New Insights into the Biogenesis of \textit{Lactobacillus rhamnosus} GG Pili and the \textit{In Vivo} Effects of Pili

YEB

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UNIVERSITY OF HELSINKI
NEW INSIGHTS INTO THE BIOGENESIS OF \textit{Lactobacillus rhamnosus} GG PILI AND THE \textit{IN VIVO} EFFECTS OF PILI

Pia Rasinkangas

ACADEMIC DISSERTATION

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Onpa kadulla mittaa!
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Mä väistelen lätäköitä
lätäköitä ja koirankakkaa
pari vastaantulijaa
olihan se helpomppaa
kun ei tiennyt
ettei tiedä mitä haluaa
olla haluavinaan

Minne nääkin juoksee kilpaa
hätäilee ja vouhottaa
renkaat rapaa roiskuen
mua ei haittais ollenkaan
jos nää haastais vain toisiaan
kamppailemaan

Kauniimmaksi kaunis kaunistuu
kun kupla kasvaa
köyhä köyhtyy ja rohkeeta pelottaa
asetelma romahtaa
ei peltilehmä starttaakaan
(tais jäädä valot päälle)

Parempi vaan
en kai mä piittaa
parempi vaan –
onpa kadulla mittaa!
CONTENTS

LIST OF ORIGINAL PUBLICATIONS
ABBREVIATIONS
ABSTRACT
1 INTRODUCTION .................................................................1
2 REVIEW OF THE LITERATURE ................................................3
  2.1 PILI IN GRAM-POSITIVE BACTERIA .......................................3
    2.1.1 Biogenesis of sortase-dependent pili ..................................4
    2.1.2 Pili in Lactobacillus rhamnosus GG .................................11
  2.2 LACTOBACILLUS RHAMNOSUS GG AS A PROBIOTIC. ....................16
    2.2.1 Intervention trials ..................................................18
    2.2.2 Cellular level of microbial probiosis in the gastrointestinal tract. ....23
3 AIMS OF THE STUDY ..........................................................34
4 MATERIALS AND METHODS ................................................35
  4.1 OVERVIEW OF THE MOUSE TRIAL ......................................35
  4.1 OVERVIEW OF THE HUMAN TRIAL .....................................35
5 RESULTS AND DISCUSSION ................................................39
  5.1 SEARCH FOR DERIVATIVES OF LACTOBACILLUS RHAMNOSUS GG
      WITH ALTERED PILUS PRODUCTION CAPACITY (I & II) ..................39
    5.1.1 Chemical mutagenesis and enrichment of pilus derivatives ..........39
    5.1.2 Phenotypic and genotypic characterisation of the pilus-less derivatives (I) . 41
    5.1.3 Phenotypic and genotypic characterisation of the highly
        mucus-adherent and pilus-secreting derivatives (II) ..................43
  5.2 IDENTIFICATION OF FUNCTIONAL VARIANTS OF PILIN SORTING SIGNAL LPxTG (III) . 45
    5.2.1 Bioinformatic analysis ..............................................45
    5.2.2 Construction of the expression system and mutagenesis of
        the LPxTG motif ..................................................45
    5.2.3 Phenotypic characterisation of the mutants .......................46
  5.3 IN VIVO TRIALS TO ASSESS THE EFFECT OF PILI ON THE PERSISTENCE AND
      GUT MICROBIOTA MODULATION POTENTIAL OF
      LACTOBACILLUS RHAMNOSUS GG (IV) ................................47
    5.3.1 Analysis of the GIT persistence of the L. rhamnosus GG strains ..........47
    5.3.2 Gut microbiota analyses ............................................48
6 CONCLUSIONS AND FUTURE ASPECTS ..................................50
7 ACKNOWLEDGEMENTS .....................................................55
8 REFERENCES .................................................................57
LIST OF ORIGINAL PUBLICATIONS

The present thesis is based on the following publications and submitted manuscripts:


The publications are referred to in the text by their roman numerals.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin</td>
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<tr>
<td>DUOX</td>
<td>dual oxidase</td>
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<tr>
<td>EGF-R</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EHCDF</td>
<td>extensively hydrolysed casein formula</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>EMS</td>
<td>ethyl methanesulfonate</td>
</tr>
<tr>
<td>EPS</td>
<td>exopolysaccharide</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent in situ hybridisation</td>
</tr>
<tr>
<td>FPR</td>
<td>formyl peptide receptor</td>
</tr>
<tr>
<td>FUT</td>
<td>fucosyltransferase</td>
</tr>
<tr>
<td>GALT</td>
<td>gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GG</td>
<td>Gorbach Goldin</td>
</tr>
<tr>
<td>GIT</td>
<td>gastrointestinal tract</td>
</tr>
<tr>
<td>GMO</td>
<td>genetically modified organism</td>
</tr>
<tr>
<td>HITChip</td>
<td>human intestinal tract chip</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IEC</td>
<td>intestinal epithelial cell</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitor of kappa B</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IS</td>
<td>insertion sequence</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>lipoteichoic acid</td>
</tr>
<tr>
<td>Mab</td>
<td>modulator of adhesion and biofilm</td>
</tr>
<tr>
<td>MAMP</td>
<td>microbe-associated molecular pattern</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBF</td>
<td>mucus-binding factor</td>
</tr>
<tr>
<td>MPK</td>
<td>MAPK phosphatase</td>
</tr>
<tr>
<td>Msp</td>
<td>major secreted protein</td>
</tr>
<tr>
<td>MUB</td>
<td>mucus binding protein</td>
</tr>
<tr>
<td>mo-DC</td>
<td>monocyte-derived dendritic cell</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
</tr>
</tbody>
</table>
NLR  NOD-like receptor
NOD  nucleotide-binding oligomerisation domain-containing
NOX  NADPH oxidase
PA   pilus SrtA
PB   pilus bald
PG   peptidoglycan
PI3K phophoinositide 3-kinase
PKC  protein kinase C
PRR  pattern recognition receptor
PS   pilus sticky
qPCR quantitative PCR
ROS  reactive oxygen species
SCFA short chain fatty acid
SG   single glycine
Spa  sortase-mediated pilus assembly
SRP  signal recognition particle
Srt  surface protein sorting
STAT signal transducer and activator of transcription
Tat  Twin-arginine translocation
TG   triple glycine
TLR  Toll-like receptor
TNF  tumour necrosis factor
T-RFLP terminal restriction fragment length polymorphism
ABSTRACT

The present thesis studied the biogenesis of the pili of *Lactobacillus rhamnosus* GG, and examined whether pili influence the *in vivo* persistence and the ability to modulate faecal microbiota in mice and humans. *L. rhamnosus* GG is one of the most extensively studied lactic acid bacteria, and it is commonly present in so-called probiotic products. Heterotrimeric proteinaceous pili, which are able to bind for example to mucus, collagen and β-lactoglobulin, have been found and characterised on the surface of *L. rhamnosus* GG during recent years. Pili are formed by the major pilin SpaA, which forms the shaft of the pilus structure, the mucus-binding tip pilin SpaC and basal pilin SpaB. The pili are synthesised on the cell membrane by the pilin-specific transpeptidase sortase C (SrtC), and attached to the cell wall by housekeeping transpeptidase sortase A (SrtA). As the pilus is the major mucus adhesin of *L. rhamnosus* GG and, according to various studies, a crucial factor in the *L. rhamnosus* GG adherence and signalling to the host, it was deemed important to characterise the pilus biogenesis pathway and the functions of the produced pili.

Random chemical mutagenesis was used to obtain derivatives of *L. rhamnosus* GG with variable pilus production capacities to study factors affecting pilus biogenesis. Enrichment schemes for the isolation of the derivatives were devised, and consequently 10 pilus-deficient, 13 highly adherent and one pilus-secreting derivative were characterised. Genotypic and phenotypic characterisation of the derivatives revealed that a functional SrtC is essential for pilus biogenesis, as well as the sufficient expression of the major pilin SpaA. Additionally, the role of the insertion sequence-rich genomic region, containing also the pilus gene cluster, in the adaptation of *L. rhamnosus* GG to varying environments was revealed, as several derivatives were obtained in which the region was deleted. The adhesion capacity of *L. rhamnosus* GG was found to be increased either due to a mutation in the tip pilin SpaC or as a result of increased pilin production. One of the highly adherent derivatives with high pilin production harboured a mutation in the SpaA pilin, likely increasing the derivative’s capacity to secrete this pilus backbone-forming subunit. In addition, a pilus-secreting derivative was characterised, and noted to harbour a mutated housekeeping sortase SrtA, demonstrating its essential role in the attachment of pili to the cell wall.

Bioinformatic analysis revealed that the C-terminal sortase recognition motif of pilins, with the sequence LPxTG, was variable between the proteins recognised by pilin-specific transpeptidase SrtC and housekeeping transpeptidase SrtA. This
was found not only for the pilins of *L. rhamnosus* GG, but also for pilins of other sortase-dependent pili-harbouring Gram-positive bacteria, indicating a role for this motif in the regulation of pilus biogenesis. In a subsequent functional characterisation of *L. rhamnosus* GG pilins, it was demonstrated that SrtC indeed recognised the shaft pilin SpaA through a conserved triple glycine (TG) motif, and was likely to recognise also the tip pilin SpaC, which harbours the same motif. The basal pilin SpaB, containing a single glycine (SG) motif, was recognised by SrtA, leading to the attachment of pilus to the cell wall.

Finally, the *in vivo* effect of the *L. rhamnosus* GG pili was evaluated in intervention trials in mice and humans. By performing mouse and human intervention trials with one of the described pilus-deficient derivatives, *L. rhamnosus* GG-PB12 and the parental strain, it was possible to demonstrate that the presence of pili increased the persistence of *L. rhamnosus* GG in the gastrointestinal tract and led to significant changes in the faecal microbiota composition, especially in humans. Species richness increased significantly due to the consumption of piliated *L. rhamnosus* GG in the human trial, indicating potential beneficial effects for the host, as a high species richness of gut microbiota has been associated with stability and resilience towards exogenous species. In conclusion, the research described here provides new insights into pilus biogenesis in *L. rhamnosus* GG and highlights the importance of the pili in the *in vivo* adherence capacity and gut microbiota modulation ability of *L. rhamnosus* GG.
1 INTRODUCTION

*Lactobacillus rhamnosus* GG is a lactic acid bacterium which was originally isolated from the intestine of a healthy human. The name of the strain originates from Barry Goldin and Sherwood Gorbach, who isolated and characterised the strain in the 1980’s (Goldin et al. 1992). *L. rhamnosus* GG was of interest at that time due to its potential as a probiotic bacterium as suspected from its capability of binding avidly to mucosal cells of the human gastrointestinal tract (GIT) (Gorbach & Goldin. 1989). Moreover, *L. rhamnosus* GG showed resistance towards the stresses encountered in the GIT, including acid in the stomach and bile in the intestine (Goldin et al. 1992, Salminen. 1996). Other promising qualities of the strain were the ability to transiently colonise GIT, strong growth, antibacterial activity and encouraging results especially in prevention and treatment of diarrhea (Alander et al. 1999, Goldin et al. 1992, Salminen. 1996, Silva et al. 1987).

Remarkably, the molecular interaction of *L. rhamnosus* GG with the host is largely mediated by pili, also called fimbriae, which are present on the cell surface of *L. rhamnosus* GG (Kankainen et al. 2009, Reunanen et al. 2012). Of Gram-positive bacteria, pili had been previously characterised only on the surface of pathogens, implying that pili constituted a pathogenic trait (Mora et al. 2005, Nallapareddy et al. 2006, Yanagawa et al. 1968). The recent identification of pili in *L. rhamnosus* GG suggests that they are a colonisation factor rather than a virulence factor. Structurally pili of *L. rhamnosus* GG were found to resemble the heterotrimeric pili of *Corynebacterium diphtheriae* (Ton-That & Schneewind. 2003). Following the discovery and primary characterisation of the pili, further studies have clarified their biological significance in the colonisation properties and host signaling of *L. rhamnosus* GG. Most of the studies on the molecular mechanisms behind *L. rhamnosus* GG’s effects have been performed *in vitro*. Although these studies have provided important insights into the function of *L. rhamnosus* GG within the GIT, the evaluation of the effects on the host needs to be further supported by *in vivo* experimental data.

Rodents, especially mice, are often used as model organisms for humans in studying the effect of various substances; in general animal models are essential for elucidating the *in vivo* effects of targeted gene modifications in bacteria and other organisms collectively called genetically modified organisms (GMO). Induced mutagenesis, a method for producing non-targeted mutations, for example in bacteria, has been used for decades in different sectors of industry as a means to improve the production properties and other functionalities of organisms. The improved mi-
Microbial strains obtained using these mutagenesis approaches are classified as non-GMO’s and can be used for studies to be performed also in humans (The European Parliament and the Council of the European Union, 2001).

Here, the current knowledge on the biogenesis of sortase-dependent pili in Gram-positive bacteria, and especially *L. rhamnosus* GG, is summarised. Moreover, the molecular basis of *L. rhamnosus* GG interaction and effects on the host, are reviewed. The aim of this thesis is to provide new insights into the biogenesis and function of pili in *L. rhamnosus* GG. This has been achieved by the characterisation of pilus-less non-mucus-adherent, as well as highly mucus-adherent derivatives of *L. rhamnosus* GG obtained by induced mutagenesis, and through the characterisation and functional verification of conserved variants of the sortase recognition motif in pilins, the building blocks of pili. In addition, the *in vivo* effect of pili on the colonisation and gut microbiota modulation ability of *L. rhamnosus* GG has been studied in mice and humans. Finally, potential future prospects for the use of the described *L. rhamnosus* GG derivatives and possible applications will be discussed.
2 REVIEW OF THE LITERATURE

2.1 PILI IN GRAM-POSITIVE BACTERIA

Gram-staining, a method developed by Hans Christian Gram at the end of the 19th century, is a means to differentiate between two distinct types of surface structures in bacteria (Gram & Friedlaender. 1884). In this procedure, Gram-positive bacteria with a thick peptidoglycan (PG) formed cell wall are dyed purple because crystal violet, used in the staining, is retained in the cell wall, while Gram-negative bacteria have a thin PG layer unable to retain the colour and hence are stained pink by safranin. Ever since, bacteria have been classified as either Gram-positive or Gram-negative. In addition to differences in the PG layer, Gram-positive bacteria have only one cell membrane, while Gram-negative bacteria have two membranes, the inner plasma membrane and the outer membrane containing lipopolysaccharides, and in between the membranes is a mesh of PG (Duong et al. 1997, Silhavy et al. 2010). In Gram-positive bacteria, the thick PG layer serves as a shield from mechanical and osmotic stresses, but it is also a platform for presenting many glycans and proteins, such as pili (Schneewind & Missiakas. 2012, Shockman & Barren. 1983, Silhavy et al. 2010). These molecules are often bound covalently to the cell wall and are important factors in attachment and signalling with the surrounding milieu (Schneewind & Missiakas. 2012, Silhavy et al. 2010).

In Gram-positive bacteria, pili were first discovered in the 60's when Yanagawa with colleagues reported their presence on the surface of a Corynebacterium species (Yanagawa et al. 1968). Subsequently, other pilus-producing pathogenic Gram-positives such as Streptococcus pyogenes and Enterococcus faecalis were discovered (Mora et al. 2005, Nallapareddy et al. 2006). In pathogens, pili have been found to have an important role in allowing the bacteria to adhere to tissues and in biofilm formation, hence they have an important role in the course of pathogenesis (Mandlik et al. 2007, Manetti et al. 2007, Nallapareddy et al. 2006).

