Immunomodulatory role for the pili of *Lactobacillus rhamnosus* GG: possible applications towards asthma and allergies.

Immunomodulerende rol voor de pili van *Lactobacillus rhamnosus* GG: mogelijke toepassingen voor astma en allergieën.

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Preface

Throughout the five years education as Bio-engineer, we were packaged with a lot of theory and practical experience during lab work. During this final year, we were able to show what we have learned and use this to contribute in scientific research for our thesis. This would not have been possible without the help of some people, who I would like to thank in this way.

First of all I want to thank my supervisor Cynthia Garcia Vargas for all the help, good advice and cooperation in the lab. I want to thank you for the knowledge and experience you have shared with me. I learned a lot throughout this last year which will certainly help me in my future work.

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Finally, I want to say to my parents that I'm grateful that you gave me the opportunity to study this education and for your support and believe in me. I thank my younger brother, who was always very interested in my work. A sincere thank you also goes to my boyfriend for all the support he gave me. It was nice that after a tough day, you were always there for me. To all my friends, thank you for the interest and support but specially for the amusing and relaxing moments that were certainly necessary during this final year.

Thank you all!
Ilke

Abstract

The human gastrointestinal tract (GIT) is colonized with large numbers of microbes, referred as the gut microbiota. This microbiota establishes a complex symbiotic interaction with local epithelial and immune cells and contributes to digestion, pathogen exclusion, epithelial barrier functioning and immune system modulation. Their described beneficial functions have led to the selection of strains that are used as probiotics, i.e. live organisms that confer a health benefit to the host when administered in adequate amounts (FAO/WHO, 2001). During the last decades, the prevalence of atopic diseases has been dramatically increased, likely due to a reduced exposure to microorganisms and parasites, thus the use of probiotics in the prevention and/or treatment of atopic disorders has gained more attention. *Lactobacillus rhamnosus* GG (LGG) is a clinically well studied probiotic. Its reported beneficial effects include enhancing certain immune functions and decreasing the development of atopic disease in susceptible individuals. Nevertheless, outcomes of different clinical trials are heterogeneous and health and probiotic organizations point out the importance of better molecular-based research techniques. In this way, the exact molecular mechanisms underlying the health-beneficial effects of probiotics can be unravelled and their use in clinical trials can be optimized. Several molecules on the cell surface of LGG are currently under investigation as they might be putative effector molecules that contribute to its probiotic effects. Recently, pili or fimbriae, i.e. proteinaceous extracellular appendages, were discovered on the cell surface of LGG. There is extensive evidence suggesting that pili are the major structures for adhesion to intestinal epithelial cells and can even induce anti-inflammatory responses in the same type of cells. As the gut microbiota is also in constant contact with immune cells, the aim of this thesis was to investigate the interaction and possible immunomodulatory role of the SpaCBA pili in these immune cells. Furthermore, cytokine profiles of immune cells from allergic and healthy donors after stimulation with LGG were evaluated in order to determine differences between these two populations upon addition of the bacterial strains. Altogether, the results indicate that SpaCBA pili play a key role in the interaction with immune cells, although the receptor(s) involved in their recognition remain to be determined. Immunomodulation experiments on the other hand could not attribute a direct role for SpaCBA pili in the induction of pro- or anti-inflammatory cytokines. Finally, data showed that immune cells from allergic individuals were less responsive to all bacterial treatments compared to non-allergic individuals.

Samenvatting

Het menselijk darmstelsel wordt gekoloniseerd door een groot aantal bacteriële species, de darmmicrobiota genoemd. Deze microbiota vormt een complexe symbiotische interactie met lokale epitheel en immuuncellen en draagt bij tot verschillende functies zoals vertering, pathogeen-exclusie, werking van de epitheelbarrière en modulatie van het immuunsysteem. Hun beschreven voordelige functies hebben geleid tot de selectie van een aantal stammen die gebruikt worden als probiotica. Deze zijn gedefinieerd als levende organismen die een gezondheidsbevorderend effect hebben op de gastheer als ze in voldoende hoeveelheid worden toegevoegd (FAO/WHO, 2001). De afgelopen decennia is het voorkomen van atopische aandoeningen in sterke mate gestegen, een fenomeen dat wordt toegeschreven aan een verminderde blootstelling aan micro-organismen en parasieten. Bijgevolg kent het gebruik van probiotica voor de preventie en/of behandeling van atopie veel interesse. *Lactobacillus rhamnosus* GG (LGG) is één van de best bestudeerde probiotische bacteriën in klinische studies. Tot de gerapporteerde gezondheidsbevorderende effecten van LGG behoren het versterken van bepaalde immuunreacties en het verminderen van de ontwikkeling van atopische aandoeningen in vatbare personen. Desondanks leveren verschillende klinische studies heterogene resultaten. Bijgevolg wijzen gezondheidsorganisaties op het belang van beter moleculair gericht onderzoek om de exacte mechanismen die aan de basis liggen van de probiotische voordelige effecten te identificeren en zo hun gebruik in klinische studies te optimaliseren. Verschillende moleculen op het oppervlak van LGG worden momenteel onderzocht omdat deze een belangrijke rol zouden kunnen spelen in de gezondheidseffecten. Recent werden op het celoppervlak van LGG lange eiwitachtige aanhangsels ontdekt, i.e. pili of fimbriae. Studies hebben aangetoond dat pili de belangrijkste structuren zijn voor adhesie aan darmepitheelcellen en dat ze zelfs anti-inflammatoire responsen kunnen induceren in deze cellen. De darmmicrobiota is tevens continu in contact met immuuncellen en bijgevolg was het doel in deze thesis om de interactie van de SpaCBA pili met immuuncellen te bestuderen alsook een mogelijke immunomodulerende rol in deze cellen. Daarnaast werden cytokinepatronen in immuuncellen van allergische en gezonde donoren geanalyseerd na stimulatie met LGG. Op deze manier werd getracht na te gaan of er verschillen merkbaar zijn in de respons van beide populaties na behandeling met de bacteriën. De resultaten impliceren dat SpaCBA pili een sleutelrol spelen in de interactie met immuuncellen, al moeten de receptor(en) die hierbij betrokken zijn nog geïdentificeerd worden. Immunomodulatie-experimenten hebben geen directe invloed van de pili kunnen aantonen in het induceren van pro- of anti-inflammatoire cytokines. Tenslotte kon aangetoond worden dat immuuncellen van allergische individuen een lagere respons vertonen na stimulatie met de bacteriën in vergelijking met cellen van gezonde donoren.

List of abbreviations

AAD	Antibiotic associated diarrhea
AD	Atopic dermatitis
APC	Antigen- presenting cell
ATCC	American Type Culture Collection
Bee	Biofilm enhancer in *Enterococci*
CFU	Colony forming units
CMPG	Centre of Microbial and Plant Genetics
CPS	Cell-wall associated polysaccharide
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
dNTP	Deoxynucleotide triphosphate
dPBS	Dulbecco's Phosphate Buffered Saline
DTT	Dithiothreitol
Ebp	Endocarditis- and biofilm-associated pili
EFSA	European Food Safety Authority
EPS	Exopolysaccharide
Ery^r	Erythromycin resistant
FACS	Fluorescence activated cell sorting
FAO	Food and Agriculture Organization
FCS	Fetal calf serum
FRET	Fluorescence resonance energy transfer
GALT	Gut associated lymphoid tissue
GAS	Group A *Streptococcus*
GBS	Group B *Streptococcus*
GFP	Green fluorescent protein
GIT	Gastrointestinal tract
GNB2L1	Guanine nucleotide binding protein beta polypeptide 2-like 1
IBD	Inflammatory bowel disease
IDT	Integrated DNA Technologies
IEC	Intestinal epithelial cell
Ig	immunoglobulin
IL	Interleukine

LAB	Lactic acid bacteria
LB	Luria-Bertani
LGG	*Lactobacillus rhamnosus* GG
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M cell	Microfold cell
m1	Classical macrophage
m2	Alternative macrophage
Mab	Modulator of adhesion an biofilm
MALT	Mucosa-associated lymphoid tissue
MAMP	Microbe-associated molecular pattern
MRS	de Man-Rogosa-Sharpe
Msp	Major secreted protein
NICE	Nisin controlled gene expression
NLR	Nod-like receptor
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PMA	Phorbol ester 12-O-tetradecanoylphorbol-13-acetate
PPIA	Peptidylprolyl isomerase A
PRR	Pattern recognition receptor
RPMI	Roswell Park Memorial Institute
RT-qPCR	Real time quantitative polymerase chain reaction
SCFA	Short-chain fatty acid
SCORAD	Scoring Atopic Dermatitis
subsp	subspecies
Tad-pili	Tight adherence pili
Tc^r	Tetracyclin resistant
TEM	Transmission electron microscopy
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
TLR	Toll-like receptor

TNF	Tumor necrosis factor
Treg	T regulatory
UC	Ulcerative colitis
WAO	World Allergy Organization
WHO	World Health Organization

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Context and objectives

It has been estimated that the human body contains about 100 trillion microbial cells, which are ten times more than the amount of human cells and encode 100-fold more unique genes than our own genome. The great diversity of microbes that colonizes the human body are referred as the human microbiota. This microbiota lives in close harmony with the human cells, a relationship known as symbiosis, in which both host and bacteria benefit from this association. A large proportion of these microbes resides in the human gastrointestinal tract (GIT).

During the last decades, more interest was given to the advantages of harboring such a huge amount of microbes and their implications on human health. The gut microbiota fulfills different functions, for example protection against pathogens, production of vitamins and amino acids, regulation of the intestinal barrier function and modulation of the immune system, which result in maintaining homeostasis and human health. Consequently, an imbalance of the normal microbiota, known as dysbiosis, negatively influences human health and has been associated with a plethora of diseases, including inflammatory bowel diseases (IBDs), cancer, obesity, autoimmunity and related disorders such as atopic diseases. Interest in the beneficial functions of our microbiota has led to the selection of strains that are used as probiotics.

The incidence of atopic diseases has been dramatically increased during the last decades, becoming an epidemic phenomenon. This increase can be explained by the hygiene hypothesis, which describes the inverse relationship between a reduced exposure to microorganisms and parasites and the rising prevalence of atopic diseases (Strachan, 1989). Hence, different clinical studies have already been conducted in order to investigate the beneficial effects of probiotic bacteria in the prevention and/or treatment of atopic disorders. Nevertheless, clinical trials show inconsistent outcomes and there is need to determine the molecular mechanisms whereby probiotics exert their health-beneficial effects.

Lactobacillus rhamnosus GG is one of the best clinically studied probiotic bacteria and is proven to exert the largest number of health benefits (Doron *et al.*, 2005), although its exact mechanisms of action are not yet fully identified. In the GIT, the interaction between bacteria with host cells occurs through conserved microbe-associated molecular patterns (MAMPs) located on the bacterial surface or as secreted proteins that bind to pattern recognition receptors (PRRs), mostly present on intestinal epithelial cells (IECs) and immune cells. Therefore, different cell wall molecules of LGG might act as MAMPs, in this way contributing to its beneficial effects.

SpaCBA pili present on the cell surface of LGG are important structures for the interaction with IECs and might even have anti-inflammatory potential. Immune cells are in continuous contact with bacteria in our GIT, through directly sampling microorganisms present in the intestinal lumen or via specialized microfold cells (M cells). Therefore we aim to identify the contribution of the pili of LGG in the interaction with immune cells. Besides, a possible immunomodulatory effect of the SpaCBA pili on these cells will be investigated as well. Finally, since LGG is often used in clinical trials for the prevention and/or treatment of atopic disorders, cytokine expression profiles of immune cells from allergic and non-allergic individuals will be compared upon stimulation with the bacterial strains.

Chapter 1: Literature study

1.1 The human gastrointestinal tract

1.1.1 General structure and functions of the GIT

The human GIT has a length of about nine meters and is composed by different organs including the mouth, pharynx, esophagus, stomach, small and large intestine. In addition, accessory organs contribute to the GIT function such as teeth, tongue, liver, gallbladder and the salivary glands. The main function of the GIT is digestion and absorption of components from food through hydrolysis reactions. In this way, polymers are converted into monomers that can be easily transported through the wall of the small intestine into the blood and lymph (Sherwood, 2008; Fox, 2011).

The small intestine is the largest part of the human GIT and is where absorption across the epithelium takes place. Its efficiency is due to a strong vascularization and greatly enlarged contact surface area. This enlargement in the surface area is provided by villi and microvilli. Villi are microscopic folds of the mucosa and consist of three cell types: i) epithelial cells or enterocytes, ii) mucus-secreting goblet cells and iii) Paneth cells. The mucosa lines the entire length of the GIT, surrounding the lumen (i.e. open space within the tube). It is the absorptive and major secretory layer and is composed of epithelium, lamina propria (i.e. connective tissue between villi with numerous blood capillaries and lymphocytes) and a muscularis mucosa (Fox, 2011). Microvilli in turn are folds in the apical plasma membrane of enterocytes. Each enterocyte contains about 3000-6000 microvilli that together form a vague brush border (Sherwood, 2008).

Besides the two primary functions, i.e. digestion and absorption, the GIT fulfills other functions as motility, secretion of different enzymes and hormones, storage and elimination of indigestible food molecules and it plays a key role in immune system homeostasis (Fox, 2011).

1.1.2 Microbiota in the GIT

1.1.2.1 Distribution, numbers and functions

All the parts of the human GIT are in constant contact with a vast number of microbes. This microbiota forms a complex microbial ecosystem that is important to maintain human homeostasis. The bacteria benefit through the acquisition of a stable nutrient supply and an environment where they can grow and survive, while they provide the host metabolic, protective and structural functions (Neish, 2009). Sometimes commensalism occurs, in which either the host or the bacteria profit, while the other is not affected.

A detailed overview of all species belonging to the GIT microbiota was long considered unavailable by conventional microbiological techniques such as growth on defined media, basically because more than 80% of stool bacteria cannot be cultivated under standard laboratory conditions (Eckburg *et al.*, 2005). Nowadays however, due to new molecular techniques, new insights are available to elucidate the diversity and development of the intestinal microbiota. Most of this information is obtained through genome sequencing techniques. As new techniques are developed, different projects were carried out like the Human Microbiome Project in which the inter- and intra-personal diversity of the microbiota was investigated as well as the influence of endo- and exogenous factors that might impact this diversity (Prakash *et al.*, 2011; Matamoros *et al.*, 2013).

The human intestinal microbiota consists of hundreds of different species that form a complex and interactive ecosystem in the GIT. The microbiome is defined as the collective genomes of all gut microbiota (Ruemmele *et al.*, 2009). Along the different parts of the GIT, bacterial populations exhibit both quantitative and qualitative variations, due to host, non-host (e.g. nutrients and environmental factors) and bacterial factors. Figure 1.1 gives an overview of the bacterial distribution and numbers in the human GIT.
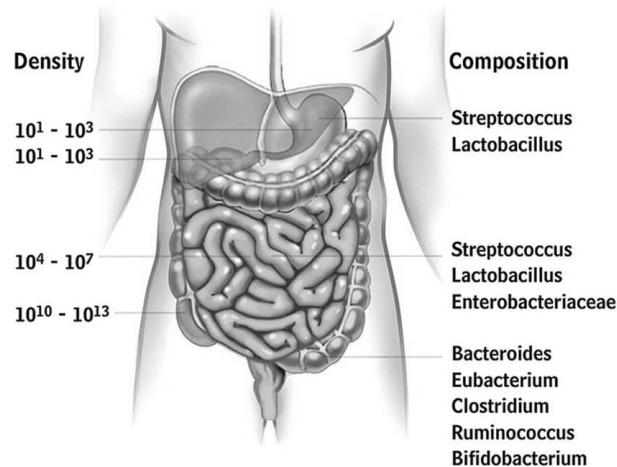
Figure 1.1: Distribution of the microbiota in the human GIT. The upper part of the GIT is typically less colonized with bacteria due to acid, bile and pancreatic secretions. Bacterial colonization increases in the small intestine and reaches its maximum in the large intestine. Different species are represented, the majority belonging to the phyla *Bacteroidetes* and *Firmicutes*. For further details see text (Simrén *et al.*, 2013).

In the stomach and duodenum, due to the presence of acid, bile and pancreatic secretions, bacterial numbers are low, approximately 10^1-10^3 cells/g content. This density increases to 10^4-10^7 cells/g in the jejunum and ileum, reaching its highest levels of 10^{10}-10^{13} cells/g in the large intestine (O'Hara & Shanahan, 2006). The majority of the population belongs to the phyla *Bacteroidetes and Firmicutes* (Backhed *et al.*, 2005). *Lactobacillus* species are most common in the upper part of the tract, while bifidobacteria and enterobacteria together with other Gram-negative species are mainly represented in the distal ileum and colon (Ruemmele *et al.*, 2009). Both aerobic and anaerobic bacteria can be found in the GIT, but anaerobes are present in several more orders of magnitudes. Common anaerobic genera are *Bifidobacterium, Clostridium* and *Bacteroides*, while *Enterococcus* and *Escherichia* are the abundant aerobic genera. The majority of the community is autochthonous, meaning that they are stable and domestic; however, transient species are also found with only a brief persistence (Neish, 2009).

Recently, it has been found that the human population can be divided into three groups, based on their gut microbiome. Arumugam, Raes and coworkers (2011) compared the gut flora of more than 200 individuals from different countries and performed comparative metagenomics. Their results indicate the presence of three main gut types in all the samples, which they defined as the human gut enterotypes. Each enterotype is characterized by a relatively high level of one of three genera; *Bacteroides* for enterotype 1, *Prevotella* for enterotype 2 and *Ruminococcus* for enterotype 3. These enterotypes are not nation nor continent specific, and can neither be simply explained by gender, body weight or age (Arumugam *et al.*, 2011). Future investigation of these enterotypes might reveal relationships between the composition of our microbiota and our health status.

Nowadays, scientific research focuses on unraveling the functions of the microbiota, which are divided into three main categories: metabolic, protective and structural (Prakash *et al.*, 2011). The metabolic functions are broad. The microbiota provides the host extended metabolic ability; for instance, enzymes that allow the host to obtain energy from the fermentation of sources that are otherwise non-digestible. Furthermore, through this process of fermentation, bacterial growth is stimulated, leading to the production of short-chain fatty acids (SCFAs), like acetate and butyrate, and gases (Wong *et al.*, 2006). These components in turn fulfill their own functions in the GIT. Moreover, the bacteria provide the host with vitamins, amino acids and help with the biotransformation of bile acid (Prakash *et al.*, 2011). The second important function of the microbiota is the protection of the gut environment against less benign and even pathogenic species. They provide a physical barrier to pathogens by competing for the same ecological niche or the same nutrients, or through the production of antimicrobial substances. This principle of pathogen exclusion is known as colonization resistance (Prakash *et al.*, 2011). Furthermore, the microbiota is

essential for the development of the host immune system. Appropriate bacterial colonization is extremely important for priming the immune system. The microbiota influences the development of the gut-associated lymphoid tissue (GALT), the production of effector molecules, the differentiation of T helper 17 (Th17) cells, the development and activation of regulatory T cells (Treg cells) and finally it is proposed that they also play a role in maintaining a balance between differentiated T helper 1 (Th1), T helper 2 (Th2) and Th17 lymphocytes (Mazmanian *et al.*, 2005; Ivanov *et al.*, 2009; Hill & Artis, 2010; Geuking *et al.*, 2011). The balance between Th1 and Th2 responses is crucial for optimal health status, as will be discussed later. Finally, microbiota exerts structural functions like the regulation of tight junctions permeability and the regulation of epithelial cell growth and differentiation (Prakash *et al.*, 2011).

The disturbance of the normal relationship between the microbiota and the GIT is referred as dysbiosis. This can be a consequence of alterations in the composition and distribution of the microbiota, but can also be the result of a change in the metabolic activity. Alterations in the normal relationship can be caused by several factors, for example the use of antibiotics, dietary changes and psychological and physical stresses (Prakash *et al.*, 2011). Dysbiosis has been intensively studied, since it has been involved in a plethora of diseases, such as IBDs (Walker *et al.*, 2011), type I diabetes mellitus (Brugman *et al.*, 2006), allergic diseases (Penders *et al.*, 2006) and even colorectal cancer (Sobhani *et al.*, 2011).

1.1.2.2 Early colonization

Before birth, the human GIT is a sterile environment but it is colonized by diverse numbers of bacterial species within few hours after birth and develops further until establishment of a stable community that remains until the adult stage (Ruemmele *et al.*, 2009). The evolution of the intestinal microbiota occurs mainly during the first two to three years of life. The initial colonization process shows some general trends. Immediately after birth, colonization starts with aerobic and facultative anaerobic bacteria as Gram-negative *Escherichia* species and Gram-positive *Staphylococcus*, *Streptococcus* and *Lactobacillus* species (Dominguez-Bello *et al.*, 2010). These are the same species found in the skin, vaginal and fecal flora of the mother. Once these organisms have depleted the oxygen supplies, the gut environment becomes anaerobic, which favors the growth of strictly anaerobes like *Bifidobacterium* and *Clostridium*. So starting from an initial low complexity and diversity, the infants gut microbiota develops and matures, until an adult stage is reached (Matamoros *et al.*, 2013).

Although there can be some general trends, defining a 'normal' gut microbiota is a difficult task since different factors influence the colonization process, resulting in interindividual variability. The most important factors that influence gut colonization in the infant are caused by the mother, for instance mode of delivery and neonatal diet. Different studies demonstrated that vaginally delivery or delivery by cesarean section has a strong influence on early colonization, especially on the number of bifidobacteria and lactobacilli (Biasucci *et al.*, 2010). Dominguez-Bello and coworkers (2010) investigated the microbiota present in the meconium (i.e. the earliest stool of a mammalian infant) in human newborns. They found a strong correlation between the first microbial species in the GIT of the newborn and the microbial communities of either the mother's vagina or skin. *Lactobacillus* species provide a natural first microbial exposure to body parts of normally delivered baby's as they are the dominant species in the mother's vaginal microbiota. Studies have shown that the colonization by *Lactobacillus* and *Bifidobacterium* in baby's born by cesarean section is delayed due to the lack off this vaginal exposure, and that they are more colonized with microbial species resembling the mother's skin microbiota such as *Staphylococcus* subsp. (Dominguez-Bello *et al.*, 2010). There is also evidence that baby's delivered by cesarean section are more likely to develop allergies and asthma, since they are less colonized with bifidobacteria and lactobacilli (Laubereau *et al.*, 2004; Negele *et al.*, 2004; Roduit *et al.*, 2009). Both species are considered to exert the most health-beneficial effects for the human host (Rastall, 2004). Another important factor influencing the colonization is the mode of feeding. Breast-feeding is associated with several benefits, like the development of the baby's immune system (Hanson, 1998). Studies have shown that the intestinal microbiota of breast-fed infants plays a dominant role for this early immune development (Hanson, 1998; Hanson *et al.*, 2003). Other studies investigating breast milk samples revealed the presence of *Streptococcus* and *Staphylococcus* species, which are early colonizers of the gut. Moreover, *Bifidobacterium* and *Lactobacillus* species are also frequently detected in breast milk samples (Fernández *et al.*, 2013). Breast-fed infants showed consequently higher amounts of these species compared to formula-fed infants, where the counts of *E. coli*, *C. difficile* and *B. fragilis* were higher (Penders *et al.*, 2006). In addition, genetics and environmental factors play a role in the colonization process (Matamoros *et al.*, 2013). Diet, for instance, directly influences the composition of the gut microbiota. De Filippo *et al*. (2010) compared the microbiota of children from Europe and Burkina Faso. European children typically consume a diet rich in animal proteins, sugars, starch and fat and low in fibers, while children from Burkina Faso mainly consume carbohydrates, fibers and non-animal proteins. The variation in these macronutrients was reflected in the composition of their microbiota, especially in the proportion *Bacteroidetes* and *Firmicutes*. These results confirm previous studies reporting that a diet rich in non-digestible carbohydrates and limited in fats and animal proteins positively influences the health state of the gut (Scott *et al.*, 2013).

1.1.2.3 Interaction mechanisms between the gut microbiota and the host

The mucosal immune system is the part of the immune system juxtaposed to the mucosal surfaces and in direct contact with the external antigenic environment. It is an extensive system of lymphoid tissues, also known as the mucosa-associated lymphoid tissues (MALT), that protect the mucosal surfaces (Murphy, 2011). An important part of the MALT is located in the GIT; the GALT comprises the largest part of the human immune system, containing about 75% of all lymphocytes (Murphy, 2011). Functionally, the GALT can be divided into inductive sites, including the lymph nodes, Peyer's patches and lymphoid follicles. These are the sites in which antigens are presented to the T and B cells. Moreover, the GALT also contains effector sites, the epithelium and the underlying lamina propria (Mowat & Viney, 1997).

The epithelium and the thick overlying mucus, lining the gut, form an important barrier against microbial invasion. The interaction between host cells and the gut microbiota occurs mainly at the level of three types of immunosensory cells: (i) enterocytes, (ii) dendritic cells (DCs) and (iii) M cells (O'Hara & Shanahan, 2006) (Figure 1.2). Enterocytes serve as sensors of danger and once activated secrete many immune modulators like cytokines and chemokines, which in turn lead to the induction of innate and adaptive immune responses at the infected site (Shanahan, 2005). DCs are professional antigen-presenting cells (APCs) that play an essential role in bridging innate and adaptive immunity. They can encounter microorganisms through two different routes (Lebeer *et al.*, 2010). DCs are able to pass their dendrites between intestinal epithelial cells (IECs) into the lumen of the gut through tight junctions between the epithelial cells and directly sense bacteria (Rescigno *et al.*, 2001). During this process, they produce tight-junction proteins such as occludin and claudin thereby preserving the integrity of the epithelial barrier (Rescigno *et al.*, 2001). These APCs then migrate to the Peyer's patches, where they activate T lymphocytes. Another route through which DCs can encounter microorganisms is via specialized M cells that directly take up bacteria. M cells are located in Peyer's patches and are responsible for the transport of microorganisms, microbial components and macromolecules from the gut lumen to the immune cells located in these patches, i.e. T and B lymphocytes, macrophages and DCs (Owen, 1999). This process is known as transcytosis, which plays an important role in mucosal immunity (Kucharzik *et al.*, 2000). In the Peyer's patches, antigens are transported to DCs, which in turn present them to naive T cells, resulting in their activation and the induction of a proper immune response (Macdonald & Monteleone, 2005; reviewed in O'Hara & Shanahan, 2006).

