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Molecular and functional analyses of lectins in gastrointestinal and vaginal *Lactobacillus* species

Moleculaire en functionele analyses van lectines in gastro-intestinale en vaginale *Lactobacillus* species

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Abstract

The numerous correlations between an imbalanced microbiota and diseases as well as the increasing resistance of pathogens against antibiotics, have catalyzed the research on probiotics. These beneficial bacteria have already shown much potential in preventing both gastrointestinal and vaginal conditions, such as diarrhea and bacterial vaginosis. However, different clinical trials on the effectiveness of probiotics have mixted outcomes and the responses vary amongst individuals. Clearly, the microbiota composes a complex network of interactions with each other as well as the host and the influence of probiotics on such a network is not predictable with our current knowledge. Many genes of probiotics are not characterized yet and may fill the gaps in our understanding of the mode of action of probiotics. This thesis investigates several putative lectin proteins present in gastrointestinal and vaginal *Lactobacillus* species, regarding their function for the physiology of the lactobacilli and their interactions with the environment. Their sugar-binding potential may allow lectins to bind pathogens and host cells and to interfere with pathogens' adhesion to human epithelium. Therefore, our main goal was to investigate whether these proteins interact with pathogens common to the gastrointestinal and vaginal niche and whether they may explain the anti-pathogenic effects often observed for probiotics. We were able to show that these proteins are binding to mannose and mannan, confirming their lectin activity. They do not appear to be involved in sugar metabolism, but this should be investigated more thoroughly. In addition, it was demonstrated that these lectins are able to bind to specific pathogens such as *Candida albicans* and *Staphylococcus aureus*, indicating they mediate direct interactions with these pathogens. The direct binding seemed to reduce their adhesion to human epithelium, but this requires further confirmation. Importantly, the research performed shows that the lectins of lactobacilli may impair the biofilm formation of *Salmonella* Typhimurium and uropathogenic *Escherichia coli* up to 85 and 95% respectively. It is now more clear than ever that a repertoire of interesting molecules, such as surface attached lectins, may be the basis of the beneficial effects of lactobacilli.

Samenvatting

De vele correlaties tussen een ongebalanceerde microbiota en ziekten, naast de toenemende antibioticaresistentie van pathogene bacteriën, hebben het onderzoek naar probiotica versneld. Deze goedaardige bacteriën hebben hun potentieel voor het voorkomen van verscheidene gastro-intestinale en vaginale infecties bewezen, waaronder bij diarree en bacteriële vaginosis. De conclusies van klinische studies over het effect van probiotica verschillen echter regelmatig en individuen reageren niet consistent op het gebruik ervan. Het is duidelijk dat de menselijke microbiota een complex netwerk vormen van onderlinge interacties en interacties met de gastheer, maar hoe probiotica dit netwerk beïnvloeden is nog niet bekend. Veel genen van probiotica zijn nog niet gekarakteriseerd en zouden onze kennis over de werkingsmechanismen van probiotica kunnen uitbreiden. In deze thesis wordt de rol onderzocht van mogelijke lectines afkomstig van gastro-intestinale en vaginale stammen op de fysiologie van deze stammen en hun interacties met de omgeving. Door het herkennen van suikers zouden lectines direct kunnen binden aan pathogene en/of gastheercellen en zo interfereren met de aanhechting van pathogenen aan gastheerepitheel. Bijgevolg was ons voornaamste doel nagaan of deze lectine-achtige eiwitten interageren met veel voorkomende gastro-intestinale en vaginale pathogenen en of ze de geobserveerde antipathogene effecten van probiotica kunnen verklaren. Het blijkt dat deze eiwitten inderdaad lectines zijn aangezien ze binden aan mannose en mannan. Vermoedelijk hebben ze geen rol in het suikermetabolisme van de overeenkomstige stammen, maar dit vraagt om verder onderzoek. Daarnaast werd aangetoond dat de lectines binden met specifieke pathogenen zoals *Candida albicans* en *Staphylococcus aureus*, wat aangeeft dat ze een rol spelen in directe interacties met pathogene cellen. De directe binding van de lectines bleek de aanhechting van deze pathogenen aan vaginale epitheelcellen te verminderen, maar dit moet nog bevestigd worden. Ten slotte was een belangrijke bevinding van deze thesis dat de lectines de biofilmontwikkeling van onder andere *Salmonella* Typhimurium en uropathogene *Escherichia coli* voorkomen tot 85 and 95% respectievelijk. Dit onderzoek benadrukt opnieuw dat een repertoire van interessante moleculen, zoals oppervlakte-lectines, aan de wortels liggen van de positieve effecten van probiotica.

Abbreviations

Ap	Ampicillin
Ap^R	Ampicillin resistant
BIg	Bacterial immunoglobin
bp	Base pairs
BSA	Bovine serum albumin
BV	Bacterial Vaginosis
CFU	Colony forming units
ConA	Concanavalin A
DC-SIGN	Dendritic cell-specific ICAM-3-grabbing non-integrin
DMEM	Dulbecco's modified eagle medium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EPS	Extracellular polysaccharides
ExPEC	Extraintestinal pathogenic *Escherichia coli*
FCS	Fecal calf serum
FITC	Fluorescein isothiocyanate
Gal	Galactose
GalNac	N-acetyl-D-galactosamine

GFP	Green fluorescent protein
GIT	Gastrointestinal tract
GlcNac	N-acetyl-D-glucosamine
h	Hour
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
ICAM	Intercellular adhesion molecule
IPTG	Isopropyl β-D-thiogalactopyranoside
Km	Kanamycin
Km^R	Kanamycin resistant
LB	Luria Bertani medium
LGG_Llp	Lectin-like protein of *Lactobacillus rhamnosus* GG
LGR-1_Llp	Lectin-like protein of *Lactobacillus rhamnosus* GR-1
Llp	Lectin-like protein
LPS	Lipopolysaccharides
MabA	Modulator of biofilm formation and adhesion
MBF	Mucus-binding factor
Msa	Mannose-specific adhesin
MOPS	morpholinepropanesulfonic acid

MRS	de Man Rogosa Sharpe medium
mMRS	Modified de Man Rogosa Sharpe medium
NeuNac	N-acetylneuraminic acid
OD	Optical density
PBS	Phosphate buffered saline
PBST	PBS with Tween20
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
SDP	Sortase-dependent protein
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
STI	Sexually transmitted infection
SraP	Serine-rich adhesion for binding platelets
TBSt20	Tris buffered saline with Tween20
TSB	Tryptic soy broth
UPEC	Uropathogenic *Escherichia coli*
VK2/E6E7	Vaginal epithelial HPB-16 E6/E7 transformed cells
VMB	Vaginal microbiota

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Context and objectives

Lactobacilli are an important group of beneficial microbes that are ubiquitously present, as they naturally colonize plants and the mucosal surfaces of animals and humans. Due to their anti-pathogenic and immune-modulating activities, certain strains are used as probiotics, which are recognized as microorganisms that confer a health benefit for the host when administered in adequate amounts. Yet, little knowledge is available on the molecular interactions between these beneficial lactobacilli and the host as well as other microorganisms, which impedes their prophylactic and/or therapeutic applications. These interactions could be mediated by lectins, which are carbohydrate-binding proteins present in all organisms and function as signal molecules involved in communication and recognition processes and hence vital for cellular physiology. Although they are little studied in bacteria, lectins are known to be involved in the adhesion of pathogenic bacteria to the host's epithelial cells. Nevertheless, there are virtually no studies available on lectins of lactobacilli and whether they play a role in the beneficial effects.

Therefore, in this thesis the lectins of several vaginal and intestinal *Lactobacillus* species, including two well-known probiotic strains, were characterized on both a molecular and functional level. The molecular analysis focused on determining the potential carbohydrate ligands of the lectin-like proteins, as the specific recognition of sugars is essential for a lectin's function. In the functional analysis, we intended to investigate a potential role of the lectin-like proteins for the endogenous sugar metabolism of the *Lactobacillus* strains. Given that lectins enable specific communication and recognition and that the lectin-like proteins of interest are surface-exposed, potential interactions with the environment of lactobacilli were studied. Here, we focused on interactions between the lectins and various pathogens naturally present in the niche of these lactobacilli. More specifically, we aimed at determining the capabilities of the lectins from probiotic lactobacilli towards inhibiting adhesion and biofilm formation of pathogens causing urogenital and gastrointestinal infections. Importantly, one of our main objectives was to recombinantly express and purify the lectin-like proteins in order to study them as entities separately from the lactobacilli. The research performed in this thesis should provide a better understanding of the fundamental principles governing the beneficial functions of lactobacilli. Ultimately, this increased understanding should be translated into the improved prophylaxis and/or treatment of urogenital and gastrointestinal infections.

Introduction

Lectins are sugar-binding proteins without catalytic activity that are prevalent in all organisms and have important functions in cell signaling and cellular interactions (Sharon and Lis, 2007). This is because each lectin has a unique sugar-binding profile that enables it to specifically recognize certain sugars. Therefore, lectins are considered to translate the "sugar code", which refers to the complex collection of sugars produced by a cell that contain information in their structure. For example, lectins enable microorganisms to specifically recognize and adhere to the host cell, which is the first step in colonization and eventually invasion of a host (Ofek *et al.*, 2013). This is of importance for both pathogenic organisms that tend to infiltrate the host, as well as commensal organisms, such as those belonging to the human microbiota, that need to attach to the host cells in order to maintain a symbiotic relationship. The human microbiota includes all microorganisms that reside in and on the human body, which under normal conditions are non-pathogenic and even beneficial for the human host by stimulating its immune system, producing useful metabolites and competing with pathogens (Robinson *et al.*, 2010). The human body can be divided into four fundamental environmental niches including the skin, oronasopharyngeal cavity, genital tract and gastrointestinal tract (GIT), which differ in their microbial composition. In different microbial niches, specific microorganisms that are exogenously applied to confer health benefits, named probiotics, are already commercially available. However, there is little knowledge on how exactly these probiotic bacteria exert their beneficial functions. For some probiotics, such as the intestinal *Lactobacillus rhamnosus* GG, several cellular surface molecules that interact with host cells are already identified (Segers and Lebeer, 2014). For others, such as for the vaginal probiotic *Lactobacillus rhamnosus* GR-1 as well as for the natural vaginal isolate *Lactobacillus plantarum* CMPG5300, very little is known about the molecules responsible for the interaction of the microorganisms with the host and the microbial community. Furthermore, how these interactions may contribute to the known health benefits, remains to be established. Recently, several genes encoding surface associated (putative) lectins were identified in these *Lactobacillus* strains. Therefore, this thesis investigates the molecular ligands of these proteins as well as their influence on pathogens commonly found in the vagina and/or GIT. More specifically, it studies whether the lectins can compete with pathogens regarding binding to epithelial surface molecules or if they influence interactions by binding to the cellular exterior of pathogenic cells. In addition, the effect of lectin proteins isolated from *Lactobacillus* strains on pathogenic biofilms is

investigated. The increased understanding of the molecular and functional properties of the tested lectins should enable the improved selection of probiotic strains for the prevention and/or treatment of relevant clinical conditions such as vulvovaginal candidiasis and bacterial vaginosis (BV), the most common vaginal disorders (Petrova *et al.*, 2015), as well as gastrointestinal disorders including diarrhoeal diseases, a major cause of mortality worldwide (Servin, 2004). Moreover, the research performed in this thesis supports the use of these lectins on their own for prophylactic goals.

Chapter 1

Literature study

Part of this chapter has been published as a review:

Mariya I. Petrova, Elke Lievens, Shweta Malik, Nicole Imholz and Sarah Lebeer

Lactobacillus species as biomarkers and agents that can promote various aspects of vaginal health

Frontiers in Physiology, 6: 81

1 Literature study

1.1 Lectins

1.1.1 Occurrence of lectins

Lectins are proteins that recognize specific carbohydrates, that are soluble or part of glycoconjugates such as glycoproteins, glycolipids and proteoglycans (Sharon and Lis, 2007). Lectins reversibly bind glycans with a high specificity but they do not possess catalytic activity on them (Sharon and Lis, 2007). Due to their very high structural diversity, glycans embody a lot of information, which is why they take part in a vast spectrum of biological functions (Bertozzi and Rabuka, 2009). Sugars are *de facto* the most common molecular mediators of cell recognition and communication (Gupta, 2012). Hence, the main function of lectins is to decipher and put into action the information encoded in the carbohydrate structure, which is commonly referred to as the 'sugar code' (Sharon and Lis, 2007). Lectins are ubiquitously present in nature and can be found in viruses, bacteria, plants and animals. Lectins have first been encountered in plant species (Varki *et al.*, 2009), where they are assumed to serve in the defense against insect herbivores and phytopathogens (Vandenborre *et al.*, 2011). They have especially high concentrations in seeds, bark and bulbs and consequently are presumed to be a form of protein storage (Michiels *et al.*, 2010). Lectin-glycan interactions also contribute to viral infections, as some viral envelopes consist out of lectins and highly glycosylated glycoproteins, which can respectively bind specific glycan or lectin targets on the host cell (Van Breedam *et al.*, 2014). For example, Influenza virus binds with its hemagglutinin to sialic acid residues on lung epithelial cells and the human immune-deficiency virus (HIV-1) binds with its highly mannosylated glycoprotein gp120 to the dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing non-integrin (DC-SIGN) receptor and CD4 receptor on T-cells (Van Breedam *et al.*, 2014). In animals, 15 structural lectin families are known at present, of which eight well established (Gupta, 2012). These include four mostly intracellular lectin families, namely the calnexin family, M-type, L-type and P-type lectins. These are found in the compartments of the secretory pathway and are involved in intracellular sorting and quality control of glycoconjugates (Gupta, 2012). The next four well-established lectin families are predominantly extracellular and contain C-type, R-type, S-type (galectins) lectins and siglecs which play a role in cell signaling, cell-cell and cell-matrix contacts and pathogen recognition. For example, the DC-SIGN receptor is a C-type lectin and pattern recognition receptor (PRR), recognizing mannose-expressing pathogens such as *Mycobacterium tuberculosis*. Besides DC-SIGN, antigen presenting cells

possess numerous other C-type lectin-like receptors as PRRs, including dectin-1 and -2 which recognize β-glucan on the cell surface of fungi. Furthermore, the immune system also contains soluble lectin PRRs such as the mannan-binding lectin that facilitates clearance of pathogens by macrophages. Bacterial lectins are divided into two functional classes: lectins that are present on the bacterial surface involved in adhesion, colonization and interactions, and secreted toxins, such as the cholera toxin from *Vibrio cholera* (Varki *et al.*, 2009). The binding between the microbes and the host cells is a complex process of protein-protein and lectin-glycan interactions, which often involves binding of multiple different ligands to different receptors. Together these interactions result in a high avidity for the host cell to allow persistence in the host and invasion of pathogenic bacteria (Virji, 2009).

1.1.2 The sugar code

Glycans contain a very high structural diversity that allows much more variation than oligonucleotides or peptides. This is based on several possible levels of variation: (1) combinations of monosaccharide building blocks, (2) different linkage positions (e.g. 5 for hexopyranose), which allows branching, (3) different ring sizes (pyranose or furanose), (4) different conformations (α or β) of the bond between monosaccharides and (5) modifications including acetylation, phosphorylation and sulfation (Gabius *et al.*, 2011). The vast number of genes related to the machinery required to produce these structures highlights both the complexity and the importance of this structural diversity. For example, it is estimated that glycosyltransferases and glycosidases, enzymes that catalyze respectively the formation and hydrolysis of glycosidic bonds, represent 1 to 3% of the gene products of prokaryotic and eukaryotic organisms (Tytgat and Lebeer, 2014). They need the enormous coding capacity of glycans for specific regulation and recognition processes. These processes take place at the glycocalyx, a sugary layer covering cells (Gabius *et al.*, 2011). Species can differ tremendously in their exposed glycan collection, both in the types of monosaccharides and their combinations through glycosidic bonds (Bishop and Gagneux, 2006). Moreover, within one individual glycans also vary in time and for multicellular organisms in space, since different cell types carry a different set of glycans. Animals generally use only 9 monosaccharides, which they combine into highly complex linear or branching structures, through various glycosidic bonds. Especially vertebrates assemble much more complex N-glycans as compared to other lineages that use more types of building blocks. In vertebrates, the high diversity is mainly caused by the terminal residues of glycan chains, which are often sialic acids, fucose, galactose, N-acetyl-D-galactosamine (GalNac) or modifications of linear

glycans in the case of proteoglycans (Bishop and Gagneux, 2006). Still, the bacterial repertoire of glycoconjugates is far more extended due to the large variation in anomeric configuration, conformations and monosaccharide building blocks (Tytgat and Lebeer, 2014). These glycoconjugates comprise mainly surface-exposed glycoproteins, exopolysaccharides, capsular polysaccharides, lipopolysaccharides, lipooligosaccharides, lipoglycans, peptidoglycan and glycosylated teichoic acids, which altogether contribute to a unique signature on the bacterial cell surface (Tytgat and Lebeer, 2014).

In order to recognize glycans specifically and distinguish them from one another, even if they only differ in the conformation of one hydroxyl group, lectins must contain a matching complexity (Gabius *et al.*, 2011).

1.1.3 Lectin glycan affinity

Several factors play a role in the affinity of a lectin for a specific glycan (Gabius *et al.*, 2011). First, the mono- or disaccharides are distinguished by their interaction with a binding site. These interactions are diverse, in which (1) the glycan's hydroxyl groups establish hydrogen bonds or coordination with cations and (2) the C-H bonds are suited for van der Waals interactions and stacking (Figure 1.1). Especially the hydrogen bonds confer specificity to the protein-carbohydrate interaction because of their directionality (Sharon and Lis, 2007). The hydroxyl groups and hydrophobic patches create a profile of potential contacts, which can be recognized by certain amino acid side chains. Therefore in lectin binding domains the amino acid distribution complements this contact profile, using a combination of planar polar amino acids (arginin, aspartate and glutamate) together with aromatic amino acids (tryptophan, histidine) for stacking. In addition, the methyl group of sugars such as fucose also interacts

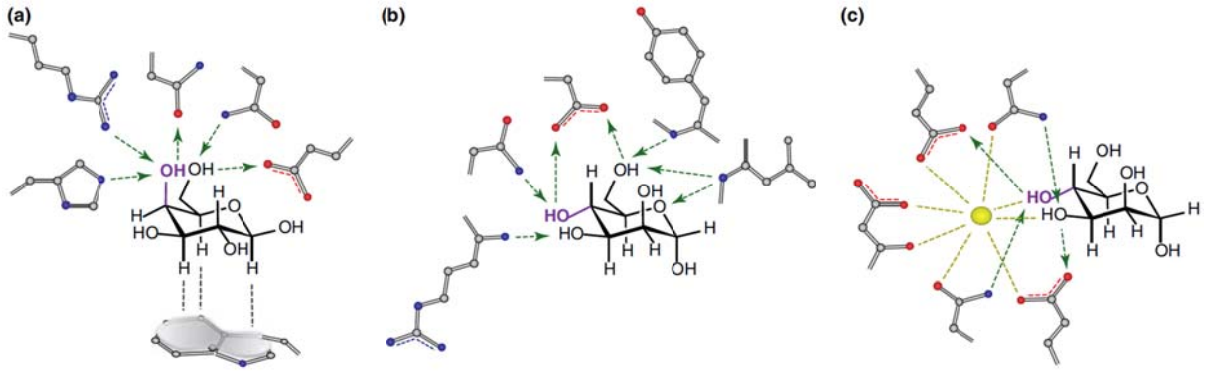

Figure 1.1 Lectins select specific monosaccharides. H-bonding is indicated with arrows (donor --> acceptor). (a) Human galectin-1 recognizes galactose specifically through its axial 4-OH and 6-OH via three and two H-bonds respectively, and through C-H/π-interactions with the indole ring of a Trp residue. (b) On the contrary, the glucose/mannose-specific leguminous lectin concanavalin A (ConA) binds specifically to the equatorial 4-OH. (c) Another strategy to sense the equatorial 4-OH of mannose uses a Ca^{2+} ion, as used by mannose-binding C-type serum lectin (rat collectin) (Gabius *et al.*, 2011).

with aromatic amino acids (Sharon and Lis, 2007). Overall, the topological configuration of amino acids and/or a cation has to match the distribution of hydroxyl groups. This principle has been conserved in diverse lectin-glycan interactions, even though there have been discovered divergent types of binding domains, ranging from flat surfaces and shallow grooves to deep pockets. So far 14 types of folds in lectins have been reported, with further phylogenetic division (Gabius *et al.*, 2011). The most studied are the legume (L-type) lectins, a structurally related group of lectins characterized by the 'jelly role' motif, which is composed of 3 sheets of β-strands (Srinivas *et al.*, 2001). Typical for this group is the variety in multimeric assemblies, despite the similarity in tertiary structure. The best known lectin, concanavalin A (ConA) exemplifies the varying modes of multimerization, given its ability to form both dimers and multimers (Srinivas *et al.*, 2001) (Figure 1.2). Although initially restricted to a single taxonomic plant family (*Leguminosae*), L-type lectins have also been discovered in animal lectins such as the membrane protein ERGIC-53, which is involved in protein transport in the secretory pathway (Etzler *et al.*, 2009).However, lectins with shallow grooves rarely have high affinities for mono- or disaccharides (Gabius *et al.*, 2011). Overall, lectins have higher affinities for oligo- and polysaccharides (Van Damme *et al.*, 2011). This implies there must be an extended binding site. The part of the complex sugar that contacts this binding site, is larger than merely the mono- or disaccharide that can inhibit binding in an activity assay, such as hemagglutination. Besides, this does not have to be a carbohydrate, also peptide interactions or substitutions may contribute to the binding (Gabius *et al.*, 2011). A third factor determining lectin-glycan affinity, are the several constellations or conformers of the glycan. In solution, oligosaccharides are present under several low-energy conformers because oligosaccharides have limited flexibility as opposed to peptides. This makes oligosaccharides "easier" to bind to, as the decrease in entropy associated with binding is less. It has been

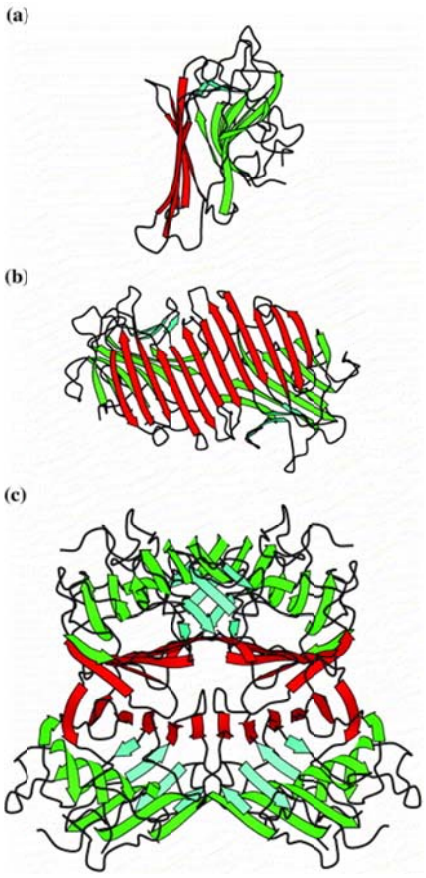

Figure 1.2 Tertiary and quaternary structure of a legume lectin (ConA). The jelly roll fold (a) consists of a back (red), front (green) and top β-sheet (blue). Monomers of ConA can assemble into dimers through side by side alignment of monomers (b) and tetramers through back to back association of dimers (c) (Srinivas *et al.*, 2001)

demonstrated for several lectins that they recognize specifically one conformer of a certain glycan. Therefore, different conformers have different biological activity and influencing the equilibria of conformers is a way to regulate the lectin binding affinity. The ability to distinguish certain conformers, may also allow to distinguish isomers, since isomers at specific sites can change the flexibility of an oligosaccharide, thereby modulating the conformational equilibria. Fourth, the affinity of a lectin also depends on the ligand part not directly bound by the lectin. In natural glycoconjugates, the shape of a glycan is influenced for example by the protein in the case of glycoproteins (Gabius *et al.*, 2011).

However, these four levels of affinity regulation cannot explain how for example PRRs of the immune system are able to differentiate a glycan covering a pathogen from the same glycan present in the host (Dam and Brewer, 2010). This is because not merely the glycan epitope determines lectin-affinity, but also the density of these glycan epitopes on a regarded surface. There appear to be lectin-specific thresholds for the glycan density needed to get a high affinity. On a molecular level, this is due to the "binding and jumping" of a lectin on neighbouring glycans, also called internal diffusion, which reduces the complete dissociation of a lectin from the target (Dam and Brewer, 2010). But the glycan density is especially important for lectins that bind to more than one glycan by containing several carbohydrate recognition domains or by non-covalent association of several lectin molecules, since the binding to several glycan epitopes simultaneously may significantly increase lectin affinity (Dam and Brewer, 2010). Therefore all the factors that influence the spatial vicinity of glycans, regulate the affinity of the lectin for the glycan. These include the type and number of branching and factors that influence the proximity of branches of the same complex glycan, such as core modification that act as molecular switches for the 3-dimensional structure. Furthermore, the glycans recognized by multivalent lectins, may be present on different, neighboring glycan chains within the same glycoconjugate or derived from different glycoconjugates. This implicates that the distribution and clustering of glycoconjugates on the cell surface also determine the affinity of a lectin for its target (Gabius *et al.*, 2011). Overall the interaction between lectin and glycan is influenced by numerous factors, ranging from molecular structure, conformational dynamics and topology to microdomains on the cell's surface (Gabius *et al.*, 2011). This is why lectin-glycan interactions are a challenge for research.

1.2 The intestinal and vaginal microbiota and the role of probiotics

As mentioned earlier, lectins play a role in adhesion and colonization of bacteria that are naturally present in and on the human body, without being pathogenic under normal conditions. The vast amount of commensal microorganisms that colonize the human body, are collectively known as the human microbiota. These are distributed in various niches, including the oral cavity, nasopharyngeal tract, skin, GIT and vaginal cavity (The Human Microbiome Project Consortium, 2012).

1.2.1 The human microbiota

1.2.1.1 Intestinal microbiota

About 10^{14} bacteria and archaea colonize the human GIT, which is 10 times the amount of human somatic cells (Lepage *et al.*, 2013). These commensal microorganisms start colonizing the human host immediately after birth and have life-long important functions for the host. These include the production of vitamins and the digestion of complex polysaccharides, (Lepage *et al.*, 2013). Traditionally, the anti-pathogenic effects of commensal microbiota have drawn most attention. Commensals can directly inhibit the growth of pathogens through the production of bacteriocins (Kamada *et al.*, 2013), which are ribosomally synthesized antimicrobial peptides and proteins with activity against closely related microorganisms (Cotter *et al.*, 2013). Other compounds indirectly affect pathogens by compromising their virulence. As an example, the metabolite butyrate was shown to down-regulate virulence genes of *S. enterica* serovar Typhimurium, thereby reducing its invasiveness (Gantois *et al.*, 2006). Additionally, commensal bacteria can affect pathogens by consuming their preferred substrates (Kamada *et al.*, 2013). Another major pathway used by commensals to outcompete pathogens is the induction of protective host immune responses. A well-known example is the antimicrobial peptide HIP/PAP, a C-type lectin secreted by host epithelial cells upon interaction with resident bacteria (Cash *et al.*, 2006). This and numerous other effects, such as the induced proliferation of epithelial cells and the constant secretion of immunoglobin A, reinforce the barrier and restrict both microbiota overgrowth as pathogenic invasion (Hooper *et al.*, 2012). However, the recognition of the fundamental role of the microbiota in tuning the immune system has grown even farther. It is now clear that the constitutive sensing of the microbiota by the immune system is vital for host homeostasis (Belkaid and Hand, 2014). Especially in the gut, the immune response has to constantly manage the exposure to microbiota, food antigens, metabolites and pathogens. In fact, the commensals and their

metabolites such as short chain fatty acids can regulate immune responses by inducing regulatory T-cells, thereby reducing mucosal inflammation and enabling tolerance (Belkaid and Hand, 2014). Overall, the combination of inflammatory and regulatory signals enables an appropriate immune tone.

It is estimated that the total number of bacterial species comprising the GIT microbiota ranges from 10000 to 40000, of which an individual only hosts about 1000 (Lepage *et al.*, 2013). Although the diversity at the strain level is enormous, the bacterial species inhabiting the GIT belong to a small number of phyla, predominately *Bacteroides* and *Firmicutes* (Robinson *et al.*, 2010). The low variety at higher phylogenetic levels indicates that a selection has occurred for certain conserved taxa. So although compositions can differ tremendously between healthy individuals, there presumably exists a functional redundancy of their microbiota (Robinson *et al.*, 2010). This hypothesis has been supported by studies of the GIT's metagenome that have described a common set of bacterial genes necessary for bacterial survival in the GIT (Lepage *et al.*, 2013). Besides the inter-individual variation, metagenomic sequencing has revealed the existence of three types of GIT microbiota composition in the human population, called 'enterotypes' (Arumugam *et al.*, 2011). Each exists of a dominant genus positively and negatively associated with several other genera, which indicates that actually a group of genera contribute to the established community. Enterotype 1 is enriched in the genus *Bacteroides*, enterotype 2 in *Prevotella* and enterotype 3 in *Ruminococcus.* Although the metabolic activities of the drivers of each enterotype were different, the enterotypes possessed equal functional richness. Importantly, the enterotypes were not associated with age, nationality or body mass index and the environmental and/or genetic factors contributing to the clustering into enterotypes still have to be determined. This metagenomic study supports the existence of well-defined, balanced and stable community types, which should however not be regarded as sharply delineated, but as "more densily populated parts of a multidimensional space of community compositions" (Arumugam *et al.*, 2011). The possible associations of these enterotypes with pathologies and the implications for treatments of diseases remain to be determined (Lepage *et al.*, 2013). However, according to Knights *et al.* (2014) there rather exist continuous gradients of dominant taxa instead of discrete enterotypes. In addition, the authors question the effectiveness of enterotypes as biomarkers for health and disease due to the compositional variability within an individual. Still, it is generally believed that in healthy conditions, the GIT microbiota is quite stable and a shifted bacterial composition or dysbiosis has been associated with a variety of disorders

(The Human Microbiome Project Consortium, 2012). These include inflammatory bowel disease, irritable bowel syndrome, obesity, autistic disorders and diabetes, although for most a causal relationship still has to be discovered (Lepage *et al.*, 2013).

1.2.1.2 Vaginal microbiota

Although most research of the human microbiota has focused on the GIT, an increasing number of studies are being conducted on the vaginal microbiota (VMB), as it is now generally believed that the VMB is essential for the prevention of several urogenital diseases, including BV, yeast infections, urinary tract infections and sexually transmitted infections (STIs), such as HIV and Herpes Simplex Virus-2 (HSV-2) (Petrova *et al.*, 2013b).