Recently, representatives of so-called probiotic, i.e. health-promoting, bacteria have been found to possess pili. In addition to Lactobacillus rhamnosus GG (Kankainen et al. 2009), pili have been characterised in Lactococcus lactis (Oxaran et al. 2012) and Bifidobacterium bifidum (Turroni et al. 2013). The genomes of 213 type strains of lactic acid bacteria have been sequenced and 51 of them were found to possess one or several pilus gene clusters. The strains belonged to Lactobacillus composti, L. casei/rhamnosus, L. ruminis, L. brevis/parabrevis or Pediococcus ethanolidurans clades (Sun et al. 2015). Host signalling dependent on the presence of...
pili is an important aspect in the pathogenesis of the above mentioned pathogens. Hence, it is not a surprise that pili seem to have a significant role also in probiosis. In addition, Gram-negative-like Type IV pili have been found in certain representatives of *Lactobacillales* (Imam *et al.* 2011, Wydau *et al.* 2006). In contrast to the sortase-dependent pili, which contain covalently linked subunits and constitute one molecule, the type IV pili consist of non-covalently linked subunit molecules and have additional functions, such as DNA uptake during transformation and enabling twitching motility of the bacterium. The structure and biogenesis pathway of Type IV pili are also very different in comparison to sortase dependent pili (Melville & Craig. 2013). Here, the biogenesis and properties of sortase-dependent pili as well as associated protein secretion pathways will be explained in detail. Finally, the current knowledge on the *Lactobacillus rhamnosus* GG pilus biogenesis and the properties of pili as well as other surface adhesins will be presented.

### 2.1.1 Biogenesis of sortase-dependent pili

Sortase-dependent pili, or fimbriae, have been characterised in two forms on the surface of Gram-positive bacteria. Short 1-2 nm wide pili with the length of 70-500 nm have been found in strains of the oral commensal *Streptococcus oralis* (Willcox & Drucker. 1989), while longer flexible pili, which can be 0.3-3 μm long and 3-10 nm wide, have been detected in species of *Corynebacterium* and pathogenic species of *Streptococcus* (Gaspar & Ton-That. 2006, Mora *et al.* 2005, Proft & Baker. 2009). Pili consist of proteins called pilins: usually in mature pili either two or three different types of pilins are present, resulting in heterodimeric or heterotrimeric pilus structures, respectively (Schneewind & Missiakas. 2012). There are indications that the pilus biogenesis and protein anchoring on the surface of bacteria are intimately linked and located at the sites of protein secretion machinery (Hu *et al.* 2008, Kline *et al.* 2009). In *Bacillus subtilis*, protein secretion seems to be confined to spiral-like clusters on the cell envelope of this rod-shaped bacterium (Campo *et al.* 2004). Hence, it seems plausible that the secretion machinery is located at sites of cell wall synthesis, which apparently ensures the availability of lipid II substrate, a precursor of peptidoglycan (PG), for the adhesion of the proteins to the cell wall (Raz & Fischetti. 2008).

**SECRETION OF SURFACE PROTEINS**

Pilins are translocated across the cell membrane by the Sec-secretion system due to the amino-terminal (N-terminal) secretion signal peptide. The signal peptide usual-
ly consists of 15-20 hydrophobic amino acids juxtaposed with N-terminal positively charged residues, and is cleaved off by signal peptidases after translocation (Blobel. 1980, Emr et al. 1980, Navarre & Schneewind. 1999, Silhavy et al. 1983). The Sec-secretion system has been studied most thoroughly in *Escherichia coli*, but is considered to be well conserved and similar in all studied bacteria (Pugsley. 1993, Schneewind & Missiakas. 2014).


As proteins secreted through SecYEG translocon have to be in an unfolded state, it is generally thought that the proteins remain unfolded until they have been translocated. However, Nouwen and colleagues demonstrated that SecA has the ability to unfold preproteins, which provides evidence that there might well be exceptions to this model (Nouwen et al. 2007). Some preproteins are known to fold already before being secreted, but these proteins are secreted through a different pathway called Twin-arginine translocation pathway (Tat). Proteins secreted through the Tat-pathway have a different secretion signal, and are hence directed to another site on the cell membrane (Natale et al. 2008). In addition, these proteins are not attached into the cell wall by sortases (DeDent et al. 2008).
Post-translational secretion can occur via three pathways: i) the canonical pathway SecA-SecYEG, which is thought to be essential for survival and in which proteins are exported with the help of SecA1 ATPase, ii) a still not fully understood accessory pathway, SecA2-SecY2, in which a different set of translocation proteins are used than in canonical pathway, and iii) a SecA2-only pathway, which most likely translocates proteins with the help of SecYEG of the canonical pathway or some other unknown protein (Bensing et al. 2014, Feltcher & Braunstein. 2012, Rigel & Braunstein. 2008, Rigel et al. 2009). Previously, it was thought that only a few pathogenic Gram-positive species harboured the SecA2-associated pathways, but currently there is increasing evidence supporting the presence of SecA2 in large part of the Gram-positives, including intestinal commensal bacteria (Bensing et al. 2014). SecA2 and SecY2 are paralogous with the corresponding proteins of the canonical pathway, but SecA2 proteins are generally smaller than their counterparts (Bensing & Sullam. 2009, Feltcher & Braunstein. 2012). The first Gram-positive bacteria, in which the SecA2-mediated secretion was characterised, included *Staphylococcus gordonii*, *Listeria monocytogenes* and *Mycobacterium tuberculosis* (Bensing et al. 2005, Bensing & Sullam. 2009, Braunstein et al. 2001, Lenz et al. 2003). According to the current knowledge, the accessory pathways are somewhat dispensable and they secrete only a subset of proteins. Nonetheless, considering their conservation among various species, it remains to be seen whether this assumption is generally applicable. At least in *Corynebacterium glutamicum*, the presence of SecA2 is essential for viability (Caspers & Freudl. 2008). In addition, the accessory pathways seem to have a major role in the virulence and colonisation ability of the bacterial strains (Bensing et al. 2014, Feltcher & Braunstein. 2012, Freudl. 2013).

One main property of the SecA2-SecY2 accessory secretion pathway (ii) is that it translocates glycosylated proteins and it has been postulated to couple glycosylation and secretion (Bensing et al. 2014, Li et al. 2008, Seepersaud et al. 2012). In addition, glycosylation prior to secretion seems to block secretion through the canonical pathway (Bensing et al. 2005). In studies on *S. gordonii* it has been discovered that in addition to SecY2 and SecA2, five additional proteins called Asps (Asp1-Asp5) are needed for the optimal translocation through the secretion pathway (Bensing & Sullam. 2002, Seepersaud et al. 2010, Takamatsu et al. 2005). The substrates are glycosylated by glycosyltransferases in the cytoplasm and cannot be translocated across the cell membrane without the presence of SecY2 translocation pore (Bensing et al. 2004, Takamatsu et al. 2004). Asp3 binds Asp1, Asp2 and SecA2, and the resulting complex is required to ensure substrate transport (Seepersaud et al. 2010). Asp4 and Asp5 are not present in all SecA2-SecY2 systems, and their
function is not well known, but *S. gordonii* Asp4 and Asp5, shown to be essential for successful secretion, harbour partial sequence homology with the translocon proteins SecE and SecG of *Bacillus subtilis* (Feltcher et al. 2013, Takamatsu et al. 2005).

The SecA2-only pathway (iii) is the least known of the post-translational secretion pathways; in fact it has been studied most thoroughly in mycobacteria (Rigel & Braunstein. 2008). In a recent study, it was noted that in *Mycobacterium smegmatis* mature domains of the secreted proteins directed them to be secreted through the SecA2-only pathway (Feltcher et al. 2013). Since it was observed that the mature regions were prone to folding, it was suggested that SecA2 acts in collaboration with the Tat-secretion pathway, although this might not be the case in other bacteria (Feltcher et al. 2013). It has been proposed that SecA2-only is a secretion route for proteins not compatible with the SecA1 enzyme, including some proteins with N-terminal secretion signals or proteins lacking canonical signal peptides (Bensing et al. 2014, Feltcher & Braunstein. 2012).

It has been postulated that in rapidly growing bacteria the post-translational pathway would be preferred, as, in general, protein synthesis is a slower process than translocation of proteins. Hence, it would be beneficial for the organism to uncouple translation and secretion for those proteins for which post-translational secretion is possible, and secrete through the SRP-pathway only the proteins that might lose their translocation ability in the cytoplasm (Hegde & Bernstein. 2006). In studies conducted with *E. coli*, it was noted that only a few proteins were secreted through the SRP-pathway, for example mult spanning membrane proteins that might aggregate in cytoplasm (Hegde & Bernstein. 2006, Huber et al. 2005). It is also possible, that the translocons, e.g. SecYEG, through which the proteins are translocated in each pathway, are positioned at different areas on the cell membrane (Hegde & Bernstein. 2006, Spirig et al. 2011).

**PROTEINS REQUIRED FOR THE BIOGENESIS OF PILI**

The pilins, which are the building blocks of Gram-positive pili, have a 30-40 residue long hydrophobic carboxy-terminal (C-terminal) domain and a tail of positively charged amino acids. This region forms the membrane anchor, which keeps the pilin attached to the cell membrane after its secretion (Davis & Model. 1985, Schneewind et al. 1992). Non-membrane proteins secreted by the same pathway are released into the extracellular space due to the absence of the membrane anchor (Schneewind & Missiakas. 2014). The C-terminal part of the pilins contains also a conserved sorting motif LPxTG, situated about 10 amino acids N-terminal from the cell membrane anchor domain (Fischetti et al. 1990, Mazmanian et al. 1999). The
LPxTG motif is essential for the recognition of the pilins by transpeptidase enzymes called sortases; these are responsible for the assembly and attachment of pili into the cell wall (Mazmanian et al. 1999). Within the LPxTG motif, x can be any amino acid residue, and the site for cleavage by sortase is between the threonine (T) and glycine (G) residues. In heterotrimeric pili, such as those found in Corynebacterium diphtheriae, three pilin types are used in the assembly of the pilus: the major pilin forming the backbone of the pilus and two minor pilins, of which one functions as a tip pilin, and the other one as the basal pilin, which has been suggested to act as a synthesis terminating pilin (Guttilla et al. 2009, Mandlik et al. 2007, Ton-That & Schneewind. 2003, Ton-That et al. 2004).

To date, six classes of sortases have been described (Spirig et al. 2011). Class A consists of housekeeping sortases present in all Gram-positive bacteria; these enzymes recognise the LPxTG motif in surface protein precursors. These housekeeping sortases link surface proteins covalently to the PG precursor lipid II, thus leading to incorporation of the surface proteins into the cell wall (Perry et al. 2002, Ton-That et al. 1999). Class A sortases are linked to the cell membrane by an N-terminal hydrophobic domain, allowing the exposure of the catalytic site in the C-terminus on the extracellular side. Secretion and sorting signal peptides are also located at the N-terminal end of the protein (Marraffini et al. 2006, Scott & Barnett. 2006). Class C sortases are pilin-specific transpeptidases and they link pilins together to form the pilus fibers. The genes of these enzymes are encoded in the same gene clusters as their substrate pilins (Budzik et al. 2008, Mandlik et al. 2008b). Class C sortases are attached to the cell membrane via their C-terminal hydrophobic domain (Hendrickx et al. 2011). Class B sortases are specialised in facilitating haem transport and have a slightly varying structure when compared to Class A enzymes, while Class D sortases are linked to spore formation (Maresso & Schneewind. 2006, Marraffini et al. 2006, Marraffini & Schneewind. 2007). Class E sortases have similar functions as Class A sortases, but have been found in bacterial species with GC-rich genomes (Spirig et al. 2011, Ton-That & Schneewind. 2003). The functions of Class F sortases found to be present in Actinobacteria have remained uncharacterised, but phylogenetically they group separately from the other sortases (Spirig et al. 2011). All sortases have a catalytic pocket, in which a TLxTC motif and the catalytic triad of amino acid residues Cys (C in TLxTC), Arg (R) and His (H) involved in the transpeptidation reaction, are located (Hendrickx et al. 2011, Manzano et al. 2008). The residue denoted by x can be any amino acid. Some Class C sortases have also been found to contain a lid region, which covers the active site (Manzano et al. 2008). Class A and C sortases are needed in the biogenesis of the pilus (Hendrickx
et al. 2011). A schematic model of post- translational secretion of pilins and biogenesis of pili on the cell envelope is shown in Figure 1.

SORTASES MAKE IT HAPPEN

Pilus biogenesis starts when the pilus-specific sortase recognises the LPxTG motif of a pilin, cleaves the motif between the T and G residues and forms an acyl bond between the cysteine (C) of its own active site, i.e. the TLxTC sequence in the catalytic pocket, and the T of the N-terminal part of the pilin, resulting in an acyl-enzyme intermediate (Guttilla et al. 2009, Hendrickx et al. 2011). When two acyl-enzyme intermediates are formed with the tip pilin and the backbone pilin, an isopeptide bond is formed between the two pilins through a nucleophilic attack by the backbone (major) pilin (Hendrickx et al. 2011, Mandlik et al. 2008a). The nucleophilic attack from the backbone pilin is due to the ε-amino group of lysine (K) in the pilin motif WxxxVxVYPKN, which results in a cleavage of the thioester bond between the sortase and the pilin in the acyl-enzyme intermediate, and the formation of a covalent bond between the two pilins (Kang et al. 2007, Ton-That & Schneewind. 2003). According to another model, it is also possible that major pilins not yet attached to a sortase might trigger a nucleophilic attack leading to pilus polymerisation, but there is experimental evidence favouring the initial model (Spirig et al. 2011). The major pilin contains another conserved motif, the E-box motif (YxLxEoAPoGY), which is essential for the formation of pili. This motif has been suggested to have a role in the stabilisation of the three dimensional structure of the pilin, as it has been shown to form an intra-protein isopeptide bond (Kang et al. 2009).

The polymerisation of the pilus continues until the synthesis-terminating basal pilin is added to the pilus fibre (Mandlik et al. 2008a). The basal pilin is recognised by a Class A sortase, which attaches the pilin to the cell wall. When the forming pilus comes into contact with an acyl-enzyme intermediate of the synthesis terminating pilin, the pilin is added to the pilus and the housekeeping sortase forms the acyl-enzyme intermediate with the pilus (Swaminathan et al. 2007). By undertaking a nucleophilic attack of lipid II, pilus is attached covalently to the cell wall (Hendrickx et al. 2011, Swaminathan et al. 2007). In C. diphtheriae, basal pilins are detected also along the length of the pili. The reason for this, according to the current model, is that sometimes basal pilin is incorporated into the pilus fibre because the Class C sortase is not entirely specific for the major pilin and is able to form an acyl-enzyme intermediate also with basal pilin and hence polymerise it into the pilus (Mandlik et al. 2008a, Spirig et al. 2011).
Figure 1. Schematic model of post-translational secretion of pilins and biogenesis of a heterotrimeric pilus on the cell envelope of a Gram-positive bacterium. Preprotein is translated at the ribosome, from where it is transferred by a chaperone to the SecYEG translocon for secretion. The pilins SpaA, SpaB and SpaC are retained at the cell membrane by their C-terminal hydrophobic anchors. Sortase C (class C sortase) synthesises the pili, while sortase A (class A sortase) covalently attaches pili into the cell wall. The figure is partly adapted from Hendrickx et al. 2011.
It has been noted that only the major pilin is essential for the formation of pili (Swierczynski & Ton-That. 2006, Ton-That et al. 2004). There are indications that pilus biogenesis is a stochastic process, meaning that the amount of different pilins on the cell membrane regulates the number and length of the pili (Mandlik et al. 2008a, Swierczynski & Ton-That. 2006). For example, it has been shown with *C. diphtheriae* SpaH pilin that a high concentration of the major pilin SpaH regulates the length of the pili, apparently because it is then able to outcompete the basal pilin (Swierczynski & Ton-That. 2006). Since the basal pilin, or the so-called synthesis-terminating pilin, is recognised by the housekeeping sortase, which attaches proteins to the cell wall, it is thought that a higher quantity of basal pilins at the membrane and consequently their incorporation to the pili leads to a halt of the pilus biogenesis and ultimate attachment of pili into the cell wall.