Both IECs and DCs are equipped with pattern recognition receptors (PRRs) to recognize conserved microbe-associated molecular patterns (MAMPs) that are mostly components of the bacterial cell surface and are widespread and conserved among different bacteria but not present in the host (Lebeer *et al.*, 2010) (Figure 1.2).
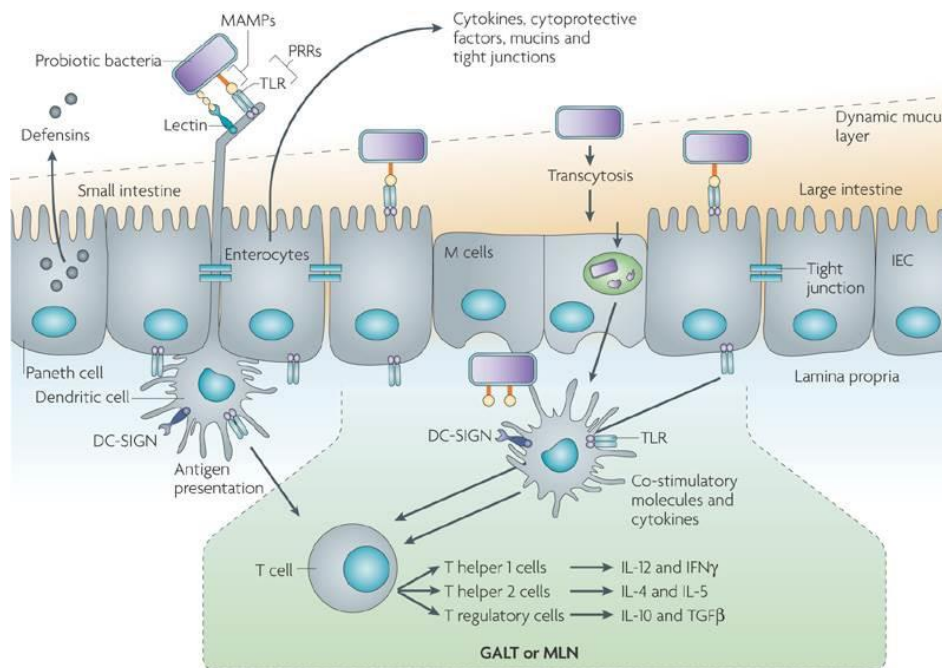
Figure 1.2: Interaction of bacteria with host cells of the GIT. Interaction between microbiota and the host in the GIT occurs mainly at the level of IECs and DCs. These cells are equipped with PRRs that recognize MAMPs, present on the cell surface of bacteria. DCs can sense the bacterial components via two different routes: (i) they can pass their dendrites through the epithelial cells and directly sample bacteria or (ii) they can interact with bacteria in the Peyer's patches when bacterial cells or products are uptaken by specialized M cells. Interaction between the PRR and MAMP activates a certain pathway in the host leading to a host molecular response whereby different effector molecules can be produced (Lebeer *et al.*, 2010).

There are several classes of PRRs but the most well-known and characterized are Toll-like receptors (TLRs), located on the cell membrane or intracellular and responsible to scan the external environment in order to recognize viral or bacterial products and even intact bacteria (Mandell *et al.*, 2004). The complete range of functions for TLRs is not yet fully determined but its ability to elicit inflammatory and antimicrobial responses after their activation is well established (Medzhitov, 2007; Neish, 2009). The human genome encodes ten different TLRs, TLR1 to TLR10 (Starckx & Opdenakker, 2005). An important TLR for the recognition of bacterial components is for instance TLR2, essential for the recognition of distinct MAMPs from Gram-positive bacteria, including lipoproteins, lipomannans and lipoteichoic acids (Lien *et al.*, 1999). TLR2 mostly acts as a heterodimer with TLR1 or TLR6. Flagellin, the structural protein that forms the filaments of bacterial flagella, interacts with TLR5 and LPS associates with TLR4 (Starckx & Opdenakker, 2005). Another important group of PRRs

are Nod-like receptors (NLRs) that are located in the cytosol and their main function is to guard the intracellular space (Neish, 2009). The PRR-MAMP interaction activates certain regulatory pathways, leading to a rapid host cell response whereby different effector molecules can be produced such as cytokines, co-stimulatory molecules and antimicrobial factors. A unique feature of PRRs is that they have a broad specificity, meaning that a limited amount of PRRs can recognize and bind a large number of MAMPs (Medzhitov, 2007). Although MAMPs are widespread and conserved, structural differences exist and this leads to differences in the interaction with the host and consequently in the cellular response (Lebeer *et al.*, 2010).

1.2 Probiotics

Since microbiota plays an essential role in the human body, interest in their beneficial functions has led to the identification of a variety of bacterial strains that are used as probiotics. The term probiotics literally means 'for life' and are *"live organisms, which when administered in adequate amount, confer a health benefit on the host"* (FAO/WHO, 2001).

In the beginning of the 20th century, the Russian microbiologist Metchnikoff was the first to observe that certain bacteria exert a positive role on the normal microbiota present in the GIT. He stated that lactobacilli were important for human health and longevity, and encouraged to consume yoghurt and other fermented food products (Metchnikoff, 1907). He mainly focused on the Gram-positive *Lactobacillus bulgaricus*, known these days as *L. delbrueckii subsp. bulgaricus*. The first commercial probiotic products contained mainly *L. bulgaricus*, but then the beneficial effects of other lactic acid bacteria (LAB) were described and new strains were successfully incorporated into probiotic preparations (Fooks & Gibson, 2002). Today, the most studied probiotics belong to the LAB and within this group, *Lactobacillus* and *Bifidobacterium* are most frequently investigated. Studies on bifidobacteria unraveled their efficacy in the prevention and treatment of human gastrointestinal disorders; for instance colonic transit disorders (Marteau *et al.*, 2002), intestinal infections (Saavedra *et al.*, 1994) and colon cancer (Singh *et al.*, 1997). An example of this ability is given by *Bifidobacterium animalis*, trade name *Bifidus Actiregularis*, in Activia yoghurt from Dannon. A commonly used probiotic additive of *Lactobacillus* species is *L. casei,* which is probably best known as *L. casei* immunitas in the commercial yoghurt-drink Actimel (*Lactobacillus casei* DN-114001), or in the dairy product Yakult (*L. casei* Shirota). *L. casei* Shirota has been shown to improve constipation (Koebnick *et al.*, 2003), to reduce the incidence of acute diarrhea (Sur *et al.*, 2011) and to influence the balance of intestinal microorganisms by increasing the number of beneficial bacteria (Matsumoto *et al.*, 2010). Nowadays, one of the most well-clinically studied probiotics is LGG that is also often used as a commercial probiotic (Doron *et al.*, 2005). Valio Ltd, one of the biggest companies in

Finland, was the first to introduce LGG in dairy products (Valio LGG®). Today, LGG is used in more than 40 countries worldwide in dairy products, juices, cheeses, and it is also available as food supplement in capsules. Culturelle for instance provides capsules containing LGG to help overcome digestive problems. LGG has proven beneficial effects on the treatment or prevention of acute diarrhea, antibiotic associated diarrhea (AAD) and traveler's diarrhea, based on its ability to restore the natural balance in the gut (Szajewska & Mrukowicz, 2001). Additionally, *Lactococcus lactis* has been extensively used as a starter culture in the dairy industry as these bacteria were found to suppress the growth of pathogens by the production of antibacterial compounds or bacteriocins (Soomro a.H., T. Masud, 2002). Nisin, for instance, is a good characterized bacteriocin, active against the Gram-positive food-borne bacteria *Listeria monocytogenes* (Rodríguez *et al.*, 2001a). Moreover, nisin also prevents the growth of spores from *Bacillus* and *Clostridium* species (Stewart *et al.*, 2000). Other bacteriocins were found to be effective against food pathogens as *L. monocytogenes*, *Staphylococcus aureus* and *E. coli* O157:H7 (Rodríguez *et al.*, 2005). Few Gram-negative probiotics exist, the best-characterized is *E. coli* strain Nissle 1917 (Saarela *et al.*, 2000; Rauch & Lynch, 2012).

There are several requirements for a microbe to be considered a probiotic. First, the genus, species and strain need to be fully identified and strains for human use should be preferably of human origin (Borchers *et al.*, 2009). Also, the species of interest must have good technological properties so that it can be manufactured and incorporated into food products while maintaining viability and functionality (Saarela *et al.*, 2000). Another criterion focuses on the survival during transit through the GIT; resistance against the acidic environment of the stomach and pancreatic enzymes and tolerance against bile salts in the small intestine (Bezkorovainy, 2001). Other functionality requirements are adherence to epithelial surfaces, persistence, immunostimulation and antagonistic activity against pathogens (Borchers *et al.*, 2009). With regard to safety criteria, probiotic strains may not carry any transmissible antibiotic resistance genes (Saarela *et al.*, 2000). Antibiotic resistance among microorganisms has become a considerable problem and it causes serious problems in the treatment of bacterial infections. Extra care should be taken into account if the microbe is capable of transmitting this resistance to other species. Like previously said, lactobacilli are often used as probiotics and they display a wide variety of antibiotic resistance naturally. However, this resistance is not transmissible; therefore, *Lactobacillus* strains do not raise a safety concern (Saarela *et al.*, 2000).

1.2.1 Effects on human health

There are several clinical trials in which probiotics have demonstrated to exert health-promoting effects, including against IBDs (Furrie *et al.*, 2005), antibiotic-associated and infectious diarrhea (Szajewska & Mrukowicz, 2001) and allergic diseases (Kalliomäki *et al.*, 2001, 2003, 2007). Therefore, probiotics have become a hot topic and their potential for the prevention and treatment of different diseases is under investigation.

The benefit of probiotic administration was extensively studied in the treatment of diarrhea. Szajewska and Mrukowicz (2001) investigated the effect of different probiotics on acute infectious diarrhea in infants and children. Their results showed a positive outcome, especially for the probiotic LGG. AAD can be prevented when probiotics are administrated simultaneously with antibiotics. McFarland (2006) showed that administration of *Saccharomyces boulardii*, LGG or probiotic mixtures (*Lactobacillus acidophilus* and *L. bulgaricus*; *L. acidophilus* and *B. animalis*; *L. acidophilus* and *Bifidobacterium infantis*) reduced the development of AAD. IBDs are a group of inflammatory conditions of the colon and small intestine; ulcerative colitis (UC) and Crohn's disease are the most abundant types of IBD. Different clinical trials investigated the impact of probiotic administration on both UC and Crohn's disease, but the results are heterogeneous (Verna & Lucak, 2010). To further investigate the potential for probiotics as therapy for UC and Crohn's disease, better controlled, on a larger scale standardized studies are necessary. Sometimes, the large intestine of UC patients is removed and replaced by ileo-anal pouches. However, in some cases this pouch can be inflamed in a similar way to the original inflammation in the large intestine, a disease called pouchitis. The administration of probiotics has been found to be effective for the prevention and treatment of pouchitis. For instance, the probiotic food supplement VSL#3 (i.e. an oil suspension containing frees-dried LAB and bifidobacteria) consistently decreased the incidence and relapse of the inflammatory response in patients with pouchitis (Mimura *et al.*, 2004). The health promoting capacity of probiotics was also investigated in allergic diseases. Several clinical trials investigated the impact of probiotic administration, specially belonging to the LAB, in the prevention of the development of allergies. Some studies showed positive results (Kalliomäki *et al.*, 2001, 2003, 2007) while others failed to confirm them (Kopp *et al.*, 2008). As with IBDs, more large-scale standardized trials are needed and insights in the basic mechanisms of allergies and probiotic action are required to optimize the studies and draw general conclusions.

One of the most important prerequisite for probiotic action is adherence to the intestinal surface and subsequent colonization (Ouwehand *et al.*, 1999). Adherent strains are likely to persist longer in the GIT and as a result exert more efficiently their metabolic and immunological properties over strains that are non-adherent (Saarela *et al.*, 2000). Another feature of adherent strains is the competitive exclusion of pathogens (Coconnier *et al.*, 1993). For instance, the adhesion capacity of LGG and *L. casei* Shirota to Caco-2 cells and intestinal mucus was investigated. LGG had an adhesion capacity that was ten times higher than *L. casei* Shirota, but nevertheless, both strains were effective in the exclusion of *E. coli* strain TG1 (Lee *et al.*, 2000).

Nevertheless, future studies are needed for the identification of molecules involved in optimal probiotic function. The exact molecular interactions between probiotic bacteria and their host is an important research topic for health and probiotic organizations. For example, the European Food Safety Authority (EFSA) has pointed out that an important step towards the approval of health claims for probiotic strains lies in better molecular-based research followed by multiple well-performed clinical trials.

1.2.2 Modes of action

The modes of action by which probiotics contribute to human health are divided into three broad categories, as can be seen in Figure 1.3. The first and best studied mechanism is the inhibition of pathogens by competition for nutrients or adhesion sites, known as competitive exclusion, and also through the production of antimicrobial substances, such as lactic and acetic acid, hydrogen peroxide and bacteriocins (Rolfe, 2000). These substances exert inhibitory effects on both Gram-positive and Gram-negative bacteria (Rolfe, 2000). A second mechanism is to enhance the epithelial barrier function, for instance by the stimulation of antimicrobial peptides as defensin, whose function is to protect the mucosal surfaces against invasion of microorganisms (Madsen, 2012). Other examples are the induction of mucus, the enhancement of tight-junction functioning, the prevention of apoptosis and the induction of cytoprotective molecules (reviewed in Madsen, 2012). The last mechanism is through modulation of host immune responses since probiotic bacteria can modulate both innate and adaptive immunity (Lebeer *et al.*, 2010). Administration of *L. casei* CRL 431, for example, modulates cytokine profiles through the stimulation of TLRs, mainly in cells from the innate immune response (Castillo *et al.*, 2011). Probiotic bacteria can also modulate the maturation of DCs toward an anti-inflammatory interleukine 10 (IL-10) profile and their role on Treg cell activity has already been demonstrated (Feleszko *et al.*, 2007; Borchers *et al.*, 2009). Other properties include the stimulation of mucosal immunoglobulin A (IgA) levels and allergen-specific B and T cell responses, which might modulate allergic diseases (reviewed in Toh *et al.*, 2012).
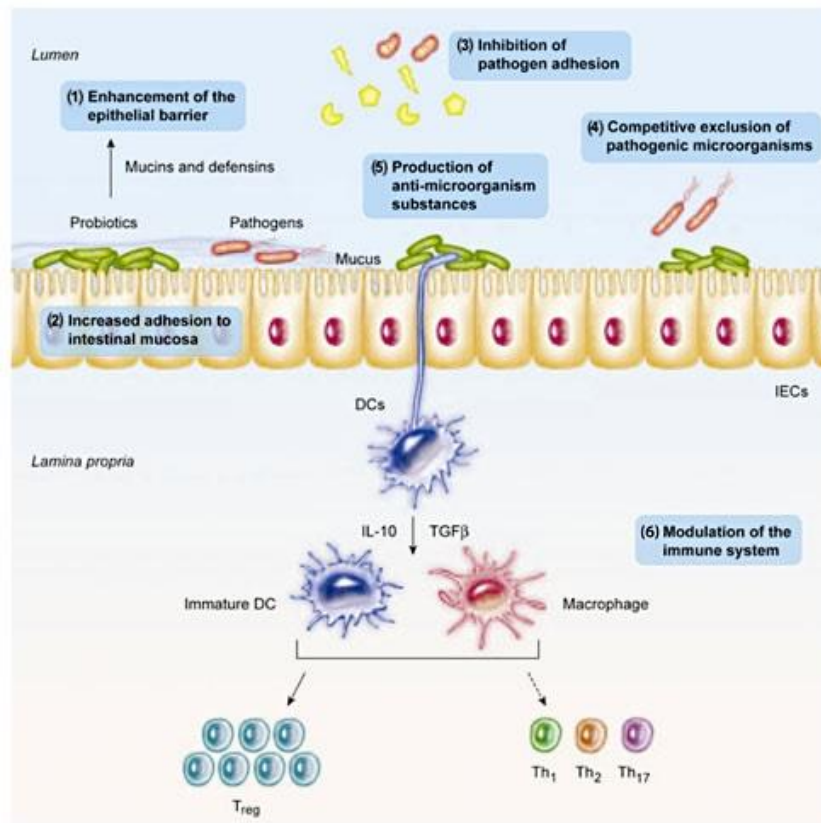
Figure 1.3: Probiotic modes of action. Probiotics are assumed to exert their health-benefial effects through different mechanisms of action. First of all, by pathogen exclusion through competition for adhesion places and nutrients and by the production of antimicrobial substances. Second, by enhancing the epithelial barrier function, for instance via the production of defensins and mucins. Finally, by modulation of host immune responses. For more details see text (Gil, 2013).

1.2.3 *Lactobacillus rhamnosus* GG - cell wall components of LGG

LGG is a Gram-positive bacterial strain that was first isolated in 1983 from human faeces by Sherwood Gorbach and Barry Goldin (Gorbach, 1996). LGG is one of the best clinically studied probiotic strains and is proven to exert the largest number of health benefits (Doron *et al.*, 2005). Different studies already investigated the clinical effects of this strain in humans, including the reduction of AAD in children (McFarland, 2006), the treatment of rotavirus and acute diarrhea in children (Guandalini *et al.*, 2000), alleviation of atopic dermatitis symptoms in children (Kalliomäki *et al.*, 2001, 2003, 2007) and the reduction of inflammation in some milder states of IBDs (Zocco *et al.*, 2006). Besides, LGG was found to suppress bacterial enzyme activity, such as β-glucuronidase and nitroreductase. These enzymes are thought to play a role in the activation of procarcinogens in the colon, suggesting a role for LGG in human colon cancer (Goldin, 1996).

During the last years, several efforts have been made to identify probiotic effector molecules of LGG. These studies are of great importance to elucidate the mechanisms of action of this probiotic strain underlying its health benefits. Studies with mutants that lack or have modified putative effector

molecules or studies with purified putative effector molecules aim to give a better understanding on the probiotic mechanisms of action. This might help to optimize clinical studies and to interpret heterogeneous clinical data.

Lactic acid for instance is known to act as antimicrobial agent against *Salmonella enterica* serovar Typhimurium (De Keersmaecker *et al.*, 2006b). LGG also secretes two major secreted proteins, Msp1 or p75 and Msp2 or p40, that mediate anti-apoptotic and protecting effects on the tight junctions (Yan *et al.*, 2007; Claes *et al.*, 2012a). Other interesting effector molecules are located on the cell wall of LGG, which is composed of a thick peptidoglycan layer, decorated with a variety of molecules. The main cell wall molecules are lipoteichoic acids (LTA), cell-wall associated polysaccharide molecules (CPS-molecules), glycoproteins and pili. All these structures are putative MAMPs that can interact with host cells (Figure 1.4).
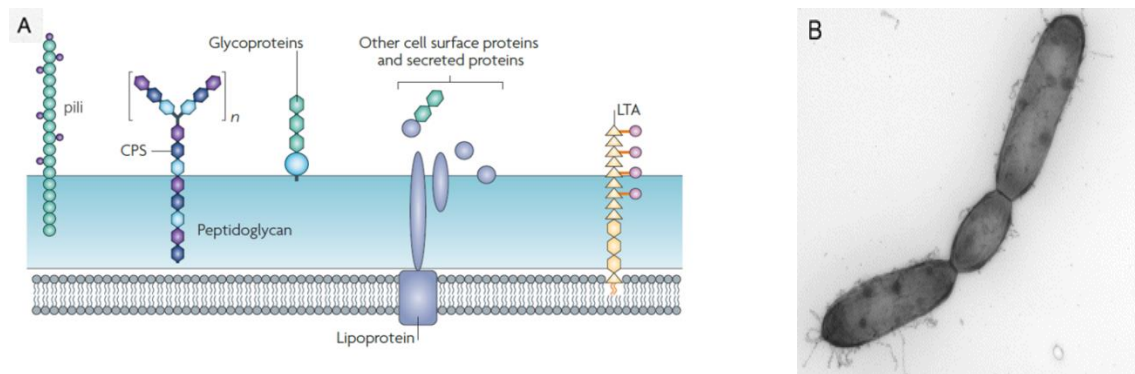
Figure 1.4: General structure of the LGG cell wall and an electron micrograph of LGG. (A) The cell wall of LGG contains a thick layer of peptidoglycan. Different cell wall molecules are embedded on the peptidoglycan, i.e. lipoteichoic acids, glycoproteins, polysaccharides and pili. These are all possible MAMPs and thus involved in the interaction with PRRs on host cells (adapted from Lebeer *et al.*, 2010). (B) Electron micrograph of LGG showing the presence of pili on the cell surface (Kankainen *et al.*, 2009).

LTA is an important pro-inflammatory molecule in Gram-positive bacteria that acts as a MAMP by binding to TLR2 in a heterodimer with TLR6 (Lebeer *et al.*, 2010). The pro-inflammatory effects of LTA are considered to be undesirable for probiotic bacteria in certain applications, for instance, in the treatment of IBDs. IBD patients already suffer a high degree of inflammation, which is increased after treatment with LGG, as shown in mice with induced colitis (Lebeer *et al.*, 2012b). The construction of a mutant with a deficiency in the *dltD* gene, which is responsible for the incorporation of D-alanine residues into LTA, showed modified immunomodulatory properties compared to the wild type in Caco-2 cells and an alleviation of colitis symptoms in a murine model (Claes *et al.*, 2012b; Lebeer *et al.*, 2012b). However, in healthy subjects, the recognition of LTA in the GIT does not result in inflammation due to a variety of microbiota- and host-associated mechanisms (Lebeer *et al.*, 2012b).

CPS-molecules of LGG are also under investigation and as seen in pathogens, these molecules are able to shield other surface molecules (Lebeer *et al.*, 2009). Lebeer and coworkers (2009) recently identified the exopolysaccharide (EPS) gene cluster of LGG and confirmed that LGG possesses both long galactose-rich EPS molecules as well as short glucose-rich EPS molecules. Through the construction of an EPS-mutant (CMPG5351), they could show that galactose-rich EPS molecules are not required for the adhesion capacity of LGG to pig gastric mucus and Caco-2 epithelial cells and could even have a negative influence on adhesion by shielding off adhesins (Lebeer *et al.*, 2009). On the other hand, these EPS molecules promote the survival of LGG in the intestinal tract by protecting against innate immune factors such as complement factors and cathelicidins (Lebeer *et al.*, 2011).

A particular characteristic of LGG is the presence of pili on its cell wall, which will be the focus of this thesis. Pili, also called fimbriae, are long filamentous surface structures, encoded within pathogenicity islands, found in both Gram-positive and Gram-negative bacteria (Reunanen *et al.*, 2012). These structures were first discovered in the 1950s using electron microscopy in the Gram-negative pathogens *E. coli*, *Pseudomonas pyocyanea* and *Proteus mirabilis* and few years later also in the Gram-positive bacteria *Corynebacterium renale* (Duguid *et al.*, 1955; Yanagawa *et al.*, 1968; Kline *et al.*, 2010). Pili of Gram-negatives were studied intensively and their organization, structure and functions are mostly unraveled. In Gram-positive species, research is more limited but in the last decade, pili have been identified in an increased number of Gram-positive species, mostly pathogens (Reunanen *et al.*, 2012).

In general, bacterial pili have a broad range of functions. They promote different types of interaction such as with other bacteria, with host cells, with environmental surfaces and with bacteriophages (Kline *et al.*, 2010). They are also involved in the transfer of genetic material among microorganisms, stimulation of protein secretion, formation of biofilms and even in twitching motility (Campos *et al.*, 2010; Filloux, 2010). In different *Streptococcus* species, pilus components are even promising candidates for vaccine development (Maione *et al.*, 2005).

1.2.3.1 Pili in Gram-positive bacteria

Gram-positive bacterial pili were first discovered in *C. renale* by Yanagawa and coworkers (1968). Morphological studies have revealed that these pili are thin flexible rod-like structures, ranging from about 1 to 10 nm in diameter. Structurally, they are generally composed by different protein subunits, called pilins, which are covalently linked to each other forming a large molecule. The best-characterized Gram-positive pili are from *Corynebacterium diphtheriae*, in which the pilus is formed by three different subunits, the major pilin subunit (SpaA) that builds up the pilus shaft and two accessory pilin subunits (SpaB and SpaC). All the genes for pili biogenesis are clustered in the same genetic loci that additionally contain an associated class C sortase or pilin sortase, and class A or housekeeping sortase. The sortases are responsible for the sortase-mediated pili assembly, which is the most common system in Gram-positive bacteria. This type of assembly is based on two conserved genetic elements that are essential for the pilus formation, the pilin motif and the cell wall sorting signal or LPXTG-motif, both present in the major pilin subunit. Moreover, a conserved E-box region determines the specificity of the pilin subunit for a specific sortase. Several sortases can indeed be produced by Gram-positive bacteria, but not all are required for the assembly process (Telford *et al.*, 2006; Danne & Dramsi, 2012; Reunanen *et al.*, 2012).

