by co-culturing polarized and differentiated Caco-2 cells and Raji B cells have been shown to express both the GP2- and β_1-integrin receptors (Kim et al., 2010; Hamzaoui et al., 2004).

(a) *14-16 days* **(b)** *3-6 days* **(c)** *45-60 minutes*
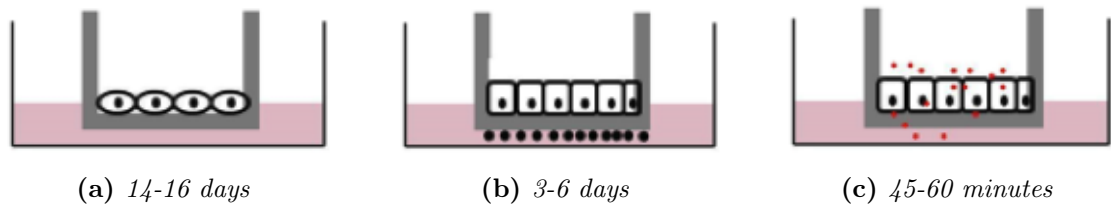
Figure 1.6. ***Schematic Drawing of the Procedure for Transcytosis by the M-Cell Model Developed by Gullberg et al. (2000).*** *Caco-2 cells are seeded on filter inserts, and incubated at 37°C for 14-16 days in order to polarize and differentiate (a). Raji B cells are added to the basal compartment to induce conversion into M-cell-like cells (b), before bacteria are added to the apical side (c). Transcytosed bacterial cells in the basal compartment are counted relative to the amount of added bacterial cells. The timescale of the procedure is indicated. The figure was made by Katarzyna Kuczkowska, IKBM.*
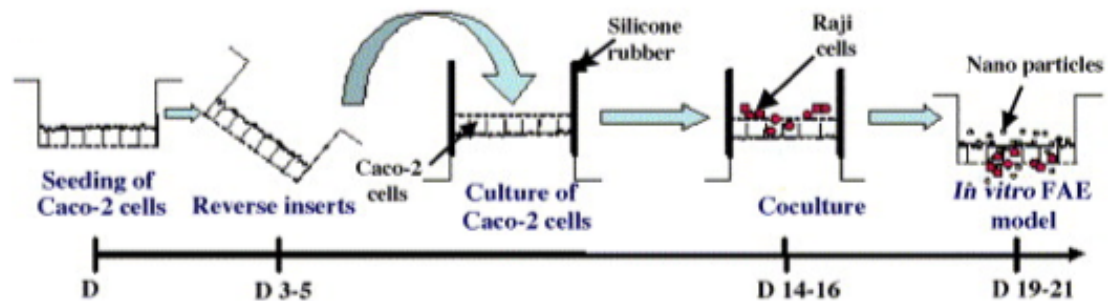
Figure 1.7. ***Schematic Drawing of the Inverted Coculture Model Developed by Des Rieux et al. (2007).*** *Caco-2 cells are seeded on filter inserts and incubated at 37°C. After 3-5 days, the filter inserts are inverted and further incubated for a total of 14-16 days. Raji B cells are then added to the basal side of the Caco-2 cells, providing physical contact between the two cell types. The co-culture is incubated for 3-6 days, before the filter inserts are turned back to their normal orientation, and nanoparticles (in this case) are added. Transcytosed nanoparticles in the basal compartment are counted relative to the amount added. The figure is taken from Des Rieux et al. (2007).*

1.6 Goals of This Study

This study is part of a larger project, where the long-term goal is to develop oral mucosal vaccines against tuberculosis, using lactic acid bacteria as a vector to deliver antigens in the gastrointestinal tract. The vaccine should ideally be more effective than the existing BCG vaccine, easier to administer and cost saving. Previous work has mostly focused on expressing antigens and on targeting antigen expressing cells to dendritic cells in the lamina propria, with promising results (Tjåland, 2011; Øverland, 2013). In an attempt to enhance the immune response, the antigens could be specifically transferred to Peyer's patches, where APCs and lymphocytes are found in high concentrations (Kim and Jang, 2014; Wang et al., 2014). The goal of the present study was therefore to target *Lactobacillus plantarum* to Peyer's patches via transcytosis by M-cells. The experimental approach had the following steps: (1) construct vectors for production of proteins with specific affinity for receptors found on M-cells, using different anchors for surface display, (2) analyse the production and correct surface display of these proteins (FimH from *S. typhimurium* and enteropathogenic *E. coli*, and Invasin from *Y. enterocolitica*) in *L. plantarum* and (3) compare the different proteins' ability to promote uptake of *L. plantarum* in *in vitro* models of M-cells.

2 Materials

2.1 Laboratory Equipment

Laboratory Equipment	Supplier
CL-Xposure^{TM} Film	Thermo Scientific
Corex Tubes	Corning Inc.
Cryovials, 1.5 ml	Sarstedt
Cell Culture Inserts, Transparent	Becton-Dickinson
PET Membrane, 3 µm pore size	
Disposable Cuvettes, 1.5 ml	Brand
Electroporation Cuvettes, Gene Pulser®, 0.2 cm	Bio-Rad
Eppendorf Tubes, 1.5 ml	Axygen
Falcon 2059 Polypropylene Round Bottom Tubes	Becton Dickinson
FastPrep® Tubes and Lids	Fisher Scientific
Glass Beads	Sigma
Macs CD14 MicroBeads (human)	Miltenyi Biotec
Macs Column	Miltenyi Biotec
Macs Separator	Miltenyi Biotec
Microwell plates, 96 V-bottom well, without Lid	Thermo Fisher Scientific
Nunc Tubes, 15 ml and 50 ml	Greiner Bio-One
Nunc UpCell Surface 24 MultiDish, Temperature-Responsive Cell Culture Surface	Thermo Scientific
PCR Tubes, 0.2 ml	Axygen
Reagent and Centrifuge Tubes, 13 ml	Sarstedt
Serological Pipettes, 5 ml, 10 ml, 25 ml	Sarstedt
Sterile Filters	
0.2 µm Pore Size	Sarstedt
0.22 µm Pore Size	Millex® GP
Syringes, 10-50 ml	Plastipak
Tissue Culture Flask, Canted Neck and Blue Vented Cap, 250 ml (75cm^2)	Becton-Dickinson
Tissue Culture Plates, 6 and 24 well, Flat Bottom with Low Evaporation Lid	Corning Inc
Various Glass Equipment	
Waterbaths	VWR

Instruments	**Supplier**
Bio-Rad GenePulser® II	Bio-Rad
Bio-Rad Pulse Controller Plus	Bio-Rad
Centrifuges	
Eppendorf Centrifuge 5418 R	Eppendorf
Eppendorf Centrifuge 5430 R	Eppendorf
Megafuge 1.0	Heraeus
Multifuge X1R	Heraeus
Table Centrifuge	Biofuge Pico, Heraeus
Coulter Counter® Z1	
Coulter® Particle Count Z1.	Nerliens Meszansky
Beckman Coulter™	
Electrophoresis Electricity Supplier	
EVOM2, Epithelial Voltohmmeter for Trans-Epithelial Electric Resistance (TEER)	WPI
FastPrep® - 24 Tissue and Cell Homogenizer	MP Biomedicals
Inverted Light Microscope, Leica DM IL	Leica Microsystems
MacsQuant® Analyzer	Miltenyi Biotec
pH-meter	Metrohm
Photo Equipment	
GelDoc Machine	Bio-Rad
Qubit™ Fluorometer	Invitrogen
SNAP i.d. Protein Detection System	Millipore
Steri-Cycle CO_2 Incubator	Thermo Electron Corporation

2.1.1 Software

Software	**Supplier**
MacsQuantify™ Software	Miltenyi Biotec
pDRAW32	www.acaclone.com
Serial Cloner	http://serialbasics.free.fr/Serial_Cloner.html

2.2   Chemicals

Chemicals	**Supplier**
Acetic acid, CH_3COOH	Merck
Agar	Difco
Agarose	
$\quad$ SeaKem® LE Agarose	Lonza
Ampicillin, $C_{16}H_{19}N_3O_4S$	Sigma-Aldrich
Calsium chloride, $CaCl_2$	Sigma-Aldrich
Disodium hydrogen phosphate, Na_2HPO_4	Merck
EDTA, $C_{10}H_{16}N_2O_8$	Merck
Erythromycin, $C_{37}H_{67}NO_{13}$	Sigma-Aldrich
Ethanol, C_2H_5OH (96% vol)	VWR
Formaldehyde	
Gentamycin, $C_{21}H_{43}N_5O_7$	Sigma-Aldrich
Glucose, $C_6H_{12}O_6$	Merck
Glycerol, $C_3H_8O_3$	Merck
Glycine, $C_2H_5NO_2$	Duchefa Biochemie
Magnesium chloride, $MgCl_2$	Merck
Magnesium sulphate, $MgSO_4$	Sigma-Aldrich
Penicillin-Streptomycin	Sigma
PeqGREEN DNA/RNA dye	VWR
Polyethylen glycol, PEG_{1500}	Sigma-Aldrich
Potassium chloride, KCl	Merck
Potassium dihydrogen phosphate, KH_2PO_4	Merck
Sodium hydrogen carbonate, $NaHCO_3$	Merck
Sodium hydroxide, $NaOH$	Merck
Sodium chloride, $NaCl$	VWR
Tris-base, $C_4H_{11}NO_3$	Sigma-Aldrich
Tris-HCl, $C_4H_{11}NO_3$ x HCl	Sigma-Aldrich
Tween-20	Sigma-Aldrich
Triton X-100	Sigma-Aldrich

2.3 Proteins and Enzymes

Protein/Enzyme	Supplier
Antibodies	
Anti-EGFR Rabbit-Polyclonal IgG	Santa Cruz Biotechnology
Anti-GP2, Rabbit-Polyclonal IgG	Atlas Antibodies
Anti-Mouse IgG FITC, Rabbit-Polyclonal	Sigma-Aldrich
Anti-Mouse IgG/HRP Rabbit-Polyclonal	Dako
Anti-myc Mouse-Monoclonal IgG	Life Technologies
Anti-Rabbit IgG FITC, Goat-Polyclonal	Sigma-Aldrich
CD29 Mouse-Monoclonal anti-Human, FITC conjugated	Life Technologies
Bovine Serum Albumin (BSA)	Sigma-Aldrich
CF^{TM}633 (Succinimidyl Ester)	Sigma-Aldrich
Fetal Bovine Serum (FBS) gold	PAA
Inducer Peptide (SppIP)	Sigma-Aldrich
Lysozyme	Sigma-Aldrich
Trypsin-EDTA	PAA
Protein Standards	
MagicMark^{TM}	Invitrogen
Restriction Enzymes and Buffers	
EcoRI	Thermo Scientific
MluI	Thermo Scientific
SalI	Thermo Scientific
10 X FastDigest Green Buffer	Thermo Scientific
Taq DNA Polymerase Master Mix Red	VWR
Q5 High-Fidelity Master Mix	NEB
Quick T4 DNA Ligase	NEB
T4 Ligation Buffer	NEB
In-Fusion® HD Enzyme Premix (5X)	Clontech

2.4 DNA

DNA	Supplier
DNA Standards	
GeneRuler^{TM} 1 kb DNA Ladder	Fermentas
Quick-Load^{TM} 1 kb DNA Ladder	NEB

2.5   Primers

The primer sequences used in this study are listed in Table 2.1, while descriptions of what the primers were used for are shown in Table 2.2.

Table 2.1: Primers Used in This Study; Sequences

Name	Sequence	Restriction Site
CWA_SeqR	GATTGTTCTATCGAAAGCGA	
SekF	GGCTTTTATAATATGAGATAA-TGCCGAC	
SeqRR	AGTAATTGCTTTATCAACTGCTGC	
1261Invasin_F	GATTGCGGCGGtcga-CGAACAAAGTTGATTTCAGAAGA	SalI
1261Invasin_R	CCGGGGTACCGaatt-CCTATTGCGGTTCGGCAC	EcoRI

The locations of the restriction sites are indicated by lowercase letters.

Table 2.2: Primers Used in This Study; Description

Name	Description
CWA_SeqR	Reverse primer for sequencing of pLp_0373FimH_S.t.cwa2
SekF	Forward primer for sequencing of pLp_0373FimH_S.t.cwa2 and plp_1261Invasin_Y.e.
SeqRR	Reverse primer for sequencing of pLp_1261Invasin_Y.e.
1261Invasin_F	Forward In-Fusion primer for amplification of Invasin_Y.e. with a 15 bp overhang
1261Invasin_R	Reverse In-Fusion primer for amplification of Invasin_Y.e. with a 15 bp overhang

2.6   Bacterial Strains, Cell Lines and Plasmids

Tables 2.3 and 2.4 list the bacterial strains, cell lines and plasmids used in this study.

Table 2.3: Bacterial Strains and Cell Lines

Strain/Cell Line	Reference
Escherichia coli TOP10	Invitrogen
Escherichia coli HST08 (Stellar Competent Cells)	Clontech
Enteropathogenic *Escherichia coli* (EPEC) 0127:H7 E2348/69	Tor Lea, IKBM
Lactobacillus plantarum WCFS1	Kleerebezem et al. (2003)
Lactococcus lactis FnBPA$^+$ N2900	Christophe Michon, INRA de Jouy-en-Josas, Institut Micalis, unité ProbiHote, France
Caco-2	ATCC
Raji B	Tor Lea, IKBM

Table 2.4: Plasmids Used in This Study

Plasmid	Description	Source
pEV	Empty vector (pSIP401-derivative without any target gene)	Fredriksen et al. (2012)
pLp_0373OFAcwa2	pSIP401-derivative for production of OFA with the Lp0373 signal sequence and a C-terminal cell wall anchor	Fredriksen et al. (2010)
pUC57FimH.S.t.*	Vector containing FimH from *Salmonella typhimurium*	Genescript
pUC57Invasin_Y.e.*	Vector containing Invasin from *Yersinia enterocolitica*	Genescript
pLp_1261ccl3gag	pSIP401-derivative for production of ccl3gag with an N-terminal Lp1261 lipoprotein anchor	Katarzyna Kuczkowska, IKBM (unpublished)
pLp_0373FimH_S.t.cwa2*	pSIP401-derivative for production of FimH from *Salmonella typhimurium* with the Lp0373 signal sequence and a C-terminal cell wall anchor	This work
pLp_0373FimH_E.c.cwa2*	pSIP401-derivative for production of FimH from enteropathogenic *Escherichia coli* with the Lp0373 signal sequence and a C-terminal cell wall anchor	Katarzyna Kuczkowska, IKBM (unpublished)
pLp_1261Invasin_Y.e.*	pSIP401-derivative for production of Invasin from *Yersinia enterocolitica* with an N-terminal Lp1261 lipoprotein anchor	This work
pLp_1452Invasin_Y.e.*	pSIP401-derivative for production of Invasin from *Yersinia enterocolitica* with an N-terminal Lp1452 lipoprotein anchor	Lise Øverland, IKBM (unpublished)
pLp_3014Invasin_Y.e.*	pSIP401-derivative for production of Invasin from *Yersinia enterocolitica* with an N-terminal 3014 lysM domain-based anchor	Lise Øverland, IKBM (unpublished)

* The plasmid includes a sequence encoding a *myc*-tag.

2.7   Kits

Kits	Supplier
iBlot^{TM} Dry Blotting System	Invitrogen
Blotting Roller	
iBlot^{TM} Transfer Stack, Regular and Mini	
iBlot^{TM} Cathode Stack, top	
iBlot^{TM} Anode, bottom	
iBlot^{TM} Disposable Sponge	
iBlot^{TM} Filter Paper	
iBlot^{TM} Gel Transfer Device	
Novex®SDS-PAGE Gel System	Invitrogen
NuPAGE®Bis-Tris Gels 10%	Invitrogen
8 cm x 8 cm x 1 cm x 1 mm, 10 wells	
1 X Tris/Glycine/SDS Buffer (TGS)	Bio-Rad
Qubit^{TM} dsDNA BR Assay	Invitrogen
Qubit^{TM} dsDNA BR Reagent (Component A)	
Qubit^{TM} dsDNA BR Buffer (Component B)	
Qubit^{TM} dsDNA BR Standard #1 (Component C)	
Qubit^{TM} dsDNA BR Standard #2 (Component D)	
Restore^{TM} PLUS Western Blot Stripping Buffer	Thermo Scientific
SNAP i.d. Protein Detection System	Millipore
SNAP i.d. Single Well Blot Holder	
SNAP i.d. Spacer	
SNAP i.d. Blot Roller	
SuperSignal® West Pico Chemiluminescent Substrate	Thermo Scientific
Luminol/Enhancer Solution	
Stable Peroxide Solution	

2.8 Agars and Media

Media	Supplier
Media	**Supplier**

BHI (Brain-Heart-Infusion)   Oxoid

    Medium
        37 g BHI
        dH_2O to 1 litre
        Sterilized in an autoclave
        for 15 min at 115°C.
    Agar
        BHI medium with 1.5% (w/v) agar.
        When the autoclaved medium was
        cooled to ~60°C, appropriate antibiotics
        was added, and the medium was poured
        into petri dishes and stored at -4°C.

Complete DMEM medium
        Dulbecco's Modified Eagle Medium   Life Technologies
        (DMEM), GlutaMAX™ + Pyruvate
        supplemented with:
        10% (v/v) Fetal Bovine Serum (FBS)   PAA
        1% (v/v) Penicillin-Streptomycin   Sigma
        25mM Monothioglycerol (50% $etOH/H_2O$)
        1% (v/v) Non-Essential Amino Acids

LB (Lysogeny Broth) medium

    Medium
        10 g Bacto™ Tryptone   Becton Dickinson
        5 g Bacto™ yeast extract   Becton Dickinson
        10 g NaCl
        dH_2O to 1 litre
        Sterilized in an autoclave
        for 15 min at 115°C
    Agar
        LB medium with 1.5% (w/v) agar
        When the autoclaved medium was
        cooled to ~60°C, appropriate antibiotics
        was added, and the medium was poured
        into petri dishes and stored at -4°C.

Media	**Supplier**
MRS (de Man, Rogosa, Sharpe)	VWR International

Medium
    55.2 g MRS
    dH_2O to 1 litre
    Sterilized in an autoclave
    for 15 min at 115°C
Agar
    MRS medium with 1.5% (w/v) agar
    When the autoclaved medium was
    cooled to ∼60°C, appropriate antibiotics
    was added, and the medium was poured
    into petri dishes and stored at -4°C.

MRSSM medium

    5.2 g MRS
    17.1 g Sucrose (0.5 M)
    2.0 g $MgCl_2$ x $6H_2O$ (0.1 M)
    dH_2O to 100 ml
    Sterililzed by filtration (0.2 µm pore size)

Complete RPMI-1640 medium:

RPMI-1640 + 2.05 mM L. Glutamine	PAA
supplemented with:	
10% (v/v) Fetal Bovine Serum (FBS)	PAA
1% (v/v) Penicillin-Streptomycin	Sigma
1% (v/v) Sodium Pyruvate	
25mM Monothioglycerol (50% etOH/H_2O)	
1% (v/v) Non-Essential Amino Acids	

S.O.C. medium

2 g Bacto^{TM} Tryptone	Becton Dickinson
0.5 g Bacto^{TM} yeast extract	Becton Dickinson
0.057 g NaCl	
0.019 g KCl	
0.247 g $MgSO_4$	
60 ml dH_2O	
Sterilized in an autoclave for 15 min at	
115°C. The medium was then cooled to	
room temperature and added the following:	
1 ml 2 M glucose, sterilized by filtration	
(0.2 µm pore size)	
Sterile dH_2O to 100 ml	

2.9   Buffers and Solutions

Buffer/Solution	Content
10 X PBS (Phosphate Buffered Saline), pH 7.4	80 g/l NaCl 2.0 g KCl 18,05 g Na_2HPO_4 x $2H_2O$ 2.4 g KH_2PO_4 NaOH to pH 7.4 dH_2O to 1 l
50 X TAE	242 g Tris base 57.1 ml Acetic acid 100 ml 0.5 M EDTA, pH 8 dH_2O to 1 L
TBS (Tris Buffered Saline), pH 7.4	0.150 M NaCl 0.010 M Tris-HCl, pH 8
TEN-Base-buffer	10 mM Tris-HCl, pH 8 1 mM EDTA-buffer, pH 8 100 mM NaCl
TTBS	TBS 0.1 % (v/v) Tween-20

3   Methods

3.1   Culturing and Growing of Bacteria

Bacteria were cultured in suitable liquid media, or spread and grown on medium-agar plates (1.5% (w/v)). Bacterial cells containing plasmids encoding an antibiotic resistance gene were selected by adding appropriate antibiotics to the media or agar plate.

Escherichia coli

Escherichia coli was cultured in liquid Brain-Heart-Infusion (BHI) medium or grown on solid BHI-agar plates. Both were incubated overnight at 37°C with and without shaking, respectively. Antibiotic concentrations used for plasmid selection in *E. coli*:

- pSIP-derivatives: 200 µg/ml Erythromycin in both liquid and solid media.
- pUC57-derivatives: 200 µg/ml Ampicillin in liquid media and 100 µg/ml in solid media.

Lactobacillus plantarum

Lactobacillus plantarum was cultured in liquid de Man, Rogosa, Sharpe (MRS) medium, and incubated overnight at 37°C without shaking. On solid media the incubation time was 1-2 days at 37°C without shaking. Antibiotic concentrations used for plasmid selection in *L. plantarum*:

- pSIP-derivatives: 10 µg/ml Erythromycin in both liquid and solid media.