As the GIT, the vagina is colonized by a community of microbial species, which begins immediately after birth and varies in composition during life. Starting at puberty and lasting until menopause, due to estrogen the vaginal mucosa is thick and deposits a lot of glycogen, which becomes available through epithelial exfoliation (Petrova *et al.*, 2013b). The release of glycogen in the vaginal environment is advantageous for glucose-fermenting bacteria. Interestingly, it has been suggested that lactobacilli can metabolize glycogen, which could explain the general prevalence of *Lactobacillus* species observed in this period of life, however this metabolic activity has not yet been proven *in vitro* or *in vivo* (Petrova *et al.*, 2013b). Lactobacilli are in fact the dominant species in approximately 70% of women. Several direct and indirect anti-pathogenic effects have been ascribed to lactobacilli. First, they maintain the vaginal pH between 4 and 5 through the production of lactic acid (Petrova *et al.*, 2013b). The low pH inhibits pathogenic microorganisms, such as uropathogenic *E. coli* (UPEC) (Tomás *et al.*, 2003), and reduces HIV and HSV-2 transmission (Petrova *et al.*, 2013b). Besides, they produce bacteriocins with sometimes broad activities. For example, the L23 bacteriocin of *L. fermentum* L23, inhibits Gram-negative and Gram-positive bacteria as well as *Candida* spp (Pascual *et al.*, 2008). In addition, lactobacilli form microcolonies which act as a physical barrier against pathogen adhesion (Petrova *et al.*, 2013b). Several vaginal *Lactobacillus* isolates have been shown to block the adhesion of vaginal pathogens to vaginal epithelial cells *in vitro*, such as by displacement and competition. The pathogens reported to be excluded by lactobacilli are *S. aureus*, Group B streptococci (Zárate and Nader-Macias, 2006), *Pseudomonas aeruginosa, Klebsiella pneumonia, E. coli* (Osset *et al.*, 2001), UPEC, *Gardenerella vaginalis* and *Prevotella bivia* (Atassi *et al.*, 2006). The genomes of vaginal lactobacilli encode various adhesins that could play a role in pathogen adhesion, such as

mucus and fibrinogen binding proteins (Anukam *et al.*, 2013). A comparative genomics approach highlights that the strong adhesive capacity of *L. crispatus* and some unique adhesins play a key role in competitive exclusion of pathogens such as *G. vaginali* (Ojala *et al.*, 2014). However the exact functions of the putative adhesins in adhesion is still unknown. Finally, the commensal microbiota will surely affect the tissue immunity of the vaginal mucosa. This however remains obscure (Belkaid and Hand, 2014).

There have been different studies on the community compositions of the VMB and similar to enterotypes, several vaginal community types have been observed, though the exact number is still under discussion. In a study by Ravel *et al.* (2011) on women from four ethnic groups- white, black, Hispanic and Asian women in North America- the VMB could be divided into five major microbial communities. The communities belonging to group I (26.2%), II (6.3%), III (34.1%) and V (5.3%) were dominated by respectively *L. crispatus, L. gasseri*, *L. iners* and *L. jensenii*, whereas group IV consisted of a heterogenous group of communities with a stronger presence of strictly anaerobic bacteria as *Prevotella*, *Dialister*, *Atopobium*, *Gardnerella*, *Megasphaera*, *Peptoniphilus, Snaethia, Eggerthella, Aerococcus, Finegoldia* and *Mobiluncus*. Smith and co-workers (2012) also observed communities corresponding to groups I-IV, but no communities dominated by *L. jensenii* (group V). In addition they reported community groups designated as VI, characterized by the presence of *G. vaginalis,* and VII, which contains both lactobacilli and *Gardnerella vaginalis* in similarly high proportions. In a study of Srinivasan and colleagues (2012) the VMB was dominated by either *L. crispatus* or *L. iners*, although *L. gasseri* and *L. jensenii* were abundantly detected as well. They also observed women with *A. vaginae*, *Leptotrichia amnionii*, *Prevotella amii* and *Fusobacterium gonidioformans* as dominant bacteria. In a more recent longitudinal study, Ravel's group further diversifies group IV into group IVa and IVb, which are characterized by a moderate presence of lactobacilli combined with low ratios of strict anaerobes and by a domination of strictly or facultatively anaerobic bacteria respectively (Gajer *et al.*, 2012). As indicated by these studies and as concluded in systematic review by van de Wijgert *et al.* (2014), *L. crispatus* and *L. iners* domination are generally recorded the most and to a lesser extent microbiota dominated by *L. jensenii, L. gasseri* or *G. vaginalis* (van de Wijgert *et al.*, 2014). These types are mainly found in white and Asian women (Ravel *et al.*, 2011). However, VMB not dominated by lactobacilli also occur and these communities are typical for black and Hispanic women. Although these communities have a reduced lactic acid production from lactobacilli, they contain other lactic acid bacilli such as *Streptococcus*,

Megasphaera, *Atopobium* (Zhou *et al.*, 2004) and *Leptotrichia* (Linhares *et al.*, 2013). Thus, even though the species composition of the vaginal microbiome can differ quite dramatically, it is possible that the functions exerted by these communities are conserved (Ravel *et al.*, 2011). The functional redundancy of several communities could contribute to an increased stability and resilience to environmental changes (Ravel *et al.*, 2011). Therefore they can all be considered as healthy, although the relative resistance and resilience to environmental disturbances may differ (Ravel *et al.*, 2011). This hypothesis is supported by a longitudinal study that investigated the temporal dynamics of the VMB (Gajer *et al.*, 2012). Indeed, *L. gasseri* dominated group II seldom undergoes transitions to other types, whereas *L. crispatus* dominated VMB tend to transform to *L. iners* dominated communities (group III) or to group IVb. *L iners* dominated groups shift more often to community type IVb than IVa. One does have to take into account that the VMB may change tremendously during menses (Lopes dos Santos Santiago *et al.*, 2012), although not in all women (Gajer *et al.*, 2012). Moreover, within one community group different women have very different variabilities, indicating that VMB variability is determined not only by community type, but also by environmental factors such as menses, sexual behavior and clearly other yet unknown factors (Gajer *et al.*, 2012).

The importance of knowledge about the composition and dynamics of the VMB is manifested in the correlation of an imbalanced microbiota, such a BV, with increased risk of preterm birth (Verstraelen *et al.*, 2005), various vaginal and urinary tract infections (Harmanli *et al.*, 2000; Koumans *et al.*, 2002), miscarriage (Eckert *et al.*, 2003) and STIs (Martin *et al.*, 1999; Cherpes *et al.*, 2003; Wiesenfeld *et al.*, 2003). BV is considered to be an ecological disorder and is generally associated with a decreased load of *Lactobacillus* species and an increase of diversity and/or levels of (facultative) anaerobic bacteria (van de Wijgert *et al.*, 2014), such as *Gardnerella vaginalis, Atopobium, Eggerthella, Megasphaera*, *Leptotrichia*, *Snaethia* and *Prevotella* spp. BV is the most frequent vaginal disorder of women of reproductive age (Linhares *et al.*, 2013) and is characterized by vaginal discomfort and homogeneous malodorous vaginal discharge (Klebanoff *et al.*, 2004). However, there is not a major difference in the prevalence of vaginal discharge and odor among women with and without BV and BV can be asymptomatic (Klebanoff *et al.*, 2004). Moreover, as indicated above, not all apparently healthy women have a *Lactobacillus*-dominated VMB. Therefore, it is still debated whether this type of microbiota is in fact reflecting a healthy state or rather an asymptomatic state of BV. It still has to be determined which microbial compositions and concentrations are really associated with BV and cause pathology (Linhares *et al.*, 2013).

However, more and more studies have pointed to *G. vaginalis* as a key pathogen in the development of BV, by being able to adhere to and to form biofilms on vaginal epithelial cells (Verstraelen and Swidsinski, 2013). In this situation, *G. vaginalis* is the primary colonizer and would provide a scaffold for other bacteria to adhere, such as *Atopobium* (Saunders *et al.*, 2007; Swidsinski *et al.,* 2010). The biofilm hypothesis is supported by the low success rates of treatment with antibiotics and the high frequency of relapses, as biofilm-associated microbes are more resistant to antibiotics (van de Wijgert *et al.*, 2014). Alves *et al* (2014) reported recently that 30 BV-related bacterial species are resistant to antibiotics used to treat BV, presumably due to the long use of antibiotics without exactly knowing the etiology of BV. Therefore the question arises whether exogenously added *Lactobacillus* bacteria known as probiotics could help to prevent the takeover of BV-associated microorganisms and the associated STIs.

1.2.2 Probiotics

Since the human microbiota have been linked to so many diseases and conditions, studies have been conducted to use bacterial strains derived from food sources and/or directly from humans to improve the health of the consumer by influencing the microbiota. These live microorganisms that are administered in adequate amounts to people in order to confer health benefits are called probiotics (Hill *et al.*, 2014). *Lactobacillus* strains are often suggested and used probiotics for both vaginal and intestinal applications due to straightforward reasons. Vaginal lactobacilli generally dominate in a healthy VMB and have various beneficial effects as discussed above. Although lactic acid bacteria, including lactobacilli, only represent 0.2-1% of the total bacteria in the GIT (Mueller *et al.*, 2006), numerous *Lactobacillus* strains have been isolated, mainly *L. ruminis*, *L. crispatus*, *L. gasseri*, *L. plantarum, L. acidophilus, L. delbrueckii, L. casei, L. paracasei, L. reuteri, L. sakei* and *L. curvatus* (Kleerebezem and Vaughan, 2009). Moreover, some species have been consumed for centuries in food products and are known to be safe.

Experts on probiotics have agreed that there exist several well-known, safe species with common core benefits and that every strain belonging to these species may be claimed to be a probiotic (Hill *et al.*, 2014). These include several *Lactobacillus* spp. such as *L. plantarum* and *L. rhamnosus*. The core benefits exerted by probiotics are competitive exclusion of pathogens, increased turnover of enterocytes, colonization resistance, normalization of perturbed microbiota, acid and short-chain fatty acid production and regulation of intestinal transit (Hill *et al.*, 2014). In addition, probiotics are known to support a balanced immune

system by preventing allergic diseases, limiting inflammation and boosting anti-infection activities (Hill *et al.*, 2014). These effects however vary amongst probiotic strains and cannot be ascribed to probiotics in general (van Baarlen *et al.*, 2013). Probiotics have been shown to strengthen the epithelial barrier by promoting the formation of tight junctions (Karczewski *et al.*, 2010) and secretion of mucus (Caballero-Franco *et al.*, 2007) and by preventing apoptosis (Yan and Polk, 2002). No single strain possesses all the functionalities described above and therefore it is necessary to investigate their effects on specific clinical conditions (van Baarlen *et al.*, 2013). Although many clinical trials with probiotics have mixed outcomes (Gareau *et al.*, 2010), a meta-analysis of 31 randomized, controlled trials concluded that *Saccharomyces boulardi* and *L. rhamnosus* GG are effective in preventing antibiotic-associated diarrhea (McFarland, 2006). Also in the treatment of vaginal disorders, probiotic lactobacilli have proved beneficial effects. *L. rhamnosus* GR-1 and *L. rhamnosus* RC-14 have been demonstrated to aid in the treatment of BV in combination with antibiotics (Martinez *et al.*, 2009) and on their own (Anukam *et al.*, 2006) through direct vaginal administration. Several studies have investigated the impact of probiotic lactobacilli on vaginal *C. albicans* infections. *C. albicans* is a commensal organisms of the GIT, oral and vaginal mucosa, present in most, possibly all healthy people (Kim and Sudbery, 2011). However, it can cause several mucosal infections such as vulvovaginal candidiasis, which occurs at least once in the lifetime of 75% of women (Kim and Sudbery, 2011). In addition, 60% of women relapse within 1 to 2 months after discontinuous treatment (Kovachev and Vatcheva-Dobrevska, 2014). Kovachev and Vatcheva-Dobrevska (2014) tested *in vivo* the effect of commercial vaginal capsules (Lactagyn, by Ecopharm) containing several *Lactobacillus* species, including *L. rhamnosus*, on vulvovaginal candidiasis. Their study showed increased curing efficacy and fewer recurrences when applying an azole therapy combined with probiotic lactobacilli.

1.2.2.1 Mode of action of anti-pathogenic effects of probiotics

The anti-pathogenic effects of probiotics have been ascribed to their ability to instigate the immune system and to their antimicrobial substances such as bacteriocins. However, this thesis deals with direct interactions of cell-surface attached proteins of lactobacilli with pathogens and therefore other mechanisms by which probiotic bacteria can presumably prevent infection by pathogens are further elaborated. These include competitive exclusion, co-aggregation and biofilm disruption, which are all related to adhesion mechanisms between probiotic, pathogenic and host cells.

Competitive exclusion

Studies of microbial ecology have demonstrated the importance of adhesion between cells and surface and amongst cells when competing with other species (Schluter *et al.*, 2015). It is hypothesized that adhesion is a key strategy to dominate a community, since merely an increased adhesion enables a genotype to displace its competitors. Also at mucosal surfaces, it has been suggested that probiotics may outcompete pathogens by using the pathogen's attachment sites or possibly by steric hindrance (Lebeer *et al.*, 2008). The most widely used probiotic strain *L. rhamnosus* GG has a high adhesion to intestinal epithelial cells and mucus, which may contribute to competitive exclusion. Various adhesins have been characterized, including the long proteinaceous appendages called pili (Lebeer *et al.*, 2012), mucus-binding factor MBF (von Ossowski *et al.*, 2011b) and the modulator of biofilm formation and adhesion MabA (Vélez *et al.*, 2010). The vaginal isolate *L. plantarum* CMPG5300 has a very high adhesion capacity to VK2/E6E7 vaginal epithelial cells, which has been correlated to its high auto-aggregation (Malik *et al.*, 2013). These properties are dependent on sortase A, which means that cell surface proteins are involved in the interaction between cells and with VK2/E6E7 (Malik *et al.*, 2013). These studies highlight the importance of surface exposed proteins for probiotics as it enables them to adhere, form biofilms and compete with pathogens.

Auto- and co-aggregation

Some probiotic strains have the ability to form aggregates with cells of the same genotype, called auto-aggregation. Besides, studies have demonstrated that certain probiotic strains aggregate with different bacteria, a phenomenon named co-aggregation. For example, Ekmekci *et al.* (2009) found a correlation between auto-aggregation and co-aggregation of 30 vaginal *Lactobacillus* isolates with *C. albicans*. The aggregation capacity was strain dependent and could only be reduced by pepsin, sonication or heat, which implicates a proteinaceous, heat-sensitive substance on the cell wall is involved in aggregation. Also the auto-aggregation and co-aggregation of *L. plantarum* isolates from cheese with *E. coli, Listeria monocytogenes* and *S. aureus* appeared to be linked, depending on the strain. However the co-aggregation with pathogens could not prevent these from adhering to intestinal epithelium *in vitro* (García-Cayuela *et al.*, 2014). Younes and co-workers (2012) measured the adhesion forces between several *Lactobacillus* strains, namely *L. reuteri* RC-14, *L. crispatus* 33820 *and L. jensenii* RC-28, and toxic shock syndrome toxin-1 producing *S. aureus* strains. They discovered that the lactobacilli and especially *L. reuteri* RC-14 had equal

or greater adhesion forces to *S. aureus* than staphylococcal pairs, and that a higher adhesion force leads to larger co-aggregates.

The effects of this auto- or co-aggregation in the GIT and vagina is still under speculation, but different scenarios have been suggested. Possibly, aggregation between lactobacilli could aid in their retention and thereby the construction of a barrier against pathogens. Similarly co-aggregation with pathogens could prevent their binding to epithelial cells and colonization (Malik *et al.*, 2013). Reid *et al.* (1988) suggested that the co-aggregation properties of endogenous or externally applied lactobacilli with *C. albicans* or *E. coli* could prevent urogenital tract infections caused by other pathogens. Moreover, co-aggregation could enable a local micro-environment of bacteriocins produced by lactobacilli around the pathogens, increasing the efficiency of certain probiotics or creating a synergy of different probiotic species (Younes *et al.*, 2012). However, a possible side effect could be that co-aggregation would retain pathogens in the GIT/vagina, increasing their persistence, which needs to be investigated. Overall, it is hard to extrapolate *in vitro* results to an *in vivo* meaning (García-Cayuela *et al.*, 2014).

Inhibition of pathogenic biofilms

Several studies report the inhibition of pathogenic biofilms by probiotic lactobacilli and ascribe this to antimicrobial and/or surface-active substances in the supernatant of lactobacilli cultures, to co-aggregation or to reduced pathogen adherence (Vuotto *et al.*, 2014). For example, Walencka and co-workers (2008) discovered that uncharacterized biosurfactants produced by *L. acidophilus* could prevent *S. aureus* and *Staphylococcus epidermidis* biofilms by impairing their adhesion but not their viability. In contrast, a study of Söderling *et al.* (2011) showed that *L. rhamnosus* GG and *L. plantarum* 299v reduce biofilms of *Streptococcus mutans* by secreting antimicrobial substances and decreasing the pH. There are however few studies on the effect of probiotic lactobacilli on biofilms of gastrointestinal pathogens (Vuotto *et al.*, 2014). More studies have been conducted on vaginal lactobacilli to elucidate how they could prevent or even cure BV. Saunders and colleagues (2007) demonstrated that *L. rhamnosus* RC-14 can eradicate *G. vaginalis* biofilms, a possible cause of BV, and that *L. rhamnosus* GR-1 can infiltrate *G. vaginalis* biofilms and dislodge it. Since this was not related to pH or H_2O_2, the authors suggest the displacement might be caused by biosurfactants produced by these strains and the killing may be due to bacteriocins and signaling molecules. This has been confirmed by McMillan *et al*. (2011), who reported that *L. rhamnosus* RC-14 and GR-1 were able to disrupt *G. vaginalis* and *A. vaginae* biofilms, which

again was suggested to be caused by the production of acids, bacteriocins or biosurfactants. Also *E. coli* biofilms were penetrated and displaced by both species. They observed elongated cells in the presence of spent medium from lactobacilli, which indicates the *E. coli* cells were stressed and even killed, also pointing to the presence of growth inhibiting bacteriocins. Moreover, in unpublished data, they noticed by atomic force microscopy that lactobacilli could displace *S. aureus* from surfaces. Overall, these studies indicate that probiotic lactobacilli may disturb biofilms of pathogenic species through several mechanisms, including bacteriocins, biosurfactants or other yet unidentified forces (McMillan *et al.*, 2011).

This illustrates the multiple effects probiotic lactobacilli can conduct. Although many phenomena of interactions between probiotic lactobacilli, host and pathogens have been described, there is little knowledge on the molecular mechanisms behind these phenomena. Given the vital roles of lectins in communication and recognition processes, lectins could possibly fill the gaps in our understanding of interactions of probiotics with pathogens and the host.

1.3 Bacterial lectins

1.3.1 Bacterial lectins involved in the infection process of pathogens

Pathogenic and symbiotic microorganisms use the host's glycan distribution for specific host recognition, adhesion and tissue tropism (Bishop and Gagneux, 2006). The diversity of the host's cell surface glycans is therefore mirrored by the variety of lectin receptors displayed by microorganisms, including toxins, soluble lectins and lectin domains attached on pili and flagella (Audfray *et al.*, 2013). Extracellular macromolecules such as lectins, polysaccharides and appendages that take part in adhesion of the bacteria they cover are called adhesins. Adhesins are fundamental for both pathogenic and symbiotic microorganisms, since bacterial adhesion is an essential step for colonization and infection (Korea *et al.*, 2011). Therefore, exposed adhesins are potential targets for interference, to inhibit the initial stage of infection (Pieters, 2011). Of several clinically relevant pathogens, the current knowledge on lectin-like adhesins will be discussed.

1.3.1.1 *Escherichia coli*

E. coli is a commensal Gram-negative bacterium, generally asymptomatically present in healthy individuals (Köhler and Dobrindt, 2011). However, several pathogenic variants exist that cause different types of diseases due to different virulence traits, including

enterotoxigenic, enteroinvasive, diffuse adherent, enteroaggregative, Shiga toxin-producing (including enterohaemorrhagic) and enteropathogenic *E. coli* (Lai *et al.*, 2013). Extraintestinal *E. coli* (ExPEC) is able to colonize other niches than the intestine, causing urogenital infections, sepsis, neonatal meningitis, airway and wound infections. Generally, ExPEC strains do not cause intestinal disease, but they are excellent at colonizing the intestines (Klemm *et al.*, 2010). The distinction between commensal *E. coli* and ExPEC is not clear, as ExPEC often do not possess specific virulence factors that cannot be regarded as adaptation factors to a commensal lifestyle in the gut (Köhler and Dobrindt, 2011).

Lectin-like adhesins of *E. coli* are usually present on the tip of pili or fimbriae, such as type I, type P, type S, and type F1C fimbriae and were reported to recognize α-linked mannosides, galabiose, sialylated galactose such as 2-3 sialyllactose and GalNac-β-1-4-Gal epitopes respectively (Pieters, 2011). These lectins may play a role in tissue tropism by specific recognition of epithelial glycans, which is exemplified by the P fimbriae. These expose PapG adhesins which are galabiose (α-Gal-1-4-β-Gal) specific and associated with pyelonephritis, an infection affecting the kidneys (Pieters, 2011). The tissue tropism of different pathovars is further manifested in the different sugar affinity of type I fimbriae of different strains. Although all type I fimbriae bind trimannose, type I fimbriae of different strains have different monomannose affinities (Sokurenko *et al.*, 1998). For example, most intestinal isolates show a low mannomannose affinity, whereas UPEC isolates have an increased monomannose affinity (Sokurenko *et al.*, 1998). However, this variation in sugar affinity is probably not related to differences in the carbohydrate-binding pocket of the tip adhesin of type I fimbriae, FimH. Rather it appears to be caused by different interactions between FimH and the fimbrial shaft proteins, which could modify the conformational stability of the binding pocket (Duncan *et al.*, 2005). How protein-protein interactions outside the lectin domain can affect sugar affinity, is illustrated by a study of Le Trong and colleagues (2010). The authors demonstrated that upon shear stress, the conformation of FimH is changed which increases its affinity for mannose, whereas it is normally maintained in a low-affinity state. This would be advantageous by allowing *E. coli* to propagate and at the same time remain adhered in niches with mechanical force such as the urethra (Le Trong *et al.*, 2010). Recent research has revealed that the genomes of *E. coli* contain numerous operons for putative fimbriae (Korea *et al.*, 2011). It is therefore hypothesized that the repertoire of fimbrial adhesins could enable binding to a variety of specific sugars, which combined allow the colonization of various niches (Korea *et al.*, 2011).

1.3.1.2 *Salmonella enterica* serovar Typhimurium

S. enterica serovar Typhimurium is spread through contaminated food and water and causes diarrheal disease in humans and animals (Fabrega and Vila, 2013). The mortality rate of the self-limiting diarrhea caused primarily by *S.* Enteridis and *S.* Typhimurium is 0,1% in developed countries, but up to 24% in developing countries (Fabrega and Vila, 2013). A prerequisite to infection is overcoming the intestinal peristalsis and adhering to its target cells, namely enterocytes and immune cells such as M-cells and dendritic cells (Wagner *et al.*, 2014). The first stable contact between host and microbe can then initiate biofilm formation or injection of toxin-like proteins, a requirement for entering the host cell. Therefore, *S. enterica* has acquired various methods to adhere to the host as reviewed by Wagner and Hensel (2011), however the knowledge on the function of most is still incomplete. It is possible that some adhesins are specific for certain hosts, tissues or niches outside a mammalian host (Wagner *et al.*, 2014). Nevertheless, several adhesins have been reported to bind to specific receptors on the host cells in a lectin-like manner and one such important class is chaperone usher assembled fimbrial adhesins (Wagner and Hensel, 2011). There exist at least 15 operons coding for fimbrial adhesins that adhere synergistically. Many of these fimbriae are not expressed *in vitro* but there is evidence of their expression *in vivo* given their requirement for intestinal persistence in mice (Weening *et al.*, 2005)**.** Besides, Ledeboer *et al.* (2006) demonstrated through mutant analysis the critical role of several fimbrial operons for the formation of biofilms on chicken intestinal epithelium. As *E. coli*, *Salmonella* species also express type I fimbria with the tip lectin FimH, although this shows little homology to the *E. coli* FimH (Duncan *et al.*, 2005). This may correspond to the observation that FimH of *E. coli* preferentially bound to bladder epithelial cells, whereas FimH of *Salmonella* to enterocytes (Duncan *et al.*, 2005). However, even within one serovar, binding affinities of FimH variants can differ. Within *S.* Typhimurium, there exist high-adhesive and low-adhesive variants. The former have a higher affinity for human epithelial cells and recognize specifically enterocytes, whereas the latter can bind to both enterocytes and human bladder cells. Other type of fimbriae, such as the Std fimbriae bind to terminal α-(1-2)-fucose receptors on human colonic epithelial cells (Fabrega and Vila, 2013).

A non-fimbrial adhesin that also binds in a lectin-like manner is SiiE, a large protein of 600 kDa with 53 repeats of bacterial immunoglobin (BIg) domains, which is secreted by the type I secretion system (Wagner *et al.*, 2014). It is assumed that these BIg domains are structural and functional homologs of the monomers of fimbriae, including *E. coli* FimH (Wagner *et al.*,

2014). It specifically binds to glycostructures containing N-acetyl-D-glucosamine (GlcNac) and/or sialic acid on the apical side of enterocytes, mediating a stable adhesion with the microvilli of the brush border. This presumably allows *Salmonella* to inject effectors required to erase the brush border and initiate its uptake by the host cell (Wagner *et al.*, 2014). SiiE is present in most serovars, including Typhimurium, and this conservation suggests that SiiE is important for infections (Wagner and Hensel, 2011). Opposed to SiiE, BapA, another large BIg containing protein with similar polypeptide sequence, is involved in biofilm formation and in intercellular interactions leading to auto-aggregation (Wagner and Hensel, 2011). Nevertheless BapA also plays a role in adhesion as it was observed that BapA mutants display decreased colonization of intestinal epithelium (Fabrega and Vila, 2013). It is possible that BapA binds with other bacterial cells through interaction between O-antigen or teichoic acids and BIg domains, or with cellulose and polysaccharides present in biofilm matrix (Wagner *et al.*, 2014).

1.3.1.3 *Pseudomonas aeruginosa*

P. aeruginosa is an opportunistic pathogen that can be dangerous for immunosuppressed individuals (Perret *et al.*, 2005).and cystic fibrosis patients due to its ability to form biofilms that are hard to treat with antibiotics (Mitchell *et al.*, 2002). Furthermore, *P. aeruginosa* is a major hospital issue due to biofilm formation on intravascular and urinary catheters (Robijns, 2013). For some strains, effective antibiotics are lacking and the number of these multidrug resistant strains is increasing (Breidenstein *et al.*, 2011). *P. aeruginosa* is one of the bacterial species with the best studied lectin molecules. *P. aeruginosa* possesses a spectrum of lectins, both excreted such as lectin-like bacteriocins (Ghequire and De Mot, 2014) and exposed on its pili, flagella and cell surface (Perret *et al.*, 2005). *P. aeruginosa* recognizes GalNac-β-1-4Gal epitopes, a part of a glycosphingolipid in the lungs, with type IV pili (Pieters, 2011). Also two lectins found on the outer membrane named LecA and LecB, which bind galactosides and fucosides respectively, are adhesion factors and take part in biofilm formation (Tielker *et al.*, 2005; Diggle *et al.*, 2006). LecA and B are cytotoxic and make the alveolar barrier more permeable, causing severe injury (Pieters, 2011). It has already been demonstrated, in a murine model for acute lung infection, that inhibiting these lectins with their glycan ligand reduces lung injury, bacterial load and bacterial dissemination into the bloodstream (Chemani *et al.*, 2009). Therefore, molecules competing with LecA and LecB could prevent adhesion and biofilm formation by *P. aeruginosa*.

1.3.1.4 *Staphylococcus aureus*

S. aureus is a Gram-positive commensal bacterium responsible for a variety of both superficial and invasive infections, including sepsis, endocarditis and pneumonia (Foster *et al.*, 2014). *S. aureus* possesses numerous lectin-like adhesins, extracellular enzymes and toxins that contribute to its virulence. The adhesins are covalently attached to its cell wall or surface-associated via hydrophobic or ionic interactions. *S. aureus* has adhesins with affinities for various glycoproteins, including fibronectin, fibrinogen, vitronectin, thrombospondin, bone-sialoprotein, collagen and von Willebrand factor, localized in extracellular matrix and blood plasma (Heilmann, 2011). However, the functions and ligands of many surface proteins still has to be elucidated and probably more ligands are yet to be discovered (Foster *et al.*, 2014). Recently, the structure of the cell wall protein SraP (serine-rich adhesion for binding platelets), which is involved in endocarditis, was characterized (Yang *et al.*, 2014). It contains a lectin module that preferentially binds N-acetylneuraminic acid (NeuNac) (Figure 1.3). Although the sequence similarity with legume lectins is less than 20%, the structural resemblance is indicative of an L-type lectin. In addition, SraP was shown to contribute to adhesion and invasion of human lung epithelial cells. Based on bioinformatics analysis, it is hypothesized that this adhesion mechanism also takes place in other *Staphylococci* and *Streptococci* (Yang *et al.*, 2014).

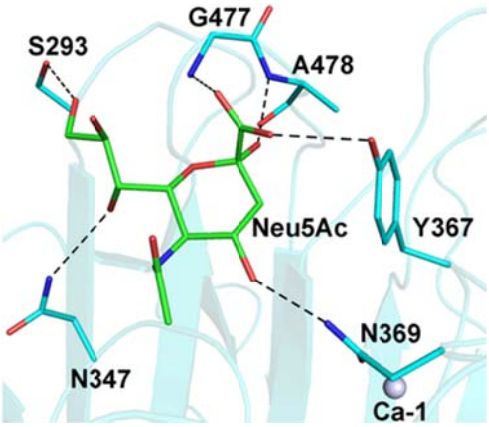

Figure 1.3 *S. aureus* SraP binding to NeuNac. A docking model shows the interactions between NeuNac (green) and the L-type lectin domain of SraP (blue). Polar interactions are represented by dashed lines. Adapted from Yang *et al*. (2014).

