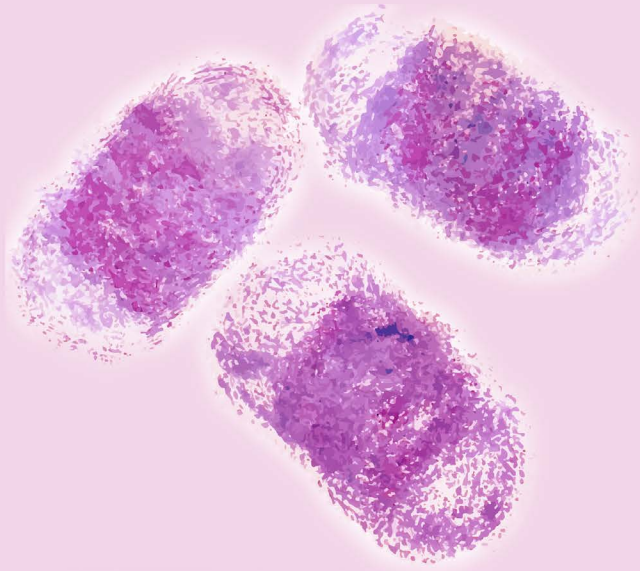
Host-interaction effector molecules of *Lactobacillus plantarum* WCFS1



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Thesis

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Chapter 1

General Introduction

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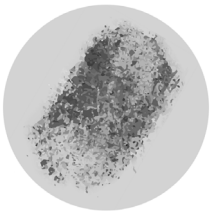

Introduction

The first consumption of fermented products by humans can be found as early as 7000BC in ancient Egypt [1]. Only in 1907, the concept that fermented foods can bring beneficial health effects to consumers was introduced by Elie Metchnikoff [2]. The definition of probiotics has evolved ever since and is currently coined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' [3]. In current commercial products lactobacilli are predominant [4, 5] and are Gram-positive, non-sporeforming, rod-shaped bacteria. This genus currently contains over 200 species, which can be found in various habitats ranging from raw and fermented dairy products, fresh and fermented plant materials, and digestive tracts and reproductive systems of humans and animals [5, 6]. Their ability to produce high levels of lactic acid lowers the pH of their environments and suppresses the growth of many other bacteria [6]. The taxonomical complexity mirrors the phenotypic diversity of the members of the *Lactobacillus* genus, which was initially divided into three phylogenetic groups: the *Lactobacillus delbrueckii* group, the *Lactobacillus casei-Pediococcus* group, and the *Leuconostoc* group. Based on subsequent phylogenetic analyses on basis of 16S rRNA sequences, the *Leuconostoc* group are now reclassified as species of the genera *Leuconostoc* or *Weissella*. The *L. delbrueckii* group was renamed the *Lactobacillus acidophilus* group, and the *L. casei-Pediococcus* group was split into further subgroups (*L. salivarius* group, *L. reuteri* group, *L. buchneri* group, and *L. plantarum* group) and the new genus *Pediococcus* [6, 7].

Genomic and genetic tools facilitate current probiotic researches

During the last decades, the growing collections of genomic sequences opened new avenues to obtain molecular insights in probiotic functionality and to decipher functional properties in bacteria and their hosts [5, 8]. With the access to genetic information available in the genome sequences in combination with comparative genomics, there is the possibility to correlate genotypic variation, to the presence and absence of a relevant probiotic function to identify potential probiotic effector genes and molecules involved in host-probiotic interactions. Successful strategies are exemplified by the identification of the gene encoding the mannose-specific adhesin in *L. plantarum* [9, 10] and the gene cluster encoding a pilin-like surface structure of *Lactobacillus rhamnosus* GG [11], which are both involved in intestinal adherence. Moreover, sequenced genomes can be employed in functional genomics approaches, including microarray-based transcriptional profiling that can provide comprehensive information on the transcriptional adaptation of a bacterium towards changes in its environment [12]. These transcriptomic profiles can provide associations of the expression of conserved genes to specific functional traits, which cannot be deduced from comparative genomic analysis. For example, such transcriptome-trait matching approach has been employed to identify genetic biomarkers of gastrointestinal (GI) survival, which facilitated the optimization of probiotic culture condition to improve their intestinal delivery in a viable form [13]. Moreover, annotated *Lactobacillus* genomes and *in silico* metabolic pathway prediction models provide comprehensive information about candidate effector genes to further identify key biological pathways that are important for the observed probiotic functions [12].

Functional validation is required to establish the link between specific phenotypes and the candidate genes selected from genome/transcriptome-trait matching approaches. Such validation is often achieved by genetic engineering, using targeted gene disruption by insertion or replacement through homologous recombination [12]. Alternatively, bacteriophage derived recombination

elements can also be employed for site-specific integration and provide high efficacy genetic engineering methodology [14, 15]. Alternative to gene disruption, (controlled) gene expression systems are also often employed in the validation of gene-function relations. Among the controlled expression systems, the nisin-controlled gene expression (NICE) system has been widely applied in *Lactobacillus* [12], but also other systems are available, including the sakacin-[16] and lactose-inducible [17] expression systems. Finally, besides controlled expression, in certain approaches constitutive gene expression systems are preferred and can be developed on basis of known constitutive promoters, such as the L-lactate dehydrogenase (*ldhL*) promoter [18, 19].

Lactobacillus plantarum *WCFS1 as a model probiotic organism*

L. plantarum is found in various environmental habitats, including a plethora of fermentations, ranging from dairy to meat and vegetables [20, 21]. Besides this dietary abundance, this species is frequently encountered as a natural inhabitant of the GI tract and specific strains are marketed as probiotics [22]. The genome sequence of several *L. plantarum* strains is available in the public domain, allowing the effective investigation of the genes and regulatory mechanisms underlying the observed GI tract persistence of the different members of this species [23, 24]. The *L. plantarum* genome appears to be the largest among the lactic acid bacteria genomes sequenced to date [25] , and contains a large amount of genes involved in carbon metabolism, as well as in regulatory and (sugar) transport functions. The overrepresentation of these functions reflects the flexibility and versatility of this species that can readily adapt to diverse environmental niches and the corresponding physicochemical conditions [24]. *L. plantarum* WCFS1 is a single isolate of strain NCIMB8826, which was originally isolated from human saliva [24]. The genome sequence of the WCFS1 strain was the first *Lactobacillus* genome to be published and is predicted to contain 3042 protein-encoding genes [24, 26]. The genome is well annotated and analyzed, including a comprehensive overview of secretome [27, 28], a genome-scale metabolic model [29], and reconstructed regulatory networks based on transcriptome data [30]. Moreover, established genetic tools for *L. plantarum* WCFS1 include gene deletion [31] and overexpression systems [16, 32], which provide the genetic engineering capacity for experimental validations of predicted gene functions. The combination of these available tools, makes this species and in particular the WCFS1 strain a suitable model for probiotic lactobacilli for the purpose of in depth investigation of the molecular mechanisms underlying probiotic functions.

Survival and persistence of L. plantarum *WCFS1 in gastrointestinal tract*

Consumption of probiotic bacteria is mostly achieved through the ingestion of freshly fermented foods or dried bacterial products in the form of dietary supplements. During passage of the GI tract, probiotic bacteria encounter various stresses, including acidity in the stomach, bile and digestive enzymes in the intestine, as well as osmotic stress and oxygen gradient throughout the digestive tract (Figure 1). The human stomach is a harsh environment where the pH can vary from 1 during fasting to 5 following food intakes [33]. The low pH in the environment results in intracellular acidification, which reduces the proton motive force, the energy source for transmembrane transport, and can damage acid-sensitive enzymes and DNA [34]. Bile is the main challenge for probiotics in the small intestine [35]; it is harmful to cells due to its detergent properties and also can cause oxidative damage by the generation of free radicals. Additionally, especially the glycine-conjugated bile acids are thought to dissipate membrane potential in a similar way as weak organic acids, and thereby can have antimicrobial activity [36]. The protonated forms of conjugated bile

acids can freely cross cell membranes and once inside the cell release protons [34], which leads to reduction of the intracellular pH, dissipating membrane potential, and thus acting similarly as organic acids.

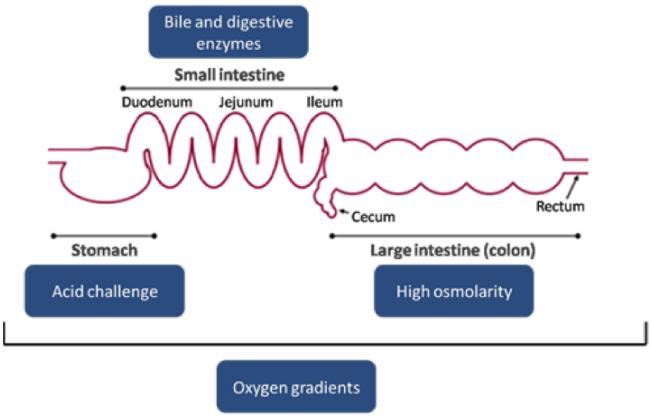

Figure 1. Stresses encountered by probiotics in the host GI tract (adapted from [28]). Probiotics experience acid stress in the stomach, bile and digestive enzymes in the small intestine and high osmolarity in the colon, as well as a highly variable oxygen concentration throughout the GI tract.

L. plantarum NCIMB8826 (alias WCFS1) was demonstrated to effectively survive passage of the human stomach, reach the ileum in high numbers, and could be detected in the colon [37]. A survival assay of 42 *L. plantarum* strains in a GI-tract mimicking environment illustrated a large diversity of the GI tract surviving capacity within this species [38]. Among the tested strains, WCFS1 was one of the better survivors and could persist at pH 2.3 for at least an hour and also displayed a strong tolerance to bile stress [38]. Notably, the *in vitro* GI survival assays employed in these studies was later shown to be in qualitative agreement with the GI persistence in a trial using healthy human volunteers [38]. Besides the survival capacity, *L. plantarum* WCFS1 adheres to intestinal molecules, including collagen I, Type II mucine, and fibronectin [39], which can also contribute to its persistence in the GI tract. The persistence of *L. plantarum* in the GI tract of healthy human volunteers enables its detection up to 3 days post-consumption, but after 7 days the levels of viable bacteria tended to decline below detection limits [37, 38], which is in agreement with the observation that probiotics are generally temporary inhabitants of the GI tract and do not exhibit long-term colonization [40].

Interaction of L. plantarum *WCFS1 and host mucosa*

After reaching the intestine, probiotic bacteria encounter the host intestinal microbiota and mucosal cells, including the activity of the mucosal immune system. The interaction between probiotics and immune cells is considered one of the core mechanisms to confer probiotic health-beneficial effects [41, 42]. *In vitro* studies have demonstrated that *L. plantarum* WCFS1 elicits an intermediate level of immune responses when co-culture with human peripheral blood mononuclear cells (PBMCs) [43] and dendritic cells (DCs) [44], in comparison to several other strains of the same species, of which some are substantially more or less immunomodulatory as compared to the WCFS1 strain. The WCFS1 strain stimulates moderate production levels of the pro-inflammatory cytokines interleukin (IL)-12 and tumor necrosis factor alpha (TNF-α), as well as the anti-inflammatory cytokine IL-10. In healthy mice, this strain was shown to increase

regulatory DCs and regulatory T cell frequencies in the spleen and tune the systemic immunity toward a more regulatory status [45]. *In vivo* mucosal responses elicited by the WCFS1 strain were also determined in healthy volunteers, by determination of the gene transcription responses in the small intestinal mucosa after exposure to *L. plantarum* WCFS1 [46, 47]. Troost *et al*. employed an intestinal perfusion technique to study the direct exposure of *L. plantarum* WCFS1 to proximal small intestine for 1-hour or 6-hour periods [46]. Their results show that the exposure has the most impact on lipid metabolism, cellular proliferation, cell death and survival and immune responses. The data show that *L. plantarum* WCFS1 inhibited lipid metabolism and cell proliferation after 1-hour exposure but these pathways were upregulated after 6-hour exposure, illustrating the dynamic response of the mucosa to microbial stimuli. Regulatory pathways associated with cell death and inflammation were also triggered, but did not appear to lead to complete activated of the corresponding cellular response networks [46]. Samples obtained from this same study [46] were also subjected to studies targeting the epithelial barrier function, showing that tight junction related proteins (scaffold protein zonula occludens 1 and transmembrane protein occludin) were significantly increase in the vicinity of the tight junction structures within duodenal tissues after 6-hour exposure to *L. plantarum* WCFS1 [48]. A parallel *in vitro* experiment using the Caco-2 epithelial cell line showed that this enhancement in tight junction function by *L. plantarum* WCFS1 is mediated through Toll-like receptor (TLR) 2 [48]. Other studies performed by van Baarlen *et al*. reported on the impact of *L. plantarum* WCFS1 consumption on the proximal duodenum of healthy adults [47]. The gene expression profiles of duodenal tissues were analyzed after 6-hour consumption of *L. plantarum* WCFS1. The results show that *L. plantarum* WCFS1 modulates innate and adaptive immune responses of small intestine, mainly genes associated with a NF-κB-related regulatory network. However, no signatures were detected of active triggering of pro-inflammatory responses, such as local immune cells infiltration [47]. Moreover, this study highlighted the distinct responses in duodenal mucosa elicited by consumption of *L. plantarum* WCFS1 bacteria harvested at specific time points during the bacterial growth (mid-logarithmic phase of growth, stationary phase of growth, or heat-killed stationary phase cells). Only consumption of stationary phase derived *L. plantarum* (live or heat-killed) leads to activation of the observed NF-κB network [47], whereas mid-logarithmic phase of growth derived cells induced pathways related to cell proliferation and biogenesis [47]. The authors pointed out that major changes take place in cell envelope component composition during transition from mid-logarithmic to stationary phase of growth which could play a critical role in the observed differential mucosal responses, which forms one of the fundaments of the work presented in this thesis [47].

Current challenges

Throughout the studies of the mechanisms underlying probiotic activity, it became apparent that the probiotic effects are often species and/or strain specific [49-52]. This situation has led more researchers to focus on the molecular characteristics of probiotic strains intending to link specific molecular structures to specific probiotic functions, and thereby deduce the mechanisms of molecular communication of probiotics. Molecules on the bacterial cell surface, such as peptidoglycan, teichoic acids, proteins and extracellular polysaccharides, not only play important roles in bacterial physiology where they are important to maintain cell integrity and morphology, but also are considered to be key-players in the interaction with the host mucosa through pattern recognition receptors (PRRs) expressed by the host cells [41, 52, 53]. These molecules are commonly present in Gram-positive bacteria, yet their structural properties can be substantially

diverse between species and also between strains of the same species, raising the core question to what extent these structural differences can influence the interaction between these bacteria and the host cells. To date, this question largely remains unanswered, where the majority of our working models of host-microbe molecular interaction are built on basis of research performed with exemplary strains of a species, rather than considering the diversity among strains as an important factor of variation.

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Outline of the thesis

This thesis focuses on potential cell envelope effector molecules involved in interaction with the mammalian host cells, including lipoteichoic acids, lipo- and glyco-proteins, and extracellular polysaccharides, of *L. plantarum* WCFS1, a probiotic model strain. The contribution of these molecules to bacterial physiology and immunomodulatory properties were assessed by gene deletion strategies and functional genomics-based approaches in order to enhance our understanding of molecular mechanisms of host-bacterial interactions.

Chapter 2 presents an overview of existing research regarding the potential roles in probiotic functionality of *Lactobacillus* surface molecules, including peptidoglycan, teichoic acids, surface polysaccharides and proteins. Biosynthesis pathways and structure variations as well as interaction with host PRRs and immunomodulatory properties of these molecules are summarized and compared among *Lactobacillus* species/strains. With this review of the current state-of-the-art in probiotic effector molecule research, the subsequent chapters focus on specific molecules that reside in the cell envelope of *L. plantarum* WCFS1, and study their role in bacterial physiology, as well as their role as ligands in TLR2 signaling and immunomodulatory properties using human-cell co-incubation models.

In **chapter 3** we focus on the lipoteichoic acid (LTA) of *L. plantarum* WCFS1. LTA is generally considered to signal through TLR2 and elicit pro-inflammatory responses in human cells, including epithelial as well as immune system cells. However, inconsistent results have been reported on this subject when using LTA molecules obtained from different Gram-positive bacteria. In our studies, two lipoteichoic acid synthase genes (*ltaS1* and *ltaS2*) were disrupted in the WCFS1 model strain, allowing the confirmation of their requirement in LTA biosynthesis by this bacterium. The deficiency of LTA also had a drastic impact on cell division, cell morphology and growth, and LTA-deficient cells elicited more pro-inflammatory responses in PBMCs rather than the expected loss of pro-inflammatory capacity as was observed with similar mutants of *L. acidophilus* NCFM. Further studies on the signaling capacity of the purified LTA from *L. plantarum* WCFS1 showed that these molecules failed to trigger TLR2 dependent responses, which is in clear contrast to the highly potent TLR2 stimulatory capacity of LTA obtained from *Bacillus subtilis*. These results imply that structural differences of the LTA produced by different bacteria are prominent determinants for their TLR2 signaling capacity and immunomodulatory properties.

Chapter 4 focuses on lipoproteins of *L. plantarum* WCFS1. Bacterial lipoproteins can be divided into two types, di-acyl and tri-acyl lipoproteins, which are recognized by TLR2/6 and TLR1/2 respectively. Despite being well-recognized TLR2 ligands, lipoproteins have mainly been studied in pathogenic bacteria and their role in probiotic functions has barely been addressed. In chapter

4, a WCFS1 derivative was constructed that is deficient in prolipoprotein diacylglyceryltransferase (Lgt), which transfers acyl chains moiety onto lipoproteins. This deletion mutant displayed normal growth and cell morphology, but released substantially more lipoproteins into its culture supernatant as compared to the wild type strain. Moreover, lipidation deficiency of lipoproteins resulted in significant reduction of TLR1/2 signaling capacity but left TLR2/6 signaling unaffected, suggesting that lipoproteins of *L. plantarum* WCFS1 are predominantly (if not exclusively) tri-acylated. The *lgt* deficient strain elicited more pro-inflammatory responses in PBMCs as compared to the wild type, indicating that the native lipoproteins could play a prominent role in dampening inflammation upon host-probiotic interaction.

Chapter 5 explores the protein glycosylation machinery in *L. plantarum* WCFS1. Glycosylation is an important post-translational modification for proteins, yet has only been discovered relatively recent in bacteria. In order to explore protein glycosylation in probiotics, we set out to identify glycosyltransferases responsible for the major autolysin (Acm2) of *L. plantarum* WCFS1, which was recently found to be *O*-glycosylated with *N*-acetylhexosamine conjugates [54]. Using sequence similarity searches with a glycosylation associated protein found in *Streptococcus parasanguinis* as a query sequence, 6 candidate glycosyltransferase genes were identified in the WCFS1 genome that could play a role in protein glycosylation but were annotated to play a role in glycosylation of teichoic acids rather than proteins. Using gene deletion and complementation in combination with a lectin-based glycan detection and mass spectrometry analysis, two glycosyltransferases, GtfA and GtfB (formerly TagE5 and TagE6, respectively), were shown to be required for the glycosylation of Acm2 and other unidentified *L. plantarum* WCFS1 proteins. These results provide the first example of a general protein-glycosylation machinery in a *Lactobacillus* species. Furthermore, the lectin-based detection revealed similar patterns of protein bands among 9 different *L. plantarum* strains, suggesting conserved protein glycosylation machineries in this species.

Chapter 6 focuses on extracellular polysaccharides (EPS) in *L. plantarum*. Two *L. plantarum* strains SF2A35B and Lp90 display an obvious ropy phenotype, implying much higher EPS production levels as compared to the model strain WCFS1. Based on genome sequence comparison, both of the ropy strains SF2A35B and Lp90 were found to possess their own, unique polysaccharide gene clusters. These gene clusters were deleted and the resulting mutants were shown to have lost the capacity to produce large amounts of EPS, and were studied in relation to their properties in host-bacteria interaction. The latter analyses included the comparative determination of bacterial surface properties and survival under GI-tract mimicking conditions, in EPS-mutants in comparison to their corresponding parent strains. Additionally, TLR2 signaling and immunomodulatory capacities of the mutant strains were compared with those obtained with their cognate wild type. The results illustrate strain-specific and variable impacts of the removal of the EPS in the background of individual *L. plantarum* strains, supporting the importance to evaluate and study the role of these cell-envelope associated molecules within the context of their producing host rather than in purified form.

Chapter 7 discusses the main findings of the thesis in the context of structural variations between bacteria and their relevance to strain specificity in host interaction.