Pili assembly in *C. diphtheriae* comprises three main steps: i) polymerization of SpaA, ii) incorporation of minor pilins and iii) assembly termination by attachment to the cell wall (Figure 1.5). First, the pilin subunits, SpaA, SpaB and SpaC, are translocated across the cell membrane by sec translocase. During this secretion across the plasma membrane, pilins are linked to the cell membrane through a membrane spanning domain at the C-terminus of the LPXTG-motif. In this first step of pili assembly, the anchored SpaA pilin is cleaved in the LPXTG-motif upon recognition by a sortase, leading to the formation of an acyl-enzyme intermediate. The thioester bond formed between the pilin threonine residue and the cysteine in the sortase undergoes a nucleophilic attack, resulting in an amide bond between the pilin threonine residue and the conserved lysine of the pilin motif of the next SpaA monomer. The formed pilin motif in the dimer can interact with other sortase-associated pilin subunits, thereby forming an elongated pilus fiber (Telford *et al.*, 2006; Kline *et al.*, 2010). The second step in which minor pilins are incorporated is not yet fully established. The third and last step involves pilus assembly termination, where the housekeeping sortase recognizes a LAFTG motif in the cell wall sorting signal of SpaB. As LPXTG is the recognition motif for the pilin sortase, LAFTG is for the housekeeping sortase. Upon recognition by the housekeeping sortase, SpaB is cleaved at this motif forming an acyl-enzyme intermediate with the sortase. This complex attacks the terminal SpaA-sortase intermediate, resulting in the transfer of the entire pilus polypeptide to the housekeeping

sortase. This sortase via a transpeptidation reaction attaches the SpaB-linked pilus fiber to the lipid II precursor, which in turn will be attached to the cell wall following the general cell wall biogenesis process (Mandlik *et al.*, 2008).
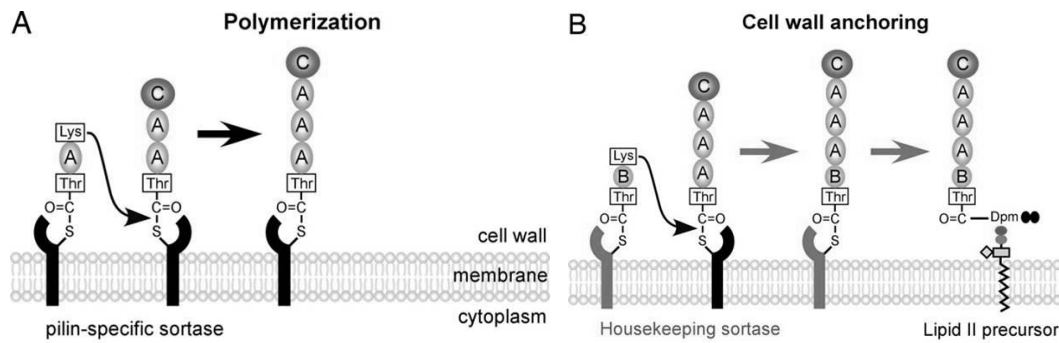
Figure 1.5: Model for sortase-mediated pilus assembly in the Gram-positive bacterium *C. diphteriae*. Pili in *C. diphteriae* are composed of three subunits, SpaA, SpaB and SpaC, and the system is dependent on two sortases: a pilus-specific and housekeeping sortase. The latter is responsible for the attachment of the pilus to the cell membrane, whereas the pilus-specific sortase is responsible for the assembly of the different subunits. (A) The SpaA subunit is the major pilin component that builds up the backbone of the pilus. Several SpaA subunits become covalently linked to each other by their conserved LPXTG- and pilin-motif. This occurs through the formation of an acyl-enzyme intermediate that undergoes a nucleophilic attack, thereby forming an amide bond between two subsequent SpaA subunits. Accessory subunits SpaB and SpaC are added to the pilus backbone, but this mechanism in not yet fully established. (B) Termination of the pilus polymerization depends on the SpaB subunit. This subunit is recognized by the housekeeping sortase, leading to their association. In this coupled conformation, the final SpaA-sortase is attacked, thereby bringing the entire pilus polypeptide to the housekeeping sortase, which attaches the pilus-fiber to the lipid II precursor. Finally, this precursor will be covalently attached to the cell wall in the general cell wall biogenesis process (Mandlik *et al.*, 2008).

The role of pili in Gram-positive bacteria is best studied in pathogenic species, such as *Streptococci* and *Enterococci*, where they are essential in the first step of infection, namely adhesion and colonization of the host tissue. Adhesins are cell-surface components, located at the tip of the pilus fiber and mediate the initial colonization process. In 1975, the adhesion properties of pili from *C. renale* to kidney epithelial cells and afterwards also to bladder epithelial cells were unraveled by Honda and Yanagawa (Honda & Yanagawa, 1975). Since then, the role of pili of pathogenic species in adherence to eukaryotic cells and in colonization and infection was further studied. For instance, infective endocarditis is a disease where the inflammation of the cardiac valves is caused by bacteria from the species *Staphylococcus* and *Streptococcus*. Besides *Staphylococcus* and *Streptococcus* species, some *Enterococci* can also be involved in the course of infective endocarditis, such as *E. faecalis*, a species that possesses two pilus loci: ebp (endocarditis- and biofilm-associated pili) and less common bee locus (biofilm enhancer in *Enterococci*). Ebp-pili mediate adherence to platelets, collagen and fibrinogen, the three main components of the cardiac valves that become colonized

during infective endocarditis, being a clear example in which pilus-mediated adhesion to the host cells leads to infection (Nallapareddy *et al.*, 2011).

Since pili are encoded in pathogenicity islands, a role in virulence should be expected. Therefore, their role as virulence factors was also investigated in human pathogens, e.g. *S. pyogenes, S. pneumoniae* and *C. diphtheria*. In fact, a general tendency was found in which pili-mutants of the different pathogens showed a decreased adhesion capacity (Danne & Dramsi, 2012). Group A *Streptococcus* (GAS)-pili of *S. pyogenes* showed impaired capacity to attach a pharyngeal cell line after deletion of the major pilin or the sortase C1 (Manetti *et al.*, 2007). In PI-1 pili of *S. pneumonia*, deletion of the adhesin RrgA resulted in decreased adherence to a human respiratory epithelial cell line (Nelson *et al.*, 2007). Finally, deficiency in the minors subunits SpaB and SpaC of the pili of *C. diphtheria* also affected the adherence to human pharyngeal cells (Mandlik *et al.*, 2007). Another important property for successful infection is the ability to modulate the innate immune system of the host to avoid clearance. Group B *Streptococcus* (GBS) for instance, possess pili that have a function in promoting virulence and resistance against killing by neutrophils and macrophages through the process of phagocytosis, leading to an increased survival (Maisey *et al.*, 2008).

Besides their role in infection, pili can induce biofilm formation by attachment to surfaces which is, for instance, an important feature for oral bacteria that colonize the human oral cavity (Danne & Dramsi, 2012). These bacteria often produce pili that are involved in the primary colonization to the tooth surface which enables further interbacterial interactions that lead to the formation of biofilms, known as human dental plaque (Rickard *et al.*, 2003).

It is proposed that pilus-mediated adhesion in Gram-positive pathogens occurs through a 'zipper-like mechanism'. This model stipulates that the initial bacterial contact with the host cell is mediated by the extended pilus structure. Then, further interaction is promoted by the adhesive properties of the SpaC subunit present in the pilus fiber and other cell wall proteins, bringing bacteria and host cells in closer contact (Telford *et al.*, 2006).

1.2.3.2 Pili in LGG

Recently, pili were identified on the cell surface of LGG. Studies have demonstrated that the main function of LGG pili is to mediate adherence to mucus glycoproteins and to human IECs (Kankainen *et al.*, 2009; Lebeer *et al.*, 2012a). In this case, adhesion does not result in infection but in a prolonged and stable persistence in the GIT, which is an important requirement to exert health benefits. Adhesion occurs through the zipper-like mechanism as in Gram-positive pathogens, described above (Lebeer *et al.*, 2012a). Besides the SpaC adhesin, also the MabA protein of LGG plays an important modulating role for the interaction with the host (Vélez *et al.*, 2010).

Based on the genome sequence, it was predicted that LGG possesses two pilus gene clusters, *spaCBA* and *spaFED*, which are located distantly from each other as individual islands in the GG genome (Kankainen *et al.*, 2009). Reunanen and coworkers (2012) used Western blotting and immunogold transmission electron microscopy (TEM) in combination with antibodies against recombinant SpaCBA and SpaFED pilins to determine the presence of these two putative pili of LGG. Based on their findings, only SpaCBA pili were present. A possible explanation for the absence of SpaFED pili could be the lack of appropriate environmental stimuli to activate the associated gene cluster of SpaFED, so these pili might still be produced in the GIT. LGG cells contain multiple SpaCBA pili, on average 10-50 per cell with a length of up to 1 μm. These pili are heterotrimeric, composed by three subunits: SpaA, SpaB and SpaC, and all participate in the assembly of the pilus fibre (Figure 1.6). SpaCBA pili are assembled through the sortase system, as described above for Gram-positive bacteria. SpaA is again the major subunit that builds up the pilus backbone, while SpaB and SpaC are the minor accessory subunits. SpaC is the adhesin pilin subunit responsible for the binding to human mucus and Caco-2 intestinal epithelial cell line and is located not only at the pilus tip but also along the length of the pilus shaft (Lebeer *et al.*, 2012a). SpaB is thought to have dualistic characteristics: (i) acting as a molecular switch that terminates pilus polymerization and predestinates mature pili to be covalently attached to the peptidoglycan by the housekeeping sortase and (ii) having a role as an adhesin allowing its binding to human intestinal mucus; this ability, however, cannot be compared to that of the SpaC subunit (von Ossowski *et al.*, 2010; Reunanen *et al.*, 2012).
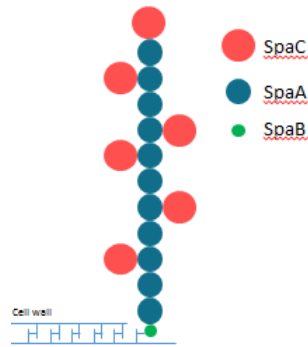
Figure 1.6: Schematic presentation of the SpaCBA pili. SpaCBA pili are composed of three different subunits. The SpaA-subunit builds up the backbone of the pilus fibre, SpaC is an important adhesin, located at the tip and along the length of the fibre and SpaB is located at the base (figure adapted from Reunanen *et al.*, 2012).

The immunomodulatory capacity of LGG-pili was also studied. Lebeer and coworkers (2012a) compared the cytokine expression profiles in a Caco-2 cell line stimulated with LGG wild type and two mutants; a pilus mutant strain CMPG5357 (*spaCBA* mutant) and an EPS-deficient mutant CMPG5351 (*welE* mutant) with an enhanced exposure of the pili. Results showed an upregulation of pro-inflammatory cytokines such as interleukine-8 (IL-8) and tumor necrosis factor α (TNF-α) and a downregulation of anti-inflammatory IL-10 when cells were treated with the *spaCBA* mutant without pili. In contrast, an inverse tendency was observed when cells were stimulated with the *welE* mutant, indicating that pili of LGG might play a key role in immunomodulatory interactions with IECs. Nevertheless, future studies are necessary to gain more insight in this pilus-mediated immunomodulatory activity.

LGG is up to now one of the few probiotics that are known to produce mucus-binding pili, providing the ability to adhere to human IECs. Pilus-like structures were also reported in some different *Bifidobacterium* species as *B. bifidum*, *B. longum* subsp. *longum* and *B. animalis* subsp. *lactis* but the function of these structures still needs to be elucidated (Foroni *et al.*, 2011). In *B. breve* UCC2003, the presence of tight adherence (Tad) pili was demonstrated to be required for colonization and persistence of the strain in the murine gut (O'Connell Motherway *et al.*, 2011). Furthermore, genes for sortase dependent pili were also discovered in *B. bifidum* PRL2010. Expression of the coding sequences in non piliated *L. lactis* increased the adherence of this strain to human enterocytes, indicating an important role for these pili in adhesion (Turroni *et al.*, 2013). Recently, a natural isolate of *L. lactis* (TIL448 strain) with high adhesion capacity to Caco-2 cells, compared to other *L. lactis* strains, was characterized. Proteomic analysis revealed the presence of pilins at the cell surface of *L. lactis* TIL448, that might be responsible for this increased adhesion capacity (Meyrand *et al.*, 2013). The production of adherent pili might contribute to the competitive advantage of LGG- and some other probiotic strains- over other species in the mucosal environment and also to exert their health-benefit effects.

1.3 Atopic diseases

1.3.1 What is atopy?

As described previously, the role of the gut microbiota in maintaining human health is of great importance and dysbiosis has been associated with different diseases, including autoimmunity and related disorders such as atopies (Robles Alonso & Guarner, 2013).

Atopy is defined as the genetic predisposition toward the development of an immediate or type I hypersensitivity reaction (Murphy, 2011; Kramer *et al.*, 2013). Type I hypersensitivity reactions are allergic reactions due to the production of high levels of IgE antibodies against innocuous antigens. After the first contact with the antigen, called sensitization, naive T-helper cells differentiate into Th2 cells that suppress Th1 cells and produce IL-4 and IL-13. These interleukins in turn activate B cells to develop into plasma cells, which start to produce IgE antibodies that bind to high affinity FcεRI receptors on mast cells and eosinophils. When IgE antibodies encounter the same antigen again, mast cells and eosinophils degranulate and release chemical mediators, such as histamine and prostaglandins, which cause the allergic symptoms in different organs (Cookson, 2004). Although the terms atopy and allergy are often used interchangeably, they do not always mean the same. Atopy refers to type I or IgE mediated allergic reactions while allergies in general are not always IgE mediated, since type II, type III and type IV hypersensitivity reactions also exist (Murphy, 2011). Most common atopic disorders are atopic dermatitis (AD), allergic rhinitis or hay fever and allergic asthma. Figure 1.7 shows the mechanism of a type I hypersensitivity reaction.

Both genetic and environmental factors seem to be responsible for the development of IgE mediated allergic reactions (Blumenthal, 2005). For example, Dold and coworkers (1992) noticed that the incidence of AD significantly increases with the number of allergic family members. The same observation was found for allergic rhinitis (Dold *et al.*, 1992). Despite the strong genetic basis, environmental factors as well play a very important role reflected in the strong increase in atopic diseases (Kramer *et al.*, 2013).
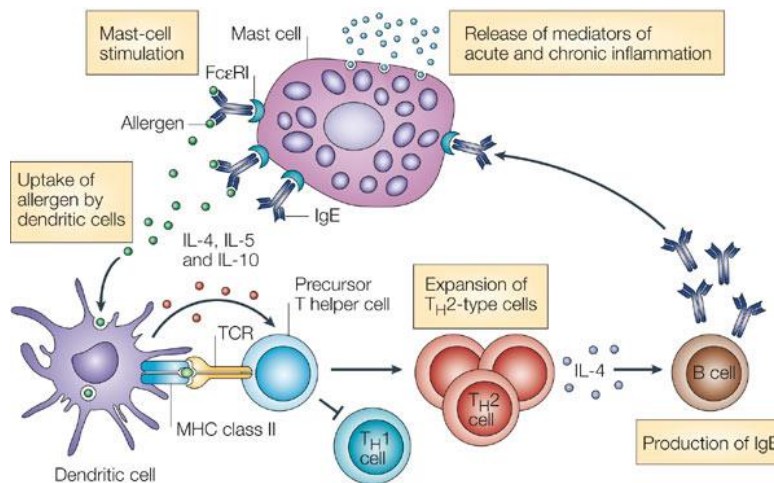
Figure 1.7: Type I hypersensitivity reaction. A type I hypersensitivity reaction starts when an antigen, located on the cell surface of a DC, is presented to naïve T helper cells, which then differentiate into Th2 cells. Next, B cells are activated and differentiate into IgE producing plasma cells. IgE antibodies bind to high affinity FcεRI receptors on the mast cells and upon contact with the same antigen, mast cells release mediators that will induce the allergic symptoms. For more details see text (Cookson, 2004).

1.3.2   Increase of atopic diseases

1.3.2.1   Some statistics

The prevalence of atopic diseases has been dramatically increased in the last decades, especially in more industrialized countries. About 40-50% of children are sensitized against one or more common allergens as pollen, grasses, dust mites and animal dander (WAO, 2013). Due to these large proportions, allergic diseases have become an epidemic phenomenon. The prevalence of AD, for instance, has tripled in industrialized countries during the last 30 years and affects about 15-30% of children and 2-10% of adults (reviewed in Okada *et al.*, 2010). In Germany, one third of the population suffers from asthma, allergic rhinitis or AD or is sensitized towards allergens (Kramer *et al.*, 2013). These numbers indicate that allergic diseases have become a major public health problem and therefore, it is important to investigate the underlying mechanisms responsible for this increased prevalence. Up to now, the mechanisms that drive the development of allergic diseases in early life are not yet fully understood.

1.3.2.2   Hygiene hypothesis

Considering that the dramatic increase in the development of atopic disorders is only observed in industrialized countries, this variation was attributed to environmental factors rather than genetics. A higher life standard -reflected in improved hygiene, reduced household size, use of antibiotics, vaccinations and dietary changes- is typically found in more westernized countries. These observations led to the onset of the hygiene hypothesis.

The hygiene hypothesis was first introduced by Strachan and states that: "*The apparent rise in the prevalence of allergic diseases could be explained if allergic diseases were prevented by infection in early childhood, transmitted by unhygienic contact with older siblings, or acquired prenatally. Over the past century declining family size, improved household amenities and higher standards of personal cleanliness have reduced opportunities for cross-infection in young families. This may have resulted in more widespread clinical expression to atopic disease*"(Strachan, 1989). Strachan collected epidemiological data for allergic rhinitis and eczema of children in Great Britain from their birth until the age of 23. The outcome of this study showed a decrease in the prevalence of allergic rhinitis and eczema as the numbers of siblings in the patient's family increased. He concluded that the presence of older sisters or brothers had a protective effect against allergic sensitization. These findings, as well as the beneficiary effect of children going to day care, were later confirmed by other studies (Celedón *et al.*, 2003; Gibbs *et al.*, 2003).

People suffering from allergies have a typical imbalance of the T cell populations, especially between Th1 and Th2 cells, leading to an inappropriate response to harmless environmental antigens (Furrie, 2005). At birth, there is a natural Th2-skewed immune system, but during childhood this imbalance is restored under normal circumstances by appropriate immune stimulation until homeostasis is reached (Furrie, 2005). However, when there is an improper priming of the immune system at young age, the imbalance is not completely recovered, which is typically associated with the development of allergies, since Th2 cells increase the production of allergen-specific IgE (Furrie, 2005; Okada *et al.*, 2010). Until recently, the imbalance between Th1 and Th2 cells was thought to be the only cause for the development of atopies. Nowadays however, more evidence suggests that Treg cells are also involved as they would provide protection against the development of allergies by controlling Th1 and Th2 subpopulations (Wills-Karp, 2008; Palomares *et al.*, 2010).

The imbalance between Th1 and Th2 cells constitutes the basis of the hygiene hypothesis. The link between this imbalance and the influence of family size can be established if the hygiene hypothesis is interpreted immunologically (Prokopakis *et al.*, 2013). Children with multiple siblings have an increased possibility of infections, because they can become more easily infected if one of the other children already suffers from an infection. This leads their immune system towards the differentiation to Th1 cells, as many bacteria and viruses elicit a Th1-mediated immune response. On the other hand, children growing up in smaller families have reduced opportunities for cross-infections, consequently resulting in the development of Th2 differentiated cells (Holt, 1995).

Even though the hygiene hypothesis indicates a direct link between a reduced microbial exposure and a rising prevalence of allergic diseases, it cannot explain the simultaneously increased incidence of other immunological disorders as multiple sclerosis, type I diabetes and Chrohn's disease, which are all mainly driven by Th1 cells. Therefore, the broader old friend hypothesis is preferred to describe the critical role of exposure to microorganisms that mediate protection from allergies, but also from other autoimmunity disorders and IBDs (Guarner *et al.*, 2006). This hypothesis comprises the effects of prenatal, neonatal and adult exposures to these organisms as well as the effects of the normal microbiota that colonizes the human body. More precisely, the old friend hypothesis states that the increase in both Th2 and Th1 driven immunodysregulatory disorders is caused by the diminished contact with harmless organisms that have been part of human microecology for millennia, such as helminthes, bifidobacteria, lactobacilli and saprophytic mycobacteria (Guarner *et al.*, 2006; Rook, 2009). The hypothesis also gives an explanation why these organisms evolved their immunomodulatory properties. Before the westernization, environmental saprophytes –including mycobacteria and lactobacilli- needed to be tolerated by the immune system, since they were present everywhere and in large quantities. Helminthic parasites, on the other hand, were not always harmless but present in large numbers. The uptake and elimination of these organisms by the immune system was likely to cause severe tissue damage; consequently, the immune system adapted in order to tolerate them (Rook, 2009). Our "old friends" exert their immunomodulatory properties as shown in Figure 1.8. Once they are recognized by the innate immune system, DCs are matured into regulatory DCs which in turn drive polarization of T cells to Treg cells rather than Th1 or Th2 cells. Then, two mechanisms take place to control inappropriate inflammation. First, the constitutive presence of the "old friends" causes a continuous activation of the DCs and Treg cells, thus providing a constant background suppression of other inflammatory responses. Second, due to these increased numbers of regulatory DCs, more self-antigens, gut content antigens and allergens are processed. Therefore, the number of Treg cells, that are specifically triggered by these antigens, are also increased, resulting in downregulation of allergies, autoimmunity and IBDs (Rook, 2009).
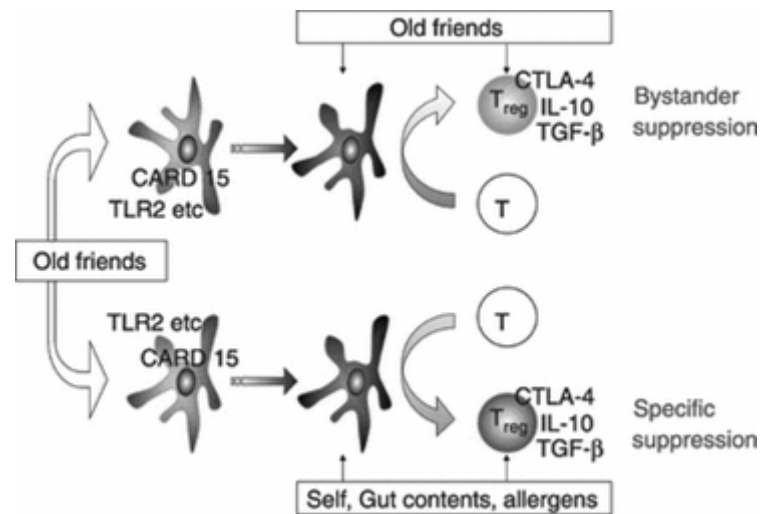
Figure 1.8: The old friend hypothesis. According to the old friend hypothesis, the lack of contact with organisms that evolved along with humans, such as helminthes, bifidobacteria and lactobacilli, leads to a diminished immunoregulation. These "old friends" influence the maturation of DCs in such a way that Treg cells are produced instead of Th1 and Th2 cells. Continuous contact between the DCs with our "old friends" provides a bystander suppression against inflammatory responses, while the processing of self and gut content antigens and allergens drives specific suppression (Rook, 2009).

Hence, the phenomenon of an increased incidence of allergies is complex and influenced by different factors. Besides the family size and a reduced exposure to our "old friends", other factors, typically associated with more industrialized countries, also have their impact; for instance, the abundant use of antibiotics. Farooqi and Hopkin (1995) reported that the use of any kind of drug in children before the age of two is related to a 50% increased possibility to develop allergic rhinitis and eczema (reviewed in Prokopakis *et al.*, 2013). The diversity of the intestinal flora seems to influence the priming of the immune response as well, which will be discussed in following section. Figure 1.9 gives an overview of how all these factors influence the differentiation of T cells and consequently the development of allergies (Wills-Karp, 2008).
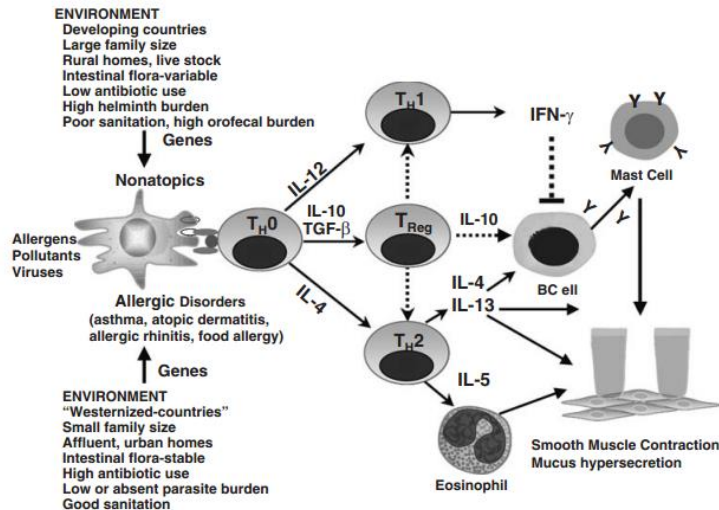
Figure 1.9: Determinants influencing the differentiation of T cells towards Th1 or Th2 subsets. The development of allergies is influenced by both environmental factors and the genetic background of an individual. However, the increased incidence of allergies in the last decades is particularly caused by environmental factors. In more industrialized countries, typically associated with higher life standards and smaller family sizes, microbial exposure is minimized, leading to a less primed immune system. Under normal circumstances, Th1 and Th2 subpopulations are in equilibrium, whereas in allergic individuals, there is an excess of Th2 polarization, leading to the suppression of Th1 cells, stimulation of B cells to produce IgE antibodies and the development of allergies. Moreover, the Treg subpopulation that controls Th1 and Th2 subsets is also affected, resulting in a skewed Th2 response (Wills-Karp, 2008).

1.4 LGG in clinical trials for prevention and/or treatment of atopic diseases

As inappropriate stimulation of the immune system by the microbiota is the major cause for the increased incidence of allergies, this has become an important issue for scientific research. Therefore, several clinical studies investigated the use of probiotic bacteria in the prevention and treatment of atopic diseases. Most of the studies have focused on eczema, as this is frequently the first indication of an allergic disease. Probiotic administration is typically given prenatally or in the first months of life, as this is the most critical time point for priming of the immune system (Prokopakis *et al.*, 2013). Several clinical trials have already been conducted, using different strains, mostly of the genus *Lactobacillus* and *Bifidobacterium*.