### 2.1.2 Pili in *Lactobacillus rhamnosus* GG

Pili were first discovered in *Lactobacillus rhamnosus* GG when its genome was sequenced and two gene clusters coding for pilins and pilin-specific sortases, *spaCBA-srtC1* and *spaFED-srtC2*, were identified (Kankainen et al. 2009). Subsequent immuno-electron microscopy analyses with SpaC antibody revealed pili for the first time in a probiotic bacterium, confirming the expression of the *spaCBA-srtC1* gene cluster. Subsequently, the pilus fibers were further characterised, and the absence of SpaFED pili was verified, although it cannot be completely ruled out that *spaFED-srtC2* is expressed under some specific conditions (Reunanen et al. 2012, Rintahaka et al. 2014).

**PILUS BIOGENESIS IN LACTOBACILLUS RHAMNOSUS GG**

Pili of *L. rhamnosus* GG are heterotrimeric and composed of pilins SpaA, SpaB and SpaC sized 31 kDa, 21 kDa and 91 kDa, respectively (Spa: Sortase-mediated Pilus Assembly) (Kankainen et al. 2009, Reunanen et al. 2012, Ton-That & Schneewind. 2004, von Ossowski et al. 2010). Pilins are present in the pilus in a ratio of 5:2:1 (SpaA:SpaC:SpaB) (Tripathi et al. 2013). The pili are synthesised by transpeptidase sortase C (SrtC, Srt: Surface protein soRTing) and attached into the cell wall by sortase A (SrtA). SpaA pilin forms the backbone of the pilus structure, while SpaC is the tip pilin, but it is also found at the sides of the pilus structure (Kankainen et al. 2009, Reunanen et al. 2012). SpaB is the synthesis-terminating pilin, but is also found along the shaft of the pilus, possibly because of the promiscuity of the SrtC as mentioned for *C. diphtheriae* (Mandlik et al. 2008a). Pilins and SrtC are believed to
be secreted through the Sec-pathway since they harbour the N-terminal secretion signal peptide, and since SpaC was recently observed to contain sugar moieties, it is possible that at least some of the pilins are secreted through the accessory SecA2-SecY2-pathway (Tytgat et al. 2016). SrtC was found to contain a GYPSY motif in its N-terminal end, which includes tyrosine (Y) and proline (P) residues essential for the formation of pili. It is likely that these residues enable SrtC to be secreted to the correct position on the cell membrane ensuring successful pilus biogenesis (Douillard et al. 2016a).

According to the currently proposed theory, pilus biogenesis starts when SpaC is bound by SrtC at the cell membrane. The pilus fibre forms as SpaA pilins are attached first to SpaC and then one by one to each other by isopeptide bonds between sorting motif LPxTG and the pilin motif WxxxVxVYPKN of the following pilin. SpaB, the base pilin, terminates the pilus synthesis, and housekeeping sortase SrtA attaches the pilus in the cell wall (Reunanen et al. 2012). It is not yet clear how SpaC pilins are attached at the sides of the pilus, and whether this attachment occurs via a covalent bond or involves some other form of interaction. It has been suggested that since SpaC harbours two putative E box elements, it could be attached to the forming pilus from the outside via some unknown mechanism. SpaC lacks a canonical pilin motif, so its incorporation into the pilus backbone is unlikely (Reunanen et al. 2012). The schematic structure of the pilus is depicted in Figure 2 in conjunction with an electron micrograph of a pilus on the surface of L. rhamnosus GG.

PROPERTIES OF THE PILUS

SpaC, the mucus-binding pilin, is essential for the binding properties of the pilus (Kankainen et al. 2009, von Ossowski et al. 2010). SpaB pilin is also able to bind mucus, but the adherence is most likely due to electrostatic interactions, and because of the position of SpaB in the mature pilus, i.e. incorporation into the pilus backbone predominantly close to the base of the pilus, it is unlikely to have a major role in the adherence (Reunanen et al. 2012, von Ossowski et al. 2010). SpaC contains 895 amino acid residues, which make up domains similar to type A von Willebrand factor domain (residues 137-262), collagen-binding protein domain B (496-551) and two Cna protein B-type domains (621-681 and 749-818) (Kankainen et al. 2009, Tripathi et al. 2013). The collagen-binding protein domain B (cd00222) and the B-type Cna protein domains (pfam05738) are similar to the domains present in the Staphylococcus aureus collagen binding protein. In the S. aureus protein, the domains do not mediate binding but form a stalk that displays the ligand-binding domain (Deivanayagam et al. 1999, Tripathi et al. 2013). A recent structural analysis
Figure 2. Model (left) and immuno-electron micrograph (right) of the <i>Lactobacillus rhamnosus</i> GG pilus structure. The major pilin SpaA forms the pilus shaft, while the mucus-binding pilin SpaC is situated at the tip and on the sides of the pilus. SpaB, the basal pilin, resides at the bottom of the structure, but is also incorporated in low quantities into the shaft. The immunoelectron micrograph was made from the stationary growth phase <i>L. rhamnosus</i> GG using antibodies against SpaC and SpaA, labelled with 5 and 10 nm protein A-gold, respectively. SpaC is indicated with a white arrow, whereas SpaA is indicated with a black arrow. The model has been partly adapted from Reunanen <i>et al.</i> 2012.

of SpaC suggested that the type A von Willebrand factor domain had an important role in the adhesion properties of SpaC (Kant <i>et al.</i> 2016). It is possible that also the glycan structures of SpaC affect the binding specificity (Tytgat <i>et al.</i> 2016).

Several studies show that pili are the major adhesins of <i>L. rhamnosus</i> GG. It has been demonstrated that pili mediate binding of <i>L. rhamnosus</i> GG to both human and porcine mucus, collagen and to intestinal epithelial cell lines Caco-2 and HT29 (Ardita <i>et al.</i> 2014, Douillard <i>et al.</i> 2013a, Douillard <i>et al.</i> 2013b, Lebeer <i>et al.</i> 2012, Tripathi <i>et al.</i> 2013, von Ossowski <i>et al.</i> 2013). Additionally, pili have been shown to mediate <i>L. rhamnosus</i> GG binding to β-lactoglobulin, one of the major proteins in whey, which is beneficial considering the commercial applications of the strain.
(Burgain et al. 2013, Guerin et al. 2016). Interestingly, there are also indications that fermentation of milk with *L. rhamnosus* GG reduces antigenicity of the milk proteins, for example β-lactoglobulin (Yao et al. 2014). In a recent study, it was shown that SpaC pilins bind each other, suggesting that pili most likely mediate binding also between *L. rhamnosus* GG cells (Tripathi et al. 2013). Since SpaC is present along the pilus length, it has been postulated that the pili bind their substrates in a zipper-like manner, which would enable intimate contact and long lasting adherence, for example in the midst of shearing forces in the GIT (Figure 3) (Lebeer et al. 2012, Telford et al. 2006). Evidence for this was obtained from studies with atomic force microscopy; it was noted that when a mature pilus was pulled using low force, the force curves generated showed gradual dissociation patterns (Tripathi et al. 2013). It was also noted that sometimes pili formed helical spring-like structures, and when pulled with a high force, stretching and spring-like behaviour curves were detected. In addition, the dissociation of SpaC was rapid, indicating that this feature might be important for colonisation and exploration of the intestinal binding sites (Tripathi et al. 2013).

Since SpaC appears to mediate binding of *L. rhamnosus* GG cells to each other, this suggests that pili are essential for the biofilm forming ability of *L. rhamnosus* GG, although environmental factors have the decisive role in whether or not the biofilm is formed (Lebeer et al. 2007, Lebeer et al. 2012). A biofilm is a layer of bacterial cells embedded in a matrix of extracellular polymeric matter, which is formed on a surface, for example on host tissue (Flemming & Wingender. 2010). The main feature of a biofilm is that cells are bound to each other and to the surface. Biofilm-like communities, such as microcolonies, are thought to play an important role in the colonisation ability of bacterial cells (Branda et al. 2005). Full biofilm formation has not been detected in the GIT of a healthy human, but rather they have been observed to assume smaller biofilm-like growth forms or become dispersed in the mucus layer (Lebeer et al. 2011b, Macfarlane et al. 2004). Biofilms seem to be less commonly present in healthy mucus than in certain gastrointestinal diseases, such as inflammatory bowel disease (IBD), which could be due to aberrant immune response towards the intestinal bacteria possibly associated with the disease (Swidsinski et al. 2005, de Vos. 2015). For example in IBD, biofilms have been detected to be mainly composed of commensal species *Bacteroides fragilis*, of which some strains can behave as opportunistic pathogens (Swidsinski et al. 2005).
PILUS AS AN ADAPTATION FACTOR

The promoter region for the spaCBA-srtC1 is located in the iso-IS30-insertion element just upstream of the gene cluster, and it has been suggested that the insertion of the element has enabled the production of pili (Douillard et al. 2013a). The spaCBA-srtC1 cluster is situated in a region in the L. rhamnosus GG genome, which is densely decorated by insertion sequence (IS) elements, making it somewhat unstable. IS elements are transposable elements less than 2.5 kilobases long and they have been recognised as an important source of genomic variation in bacteria (Chandler & Mahillon. 2002, Mahillon & Chandler. 1998, Schneider & Lenski. 2004). The region around the spaCBA-srtC1 cluster includes 2 of the 5 genome islands (GG-ISL1-GGISL5) in L. rhamnosus GG genome, namely GGISL1 and GGISL2 (Kankainen et al. 2009). GGISL2 contains the spaCBA-srtC1 gene cluster. These genome islands have also been referred to as lifestyle islands, reflecting the role of the regions in the adaptation of the strain to varying environmental conditions and special niches (Douillard et al. 2013a). Recently it was demonstrated that L. rhamnosus strains adapted to niches other than those found in the intestinal tract, such as strains isolated from dairy products, have often lost genomic regions including GGISL2 as well as other genes involved for example in metabolism of certain carbohydrates (Douillard et al. 2013a, Sybesma et al. 2013). The long-term stability of the spaCBA-srtC1 region was examined under various environmental stress conditions, revealing that prolonged bile stress caused deletion of this same region via an IS-mediated deletion event (Douillard et al. 2016b). It is possible that such events affect the long-term GIT colonisation ability of L. rhamnosus GG.

CHARACTERISATION OF THE SpaFED PILI

As mentioned before, SpaFED pili have not been detected on the surface of L. rhamnosus GG cells. Nevertheless, the pilins encoded by the genes have been characterised, as well as the whole pili, which were studied in a Lactococcus lactis model (Rintahaka et al. 2014, von Ossowski et al. 2010). In general, SpaFED pilins are slightly larger than SpaCBA pilins, but as with the SpaCBA pili the tip pilin SpaF is the largest (104 kDa), while major pilin SpaD is the second largest (51 kDa) and the predicted synthesis-terminating pilin SpaE is the smallest (45 kDa). SpaF possesses mucus-binding properties similar to SpaC, but SpaE is not mucus-adherent in contrast to SpaB. SpaD does not bind mucus similarly to its counterpart SpaA (von Ossowski et al. 2010). Characterisation of the structure, adherence features and immunological effects of the SpaFED pili was performed by expressing the pili on the surface of L. lactis cells. SpaFED pili were structurally similar to SpaCBA pili, and
were adherent to mucus, Caco-2 and HT29 intestinal epithelial cell lines and proteins of mammalian cells such as collagen. On the other hand, SpaFED pili did not induce similar immune signalling as SpaCBA pili, but rather dampened the effects of L. lactis, which was used as the expression host (Rintahaka et al. 2014).

**OTHER SURFACE ADHESINS OF LACTOBACILLUS RHAMNOSUS GG**

In addition to pili, other surface adhesins have been detected on the surface of L. rhamnosus GG cells. According to the current model of the adhesion of L. rhamnosus GG to the host tissue, initial contact is achieved via the pili and as the intimate contact forms with the help of the spring-like nature of the pilus and sequential adhesions by the other SpaC pilins in the pili, other molecules at the surface have the possibility to adhere. Few adhesins other than pili have been thoroughly characterised. Of those, modulator of adhesion and biofilm (MabA) is a very large protein of 2419 residues encoded by the gene with locus tag LGG_01865. The strain in which the mabA gene has been knocked-out displays a reduced biofilm formation capacity and decreased adhesion to intestinal epithelial cell lines and mouse intestinal tissues (Velez et al. 2010). Mucus binding factor (MBF) is encoded by the gene LGG_02337 and is an approximately 40 kDa protein, which can adhere to human mucus and it has been demonstrated in vitro to contribute to the total adherence capability of L. rhamnosus GG (von Ossowski et al. 2011). Additionally, other proteins with potential adhesive functions, such as several moonlighting proteins, have been identified on the surface of L. rhamnosus GG cells, but their functions require further characterisation (Espino et al. 2014). Although in vitro studies have shown the pili to have the highest effect on the adherence properties of L. rhamnosus GG, their impact has to be evaluated under in vivo conditions, since the situation might be quite different considering all the environmental factors possibly affecting the interactions, such as the presence of other microbes, host metabolism and the response of L. rhamnosus GG to the milieu. The schematic model of L. rhamnosus GG adhesion is shown in Figure 3.

**2.2 LACTOBACILLUS RHAMNOSUS GG AS A PROBIOTIC**

Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al. 2014). Probiotics are used mainly in promoting GIT health, but they are used as well for example in balancing disturbed vaginal and oral microbiota (Borges et al. 2014, Haukioja. 2010). Traditionally probiotics are representatives of lactobacilli and bifidobacteria, but due to
Figure 3. Model of adhesion of *L. rhamnosus* GG in the gastrointestinal tract. According to the current theory, when arriving into the GIT of the host, *L. rhamnosus* GG attaches to the host mucus via its pili, and binds host mucus and possibly epithelial cells in a zipper-like manner. This ensures intimate contact and signalling between the microbe-associated molecular patterns (MAMPs) on the surface of *L. rhamnosus* GG and host pattern-recognition receptors (PRRs) on the intestinal wall. PRRs are shown as curved Y-shaped receptors on the intestinal wall interacting with the MAMPs on the surface of *L. rhamnosus* GG.

Recent advances in the knowledge on the human gut microbiota composition, novel potential probiotic strains belonging to other taxa have been found and are being characterised, such as *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* (Derrien *et al.* 2004, Everard *et al.* 2013, Sokol *et al.* 2008). Probiotics are thought to promote health by normalising perturbed microbiota (sometimes designated as dysbiosis), strengthening colonisation resistance by inducing tight junction protein and mucus production, and excluding pathogens by occupying their binding sites. In addition, probiotics produce substances, such as short chain fatty acids (SCFA), which are important for the health and growth of the host cells, and lactic acid, which inhibits the growth of harmful bacteria (Hill *et al.* 2014). *L. rhamnosus* GG is one of the most studied and generally used probiotic strains. Clinical evidence on its function as a probiotic and current knowledge on the molecular background of the probiotic action is reviewed in the following chapters. *L. rhamnosus* GG has been studied also in prevention and treatment of oral and vaginal conditions, but here
the emphasis will be on the GIT-related benefits in human (Gardiner et al. 2002, Hatakka et al. 2007, Näse et al. 2001).