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Chapter 2

The quest for probiotic effector molecules - Unraveling strain specificity at the molecular level

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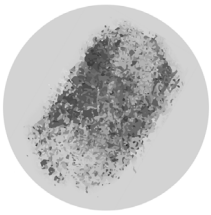

Abstract

Pharmaceutical agents are widely applied for the treatment of gastrointestinal (and systemic) disorders and their role as modulators of host cell responses is relatively well characterized. By contrast, we are only beginning to understand the molecular mechanisms by which health-promoting, probiotic bacteria act as host cell modulators. The last decade has seen a rapid development of the genomics field for the widely applied probiotic genus *Lactobacillus*, and nowadays dozens of full genome sequences are available, as well as sophisticated post genomic and genetic engineering tools. This development has enabled comparative (functional) genomics approaches to identify the bacterial effector molecules involved in molecular communication with the host system that may underlie the probiotic effects observed. These efforts can also be complemented with dedicated mutagenesis approaches to eliminate or alter these effector molecules, followed by assessment of the host interaction consequences thereof, allowing the elucidation of the molecular mechanisms involved in probiotic health effects. Many of these approaches have pinpointed that the *Lactobacillus* cell envelope contains several effector molecules that are pivotal in the direct signaling capacity of these bacteria that underlies their immunomodulatory effects, including lipoteichoic acid, peptidoglycan, and (glyco)proteins. Moreover, the cell envelope contains several compounds such as wall teichoic acid and capsular polysaccharides that may not be involved in direct signaling to the host cell, but still affect signaling through shielding of other bacterial effector molecules. Initial structural studies revealed subtle strain-and species-specific biochemical differences in the canonical cell envelope compounds that are involved in these host interactions. These biochemical variations include the degree and positioning of d-alanyl and glycosyl substitution in lipoteichoic acids, and acetylation of peptidoglycan. Furthermore, specific peptides derived from peptidoglycan and envelope associated (glyco)proteins were recently identified as potent immunomodulators. These findings are exciting in the light of the possibility of more pharmacological application of these bioactive probiotic molecules, and especially cost-effective production and targeted delivery of bioactive peptides seems to emerge as a feasible strategy to harness this knowledge.

Introduction

The word 'probiotic' is derived from the two Greek words 'προ' (pro) and 'βιοτος' (biotic), which translates to 'for life' [1]. Nowadays, probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [2]. The most extensively commercialized probiotic genera are those of *Lactobacillus* and *Bifidobacterium* [3-5], although other microorganisms are also employed, including the Gram-negative bacterium *Escherichia coli* str. Nissle 1917 [6, 7] and the yeast *Saccharomyces boulardii* [8, 9].

A variety of health benefits has been proposed to be associated with the consumption of probiotics, which are frequently related to prophylactic or therapeutic effects in specific disorders and diseases. Several of the proposed health benefits have been observed relatively consistently in clinical studies that targeted (intestinal) diseases, including the prevention of antibiotic associated diarrhea [10], prevention of *Clostridium difficile*-associated diseases [11], prevention of pouchitis after ileal pouch anal anastomosis [12], increased eradication rates of anti-*Helicobacter pylori* therapy [13], prevention of severe necrotizing enterocolitis [14], symptom alleviation in irritable bowel syndrome [15], and reduction of respiratory tract infection incidence [16]. By contrast, other diseases that were targeted by probiotic supplementation have generated less consistent clinical results and/or were less reliably observed in different studies, including prevention of atopic dermatitis in infants [17], extension or induction of remission periods in Irritable Bowel Disease patients [18-20], Traveler's diarrhea [21], reduction of duration of active diarrhea [22], and relieve of childhood constipation [23]. Importantly, accurate comparison of these clinical studies is often hampered by substantial differences in study design, including the probiotic strain and dosage tested, the duration of intervention, the clinical read-outs employed to assess the intervention efficacy, and the target population included in the intervention. Therefore, at this stage it is virtually impossible to draw general conclusions on the health impact of probiotics. Additionally, although some health benefits may be less dependent on the probiotic strain used, many of the beneficial effects of probiotics are expected to be strain specific, implying that the proposed efficacy of a particular strain cannot be extrapolated to other probiotic strains or species [24]. Analogously, many of the referred meta-analyses conclude that further studies are needed to draw strong conclusions. As a consequence, the European Food Safety Agency (EFSA) has deemed the vast majority of probiotic health claims as insufficient, in probiotic strain characterization and/or the lack of biomarkers to demonstrate a cause-effect relationship [25]. This situation has fueled the ambitions of the industrial and scientific community to unravel the molecular characteristics of probiotics and identify the effector molecules underlying the (strain-specific) clinical effects of probiotics.

Probiotic modes of action

Probiotics can potentially influence gastrointestinal and systemic health in various ways. One of their modes of action may be indirectly elicited via their influence on the endogenous microbiota of the gastrointestinal tract [26]. For example, probiotics can protect the host from pathogens by competitive exclusion, thereby hindering the adhesion on the intestinal surface and subsequent infection by pathogens [27]. Probiotics can also inhibit pathogens by acidification of the gut microenvironment through production of organic acids or by secretion of antimicrobial

compounds [28, 29]. The latter concept has elegantly been evidenced for a bacteriocin produced by *Lactobacillus salivarius* which was shown to be solely responsible for the efficient reduction of *Listeria monocytogenes* infection in mice [30].

Probiotics may also directly interact with the different cell lineages that reside in the intestinal tract mucosa. These interactions may involve epithelial cell lineages like enterocytes, Goblet cells and / or entero-endocrine cells (Figure 1), which play various roles in controlling local innate immune activity as well as in systemic control of defense-associated responses [4, 5, 31, 32]. Additionally, probiotics may interact with the adaptive immune system of the intestinal tract, which is contained predominantly in the gut-associated lymphoid tissues (GALT) of the lamina propria [5, 31, 33]. The small intestine lymphoid tissues are especially dominant in the Peyer's patches (Figure 1), which are regions that contain follicle centers that are covered by specific, follicle-associated epithelium composed of 'M'-cells that play a role as primary portal for antigen-entry into follicle centers. Besides these dedicated follicle centers, the lamina propria of the mucosa is populated by various immune cells, including macrophages, dendritic cells (DCs), and T-cells, but are predominated by antigen presenting B cells that produce antibodies (Figure 1) [5, 31, 33]. Among the T-cells the most important classes of lamina propria associated T cells in the context of probiotic function belong to the T helper (Th) and regulatory T (Treg) cells [5]. Tregs are the major producers of the anti-inflammatory interleukin (IL)-10, and play a key-role in the control of appropriate T cell responses, which includes the suppression of auto-reactive T cells and maintenance of tolerance [34]. The role of macrophages and DCs is related to their phagocytotic capacity, where macrophages are important for the removal of cell-debris and pathogens [35] and DCs act as dedicated antigen presenting cells that control both adaptive and innate immune responses [5]. Immature DCs are present in the lamina propria throughout the intestinal mucosa as well as in the Peyer's Patches that upon stimulation with microbes or other factors (e.g., diet derived) can mature via activation of the nuclear factor (NF)-κB pathway and in their activated form can modulate T-cell activity via T-cell clonal expansion and/or differentiation [36, 37]. These direct interactions of probiotics with different cell lineages within the mucosa of the intestine are considered to involve direct molecular recognition of specific microbial components, designated Microbe Associated Molecular Patterns (MAMPs) that are recognized by corresponding Pattern Recognition Receptors (PRRs) expressed by host cells (Figure 2). These PRRs include Toll-Like Receptors (TLRs), Nucleotide Oligomerization Domain (NOD)-Like Receptors (NLRs) and C-type Lectin Receptors (CLRs) [4, 5, 38].

In a healthy situation, the integrated interactions of the endogenous intestinal microbiota with the mucosal tissue leads to a state of homeostasis that sustains an adequate and balanced mucosal barrier function and prevents excessive immune responses. This homeostasis or balance involves all components of the mucosal defense systems, including mucin production, antimicrobial activities produced by the innate immune system, and specific responses elicited in the adaptive immune system [5, 31, 32]. However, this situation may be (transiently) compromised, for example by excessive abundance of certain microbial groups in the endogenous microbiota or specific pathogens [5, 39]. This state is referred to as dysbiosis, in which defense responses are unbalanced and may eventually lead to disease [5, 40]. Direct interactions between probiotic bacteria and the mucosa of the intestine may play a role in restoration and / or maintenance of homeostasis or prevention of deterioration of barrier functions in dysbiosis situations that can eventually contribute to the prevention of disease development [41]. Therefore, it is of great importance

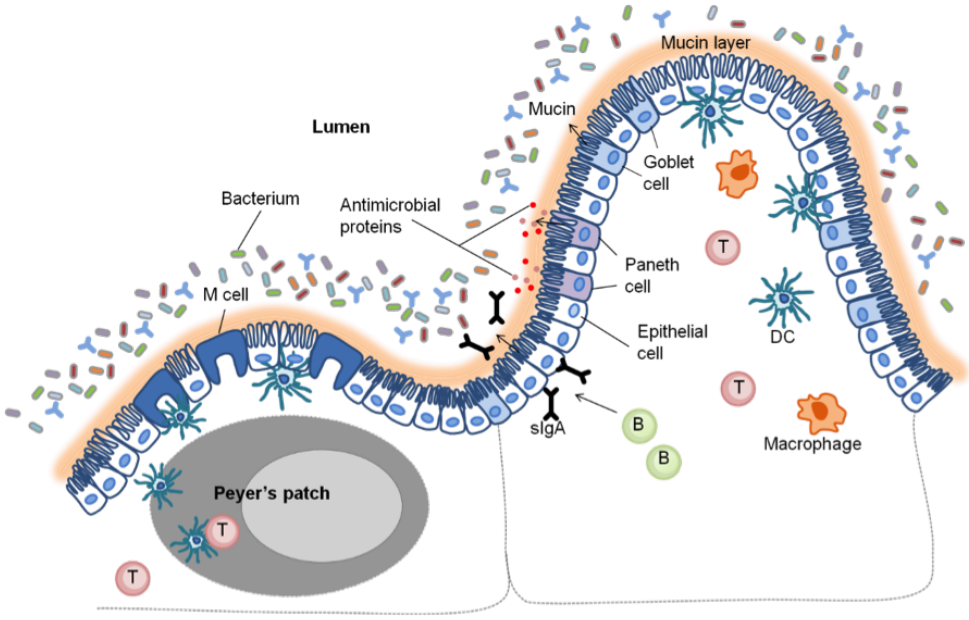

Figure 1. The intestinal mucosa tissue (Modified from [5]). The intestinal mucosa consists of a one-cell-thick epithelium layer, separating the highly colonized intestinal lumen from the lamina propria. Specialized epithelial cells, including mucin-producing Goblet cells and antimicrobial-protein-secreting Paneth cells, limit the exposure of mucosa tissue to the bacteria. The small intestine lymphoid tissues are dominant in the Peyer's patch, which is a region containing follicle centres covered by follicle-associated epithelium. The follicle-associated epithelium contains Microfold (M) cells, which transports microbial antigen across the epithelial barrier to antigen-presenting cells, such as dendritic cells (DC) and macrophages underneath. The lamina propria of the mucosa is populated by various immune cells, including macrophages, DCs, T-cells (T) and predominately the antibody-producing B-cells (B). slgA: secreted IgA.

to better understand the precise biochemical nature of the interactions between the probiotic MAMPs or effector molecules and their cognate PRRs. Especially the molecular mechanisms and corresponding effector molecules (MAMPs) that underlie strain specific probiotic effects in the mucosa remain largely unknown. Moreover, it is also poorly understood how the multi-factorial PRR-MAMP interactions that trigger a complex network of signal transduction cascades is integrated into the overall response that they elicit in terms of changed transcriptional patterns in the cell nucleus, which are the ultimate determinant of the host (immunomodulatory) responses.

It is generally considered plausible that a prominent fraction of the probiotic effector molecules resides in the bacterial cell envelope, as this part of the microbial cell is the first to interact with intestinal host cells [4, 31, 38]. Analogously, several PRRs have been reported to specifically recognize certain microbial cell envelope components or fragments thereof, including lipopolysaccharides, peptidoglycan, teichoic acids, lipoproteins or other surface (glyco)proteins, and cell envelope associated polysaccharides [38]. The remainder of this review will focus on the state-of-the-art knowledge on *Lactobacillus*-derived cell envelope associated components that may vary in terms of their biochemistry and structural properties and as a consequence may play distinct roles as probiotic effector molecules in different probiotic strains and species which might provide the molecular basis to explain the strain- and species-specific probiotic effects observed.

Probiotic surface effector molecules

Peptidoglycan

The cell wall of Gram-positive bacteria, including lactobacilli, consists of a thick peptidoglycan layer, which is a multilayered, cross-linked glycan chain with a repeating unit of β-1,4-linked *N*-acetylglucosamine (Glc*N*Ac)and *N*-acetylmuramic acid (Mur*N*Ac) [4, 42]. These multilayered and stacked glycan strands are linked via peptide bridges and form a rigid structure to maintain the integrity of the underlying protoplast against internal turgor pressure [38, 42-44]. This layer also serves as a platform for the anchoring of cell surface molecules (Figure 2), such as wall teichoic acids (WTA), wall polysaccharides and surface proteins [38, 42, 45]. In lactobacilli, the disaccharide unit of peptidoglycan can undergo a wide range of modifications, which have important consequences for bacterial physiology. For example, *N*-deacetylation of Glc*N*Ac and/or Mur*N*Ac in *Lactobacillus fermentum* [46] and 6-*O*-acetylation of Mur*N*Ac in *Lactobacillus casei*, *L. acidophilus* and *L. fermentum* [38, 42, 47] have been described. The *N*-deacetylation and 6-*O*-acetylation in Glc*N*Ac/Mur*N*Ac have shown to reduce the susceptibility for autolysis by lysozyme, which is a bacteriolytic enzyme present in the host innate immune system [38, 47]. An *in silico* analysis of the complete genome sequences of 12 *Lactobacillus* species showed that most lactobacilli lack the *pgdA* gene, encoding a peptidoglycan Glc*N*Ac deacetylase [48], but 10 out of the 12 species have at least one copy a peptidoglycan *O*-acetyltransferase gene, *oatA* [38, 49]. This analysis implies that *O*-acetylation of peptidoglycan occurs in the majority of *Lactobacillus* species.

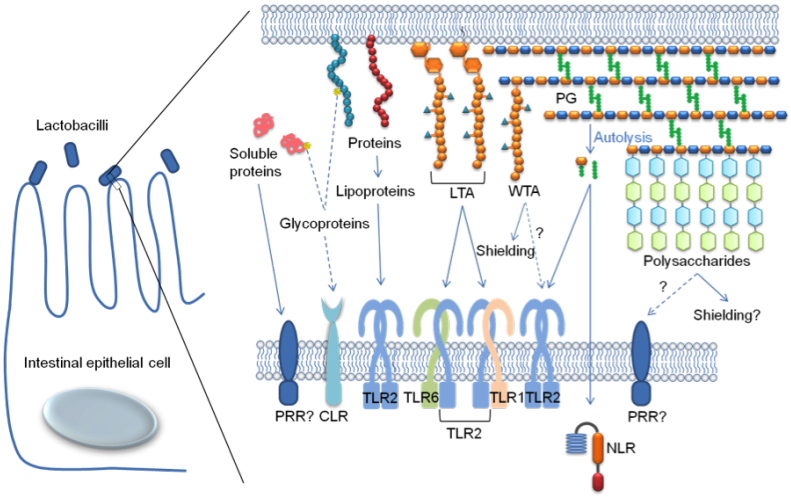

Figure 2. Molecular interaction of *Lactobacillus* surface components and epithelial Pattern Recognition Receptors (PRRs). (Modified from [5] and [57]). The *Lactobacillus* cell envelope components or fragments thereof, such as polysaccharides, peptidoglycan (PG), teichoic acids, lipoproteins or surface (glyco) proteins, have been reported to be specifically recognized by PRRs, including Toll-like receptors (TLRs), Nucleotide Oligomerization Domain (NOD)-Like Receptors (NLRs), and C-type Lectin Receptors (CLRs). Pentapeptides derived from PG autolysis or enzymatic degradation as well as lipoproteins are signaling through TLR2. Lipoteichoic acid (LTA) commonly contains di-acyl and/or tri-acyl glycolipids, which are proposed to signal through heterodimers TLR2/6 and TLR2/1, respectively. The capability of wall teichoic acid (WTA) to induce TLR2 signaling still remains debated yet it is thought WTA might shield other surface molecules from recognition and / or signaling. Glycoproteins might be recognized and signal through C-type Lectin Receptors (CLRs). *Lactobacillus* surface polysaccharides are thought to contribute by a shielding effect for other microorganism-associated molecular patterns (MAMPs).

There are two paralogues of *O*-acetyltransferase found in *L. plantarum* WCFS1 and *L. sakei* 23K [38]. Bernard *et al*. found that in *L. plantarum* WCFS1 these *O*-acetyltransferases, OatA and OatB, are responsible for *O*-acetylation of Mur*N*Ac and Glc*N*Ac, and consequently results in resistance against lysozyme and endogenous autolysins, respectively. More specifically, OatB reduces the peptidoglycan autolysis by inhibiting the activity of the *acm2*-encoded *N*-acetylglucosaminidase, the major *L. plantarum* autolysin [50]. The impact of these two modifications on peptidoglycan degradation and remodeling, as well as immunomodulation still remain to be determined.

The peptide bridge of peptidoglycan consists of a pentapeptide chain, containing l- and d-amino acids and one diamino acid (either l-Lys or *meso*-diaminopimelic acid) to allow cross-linkage. In most lactobacilli, the peptide chain consist of l-Ala-d-Glu-l-Lys-d-Ala-d-Asp (position 1 to 5), and the l-Lys at position 3 is cross-linked to d-Asp of an adjacent stem peptide (Figure 3A). Other types of peptide bridges have also been found, for example the linkage via l-Orn (l-ornithine), instead of l-Lys (Figure 3A), with d-Asp in *L. fermentum* and *Lactobacillus cellobiosus* or direct linkage through *meso*-diaminopimelic acid (*meso*-A_2pm) in *L. plantarum* (Figure 3B) [38, 44, 51]. The l-Lys/l-Orn-d-Asp bridge first requires RacD, an aspartate racemase, to generate d-Asp from l-Asp, followed by cross-linking that is catalyzed by a ligase, AslA [52, 53]. The analysis of sequenced *Lactobacillus* genomes confirms that orthologues of RacD and AslA are found in all l-Lys/l-Orn-d-Asp types lactobacilli, but AslA is absent in *L. plantarum* strains that harbor peptidoglycan containing *meso*-A_2pm. The *racD* gene, however, is also found in *meso*-A_2pm type species, suggesting that d-Asp might have a metabolic function besides peptidoglycan biosynthesis [38]. Another important feature of the peptide stems of many *Lactobacillus* species, including *L. casei* and *L. plantarum*, is the substitution of the terminal d-Ala with d-lactate [38, 42]. This determines the bacterial resistance to vancomycin, which acts as an antibiotic by binding to d-Ala containing peptidoglycan precursors, thereby preventing the proper assembly of peptidoglycan. The d-lactate containing peptidoglycan precursors displays a 1000-fold decreased affinity to vancomycin as compared to d-Ala-containing peptidoglycan [42]. Besides the compositions and linkage types of the peptide bridges, post-assembly modifications allow even more diversity in peptidoglycan structures, including amidations of d-Glu, *meso*-A_2pm and d-Asp which have been reported in *L. casei* and *L. plantarum* [54, 55]. An asparagines synthase, encoded by *asnH*, has recently been identified and predicted to be involved in d-Asp amidation in *Lactococcus lactis* [56]. Homologues of *asnH* are found in sequenced *Lactobacillus* genomes, even in the *meso*-A_2pm type *L. plantarum*, which indicates d-Asp amidation is a general feature of lactobacilli and AsnH might also be responsible for amidation other than d-Asp in pentapeptide bridges of peptidoglycan (such as *meso*-A_2pm or d-Glu) [38].

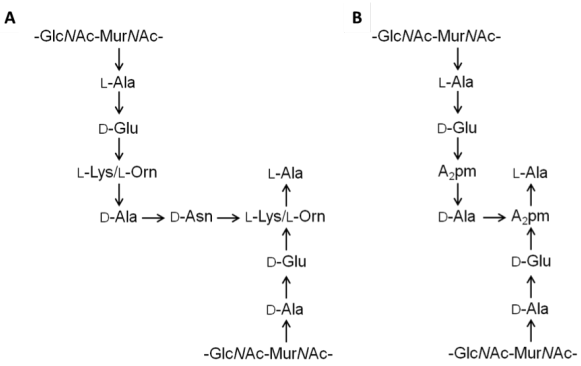

Figure 3. Two types of peptide bridge of peptidoglycan in *Lactobacillus*. (A) The peptide chain in most lactobacilli consists of l-Ala-d-Glu-l-Lys-d-Ala-d-Asp with l-Lys cross-linked to d-Asp of an adjacent stem peptide. However, the linkage via l-Orn (l-ornithine), instead of l-Lys, with d-Asp has been found in *Lactobacillus fermentum* and *Lactobacillus cellobiosus*. (B) Another type of peptide bridge, present in *L. plantarum*, is directly linked through *meso*-diaminopimelic acid (*meso*-A_2pm).