1.4.1 Allergy treatment studies

Allergy treatment studies are performed in infants that already show symptoms of AD. Initial studies demonstrated a therapeutic effect by probiotic administration, although it should be noted that these studies involved relatively small sample sizes (reviewed in Toh *et al.*, 2012). Isolauri *et al.* (2000) investigated the effect of LGG supplementation in infants who manifested AD in a randomized double-blind placebo-controlled study. After two months, they observed a decrease in the Scoring Atopic Dermatitis (SCORAD) score, which reflects the extent and severity of AD, in infants given LGG

supplemented formulas compared to the control group. Hattori and coworkers also found an improvement in SCORAD after administration of *B. breve* M-16V in children with AD relative to the control group (Hattori *et al.*, 2003). However, it should be noted that for both studies, the improvement in SCORAD was only manifested at early time points, since after six months, there were no significant differences anymore in treated and placebo group (Tang *et al.*, 2010). Even though these results were promising, other larger scale studies failed to confirm them (Tang *et al.*, 2010). Grüber and coworkers (2007) investigated the therapeutic effect of LGG as a food supplement in infants that suffered from mild-to-moderate AD. Although the severity of AD showed a decreased tendency over time, no therapeutic effect of LGG could be observed, as the decrease was manifested both in the tested as the control group. This general improvement is likely to be the result of the natural course of the disease, since AD is common in early infancy, but disappears around the age of three in more than 40% of the patients (Illi *et al.*, 2004). Fölster-Holst *et al*. (2006) also failed to observe advantageous effects of LGG in infants with moderate-to-severe AD during an intervention of eight weeks. Their results showed no significant differences between the test and placebo group. Besides these two studies with LGG, different other clinical trials with other probiotic strains failed to observe any beneficial effect on the severity of AD-symptoms after probiotic administration (reviewed in Toh *et al*., 2012). For instance, the administration of *B. breve* M-16V with a prebiotic to 90 infants with AD in the first six months of life had no beneficial effect on the severity of AD (van der Aa *et al.*, 2010). Administration of *L. fermentum* VR1-003PCC in children with eczema showed improvements in eczema severity and extent, however, SCORAD and parental perception of the eczema were not significantly different between the test and control group (Weston *et al.*, 2005).

In addition to the studies on AD, a limited amount of other studies investigating the impact of probiotics on asthma were performed. In a double-blind study carried out by Rose *et al.* (2010), the impact of LGG on allergic sensitization was investigated in infants with recurrent wheezing and also with a family history of atopy. The outcome showed no effect on eczema or wheezing, since the symptoms of the patients were not alleviated. The authors even noticed a mild negative influence of LGG supplementation, as the wheezing episodes slightly increased. Helin *et al.* (2002) studied the effects of LGG in adults and teenagers (age 14-36 year), suffering from birch pollen and apple food allergy with intermittent symptoms of atopic allergy and/or asthma. Their results showed as well no beneficial impact of LGG on allergy and asthma symptoms. Moreover, one year administration of *L. casei* DN-114 001 in children (2-5 years) suffering from asthma likewise had no improving effects (Giovannini *et al.*, 2007).

So despite the promising results in the beginning, recent data cannot find strong evidence for a therapeutic influence of LGG or other probiotics in the treatment of AD. This might be caused by the heterogeneity between studies (i.e. the study populations, the duration of treatment and the dose used). Therefore, a health beneficial effect of LGG on AD cannot be completely excluded. The use of probiotics for the treatment of asthma is not proven, since the amount of studies is quite limited and results are conflicting (reviewed in Toh *et al.*, 2012).

1.4.2 Allergy prevention studies

Besides treatment studies, several prevention studies were performed as well, in which the probiotic is administrated before the symptoms occur. These studies are typically performed with patients with a family history of atopic disease, hence the infants are at high risk of developing AD, allergic rhinitis or asthma.

One of the first clinical studies that proved the preventive effects of LGG supplementation on atopic disease was performed by Kalliomäki *et al.* (2001). LGG was given prenatally to mothers (2-4 weeks before delivery) and postnatally for six months. The infants were examined at different time points until the age of two. The outcome of the study showed that the frequency of AD was reduced by half in LGG treated infants compared to the placebo group. To investigate if this preventive effect of LGG was extended after the first two years of life, Kalliomäki and coworkers (2003) examined the same population again but now until the age of four. Their results indicate that the positive effect of LGG on AD is not only observable in infancy (two years), but prolongs during childhood. To evaluate whether the effect of the probiotic intervention further proceeded until the age of seven, they re-examined the study cohort and noted that the risk for the development of eczema was significantly lower in the LGG group than placebo group (Kalliomäki *et al.*, 2007). A possible preventive effect of LGG on inhalant allergen sensitization and respiratory allergy, however, cannot be concluded from these studies, since these diseases typically manifest at older age. In another study, LGG was given in combination with *L. acidophilus* La-5 and *B. animalis* subsp*. lactis* Bb-12 to pregnant women three weeks before delivery and three months postnatally. The treatment reduced the incidence of AD among their infants at the age of two, but showed no effect on asthma (Dotterud *et al.*, 2010). Tang *et al.* (2010) performed a meta-analysis of 13 randomized controlled trials, testing different probiotic strains, and revealed an overall protective effect for the treatment for eczema, but not for atopic or IgE mediated eczema.

Most of the studies that have proven an advantageous effect of probiotic administration combined prenatal and postnatal treatment (Tang *et al.*, 2010). Boyle and coworkers (2011) failed to observe a preventive effect of LGG in AD when the probiotic was given only prenatally. They assumed that prenatal administration alone is not sufficient to induce beneficial effects. In addition, when LGG in

combination with *B. lactis* Bb-12 were administrated only postnatally, also no health-promoting effects were found (Rautava *et al.* 2006). Daily administration of *L. acidophilus* (LAVRI-A1) to newborns, whose mothers have allergies, during the first six months of life also showed no reduction in the risk of developing AD (Taylor *et al.*, 2007). Therefore, a combination of pre- and postnatal supplementation might be required, as initially showed by Kalliomäki *et al*. (2001,2003,2007).

Nonetheless, it should be mentioned that Kopp and coworkers (2008) investigated supplementation of LGG during pregnancy and early infancy in a German population and no reduction in the incidence of AD was found, although they used a similar protocol and dose to the study of Kalliomäki (reviewed in Toh *et al.*, 2012). This is probably due to differences in some confounding variables, as mentioned by the authors. First, the number of atopic family members per individual was lower in the study of Kalliomäki than in the study of Kopp, leading to a higher risk of developing AD in the latter population. Second, Kopp and coworkers used more infants with older siblings. Finally, the genetic background of the participants in both studies differs considerably, i.e. German and Finnish population (Kopp *et al.* 2008). Also other studies failed to prove the beneficial effects of probiotic administration to infants at risk for atopic disorders (Soh *et al.*, 2009; Ou *et al.*, 2012). For example, Soh and coworkers (2009) investigated whether daily administration of *B. longum* and *L. rhamnosus* during the first six months of life reduced eczema symptoms and allergic sensitization. They failed to observe any significant effects. A recent study by Ou and coworkers (2012) examined the impact of LGG supplementation in pregnant women with atopic disease. They started the probiotic intervention at the beginning of the second trimester of the pregnancy and six months postnatally. Their outcome showed no preventive effect on the development or severity of AD in the infants at risk. However, a significant improvement was found in the mothers that received the probiotic, compared to the placebo group. Finally, Wickens *et al.* (2008) evaluated the effects of two different probiotic strains, *L. rhamnosus* HN001 and *B. animalis* subsp. Lactis, for the prevention of eczema and sensitization. They found a protective effect for *L. rhamnosus* HN001 but not for *B. animalis* subsp. Lactis, indicating that strain specificity is important for the outcomes of clinical trials.

Taken into account all these findings, it can be concluded that different factors influence the outcome of a study. More double-blind placebo-controlled studies are needed in order to gain more insight into the preventive effects of LGG.

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Bacterial strains

The bacterial strains that were used in the experiments of this master thesis are shown in Table 2.1.

Table 2.1: Overview of the used bacterial strains.

Strain/ species	Relevant properties	Reference
L. rhamnosus GG (LGG) (ATCC53103)	Wild type strain, isolated from human faeces	Doron *et al.*, 2005
L. rhamnosus CMPG5357	*spaCBA* mutant, knockout of the SpaCBA pilus of LGG. Double homologous recombination of *spaCBA* eliminates the binding capacity of LGG almost completely. Resistant to tetracyclin (Tc^r)	Lebeer *et al.*, 2012a
L. rhamnosus CMPG5351	*welE* knockout mutant of LGG. Mutant hit in the priming glycosyl transferase necessary for the production of galactose-rich EPS; Tc^r	Lebeer *et al.*, 2009
L. rhamnosus CMPG5365	Double knockout mutant of the *spaCBA* operon and the *welE* gene. Resistant to erythromycin (Ery^r) and Tc^r	Lebeer *et al.*, 2012a
Salmonella enterica serovar Typhimurium ATCC14028	Wild type strain, isolated from animal tissue	Miller *et al.*, 1989
FAJ1906/pMEC45	*L. rhamnosus* GG with chromosomal insertion of pFAJ1934 in the *attB* sequence of phage A2; Ery^r	De Keersmaecker *et al.*, 2006a
CMPG1907/pMEC45	*welE* mutant with chromosomal insertion of pMEC10 in the attB sequence of phage mv4; Ery^r	Vargas *et al*., submitted for publication
CMPG1908/pMEC45	*spaCBA* mutant with chromosomal insertion of pMEC10 in the attB sequence of phage mv4; Ery^r	Vargas *et al*., submitted for publication

Table 2.2: Properties of the plasmids used to generate green fluorescent protein (GFP)-labeled LGG and its mutant strains.

Plasmid	Properties	Reference
pMEC10	Integration plasmid (*attB* located at the 3' end of the tRNA^{Ser}locus) containing *int-attP* cassette (of phage mv4) of pMC1 cloned into pNZ9500 (pUC19 derivative carrying a 2.7-kb chromosomal DNA fragment of *L. lactis* NZ9700 containing the 3' end of *nisP* and *nisRK*); expression driven by *ery* read-through; Ery^r	Pavan *et al.*, 2000
pMEC45	*L. lactis* pSH71 replicon; pNZ8037 derivative with *gfp*$_{uv}$ cloned downstream of the *nisA* promoter from *L. lactis* NZ9800; Resistant to chloramphenicol	Geoffroy *et al.*, 2000
pFAJ1934	2,634-bp PCR fragment (flanking EcoRI sites) carrying the *nisRK* genes of pMEC10 cloned into the EcoRI site of pEM40 downstream of the *ery* gene in the opposite orientation; Resistant to ampicillin and Ery^r	De Keersmaecker *et al.*, 2006a

2.1.2 Bacterial culture media

Table 2.3 lists the different media and their composition for the growth of the bacterial strains shown in Table 2.1. All MRS-media were manually sterilised for 20 minutes at 15 psi in a pot to avoid Maillard-reactions. LB-medium was autoclaved. Solid MRS- and LB- medium were obtained by adding 15 g/L agar (Invitrogen). Test tubes were prepared with 10 ml liquid MRS-medium for inoculation of the *Lactobacillus* species or with 5 ml liquid LB-medium for inoculation of *Salmonella* species. Solid medium in petridishes (20 ml) were used to plate out.

Table 2.3: Bacterial culture media.

Medium	Composition	Species
Difco™ Lactobacilli broth MRS (de Man-Rogosa-Sharpe)	55.0 g MRS in 1 L distilled water Approximate formula for 1 L: 10.0 g proteose pepton No.3, 10.0 g beef extract, 5.0 g yeast extract, 20.0 g dextrose, 1.0 g polysorbate 80, 2.0 g ammonium citrate, 5.0 g sodium acetate, 0,1 g magnesium sulphate, 0.05 g manganese sulphate en 2.0 g dipotassium phosphate	*Lactobacillus* species
LB (Luria – Bertani)	In 1 L distilled water: 10 g Tryptone, 10 g sodium chloride and 5 g yeast extract	*Salmonella* species

2.1.3 Host cell lines

The human monocytic THP1 and the murine macrophage-like Raw 264.7 cell lines were chosen as host cells for the experiments. Culture media in which the cells were grown are described in Table 2.4.

Table 2.4: Overview of the culture media used for the cell lines.

Cell line	Culture medium	Growth mode	Split ratio	Reference
THP1 monocytes	Roswell Park Memorial Institute (RPMI)1640 (Gibco®) In 500 ml: 2 mM L-glutamine (Invitrogen), 10 % heat inactivated fetal calf serum FCS (30 min at 56°C) (Perbio Science BVBA), 1 mM sodium pyruvate (Gibco®), 100 mM β-mercaptoethanol (Sigma), 1X non-essential amino acids (Gibco®), 100U/ml penicillin and 100 µg/ml streptomycin (Sigma)	in suspension	1:12 to 2:12 every 3 days	ATCC (TIB-202™)
Raw 264.7 macrophages	Dulbecco's Modified Eagle's Medium (DMEM) (Gibco®) In 500 ml: 2 mM L-glutamine (Invitrogen), 10 % heat inactivated fetal calf serum (FCS) (30 min at 56°C) (Perbio Science BVBA), 1 mM sodium pyruvate (Gibco®), 100 mM β-mercaptoethanol (Sigma), 1X non-essential amino acids (Gibco®) , 50 µg/ml gentamycin (Sigma)	adherent	1:2 to 1:8 every 3 days	ATCC (TIB-71™)

2.1.4 Adhesion and phagocytosis assays

Table 2.5: Reagents used for the adhesion assay.

Suspension	Composition
Phosphate Buffered Saline (PBS) 10X	For 1 L: 80 g NaCl, 2 g KCl, 17.8 g Na_2HPO_4-$2H_2O$ and 2.4 g KH_2PO_4 resuspended in 800 ml distilled water (pH 7.4)
PBS 1X	For 1 L: 100 ml of the PBS 10X suspension resuspended in 900 ml distilled water

Table 2.6: Reagents used for the phagocytosis assay.

Suspension	Composition
PBS 10X	For 1 L: 80 g NaCl, 2 g KCl, 17.8 g Na_2HPO_4-$2H_2O$ and 2.4 g KH_2PO_4 resuspended in 800 ml distilled water (pH 7.4)
PBS 1X	For 1 L: 100 ml of the PBS 10X suspension resuspended in 900 ml distilled water
Triton X-100 (0.2%)	For 20 ml: 40 µl Triton (0.0004%) in 20 ml milli-Q water
Streptomycin/Penicillin	For 20 ml: 200 µl of the stock (10 mg/ml streptomycin and 10000 U/ml penicillin) in 20 ml RPMI1640 without FCS nor antibiotics

Table 2.7: Antibiotics used for the phagocytosis assay.

Antibiotic	Stock	Final concentration
Streptomycin	10 mg/ml	100 µg/ml
Penicillin	10000 U/ml	100 U/ml

2.1.5 Fluorescence activated cell sorting (FACS)

Table 2.8: Reagents used for FACS experiment.

Suspension	Composition
FACS-Wash	For 50 ml: 1.5 ml FCS serum was added to 48.5 ml sterile Dulbecco's Phosphate Buffered Saline (dPBS)
3% PFA	3g paraformaldehyde (PFA) was added to 90 ml milli-Q water together with 1 drop NaOH. Next, ten ml 10X PBS was added to the solution (pH 7.4)

2.1.6 Adhesion competition assays

Table 2.9: Sugars used for the adhesion competition assay.

Sugar	Stock concentration
Fucose	100 µg/ml
Mannose	100 µg/ml

2.1.7 Immunomodulation experiments in THP1 cells

2.1.7.1 RNA isolation

For the isolation of RNA, the commercial High Pure RNA Isolation Kit (Roche) was used. Materials are
shown in Table 2.10.

Table 2.10: Components of the High Pure RNA Isolation Kit (Roche).

Contents	Use
Lysis/Binding buffer	To lyse the cells
DNase I, recombinant, lyophilizate	To avoid DNA contamination
DNase incubation buffer	To avoid DNA contamination
Wash buffer I	To wash the cells and remove contaminants
Wash buffer II	To wash the cells and remove contaminants
Elution buffer	To elute the RNA

2.1.7.2 cDNA synthesis

Table 2.11: Components in the reaction mixture for the synthesis of cDNA.

Component	Volume/ sample	Final concentration
total RNA (1 µg/ml)	n µl (n = maximal 11)	
Oligo(dT)$_{20}$ (50 µM)	1 µl	0.5 µM
Deoxynucleotide triphosphate(dNTP)-mix (10 mM)	1 µl	0.1 mM
Distilled RNase-free water	Up to 13 µl	

Table 2.12: Buffers and transcriptase used for the synthesis of cDNA.

Component	Volume/sample	Final concentration
First Strand Buffer (5X)	4 µl	0.2X
Dithiothreitol (DTT) (0.1 M)	1 µl	1mM
RNaseOUT™ Recombinant RNase Inhibitor (40 U/µl)	1µl	0.4 U/µl
Superscript™ III RT (200 U/µl)	1 µl	2 U/µl

2.1.7.3 Real-time quantitative polymerase chain reaction (RT-qPCR)

For the qPCR reaction, mastermixes with a final volume of 20 µl per sample were prepared (Table
2.13). Afterwards, 5 µl of the target DNA was added.

Table 2.13: Materials necessary for the preparation of the mastermix for the qPCR reaction.

Component	volume/ well	Final concentration
Taqman universal PCR master mix (Applied Biosystems) (2X)	12.5 µl	1.25X
Forward primer (30µM)	0.25 µl	0.375 µM
Reverse primer (30 µM)	0.25 µl	0.375 µM
Probe (5 µM)	1 µl	0.25 µM
RT PCR grade water (Ambion)	6 µl	Up to 20 µl

Table 2.14: Sequences for the primers and probes of the tested cytokines.

Marker	Forward primer	Reverse primer	Probe
hPPIA	CGCGTCTCCTTTGAGCTGTT	CTGACACATAAACCCTGGAATAATTC	CAGACAAGGTCCCAAAGACAGCAGAAAATTT
hTNF-α	TCTTCTCGAACCCCGAGTGA	CCTCTGATGGCACCACCAG	TAGCCCATGTTGTAGCAAACCCTCAAGCT
hIL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC	TGTTACTCTTGTTACATGTCTCCTTTCTCAGGGCT
hIL-1β	CTGATGGCCCTAAACAGATGAAG	GGTCGGAGATTCGTAGCAGCTGGAT	TTCCAGGACCTGGACCTCTGCCCTC
hIL-10	GTGATGCCCCAAGCTGAGA	CACGGCCTTGCTCTTGTTTT	CCAAGACCCAGACATCAAGGCGCA

2.1.8 Immunomodulation experiments in peripheral blood mononuclear cells (PBMCs)

2.1.8.1 RNA isolation

RNA was isolated using the commercial Mini RNeasy Kit (QIAGEN), contents are listed in Table 2.15.

Table 2.15: Components of the Mini RNeasy Kit (QIAGEN).

Contents	Use
Buffer RLT + β-mercapthomethanol	To lyse the cells
70% ethanol	Added to the lysate to provide ideal binding conditions
Buffer RW1	Washing of the cells to remove contaminants
Buffer RPE	Washing of the cells to remove contaminants
RNase-free water	To elute the RNA

2.1.8.2 cDNA synthesis

Table 2.16: Components in the reaction mixture for the synthesis of cDNA.

Component	Volume/sample	Final concentration
total RNA (1-5 µg)	n µl (n = maximal 18)	
dNTP-mix (10 mM)	1 µl	0.1 mM
Oligo(dT)$_{12-18}$ (50 µM)	1 µl	0.5 µM
RNase-free water	Up to 20 µl	

Table 2.17: Buffers and transcriptase used for the synthesis of cDNA.

Component	Volume/sample	Final concentration
First Strand buffer (5X)	6 µl	0.3X
DTT (0.1 M)	3 µl	3 mM
Superscript II (50 U/µl)	1 µl	0.5 U/µl

2.1.8.3 Real-time quantitative polymerase chain reaction

For the qPCR reaction, mastermixes with a final volume of 20 µl per sample were prepared (Table 2.18). Afterwards, 5 µl of the target DNA was added.

Table 2.18: Materials necessary for the preparation of the mastermix for the qPCR reaction.

Component	Volume/ well	Final concentration
TaqMan universal PCR master mix (Applied Biosystems) (2X)	12.5 µl	1.25X
Forward primer (30 µM)	0.25 µl	0.375 µM
Reverse primer (30 µM)	0.25 µl	0.375 µM
Probe (5 µM)	1 µl for cytokines	0.25 µM
	0.5 µl for housekeeping gene	0.125 µM
RT PCR grade water (Ambion)	6 µl for cytokines	Up to 20 µl
	6.5 µl for housekeeping gene	

Table 2. 19: Sequences for the primers and probes of the tested cytokines.

Marker	Forward primer	Reverse primer	Probe
hGNB2L1	CACTGTCCAGGATGAGAGCCA	CATACCTTGACCAGCTTGTCCC	TCCGCTTCTCGCCCAACAGCAG
hTNF-α	TCTTCTCGAACCCCGAGTGA	CCTCTGATGGCACCACCAG	TAGCCCATGTTGTAGCAAACCCTCAAGCT
hIL-6	CCAGGAGCCCAGCTATGAAC	AAGGCAGCAGGCAACACC	CCTTCTCCACAAGCGCCTTCGGT
hIL-1β	TTGCTCAAGTGTCTGAAGCAGC	CAAGTCATCCTCATTGCCACTG	TACCTGAGCTCGCCAGTGAAATGATGG
hIL-10	GTGATGCCCCAAGCTGAGA	CACGGCCTTGCTCTTGTTTT	CCAAGACCCAGACATCAAGGCGCA

2.2 Methods

2.2.1 Bacterial growth conditions

Lactobacillus species were grown under micro-aerobic conditions. Strains were weekly streaked out from the glycerol stock on solid MRS-medium in petridishes and stored at 37°C for 48h. Cultures were incubated under non-shaking conditions at 37°C in liquid MRS-medium .

For *Salmonella* ATCC 14028, a portion from the top of the frozen glycerol stock was scraped off, streaked onto solid LB-petridishes and incubated overnight at 37°C. Cultures were inoculated in liquid LB-medium and kept at 37°C overnight in shaking conditions.

2.2.2 Preparation of the bacterial strains

Two days before the experiments, LGG wild type and mutant strains were inoculated in liquid MRS-medium. After 48 hours, one hundred microliters of bacteria from this preculture were inoculated into fresh MRS-medium at 37°C overnight. The day of the experiment, optical density (OD) of the bacterial cultures was measured at 595 nm using Genesys UV-VIS spectrometer (Thermo Electron Corporation). MRS-medium was used as a blank, bacteria were diluted 1/10 in medium. Starting from the OD and the wanted final concentration of the bacteria, the corresponding volume from the

culture was calculated and transferred to eppendorf tubes or 15 ml falcon tubes. Bacteria were centrifuged at 2000 x *g* for 15 minutes at 4°C (Eppendorf centrifuge 5415R). The supernatant was discarded and the pellet was washed once in one ml dPBS (Gibco® by Life Technologies), followed by centrifugation at the same conditions. Finally, the pellet was resuspended in cell culture medium without FCS nor antibiotics.

GFP-labeled LGG mutants were obtained by implementation of the nisin-controlled gene expression (NICE) system through an electroporation protocol, as previously described by De Keersmaecker *et al.* (2006a) for the wild type, with minor modifications. First, the *nisRK* genes were integrated in the chromosome of the *spaCBA* mutant CMPG5357 and *welE* mutant CMPG5351. Site-specific integration was accomplished by electroporation of the pMEC10 plasmid and confirmed by PCR (Alvarez *et al.*, 1998). Next, the pMEC45 plasmid, containing *gfp* under the control of the *nisA* promoter, was electroporated in the CMPG5357/pMEC10 and CMPG5351/pMEC10 mutant. Transformants were selected via PCR analysis. GFP-expression was induced as previously described (De Keersmaecker *et al.*, 2006a). Overnight cultures (FAJ1906/pMEC45, CMPG1907/pMEC45 or CMPG1908/pMEC45) were inoculated to fresh MRS-medium (diluted 1:50) and after 30 minutes incubation at 37°C in non-shaking conditions, 500 ng/ml nisin (Sigma) was added to the cultures, followed by additional incubation at 37°C for 2.5 – 3 hours. After the nisin induction, OD was measured. The necessary volume of the culture was calculated, transferred to eppendorf tubes or 15 ml falcon tubes, and centrifuged at 2000 x *g* for 15 minutes at 4°C. The pellet was washed one time with dPBS, centrifuged under the same conditions and resuspended in cell culture medium without FCS nor antibiotics.

To measure the OD of *Salmonella* cultures, LB-medium was used as a blank and bacterial strains were diluted 1/10 in medium. Again the necessary volume from the culture was calculated based on the OD and the desired final concentration of the bacteria. Bacteria were centrifuged at 6000 rpm for 5 minutes at room temperature (Eppendorf centrifuge 5418). The pellet was washed once with dPBS, followed by another centrifugation step under the same conditions. The final pellet was then resuspended in cell culture medium without FCS nor antibiotics.

2.2.3 Treatment of the cell cultures

THP1 and Raw 264.7 cells were originally obtained from American Type Culture Collection (ATCC) and stored in the Laboratory of Pediatric Immunology (Prof. D. Bullens, K.U.Leuven). In the cell culture room of the Centre of Microbial and Plant Genetics (CMPG), cells were routinely maintained at 37°C with 5% CO_2 and 90% relative humidity in 25cm^2 tissue culture flasks. THP1 cells were grown in complete RPMI1640 medium and Raw cells in complete DMEM medium as described in Table 2.4.

2.2.3.1   THP1 cells

Cells were split every three days at a ratio of 1:12 to 2:12 (cells:medium) by addition of fresh complete RPMI1640 medium. The medium was prewarmed to 37°C to avoid thermal shock of the cells. Cultures were maintained in small flasks (25 cm²) by adding 10 ml fresh medium with 1.5 ml of cells, which were first gently mixed in order to homogenize the cell suspension. Two or three days before an experiment, cells were split in big flasks (75cm²) with a ratio of 1:6 (cells:medium).

The day before the experiment, THP1 cells, were seeded in 12-well culture plates with a final concentration of 1 x 10^6 cells per ml. Cells were first counted using a Neubauer chamber. Herefore, 100 µl of cells were transferred to an eppendorf tube together with 10 µl tryptan blue. Tryptan blue is used to distinguish between live and dead cells. Ten µl of the cell suspension was gently loaded on the loading groove of the coverslip, thereby allowing the sample to be drawn out of the pipette by capillary action. The system is designed in such a way that the number of cells in one set of 16 corner squares is equivalent to the number of cells x 10^4. The necessary volume to obtain a final concentration of 10^6 cells per ml was calculated, transferred into a 50 ml falcon tube and centrifuged at 1800 rpm for 5 minutes at room temperature (Thermo Scientific, SL 16R Centrifuge). The supernatant was discarded and the pellet resuspended in complete RPMI1640 medium. Monocytic differentiation of THP1 cells was stimulated by the addition of phorbol ester 12-O-tetradecanoylphorbol-13-acetate (PMA)(Sigma) to a final concentration of 10 ng/ml. Cells were seeded in a 12-well culture plate (1 ml/well) and kept overnight in a 5% CO_2-humidified incubator.