Lactococcus lactis

Lactococcus lactis was cultured in liquid M17 medium with 0.5% (w/v) glucose. The culture was incubated overnight at 30°C without shaking. Antibiotic concentrations used for plasmid selection in *L. lactis*:

- *L. lactis*: 5 µg/ml Erythromycin and 10 µg/ml Chloramphenicol in liquid media.

3.2   Long Term Storage of Bacteria

Bacterial cultures were stored at -80°C as glycerol stocks, preserving the cells by preventing cell disruption. The stocks were made by adding 300 µl 85% (v/v) glycerol to 1 ml bacterial culture in 1.5 ml cryovials. The cryovials were inverted a few times to mix the contents, and immediately transferred to a -80°C freezer. When bacteria from a glycerol stock were needed, a sterile toothpick was used to transfer a small amount of frozen culture to a culture tube containing the appropriate growth medium.

3.3   Plasmid Isolation from Bacterial Cultures

Plasmid DNA from *Escherichia coli* was isolated using Pure Yield^{TM} Plasmid Miniprep System from Promega. The protocol provided by the supplier, "PureYield^{TM} Plasmid Miniprep System Protocol; Centrifugation Protocol" was followed and the procedure was performed at room temperature:

1. 3 ml of a bacterial culture grown overnight was centrifuged in an Eppendorf tube at 13 000 x g for 30 seconds. All of the supernatant was discarded.

2. The remaining cell pellet was resuspended in 600 µl water

3. Cell Lysis Buffer (100 µl) was added and the solution was mixed by inverting the tube 6 times. A clear blue solution indicated complete cell lysis.

4. To restore the pH from the alkaline cell lysate, 350 µl Neutralization Solution (4-8°C) was added within 2 minutes, followed by mixing by inverting the tube. Plasmid DNA was thereby renatured while genomic DNA and protein were precipitated.

5. The solution was centrifuged at 13 000 x g for 3 minutes, and 900 µl of the supernatant was transferred to a PureYield^{TM} Minicolumn. The Minicolumn was placed in a PureYield^{TM} Collection Tube and centrifuged at 13 000 x g for 15 seconds.

6. The flow through was discarded and 200 µl Endotoxin Removal Wash was added in order to remove endotoxins that could inhibit the efficiency of subsequent transformation (see section 3.11). The tube was further centrifuged at 13 000 x g for 15 seconds.

7. To remove other contaminants (all parts of the bacterial cell other than DNA), 400 µl Column Wash solution containing ethanol was added. The ethanol keeps the DNA bound to the column. The tube was centrifuged at 13 000 x g for 30 seconds.

8. The minicolumn was then transferred to a new 1.5 ml Eppendorf tube. Elution buffer or warm water was heated in advance to 65°C, and 30 µl was added to the minicolumn to elute the DNA. The column was incubated for 5 minutes at 65°C, and then centrifuged at 13 000 x g for 1 minute.

9. The eluted DNA was stored at -20°C.

3.4   Determination of DNA Concentration

The concentration obtained when purifying plasmid DNA was determined with the Qubit® dsDNA BR Assay Kit (Invitrogen).

Materials:
Qubit™ dsDNA BR Assay (Invitrogen)
    Qubit™ Assay Tubes
    Qubit™ dsDNA BR Buffer
    Qubit™ dsDNA BR Reagent
    Qubit™ dsDNA BR Standard 1 and 2
Qubit™ Fluorometer

Procedure:
Invitrogen's "Instruction Manual" was followed to prepare the standards and samples, and to determine DNA concentrations.

1. The Qubit™ reagent was diluted 1:200 in Qubit™ buffer to make a stock solution of 200 µl per sample.

2. To calibrate the Qubit™ fluorometer, two standards (one blank solution and one containing 100 ng/µl dsDNA) were prepared by mixing 190 µl stock solution with 10 µl of each of the standards. For samples with unknown concentration, 1 µl was mixed with 199 µl stock solution. All samples, including the standard solutions, were vortexed and incubated at room temperature for 2 minutes prior to analysis.

3. The prepared standards were read to calibrate the Qubit™ fluorometer, and then the samples were read to determine the DNA concentration.

3.5   Restriction Enzyme Digestion

DNA can be cleaved at specific sites by enzyme digestion, using appropriate restriction enzymes. The restriction enzymes recognize restriction sites in the DNA sequence, where they cleave the two strands of DNA. The two strands can be cleaved at the same position, which generates blunt ends, or a few base pairs apart, creating

sticky ends with overhang. This method can for instance be used to cut out a specific DNA fragment from a plasmid, and then clone it into another plasmid, which has been digested with the same restriction enzymes. Different enzymes require different buffers and varying conditions, meaning that two enzymes cannot necessarily be used at the same time. The incubation time is quite long (1 hour to overnight) and there are risks of star activity, which implies that the enzymes cleave the DNA in an uncontrolled manner. For this project, fast digest restriction enzymes from Thermo Scientific were used. These enzymes are all compatible with one universal buffer (10X Fast Digest® Green Buffer, Thermo Scientific), which means that any enzyme can be used in one reaction. The incubation time is also much shorter (5-15 minutes), and as opposed to other enzymes, there is no star activity. Furthermore, the digestion reaction can be loaded directly onto an agarose gel, as the green reaction buffer contains a loading dye. The fast digestion procedure was performed according to the manufacturer's protocol; "Fast Digestion of Different DNA".

Materials:

DNA

Restriction Enzymes (section 2.3)

10X FastDigest® Green Buffer (Thermo Scientific)

Water, nuclease-free

Procedure:

Reaction mixtures were prepared at room temperature as shown in Table 3.1.

Table 3.1: Fast Digestion of DNA

Component	Volume (µl)
Water, Nuclease-free	Regulated to keep the total reaction volume
10X FastDigest® Green Buffer	5
Plasmid DNA	up to 1 µg
FastDigest® Enzymes	5
Total Volume	50

The components were mixed gently and collected at the bottom of the tube by a short centrifugation. The mixture was then incubated in a water bath at 37°C for 5-15 minutes. To separate the digested DNA fragments, the mixture was run on an agarose gel, as described in section 3.6.

3.6   Agarose Gel Electrophoresis

DNA fragments can be separated according to their size with a method called agarose gel electrophoresis. First, DNA samples mixed with a loading dye, are loaded onto the gel. Then, by applying an electric current, the DNA fragments migrate towards the positive pole of the agarose gel. As the fragments have a uniform mass/charge ratio, the current will affect each fragment equally. However, the pores in the gel vary in size, forcing larger fragments to take detours, while smaller fragment can migrate straight through towards the positive pole. This causes small fragments to move faster through the gel than larger ones. A linear DNA ladder containing DNA fragments of known molecular weights is loaded onto the gel along with the DNA samples. A fluorescent nucleic acid gel stain, like peqGREEN, is used to visualize the DNA, and the size of the fragments in the samples can be determined, using the DNA ladder as a reference. A 1.2% agarose gel was used to separate fragments larger than 200 bp.

Materials:
SeaKem® LE Agarose (Lonza)
1X TAE Buffer (section 2.9)
peqGREEN DNA/RNA Dye (VWR)
10X FastDigest® Green Buffer (Thermo Scientific)
GeneRuler™ 1 kb DNA Ladder (Fermentas) or Quick-Load™ 1 kb DNA Ladder (NEB)

Procedure:

1. Stock solutions of 1.2% agarose were prepared by dissolving 12 g SeaKem® LE Agarose in 1 l TAE buffer (1X). The stock solutions were autoclaved at 115°C for 15 minutes and were further kept at 50°C.

2. 2.5 μl peqGREEN was mixed with 60 ml of the agarose stock solution. The mixture was then poured into a gel rack, and wells were made by inserting well combs. The gel solidified during approximately 20 minutes.

3. The well combs were gently removed, and the gel was transferred to an electrophoresis chamber. 1X TAE buffer was added to cover the gel.

4. Prior to application, 10X FastDigest® Green buffer was mixed with the DNA samples to a 1X final concentration. The samples, together with a suitable DNA ladder, were then applied to different wells. The voltage was set to 90 V, and the

gel was run for 40-60 minutes.

3.7   DNA Extraction from Agarose Gels

To extract DNA from agarose gels, the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) was used. The extraction steps suggested by the supplier's protocol 5.2, "DNA extraction from agarose gels" were followed and are described below.

Procedure:

1. Using UV-light for visualization and a clean scalpel, the DNA fragments were carefully excised from the agarose gel, and transferred to an Eppendorf tube. This step was done quickly to limit the time of UV-light exposure and its damaging effect on the DNA.

2. The weight of the gel slice was determined, and for each 100 mg of agarose gel, 200 µl binding buffer, NTI, was added. The binding buffer helps maintain a pH of 5-6, which ensures optimal binding conditions for the DNA to the silica membrane used for purification. The tubes were then incubated in a water bath at 50°C for 5-10 minutes. To completely dissolve the gel slice, the tubes were vortexed every 2-3 minutes.

3. A NucleoSpin® Gel and PCR Clean-up Column containing a silica membrane, was placed in a 2 ml Collection Tube, and 700 µl sample was transferred to the column. To bind the DNA to the silica membrane, the column was centrifuged for 30 seconds at 11000 x g and the flow through was discarded. Remaining sample was loaded if needed, and the centrifugation step was repeated.

4. The column was washed with 700 µl buffer NT3, and the column was centrifuged at 11000 x g for 1 minute. The flow-through was discarded and the column was centrifuged once more at 11000 x g for 1 min to dry the silica membrane.

5. The column was placed in a clean Eppendorf tube, and 15-30 µl preheated buffer NE (70°C) was added to the column. The column was incubated with the elution buffer (NE) at 70°C for 5 minutes, and was then centrifuged at 11000 x g for 1 minute.

6. The resulting DNA eluate was kept at -20°C.

3.8   DNA Cloning

The first step to obtain *L. plantarum* bacteria expressing different proteins on their surface was to establish plasmid constructs in *E. coli*. To make these plasmid constructs, DNA fragments coding for specific proteins were cloned into derivatives of the pSIP401-vector (Sørvig et al., 2005) with different signal peptides and anchor sequences (Fredriksen et al., 2010, 2012). This was done either by Quick T4 Ligation or In-Fusion Cloning.

3.8.1   Quick T4 DNA Ligation

Quick T4 DNA Ligation is a method used to clone DNA fragments into linearized plasmid vectors using an enzyme called Quick T4 DNA Ligase (NEB). This ligase functions as a catalyst, which drives the formation of a phosphodiester bond between the 5'phosphate- and the 3'hydroxyl ends of DNA chains, and in this way couples DNA fragments. The ligase can be used to combine both sticky- and blunt end DNA. To ensure optimal conditions for the ligase, a T4 Ligation Buffer (NEB), which contains the required cofactor Mg^{2+} and ATP, is added to the reaction mix.

Materials:
DNA fragment and vector
2X Quick Ligation Buffer (NEB)
Quick T4 DNA Ligase (NEB)

Procedure:
Ligation of DNA was performed according to the "Quick Ligation Protocol (M2200)" provided by NEB:

1. Approximately 50 ng linearized vector was combined with 3 times more (approximately 150 ng) DNA fragment (insert) in an Eppendorf tube. The volume was adjusted to (10 µl) with dH_2O.

2. The 2X Quick Ligation Buffer was added to a 1X final concentration (10 µl), and 1 µl of the Quick T4 Ligase was added, before the reaction was mixed thoroughly.

3. The reaction mixture was then briefly spun, and further incubated at room temperature for 5 minutes. The tubes were then chilled on ice.

4. The ligation mixture was either transformed into competent *E. coli* cells (section 3.11) or stored at -20°C.

3.8.2   In-Fusion Cloning

For In-Fusion Cloning, the DNA fragment (insert) must be generated by PCR, using primers designed to produce a 15 bp overlap at each end, which is complementary to the ends of the linearized vector sequence. The In-Fusion enzyme, which is a 3' exonuclease, creates single-stranded regions at the ends of the PCR insert and linearized vector. The 15 bp complementary regions on the insert and vector are then exposed, and spontaneously anneal/fuse through base pairing.

Materials:

In-Fusion® HD Cloning Kit (Clontech):

> Purified PCR Fragment
> Linearized Vector
> 5X In-Fusion HD Enzyme Premix
> Deionized Water

Procedure:

In-Fusion Cloning was performed according to Clontech's "Protocol I B: In-Fusion Cloning Procedure for Spin-Column Purified PCR Fragments". The components were added to an Eppendorf tube as listed in Table 3.2.

Table 3.2: In-Fusion Cloning Set-up

Component	Cloning Reaction	Negative Control Reaction
2X In-Fusion HD Enzyme Premix	2 µl	2 µl
Linearized Vector	x µl*	x µl*
Purified PCR Fragment	x µl*	x µl*
dH$_2$O (as needed)	x µl	x µl
Total volume	10 µl	10 µl

> * The amount of purified DNA fragment and linearized vector was determined using Clontech's "In-Fusion® Molar Ratio Calculator"

The reaction mixture was incubated in a water bath at 50°C for 15 minutes, and then placed on ice. Further, the reaction mixture was either transformed into *E. coli* (section 3.11) or stored at -20°C.

3.9   Polymerase Chain Reaction

DNA fragments needed for cloning purposes were obtained by the Polymerase Chain Reaction (PCR). PCR is a method developed to exponentially amplify specific DNA sequences *in vitro*. The method utilizes synthetically designed oligonucleotide primers, deoxynucleotides (dNTPs) and a DNA Polymerase, together with thermocycling conditions as follows:

Denaturation Double stranded DNA becomes separated as the reaction mixture is heated. Each strand can then be used as a template for the synthesis of new DNA strands.

Annealing As the temperature decreases, the primers are able to bind to the complementary nucleotides on the DNA template.

Elongation The primers provide binding sites for a thermostable DNA polymerase, which synthesizes new DNA strands by inserting dNTPs.

A thermal cycling device repeats these cycles of denaturation, annealing and extension, so that newly synthesized strands are used as templates in the next cycle. In this way the original DNA template is exponentially amplified.

3.9.1   Q5® High-Fidelity 2X Master Mix

DNA fragments intended for cloning must contain as little errors as possible, and a DNA polymerase with a low error rate is therefore required. The Q5 High-Fidelity Master Mix meets this requirement (as it has more than a 100-fold lower error rate than that of Taq DNA Polymerase, section 3.9.2) and is also very convenient as it contains dNTPs, Mg^{2+} (a cofactor for the polymerase) and a broad-use buffer.

Materials:
Q5 High-Fidelity 2X Master Mix (NEB)
Primers (section 2.5)

Procedure:
New England Biolabs' protocol for Q5® High-Fidelity 2X Master Mix was followed when preparing the PCR reactions. All reactants were assembled on ice and mixed in a 0.2 ml PCR tube according to Table 3.3.

Table 3.3: Q5 PCR Components

Component	Volume (µl)	Final Concentration
Q5 High-Fidelity 2X Master Mix	25	1X
10 µM Forward Primer	2.5	0.5 µM
10 µM Reverse Primer	2.5	0.5 µM
Template DNA	Variable	<1000 ng
Nuclease-free Water	to 50	

The reaction mixture was then transferred to a thermal cycling device, applying the settings shown in Table 3.4.

Table 3.4: Thermocycling Conditions for PCR Reactions Using Q5 High-Fidelity 2X Master Mix

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	98	30 seconds	1
Denaturation	98	5-10 seconds	25-35
Annealing	50-72*	10-30 seconds	25-35
Elongation	72	20-30 seconds/kb	25-35
Final Extension	72	2 minutes	
Hold	4		

*The annealing temperature was adjusted according to the primers used. The temperature should be 3-5°C lower than the melting temperature (T_m) of the primer with the lowest T_m.

3.9.2   VWR Red Taq DNA Polymerase Master Mix

Colony PCR was used to verify that the correct DNA insert was cloned into a plasmid. For this purpose, a polymerase with a higher error rate was adequate, and the less expensive VWR Red Taq DNA Polymerase Master Mix was used. VWR Red Taq DNA Polymerase Master Mix contains dNTPs, $MgCl_2$, and buffer. In addition, the PCR product can be directly applied onto a gel for analysis, as an inert red dye and stabilizer are also present in the mix.

Materials:
Taq 2X Master Mix RED (VWR)
Primers (see materials, section 2.5)

Procedure:

VWR's "Suggested Protocol Using VWR Red Taq Master Mix" was followed when preparing PCR reactions for colony PCR.

The Master Mix and the primers were first thawed completely, and to ensure evenly distributed salt concentrations, the solutions were mixed thoroughly. All components shown in Table 3.5 were kept on ice before they were added and gently mixed by pipetting up and down.

Table 3.5: Red Taq PCR Components

Component	Volume (µl)	Final Concentration
Taq 2X Master Mix RED	25	1X
10 µM Forward Primer	1	0.2 µM
10 µM Reverse Primer	1	0.2 µM
Distilled Water	23	
Template DNA	Variable	

A pipette tip was used to transfer bacterial cells from a colony on an agar plate to a 0.2 ml PCR tube containing the 50 µl Red Taq PCR reaction mixture (Table 3.5). Cells from the same colony were also inoculated in 10 ml BHI medium containing the appropriate antibiotics, and incubated overnight to obtain a culture of the tested colony. The PCR tube was then transferred to a thermal cycling device, applying the settings shown in Figure 3.6.

Table 3.6: Thermocycling Conditions for PCR Reactions Using Red Taq 2X Master Mix

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	95	2 minutes	1
Denaturation	95	20-30 seconds	35
Annealing	50-65*	20-40 seconds	35
Elongation	72	30 seconds/kb	35
Final Extension	72	5 minutes	1

*The annealing temperature was adjusted according to the primers used. The temperature should be 3-5°C lower than the melting temperature (T_m) of the primer with the lowest T_m.

3.10   Competent Cells

Bacterial cells that can take up foreign plasmid DNA are called competent cells. By transformation procedures, cloned plasmid DNA can be transferred into these competent cells. To become competent, the cell wall is made more permeable, and this is most easily done if the bacterial cells are harvested in the exponential growth phase.

3.10.1   Preparation of Electrocompetent *Lactobacillus plantarum* WCFS1

To prepare electrocompetent *L. plantarum*, the cells were grown in a medium containing glycine. In this way, L-alanine in the cell wall is exchanged with glycine, making the cell wall more permeable for foreign plasmids during electroporation. The procedure was performed in accordance with the protocol described by Aukrust et al. (1995).

Materials:
L. plantarum WCFS1
MRS medium (VWR)
2X MRS medium (VWR)
20 % glycine (w/v) (Duchefa Biochemie)
1 mM $MgCl_2$ (Merck)
PEG_{1500} (Polyethylen Glycol), sterile filtrated (Sigma-Aldrich)
TEN-buffer (section 2.9)
Multifuge X1R (Heraeus)

Procedure:

1. *L. plantarum* from a glycerol stock was inoculated in 10 ml MRS medium, and grown overnight at 30°C.

2. The culture was then diluted in series from 10^{-1} to 10^{-10} in MRS medium and incubated overnight at 30°C.

3. The diluted overnight culture showing an OD_{600} of 0.5-0.7 was selected, and 5-10 ml were transferred to a 50 ml Nunc tube. 20 ml 2X MRS medium, 5 ml 20% glycine and 5-10 ml sterile water were added to the same tube, to a total volume of 40 ml.

4. The OD_{600} was measured, and the cells were grown until the OD_{600} had increased 2.5 times. The culture was then chilled on ice for 5 minutes.

5. To harvest the cells, the culture was centrifuged at 5200 rpm, at 4°C for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 10 ml ice-cold TEN-buffer (section 2.9).

6. The cell solution was centrifuged at 5200 rpm, at 4°C for 5 minutes, and the supernatant was discarded. The cell pellet was then resuspended in 40 ml 1mM $MgCl_2$ and centrifuged at 5200 rpm, 4°C for 5 minutes.

7. The supernatant was discarded, and 5 ml 30% PEG_{1500} was added to the cell pellet. The resulting suspension was transferred to a chilled Corex-tube. The tube was centrifuged at 6000 rpm, 4°C for 10 minutes. The supernatant was discarded.

8. The cells were resuspended in 400 µl 30% PEG_{1500} and aliquoted into 40 µl portions in chilled Eppendorf tubes.

9. The tubes were stored at -80°C.

3.11   Transformation of Chemically Competent *E. coli*

Both competent *E. coli* TOP10 cells from Invitrogen, and Stellar Competent cells from Clontech Laboratories were used for transformation procedures. The TOP10 cells were used to transform plasmids made by Quick T4 DNA Ligation (section 3.8.1), and the Stellar Competent cells were used to transform plasmids made by in-fusion cloning (section 3.8.2).
The transformation procedure for the ligated plasmids was performed according to "*E. coli* TOP10 One Shot Chemical Transformation procedure" (Invitrogen), with modifications. For in-fusion cloned plasmids, Clontech's "Transformation Protocol" was followed.

Materials:
Competent *E. coli* cells
Ligation mixture or in-fusion reaction mixture
S.O.C. medium (section 2.8)
BHI agar plates containing 200 µg/ml Erythromycin

Procedure:

1. The competent *E. coli* cells were thawed on ice, and then gently mixed to evenly distribute the cells.

2. To transform ligated plasmids, 100 µl TOP10 cells (Invitrogen) were added to a 14 ml falcon tube together with all of the ligation mixture (see section 3.8.1).

To transform in-fusion cloned plasmids, 50 µl Stellar Competent cells (Clontech) and 2.5 µl in-fusion reaction mixture (see section 3.8.2) were added to a 14 ml falcon tube.