The precise role of amidation remains to be established. However, the degree of d-Asp amidation strongly affects the sensitivity of the bacterium to endogenous autolysins but also to lysozyme and the cationic antimicrobial nisin [56]. Taken together, these studies underline the notion that despite the universal conservation of peptidoglycan in Gram-positive bacteria, its composition, cross-linking and modification varies considerably among different *Lactobacillus* species [42, 44, 45, 51, 54, 55]. Moreover, it requires further investigation to determine whether these variations also occur among different strains of the same species. Importantly, the biochemical differences in peptidoglycan affect the susceptibility to lysozyme and endogenous autolysins, which affects the formation of fragments recognizable by the host immune system (see below).

It has been suggested that specific small fragments of peptidoglycan derived from enzymatic processing of peptidoglycan or normal cell wall remodeling, are recognized by TLR2 and NLRs of the host innate immune system [38, 55]. Highly *O*-acetylated peptidoglycan has been shown to be more resistant to hydrolysis by human lysozyme [56], thereby reducing the release of NLRs recognizable peptidoglycan fragments which may result in altered innate immune responses of antigen presenting cells, such as DCs and macrophages [57]. One example is the sensitivity of peptidoglycan to *N*-acetylmuramidase that is negatively correlated to the ability of *Lactobacillus* strains to induce IL-12 in mouse-derived macrophages [58]. Moreover, peptidoglycan from *N*-acetylmuramidase sensitive strains was demonstrated to be able to inhibit the IL-12 production induced by *L. casei* peptidoglycan, which is a strain insensitive to *N*-acetylmuramidase, in a TLR2-dependent- and independent manner [59]. The fact that the suppression of IL-12 production was also observed in TLR2-deficient macrophages suggests that NLRP2 (NOD-, LRR- and pyrin domain-containing 2) is also involved in this inhibitory mechanism [59]. Furthermore, host receptors have different affinities to distinct structures of peptidoglycan fragments. It has been established that TLR2 binds more favorably to A_2pm-containing peptidoglycan fragments than to lysine-containing ones [55, 60]. However, other studies debate that peptidoglycan fragments can induce TLR2 signaling [61]. In this respect our view might be blurred by minute contaminations in isolated peptidoglycan with other highly immunogenic cell wall components, such as lipoproteins [62].

It is more generally accepted that peptidoglycan fragments are recognized by NLRs after internalization into host cells [61, 63]. The NLR protein NLRP1 (NOD-, LRR- and pyrin domain-containing 1) recognizes γ-d-glutamyl-*meso*-A_2pm, which is present in all Gram-negative bacteria and specific Gram-positive probiotics (e.g. *L. plantarum*), while NLRP2 interacts with muramyl dipeptides (Mur*N*Ac-l-Ala-d-Glu), which are found universally in all peptidoglycan variants [31, 42]. NLRP1 and NLRP2 belong to the NLR family, which is composed of cytoplasmic proteins and characterized by a C-terminal domain with leucine-rich repeats, nucleotide-binding oligomerization domain, and an N-terminal caspase-recruitment domain [64]. An elegant study illustrated an NLRP2-dependent anti-inflammatory capacity of peptidoglycan-derived muropeptides (Mur*N*Ac-l-Ala-d-Glu-l-Lys) from *Lactobacillus salivarius* Ls33 [63]. The peptidoglycan purified from the strain Ls33 displayed a protective effect in a trinitrobenzene sulfonic acid (TNBS)-induced colitis mouse model, whereas peptidoglycan isolated from *L. acidophilus* NCFM lacked this capacity. This protective effect appeared mediated by local IL-10 production [63, 65]. This distinct property could be pinpointed to the difference of muropeptides released from peptidoglycan of these strains, even though they possess the same l-Lys-d-Asn type linkage. While NCFM released the muropeptide Glc*N*Ac-Mur*N*Ac-l-Ala-d-Glu-l-Lys-d-Asn

exclusively, carrying an epsilon-linked d-Asn, Ls33 produced an additional muropeptide without d-Asn (Glc*N*Ac-Mur*N*Ac-l-Ala-d-Glu-l-Lys). Both muropeptides were chemically synthesized, confirming that this strain-specific immunomodulatory property is determined by the subtle difference in the structure of the muropeptides [63, 65]. The authors proposed that different peptidoglycan turnover, in relation to amidases and γ-d-glutaminyl-Lysine endopeptidase, might be the reason of the observed differentiated release of muropeptides. In addition, the peptide transporters, such as PepT1 or PepT2, might influence the internalization of muropeptides, which is required for the intracellular NLRP2 recognition. Interestingly, the mutations in leucine-rich repeats of NLRP2 are associated with Crohn's disease [66]. These mutations also defect the NLRP2 recognition of muramyl dipeptide [67]. This hints towards a connection between peptidoglycan and probiotic effects in Crohn's disease. Taken together, these studies clearly demonstrate the important roles of *Lactobacillus* peptidoglycan components in host NLR signaling, as well as the significant impact of species-specific variations in peptidoglycan on its immunomodulatory effects, and consequently on probiotic functionality of lactobacilli [5, 63, 65].

Teichoic acid

Lactobacilli and most other Gram-positive bacteria synthesize teichoic acids (TAs) which are anionic polymers consisting of repeating units of alditol-phosphate. TAs are typically present in substantial amounts in the cell envelope and provide a net anionic charge at the cell surface, contributing to the mechanical strength of the cell wall together with peptidoglycan [38, 42]. The anionic charge of TAs is also involved in functional aspects of the cell wall, e.g. playing a role in processes such as cell division [68], metal cation homeostasis (particularly Mg^{2+}) [69, 70], and resistance toward antimicrobial substances [71, 72]. TAs are produced in two distinct forms, lipoteichoic acid (LTA) and wall teichoic acid (WTA). LTA is anchored in the cytoplasmic membrane through a terminal glycolipid moiety, whereas WTA is covalently linked to Mur*N*Ac of peptidoglycan via a disaccharide linkage unit [73]. The LTA polymer typically consists of glycerol-phosphate (Gro-P) repeating units, whereas WTA of lactobacilli is commonly composed of either Gro-P or ribitol-phosphate (Rbo-P) repeating units [74-76]. The backbone polymer of both WTA and LTA can be substituted with D-Alanine and glycosyl moieties. These glycosylations can involve various glycosyl residues, e.g. glucose, galactose, rhamnose, Glc*N*Ac, and Gal*N*Ac in either α- or β-configurations [77, 78].

Despite the structural similarity of the backbone of both molecules, the LTA and WTA backbone polymers are synthesized via independent pathways using different precursor molecules [77, 79]. LTA is directly polymerized on a glycolipid carrier, typically a di- or trihexosyldiacylglycerol, in which the composition and conformation of the glycoside moiety may vary between species [80-82]. The biosynthetic pathway of LTA is well established [83-86]. First, glycolipid anchors are synthesized in the cytoplasm, followed by translocation from the inner to the outer face of the cytoplasmic membrane by a flippase, encoded by *ltaA* [83]. The subsequent generation of the poly(Gro-P) chain involves a primase (LtaP) that adds the initial Gro-P residue, and a polymerase (LtaS) that polymerizes the Gro-P backbone using phosphatidylglycerol as substrate [79, 86]. The presence of at least one *ltaS* homologous in all completed *Lactobacillus* genomes suggests that LTA is present universally among these species [38]. Recently, an *ltaS* deletion mutant was constructed in *L. acidophilus* NCFM and its LTA-deficient phenotype confirmed the involvement of *ltaS* in LTA biosynthesis. The structure of LTA has been determined in *L. casei*, *L. rhamnosus*, *L. delbrueckii*,

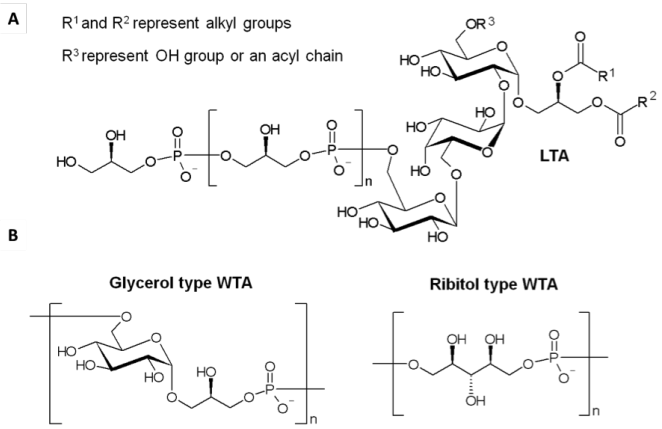

Figure 4. *Lactobacillus* teichoic acid backbones. (A) The structure of lipoteichoic acid (LTA) backbone in *Lactobacillus*, which consists of 1, 3-linked poly(glycerol-phosphate) and a glycolipid anchor which consists of Glc-β-1→6Gal-α-1→2Glc-α-1→3-diacylglycerol. Some LTAs contain an extra acyl chain (R^3) attached to the C6 carbon of the glucose linked to di-acyl chains. (Modified from [88]). (B) Two types of wall teichoic acid (WTA), glycerol-type and ribitol-type. In *Lactobacillus plantarum*, glycerol-type WTA has glucose substitutions being parts of the repeating backbones. Ribitol-type WTA has also glucose substitutions added to the backbone, possibly on the –OH group of ribitol C2 to C4.

and *L. plantarum* and all these polymers are composed of 1,3-linked poly(Gro-P) and contain a glycolipid anchor which consists of Glc-β-1→6Gal-α-1→2Glc-α-1→3-diacylglycerol (Figure 4A) [82, 87-89]. The LTA isolated from *L. plantarum* KCTC 10887BP contains an extra acyl chain attached to the C6 carbon of the glucose linked to di-acyl chains [82]. Similarly, the *L. casei* LTA was also shown to contain a lipid anchor which is partially substituted with an additional, third fatty acid connected at the same position [87]. This additional acyl chain is thought to be important for TLR2 signaling, as the acyl chains of lipopeptides interact with the hydrophobic pocket in the extracellular domain of TLR2 [57, 82, 90]. The diacyl lipid chains are recognized by TLR2/6 heterodimers, whereas triacylated lipoproteins are recognized by TLR2/1 heterodimers [57, 90]. However, how this structural difference in LTA contributes to immunomodulatory effects is not determined yet.

Although the LTA backbone polymer structure appears conserved in lactobacilli, it is likely that the LTA molecules produced vary between different species or even strains of the same species. Structural variations are anticipated to include different glycolipid anchors, chain length, and the degree and composition of the polymer substitutions with glycosyl moieties and d-alanyl residues [38, 77, 91-95]. As an illustration of these variations, the LTA polymer contains 20-22 Gro-P residues in *L. plantarum* [96] and *L. reuteri* [97], but has 37-44 residues in *L. casei* DSM20221 [87], and 33 and 50 residues in *L. delbrueckii* [88] and *L. rhamnosus* [89], respectively. Moreover, the LTAs of different strains of *L. delbrueckii* appeared to vary in their α-glucosyl- and D-alanyl-substitution level, which impacted on strain specific phage sensitivity, suggesting a role for LTA as a phage-docking molecule [88]. The *dltABCD* genes have been demonstrated to be responsible for D-alanine activation, transport, and substitution to LTA [77, 79]. The *dlt* operon is highly conserved among lactobacilli, and the genetic and enzymatic characteristics of this system have been studied in detail in *L. casei* [98-102]. Mutants of the *dlt* system have been described in *L. rhamnosus* [89, 103], *L. reuteri* [97], and *L. plantarum* [104], revealing the impact of d-alanyl depletion on cell morphology, biofilm

formation, colonization in the gastrointestinal tract, and host immune responses. By contrast to the mechanism of d-alanylation of LTA which is well understood, the genes and enzymes involved in LTA glycosylation are poorly characterized, which in part may be due to the high variation of the glycosyl components involved, as well as their substitution linkage type and α-/β-configurations.

The immunomodulatory effects of purified LTAs have been studied in various lactobacilli, including *L. plantarum* WCFS1 [104], *L. plantarum* KCTC10887BP [105], *L. casei* YIT9029 [106] and *L. fermentum* YIT0159 [106]. The LTA purified from WCFS1, YIT9029 and YIT0159 induces TNF-α production in a TLR2-dependent manner [104, 106], whereas LTA from *L. plantarum* KCTC10887BP, attenuates the TNF-α production induced by LTA from *S. aureus* [105]. Jang *et al.* suggested that the third acyl chain and the unsaturated fatty acids in the lipid anchor as well as the glucose and galactose substitutions of *L. plantarum* KCTC10887BP LTA may be responsible for this attenuating effect [82]. Yet, LTA of *L. plantarum* WCFS1 also consists of tri-acylated lipid anchors while other LTA structures (from *L. casei* YIT9029 and *L. fermentum* YIT0159) have not been determined. Hence, it is premature to draw a conclusion on the exact factors that determine the immunomodulatory effects of LTAs.

Besides studies involving purified LTA, the impact of LTA modifications when it resides in the cell envelope of intact cells has also been studied in several lactobacilli. An LTA deficient mutant of *L. acidophilus* NCFM exhibits enhanced anti-inflammatory capacity in an *in vitro* assay employing DCs, but also *in vivo* in a mouse model for colitis, where this mutant was demonstrated to protect mice from dextran sulfate sodium (DSS)-established colitis [107, 108]. In addition, the same group further tested the ability of this LTA-deficient mutant to regulate inflammation in a mouse colonic polyposis model [109]. They employed this unique mouse model in which the adenomatous polyposis coli gene (*apc*), the gate-keeper of colonic epithelial proliferation, was truncated specifically in the colon and distal ileum which resulted in extensive polyposis throughout the colon and distal ileum. Oral treatment with the LTA deficient mutant was shown to result in a reduced frequency of interferon-γ-producing CD4+ T-cells and attenuation of colonic inflammation [109]. Studies that employed *dlt* mutants that produce LTA devoid of d-alanylation, indicate that TLR2-mediated LTA-signaling requires the presence of d-alanylation in LTA in the case of *L. plantarum* NCIMB8826 [104]. However, the influence of d-alanylation with respect to its signaling capacity appears to be less profound in *L. rhamnosus* GG [89]. Moreover, the *dlt* mutant of *L. plantarum* NCIMB8826, had significantly enhanced anti-inflammatory capacity compared to the wild-type strain, which was shown in both *in vitro* immune assays as well as in a mouse colitis model [104]. Notably, the *dlt* mutant of *L. rhamnosus* GG suppresses colitis symptoms in a mouse model as well, but the *in vitro* immunomodulation data obtained did not consistently show enhanced anti-inflammatory capacities for this strain in comparison with the wild-type strain [89, 110]. In general, loss of d-alanylation of LTA appears to generate more anti-inflammatory capacities for the producing *Lactobacillus* species, yet different *in vitro* and *in vivo* experimental set-ups (e.g. mouse-derived macrophages, DCs, or human peripheral blood mononuclear cells (PBMCs)) as well as various LTA sources (e.g. purified LTA, heat-killed bacteria or whole bacterial cells) could contribute to the results that are obtained. Hence, the direct link between subtle structural differences of LTA and differential host immune responses have not yet reached unambiguous conclusions.

In contrast to LTA, WTA is not universally synthesized in the cell wall of lactobacilli, e.g. the

genomes of *L. rhamnosus, L. casei*, *L. fermentum*, and *L. reuteri* lack the genetic capacity to produce WTA [38, 111]. Lactobacilli that do produce WTA typically synthesize the Gro-P type backbone polymer [38, 111], with the notable exception of *L. plantarum* for which strains have been reported to produce either Gro-P or Rbo-P type WTA [112-115]. Moreover, the frequent decoration with a-glucosyl residues distinguishes the WTA at the strain level [116]. Unlike LTA synthesis, WTA is assembled on undecaprenylphosphate as lipid carrier during the biosynthesis process [77, 79, 117, 118]. Prior to the main chain formation, the linkage unit between the main chain and peptidoglycan, which is commonly (Gro-P)2-3-4ManNAc-β-1→4GlcNAc, is generated on the lipid carrier by sequential reactions catalyzed by TagO, TagA, and TagB [77, 79]. Subsequently, the main chain biosynthesis is performed through different pathways for poly(Gro-P) and poly(Rbo-P) backbone polymer chains (via the *tagDF* and *tarIJKL* gene clusters, respectively [117, 119-121]), while for both backbone types the repeating units are derived from nucleotide-activated alditol phosphate as substrates for the polymerization reaction. In *L. plantarum* strains the presence or absence of the *tag* and *tar* clusters in the genome is directly related to the type of WTA synthesized by the strain (Figure 4B)[115]. The *tar* locus is commonly conserved among the strains regardless of the WTA backbone polymer variant synthesized, whereas the *tag* locus is present only in the strains that produce the Gro-P type WTA [115]. Hence, the strains with Gro-P type WTA harbor both loci in the genome, however the *tar* locus has been shown to be inactive at the transcriptional level [115]. Recent work in our laboratory included the mutation of the *tag* locus in the Gro-P type WTA producing *L. plantarum* WCFS1, which led to a marked up-regulation of the *tar* locus and resulted in switching of WTA type from the conventional Gro-P type to an alternative Rbo-P type. These results demonstrated that the strains with Gro-P type WTA possesses the capacity to produce Rbo-P type WTA, but this capacity appears to be repressed under the conditions tested so far. Moreover, a mutant devoid of any WTA was also constructed by deleting *tagO* [122], the gene encoding for the enzyme that catalyzes the initial reaction in WTA biosynthesis. The deficiency as well as the artificial backbone switching of WTA corresponded to differential immunomodulatory properties in comparison with the wild-type bacterium [123]. A study on the structural diversity of WTA revealed that within a collection of *L. plantarum* strains six variants of the WTA repeating units could be identified (3 Gro-P and 3 Rbo-P backbone polymers with glucosyl substitutions in variable positions), suggesting that variation in WTA structure is important for the lifestyle of this bacterium [124].

Despite the complete separation in terms of their biosynthetic pathways, WTA and LTA backbones share the same d-alanine incorporation machinery [79]. Moreover, several additional genes that are involved in WTA glycosylation have been identified; tagE of B. subtilis is involved in α-Glc decoration of its Gro-P type WTA [125, 126], tarM of S. aureus is involved in α-GlcNAc decoration of the Rbo-P type WTA [127], and the gltA−gltB and gtcA−lmo2550 genes of Listeria monocytogenes are involved in the β-Glc and α-GlcNAc decoration of the serotype-specific WTAs of this species, respectively [128-130]. In the six different WTA structures identified in different strains of L. plantarum, α-glucosyl residues in various positions within the polymers contribute to the structural diversity [116]. Interestingly, many tagE and gtcA homologues (tagE1−tagE6 and gtcA1−gtcA3, respectively) are conserved among the genomes of L. plantarum WCFS1 [131, 132], JDM1 [133], and ST-III [134]. Based on comparative genome hybridization data using L. plantarum WCFS1 DNA microarrays, all 42 analyzed L. plantarum strains have multiple but variable tagE and gtcA homologues [135]. This observation suggests that variations in presence/absence patterns and expression levels of the tagE and gtcA gene sets among the different strains

plays a role in the production of the varied WTA structures. Genetic engineering may resolve the role of these genes in the determination of the WTA structural properties and may enable the elucidation of the impact of WTA structure on the immunomodulatory capacities of individual strains in an isogenic background.

In contrast to LTA, the immunomodulatory roles of WTA have been less extensively investigated and remain poorly understood. The role of WTA in TLR2-dependent immunomodulation remains debated [136], which may in part be due to the lack of lipid-anchoring of WTA, which was reported to be essential for TLR2 recognition of LTA [57]. However, the anionic property and d-alanylation of WTA may still contribute to immune signaling [57, 137]. It has recently been proposed that the WTAs of *L. casei* Shirota and *L. plantarum* subsp. *plantarum* ATCC14917 induce IL-10 production synergistically with LTA [138]. Furthermore, recent results in our group revealed that a WTA-deficient mutant of *L. plantarum* WCFS1 displayed increased NF-κB activation in an assay using TLR2/6 expressing HEK cells but no signaling was detected using WTA purified from the parental strain, suggesting a shielding effect of WTA rather than a direct signaling activity [123]. Moreover, mutation of a transcriptional regulator in *L. plantarum* WCFS1 (Lp_2991) that is anticipated to be involved in regulation of an immediately downstream encoded glycosyltransferase (GtcA3) which might be involved in TA glycoyslation, elicited higher IL-10 and TNF-α secretion in human monocyte-derived DCs [139]. Intriguingly, the mutation of this regulator also affects the expression of *tagO*, the gene involved in the initial step of WTA biosynthesis. In conclusion, further expansion of our knowledge of the subtle structural diversity among LTA and WTA molecules and its consequences for immunomodulation is required to eventually fully elucidate and appreciate their role in species and / or strain specificity in terms of immunomodulation.