1.3.2 The lectins of probiotic bacteria

Probiotic bacteria possess cell surface associated molecules, including lectins that may interact with host cells and pathogens and are therefore hypothesized to be involved in pathogen exclusion. Lectins could reduce a pathogen's virulence 1) by blocking receptors on host cells which are recognized by the pathogen or 2) by directly binding to the pathogen or its secreted products, influencing its adhesion and invasion (Petrova *et al*, 2013) and potentially its abilities to aggregate or form biofilms. However, very little is known on lectin-like proteins expressed by lactobacilli (Petrova *et al,* 2013). For example it has been reported that *L. plantarum* can adhere to intestinal epithelial cells in a mannose-specific manner and the supposedly key gene responsible for this interaction has been identified in *L. plantarum*

WCFS1 and termed *msa* (mannose-specific adhesin) (Pretzer *et al.*, 2005). Comparison of the *msa* gene and mannose-adhesion among several *L. plantarum* strains has pointed out that mannose-binding capacities vary, presumably due to variations in domain composition rather than gene expression of *msa* or other adhesion factors (Gross *et al.*, 2010). However, no correlation has been made between domain variation and mannose-binding capacity so far (Gross *et al.*, 2010). It is hypothesized that small differences in genetic sequence, leading to differences in specific amino acids, might cause the varying mannose-binding capacity. Also the genetic background of the strains (e.g. different cell wall characteristics) might be involved in the functioning of Msa (Gross *et al.*, 2010). In a study of 11 *msa* negative *L. plantarum* strains by Guidone *et al*. (2014), 64% was shown to agglutinate yeast cells. Also Turchi *et al*. (2013) reported that out of 21 *L. plantarum* strains able to agglutinate yeast cells, only 5 contained the *msa* gene. Therefore other gene products besides Msa may be involved in mannose-adhesion. Overall, the exact function(s) of Msa for *L. plantarum* remains to be determined.

Interestingly, many mucus-binding domains have been identified in proteins of lactic acid bacteria (Boekhorst *et al.*, 2006). One of the pilin proteins of *L. rhamnosus* GG, SpaC, as well as the MBF are established mucus-binding proteins (von Ossowski *et al.*, 2011a). Given that mucus is composed of highly glycosylated proteins (Strous and Dekker, 1992), it is plausible that these proteins are in fact lectins. However, the binding mechanism is not yet elucidated. Increased knowledge of lectins could decipher the complex interactions between microbiota and pathogens and the host and could improve the application of probiotics. In this thesis, several lectins of three *Lactobacillus* strains were investigated, namely *L. plantarum* CMPG5300, *L. rhamnosus* GG and *L. rhamnosus* GR-1.

1.3.2.1 Cell surface attached lectin Cmpg5300_05.29 of *L. plantarum* CMPG5300

L. plantarum CMPG5300 is a natural vaginal isolate with probiotic potential due to its high auto-aggregation capacity and adherence to vaginal epithelial cells (Malik *et al.*, 2013). These properties have been attributed to sortase-dependent proteins (SDPs). SDPs are surface associated proteins containing a C-terminal LPxTG motif, which is cleaved and subsequently covalently linked to the peptidoglycan by a sortase enzyme. At least 30 putative SDPs are encoded in the genome of *L. plantarum* CMPG5300, including seven putative mucus-binding proteins, four putative collagen-binding proteins and one putative mannose-specific adhesion. This last is called Cmpg5300_05.29 and is 62% identical to the Msa of *L. plantarum* WCFS1. It was annotated to contain an N-terminal L-type lectin domain, followed by one type I

mucus-binding domain and four type II mucus-binding domains (Figure 1.4a). The lectin domain of this protein was purified and shown to bind mannose and the gp120 capsid protein of HIV (Malik, 2014). Therefore, Cmpg5300_05.29 may be involved in the aggregation and adhesion capacities of this strain as well as interactions with its environment, which is investigated in this thesis.

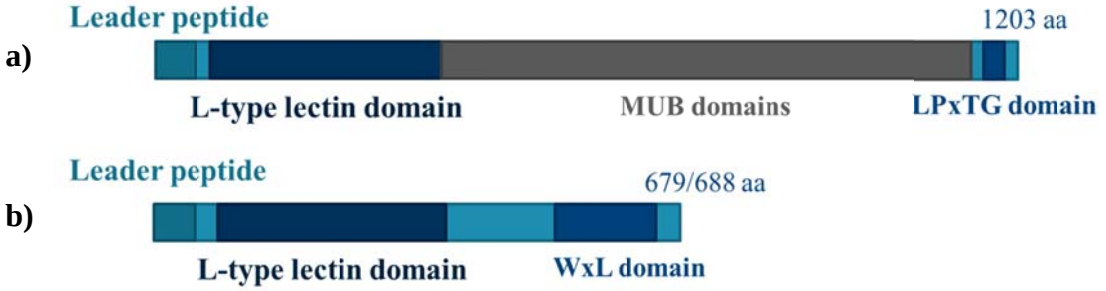

Figure 1.4 Protein domain organization of cell surface attached lectin and lectin-like proteins of *Lactobacillus* species. a) Cmpg5300_05.29 of ***L. plantarum*** CMPG5300 and b) LGG_Llp1, LGG_Llp2 and LGR-1_Llp1 of *L. rhamnosus* GG and *L. rhamnosus* GR-1. All contain L-type lectin domains and Cmpg5300_05.29 also mucus-binding (MUB) domains. The N-terminal domains contain signal leader peptides responsible for transport out of the cell, whereas the C-terminal domains cause the covalent attachment (LPxTG) or non-covalently anchoring (WxL) on the cell wall. Adapted from Malik (2014) and Petrova (2013).

1.3.2.2 Lectin-like proteins of *Lactobacillus rhamnosus* GG and GR-1

L. rhamnosus GG and GR-1 are respectively an intestinal and vaginal probiotic strain. *L. rhamnosus* GG was originally selected as a probiotic because of its resistance to acid and bile, strong adhesion to intestinal epithelium, good growth and production of antimicrobial substances (Doron *et al.*, 2005). Now it is one of the most used probiotic strains, present in numerous food products (Doron *et al.*, 2005). The molecular mechanisms underlying its high adhesion is well established and includes the use of pili that create a zipper-like tight adhesion, supported by other adhesins such as MabA and MBF (Segers and Lebeer, 2014). *L. rhamnosus* GR-1 has been known for decades for its high adhesion capacity to uroepithelial and vaginal cells (Reid and Bruce, 2001) and its persistence in the vagina (Gardiner *et al.*, 2002). This strain is also bile-resistant, survives passage through the GIT and could be formulated in yoghurt (Hekmat *et al.*, 2009). As indicated above, several studies have shown the ability of *L. rhamnosus* GR-1 to inhibit urogenital pathogens and potentially cure BV. When the genomes of these strains were sequenced, genes with potential lectin activity were identified and named lectin-like proteins (Llps) (Petrova, 2013). The genes under investigation in this thesis are called LGG_Llp1 and LGG_Llp2 for Llps of *L. rhamnosus* GG and LGR-1_Llp1 for an Llp of *L. rhamnosus* GR-1. All corresponding proteins contain an N-terminal signal leader peptide indicating its secretion, followed by an L-type lectin domain

and a C-terminal WxL motif assumed to keep the protein at the bacterial surface through non-covalent interactions (Brinster *et al.*, 2007) (Figure 1.4b). LGG_Llp1 and LGR-1_Llp1 show 98% amino acid similarity and they differ only in 4 amino acids in the L-type lectin domain. LGG_Llp1 and LGG_Llp2 share only 35% of their amino acid sequences, implying they may have evolved from a gene duplication event and subsequent diversification (Petrova, 2013). Petrova (2013) observed that LGR-1_Llp1 is involved in *in vitro* adhesion of *L. rhamnosus* GR-1 to vaginal epithelial cells (VK2/E6E7), but not to cervical (Hela) and colon carcinoma (Caco-2) cells, and could therefore contribute to this strain's tissue tropism. On the contrary LGG_Llp1 and LGG_Llp2 were shown to contribute to *L. rhamnosus* GG adhesion capacity to all these tissues. In addition, *L. rhamnosus* GR-1 has been shown to reduce adhesion of *C. albicans,* which appeared not to be related to Llp1. Since the Llp's bind to specific receptors on epithelial cells, it is possible that they compete with adhesion factors of other pathogens, thereby excluding them (Petrova, 2013).

The studies of Malik (2014) and Petrova (2013) as well as the prevalent role of surface-associated factors as probiotic effector molecules (Lebeer *et al.*, 2008), suggest several roles for surface attached lectins in the interaction of lactobacilli with their environment. These are depicted in Figure 1.5. However, the exact functional roles as well as the exact ligands of these proteins remain to be elucidated, which will be investigated in this thesis.

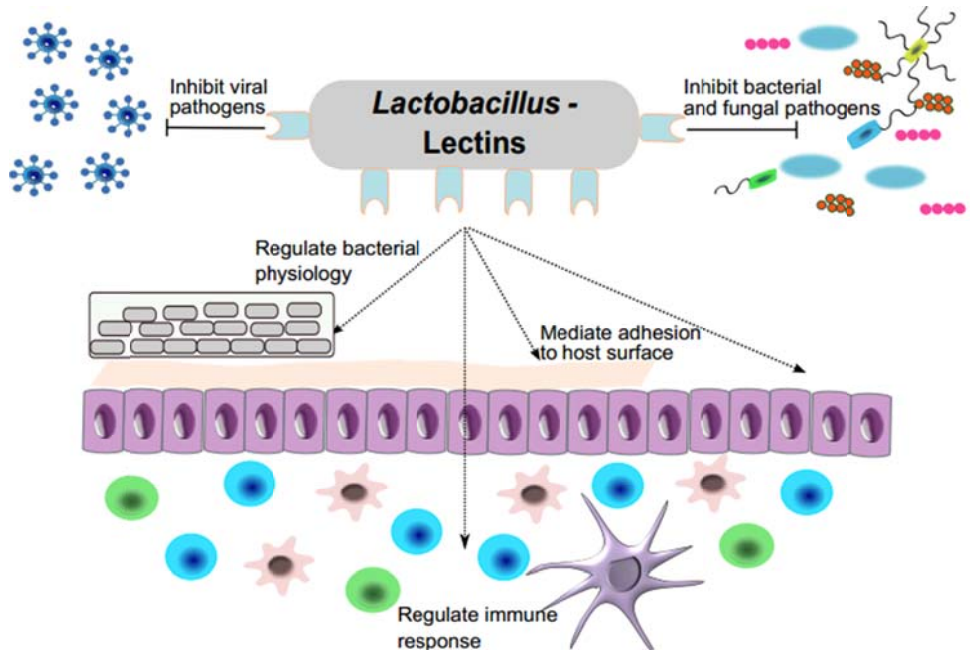

Figure 1.5 Potential roles of *Lactobacillus* lectins in interactions with pathogens and the host in the GIT or the vagina. The lectins may bind and thereby exclude viruses, bacteria and fungi from adhering to the host. In addition, the *Lactobacillus* strains use lectins to adhere to the host surface. This interaction may trigger immune responses in the host. Besides, some lectins such as Cmpg5300_05.29 are known to be involved in physiological processes of lactobacilli, such as auto-aggregation and biofilm formation. Adapted from Petrova *et al*, 2013.

1.3.3 Bacterial extracellular polysaccharides as adhesion factors and lectin targets

Lectins are for all bacteria described important for interactions with host cells, bacterial cells and extracellular matrix, which enables adherence, colonization and biofilm formation. Also surface glycans may contribute to these interactions and therefore could be interesting lectin targets. In fact, many bacteria produce and secrete a spectrum of extracellular polysaccharides (EPS), which can become covalently attached to the cell via a phosphodiester bond with lipid A or phospholipids, then called capsular polysaccharides. It does not have to be anchored to the cell and can be found as "free EPS" or slime. Besides their protective role, EPS are involved in adhesion to various both biotic and abiotic surfaces, including other cells, which allows auto- or co-aggregation. These interactions are established by van der Waals forces, covalent or ionic bonding, steric interactions and hydrophobic associations. Bacterial species can differ tremendously in their capsular polysaccharides, as an illustration, 80 capsular serotypes have been observed for *E. coli* (Bazaka *et al.*, 2011).

Lipopolysaccharides (LPS) in the outer membrane of Gram-negative bacteria consist of a conserved lipid A anchor and a core oligosaccharide, followed by a highly variable polysaccharide, termed the O-antigen. For example, *S. enterica* is divided into 67 serogroups based on its O-antigen (Fabrega and Vila, 2013). All this variation has implications for the strain's ability to interact with the host, including its susceptibility to chemicals and its virulence towards host's cells. The abundant LPS also play an important role in adhesion to surfaces. For example, hydrogen bonds established between the O-antigen and inorganic molecules enable the colonization of these surfaces and also promote aggregation with other cells (Bazaka *et al.*, 2011). Although there have been several indications that LPS on Gram-negative bacteria can bind to epithelium and the covering mucus, there is no conclusive proof of a mucosal lectin binding bacterial carbohydrates (Ofek *et al.*, 2013). Gram-positive bacteria contain teichoic and lipoteichoic acid, which are also involved in adhesion and biofilm formation (Pieters, 2011). For example, *S. aureus* exposes polysaccharide intercellular adhesion (PIA) molecules, which is linear β-1,6-N-acetylglucosaminoglycan containing 15-20% non-acetylated residues. Due to its positive charge, it mediates intercellular interactions probably via electrostatic attraction with negatively charged teichoic acid (Pieters, 2011). Also in *C. albicans*, cell wall carbohydrates may play a role in adhesion although the most important effectors of adhesion are generally assumed to be proteins. The *C. albicans* cell wall is for 90 % composed of carbohydrates (Gow and Hube, 2012), which are mainly

mannans, chains of hundreds of mannose residues, post-translationally added to cell wall proteins (Ferrreira *et al.*, 2012).

By competing with the pathogen's adhesins for common ligands or by binding to the pathogens surface glycans, exogenously applied lectins could interfere with adhesion, aggregation and biofilm processes and therefore have therapeutic/preventive potential. Lectins derived from the human microbiota or probiotics may fulfill this role, as they by nature compete with these pathogens regarding adhesion and persistence in the host.

1.4 Applications of lectins

Knowledge of lectins has been used to prevent infections by targeting pathogenic lectins with glycans or glycomimetic compounds to impair microbial colonization (Virji, 2009). Blocking bacterial adhesins is a strategy used by the human host in several ways. For example, the major receptor of intestinal pathogens *E.* coli and *Salmonella* (CEA) is shed in large quantities in the faces to block binding of these bacteria to the actual receptors on the epithelial cells (CEACAMs, CEA-related cell adhesion molecules) and is therefore considered as part of the innate immune system (Virji, 2009). Also the complex sugars in breast milk protect the infant in a similar manner against intestinal infections, again by lowering the pathogen load. Moreover, a connection has been demonstrated between the mother's genes encoding the machinery to produce several oligosaccharides and the occurrence of infant's diarrhea (Perret *et al.*, 2005). These examples of evolutionary strategies against pathogens confirm the prophylactic potential of artificial glycans or peptides that block bacterial adhesins. Several studies in multiple animal models have supported the use of saccharides in preventing experimental infections, but clinical studies have had limited success (Ofek *et al.*, 2013).

Similarly, bacterial adhesion to epithelial cells may be prevented by blocking the host receptors with adhesin analogs such as lectins (Ofek *et al.*, 2013). *In vitro* studies have reported that peptides mimicking an adhesin can inhibit pathogenic adhesion (Ofek *et al.*, 2013). However, they may interfere with the natural functions of the receptors and thereby possibly create side effects, which needs to be investigated (Virji, 2009). Instead of using external/exogenous blocking agents, it would probably be more efficient to block adhesins with antibodies produced by the host. Nevertheless, immunization with the adhesion requires knowledge of the binding mechanisms of the bacteria to the host and requires the adhesion to be invariant. Therefore vaccines have only been successful against FimH and PapG of pathogenic *E. coli* in animal models (Virji, 2009).

External lectins are also attractive candidates as prophylactic or therapeutic agents against viruses, by blocking the viral glycoproteins that a lot of enveloped viruses such as HIV and Influenza use to initiate infection (Balzarini, 2007). A great advantage of this antiviral approach is that the virus is forced to mutate its exposed glycans. This might uncover epitopes recognized by the immune system. So the use of lectins has a dual antiviral mechanism: blocking entry and transmission of the virus and forcing the virus to become more susceptible for the immune system (Balzarini, 2007). For example, the mannose-specific lectin Griffithsin, derived from red algae *Griffithsin* sp., has been shown to inhibit HIV-1 and -2 and HSV-2 at picomolar concentrations. Combining several lectins even increases the antiviral effect (Ferir *et al.*, 2012). Similarly, Msa from *L. plantarum* WCFS1 may be interesting for prevention and/or treatment of pathogens that exhibit mannose on their surface, for example HIV and *C. albicans* (Petrova *et al.*, 2013b).

Finally, lectins can be used as tools for the detection of glycosylated proteins and lipids and therefore for cytochemistry and histochemistry. They are less costly than antibodies and possess specificity for a range of complex carbohydrates. They enable the study of cellular surfaces, as in tissue identification, blood typing and the study of cellular differentiation or malignant transformation into tumors (Akimoto & Kawakami, 2014). Lectins can also replace antibodies in a western blot analysis of glycoproteins. Another emerging field for the use of lectins is glycomics, the study of the whole collection of glycans, glycopeptides and glycolipids produced by a cell, related to its environment. A lectin microarray is a high-throughput technique to examine multiple glycan-lectin interactions in order to determine the glycome (Hirabayashi, 2014).

Chapter 2

Materials and methods

2 Materials and methods

2.1 Microbial strains and growth conditions

The bacterial and fungal strains used in this thesis are listed in Table 2.1. The *Lactobacillus* wild type strains and the corresponding mutants were routinely grown non-shaken in de Man Rogosa Sharp (MRS) medium (Difco) at 37°C (De Man *et al.*, 1960). *Escherichia coli* strain TG1 and strain UTI89*, Staphylococcus aureus* strain Rosenbach and strain SH1000*, Pseudomonas aeruginosa* PA14 and *Salmonella enterica* serovar Typhimurium ATCC14028 were grown in Luria Bertani (LB, Table 2.2) medium with aeration at 37°C (Sambrook *et al.*, 1989). *Candida albicans* SC5314 and *Saccharomyces cerevisiae* BY4741 were grown in yeast-extract peptone dextrose (YPD) medium (Table 2.2) under aerobic conditions at 37°C. If required, kanamycin was used for *E. coli* BL21 (DE3) at a concentration of 50 µg/ml. During biofilm and bioscreen experiments, *E. coli* TG1 and UTI89, *S.* Typhimurium ATCC14028 and *P. aeruginosa* PA14 were grown in 1/20 diluted tryptic soy broth (TSB, Gibco) and *S. aureus* Rosenbach and SH1000 in undiluted TSB (Table 2.2).

Table 2.1. Strains and plasmids used in this study

Strain	Genotype/ description	Reference/ source
***Lactobacillus* strains**		
L. plantarum CMPG5300	Wild type, human vaginal isolate	Malik *et al.*, 2013
L. plantarum CMPG11201	*cmpg5300_05.29* knock-out mutant of *L. plantarum* CMPG5300, *cmpg5300_05.29::Cm*^{R}	Malik, 2014
L. rhamnosus GG (ATCC 53103)	Wild type, faecal isolate	Kankainen *et al.,* 2009
L. rhamnosus CMPG10701	*llp1* knock-out mutant of *L. rhamnosus* GG, *llp1::tet*^{R}	Petrova, 2013
L. rhamnosus CMPG10706	*llp2* knock-out mutant of *L. rhamnosus* GG, *llp2::ery*^{R}	Petrova, 2013
L. rhamnosus CMPG10707	*llp1-llp2* double knock-out mutant of *L. rhamnosus* GG, *llp1::tet*^{R}*; llp2::ery*^{R}	Petrova, 2013
L. rhamnosus GR-1 (ATCC 55826)	Wild type, female urethra isolate	Chan *et al.*, 1985; Reid, 1999
L. rhamnosus CMPG10744	*llp1* knock-out mutant of *L. rhamnosus* GR-1, *llp1::tet*^{R}	Petrova, 2013

Strain	Genotype/ description	Reference/ source
Pathogens		
Candida albicans SC 5314 (ATCC MYA-2876)	Wild type, human clinical isolate	Gillum *et al.,* 1984
Escherichia coli UTI89	Wild type, clinical isolate	Hunstad *et al.,* 2005
Escherichia coli MG1655	Wild type	Blattner *et al.*, 1997
Escherichia coli TG1	Wild type	Sakamoto *et al.,* 1994
Pseudomonas aeruginosa PA14	Wild type, human isolate	Rahme *et al.,* 1995
Staphylococcus aureus SH1000	*rsbU* positive derivative of *S. aureus* 8325-4	Horsburgh *et al.,* 2002
Staphylococcus aureus Rosenbach (ATCC 33591)	Wild type, clinical isolate	ATCC
Salmonella enterica serovar Typhimurium ATCC 14028	Wild type, isolated from chicken tissue	ATCC (Fields *et al.*, 1986)
S. Typhimurium ATCC 14028 carrying pFPV25.1	Mutant constitutively expressing GFP	Robijns, 2014
Other		
Saccharomyces cerevisiae BY 4741	*MATa*; *his3Δ1; leu2Δ0, met15Δ0; ura3Δ0*	Brachmann *et al.,* 1998
E. coli Top10	F' (*lacI*^{q}, Tn^r) *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*LacZ*ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(St^r) *end*A1 *nup*G	Invitrogen
E. coli BL21 (DE3)	*E. coli* B F- *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ (DE3)	Invitrogen
CMPG11209	*E. coli* BL21 (DE3) carrying the overexpression plasmid pCMPG11209 for secretion of N-His$_6$ tagged lectin domain of *cmpg5300_05.29* of *L. plantarum* CMPG5300, Km^R	Malik, 2014
CMPG11210	*E. coli* BL21 (DE3) carrying the overexpression plasmid	Malik, 2014

Strain	Genotype/ description	Reference/ source
	pCMPG11210 for secretion of N-His_6 tagged lectin domain of *msa* of *L. plantarum* WCFS1, Km^R	
CMPG10708	*E. coli* BL21 (DE3) carrying the overexpression plasmid pCMPG10708 for secretion of N-His_6 tagged Llp1 protein of *L. rhamnosus* GG, Km^R	Petrova, 2013
CMPG10709	*E. coli* BL21 (DE3) carrying the overexpression plasmid pCMPG10709 for secretion of N-His_6 tagged Llp2 protein of *L. rhamnosus* GG, Km^R	Petrova, 2013
CMPG10712	*E. coli* BL21 (DE3) carrying the overexpression plasmid pCMPG10712 for secretion of N-His_6 tagged Llp1 lectin-like domain from *L. rhamnosus* GG, Km^R	Petrova, 2013
CMPG10713	*E. coli* BL21 (DE3) carrying the overexpression plasmid pCMPG10713 for secretion of N-His_6 tagged Llp2 lectin-like domain from *L. rhamnosus* GG, Km^R	Petrova, 2013
CMPG10774	*E. coli* BL21 (DE3) carrying the overexpression plasmid pCMPG10774 for secretion of N-His_6 tagged Llp1 protein of *L. rhamnosus* GR-1, Km^R	This study
CMPG10775	*E. coli* BL21 (DE3) carrying the overexpression plasmid pCMPG10775 for secretion of N-His_6 tagged Llp1 lectin-like domain from *L. rhamnosus* GR-1, Km^R	This study
Plasmids		
pET28 (a+)	Km^R, T7 lac, N and C-terminal His Tag	Novagen
pCMPG00685/686	pET28 (a+) derivative carrying the N-His_6 tagged lectin-like domain of the *llp2* gene of *L. rhamnosus* GG in the *Sal*I/*Not*I site, Km^R	This study
pCMPG00685/707	pET28 (a+) derivative carrying the N-His_6 tagged lectin-like domain with 14 amino acids extension of the *llp2* gene of *L. rhamnosus* GG in the *Sal*I/*Not*I site, Km^R	This study
pCMPG00685/621	pET28 (a+) derivative carrying the N-His_6 tagged lectin-like domain with 22 amino acids extension of the *llp2* gene of *L. rhamnosus* GG in the *Sal*I/*Not*I site, Km^R	This study
pFPV25.1	(promotor of *rpsM)-gfpmut3*, *mob*, Ap^R	Valdivia and Falkow, 1996

Table 2.2 Growth media used in this thesis

Medium	Component	Concentration
LB	Sodium chloride	1%
	Peptone	1%
	Yeast extract	0.5%
MRS	Proteose peptone No. 3	10.0 g/l
	Beef extract	10.0 g/l
	Yeast extract	5.0 g/l
	Dextrose	20.0 g/l
	Polysorbate 80	1.0 g/l
	Ammonium citrate	2.0 g/l
	Sodium acetate	5.0 g/l
	Magnesium sulfate	0.1 g/l
	Manganese sulfate	0.05 g/l
	Dipotassium phosphate	2.0 g/l
YPD	Peptone	2%
	Yeast Extract	1%
	Glucose	2%
TSB	Bacto™ Tryptone	17.0 g/l
	Bacto Soytone	3.0 g/l
	Glucose	2.5 g/l
	Sodium chloride	5.0 g/l
	Dipotassium hydrogen phosphate	2.5 g/l

2.2 Growth assay of lactobacilli and pathogens

To investigate a potential role of the putative lectin proteins in sugar metabolism, the growth of *L. rhamnosus* GG, *L. rhamnosus* GR-1 and *L. plantarum* CMPG5300 and the corresponding knock-out mutants of the lectin genes was compared, while growing on various sugars. The assay was performed in 100-well microtiter plates (Honeycomb, Oy Growth Curves Ab Ltd.). The sterile wells were supplied with 300 µl modified MRS, in which the glucose (2 g/l) is replaced by one of the sugars of interest, namely D-glucose, D-fucose, D-lactose, D-mannose, GlcNac (Sigma-Aldrich) and D-sorbitol (VEL). A 4.5/100000 dilution of overnight culture of *L. rhamnosus* GG, *L. rhamnosus* GR-1, *L. plantarum* CMPG5300 and their knock-out mutant strains CMPG11201, CMPG10701, CMPG10706, CMPG10707 and CMPG10744 was added to the wells. The microtiter plate was incubated for 3 days at 37°C without agitation in a Bioscreen (Oy Growth Curves Ab Ltd.), which measured the OD at 600 nm every 10 minutes. Each bacterial strain and sugar was tested in triplicate and each experiment was performed at least three times.

In order to verify whether the lectins of lactobacilli affect the growth of pathogens, bioscreen experiments were performed. Overnight cultures of *S. aureus* SH1000, *S. aureus* Rosenbach, *E. coli* UTI89, *E. coli* TG1 and *S.* Typhimurium ATCC14028 were 200-fold diluted in TSB

(for SH1000, Rosenbach, TG1) or 1/20 TSB (for UTI89 and ATCC14028) and 200 µl was added to sterile wells of 100-well microtiter plates (Honeycomb, Oy Growth Curves Ab Ltd). The purified lectin domains were added at concentration 50 and 200 µg/ml (Table 2.3) except for the negative controls, to which no lectins were supplied. These were incubated at 25°C (for strains TG1 and ATCC14028) or 37°C (for strains SH1000, Rosenbach and UTI89) for 3 days under continuous shaking in a Bioscreen (Oy Growth Curves Ab Ltd), which measured the OD at 600 nm every 10 minutes to monitor the growth. TSB and 1/20 TSB were used as blanks. Each strain and lectin domain concentration was tested in triplicate.

Table 2.3 Concentrations (molarity) of purified lectin domains used in this study

	5 µg/ml	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**200 µg/ml**
Cmpg5300_05.29	0.169 µM	0.337 µM	1.69 µM	3.37 µM	6.74 µM
LGG_Llp1	0.144 µM	0.287 µM	1.44 µM	2.87 µM	5.75 µM
LGR-1_Llp1	0.143 µM	0.287 µM	1.43 µM	2.87 µM	5.74 µM
LGG_Llp2	0.155 µM	0.310 µM	1.55 µM	3.10 µM	6.21 µM

2.3 Optimizing the construct of the lectin domain of Llp2 of *L. rhamnosus* GG

As the recombinant expression of the available construct of the lectin domain of LGG_Llp2 resulted in low protein concentrations (Petrova, 2013), different genetic constructs of the lectin domain were developed, containing different extensions of the predicted lectin domain (Figure 2.1). Hereto, the *LGG_RS02750* gene of *L. rhamnosus* GG was amplified using polymerase chain reaction (PCR) and the primers listed in Table 2.4**Table 2.4**. The different gene fragments of *LGG_RS02750* were cloned into pET28a(+) vector (Novagen), which was subsequently transformed in *E. coli* Top10 cells. After overnight incubation at 37°C, the clones resistant to Kanamycin were verified for containing the construct by PCR using primers S&P-0044 and S&P-0045 (Table 2.4). The plasmids were purified from Top10 cells using a QiaPrep® spin miniprep kit (Qiagen) and transformed into competent *E. coli* BL21 (DE3). Again clones resistant to Kanamycin were selected and tested for carrying the correct constructs. The correct colonies carrying the different variants of the lectin domain of

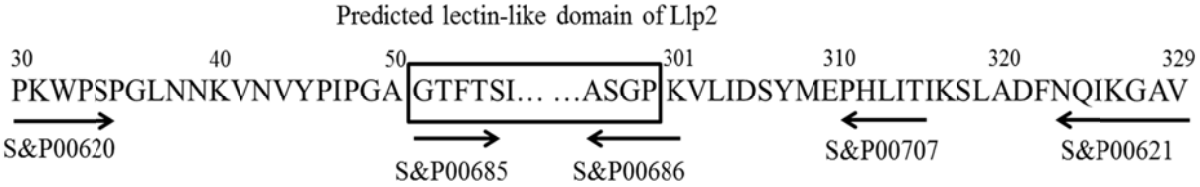

Figure 2.1 Primary structure of part of the LGG_Llp2 protein comprising the predicted lectin-like domain (amino acids 51-300). Primers are indicated with arrows and when combined lead to a variety of constructs of the lectin-like domain with different extensions upstream and downstream.

LGG_Llp2 were subjected to protein induction and detection by using Western blotting as described below (see 2.4).