2.2.1 Intervention trials

The effects of *L. rhamnosus* GG have been evaluated in the prevention and treatment of several diseases and conditions. The most convincing results have been obtained in the prevention of infectious, antibiotic-associated and traveller’s diarrhea in children and adults (Sanders et al. 2013, Szajewska & Kołodziej. 2015, Wolters et al. 2010). In addition, *L. rhamnosus* GG seems to be useful in reducing GIT related symptoms and diarrhea caused by the chemo- and radiotherapy used for the treatment of cancer (Österlund et al. 2007, Urbancsek et al. 2001). Promising results have been obtained also when *L. rhamnosus* GG has been administered to prevent respiratory tract infections in children (Hojsak et al. 2010, Kumpu et al. 2012, Luoto et al. 2014). *L. rhamnosus* GG has been investigated intensively in the prevention and treatment of allergic diseases, including atopy and food allergy, but the results have been variable (Földer-Holst et al. 2006, Isolauri et al. 2012, Kalliomäki et al. 2010, Viljanen et al. 2005). A large prospective study indicated that consumption of *L. rhamnosus* GG by the mother before birth and during lactation reduced the occurrence of eczema in her offspring at least during the first 7 years of life, and that the consumption of the probiotic could reduce the severity of symptoms in atopic eczema/dermatitis patients (Kalliomäki et al. 2001, Kalliomäki et al. 2003, Kalliomäki et al. 2007). Encouraging results have been obtained in the treatment of allergy in infants by supplementing with *L. rhamnosus* GG the extensively hydrolysed whey formulas used for weaning. Isolauri et al. reported that when infants with atopic eczema were weaned with the abovementioned *L. rhamnosus* GG-supplemented formula, this achieved a significant improvement in the eczema symptoms (Isolauri et al. 2000). Recent findings indicate that extensively hydrolysed casein formula (EHCF) in combination with *L. rhamnosus* GG may induce tolerance to cow’s milk in allergic children (Berni Canani et al. 2013). *L. rhamnosus* GG has been studied also when combined with other probiotics, as multiprobiotics, for example in a large trial in which it was consumed as a combination with *Bifidobacterium lactis* Bb12 by mothers before labour and during lactation. The results revealed the potential of the probiotic combination to reduce atopic eczema and early life weight gain in children, as well as increasing glucose tolerance and decreasing adiposity in the mothers (Huurre et al. 2008, Ilmonen et al. 2011, Laitinen et al. 2009, Luoto et al. 2010). Although the results obtained with multiprobiotics cannot be extrapolated to the effects obtained with single strains, there are interesting indications that also
*L. rhamnosus* GG alone has the potential to modify serum lipid profiles in addition to influencing inflammatory markers in healthy adults (Kekkonen *et al*. 2008). In general, it is clear that more studies will be needed to establish these findings.

**EFFECT ON THE HOST GUT MICROBIOTA**

Gut microbiota composition varies between individuals, due to genetics, colonisation and other environmental factors, such as diet (Bäckhed *et al*. 2012, Benson *et al*. 2010). Currently, it is thought that gut microbiota consists of an individual core microbiota, containing bacteria present most of the time in the GIT of an individual, accompanied by variable and transient colonisers affected by environmental factors (Derrien & van Hylckama Vlieg. 2015, Jalanka-Tuovinen *et al*. 2011, Rajilić-Stojanović *et al*. 2013). The determinants of a healthy gut microbiota are thought to be high diversity, high temporal stability and functional redundancy, while disturbance of these states might cause aberrations and possibly lead to illness (Jalanka. 2014). The species composition of a healthy microbiota is difficult, if not impossible to define. However, when differences between age and nationality are controlled, it may be feasible to identify a common core microbiota (Jalanka-Tuovinen *et al*. 2011, Lozupone *et al*. 2012). The microbiota composition varies greatly during life, from bifidobacteria-driven communities in infancy through diverse communities in adulthood to decreased diversity in the elderly. However, it is not currently known whether the decrease in diversity is a cause or a consequence of increased prevalence of diseases in the elderly (Jeffery *et al*. 2015, Turroni *et al*. 2012). Prevention and treatment of diseases by modulating the gut microbiota composition, for example by using probiotics, is an interesting possibility, and the subject is currently under intensive research.

For now, the studies, in which the effect of *L. rhamnosus* GG consumption on the gut microbiota composition has been studied, are sparse. As the community level microbiota analysis methods using 16S rRNA-based approaches are still relatively new, there are only a few published papers, which have employed these methods in this type of work. Most of the research has been done using conventional methods, such as cultivation, fluorescent in situ hybridisation (FISH), quantitative PCR (qPCR) and terminal restriction fragment length polymorphism (T-RFLP). Community level studies of the GIT include phylogenetic analyses, which target 16S rRNA, as well as metagenomic analyses, which investigate all of the DNA in an ecosystem. The latter approach is relatively unbiased as it does not include regular PCR amplification reactions but is rather expensive as the genomic complexity in bacteria is approximately 1000-fold higher than that of 16S rRNA genes. Moreover, advanced
bioinformatics tools are needed as the GIT metagenome includes several millions of genes (Qin et al. 2010). Hence, most studies have targeted 16S rRNA gene sequences and are performed with phylogenetic microarrays, such as HITChip (Human Intestinal Tract Chip) or PhyloChip, or 16S rRNA sequencing, usually using the Roche 454 pyrosequencing, the Illumina MiSeq or HiSeq platforms, and the Applied Biosystems SOLiD platform (DeAngelis et al. 2011, Rajlić-Stojanović et al. 2009). The effect of *L. rhamnosus* GG consumption on the gut microbiota composition was studied for the first time in a trial concerning 6 months old infants. The trial, using the PhyloChip microarray as the analysis method, revealed significant increase in several species of *Lactobacillus* and a member of *Bifidobacteriaceae* upon administration of *L. rhamnosus* GG (Cox et al. 2010). However, in healthy adults, no major intervention-associated changes at the community level or in the bacterial taxa have been discovered following consumption of *L. rhamnosus* GG. In a study using the HITChip phylogenetic microarray, no overall community changes were observed, although an increase in *L. rhamnosus*-related bacteria was detected, and in another study applying pyrosequencing, only intraindividual changes in bacterial groups were detected (Kim et al. 2013, Lahti et al. 2013). Intraindividual changes were also detected using the HiSeq platform in a recent study in elderly subjects (Eloe-Fadrosh et al. 2015).

In children and in adults with health issues, gut microbiota modulation due to consumption of *L. rhamnosus* GG has been detected. In a well-controlled study investigating the acquisition of tolerance in children with cow’s milk allergy due to consumption of EHCF or EHCIF in combination with *L. rhamnosus* GG (EHCF-LGG), a number of changes in bacterial genera were detected by 16S rRNA sequencing with the MiSeq platform (Berni Canani et al. 2015). Tolerance was induced in 5 of 12 children in the EHCF-LGG group, whereas in the EHCF group none became tolerant. *Blautia, Coprococcus* and *Roseburia* were increased after EHCF-LGG treatment, although *Roseburia* was increased also after EHCF treatment. When comparing the post-treatment samples, *Roseburia* and *Anaerostipes* were increased in EHCF-LGG group in comparison to the EHCF group. The amount of faecal butyrate was increased in children who became tolerant, and study subjects with highest amounts of butyrate displayed an increased abundance of *Blautia* and *Roseburia*. The abundance of *Oscillospira* was significantly enriched in the allergic samples. No significant differences were detected in the pre-intervention samples between those children who became tolerant and the children that remained allergic. Single *Coprococcus* and *Roseburia* oligotypes were significantly enriched in the tolerant subjects and these correlated positively with butyrate quantities in faecal speci-
mens. It is possible that *L. rhamnosus* GG increased the abundance of butyrate producers and hence the quantity of butyrate in the GIT, which in part influenced the tolerance acquisition (Berni Canani *et al.* 2015).

A recent study using the HITChip microarray showed that *L. rhamnosus* GG consumption could protect against gut microbiota changes induced by antibiotic use in preschool children, especially in the case of penicillin. The results indicated also that the use of macrolide- and sulphonamide-trimethoprim-antibiotics was significantly reduced in the *L. rhamnosus* GG intervention group for up to 3 years after the intervention (Korpela *et al.* 2016). In a trial studying the effects of *L. rhamnosus* GG consumption in liver cirrhosis patients, gut microbiota alterations were observed as well as reduction in endotoxia. Endotoxia, i.e. the presence of endotoxins from Gram-negative bacteria in the blood stream, has been noted to be associated with a poorer disease prognosis (Bellot *et al.* 2013). The gut microbiota analyses were performed using multi-tag pyrosequencing. *L. rhamnosus* GG consumption resulted in a decrease in the abundance of *Enterobacteriaceae*, whereas Clostridiales Incertae Sedis XIV and *Lachnospiraceae* were increased. Additionally, a decrease in endotoxia was detected (Bajaj *et al.* 2014).

**RESPONDER EFFECT IN PROBIOTIC TRIALS**

Trials studying the effect of probiotics in the prevention and treatment of different disorders as well as on metabolic markers and gut microbiota have often produced controversial results. This is most likely partly due to differences in the study designs and analytical methods, but the responder effect, i.e. the effect of individual genetics and gut microbiota composition on the response of the person consuming the studied probiotic, is becoming increasingly acknowledged (Tims *et al.* 2011). The host lives in a symbiotic relationship with his/her gut microbiota: the microbiota makes its own contribution, for example in metabolising food ingredients and producing vitamins, the host in turn offers nutrients to the microbiota. The members of the gut microbiota might be mutualistic, offering benefits to both host and the microbe itself, commensal, providing benefits to the microbe, or pathogenic, benefiting the microbe by harming the host (Reid *et al.* 2011). The availability of nutrients and binding sites are the prerequisite allowing microbes to colonise the gut. Nutrients can be produced by the host or obtained from the host diet, or they might be products of the metabolism of other microbial species. Binding sites can be found in the mucus layer covering the intestinal wall, on the epithelial cells and possibly also on other bacteria. The host genetics affects the gut microbiota composition through variants of the molecules mediating contact with the members of
the microbiota, such as polymorphism in cell surface receptors or genes affecting mucus glycan structures.

Certain cell surface receptor variants, especially those regulating the immune responses, such as nucleotide-binding oligomerisation domain-containing 2 receptor (NOD2), have been associated with particular diseases as they might result in a disturbed immune reaction. For example, NOD2 variants have been associated with Crohn’s disease, an inflammatory bowel disease, and NOD2 mutations have been claimed to lead to reduced gut microbiota diversity. This may be attributable to their inability to regulate commensal microbes due to insufficient immune response, which leads to an increased susceptibility to infections (Lauro et al. 2016). ABO and Lewis b histo-blood group antigen precursors, which are present in secretions such as secreted intestinal mucus, are fucosylated by fucosyltransferase 2 (FUT2). Three variants in the FUT2 genotype give rise to two secretor phenotypes, secretors (two genotypes) and non-secretors (one genotype), of which the latter does not secrete the fucosylated antigen. In a recent study, exploiting pyrosequencing and iChip microarray analysis, the secretor status and the FUT2 genotype were linked to variations in the composition of GIT microbiota (Wacklin et al. 2014). A variation in gut microbiota composition and diversity was detected also between individuals belonging to different ABO blood groups, especially in individuals expressing B-antigen (Mäkivuokko et al. 2012). Additionally, the quantity and composition of bile acids have been noted to regulate the gut microbiota composition due to the antimicrobial properties of some bile acids, and, on the other hand, due to the ability of some microbes to use bile acids in their metabolism. For example, it seems that decreased levels of bile acids support the growth of Gram-negative bacteria, whereas high levels support the growth of Gram-positive Firmicutes (Fiorucci & Distrutti. 2015, Inagaki et al. 2006, Islam et al. 2011). The intestinal mucus layer consists of both membrane-bound and secreted mucins, which are encoded by genes of the MUC family. Mucins are heavily O-glycosylated, and the glycan structures are complex and variable along the GIT, in part impacting on the GIT microbiota composition (Etzold & Juge. 2014). Mucin polymorphisms and alterations have been associated with GIT diseases, which are thought to be influenced by deviations in the GIT microbiota. This highlights the importance of mucin glycans in maintaining the mucosal homeostasis (Bergström & Xia. 2013).

Lactobacilli are some of the first colonisers of the developing human GIT. Some species colonise the GIT for the entire life of the host, while others are more transient visitors. Probiotic lactobacilli are often transient colonisers, although many strains have acquired genes increasing their GIT colonisation ability, reflecting their
adaptation to the mucus-dwelling niche of the GIT (Douillard et al. 2013b, Etzold et al. 2014). In addition to *L. rhamnosus* GG, which expresses mucus-binding pili and mucus binding protein MBF, many lactobacilli, such as *L. reuteri*, harbour mucus-binding proteins (MUB) consisting of several Mub1 and Mub2 subunits (Etzold et al. 2014, Kant et al. 2014b, MacKenzie et al. 2010). MucBP-domain containing proteins, such as MUB, have been found almost exclusively in lactic acid bacteria, especially in long-term gut-colonising lactobacilli (Kleerebezem et al. 2010). Additionally, many lactobacilli from the intestinal niche harbour large amount of genes with sugar importing and utilisation functions (Becerra et al. 2015, Ventura et al. 2009). Recent evidence shows that *L. rhamnosus* GG has adapted to GIT conditions by acquiring pathways for L-fucose fermentation, which is a common constituent of mucosa. As *L. rhamnosus* GG is not able to degrade mucus, it most likely relies on other bacteria for its supply of L-fucose (Becerra et al. 2015, Douillard et al. 2013a, Sanchez et al. 2010). It is possible that the composition of the glycans in the intestine affects also the availability of nutrients, thus influencing the composition of the gut microbiota.

In addition to other factors, the above-mentioned host-dependent features in the gut milieu - the variable glycan structures in mucus, protein polymorphisms, bile acids and availability of nutrients - might affect the colonisation ability and the effects of probiotic bacteria. Establishing biomarkers, which could predict the responder status for example in participants in a probiotic trial and choosing participants accordingly, would be likely to help in obtaining more valid and repeatable results.

### 2.2.2 Cellular level of microbial probiosis in the gastrointestinal tract

The basis of probiotic functions of *L. rhamnosus* GG, i.e. the effects induced at the cellular level, have been studied mainly with human derived intestinal epithelial cell lines and immune cells, mouse models, and also in a few studies, insect models have been utilised. Recently, a human trial was performed, in which the transcriptional response of small intestinal mucosa to the consumption of *L. rhamnosus* GG was studied in adult volunteers, providing an interesting new viewpoint on the probiotic-induced cellular signalling (van Baarlen et al. 2011). In the following chapters, the basic details of the intestinal milieu and signalling between the GIT microbiota and the host will be reviewed. In addition, current knowledge on signalling induced by *L. rhamnosus* GG, especially how the presence of pili modulate its probiotic effects, is summarised.
FEATURES OF THE INTESTINAL WALL AND THE ASSOCIATED MUCUS LAYER

Microbial cells in the GIT interact with the host intestinal epithelial cells (IEC) and with cells of the immune system. IECs form the intestinal wall, which is a monolayer of polarised epithelial cells folded into villi and crypts. Proliferative cells in the crypts renew the IEC layer, and IECs are fully matured and functional in the upper parts of the crypts and in villi, except for the Paneth cells which migrate to the bottom of the crypts (Al-Dewachi et al. 1975, Al-Dewachi et al. 1979, McCracken & Lorenz. 2001, Potten et al. 1997). IECs are bound together by tight junctions enabling the intestinal wall to function as a selective barrier towards microbes and food particles in the intestinal lumen (Balda & Matter. 2008). In addition to columnar IECs, which form the majority of the intestinal wall, the intestinal wall is decorated by specialised cell types such as goblet cells, which produce mucus, Paneth cells, which produce antimicrobial compounds such as defensins and lysozyme to protect proliferative cells in the crypts, and enteroendocrine cells integrating the intestine with the nervous system (Cheng & Leblond. 1974, Furness et al. 1999, McCracken & Lorenz. 2001).