Cell surface polysaccharides

Polysaccharides are ubiquitously found in *Lactobacillus* cell walls. They can be covalently linked to Mur*N*Ac of peptidoglycan (wall polysaccharides, WPS), loosely attached to the cell envelope (capsular polysaccharides, CPS) or released into the environment (exopolysaccharides, EPS). Despite the different terminologies, the localization and abundance of polysaccharides depends strongly on the bacterial growth conditions, thereby making the differentiation of WPS, CPS and EPS quite arbitrary [29, 42]. Therefore, we will discuss this group of molecules as cell surface polysaccharides without distinguishing their specific (predicted) positioning or linkage to the other components of the cell envelope. The structures of cell surface polysaccharides vary considerably in terms of the sugar compositions, modes of sugar-linkages, polymer branching, as well as their modifications such as phosphorylations, acetylations, and pyruvylations. The majority of surface polysaccharides known in lactobacilli are heteropolysaccharides, in which the polymer is composed of regular repeating units that contain different sugars, commonly including d-glucose, d-galactose and l-rhamnose, and in a few cases also *N*-acetylglucosamine (Glc*N*Ac), *N*-acetylgalactosamine (Gal*N*Ac) or glucuronic acid (GlcA) [140].

The polysaccharide biosynthesis genes are organized in monocistronically-transcribed operons [141]. The sugar repeat unit builds up intracellularly using nucleotide-sugars as precursors and undecaprenyl phosphate as a recycling lipid carrier. The biosynthesis is initiated by a priming glycosyl transferase (WchA) that couples the first monosaccharide phosphate to the membrane-associated lipid carrier. Subsequently the additional glycosyltransferases encoded by the gene cluster sequentially add monosaccharides to this membrane-anchored intermediate to generate

the repeat unit. The lipid-linked repeat unit is transported across the cytoplasmic membrane by the Wzx flippase, followed by the polymerization (Wzy) of individual repeat units to form the mature surface polysaccharide, which can then be attached to the peptidoglycan by the Wzd-Wze complex [141].

The genes involved in cell surface polysaccharide biosynthesis in lactobacilli are organized in gene clusters that share substantial structural similarity with those found in streptococci, and in many other bacteria. For example, four gene clusters involved in cell-surface polysaccharide biosynthesis that are encoded in *L. plantarum* WCFS1 have recently been characterized [38, 122, 142]. Individual deletions of these clusters led to altered surface polysaccharide compositions in these *L. plantarum* WCFS1-derivatives, supporting their roles in production of cell surface associated polymeric glycan structures. Notably, the deletion of all four clusters (*cps1A-I*, *2A-K*, *3A-I* and *4A-J*) in the same background led not only to a substantial reduction of cell-surface polysaccharide production but also had dramatic impacts on cell morphology, and growth. Moreover, this surface polysaccharide biosynthesis-cluster deficient derivative displayed altered signaling capacities when applied to TLR2-reporter cell lines, suggesting either a direct role of these polysaccharides in host communication or an indirect role via their capacity to shield alternative signaling compounds that reside in the bacterial cell envelope [Remus D.M. PhD thesis Wageningen University, submitted]. *L. rhamnosus* GG, one of the best documented probiotic lactobacilli, produces two major types of cell-wall associated polysaccharides. The most abundant polymer is a long and galactose-rich polysaccharides, while the lower abundant polymer is significantly smaller-sized and glucose-rich [143]. The biosynthesis cluster of the galactose-rich polysaccharide of *L. rhamnosus* GG was recently characterized and has a genetic organization that resembles the typical polysaccharide biosynthesis clusters as have also been described for *S. pneumonia*. By contrast, the gene cluster involved in production of the glucose-type surface polysaccharide of strain GG has not been functionally characterized [144]. The polysaccharide biosynthesis clusters display substantial diversity between individual strains of a species, as is exemplified by *L. plantarum*, where one of the polysaccharide biosynthesis clusters belongs to the least conserved regions of the WCFS1 genome [135]. Hence, the polysaccharide producing capacity of lactobacilli can create an immense diversity of surface decorations in individual strains, which may be of great relevance for their molecular communication with the host mucosal system [145].

The biological functions of the cell surface polysaccharides in lactobacilli have been reported to be related to phage absorption (*L. plantarum* [146] and *L. casei* [147]), the attachment of surface layer proteins (*Lactobacillus buchneri* [148]), and immunomodulation [145] (for recent reviews see [29, 42]). Although some examples have been reported, our overall knowledge about the immunomodulatory properties of surface polysaccharides remains very fragmented. For example, the polysaccharides in *L. casei* Shirota reduced the susceptibility of its peptidoglycan to *N*-acetylmuramidase digestion, which influences IL-12 production of macrophages [59], while the isolated polysaccharide of this strain suppressed pro-inflammatory responses in macrophages [145]. Moreover, *L. casei* NIZO B255 and *L. reuteri* ASM20016 stimulate human DC maturation and regulatory T cell differentiation through a C type lectin DC-specific ICAM3-gabbing non-integrin (DC-SIGN) [149]. The fact that DC-SIGN recognizes mannose or fucose-containing structures [150] suggests the presence of these sugar moieties on the surface of these bacteria. Although the exact nature of these carbohydrate containing molecules remains uncharacterized, a role for the cell surface associated polysaccharides can certainly not be excluded [149]. In addition, deprivation

of the long, galactose-rich type polysaccharide in *L. rhamnosus* GG appeared to promote adherence to intestinal epithelial cells [144], yet increases the sensitivity of this bacterium toward innate immune defensive factors, such as antimicrobial peptide LL-37 [151].

For many of the polysaccharides derived from lactobacilli, their role in host-cell interaction is likely dependent on the shielding effect they may provide for other surface molecules such as LTA or peptidoglycan. However, a direct role of some polysaccharide molecules of specific *Lactobacillus* strains has also been proposed for the polysaccharide derived from *L. casei* Shirota [145]. Taking into account the immense chemical diversity of the polysaccharides that can be produced by lactobacilli, in combination with their variations in terms of exact subcellular location, polymer size, and conformational properties, it is likely that eventual direct signaling effects depend on the exact characteristics of the molecule in the cellular context. Moreover, the lack of knowledge on the corresponding PRRs or alternative recognition pathways involved in eventual host interactions leaves the immunomodulatory effects of lactobacilli derived polysaccharide largely obscure. Nevertheless, some examples of other bacteria, e.g., the polysaccharide A produced by *Bacteroides fragilis* [152], exemplify the huge potential of these cell surface molecules when it comes to host response modulation, which implies that substantial investigation of these molecular structures in lactobacilli in the perspective of their role in probiotic functionality is fully justified.

Surface Proteins

Proteins of Gram-positive bacteria are transported to the cell surface via seven main protein secretion mechanisms, namely the main secretion machinery (Sec), twin-arginine translocation (tat), the flagella export apparatus (FEA), the fimbrilin-protein exporter (FPE), the holin mechanism (pore-forming), peptide-efflux ABC transporters, and the WXG100 secretion system (Wss) (for reviews, see [153-156]). Mining 13 complete genomes of lactobacilli revealed that this group of bacteria appeared to possess genes encoding the Sec, RPE, peptide-efflux ABC and holin systems (for a recent review see [38]). The secreted proteins can be divided into two major types, firstly the proteins that are released into the environment and secondly those that are covalently or non-covalently anchored to the cell surface. Among the latter group of proteins several modes of anchoring have been described, including anchoring in the cytoplasmic membrane by single or multiple membrane spanning protein-domains, or by N-terminal linkage to long-chain fatty acids (i.e. lipoproteins). Alternatively, C-terminal LPxTG motifs target proteins to the sortase dependent anchoring machinery that covalently attaches these proteins to the peptidoglycan. Finally, a variety of domains has been recognized that enable secreted proteins to non-covalently bind to different cell envelope structures like peptidoglycan or LTA (for a review see [38]). Overall, the cell envelope is richly decorated with proteinaceous compounds that are either firmly or loosely attached to the different structural components that collectively form the cell envelope. These proteins may play important roles in host cell communication and could be playing a central role in the beneficial effects of probiotic lactobacilli which are discussed below.

Two secreted proteins of *L. rhamnosus* GG, originally designated p75 and p40 but recently renamed to Msp1 and Msp2 [110], inhibit cytokine-induced apoptosis of epithelial cells, reduce TNF-induced epithelial damage in the colon and as a result promote epithelial homeostasis [157]. Homologues proteins that can also elicit similar host responses have also been identified in *L. casei* strains ATCC 334, ATCC 393 [157], and BL 23 [158]. Less specific is the observation that the probiotic mixture VSL#3, which contains *L. casei*, *L. plantarum*, *L. acidophilus* and *L. delbureckii*

subsp. *bulgaricus*, was reported to promote epithelial barrier functions (e.g. tight junctions and mucin production) via an unidentified soluble proteinaceous factors [159]. Although bacteriocins may fulfill probiotic functions via their primary function as antimicrobial components that inhibit the growth of pathogens (see also above and [30]), they were recently reported to also play a direct role in immunomodulation [160]. These bacteriocin (plantaricin) encoding genes were identified as potential effector molecules by a gene trait matching approach using 42 *L. plantarum* strains, and subsequent mutation analysis established their importance in anti-inflammatory properties of this species in both PBMCs and DCs [135, 139, 160, 161]. The importance of plantaricin is further supported by the notion that a specific plantaricin gene (*plnI*) is induced *in vivo* during gastrointestinal passage of *L. plantarum* [162-164]. An analogous gene-trait matching approach also identified the mannose-specific adhesin (Msa) in *L. plantarum* WCFS1, which is a LPxTG-anchored protein involved in mannose specific recognition and binding to epithelial surface glycan-structures in the host mucosa [165]. Subsequent work showed that this protein is important for the induction of specific innate immune responses in pig intestinal tissues *in vivo* [166]. Moreover, purified STp, a recently identified serine and threonine rich peptide (STp) harbored by protein D1 that is secreted by *L. plantarum* BMCM12, was shown to stimulate regulatory responses in human intestinal DCs *in vitro* [167]. Using polyclonal serum generated against purified STp, it could also be established that proteins containing regions homologous to STp can be found in the healthy human colonic microenvironment, whereas STp-containing proteins appeared absent in the intestinal microenvironment from inflammatory bowel disease patients, suggesting that STp can be employed as a biomarker for gut homeostasis [167].

Specific lactobacilli, such as *L. acidophilus*, *L. helveticus* and *L. brevis,* produce a paracrystalline protein-monolayer as the most exterior layer of their cell envelope, termed surface layer [29, 168-171]. Notably, surface layer protein A (SlpA) of *L. acidophilus* NCFM is recognized by DC-SIGN and modulates human DCs and T cell functions, leading towards more regulatory differentiation through increased IL-10 and reduced IL-12p70 production [172]. On the other hand, many lactobacilli lack surface layer proteins but might still stimulate immune responses through DC-SIGN by glycoproteins other than surface layer proteins [29, 32]. These examples support a prominent role of (surface-associated) proteins in the interaction with the host cells in the mucosa, but for the majority of these proteins it remains relatively unclear how they achieve these effects, and how important their contribution to eventual probiotic effects is *in vivo*.

Despite the long-existing belief that protein glycosylation is restricted to eukaryotic organisms, more and more evidence emerges that this form of post-translational modification also occurs frequently in the bacterial world. The first bacterial *O*-glycosylation, i.e. glycan attachment to Ser or Thr residues, was reported for surface layer proteins of hyperthermophilic *Clostridium* species, while the first bacterial *N*-glycosylation machinery, i.e., attaching glycans to asparagine residues, was discovered in *Campylobacter jejuni* (for reviews see [38, 173]). Bacterial protein glycosylation has been studied mainly in pathogenic organisms, but is also found in certain human intestine commensals, including several *Bacteroides* species [173]. Interestingly, glycoproteins were recently discovered in *L. plantarum* WCFS1 [174] and *L. rhamnosus* GG [175] as well. In *L. plantarum* WCFS1 the major autolysin, Acm2, was shown to be *O*-glycosylated with Glc*N*Ac moieties in its N-terminal AST domain, which is an alanine, serine and threonine rich region. This *O*-glycosylation occurs intracellularly, prior to Acm2 export to the cell surface. Although the biological significance of glycosylation has not yet been elucidated, one may speculate that *O*-glycosylation has a regulatory

role in modulation of the Acm2 function in peptidoglycan turn-over, which is supported by the dynamic nature of its glycosylation [174]. Intriguingly, the N-terminal AST domain is also present in 10 other peptidoglycan hydrolases that are encoded within the *L. plantarum* WCFS1 genome, suggesting that many of these proteins could also be subject to glycosylation [174]. Another AST-domain containing protein is encoded by *lp_2145* and this protein displayed a smearing pattern on SDS-PAGE gels, which is in agreement with its postulated glycosylation and was speculated to depend on glycosylation of Lp_2145 by the genetically linked glycosyltransferase encoding gene *ica2* (*lp_2142*). Moreover, heterologous expression of Lp_2145 in *Lc. lactis* MG1363, led to a discrete band of the expected molecular weight on SDS-PAGE gels, suggesting that the proposed glycan modification does not take place in this host background and supporting a role of an *L. plantarum* specific gene in this post-translational modification, e.g. *ica2* [32].

Protein glycosylations were also found in the secreted protein Msp1 of *L. rhamnosus* GG that, similar to the Acm2 protein of *L. plantarum*, functions as a peptidoglycan hydrolase. The Msp1 protein is *O*-glycosylated at serine residues 106 and 107 and its glycan moieties are recognized by the Concanavalin A (ConA) lectin, which is specific for α-mannose and α-glucose [175]. Interestingly, the Msp1 homologue of two other *L. rhamnosus* strains also contain these serine residues, whereas 3 *L. casei* strains analyzed lack these residues. Concomitantly, ConA recognition appeared specific for the *L. rhamnosus* strains, suggesting species-specific glycosylation of Msp1 [175]. Glycosylation in *L. rhamnosus* GG did not play a role in peptidoglycan hydrolyase activity, nor in the activation of Akt signaling that is related to its effect on epithelial cell-apoptosis [157]. On the other hand, glycosylation was demonstrated to be of importance for the resistance to protease degradation and overall stability of Msp1 [175]. Moreover, the glycosylation of Msp1 was found in supernatant but not in cytosolic or cell wall fractions [175], which suggests a link between the glycosylation of Msp1 and its subcellular localization [175]. Notably, this also hints towards a different glycosylation machinery in *L. rhamnosus* GG as compared to the glycosylation of Acm2 in *L. plantarum* which occurs intracellularly [174], reiterating the species-specificity of *Lactobacillus* protein glycosylation. Experimental evidence also suggests the presence of additional glycosylated proteins in *L. rhamnosus* GG, such as LGG-02225, another putative cell wall hydrolase. Taken together, the existing data support the glycosylation of specific proteins in lactobacilli, but it is anticipated that many other proteins (containing AST or other glycosylation domains) are subject to post-translational glycosylation in lactobacilli [175]. Importantly, SlpA of *L. acidophilus* NCFM has shown its regulatory effects on DC functions through DC-SIGN, which is a C-type lectin recognizing mannose- and fucose-containing glycans [172], strongly suggesting SlpA is glycosylated. The other surface protein (SlpB) of *L. acidophilus* NCFM failed to interact with DC-SIGN and displayed dramatically different immunomodulatory properties than that of SlpA [172], implying the glycosylation of SlpA may be essential in the modulation of DCs and T cells functions.

An *in silico* analysis performed with the *L. rhamnosus* GG genome led to a list of 50 genes encoding glycosyltransferases potentially involved in protein glycosylation. Unfortunately, initial analysis of a subset of corresponding mutants revealed identical ConA-reactive glycosylation of Msp1 as was observed for the wild-type strain, leaving the glycosylation machinery and the glycosyl transferase(s) involved to be identified [176]. Similarly, six putative glycosyltransferases that share sequence similarities with Gtf1, involved in *O*-Glc*N*Ac glycosylation of the streptococcal Fap1 adhesin [177, 178] were identified within the *L. plantarum* WCFS1 genome, identifying these genes as tempting candidates for further research [174]. Development of *in silico* tools allowing bioinformatics-based

predictions of substrate specificity of glycosyltransferases could significantly constrain the amount of candidate genes encoding protein glycosyltransferases, thereby accelerating the identification of *Lactobacillus* protein glycosylation machineries.

The road ahead; molecular cross-talk makes the difference

The line of research described in this review is bound to deliver a more detailed structure-function relationship for probiotic effector molecules, which when combined with advanced understanding of how these molecules may elicit their effects in specific host cells can provide the mechanistic understanding of the (species-specific) health benefits that these bacteria can confer. An enhanced characterization and understanding of probiotic effector molecules is not only relevant for the food industry but might also enable targeted pharmaceutical applications. Evidence is emerging that subtle structural differences in the effector molecules expressed by individual strains are responsible for the strain-specificity of probiotic functions. As this field is still in its infancy, identification of the true effector structures in the canonical MAMPs is essential, while it is also crucial to understand their molecular interaction with their corresponding PRRs expressed in host tissues. Detailed investigation of the biosynthesis pathways for the canonical cell wall associated effector molecules including peptidoglycan, TAs and polysaccharides, and their modifications will greatly improve our capacity to accurately predict probiotic function.

The growing availability of genome sequences as well as the corresponding molecular tools allows us to pursue more knowledge driven approaches. The growing availability of *Lactobacillus* genome sequences and annotations provides information for the encoded gene functions, including their metabolic and biosynthetic pathways, stress responses, cell-wall associated proteins, and potential host interaction factors [38, 179]. Gene-trait matching (GTM) approaches may decipher strain-specific functions that are involved in host interactions, as has been successfully illustrated in the identification of pili formation in *L. rhamnosus* GG and its role in mucus binding and intestinal persistence [180, 181], the mannose binding capacity of *L. plantarum* [165] and some of the *L. plantarum* genes involved in immunomodulation [139]. Transcriptome-trait matching (TTM) offers a further possibility to correlate genes to specific functionalities, and compensates for the blind spots of GTM with respect to genes that are conserved among the strains tested [182, 183]. Candidate genes selected on basis of GTM or TTM still require functional verification in relation to specific phenotypes, which can be achieved by studies using genetically engineered strains [139, 161, 165, 166, 171, 180, 183]. Many genetic engineering tools have been developed for the construction of gene deletion derivatives, as well as (controlled) gene expression in lactobacilli, which have been reviewed elsewhere [29, 179]. Mutational analysis has the potential drawback that the changed genetic make-up has pleiotropic effects that exceed the primary effects of the gene targeted by mutation. Especially when targeting cell envelope associated functions, such pleiotropic or compensatory effects have been observed. For instance, a WTA-deficient mutant of *L. plantarum* WCFS1 also displays altered growth and morphology [122]. Moreover, a *dltB* mutant of *L. plantarum* WCFS1 synthesizes LTA that lacks d-ala substitutions, but was also reported to produce LTA of three-fold increased length which contained 25% glucose substitution, which is absent in LTA derived from the wild-type [96]. Notably, a recent study from our group showed that a *dlt* mutant devoid of the complete *dlt* operon constructed in the same strain did not show altered LTA chain length [123]. These changes have potential impact on host immune response, e.g. additional glucose substitutions of LTA might be recognized and interact with C-type lectins

and may affect the immune responses of the host. It is important to establish such pleiotropic consequences and take them into account, since they may lead to drastic changes in surface associated biochemical structures and the corresponding consequences in terms of host responses.

The modulation of human mucosa transcriptomic profiles was shown to be affected by the growth stage from which *Lactobacillus plantarum* was harvested [184, 185], implying that the outcome of microbial interaction with the mucosa is potentially influenced by the way probiotics are produced. Therefore, genetically modified lactobacilli may be an important strategy to enhance or ensure their functionality *in situ* in humans after consumption. However, their application in consumer products is currently not considered due to legislation constraints and consumer concerns about genetically modified food ingredients, although food-grade genetic engineering strategies may be possible [186]. Finally, the consumption of live-microorganisms with a safe status like the probiotics that are currently marketed, is virtually without risk in a healthy population, but may not be desired for the application in certain (severely) health-compromised populations. These considerations support an approach that is built on the concept of 'synthetic' probiotic effectors that allows bypassing of these potential problems. Among the probiotic effector molecules, proteins but especially peptides are probably the most straightforward and cost-effective to chemically synthesize, and effectors such as the muropeptide produced by *L. salivarius* Ls33 [63] and a peptide, STp, secreted by *L. plantarum* BMCM12 [167] might have potential for applications that follow more pharmacological production and administration regimes. However, if glycosylation of proteinaceous molecules in some instances is required for their functionality in interactions with the host system, their *in vitro* synthesis may become more complicated. Nevertheless, these hurdles may be taken by identifying suitable biotechnological or synthetic biology approaches to produce such glycosylated protein or peptide compounds in an efficient and cost-effective way. Identification of the mechanisms involved in protein glycosylation is bound to stimulate this route.