3. The falcon tubes were tapped gently to mix the reactions, and were then placed on ice for 30 minutes.

4. The tubes were quickly transferred to a water bath at 42°C, and held there for 45 second (TOP10 cells with ligated plasmids) or 1 minute (Stellar Competent cells and in-fusion cloned plasmids). After the heat shock, the tubes were immediately transferred to ice, and chilled for 1-2 minutes.

5. Room tempered S.O.C. medium was then added to the transformation mixtures, 250 µl to the transformed ligation mixture, and to a total of 500 µl for the transformed in-fusion reaction mixture.

6. The transformation mixtures were incubated at 37°C, with shaking (160-225 rpm), for 1-2 hours.

7. The transformation mixtures were spread on BHI-agar plates containing appropriate antibiotics (50-100 µl per plate). The plates were incubated overnight at 37°C.
   The rest of the transformation mixture was kept at room temperature overnight, and then spread on agar plates if necessary.

Colonies, i.e. potential transformants, were analysed by colony PCR as described in section 3.9.2.

3.12   Transformation of Electrocompetent *Lactobacillus plantarum*

Materials:
Electrocompetent *L. plantarum* cells
Electroporation cuvette
Isolated plasmid
Bio-Rad GenePulser® II
Bio-Rad Pulse Controller Plus
MRSSM medium (2.8)
MRS agar plates containing 10 µg/ml Erythromycin

Procedure:

1. Electrocompetent *L. plantarum* WCFS1 cells were thawed on ice and 40 µl were carefully mixed with 5 µl isolated plasmid.

2. The cell mixture was transferred to an ice-cold electroporation cuvette, which was inserted into the Bio-Rad GenePulser® II and given an electric pulse. The following settings were used:

   Capitance: 25 µF
   Voltage: 1.5 kV
   Resistance: 400 Ω

3. After adding 950 µl ice-cold MRSSM medium to the cuvette, the mixture was transferred to a sterile Eppendorf tube and incubated at 37°C for 2 hours.

4. The transformation mixture was spread on MRS agar plates containing appropriate antibiotics (100-150 µl per plate).

3.13 Cultivation and Harvesting of *Lactobacillus plantarum*

For overproduction of target proteins, derivatives of the pSIP401 expression vector were used (see introduction, section 1.2). An inducer peptide was added to cultures of *L. plantarum* harbouring expression plasmids. The inducer peptide induces protein expression, and after an incubation period, the cells were harvested.

Materials:
MRS medium containing 10 µg/ml Erythromycin
Inducing peptide SppIP (100 mg/ml in dH$_2$O)
PBS (section 2.9)

Procedure:

1. *L. plantarum* strains were grown overnight in MRS medium containing the appropriate antibiotics at 37°C.

2. The overnight cultures were diluted in 50 ml MRS containing the appropriate antibiotics to an OD$_{600}$ of 0.1. The diluted cultures were further incubated at 37°C until they reached an OD$_{600}$ of 0.3.

3. To induce protein expression, the inducing peptide SppIP was added to a final concentration of 25 ng/ml. The induced bacterial strains were then incubated at 37°C for 3 hours. After the incubation, the induced cultures were chilled on ice for 10 minutes to cease the growth.

4. The bacterial cells were then harvested by centrifugation at 5000 x g for 10 minutes at 4°C. The cells were washed twice with 1X PBS, and the supernatant was removed after each centrifugation. The cell pellets were either used directly for further analyses, or stored at 4°C overnight, or at -20°C (resuspended in PBS containing 20% glycerol).

3.14   Disruption of Bacterial Cells

To analyse protein expression in the induced *L. plantarum* strains, cell free protein extracts were obtained by disrupting the harvested cells with glass beads. The resulting extract contains both intracellular proteins and surface proteins.

Materials:
PBS (section 2.9)
FastPrep tubes
Glass beads (106 microns and finer)
FastPrep® - 24 Tissue and Cell Homogenizer

Procedure:

1. The harvested cell pellet was resuspended in 1 ml 1X PBS and transferred to a FastPrep tube containing glass beads.

2. A FastPrep® - 24 Tissue and Cell Homogenizer was used to shake the FastPrep tubes at 6.5 m/s for 45 seconds.

3. The FastPrep tubes were further centrifuged at 5000 x g for 5 minutes at 4°C.

4. The supernatant containing proteins was transferred to a clean Eppendorf tube and then recentrifuged to remove any remaining glass beads. The supernatant was once again transferred to a new Eppendorf tube to obtain a cell-free protein extract. The extract was separated by protein gel electrophoresis (section 3.15).

3.15   Protein Gel Electrophoresis

Proteins can be separated according to their size by a widely used method called Polyacrylamide Gel Electrophoresis (PAGE). The NuPAGE® Novex Bis-Tris Electrophoresis System (Invitrogen™) used in this study provides precasted Bis-Tris-HCl buffered polyacrylamide mini gels.

The migration velocity is proportional to the ratio between the charges of the protein and its mass. The tertiary structure of the protein will however affect the

migration, so to be able to separate the proteins according to their size, the proteins must be denatured. To denature the proteins, they are mixed and boiled with a sample buffer containing lithium dodecyl sulphate (LDS) and a reducing agent such as dithiothreitol (DTT). The reducing agent breaks the disulphide bonds, while the LDS disrupts non-covalent interactions. LDS also gives the proteins a fixed negative charge per residue.

After boiling the proteins in sample buffer, the negatively charged, denatured proteins can be loaded onto the gel. By applying an electric current, the proteins move towards the positive pole, and in this way the proteins are separated, because smaller ones move faster than larger ones. A suitable protein ladder is used as a reference to determine the size of the proteins. In this study, proteins were visualized by western blotting (see section 3.16).

Materials:
NuPAGE® LDS Sample Buffer (4X) (Invitrogen)
NuPAGE® Reducing Agent (10X) (Invitrogen)
NuPAGE® Novex Bis-Tris gels (Invitrogen)
1X TGS Running Buffer (Bio-Rad)

Procedure:

1. A stock solution of sample buffer and reducing agent was made and kept at room temperature by mixing 2.5 ml 4X sample buffer, 1 ml 10X reducing agent and 1.5 ml dH_2O.
   30 µl of the stock solution was added to 30 µl the cell-free protein extract sample (see section 3.14).

2. The samples were boiled in a water bath at 100°C for 5 minutes to denature the proteins.

3. The gel was placed in the inner chamber of the assembled electrophoresis chamber, and both chambers were filled with 1X TGS running buffer.

4. A reference sample ("protein ladder") and the other samples were loaded onto the gel, and the gel was run at 300 V for 15 minutes.

5. The chambers were disassembled, and the gel was carefully transferred to a plastic tray with dH_2O. The gel was then analysed by western blotting.

3.16   Western Blotting

Western blotting is a method used to detect proteins in a sample by hybridizing these proteins with specific antibodies. The proteins are first separated according to size, using protein gel electrophoresis (see section 3.15). The next step is called "blotting". Here, an electric current is applied, which transfers the proteins from a gel to the membrane. The use of a blocking solution, containing BSA, prevents antibodies from binding unspecifically to the membrane. After blocking the membrane, it is incubated with a primary antibody, which specifically binds to an epitope on the protein of interest. Unbound antibodies are removed by washing steps, before a secondary antibody with specificity for the primary antibody is added. The secondary antibody is labelled with a reporter enzyme, and by adding an appropriate substrate, a detectable signal can be generated. In this study chemiluminescent detection was used. The principle of western blotting is illustrated in Figure 3.1.
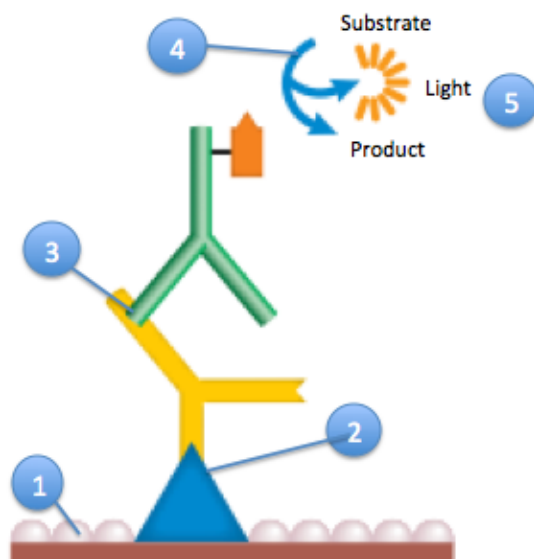

Figure 3.1. *The Principle of Western Blotting.*
(1) The blocking reagent blocks unoccupied sites on the membrane to avoid unspecific binding of antibodies. (2) The primary antibody binds to an epitope on the protein on the membrane. The antibody and the protein are thus hybridized. (3) The secondary antibody, which is linked to a reporter enzyme, binds to a specific part of the primary antibody. (4) The reporter enzyme catalyzes the reaction of an added substrate to a product. (5) The reaction gives a detectable (for example a chemiluminescent) signal. The figure is a modified version of a figure from the BioRad Protein Blotting Guide.

3.16.1   iBlot^{TM} Dry Blotting System

Materials:

iBlot^{TM} Dry Blotting System (Invitrogen) (section 2.7)

      Blotting roller

      iBlot^{TM} Gel Transfer Stack

            iBlot^{TM} Cathode Stack, top

            iBlot^{TM} Anode, bottom

            iBlot^{TM} Disposable Sponge

            iBlot^{TM} Filter Paper

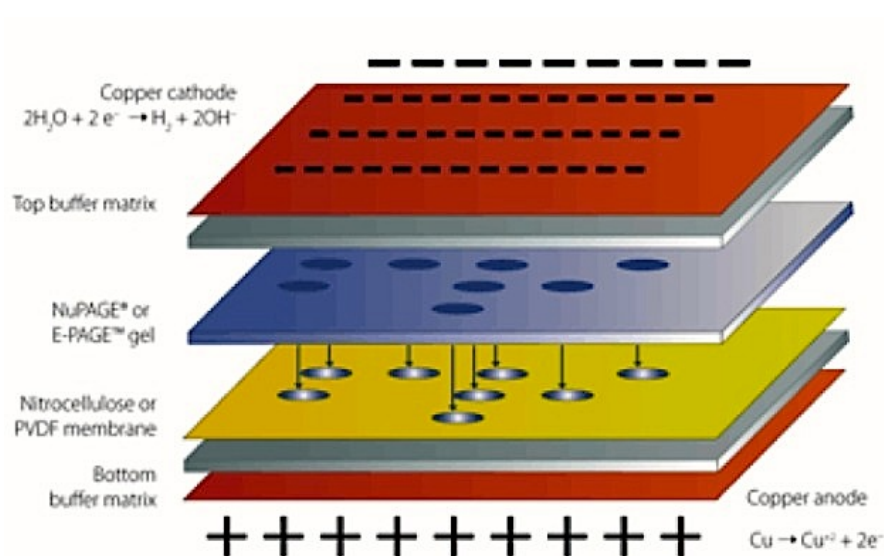   iBlot^{TM} Gel Transfer Device

Figure 3.2. *The iBlot^{TM} Transfer Stack.*
The iBlot^{TM} Transfer Stack consists of three parts. First there is a bottom stack with a copper anode, a gel matrix with anode buffer incorporated, and a nitrocellulose membrane. Secondly, the gel is placed on top of the membrane, and covered by a wet filter paper. The top stack consists of a copper cathode and a gel matrix with cathode buffer incorporated. The figure is taken from the iBlot Dry Blotting System user manual (Invitrogen).

Procedure:

1. The bottom anode stack (as shown in Figure 3.2) was placed in the iBlot^{TM} Gel Transfer Device. The gel, which was kept in a plastic tray with dH_2O, was then placed on top of the nitrocellulose membrane. Air bubbles between the gel and the membrane were removed using a blotting roller.

2. A filter paper was wetted in dH_2O and placed on top of the gel. The top (cathode) stack (Figure 3.2) was then placed on top of the filter paper, with the copper

electrode side facing upwards.

3. A disposable sponge was attached to the lid of the transfer device. The sponge has an aluminium metal contact which ensures proper contact between the electrical contact on the lid and the electrode on the assembled iBlot$^{\text{TM}}$ Gel Transfer Stack.

4. The gel transfer device lid was closed, and the blotting was carried out using program 3 (20 V for 7 minutes).

5. After the blotting, the membrane was transferred to a plastic tray with dH$_2$O. The protein side facing up.

3.16.2 SNAP i.d.® Immunodetection

The SNAP i.d.® Protein Detection System is used to hybridize antibodies to proteins on the membrane and to visualize the hybridized antibodies. This system utilizes vacuum to efficiently transfer the reagents through the membrane, and is thus less time consuming than traditional immunodetection where longer incubation periods are necessary for the reagents to diffuse through the membrane.

Materials:
SNAP i.d.® Protein Detection System (Millipore)
        SNAP i.d. Single Well Blot Holder
        SNAP i.d. Spacer
        SNAP i.d. Blot Roller
TTBS (section 2.9)
TTBS/ 0.5% BSA
Blocking buffer (TTBS/ 1% BSA)
Primary antibody: Anti-myc Mouse-Monoclonal IgG (Life Technologies)
Secondary antibody: Anti-Mouse IgG/HRP Rabbit-Polyclonal (Dako)

Procedure:

1. The blot holder was opened and wetted with dH$_2$O. To prevent movement of the membrane, excess water was removed using the blot roller. The membrane was then transferred from the plastic tray with dH$_2$O to the blot holder, with the protein side facing down. A spacer was placed on top of the membrane, and a blot roller was gently used to remove any air bubbles.

2. The blot holder was closed, and placed in the system chamber of the SNAP i.d. Protein Detection System device.

3. Immediately after adding 30 ml of blocking buffer, the vacuum was turned on. The vacuum was applied until the well had emptied, and was then turned off.

4. A prepared solution of 3 ml TTBS/ 0.5% BSA and 0.6 µl primary antibody (diluted 1:5000) was evenly added to the well, followed by incubation for 10 minutes at room temperature.

5. After the incubation with primary antibodies, the vacuum was turned on while the blot was washed 3 times with 10 ml TTBS. The vacuum was turned off after the last wash.

6. A prepared solution of 3 ml TTBS/ 0.5% BSA and 0.3 µl secondary antibody (diluted 1:10000) was then evenly added to the well and incubated for 10 minutes. The 3 washing steps (step 5) were then repeated.

7. The system chamber was opened and the membrane was removed from the blot holder for incubation with a detection agent.

3.16.3   Chemiluminescent Detection of Proteins

In order to visualize the proteins of interest on the membrane, chemiluminescent detection can be used. The secondary antibody used in the immunoblotting (section 3.16.2) is linked to a horseradish peroxidase (HRP). When a chemiluminescent substrate (SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific)) is added, the HRP will oxidize the substrate, resulting in emission of light. The emitted light can be detected on an x-ray film.

Materials:
SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific)
      Luminol/Enhancer Solution
      Stable Peroxide Solution
CL-Xposure$^{\text{TM}}$ Film
Developer Solution
Fixing Solution

Procedure:

1. The membrane was transferred to an empty tray, and a working solution consisting of 3.3 ml Luminol/Enhancer Solution and 3.3 ml Stable Peroxide Solution was added.

2. After 5 minutes incubation, plastic foil was used to carefully cover the membrane, avoiding air bubbles. The plastic covered membrane was then placed in a light-proof film cassette with the protein side facing up, and then transported to a dark room.

3. A CL-Xposure™ Film was placed on top of the membrane inside the film cassette, and the cassette was quickly closed. The film was incubated with the membrane for 1-15 minutes. The incubation time depended on the strength of the chemiluminescent signal.

4. The film was quickly transferred to a tray containing developer solution and incubated until the bands became visible.

5. The developed film was transferred to a fixing solution, and incubated for at least 2 minutes.

6. The film was washed in a tray with water and then left to air dry.

3.16.4 Stripping and Reprobing of Nitrocellulose Membrane Probed by Western Blotting

The western blotting procedure often needs to be optimized for different blots. If the western blot procedure fails or needs optimization, nitrocellulose membranes can be stripped for hybridized antibodies and reprobed. In this method, the primary- and secondary antibodies are stripped from the membrane by a Western blot stripping buffer containing detergents to dissociate the affinity interactions between the antibodies and the target sample protein. This method can save considerable amounts of time. However, the signals from the reprobed membrane can be decreased due to incomplete antibody removal.

Materials:
Restore™ PLUS Western Blot Stripping Buffer (Thermo Scientific)
TTBS (section 2.9)
PBS (section 2.9)
SNAP i.d.® Protein Detection System (Millipore)
          SNAP i.d. Single Well Blot Holder
          SNAP i.d. Spacer
          SNAP i.d. Blot Roller
Blocking buffer (TTBS/ 1 % BSA)

Procedure:

1. The western blot membrane, previously blocked, probed and treated with Super-Signal® West Pico Chemiluminescent Substrate (Thermo Scientific), was washed for 5 minutes in PBS, and either stripped directly or stored overnight in PBS at 4°C, before starting the stripping procedure.

2. The membrane was placed in a plastic tray and completely covered with Restore Plus Western Blot Stripping Buffer, and incubated for 5-15 minutes at room temperature.

3. The membrane was washed in PBS and then transferred to a blot holder and inserted into the system chamber of the SNAP i.d. Protein Detection System device as described in step 1-2 in the SNAP i.d.® Immunodetection procedure (section 3.16.2).

4. 30 ml blocking buffer was added to the well, followed by incubation for 45 minutes.

5. The membrane was washed with TTBS, and a new immunoblotting and detection procedure could continue as described in sections 3.16.2 and 3.16.3.

3.17   Detection of Proteins Anchored to the Cell Surface Using FITC-Labelled Secondary Antibody

To detect proteins anchored to the surface of *L. plantarum* WCFS1, an anti-mouse IgG secondary antibody, labelled with the fluorochrome fluorescein isothiocyanate (FITC), was used. This antibody binds to the primary mouse antibody that again specifically binds to the protein of interest. The FITC-label can be detected by several methods, such as flow cytometry and fluorescent microscopy, offering direct visualization of the target proteins. In this study, flow cytometry was used.

A flow cytometer analyses cells as they flow through a beam of laser light, one at a time. Light hitting a cell, will be scattered in all directions and is then detected by different detectors. The forward-scattered light (mostly diffracted light) is proportional to the cell-surface area or the size, while side-scattered light (mostly refracted and reflected light) is proportional to the cell granularity. Information about size and granularity makes it possible to differentiate between different cell types and bacteria.

Fluorochrome-labelled cells can be detected using a laser light with a wavelength that excites the specific fluorochrome. When the fluorochrome has been excited by absorbing the laser light, light with a slightly higher wavelength than the absorbed

light, is emitted. The emitted light is then detected. In this way, the flow cytometer will give a signal if the FITC-labelled secondary antibody has bound a primary antibody bound to the protein of interest.

3.17.1   Staining Cells for Flow Cytometry

Materials:
PBS (section 2.9)
PBS/ 2% BSA
Primary antibody: Anti-myc Mouse-Monoclonal IgG (Life Technologies)
Secondary Antibody: Anti-Mouse IgG FITC, Rabbit-Polyclonal (Sigma-Aldrich)

Procedure:

1. *L. plantarum* WCFS1 strains harbouring different plasmids were cultured and induced for 3 hours as described in section 3.13, step 1-3.

2. At the time of harvest, the OD_{600} of each bacterial culture was measured, and the amount needed to obtain 10^8 cells was calculated according to CFU-curves (Appendix 5.6, Figure A.1), which gave the number of bacteria per ml at the given OD_{600}.

3. The calculated amounts of bacterial culture were transferred to Eppendorf tubes and centrifuged at 5000 x g for 3-5 minutes. The supernatant was discarded.

4. The harvested cell pellets were washed twice with 1 ml PBS, and centrifuged at 5000 x g for 3-5 minutes between each wash. The supernatant was discarded after the final wash.

5. The cells were resuspended in 50 µl PBS/ 2% BSA and 0.4 µl primary antibody, and incubated for 40 minutes at room temperature.

6. The samples were washed 5 times with 1 ml PBS/ 2% BSA, and centrifuged at 5000 x g between each wash. After the final wash, all of the supernatant was discarded.

7. The samples were resuspended in 50 µl PBS/ 2% BSA and 0.2 µl FITC-labelled secondary antibody. The samples were then incubated in the dark for 30 minutes.

8. The samples were washed 4 times as described in step 6.

9. The resulting stained pellets were resuspended in 100 µl PBS and kept dark until flow cytometry analysis using the MacsQuant® Analyzer (Miltenyi Biotec) and the MacsQuantify™ software.

3.18   Lysozyme Treatment

A lysozyme is an enzyme that degrades the peptidoglycan in the cell wall of bacteria. When analysing surface display of proteins, the epitope that the antibody binds to, could be embedded in the cell wall. In such cases, the protein may be present, but the epitope is unavailable for antibody-binding, giving false negative results. In an attempt to enhance the fluorescent signal detected by flow cytometry analysis, the lysozyme characteristics of degrading the cell wall and thereby revealing any hidden epitope, were utilized.

Materials:
Lysozyme, 100 mg/ml in dH$_2$O (Sigma-Aldrich)
PBS (section 2.9)

Procedure:

1. *L. plantarum* WCFS1 harbouring different plasmids were harvested and washed as described in section 3.17.1, step 1-4.

2. The cell pellet was resuspended in 300 µl PBS and 200 µl lysozyme solution. The control sample was resuspended in 500 µl PBS. All samples were then incubated in a water bath at 37°C for 30 minutes.