Table 2.4 List of primers used in this thesis

Primer	Sequence	Restriction site	Remarks
S&P-00620	ATGTCGACAACCAAAATGGCCAAGCCC	*Sal*I	Forward primer upstream of lectin-like domain of *llp2* gene
S&P-00685	ATGTCGACAAGGGACGTTCACTAGCAT	*Sal*I	Forward primer lectin-like domain of *llp2* gene
S&P-00686	ATGCGGCCGCTTATTTAGGGCCGGATG CA	*Not*I	Reverse primer lectin-like domain *llp2* gene
S&P-00707	ATGCGGCCGCTTAGGTAATGAGATGCG GTT	*Not*I	Reverse primer downstream of lectin-like domain of *llp2* gene
S&P-00621	ATGCGGCCGCTTATACGGCGCCTTTAAT TTGATT	*Not*I	Reverse primer downstream of lectin-like domain of *llp2* gene
S&P-0044	TGGCAGCAGCCAACTCAGCTT	/	Reverse primer for MCS of pET28a(+)
S&P-0045	TATAGGCGCCAGCAACCGCA	/	Forward primer for MCS of pET28a(+)

2.4 Production, purification and quantification of recombinant proteins

2.4.1 Production of recombinant lectins and lectin domains

The recombinant *E. coli* BL21 (DE3) (Table 2.1) expressing the full length lectins or the corresponding lectin domains of Cmpg5300_05.29, LGG_Llp1, LGG_Llp2 and LGR-1_Llp1 were grown overnight in LB with 50 µg/ml Kanamycin. Due to its size (1203 amino acids), it was not possible to express the full length lectin Cmpg5300_05.29, therefore only the lectin domain of this protein was used. Each culture was diluted 100-fold in 1 or 2 L LB (depending of the experiment) with Kanamycin and grown for 2 to 3 hours at 37°C under shaking conditions until an optical density (OD) (595 nm) between 0.3 and 0.4 was reached. Then the production of recombinant protein was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and the cultures were incubated at 25°C under shaking. Cmpg5300_05.29 was produced overnight, whereas *E. coli* BL21 (DE3) strains expressing

LGG_Llp1, LGG_Llp2 and LGR-1_Llp1 were grown until an OD of 0.8 to prevent the formation of inclusion bodies, which was about 4 hours until the OD reached 0.8 to 1. Hereafter the cells were pelleted and incubated overnight at -80°C. The next day, the pellets were suspended in 20 ml of non-denaturing lysis buffer (Table 2.5) per liter of original culture and incubated for 30 minutes at room temperature while swirling. Subsequently the cell lysate was sonicated during 4 minutes in cycles of 30 sec on and 30 sec off (amplitude 18%) to release the soluble recombinant lectin from the cells.

Table 2.5 Buffers used in this thesis

Paragraph	Buffer	Component	Concentration
2.4.1	Non-denaturing lysis buffer (pH 8.0)	Sodium dihydrogen phosphate	50 mM
		Sodium chloride	300 mM
		Imidazole	10 mM
2.4.2	Running/washing buffer (pH 8.0)	Sodium dihydrogen phosphate	50 mM
		Sodium chloride	300 mM
		Imidazole	20 mM
	Elution buffer (pH 8.0)	Sodium dihydrogen phosphate	50 mM
		Sodium chloride	300 mM
		Imidazole	250 mM
2.4.3	MOPS buffer (pH 7.7)	Morpholinepropanesulfonic acid	50 mM
		Tris base	50 mM
		Sodium dodecyl sulfate	0.1%
		EDTA	1 mM
	Coomassie staining solution	Coomassie Brilliant Blue R-250	0.2%
		Methanol	45%
		Acetic acid	1%
	TBSt20 (pH 7.5)	Tris base	50 mM
		Sodium chloride	0.9%
		Tween20	0.05%
2.5.1	PBS(T)	Dipotassium phosphate	1.24 g/l
		Monopotassium phosphate	0.39 g/l
		Sodium chloride	8.8 g/l
		(Tween20)	500 µl/l
	Carbonate-bicarbonate buffer (pH 9.6)	Sodium carbonate	64 mM
		Sodium hydrogen carbonate	50 mM
	ELISA substrate buffer (pH 10.6)	Magnesium dichloride hexahydrate	50 nM
		Sodium hydrogen carbonate	35 mM
		Sodium carbonate	23.4 mM
2.5.2	Binding buffer (pH 6.0)	MES	25 mM
		Sodium chloride	25 mM
		Polyvinylalcohol	1%
	Wash buffer (pH 6.0)	MES	25 mM
		Sodium chloride	25 mM

2.4.2 Purification

The full length lectins or the corresponding lectin domains were purified from the cell lysate using affinity chromatography. Hereto, the lysate was first filtered with 0.2 µM filters. The filtered lysate was run through a HisTrap™ HP column (GE Healthcare), which contains Nickel ions embedded in a matrix of sepharose. The full length lectins or the corresponding lectin domains contain N-terminal His tags that bind to these ions and therefore they remain on the columns while other proteins run through using a running/washing buffer (Table 2.5). The lectin (domain) was eluted using an elution buffer (Table 2.5). Different fractions were collected and sample purity was analyzed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see 2.4.3). The lectin (domain) was further purified from the eluted sample using size exclusion chromatography. Hereto the sample was applied on a Highload™ 16/60 column packed with a matrix of Superdex™ prep grade (GE Healthcare). Fractions containing the lectin (domain) were collected, analyzed using SDS-PAGE, pooled together and concentrated.

2.4.3 SDS-PAGE and Western blot

To verify if the eluted fractions contained the pure lectin (domain), the proteins in each fraction were separated by SDS-PAGE in Bolt 12% Bis-Tris Plus gels (Life sciences). The protein samples were incubated for 5 min at 95°C in loading buffer (Fermentas, Life sciences) containing SDS and reducing agent and loaded onto the wells of the protein gel. The gel was run submerged in morpholinepropanesulfonic acid (MOPS) buffer (Table 2.5) for 45 minutes at 400 mA and 200 V. Hereafter the gel was used for a Western blot or stained with Coomassie Brilliant Blue R-250 (Table 2.5) (Bio Rad) or Sypro® Ruby protein gel stain (Invitrogen).

For performing the Western blot, the proteins from the gel were transferred to a polyvinyldifluoride (PVDF) membrane by electroblotting at 500 mA and 30 V for 1 h. Hereafter, the membrane was washed with tris buffered saline containing Tween20 (TBSt20) (Table 2.5) and blocked with TBSt20 supplied with 10% milk powder (Carl Roth, GmbH) for 2 h at room temperature. Subsequently the membrane was washed thrice for 10 minutes with TBSt20. During each washing step the membrane was shaking on a platform at 100 rpm at room temperature. The membrane was incubated overnight at 4°C in the presence of 0.2 µg/ml primary mouse monoclonal anti-His_6 antibodies (serial no. 11922416001, Roche) in 20 ml 0.3% bovine serum albumin (BSA) (Sigma-Aldrich). After washing 3 times 10 minutes

with TBSt20, the membrane was incubated with 1:10000 diluted secondary anti-mouse antibodies conjugated with alkaline phosphatase (A3562-25ML, Sigma-Aldrich). Finally, the membrane was washed thrice with TBSt20 for 10 minutes, before detection by adding nitro blue tetrazolium and bromo-chloro indolyl phosphate as substrate. The reaction was stopped using 1x phosphate buffered saline (PBS) with 25 mM ethylenediaminetetraacetic acid (EDTA) (Table 2.5).

2.5 Sugar-binding assays

2.5.1 ELISA-based assay

To quantify the affinity of the purified lectin domains towards a variety of sugars and glycosylated proteins, an enzyme-linked immunosorbent assay (ELISA)-based assay was performed. Hereto, the wells of microtiter plates (X50 Immulon 4HBX 96 well plate, Fischer Scientific) were coated by adding 100 µl of 100 µg/ml sugar or protein solutions in coating buffer and incubated overnight at 4°C. To optimize the assay, different coating buffers were used, namely carbonate-bicarbonate buffer and PBS (Table 2.5**Table 2.5**). The used sugars were D-glucose (Merck), D-galactose (VWR), mannan of *S. cerevisiae*, D-mannose, D-lactose, D-fucose, NeuNac, GlcNac, GalNac, D-cellobiose, Oyster glycogen and the used proteins were BSA, ovalbumin, human albumin, mucin of porcine stomach type II, and fetuin (all from Sigma-Aldrich). After the coating, the wells were washed thrice with distilled water and blocked by incubating the wells with 200 µl of blocking buffer (TBSt20 with 0.5 % blocking agent) at room temperature for 2 h. Different blocking agents were applied to optimize the assay, including milk powder (Carl Roth, GmbH) and polyvinylalcohol (PVA) (Sigma-Aldrich). After washing three times with distillated water, 100 µl of 200 µg/ml, 100 µg/ml or 50 µg/ml of purified lectin domains (Table 2.3) dissolved in PBS were added to the wells. This was incubated at 37°C for 3 h. In a variant of the assay, D-mannose or D-fucose was added to the solution with lectin domains at the same molarity and incubated for 30 minutes at room temperature before adding this to the wells.

The assay included several controls to take into account false positive signals. To monitor an affinity of the lectins towards the plastic microtiter plates or the blocking agent, additional wells were coated with coating or blocking buffer. Negative controls were wells to which PBS instead of lectin domains was added after the blocking step, in order to detect unspecific binding of the antibodies. Positive controls were wells to which 100 µl of 5 µg/ml griffithsin (in PBS) was added after the blocking step.

After lectin binding, the wells were washed thrice with PBS containing Tween20 (PBST) (Table 2.5) and incubated at 37°C for 1 h with 1/500 anti-griffithsin antibody for the positive control or 0.3 µg/ml anti-His tag antibody for the other wells. After washing three times with PBST, the wells were incubated at 37°C for 1 h with 1/5000 diluted anti-rabbit or anti-mouse antibodies for the positive control and other wells, respectively. After a final triple wash with PBST (Table 2.5), 100 µl of 1 mg/ml 4-nitrophenyl phosphate disodium salt hexahydrate dissolved in ELISA substrate buffer (Table 2.5) was added to each well and allowed to react for 10 to 30 min while swirling at room temperature protected from light. Using the Synergy MX Multimode Reader (BioTek), the absorbance at 405 nm was measured.

2.5.2 Pull-down sugar-binding assay using sepharose beads

Sepharose® 6B beads (Sigma-Aldrich) were coated with mono- and polysaccharides as previously described with little modification (Fornstedt and Porath, 1975). Briefly, 1 ml of sepharose beads was washed in 10 ml distilled water and dissolved in 1 ml 0.5 M sodium bicarbonate buffer (pH 11.0). Subsequently 100 µl of divinylsulfone (Sigma-Aldrich) was added and the suspension was incubated for 70 minutes at room temperature under gentle swirling. Hereafter the beads were washed with distilled water and dissolved in 1 ml 0.5 M sodium bicarbonate buffer (pH 10.0) containing 0.2 g of D-glucose (Merck), GlcNac, GalNac, D-mannose, D-fucose, D-lactose, mannan of *S. cerevisiae*, D-cellobiose and bovine serum albumin (all from Sigma-Aldrich), D-galactose (VWR); 0.1 g mucin of porcine stomach type II (Sigma-Aldrich) or 0.066 g of Barley β-glucan (Megazyme). After overnight incubation at room temperature under gentle swirling, the beads were washed in 10 ml of distilled water and resuspended in 1 ml of 0.5 M sodium bicarbonate buffer (pH 8.5). Next, 20 µl of β-mercaptoethanol (Sigma-Aldrich) was added and the suspension incubated at room temperature for 2 h. Finally, the beads were washed and dissolved in 1 ml of distilled water. The control beads underwent the same treatment, except that no sugars were added. All beads were stored at 4°C.

For the sugar-binding assay, 25 µl of each functionalized bead was washed with binding buffer (Table 2.5) as previously described (Cash *et al.*, 2006). 1 ml of binding buffer containing 50 µg of the purified lectin domain was added to each bead. Hereafter, the mixture was incubated at 4°C for 2 h. The beads were washed twice with 1 ml of wash buffer (Table 2.5**Table 2.5**) and bound lectin domains were eluted by boiling the beads in SDS-PAGE loading buffer (Fermentas, Life Sciences) for 10 minutes at 95°C. The bound lectin domains were resolved by SDS-PAGE through 12% polyacrylamide gels (Life Sciences), which were

stained with Sypro® Ruby protein gel stain (Invitrogen) and scanned by using the Typhoon scanner (GE Healthcare Life Sciences).

2.6 Microscopic assays of microbial strains incubated with lectin (domains)

2.6.1 Agglutination assay

The agglutination assay was based on a previously described protocol (Malik, 2014). Overnight cultures of *S. aureus* Rosenbach, *S. aureus* SH1000, *E. coli* UTI89, *S.* Typhimurium ATCC14028, *S. cerevisiae* BY4741, and *C. albicans* SC5314 were washed twice with PBS and dissolved in PBS. The *C. albicans* SC5314 and *S. cerevisiae* BY4741 cells were diluted to obtain a cell density of 0.5 and 1 w/v % respectively. 50 µl of these cell suspensions was added to the wells of 96-well U-bottomed plates (Cellstar® 650180, Greiner bio-one) together with 50 µl of a 400 µg/ml lectin domain solution, obtaining a final lectin concentration of 200 µg/ml (Table 2.3). The cell suspensions of the other strains were added at 200 µl per well and supplied with 200 µg/ml of purified lectin domains. Control wells did not contain any lectin domain. The wells were incubated at room temperature for 15 minutes while gently swirling. Finally, the mixtures were spotted on glass slides and visualized by phase-contrast microscopy at 400-fold for *S. cerevisiae* BY4741 and *C. albicans* SC5314 and 1000-fold magnification for the bacterial strains, using the Zeiss Axio Imager Z1 microscope equipped with an AxioCam MRm Rev.3 monochrome digital camera.

2.6.2 Immunofluorescence assays

The immunofluorescence assays were performed as previously described (Claes, 2011) with small modifications. The OD of overnight cultures of *S. cerevisiae* BY4741 and *C. albicans* SC5314 was measured at 595 nm, washed twice with PBS and resuspended in PBS. In U-bottomed 96 well plates (Cellstar® 650180, Greiner bio-one) 200 µl of the cell suspension was added to each well, using three wells per treatment. To the negative control, no lectin domains were added, whereas the treatments contained 200 µg/ml of a purified lectin domain (Table 2.3). Lectin domains and cells were incubated for 30 minutes at room temperature while gently swirling. Hereafter, the three repetitions were pooled together and washed once with PBS. Subsequently the cells were fixed for 1 h at room temperature in PBS with 2.5% formaldehyde and 0.05% gluteraldehyde. After washing with PBS once, the cells were incubated for 10 minutes in blocking buffer containing PBS and 0.5% blocking reagent (Cat. No. 1096176, Roche). The cells were then incubated for 15 minutes with anti-His mouse antibodies (1:400 dilution) at room temperature. The cells were washed thrice with PBS and

incubated with 1:200 diluted goat anti-mouse IgG antibody conjugated with Alexa Fluor® 488 (A21121, Invitrogen) for 15 minutes at room temperature, covered with aluminum foil. Finally, cells were washed thrice with PBS and transferred to a microscopic slide. The cells were visualized with a Zeiss Axio Imager Z1, equipped with an AxioCam MRm Rev.3 monochrome digital camera.

2.6.3 Fluorescence assay with FITC-labelled lectin domains

The lectin domains of Cmpg5300_05.29, LGG_Llp1, LGG_Llp2 and LGR-1_Llp1 were labelled with fluorescein isothiocyanate (FITC) using the FluoReporter® FITC Protein Labeling Kit (Life Technologies) according to the producer's manual. Briefly, dimethyl sulfoxide was added to the reactive dye, which was then added to 200 µl PBS containing 5 mg/ml of purified lectin domain and 20 µl of 1 M sodium bicarbonate buffer (pH 9.0). The molar ratio of added dye to the lectin domain was 30 and according to the manual's recommendations. After 1 h stirring at room temperature protected from light, the sample was loaded on a prepared spin column. After centrifuging 5 minutes at 1100 g, the centrifugate was collected, which contained approximately 200 µl of purified labeled lectin domain. The concentration was estimated using the Nanodrop (Thermo Scientific). The labelled lectin domains were used in an assay performed on *S. cerevisiae* BY4741*, C. albicans* SC5314*, E. coli* UTI89 and *S.* Typhimurium ATCC14028, which is similar to the immunofluorescence assay described above. The only difference is that after fixation, the cells were washed twice with PBS and directly visualized, instead of the several antibodies interaction steps.

2.7 *In vitro* adhesion assays to human epithelial cell lines

In order to investigate whether the lectins of *L. rhamnosus* GG, *L. rhamnosus* GR-1 and *L. plantarum* CMPG5300 affect the adhesion of pathogens to host epithelium, vaginal epithelial VK2/E6E7 (ATCC CRL-2616^{TM}) cells were used. The cells were routinely grown in 75 cm^2 culture flasks at 37°C and 5% CO_2 – 95% air atmosphere in keratinocyte serum-free medium (GIBCO-Invitrogen) supplied with 0.1 ng/ml epithelial growth factor (GIBCO-Invitrogen) and 25 µg/ml bovine pituitary extract (GIBCO-Invitrogen). Every 3 days (at 70-80% confluence) cells were passaged at a split ratio of 1:7. For the adhesion assays, the cells were grown in 12-well Multiwell Plates (665180, Greiner-bio one) at a density of 4.10^4 cells/cm^2 per well. The experiments were performed on 7 days old confluent monolayers as previously described (Petrova *et al.*, 2013a). Two types of adhesion assay were executed. In the first type, the lectin domains were incubated with the VK2/E6E7 cells to verify if the lectins can

block adhesion of pathogenic strains by binding to common receptor sites on VK2/E6E7. Hereto, the VK2/E6E7 monolayers were washed twice with pre-warmed PBS and incubated for 1 hour at 37°C and 5% CO_2 with 100 µg/ml of purified lectin domains of LGG_Llp1 and LGR-1_Llp1 and Cmpg5300_05.29 (Table 2.3) dissolved in DMEM medium without supplemented fecal calf serum (FCS). Hereafter, the VK2/E6E7 monolayers were washed once with pre-warmed PBS to remove unbound lectin domains and subsequently incubated at 37°C with 1 ml per well of DMEM (without added FCS) containing the bacterial or fungal strain of interest. The used concentrations were as follows: 10^6 CFU/ml for *S. aureus* SH1000 and Rosenbach and 10^5 CFU/ml for *C. albicans* SC5314. After 1 hour, the VK2/E6E7 cells were washed twice with pre-warmed PBS and each well was supplemented with 100 µl of trypsin-EDTA (1x) (Invitrogen). After incubation for 10 minutes at 37°C, 900 µl PBS was added to each well and serial dilutions were plated out. Plates were incubated at 37°C for 24 h. The adhesion ratio was calculated by dividing the number of adherent microorganisms by the number of microorganisms added to the VK2/E6E7 cells. The effect of the lectin domains was determined by comparing the adhesion ratio of the strain without adding the lectin domains (positive control) to the adhesion ratio of the strain after incubation with the lectin domains. Each lectin and pathogen combination was evaluated in three technical repeats and each experiment was performed at least three times.

In a second version of the assay, the lectin domains of LGG_Llp1, LGR-1_Llp1 and of Cmpg5300_05.29 were first incubated with the pathogens, before adding these to the VK2/E6E7 epithelial cells. This was performed in order to investigate whether the lectins could directly bind to the strains of interest and therefore block their adhesion to vaginal epithelium. Hereto, the pathogenic bacterial and fungal strains were first washed with PBS and dissolved in DMEM without FCS according to above mentioned concentrations, supplemented with 100 µg/ml of lectin domain (Table 2.3) and incubated for 30 minutes at room temperature while gently shaking. Hereafter, the pathogens were incubated with the VK2/E6E7 (1 ml per well) for 1 h at 37°C in presence of CO_2. The rest of the assay was performed as described in the previous version. Again each combination of lectin domain and pathogenic strain was performed in three technical repeats and each assay was executed thrice.

2.8 Biofilm assay of *S. aureus*, *S.* Typhimurium, *E. coli* and *P. aeruginosa*

2.8.1 Static peg assays (Calgary method)

The static peg assays were performed as previously described with minor modifications (Robijns, 2013). Biofilms were grown on a platform carrying 96 polystyrene pegs (Nunc no. 445497) that fits as a lid on microtiter plates (Nunc no. 269789), allowing each peg to hang in a microtiter plate well. Hereto, *E. coli* UTI89, *E. coli* TG1, *P. aeruginosa* PA14, *S. aureus* SH1000, *S. aureus* Rosenbach and *S.* Typhimurium ATCC14028 were grown overnight at 37°C at 250 rpm and diluted 100-fold in 1:20 TSB or TSB for *S. aureus* Rosenbach and SH1000. Of this dilution 75 µl was added to each microtiter plate well together with 75 µl of purified full length lectins or lectin domains diluted in 1/20 TSB or TSB for *S. aureus* Rosenbach and SH1000, with a final lectin concentration of 10, 50, 100 or 200 µg/ml (Table 2.3). Each treatment and negative control (no lectins) contained 8 technical repeats. One row was filled with medium only to serve as a blank later on during spectrophotometry. The pegged lid was placed on the plate and the plates were incubated for 24 hours at 25°C for *S. aureus* SH1000 and Rosenbach and *S. enterica* Typhimurium ATCC14028 and 37 °C for *P. aeruginosa* PA14 and *E. coli* TG1 and UTI89. The plates of *P. aeruginosa* PA14 were incubated in a sealed container with wet paper towels to create a constant humid environment. After 24h, all peg platforms were placed in a new microtiter plate with 150 µl fresh medium per well and incubated in the same conditions for another 24h. The OD at 595 nm of the used microtiter plates was measured to monitor the growth of the planktonic phase.

Several alternative versions of the assay were performed in order to understand in which phase of biofilm formation the lectins display an effect. In one version, the lectins were added after the adhesion phase. Hereto, the strains were first grown on pegs in appropriate medium without lectins for 1.5 hours under conditions mentioned above. Subsequently, the pegs were placed in a new microtiter plate with fresh medium containing the corresponding lectins. In a second version of the assay, the lectins were added with fresh medium after 24 hours to verify if the lectins could disrupt an already developed biofilm. In a third variant, the lectins were added continuously with fresh medium to the biofilm after 0, 12, 24 and 36 hours.

After 48 hours of growth, the biofilm formation was quantified as follows. The pegs were washed once with PBS, followed by coloring with crystal violet (0.1 w/v% in 5% methanol, 5% isopropanol and 90% PBS) for 30 minutes. Hereafter the pegs were washed in distilled water and let to dry at the air for 30 minutes. The biofilms were destained by hanging the pegs in microtiter plates containing 200 µl 30% acetic acid per well. The OD of these microtiter

plates was measured at 570 nm using a Synergy MX multimode reader (BioTek, USA). For each strain and lectin domain, the experiment was performed at least three times with 8 technical repeats. For *S.* Typhimurium ATCC14028 and *E. coli* UTI89 the first variant of the experiment was once performed with the purified full length lectins as well as the FITC-labeled lectin domains with 3 technical repeats.

2.8.2 Total cell count of biofilms

An overnight culture of *S.* Typhimurium ATCC14028 or *E. coli* UTI89 was diluted 200 fold in 1/20 TSB and 1 ml per well was added to 12-well cell culture plates (Cellstar®). Lectin domains were added at 50 µg/ml (Table 2.3). No lectins were added to the control wells. The plate with lid was taped to maintain constant humidity and incubated at 37 °C for *E. coli* UTI89 and 25 °C for *S.* Typhimurium ATCC14028, to allow biofilms to form on the bottom of the wells. After 24 hours, the medium was carefully removed and the OD at 595 nm of the used medium was measured. Then 1 ml of fresh 1/20 TSB was added to each well. After incubating another 24 hours at 25°C or 37°C, the medium was again removed and its OD_{595} measured. All biofilms were washed once with PBS before adding 1 ml PBS to each well. The biofilm was detached from the bottom of the wells using scrapers (Greiner bio-one) and pushed through a needle (25G, 0.5 x 16 mm, Terumo) to dissolve cellular aggregates. The dissolved biofilms were serially diluted in PBS and plated on LB. After overnight incubation at 37°C, the colonies were counted and the CFU/ml of the biofilms was calculated. For each strain, the experiments were performed at least three times with 3 technical repeats.

2.8.3 Fluorescence microscopy of biofilms

For the visualization of *S.* Typhimurium biofilms, *S.* Typhimurium ATCC14028 carrying the pFPV25.1 plasmid was used, which constitutively expresses the *gfp*mut3 gene (Robijns, 2013). Biofilms of this strain were grown on the bottom of wells of black polystyrene, clear bottomed microtiter plates (Greiner bio-one 655096). Hereto 150 µl 1/20 TSB containing a 1/200 dilution of an overnight culture of the strain was added to each well together with 50 µg/ml of the purified lectin domains (Table 2.3). After covering and taping the plate, it was incubated for 48 hours at 25°C. After 24 hours the medium was carefully removed in order not to disturb the biofilm and fresh 1/20 TSB was added. Before fluorescence microscopy, the biofilm was washed once with PBS and the medium on top of the biofilms was removed to discard the planktonic phase. Microscopic epifluorescence imaging was performed using a Zeiss Axio Imager Z1 microscope with an EC Plan Neofluar (X40 magnification/0.3

numerical aperture) objective (excitation 488 nm, emission 511 nm). Pictures were acquired with an AxioCam MRm and the AxioVision software. In an alternative version of this experiment, wild type *S.* Typhimurium ATCC14028 and FITC-labeled lectin domains were used instead of *S.* Typhimurium ATCC14028 expressing the GFP and unlabeled lectin domains. This version of the experiment was performed exactly as described above.

2.9 Biofilm assay of *Candida albicans* SC5314

Biofilms of *C. albicans* SC5314 were grown in round-bottomed 96 well microtiter plates (TPP Techno Plastic Products AG, Switzerland). Hereto, an overnight culture of *C. albicans* SC5314 was grown until an OD_{600} between 20 and 30 and washed twice with PBS. Subsequently it was diluted until an OD_{600} of 0.1 (approximately 10^6 CFU/ml) in RPMI 1640 medium (pH 7.0) with L-glutamine and without sodium bicarbonate (Sigma-Aldrich), buffered with MOPS (Sigma-Aldrich). Of this suspension 100 µl was added to each well of the microtiter plate. The assay was performed in two versions to verify if the lectin domains were involved in the adhesion phase of the forming biofilm. Therefore, in the first version the purified lectin domains were added to the *C. albicans* SC5314 suspension at the start of the biofilm formation, whereas in the second version no lectins were added. The rest of the assay was identical for both assays. The lectin domains of LGG_Llp1, LGR-1_Llp1 and Cmpg5300_05.29 were added at concentrations of 200 µg/ml, 100 µg/ml and 50 µg/m (Table 2.3).

After 1 h incubation at 37°C to allow the cells to adhere, the medium was carefully removed and the biofilm gently washed with PBS to remove nonadherent cells. Subsequently 100 µl of fresh RPMI with the purified lectins domains was added per well and the biofilms were incubated at 37°C for 24 h. The negative control was supplied with RPMI without lectins domains. Hereafter, the biofilms were again washed once with PBS and quantified using Cell-Titer Blue (CTB) (Promega, USA) by adding 100 µl of CTB 1/10 diluted in PBS to each well. After incubation at 37°C in the dark for 1 h, the fluorescence was recorded using the Synergy Mx multimode reader (BioTek, USA) (excitation at 535 nm and emission at 590 nm). The measured values were corrected by subtracting the fluorescence value of uninoculated wells (blank). Of blank, negative control and treatments 8 technical repeats were performed. The average CTB-value of biofilms formed in the presence of lectin domains was compared to the average CTB-vale of the negative control. Each assay was performed three times.

Chapter 3

Results

3 Results

3.1 Growth assay of lactobacilli

Given that the lectin-like proteins are predicted to bind sugars, it is possible that they have a function in sugar metabolism of the *Lactobacillus* strains. Therefore, growth assays were performed, in which both the wild type strains and the corresponding knock-out mutants lacking the lectin-like proteins were grown in the presence of specific sugars. All of the tested strains grew well on D-glucose (Figure 3.1, a, b and c), GlcNac and D-mannose (data not shown), without a significant growth difference between wild type *Lactobacillus* and the corresponding mutants. For none of the strains an optimal growth was observed in the presence of fucose. Especially *L. rhamnosus* GR-1 and *L. plantarum* CMPG5300 did not grow on this type of sugar, whereas *L. rhamnosus* GG showed an intermediate growth (Figure 3.1a). The LGG_Llp1 and LGG_Llp2 knock-out mutants grew less than the wild type, but when the experiment was repeated, neither the wild type nor the mutants grew well. However the experiments need to be confirmed. As expected *L. plantarum* CMPG5300 and *L. rhamnosus* GG also grew sub optimally on lactose, although *L. rhamnosus* GR-1 grew well on lactose (Malik, 2014), (Petrova, 2013). For *L. plantarum* CMPG5300, there was a delayed growth peak of the wild type compared to the Cmpg5300_05.29 knock-out mutant, which could be due to the metabolic burden of the lectin (Figure 3.1c). The growth difference could also be caused by the increased clumping of the wild type as the cells inside clumps have less access to nutrients. Finally, *L. rhamnosus* GG, GR-1 and *L. plantarum* CMPG5300 were characterized by different growth curves when grown on sorbitol. As observed in previous studies, the growth curve of *L. rhamnosus* GR-1 was sigmoidal, indicating a metabolic shift, but no difference between mutant and wild type was observed (Figure 3.1b) (Petrova, 2013). For *L. rhamnosus* GG, the LGG_Llp2 knock-out mutant grew less than the wild type or LGG_Llp1 knock-out mutant (Figure 3.1d). The sorbitol growth curve of *L. plantarum* CMPG5300 wild type and knock-out mutant was similar to lactose and displayed the same delay.