On the other hand, it is important to better understand the basis of variations seen in individual humans. Notably, both the molecular and cellular characteristics of the human mucosal tissues [184, 185], as well as those of the luminal microbiota [187, 188] are considerably different between individual humans. The endogenous microbiota may play a crucial role in determining the differences in overall intestinal functionality in individual humans, which is supported by recent metagenomics studies that discovered three distinct microbiome types, termed enterotypes [187, 188]. Besides differential microbiomes, also the genotype, dietary habits, life-style, or a combination of all these factors may determine the molecular status of the human mucosal tissues, which we previously proposed to designate as "the human band-width of health" [5]. These components of human individuality may significantly influence the molecular responsiveness to probiotic interventions or probiotic-effector molecule treatment, as well as their relevance for the health status of the individual consumer [32, 189]. Despite this complexity, the increasing knowledge on the structural details of probiotic effectors and on their consequent immunoregulatory properties enlighten the molecular mechanisms of probiotic function. A better understanding of the precise mechanism of effector molecule interaction and the cognate responses elicited may help to improve the effector application in specific susceptible population subgroups that are predictably responsive to this treatment. As some probiotic effector molecules, for example Msp1 and Msp2 of *L. rhamnosus* GG, SlpA of *L. acidophilus* NCFM, and STp of *L. plantarum* BMCM12, have been identified as important immunomodulators, their application holds great promise especially providing we are able to recognize the susceptible / responsive

subgroups in the human population that can benefit from such treatment.

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2

Chapter 3

The impact of lipoteichoic acid-deficiency on the physiological and immunomodulatory properties of *Lactobacillus plantarum* WCFS1

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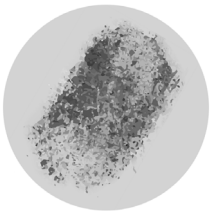

Abstract

Lipoteichoic acid (LTA) is one of the major surface components of Gram-positive bacteria and plays many important roles in bacterial physiology, including ion homeostasis, physicochemical surface properties, and cell division. Pro-inflammatory responses in mammalian immune cells can commonly be elicited by LTA from various bacterial species, such as *Staphylococcus aureus* and *Bacillus subtilis*. Conversely, LTA from specific lactobacilli can inhibit inflammation and act as an antagonist of LPS or pro-inflammatory LTA. However, different LTA purification protocols employed may lead to different levels of contamination with lipoproteins, which may confound these conclusion. Here, we focus on the LTA of the model probiotic *Lactobacillus plantarum* WCFS1, to study the role of LTA in bacterial physiology, TLR2 signaling capacity, and immunomodulatory properties upon co-incubation with human peripheral blood mononuclear cells (PBMCs). To this end, we established a purification procedure that minimizes the lipoprotein contamination of LTA preparations, while maintaining the structural integrity of the molecule. Purified LTA from *L. plantarum* WCFS1 and from *B. subtilis* 168 displayed opposite TLR2 signaling capacities and elicited different immunomodulation responses in PBMCs. In a gene deletion approach we targeted the two LTA synthase encoding genes *lp_1283* (*ltaS1*) and *lp_2580* (*ltaS2*), which both were shown to be required for LTA synthesis. Moreover, both mutants displayed defects in growth and cell division, and alterations in their physicochemical surface properties, supporting the important role of LTA in bacterial physiology. The deletion mutants elicited similar pro-inflammatory cytokine production but less anti-inflammatory IL10 production in PBMCs as compared to the wild type. However, the considerable pleiotropic effects observed in both mutants makes it unclear whether these observations can be exclusively assigned to LTA. Nevertheless, the differences in the structural properties of purified LTA from *B. subtilis* and *L. plantarum* are likely to play a key role in the differential immunomodulatory properties elicited by these molecules.

Introduction

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [1]. One of the most commonly proposed modes of action via which probiotics exert their beneficial effects is through immunomodulation [2]. Bacterial surface molecules are logic candidates to be the first components communicating with the immune system [3, 4] and as a consequence are considered to play a key role in the immunomodulatory capacity of probiotic and other bacteria. Lipoteichoic acid (LTA) is one of the major structural components of the cell envelope of most Gram-positive bacteria [5], including the most commercialized probiotic genus *Lactobacillus* [2]. This major surface component typically consists of a backbone of polyglycerol-phosphate [poly(Gro-P)], decorated with amino acid and/or glycosyl-substitutions and is anchored to the cell membrane through a basal glycolipid moiety [6]. Importantly, the structure of LTA varies between species and strains, including variations in the length of the backbone polymer, the chemical nature and degree of its substitutions, and the cell membrane anchoring glycolipid structure, including its acyl chains (di- or tri- acylation, and degree of saturation) [5, 7, 8]. LTA of lactobacilli has the canonical glycerol-phosphate backbone, with glucosyl- and D-alanyl substitutions and is commonly anchored to the cell membrane via a di- or tri-acyl glycolipid moiety [9-13].

LTA plays many important roles in bacterial physiology. For example, the charged repeating unit of the LTA backbone contributes to surface charge and can act as a scavenger for cations, especially Mg^{2+} [14, 15]. Moreover, the D-alanine ester substitutions of LTA have been suggested to play a prominent role in the determination of surface hydrophobicity [16, 17] and contribute to bacterial adhesion to epithelial cells [18] and to colonization in the murine gastrointestinal tract [19]. In addition, LTA has also been reported to regulate autolysin activity, a process in which D-alanylation of LTA plays a pivotal role [20-22].

The biosynthesis of LTA has been studied in various bacteria, including *Staphylococcus aureus* [23, 24], *Bacillus subtilis* [25] and *Listeria monocytogenes* [26]. As a first step, glycolipid anchors are synthesized in the cytoplasm, followed by their translocation from the inner to the outer face of the cytoplasmic membrane by a flippase, encoded by *ltaA* [23]. The subsequent polymerization of phosphatidylglycerol into the poly(Gro-P) chain is catalyzed by a LTA synthase (LtaS) [24, 25, 27]. In *Listeria monocytogenes* 10403S, the polymerization step is performed by a two-enzyme system in which a LTA primase (LtaP) adds the initial Gro-P residue to the glycolipid, followed by polymerization of the poly-Gro-P backbone by LtaS [26]. The *dltABCD* genes are responsible for D-alanylation of LTA [6, 27]. By contrast to the well-characterized mechanism of D-alanylation of LTA, the genes and enzymes involved in the glycosyl-substitution remain unknown. LTA biosynthesis has also been studied in lactobacilli, and mining of their genomes identified at least one *ltaS* gene homologue and a high degree of conservation of the *dlt* operons among all lactobacilli, which underpins the important biological role of LTA in this species [5, 7]. Given the multiple roles that have been established for LTA, mutations in genes involved in LTA biosynthesis often severely impact on bacterial physiology [22, 25, 28].

Since LTA is one of the major components in the bacterial cell envelope, many studies focused on the immunomodulatory properties of LTA. Typically, LTA is able to stimulate the production of pro-inflammatory cytokines by mouse and human immune cells, such as tumor necrosis factor-alpha (TNFα) [29-31], interleukin (IL)-1β [32, 33], and IL-6 [33, 34]. The immunomodulation

of LTA is generally thought to be mediated by Toll-like receptor (TLR) 1/2 and 2/6 heterodimer complexes, because of the similarity of the acyl chains structures of LTA and lipoproteins, where TLR 1/2 and 2/6 complexes recognize di- and tri-acylated LTA molecules, respectively [33, 35, 36]. However, LTA from *Lactococcus lactis* G121 induces IL-6 secretion in human monocytes in a TLR2- and TLR4-independent manner [37]. Additionally, LTA purified from *L. plantarum* KCTC10887BP elicited much less immunostimulation as compared to the LTA purified from *S. aureus* ATCC 6538 or *B. subtilis* ATCC 6633, including reduced TLR2 stimulation and NO production in macrophages [38]. It was also shown that LTA from *L. plantarum* KCTC10887BP can inhibit TNFα production induced by *S. aureus* LTA [35] and LPS [39] in monocytes as well as down-regulate the inflammation induced by *Shigella flexneri* peptidoglycan [40]. Analogously, LTA from *Lactobacillus johnsonii* La1 and *Lactobacillus acidophilus* La10 were shown to act as antagonists of LPS- and Gram-negative bacteria-induced pro-inflammatory cytokine productions in human intestinal epithelial cells [41]. Recently, structural comparison of LTA from *S. aureus* and *L. plantarum* KCTC10887BP shed new light on the role of structural diversity of LTA molecules, which may explain the diverse results obtained in immune-cell interaction studies using different bacterial LTA sources [13]. Furthermore, Hashimoto *et al*. suggested that contamination of lipoproteins is the main cause of the observed immunmodulatory activity in purified LTA fractions [42].

Here we focus on the LTA from *L. plantarum* WCFS1, a model probiotic for which a well-annotated genome and sophisticated genetic tools for mutagenesis are available. We identified two *ltaS* homologues, *lp_1283* and *lp_2580* (named, *ltaS1* and *ltaS2*, respectively), in the WCFS1 genome, and could show that both are required for LTA backbone synthesis by mutation analysis. We also report on the consequences of the deletion of the *ltaS* genes on bacterial physiology, surface characteristics, TLR-signaling capacity and immunomodulatory properties. Furthermore, purified LTA of *L. plantarum* WCFS1 was analyzed in TLR2-signaling capacity assays and immunomodulation in PBMCs. The results strongly contrast with those obtained with purified LTA from *B. subtilis* 168 that was isolated using the same procedure. These results suggest that structural differences between LTA from *B. subtilis* and *L. plantarum* are key determinants in the immunomodulatory properties of these molecules.

Material and Method

Bacterial strains and culture conditions. Bacterial strains used in this work are listed in Table 1. *Lactobacillus plantarum* WCFS1 and its derivatives were grown at 37°C in MRS broth (Difco, West Molesey, United Kingdom) without aeration. *Bacillus subtilis* 168 was grown at 37°C in LB-broth with aeration. *Escherichia coli* strain TOP10 (Invitrogen, Bleiswijk, The Netherlands) was used as an intermediate cloning host, and was grown at 37°C in TY broth [43] with aeration [44]. Solid media were prepared by adding 1.5% (w/v) agar to the broths. Antibiotics were added where appropriate and concentrations used for *L. plantarum* and *E. coli* were 10 μg/ml chloramphenicol (Cm), and 30 and 200 μg/ml erythromycin (Ery), respectively.

DNA manipulations. Plasmids and primers used are listed in Table 2. Standard procedures were used for DNA manipulations in *E. coli* [44]. Plasmid DNA was isolated from *E. coli* using a JETSTAR kit (Genomed GmbH, Bad Oberhausen, Germany). *L. plantarum* DNA was isolated as described previously [45]. PCR amplifications were performed using hot-start KOD polymerase (Novagen,

Table 1. Bacterial strains and plasmids used in this study

Strains	Characteristics[a]	Reference
L. plantarum		
WCFS1	Single-colony isolate of *L. plantarum* NCIMB8826. Isolate from human saliva, UK.	[66]
NZ3400Cm	Cm^r; WCFS1 derivative; chromosomal integration of *cat* cassette into H-locus	[61]
NZ3537Cm	Cm^r; derivative of WCFS1 containing a *lox66*-P_{32}-*cat*-*lox71*-tagF2 replacement of *ltaS1 (lp_1283)* (*ltaS1*::*lox66*-P_{32}-*cat*-*lox71*-tagF2)	this work
NZ3538Cm	Cm^r; derivative of WCFS1 containing a *lox66*-P_{32}-*cat*-*lox71*-tagF4 replacement of *ltaS2 (lp_2580)* (*ltaS2*:: *lox66*-P_{32}-*cat*-*lox71*-tagF4)	this work
NZ8200CmEry	Cm^r, Ery^r; NZ3537Cm derivative; chromosomal integration of a single copy of *ltaS1* gene mediated by pMEC10 vector	this work
NZ8201CmEry	Cm^r, Ery^r; NZ3538Cm derivative; chromosomal integration of a single copy of *ltaS2* gene mediated by pMEC10 vector	this work
B. subtilis		
168		[88]
E. coli		
TOP 10	Cloning host; F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen

[a] Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant

Madison, USA) according to the protocol of the manufacturer. Amplicons were purified using the Wizard®SV Gel and PCR Clean-Up System (Promega, Leiden, The Netherlands). Restriction endonucleases (Fermentas GmbH, St. Leon-Rot, Germany), MSB® Spin PCRapace (Invitek GmbH, Berlin, Germany), PCR Master Mix (Promega) and T4 DNA ligase (Invitrogen) were used as specified by the manufacturers.

Construction of *ltaS* deletion mutants. The *ltaS1* and *ltaS2* deletion mutants were constructed essentially as described previously [46], using a double crossing-over strategy to replace the target genes by a chloramphenicol resistance cassette (*lox66*-P_{32}*cat*-*lox71*)[46]. In this study, a derivative of the mutagenesis vector pNZ5319 [46], designated pNZ5319TAG was used to introduce a unique 42-nucleotide tag into chromosome during gene deletion, which can be used for mutant tracking purposes in mixed populations (not relevant for the study presented here). The upstream and downstream flanking regions of *ltaS1* (*lp_1283*) and *ltaS2* (*lp_2580)* genes were amplified by PCR using corresponding primer sets (Table 2). Each amplicon generated was subsequently joined by a second PCR to *lox*66-P_{32}*cat*-*lox*71-tag by a splicing by overlap extension strategy [47], using targets-Up-F/targets-Down-R primer pairs (Table 2). The resulting PCR products were digested with SwaI and Ecl136II, and cloned into similarly digested pNZ5319TAG. The obtained mutagenesis plasmids were transformed into *L. plantarum* WCFS1 as described previously [45]. The resulting transformants were assessed for a double cross over integration event by selecting for colonies with a Cm resistant and Ery sensitive phenotype, in which the anticipated chromosomal organization of the mutated *ltaS1* or *ltaS2* locus was confirmed by PCR using targets-out-F/R primers (Table 2). For each of the mutant constructions a single colony displaying the anticipated genotype was selected, yielding the mutants NZ3537Cm (Δ*ltaS1*), and NZ3538Cm (Δ*ltaS2*).

For complementation of Δ*ltaS1* and Δ*ltaS2*, the *ltaS1* and *ltaS2* genes of *L. plantarum* WCFS1 were amplified, including their native promoters, using primers IS311/IS312 and IC004/IC005, respectively. A SacI site was introduced by primer IS312 and IC005 downstream of *ltaS1* and

ltaS2, respectively. pMEC10 was digested by SacI and SfoI, whereas PCR products of *ltaS1* and *ltaS2* were digested with SacI. Digested vector and inserts were ligated using T4 DNA ligase. Subsequently, the ligation mixtures were transformed into *E. coli* TOP10; positive clones were selected by colony PCR (52) using primers Ip_1283F/IS260 for *ltaS1* and Ip_2580F/IS260 for *ltaS2*. Resulting plasmids were designated pNZ8200 and pNZ8201 for the complementation

Table 2. Plasmids and primers used in this study

Plasmids	Description[a]	Reference
pNZ5319	Cm^r Em^r; Mutagenesis vector for gene replacements in *L. plantarum*	[46]
pMEC10	Em^r; integration plasmid	[67]
pNZ3537	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of *ltaS1* (*lp_1283*)	this work
pNZ3538	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of *ltaS2* (*lp_2580*)	this work
pNZ8200	Em^r; pMEC10 derivative containing a single copy of ltaS1 gene and its own promoter	this work
pNZ8201	Em^r; pMEC10 derivative containing a single copy of ltaS2 gene and its own promoter	this work
Primers	**Sequence[b]**	**Reference**
is128 tag-lox66-F3	5'-AAATCTACCGTTCGTATAATGTATG-3'	[69]
is129 tag-lox71-R3	5'-CTCATGCCCGGGCTGTAACCG-3'	[69]
IS169	5'-TTATCATATCCCGAGGACCG-3'	[89]
87	5'-GCCGACTGTACTTTCGGATCC-3'	[46]
CreF	5'-CGATACCGTTTACGAAATTGG-3'	[46]
CreR	5'-CTTGCTCATAAGTAACGGTAC-3'	[46]
EryintF	5'-TCAAATACAGCTTTTAGAACTGG-3'	[46]
EryintR	5'-ATCACAAACAGAATGATGTACC-3'	[46]
ltaS1-Up-F	5'-GTGCTTCTTGAATGATGGGG-3'	this work
ltaS1-Up-R	5'-GCATACATTATACGAACGGTAGATTTTTTAGGCATGGTA-ATTTCTTCC -3'	this work
ltaS1-Down-F	5'-CGGTTACAGCCCGGGCATGAGAGTAGTTCTGATTAATC-GAACTCG-3'	this work
ltaS1-Down-R	5'-TTTCCCACGTGTTACTCACC-3'	this work
ltaS1-out-F	5'-AAATCGTTAACGCCTGTATCC-3'	this work
ltaS1-out-R	5'-CGGTCCAAGTTGTTATGCGG-3'	this work
ltaS2-Up-F	5'-AGCTCCATATGTACAATACTGC-3'	this work
ltaS2-Up-R	5'-GCATACATTATACGAACGGTAGATTTCGGGTTCACGAGCAT-CAGG -3'	this work
ltaS2-Down-F	5'-CGGTTACAGCCCGGGCATGAGTCCAAATAGCAACAAGCAT-TCC -3'	this work
ltaS2-Down-R	5'-GAATTCCAAGGTAACCAGCC-3'	this work
ltaS2-out-F	5'-CACCAGTTAGTGATTCACGC-3'	this work
ltaS2-out-R	5'-CCGTTTTCCATGGAAGCCG-3'	this work
IS311	5'-GCGATAGCTTCAGTCGCTCC-3'	this work
IS312	5'-AAATTGAGCTCACCATTCCAACTTTGCATTTACTTGG-3'	this work
IC004	5'-GAGAATACGCGTTTCACCGGTTATCG-3'	this work
IC005	5'-TCGGAGAGCTCTGAACGGCACTGAACTTAAGAGATGG-3'	this work
lp_1283F	5'-ACTGTCGAAACAGCATAACC-3'	this work
lp_2580F	5'-TTATGGCGGTGAAATTGACG-3'	this work
IS260	5'-GTTGAAAGAACCTGTACTCTCC-3'	[90]
TRNA	5'-GCGAACCGGCTAATACCGGC-3'	[91]

[a] Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant

[b] Underlined nucleotides indicate parts of the primers that are complementary to the is128-lox66-F3 and is129-lox71-R3 primers.

plasmid of *ltaS1* and *ltaS2*, respectively. Integrity of nucleotide sequences for each construct was confirmed by sequence analysis. Subsequently, the complementation plasmids were introduced into the corresponding deletion strains by electroporation as described previously (49). Transformants were screened for chloramphenicol and erythromycin resistance, followed by PCR amplifications to confirm the chromosomal integration of introduced plasmid using primers lp_1283F/TRNA and lp_2580/TRNA for *ltaS1* (NZ8200CmEry) and *ltaS2* complementation (NZ8201CmEry) strains, respectively.

SDS-PAGE and western blot analyses. SDS-PAGE and wet blotting were performed using the NuPAGE® electrophoresis system (Invitrogen) and XCell II™ Blot Module (Invitrogen), respectively, as described in the user manuals. Whole cell extracts were mixed with NuPAGE sample buffer and were separated under denaturing condition on NuPAGE®Novex® 4-12% Bis-Tris gels with MOPS SDS running buffer (Invitrogen).

For western blots, the gels were transferred to nitrocellulose membranes (Thermo Scientific, Bremen, Germany) using the wet blotting method described in the NuPAGE manual (Invitrogen). The membranes were blocked with 3% bovine serum albumin (BSA) in PBS containing 0.05% Tween20 (PBST) for 1 hour at room temperature. The membranes were then incubated with a final concentration of 0.2 μg/ml (1:1000 dilution) of monoclonal mouse IgG_3 against LTA Clone 55 (HyCult Biotechnology, Uden, The Netherlands) in blocking solution, followed by incubation with 1:5000 dilution Horse anti-mouse IgG, horseradish peroxidase conjugated (Cell Signaling Technology, Beverly, USA). In between the incubations the membranes were washed three times with PBST for 15min. Precision Plus Protein™ Dual Color Standard (Bio-Rad, Richmond, USA) was used as a reference of molecular size. After the membranes were washed, they were developed by using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) and Kodak BioMax Light film (Kodak, Rochester, USA).

Microscopy. For phase-contrast microscopy, mid-logarithmic cultures (OD_{600} = 1.0) of *L. plantarum* strains were negatively stained with nigrosin [48] and spotted onto glass slides. After drying, the cultures were examined at 1250× magnification using a Dialux 20 microscope (Leitz, Wetzlar, Germany). Fluorescence microscopy was performed as described previously [49] with some modifications. Mid-logarithmic cultures of *L. plantarum* strains were incubated with 20 μg/ml FM4-64 and 0.5 μg/ml Syto9 (Both from Molecular Probes, Eugene, USA) for 30 min and imaged by oil immersion fluorescence microscopy (BX51TRF Fluorescence Microscope, Olympus Corporation, Tokyo, Japan) at a 500x magnification.