3. The cells were washed 3 times with PBS, and centrifuged at 5000 x g for 3 minutes between each wash. The procedure for staining cells for flow cytometry was continued as described in section 3.17.1, step 5-9.

3.19   *In Vitro* Studies of Transcytosis via M-Cells

Caco-2 cells are continuous lines of heterogeneous human epithelial colorectal adenocarcinoma cells, and are widely used to study the transport of drug candidates across the intestinal epithelial barrier (Lakshmana Rao and Sankar, 2009). In this study, non-polarized, undifferentiated Caco-2 cells were used as an *in vitro* model of M-cells, and different experiments were performed to evaluate the internalization of the recombinant bacteria harbouring different plasmids. In addition, differentiated Caco-2 cells were co-cultured with the continuous human lymphoma cell line, Raji B, to induce expression of a more M-cell-like phenotype (Gullberg et al., 2000). See section 3.19.7 and the introduction (section 1.5) for more details.

3.19.1   Cultivation of Caco-2 Cells

Caco-2 cells are adherent cells, meaning that they grow on a surface, and not in suspension. Trypsin-EDTA is used to detach the cells from the surface when necessary. To keep the cells viable over time, they must be subcultivated into new passages regularly (every 3-5 days). A new passage is made by taking a small number of cells to seed a new culture.

Materials:
Caco-2 Cells (ATCC)
Complete RPMI-1640 medium (section 2.8)
PBS (section 2.9)
Trypsin-EDTA Solution (PAA)
Tissue Culture Flask, 250 ml, 75cm^2 (Becton-Dickinson)
Megafuge 1.0 (Heraeus)

Procedure:
The Caco-2 Cells were grown in a tissue culture flask containing complete RPMI-1640 medium. The flasks were incubated in a humidified incubator at 5% CO_2 and 37°C. The cells were subcultivated into new passages every 3-5 days by the following steps:

1. PBS and complete RPMI-1640 medium was prewarmed in a water bath at 37°C.

2. The growth medium was aspirated, and the adherent cells were washed with PBS.

3. The washing solution was aspirated and 2.5 ml of trypsin-EDTA solution was added. The tissue culture flask was then incubated at 5% CO_2 and 37°C for 5-10 minutes. An inverted microscope was used to see whether the cells had fully detached from the plastic surface.

4. Once the cells were detached from the plastic surface, 8 ml complete RPMI-1640 medium was added. The trypsination reaction is then inhibited because trypsin starts acting on the serum proteins (FBS) in the medium instead of the cells.

5. The cell suspension was transferred to a 15 ml Nunc tube and centrifuged in a Megafuge at 1300 rpm for 10 minutes.

6. The supernatant was discarded and the cell pellet was resuspended in 1 ml complete RPMI-1640 medium. 20 µl of the resuspension was used to count cells with a size between 4 and 12 µm in a Coulter Counter® Z1. The resulting number was used to calculate the amount of resuspended cells needed for the new culture, or for *in vitro* experiments.

7. 1 million cells were transferred to a new tissue culture flask, containing 30-50 ml complete RPMI-1640 medium. The new culture/passage was incubated in a humidified incubator at 5% CO_2 and 37°C.

3.19.2   Cultivation of Raji B Cells

Raji B cells were grown in suspension and were kept viable by subcultivating the cell culture every 5-7 days.

Materials:
Raji B Cells
Complete DMEM medium (section 2.8)
Tissue Culture Flask, 250 ml, 75cm^2 (Becton-Dickinson)
Megafuge 1.0 (Heraeus)

Procedure:
Raji B cells were grown in a tissue culture flask containing complete DMEM medium. The flasks were incubated in a humidified incubator at 5% CO_2 and 37°C. The cells were subcultivated into new passages every 5-7 days by the following steps:

1. Complete DMEM medium was prewarmed in a water bath at 37°C.

2. The Raji-B cell suspension was transferred to a 50 ml Nunc tube, and centrifuged in a megafuge at 1300 rpm for 10 minutes.

3. The supernatant was discarded and the cell pellet was resuspended in 1 ml complete DMEM medium. 20 µl of the resuspension was used to count cells with a size between 3 and 9 µm in a Coulter Counter® Z1. The resulting number was used to calculate the amount of resuspended cells needed for the new culture, or for *in vitro* experiments.

4. 1 million cells were transferred to a new tissue culture flask, containing 40-50 ml complete DMEM medium. The new culture/passage was incubated in a humidified incubator at 5% CO_2 and 37°C.

3.19.3   Test for M-Cell Receptors on Non-Polarized, Undifferentiated Caco-2 Cells

The plasmid constructs used in this study, encode the proteins Invasin from *Yersinia enterocolitica* and FimH from *Salmonella typhimurium* and enteropathogenic *E. coli*. Invasin has high affinity for a subset of β_1-integrin receptors located on the apical side of M-cells (Uliczka et al., 2011), while FimH binds to Glycoprotein 2 (GP2)

receptors (Hase et al., 2009). Research claims that non-polarized, undifferentiated Caco-2 cells express these receptors (Coconnier et al., 1994; Werner et al., 2012), although, in the case of GP2, only in modest amounts.

To verify the presence of these receptors on the Caco-2 cells used in this study, the cells were incubated with receptor specific FITC-labelled antibodies, either in one step, using a FITC-labelled primary antibody, or in two steps, using a primary antibody and subsequently a secondary FITC-labelled antibody. The results were analysed using flow cytometry. The epidermal growth factor receptor (EGFR), is often overexpressed on tumor cells (Shigeta et al., 2013), and is also known to be expressed on Caco-2 cells. The EGFR receptor was used as a positive control in the receptor-test experiment described below.

For the receptor-test and the internalization experiments with Caco-2 cells performed in this study, temperature-responsive Nunc Upcell Surface 24 Multidish plates (Thermo Scientific) were used. The surface of these plates becomes slightly hydrophobic at temperatures above 32°C, allowing cells to adhere. At temperatures below 32°C, the surface becomes hydrophilic, which causes the cells to detach. The temperature-responsive plates were used in order to avoid trypsination, which could remove receptors from the Caco-2 cell surface.

Materials:
Caco-2 cells (ATCC), passage 28
Complete RPMI-1640 medium (section 2.8)
Temperature-Responsive Nunc UpCell Surface 24 MultiDish (Thermo Scientific)
Microwell plates, 96 V-bottom well, without Lid (Thermo Fisher Scientific)
Antibodies (section 2.3):
>   CD29 (anti-β_1-integrin), Mouse-Monoclonal anti-Human, FITC
>   conjugated (Life Technologies)
>   Anti-EGFR, Rabbit-Polyclonal IgG, 200 µg/ml (Santa Cruz Biotechnology)
>   Anti-GP2, Rabbit-Polyclonal IgG, 0.2 mg/ml (Atlas Antibodies)
>   Anti-Rabbit IgG FITC, Goat-Polyclonal (Sigma-Aldrich)

1% (v/v) formaldehyde
Megafuge 1.0 (Heraeus)

Procedure:

1. Caco-2 cells were detached as described in section 3.19.1, and cells were transferred to a temperature-responsive Nunc Upcell Surface 24 Multidish (3 x 10^5 cells resuspended in 1 ml complete RPMI-1640 in each well). The cells were

incubated overnight in a humidified incubator at 5% CO_2 and 37°C.

2. The next day, the medium was removed and 200 µl cold complete RPMI-1640 medium was added. The plate was then placed on ice until the cells had detached from the surface.

3. The detached cells were transferred to two 96 well V-bottom Microwell plates and centrifuged at 1300 rpm for 3 minutes. The supernatant was discarded and the cells were washed 3 times with 200 µl PBS/1% BSA. The cells were centrifuged at 1300 rpm for 3 minutes after each wash. The supernatants were discarded.

4. The cells were resuspended in 100 µl PBS/1% BSA containing the different antibodies as shown in Table 3.7. Non-stained Caco-2 cells (incubated in PBS/1% BSA) were included to adjust the instrument settings of the flow cytometer, and Caco-2 cells only incubated with the secondary Anti-Rabbit IgG FITC antibody were included to correct for unspecific binding. These latter cells only incubated with FITC-conjugated secondary antibody, were incubated in PBS/1% BSA, while the other cells were incubated with primary antibody.

Table 3.7: Test for M-cell Receptors: Receptor name, and antibodies used

Receptor	Primary Antibody	Secondary Antibody
CD29 (β_1-integrin)	15 µl CD29 Mouse-monoclonal anti-Human, FITC conjugated	
GP2	2 µl Anti-GP2 Rabbit-Polyclonal IgG	2 µl Anti-Rabbit IgG FITC
EGFR (positive control)	4 µl Anti-EGFR Rabbit-Polyclonal IgG	2 µl Anti-Rabbit IgG FITC

The non-stained Caco-2 cells and the cells incubated with FITC-labelled anti-β_1-integrin were kept in the same microwell plate, while the other cells which also needed a secondary antibody were kept in another microwell plate.

5. The microwell plates were covered with plastic, and incubated with the primary antibody for 1 hour at 5% CO_2 and 37°C. The microwell plate with non-stained Caco-2 cells and Caco-2 cells incubated with FITC-labelled anti-β_1-integrin were also covered with aluminium foil to protect the fluorochromes from light.

6. After the incubation, the cells were centrifuged and washed 3 times as described in step 3.

7. The pellets of the non-stained Caco-2 cells and the cells incubated with FITC-conjugated anti-β_1-integrin were resuspended in 50 µl 1% formaldehyde and analysed using the MacsQuant® Analyzer (Miltenyi Biotec) and the MacsQuantify™ software.

8. The other cells were resuspended in 100 µl PBS/1% BSA containing Anti-Rabbit FITC-labelled IgG. The microwell plate were covered in plastic and aluminium foil, and incubated at 37°C for 30 minutes.

9. The microwell plate was then centrifuged and washed 3 times as described in step 3.

10. The cell pellets were resuspended in 50 µl 1% formaldehyde and analysed using the MacsQuant® Analyzer (Miltenyi Biotec) and the MacsQuantify™ software.

3.19.4   Verifying Internalization - The Gentamicin Survival Assay

A method to analyse the internalization of bacteria by Caco-2 cells, is the so-called gentamicin survival assay. The procedure used in this study was based on the method developed by Innocentin et al. (2009). In the gentamicin survival assay, Caco-2 cells are first incubated with bacteria, and then gentamicin is added to kill bacteria that have not been internalized. Triton is then added to lyse the Caco-2 cells, releasing internalized bacteria. The lysed cell solution is plated on agar plates, and the number of internalized bacteria is counted.

Materials:
Tissue Culture Plates, 24 well (Corning Inc) Complete RPMI-1640 medium (section 2.8)
Gentamicin, 10 mg/ml (Sigma-Aldrich)
PBS/0.1% triton
MRS agar plates containing 10 µg/ml Erythromycin

Procedure:

1. Caco-2 cells were detached as described in section 3.19.1, and cells were transferred to a 24 well tissue culture plate (2×10^5 cells resuspended in 1 ml complete RPMI-1640 in each well). The cells were incubated overnight in a humidified incubator at 5% CO_2 and 37°C.

2. *L. plantarum* WCFS1 harbouring different plasmids were cultured, induced, harvested and washed as described in section 3.17.1, step 1-4. Here, 2×10^9 bacterial cells were harvested (calculated according to CFU-curves, Appendix 5.6, Figure A.1) and resuspended in 1 ml PBS.

3. 2×10^8 bacterial cells was to be added to 2×10^5 Caco-2 cells, and therefore, to obtain the correct number of bacteria, 200 µl of the 1 ml resuspension containing 10^9 bacterial cells, was centrifuged at 5000 x g for 3-5 minutes. The supernatant was discarded, and the bacterial cell pellets were resuspended in 1 ml complete RPMI-1640 medium.

4. To verify that the 200 µl bacterial cell suspensions actually contained 2×10^8 cells, another 200 µl of the 1 ml bacterial resuspension containing 10^9 bacterial cells, was diluted in series in Ringers solution, and 100 µl from 10^{-5} and 10^{-6} dilutions was dispersed on MRS agar plates containing the appropriate antibiotics.

5. The medium from the wells containing Caco-2 cells was aspirated, and the bacterial cell suspensions were added to each well containing Caco-2 cells.

6. The tissue culture plate containing Caco-2 cells and bacteria was incubated in a humidified incubator at 5% CO_2 and 37°C for 2-3 hours.

7. After the incubation, the cells were washed 4 times with 1 ml complete RPMI-1640 medium. 1 ml of complete RPMI-1640 medium containing 400 µg/ml gentamicin was then added to each well. The cells were incubated with gentamicin in a humidified incubator at 5% CO_2 and 37°C for 2 hours.

8. After the incubation with gentamicin, the cells were washed 3 times with 1 ml complete RPMI-1640 medium. 100 µl of the last washing solution was plated on MRS agar plates containing the appropriate antibiotics to check for surviving bacteria.

9. The Caco-2 cells were then lysed by adding 300 µl PBS/0.1% triton to each well, and an inverted light microscope were used to confirm that the cells were lysed. 2 replicates of 100 µl of the triton solution were plated directly on MRS agar plates containing the appropriate antibiotics, whereas the last 100 µl was diluted 10 times in Ringers solution before plating.

10. The MRS agar plates were incubated at 37° for 1-2 days, and then the bacterial colonies were counted.

3.19.5   Verifying Internalization - Uptake of Stained Bacteria

L. plantarum WCFS1 harbouring different plasmids were stained with a CF^{TM}633 (Succinimidyl Ester) dye and then co-incubated with Caco-2 cells. CF^{TM}633 binds to amine groups through its succinimidyl ester groups, forming a stable amide linkage. The dye can be excited by a 633 nm or 635 nm laser light, and is quite photostable.

Caco-2 cells with internalized stained bacteria were detected using flow cytometry.

Lactococcus lactis expressing Fibronectin binding protein A (FnBPA) was used as a positive control for the Caco-2 internalization assay. FnBPA binds to fibronectin, fibrinogen and elastin in the extracellular matrix and connective tissues of the Caco-2 cells. FnBPA is known to mediate adhesion and bacterial uptake into nonphagocytic cells (Innocentin et al., 2009).

Materials:
Sodium hydrogen carbonate (Merck), sterile filtrated
CF^{TM}633 (Succinimidyl Ester), 10 mM (Sigma-Aldrich)
Caco-2 cells (ATCC), passage 29-39
Complete RPMI-1640 medium (section 2.8)
Temperature-Responsive Nunc UpCell Surface 24 MultiDish (Thermo Scientific)
Microwell plates, 96 V-bottom well, without Lid (Thermo Fisher Scientific)
1% (v/v) formaldehyde
Megafuge 1.0 (Heraeus)

Procedure:

1. Caco-2 cells were detached as described in section 3.19.1, and cells were transferred to a temperature-responsive Nunc Upcell Surface 24 Multidish (3 x 10^5 cells resuspended in 1 ml complete RPMI-1640 in each well). The cells were incubated overnight in a humidified incubator at 5% CO_2 and 37°C.

2. *L. plantarum* WCFS1 strains harbouring different plasmids were cultured, induced and harvested as described in section 3.17.1, step 1-3. Here, 2 x 10^8 bacterial cells were harvested (the amount of bacterial culture needed to obtain the correct number of cells was calculated according to CFU-curves, Appendix 5.6, Figure A.1). *L. lactis* was cultured as described in section 3.1. 100-500 µl of culture with an OD$_{600}$ of 1 was harvested (Katarzyna Kuczkowska, personal communication).

3. The harvested cell pellets were washed twice with 1 ml 0.1 M sodium hydrogen carbonate, and centrifuged at 5000 x g for 3-5 minutes between each wash. The supernatant was discarded after the final wash.

4. The cell pellets were resuspended in 1 ml sodium hydrogen carbonate and 1 µl CF^{TM}633 was added for every ml of sample.

5. The samples were incubated in the dark for 1 hour at room temperature.

6. The samples were centrifuged at 5000 x g for 3-5 minutes. The supernatants were discarded and the cell pellets were washed 4 times with 1 ml PBS. The supernatants were discarded and the cell pellets were resuspended in 0.5 ml PBS or complete RPMI medium without antibiotics.

7. The Caco-2 cells in the temperature-responsive Nunc Upcell Surface 24 MultiDish were gently washed by first removing the growth medium and then adding 1 ml PBS/1% BSA. The BSA is needed to keep the cells attached to the bottom of the wells and to reduce unspecific binding. The washing step was repeated once.

8. The 1 ml resuspension containing 2×10^8 CF^{TM}633-stained bacterial cells were added to the 3×10^5 Caco-2 cells and incubated in a humidified incubator at 5% CO_2 and 37°C for 2 hours.

9. After the incubation, the temperature-responsive multidish was placed on ice until the cells had detached from the surface (the principle for cell detachment from the temperature-responsive multidish is described in section 3.19.3).

10. The cell-bacteria suspension was transferred to a 96 well V-bottom Microwell plate and centrifuged at 1300 rpm for 3 minutes. The supernatant was discarded and the cells were washed 3 times with $200 \, \mu l$ PBS/1% BSA. The cells were centrifuged at 1300 rpm for 3 minutes after each wash. The supernatants were discarded.

11. The cell pellets were resuspended in $50 \, \mu l$ 1% formaldehyde and analysed using the MacsQuant® Analyzer (Miltenyi Biotec) and the MacsQuantify^{TM} software.

3.19.6   Competition Assay for Caco-2 Internalization

Competition assays are widely used as a tool to determine binding affinities, binding specificities, or the concentration of receptors or antigens. In this study, a competition assay was set up to analyse the binding specificity/affinity between the receptors (β_1-integrin and GP2) and the proteins displayed on the lactobacilli. Before incubating the Caco-2 cells with the stained bacteria (section 3.19.5), antibodies with specificity for the mentioned receptors, were added. In theory, the antibodies will bind to the receptors, and thereby block the bacteria from binding.

Materials:
Sodium hydrogen carbonate (Merck), sterile filtrated
CF^{TM}633 (Succinimidyl Ester), 10 mM (Sigma-Aldrich)
Caco-2 cells (ATCC), passage 29-39
Complete RPMI-1640 medium (section 2.8)

Temperature-Responsive Nunc UpCell Surface 24 MultiDish (Thermo Scientific)
Microwell plates, 96 V-bottom well, without Lid (Thermo Fisher Scientific)
Antibodies (section 2.3):

      CD29, Mouse-Monoclonal anti-Human, FITC conjugated, 1 mg/ml
      (Life Technologies)

      Anti-GP2, Rabbit-Polyclonal IgG, 0,2 mg/ml (Atlas Antibodies)

1% (v/v) formaldehyde
Megafuge 1.0 (Heraeus)

Procedure:

The procedure was performed as described in section 3.19.5 for Caco-2 Internalization with the following changes:

1. When the bacteria were harvested and washed (section 3.19.5, step 2-3), the cell pellets were kept on ice.

2. The RPMI-1640 medium were removed from the temperature-responsive Nunc Upcell Surface 24 MultiDish, and 1 ml complete RPMI-1640 containing 4 µl antibody solution was added to each well with Caco-2 cells.

3. The Caco-2 cells were incubated with antibodies in a humidified incubator at 5% CO_2 and 37°C for 2 hours.

4. In the meantime, the washed bacteria were incubated with $CF^{TM}633$-dye for 1 hour and washed with PBS as described in section 3.19.5, step 6-8. The pellets were resuspended in 200 µl PBS.

5. The antibody-containing RPMI-1640 medium was removed from the Caco-2 cells, and 200 µl PBS/1% BSA was added before the plate was placed on ice to detach the cells from the well surface (the principle for cell detachment from the temperature-responsive multidish is described in section 3.19.3). The cells were then transferred to a 96 well V-bottom microwell plate and centrifuged at 1300 rpm for 3 minutes.

6. The supernatants were discarded and the cells were washed 3 times with PBS/1% BSA. When the supernatants had been discarded after the final wash, the Caco-2 cells were resuspended with the corresponding 200 µl stained bacterial-solution from step 4. The 96 well V-bottom microwell plate was then placed in a humidified incubator at 5% CO_2 and 37°C for 2 hours.

7. After the incubation, the Caco-2 cells and bacteria were centrifuged at 1300 rpm for 3 minutes. The supernatants were discarded and the cell pellets were washed 3 times with PBS/1% BSA.

8. After the final wash, the resulting pellets were resuspended in 50 µl 1% formalde-
   hyde and analysed using the MacsQuant® Analyzer (Miltenyi Biotec) and the
   MacsQuantify™ software.

3.19.7   Transcytosis via M-Cell-Like Cells

A method for growing cells with M-cell characteristics were developed by Kernéis
et al. (1997) and further developed by Gullberg et al. (2000). Caco-2 cells are first
grown for 14-16 days on semi permeable filter inserts (see section 1.5, Figure 1.6).
This time allows the Caco-2 cells to reach confluence, and thereby polarize and
spontaneously differentiate into monolayers with characteristics similar to the small
intestinal epithelium. Raji B cells are then co-incubated with the differentiated
Caco-2 cells, causing conversion of the Caco-2 cells into cells with an M-cell-like
phenotype (see introduction, section 1.5 for more details). In this study, M-cell-like
cells were used to analyse transcytosis of *L. plantarum* with surface displayed FimH
and Invasin via M-cells *in vitro*.

To ensure that translocation of the recombinant bacteria actually occurred via tran-
scytosis through the M-cell-like cells, and not through leaks in the cell monolayer,
the transepithelial electrical resistance (TEER) was measured, using an EVOM2
voltohmeter. The TEER represents the resistance to ion flow across the cell mono-
layer on the filter insert, and is used as a measure of cell layer integrity or confluence.
TEER values over 200 Ω x cm^2 are considered high, and should ensure a confluent
cell layer (Wilhelm et al., 2011).