2.2.3.2   Raw 264.7 cells

As with the THP1 cells, Raw cells were split every three days in a ratio of 1:2 to 1:8 (cells:medium). Fresh complete DMEM medium (7ml), prewarmed to 37°C, was added to new flasks together with the cells. These were first gently detached using a cell scraper and the suspension was homogenized before transferring 3 ml to the flask.

Similar to the THP1 cells, Raw cells were seeded in 12-well culture plates one day before the experiment. Cells were counted by using a Neubauer chamber and the volume to obtain a final concentration of 1x10^6 cells per ml was calculated and transferred to a 50 ml falcon. After centrifugation at 400 x g for 5 minutes at room temperature (Thermo Scientific, SL 16R Centrifuge), the supernatant was discarded and the pellet resuspended in complete DMEM medium. Cells were seeded in a 12-well culture plate (1 ml/well) and kept overnight in a 5% CO_2-humidified incubator.

2.2.4   Adhesion and phagocytosis assays

2.2.4.1   Adhesion assay to THP1 cells

To investigate the interaction between bacteria and the cell lines, adhesion assays were carried out, similarly as described by Lebeer and coworkers (2012), with minor modifications. Bacteria and THP1 cells were prepared according to section 2.2.2 and 2.2.3.1, respectively.

The medium in which the cells were grown overnight was removed with the Vacusafe (Integra Biosciences) and cells were rinsed once with prewarmed dPBS. One ml of the bacterial suspension (5×10^7 CFU/ml) was added to 12-well culture plates containing THP1 cells, previously stimulated with PMA. All strains were tested in triplicate, only medium was used as negative control and *Salmonella* ATCC 14028 as positive control. After the incubation time, all supernatants were discarded using the Vacusafe (Integra Biosciences). Cells were washed three times with prewarmed dPBS in order to remove non bound bacteria and extracellular components. Next, one ml of dPBS was added to each well, cells were scraped with cell scrapers and the suspension was homogenized by pipetting up and down. A set of serial dilutions was prepared and plated out on solid MRS-medium for *Lactobacillus* species and on solid LB-medium for *Salmonella*. Plates with the lactobacilli were stored at 37°C for 48h. *Salmonella* plates were only incubated overnight. The initial concentration of the bacteria (5×10^7 CFU/ml) was checked by plating out the bacterial suspension that was added to the cells in the beginning of the experiment. The adhesion ratio, expressed as a percentage, was calculated by comparing the total number of bacterial colonies counted after adhesion to the number of cells in the bacterial suspension added originally.

The experiment was performed at different incubation time points (1, 2 and 3 hours). Besides incubation at 37°C, which is the optimal temperature for the cells, the experiment was also performed at 4°C to avoid phagocytosis as previously described (Rodríguez *et al.*, 2001b). The protocol for both conditions was the same with one exception; after incubation at 4°C, cells were not washed with prewarmed but ice-cold dPBS.

2.2.4.2 Phagocytosis assay to THP1 cells

Besides bacterial adhesion, phagocytosis or bacterial uptake by THP1 cells previously stimulated to macrophages was investigated as well. Bacterial strains and monocytes were prepared as described in paragraph 2.2.2 and 2.2.3.1, respectively.

The initial steps of the experiments were similar to those in the protocol of the adhesion assay (2.2.4.1.). Bacteria were incubated with the cells at 37°C in a ratio 1 to 50 (1 x 10^6 cells/ml : 5 x 10^7 CFU/ml). The experiment was performed with different incubation time points (1, 2 and 3 hours). Following incubation, all supernatants were discarded using the Vacusafe (Integra Biosciences) and cells were washed three times with ice-cold dPBS to stop the reaction. Penicillin (100 U/ml) and streptomycin (100 µg/ml) were added for 1 hour to kill extracellular bacteria. Afterwards, cells were again washed three times with dPBS to remove any antibiotic residues. Finally, 500 µl Triton X-100 was added to the cells for 15 minutes in shaking conditions to lyse the cells. The cellular suspension was mixed by pipetting up and down and dilution series were prepared before plating out. *Lactobacillus* colonies were countable after two days and *Salmonella* after 1 day. The amount of phagocytosed bacteria was quantified by comparing the total number of bacterial colonies counted after phagocytosis to the number of cells in the bacterial suspension added originally.

2.2.5 Fluorescence activated cell sorting

Further assessment of the interaction of LGG wild type and mutants with THP1 cells was performed by using FACS. GFP-labeled bacterial cultures were prepared as described in section 2.2.2 and THP1 cells according to paragraph 2.2.3.1.

Stimulated THP1 cells were incubated with bacteria (1 x 10^8 CFU/ml) for 2 hours at 37°C. Bacterial strains were added in triplicate. Incubation was followed by three washing steps with dPBS and finally, 500 µl dPBS was added to each sample. Cell scrapers were used to detach the cells and the content of the wells was transferred to a 96-well plate. Transferring the whole content required different steps, each followed by a centrifugation at 1800 rpm for 5 minutes at room temperature. Next, the cells were washed twice with FACS-Wash (PBS + 3% serum) and 3% of PFA was added for fixation for 5-10 minutes in the dark. Afterwards, the THP1 cells were again washed twice with FACS-Wash, followed by centrifugation between each step. Finally, 75 µl FACS-Wash was added to the samples. The 96-well plates were wrapped in aluminium foil to prevent degradation of the GFP$^+$ signal and stored at 4°C. Before the FACS analysis, samples were transferred from the 96-well plate to mini-FACS tubes.

2.2.6 Adhesion competition assay to Raw cells

Up to now, it is not known yet how the pili of LGG interact with cells. As described in literature for the pili of other species (Stimson *et al.*, 1995; Lévesque *et al.*, 2001; Hegge *et al.*, 2004), pili of LGG might be glycosylated (PhD work Hanne Tytgat). Here, a competition experiment was performed to investigate whether some sugars, possibly present on the SpaCBA pili of LGG are involved in the interaction with macrophages. Two different sugars (fucose and mannose) were tested due to their possible role in the bacterial adhesion capacity as earlier described (Jones & Freter, 1976; Salit & Gotschlich, 1977; Gross *et al.*, 2010). Working solutions were prepared from the stock (100 µg/ml) in sterile dPBS and were filter sterilized. Sugars were added to Raw cells (prepared according to 2.2.3.2) for one hour at 37 °C. Cells were then washed twice with dPBS. Next, one ml of bacteria (5 x 10^7 CFU/ml) were added and incubated for one additional hour at 37°C. Raw cells without treatment of the sugar were used as negative control. The following steps of the experiment were similar to those from the protocol of the adhesion assay (2.2.4.1). A preliminary study was performed with different concentrations of sugars (5, 10, 50 and 100 µg/ml) to determine their optimal concentration.

2.2.7 Immunomodulation experiments in THP1 cells

To investigate a possible immunomodulatory effect of the pili of *L. rhamnosus* GG, a profile of different pro- and anti-inflammatory cytokines, secreted by the THP1 cells after treatment with bacteria, was performed. This was done by using RT-qPCR, a technique used to simultaneously amplify and quantify a targeted DNA molecule. Preparation of bacteria and THP1 cells was according to 2.2.2 and 2.2.3.1, respectively. Cells were incubated together with bacteria (1 x 10^8 CFU/ml). For each strain, three repetitions were tested. Initially, time kinetics experiments showed that an incubation of 2 hours was optimal. After this incubation time, cells were washed three times with dPBS and 200 µl dPBS was finally added to each well.

2.2.7.1 RNA isolation

RNA was isolated using the commercially available High Pure RNA Isolation Kit (Roche) following the manufacturer's protocol. Cells resuspended in 200 µl dPBS were mixed with 400 µl lysis buffer and vortexed for 15 seconds to lyse the cells. Each sample was then transferred to the upper reservoir of High Pure Filter Tubes and centrifuged for 15 seconds at 8000 x *g* (eppendorf centrifuge 5418). The flow through liquid was discarded and filter tubes were incubated for 15 minutes with DNase I (10KU/0.55 ml), followed by different washing steps to remove contaminants. After the last wash step, filter tubes were placed on sterile 1.5 ml eppendorfs, and 50 µl of the elution buffer was added to each sample. The concentration of the eluted RNA was measured by using the NanoDrop.

The quality of the isolated RNA was verified by checking the 280/260 nm ratio. RNA samples were stored at -80°C until further use.

2.2.7.2 cDNA synthesis

One µg/µl of RNA was mixed with oligo (dT)$_{20}$ (0.5 µM) and dNTP-mix (0.1 mM) and incubated for 5 minutes at 65°C. Samples were then cooled down for at least one minute on ice. Buffers and reverse transcriptase (indicated in Table 2.12) were added and incubation of 30 min at 50°C was followed. Next, the reaction was stopped by an additional incubation at 70 °C for 15 minutes. RT PCR grade water was added until a final volume of 100 µl. Finally, samples were stored at -20°C.

2.2.7.3 Real-time quantitative PCR

All primers and probes were designed based on published sequences and chemically synthesized by Integrated DNA Technologies (IDT; USA). TNF-α, IL-6 and IL-1β, were tested as pro-inflammatory cytokines and IL-10 as an anti-inflammatory cytokine. Peptidylprolyl isomerase A (PPIA) was used as housekeeping gene (i.e. a gene with an almost constant rate of expression) to normalize gene expression levels. Mastermixes were prepared with the reagents shown in Table 2.13. Twenty µl volume of the mastermix and five µl of the target cDNA were added to 96-well plates (MicroaAmp®, Applied Biosystems). During the whole procedure, the plates were kept on ice. Each PCR amplification was performed in duplicate wells under the following conditions: 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute . Taqman probes were used to detect and quantify the PCR products. These are sequence-specific DNA probes that bind to the sequence of interest and are joined by 2 different molecules; a reporter fluorophore at the 5'-end and a quencher molecule at 3'-end. The basic principle of Taqman probes relies on fluorescence resonance energy transfer (FRET). After excitation of the reporter by light, the quencher molecule quenches the fluorescence emitted by the reporter. This only happens when quencher and reporter are in each other's proximity and consequently there is no fluorescence signal. However, when the template DNA is replicated by the Taq polymerase, the 5' to 3' exonuclease activity of the latter degrades the Taqman probe, thereby releasing the reporter molecule. As the reporter and quencher are no longer in close proximity, the quenching effect is disbanded and fluorescence can be detected. Thus, the amount of fluorescence is a direct measurement for the amount of amplified DNA template. Quantitative PCR data were analyzed by the comparative Ct-method, described by Schmittgen & Livak (2008). Ct is defined as the cycle where the fluorescent signal of the reporter dye exceeds an arbitrary threshold. The method assumes that the efficiency of the PCR is close to one and that the PCR efficiency of the targeted gene is similar to the housekeeping gene.

2.2.8 Immunomodulation experiments in PBMCs

To further investigate the possible immunomodulatory role of the pili of *L. rhamnosus* GG, experiments with human PBMCs were performed, i.e. any blood cell having a round nucleus as monocytes, lymphocytes and macrophages. PBMCs were isolated from blood samples of both healthy and allergic donors by Ficoll density gradient centrifugation in collaboration with Prof. D. Bullens (study S54539,ML8484). Healthy donors were tested for no allergies, in case they were not tested before, by using the scratch or prick test (Heinzerling *et al.*, 2013). Hereto, the skin on the forearm was first cleaned with ethanol and some areas were marked for further identification of each tested allergen. A drop of the extract of each potential allergen was added on the corresponding mark and a pricking device was used to allow its penetration into the outer layer of the skin. After 15 minutes, the results were visible. Potential tested allergens were grass and birch pollen, house dust mite, cats and dogs. Allergic donors were selected based on positive skin-prick test results.

After isolation of PBMCs, cells were counted automatically (ABX Micros 60) and resuspended in a final concentration of 1×10^6 cells per ml. Cells were incubated with LGG wild type and its knockout mutants followed by analyzing the expression of pro- and anti-inflammatory cytokines secreted by the cells as previously described (Donkor *et al.*, 2012). Bacterial strains (1×10^8 CFU/ml) were added to the cells and incubated in glass tubes, medium served as negative control and 1 μg/ml LPS from *E. coli* O111:B4 (Sigma) as positive control. To determine the optimal incubation time, a time kinetics experiment was first performed with PBMCs from a healthy donor, and 3 hours were chosen for further experiments. After 3 hour incubation at 37°C, samples were centrifuged for 5 minutes at 1600 rpm. Finally, pellets were resuspended in 350 μl RLT lysis buffer with β-mercaptoethanol, vortexed at maximal speed for about 1 minute and stored at -80°C.

2.2.8.1 RNA isolation

RNA was isolated using the commercial Mini RNeasy Kit (QIAGEN) following the manufacturer's protocol. First, one volume (350 μl) of 70% ethanol was added to each cell lysate, mixed very well and transferred to a RNeasy mini spin column. After centrifugation (15 seconds, 14800 rpm), the flow-through was discarded and the filter tube was washed three times; once with buffer RW1, and two times with buffer RPE. After the final washing step, the collection tube was replaced by a new one, followed by another centrifugation step. The filter columns were transferred to sterile eppendorf tubes and 40 μl RNase-free water was added on the membrane and centrifuged for one additional minute at 10000 rpm. Eluted RNA concentrations were measured using the Nanodrop. The quality of the isolated RNA was verified by checking the 280/260 nm ratio.

2.2.8.2 cDNA synthesis

cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen), following the manufacturer's protocol. For each sample, RNA was mixed with oligo (dT)$_{12-18}$ (0.5 µM) and dNTP-mix (0.1 mM) in RNase-free tubes and incubated at 65°C for five minutes. Next, a cooling down step for at least one minute on ice was followed. Samples were shortly centrifuged to collect the content. Then, First Strand buffer and DTT (3 mM) were added and incubated for another two minutes at 42°C. Finally, Superscript II reverse transcriptase was added and incubated for one hour at 42°C. The reaction was stopped by incubation at 70°C for 15 minutes. Following a shortly centrifugation in order to collect the content, RNase-free water was added up to a final volume of 100 µl. Samples were stored at -20°C.

2.2.8.3 Real-time quantitative PCR

Mastermixes for the different cytokines and the housekeeping gene were prepared with the materials shown in Table 2.18. The experiment was optimized by determining the optimal incubation time and selecting the best housekeeping gene (PPIA, guanine nucleotide binding protein beta polypeptide 2-like 1 (GNB2L1) and β-actin were tested). The stability of these three housekeeping genes was compared by using the software program Bio-Rad CFX Manager of the qPCR machine (CFX connect) and GNB2L1 was found to be the most stable. During the whole procedure, 96-well plates (MicroaAmp®, Applied Biosystems) were kept on ice and if not directly analyzed, wrapped in aluminium foil and stored at 4°C. The qPCR reaction was performed as previously described in paragraph 2.1.7.3. Quantification of the target genes was done by the use of cDNA plasmid standards that consist of purified plasmid DNA, specific for each tested target. The results were normalised to GNB2L1 in order to compensate for differences in the amount of cDNA.

2.2.9 Statistics

Significant differences were determined by the Student's *t*-test for two samples with unequal variances. This test assigns whether two sets of data have a significant difference between their means. The null hypothesis for the Student's *t*-test states that both means are equal, thus, rejection of the null hypothesis indicates a statistically significant difference between the data set means. The *t*-test should be used only when both data sets follow a normal distribution (McDonald, 2008).

Chapter 3: Results

3.1 Objectives and background information

The purpose of this thesis was to investigate how pili of *L. rhamnosus* GG interact with immune cells and what their role could be in immunomodulation. LGG is one of the few probiotics that produces pili on its cell surface (Kankainen *et al.*, 2009). These pili might be putative effector molecules that contribute in the mechanisms of action of this probiotic strain. Based on the genome sequence, two gene clusters were identified (*spaCBA* and *spaFED*), however, in experimental setups, only SpaCBA pili appear to be present under standard culture conditions (Reunanen *et al.*, 2012). SpaCBA pili are heterotrimeric, containing a major SpaA subunit that builds up the pilus backbone, and two minor subunits: SpaC is an important adhesin, while SpaB is thought to act as a molecular switch in the termination of pilus polymerization and has a minor role as adhesin. It has already been demonstrated that SpaCBA pili of LGG mediate adherence to mucus glycoproteins (Kankainen *et al.*, 2009) and to human IECs (Lebeer *et al.*, 2012a). Moreover, Lebeer *et al.* (2012a) studied the immunomodulating role of the pili of LGG in a Caco-2 cell line. Their results showed that a non-piliated *spaCBA* mutant of LGG (CMPG5357) increased the expression of the pro-inflammatory cytokine IL-8 mRNA compared to the wild type. In contrast, the EPS-deficient *welE* mutant (CMPG5351) showed a reduced IL-8 mRNA induction, likely due to the increased exposure of the pili. Based on these results, pili might play an important role in immune modulating interactions with IECs. While the interplay of LGG pili with IECs and intestinal mucus was already previously studied (von Ossowski *et al.*, 2010; Lebeer *et al.*, 2012a), as also discussed in the literature study, the interaction with immune cells was not yet investigated in detail. However, microorganisms in the GIT are not only in close contact with IECs, but also with immune cells present in the GALT, such as DCs and macrophages. This contact is mediated by specialized M cells and DCs. DCs can directly sense bacteria by passing their dendrites through IECs, while M cells take up bacteria and transfer them to the Peyer's patches in the GALT (Rescigno *et al.*, 2001).

Therefore, in this thesis we investigated the interaction between SpaCBA pili of LGG and immune cells and their possible immunomodulatory role by comparing the wild type and two LGG knockout mutants, one without pili (*spaCBA* mutant CMPG5357) and one showing enhanced exposure of pili (*welE* mutant CMPG5351). The *welE* mutant, due to a deficiency in the priming glycosyltransferase, has no galactose-rich molecules on its surface (Lebeer *et al.*, 2009). Consequently, other surface molecules, such as pili, are overexposed. The *spaCBA* mutant is deficient in the production of the SpaCBA pili (Lebeer *et al.*, 2012a). Interaction between the wild type and its knockout mutant

derivatives with immune cells was analyzed by adhesion and phagocytosis experiments. A possible immunomodulatory role on the other hand, was mainly investigated by cytokine expression studies. Two different cell lines were used in these experiments, the murine macrophage Raw 264.7 cell line and the human monocytic THP1 cell line. THP1 is a human monocytic cell line derived from the peripheral blood of an acute monocytic leukemia patient (Tsuchiya *et al.*, 1980). Before each experiment, THP1 cells were differentiated into macrophage-like cells by stimulation with PMA. Raw cells were obtained from a tumour, induced by intraperitoneal injection of the Abelson murine leukaemia virus, from a male mouse (Raschke *et al.*, 1978). In addition to the use of artificial cell lines, human PBMCs were also investigated in cytokine expression assays. Experiments were performed with PBMCs of healthy and allergic individuals in order to investigate the influence of SpaCBA pili in the cytokine expression profiles from both populations. This might reveal new insights on the immune modulating role of LGG and can be interesting for optimizing the use of this probiotic strain in clinical trials for the treatment and/or prevention of atopic diseases.

3.2   Adhesion and phagocytosis assays

The role of LGG pili in the bacterial recognition by APCs was investigated by adhesion and phagocytosis assays in the THP1 cell line.

In adhesion assays, LGG wild type and its knockout mutant derivatives were incubated with stimulated THP1 cells. A time kinetics experiment was performed with one, two and three hours incubation at 37°C. Unbound bacteria were washed away and the adhesion ratio, expressed as a percentage, was calculated by comparing the total number of bacterial colonies counted after adhesion to the number of cells in the bacterial suspension added originally. The adhesion percentages for all strains to THP1 cells are shown in Figure 3.1. Results showed the same tendency between the bacterial strains for all incubation times compared to LGG wild type. The *spaCBA* mutant CMPG5357 showed a markedly reduced adhesion capacity. The adherent behaviour of the EPS-deficient mutant CMPG5351 was not significantly different with respect to LGG wild type, except for an incubation time of two hours, where the adhesion capacity of *welE* was slightly higher. Moreover, a double knockout mutant of the *spaCBA* operon and *welE* gene (CMPG5365) was tested in order to analyze whether other surface structures overexposed on the cell surface of the *welE* mutant contribute to the bacterial adhesion capacity. The adherent capacity of this mutant to THP1 cells was also drastically impaired compared to wild type, indicating that the pili are mainly involved in adherence. Figure 3.2 gives a schematic presentation of the used bacterial strains.
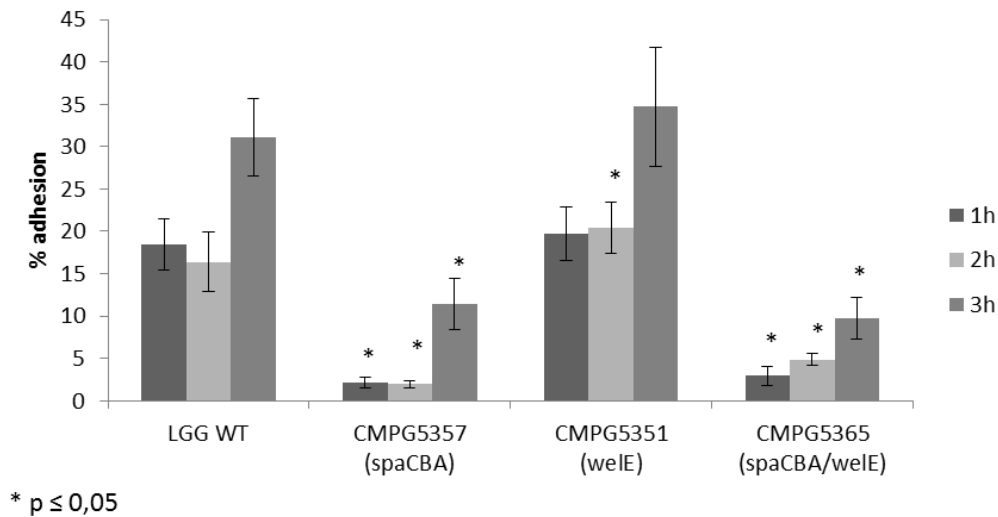
Figure 3.1: Adhesion capacity of LGG wild type and its knockout mutant derivatives to THP1 cells. An overnight culture of LGG wild type, *spaCBA* mutant CMPG5357, *welE* mutant CMPG5351 or double mutant CMPG5365 (5 x 10^7 CFU/ml) was incubated with THP1 cells for 1, 2 or 3 hours, followed by quantification of the proportion of adhered bacteria, expressed as percentages. The experiments were done in triplicate and corresponding standard deviations are indicated by error bars. Statistical differences (p ≤ 0.05) between mutant strains versus wild type are indicated with an asterisk.
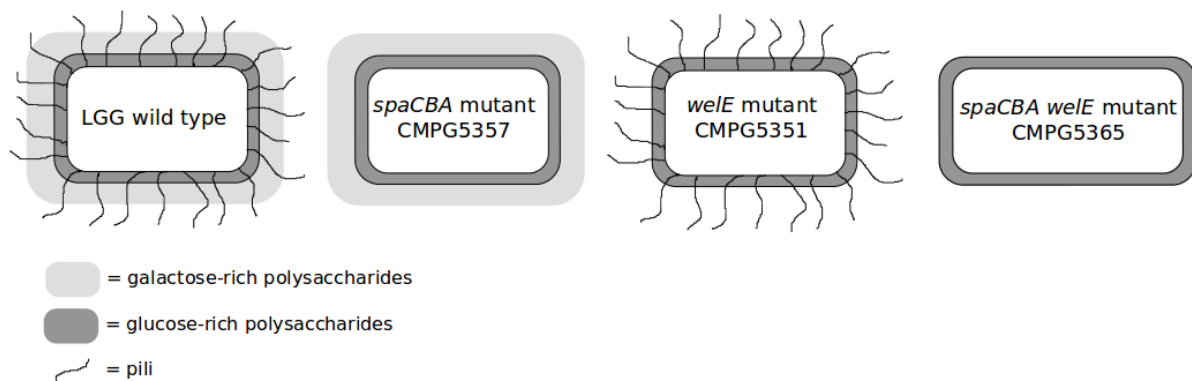
Figure 3.2: Schematic presentation of the used bacterial strains. LGG wild type consists of both short glucose-rich and long galactose-rich polysaccharides and has pili on its surface. The *spaCBA* mutant CMPG5357 has no pili on its cell surface while the *welE* mutant CMPG5351 has the pili and other surface structures overexposed, due to the lack of galactose-rich EPS. The double knockout mutant CMPG5365 has no pili nor galactose-rich EPS, but has increased exposure of other surface molecules.