Scanning electron microscopy (SEM) analysis was performed as described previously [49]. Briefly, 8-mm circular coverslips were coated with 0.01% (w/v) Poly-L-lysine (in water) and incubated for 30 min in *L. plantarum* mid-logarithmic cultures (OD_{600} = 1). The bacteria-adhered coverslips were then fixed with 4% (v/v) glutaraldehyde for 30 minutes, rinsed with water, followed by dehydration in serial acetone solutions. The samples were subsequently critical point dried with carbon dioxide (CPD 030, BalTec, Liechtenstein). The cover slips were then sputter coated with 5 nm platinum in a dedicated preparation chamber (CT 1500 HF, Oxford Instruments, Oxford UK). The bacteria were analyzed with a field emission scanning electron microscope (FEI Magellan 400 FESEM, Hillsboro, USA) at room temperature at a working distance between 4 and 5 mm, with SE detection at 2.0 kV. Images were digitally recorded (Orion 6 PCI, E.L.I. sprl. Belgium).

Zeta potential and hydrophobicity measurements. Overnight cultures were washed twice with 10 ml PBS and bacteria were resuspended in PBS at an optical density at 600 nm (OD_{600}) of 1. Zeta potential was measured according to the manufacturer's manual at 20°C using ZetaSizer cuvettes DST1070 (Malvern Instruments, Malvern, UK) in a Zetasizer nano series (Malvern Instruments).

Surface hydrophobicity was determined using microbial adhesion to solvents (MATS) method [50]. Briefly, overnight cultures of *L. plantarum* strains were harvested by centrifugation at 5,000 x g for 10 min, washed twice and resuspended to OD_{600} of 1.0 (A_0) in PBS. 5 ml of bacterial suspension was mixed with 2 ml petroleum ether (the solvent) by 2 min vortexing in a 10 ml glass tube. The tubes were incubated statically for 15 min at room temperature to allow phase separation of the mixture. The aqueous phase was collected and its OD_{600} was measured (A_1). The cell surface hydrophobicity (CSH) was presented as the percentage of microbial retained in the solvent, and calculated as $(1 - A_1/A_0) \times 100$.

RNA isolation and DNA microarray analysis. RNA isolation, labeling and hybridization were performed according to previously described methods [51, 52]. Briefly, *L. plantarum* WCFS1 and its *ltaS1* deletion derivative (NZ3537Cm) were grown in MRS with chloramphenicol and cells were harvested by centrifugation, following quenching and cell disruption by bead beating. RNA was purified using the High Pure Isolation Kit (Roche Diagnostics, Mannheim, Germany) including 1 h treatment with DNaseI (Roche Diagnostics). Five microgram of total RNA was used for cDNA synthesis. Cyanine-3 (Cy3) and cyanine-5 (Cy5) cDNA labeling was performed as described previously [51], using the CyScribe Post-Labeling and Purification kits according to the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, UK). Cy-dye-labeled cDNAs (0.5 µg each) were hybridized to *L. plantarum* WCFS1 printed-oligonucleotide DNA microarrays (Agilent Technologies, Amstelveen, the Netherlands). Hybridization and scanning procedures were performed as previously described [51]. The data were normalized using the Lowess normalization as available in MicroPrep [53]. CyberT [54] was used to compare the transcriptomes of WCFS1 and *ltaS1* mutant, resulting in a gene expression ratio and Benjamini and Hochberg's False Discovery Rate (FDR) [55] for each gene. The expression of a gene was considered significantly altered when the FDR-adjusted p value was < 0.05. The transcript data of the *ltaS2* mutant were analyzed by employing the CyberT package and were compared with those of WCFS1 and the *ltaS1* mutant by Pearson correlation analysis and visualized using maximum likelihood trees (Phyml) for all genes or for genes within specific functional classes.

LTA purification and structure analysis by NMR spectroscopy. LTAs of *L. plantarum* and *B. subtilis* were extracted by the 1-butanol method as described in previous studies [56] with some modifications. Using a Techfors-S fermentor (Infors, Bottmingen, Switzerland), *L. plantarum* cells were cultured in 10 L of MRS-broth at 37°C at a constant pH of 6.8 and harvested after 20 hr incubation, while *B. subtilis* was aerobically grown in 10 L of LB-broth [57] at 37°C and harvested after 8 hr incubation when the rising medium pH reached 7.2. The earlier harvesting time for *B. subtilis* was chosen to avoid degradation of D-Ala residues under alkaline conditions. Harvested cells (ca. 100 g and 30 g wet weight biomass were obtained for *L. plantarum* and *B. subtilis*, respectively) were washed with PBS and resuspended in the same buffer containing DNase and RNase (1 mg each, Roche diagnostics, Basel, Switzerland), followed by 5 cycles of cell disruption by passage through a French press cell (SLM Instruments Inc., Urbana, USA) at

10000 psi. The suspensions were centrifuged at 12000 rpm for 30 min at 4°C, and 100 ml of the supernatants were collected. The supernatants were mixed with an equal volume of 1-butanol and then stirred at room temperature for 30 min, followed by centrifugation for phase separation. The lower aqueous phase was collected and lyophilized. Subsequently, LTAs in the lyophilized butanol extracts were purified by hydrophobic interaction chromatography and anion-exchange chromatography. Briefly, the extracts were dissolved in 20 ml of 15% (v/v) 1-propanol in 100 mm sodium acetate (NaOAc) buffer (pH 4.7) and subjected to a column packed with Octyl-Sepharose 4 FastFlow (GE Healthcare, Chalfont St. Giles, England; i.d. 50 mm × 10 cm). The column was eluted with 1–2 column volume (CV) of 15%, 20%, 25%, 35%, and 45% (v/v) 1-propanol in NaOAc buffer. The LTA-containing eluate was identified by their organic phosphate contents, which are determined by the method described previously by Tomita *et al*. [58]. The LTA-containing eluate (35% 1-propanol fraction in most cases) was dialyzed and lyophilized, and the isolate was dissolved with NaOAc buffer and was further subjected to fractionation on a column packed with DEAE-Sepharose 4 FastFlow (GE Healthcare; i.d. 26 mm × 11 cm). The column was washed with 2 CV of NaOAc buffer and eluted with a linear gradient elution of sodium chloride (0–1.0 M, 5 CV) using an AKTA fast performance liquid chromatography system (GE Healthcare). The fractions were measured for the presence of hexose [59] and organic phosphate [58] to identify LTA-containing fractions, which were pooled, dialyzed, and lyophilized to obtain the purified LTA. For structural comparison, proton nuclear magnetic resonance (^{1}H NMR) spectrum of purified LTAs was recorded on an Avance III 500 MHz NMR spectrometer (Bruker BioSpin, Karlsruhe, Germany) under the condition described previously [60].

Toll-like receptor (TLR) assay. The assay was performed as described previously [61]. Briefly, human embryonic kidney (HEK)-293 TLR reporter cell lines expressing human TLR1/2, TLR2/6, or TLR4, and pNIFTY, a NF-κB luciferase reporter construct (InvivoGen, Toulouse, France) [62], were used. The HEK-293 reporter cell lines were seeded at 6×10^4 cells/well in 96-well plates and incubated overnight under standard culture conditions. Cells were then stimulated with purified LTA or bacterial cells isolated from the stationary phase of growth of the *L. plantarum* wild type WCFS1 and its LtaS1- and LtaS2-deficient derivatives (NZ3537Cm and NZ3538Cm, respectively) at a MOI of 1:10, HEK cell to bacteria. The TLR1/2 agonist Pam3CSK4 (5 μg/mL, InvivoGen) and TLR2/6 agonist Pam2CSK4 (5 μg/mL, InvivoGen) were used as positive controls, whereas PBS served as the negative control.

For signal-attenuation assays, the LTA from *L. plantarum* WCFS1 (0.1, 1.0, and 10 μg/ml), control IgA2 antibody and neutralizing monoclonal IgA antibody to human TLR2 (both from InvivoGen, San Diego, USA; 5 μg/ml), were added to the TLR expressing reporter cell lines 15 min prior to the addition of the stimulant, 1 μg/ml of purified LTA from *B. subtilis*. Following a 6 hr incubation period, the medium was replaced with Bright-Glo™ (Promega Benelux BV, Leiden, The Netherlands), the plate was vortexed for 5 min, and the luminescence was measured using a Spectramax M5 (Molecular Devices, Sunnyvale, USA).

Peripheral blood mononuclear cells (PBMCs) assay. The assay was performed as described previously [63] and was approved by Wageningen University Ethical Committee and was performed according to the principles of the Declaration of Helsinki. Peripheral blood of healthy donors was obtained from the Sanquin Blood Bank, Nijmegen, The Netherlands. PBMCs were separated from the blood using Ficoll-Paque Plus gradient centrifugation according to the

manufacturer's description (Amersham biosciences, Uppsala, Sweden). The mononuclear cells were collected, washed in Iscove's Modified Dulbecco's Medium (IMDM) + glutamax (Invitrogen, Breda, The Netherlands) and adjusted to 1×10^6 cells/ml in IMDM + glutamax supplemented with penicillin (100 U/ml) (Invitrogen), streptomycin (100 μg/ml) (Invitrogen), and 1% human AB serum (Lonza, Basel, Switzerland). PBMCs (1×10^6 cells/well) were seeded and cultured overnight prior to the experiment in 48-well tissue culture plates, incubated at 37°C in 5% CO_2. Bacteria or purified LTA were added to PBMCs at a MOI of 1:10, PBMCs to bacteria or at 1 μg/ml, respectively. PBMCs from 3 different donors were used in the assay. Following 24 hr incubation at 37°C in 5% CO2, culture supernatants were collected and stored at -20°C until cytokine analysis. Cytokines were measured using a FACS CantoII flow cytometer (BD Biosciences, New Jersey, USA) and BD Cytometric Bead Array Flexsets (BD Biosciences) for interleukin (IL)10 and IL12p70, TNFα, IL6, IL1β, and IL8 according to the manufacturer's procedures. Concentrations of cytokines were calculated based on the standard curves in the BD Biosciences FCAP software.

Statistical analysis. The hydrophobicity, TLR and PBMCs assays analyses were performed in triplicate, while zeta potential was measured in quarduplicate. The One-way ANOVA followed by Tukey's multiple comparison test was used to compare the means of surface properties and TLR2 activations between strains. The paired *t* test was used to determine the Log10 values of PBMCs cytokine productions after stimulated with wild-type verse mutant strains with respects of individual donors. GraphPad Prism 5 software (GraphPad Software, San Diego, CA) was used for all determinations, and a P value <0.05 was considered significant.

Results

Identification of LtaSs involved in LTA synthesis in L. plantarum *WCFS1*

LtaS is responsible for the synthesis of polyglycerolphosphate [poly(Gro-P)] backbone of LTA [24]. A BlastP analysis [64] using the sequences of known LtaS enzymes from *S. aureus* [24], *B. subtilis* [25] and *L. acidophilus* NCFM [65], revealed two homologous genes, *lp_1283* and *lp_2580* (named *ltaS1* and *ltaS2*, respectively) in *L. plantarum* WCFS1. Both Lp_1283 and Lp_2580 are annotated as a membrane-bound sulfatase of the alkaline phosphatase superfamily [66] and share high protein sequence similarity with known LtaS (45% identity with LtaS from *S. aureus*, 48% for LtaS1 and 46% for LtaS2 with LtaS from *B. subtilis*, and 53% with LBA0447 from *L. acidophilus* in protein sequences). Gene deletion mutants for both genes were constructed using double cross-over gene replacement of either *ltaS1* or *ltaS2* by the chloramphenicol acetyltransferase gene (*cat*), resulting in *L. plantarum* WCFS1 Δ*ltaS1* (NZ3537Cm) and Δ*ltaS2* (NZ3538Cm), respectively. To complement the mutants, single copies of the respective genes were inserted in the chromosome downstream of the serine tRNA encoding gene under control of their own promoter, using the pMEC10 vector for targeted integration [67]. Notably, the growth and accessibility for genetic manipulations of the mutants were much compromised, prohibiting the construction of a double deletion mutant that lacks both *ltaS1* and *ltaS2*.

To confirm that LtaS1 and LtaS2 are involved in LTA poly(Gro-P) synthesis, whole cell extracts of the deletion and complementation strains were subjected to western blotting using a primary antibody against poly(Gro-P). Both the *ltaS1* and *ltaS2* deletion strains did no longer contain the

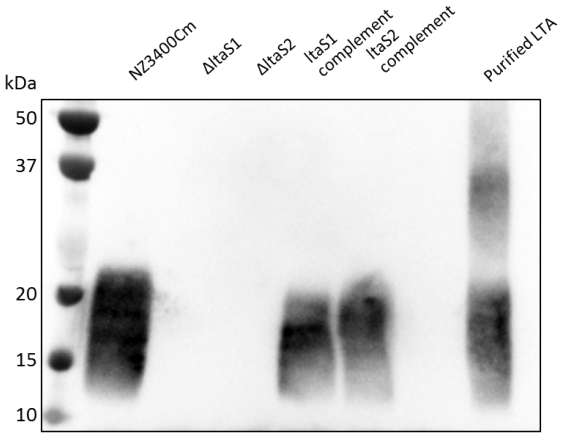

Figure 1. Whole cell extracts of wild type, Δ*ltaS1*, Δ*ltaS2*, and complemented mutants were analyzed by the western blot using a polyglycerolphosphate-specific monoclonal antibody to detect the poly-GroP LTA backbone. On the left side of the blot the protein sizes (kDa) are indicated based on the Precision Plus Protein™ Dual Color Standards (Bio-Rad) molecular marker. 0.1 μg/ml of purified LTA from wild type was loaded.

component recognized by the poly(Gro-P) antibody, which is assumed to represent LTA, and was detected in the wild type and the complemented strains (Figure 1). Taken together we identified LtaS1 (Lp_1283) and LtaS2 (Lp_2580), in *L. plantarum* WCFS1 and demonstrated that the LTA backbone synthesis in this strain requires both enzymes.

The role of LTA in bacterial physiology and surface properties

While the deletion of *ltaS* (LBA0447) in *L. acidophilus* resulted in unaffected growth characteristics of the mutant strain relative to its parental strain [65], in other organisms, including *S. aureus* [24], *B. subtilis* [25] and *Listeria monocytogenes* [26] deficiency of this function had a substantial impact on growth and cell division. To investigate what the consequences of mutation are in *L. plantarum*, we investigated growth rate and cell morphology of the *ltaS1* and *ltaS2* deletion mutants. The maximum growth rate of the wild type is $0.39 \pm 0.006\ h^{-1}$, whereas the growth rate of Δ*ltaS1* and Δ*ltaS2* were strongly reduced, and were approximately half of that of wild type (0.18 ± 0.027 and $0.29 \pm 0.162\ h^{-1}$, respectively). Moreover, while the *ltaS1* mutant reached a final optical density (OD_{600}; 8.39 ± 0.042) similar to the parental strain *L. plantarum* WCFS1 (8.50 ± 0.651) at 37°C in MRS, the *ltaS2* mutant displayed a much lower final density under the same culture condition (6.33 ± 1.032). Subsequently, we investigated possible defects in cell division and morphology by microscopy. Light microscopy of Δ*ltaS1* and Δ*ltaS2* indicated that both mutants had lost the normal rod shape observed in the parental strain, and displayed distinct morphologies. The *ltaS1* mutant displayed long and curly shaped cells, whereas the *ltaS2* mutant appeared to form filamentous cells (Figure 2) that had a tendency to clump together (data not shown). The filamentous and clumpy phenotype of Δ*ltaS2* may be the cause of the high standard deviations observed in growth rate and OD_{600} measurements, since the culture is not homogeneous. Scanning electron microscope (SEM) analysis clearly showed that Δ*ltaS1* displayed abnormal positioning of cell separation, resulting in curly cell shapes, whereas Δ*ltaS2* grew in very long cells due to an apparently strongly impaired cell division (Figure 2). By visualization of cell membranes using the lipophilic dye FM4-64, it

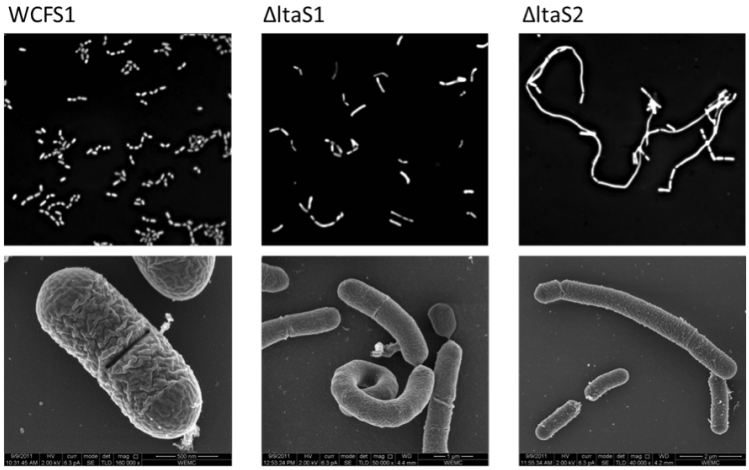

Figure 2. Images of *L. plantarum* WCFS1 wild type and *ltaS* deletion derivatives under phase-contrast microscopy (upper panel) and scanning electron microscopy (lower panel). The phase-contrast microscopy shows 1250x magnified views of nigrosin-stained cells. The scanning electron microscopy shows images under different magnifications (160000x, 50000x, and 40000x for wild type, ΔltaS1, and ΔltaS2, respectively) to capture the morphology.

became apparent that the cells of both *ltaS* mutants are longer than *L. plantarum* WCFS1 and Δ*ltaS2* forms much more elongated cells as compared to Δ*ltaS1* (Figure S1). We also observed normal rod-shaped cells in both mutants, indicating that the cell division is delayed or less efficient but not completely lost. The complementation strains seem only partially restored in terms of their morphology. For instance, the *ltaS2* complemented strain does not show filamentous morphology but forms a chain of rod-shaped cells (Figure S2). It is likely that the expression levels of LtaS2 is slightly different in the complemented strain as compared to the wild-type situation which could lead to these altered phenotypes, albeit less distinct as in the Δ*ltaS2* mutant. These observation indicate that both growth and cell division are substantially, but distinctly modulated by deletion of either of the LtaS encoding genes in *L. plantarum* WCFS1.

The surface properties of bacterial cells are considered to play an important role in the capacity of bacteria to physically interact with specific surfaces, including their capacity to interact with host cells in the intestinal tract. Important bacterial surface characteristics include its net-charge and hydrophobicity. Since LTA is one of the major cell envelope components and the fact that the poly(Gro-P) backbone contains a high amount of negative charges, as well as substitutions of D-alanine and glucose on the backbone [33, 68], it is likely that removal of mature LTA impacts on surface properties. To this end, surface charge and hydrophobicity of *L. plantarum* WCFS1 was compared to that of the *ltaS*1/2-deficient mutants. Notably, despite obvious changes in growth and cell division, Δ*ltaS1* does not alter surface charges nor hydrophobicity (Figure 3). Conversely, *ltaS2* mutation led to a strong increase in negative surface charge as well as the surface hydrophobicity (Figure 3). Importantly, although deletion of both *ltaS1* and *ltaS2* led to loss of normal LTA production (Figure 1), the impact of these mutations on the bacterial surface properties is quite distinct, strongly suggesting distinct functions for these two enzymes.