In this study, wild type enteropathogenic *Escherichia coli* (EPEC) 0127:H7 E2348/69
was used as a positive control as it is known to be transcytosed through M-cell-like
cells (Tahoun et al., 2011). Notably, the gene sequence encoding FimH used in the
plasmid pLp_0373FimH_E.c.cwa2 used in this study, was taken from this EPEC
strain. Therefore, the EPEC strain is also a control for the functionality of the
FimH protein.

Materials:
Caco-2 cells (ATCC), passage 28-32
Raji B cells, passage 4-7
Cell Culture Filter Inserts, 3 µm pore size (Becton-Dickinson)
Tissue Culture Plates, 6 and 24 wells (Corning Inc)
Complete DMEM medium (section 2.8)
Complete DMEM medium without antibiotics
MRS agar plates, containing 10 µg/ml Erythromycin

Procedure:

1. Cell culture filter inserts were placed in a 6 well tissue culture plate (see section 1.5, Figure 1.6).

2. Caco-2 cells were subcultivated as described in section 3.19.1, and 3 x 10^5 cells resuspended in 600 µl complete DMEM medium were added to each cell culture filter insert. 3 ml complete DMEM medium was added to each well.

3. The plate with filter inserts were incubated in a humidified incubator at 5% CO_2 and 37°C for 14-16 days to polarize and differentiate. The medium in both the wells and in the filter inserts was changed every second day.

4. The confluence of the cell monolayers was measured by the transepithelial electric resistance (TEER) in the filter inserts, using an EVOM2 voltohmeter. After 14-16 days, when the resistance was between 2-300 Ω x cm^2, the Caco-2 cells were considered ready for co-culturing with Raji B cells.

5. Raji B cells were detached as described in section 3.19.2, and 5 x 10^5 cells resuspended in 700 µl complete DMEM were added to different wells in a 24 well tissue culture plate.

6. The medium in the filter inserts and wells in the 6 well tissue culture plate was removed and the filter inserts were carefully transferred to the 24 well tissue culture plate containing Raji B cells, using sterile tweezers.

7. The filter inserts were filled with 600 µl complete DMEM medium, and the Caco-2 cells were incubated with Raji B cells in a humidified incubator at 5% CO_2 and 37°C for 5 days. The medium in the filter inserts was changed every day, and extra medium was added to the Raji-B cells.

8. When the co-culture had been incubated for 5 days, *L. plantarum* WCFS1 harbouring different plasmids were cultured, induced and harvested as described in section 3.17.1, step 1-3. Here, 2 x 10^7 cells were harvested (calculated according to CFU-curves, Appendix 5.6, Figure A.1). EPEC was cultured in liquid Brain-Heart-Infusion (BHI) medium without antibiotics, and incubated overnight at 37°C with shaking. 2 x 10^7 cells were harvested (Appendix 5.6, Figure A.2).

9. The bacterial cell pellets were washed 3 times with 1 ml DMEM medium without antibiotics. The resuspensions were centrifuged at 5000 x g for 3-5 minutes. After the final supernatants had been discarded, the pellets were resuspended in 1 ml DMEM without antibiotics.

10. To determine the actual number of bacteria used, 100 µl of the bacterial suspension was diluted in series in Ringers solution and 100 µl from 10^{-3} and 10^{-4} dilutions was dispersed on MRS agar plates containing the appropriate antibiotics. EPEC was dispersed on BHI plates without antibiotics.

11. The TEER in the Caco-2 cells on the filter inserts was measured to ensure that the resistance was still 2-300 Ω x cm^2. The media were then removed from all filter inserts and the filter inserts were transferred to a new well containing fresh medium to wash the cells.

12. After this washing step, the filter inserts were transferred to yet another well containing 600 µl fresh medium. 600 µl of each bacterial suspension was added to the filter inserts, and the cell mixture was incubated in a humidified incubator at 5% CO_2 and 37°C for 45-60 minutes.

13. The filters were then carefully removed, and the media in the well (containing transcytosed bacterial cells) were either dispersed directly, or diluted 10 times in Ringers solution and then dispersed on MRS agar plates containing the appropriate antibiotics. EPEC was dispersed on BHI plates without antibiotics.

14. All the MRS and BHI plates were incubated at 37° for 1-2 days before counting of bacteria.

4 Results

4.1 Plasmid Constructs

The protein FimH from *Salmonella typhimurium* and enteropathogenic *Escherichia coli* and the protein Invasin from *Yersinia enterocolitica* were selected to target *Lactobacillus plantarum* to Peyer's patches through M-cells. These proteins are known to promote transcytosis to Peyer's patches by binding specific receptors on the apical side of M-cells (Kisiela et al., 2011; Uliczka et al., 2011). Derivatives of the pSIP401 expression vector originally developed by Sørvig et al. (2003) were used to make plasmid constructs for production of the proteins in *L. plantarum*, using different anchors to bind the proteins to the surface. The sequences were codon optimized for *L. plantarum*, and for all the plasmid constructs, a sequence encoding a *myc*-tag was located between the sequence encoding the protein of interest and the anchor sequence. The *myc*-tag was used for immunodetection of the protein.

T4 ligation (section 3.8.1) and in-fusion cloning (section 3.8.2) were used to construct two of the plasmids used in this study. They were first made in *E. coli* TOP10 cells, and then transformed into *L. plantarum* (section 3.11). The construction of the two plasmids is described in more detail below.

4.1.1 Construction of a plasmid for Cell Wall Anchoring of FimH from *Salmonella typhimurium*

The adhesive domain of FimH from *S. typhimurium* (*St*FimH) is located at the N-terminus of the proteins, and a plasmid for C-terminal cell wall anchoring was therefore constructed to enable the adhesive domain of FimH to protrude into the extracellular environment. The gene sequences encoding *St*FimH domains and a *myc*-tag were obtained by enzyme digestion of pUC57FimH_S.t. using SalI and MluI. The same enzymes were also used for digestion of pLp_0373OFAcwa2 (Figure 4.1), so that OFA could be exchanged by *St*FimH and *myc* using the Quick T4 ligation kit. The resulting plasmid pLp_0373FimH_S.t.cwa2 (Figure 4.1), encodes C-terminal anchoring of the *St*FimH protein through a LPxTG domain-based anchor called Cwa2 (see introduction, section 1.3 for more details).
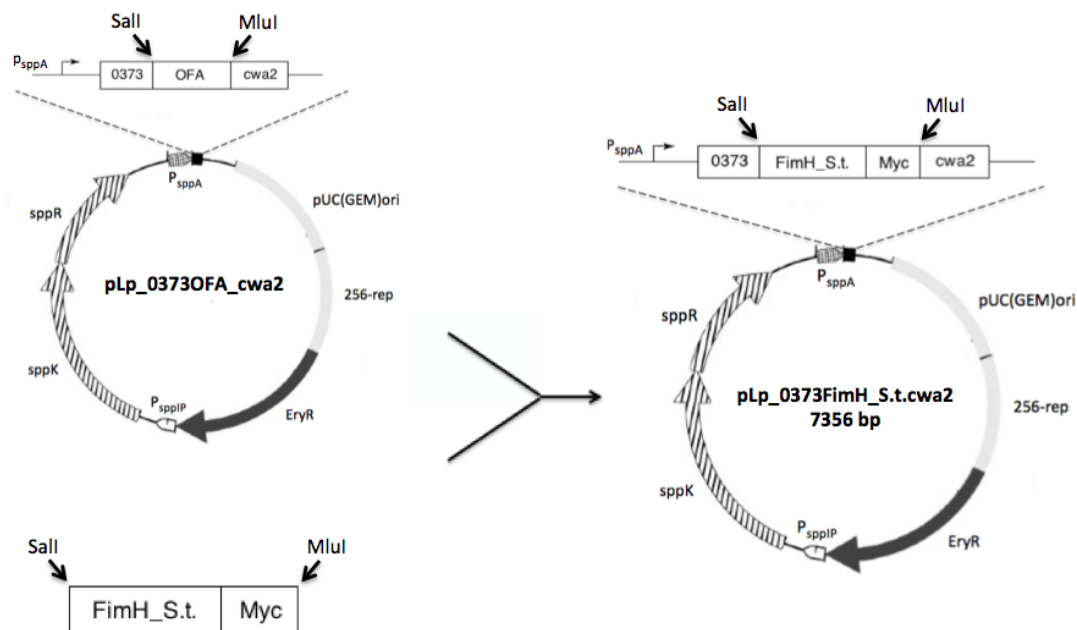
Figure 4.1. *Schematic Representation of the Construction of pLp_0373FimH_S.t.cwa2.* *The figure illustrates the ligation of the gene sequences encoding a myc-tag and StFimH with a vector containing the Lp0373 signal sequence and a C-terminal cell wall anchor sequence. The sequence lengths shown do not display the correct proportions. The figure is a modified version of a figure from Sørvig et al. (2005).*

4.1.2   Construction of a Plasmid for Lipo-Anchoring of Invasin from *Yersinia enterocolitica*

As opposed to FimH, the adhesive domain of Invasin from *Y. enterocolitica* (*Ye*Invasin) is located C-terminally, and a plasmid for N-terminal anchoring, through a lipoprotein anchor, was therefore constructed. The gene sequences encoding *Ye*Invasin and a *myc*-tag was amplified by PCR with the primer pair 1261Invasin_F and 1261Invasin_R (Table 2.1), using pUC57Invasin_Y.e. as a template. The PCR fragment was subsequently In-fusion cloned (section 3.8.2) into SalI/EcoRI digested pLp_1261CCL3gag (Katarzyna Kuczkowska, unpublished) (Figure 4.2), yielding pLp_1261Invasin_Y.e.

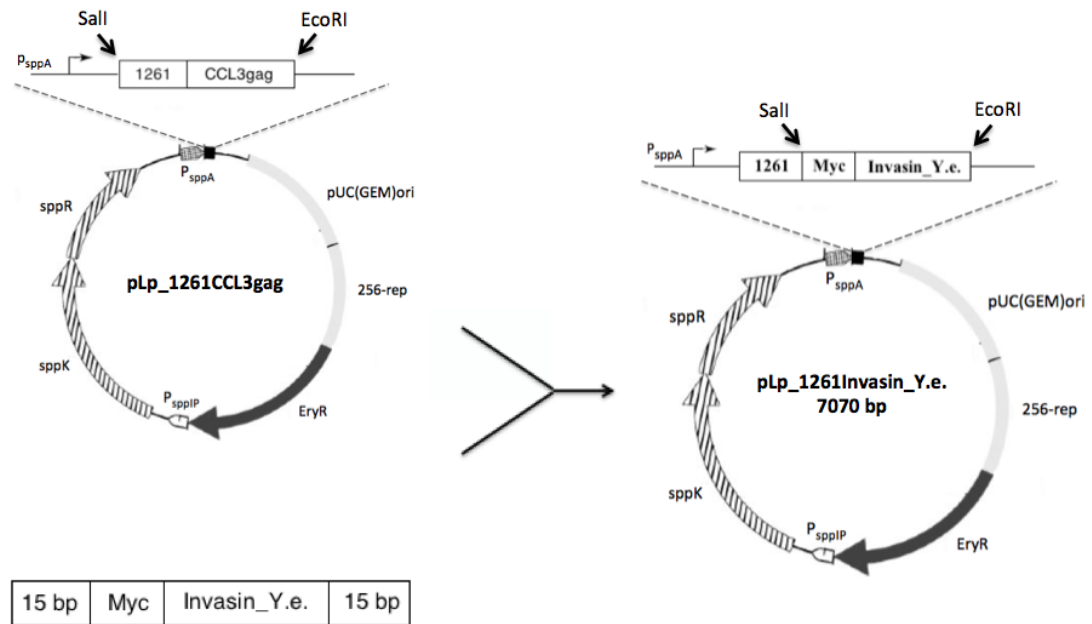**Figure 4.2.** *Schematic Representation of the Construction of pLp_ 1261_ Y.e.*
The figure illustrates in-fusion cloning of PCR amplified gene sequences
encoding a myc-tag and YeInvasin, with a plasmid vector containing an
N-terminal Lp1261 lipoprotein anchor. The PCR amplified gene sequence
has a 5' and 3' 15 bp overlap complementary to the vector. The restriction
sites SalI and EcoRI are included in the 15 bp, respectively. The sequence
lengths shown do not display the correct proportions. The figure is a
modified version of a figure from Sørvig et al. (2005).

4.1.3   The Plasmid Constructs used in This Study

In addition to the plasmids constructed in this study, three other plasmids were
included for further analysis, one for C-terminal cell wall anchoring of FimH from
enteropathogenic *E. coli* (pLp_0373FimH_E.c.cwa2) and two more plasmids for
production of Invasin from *Y. enterocolitica*. The two additional Invasin plasmids
were meant to lead to anchoring of Invasin N-terminally through a LysM-domain
(pLp_3014Invasin_Y.e.), or through another lipoprotein anchor (pLp_1452Invasin-
_Y.e.). Lp1261 and Lp1452 are both lipoprotein anchors, but the signal sequences
differ. A sequence encoding a *myc*-tag was located between the sequence for the
protein of interest and the anchor sequence. The five plasmid constructs used in
this study are summarized in Table 4.1 below.

Table 4.1: The Plasmid Constructs Used in This Study

Plasmid	Description	Source
pLp_0373FimH_S.t.cwa2	pSIP401-derivative for production of *St*FimH with the Lp0373 signal sequence and a C-terminal cell wall anchor	This work
pLp_0373FimH_E.c.cwa2	pSIP401-derivative for production of *Ec*FimH with the Lp0373 signal sequence and a C-terminal cell wall anchor	Katarzyna Kuczkowska, IKBM (unpublished)
pLp_1261Invasin_Y.e.	pSIP401-derivative for production of *Ye*Invasin with an N-terminal Lp1261 lipoprotein anchor	This work
pLp_1452Invasin_Y.e.	pSIP401-derivative for production of *Ye*Invasin with an N-terminal Lp1452 lipoprotein anchor	Lise Øverland, IKBM (unpublished)
pLp_3014Invasin_Y.e.	pSIP401-derivative for production of *Ye*Invasin with an N-terminal 3014 lysM domain-based anchor	Lise Øverland, IKBM (unpublished)

For all plasmids, a sequence encoding a *myc*-tag was located between the protein and the anchoring sequence.

4.2 Growth Characteristics of *L. plantarum* Harbouring Different Plasmids

Cloning of foreign plasmids may affect the growth of the host organism, because increased metabolic energy is needed for maintenance and function of the plasmid (Fakruddin et al., 2013). Furthermore, the expressed proteins, the combination of the protein sequence and the signal peptide, as well as secretion stress can also affect the growth (Gellissen, 2005; Mathiesen et al., 2008, 2009). Such decreases in growth rate of *L. plantarum* harbouring different plasmid constructs for expression of heterologous proteins have previously been shown by Fredriksen et al. (2012), Tjåland (2011) and Øverland (2013). In order to investigate whether, and to what extent, the different recombinant plasmids used in this study affected the growth rates of *L. plantarum*, growth analyses were performed.

Overnight cultures of *L. plantarum* harbouring the different plasmids were grown to OD$_{600}$ 0.3, and induced with inducer peptide pheromone to a final concentration of

25 ng/ml (see section 3.13 for details). The OD_{600} of the cultures was measured and recorded every hour until the growth ceased (Figure 4.3). Three replicates of both induced and non-induced cultures were included for each plasmid. *L. plantarum* harbouring an empty vector with no target gene (pEV), was included as a control.

The growth curves (Figure 4.3) demonstrated a decrease in growth for all the recombinant strains compared to the control strain (pEV). Although the difference between the strains was quite small, *L. plantarum* harbouring the plasmid for cell wall anchoring of FimH from enteropathogenic *E. coli*, seemed to grow slightly slower than the other strains.

Non-induced cultures (data not shown) showed little difference in growth rate compared to the control strain (pEV).
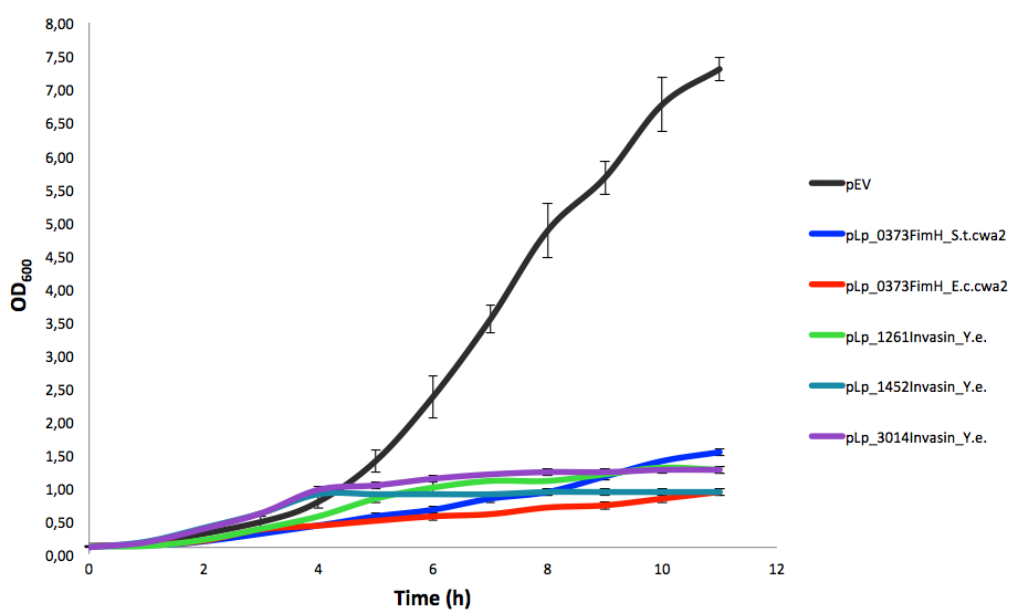

Figure 4.3. *Growth Curves for L. plantarum Harbouring Different Plasmids. The cultures were induced with 25 ng/ml SppIP at OD_{600} 0.3, at time points between 2-2.5 hours, and incubated at 37°C. The curves shown are the average of triplicates.*

4.3   Western Blot Analysis of Protein Production

To investigate whether the different recombinant *L. plantarum* strains produced protein after induction, western blotting was performed as described in section 3.16. The induced cultures were harvested 3 hours after induction and cell-free protein extracts were used for analysis.

Figure 4.4 and 4.5 show the western blots of the cell-free protein extracts of the *L. plantarum* strains harbouring the different plasmids. The bands obtained, showed that all strains had produced proteins after 3 hours induction. When comparing the bands representing the FimH proteins (Figure 4.4, lane 4 and 6) to the MagicMark Protein ladder (lane 1), the two proteins seemed to be larger than the theoretical size indicated in the figure caption. Similar results have previously been seen for cell wall anchored proteins (Fredriksen et al., 2010; Øverland, 2013). The western blots also showed an extra band of a larger size both for pLp_0373FimH_S.t.cwa2 (Figure 4.4, lane 6), and for pLp_3014Invasin_Y.e. (Figure 4.5, lane 4).

The strain harbouring pEV and the non-induced strains did not show any bands on the western blots.
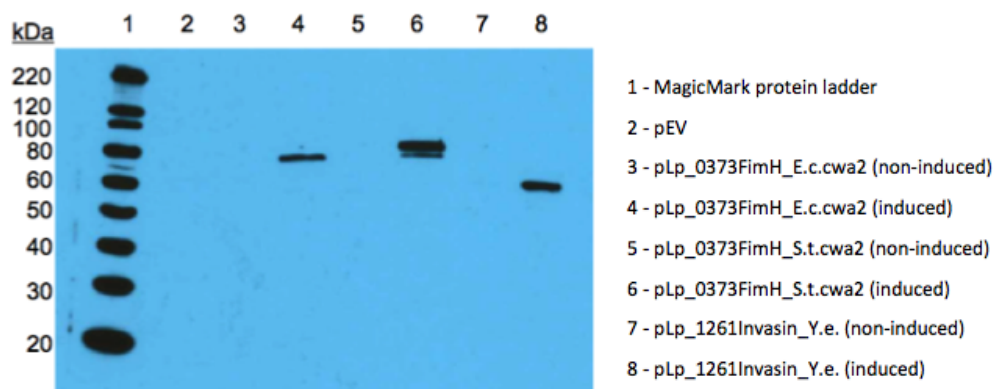

Figure 4.4. *Western Blot Analysis of Cell-Free Protein Extracts from L. plantarum Strains Harbouring Different Plasmids.* *The blot shows cell-free protein extracts from L. plantarum harbouring the plasmids: pEV (empty vector), pLp_0373FimH_E.c.cwa2 (∼61 kDa), pLp_0373FimH_S.t.cwa2 (∼65 kDa) and pLp_1261Invasin_Y.e. (∼51 kDa). The theoretical molecular masses of the recombinantly produced proteins are indicated in parentheses.*
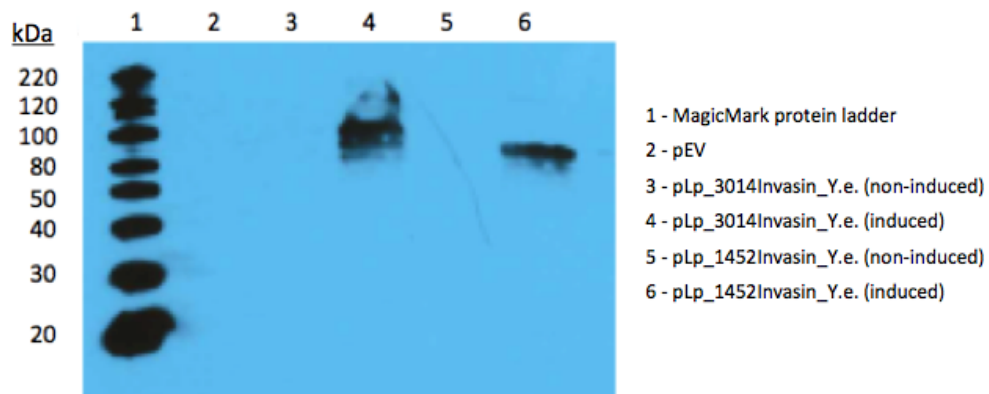
Figure 4.5. *Western Blot Analysis of Protein Extracts from L. plantarum Strains Harbouring Different Plasmids. The blot shows cell-free protein extracts from L. plantarum harbouring the plasmids: pEV (empty vector), pLp_3014Invasin_Y.e. (∼64 kDa) and pLp_1452Invasin_Y.e. (∼58 kDa). The theoretical molecular masses of the recombinantly produced proteins are indicated in parentheses.*

4.4   Detection of Surface Displayed Proteins in *L. plantarum* Using Flow Cytometry

To investigate whether FimH and Invasin were displayed on the surface of *L. plantarum*, induced bacteria were analysed using flow cytometry (section 3.17.1). The recombinant *L. plantarum* strains were induced and harvested 3 hours after induction, and the bacterial cells were stained with mouse monoclonal anti-myc primary antibody. Rabbit polyclonal anti-mouse FITC-con- jugated IgG secondary antibody was further added to hybridize the already bound primary antibody.