Since macrophages are important phagocytic cells (Bron *et al.*, 2012), the role of the SpaCBA pili in the uptake of LGG by these cells was investigated by phagocytosis assays. Hereto, a time kinetics experiment was performed in which stimulated THP1 cells were incubated with bacteria at 37°C for one, two or three hours. Following incubation, remaining extracellular bacteria were killed by addition of streptomycin (100 µg/ml) and penicillin (100 U/ml) for one hour. Next, cells were lysed and the amount of internalized bacteria was quantified by comparing the number of phagocytosed

bacterial cells to the number of bacteria in the original bacterial suspension added. The percentages of phagocytosed bacteria were very low after one hour incubation (< 0.5%). Nevertheless, strains expressing the SpaCBA pilus were internalized to a significant higher degree by THP1 cells than the SpaCBA deficient mutant. Our results also showed that phagocytosis was stimulated from the two hours incubation time onwards. The *spaCBA* mutant was less phagocytosed compared to the wild type at one and two hours incubation, while the *welE* mutant was clearly more taken up. An incubation time of three hours showed the same tendency as the two hour incubation. However, already a decrease in the amount of phagocytosed bacteria was observed, likely because some internalized bacteria were already killed inside the macrophages. Therefore, an incubation time point of two hours was selected for future experiments. Figure 3.3 shows the results for the phagocytosis assay for an incubation time of one, two and three hours.
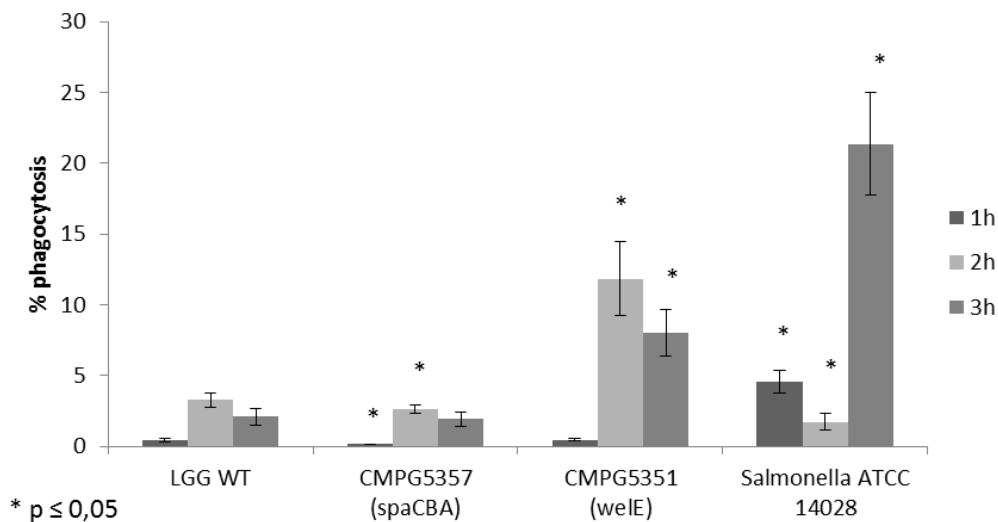
Figure 3.3: Phagocytosis capacity of LGG wild type and its mutant derivatives to THP1 cells. An overnight culture of LGG wild type, *spaCBA* mutant CMPG5357 or *welE* mutant CMPG5351 (5 x 10^7 CFU/ml) was incubated with THP1 cells for 1, 2 or 3 hours. *Salmonella* ATCC 14028 was used as positive control (5 x 10^7 CFU/ml). Remaining extracellular bacteria were killed by addition of streptomycin (100 µg/ml) and penicillin (100 U/ml) for 1 hour, followed by lysing of the cells. The proportion of internalized bacteria, expressed as percentages, was determined. The experiments were done in triplicate and corresponding standard deviations are indicated by error bars. Statistical differences (p ≤ 0.05) between mutant strains or positive control versus wild type are indicated with an asterisk.

The interaction between LGG wild type and its mutant derivatives with THP1 cells was further investigated with FACS. Therefore, GFP-labeled bacteria were incubated with THP1 cells for two hours. Figure 3.4 depict the level of GFP-fluorescence (*x*-axis) versus the number of cells (*y*-axis). Confirming our previous results, the *spaCBA* pilus mutant (CMPG1908/pMEC45) showed a lower interaction capacity with THP1 cells compared to the wild type, while the EPS-mutant with an increased exposure of pili (CMPG1907/pMEC45) interacted more than LGG wild type.
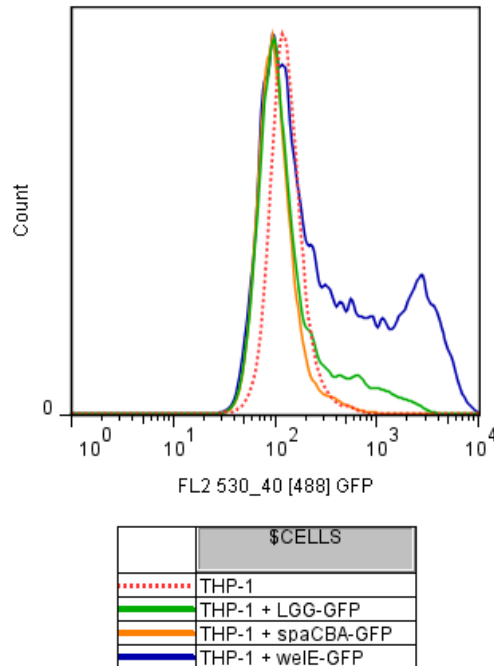
Figure 3.4: Interaction between LGG wild type and its knockout mutants with THP1 cells using FACS. Nisin-induced LGG wild type FAJ1906/pMEC45, *spaCBA* mutant CMPG1908/pMEC45 and *welE* mutant CMPG1907/pMEC45 (1 x 10^{8} CFU/ml) were grown for 2.5 - 3 hours, followed by 2 hours incubation with THP1 cells. The experiments were done in triplicate. THP1 cells without bacteria were used as negative control.

Since it is described in literature that adhesion at 4°C would avoid phagocytosis (Rodríguez *et al.*, 2001b), the bacterial adhesion capacity of LGG to THP1 cells was investigated at this temperature. The results, shown in Figure 3.5, are representative for all repeated experiments. Compared to LGG wild type, the pilus *spaCBA* mutant showed a markedly reduced adhesion capacity to THP1 cells, similar to the results at 37°C. Strikingly, a drastic reduction was observed in the adherent behaviour of the EPS-deficient mutant CMPG5351 to the cells at 4°C in comparison with 37°C (Figure 3.5). While at 37°C, the adhesion of the *welE* mutant to THP1 cells was similar to the wild type, it decreased almost five-fold at 4°C. However, no variation was observed in the adhesion capacity of LGG wild type and *spaCBA* mutant to THP1 cells at 4 and 37°C (Figure 3.5).

Figure 3.5: Adhesion capacity of LGG wild type and its knockout mutant derivatives to THP1 cells: comparison between incubation at 4°C and 37°C. The proportion of adhered bacteria to THP1 cells, expressed as percentages, is compared for an incubation temperature at 37°C and 4°C. The experiments were done in triplicate and corresponding standard deviations are indicated by error bars. Si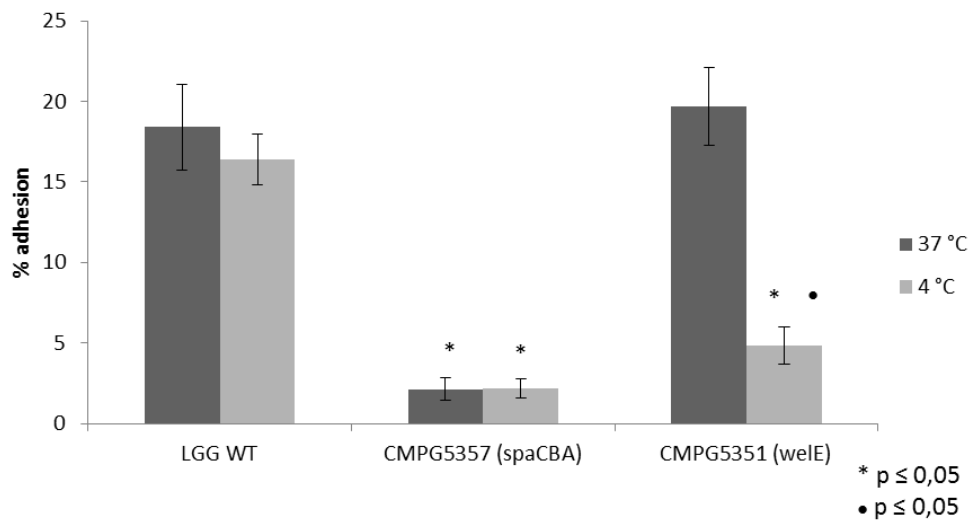gnificant differences between the mutants strains versus the wild type are indicated with an asterisk. The data set comparisons (4°C incubation versus incubation at 37°C) considered significant are indicated with a black circle (●).

3.3 Adhesion competition assay

Based on the results from adhesion and phagocytosis assays, we could demonstrate that SpaCBA pili of *L. rhamnosus* GG are essential for its adhesion capacity to macrophages and that this interaction stimulates bacterial uptake. However, up to now the host receptors involved in the recognition of SpaCBA pili of LGG have not been identified. It is described in literature that pili of some species are glycosylated (Stimson *et al.*, 1995; Lévesque *et al.*, 2001; Hegge *et al.*, 2004). Therefore, it might be that pili of LGG are glycosylated (PhD work Hanne Tytgat). Consequently sugars might be involved in the interaction with macrophages. To investigate whether some sugars, possibly present on the LGG pili (H. Tytgat, personal communication), are involved in this interaction, an adhesion competition experiment was performed. We decided to investigate the sugars mannose and fucose, as it is described in literature that these sugars can be involved in adhesion to eukaryotic cells (Jones & Freter, 1976; Salit & Gotschlich, 1977; Gross *et al.*, 2010). The experiments were performed using the murine Raw 264.7 macrophage cell line.

Initially, the assay was done with different concentrations of sugars (5, 10 , 50 and 100 µg/ml). Raw cells were first incubated with the sugars at 37°C for one hour, followed by addition of LGG wild type or *spaCBA* mutant CMPG5357 to the cells for another hour at 37°C. Raw cells without sugars were used as negative control. Afterwards, the amount of adhered bacteria was quantified by comparing the total number of bacterial colonies counted after adhesion to the number of cells in the bacterial suspension added originally. Results for mannose and fucose are shown in Figure 3.6 and Figure 3.7, respectively. A decreased adhesion capacity of the *spaCBA* mutant CMPG5357 to Raw cells was observed compared to LGG wild type. The competition assay with mannose showed no significant differences between the untreated cells (no sugar) and the treatment with mannose with all tested concentrations for LGG wild type. The *spaCBA* mutant CMPG5357 showed reduced adhesion only when Raw cells were pretreated with 10 µg/ml mannose compared to the negative control, but higher concentrations of the sugar did not have an impact in bacterial adhesion to cells. Results with fucose could not show a significant decrease in the adhesion capacity for LGG wild type after treatment with fucose compared to untreated cells, except for a concentration of 100 µg/ml fucose. The *spaCBA* mutant adhered less to cells when previously treated with concentrations from 10 to 100 µg/ml fucose.

Figure 3.6: Adhesion capacity of LGG wild type and *spaCBA* knockout mutant to Raw cells after coincubation with mannose. Raw cells were incubated with different concentrations of mannose for 1 hour. Raw cells without mannose were used as negative control. Next, an overnight culture of LGG wild type or *spaCBA* mutant CMPG5357 (5×10^{7} CFU/ml) was added to the cells for an additional hour. The proportion of adhered bacteria, expressed as percentages, was quantified. The experiments were done in triplicate and corresponding standard deviations are indicated by error bars. The data set comparisons (impact of mannose on adhesion capacity versus negative control) considered significant ($p \leq 0.05$) are indicated with an asterisk.
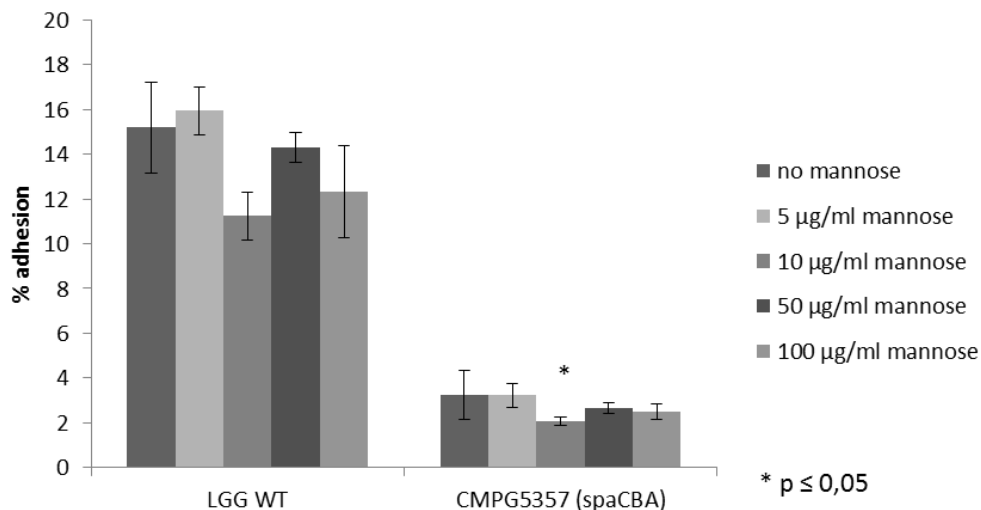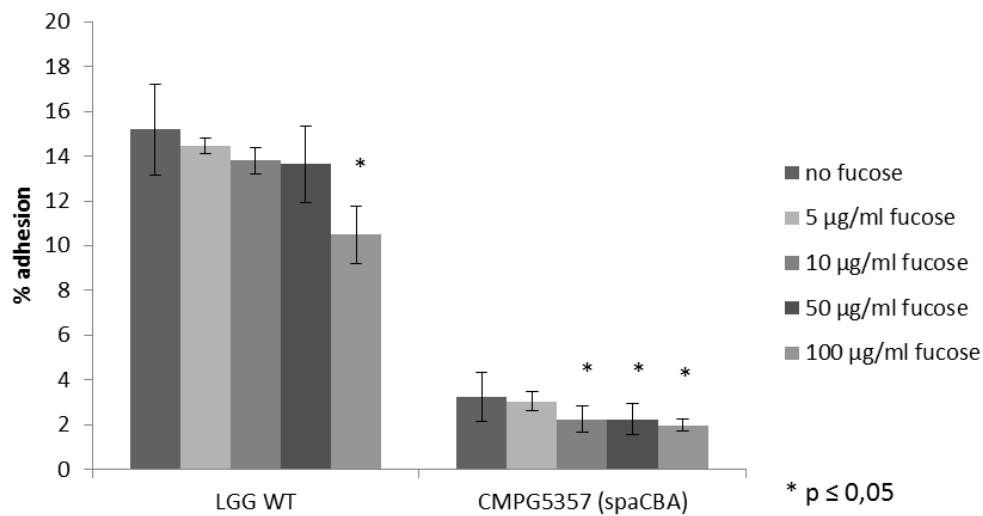
Figure 3.7: Adhesion capacity of LGG wild type and *spaCBA* knockout mutant to Raw cells after coincubation with fucose. Raw cells were incubated with different concentrations of fucose for 1 hour. Raw cells without fucose were used as negative control. Next, an overnight culture of LGG wild type or *spaCBA* mutant CMPG5357 (5 x 10^7 CFU/ml) was added to the cells for an additional hour. The proportion of adhered bacteria, expressed as percentages, was quantified. The experiments were done in triplicate and corresponding standard deviations are indicated by error bars. The data set comparisons (impact of fucose on adhesion capacity versus negative control) considered significant (p ≤ 0.05) are indicated with an asterisk.

Based on these preliminary results, we decided to repeat the experiment with fucose as for mannose, no significant differences were found compared to the negative control. A concentration of 100 µg/ml fucose was chosen, since at this concentration, the adherence capacity of both wild type and *spaCBA* mutant was reduced. In the following experiment, the EPS-mutant CMPG5351 was included. LGG wild type and the *spaCBA* pilus mutant CMPG5357 showed no significant differences when comparing the treatment with fucose (100 µg/ml) and untreated cells (no sugar), indicating that a concentration of 100 µg/ml fucose does not inhibit the adhesion capacity of these strains to Raw cells. However, after fucose treatment, the EPS-mutant showed a markedly increased adhesion to macrophages (Figure 3.8).
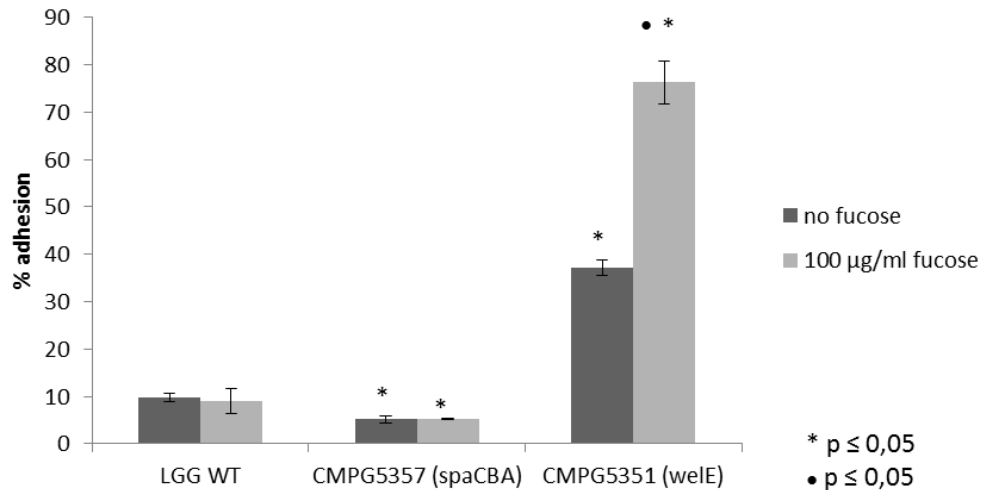
Figure 3.8:. **Adhesion capacity of LGG wild type and its mutant derivatives to Raw cells after coincubation with 100 µg/ml fucose.** Raw cells were incubated with 100 µg/ml fucose for 1 hour. Cells without fucose were used as negative control. Next, an overnight culture of LGG wild type or mutants -*spaCBA* CMPG5357 and *welE* CMPG5351- (5 x 10^{7} CFU/ml) was added to the cells for an additional hour. The proportion of adhered bacteria, expressed as percentages, was quantified. The experiments were done in triplicate and corresponding standard deviations are indicated by error bars. The data set comparisons (impact of fucose on adhesion capacity versus negative control) considered significant (p ≤ 0.05) are indicated with a black circle (●). Significant differences between the mutants strains versus the wild type are indicated with an asterisk.

3.4 Immunomodulation experiments

As discussed in the literature study, probiotics are known to modulate several aspects of the host immune system. They can influence humoral immune responses as well as non-specific immunity by the enhancement of phagocytosis of pathogens (Erickson & Hubbard, 2000) and modification of cytokine production (Marin *et al.*, 1998; Miettinen *et al.*, 1998; Tejada-Simon *et al.*, 1999). Among the documented molecules of LGG, SpaCBA pili were found to have immunomodulatory effects in IECs (paragraph 3.1). However, the underlying molecular mechanisms by which pili exert their immunomodulatory activity are largely unknown, pointing out the importance of further studies on these surface structures. Therefore, this thesis also aimed to investigate the role of the spaCBA pili of LGG in immunomodulation in immune cells. Stimulated THP1 cells, as well as isolated human PBMCs were used for this purpose. Even though LGG is often used in clinical trials for the prevention and/or treatment of atopic disease (Doron *et al.*, 2005), clinical outcomes are not consistent, as mentioned in the literature study. This is possibly due to the lack of knowledge of how this probiotic exert its immune modulating effects. For this reason, in this thesis, LGG wild type and its derivative mutants were incubated with PBMCs from both allergic and healthy donors, in order to analyze whether cytokine profiles between these two populations differ upon addition of the bacterial strains.

3.4.1 Immunomodulation experiments with THP1 cells

In this section, we investigated whether the SpaCBA pili-mediated adhesion influences the induction of pro- and anti-inflammatory cytokines in stimulated THP1 cells. First, a time kinetics experiment was performed to determine the optimal incubation time between bacteria and cells, and two hours were chosen for the following experiments (data not shown). After RNA isolation and conversion into cDNA, cytokine levels were quantified with RT-qPCR. Figure 3.9 shows the results for mRNA expression of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 and anti-inflammatory IL-10. Data represent all repeated experiments. For all tested cytokines, the EPS-mutant CMPG5351 showed a significant induction in mRNA expression in THP1 cells with respect to LGG wild type. Conversely, there were no significant differences in cytokine expression when THP1 cells were treated with *spaCBA* knockout mutant CMPG5357 compared to wild type.
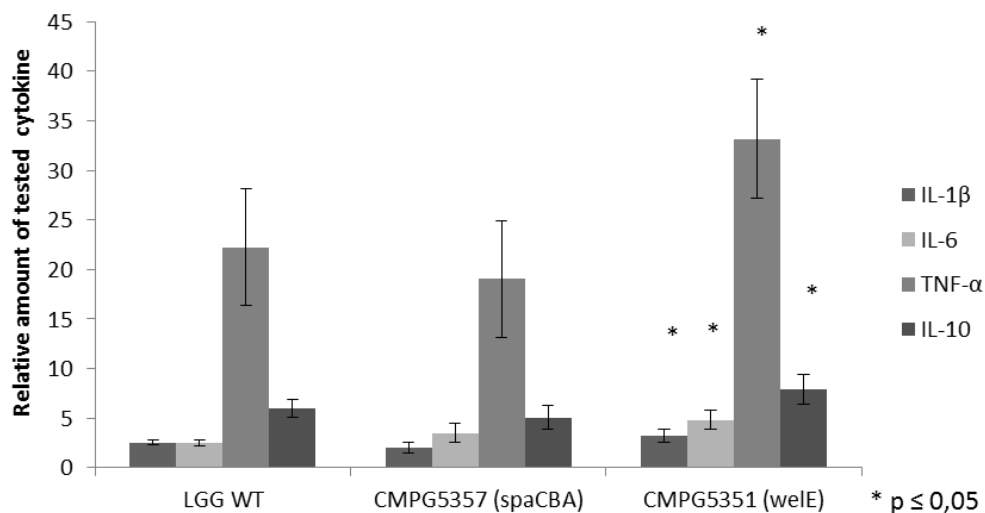
Figure 3.9: IL-1β, IL-6, TNF-α and IL-10 mRNA levels in THP1 cells after treatment with LGG wild type and its knockout mutant derivatives. An overnight culture of LGG wild type, *spaCBA* mutant CMPG5357 or *welE* mutant CMPG5351 (5 x 10^7 CFU/ml) was incubated with THP1 cells for 2 hours. IL-1β, IL-6, TNF-α and IL-10 mRNA was quantified by RT-qPCR and normalized to PPIA. The experiments were done in triplicate, corresponding standard deviations are indicated by error bars. Statistical differences (p ≤ 0.05) between mutant strains versus wild type are indicated with an asterisk.

3.4.2 Immunomodulation experiments with PBMCs

Cytokine expression was also quantified after incubation of LGG wild type and its derivative mutants with PBMCs from both healthy and allergic individuals. First, a time kinetics experiment using PBMCs from a healthy donor was performed to determine the optimal incubation time (data not shown). Three hours were chosen for further experiments. The experiment was repeated for the same healthy donor on a relatively short period of time (< 2 weeks) in order to evaluate intra-individual biological variability. Then, two other non-allergic donors were tested. Despite the biological variation, cytokine expression levels were in the same range for the three tested donors. After this, cytokine profiles in PBMCs from three allergic individuals were tested as well.

First, data from healthy donors were processed. Figure 3.10 - Figure 3.13 show the results of pro- and anti-inflammatory cytokine mRNA expression from healthy donors. LGG wild type and the mutant strains induced the expression of all tested cytokines in PBMCs compared to the negative control (only cells). Moreover, the *welE* mutant CMPG5351 showed a significant downregulation in the expression of IL-6 mRNA in PBMCs compared to the wild type (p = 0,02). However, no statistical differences in mRNA induction of IL-1β, TNF-α, and IL-10 were found when comparing LGG wild type and the mutants CMPG5357 (*spaCBA* mutant) and CMPG5351 (*welE* mutant). Nevertheless, it should be noted that although results showed no significant differences between the three strains, an overall decrease in mRNA expression for all tested cytokines was found after stimulation with the *welE* mutant CMPG5351 compared to the wild type.