Mutation of ltaS *elicits pleiotropic transcriptional changes*

To further assess the impact on overall physiology, genome-wide transcriptional profiles of

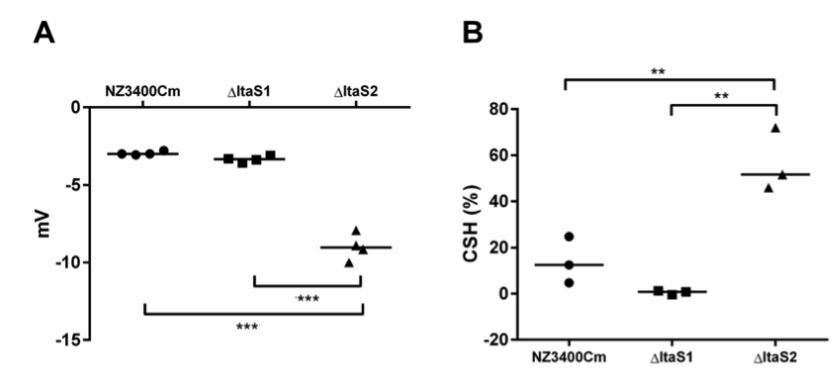

Figure 3. (A) Zeta potential and (B) cell surface hydrophobicity (CSH) of *L. plantarum* WCFS1 (NZ3400Cm) and its deletion mutant derivatives. All individual data points are shown (n = 4 for zeta potential and n = 3 for hydrophobicity measurements) as well as the median (bar). Statistical significance of observed differences was determined by applying Kruskal-Wallis test; $P \leq 0.05$ (overall ANOVA); ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.

the *ltaS1* deletion strain were generated and compared with those of the wild-type strain. The transcriptome analysis confirmed the abolishment of *ltaS1* expression in the mutant strain and showed the expression of a large number of genes were significantly altered by the deletion. The *ltaS1* deletion affected the expression of a total of 1258 genes; 709 genes were significantly down-regulated and 576 genes were up-regulated compared to the wild-type strain, reflecting a substantial role of LTA in bacterial physiology. Among these altered genes, genes involved in energy metabolism (58 up-regulated and 21 down-regulated) and stress/environment adaptation (16 up-regulated and 2 down-regulated) appeared mainly up-regulated, whereas genes involved in biosynthesis of proteins (14 up-regulated and 51 down-regulated), nucleotides (17 up-regulated and 31 down-regulated), cofactors (7 up-regulated and 14 down-regulated), and fatty acids (2 up-regulated and 16 down-regulated)were mostly down-regulated. The *ltaS1* deletion also affected a large group of genes (134 genes) involved in the biosynthesis of other cell surface components, including peptidoglycan, surface polysaccharides and proteins. Notably, while the genes involved in peptidoglycan and surface polysaccharide synthesis were both down- and up-regulated without a clear consistent pattern, the genes encoding surface proteins were almost exclusively up-regulated. The genes involved in D-alanylation of LTA and wall teichoic acids, WTA, (*dltA*, *dltB*, *dltC1*, *dltD*, and *dltX*) were up-regulated in the *ltaS* mutant, suggesting a potential influence of LTA on WTA structures. Interestingly, two genes, *tagF2* (*lp_0269*), *tagD2* (*lp_1248*), involved in glycerol-type wall teichoic acid (WTA) biosynthesis were down-regulated while *tagL* (*lp_1819*) that is involved in ribitol-type WTA synthesis, was up-regulated. It has been shown that *L. plantarum* WCFS1 has the ability to synthesis both types WTA but under tested conditions only produce glycerol-type WTA [69]. Comparative analysis showed that the transcriptomes of both the Δ*ltaS1* and Δ*ltaS2* strains were substantially different from the wild-type and also quite distinct from each other (Figure S3A). However, the evaluation specifically focusing on changes in cell envelope-associated genes in the *ltaS2* mutant revealed a significant degree of coherence with *ltaS1*, in comparison to the wild-type strain (Figure S3B), suggesting a similar impact on surface molecules by both LTA deficiency causing mutations. These data indicate the pleiotropic and distinct impact of the *ltaS* deletions on overall bacterial physiology and underpin the stress induced by *ltaS1* deletion that drives the reduction of biosynthetic pathways and the increase of energy generating metabolism, while both *ltaS* mutations broadly affect a variety of surface molecules.

LTA and TLR2 signaling

Several studies have shown that LTA signals through TLR2 [29, 33, 70]. Therefore the effect of LTA-deficiency in *L. plantarum* WCFS1 on host cell signaling was investigated by employing a TLR2 activation reporter cell assay. The dosage of bacterial cells to which the reporter cells were exposed was normalized on basis of total bacterial biomass rather than on bacterial colony forming units (CFU), with the intention to avoid confounding of the data due to the altered morphology and tendency towards cell-clumping observed in the LTA deficient strains. Notably, the LTA-deficient mutants stimulated significantly higher TLR1/2 and TLR2/6 signaling (Figure 4A and 4B, respectively) as compared to the wild-type strain (WCFS1). Deletion of *ltaS1* led to an approximately 5-fold increased TLR1/2, and approximately 1.5-fold increased TLR2/6 signaling in the respective reporter cell lines. The *ltaS2* deletion resulted in an even stronger increase in TLR1/2 (ca. 10-fold) and TLR2/6 (ca. 5-fold) signaling, respectively, relative to the activation by WCFS1 (Figure 4). These results apparently exclude a direct role of *L. plantarum* LTA in TLR1/2 or TLR2/6 signaling pathway activation, although this conclusion should be taken with caution and the differences are possibly not only assigned to a lack of LTA but may involve other compensatory changes in these mutants that display quite pleiotropic differences in cell-envelope pathways compared to the parental strain (see above).

To further investigate the TLR2 signaling role of *L. plantarum* LTA, we purified LTA from *L. plantarum* (LTA-Lp) and tested this component in the TLR2 signaling reporter cell assay. As a positive control, LTA from *Bacillus subtilis* 168 was purified (LTA-Bs) using the same purification procedure. This preparation was considered as a suitable positive control, since LTA-Bs is commercially available as a TLR2 ligand (InvivoGen), although the purification procedure for this material is not clearly described. Purified LTA-Lp stimulates only very low TLR1/2 activation and fails to activate TLR2/6 signaling. By contrast, LTA-Bs activates strong TLR1/2 and TLR2/6 signaling, already at low concentrations (0.1 μg/ml), which is comparable to the stimulants

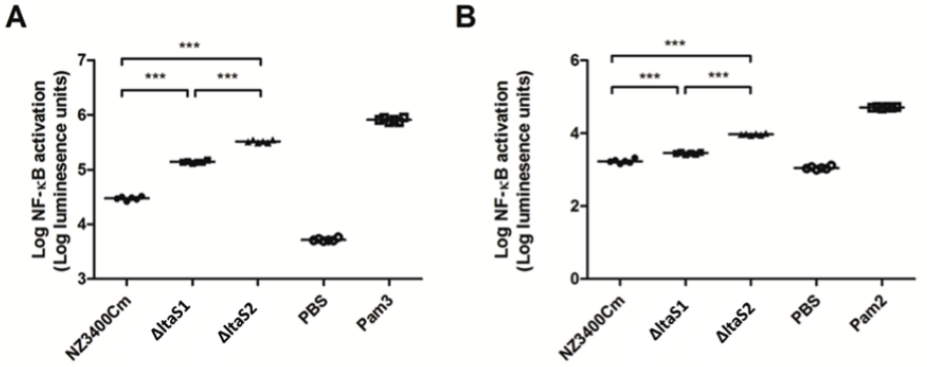

Figure 4. TLR1_2 (A) and TLR2_6 (B) signaling stimulated by *L. plantarum* wild type and *ltaS* deletion derivatives (ΔltaS1 and ΔltaS2), using TLR-expressing HEK cell lines, containing a NF-kB responsive luciferase reporter system. Measurements were performed in two independent experiments each with three technical replicates (n = 6) and are presented as Log values, and individually displayed and the bar indicates the median. PBS serves as negative control, and Pam3CysSK4 (Pam3) and Pam2CysSK4 (Pam2) are the positive stimulants of TLR1_2 and TLR2_6 activation, respectively. Data comparison of the *ltaS* deletion strains and the wild-type strains were tested for significant differences using one-way ANOVA followed by Tukey's multiple comparison correction and significant differences are indicated; ***, $P \leq 0.001$.

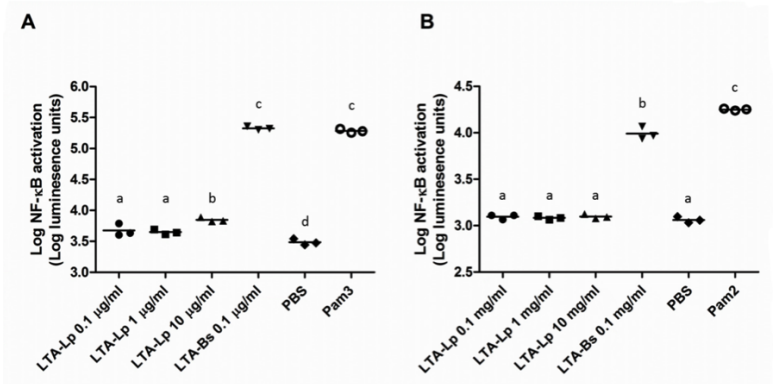

Figure 5. TLR1_2 (A) and TLR2_6 (B) signaling stimulated by purified LTA from *L. plantarum* WCFS1 (LTA-Lp) or *B. subtilis* 168 (LTA-Bs). Measurements were performed in triplicate and are presented as Log luminescence units, and individually displayed (n = 3) and the bar indicates the median. PBS serves as negative control, and Pam3CysSK4 and Pam2CysSK4 are the positive stimulants of TLR1_2 and TLR2_6 activation, respectively. LTA-Lp was tested in 0.1, 1, and 10 µg/ml while LTA-Bs was tested in 0.1 µg/ml. Data were tested for significant differences using one-way ANOVA followed by Tukey's multiple comparison correction and samples showed significant differences are marked with different letters.

Pam3CSK4 and Pam2CSK4 that are the synthetic tri-acylated and di-acylated lipopeptide agonists of TLR1/2, and TLR2/6, respectively (Figure 5). These results indicate that contrary to LTA of *B. subtilis*, which is an effective TLR2 signaling ligand, the LTA molecule produced by *L. plantarum* WCFS1 is a poor TLR2 stimulator and elicits more than 40- and 8-fold reduced TLR1/2- and TLR2/6-mediated NF-κB activation in the HEK-reporter cell line, respectively (Figure 5).

The low TLR2-stimulating capacity of the purified *L. plantarum* LTA in combination with the enhanced TLR2 signaling elicited by *L. plantarum* cells in which the LTA synthesis is eliminated by *ltaS1/2* mutation, possibly implies that the LTA molecules of *L. plantarum* WCFS1 plays an attenuating role in molecular signaling through the TLR2-dependent pathway. Such attenuation effect would most likely involve binding of the *L. plantarum* LTA to the TLR-receptor without eliciting the signal transduction activated by LTA molecules of other species (e.g., *B. subtilis*). To examine the postulated attenuation effect, the TLR1/2 and TLR2/6 reporter cell lines were pre-incubated with LTA-Lp in different concentrations and subsequently stimulated with LTA-Bs. As a positive control for TLR2 activation, a neutralizing IgA monoclonal antibody directed against human TLR2 was used [71], whereas human IgA isotype was included as a negative control. Of these controls, only the TLR2-specific IgA controls effectively attenuated the LTA-Bs activation of TLR2 signaling in both the TLR1/2 and TLR2/6 expressing reporter cell lines, illustrating the validity of the approach chosen. However, the pre-incubation of the TLR1/2 and TLR2/6 reporter cells with LTA-Lp prior to stimulation with LTA-Bs failed to affect the LTA-Bs signaling capacity, indicating that LTA-Lp does not display attenuation capacity in this assay or at least not at the concentrations employed (Figure S4A and S4B, respectively).

The impact of LTA on immunomodulatory properties of L. plantarum

To evaluate whether LTA can modulate immune responses via other PRRs, the general immunomodulatory properties of LTA-deficient mutants and of purified LTA were investigated using PBMCs assay and the production of inflammation-related cytokines, namely interleukin (IL) 12p70, tumor necrosis factor (TNF) α, IL1β, IL8, IL6, and IL10, were measured. The deletion

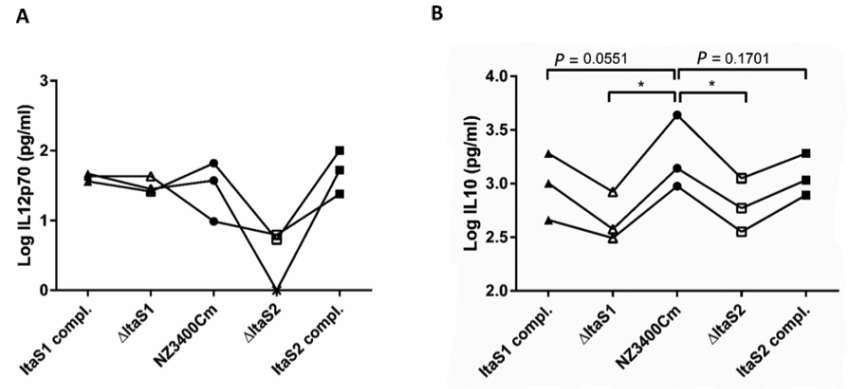

Figure 6. Immunomodulatory effect of *L. plantarum* WCFS1 and, *ltaS* deletion derivatives, and complementated strains in PBMC stimulation assays (n=3 donors), measuring inflammation-related cytokine secretions. The productions of pro-inflammatory cytokine IL12p70 (A) and anti-inflammatory cytokine IL10 (B) are presented in Log values. One measurement, labeled as 'x', of IL12p70 in ΔltaS2 was under the detection limit and was set at the detection limit. The cytokine levels for individual donors stimulated with wild-type and deletion mutants bacteria were connected by a line to focus the read-outs on changes elicited by the mutations. Significant differences between cytokine levels induced by wild-type strains and mutants (paired t-test) are indicated; *, $P \leq 0.05$. No significant differences were found between samples in IL12 measurements. The measurements of TNFα, IL1β, IL6 and IL8 from the same assays are presented in supplemental figure S3.

mutants, Δ*ltaS1* and Δ*ltaS2*, both induced similar amounts of pro-inflammatory cytokines as the wild type strain (Figure 6A and S5), except in one donor where Δ*ltaS1* induces about 4.5-fold higher amount of IL12 than WCFS1 (Figure 6A). On the other hand, the productions of anti-inflammatory cytokine IL10 induced by LTA-deficient mutants were significantly lower than by wild type strain while complemented strains stimulated similar level of IL10 as wild type (Figure 6B). The IL10/IL12 ratio, which has been reported as an indicator for *in vivo* performance in a mouse colitis model, displayed no significant differences between the mutants and the wild type (Figure S6). Overall, despite the enhanced TLR2 activations by LTA-deficient *L. plantarum* strains, they do not trigger more pro-inflammatory cytokine productions in PBMCs. Notably, it seems LTA plays a role in stimulating the production of anti-inflammatory cytokine IL10 in PBMCs since reduced IL10 productions observed with LTA-deficient strains. However, given the pleiotropic impacts of *ltaS* deletions on *L. plantarum* cells, we measured cytokine productions of PBMCs using purified LTA to evaluate the direct involvement of LTA in stimulating IL10 production.

The purified LTA from *L. plantarum* WCFS1 (LTA-Lp) and from *Bacillus subtilis* 168 (LTA-Bs) were tested for their immunomodulation responses in PBMCs assay and the responses were compared with those stimulated by *L. plantarum* WCFS1 cells. Despite the large difference in TLR2 signaling, LTA-Lp and LTA-Bs stimulate similar levels of all cytokines measured (Figure 7 and S7), suggesting TLR1/2 and TLR2/6 alone are not the major contributors to LTA recognition in inflammatory responses of PBMCs. Notably, the production of IL12p70 was below the detection limit.

Although individual cytokine production does not significantly differ between LTA-Lp and LTA-Bs, LTA-Lp stimulates generally less cytokines, both pro- and anti-inflammatory, as compared to LTA-Bs (Figure S7), which demonstrates an overall difference in immunomodulatory properties between LTA isolated from *L. plantarum* and *B. subtilis*. When comparing the responses of PBMCs stimulated by the cells and purified LTA of *L. plantarum* WCFS1, the purified LTA stimulates lower

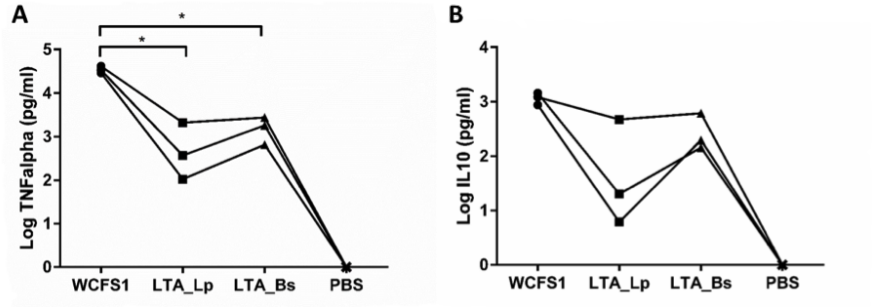

Figure 7. Immunomodulatory effect of *L. plantarum* WCFS1 cells, purified LTA from *L. plantarum* WCFS1 (LTA-Lp) or *B. subtilis* 168 (LTA-Bs) in PBMC stimulation assays (n=3 donors), measuring inflammation-related cytokine secretions. The productions of pro-inflammatory cytokine TNFalpha (A) and anti-inflammatory cytokine IL10 (B) are presented in Log values. Measurements of PBS were all under the detection limit and thus were set at the detection limit and labeled as 'x'. The cytokine levels for individual donors stimulated with WCFS1 strain or purified LTA were connected by a line to focus the read-outs on changes elicited by the origin of LTA. Significant differences between cytokine levels induced by wild-type strains and mutants (paired t-test) are indicated; *, $P \leq 0.05$.

production of the pro-inflammatory cytokines, IL12 (below the detection limit), TNFα (Figure 7A) and IL1β (Figure S7A), relative to whole bacterial cells. The purified LTA does not stimulate elevated IL10 production in PBMCs as compared to the bacterial cells of *L. plantarum* WCFS1 (Figure 7B), suggesting that LTA is not directly involved in IL10 production in PBMCs and the reduced IL10 level stimulated by LTA-deficient mutants may be caused by other factors, which are likely other surface-exposed components that are affected by *ltaS* deletion. Taken together, our results implies that LTA-Lp is not signaling actively in purified form and also does not contribute significantly to immune signaling in the context of the whole cell interaction with PBMCs.

Discussion

LtaS plays an essential role in LTA biosynthesis in various bacteria, such as *S. aureus* [24], *B. subtilis* [25], *Listeria monocytogenes* [26], and *L. acidophilus* [65]. While only a single copy of *ltaS* is found in the genome of *S. aureus*, *Streptococcus pyogenes*, and *Streptococcus agalactiae* [24], there are 4 homologues (*ltaS*, *yfnI*, *yqgS*, and *yvgJ*) found in *B. subtilis* [25]. Our analysis concludes that most lactobacilli, including *L. plantarum* WCFS1, *L. acidophilus* NCFM and *Lactobacillus rhamnosus* GG, possess 2 homologues of *ltaS* encoding gene in their genome (Table S1), showing that multiple copies of *ltaS* genes are commonly found in *Lactobacillus* species. While one of the four LtaS-like proteins of *B. subtilis* (designated LtaS) plays the major role in LTA biosynthesis [25], our study shows that both LtaS1 and LtaS2 in *L. plantarum* WCFS1 are required for LTA backbone synthesis, and deletion of either of their encoding genes eliminates LTA production. Notably, a two-enzyme system for LTA backbone synthesis has been proposed for *Listeria monocytogenes*, involving a LTA primase (LtaP) that produces the priming molecule Gro-P-glycolipid and a LTA synthase (LtaS) that catalyzes the formation of the poly(Gro-P) backbone [26]. To evaluate whether it is likely that LtaS1 and LtaS2 of *L. plantarum* WCFS1 also function in a similar concerted manner, the protein sequences of LtaS1 and LtaS2 were aligned with the LtaS and LtaP of *Listeria monocytogenes*, revealing that both *L. plantarum* proteins are significantly more homologous to LtaS of *Listeria monocytogenes*, disallowing their segregation in the possible LtaP- and LtaS-like functions. Moreover,

no Gro-P-glycolipid intermediate could be detected in either the *ltaS1* or the *ltaS2* mutant strain (Figure S8) underpinning the suggestion that neither of the genes encodes a typical LtaP function. Nevertheless, Δ*ltaS1* and Δ*ltaS2*, are not phenotypically identical, and displayed clearly distinct cell-morphology characteristics and transcriptomes, indicating phenotypic differences between these strains, despite their consistent loss of LTA backbone synthesis. Taken together, both LtaS1 and LtaS2 functions of *L. plantarum* are required for LTA backbone synthesis but have a differential role in bacterial cell division and surface properties.