Figure 4.6 shows flow cytometry histograms of the induced recombinant *L. plantarum* strains. Increase of the fluorescence signal intensity was observed for pLp_-0373FimH_E.c.cwa2 (red) and pLp_0373FimH_S.t.cwa2 (blue), compared to the pEV as shown by positive shifts of the peaks in Figure 4.6a. The strain harbouring pLp_1452Invasin_Y.e. (Figure 4.6b) also showed an increased signal. This indicates that the myc-tag, located between the anchor and the protein, was present for antibody binding on the surface. The bacterial cells harbouring pLp_1261Invasin_Y.e. (Figure 4.6a, light green) and pLp_3014Invasin_Y.e. (Figure 4.6b, purple) did not give a clear shift in fluorescent signal intensity. However, the histograms showed a small additional peak for the two strains, with higher fluorescent signal intensity, indicating that some proteins were detected on the surface.
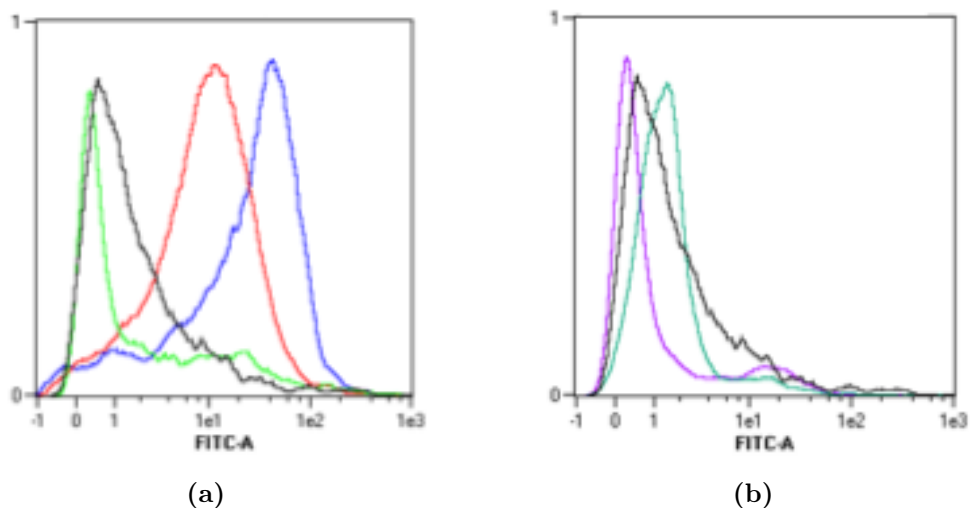
(a)   **(b)**

Figure 4.6. *Flow Cytometry Histograms of FITC-Stained L. plantarum Cells Harbouring Different Plasmids. The figure shows flow cytometry histograms of detected surface displayed heterologous proteins in induced recombinant L. plantarum harbouring the following plasmids: Panel (a): pEV (black), pLp_1261Invasin_Y.e. (light green), pLp_0373FimH_E.c.-cwa2 (red) and pLp_0373FimH_S.t.cwa2 (blue). Panel (b) pEV (black), pLp_3014Invasin_Y.e. (purple) and pLp_1452Invasin_Y.e. (green). The x-axis shows the fluorescence intensity.*

The MacsQuantify™ software used to analyse the flow cytometry results offers various functions to analyse the data. One option is to view the results in dotplots instead of histograms, which give more information about the cell population, and not only about the cells that give fluorescent signals. Dotplots of the strains harbouring pLp_1261Invasin_Y.e. and pLp_3014Invasin_Y.e., i.e. the strains not showing clear shifts in Figure 4.6, were used to get a deeper look into the fluorescent population. The dotplots (Figure 4.7) confirmed that a small part of the bacterial cell population had bound antibodies compared to the strain harbouring pEV.
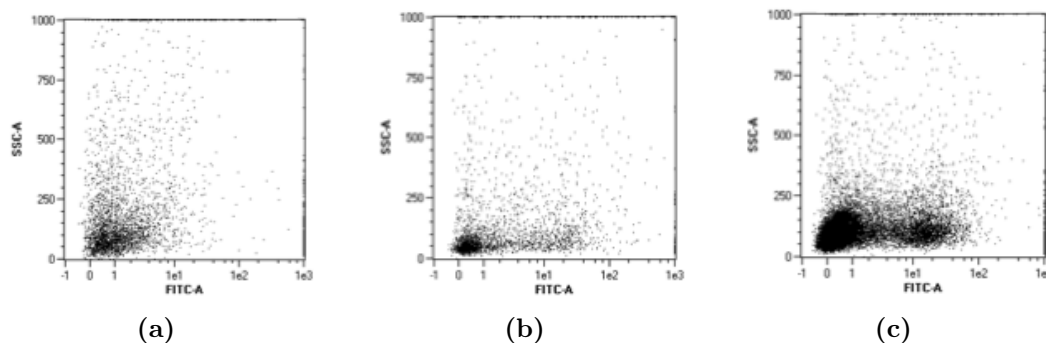
(a)   (b)   (c)

Figure 4.7. *Flow Cytometry Dotplots of FITC-Stained L. plantarum Cells Harbouring Different Plasmids. The figure shows flow cytometry dotplots of detected surface displayed hetereologous proteins in induced recombinant L. plantarum harboring the following plasmids: pEV (a), pLp_ 1261Invasin_ Y.e. (b) and pLp_ 3014Invasin_ Y.e. (c). The x-axis shows the fluorescence intensity; the y-axis shows side-scattered cells (i.e. cell granularity).*

One possible explanation for the low fluorescent signal intensity for the strains harbouring pLp_1261Invasin_Y.e. and pLp_3014Invasin_Y.e. could be that the myc-tag was hidden in the bacterial cell wall, and was therefore less available for antibody binding. To investigate whether the proteins were hidden in the cell wall, the bacterial cells were treated with lysozyme, which partially breaks down the cell wall, and may reveal the myc-tag. The lysozyme treatment (section 3.18) was performed five times for the strain harbouring pLp_1261Invasin_Y.e., and increased fluorescent signal intensities (indicating surface display of Invasin) were detected in two of the attempts (results not shown).

Flow cytometry analyses were repeated five times for the strains harbouring pLp_-1261Invasin_Y.e., pLp_0373FimH_E.c.cwa2 and pLp_0373FimH_S.t.cwa2, and once for the strains harbouring pLp_3014Invasin_Y.e. and pLp_1452Invasin_Y.e. Analysis of the two latter strains should be repeated further to confirm the results.

As the recombinant bacteria are intended for use in an oral vaccine, easy storage of induced and harvested bacteria is important. Therefore, bacterial cells were kept overnight both at 4°C (cell pellet) and at -20°C (resuspended in 20% glycerol in PBS), and subsequently analysed in order to confirm that the proteins were still displayed on the surface. The flow cytometry results (Appendix 5.6) showed little difference between the stored bacteria and fresh bacteria harvested the same day, although the strain harbouring pEV showed more unspecific antibody-binding. Similar results were obtained for cells stored at -20°C for one week. This indicates that the surface display of proteins is unaffected by storage, and this provides the

opportunity to use the same batch of cells in different analyses.

4.5   Test for Specific Receptors on Non-Polarized, Undifferentiated Caco-2 Cells

Studies show that the β_1-integrin and GP2 receptors which Invasin and FimH bind to, respectively, are present on Caco-2 cells (Werner et al., 2012). To verify the presence of the receptors on the Caco-2 cells used in this study, Caco-2 cells were incubated with receptor-specific antibodies, and subsequently analysed by flow cytometry.

To detect β_1-integrin receptors, Caco-2 cells were incubated with antibody against β_1-integrin (CD29 mouse monoclonal FITC-conjugated anti-human antibody). Non-stained Caco-2 cells were included as a negative control. GP2 receptors were detected by two-step staining, with the polyclonal rabbit-GP2 primary antibody and, subsequently, the polyclonal goat FITC-conjugated anti-rabbit secondary antibody. As a control for unspecific binding of the secondary antibody, Caco-2 cells were incubated only with the FITC-conjugated secondary antibody. Epidermal growth factor receptors (EGFR) are known to be expressed on Caco-2 cells (Shigeta et al., 2013). Therefore, Caco-2 cells incubated with polyclonal rabbit anti-EGFR primary antibody and, subsequently, polyclonal goat FITC-conjugated anti-rabbit secondary antibody, were used as a technical control. The flow cytometry results are shown in Figure 4.8.
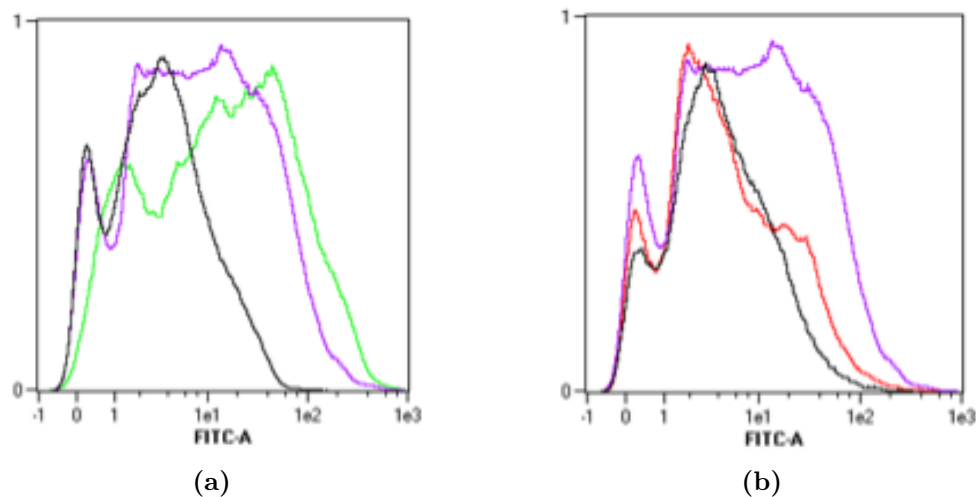
(a)   (b)

Figure 4.8. *Flow Cytometry Analysis of Caco-2 Cell Receptors. The β_1-integrin receptor (a) was detected in one-step staining using FITC-conjugated primary antibody (a), and the GP2-receptor was detected in two-step staining with a receptor-specific primary antibody (b), and a FITC-conjugated secondary antibody. Panel (a): black, non-stained Caco-2 cells; purple, anti-EGFR; light green, FITC-conjugated anti-β_1-integrin. Panel (b): black, Caco-2 cells stained with FITC-conjugated secondary antibody; purple, anti-EGFR; red, anti-GP2. The x-axis shows the fluorescence intensity.*

Caco-2 cells incubated with anti-β_1-integrin (Figure 4.8a) showed high fluorescent signal intensity compared to the non-stained Caco-2 cells, while the signal for the GP2-receptor (Figure 4.8b) was only slightly increased compared to the negative control. This confirmed the results from Werner et al. (2012) study, that both receptors are present, but GP2 only in modest amounts.

4.6   Caco-2 Internalization of *L. plantarum* Strains - The Gentamicin Survival Assay

To investigate the functionality of surface displayed proteins *in vitro*, only the following strains were selected: pLp_0373FimH_S.t.cwa2, pLp_0373FimH_E.c.cwa2 and pLp_1261Invasin_Y.e. The reason for this was that these plasmids were made at an earlier stage of the study than pLp_3014Invasin_Y.e. and pLp_1452Invasin_-Y.e.

Caco-2 cells are known to be able to internalize bacteria (Critchley et al., 2004; Innocentin et al., 2009), and were therefore used to examine the ability of surface displayed FimH and Invasin to promote internalization of *L. plantarum in vitro*, using the gentamicin survival assay as described in section 3.19.4.

Bacteria were incubated with Caco-2 cells for 3 hours, and gentamicin was subsequently added to kill bacteria that had not been internalized. The amount of bacterial cells used was calculated based on CFU-curves (Appendix 5.6, Figure A.1) and the Caco-2:bacteria ratio was set to 1:1000. The actual bacterial number used in the experiment was determined by plating on agar plates, before adding them to the Caco-2 cells. Internalized bacteria were quantified by counting CFU on agar plates. The results shown in Figure 4.9 are presented as the relative number of internalized bacteria (the number of internalized bacteria divided by the number of bacteria added). Surprisingly, *L. plantarum* harbouring the empty vector (pEV) showed the decidedly highest internalization. The experiment was only performed once and should be repeated to be able to draw any conclusions. However, as this would require several optimization steps, it was decided to use the Caco-2 internalization Assay (section 4.7) instead.
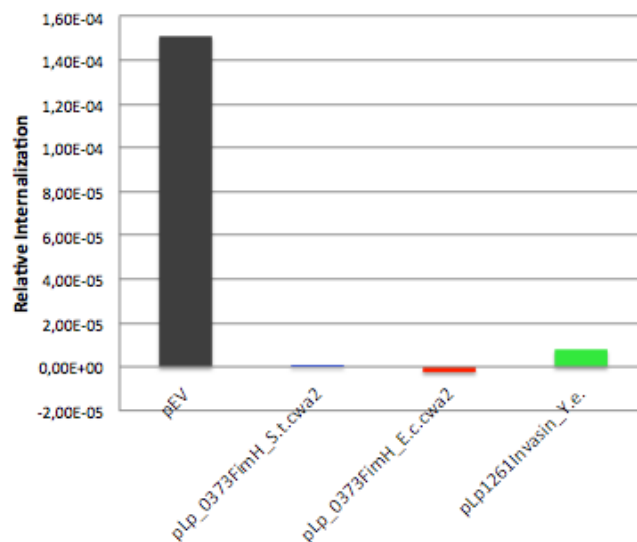

Figure 4.9. *Internalization of L. plantarum strains by Caco-2 cells using the Gentamicin Survival Assay. Induced recombinant L. plantarum cells were added to non-polarized, undifferentiated Caco-2 cells with a Caco-2: bacteria ratio set to 1:1000. The bacterial cells were incubated with the Caco-2 cells for 3 hours at 37°C. The bars represent the relative number of internalized bacteria (the number of internalized bacteria divided by the number of bacteria added to the Caco-2 cells). The experiment was only performed once.*

4.7 Caco-2 Internalization of *L. plantarum* Strains Analysed by Flow Cytometry

The Caco-2 internalization assay is another method to analyse the internalization of bacterial cells by Caco-2 cells (see section 3.19.5). Stained bacteria were incubated with Caco-2 cells, and the Caco-2 cells were subsequently analysed by flow cytome-

try. Only cells with internalized or bound bacteria could give fluorescent signals.

Representative results from the internalization experiments are shown in Figure 4.10. *Lactococcus lactis* expressing Fibronectin Binding Protein A (*L. lactis* FnBPA$^+$) had previously been shown to be internalized by Caco-2 cells (Innocentin et al., 2009), and was therefore used as a positive control. Figure 4.10c shows increased fluorescence signal intensity for Caco-2 cells incubated with *L. lactis* FnBPA$^+$, demonstrating that the experiment was technically successful. The intensity of the fluorescent signal for the Caco-2 cells incubated with the recombinant *L. plantarum* strains was also stronger than what was observed for those incubated with *L. plantarum* harbouring the empty vector (pEV). The experiment was performed 4 times, and although the numbers of cells and bacteria differed, the same tendency was observed in all experiments. This indicates that FimH and Invasin can promote internalization by Caco-2 cells, or at least binding, to Caco-2 cells.

As no standard protocol was available, attempts were made to optimize the conditions for internalization of bacteria. The experiment was performed in both complete RPMI-1640 medium and PBS, and no effect on the Caco-2 cells was observed (data not shown). Moreover, various incubation times were analysed, and increased incubation time did not increase the fluorescence signal (data not shown).
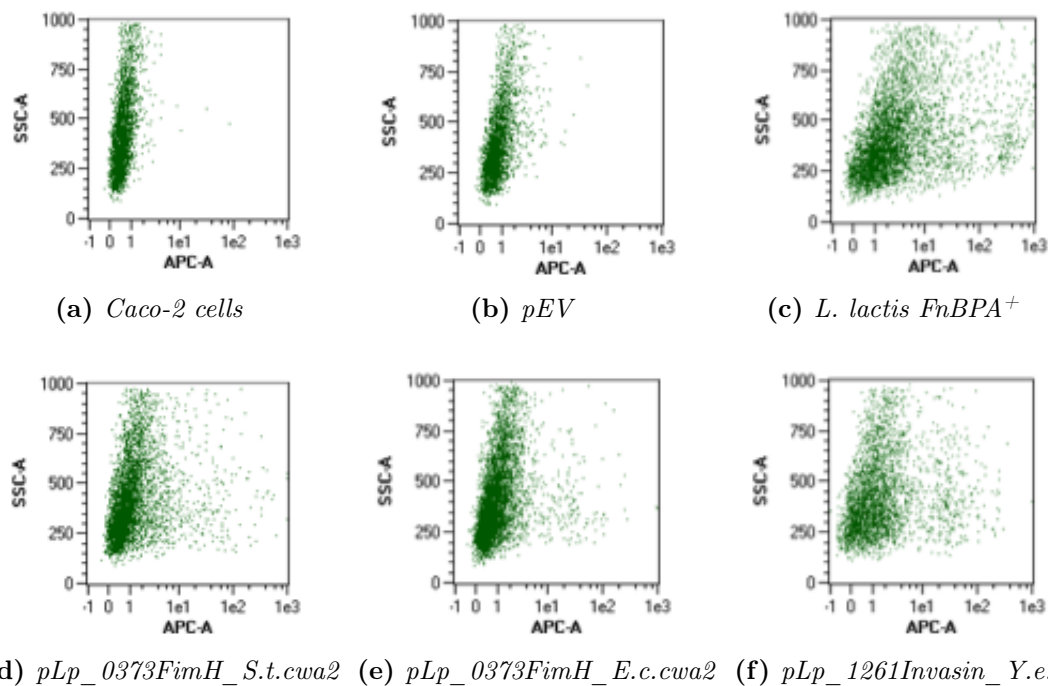
(a) *Caco-2 cells* **(b)** *pEV* **(c)** *L. lactis FnBPA$^+$*

(d) *pLp_0373FimH_S.t.cwa2* **(e)** *pLp_0373FimH_E.c.cwa2* **(f)** *pLp_1261Invasin_Y.e.*

Figure 4.10. ***Flow Cytometry Dotplots of Caco-2 Internalization of L. plantarum Strains.*** *The figure shows dotplots of Caco-2 internalization of stained L. plantarum strains. The strain harbouring the empty vector (pEV) was used as a negative control (b), and L. lactis FnBPA$^+$ was used as a positive control (c). The experiment was repeated four times, and two parallels of each strain were included in each experiment. The x-axis shows the fluorescence intensity; the y-axis shows side-scattered cells (i.e. cell granularity).*

4.8 Competition-Assay for Caco-2 Internalization

To investigate the binding specificity/affinity between the proteins and the β_1-integrin and GP2 receptors, a competition assay was set up. The Caco-2 cells were first incubated with receptor-specific antibodies, and subsequently with the induced bacterial strains producing recombinant proteins. In theory, bacterial internalization should be substantially reduced after pre-incubation with specific antibodies.