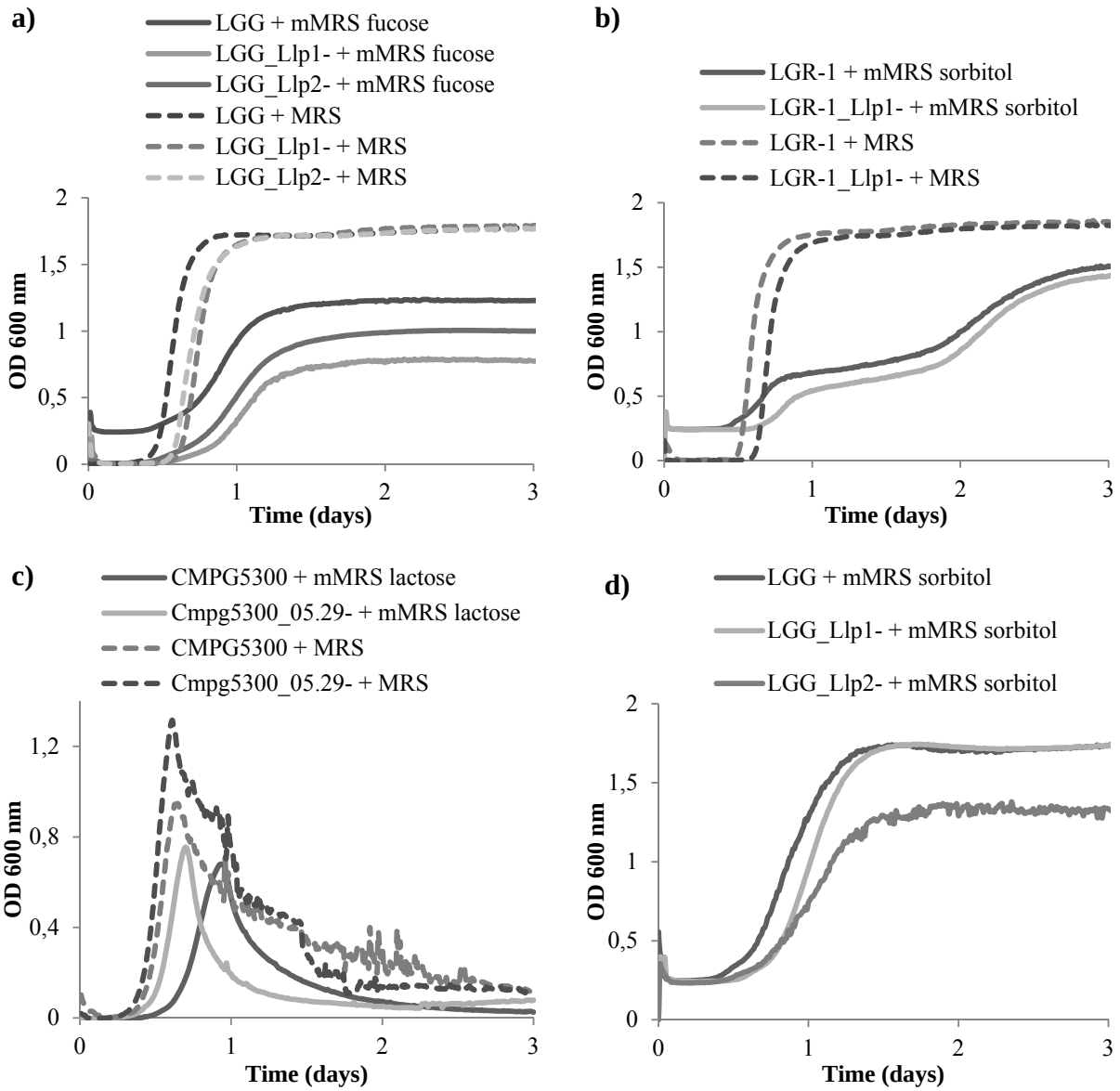

Figure 3.1 Carbohydrate utilization of *L. rhamnosus* GG, *L. rhamnosus* GR-1 and *L. plantarum* CMPG5300: comparison of wild type and lectin protein deletion mutants. a) *L. rhamnosus* GG (LGG) growing on glucose (MRS) and fucose; b) *L. rhamnosus* GR-1 (LGR-1) growing on glucose and sorbitol, c) *L. plantarum* CMPG5300 growing on glucose and lactose; d) *L. rhamnosus* GG (LGG) growing on sorbitol.

3.2 Optimization of recombinant expression of LGG_Llp2 lectin domain in *E. coli*

The overexpression of the lectin domain of LGG_Llp2 has been previously initiated. However, this resulted in a low concentration of the protein of interest in the soluble cytosolic fraction, but rather high concentration as inclusions bodies (Petrova, 2013). Therefore, in order to obtain a higher concentration of the lection domain of Llp2 three new constructs were designed with different downstream extensions of the lectin domain (Figure 2.1). The production of the three

new constructs of the lectin domain of LGG_Llp2 was compared with the initially available construct in a Western blot (Figure 3.2). This shows that the production of recombinant lectin domain in *E. coli* BL21 (DE3) was the highest for the shortest construct based on primers S&P-00685 and S&P-00686 (Figure 3.2, lane 4). However, a second larger protein was also produced with this construct. No expression of the lectin domain was observed for the two other constructs based on S&P-00685/ S&P-00686 and S&P-00685/ S&P-00707 (Figure 3.2, lanes 2 and 3). Therefore, the original construct (Figure 3.2, lane 1) of the domain of LGG_Llp2 was used for recombinant production, purification and the other experiments.

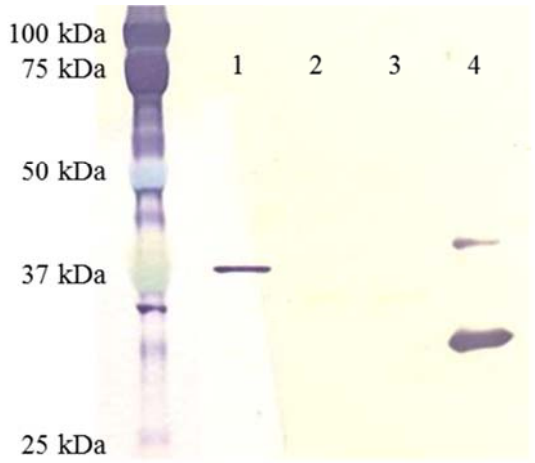

Figure 3.2 Western blot showing the expression of the different constructs of the lectin-domain of LGG_Llp2. Lane 1: original construct based on primers S&P-00620 and S&P-00621, lane 2: construct based on primers S&P-00685 and S&P-00621, lane 3: construct based on primers S&P-00685 and S&P-00707, lane 4: construct based on primers S&P-00685 and S&P-00686.

3.3 Production and purification of lectins and corresponding lectin domains

In order to obtain a high amount of pure recombinant protein, the lysate of *E. coli* BL21 (DE3) expressing the lectin domains was purified using both His-trap affinity chromatography and size exclusion chromatography. On average, the purification of 2 L *E. coli* BL21 (DE3) culture resulted in roughly 30 to 40 mg lectin domain of LGR-1_Llp1 and Cmpg5300_05.29, 15 to 20 mg lectin domain of LGG_Llp1 and 5 mg lectin domain of LGG_Llp2. The full length lectins LGG_Llp1, LGG_Llp2 and LGR-1_Llp1 yielded approximately between 0.1 and 0.3 mg protein per 1 L culture.

3.4 Sugar-binding assays

3.4.1 ELISA-based assay

The sugar-binding affinity of the purified lectin domains derived from *L. rhamnosus* GG, *L. rhamnosus* GR-1 and *L. plantarum* CMPG5300 was first investigated using an ELISA-based assay. Hereto, the wells of microtiter plates were coated with various sugars and glycosylated proteins and bound lectin domains were detected with anti-His tag antibodies. Since different

repeats of the assay often had different outcomes, a variety of experimental conditions were performed to optimize the assay and to reduce aspecific adsorption, by varying the blocking buffers, coating buffers and lectin concentrations. In a first variant of the assay, carbonate bicarbonate buffer was used as coating buffer and the blocking buffer was based on milk powder. The results of this assay are presented in Figure 3.3a. As expected the positive control griffithsin was shown to have a very high affinity for mannan, confirming its known binding to oligomannose (Huskens and Schols, 2012). BSA is not glycosylated and therefore used as a negative control (Thompson *et al.*, 2011). For Cmpg5300_05.29, the signal was generally low and not significantly different from BSA (two-sided t-test, $P > 0.05$). LGG_Llp1 had a significantly ($P < 0.05$) high affinity for D-mannose and D-fucose and a significantly low affinity for D-galactose and fetuin compared to BSA. The high binding to BSA may be due to contaminating glycoproteins present in the BSA product (Thompson *et al.*, 2011). LGR-1_Llp1 had a significant affinity ($P < 0.05$) for ovalbumin, D-galactose, D-mannose and D-fucose. However, all lectin domains bound considerably to wells not coated with sugars but still blocked with milk blocking buffer (shown as 'Milk blocking' in Figure 3.3a). As the milk blocking buffer contains many contaminating glycans, these signals may be false positive. Therefore instead of milk powder the blocking agent PVA was used, which is recommended as the best blocking agent for lectin assays (Thompson *et al.*, 2011; Tytgat *et al.*, 2012). Again, all purified lectin domains bound significantly to wells not coated with sugars yet blocked with PVA blocking buffer (PVA in Figure 3.3b and c). For Cmpg5300_05.29 and Msa, the signal was constantly high for all tested sugars and proteins, except for mannan and fetuin (Figure 3.3b). This pattern was observed in multiple repeats of this assay, and with both PBS and carbonate-bicarbonate coating buffers. Moreover, the same tendency was observed for LGG_Llp1 and LGR-1_Llp1 in several repeats of the experiment, such as in Figure 3.3c. Control wells to which PBS without lectin domains was added, demonstrated that the antibodies did not bind to PVA or any of the protein or sugar coatings.

In another repetition, the lectin domains were observed to bind more to uncoated wells than wells coated with PVA (data not shown). Therefore, it is possible that under the used conditions, PVA did not sufficiently block the wells and that the lectins bound to the plastic surface. This could explain the overall high signal for almost all sugars. The lower signals for mannan and fetuin could be due to a more efficient coating of the polysaccharide and glycoprotein.

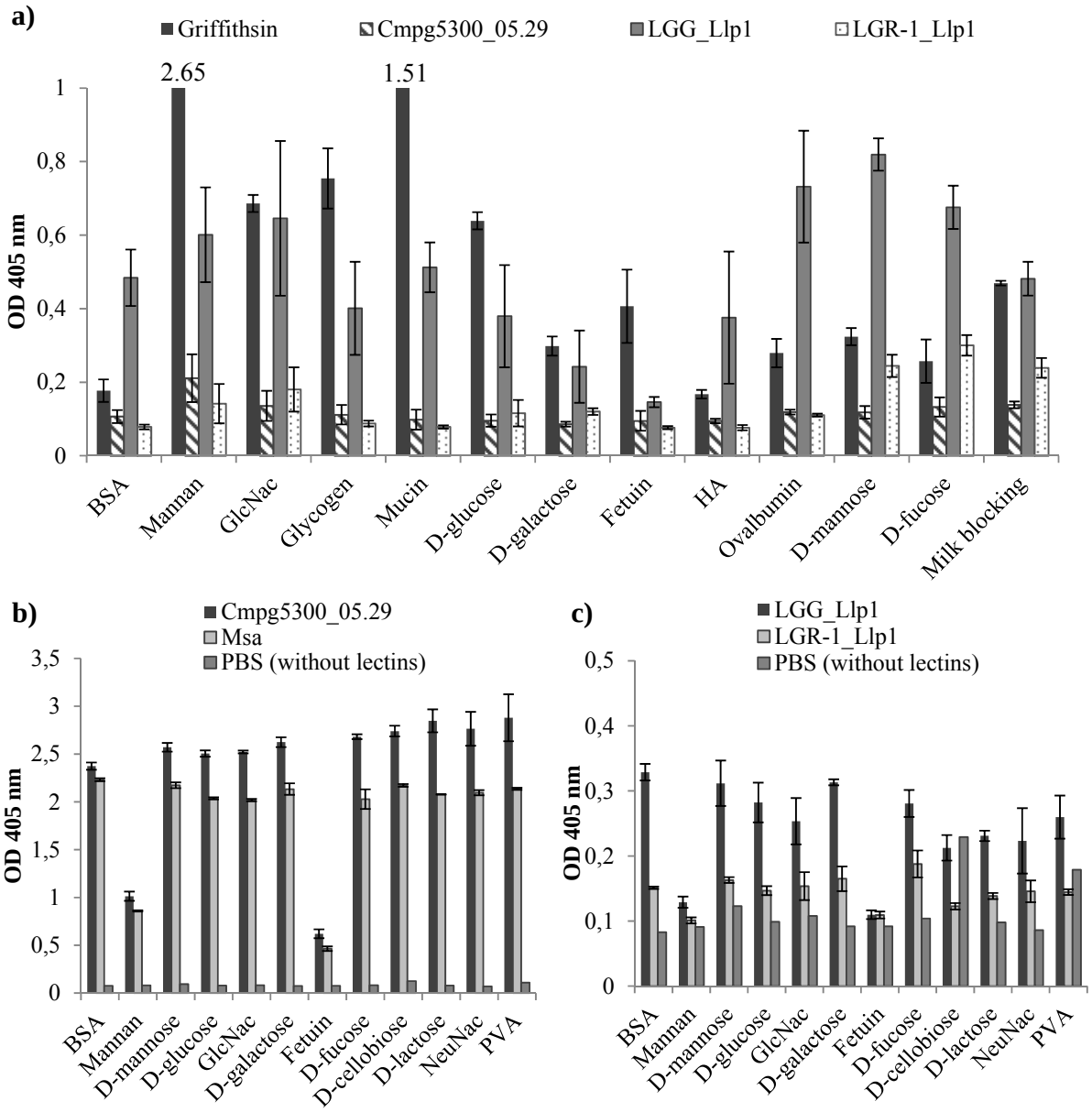

Figure 3.3 Affinity of the purified lectin domains towards various sugars and (glyco)proteins. a) Griffithsin, Cmpg5300_05.29, LGG_Llp1 and LGR-1_Llp1 (carbonate bicarbonate coating buffer, milk blocking buffer). All lectin domains show a relatively high affinity for uncoated wells (Milk blocking).; b) Cmpg5300_05.29 and Msa (PBS coating buffer, PVA blocking buffer). Both lectin domains (dissolved in PBS) have a high affinity for all coatings, including PVA, except for mannan and fetuin. As a type of negative control, wells were incubated with PBS without lectins to monitor the capacity of the antibodies to bind to the coated surfaces.; c) LGG_Llp1 and LGR-1_Llp1 (PBS coating buffer, PVA blocking buffer).

To investigate whether the lectins bind unspecifically or via lectin-glycan interactions with the coated wells, the lectin domains were incubated with D-mannose or D-fucose prior to binding to the wells. For Cmpg5300_05.29 the observed binding pattern and signal remained the same in several repeats, indicating that competition with D-mannose or D-fucose did not impair the interaction with the wells (data not show). In several repetitions of this experiment, the signals of LGG_Llp1 and LGR-1_Llp1 were also not reduced after incubation with D-mannose. In the repetition shown in Figure 3.4, the binding of LGR-1_Llp1 to mannan, GlcNac and GalNac is significantly (two-sided t-test, $P<0.05$) reduced upon competition with D-mannose. Also LGG_Llp1 showed significantly decreased binding to BSA and D-fucose after incubation with D-mannose. However, these results could not be reproduced.

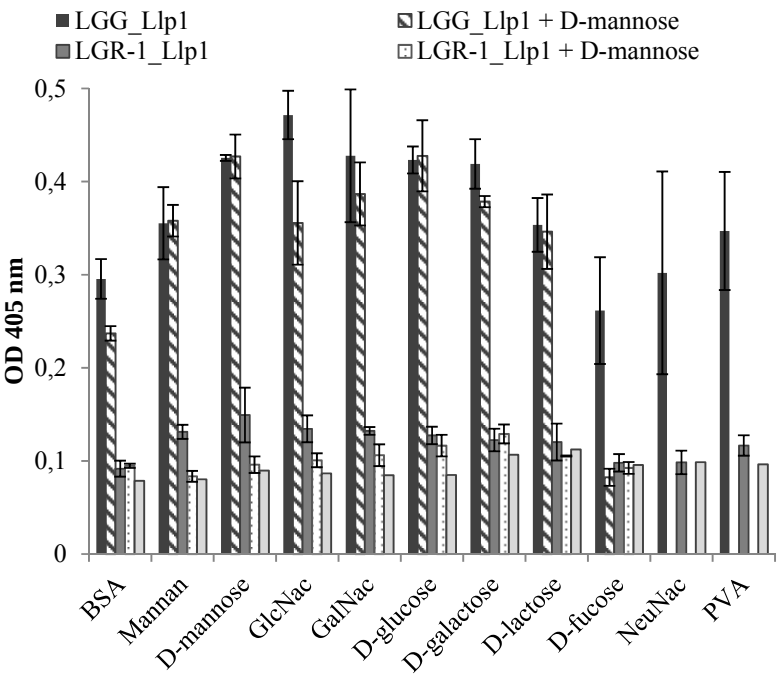

Figure 3.4 Sugar- affinity of LGG_Llp1 and LGR-1-Llp1 without and with preincubation with D-mannose (PBS coating buffer, PVA blocking buffer). Soluble D-mannose appears to compete with the binding of LGG_Llp1 to BSA and D-fucose and the binding of LGR-1_Llp1 to D-mannan, GlcNac and GalNac.

Due to the high affinity towards uncoated wells and the variation among different repeats of the experiments, we sought for an alternative assay to investigate the sugar-affinity of the lectin domains.

3.4.2 Sugar-binding assay with sepharose beads

The affinity of the purified lectin domains towards various sugars was examined by allowing the proteins to bind to sugar-coated sepharose beads and visualizing the bound proteins with SDS-PAGE. The sugars and glycosylated proteins tested were BSA, mannan, D-mannose, D-glucose, D-fucose, D-galactose, D-lactose, GlcNac, GalNac, D-cellobiose, mucin and β-glucan. As this

experiment was optimized during this thesis and performed for a first time in our lab, ConA was included as a positive control and was shown to bind significantly to mannan, D-mannose and D-glucose (Figure 3.5, lane 2, 3 and 4 respectively), confirming its known sugar specificity (Sharon and Lis, 2007).

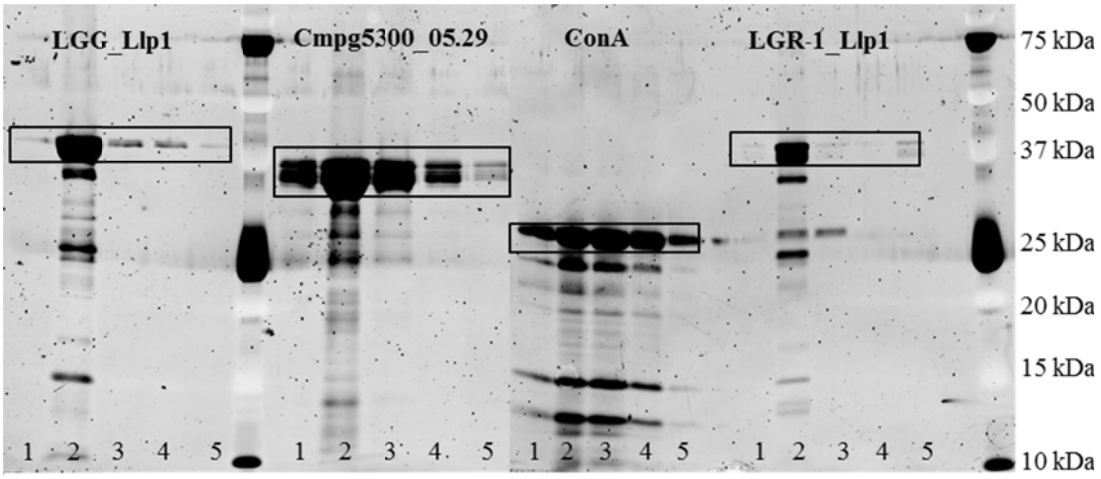

Figure 3.5 Sugar-binding of ConA and lectin domains of LGG_Llp1, Cmpg5300_05.29, LGR-1_Llp1 and LGG_Llp2. Proteins that bound to sugar-coated sepharose beads were separated by SDS-PAGE. Sepharose beads were not coated (lanes 1), coated with mannan (lanes 2), D-mannose (lanes 3), D-glucose (lanes 4) and D-fucose (lanes 5). All lectins bind predominantly to mannan. ConA binds to mannan, D-mannose and D-glucose.

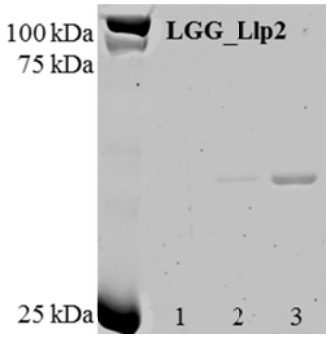

Figure 3.6 Sugar-binding of lectin domain of LGG_Llp2 to uncoated beads (lane 1), D-mannose (lane 2) and mannan (lane 3). It binds mostly to mannan.

Furthermore, all purified lectin domains showed the highest affinity for mannan (Figure 3.5, lanes 2). LGG_Llp1 also bound to D-mannose and D-glucose (lane 3 and 4), whereas LGR-1_Llp1 had a minor affinity for D-fucose (lane 5). In addition to mannan, Cmpg5300_05.29 showed a significant binding to D-mannose (lane 3) compared to the control (lane 1). As shown in Figure 3.6, the lectin domain of LGG_Llp2 had a significant binding to mannan and to lesser extent to D-mannose. For all lectin domains, there was no significant binding to any of the other tested sugars or glycosylated proteins under the used experimental conditions. Notably, all the bands corresponding to the bound lectin domains consist of two closely spaced bands. In fact, after size exclusion chromatography the lectin domains were purified together with a slightly shorter protein, which corresponds to 2 stacked bands on the protein gel. We consider that these bands are corresponding to the same protein and that the 2 different lengths are the result of a premature termination of transcription or translation in *E. coli* BL21 (DE3).

3.5 Agglutination and (immuno)fluorescence assays

3.5.1 *Candida albicans* SC5314 and *Saccharomyces cerevisiae* BY4741

The agglutination assay of *S. cerevisiae* BY4741 showed that the cells aggregate in the presence of the lectin domain of Cmpg5300_05.29 (Figure 3.7a). The lectin domains of LGG_Llp1 (data not shown) and LGR-1_Llp1 (Figure 3.7a) did not seem to cause a significant aggregation compared to the control. For *C. albicans* SC5314 cells also clumped in the presence of lectin domain of Cmpg5300_05.29, although less than *S. cerevisiae* BY4741 (Figure 3.7b). In addition, *C. albicans* SC5314 formed aggregates when the lectin domain of LGR-1_Llp1 was added.

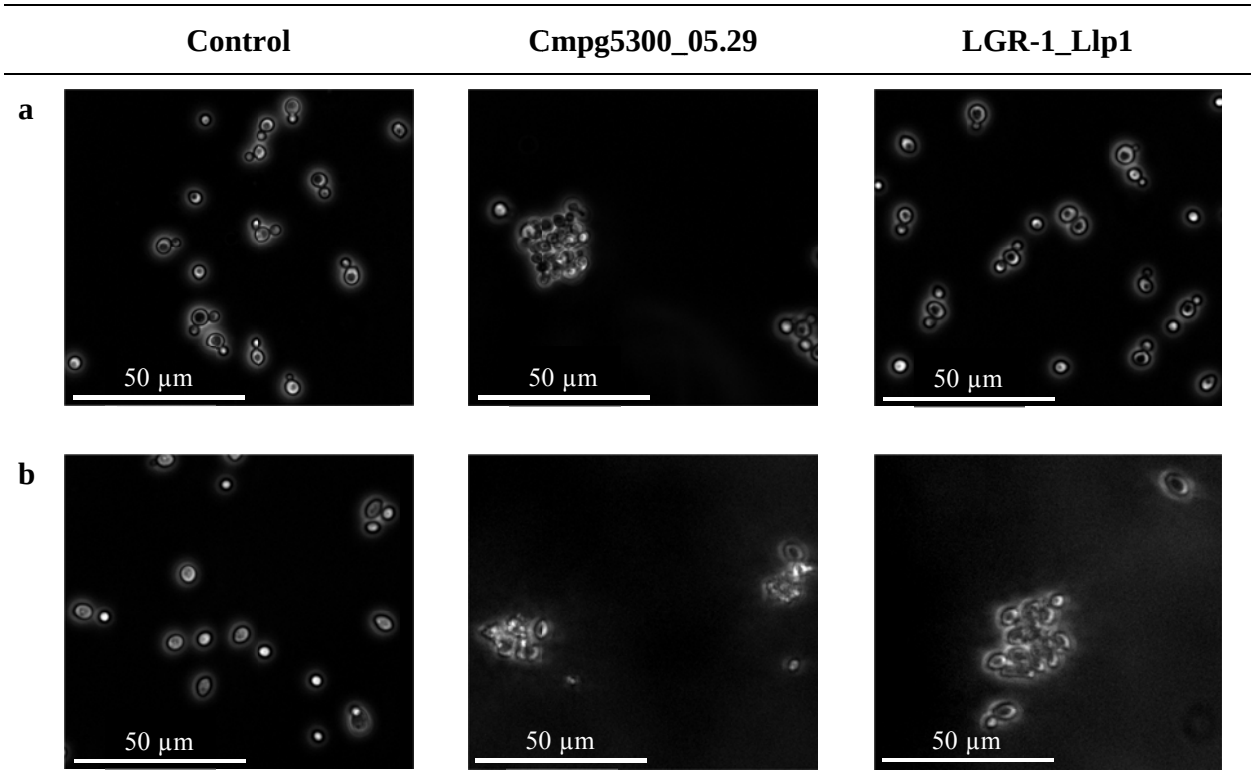

Figure 3.7 Phase contrast images of the agglutination assay of *S. cerevisiae* BY4741 and *C. albicans* SC5314. a) *S. cerevisiae* BY4741 forms aggregates in the presence of Cmpg5300_05.29 lectin domain, whereas *C. albicans* SC5314 (b) in the presence of lectin domains of Cmpg5300_05.29 and LGR-1_Llp1. (400-fold magnification)

However, the results of these agglutination assays can be subjective, as can be the decision whether cells are clumping significantly more than the control. Moreover, it is not possible to demonstrate that the aggregates are caused by the lectin domains instead of auto-aggregation. Therefore, we sought for alternative assays in which we visualized the lectin domains, under the hypothesis that if the lectin domains cause the aggregation, they should be present in the

aggregates. Hereto, an immunofluorescence assay was performed, in which the lectin domain of Cmpg5300_05.29, as one of the most promising lectins, was detected using mouse anti-His tag antibodies followed by anti-mouse AlexaFluor^{TM} conjugated antibodies. Similar to the previous agglutination assay, the cells aggregate when the lectin domains were added (Figure 3.8). Due to the fluorescent antibodies, the cells to which Cmpg5300_05.29 has bound have become fluorescent. The pictures demonstrate that especially the center of clumps is fluorescent, whereas single cells are not or less fluorescent.

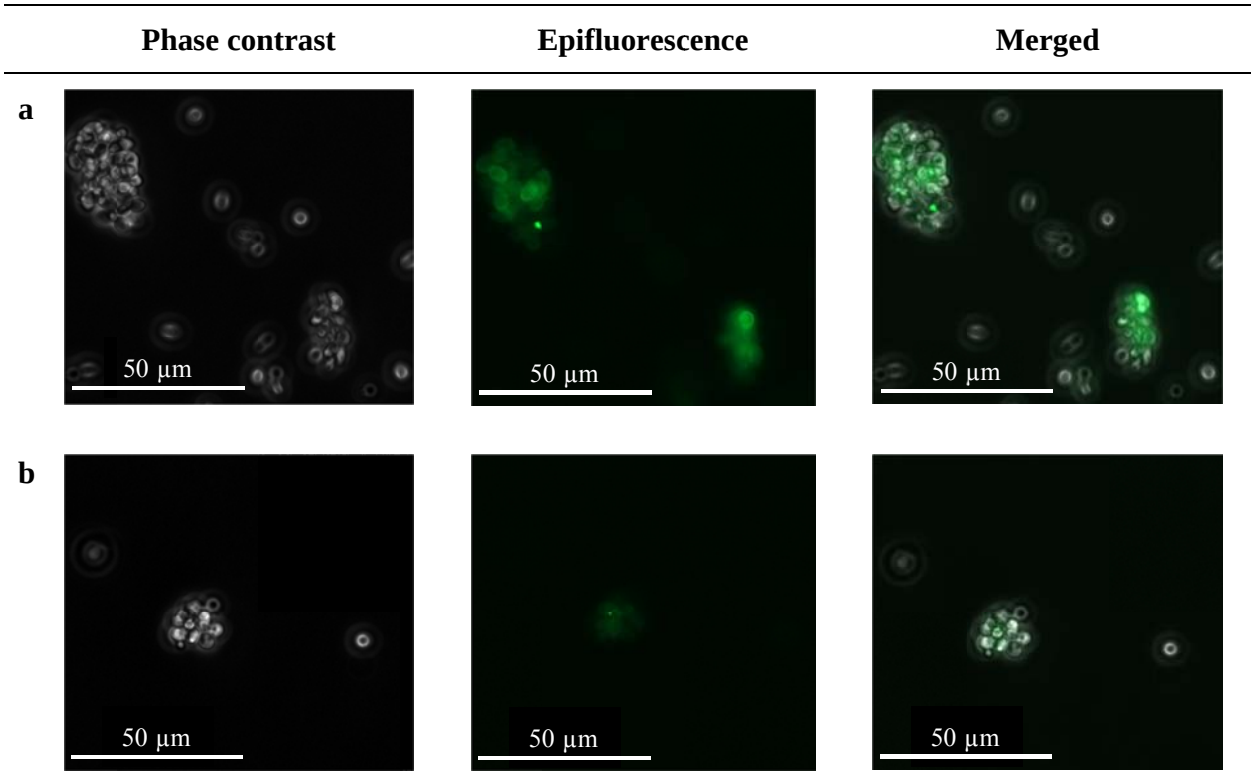

Figure 3.8 Phase contrast and fluorescence microscopy of the immunofluorescence assay of *C. albicans* SC5314 (a) and *S. cerevisiae* BY4741 (b). After incubation with 200 µg/ml of purified lectin domain of Cmpg5300_05.29, both species form aggregates instead of single cells. The fluorescence indicates the presence of the lectin domain in the aggregates. (400-fold magnification)

However, the immunofluorescence agglutination assay involves numerous washing steps and interdependent binding steps due to the use of primary and secondary antibodies, which considerably reduces the amount of cells and results in a low signal. This assay did not work well with the bacterial strains and the other lectin domains. Therefore, the lectin domains were directly labeled with FITC and used in a third type of agglutination assay. One concern was the possibility that the FITC-tag changed the activity of the lectin domains (Chersi *et al.*, 1997).

Nevertheless, the results confirmed that the lectin domain of Cmpg5300_05.29 still causes aggregation of *S. cerevisiae* BY4741 and *C. albicans* SC5314 as can be seen in Figure 3.9b and Figure 3.10b respectively. Regarding the other lectin domains, in the presence of FITC-labeled LGR-1_Llp1 *C. albicans* SC5314 developed a few fluorescent aggregates (Figure 3.10d), but *S. cerevisiae* BY4741 did not (data not shown), which confirms previous experiment. LGG_Llp1 led to minor aggregation of *C. albicans* SC5314, which was associated with a fluorescent signal, indicating that LGG_Llp1 may interact with the cells and may cause the clumping (Figure 3.10c). LGG_Llp1 did not cause any clumping or significant fluorescent signal of *S. cerevisiae* BY4741. LGG_Llp2 did not lead to any aggregation or fluorescent signal, indicating this lectin domain does not interact with these species (data not shown). Overall, the (immuno)fluorescence agglutination assays showed that Cmpg5300_05.29 causes agglutination of both species, whereas LGR-1_Llp1 only of *C. albicans* SC5314 to a minor extent. Moreover, the assays support the hypothesis that the agglutination is caused by direct interaction of the lectin domains with the cells, and that the aggregates are not due to auto-aggregation.