Figure 3.10: Pro-inflammatory IL-6 mRNA levels in PBMCs from healthy donors after treatment with LGG wild type and its knockout mutant derivatives. PBMCs were isolated from three non-allergic patients. An overnight culture of LGG wild type, *spaCBA* CMPG5357 or *welE* CMPG5351 (1 x 10^8 CFU/ml) was incubated with PBMCs for 3 hours. RPMI1640 medium was used as negative control and LPS (1 µg/ml) as positive control. IL-6 mRNA was quantified by RT-qPCR and normalized to GNB2L1. Data are expressed as means ± standard deviations indicated by error bars. Significant differences between the mutant strains versus the wild type are indicated with an asterisk.
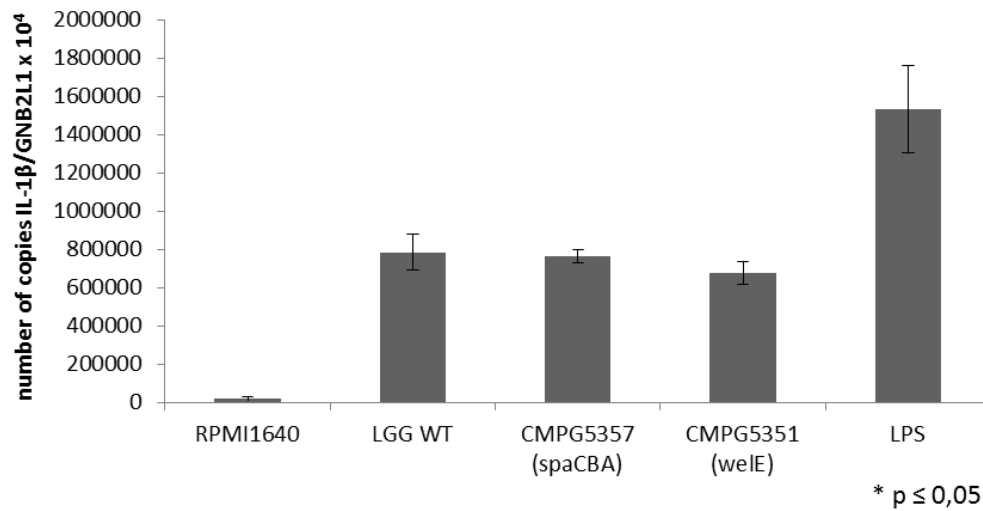
Figure 3.11: Pro-inflammatory IL-1β mRNA levels in PBMCs from healthy donors after treatment with LGG wild type and its knockout mutant derivatives. PBMCs were isolated from three non-allergic patients. An overnight culture of LGG wild type, *spaCBA* CMPG5357 or *welE* CMPG5351 (1×10^8 CFU/ml) was incubated with PBMCs for 3 hours. RPMI1640 medium was used as negative control and LPS (1 μg/ml) as positive control. IL-1β mRNA was quantified by RT-qPCR and normalized to GNB2L1. Data are expressed as means ± standard deviations indicated by error bars. Significant differences between the mutant strains versus the wild type are indicated with an asterisk.
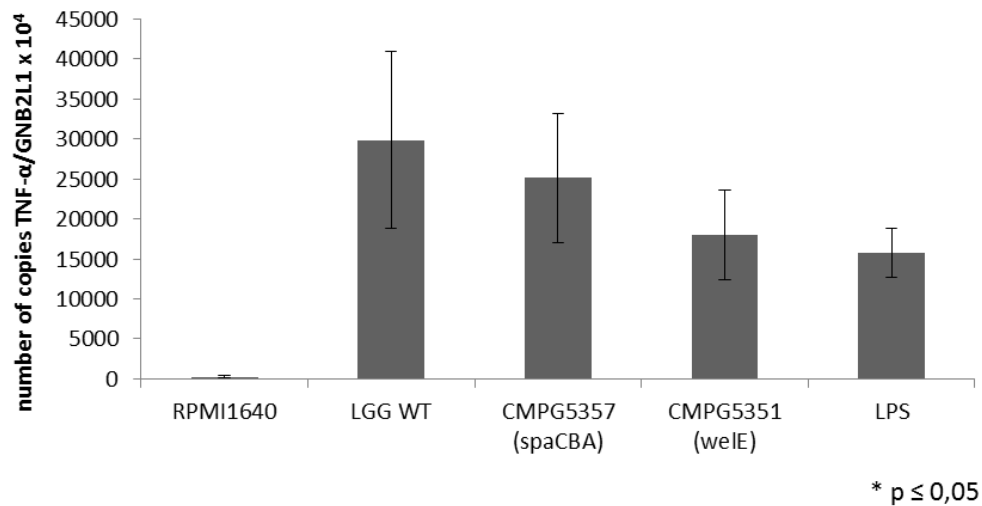
Figure 3.12: Pro-inflammatory TNF-α mRNA levels in PBMCs from healthy donors after treatment with LGG wild type and its knockout mutant derivatives. PBMCs were isolated from three non-allergic patients. An overnight culture of LGG wild type, *spaCBA* CMPG5357 or *welE* CMPG5351 (1×10^8 CFU/ml) was incubated with PBMCs for 3 hours. RPMI1640 medium was used as negative control and LPS (1 μg/ml) as positive control. TNF-α mRNA was quantified by RT-qPCR and normalized to GNB2L1. Data are expressed as means ± standard deviations indicated by error bars. Significant differences between the mutant strains versus the wild type are indicated with an asterisk.
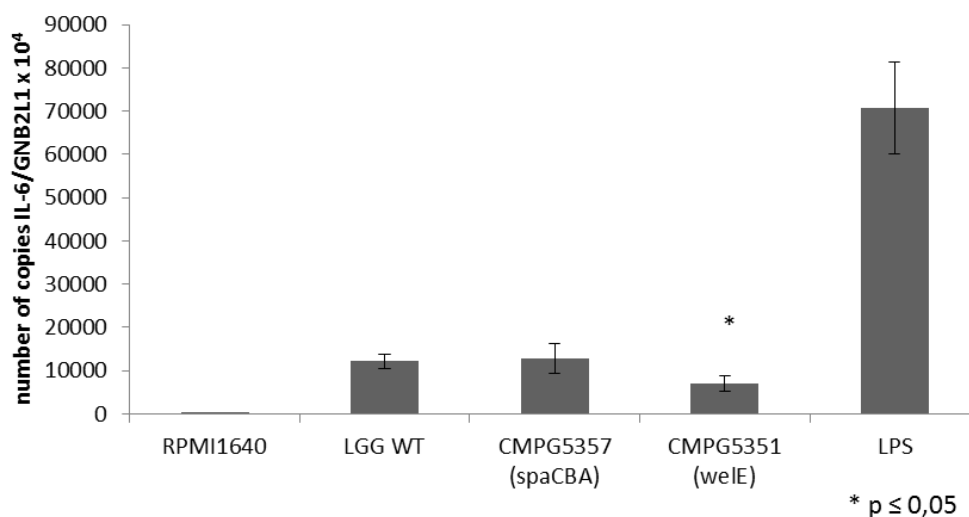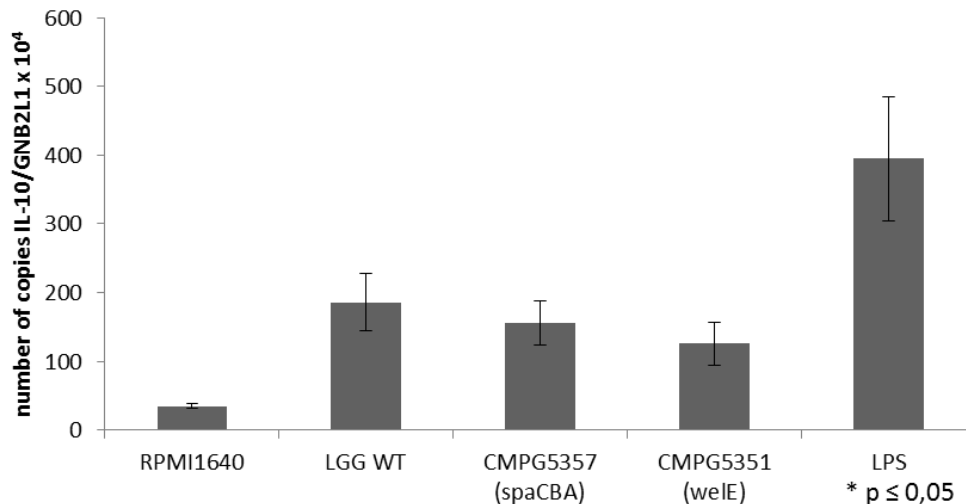
Figure 3.13: Anti-inflammatory IL-10 mRNA levels in PBMCs from healthy donors after treatment with LGG wild type and its knockout mutant derivatives. PBMCs were isolated from three non-allergic patients. An overnight culture of LGG wild type, *spaCBA* CMPG5357 or *welE* CMPG5351 (1 x 10^8 CFU/ml) was incubated with PBMCs for 3 hours. RPMI1640 medium was used as negative control and LPS (1 µg/ml) as positive control. IL-10 mRNA was quantified by RT-qPCR and normalized to GNB2L1. Data are expressed as means ± standard deviations indicated by error bars. Significant differences between the mutant strains versus the wild type are indicated with an asterisk.

To investigate whether LGG modulates the expression of different cytokines in allergic individuals, cytokine profiles of PBMCs from allergic individuals upon pretreatment with bacterial strains were compared to the expression profiles of PBMCs from non-allergic donors. Figure 3.14- Figure 3.17 represent the results for IL-6, IL-1β, TNF-α and IL-10 mRNA expression of PBMCs from allergic and non-allergic donors after treatment with LGG wild type, *spaCBA* mutant CMPG5357 and *welE* mutant CMPG5351. The strains induced the expression of anti-inflammatory IL-10 mRNA compared to the unstimulated cells in PBMCs only from healthy donors. The induction of IL-10 mRNA by all bacterial strains was not significantly induced in allergic donors when compared to cells that were not stimulated with bacteria. The positive control (1 µg/ml LPS) induced IL-10 mRNA expression in PBMCs from allergic donors compared to the negative control, but this induction was significantly lower than in healthy donors (Figure 3.17). Furthermore, we found no significant differences in the expression of IL-6 mRNA between LGG wild type and *spaCBA* mutant CMPG5357 in the allergic and non-allergic populations, however, a downregulation in IL-6 mRNA expression after treatment with the *welE* mutant CMPG5351 compared to wild type was observed in both subsets (Figure 3.14). Data set comparisons showed no significant differences in IL-6 mRNA induction between PBMCs from allergic versus non-allergic donors after treatment with LGG wild type or *welE* mutant CMPG5351, although the expression of IL-6 mRNA in PBMCs from allergic individuals upon addition of the *spaCBA* mutant CMPG5357 was significantly reduced compared to non-allergic donors (Figure 3.14). In both populations of PBMCs (allergic and healthy donors), no significant differences in the induction of IL-1β mRNA were found among the bacterial strains nor between the allergic versus non-allergic

subsets (Figure 3.15). Nevertheless, although not significant, a reduced induction of IL-6 and IL-1β mRNA in PBMCs from allergic donors was found for all strains as compared to non-allergic individuals. For TNF-α mRNA expression, no significant differences were found among the strains in both subpopulations and also no differences were seen between PBMCs from allergic and healthy donors upon bacterial treatment.

Figure 3.14: IL-6 mRNA levels in PBMCs after stimulation with LGG wild type and its mutant derivatives: comparison between healthy controls and allergic donors. PBMCs were isolated from three allergic and three healthy patients. An overnight culture of LGG wild type, *spaCBA* CMPG5357 or *welE* CMPG5351 (1 x 10^8 CFU/ml) was incubated with cells for 3 hours. RPMI1640 medium was used as negative control and LPS (1 µg/ml) as positive control. IL-6 mRNA was quantified by RT-qPCR and normalized to GNB2L1. Data are expressed as means ± standard deviations, indicated by error bars. Statistically significant induction of IL-6 mRNA expression by the mutant strains and positive control compared to the wild type is indicated with an asterisk. The data set comparisons (impact of bacterial strains or positive control LPS on mRNA expression in PBMCs from allergic versus healthy donors) considered significant (p ≤ 0.05) are indicated with a black circle (●).

Figure 3.15: IL-1β mRNA levels in PBMCs after stimulation with LGG wild type and its mutant derivatives: comparison between healthy controls and allergic donors. PBMCs were isolated from three allergic and three healthy patients. An overnight culture of LGG wild type, *spaCBA* CMPG5357 or *welE* CMPG5351 (1 x 10^{8} CFU/ml) was incubated with cells for 3 hours. RPMI1640 medium was used as negative control and LPS (1 μg/ml) as positive control. IL-1β mRNA was quantified by RT-qPCR and normalized to GNB2L1. Data are expressed as means ± standard deviations, indicated by error bars. Statistically significant induction of IL-1β mRNA expression by the mutant strains and positive control compared to the wild type is indicated with an asterisk. The data set comparisons (impact of bacterial strains or positive control LPS on mRNA expression in PBMCs from allergic versus healthy donors) considered significant (p ≤ 0.05) are indicated with a black circle (●).

Figure 3.16: TNF-α mRNA levels in PBMCs after stimulation with LGG wild type and its mutant derivatives: comparison between healthy controls and allergic donors. PBMCs were isolated from three allergic and three healthy patients. An overnight culture of LGG wild type, *spaCBA* CMPG5357 or *welE* CMPG5351 (1 x 10^{8} CFU/ml) was incubated with cells for 3 hours. RPMI1640 medium was used as negative control and LPS (1 μg/ml) as positive control. TNF-α mRNA was quantified by RT-qPCR and normalized to GNB2L1. Data are expressed as means ± standard deviations, indicated by error bars. Statistically significant induction of TNF-α mRNA expression by the mutant strains and positive control compared to the wild type is indicated with an asterisk. The data set comparisons (impact of bacterial strains or positive control LPS on mRNA expression in PBMCs from allergic versus healthy donors) considered significant (p ≤ 0.05) are indicated with a black circle (●).
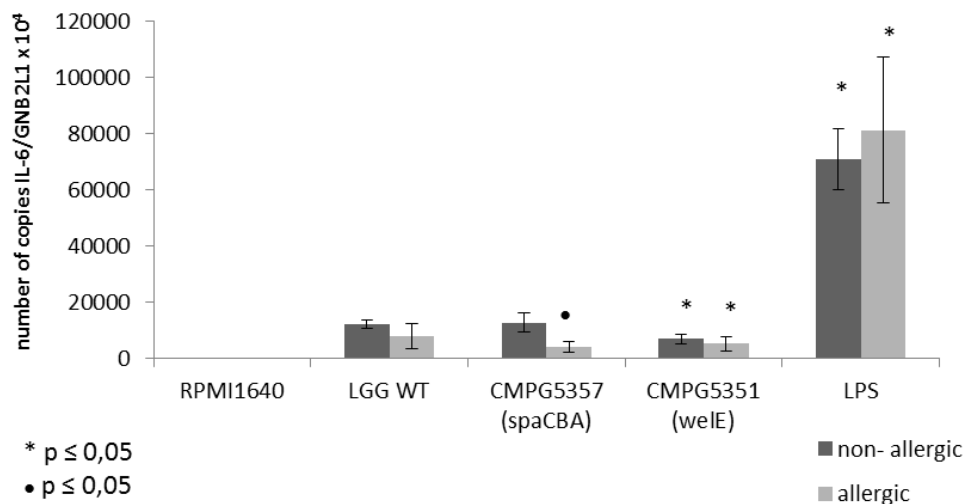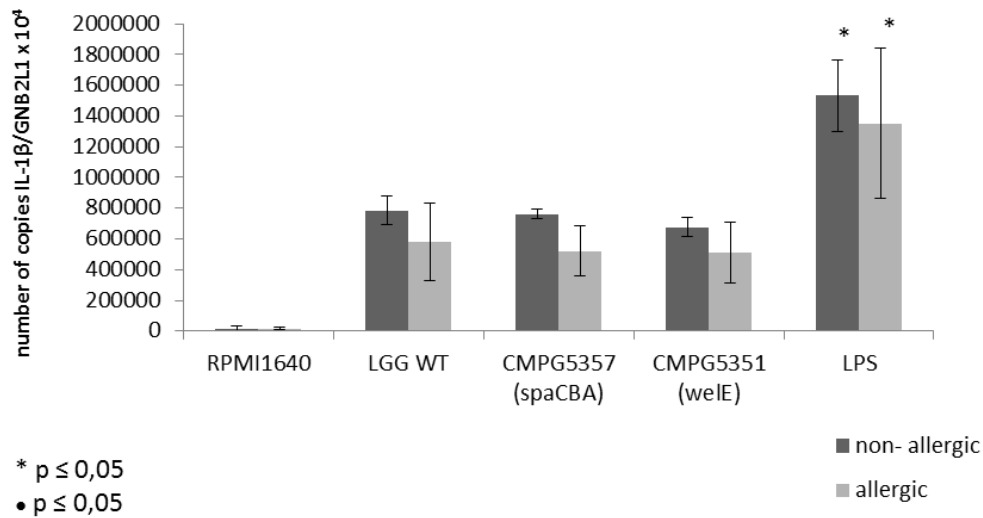
Figure 3.17: IL-10 mRNA levels in PBMCs after stimulation with LGG wild type and its mutant derivatives: comparison between healthy controls and allergic donors. PBMCs were isolated from three allergic and three healthy patients. An overnight culture of LGG wild type, *spaCBA* CMPG5357 or *welE* CMPG5351 (1 x 10^8 CFU/ml) was incubated with cells for 3 hours. RPMI1640 medium was used as negative control and LPS (1 µg/ml) as positive control. IL-10 mRNA was quantified by RT-qPCR and normalized to GNB2L1. Data are expressed as means ± standard deviations, indicated by error bars. Statistically significant induction of IL-10 mRNA expression by the mutant strains and positive control compared to the wild type is indicated with an asterisk. The data set comparisons (impact of bacterial strains or positive control LPS on mRNA expression in PBMCs from allergic versus healthy donors) considered significant (p ≤ 0.05) are indicated with a black circle (●).
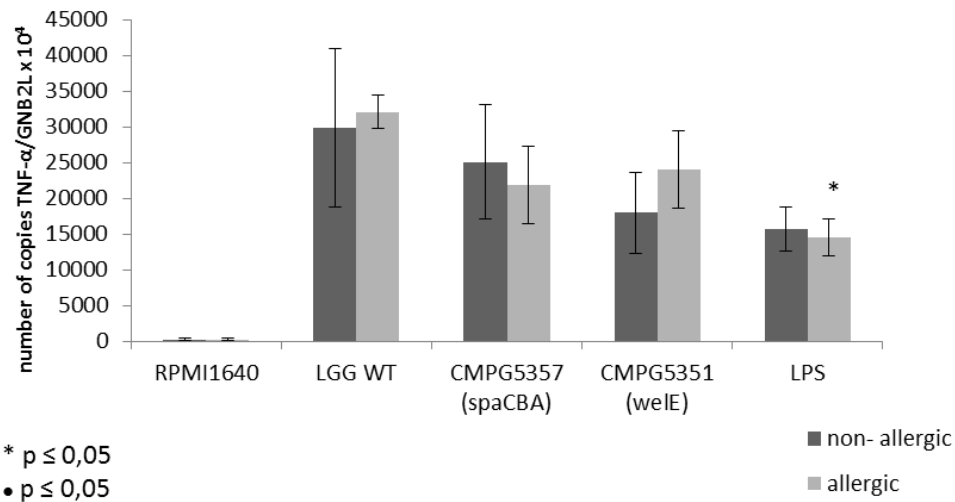
Further analyses of the results were done by calculating the ratios of the tested pro-inflammatory cytokines to the anti-inflammatory IL-10, which is an important biomarker involved in the modulation of different cell and effector functions associated with allergies (Hawrylowicz & O'Garra, 2005). For instance, previous studies have demonstrated the role of IL-10 in the regulation of Th2 cell activity and allergic responses, in the inhibition of the activity and cytokine expression by mast cells and eosinophils (Takanaski, 1994; Royer *et al.*, 2001) and in the enhancement of isotype switching on B cells leading to changes in IgG to IgE ratios (Jeannin *et al.*, 1998). Furthermore, in several animal models, it has been shown that IL-10 is effective in the intervention of allergic inflammation (Stämpfli *et al.*, 1999; Oh *et al.*, 2002). Thus, IL-10 is an important cytokine to take into account when investigating allergies. Although IL-10 expression was statistically not significant induced in PBMCs from allergic donors by the bacterial strains compared to the negative control, ratios were still calculated to get an idea whether the cytokine profile in allergic and non-allergic individuals upon addition of LGG cells differs. Results for both populations are shown in Figure 3.18 - Figure 3.20.

First, we analyzed the results by comparing the ratios between the three bacterial strains in each population in order to see whether the mutants induced a more pro- or anti-inflammatory profile compared to LGG wild type. For the healthy group, none of the ratios showed any significant differences between LGG wild type and the mutants *spaCBA* CMPG5357 and *welE* CMPG5351. However, in the allergic population, *spaCBA* mutant CMPG5357 showed a more pro-inflammatory profile as compared to the wild type, based on IL-6/IL-10 and IL-1β/IL-10 ratios.

Subsequently, we compared the allergic versus non-allergic group in order to examine whether PBMCs from these two populations showed a different cytokine profile upon treatment with bacteria. TNF-α/IL-10 ratios showed no significant differences between the two tested populations for the three bacterial strains. On the other hand, the EPS-mutant CMPG5351 exhibited an increased IL-6/IL-10 ratio in the allergic population as compared to the non-allergic one (p = 0.04). Likewise, the ratio IL-1β/IL-10 was higher in PBMCs from allergic donors upon treatment with the *spaCBA* mutant CMPG5357 with respect to non-allergic donors (p = 0.02).
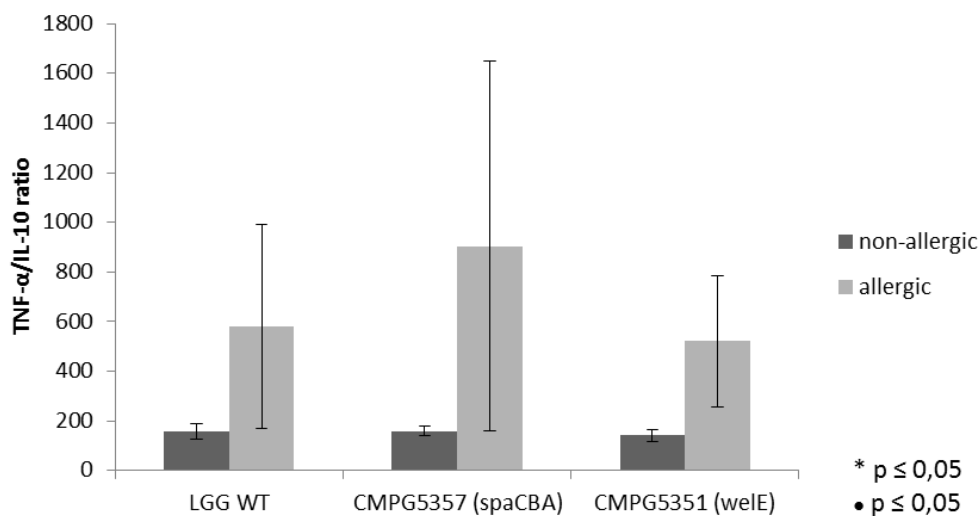
Figure 3.18: Ratio of TNF-α to IL-10 mRNA expression in PBMCs after treatment with LGG wild type and its derivative mutants. TNF-α/IL-10 ratios were calculated for both the healthy and allergic population. Results are the average of three tested donors expressed as means ± standard deviations indicated by error bars. Statistical differences between LGG wild type and mutant strains, *spaCBA* CMPG5357 and *welE* CMPG5351, are indicated with an asterisk. The data set comparisons (non-allergic versus allergic donors) considered significant different (p ≤ 0.05) are indicated with a black circle (●).
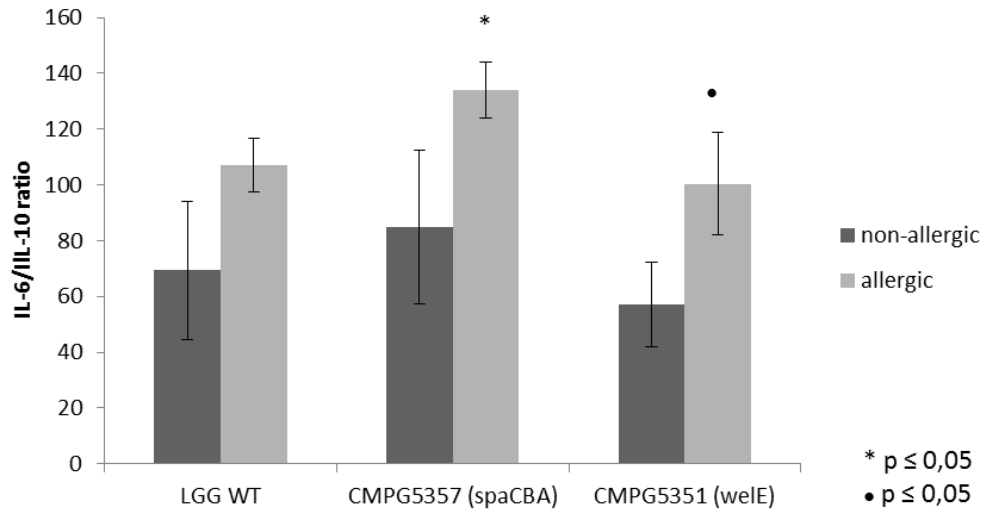
Figure 3.19: Ratio of IL-6 to IL-10 mRNA expression in PBMCs after treatment with LGG wild type and its derivative mutants. IL-6/IL-10 ratios were calculated for both the healthy and allergic population. Results are the average of three tested donors expressed as means ± standard deviations indicated by error bars. Statistical differences between LGG wild type and mutant strains, *spaCBA* CMPG5357 and *welE* CMPG5351, are indicated with an asterisk. The data set comparisons (non-allergic versus allergic donors) considered significant different (p ≤ 0.05) are indicated with a black circle (●).
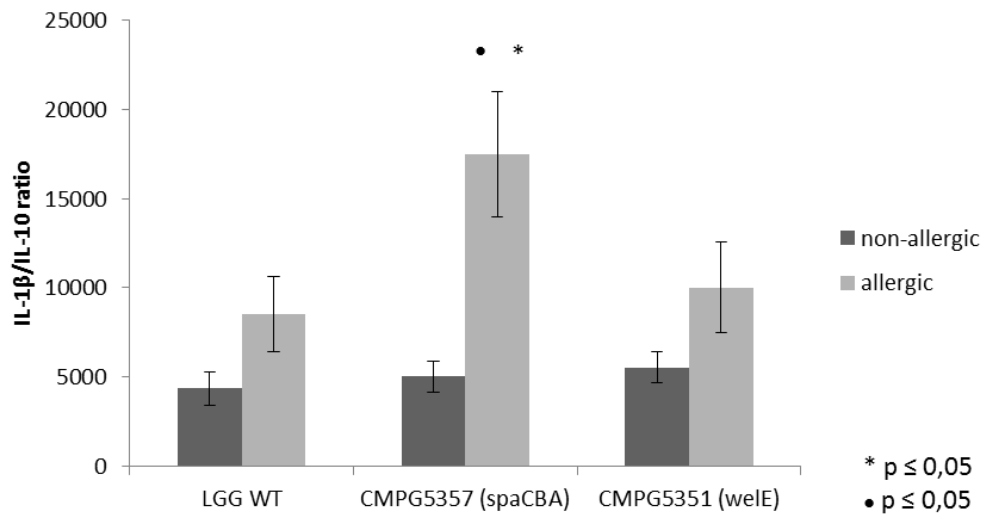
Figure 3.20: Ratio of IL-1β to IL-10 mRNA expression in PBMCs after treatment with LGG wild type and its derivative mutants. IL-1β/IL-10 ratios were calculated for both the healthy and allergic population. Results are the average of three tested donors expressed as means ± standard deviations indicated by error bars. Statistical differences between LGG wild type and mutant strains, *spaCBA* CMPG5357 and *welE* CMPG5351, are indicated with an asterisk. The data set comparisons (non-allergic versus allergic donors) considered significant different (p ≤ 0.05) are indicated with a black circle (●).

Chapter 4: Discussion

As described in the literature review, probiotic bacteria are defined as *" live organisms, which when administered in adequate amount, confer a health benefit on the host"* (FAO/WHO,2001). Although the use of probiotics is common these days, their mechanisms of action are still not yet completely elucidated.

One of the mechanisms whereby probiotics are known to exert their health benefits is through the modulation of host immune responses (as previously explained in section 1.2.2). Probiotics can influence both innate and adaptive immunity through interactions with IECs and DCs in the GALT (Lebeer *et al.*, 2010). Their effects on the immune system are broad and dependent of the strain, dosage, as well as the health state of the host. Recent studies, which intent to unravel their exact mechanisms of action, have focused on cell wall molecules present on the cell surface of the probiotic bacteria, since these molecules might be putative effectors contributing to the beneficial effects. As LGG is one of the most studied and clinically used probiotic strains (Doron *et al.*, 2005), different cell surface structures of this model organism are under investigation, including pili, which are long filamentous structures recently discovered on the LGG cell surface (Lebeer *et al.*, 2009). Hence, in this thesis, we aimed to investigate the role of the SpaCBA pili of LGG in the interaction with immune cells, as well as their possible immune modulating effects on these cells.