Figure 4.11 shows the flow cytometry dotplots from a regular Caco-2 internalization assay (panels c-e) and the competition assay (panels f-h). The experiments were performed simultaneously. The dotplots showed no differences between the results from the competition assay and the regular internalization assay.
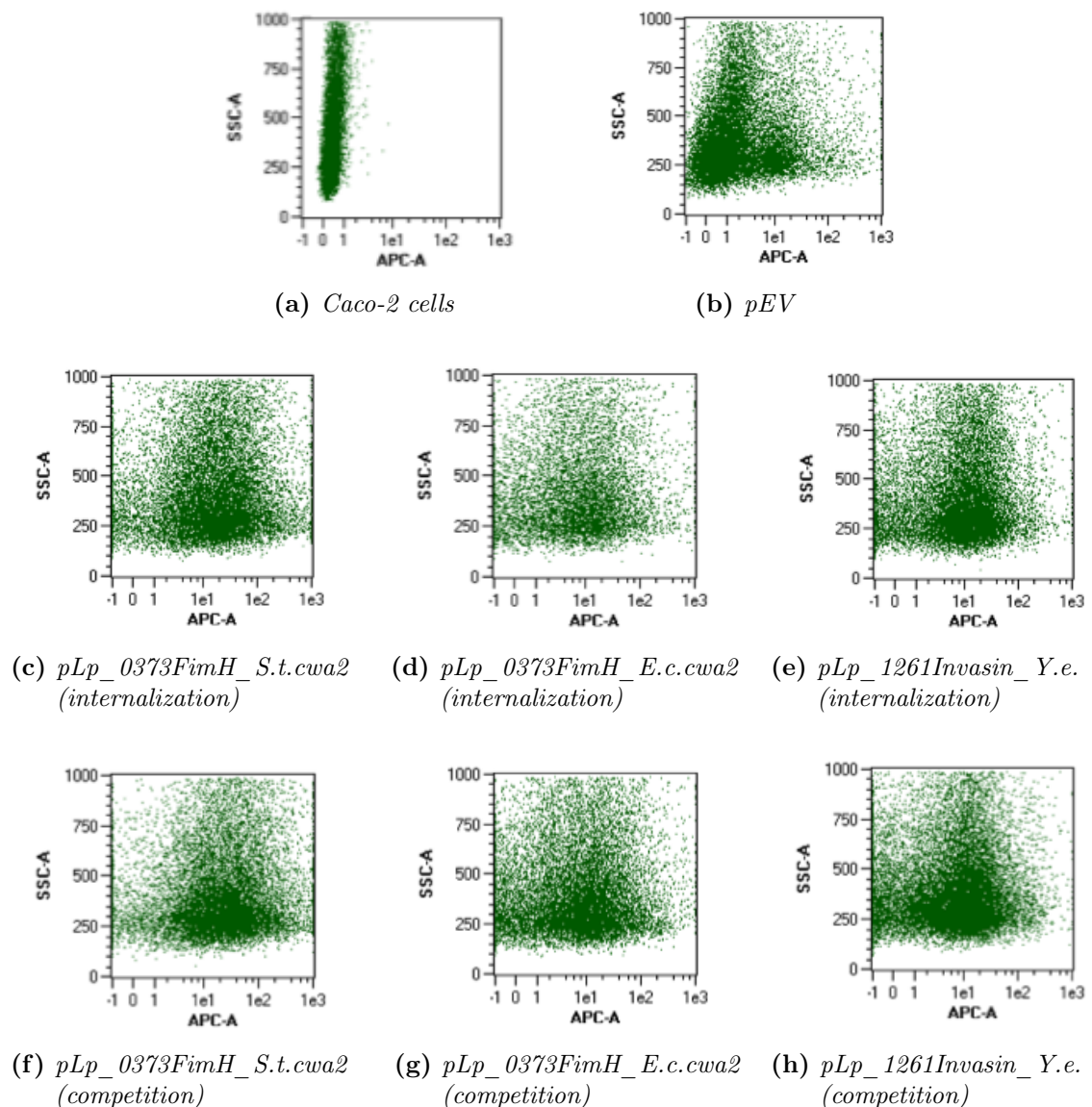
(a) *Caco-2 cells*

(b) *pEV*

(c) *pLp_0373FimH_S.t.cwa2 (internalization)*

(d) *pLp_0373FimH_E.c.cwa2 (internalization)*

(e) *pLp_1261Invasin_Y.e. (internalization)*

(f) *pLp_0373FimH_S.t.cwa2 (competition)*

(g) *pLp_0373FimH_E.c.cwa2 (competition)*

(h) *pLp_1261Invasin_Y.e. (competition)*

Figure 4.11. ***Flow Cytometry Dotplots of Competition Assay and Internalization Assay of L. plantarum strains by Caco-2 Cells.*** *The figure shows dotplots of Caco-2 cells with internalized stained recombinant L. plantarum cells using the internalization assay (panel c-e), and the competition assay (panel f-h) as indicated in parenthesis. The x-axis shows the fluorescence intensity; the y-axis shows side-scattered cells (i.e. cell granularity).*
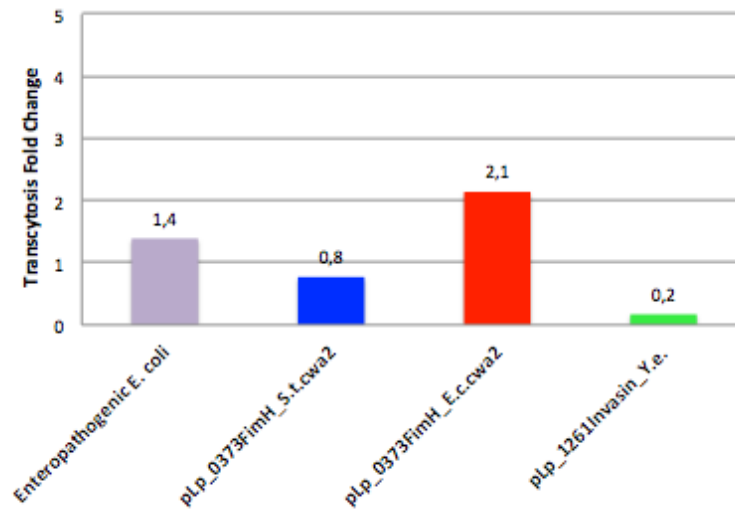
4.9   Transcytosis via M-Cell-Like Cells

An *in vitro* model of M-cells was developed by Kernéis et al. (1997), who managed to convert Caco-2 cells into cells with M-cell characteristics (see introduction, section 1.5 for more details). This M-cell model has been shown to significantly improve transcytosis of bacteria, compared to monolayers of polarized and differentiated Caco-2 cells (Kernéis et al., 1997; Maresca et al., 2007; Martinez-Argudo and

Jepson, 2008; Paixão et al., 2009; Finn et al., 2014).

The ability of FimH and Invasin to promote transcytosis through M-cells was therefore further investigated using an *in vitro* assay with M-cell-like cells. Polarized and differentiated Caco-2 cells were co-incubated with Raji B cells to induce conversion of Caco-2 cells into M-cell-like cells as described in section 3.19.7. The recombinant bacterial strains were added to the apical side of the M-cell-like cell layer, and transcytosed bacteria on the basal side were counted (see section 1.5, Figure 1.6). This method, as opposed to the Caco-2 internalization assay, enables counting of transcytosed bacteria only, while bacteria that are bound to the cells on the filter inserts, but not transcytosed to the basal compartment, are excluded. Wild type enteropathogenic *Escherichia coli* (EPEC) was used as a positive control as it is known to be transcytosed by M-cell-like cells (Tahoun et al., 2011).

Figure 4.12 shows the results of two independent experiments, where the bars represent the fold change of transcytosed bacteria relative to the negative control (pEV). The first experiment (Figure 4.12a) showed quite low fold change for all strains, with pLp_0373FimH_E.c.cwa2 showing the highest number of transcytosed bacteria, i.e. about two times higher compared to pEV. For the next experiment, more bacteria were added in an attempt to increase the number of transcytosed bacteria. The results from this second optimized experiment (Experiment 2, Figure 4.12b) showed much higher numbers of transcytosed bacteria, and here pLp_1261Invasin_Y.e. showed the decidedly highest fold change relative to pEV. Although the experiments should be repeated, experiment 2 indicates that lipo-anchored Invasin (pLp_1261Invasin_Y.e.) promotes the most efficient transcytosis of *L. plantarum* compared to FimH that is displayed by the other two strains used in the experiment. *L. plantarum* harbouring pLp_1261Invasin_Y.e. was also added to a monoculture of polarized and differentiated Caco-2 cells, resulting in very few transcytosed bacteria. This indicates that the conversion of Caco-2 cells into M-cell-like cells was successful. The positive control (wild type enteropathogenic *E. coli*) showed notably low numbers of transcytosed bacteria.

(a) *Experiment 1*
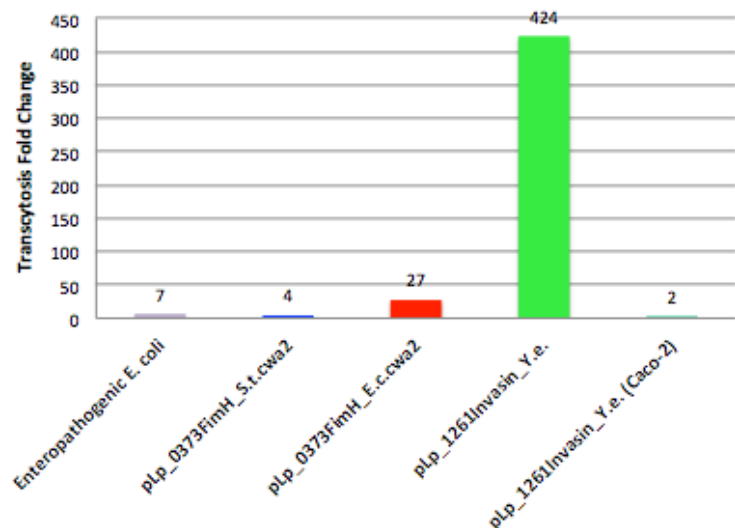
(b) *Experiment 2*

Figure 4.12. ***Transcytosis through M-Cell-Like Cells.*** *Panels (a) and (b) show the results of two independent experiments. More bacteria were used in experiment 2 (b). The relative number of transcytosed bacteria was calculated by dividing the number of transcytosed bacteria in the basal compartment by the number of bacteria added. The bars represent the fold-change of transcytosed bacteria, calculated by dividing the relative number of transcytosed recombinant bacteria by the relative number of transcytosed bacteria for the strain harbouring pEV. The fold change for each strain is indicated above the bars.*

5 Discussion

In an attempt to enhance the immune response of orally administered mucosal vaccines, vaccine antigens could be specifically transferred to high concentrations of immune cells found in Peyer's patches, via transcytosis by M-cells. Various pathogens utilize M-cells to invade the underlying tissues, by expressing certain proteins that bind receptors on M-cells, which promotes transcytosis of the pathogen (Azizi et al., 2010). Examples of such proteins are FimH from *Salmonella typhimurium* and enteropathogenic *Escherichia coli* which binds an M-cell glycoprotein receptor called GP2, and Invasin from *Yersinia enterocolitica* which binds β_1-integrin receptors located on M-cells (Hase et al., 2009; Uliczka et al., 2011). This study explored the possibility to use these proteins to target the vaccine delivery vector *Lactobacillus plantarum* to Peyer's patches. (Azizi et al., 2010; Kim and Jang, 2014; Wang et al., 2014).

5.1 Construction of Plasmids

The gene sequence encoding FimH from *S. typhimurium* was successfully ligated into a derivative of the pSIP401 expression vector with an N-terminal Lp0373 signal peptide and a C-terminal LPxTG-anchor called cwa2. In-fusion cloning was used to obtain a pSIP401-derivative expressing Invasin from *Y. enterocolitica* with an N-terminal Lp1261 lipoprotein anchor. The resulting plasmids, pLp_0373FimH_S.t.-cwa2 and pLp_1261Invasin_Y.e., were subsequently transformed into *L. plantarum* for production, secretion and surface display of the two proteins. Both gene constructs also contained a sequence encoding a so-called *myc*-tag, i.e. a peptide sequence originally encoding an oncogenic transcription factor in humans (Boxer and Dang, 2001), which was used for immunodetection. The *myc*-tag was located between the protein and the anchoring sequence, where it should be available for detection, without disrupting the conformation and functionality of the heterologous protein. These constructs were designed by analogy to previously designed constructs for cell wall anchoring of the cancer antigen OFA (Fredriksen et al., 2010) and N-terminal anchoring of Invasin from *Yersinia pseudotuberculosis* (Fredriksen et al., 2012). Obviously, many variations are possible in this type of constructs as both the signal peptide sequence, the anchor sequence and the sequence encoding the protein of interest can be easily exchanged. The LPXTG-anchor used in this study was selected for its ability to anchor FimH C-terminally, which was essential to let the N-terminal adhesive domain of FimH protrude out into the external environment. The adhesive domain of Invasin was located C-terminally, thus N-terminal anchoring was necessary. Three additional constructs were used in this study, which were generated by other members of the research group, as indicated in Table 4.1.

An overview of the plasmid constructs are shown in Table 5.1.

Table 5.1: Overview of the Plasmid Constructs Used in This Study

Plasmid	Description
pLp_0373FimH_S.t.cwa2	pSIP401-derivative for production of *St*FimH with the Lp0373 signal sequence and a C-terminal cell wall anchor
pLp_0373FimH_E.c.cwa2	pSIP401-derivative for production of *Ec*FimH with the Lp0373 signal sequence and a C-terminal cell wall anchor
pLp_1261Invasin_Y.e.	pSIP401-derivative for production of *Ye*Invasin with an N-terminal Lp1261 lipoprotein anchor
pLp_1452Invasin_Y.e.	pSIP401-derivative for production of *Ye*Invasin with an N-terminal Lp1452 lipoprotein anchor
pLp_3014Invasin_Y.e.	pSIP401-derivative for production of *Ye*Invasin with an N-terminal 3014 lysM domain-based anchor

For all plasmids, a sequence encoding a *myc*-tag was located between the protein and the anchoring sequence.

5.2   Growth Effects of Protein Production

Bacteria harbouring foreign plasmids may experience considerable stress, due to the increased metabolic energy needed for production of heterologous proteins (Fakruddin et al., 2013), and/or stress on the bacterial translation and translocation machineries. Notably, the proteins expressed in this study needed to be secreted, which may lead to secretion stress (Bolhuis et al., 1999). These stresses are often manifested in reduced growth rates, and have previously been demonstrated in recombinant lactobacilli expressing heterologous proteins coded on pSIP vectors (Fredriksen et al., 2012; Tjåland, 2011; Øverland, 2013). Therefore, the growth characteristics of the recombinant *L. plantarum* strains used in this study were analysed. Substantial decreases in the growth rate were observed for all induced strains expressing FimH and Invasin (Figure 4.3) compared to non-induced strains and the strain harbouring the empty vector (pEV). This observation indicates that *L. plantarum* was affected by the production of heterologous proteins, which may be explained by the extra energy needed for secretion and anchoring of the proteins, "clogging" of the secretion machinery, or intracellular accumulation of proteins.

The pSIP expression system has previously been shown to produce high levels of intracellular proteins (Sørvig et al., 2003), and if the protein production exceeds the capacity of the secretion machinery, proteins may accumulate intracellularly, and stress the bacteria (Gellissen, 2005). In addition, the combination of the signal peptide and the heterologous protein may also affect the secretion capacity in *L. plantarum* (Brockmeier et al., 2006). Furthermore, if large amounts of proteins are successfully secreted and anchored to the bacterial surface, overloading the surface can cause stress as well. The growth of the strain harbouring the plasmid pLp_0373FimH_E.c.cwa2 (Figure 4.3) for cell wall anchoring of *Ec*FimH, seemed to be slightly more affected compared to the other strains. Based on the above-mentioned theories, this strain may have had more intracellularly accumulated proteins, or the combination of the signal peptide (Lp0373) and FimH from enteropathogenic *E.coli* may have been less optimal than the combination used for the other strains.

Non-induced cultures showed small differences in growth rate compared to the negative control. This indicates that protein production was repressed in the absence of inducer peptide, and that the decrease in growth rate was an effect of the burden caused by protein overexpression.

Although protein overexpression affected the growth rates, the recombinant bacteria may still have secreted and anchored the heterologous proteins in sufficient amounts to promote transcytosis by M-cells. Therefore, production and surface display of the proteins were further investigated using western blot and flow cytometry analyses.

5.3 Western Blot Analysis of Protein Production in *L. plantarum*

Western blot analyses of cell-free protein extracts were performed in order to investigate whether the recombinant *L. plantarum* strains (Table 5.1) produced the heterologous proteins after induction. The analyses showed that proteins were produced by all the induced recombinant *L. plantarum* strains (Figures 4.4 and 4.5). However, as mentioned in section 4.3, the sizes of the cell wall anchored FimH proteins appeared to be larger than the expected theoretical sizes. Similar results have previously been obtained for other proteins anchored to the cell wall through LPxTG motifs (Fredriksen et al., 2010; Øverland, 2013). Fredriksen et al. (2010) suggested that the increased mass could be due to the formation of dimers. The relative charge of the proteins, determined by the composition of charged and non-charged amino

acids can also affect the migration, despite the presence of LDS (Ospinal-Jiménez and Pozzo, 2012).

As mentioned, the reduced growth rates of the different strains could be a result of intracellular accumulation. 19 genes in the *L. plantarum* genome are predicted to encode intracellular proteases (Kleerebezem et al., 2003), and these might degrade accumulated and possibly incorrectly folded intracellular proteins. Protein degradation would result in bands of smaller sizes on the western blots, and since the blots mostly showed one band for each protein, most of the proteins were probably not degraded, at least not to a large extent. For pLp_0373FimH_S.t.cwa2 and pLp_3014Invasin_Y.e., two bands were present. The sizes of the two bands only differed by a few kDa, and therefore the two bands probably represented protein with and without the signal peptide, indicating that some non-processed, and therefore most likely not secreted, protein existed. The higher band, i.e. representing the variant with the highest mass, also seemed to be more intense than the lowest, indicating that there were more proteins with uncleaved signal peptide than correctly processed proteins.

5.4   Detection of Surface Displayed Proteins in *L. plantarum*

Flow cytometry was used to detect proteins anchored to the surface of induced and FITC-stained *L. plantarum* cells (see Table 5.1 for an overview of the different proteins and anchors used). FITC-staining was achieved by two-step staining using mouse monoclonal anti-myc primary antibody, and subsequently rabbit polyclonal anti-mouse FITC-conjugated IgG secondary antibody. The histograms presented in Figure 4.6 showed clear fluorescent signals for the strains harbouring the plasmids encoding the *FimH* genes, indicating that FimH from both *S. typhimurium* and enteropathogenic *E. coli* were successfully anchored and displayed on the surface of *L. plantarum*. The peak representing pLp_1452Invasin_Y.e. also showed a positive shift in the fluorescent signal intensity compared to the strain harbouring the empty vector (pEV). The two remaining strains harbouring the plasmids pLp_1261Invasin_Y.e. and pLp_3014Invasin_Y.e. did not show clear shifts of the main peaks (Figure 4.6). However, small additional peaks did show a shift, indicating that some bacterial cells had bound antibodies. Dotplots (Figure 4.7), which give an image of the whole bacterial cell population, further confirmed that a subset of the cells had bound antibodies.

In an attempt to enhance the fluorescent signal for the strain harbouring the plasmid pLp_1261Invasin_Y.e., the cells were treated with lysozyme because it was

envisaged that the myc-tag could be covered by or embedded in the bacterial cell wall. Lysozyme partly degrades the cell wall, and could, in theory, reveal hidden proteins, thus allowing antibody binding. Lysozyme treatment has previously been shown to increase the fluorescent signals for antigens anchored to the surface of *L. plantarum*, using the Lp1261 lipoprotein anchor and an LPxTG-anchor (cwa2) (Øverland, 2013). In the present study, lysozyme treatment enhanced the signal for surface displayed Invasin in the strain harbouring pLp_1261Invasin_Y.e. in two out of five attempts. The varying results may indicate that the Invasin protein was present in small amounts and only on the surface of a subset of the bacterial cells, or that the lysozyme treatment was insufficient to degrade the bacterial cell wall, keeping the myc-tag unavailable for antibody binding. Overall, the results indicate that the surface display of Invasin in *L. plantarum* harbouring pLp_1261Invasin_Y.e. was limited. The proteins were produced intracellularly, as shown by the western blot analysis, but maybe only a subset was secreted. Another possibility is that the proteins were secreted and anchored, but their conformation hindered antibody binding. The weak signals could also be a result of too low antibody concentrations in relation to the amount of bacterial cells, and further optimization of the experiment could perhaps have increased the signal intensity. Surface display analyses of *L. plantarum* harbouring pLp_3014Invasin_Y.e. and pLp_1452Invasin_Y.e. was only performed twice and should be confirmed, both with and without lysozyme treatment. All in all it seems safe to conclude that there is convincing biochemical evidence for surface display of FimH from *S. typhimurium* and enteropathogenic *E. coli* and of Invasin from *Y. enterocolitica* with the Lp1452 lipoprotein anchor, whereas the situation is less clear for the strains harbouring pLp_1261Invasin_Y.e. and pLp_3014Invasin_Y.e.

5.5 Transcytosis of Recombinant *L. plantarum* Using *In-Vitro* M-cell Models

5.5.1 Internalization of Recombinant *L. plantarum* by Caco-2 Cells

Caco-2 cells are known to internalize bacteria (Critchley et al., 2004; Innocentin et al., 2009), and have also been shown to express the GP2- and β_1-integrin receptors that bind FimH and Invasin, respectively (Werner et al., 2012). In this study, Caco-2 cells were therefore used to investigate the ability of the surface displayed proteins to promote internalization of *L. plantarum in vitro*. The two strains expressing cell wall anchored FimH and the strain expressing Invasin with the Lp1261 lipoprotein anchor were included in this and the remaining experiments. Stained, induced recombinant bacteria were incubated with non-polarized, undifferentiated

Caco-2 cells, and the Caco-2 cells, or the occurrence of stained cells (indicating internalized stained bacteria), were subsequently analysed by flow cytometry. The results (Figure 4.10) showed increased fluorescent signal intensity for the tested strains compared to the negative control (pEV), and the results were confirmed in four individual experiments. As the number of bacteria and Caco-2 cells differed, one cannot draw any conclusions regarding which strain promoted the most efficient internalization, but the clear difference in signal between the tested strains and the strain harbouring pEV, clearly indicates that the anchored target proteins did influence the interaction with Caco-2 cells.