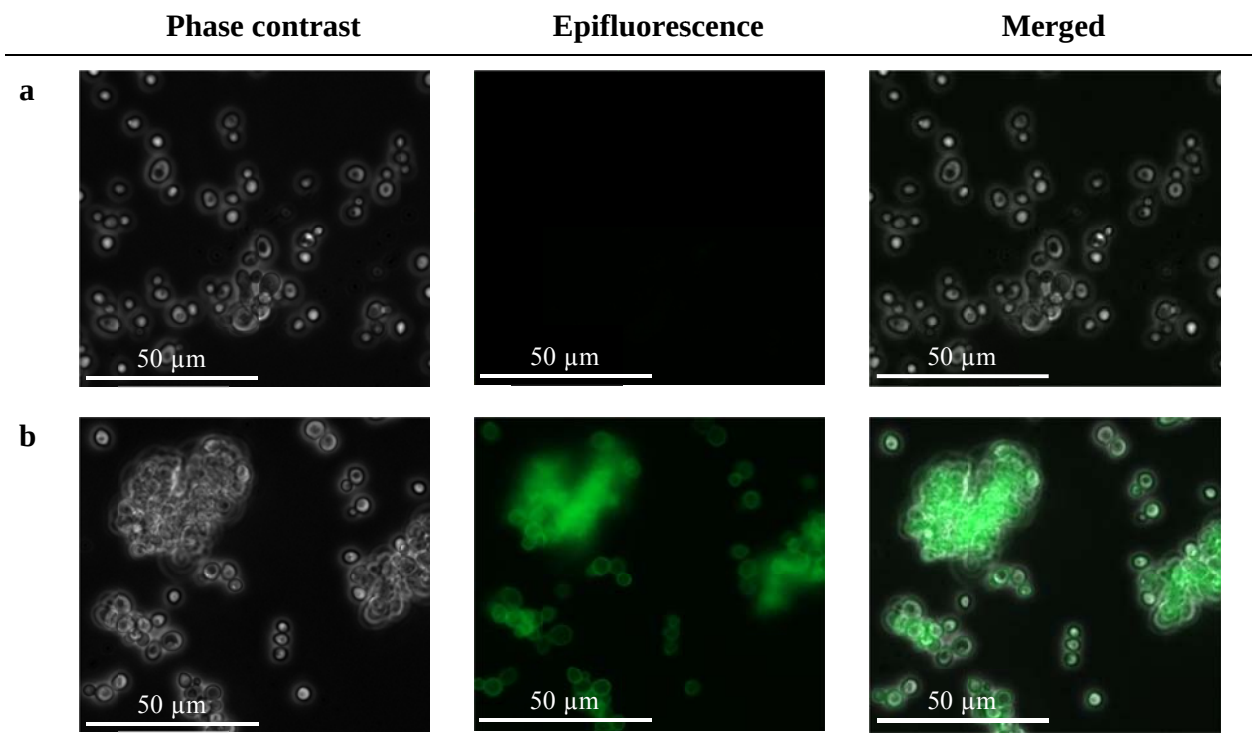

Figure 3.9 Phase contrast and epifluorescence microscopy of the agglutination of *S. cerevisiae* BY4741 due to FITC-labeled Cmpg5300_05.29 lectin domain. a) Control without lectin domains shows single, not fluorescent cells. b) After incubation with FITC-labeled lectin domains of Cmpg5300_05.29 (200 µg/ml), the cells form fluorescent aggregates. (400-fold magnification)

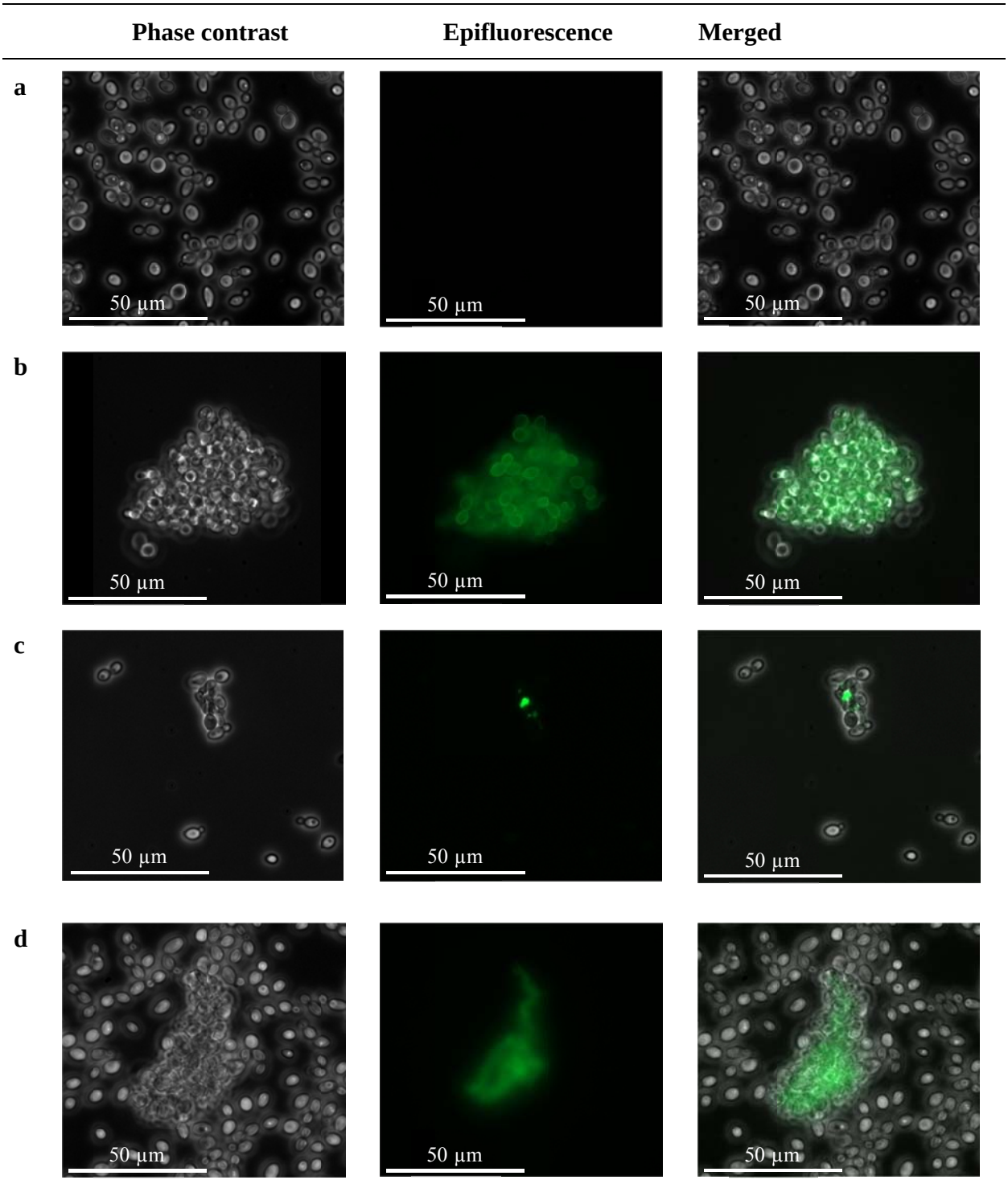

Figure 3.10 Phase contrast and epifluorescence microscopy images of *C. albicans* SC5314 incubated with FITC-labeled lectin domains. a) Control without lectin domains. Cells exhibit a varying aggregation and corresponding fluorescence after incubation with FITC-labeled lectin domains of Cmpg5300_05.29 (b), LGG_Llp1 (c) and LGR-1_Llp1 (d) (200 µg/ml). (400-fold magnification)

3.5.2 *Salmonella* Typhimurium ATCC14028

The agglutination assay of *S.* Typhimurium ATCC14028 showed that these cells aggregate in the presence of lectin domains of Cmpg5300_05.29 (Figure 3.11), but not significantly in the presence of LGG_Llp1 or LGR-1_Llp1 (data not shown). In addition, FITC-labeled lectin domains of LGG_Llp1, LGG_Llp2 and LGR-1_Llp1 did not cause any significant aggregation or fluorescence compared to the control. In contrast with the agglutination assay with unlabeled lectin domain, the FITC-labeled lectin domain of Cmpg5300_05.29 did not lead to the formation of clumps or a fluorescent signal (data not shown). The lack of fluorescent signal indicates this lectin domain did not bind to the cells and therefore the aggregates formed in the agglutination assay with the unlabeled lectin domain (Figure 3.11) could be *Salmonella* auto-aggregates. Hence, caution is needed when interpreting the agglutination in the first experimental set up as caused by the lectin domain of Cmpg5300_05.29. Presumably none of the lectin domains bound to *S.* Typhimurium ATCC14028 under the experimental conditions.

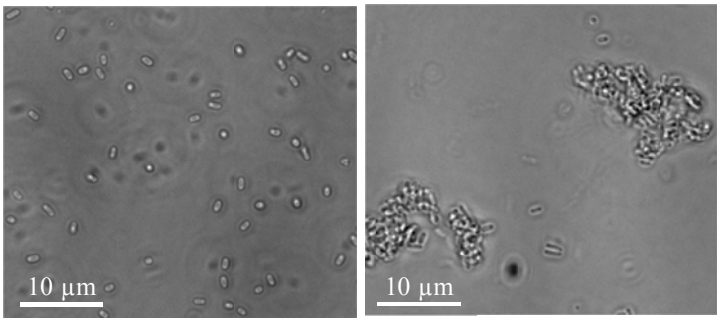

Figure 3.11 Phase contrast image of an agglutination assay of *S.*Typhimurium. Cells aggregate in the presence of 200 µg/ml lectin domain of Cmpg5300_05.29 (right) compared to the control (left). (1000-fold magnification)

3.5.3 Uropathogenic *Escherichia coli* UTI89

In the presence of LGG_Llp1 and LGG_Llp2, *E. coli* UTI89 formed some aggregates, but not in the presence of Cmpg5300_05.29 or LGR-1_Llp1 (data not shown). However, when performing the agglutination assay with FITC-labeled lectin domains, we were not able to prove that the observed clumps are due to the presence of LGG_Llp1 or LGG_Llp2 (data not shown). Therefore we believe that the observed aggregates are auto-aggregates and not caused by the lectin domains, similar to the case with *S.* Typhimurium ATCC14028.

3.5.4 *Staphylococcus aureus*

The agglutination assays of *S. aureus* Rosenbach did not show a significant aggregation under the influence of the lectin domains (data not shown). However, *S. aureus* SH1000 showed significant clumping when lectin domains of Cmpg5300_05.29 or Msa from *L. plantarum* WCFS1 were

added (Figure 3.12). When using the FITC-labeled lectin domain of Cmpg5300_05.29, there was no increased clumping as compared to the control, where aggregates were observed as well Figure 3.13. This was presumably caused by the fixation as the control of the agglutination assay was performed without a fixation step (Figure 3.12) did not display aggregates. Therefore it is not possible to conclude based on the fluorescence agglutination assay whether the lectin domain caused aggregation. However, the fluorescence assay demonstrates the presence of the lectin domain in the clumps, as these were highly fluorescent (Figure 3.13b). Hence, it is likely that the lectin domain of Cmpg5300_05.29 causes aggregation of *S. aureus* SH1000, but that the clumping is masked by the auto-aggregation of the control in this assay.

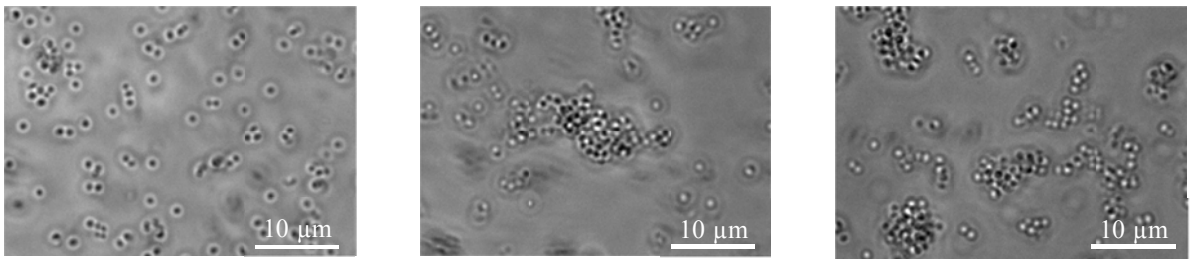

Figure 3.12 Phase contrast image of an agglutination assay of *S. aureus* SH1000. Left: Control without added lectin domains, center: with lectin domain of Cmpg5300_05.29 and right: with lectin domain of Msa of *L. plantarum* WCFS1 (right) at 200 µg/ml. (1000-fold magnification)

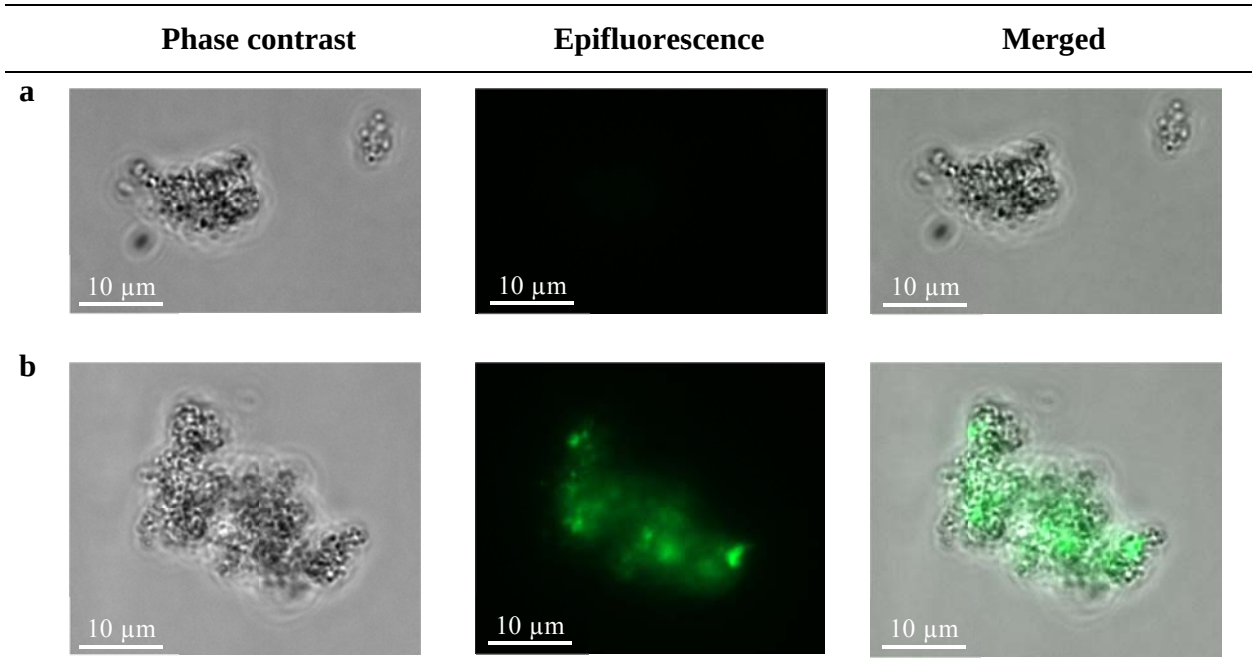

Figure 3.13 Agglution assay of *S. aureus* SH1000 with FITC-labeled Cmpg5300_05.29 lectin domain. a) Phase contrast and fluorescence microscopy image of *S. aureus* SH1000 without lectin domains and b) after incubation with FITC-labelled lectin domains of Cmpg5300_05.29 (200 µg/ml). (1000-fold magnification)

3.6 Adhesion assays

The proteins Cmpg5300_05.29, LGG_Llp1 and LGR-1_Llp1 have all been demonstrated to be important to a different extent for adhesion of their corresponding strains to epithelial cell lines. This adhesion competes with the adhesion of other microorganisms that intend to colonize the epithelial cells. To investigate if the lectins are directly involved in this competition, adhesion assays were performed to study the impact of the lectins on the adhesion of several pathogens. If the purified lectin domains can block their adhesion, this could explain on a molecular level how lactobacilli may exclude pathogens.

3.6.1 *Candida albicans* SC5314

The adhesion of *C. albicans* SC5314 to VK2/E6E7 was tested after the pathogen was incubated with the lectin domains of Cmp5300_05.29, LGG_Llp1 and LGR-1_Llp1 to monitor the effect of the lectins on its adhesion capacity. However, its adhesion to VK2/E6E7 was not significantly impaired by the treatments with LGG_Llp1 (P-value 0.070) and LGR-1_Llp1 (P- value 0.234) as compared to the control adhesion of *C. albicans* SC5314, which was incubated without the lectin domains (Figure 3.14). The treatment with Cmpg5300_05.29 led to adhesion percentages between 191 and 2132% (data not shown). This overestimation of adhering cells is presumably caused by the clumping of *C. albicans* SC5314 in the presence of Cmpg5300_05.29, which leads to an underestimated count of the *C. albicans* SC5314 cells added to the VK2/E6E7 (see 3.5.1). In another variant of the experiment, the lectin domains were first incubated with the VK2/E6E7 before adding *C. albicans* SC5314 to verify whether the lectins bind to and thereby block the pathogen's ligands. Here as well the lectin domains did not impair the adhesion of the pathogen, but this experiment was only performed once (data not shown).

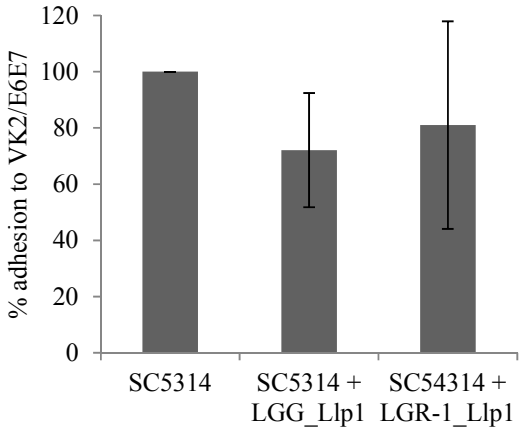

Figure 3.14 Adhesion of *C. albicans* SC5314 incubated with lectin domains to VK2/E6E7. The lectin domain of LGG_Llp1 in particular may bind to and impair the adhesion of *C. albicans*, but the reduction is not significant. The used lectin domain concentration was 100 µg/ml. No lectins were added to the control (SC5314).

3.6.2 *Staphylococcus aureus* Rosenbach and SH1000

As for *C. albicans* SC5314, *S. aureus* Rosenbach and SH1000 were incubated with the purified lectin domains of Cmpg5300_05.29, LGG_Llp1 and LGR-1_Llp1 and subsequently added to the VK2/E6E7. The percentage of cells added that adhered to the VK2/E6E7 was for each lectin treatment normalized with respect to the treatment without lectin. For *S. aureus* Rosenbach, one biological repeat was omitted from the results, as the adhesion ratio of the control treatment was 4030%, which is probably due to random variation. The high adhesion ratio of the control would lead to a severe underestimation of the relative adhesion of the lectin treatments. For the same reasons, 2 repeats of the experiment with *S. aureus* SH1000 also had to be excluded. The results of the remaining repeats of both strains are represented in Figure 3.15**Figure** . Only LGR-1_Llp1 caused a significant reduction in adhesion of *S. aureus* Rosenbach compared to the control treatment. For *S. aureus* SH1000, Cmpg5300_05.29 caused a 37% reduction of adhesion relative to the control. However more experiments are necessary to confirm the results of both strains.

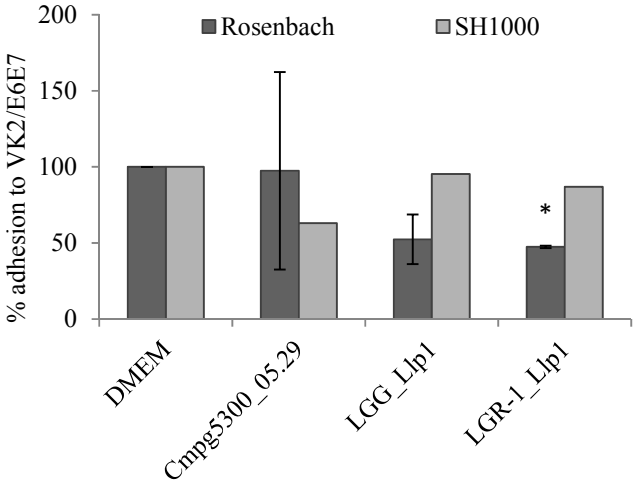

Figure 3.15 Adhesion of *S. aureus* Rosenbach and SH1000 to VK2/E6E7. Before adhesion, the strains were first incubated with the lectin domains of Cmpg5300_05.29, LGG_Llp1 and LGR-1_Llp1 dissolved in DMEM. The control was incubated with DMEM. Only the adhesion of *S. aureus* Rosenbach after treatment with LGR-1_Llp1 is significantly lower than the control. * $P < 0.01$

In a variant of the experiment, the lectin domains were added to the VK2/E6E7 cells before *S. aureus* was added, since the lectins may potentially block the ligands to which *S. aureus* binds. The adhesion of both strains was not impaired after the epithelial cells had been incubated with the lectin domains (data not shown). However, the experiment was only performed once for *S. aureus* Rosenbach.

3.7 Biofilm assays and bioscreens

As described above, lectins may be mediators of interactions between lactobacilli and microorganisms in their environment. To monitor interactions between the putative lectins or

their corresponding lectin domains and pathogens naturally present in the GIT and the vagina, biofilm assays were performed, in which the pathogenic microorganisms were allowed to form a biofilm in the presence of the lectins/ lectin domains. In the case the lectin domains affected the biofilm, bioscreens were performed to verify if the lectin domains influence the biofilm and/or the planktonic phase.

3.7.1 *Candida albicans* SC5314

The outcome of the biofilm assays is represented in Figure 3.16 and shows that the biofilm formation of *C. albicans* SC5314 is not significantly affected by the lectins of the probiotic strains. This holds for both variants of the assay, in which the lectins were added before and after or only after the adhesion phase of biofilm formation.

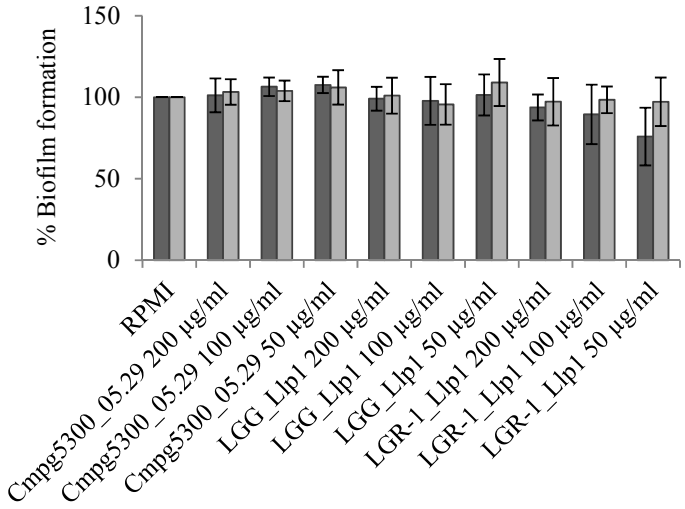

Figure 3.16 Biofilm assay of *C. albicans* SC5314 in the presence of lectins. These were added at different concentrations. Dark bars: lectins added before (t=0) and after adhesion phase (t=90'), light bars: lectins added after adhesion phase (t=90').

3.7.2 *Salmonella* Typhimurium ATCC14028

When the lectins were added at the start of the static peg biofilm assay (t=0), all lectins led to a significant reduction in biofilm formation, which was on average 33.3% for LGG_Llp1 and 68.2% for LGR-1_Llp1 at a concentration of 50 µg/ml (Figure 3.17, a and b). At the same concentration, the homologous proteins Cmpg5300_05.29 of *L. plantarum* CMPG5300 and Msa of *L. plantarum* WCFS1 caused an average reduction of biofilm formation of respectively 77.8% and 79.0% (Figure 3.17c). When higher concentrations were tested (200 and 100 µg/ml), these did not cause a significantly higher biofilm reduction. At 50 µg/ml, LGG_Llp2 caused an average biofilm reduction of 84.2% (Figure 3.17d). Also at 10 µg/ml of this lectin domain, a high biofilm reduction was observed, which was statistically similar to the reduction caused by 50 µg/ml (data not shown). As LGG_Llp1 and LGG_Llp2 are isolated from the same strain, a possible synergistic effect of Llp1 and Llp2 of LGG was investigated. However, when LGG_Llp1 and LGG_Llp2 were added together at the start of the biofilm formation (Figure 3.17d), each at a

concentration of 50 µg/ml, the biofilm formation was higher than when only LGG_Llp1 or LGG_Llp2 was added (Figure 3.17c and d). Yet, when these lectin domains were added at a concentration of 5 µg/ml, a biofilm reduction of on average 45.6% was observed (Figure 3.17d). When the lectin domains were added after 1.5 hours to allow the cells to adhere to the pegs, none of them caused a significant decrease in biofilm formation (Figure 3.17). In a third variant of the assay, the lectins were added after 24 hours of biofilm growth. Here as well, the lectins had no effect on the biofilm formation of *S.* Typhimurium ATCC14028 (Figure 3.17).

In a fourth variant of the static peg assay, lectin domains were added at several time points instead of only once, to study the impact of continuous administration of the lectin domains. Hereto, lectin domains were added at 0 h, 12 h, 24 h and 36 h. In contrast with previous assays, Cmpg5300_05.29 did not cause a significant reduction in biofilm formation (P = 0.09) when the lectins were added only at 0 h (Figure 3.18). Still there is a tendency observable that the biofilm

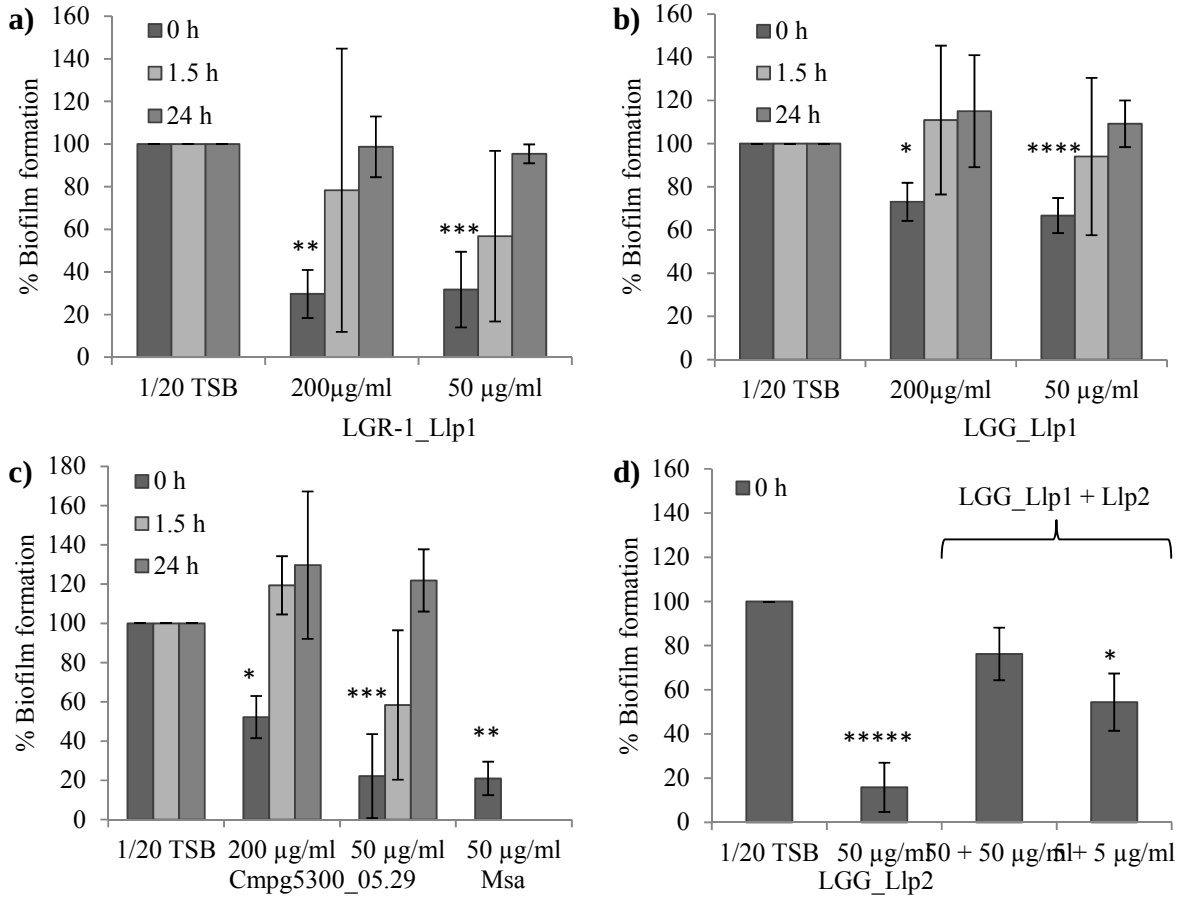

Figure 3.17 Effect of lectin domains on *S.* Typhimurium ATCC14028 biofilms. The purified lectin domains of LGR-1_Llp1 (a), LGG_Llp1 (b), Cmpg5300_05.29 (c), Msa (c) and LGG_Llp2 (d) were added after 0, 1.5 and 24 hours to the biofilms. (For Msa and LGG_Llp2 only after 0 h.) Also combinations of LGG_Llp1 and LGG_Llp2 (d) were tested. *: P < 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001, *****: P < 0.00001.

is prevented the most when the lectin domain was administered only at 0 h. Additional adminstrations at later time points seemed to reduce the biofilm inhibition. As can be seen in Figure 3.18, the lectin domain of LGG_Llp1 caused an average reduction of 40.5% when added at 0 h, consistent with the first static peg assay. When LGG_Llp1 was added at 0 h and again 12 h later, there was a significant extra decrease of biofilm formation. However additional administrations of the lectin domain of LGG_Llp1 at 24 or 36 h could not inhibit the biofilm further. The lectin domain of LGR-1_Llp1 added at 0 h could prevent biofilm formation for 76.4%, which did not change significantly when the lectin domain was additionally administered at 12, 24 and 36 h (Figure 3.18).

In order to confirm the results of these static peg assays, the absolute CFU count of *S.* Typhimurium biofilms grown in the presence of the lectin domains was determined. Hereto, *S.* Typhimurium biofilms were grown in 12 well plates containing 1 ml of medium per well. As shown in Figure 3.19, LGG_Llp1 and LGR-1_Llp1 resulted in a reduction of biofilm growth of respectively 50.9% and

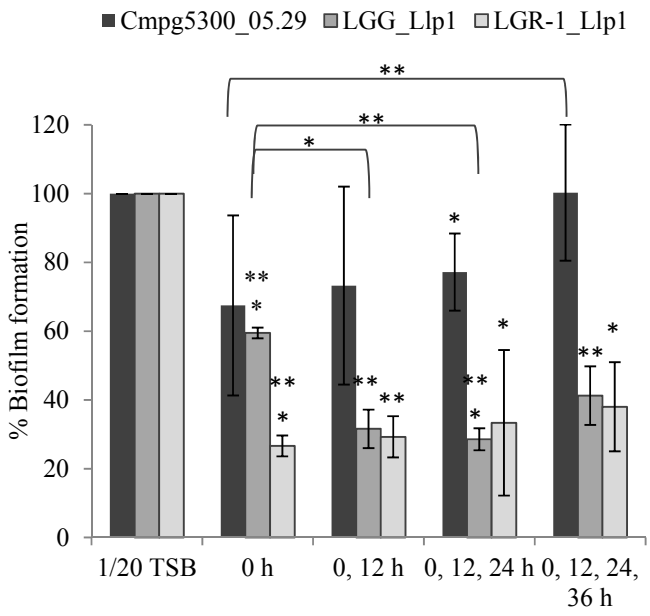

Figure 3.18 Time-dependency of the activity of the lectin domains against biofilms of *S.* Typhimurium ATCC14028. The purified lectin domains were added after 0, 12, 24 and 36 hours in fresh medium (50 µg/ml in 1/20 TSB). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. T-test compared to control (1/20 TSB) or else as indicated by brackets.