The intestinal wall is covered at the luminal side by a layer of mucus, which is formed by mucin glycoproteins; its structure and thickness varies along the length of the GIT (Bron et al. 2012, El Aidy et al. 2015, Juge. 2012). The mucus functions as a lubricant and offers protection towards shearing caused by intestinal contents, and in some parts of the intestine, it also protects from microbes. Human mucus is constructed of up to 20 mucin glycoproteins with varying compositions in different areas of the body. MUC2 is the most common mucin in the GIT (Johansson et al. 2011). In the small intestine, the mucus layer is thin and loose, and penetrable by bacteria allowing contact between host cells and microbiota (Juge. 2012). At this site, where there should be rapid uptake of nutrients, also communication between host cells and luminal microbiota is important for the development and proper function of the immune system (Chu & Mazmanian. 2013, El Aidy et al. 2015). In the colon, two layers of mucus are present: a firm inner layer and a loose outer layer. In the mouse proximal colon, the inner layer has been found to be partly permeable to particles of the size of bacteria, while in the distal colon the inner layer was impenetrable (Ermund et al. 2013). It is possible that the mucus layer is somewhat different in the human GIT, but most of the studies on mucus structure have been performed in rodents. Some bacterial species live in the outer mucus layer and feed on the host mucus, producing SCFAs, which are used as an energy source by the host cells (den Besten et al. 2013, Johansson et al. 2013). In addition to the ability of the mucus layer to influence which bacteria are able to exist in the GIT, it has also
been noted that the gut microbiota composition can affect the mucus composition (Jakobsson et al. 2015). The lamina propria is the tissue below the intestinal wall; it is a layer of connective tissue, which contains various types of immune cells, such as B and T lymphocytes, dendritic cells (DC) and macrophages as a part of the so-called gut-associated lymphoid tissue (GALT) (Bron et al. 2012). Peyer’s patches, aggregates of lymphoid cells in the small intestine, are part of GALT. On top of a Peyer’s patch at the intestinal wall, there is a follicle formed by epithelial cells and specialised microfold cells that are able to take up antigens and present them to the immune cells in the Peyer’s patch (Bron et al. 2012, McCracken & Lorenz. 2001). In addition to the portal between the intestinal microbiota and immune system provided by the M cells, the DCs and macrophages can sample microbes by inserting dendrites through the intestinal wall, and recently it was discovered that also small intestinal goblet cells can deliver antigens to DCs in lamina propria (Guilliams et al. 2014, McDole et al. 2012, Peterson & Artis. 2014, Rescigno et al. 2001).

COMMUNICATION BETWEEN THE HOST AND THE MICROBIOTA

The intestinal microbiota connect with host cells through receptors collectively called pattern recognition receptors (PRR). The molecules recognised by PRRs on microbes are called microbe-associated molecular patterns (MAMP) (Mackey & McFall. 2006). There are many types of MAMPs, e.g. lipoteichoic acids (LTA) and PG in Gram-positive bacteria, lipopolysaccharides (LPS) in Gram-negatives, and pili in both groups (Delcour et al. 1999, Lebeer et al. 2010, Neuhaus & Baddiley. 2003). PRRs recognising bacteria consist of receptors such as Toll-like receptors (TLR), intracellular NOD–like receptors (NLR), extracellular C type lectin receptors (CLR) and formyl peptide receptors (FPR) (Figgdon et al. 2002, Medzhitov. 2007, Migeotte et al. 2006, Strober et al. 2006). Although there are several types of PRRs, TLRs have been the most extensively studied. They are transmembrane receptors and can be present in both cell surface and intracellular membranes (Medzhitov et al. 1997). The DC-specific intercellular adhesion molecule 3-grapping non-integrin (DC-SIGN) is a CLR found on the surface of DCs that recognises glycosylated proteins (Geijtenbeek et al. 2000). FPRs are G-protein coupled surface receptors, for which there are both exogenous (microbial) and endogenous ligands. The signal cascades triggered after ligand binding depend on the ligand modifications, such as glycosylation, and they are dose and time-dependent (Jones et al. 2012). Surface PRRs are often situated close to each other on lipid rafts and they may function as multi-receptor clusters (Lebeer et al. 2010, Triantafilou & Triantafilou. 2002).
Due to the interaction of MAMPs with PRRs, signal cascades are induced, for example ones involving nuclear factor κB (NF-κB) transcription factor or extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) mediated pathways. These pathways lead to either transcription or inhibition of transcription of certain genes (Bron et al. 2012). The signalling affects the production of various compounds, including some of the proteins needed for the intestinal barrier function, such as tight junction proteins, mucin glycoproteins, secretory immunoglobulin A (IgA), cytokines and other proteins like the TLRs capable of modulating the immune responses. For example, interleukins (IL), a subgroup of cytokines, induce the maturation of T and B lymphocytes and DCs, thus affecting the composition of different immune cells recruited for the immune response at hand (Bron et al. 2012, Wells et al. 2011). IgAs are secreted by B lymphocytes, which have matured to plasma cells due to antigen presentation for example by an M cell, and they have an important role in the maintenance of immune tolerance between the host and the microbiota, as they reduce pro-inflammatory signalling and bacterial epitope expression (Peterson et al. 2007).

The induced signalling cascades depend on which MAMP signature is encountered, as for example pathogens trigger different signalling cascades than commensals and probiotics (Lebeer et al. 2010). On the other hand, probiotics are also taught to differ from commensals in that they are recognised by the host immune system as ‘non-self’ entities, which is likely to be important for the probiotic functions (van Baarlen et al. 2013). Regulation of the inflammatory signalling induced by commensals is essential for the health of the host as inflammation can damage the tissues and might even be lethal to the host (Elinav et al. 2013, Peterson & Artis. 2014). One important means to regulate the inflammatory responses is via the polarisation of the IECs and its effect on the mode of signalling. For example on the apical (luminal) side, contact of TLR9 with MAMPs have been detected to lead to inhibition of NF-κB signalling and hence anti-inflammatory responses, whereas contact on the basolateral side induces NF-κB signalling and pro-inflammatory responses (Gewirtz et al. 2001, Lee et al. 2006). This is probably not the same for all TLRs, instead it is only one example of the possible ways to maintain tolerance of commensal microbes while inducing efficient protection towards pathogens (Peterson & Artis. 2014).

Another important aspect in the regulation of immune tolerance and control of inflammation in the gut involves the production of reactive oxygen species (ROS) by the host cells as a response to microbes. This mechanism seems to be evolutionarily conserved: in studies conducted with plants examining their relationship
with environmental stresses, such as pathogens, it has been noted that ROS are not solely a response to stress or an accidental by-product of aerobic metabolism, but instead have an important role in environmental adaptation and defence system. For example ROS might function as signalling molecules or reinforce the cell wall through oxidative crosslinking (Kotchoini & Gachomo. 2006). It has been observed that commensal bacteria are able to induce physiological levels of ROS, which in turn regulate certain signalling pathways in the epithelial cells, as some enzymes and signalling molecules are activated or inhibited through reduction-oxidation (redox)-reactions (Aviello & Knaus. 2016, Kumar et al. 2007). For example, some of the non-radical forms of ROS, such as $H_2O_2$, have been observed to function as signalling molecules in mediating the function of for example redox-sensitive thiols, including MAPK phosphatase, an inhibitor of the anti-inflammatory ERK/MAPK-mediated pathways (Holmstrom & Finkel. 2014, Wentworth et al. 2011). On the other hand, the appearance of pathogens may induce an oxidative burst, a release of high quantities of ROS, which limits the growth of microbes in the gut (Ha et al. 2005). Following the adhesion of, for example a bacterial ligand by an FPR receptor, ROS is produced by an enzyme belonging to a family called nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) (Jones et al. 2012). One of the members of this family, NOX2, has been determined to be responsible for the oxidative burst generated by phagocytes; a similar enzyme, dual oxidase (DUOX) undertakes this property in fly enterocytes (Chakrabarti et al. 2012, Ha et al. 2005, Lambeth. 2004). According to studies conducted in Drosophila, murine models and human cell lines, lactobacilli induce physiological levels of ROS via the stimulation of NOX1 in epithelial cells (Jones et al. 2013). The presence of DUOX2 has been detected in IECs throughout the intestine, but its function is still unclear, as is the impact of mitochondrially produced ROS on the ROS mediated signalling as a whole (Bedard & Krause. 2007, Jones et al. 2012). Physiological levels of ROS exert many effects on the host cells including cellular proliferation, migration and differentiation, and ROS have been shown to influence mucosal repair and homeostasis (Alam et al. 2014, Jones et al. 2013, Owusu-Ansah & Banerjee. 2009, Swanson et al. 2011).

**MOLECULES CONTRIBUTING TO THE HOST-INTERACTION OF LACTOBACILLUS RHAMNOSUS GG**

*L. rhamnosus* GG shares many signalling molecules with other Gram-positive bacteria, especially lactobacilli. Pili are among the longest molecules, with sizes up to a few micrometers. While pili have been extensively characterised in *L. rhamnosus* GG, a recent survey of the genomes of 213 type strains of lactic acid bacteria pre
Figure 4. *L. rhamnosus* GG cell surface and secreted molecules contributing to host interactions. Above is *L. rhamnosus* GG cell, and molecules it produces known to contribute to its host interactions. Below are the host cell and the pathways known to be induced due to the presence and most likely attachment of *L. rhamnosus* GG. *IkB*: inhibitor of NF-κB; MPK: MAPK phosphatase, inhibitor of MAPK; PKC: protein kinase C; PI3K: phosphoinositide 3-kinase. The figure is partly adapted from Segers et al. 2014 and van Baarlen et al. 2011.
dicted the presence of pili in 51 of the studied strains, and one third of these had pilus gene clusters similar to *L. rhamnosus* GG (Sun et al. 2015). Due to their importance in *L. rhamnosus* GG signalling, pili are now receiving considerable attention (Ardita et al. 2014, Lebeer et al. 2012). The studies considering the effect of pili on the host interaction will be discussed in detail in the next chapter.

LTA, a surface molecule of Gram-positive bacteria, is one of the most studied MAMPs of *L. rhamnosus* GG. It has been observed to induce pro-inflammatory signalling in host cells *in vitro* by activating IL-8 production and TLR2/6 receptor mediated NF-κB signalling (Claes et al. 2012). Major secreted proteins Msp1 (p75) and Msp2 (p40) have been noted to prevent cytokine induced epithelial damage and apoptosis, and to protect epithelial barrier function *in vitro* (Seth et al. 2008, Yan et al. 2007, Yan & Polk. 2002). Msp2 is currently thought to be more potent inducer of the effects than Msp1, and its ability to prevent and ameliorate intestinal epithelial damage has been demonstrated also in a mouse trial (Yan et al. 2011). Msp2 has been detected to exert its functions at least partly through activation of epidermal growth factor receptor (EGF-R) activation (Wang et al. 2014, Yan et al. 2011, Yan & Polk. 2012). These proteins have been characterised also in other bacteria, for example *Lactobacillus casei* BL23 (Bauerl et al. 2010). In addition, *L. rhamnosus* GG produces antimicrobial compounds, such as lactic acid, which regulates the growth of other bacteria, but can also be used by other bacteria to produce SCFAs for host nutrition(Duncan et al. 2004, Silva et al. 1987). It is possible that the exopolysaccharide (EPS) layer on the surface of *L. rhamnosus* GG is also involved in host-signalling, since isolated EPS has been shown to protect host cells from cytotoxicity caused by bacterial toxins *in vitro* (Ruas-Madiedo et al. 2010). However, more studies are needed to verify the results (Lebeer et al. 2012, Segers & Lebeer. 2014). The RNA and DNA, which are released when bacterial cells lyse in the intestine, are also thought to act as signalling molecules. Unmethylated CpG-rich DNA motifs released from bacteria have been claimed to be involved in commensal versus pathogen recognition and signalling, for example genomes of probiotics such as *L. rhamnosus* GG have been noted to be rich in these motifs in comparison to the genomes of pathogens such as *Clostridium difficile* (Kant et al. 2014a). According to the current knowledge, CpG-motifs react with apical TLR9 and hence inhibit pro-inflammatory signalling (Hemmi et al. 2000). There are indications that *L. rhamnosus* GG RNA induces signalling through TLR8 (Fong et al. 2015a). In a transcriptomics study, evaluating the response of the host small intestine on the consumption of *L. rhamnosus* GG, activation of signal transducer and activator of transcription 4 (STAT4) and JUN pathways promoting cellular proliferation and ion homeostasis was detected (van
Baarlen et al. 2011). The signalling molecules produced by L. rhamnosus GG according to the current knowledge and their likely signalling routes on host cells are depicted in Figure 4. As is the case for other probiotic microbes, L. rhamnosus GG induces a combination of anti-inflammatory and pro-inflammatory signalling. According to one theory, probiotics activate the host immune responses, and hence confer protection by evoking a more efficient immune response towards pathogens (Lebeer et al. 2010, van Baarlen et al. 2013). The induction of type 1 T helper lymphocytes (T_{h,1}) has been detected in a few recent studies, indicating that L. rhamnosus GG drives the immune responses towards T_{h,1} mediated immunity (Fong et al. 2015b, Fong et al. 2016, van Baarlen et al. 2011). In general, L. rhamnosus GG induced effects often seem to involve the promotion of health and proliferation of host cells, as well as immune functions, and strengthening of the epithelial barrier function (Segers & Lebeer. 2014, Seth et al. 2008, Wang et al. 2014).

**SpaCBA-PILI – ESSENTIAL FOR LACTOBACILLUS RHAMNOSUS GG INDUCED SIGNALLING?**

Further studies will be needed to elucidate the role of pili in the probiosis of L. rhamnosus GG in humans, but in vitro and in vivo studies in mice with mutants possessing varying pilus phenotypes have given some indications of the mechanisms involved. Immunomodulatory interactions of L. rhamnosus GG with and without pili have been studied with the IEC cell line Caco-2 and macrophages (Lebeer et al. 2012, Vargas Garcia et al. 2015, von Osowski et al. 2013). In IECs pilus-knockout strain of L. rhamnosus GG induced greater production of the pro-inflammatory cytokine IL-8 and tumor necrosis factor (TNF) while that of anti-inflammatory cytokine IL-10 was lower in comparison to wild-type L. rhamnosus GG (Lebeer et al. 2012). On the other hand, IL-8 production was still much lower than the quantities detected after exposure to the pathogen Salmonella enterica serovar Typhimurium, emphasising the differences in the pathogen and probiotic induced immune responses. In addition, the IL-8 production seemed to be inversely correlated with the level of the pilus-mediated interaction: the welE-mutant of L. rhamnosus GG lacking the EPS surface layer present in the wild-type strain, and consequently displaying more exposed pili, exerted a stronger anti-inflammatory impact than the wild-type strain by inducing a decrease of IL-8 production in comparison to the wild-type strain (Lebeer et al. 2012). Hence, it is likely that IL-8 is induced by factors other than pili or EPS, for example by LTA. The welE-mutant was tested in vivo in mice and was found to lack resistance to the harsh milieu of the GIT, most likely because
of its lack of EPS. Further studies indicated that EPS shields the bacterial cells from detrimental innate immune response mounted by the host (Lebeer et al. 2011a).