A major drawback of this method is that the fluorescent signals may be the result of binding to the Caco-2 cell surface, and not internalization. To be able to distinguish between bound and internalized bacteria, the samples should be further investigated using Confocal Laser Scanning Microscopy (CSLM). CLSM would give a three dimensional image of the sample, and by fluorescent staining of the different cells and proteins, Caco-2 cells with both bound and internalized bacteria could be viewed directly. CLSM images would also reveal the distribution of proteins displayed on the bacterial cell surface.

The specificity/affinity between the FimH- and Invasin proteins and their receptors, was further analysed using a competition assay. Specific antibodies against GP2- and β_1-integrin receptors were added to the Caco-2 cells prior to incubation with the bacteria expressing cell wall anchored FimH and Lp1261 lipoprotein anchored Invasin, in order to competitively inhibit binding of the recombinant bacteria. The regular Caco-2 internalization assay, without the prior incubation with antibodies, was performed simultaneously as a control for the competition assay. Surprisingly, the results (Figure 4.11) showed no difference between the regular internalization assay and the competition assay. *L. plantarum* is predicted to express several adhesion factors on its surface (Kleerebezem et al., 2003) (see introduction, section 1.1.1), and several strains are known to adhere to Caco-2 cells (Duary et al., 2011). Therefore, the recombinant strains used in this study may bind Caco-2 cells independently of β_1-integrin and GP2 receptors. However, as increased fluorescent signal intensities were observed for the strains that express these proteins, compared to the strain harbouring pEV, the presence of additional adherence factors does not entirely explain the results from the competition assay. For the strains expressing FimH, adherence to Caco-2 cells could be explained by the fact that FimH not only binds GP2 receptors, but glycoproteins containing mannose, in general. For the Invasin producing strain, one possible explanation is that the affinity between the recombinant bacteria and the receptors is higher than the affinity between the an-

tibodies used and the receptors. The antibody concentrations may also have been too low, and thereby insufficient to bind all available receptors. Another possibility is that the antibodies used bind different epitopes than FimH and Invasin. In that case, the proteins would be able to promote internalization regardless of the preincubation with antibodies. The competition assay was only performed once, and as discussed above, optimizations of the procedure may be necessary.

A gentamicin survival assay was also used in an attempt to analyse FimH and Invasin's ability to promote internalization by Caco-2 cells. As shown in Figure 4.9, the most prominent result from this assay was the high number of internalized *L. plantarum* harbouring pEV. The high numbers could represent bacteria that had bound to the Caco-2 cells by other adhesion factors than the proteins used in this study, but these bacteria should in theory be killed by the gentamicin. However, the gentamicin concentration may have been too low to effectively kill all bacteria that had not been internalized. Another possibility is that the bacterial cells were somehow covered by the Caco-2 cells, and thereby protected from the gentamicin. Still, this should apply for all the bacterial strains, not only the strain harbouring pEV. The gentamicin assay clearly needs optimization, and should be repeated to be able to draw any conclusions.

5.5.2   Transcytosis via M-Cell-Like Cells

The formation of M-cell-like cells by Caco-2 cells was induced according to the method developed by Kernéis et al. (1997) and further by Gullberg et al. (2000). Caco-2 cells that are grown to polarize and differentiate on filter inserts and subsequently co-incubated with Raji B cells (see introduction, Figure 1.6) will exhibit M-cell characteristics, and have been shown to significantly enhance transcytosis of bacteria, compared to monocultures of polarized and spontaneously differentiated Caco-2 cells (Kernéis et al., 1997; Maresca et al., 2007; Martinez-Argudo and Jepson, 2008; Paixão et al., 2009; Finn et al., 2014). GP2 and β_1-integrin receptors have previously been shown to be expressed by such M-cell-like cells (Kim et al., 2010; Hamzaoui et al., 2004), and the presence of these receptors was verified for the Caco-2 cells used in the internalization assays discussed in section 5.5.1 (results shown in Figure 4.8). The presence of these receptors was not verified for the M-cell-like cells, and this should have been done prior to further analysis. A similar experiment as the one used to detect the receptors on Caco-2 cells using flow cytometry (see section 3.19.3) could be used, or preferably CLSM, which would provide knowledge about both the presence, and the distribution of the receptors on the cell surface.

Using the M-cell model, the transcytosed bacteria on the basal side were counted

relative to the number of bacteria added to the apical side, meaning that bound bacteria, which had not been transcytosed, were not detected. The experiment was performed twice, and the results obtained from the first experiment (Figure 4.12a) showed quite low numbers of transcytosed bacteria. Therefore, the number of added recombinant bacteria had to be increased in an attempt to optimize the procedure. The second experiment showed a much increased number of transcytosed bacteria (Figure 4.12b), especially for the strain harbouring pLp_1261Invasin_Y.e. which showed more than 400 times higher transcytosis relative to the negative control. This result, together with the results from the Caco-2 internalization assays, also imply that Invasin was successfully displayed on the bacterial cell surface. The reason for the varying results of surface displayed proteins in this strain, detected by flow cytometry (see section 4.4), may therefore be that the myc-tag was somehow unavailable for antibody binding as discussed in section 5.4. Another possibility is that Invasin was only expressed in limited amounts, but sufficiently to promote transcytosis via M-cell-like cells.

As a control of the M-cell model, the strain harbouring pLp_1261Invasin_Y.e. was also incubated with a monoculture of polarized and differentiated Caco-2 cells without M-cell features, that is, Caco-2 cells that had grown on filter inserts for approximately three weeks, without addition of Raji-B cells. The transcytosis of bacteria by these cells, was very low compared to the transcytosis across the M-cell-like cells (Figure 4.12b). As opposed to the non-polarized, undifferentiated Caco-2 cells used for the internalization experiments, the Caco-2 cells that had time to polarize and differentiate, obtained apical and basal domains. Previous studies have shown that the subtype of β_1-integrin receptors that Invasin bind to, are only present on the basal side of such polarized and differentiated Caco-2 cells (Coconnier et al., 1994; Liévin-Le Moal and Servin, 2013; Uliczka et al., 2011). The low number of transcytosed bacteria across the differentiated Caco-2 cells is therefore consistent with these previous findings.

In the first experiment, the FimH expressing strains showed higher numbers of transcytosed bacteria than the Invasin expressing strain. These results were not reproduced in the second experiment where the Invasin expressing strain showed the clearly highest number of transcytosed bacteria. Martinez-Argudo and Jepson (2008) observed similar variability when investigating transcytosis of *Salmonella enterica*, and an explanation for the variable results may be that the conversion of Caco-2 cells into M-cell-like cells was nonuniform, meaning that the number of M-cell-like cells in the co-cultures varied between the experiments, as observed by Gullberg et al. (2000). Still, in both experiments, the strain harbouring pLp_0373FimH_-

E.c.cwa2 showed higher numbers of transcytosed bacteria than the strain harbouring pLp_0373FimH_S.t.cwa2.

Enteropathogenic *E. coli* (EPEC) was selected as a technical control for the experiments investigating transcytosis through M-cell-like cells. EPEC was also a control for the functionality of the FimH protein used in the plasmid pLp_0373FimH_E.c.-cwa2, as the gene sequence encoding FimH used in the plasmid was taken from this EPEC strain. The transcytosis experiments using M-cell-like cells showed that transcytosis of the strain harbouring pLp_0373FimH_E.c.cwa2 was higher than the transcytosis of EPEC. Tahoun et al. (2011) showed that EPEC was transcytosed by M-cell-like cells, but not at very high levels. The same study also found that the effector protein EspF, which is expressed by the EPEC strain, inhibits transcytosis, and this could explain why EPEC showed a lower number of transcytosed bacteria than the strain harbouring pLp_0373FimH_E.c.cwa2. The results are also consistent with a different study by Martinez-Argudo et al. (2007), who found that wild type EPEC was transcytosed at low levels across both polarized and differentiated Caco-2 monolayers and Caco-2/Raji-cocultured layers.

The transepithelial electrical resistance (TEER) of the co-culture monolayer was measured immediately prior to addition of bacteria, showing a resistance of 2-300 Ω x cm^2, which is considered high (Wilhelm et al., 2011). This should ensure cellular confluence in the monolayers, and bacteria should not be able to leak through and into the basal compartment. This means that the bacteria counted in the basal compartment were most likely transferred by transcytosis by the M-cell-like cells.

If further transcytosis experiments using M-cell-like cells keep giving variable results, a more reproducible M-cell model could be considered. As mentioned in section 1.5, Des Rieux et al. (2007) demonstrated increased translocation of nanoparticles across inverted co-cultures of Caco-2 and Raji B cells compared to the normal co-cultures developed by Kernéis et al. (1997) and Gullberg et al. (2000). Des Rieux et al. (2007) claimed that this method provided a more functional and more reproducible model, and this model could thus perhaps be more suitable for analysis of transcytosis of *L. plantarum* than the normally oriented model used in this study. However, M-cells seem to take up nanoparticles by non-specific absorption endocytosis, and transcytosis of recombinant bacteria by this model has not been tested. Still, the inverted model could be worth investigating for future analyses. Furthermore, Des Rieux et al. (2007) estimated the percentage of M-cell-like cells in the co-culture, using transmission electron microscopy (TEM) and scanning electron microscopy (SEM) to determine the surface area consisting of cells morphologically similar to M-cells.

They found that 15-30% of the cells in the co-cultures were M-cell-like cells, and this number has not been determined for the normally oriented models used in the present study. Knowing the share of M-cell-like cells in the co-cultures, provides the ability to interpret the results and compare them with *in vivo* data.

5.6   Conclusion and Future Perspectives

The experiment investigating internalization of the bacterial strains by non-polarized, undifferentiated Caco-2 cells clearly showed that FimH and Invasin were involved in binding of *L. plantarum* to Caco-2 cells, but the experiment could not distinguish between binding and internalization, and little differences were seen between the different bacterial strains (section 4.7). The M-cell model developed by Gullberg et al. (2000) provided an improved method, as it enabled to distinguish bound and transcytosed bacteria. However, the results from the two experiments performed using the M-cell model varied. The transcytosis experiments should be repeated, and further improved models like the inverted M-cell model developed by Des Rieux et al. (2007) (discussed above) could also be considered.

If the delivery system explored in the present study should prove to efficiently deliver antigens to the Peyer's patches of the gastrointestinal tract, there is no guarantee that the antigens will elicit an immune response. There may be a risk that *L. plantarum* with its GRAS status will even induce tolerance against the antigens, or that the antigens will break the tolerance against *L. plantarum* (Detmer and Glenting, 2006). There are promising data in the literature. For example, immune responses have been elicited in mice after oral delivery of LPxTG-anchored E7 oncoprotein in *Lactococcus lactis* (Bermúdez-Humarán et al., 2004). Notably, although these results are promising, there are significant differences between the mucosae of humans and that of other species, making clinical trials on humans the ultimate test (Czerkinsky and Holmgren, 2009).

In conclusion, the results from this study indicate that FimH and Invasin are involved in promoting transcytosis through M-cells *in vitro*, and further analyses should be carried out to evaluate the use of the proteins to deliver vaccine antigens. In addition, other M-cell receptors, and other receptor-specific proteins should be considered, and future detection of specific M-cell markers may also open for alternative approaches for specific targeting of Peyer's patches (Casteleyn et al., 2013).

Future challenges include *in vivo* testing of the functionality of the delivery system, meaning that disease antigens must be co-expressed with the M-cell targeting proteins in *L. plantarum*. There is still a long way to go before a potential vaccine is ready, but hopefully, this delivery system can some day be used in several mucosal vaccines to fight various infectious diseases.

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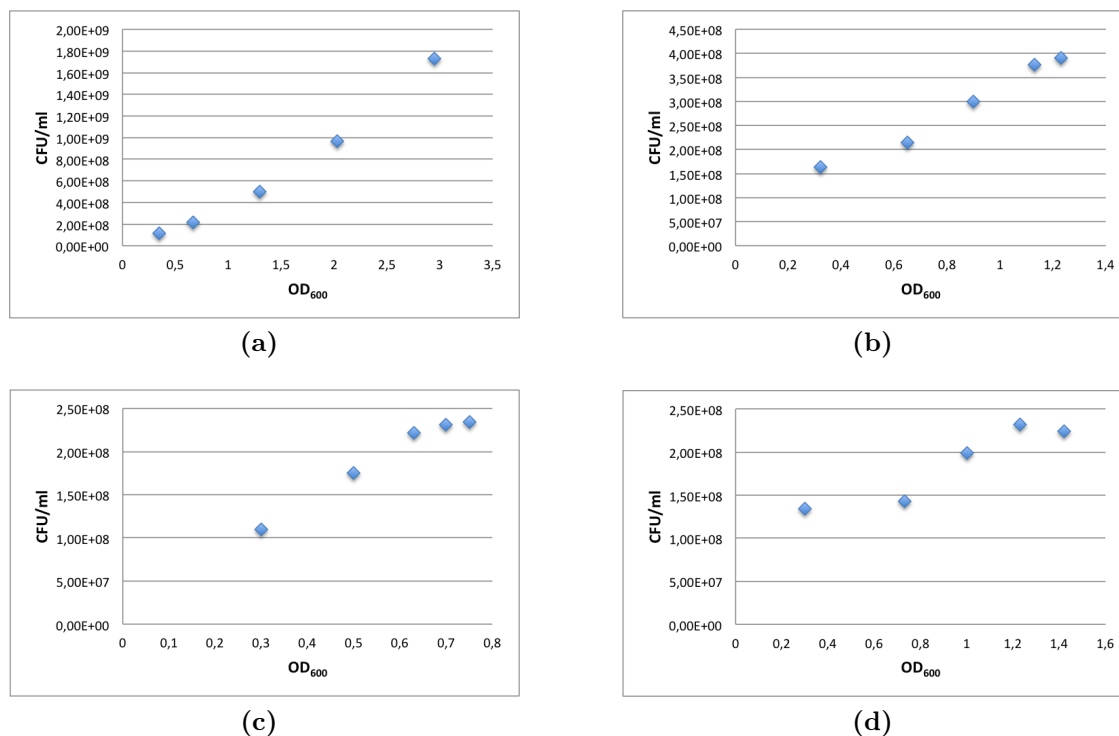
Appendix A

Figure A.1. *The Relationship Between OD_{600} and CFU/ml for L. plantarum Harbouring Different Plasmid Constructs.* *The figure shows CFU-OD_{600} curves for L. plantarum harbouring pEV (a), pLp_0373FimH_S.t.cwa2 (b), pLp_0373FimH_E.c.cwa2 (c), and pLp_1261Invasin_Y.e. (d). The OD_{600} values are the average of 3 culture replicates, and the CFU/ml values are the average of two replicates of two dilutions from each of the 3 culture replicates. The graphs were used to calculate the amount of culture needed to harvest the same number of cells, based on the OD_{600} value at the time of harvest for L. plantarum harbouring the different plasmid constructs.*
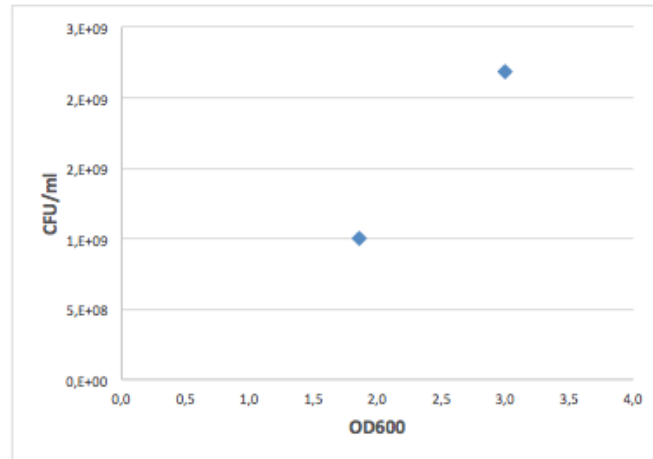
Figure A.2. ***The Relationship Between OD_{600} and CFU/ml for wild type enteropathogenic E. coli.*** *The graphs were used to calculate the amount of culture needed to harvest the same number of cells, based on the OD_{600} value at the time of harvest for wild type enteropathogenic E. coli. The graph was provided by Lise Øverland.*
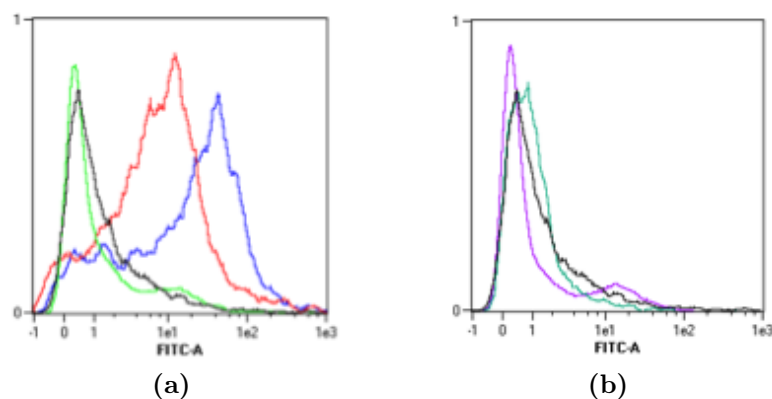
Appendix B

(a)　　　　　　　　　(b)

Figure B.1. *Flow Cytometry Histograms of L. plantarum Cells Stored at 4°C for One Night. Recombinant L. plantarum strains were cultured and induced, and harvested cell pellets were kept overnight at 4°C before flow analysis were performed. Panel (a) shows pEV (black), pLp_1261Invasin_Y.e. (light green), pLp_0373FimH_E.c.cwa2 (red) and pLp_0373FimH_S.t.cwa2 (blue). Panel (b) shows pEV (black), pLp_3014Invasin_Y.e. (purple) and pLp_1452Invasin_Y.e. (green). The x-axis shows the fluorescence intensity.*
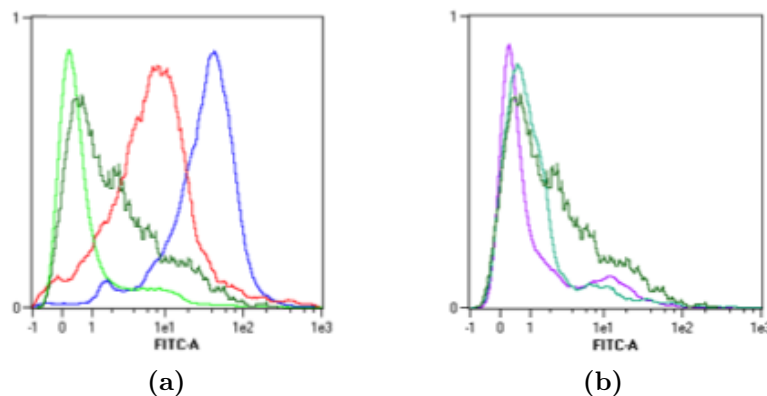

(a)　　　　　　　　　(b)

Figure B.2. *Flow Cytometry Histograms of L. plantarum Cells Frozen in PBS with 20% Glycerol at -20°C for One Night. Recombinant L. plantarum strains were cultured and induced, and harvested cell pellets were resuspended in PBS containing 20% glycerol and kept at -20°C overnight before flow analysis were performed. Panel (a) shows pEV (dark green), pLp_1261Invasin_Y.e. (light green), pLp_0373FimH_E.c.cwa2 (red) and pLp_0373FimH_S.t.cwa2 (blue). Panel (b) shows pEV (dark green), pLp_3014Invasin_Y.e. (purple) and pLp_1452Invasin_Y.e. (green). The x-axis shows the fluorescence intensity.*
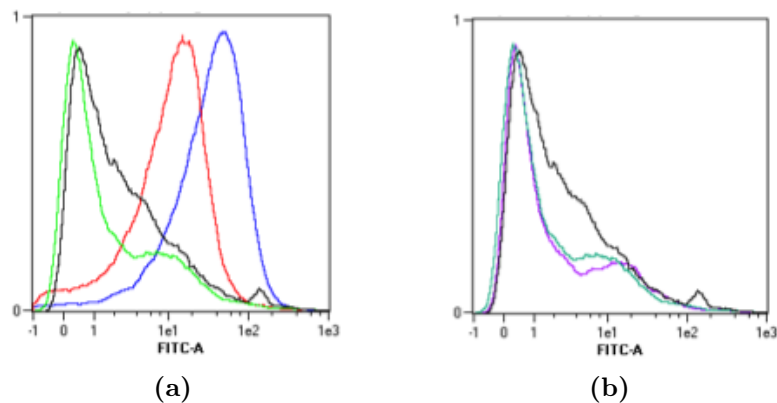
(a) (b)

Figure B.3. *Flow Cytometry Histograms of L. plantarum Cells Frozen in PBS with 20% Glycerol at -20°C for One Week. Recombinant L. plantarum strains were cultured and induced, and harvested cell pellets were resuspended in PBS containing 20% glycerol and kept at -20°C for one week before flow analysis were performed. Panel (a) shows pEV (black), pLp_1261Invasin_Y.e. (light green), pLp_0373FimH_E.c.cwa2 (red) and pLp_0373FimH_S.t.cwa2 (blue). Panel b shows pEV (black), pLp_3014Invasin_Y.e. (purple) and pLp_1452Invasin_Y.e. (green) (b). The x-axis shows the fluorescence intensity.*