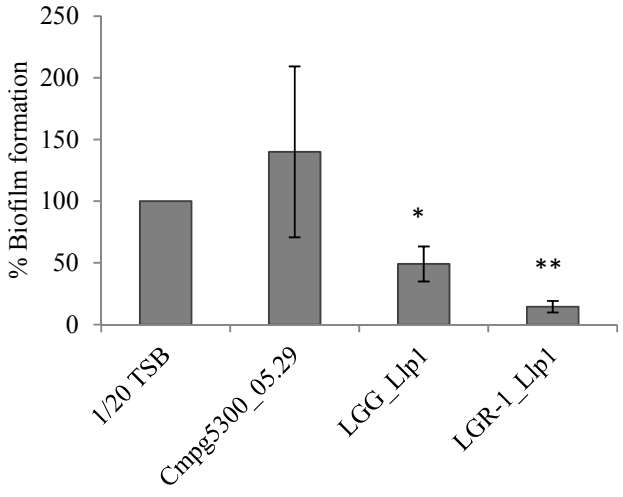

Figure 3.19 Effect of lectin domains on biofilms of *S.* Typhimurium ATCC14028 based on absolute CFU count. Biofilms were grown in 12 well cell culture plates, containing 1 ml 1/20 TSB with lectins added at t=0 at a concentration of 50 µg/ml. Absolute CFU counts of the treatments was normalized towards the control, which was grown in 1/20 TSB without lectins. *: $P < 0.05$, **: $P < 0.01$

85.5% compared to the biofilm grown without lectins. Of notice, while the assay was performed, the biofilms grown in the presence of LGR-1_Llp1 could be seen to dissolve and detach from the wells, whereas the control biofilms did not. This was to a lesser extent also observable for LGG_Llp1, but not for Cmp5300_05.29. The lectin domain of Cmpg5300_05.29 did not cause the same biofilm reduction as in the first peg assay (Figure 3.19). However, this could be masked by the varying results of the different repetitions.

All experiments discussed so far were performed using the purified lectin domains of the respective lectins. However, in their natural context, the lectin domains are part of full length lectins, which could as a whole have different activities than the lectin domains on their own. To verify that the results of the biofilm assays also hold for the lectins as how they are naturally present in lactobacilli, the static peg assay was performed with purified full-length lectins when these were available. In addition, as mentioned before in 3.4.2, each purified lectin domain as well as full length lectin appeared as two closely spaced bands in SDS-PAGE. For the biofilm assays with the lectin domains, both proteins corresponding to these bands were used together. To investigate whether both bands in fact represent the lectins and share the same activity, the proteins corresponding to these bands were collected as separate fractions during size exclusion of the full length lectins. The different fractions were then used in a static peg biofilm assay. Both fractions of LGR-1_Llp1, LGG_Llp1 and LGG_Llp2 had similar effects on *S.* Tyhimurium biofilms, indicating the two bands indeed correspond to the lectin. For each full length lectin, the average effect of the two fractions is shown in Figure 3.20**Figure** . The full length lectins LGR-1_Llp1, LGG_Llp1 and LGG_Llp2 caused average biofilm reductions of 78.0, 79.0 and 77.4%. These reductions are similar to those observed for the corresponding lectin domains, though full length LGG_Llp1 seems to be more active than its lectin domain (Figure 3.17b). However, these results need to be confirmed.

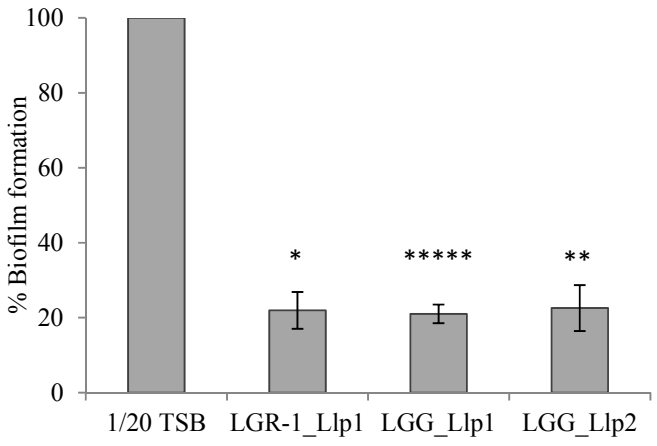

Figure 3.20 Biofilm formation of *S.* Typhimurium ATCC14028 in the presence of purified full length lectins. These were added at 50 µg/ml to the medium at the start of the assay (t=0). **: $P < 0.01$, ***** $P < 0.00001$.

Given the capacity of the lectins to prevent *S.* Typhimurium ATCC14028 biofilms, bioscreens were performed to investigate if the lectins reduce the growth of this strain. Hereto the strain was grown with and without purified lectin domains under the same conditions as the biofilm assays. As Figure 3.21 demonstrates, the growth is not impaired by any of the lectin domains. In fact, it appears that the strains grow better by adding the lectin domains, especially LGG_Llp2, which might be based on a shift in the metabolism and eventual utilization of the proteins (Figure 3.21a). When grown in a rich medium, *S.* Typhimurium grown with LGG_Llp2 displays a sigmoidal curve, typical for a metabolic shift. This was also observed for LGR-1_llp1 but not for LGG_Llp1 and CMpg5300_05.29 (Figure 3.21b). Nevertheless, the bioscreens demonstrate that the biofilm inhibition effect of the lectin domains is not due to an inhibition of the planktonic growth of *S.* Typhimurium ATCC14028.

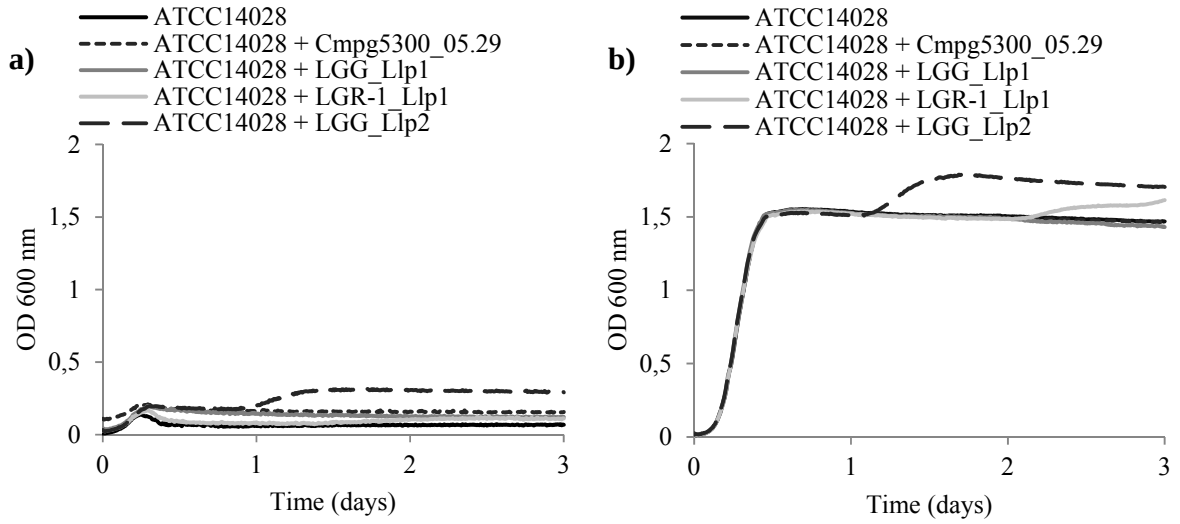

Figure 3.21 Growth of *S.* Typhimurium ATCC14028 in the presence of lectin domains. These were dissolved in 1/20 TSB (a) and TSB (b) at a concentration of 50 µg/ml.

Finally, the effect of the lectins on *S.* Typhimurium biofilms was visualized in two different ways. First, *S.* Typhimurium ATCC14028 carrying the plasmid pFPV25.1, which allows constitutive expression of GFP, was grown into biofilms in medium supplied with the corresponding lectin domains. After 48h, the biofilms were washed to remove the planktonic phase and visualized with phase contrast and epifluorescence microscopy. The obtained images are presented in Figure 3.22 Compared to the control, the lectin domains of LGR-1_Llp1 and LGG_Llp2 led to the formation of large holes in the biofilm. When the assay was performed without washing, there was no significant difference between the control and treated biofilms

(data not shown). Therefore it is assumed that the holes are due to the washing and that the lectin domains destabilize the biofilm. Although not in this experiment, the lectin domain of Cmpg5300_05.29 was also observed to form holes in the biofilm as well as Msa of *L. plantarum* WCFS1 (data not shown). The biofilms treated with LGG_Llp1 generally appeared similar as the control (Figure 3.22d).

In a second assay, biofilms of *S.* Typhimurium ATCC14028 were developed in medium containing FITC-labeled lectin domains. This allowed the visualization of the distribution of the lectin domains within the biofilm. As shown in Figure 3.23, all lectin domains caused the formation of very large holes. Especially for the treatments with Cmpg5300_05.29, LGR-1_Llp1 and LGG_Llp2, the biofilms were almost completely rinsed away after washing, which was even visible with the naked eye (data not shown). In contrast with previous results, LGG_Llp1 also caused large holes in the biofilm, although not to a similar degree as the other lectin domains.

As the epifluorescence images show in Figure 3.23, all lectin domains caused a highly fluorescent signal which appears to be corresponding with the biofilm thickness, when comparing with the phase contrast images. Since the control was not fluorescent, the fluorescence of the lectin-treated biofilms must be due to the FITC-lectin domains. Therefore, this assay demonstrates the presence of the lectin domains within the biofilm phase.

3.7.3 Uropathogenic *Escherichia coli* UTI89

As for *S.* Typhimurium ATCC14028, various biofilm assays were performed on *E. coli* UTI89 to investigate whether the lectins from lactobacilli can affect this strain's biofilm formation. First, purified lectin domains were added to the bacteria at the onset of biofilm development. As shown in Figure 3.24, all lectin domains significantly prevented the biofilm formation. At a concentration of 50 µg/ml, the lectin domains of LGR-1_Llp1, LGG_Llp1, Cmpg5300_05.29 and LGG_Llp2 reduced the biofilm on average for 85.0, 85.5, 94.8 and 88.8% respectively (Figure 3.24, **Figure** a, b, c, d). Again, the lectin domains of LGG_Llp1 and LGG_Llp2 were added together to investigate a potential synergism of these lectins. In contrast with *S.* Typhimurium, the biofilm reduction of *E coli* UTI89 was similar when LGG_Llp1 and LGG_Llp2 were added separately or together, both at 50 µg/ml and 10 µg/ml. In addition, adding 5 µg/ml of each of these lectin domains exerted a similar effect as adding 10 µg/ml of one of these lectin domains (Figure 3.24d).

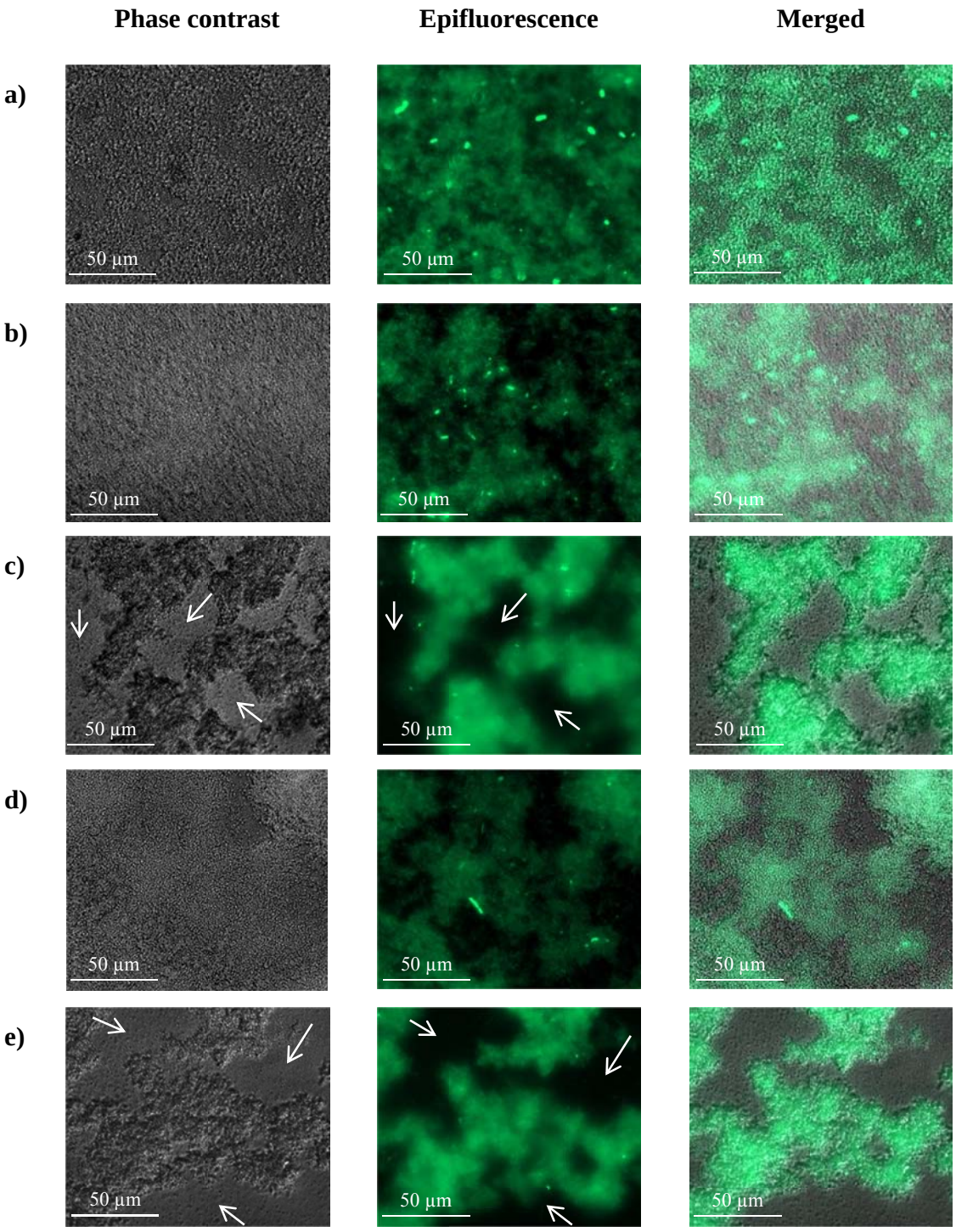

Figure 3.22 Phase contrast and fluorescence images of biofilms of GFP-expressing *S.* Typhimurium ATCC14028 developed in the presence of lectin domains. The biofilms were grown in 1/20 TSB (a) containing 50 µg/ml lectin domains of Cmpg5300_05.29 (b), LGR-1_Llp1 (c), LGG_Llp1 (d) and LGG_Llp2 (e). LGR-1_Llp1 and LGG_Llp2 cause the formation of large holes after washing with PBS, which are indicated with arrows.

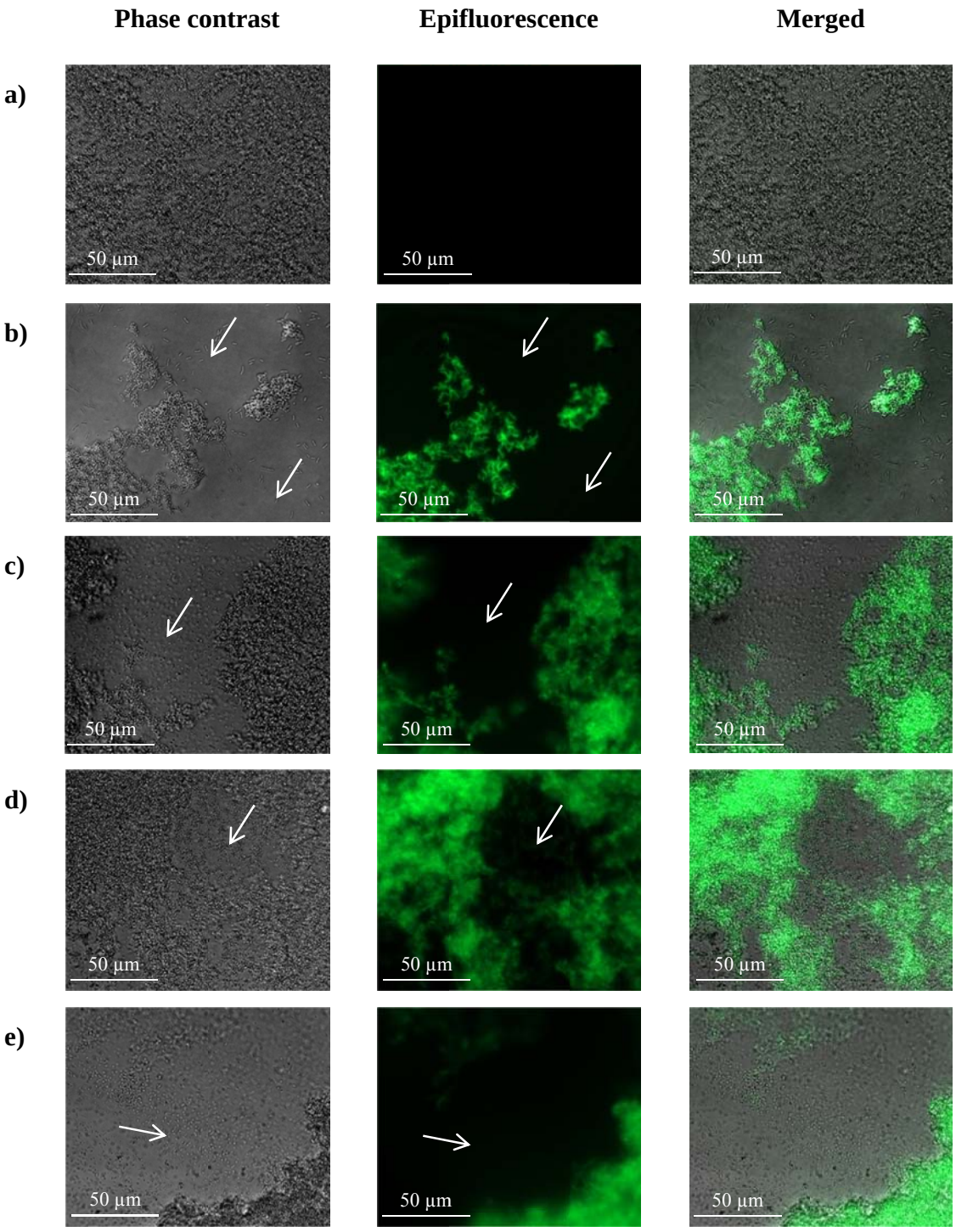

Figure 3.23 Phase contrast and fluorescence images of biofilms of *S*. Typhimurium ATCC14028 developed in the presence of FITC-labeled lectin domains. The biofilms were grown in 1/20 TSB (a) with 50 µg/ml of FITC-labelled lectin domain of Cmpg5300_05.29 (b), LGR-1_Llp1 (c), LGG_Llp1 (d) and LGG_Llp2 (e). The biofilms formed in the presence of lectin domains easily detach from the wells, leaving only parts of the biofilms after washing with PBS, whereas the control (a) remained a complete biofilm. Holes are indicated with arrows.

Second, the lectin domains were added after 1 h to allow the cells to adhere to the pegs. This was to verify if the lectins prevent adhesion to the substrate or a later phase of biofilm development. Figure 3.24 demonstrates that all lectins still reduce the biofilm formation as compared to the control, although they are generally slightly less effective. In a third type of the experiment, the lectin domains were added after 24 h to test their ability to eradicate established biofilms. As opposed to *S.* Typhimurium ATCC14028, the lectin domains of LGR-1_Llp1, LGG_Llp1 and Cmpg5300_05.29 are still active at a concentration of 50 µg/ml, with average biofilm reductions of 51.2, 44.9 and 50.7%, respectively (Figure 3.24, a, b and c). LGG_Llp2 at a concentration of 10 µg/ml could reduce the biofilm for 45.6% (Figure 3.24d).

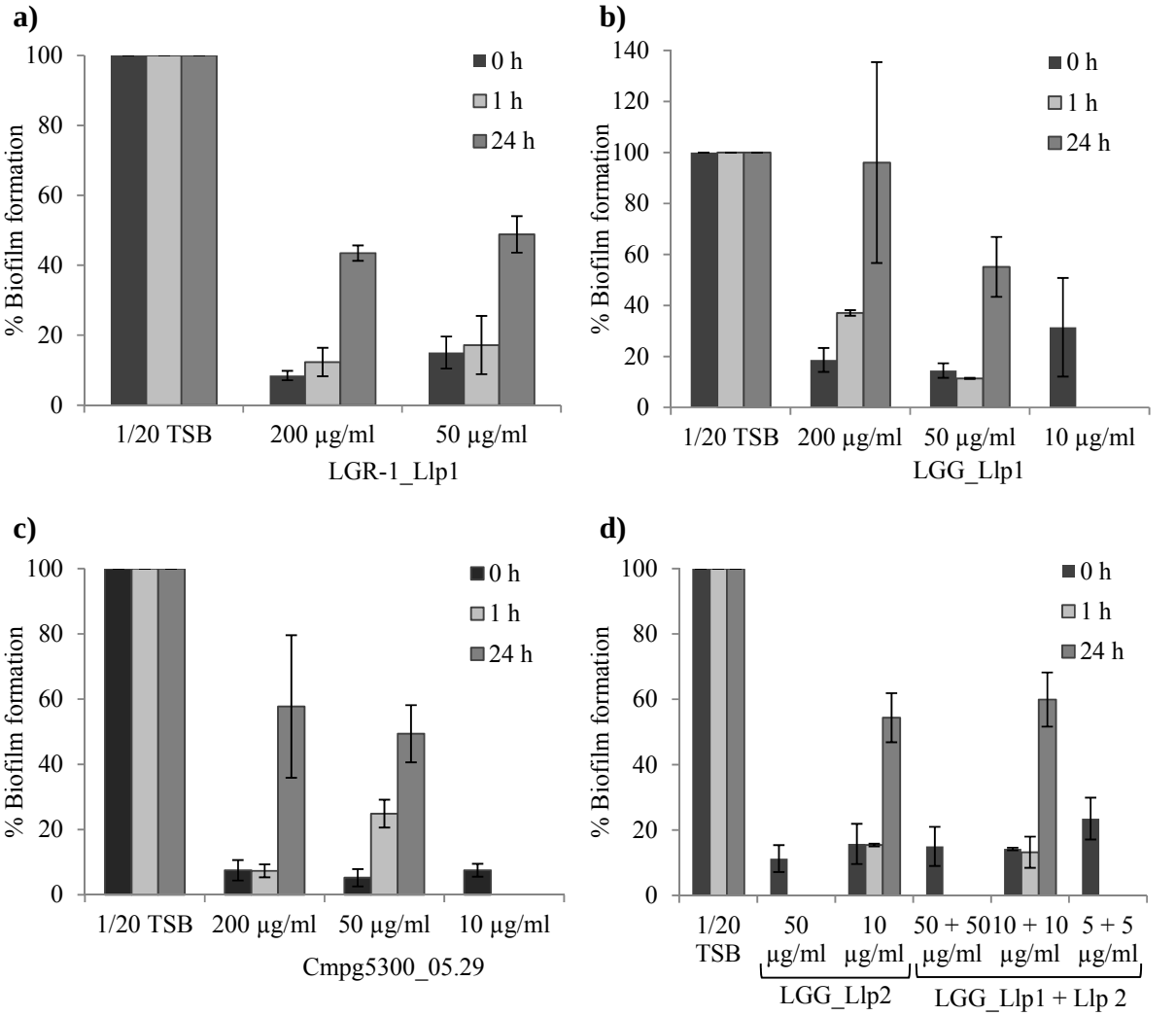

Figure 3.24 Effect of lectin domains on *E. coli* UTI89 biofilms. The purified lectin domains of LGR-1_Llp1 (a), LGG_Llp1 (b), Cmpg5300_05.29 (c) and LGG_Llp2 (d) were added after 0, 1 and 24 hours to the biofilms. Also combinations of LGG_Llp1 and LGG_Llp2 (d) were tested. (For Cmpg5300_05.29 10 µg/ml, LGG_Llp1 10 µg/ml, LGG_Llp2 50 µg/ml, LGG_Llp1 + Llp2 50 + 50 µg/ml and 5 + 5 µg/ml only addition after 0h was tested.)

To confirm these results, biofilms were grown in wells of cell culture plates and plated out to determine the absolute CFU count. It was expected that the biofilm reduction observed in the static peg assay should correspond to a reduced cell count of the biofilm. Indeed, both LGG_Llp1 and LGR-1_Llp1 caused significant reductions of *E. coli* UTI89 biofilm development of on average 41.7% and 58.4% respectively (Figure 3.25). However, for Cmpg5300_05.29 no significant decrease in cell count was observed.

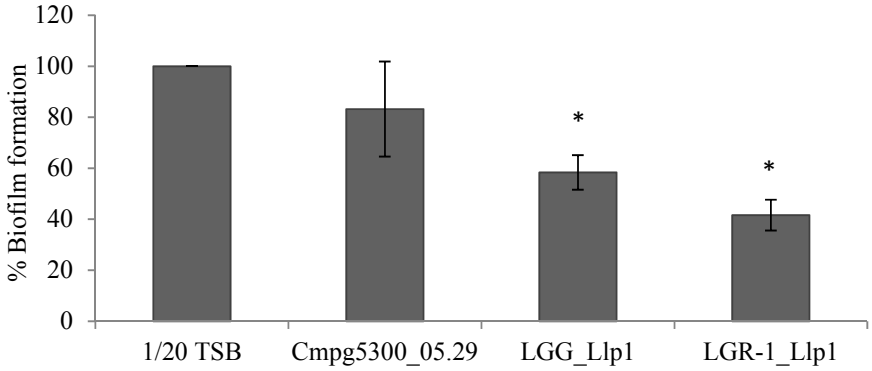

Figure 3.25 Biofilm formation of *E. coli* UTI89 based on absolute cell counts. Biofilms were grown in 1/20 TSB without (control) or with 50 µg/ml of lectin domain of Cmpg5300_05.29, LGG_Llp1 and LGR-1_Llp1. * P<0.01

All *E. coli* UTI89 biofilm experiments discussed so far were performed using the lectin domains of the corresponding lectins. However, one of our goals was to validate whether the anti-pathogenic effects also hold for the *Lactobacillus* strains from which the lectins were derived. Hereto, the full length lectins were purified and used in a static peg biofilm assay. As for *S.* Typhimurium ATCC14028, both protein fractions (called A and B) that were obtained after the protein purification of each full length lectin were tested separately. The results are presented in Figure 3.26**Figure** and demonstrate that the full length lectins are indeed able to prevent *E. coli* UTI89 biofilm formation, though they appear to be less active than their corresponding lectin domains (Figure 3.24). The different effect of the A and B fractions of LGR-1_Llp1 and LGG_Llp2 (Figure 3.26**Figure**) are not necessarily significant as there was quite some variety

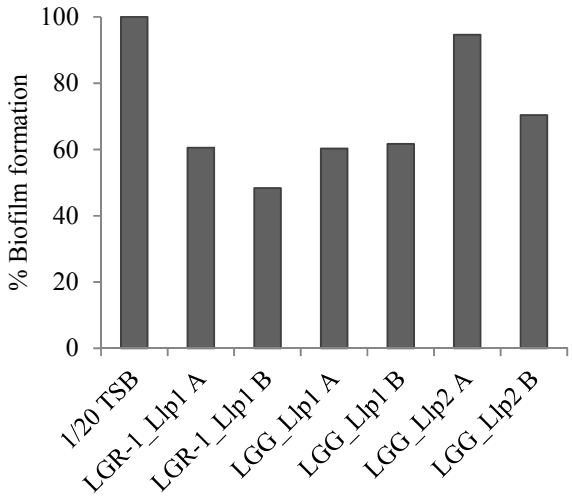

Figure 3.26 Biofilm formation of *E. coli* UTI89 in the presence of full length lectins. LGR-1_Llp1, LGG_Llp1 and LGG_Llp2 (50 µg/ml) were added at 0 h. During the purification process, two fractions possibly containing the lectin were collected for each lectin. These fractions, called A and B, were tested separately.

among the technical repeats. In fact, only one experiment with little technical repeats could be performed. Therefore more experiments are needed in order to decide whether i) the two fractions have a significantly different action and ii) whether the biofilm inhibition of the full length lectins differs from that caused by the corresponding lectin domains. Finally, a bioscreen of *E. coli* UTI89 pointed out that the lectin domains did not affect the planktonic growth (data not shown).

3.7.4 *Escherichia coli* MG1655 and TG1 and *Pseudomonas aeruginosa* PA14

Since the lectins dramatically affect the biofilm formation of *E. coli* UTI89, other *E. coli* strains were included to investigate whether the lectin activity is strain-specific or not. Hereto, biofilm assays were performed on two laboratory strains originally derived from *E. coli* K-12, namely *E. coli* MG1655 (Blattner *et al.*, 1997) and *E. coli* TG1 (Casali, 2003). The biofilm assay of *E. coli* MG1655 was not optimized yet at the end of this thesis. However, preliminary results of the *E. coli* TG1 biofilm assay indicate that both LGG_Llp1 and LGR-1_Llp1 can prevent this strain's biofilm formation (Figure 3.27a). Cmpg5300_05.29 seemed to increase the biofilm formation when 1/20 TSB was used as growth medium. However this effect was reversed when the experiment was performed with TSB (Figure 3.27a). Nevertheless more experiments are needed to confirm these results. The effect of the lectin domains on the planktonic growth of *E. coli* TG1 was studied in a bioscreen. As shown in Figure 3.27b, the lectin domains did not inhibit the growth of *E. coli* TG1.