The immunomodulatory role of the \textit{L. rhamnosus} GG pili \textit{per se} has been addressed in a few studies, but the results have been inconclusive and somewhat contradictory. In one study, in which SpaCBA pili were expressed on the surface of \textit{Lactococcus lactis} subsp. \textit{cremoris} to examine pilus-induced immune modulation, pili were found to enhance NF-κB-activation via TLR2 in HEK-TLR2 reporter cell line (Figure 4.) and also to increase the production of cytokines TNF-α, IL-12, IL-10 and IL-6 in monocyte-derived DCs (mo-DCs) (von Ossowski et al. 2013). However, in an analysis of 100 \textit{L. rhamnosus} strains, TLR2 signalling was not associated with the presence of pili, indicating that further studies are needed to elucidate the role of pili in TLR2-mediated NF-κB-activation (Douillard et al. 2013a). As the used \textit{L. lactis} strain itself induced immunomodulatory signalling, the different MAMP combination present in the pilus producing \textit{L. lactis} should be considered when comparing the results to those obtained with \textit{L. rhamnosus} GG. In a recent study, immune signalling in DCs through DC-SIGN was induced by detached, mature pili (Tytgat et al. 2016). DC-SIGN is a receptor that detects glycosylated proteins, and fucosylation, such as that present in pili, leads to the activation of anti-inflammatory signalling via a yet unknown pathway (Figure 4.) (Gringhuis et al. 2009). The pili induced production of IL-6, IL-10, IL-12p40 and IL-12p35 expression. In another study, it was also noted that the presence of recombinant SpaC tip pilin reduced IL-1β-induced and increased TNF-α induced IL-6 expression in a dose dependent manner in human fetal intestinal organ culture. The results indicate that pili and especially the tip pilin SpaC might indeed have immunomodulatory properties. However, in the case of SpaC, there could well be structural differences between a recombinant SpaC and an endogenous SpaC attached to a pilus structure, which might affect the results (Ganguli et al. 2015).

When the role of pili in the interaction between \textit{L. rhamnosus} GG and murine macrophages was studied with the help of \textit{L. rhamnosus} GG mutants and the SpaC-BA pilus-expressing \textit{L. lactis} mentioned above, it was noted that the presence of pili increased binding and phagocytosis (Vargas Garcia et al. 2015). Interestingly, the \textit{welE}-mutant of \textit{L. rhamnosus} GG was found to bind twice as much as the wild-type strain and was phagocytosed as much as 11 times more efficiently. In addition, mRNA levels for anti-inflammatory IL-10 were increased by the \textit{welE}-mutant and pilus-producing \textit{L. lactis}, while the induction was at a similar level between pilus-less mutant and wild-type \textit{L. rhamnosus} GG. The extent of the induction of pro-inflammatory IL-6 was slightly decreased in the presence of pili, also when
measured with *L. lactis* strains. This is controversial to the original study (von Ossowski *et al.* 2013), in which pili induced IL-6 production, but the cell line used and the measurement done (mRNA vs. protein) were also different, making comparison difficult. These data suggest that signalling through induction of IL-10 and IL-6 are not mediated by pili, but the presence of pili affects the induction, most likely through close contact with cells (Vargas García *et al.* 2015).

Recently, an isogenic SpaC mutant of *L. rhamnosus* GG was used as a control while studying the importance of the presence of SpaC for the ability of *L. rhamnosus* GG ability to induce ROS production, ERK/MAPK mediated signalling, cell proliferation and cytoprotection against radiological insult in the Caco-2 cell line and murine small intestinal tissue. Interestingly, SpaC was noted to be essential for all the *L. rhamnosus* GG induced effects, further emphasising the importance of pili and their property to adhere to mucus and epithelial cells on the ability of *L. rhamnosus* GG to function as a probiotic bacterium (Ardita *et al.* 2014). However, the question about which exact molecule induces these effects remains unanswered, for now (Figure 4).

To conclude, the results demonstrate that *L. rhamnosus* GG induces a combination of anti-inflammatory and pro-inflammatory signalling, as it was mentioned earlier to be a general feature in probiotics (Lebeer *et al.* 2010). The significance of pili in *L. rhamnosus* GG induced signalling results most likely from the ability of pili to bring the bacteria into close contact with the host cells. Pili are believed to be the longest surface structures of *L. rhamnosus* GG and are thought to make the initial contact with host cells with the help of the tip pilin SpaC as the bacterial cells arrive to mucosal surfaces, which then results in contact with other surface molecules (Figure 3.). The importance of intimate contact mediated by pili has been emphasised by some of the above-mentioned studies using a Transwell-system, in which bacteria and cells share the growth medium but are not in physical contact; if there is no physical contact between the bacteria and the cells, then immune signalling is abolished (Lebeer *et al.* 2012, von Ossowski *et al.* 2013). Additionally, as the non-piliated strains also induced immune signalling when in contact with cells, it has been postulated that pili have a mediating role, rather than a major contribution in cellular signalling. Thus, it is likely that the MAMP signature of *L. rhamnosus* GG is formed mainly by surface molecules other than pili. However, some studies do indicate that also pili might have signalling functions. It is probable that the contact with the host cells by the non-piliated *L. rhamnosus* GG is much more prominent in the artificial *in vitro* setting than it would be *in vivo*. Consequently, it is likely that the impact of pili on the overall probiotic function of *L. rhamnosus* GG will be better
revealed when examined in human *in vivo* trials performed using derivatives of *L. rhamnosus* GG with varying pilus production capacities.
3 AIMS OF THE STUDY

Understanding the biogenesis and function of the *Lactobacillus rhamnosus* GG pili is important in evaluating their role in the probiotic properties of the strain. Consequently, the aim of this thesis was to investigate the genes and motifs involved in pilus biogenesis and pilus-mediated adherence of *Lactobacillus rhamnosus* GG. Additionally, the *in vivo* role of *L. rhamnosus* GG pili was addressed.

In detail, the following aims were investigated:

I) Phenotypic and genotypic characterisation of the pilus-less derivatives obtained by chemical mutagenesis and enrichment to identify genes affecting the production of pili.

II) Phenotypic and genotypic characterisation of highly mucus adherent and pilus-secreting derivatives to identify genes promoting adherence

III) Characterisation of conserved variants of the LPxTG motifs leading to differential recognition by either the pilin-specific sortase C or the housekeeping sortase A.

IV) Intervention trials in mouse and human to study the effect of pili on the colonisation ability of *L. rhamnosus* GG and its capabilities to modulate the composition of the faecal microbiota
4 MATERIALS AND METHODS

The methods used in the individual studies are shown in Table 1. A more thorough depiction of the methods and materials used can be found in the publications and manuscripts implicated in the table. Bacterial strains and plasmids used are listed in Table 2. Plasmids modified from the original plasmid pNZ44 in the Study III are listed in detail in the corresponding publication. The basic information on the mouse and human intervention trials are shown here.

4.1 OVERVIEW OF THE MOUSE TRIAL

The mouse feeding trial was performed in the animal facilities of University College Cork adhering to local regulations. The use of the mice was approved by the Animal Experimentation Ethics Committee of the UCC (permit 2011/#017). A schematic depiction of the trial is shown in figure 5. Ten 8-10 weeks old Balb/c mice were included in the trial and were divided into two groups, one consuming L. rhamnosus GG and the other receiving L. rhamnosus GG-PB12. The mice consumed the bacteria in their drinking water on average 8x10^10 CFU per day. The study products were freeze-dried bacteria produced by Valio Ltd. Both the feeding period and follow-up lasted for two weeks, during which time, faecal samples were collected 4 times a week. At the end of the trial, the mice were sacrificed and the ileum, caecum and colon were collected for further analyses.

4.1 OVERVIEW OF THE HUMAN TRIAL

The human intervention trial was approved by the Coordinating Ethics Committee of the Helsinki and Uusimaa Hospital District (HUS 245/13/03/00/13). A schematic overview of the trial is shown in figure 5. The study subjects provided their informed consent before participating in the trial. The study followed the form of a randomised double-blind trial. The participants were recruited from the Finnish population, and ultimately, 27 participants aged between 24-53 years were enrolled in the study. The participants were chosen so that they had not received antibiotic courses during 4 months before the start of the trial, and they did not have any severe gastrointestinal diseases. In addition, the participants were not allowed to consume L. rhamnosus GG containing products during the trial. The participants were divided into three groups, with the first group consuming L. rhamnosus GG, the second group consumed L. rhamnosus GG-PB12 and the third consumed a mixture of both strains, the total amount equalling to the amount consumed in
Table 1. Methods used in the thesis.

<table>
<thead>
<tr>
<th>Method</th>
<th>Study</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>DNA extraction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extraction of DNA for genome sequencing</td>
<td>I, II</td>
<td>Studies I &amp; II</td>
</tr>
<tr>
<td>(Promega Wizard kit)</td>
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<td></td>
</tr>
<tr>
<td>DNA extraction from faecal material</td>
<td>IV</td>
<td>Salonen et al. 2010</td>
</tr>
<tr>
<td><strong>RNA extraction</strong></td>
<td></td>
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<tr>
<td>RNA extraction from <em>L. rhamnosus</em> GG cultures</td>
<td>II</td>
<td>Study II</td>
</tr>
<tr>
<td><strong>Cloning</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Construction of minimal pilus-cassette</td>
<td>III</td>
<td>Study III</td>
</tr>
<tr>
<td><strong>Mutagenesis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical mutagenesis</td>
<td>I, II</td>
<td>Studies I &amp; II</td>
</tr>
<tr>
<td>Site-directed mutagenesis</td>
<td>III</td>
<td>McGrath et al. 2001</td>
</tr>
<tr>
<td>Enrichment of pilus-derivatives and high throughput dot blot screening</td>
<td>I, II</td>
<td>Studies I &amp; II</td>
</tr>
<tr>
<td><strong>Phenotypic characterisation of strains</strong></td>
<td></td>
<td></td>
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<tr>
<td>Extraction of cell wall-associated proteins</td>
<td>I, II</td>
<td>Åvall-Jääskeläinen et al. 2008</td>
</tr>
<tr>
<td>Western blot</td>
<td>I, II, III</td>
<td>Studies I &amp; III</td>
</tr>
<tr>
<td>Immuno-fluorescence labeling, intensity quantification and microscopy</td>
<td>I, II</td>
<td>Studies I &amp; II</td>
</tr>
<tr>
<td>Mucus binding assay</td>
<td>I, II</td>
<td>Vesterlund et al. 2005</td>
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<td><strong>Quantitative-PCR</strong></td>
<td></td>
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<tr>
<td>Quantification of <em>L. rhamnosus</em> GG strains from faeces samples</td>
<td>IV</td>
<td>Study IV</td>
</tr>
<tr>
<td>Quantification of total bacteria from faeces samples</td>
<td>IV</td>
<td>Salonen et al. 2010, Nadkarni et al. 2002</td>
</tr>
<tr>
<td><strong>Sequencing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genome re-sequencing</td>
<td>I, II</td>
<td>Studies I &amp; II</td>
</tr>
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<td>High-throughput 16S rRNA sequencing (MiSeq)</td>
<td>IV</td>
<td>Klindworth et al. 2013, Illumina</td>
</tr>
<tr>
<td>RNA sequencing (HiSeq)</td>
<td>II</td>
<td>Study II, Illumina</td>
</tr>
<tr>
<td><strong>Bioinformatics</strong></td>
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<td>Protein sequence alignments</td>
<td>III</td>
<td>Bailey et al. 2009</td>
</tr>
<tr>
<td>Genome re-sequencing; read mapping, alignment and annotation of mutation</td>
<td>I, II</td>
<td>Kurtz et al. 2004, Rutherford et al. 2000, Chevreux et al. 1999</td>
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<tr>
<td>Gut microbiota community analyses</td>
<td>IV</td>
<td>Korpela 2016, R Core Team 2014</td>
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<tr>
<td>Transcriptome analyses</td>
<td>II</td>
<td>Langmead et al. 2012, Love et al. 2014</td>
</tr>
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### Table 2. Bacterial strains and plasmids used in the thesis.

<table>
<thead>
<tr>
<th>Bacterial strain/plasmid</th>
<th>Origin/features</th>
<th>Study</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus rhamnosus</em> GG (ATCC 53103)</td>
<td>Human intestine, Valio Ltd. culture collection</td>
<td>I, II, III, IV</td>
<td>Goldin et al. 1992</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> LC705 (DSM 7061)</td>
<td>Cheese, Valio Ltd. culture collection</td>
<td>I</td>
<td>Valio Ltd.</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> GG-PBxx</td>
<td>Pilus-deficient derivative of <em>L. rhamnosus</em> GG</td>
<td>I, II, IV</td>
<td>Study I</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> GG-PSxx</td>
<td>Highly mucin-adherent derivative of <em>L. rhamnosus</em> GG</td>
<td>II</td>
<td>Study II</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> GG-PAXx</td>
<td>Pilus secreting derivative of <em>L. rhamnosus</em> GG</td>
<td>II</td>
<td>Study II</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> subsp. cremoris NZ9001</td>
<td>Strain MG1363 containing <em>nis</em> RK genes for nisin-regulated gene expression</td>
<td>III</td>
<td>Kuipers et al. 1998</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> IL1403 ΔsrtA pNZ44</td>
<td>Strain IL1403 in which the gene of the housekeeping sortase SrtA is deleted, <em>L. lactis</em> expression vector containing constitutive promoter P44 and chloramphenicol resistance cassette</td>
<td>III</td>
<td>McGrath et al. 2001</td>
</tr>
</tbody>
</table>

ATCC: American Type Culture Collection  
DSM: strain from DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
the single strain groups. The study consisted of a 4-week run-in period, a 2-week intervention and 3-week follow-up. A quantity of $6 \times 10^{10}$ CFU of freeze-dried bacteria was consumed daily as a mix with a cold drink during the intervention. In the course of the intervention and follow-up period, 7 faecal samples were collected. A questionnaire enquiring about health and eating habits was filled in at the beginning and end of the trial.

![Study flows of the mouse and human intervention trials.](image)

Figure 5. Study flows of the mouse and human intervention trials.
5 RESULTS AND DISCUSSION

5.1 SEARCH FOR DERIVATIVES OF LACTOBACILLUS RHAMNOSUS GG WITH ALTERED PILUS PRODUCTION CAPACITY (I & II)

The aim of the study was to obtain derivatives of *L. rhamnosus* GG, which would have either no ability to produce pili or alternatively would produce more pili than the parental strain. Naturally occurring pilus-production variants were first searched through screening of *L. rhamnosus* GG culture by cultivation and dot blot analysis with anti-SpaC antibodies and flow cytometry. Since natural variants could not be found, mutagenic substances were tested and finally used to produce derivatives with altered pilus-production capacities.