4.1 Importance of SpaCBA pili of LGG in the interaction with immune cells

In this thesis, the contribution of LGG pili in the interaction with immune cells was analyzed by adhesion and phagocytosis assays. Results from adhesion experiments point out an important role of the SpaCBA pili in the adhesion capacity of LGG to human stimulated THP1 cells. The pilus *spaCBA* mutant CMPG5357 showed markedly reduced adhesion capacity to the cells compared to the wild type. The *welE* mutant CMPG5351 on the other hand, with pili overexposed, showed a similar adherent behaviour as the wild type, while the double knockout mutant CMPG5365 again showed a reduced adhesion capacity. Thus, mutant strains expressing the SpaCBA pili showed a significant higher degree of adherence to THP1 cells than the SpaCBA deficient mutants. Previous studies on an additional adhesin protein produced by LGG i.e. MabA, which acts as a modulator in the adherence to IECs and biofilm formation (Vélez *et al.*, 2010), have shown that the adhesion capacity of LGG can be mainly attributed to the SpaCBA pili. Nevertheless, the role of MabA in adhesion cannot be neglected as it might play a role in later steps in the adhesion process, as hypothesized by Vélez and coworkers (2010).

Since the pili of LGG were only recently discovered, their role in the interaction with immune cells is still under investigation and similar studies describing this interaction are scarce. However, the results in this thesis are in line with the data obtained by Lebeer and coworkers (2012a), who studied the adhesion capacity of LGG wild type and its knockout mutants (CMPG5357 and CMPG5351) to Caco-2 intestinal epithelial cells. They concluded that SpaCBA pili exert an important role in the adherence of LGG to human IECs, since they found an impaired adhesion to the Caco-2 cells for the *spaCBA* mutant, while the adhesion capacity for the *welE* mutant was increased with respect to the wild type. The drastic reduction in the adherent behaviour of the *spaCBA* mutant CMPG5357 was also observed in our experiments with THP1 cells. However, the adhesion properties of the *welE* mutant CMPG5351 to THP1 cells differed, as its adhesion capacity was the same as for wild type and not strongly increased as Lebeer and coworkers (2012a) found in Caco-2 cells.

Next, phagocytosis assays in THP1 cells were carried out to analyze the role of the pili in bacterial internalization by these immune cells. Results indicated a contribution of the SpaCBA pili in bacterial uptake by stimulated THP1 cells. Strains expressing the SpaCBA pili were internalized to a significant higher degree than the pilus deficient mutant. The *welE* mutant CMPG5351 even showed an increased uptake by THP1 cells as compared to the wild type, likely due to the overexposure of pili as well as the absence of the galactose-rich EPS-layer in this mutant. In pathogens, the EPS-layer has the capacity to act as a protective shield, thereby preventing the uptake and removal of the pathogens by immune cells (Lerouge & Vanderleyden, 2002; Comstock & Kasper, 2006). Furthermore, the presence of a polysaccharide pellicle in *L. lactis* has been proven to exert anti-phagocytic properties (Chapot-Chartier *et al.*, 2010). Lebeer and coworkers (2011) already confirmed that the EPS-layer of LGG indeed acts as a protective shield against host innate defence molecules. Therefore, the higher amount of internalized *welE* bacteria is possibly caused by the lack of this EPS-layer, which makes them more susceptible for phagocytosis.

Furthermore, the interaction of the three strains to THP1 cells was investigated by FACS and a higher interaction for the *welE* mutant CMPG1907/pMEC45 was observed with respect to wild type. Even though the adhesion capacity of this mutant was found to be similar as LGG wild type, the FACS results are in agreement with our previous results that *welE* is more easily internalized. This higher sensitivity towards phagocytosis might explain why we couldn't find an increased adherent capacity of *welE* to THP1 cells, as earlier described by Lebeer *et al.* (2012a) in Caco-2 cells. The higher ability of the *welE* mutant to adhere to IECs than to THP1 cells relies in the nature of the cells, the latter are professional APCs with the ability to take up bacteria. The *spaCBA* pilus mutant CMPG1908/pMEC45 showed barely interaction with the cells, which was also in line with our observations from the adhesion and phagocytosis assays.

In conclusion, the results altogether suggest a clear contribution of SpaCBA pili of LGG in the interaction with THP1 cells, by first providing adherence, followed by stimulation of bacterial internalization.

The effect of temperature in the phagocytosis process of LGG cells was investigated as well, as previous studies have revealed that internalization is avoided at 4°C (Rodríguez *et al.*, 2001b). Therefore, the adhesion capacity of LGG wild type, *spaCBA* mutant CMPG5357 and *welE* mutant CMPG5351 to stimulated THP1 cells at 37°C and 4°C was compared. However, only the *welE* mutant showed a strong decrease in the ability to adhere at 4°C compared to 37°C, suggesting that galactose-rich EPS might give thermal stability to the bacteria as when the EPS is removed, bacteria appear to be more sensitive to temperature. As previously mentioned, the *welE* mutant is more sensitive towards host innate defence molecules, since the EPS-layer acts as a protective shield (Lebeer *et al.*, 2011). These findings point out a combined effect of the temperature and the lack of EPS that affects the adhesion ability of the *welE* mutant. Therefore, we hypothesized that at 4°C, being a stress condition for eukaryotic cells, cells might start to produce compounds in response to the temperature stress that can affect the bacteria. As the *welE* mutant is more sensitive towards host factors due to the lack of EPS, this mutant might consequently be more susceptible towards this components compared to the wild type and *spaCBA* mutant. This hypothesis however was tested but could not be confirmed. Furthermore, it was evaluated whether the *welE* mutant itself is sensitive towards low temperatures which leads to its death, but this was neither the case. Another explanation for the aberrant behaviour of the *welE* mutant can be searched in the receptors the cells express on their surface. It might be that different receptors are exposed on the cell surface or some can be less available or accessible at 4°C. Wang and coworkers, for instance, demonstrated that TLR4 mRNA expression of macrophages was reduced upon hypothermia (Wang *et al.*, 2014). It is not yet known to which receptor(s) the SpaCBA pili bind and it might be that the *welE* mutant CMPG5351 uses other or additional receptors than LGG wild type. If these receptors are impaired at lower temperatures, this may explain the reduced ability of the *welE* mutant to adhere to the THP1 cells at 4°C. Future experiments, however, are needed to gain more insights in the receptor(s) involved in the interaction of the SpaCBA pili of LGG with eukaryotic cells.

4.2 Putative receptor(s) involved in the interaction of SpaCBA pili of LGG with immune cells

As previously mentioned, pili of some pathogenic species are glycosylated, so we hypothesized that SpaCBA pili of LGG might be glycosylated as well. In eukaryotes, glycosylation is important in a wide range of cell-cell and cell-matrix interactions in the immune system. These interactions are achieved by binding of the sugar moieties to sugar binding proteins, i.e. lectins. Glycosylated proteins in bacterial pathogens have been proven to be involved in a broad range of functions, including adherence (Lindenthal & Elsinghorst, 2001), protection against proteolytic cleavage (Herrmann *et al.*, 1996), protein assembly (Grass *et al.*, 2003), protective immunity (Romain *et al.*, 1999) and antigenic variation (Doig *et al.*, 1996). For instance, the contribution of glycosylated pili in adhesion to eukaryotic cells was described in the pathogen *Pseudomonas aerigunosa* 1422. The influence of pilin glycosylation in the pilus function of this pathogen was studied and results showed that pilus glycosylation enhances virulence (Smedley III *et al.*, 2005).

A possible contribution of sugars, that might be present on SpaCBA pili, in the interaction with murine macrophages was analyzed by adhesion competition assays. Since it is described in literature that fucose and mannose are involved in adhesion to eukaryotic cells (Jones & Freter, 1976; Salit & Gotschlich, 1977), these two sugars were tested. In addition, a mannose specific adhesin encoding gene (msa) was recently identified in the probiotic strain *Lactobacillus plantarum* and showed to be involved in mannose-adhesion capacity to yeast cells, as showed by a yeast agglutination assay (Gross *et al.*, 2010). However, our results could not show significant differences in the bacterial adhesion capacity to Raw cells upon treatment with the sugars versus the negative control (no sugar). Nevertheless, although not significant, a decreased tendency was seen upon addition of rising concentrations of fucose. Therefore it would be interesting in future experiments to investigate the tendency when higher concentrations of this sugar are used, since a role of fucose in the interaction with macrophages cannot be completely excluded.

More experiments are needed to get better insights into the possible contribution of sugars in the interaction with macrophages. For instance competition assays with lectins should give more information about which lectins are involved in the recognition of certain sugars on the pili. Figure 4.1 represents a proposed model of how the SpaCBA pili of LGG might interact through sugars, possibly present on these pili, with their corresponding lectins on the macrophages.
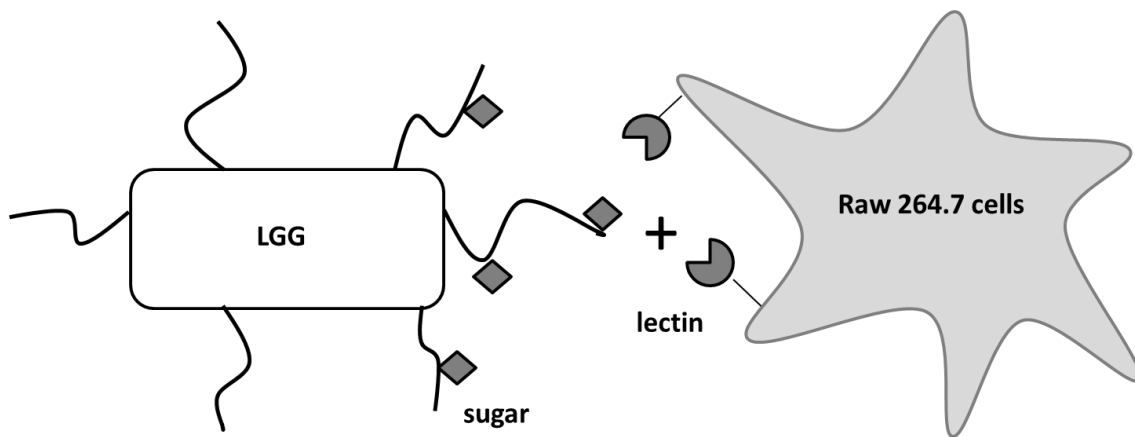
Figure 4.1: Proposed model for the interaction of LGG with murine macrophage cells. Pili of LGG might be glycosylated (H. Tytgat *et al.,* in preparation) and therefore it is hypothesized that certain sugars, present on the SpaCBA pili, might interact with their corresponding lectins on the macrophages.

4.3   Immunomodulatory properties of SpaCBA pili of LGG on immune cells

To study an immune modulating role of the pili of LGG, cytokine expression profiles of immune cells were compared after treatment with LGG wild type and its knockout mutant derivatives. Three pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) were screened, together with the anti-inflammatory IL-10. The immunomodulation experiments were performed in both THP1 cells and human PBMCs from both allergic and non-allergic donors. Results for THP1 cells will first be discussed, as well as the results of PBMCs from healthy donors, followed by a comparison between these data. This might give a better idea whether bacteria behave different *in vitro* (THP1 cells) or in conditions that represent more an *in vivo* state (PBMCs). Next, data of PBMCs from allergic donors will be compared with the data obtained from non-allergic donors to evaluate whether these two populations respond different upon stimulation with the bacteria.

Results in stimulated THP1 cells showed no direct effect of the SpaCBA pili on the induction of cytokine mRNA expression. No significant differences were found in the induction of all cytokines tested between *spaCBA* knockout mutant CMPG5357 versus LGG wild type. Although the *welE* mutant CMPG5351 significantly upregulated mRNA expression of the tested cytokines compared to the wild type, no different profile was seen between pro-inflammatory and anti-inflammatory cytokines. An anti-inflammatory induction of the SpaCBA pili in Caco-2 cells was earlier demonstrated by Lebeer and coworkers (2012a). They found a drastically reduced IL-8 mRNA expression in piliated LGG cells compared to the non-piliated *spaCBA* mutant CMPG5357. However, it is not known yet whether pili directly induce this anti-inflammatory effect, or whether the effect is an indirect role for

instance by shielding off other pro-inflammatory components. The immunomodulatory capacity of the SpaCBA pili was also studied in the macrophage Raw 264.7 cell line by cytokine expression assays. No direct role for the pili in the induction of anti-inflammatory pathways was found but it seems that pili are important to promote close contact with Raw cells, which might facilitate the delivery of other anti-inflammatory components (Vargas *et al.*, submitted for publication). The results obtained in this thesis could not confirm a direct anti-inflammatory potential for SpaCBA pili neither but suggested a more immune stimulating capacity of the *welE* mutant CMPG5351 compared to wild type. This cannot be directly attributed to the pili though, as no effects were observed in the *spaCBA* mutant. Since other structures on the cell surface of the *welE* mutant are also overexposed due to the lack of galactose-rich EPS, it might be that the more pro-inflammatory profile induced by *welE* is caused by another important surface structure on the LGG surface, i.e. LTA, which is known to be an important pro-inflammatory regulator through binding to TLR2/6 (Claes *et al.*, 2012b). The pili play a crucial role in promoting bacterial interaction with the cells as demonstrated by the interaction assays, therefore they might indirectly influence immune stimulating reactions by bringing other surface structures as LTA closer to the eukaryotic cells. Taken this into account, it would be interesting in future experiments to study the behavior of an additional mutant with no functional LTA (*dltD* mutant CMPG5540) in order to investigate whether the observed pro-inflammatory tendency is reduced.

To test the effects of bacterial stimulation in human PBMCs, cells were isolated from three healthy donors and coincubated with LGG wild type and its pilus mutants. Induction of TNF-α, IL-1β, IL-6 and IL-10 mRNA expression in PBMCs was profiled. As described earlier, LAB are efficient in the induction of IL-6, IL-10 and TNF-α in PBMCs (Miettinen *et al.*, 1996). No statistical differences among the strains could be observed for any of the tested cytokines, except for the *welE* mutant CMPG5351 which induced a downregulation in the expression of IL-6 mRNA compared to the wild type. It was found, although not significant, a decreased expression for all tested cytokines when cells were treated with the *welE* mutant CMPG5351 as compared to the wild type. Since the tested population was limited (only three donors) and biological variation needs to be taken into account, the decreased tendency might become significant when the amount of donors in the population is higher. Strikingly, our results showed that LGG wild type and the mutants were able to induce the expression of TNF-α to a same degree as the positive control LPS. Findings that were also previously reported for several other strains of LAB by Miettinen and coworkers (1996). None of the mutants exhibited a more pro- or anti-inflammatory cytokine profile compared to the wild type, as seen from the ratios of pro-inflammatory cytokines to IL-10. Thus, the results obtained from PBMCs from healthy donors do not indicate a clear immune modulating role of the SpaCBA pili of LGG.

When comparing the data of THP1 cells and PBMCs from healthy donors, we found no statistical differences in the induction of all tested cytokines between the *spaCBA* knockout mutant CMPG5357 and LGG wild type. The effect of the EPS-deficient mutant CMPG5351 on cytokine induction, however, was different in THP1 cells than in PBMCs from healthy donors. While in THP1 cells, treatment with the *welE* mutant showed a significant upregulation in the expression of all tested cytokines with respect to the wild type, we could detect a significant downregulation in the expression of IL-6 mRNA in PBMCs as compared to the wild type. Moreover, when statistics were not taken into account, the EPS-mutant showed a reduced capacity to induce IL-10, IL-1β and TNF-α mRNA expression in PBMCs as well compared to LGG wild type. A possible explanation for this inverse tendency seen in THP1 cells and PBMCs might rely in the fact that PBMCs are a mixture of cell types (i.e. monocytes, macrophages and lymphocytes), while the THP1 cell line consists of only one cell type (i.e. monocytes). Consequently, it cannot be excluded that when bacteria are incubated with PBMCs, different interactions between the cell types may occur. These reactions might result in stimulating or inhibiting effects on the induction of the tested cytokines or might counteract each other's effects, leading to changes in cytokine expression.

Furthermore, the effect of bacterial treatment in PBMCs from allergic donors was studied as well to determine whether bacteria induce different responses in this population compared to healthy donors. Data set comparisons between the two subpopulations (allergic versus non-allergic) did not show significant differences in the induction of pro-inflammatory cytokines by PBMCs, although a general decreased expression for IL-6 and IL-1β was seen in allergic donors compared to healthy individuals for the three bacterial strains. As mentioned earlier, since the amount of donors in the tested populations was limited, it would be interesting to repeat the experiment with more donors to evaluate whether this decreased tendency is maintained and might even become significant. In the allergic donors, all bacterial strains were not able to significantly induce IL-10 mRNA expression compared to unstimulated cells. The positive control LPS induced IL-10 mRNA expression but this induction was significantly lower compared to the induction in healthy donors. However, when individual data sets of the allergic donors were processed, IL-10 mRNA expression was significantly induced compared to the negative control, although the induction was not that distinct as in healthy donors. Thus, treatment of PBMCs with LGG cells seems to have different effects in PBMCs from allergic donors compared to those from non-allergic, as in general, a decreased cytokine expression pattern is seen in PBMCs from allergic individuals. Bacterial recognition by APCs such as macrophages occurs through PRR-MAMP interactions. TLRs are important PRRs and studies already demonstrated that TLRs might be impaired in atopic individuals. For instance, an impaired TLR4-mediated innate immune function in peripheral monocytes is associated with children having atopic histories

(Préfontaine *et al.*, 2010). TLR2 signaling in monocytes is also attenuated in five-year old children with atopic diseases, as demonstrated by Amoudruz *et al.* (2009). Even though receptors responsible for the recognition of SpaCBA pili of LGG have not yet been identified, a possible impaired functioning of TLRs might influence the interaction between bacteria and the cells. Consequently, PBMCs of healthy donors may respond different upon addition of LGG cells compared to PBMCs of allergic donors. Another explanation for the differences observed between the two populations might rely on differences in macrophage polarization between allergic and non-allergic individuals. Macrophages can differentiate into classical (m1) or alternative (m2) macrophages, therefore different receptors are expressed on their surface promoting diverse reactions (reviewed in Sica & Mantovani, 2012). It is found that allergy is associated with m2 polarization (Kim *et al.*, 2010; Melgert *et al.*, 2011). Hence, it might be that differences in macrophage polarization were reflected in the PBMC fractions of our tested donors and have led to variation in the responses upon bacterial stimuli.

Finally, the ratios of the pro-inflammatory cytokines to IL-10 between allergic and non-allergic donors were compared in order to further evaluate any differences in cytokine profiles. We could observe a higher IL-6/IL-10 ratio in allergic donors after stimulation with the EPS-mutant CMPG5351 as compared to non-allergic individuals. Similarly, *spaCBA* mutant CMPG5357 induced a more pro-inflammatory response, based on the IL-1β/IL-10 ratio, in PBMCs from allergic donors with respect to non-allergic ones. Although not significant, based on higher ratio numbers, higher pro-inflammatory profiles were observed in the allergic population after treatment with all bacterial strains. This might indicate that in allergic individuals, LGG cells are less efficient in counteracting a pro-inflammatory state.

In conclusion, we could demonstrate that LGG cells are able to induce the expression of cytokines in THP1 cells and PBMCs. However, we did not find differences in the induction of all tested cytokines between the *spaCBA* mutant CMPG5357 and the wild type. Immunomodulation experiments could hence not indicate a direct immune modulating role for SpaCBA pili in THP1 cells and human PBMCs. We could, however, show that the *welE* mutant has a greater immune stimulating effect in THP1 cells compared to the wild type, while an inverse tendency for this mutant was observed in PBMCs, which might rely in the fact that PBMCs are a mixture of cell types and THP1 cells not. Our results from THP1 cells indicate that when pili are overexposed, bacteria interact to a higher extend with cells as compared to the wild type, in this way, facilitating the interaction of other molecules present on the cell surface of LGG and their corresponding receptors. Further analysis on the behaviour of this mutant might confirm our hypothesis that SpaCBA pili have an indirect role on immune stimulatory or inhibitory effects on immune cells.

Furthermore, we observed differences in the induction of cytokine mRNA in immune cells from allergic donors compared to non-allergic individuals after stimulation with LGG. Further analyses should be performed in order to better understand the underlying cause(s) for these differences. This might be interesting to optimize the use of LGG in clinical trials for the prevention and/or treatment of atopic diseases.

Taken our results together, SpaCBA pili do not act as active MAMPs that induce pro- or anti-inflammatory pathways in immune cells, but based on our results from the EPS-deficient mutant, it seems that pili can indirect influence responses in immune cells by bringing other molecules of LGG in close contact.

Chapter 5: General conclusion

Although *Lactobacillus rhamnosus* GG is one of the most well-studied and clinically used probiotic strains, clinical trials often fail or have inconsistent results. Therefore, there is need to unravel the basic mechanisms by which LGG exerts its beneficial effects on the host's health in order to optimize its use. Different molecules on the cell surface of this model organism are currently under investigation since they might be important MAMPs that contribute to the health benefits. In this thesis, we focused on the recently discovered SpaCBA pili present on the cell surface of LGG. We aimed to study the interaction of these pili with immune cells as well as an immunomodulatory role in these cells.

The contribution of the SpaCBA pili in the interaction with immune cells was studied by incubating LGG wild type and pilus knockout mutants with stimulated THP1 cells and their adherent and phagocytosing behaviour was finally compared. Our results indicate an important role of the SpaCBA pili in the interaction with THP1 cells, suggesting that pili are important MAMPs for initial interactions with immune cells and also for further internalization by these cells. In addition, the results also demonstrate that galactose-rich EPS is an important surface structure to protect LGG from cellular uptake, a finding that is in accordance with previously described observations that EPS acts as a protective shield (Lebeer *et al.*, 2011). Although SpaCBA pili are important structures for the interaction with immune cells, the host receptor(s) involved in this interaction are not yet determined. We tried to investigate whether possible sugar moieties on the pili might be involved. Preliminary competition adhesion experiments with fucose and mannose were performed but results could not directly show an influence of these sugars on the interaction with murine macrophages in the concentrations that were tested. Nevertheless, it would be interesting to analyze this further in future experiments, as promising trends were found. Moreover, competition assays with lectins should give more information about which lectins, present on the macrophages, are involved in the recognition of certain sugars on the pili.

We also studied the immunomodulatory capacity of SpaCBA pili of LGG in immune cells, as it was previously reported that SpaCBA pili might have anti-inflammatory potential in IECs (Lebeer *et al.*, 2012a). The expression of pro-and anti-inflammatory cytokines in stimulated THP1 cells and human PBMCs was therefore compared upon stimulation with LGG wild type and its mutant derivatives. Results from both THP1 cells as PBMCs suggest that SpaCBA pili do not directly induce the expression of anti-inflammatory cytokines. A remarkable finding was that the mutant with enhanced exposure of pili, i.e *welE* mutant CMPG5351, upregulated the expression of pro- and anti-inflammatory cytokines compared to the wild type in THP1 cells. However, this behaviour was different in PBMCs,

presumably because PBMCs are a mixture of cell types (monocytes, macrophages and lymphocytes), while THP1 cells are monocytes. Thus, the *welE* mutant seemed to exert more immune stimulating effects in THP1 cells, probably due to the action of other surface molecules that were brought into close contact with the cells by the SpaCBA pili. Future experiments need to be conducted in order to further investigate the behaviour of this mutant as it might give a better understanding about the possible indirect role of the SpaCBA pili of LGG in immune stimulating or inhibiting effects. Furthermore, experiments with purified pili will help to elucidate which responses are caused by the pili themselves.

Finally, since LGG is often used in clinical trials for the prevention and/or treatment of atopic diseases, we aimed to investigate whether stimulation of PBMCs from healthy and allergic donors with LGG cells induced different responses. Although results were not always significantly different, likely due to biological variation, a general downregulation of cytokine mRNA expression was found when PBMCs of allergic donors were treated with the three tested strains (LGG wild type, *spaCBA* mutant CMPG5357, *welE* mutant CMPG5351). In particular, the induction of anti-inflammatory IL-10 mRNA differed markedly between allergic and non-allergic individuals. Hence, a follow-up of these experiments with larger test populations will give more insights into the differences we observed in these small-scale experiments.

In conclusion, SpaCBA pili are important molecules on the LGG surface that facilitate adhesion to immune cells as well as further internalization. Although no direct immunomodulatory role was observed, results with the *welE* mutant suggest that pili might have an indirect role on stimulation in immune cells by promoting or inhibiting interactions of other LGG surface molecules.

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Vulgarising summary

The human body is continuously in contact with microorganisms, not only on the outer surfaces such as the skin, but also internally. One of the body parts that hosts the largest number of bacteria is the gastrointestinal tract (GIT). When talking about bacteria this is often associated with a negative connotation, however, not all bacteria are harmful. Moreover, in the gut, many bacteria actually exert health-beneficial effects. Such bacteria are called probiotics and due to their positive influence on human health they are being studied intensively in order to use them for the treatment and/ or prevention of some diseases, for instance allergies. These days, more and more people suffer from allergic disorders due to a diminished contact with good bacteria, a theory named the hygiene hypothesis. Although probiotic bacteria are believed to be good for our health, it is not known yet how exactly they exert their effects. A very well-studied probiotic bacterium is *Lactobacillus rhamnosus* GG (LGG), which is often used in clinical studies for the prevention and/or treatment of allergies. An important feature of LGG is the presence of pili on its cell surface, structures that can be compared with small tails. It is likely that pili might be involved in the positive effects exerted by LGG on the human body. In our GIT, bacteria are in contact with local epithelial and immune cells. Studying these pili in the interaction with cells of our immune system was the main focus of this thesis. Our results could show that the pili of LGG are very important to bring the bacteria in close contact with immune cells. Besides, we tried to investigate whether the pili were also able to control certain products that are produced by these immune cells, i.e. an immunomodulatory role, but no such direct influence of the pili could be found. Finally, as LGG is used in studies for the prevention of allergies, we tried to investigate whether immune cells of allergic and non-allergic individuals respond differently upon stimulation with the bacteria. Our tested populations were small, but nevertheless we were able to observe higher responses in cells from non-allergic donors after bacterial treatment.