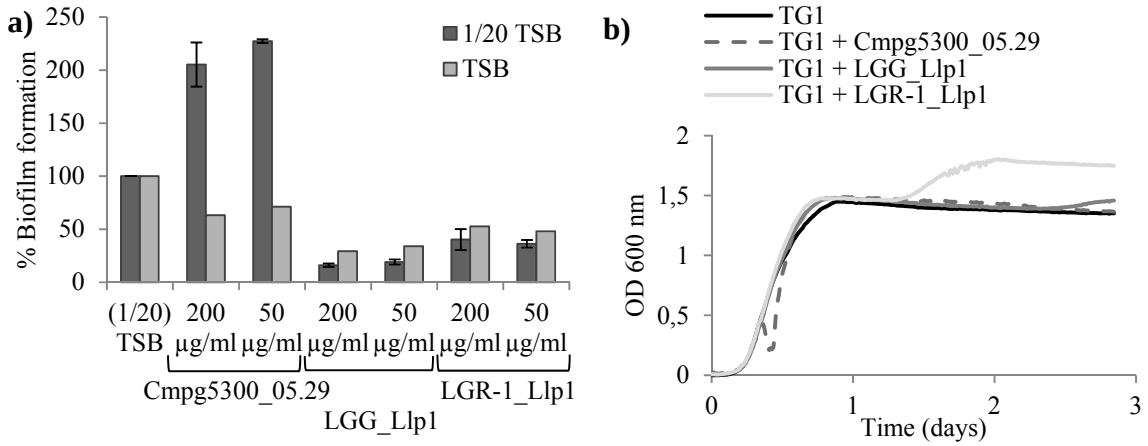

Figure 3.27 Effect of lectin domains of Cmpg5300_05.29, LGG_Llp1 and LGR-1_Llp1 on biofilm formation and planktonic growth of *E. coli* TG1. a) Biofilms were grown in 1/20 TSB (2 experiments) and TSB (1 experiment) supplemented with lectin domains. b) The bioscreen was performed in TSB with 200 µg/ml of lectin domains.

P. aeruginosa PA14 was also included as a potential target of the lectin domains. However, the biofilm assays of *P. aeruginosa* PA14 were not established yet, therefore no conclusions can be made about a potential inhibitory effect of the lectins towards this strain.

3.7.5 *Staphylococcus aureus* Rosenbach and SH1000

The lectin domains had no effect on the biofilm formation of *S. aureus* SH1000 and Rosenbach, with exception of Cmgp5300_05.29 (Figure 3.28). This lectin domain caused average biofilm reductions up to 35.5% in the case of *S. aureus* SH1000 and 40.5% in the case of *S. aureus* Rosenbach. However, when the lectin domains were added after 1 h or 24 h, there was no significant anti-pathogenic activity (data not shown). Bioscreens of both strains showed no effect of the lectin domains on the planktonic growth of either strain (data not shown).

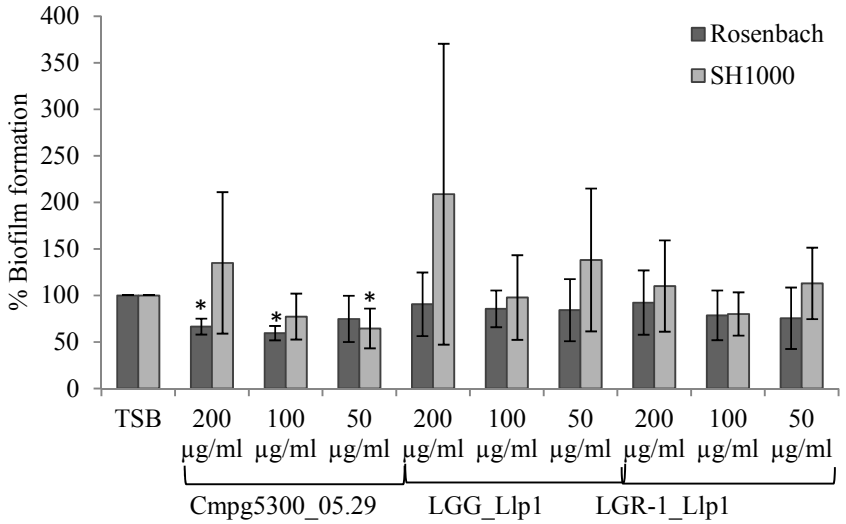

Figure 3.28 Effect of lectin domains of biofilms of *S. aureus* Rosenbach and SH1000. Biofilms were grown in TSB with different concentrations of the lectin domains of Cmpg5300_05.29, LGG_Llp1 and LGR-1_Llp1. Only Cmpg5300_05.29 shows anti-biofilm activity. *P < 0.05.

Chapter 4

Discussion and conclusion

4 Discussion

Although probiotic bacteria have been extensively studied, little is known about the molecular mechanisms behind the benefits ascribed to them. However, it is clear that surface associated structures are involved in the adaptation to their environment and the beneficial effects of probiotics (Lebeer *et al.*, 2008). As lectins are known to mediate specific intercellular and interspecies interactions throughout the tree of life, these proteins may play a key role in the probiotic factors of lactobacilli. In *L. rhamnosus* GG, *L. rhamnosus* GR-1 and *L. plantarum* CMPG5300 putative lectins were identified in previous studies (Petrova, 2013; Malik, 2014). Given the presumed sugar-binding activity of these proteins, a first goal of the thesis was to determine whether these genes are involved in the sugar metabolism of the strains. Furthermore it was our aim to confirm their lectin activity and determine their carbohydrate-binding profiles. Finally, it was investigated whether the lectins mediate interactions with GIT and urogenital pathogens and therefore may exclude pathogens and impair their biofilm development.

4.1 Lectins (not) involved in sugar metabolism of *Lactobacillus* strains

The growth assays of *L. rhamnosus* GG, *L. rhamnosus* GR-1, *L. plantarum* CMPG5300 and the corresponding lectin deletion mutants point out that the lectins are not involved in the metabolism of glucose, mannose, GlcNac and lactose. As previously reported, *L. rhamnosus* GR-1 and *L. plantarum* CMPG5300 are able to metabolize lactose although *L. rhamnosus* GG is not (Petrova, 2013; Malik, 2014). Interestingly, in one experiment, *L. rhamnosus* GG was able to grow on fucose, whereas *L. rhamnosus* GR-1 and *L. plantarum* CMPG5300 did not show that capacity. Given that the intestine is rich in fucose and that fucosylation is a mechanism by which the host promotes its endogenous microbiota (Pickard *et al.*, 2014), the fucose metabolism of *L. rhamnosus* GG could be an adaptation to the gastrointestinal niche. In contrast with *L. rhamnosus* GR-1 and *L. rhamnosus* LC705 (a cheese isolate), several genes with potential functions for fucose metabolism have been identified in *L. rhamnosus* GG, namely an L-fucose isomerase (*LGG_02685*) and an α-L-fucosidase (*LGG_02652*) (Petrova, 2013). Moreover, the growth on fucose was particularly reduced for the LGG_Llp2 deletion mutant. Of interest LGG_Llp2 is markedly different form the corresponding gene in *L. rhamnosus* GR-1 and *L. rhamnosus* LC705 (Petrova, 2013), indicating that this lectin may have evolved as a specifically intestinal adaption factor. However, the *L. rhamnosus* GG

growth curve on fucose could not be repeated and in literature this strain has been reported not to grow on fucose (Sanchez *et al.*, 2010). Therefore further studies are needed to elucidate the role of lectins in the fucose metabolism of *L. rhamnosus* GG.

Sorbitol is naturally present in fruits and is often used by the food industry as a sugar alternative due to its reduced absorption in the intestine (Yao *et al.*, 2014). In contrast with the other studied lectins, LGG_Llp2 could be involved in sorbitol metabolism as the deletion mutant showed reduced growth compared to the wild type in the presence of this sugar. However, this mutant still grew considerably, indicating LGG_Llp2 is not absolutely required for survival on sorbitol. Interestingly, *L. rhamnosus* GR-1 was characterized by a bi-phase growth curve. Thus, the sorbitol metabolism between *L. rhamnosus* GG and GR-1 appears to be markedly different, as also observed previously (Petrova 2013).

In theory LGG_Llp2 could be a moonlighting protein, meaning it performs multiple, mechanistically different functions, which are not conducted by different protein domains or a result of gene fusion or alternative splicing (Huberts and van der Klei, 2010). Moonlighting proteins arise by chance when a mutation promotes interactions between the protein and novel (macro)molecules (Copley, 2012). If these interactions contribute to the species fitness, organisms bearing the mutation will be favored. Over the past years, several bacterial moonlighting proteins have been identified, including in pathogenic and probiotic Gram-positive bacteria (Wang *et al.*, 2013). These are often enzymes active in sugar metabolism. However, through an unknown signal-peptide independent mechanism, they are secreted and exposed on the cell surface, where they function as adhesins and inductors of immune reponses (Wang *et al.*, 2013). It has also been noticed that gene duplication events go hand in hand with moonlighting proteins, as they allow a protein to mutate leading to different functions without jeopardizing the original function. (Huberts and van der Klei, 2010). Therefore it is possible that moonlight proteins represent in fact an intermediate stage in the evolutionary process towards separate proteins with individual, specialized functions (Copley, 2012). LGG_Llp2 indeed shows characteristics of a moonlighting protein, as it is able to bind mannose, involved in adhesion to epithelial cells (Petrova, 2013) and presumably the result of a gene duplication. A marked difference with common moonlighting proteins discovered so far, is that these are not exported via signal-peptide dependent pathways (Copley, 2012; Wang *et al.*, 2013). On the other hand, LGG_Llp1 and LGG_Llp2 show a high similarity with the CscC protein of *L. paracasei* subspecies *tolerance* LpL7 (Petrova, 2013). The Csc proteins

are present in operon-structure in many Gram-positive bacteria, including *L. plantarum,* and are suggested to form a cell-surface associated complex involved in the degradation of complex polysaccharides (Siezen *et al.*, 2006). Therefore it may be interesting to investigate the growth of *L. rhamnosus* GG and *L. rhamnosus* GR-1 on polysaccharides in the future.

4.2 Carbohydrate affinity of the lectins

Determining the carbohydrate-binding of lectins is not an easy task. One of the simplest methods that requires little amount of lectin is the ELISA-like assay (Sharon and Lis, 2007). However, it has become clear in this thesis that this assay has major drawbacks and is not a universal method. Although several attempts were performed to optimize the assay, it still generated false positive results as the signal was similarly high for nearly every sugar tested. Moreover, this binding could not be reduced by competition with mannose or fucose, although later assays indicated these as the primary sugars of the lectins. It is hypothesized this is caused by unspecific interactions between the plastic wells and the lectins and an ineffective blocking with the used blocking buffers, namely milk powder and PVA.

Therefore a second type of sugar-binding assay was performed, in which the affinity of the lectins towards sugar coated beads was assessed. One important advantage is that the many possible conformations of the sugars on the beads likely resemble their natural conformation on cells better than when coated on a surface. The PVA blocking functioned well here, as none of the lectin domains of the Llp's bound to the control (sepharose beads not coated with sugars). It was shown that LGG_Llp1 and LGG_Llp2 bind mannose and even better to mannan. The lectin domain of Cmpg5300_05.29 also exhibits a high affinity for mannan, followed by mannose although there was a high signal for the control. This confirms the fact that lectins have higher affinities for oligo- and polysaccharides than monosaccharides (Van Damme *et al.*, 2011). LGG_Llp1 additionally binds to glucose and may be glucose/mannose-specific similar to the specificity of ConA. In addition, lectins exist with only a high affinity for oligo- and polysaccharides (Sharon and Lis, 2007) and the LGR-1_Llp1 lectin may belong to this group as it did not bind significantly to any of the tested monosaccharides, whereas it did bind mannan. Although LGG_Llp1 and LGR-1_Llp1 lectin domains only differ in four amino acids, they appear to have different sugar affinities. Three of these residues have markedly different charge and/or polarity, which are asparagine, glycine and arginine for LGG_Llp1 and lysine, aspartate and glutamine for LGR-1_Llp1. Given these amino acids are located near to one another, namely positions 287, 294 and 300, it may be possible that this

part composes the carbohydrate-binding site. This is however speculative because interactions far away from the carbohydrate-binding site have been shown to influence the conformation of the lectin domain and thereby can tune the sugar affinity (Duncan *et al.*, 2005). Hence, the exact roles of these amino acids can only be assured by analysis of point-mutations.

Agglutination assays with yeast present a simple alternative to test sugar-binding, as yeast cell walls exist for > 90% out of carbohydrates (Latgé, 2007). The outer layer of the cell wall of *C. albicans* and *S. cerevisae* are in fact decorated with branched mannan which is covalently attached to proteins (Bowman and Free, 2006; Hall and Gow, 2013). Therefore, agglutination assays were performed with FITC-labeled lectins to allow visualization of the binding to the yeast cells. The result of these assays only partially confirm the previous binding assay, since only Cmpg5300_05.29 was shown to cause agglutination and bind to *S. cerevisiae* and *C. albicans*. LGR-1_Llp1 and to a minor extent LGG_Llp1 did bind to *C. albicans*, but not to *S. cerevisiae*. LGG_Llp2 bound to neither yeast species. The apparent discrepancy between the sepharose bead assay and the yeast agglutination assay may be explained by subtle differences in sugar-binding affinity of the lectins. Lectins have in fact a unique saccharide binding profile, despite common recognition of monosaccharides (Sharon and Lis, 2007). Also when different lectins bind the same oligosaccharide, this does not mean they have the same affinity or binding profile, because they may interact with the saccharide in different ways, for example recognizing different conformers (Gabius *et al*., 2011). In addition, a ligand behaves differently when in solution, absorbed on a surface, exposed on sepharose beads or on a cell. This means that the *in vitro* interaction between a ligand and lectin may differ from the interaction in a physiological context (Sharon and Lis, 2007), as we observed here. In order to fully understand the sugar-binding profile of the lectins, the lectin domains were sent for a glycan array, but the results were not available before completion of this thesis.

4.3 Interaction with pathogens – reducing adhesion and biofilm formation

A property often ascribed to probiotics is competitive exclusion, which means that through binding the host ligands of pathogens, probiotics can prevent their adhesion to the host (Lebeer *et al.*, 2008). Although it should be repeated, the *in vitro* binding of *C. albicans* and *S. aureus* to vaginal epithelial cells was not impaired by blocking the epithelium with lectins. However the used concentrations of lectins (100 µg/ml or approximately 3 mM) may have been insufficient to block enough ligands to really observe a difference.

Pathogen exclusion may also be achieved through a mechanism opposite to that described above. It may be possible that through direct interaction with pathogens, probiotics may block their adhesion. It has in fact been suggested that co-aggregation with pathogens may impede binding to host epithelium (Spurbeck and Arvidson, 2011). Therefore, as described above, agglutination assays were performed to determine whether the lectin domains of the lectins directly bind to the cells. Opposed to *C. albicans*, it appears that none of the lectin domains is able to bind to *S.* Typhimurium, UPEC and *S. aureus* SH1000 and Rosenbach, except for Cmpg5300_05.29 which could bind to *S. aureus* SH1000. Whether the lectin domain of Cmpg5300_05.29 in fact causes the agglutination of *S. aureus* still has to be ascertained. Previously it was demonstrated that *L. plantarum* CMPG5300 co-aggregates with both *S. cerevisiae* as *C. albicans* (Malik, 2014). It is now clear that the lectin Cmpg5300_05.29 is at least partially responsible for this interaction. It would be interesting to test the co-aggregation capacity of *L. plantarum* CMPG5300 with *S. aureus* and to verify if the other *Lactobacillus* strains co-aggregate with *C. albicans,* as these results imply this. Subsequently, we performed adhesion assays in which the pathogens *C. albicans* and *S. aureus* were first 'blocked' with the lectin domains before adhering to vaginal epithelial cells. Indeed, preliminary results indicate that Cmpg5300_05.29 reduces the adhesion of *S. aureus* SH1000, but this needs to be repeated. *S. aureus* Rosenbach was also shown to be significantly impaired in adhesion by LGR-1_Llp1. This was unexpected since the lectin domain did not bind to *S. aureus* in the agglutination assay. In contrast, *C. albicans* was not significantly hindered by the lectin domains in adhesion to the vaginal epithelium. Again it may be possible that the concentration was not high enough, given that *C. albicans* cells are much larger than *S. aureus*. A marginal reduction in adhesion was in fact observed, so it would be interesting to investigate whether a higher lectin concentration can further reduce adhesion of *C. albicans*. In the future it could be useful to include more strains in these experiments, since the lectins may have different specificity and since pathogens vary in their surface glycosylation. Especially mannose-exposing pathogens, such as *Mycobacterium tuberculosis* (Gupta, 2012), may confirm the capacity of lectins to prevent adhesion.

Various studies have reported the ability of probiotic lactobacilli to interfere with pathogenic biofilms (Saunders *et al.*, 2007; McMillan *et al.*, 2011; Woo and Ahn, 2013). However, the mechanisms underlying this property have not been discovered. We investigated whether lactobacilli lectins could be involved in the process. Our results show that all lectin domains had major impacts on the biofilm development of some or multiple pathogens. First, all lectin

domains remarkably prevented the UPEC biofilm formation on pegs, with reduction percentages between 85 and 95%, even at concentrations of approximately 0.3 µM (10 µg/ml). *S.* Typhimurium biofilms were also impaired albeit slightly less effectively, with reductions between approximately 70 and 85% for all lectin domains, except for LGG_Llp1 which only prevented the biofilm for 33%. Furthermore, only Cmpg5300_05.29 could moderately affect biofilms of *S. aureus* strains, whereas none of the lectins impacted the biofilms of *C. albicans*. Interestingly, *E. coli* TG1 biofilms showed high reductions caused by LGG_Llp1 and LGR-1_Llp1, but not as much by Cmpg5300_05.29, however these results are still preliminary. It should be noted that LGG_Llp2 was not tested on *S. aureus*, *C. albicans* and *E. coli* TG1 due to difficulties with its recombinant expression. Overall, it seems that each lectin domain has a specific activity against each of the pathogens and that each pathogen has a specific lectin inhibition profile, meaning the activity is both lectin and strain specific.

This strain-specific activity is further exemplified by the time-dependency of the lectin activities. The lectin domains only impair the *S.* Typhimurium biofilms when added at the onset of biofilm formation, whereas they are still significantly active against UPEC when added after 24h. This implies that *S.* Typhimurium biofilms can only be prevented by affecting their adhesion phase, although UPEC biofilms can be eradicated after establishment. It should be however kept in mind that in the peg assay UPEC did not develop into such an extensive biofilm as *S.* Typhimurium. Hence, it is possible that while *S.* Typhimurium develops into a thick impermeable biofilm within 24 h, UPEC biofilms remain sensitive to exogenous compounds much longer. On the other hand, these effects were only observed *in vitro* and it may be possible that *in vivo* biofilms of *S.* Typhimurium are susceptible much longer, although this is hard to predict. The lectin-specific activity manifested in different outcomes for the different biofilm assays. For example, the CFU count assays confirmed the activity of LGG_Llp1 and LGR-1_Llp1 for both *S.* Typhimurium and UPEC, but could not confirm the activity of Cmpg5300_05.29. Similarly, in the microscopic assays of *S.* Typhimurium biofilms, the biofilms were clearly destabilized by LGG_Llp2 and LGR-1_Llp1 and less by LGG_Llp1, confirming the previous biofilm assays. Again, biofilms treated with the lectin domain of Cmpg5300_05.29 appeared similar to the control. It is known that different assays may give rise to very different biofilm phenotypes of *S.* Typhimurium (Steenackers *et al.*, 2012), indicating that possibly in our scenario Cmpg5300_05.29 is only effective against the biofilms developed on pegs but not when grown in wells.

The lectin-specific activity is emphasized the most in the assay in which lectin domains were added at specific time point (0, 12, 24, 36 h) to *S.* Typhimurium biofilms. For example, it does not matter for LGR-1_Llp1 if the lectin domain is added at 12 h or later. As long as the lectin domain is present at the onset of biofilm formation, the preventive activity remains similar. In contrast, the lectin domain of LGG_Llp1 impairs the biofilm formation significantly more when it is added at the onset as well as after 12 h of biofilm development.

Several hypotheses can be made about the mode of action of the lectin domains against the biofilms. A first hypothesis assumes that the lectins bind to the pathogenic cells. The strain-specific inhibition could be explained by different carbohydrate signatures the pathogens expose on their cell surface and the lectin-specific action by their different sugar-binding profiles. For example, the broad-spectrum Cmpg5300_05.29 might be a more promiscuous lectin that recognizes multiple different oligosaccharides or carbohydrates common to many pathogens. However, none of the lectin domains were shown to bind directly to *S.* Typhimurium and UPEC in the agglutination assays. Actually this does not necessarily refute this hypothesis as it is known that the expression of various cell-surface associated molecules drastically changes at the onset of biofilm formation (Van Houdt and Michiels, 2005; Steenackers *et al.*, 2012). Therefore, it may be possible that the lectins bind to surface structures involved in the establishment of the biofilm but not present in the planktonic phase. This was in fact observed in the *S.* Typhimurium biofilms grown with FITC-labeled lectin domains, in which cells inside the biofilm were fluorescent and planktonic cells were not. Hence, it is plausible the lectins may block the adhesion of the pathogens, as was established in the previous paragraph. Another possibility is that the interaction with the lectins triggers a signaling cascade within the *S.* Typhimurium cells that counteracts processes involved in biofilm formation. Multiple examples exist, in which secreted compounds of probiotics are indeed able to decrease the virulence of pathogens, such as the expression of fimbriae in *E. coli* (Cadieux *et al.*, 2009)**.** In this respect, it would be interesting to investigate the expression of biofilm-related genes in the pathogens upon exposure to the lectins through RNA sequencing. A logical target would be the *CsgD* gene, as CsgD is considered the major regulator of the transition between planktonic and biofilm behavior (Steenackers *et al.*, 2012).

Nevertheless, the presence of the lectin domains within the biofilms does not assure they bind to the cells. It is possible the lectin domains interact with components of the biofilm matrix, which is composed of extracellular polymeric substances, including polysaccharides, proteins

such as fimbriae and lectins, DNA and lipids (Flemming and Wingender, 2010). These vary among strains and may also explain the observed strain-specific activity. A few of the functions of extracellular polymeric substances are adhesion to substrates, enabling aggregation and bridging of cells and conferring mechanical stability (Flemming and Wingender, 2010). In particular, cell surface associated and extracellular lectins play a role in the crosslinking of the polysaccharides and connecting the bacterial cells with the matrix. For example**,** outer membrane lectins LecA and LecB of *P. aeruginosa* are known to stabilize the biofilm (Tielker *et al.*, 2005; Diggle *et al.*, 2006). Both *S.* Typhimurium and *E. coli* biofilm matrices contain the polysaccharides cellulose (β-1-4-D-glucose polymer) and colonic acid (polymer of glucose, galactose, fucose and glucuronic acid) (Van Houdt and Michiels, 2005; Steenackers *et al.*, 2012). For *S.* Typhimurium and *E. coli* strains isolated from human faeces, it was demonstrated that aggregative fimbriae and cellulose together form a tight network stabilizing the biofilm matrix (Zogaj *et al.*, 2001). In addition, biofilms of *S.* Typhimurium, *E. coli* and *S. aureus* all contain the homologous proteins BapA, Yeej and Bap, which are presumed to be important for stabilizing this network via long-distance intercellular connections (Lasa and Penadés, 2006). Interestingly, it has been suggested BapA binds to carbohydrates on the cell surface or of the biofilm matrix (Wagner *et al.*, 2014). The exogenous lectin domains from *Lactobacillus* may compete with these crosslinking interactions and thereby destabilize the biofilm. To verify that the mode of action is indeed linked to sugar-binding, it would be interesting to perform the bioflm assays with mannose-competition, as this may point out whether the lectins really bind through specific sugar-binding instead of non-specific interactions.

4.4 Multimerization?

Besides sugar specificity, the yeast agglutination assay also sheds light on another interesting feature of the lectins. The fact that each lectin domain presumably only binds to one sugar moiety on one cell, implicates that the agglutination is caused by multimerization of the lectin domains. This is supported by the genetic structure of the domain given it was predicted to contain a homotetramerization domain. Moreover, L-type lectins are typically dimers or tetramers (Srinivas *et al.*, 2001). The anti-biofilm inhibition is presumably not related to the multimerization of the lectin domains. In fact, the multimerization may be decreasing the activity of the lectins, as the biofilm prevention is not enhanced at higher concentrations. This is especially highlighted by the fact that 50 µg/ml of LGG_Llp2 and 50 µg/ml of LGG_Llp1 together have a lower activity than 50 µg/ml LGG_Llp2, which indicates that LGG_Llp1

interferes with the activity of LGG_Llp2. It might be possible that LGG_Llp1 and LGG_Llp2 form heterodimers/tetramers. However in the full length lectin, the multimerization sites within the lectin domain may be buried, meaning that the full length lectin as it naturally is present on the lactobacilli cell wall does not necessarily multimerize. This could explain the descreased activity of the LGG_Llp1 lectin domain against *S.* Typhimurium compared to the full length protein.

4.5 Future prospects

The research in this thesis may serve as a starting point for various new research topics, as so many questions arise based on the results. Future investigation may be divided into two main categories. A first is to deepen our understanding of the fundamental role of lectins for bacteria. Interestingly, in many *Lactobacillus* species genes homologous to the lectin-like proteins have been discovered (Petrova, 2013) as well as many *L. plantarum* strains expressing the Msa lectin, which is similar to Cmpg5300_05.29. It is possible that surface-associated lectins may pose a general mechanism to prevent pathogen adhesion and biofilm formation in a variety of niches. The observations made in this thesis, may be merely scratches on the surface of *Lactobacillus* lectins. In the case of probiotics, it would be interesting to investigate the immune-modulating potential, given their lectins are involved in adhesion (Petrova, 2013). These strain-specific interactions with the host are crucial in determining which strain is a good probiotic in specific hosts and specific conditions, such as irritable bowel disease (Lebeer *et al.*, 2010) and BV (Petrova *et al.*, 2015). A second major area of research instigated by this thesis, is the applications of the lectins on their own for prophylactic goals. The clear biofilm inhibiting effect is worth to be explored, given the prevalence of problems associated with biofilms and the increased resistance of many bacteria against antibiotics. Resistance to antimicrobial agents is increasing and multidrug resistance has occurred in more than 55% of *S.* Typhimurium isolates (Fabrega and Vila, 2013). Therefore in the future we aim at testing the activity of the lectins against a variety of clinical isolates as well. The first company (LectinLabs, Nevada) specialized solely in plant lectins as alternatives for antibiotics, illustrates the enormous potential of lectins. However for lectin application, many safety issues first have to be addressed including their stability, cytotoxicity, allergenicity and effect on the endogenous microbiota. Regarding these issues, the lectins discovered in this thesis may pose additional advantages as compared to plant lectins, given their origin from *Lactobacillus* species naturally present in humans.

4.6 Conclusion

The functions as well as molecular ligands of two lectin-like proteins in *L. rhamnosus* GG and *L. rhamnosus* GR-1 and one surface lectin of *L. plantarum* CMPG5300 were analyzed. The investigated putative lectins were confirmed to be lectins, able to bind mannose and/or mannan. Although they are likely not involved in the metabolism of monosaccharides, one lectin of *L. rhamnosus* GG possibly plays a role in fucose metabolism, which could mean it evolved as a specific adaptation factor for the intestinal niche. However, this hypothesis requires further investigation and also the potential role of the lectins in the metabolism of polysaccharides is a path worth tracking. Furthermore, our hypothesis of surface lectins mediating interactions with other microorganisms was shown to be correct, as the lectins bind to *C. albicans* and *S. aureus* and appear to reduce their adhesion to vaginal epithelium. Importantly, the purified lectins all had the potential to impair the biofilm formation of specific pathogens, including *S.* Typhimurium and UPEC. Whether these clearly inhibitory activities against pathogens will still hold in the actual human body with the complete lactobacilli cells, is an interesting question that should be addressed. Notwithstanding, this thesis has contributed to the molecular proof underlying the probiotic action of gastrointestinal and vaginal lactobacilli. This increased knowledge will move us another step closer to the credibility and implementation of probiotics, with the ultimate goal to improve the health of both men and women.

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Vulgariserende samenvatting

Naast heel wat bacteriën die ziekten veroorzaken, zijn er ook zeer veel goede bacteriën die in en op ons leven, genaamd de microbiota. Deze vormen een complexe samenleving die zorgt voor het onderhouden van ons immuunsysteem, het verteren van ons voedsel in de darm en bescherming tegen ziekteverwekkers. Nu blijkt dat heel wat ziekten en aandoeningen gelinkt zijn met een ongebalanceerde microbiota. Een veelbelovende manier om ziekten te voorkomen of te genezen, is bijgevolg het bewust toevoegen van goede bacteriën aan ons lichaam om de samenleving weer in balans te brengen en te beschermen tegen gevaarlijke indringers. Deze bewust opgenomen bacteriën worden probiotica genoemd. Probiotica lijken echter niet altijd te werken en het optimaal gebruik van probiotica wordt nu tegengehouden door het gebrek aan kennis erover. Wat probiotica eigenlijk doen voor de mens en wat hun komst betekent voor de andere bacteriën aanwezig in het lichaam, is nog niet duidelijk. Om de gezondheidseffecten van probiotica te verklaren en om te bepalen welk probioticum geschikt is voor een specifieke aandoening, moet onderzocht worden wat zo'n bacterie op moleculair niveau teweeg brengt in ons lichaam. Dit is het domein waarbinnen deze thesis zich situeert. We hebben namelijk aangetoond dat er eiwitten zijn op het oppervlak van sommige goede bacteriën die kunnen binden aan suikers. Het belang hiervan ligt in het feit dat alle cellen bedekt zijn met een laag suikers. Onze hypothese was dan ook dat goede bacteriën deze eiwitten gebruiken om te binden aan andere cellen. Inderdaad, het blijkt dat deze eiwitten kunnen binden aan het oppervlak van bekende ziekteverwekkers zoals *Candida albicans* en *Staphylococcus aureus*. Bovendien observeerden we dat door te binden met deze eiwitten, deze ziekteverwekkers zich minder goed vasthechten aan epitheelcellen in de vagina. Ten slotte heeft het onderzoek beschreven in deze thesis aangetoond dat deze suiker-bindende eiwitten biofilmen – dit zijn meercellige bacteriegemeenschappen - van *Salmonella* Typhimurium en gevaarlijke *Escherichia coli* kunnen voorkomen. Hoe dit werkt is ons echter nog onbekend. De eiwitten van goede bacteriën laten ons dus toe te verklaren hoe probiotica een voordeel kunnen zijn voor onze gezondheid. Nu rest ons de uitdaging om deze kennis over moleculen samen te leggen met kennis over talrijke andere moleculen van goede, maar ook slechte bacteriën en de mens zelf. Hierdoor zouden we de effecten van probiotica kunnen verklaren en hopelijk ook voorspellen, om uiteindelijk de gezondheid van mensen optimaal te kunnen ondersteunen.