5.1.1 Chemical mutagenesis and enrichment of pilus derivatives

Random mutagenesis is routinely used in industry as a means to improve microbial strains so that they have certain beneficial features important for the production process (Bron & Kleerebezem. 2011). In comparison to targeted genetic manipulation, in random mutagenesis, one does not need to have any information on the genes affecting a particular trait. In addition, targeted mutagenesis is not allowed to be used by food industry in the European Union, as strains obtained through modification with molecular techniques are considered as genetically modified organisms (GMO) (The European Parliament and the Council of the European Union. 2001). Random mutagenesis can be performed chemically, for example with mutagens or physically, for example with UV radiation. After mutagenesis, the culture can be grown in a way that enhances the enrichment of the features of interest or the strains can be enriched with other methods, after which the strains are screened and characterised (Margolles & Sanchez. 2012, Tillich et al. 2012). In research, random mutagenesis can be used as a tool to reveal underlying interesting mechanisms, for example in protein production pathways. Here, ethyl methanesulfonate (EMS), a mutagen causing transition mutations was used in random mutagenesis (Sega. 1984). Ten pilus-less derivatives were obtained from the mutagenesis of *L. rhamnosus* GG culture and enrichment for non-piliated strains. The basic concept underpinning the enrichment system was to attach piliated cells to the the bottom of a microtitre plate well harbouring anti-SpaC IgG molecules, leaving non-piliated cells suspended in the liquid. Sample taken from the liquid phase was grown overnight in broth and plated the next day, after which colonies were picked for fur-
ther ening. In the same screening procedure, a pilus-secreting strain was identified. Thirteen highly mucus-adherent derivatives were obtained from the screening of highly piliated strains. The *L. rhamnosus* GG culture treated with the same amount of mutagen as for the pilus-less enrichment was used. Here, a concentrated bacterial suspension was used to increase the possibility to isolate the most adherent cells. The microtiter wells were coated similarly with anti-SpaC IgG as with the pilus-less enrichment, but after incubation, the wells were washed intensively, and the bottoms were scraped during the last washing in order to detach highly adherent cells. The last washing liquid was used for further screening of the rains. The enrichment systems are depicted in Figure 6.

![Enrichment systems](image)

**Figure 6. The enrichment systems for *L. rhamnosus* GG derivatives with varying pilus production abilities.** The saturated system for enrichment of highly-piliated strains is shown on the left with unsaturated system for enrichment of pilus-less strains on the right. Saturated system means, that the bacteria in the system are much more abundant than the binding sites in the bottom of the well, and for the unsaturated system this ratio is reversed. The Y-markings at the bottom of the wells denote the IgG molecules to which the cells adhere.

As expected, mutagenesis increased the quantity of mutated cells in the culture and this made it possible to find derivatives with variable pilus production capacities, a process further helped by the efficient enrichment scheme. Enrichment reduced the need for strain screening substantially: for the ten pilus-less strains, 384 colo-
nies had been screened from the enriched sample, making the discovery rate 2.6%, while for the thirteen highly mucus-adherent strains 384 colonies were screened and hence the discovery rate was 3.4%. Thus, the desired derivatives were obtained at a similar rate in both approaches.

5.1.2 Phenotypic and genotypic characterisation of the pilus-less derivatives (I)

The pilus-less derivatives were genotypically characterised by genome re-sequencing and Sanger sequencing of the mutations in the pilus gene cluster, and phenotypically characterised by Western blotting of the cell surface, cytosolic and secreted proteins, in order to determine whether pilins were being expressed. In addition, immuno-electron microscopy (immuno-EM) was applied to visualise the cells, and immuno-fluorescence labelling was exploited to quantify the pilins. Finally, the mucus binding properties of the strains were determined. The derivatives were found to divide into three classes: sortase C-mutants (Class I), deletion mutants (Class II) and SpaA-mutants (Class III). Sortase C is the enzyme, which assembles the pili and SpaA is the pilin forming the pilus backbone (Table 1/I; refers to the Table 1 of Study I).

Of the Class I derivatives, having a mutation in the srtC1 gene, PB11 (PB: Pilus Bald) and PB12 both had a mutation in the codon of residue Trp256 (TGG), although the position of the mutation differed. PB11 harboured the mutation in the second base of the codon (TAG), whereas PB12 displayed the mutation in the third base (TGA). In PB13, the codon of residue Trp252 was mutated (TGG → TAG) (Table 1/I). In each strain the mutation caused the appearance of a STOP-codon in the C-terminal end of the protein, and hence resulted in a truncated enzyme and dysfunctional pilus biogenesis. Since the mutation led to the removal of approximately 100 residues, it is likely that the membrane anchor of the enzyme had been disrupted and the enzyme could not remain at the membrane long enough to allow proper pilus synthesis. The active site TLxTC was not removed, and it is possible that some residual activity was retained by the enzyme, as small, 2-4 residue long, pilin oligomers were detected in the electron micrographs (Fig 1/I) and Western blots (Fig 2/I). These oligomers were not detected at the cell surface in EM, as they were apparently covered by the EPS layer. In these strains, it was also noted in Western analysis that, as the pilin synthesis was not hampered, pilins accumulated on the cell surface and to the milieu of the cells (Fig 2/I).

Class II derivatives contained a large deletion in their genome in the region between 348700 and 526400 bp, resulting in the loss of the spaCBA-srtC1 pilus gene.
cluster (Table 1/I). This derivative class was the most common one, consisting of five strains. The deleted area varied in length, but always resided between two IS elements of family IS5. It is possible that stress resulting from the mutagenesis evoked an activation of the transposable elements, or the deletions were the product of a homologous or illegitimate recombination event (Aras et al. 2003, Bierne et al. 1997, Levinson & Gutman. 1987, Tillier & Collins. 2000). Large deletions were detected in the same region by Sybesma and colleagues in *L. rhamnosus* GG strains isolated from dairy products, and in our unpublished study, in which *L. rhamnosus* GG was grown in the presence of varying stress factors for 1000 generations (Sybesma et al. 2013). In another study, in which 100 *L. rhamnosus* strains were sequenced and compared, the area was noted to be highly variable. These findings emphasise the significance of the region in adaptation to changing environmental conditions. As expected, no pilins or pilin oligomers were detected in Western (Fig 2/I) or EM (Fig 1/I).

Two derivatives belonging to the Class III had a mutation in gene *spaA* (Table 1/I). PB31 had a mutation in the start codon of the gene (ATG → ATA). The mutation seemed to reduce the translation initiation efficiency, as a lower amount of SpaA was detected in Western blot than in the parental strain (Fig 2/I). Additionally, pili seemed to be produced in low amounts according to Western analysis, but they were not of mature length. In EM, the oligomeric pili were detected in the milieu of the cells, but again not on the surface of the cells as they were most likely covered by the EPS layer (Fig 1/I). It is plausible that the mutated start codon retained some function, and normal sized SpaA was produced in low amounts. In PB32, the mutation was in codon 247 (TCT → TTT), resulting in an amino acid change from serine to phenylalanine. The quantity of pili was significantly reduced according to Western analysis, and a possible explanation is that due to the mutation, pili were polymerised less efficiently. In addition, the electrophoretic mobility of the SpaA monomer was reduced, indicating the possible presence of an uncleaved signal peptide. In EM, a few gold particles were detected on the surface of cells, which were possibly attached to the tips of the short pili produced by this strain (Fig 1/I). Additionally, pilin oligomers were found in the milieu of the cells and in Western, which was prepared of the growth medium of the strain, a large amount of immature pili were found. These results indicate that the attachment of the pili to the cell wall by SrtA was disturbed due to the mutation. The results obtained from the SpaA derivatives support the model that pilus biogenesis is regulated by the amount of available pilins: when SpaA is readily available, long pili are produced, but when SpaA is scarce, more SpaB is added into the forming pilus, leading finally to recogni-
tion by SrtA and subsequent halt in the pilus synthesis and attachment of the pilus into the cell wall. As SpaA is scarce in the SpaA derivatives, more SpaB is added into the pili and this results in early recognition by SrtA and the formation of short pili.

None of the derivatives bound porcine mucus, and pilin quantities were clearly reduced when determined by immunofluorescence labeling with SpaC and SpaA antibodies. In the derivative PB32, slightly higher quantities of pilins were detected, as it produced immature pili in low quantities, but this feature did not increase the mucus binding ability of the strain. All of the derivatives were unique and harboured different combinations of mutations, although it could be suspected from the similarity of the SNP patterns that the derivatives of each class had derived from a common ancestor. The results of the study pointed out the crucial elements needed for a successful pilus biogenesis in *L. rhamnosus* GG: functional pilus assembling enzyme SrtC and backbone pilin SpaA. In addition, further evidence was obtained of the relevance of the genomic region, in which the pilus gene cluster is situated, in the adaptation of *L. rhamnosus* GG to varying environmental factors.

### 5.1.3 Phenotypic and genotypic characterisation of the highly mucus-adherent and pilus-secreting derivatives (II)

The derivatives were genotypically characterised by genome re-sequencing and Sanger sequencing of the pilus gene cluster, and phenotypically characterised by Western blotting of the cell surface proteins, immuno-EM, immunofluorescence labelling and mucus binding, similarly as conducted with the pilus-less mutants. Class I derivatives (PS11-PS17, PS: Pilus Sticky) harboured a mutation in the *spaC* gene, resulting in an amino acid change (Pro → Ser) in residue 552 (Table 1/II). This residue is located in the C-terminal end of the collagen-binding domain of SpaC (Tripathi *et al.* 2013). When the pilins were quantified by immuno-fluorescence, the expression of SpaA and SpaC were at a similar level as encountered in the parental strain (Fig 2/II). The same result was observed using immuno-EM (Fig 4/II). As the mucus binding levels were higher than in the parental strain (Fig 1/II), the results suggest that the mutation occurring in SpaC had been responsible for the increased mucus binding ability of the strains, although the effect of other mutations can not be ruled out (Table 1/II). A possible disturbed recognition by the SpaC antibody affecting the results is not likely as the strains were enriched using the anti-SpaC IgG, indicating that all of these strains bind the antibody avidly.

The mutation responsible for the high mucus binding phenotype of the Class II (PS21-PS24) and Class III (PS31) derivatives could not be identified (Table 1/II). Nevertheless, due to extensive differences in the SNP profiles between the strains,
they could be grouped into different classes. According to the immuno-fluorescence
labelling (Fig 2/II) and EM-data (Fig 4/II), the strains seem to have more pili than
the parental strain, and in the immuno-fluorescence labeling, the increased quantity
especially of SpaC was noted. Class III derivative PS31 seemed to produce SpaA
at a similar quantity as the parental strain, but had increased production of SpaC
(Fig 2/II). One possible explanation for these phenotypes is that they have more
efficient secretion of the pilins and/or sortases to the cell membrane, resulting in a
higher pilus production. The strains contained several single nucleotide mutations
in possible effector genes, including ribosomal, transporter, transcriptional regulator
and ATP-dependent protein genes. Transcriptomic data of L. rhamnosus GG in
different growth phases was used to rule out non-transcribed genes (Table 2/II).
The results suggested several possible effector genes, including transcriptional reg-
ulators from families GntR and MarR. Transcriptional regulators belonging to these
families have been indicated to affect protein secretion, adhesion and virulence in

The Class IV derivative PS41 had a mutation in the spaA gene, which resulted in
an amino acid change from (Thr → Met) in the residue 35 (Table 1/II). According
to the phenotypic characterisation, this strain seemed to have a higher quantity
and perhaps even longer pili than the parental strain (Fig 2 & 4/II). It is possible
that the mutation increased the secretion of the SpaA pilus monomer, resulting in
formation of longer pili, as the pili have been shown to become longer when the
backbone pilin is abundant in comparison to the basal pilin (Mandlik et al. 2008a).
Another interesting finding was the identification of so-called secretor derivative
of L. rhamnosus GG. Secretor PA11 (Pilus SrtA) harboured a mutation in the gene
of SrtA, which was situated at the C-terminal end of the protein, 10 residues to the
C-terminal direction from the TLxTC catalytic motif (Table 1/II). As it was detected
from the phenotypic analysis that this strain secreted mature pili and bound mucus
at low levels (Fig 1, 4 & 5/II), it is probable that the mutation had disrupted the
structure of the SrtA enzyme, preventing it from functioning properly during the
attachment of the pili into the cell wall.

Interestingly, the enrichment system for highly piliated strains did not result
only in highly piliated L. rhamnosus GG, but also strains with higher mucus-bind-
ing ability without a pronounced increase in the quantity of pilins produced by the
strain. This result suggests that the mutation in SpaC in Class I derivatives resulted
in a higher binding ability of SpaC in general, but not only towards mucin, since
in the enrichment scheme purified anti-SpaC IgG was used in binding the bacteria
to the bottom of the wells. Little is known about the protein secretion system in
5.2 IDENTIFICATION OF FUNCTIONAL VARIANTS OF PILIN SORTING SIGNAL LPxTG (III)

The study aimed at characterising the variants of the sortase recognition motif LPxTG present in pilins and other cell surface proteins. Motif variants were discovered by bioinformatic analysis of L. rhamnosus GG surface proteins and pilins of other Gram-positive piliated bacteria (Bailey et al. 2009). For functional verification of the motif variants, L. rhamnosus GG pili composed of modified major pilin SpaA containing different LPxTG motifs were expressed in L. lactis.

5.2.1 Bioinformatic analysis

In the bioinformatic comparison of L. rhamnosus GG proteins predicted to harbour an LPxTG motif, the motif was observed to contain conserved residues, which had two variants in the different pilin genes of L. rhamnosus GG (Fig 1/III). The pilins SpaA, SpaC, SpaD and SpaF contained a motif with three glycine (G) residues, in positions +5, +6 and +8 (lysine (L) in LPxTG motif is +1), named as the triple G motif (TG). The pilins SpaB and SpaE, and other predicted surface proteins of L. rhamnosus GG, contained a conserved glutamic acid (E) or aspartic acid (D) residue in the position +6, and this motif was named as the single G motif (SG). Since the TG motif was present in backbone and tip pilins, whereas SG was present in basal pilin, evidence for the putative role of these variants in influencing the recognition by sortase was obtained. An analysis of pilins from other Gram-positive bacteria, such as C. diphtheriae and E. faecalis, revealed the conservation of the TG and SG motifs, although more variance was detected for the SG motif as in L. rhamnosus GG. Moreover, as in L. rhamnosus GG, TG motif was found in backbone and tip pilins, while SG was found in basal pilins (Fig 2 & Table 2/III).

5.2.2 Construction of the expression system and mutagenesis of the LPxTG motif

L. lactis was chosen as the expression host as it has been previously used successfully in the expression and investigation of sortase-dependent pili (Quigley et al.
2010, von Ossowski et al. 2013). A minimal pilus-operon containing only spaA and srtC1 was constructed and placed under the constitutive L. lactis promoter P44. The mutations were produced by directed mutagenesis using a previously described method (Holo & Nes. 1989, McGrath et al. 2001). The expression in the L. lactis sub-sp. cremoris strain NZ9000 (Kuipers et al. 1998) was observed to be at a high level when examined with Western blot and immuno-EM (Fig 3 & 4/IV). To exclude the possible role of SrtA of the host strain in the biogenesis of the pilus, an expression cassette containing only spaA was also constructed. The expression of this cassette resulted in a phenotype in which SpaA monomers accumulated on the cell surface, indicating that SrtA does not participate in the synthesis of pili (Fig 3 & 4/IV). The relevance of the residues in the LPxTG motif on the recognition by sortase was tested with mutagenesis. The tested variants are listed in Table 1/III.

5.2.3 Phenotypic characterisation of the mutants

The phenotypes of the mutants were characterised using Western blot analysis of the cell surface proteins in conjunction with immuno-EM. The results are summarised in Table 3/III. Different combinations of mutations in the residues of the LPxTG motif were tested. Altering the x residue in the LPxTG motif did not affect pilus production, as previously reported (Fig 5/III) (Gaspar et al. 2005). The residues +3, +6 and +8 in the SpaA LPxTG motif were interchanged with the residues of SpAB, which resulted in disturbed pilus biogenesis. The substitution of residue 8 with valine (V) resulted in short pili, while the substitution of residue +6 with aspartic acid abolished pilus assembly. A similar result was obtained when +6 was substituted with glutamic acid, indicating that residue +6 is essential for successful pilus biogenesis (Fig 5/III). When the constructs were expressed in the srtA-null strain L. lactis IL1403 (Dieye et al. 2010), all recombinant strains produced pili, indicating that SrtA is not required for the pilus synthesis (Fig 8/III). It is not known how the pili were retained at the cell surface without attachment to the cell wall by SrtA. The most likely explanation is that they had formed acyl-enzyme intermediates with SrtC. Surprisingly, the construct harbouring the mutation in residue +6 (E) was able to produce pili in the srtA-null strain, indicating that in the absence of SrtA, SrtC can synthesise pili in spite of the mutation. The pili were found also in the culture media, indicating eventual release from the cell surface.

The importance of residue +6 was characterised further by substituting it with all other amino acids (Table 3/III). In most cases, the mutation led to disruption of pilus synthesis, but wild type-like pili were observed when the substitution was made with alanine (A) or serine (S). Lysine (K) and arginine (R) led to the forma-
tion of shorter pili in low amounts (Fig 6 & 7/III). These results indicate that in *L. rhamnosus* GG, shaft and tip pilins are preferentially recognised by SrtC, while basal pilin is recognised by SrtA, as reported before, for example, in *C. diphtheriae* (Mandlik *et al.* 2008a). SrtA recognition leads to the attachment of pilus into the cell wall (Fig 9/III). However, this preference did not exclude the recognition by the other enzyme. Three glycine residues in the TG motif provide the motif region with greater flexibility in comparison to the residues present in the SG, possibly affecting its sortase recognition. Due to the conservation of TG motif, these results might be applicable also to other Gram-positive bacteria. The study provided novel insights into the mechanisms underpinning the function of the sortases in the biogenesis of the pili in *L. rhamnosus* GG.

**5.3 IN VIVO TRIALS TO ASSESS THE EFFECT OF PILI ON THE PERSISTENCE AND GUT MICROBIOTA MODULATION POTENTIAL OF LACTOBACILLUS RHAMNOSUS GG (IV)**

The aim of the study was to determine whether piliation exerted any effect on the GIT persistence and gut microbiota modulation ability of *L. rhamnosus* GG. Previously characterised pilus-less *L. rhamnosus* GG-PB12 was used as a control to study especially the role of pili on the *L. rhamnosus* GG induced effects, as the derivative is phenotypically similar in comparison to the parental strain and harbours only 24 single nucleotide mutations revealed by genomic re-sequencing. Trials were performed in both mice and humans, with the difference that in the mouse trial, the mice consumed either *L. rhamnosus* GG or PB12, while in the human trial an additional mixed group was included, in which the participants consumed both strains as a combination. The mouse trial was performed in part to verify the safety of the newly isolated bacterial strain PB12.

**5.3.1 Analysis of the GIT persistence of the *L. rhamnosus* GG strains**

The persistence of the strains in the GIT was measured by following the reduction of the quantity of the strains in faecal samples during the trial. The quantity of the strains was measured using hydrolysis probe quantitative PCR (qPCR) assays developed for the study. The results indicated that the piliated *L. rhamnosus* GG persisted in the GIT longer than the non-piliated strain, and this was detected especially in the human trial (Fig 1/IV). The mouse trial gave a similar result, but with a larger number of mice the results might have been more distinct. In the human trial, it was observed both in the single strain and combination groups, that at first,
PB12 was secreted in higher amounts, but the situation became reversed towards the end of the trial and the parental strain was secreted in higher amounts, indicating that the piliated *L. rhamnosus* GG was able to persist in the GIT for a longer time than its non-piliated counterpart. In all study groups, the interindividual variation between the persistence times was evident. Hence, the results from the combination group are likely to be more accurate, as the host effect had been minimised. On the other hand, the effect of the consumption of both strains at the same time might have influenced the results as the two strains were supposedly competing for the same resources in the GIT.

### 5.3.2 Gut microbiota analyses

Statistically significant differences in the gut microbiota profiles between the treatment groups were detected in both the mouse and the human trial. In the mouse trial several differences in bacterial taxons were observed (Fig 3/IV), while in the human trial significant community level differences were also detected (Fig 2 & 3/IV).

In the mouse trial, statistically significant community level differences were not observed, which is most likely due to the strong increase in lactobacilli during the intervention dominating the community level effect of both treatments. The effects were somewhat contradictory between the mouse and human trials, as in the mouse trial, the abundance of Actinobacteria increased and that of Clostridia decreased in the GG group, while the shifts for these classes were the opposite in the human trial (Fig 3A/IV). On the other hand, it was clear in both trials that GG induced stronger changes than PB12. The relative abundance of lactobacilli was clearly increased in all study groups in both mouse and human trials indicating the effect of the consumed bacteria, but possibly also an increase in other lactobacilli (Fig 3A & B/IV). In the GG group of the human trial the clearest increase, in addition to the increase in lactobacilli, was detected in Erysipelotrichi, and especially in the genus *Turicibacter* (Fig 3/IV). This bacterial genus was also increased significantly in the mixed group after the intervention, possibly reflecting a prolonged effect of *L. rhamnosus* GG still remaining in the GIT. *Turicibacter* is known to be present in the small intestine in high quantities, and since this section of the intestine is believed to be portion of the GIT in which *L. rhamnosus* GG exerts its largest effects, it is likely that these genera affect each other (Derrien & van Hylckama Vlieg. 2015, Kamada *et al.* 2013).

The correlation between the quantity of *L. rhamnosus* GG strains measured using quantitative PCR and the relative abundance of bacterial genera in the faecal
samples was determined. In addition to the expected significant positive correlation of the quantity of both strains to the genus *Lactobacillus*, GG displayed a significant negative correlation with *Barnesiella, Ruminococcus* and unassigned genera of Firmicutes and Clostridia in the human trial, whereas the quantity of PB12 correlated negatively with the abundance of *Enterorhabdus* and *Enterococcus* in the mouse trial (Table 1/IV).

In the human trial, significant community level changes were observed between the treatment groups. The community shift was significant in the GG and mixed groups, but not in the PB12 group and, interestingly, the shift happened in opposite directions in the GG and mixed groups (Fig 2A & 2B/IV). This result implies that when bacterial strains are consumed at the same time, they might modify each other’s effect, possibly by niche competition. This should be taken into account when considering the potential effects of multiprobiotics. The altered state of the communities was retained until the end of the trial. Additionally, species richness was increased significantly in the GG group, but not in other groups, and it remained elevated until the end of the trial (Fig 2C/IV). High species richness has been associated with healthy gut microbiota, as high richness has been shown to promote stability and resilience against pathogens (Jalanka-Tuovinen et al. 2011). The results suggest that piliated *L. rhamnosus* GG possesses the potential to promote the maintenance of a balanced gut microbiota. To conclude, the results highlight the crucial role for pili in the GIT persistence and gut microbiota modulation properties of *L. rhamnosus* GG especially in humans, but also in mice. Additionally, a novel approach was used by studying the effect of modification of a bacterial surface structure *in vivo* in humans.
6 CONCLUSIONS AND FUTURE ASPECTS

The research presented in this thesis has revealed new aspects of the pilus biogenesis of *L. rhamnosus* GG, including factors determining the success of the pilus production, but also factors which increase the adhesion capacity of the pili. The derivatives with altered pilus expression capacities obtained in the Studies I and II represent novel tools for analysing the effect of different pilus phenotypes *in vivo* in humans. As an example, the importance of pili for the persistence of *L. rhamnosus* GG in the mouse and human gastrointestinal tracts (GIT) was studied using a pilus-less derivative characterised in Study I as a control strain. Furthermore, faecal microbiota of mice and humans were analysed at different time points during the trial to evaluate the effects of the intervention on the faecal microbiota composition. This study is the first to detect *L. rhamnosus* GG consumption induced changes in the faecal microbiota of healthy adult study subjects. In addition, the results emphasised the importance of the pili in the GIT persistence of *L. rhamnosus* GG.

Study I introduced an approach to obtain derivative strains of bacteria with altered protein production features and importantly, these are not considered as genetically manipulated organisms (GMO), hence enabling their use also in human trials (The European Parliament and the Council of the European Union. 2001). Clearly, when designing a study in humans exploiting these types of organisms, one has to choose a microbial species, which is recognised as being safe for humans, such as probiotic species from lactobacilli and bifidobacteria, and additionally the strains have to be characterised genotypically and phenotypically in order to obtain information of their features. In Study I, 10 newly isolated pilus-less, non-mucus adherent derivatives were characterised and it was observed that pilus biogenesis could be hampered if the pilus-specific sortase C (srtC) was not functional (Class I), if the pilus gene cluster had been deleted (Class II) or if production of shaft pilin SpaA was reduced (Class III). The deleted region characterised in Class II mutants has been noted to be responsive to environmental stress, such as bile stress, and consequently it is thought to be important in environmental adaptation of the strain (Douillard *et al.* 2013a, Douillard *et al.* 2016b, Sybesma *et al.* 2013). Here, it is possible that the stress caused by the mutagenesis led to deletion of the region.

In Study II, highly mucus-adherent *L. rhamnosus* GG derivatives were characterised. Class I derivatives harboured a mutated residue in the mucus-binding pilin SpaC, which is likely to be responsible for the increased binding ability of the strains, without there being any clear increase in the quantity of pilins. Other three classes displayed an increase in the pilin quantities, but their mutation profiles var-
cluded. Although the mechanisms behind the high quantity of pilins in Classes II and III could not be determined, further studies on the mutations are likely to reveal new molecules affecting the efficiency of pilin transcription and secretion in *L. rhamnosus* GG, since none of the pilus-associated genes were affected. It was found that the Class IV derivative carried a mutation in the region of the secretion signal peptide of the backbone pilin SpaA, which most likely enhanced the secretion of the pilin, resulting in a higher amount of pili or possibly longer pili. Furthermore, the pilus-secreting derivative verified the essential contribution of sortase A (SrtA) in the attachment of pili into the cell wall in *L. rhamnosus* GG, as has been described previously for other bacteria (Mazmanian et al. 1999, Swaminathan et al. 2007). Figure 7. shows a schematic overview of how the different mutations detected in the studies could affect the pilus biogenesis.

In Study III, the pilus biogenesis of *L. rhamnosus* GG was approached from another perspective by inducing specific mutations in the pilin genes and characterising the resulting phenotype. Through bioinformatic analyses of membrane proteins of *L. rhamnosus* GG and pilins of several Gram-positive species, conserved variants of the pilin sorting motif LPxTG were detected, after which, by modifying different residues of the motif, the variants were functionally characterised in *L. lactis*. The characterisation revealed that a triple glycine (TG) motif is present in the tip pilin SpaC and backbone pilin SpaA, and this is recognised by the pilus-specific SrtC. TG motif is replaced by single glycine (SG) motif in the basal pilin SpaB, and SG motif is recognised by the housekeeping SrtA attaching the pilus into the cell wall. These motifs, by directing recognition of sortases with variable functions, are essential factors for successful pilus biogenesis in *L. rhamnosus* GG and most likely in other Gram-positive bacteria harbouring sortase dependent pili. In conclusion, the studies presented here increased knowledge of the aspects affecting the biogenesis of sortase-dependent pili in general.

In Study IV the significance of pili in the in vivo persistence of *L. rhamnosus* GG was studied with the help of a non-piliated derivative, *L. rhamnosus* GG-PB12. The derivative was chosen for these studies due to its phenotypic similarity to the parental strain, good growth and low quantity of additional mutations. The study scheme was first tested in mice, after which the human trial was performed. Higher persistence was detected for the piliated *L. rhamnosus* GG in both trials. Alterations in the faecal microbiota composition were also detected, although the changes were clearer in the human trial. One likely explanation for this is the high bacterial dose used in the mouse trial, which possibly hindered observation of subtle but more informative effects of the trial. The overall community in the human trial...
Figure 7. Illustration of the possible ways the mutations in the obtained *L. rhamnosus* GG derivatives could affect pilus biogenesis. On the left is the probable situation in the secretor derivative, as deficient SrtA is unable to attach pili into the cell wall. Next two panels depict the pilus-less derivatives, excluding the deletion class. In the SrtC mutants due to the lack of C-terminal anchor, SrtC and pilins attached to it are released into the culture medium (Class I). The lack of proper expression of SpaA in SpaA mutants results in short, SpaB dominated pilin oligomers (Class III). Of the highly adherent derivatives, SpaC mutants (Class I) adhere in high quantities to mucus even though they express equal amounts of pilins as the parental strain. In SpaA mutant, due to the presence of a mutation close to the secretion signal peptide, SpaA pilins are likely to be secreted efficiently to the cell membrane, resulting in a higher amount and/or longer pili (Class IV). The derivatives of the unknown classes are likely to have high amounts of pili due to efficient secretion or transcription, but no obvious mutation causing these changes could be characterised (Classes II and III).
shifted significantly due to the consumption of piliated *L. rhamnosus* GG, while consumption of the non-piliated strain did not cause this kind of alteration, highlighting the importance of pili in the ability of a bacterial strain to modulate microbiota composition. It was also observed that when piliated and non-piliated strains were consumed in combination, the community was again altered significantly, but in the opposite direction in comparison to that encountered in the piliated group. This is likely to be attributable to niche competition between the strains, and underlines the need to consider this possibility when probiotics are used in combinations instead of as single strains: effects caused by single strains cannot be extrapolated to guarantee that they will be reproduced when used in combinations, and vice versa. Additionally, in the human trial species richness was increased due to the intervention with the piliated *L. rhamnosus* GG. High richness is considered as a marker of a healthy gut microbiota, and consequently, the results indicate the significant role of pili in mediating *L. rhamnosus* GG induced probiotic effects (Jalanka-Tuovinen *et al.* 2011).

In the human trial, inter-individual differences in the persistence patterns of both piliated and non-piliated strains were observed, for example in some participants the strains were detected from faeces only during the intervention, while in some other subjects, they were still detected at the end of the follow-up. It would be advantageous to obtain markers, which would predict the ability of certain probiotics to colonise the GIT of a certain person and choose study subjects accordingly. For example, the connection between the ABO blood types and the colonisation ability of probiotics would be worthwhile to elucidate. However, it is not entirely certain whether a long colonisation time is associated with greater benefits of the probiotic, and clearly this would be another important factor requiring clarification.

According to the results obtained here and in previously published studies, pili seem to be essential for the probiotic properties of *L. rhamnosus* GG. They are likely to be more important as mediators of contact between the microbe-associated molecular patterns (MAMPs) of *L. rhamnosus* GG and host pattern recognition receptors (PRRs) than as signalling molecules, although some results indicating the signalling ability of pili *per se* have been published (Ganguli *et al.* 2015, Tytgat *et al.* 2016, von Ossowski *et al.* 2013). More informative results would be obtained from *in vivo* trials, and especially human trials, using the pilus-less and highly adherent derivatives from the studies presented here as reference strains. For example *in vivo* transcriptome studies would offer more information on the signalling induced especially by pili (van Baarlen *et al.* 2011). Recently, a study was performed in which the piliated
and non-piliated *L. rhamnosus* GG were studied as a synbiotic with prebiotic soluble corn fibre (SCF) for their ability to modulate gut microbiota composition, immune function and metabolism in healthy elderly subjects. In addition to the symbiotic treatment, the study included only-SCF and placebo treatment. Significant microbiota changes were observed, especially due to piliated *L. rhamnosus* GG-SCF treatment, similarly to the trial presented in the thesis (Costabile *et al.* in preparation). and the treatment also induced a significant decrease in the serum total cholesterol and low-density lipoprotein levels (LDL) in study subjects that had high cholesterol levels at the beginning of the trial. These results highlight the ability of *L. rhamnosus* GG to lower cholesterol levels in a beneficial way through a mechanism possibly associated with an alteration of the gut microbiota, and furthermore that pili are needed for this effect. In addition, the results demonstrate the need to target future trials to participants who are likely to respond most strongly to the intervention; in this case, they would be individuals with elevated cholesterol levels.
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74 | References

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