Previous studies employing *ltaS* deletion mutants have shown the link between LTA and cell division in *Staphylococcus aureus* [24, 28], *B. subtilis* [25], and *Listeria monocytogenes* [26], in which *ltaS* mutants display a filamentous phenotype. One exceptional appears the *ltaS* (*LBA0447*) deletion derivative of *L. acidophilus* NCFM that displays normal growth and morphology [65]. We observed elongated and curly cells in the *ltaS1* mutant and filamentous cells in the *ltaS2* mutant, indicating that cell division is affected by LTA in *L. plantarum* WCFS1. Besides cell division, *ltaS* deletion also has been shown to link with lipid metabolism in *S. aureus* [72] and *B. subtilis* [73]. It has been described in *S. aureus* that the membrane lipid pool of phosphatidylglycerol (PG) sustains a high turnover rate to support LTA synthesis, based on the LtaS-catalized hydrolysis of PG to extend LTA by a single Gro-P unit and generating a diacylglycerol (DAG) molecule [72], which is subsequently recycled to PG or is used for glycolipid synthesis [74]. Diacylglycerol kinase (DgkB) is responsible for the first step of DAG recycling [74] and deletion of *dgkB* is lethal in *B. subtilis*, due to accumulation of DAG [73]. The lethality of *dgkB* deletion could be avoided by deletion of either of the two *ltaS* homologues in *B. subtilis* (*ltaS* and *yfnI*), showing the impact of LtaS in lipid turnover [73]. We also observed that many of the genes involved in lipid metabolism were down-regulated in *ltaS1* deletion mutant, including the gene annotated as diacylglycerol kinase (*lp_1968*), suggesting an intimate link between LTA biosynthesis and lipid metabolism in *L. plantarum*. Notably, LTA-deficiency in *L. plantarum* WCFS1 enhanced the expression of the *dlt* operon, which is responsible for D-alanylation of both LTA and WTA. A previous study showed D-alanylation of LTA becomes essential when WTA is lacking in *S. aureus*, suggesting D-alanylation of LTA and WTA could have a redundant role in bacterial physiology [75]. On the other hand, WTA isolated from LTA-deficient *S. aureus* strain has lower D-alanine decoration isolated than that from wild type. The authors suggested that the presence of LTA affects the efficiency of D-alanylation in WTA [76]. Thus, the enhanced *dlt* operon expression in *ltaS1* strain of *L. plantarum* WCFS1 could suggest a higher degree of WTA D-alanylation as a compensatory mechanism for absence of LTA, or alternatively a compensation for the reduced efficiency of D-alanylation in WTA.

It has been reported that *Lactobacillus* mutants with modified LTA stimulate anti-inflammatory responses. A reduction of D-alanyl substitutions in LTA of *L. plantarum* NCIMB8826 [33] and a complete removal of D-alanyl substitutions in LTA of *L. rhamnosus* GG [77] resulted in enhanced anti-inflammatory capacity and improved effects in treating murine colitis models. Moreover, a complete removal of LTA in *L. acidophilus* NCFM resulted in a strain that exhibits enhanced anti-inflammatory capacities, which was illustrated by suppressed production of IL12 and TNFα and enhanced production of IL10 in dendritic cells. Moreover, the LTA deficient *L. acidophilus* strain was shown to ameliorate colonic inflammation in mouse colitis [65] as well as reduced local DC cell densities and pro-inflammatory cytokines and reduced the formation of colonic polyposis in a mouse model [78]. These findings suggest LTA acts as a pro-inflammatory molecule in the cell envelope of lactobacilli. However, this role of LTA was not observed for *L. plantarum* WCFS1,

where LTA-deficient derivatives do not suppressed pro-inflammatory cytokine productions nor stimulate more anti-inflammatory cytokine production in PBMCs as compared to the wild type strain. Importantly, the alteration of LTA leads to pleiotropic changes on bacterial surface components, including polysaccharides, proteins, and teichoic acids, based on the transcriptome analysis. In addition, purified LTA from *L. plantarum* WCFS1 does not elicit clear pro- nor anti-inflammatory responses in PBMCs. Taken together, it is likely that the changes in immune properties of the LTA-deficient mutants are driven by complex and multifactorial changes in a variety of surface molecules, rather than by LTA removal alone. Also, the enhanced TLR2-signaling in LTA-deficient derivatives of *L. plantarum* WCFS1 may come from other TLR2 ligands, such as lipoproteins/lipopeptides. The indirect impact of LTA modification on surface components also has been reported in *L. rhamnosus* GG, in which abolishing the D-alanine substitution of LTA by deleting *dltD* was shown to elevate the level of secreted Msp2 (formerly p40) [77]. Msp2 is a soluble protein that was shown to prevent cytokine-induced apoptosis in intestinal epithelial and DSS-induced acute colitis in a mouse model via epidermal growth factor receptor (EGFR)-dependent mechanism [79]. However, the link between increased Msp2 secretion and the enhanced anti-inflammatory properties by *dltD* mutant of *L. rhamnosus* GG remains to be verified. Overall, purified LTA of *L. plantarum* WCFS1 is not a potent immune stimulator in PBMCs.

The purified LTA from *L. plantarum* WCFS1 stimulates a very low TLR1/2 signaling and no TLR2/6 signaling. These findings could imply a remaining trace of lipoproteins in LTA purified fraction, since lipoproteins of *L. plantarum* WCFS1 are mainly signaling via TLR1/2 but not TLR2/6 (chapter 4). Previously, Grangette *et al*. have reported that purified LTA from *L. plantarum* NCIMB8826 (*L. plantarum* WCFS1 is a single colony isolate of strain NCIMB8826) induced TNFα production in mouse bone marrow cells in a TLR2-dependent manner [33]. The apparent difference in these observations could be the result of different LTA purification procedures; Grangette *et al*. employed a method that lacks the anion-exchange chromatography and thereby may lead to a higher residual lipoprotein level [33]. Alternatively, the TLR-2 signaling capacity of *L. plantarum* NCIMB8826 LTA was determined using mouse bone marrow cells [33], whereas we employed a TLR-2 reporter cell line derived from human embryonic kidney (HEK) 293cells, which lacks potentially essential co-receptors for the TLR-2 signaling by LTA such as CD14 and CD36 [80]. Notably, the latter explanation appears in agreement with the observation that the LTA we purified from WCFS1 was capable of inducing cytokine production in PBMCs, implying that LTA can be sensed by these cells through other receptors. Furthermore, *dltD* deletion derivatives of *L. plantarum* NCIMB8826 that have lost the D-alanylation capacity, were shown to be more anti-inflammatory as compared to their cognate wild-types [33]. The LTA of this *dltD* strain has also been reported to have increased glucose substitution and an extended Gro-P backbone compared to the LTA of the parental strain [33]. However, in a mutant of *L. plantarum* WCFS1 that lacks the entire *dlt* operon (Δ*dltD-X*), no extended LTA molecules were detected and no enhanced anti-inflammatory properties were observed *in vivo* in healthy mice [81]. Moreover, the *dlt* mutant of *Lactobacillus rhamnosus* GG did not display enhanced anti-inflammatory properties *in vitro* [22], but still suppressed disease symptoms in a mouse colitis model [77]. Based on these results, differences in bacterial species, construction of mutations, and the choice in animal and *in vitro* models could all contribute to differences in the immunological readouts obtained.

Following the same purification procedure, remarkable differences were observed between LTA-Lp and LTA-Bs in TLR2 signaling, suggesting that structural differences between these LTA

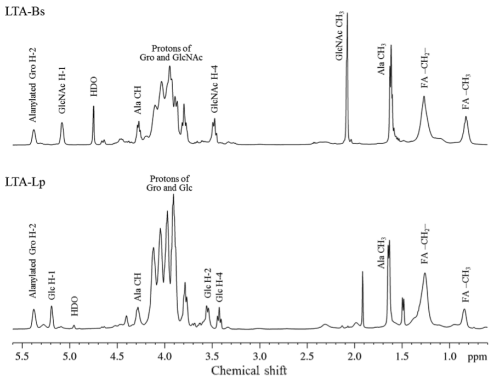

Figure 8. ^{1}H NMR spectrum of purified LTA from *B. subtilis* (LTA-Bs, top) and *L. plantarum* WCFS1 (LTA-Lp, bottom). Intensity of the spectra was approximately adjusted by the signal intensities of fatty acid CH_2 and CH_3. Chemical shift was calibrated to trimethylsilyl propanoic acid (TMSP) signal (0.00 ppm). Abbreviations stand for: Ala, D-alanine; FA, fatty acids; Glc, α-D-glucose; GlcNAc, *N*-acetyl-α-D-glucosamine; Gro, glycerol.

molecules may underlie the differential immunomodulatory properties. Structural analysis of LTA reveals extensive non-uniformity of this molecule with at present 5 reported LTA structure-types [for reviews, see [82, 83]]. Both LTA-Lp and LTA-Bs belong to type-I LTA, the best characterized LTA type with an unbranched 1–3 linked GroP backbone, which can be found in a wide range of Gram-positive bacteria, including *S. aureus* and *Listeria monocytogenes* [82]. We compared the LTA structure of *L. plantarum* WCFS1 and *B. subtilis* 168 using proton-NMR spectroscopy. Using published structures of LTA from *L. plantarum* [13] and *B. subtilis* [84, 85] in a comparative mode, our analyses revealed that both LTAs contain D-alanine substitutions (Figure 8), which is in agreement with previous studies [13, 84, 85]. However, the glycoside-substitution of the LTA backbone appeared to differ substantially, where *L. plantarum* LTA is glucosylated and *B. subtilis* LTA is substitutes with *N*-acetyl-α-D-glucosamine residues (GlcNAc) (Figure 8). In addition, the LTA-Bs has a shorter poly(Gro-P) backbone as compared to LTA-Lp, which is approximately 20-22 Gro-P residues in length [21]. These structural differences are relevant targets for further study, particularly in the context of their role in TLR2 signaling and immunomodulation, which is clearly different between the two LTA molecules. Importantly, since the glycol-substitutions of LTA seem to be an important structure distinction between strong and weak immune-stimulatory LTAs, the identification of the pattern recognition receptor (PRR) involved in glycoside-substitutions recognition is important for a better understanding of the immunomodulatory capacity of LTA. Candidate PRRs for such role may be discovered among the C-type lectin receptors, a PRR family that currently has been proposed to have at least 17 groups, and is proposed to be involved in glycoside recognition through their lectin-like domains [86].

In conclusion, we have shown that LTA plays important roles in bacterial growth, cell division, and surface properties in *L. plantarum* WCFS1, analogous to what has been observed in other Gram-positive bacteria. Interestingly, we found that the deletion mutants exhibit more pro-inflammatory properties relative to their parental strain, which is in apparent contradiction with the suggested pro-inflammatory characteristics of the LTA from other Gram-positive bacteria. Our work pointed out that the pleiotropic impact of LTA deficiency, complicates the interpretation of the immunomodulation capacities of LTA in studies that employ LTA deficient mutants as we have constructed here and as were also reported for *Staphylococcus aureus* [24, 28], *B. subtilis* [25], and

Listeria monocytogenes [26]. For strict immunomodulatory analysis of LTA itself, purified molecules are required. We developed a purification procedure that minimizes lipoprotein contamination while maintaining the structural integrity of LTA, and appears applicable in different Gram-positive bacteria. Notably, purified LTA from *L. plantarum* WCFS1 and from *B. subtilis* 168 are ineffective and effective TLR2 ligands, respectively. The differences in TLR2-signaling capacity are likely explained by structural differences of the LTA molecules, particularly their differential glycoside substitution, and could possibly also involve their acyl chain anchors. Importantly, the differences between purified LTA-Lp and LTA-Bs are much smaller in terms of stimulating cytokine responses of PBMCs as compared to their TLR2 signaling capacities. This observation strongly suggests the participation of other PRRs besides TLR2 in inflammation responses in immune cells and recognition of LTA. Our study strongly underpins that the commonly accepted perspective that Gram-positive LTA molecules act as pro-inflammatory compounds through TLR-mediated signaling is invalid and quite naive. This perspective ignores the substantial diversity that exists in the chemical structures that are present in LTA from different bacteria, which is a subject that deserves much more research in order to explain the role of different bacterial LTA molecules in host-microbe communication. Remarkably, a similar conclusion is reached when the canonical TLR4 signaling capacity of Gram-negative lipo-polysaccharides (LPS) is evaluated, revealing that LPS structural diversity (e.g., penta- or hexa-acylation forms) critically affects the LPS-TLR-4 interaction [87]. Taken together, these findings illustrate that generalization of PRR-ligand interactions is unreliable and requires much more insight in ligand structure-function requirements in the context of PRR signaling. Overall, this study supports the critical role of bacterial cell envelope components in the species- and strain-specific interactions with the extensive PRR repertoire of the host cells.

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Supplemental Material

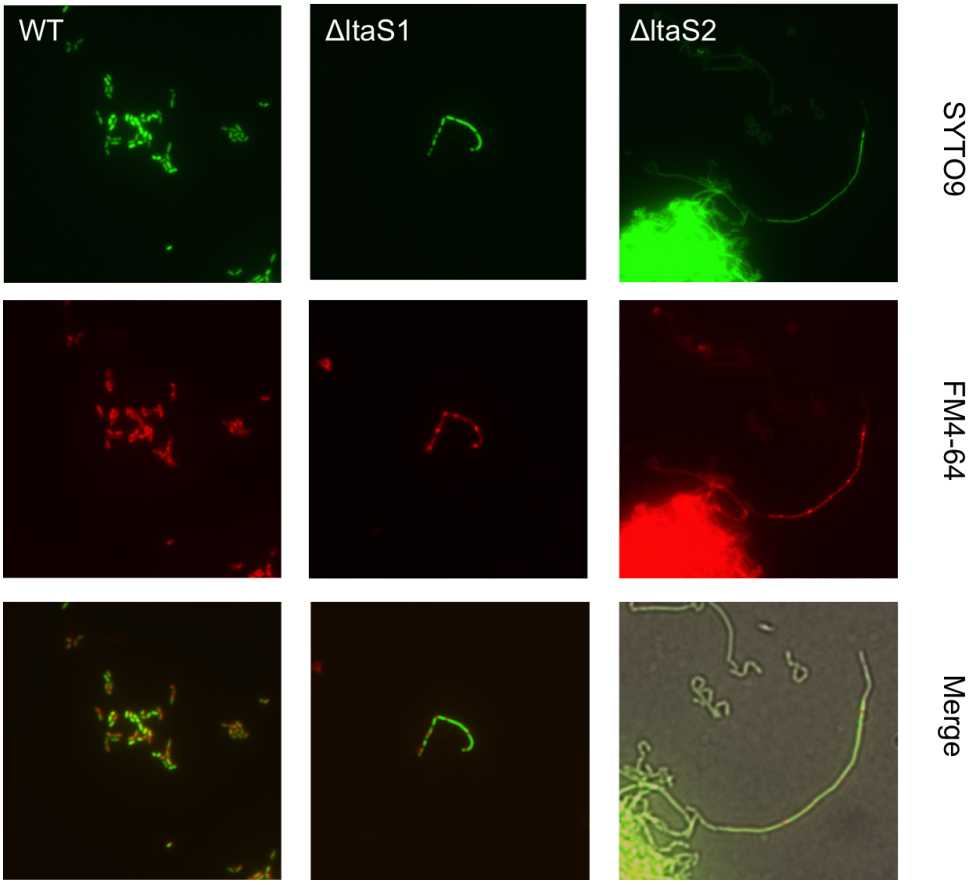

Figure S1. Images of *L. plantarum* WCFS1 and *ltaS* deletion derivatives under fluorescence microscope at a 500x magnification. Visualization of DNA by Syto9 and membrane by FM4-64 are shown in upper and middle panels, respectively. The lower panel shows the merge image of the Syto9 and FM4-64 images.

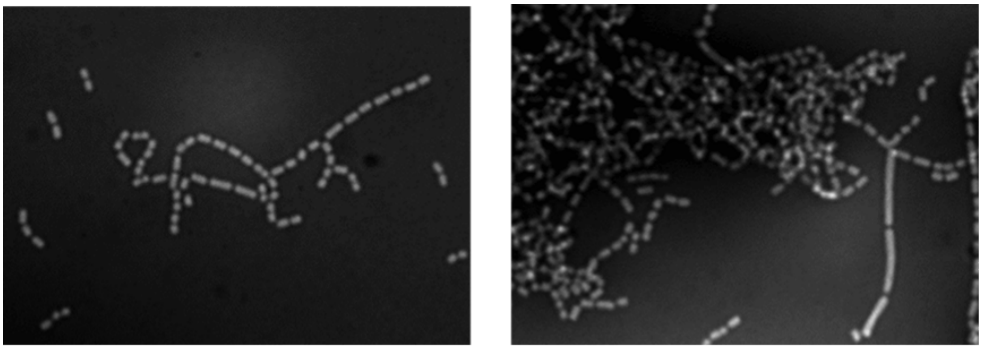

Figure S2. Images of *L. plantarum ltaS2* complementation strain under phase-contrast microscopy. The phase-contrast microscopy shows 1250x magnified views of nigrosin-stained cells.

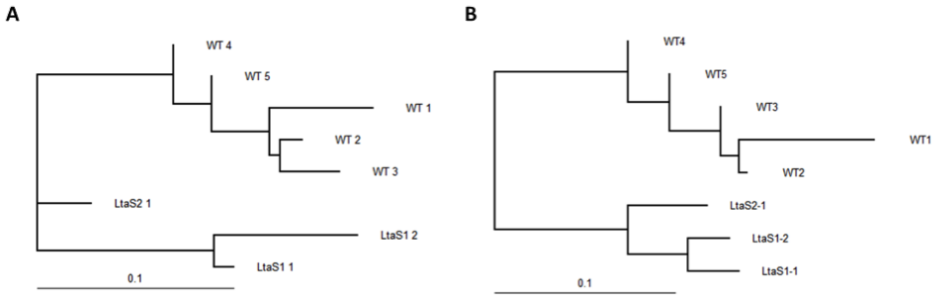

Figure S3. Dendrograms visualizing Pearson correlation analyses of the transcriptomes of *L. plantarum* WCFS1 (WT), *ltaS1* and *ltaS2* mutants based on the transcript levels of all genes (A) or of cell envelope-associated genes (B).

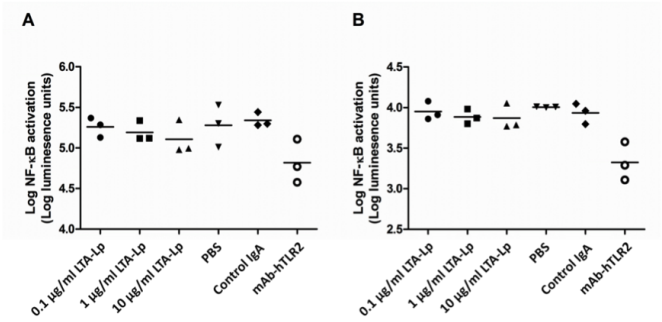

Figure S4. Attenuation assay of purified LTA from *L. plantarum* WCFS1 (LTA-Lp) in TLR1/2 (A) and TLR2/6 (B) signaling. The tested attenuating substances, LTA-Lp (0.1, 1, or 10 µg/ml), were added to TLR1/2 and TLR2/6 cell lines prior to the stimulant, purified LTA from *B. subtilis* (1 µg/ml). Measurements were performed in triplicate and are presented as Log values, and individually displayed (n = 3) and the bar indicates the median. The PBS and control IgA (InvivoGen) serve as negative controls while a monoclonal antibody blocking human TLR2 (mAb-hTLR2, InvivoGen) serves a positive control.

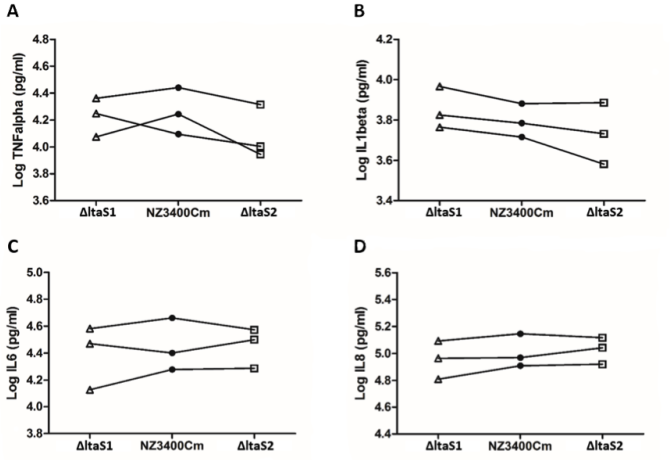

Figure S5. Immunomodulatory effect of *L. plantarum* WCFS1 and strains and *ltaS* deletion derivatives in PBMC stimulation assays (n=3 donors), measuring inflammation-related cytokine secretions. The productions of pro-inflammatory cytokine TNFα (A), IL1β (B), IL6 (C) and IL8 (D) are presented in Log values. The cytokine levels for individual donors stimulated with wild-type and deletion mutants bacteria were connected by a line to focus the read-outs on changes elicited by the *ltaS* deletion. No significant differences between cytokine levels induced by wild-type strains and mutants (paired t-test) are found.

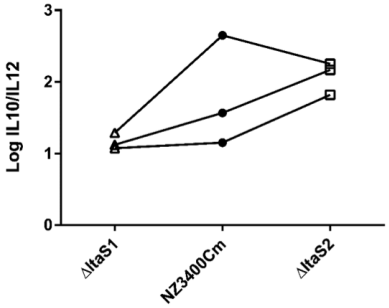

Figure S6. IL10/IL12 was also compared of between *L. plantarum* wild type (NZ3400Cm) and the deletion mutants (ΔltaS1 and ΔltaS2). The cytokine levels for individual donors stimulated with *L. plantarum* strains were connected by a line to focus the read-outs on changes elicited by the mutations. The differences between wild-type and corresponding mutant were statistically analyzed by using paired *t* test. No significant difference was found between the IL10/IL12 ratios stimulated by the strains.

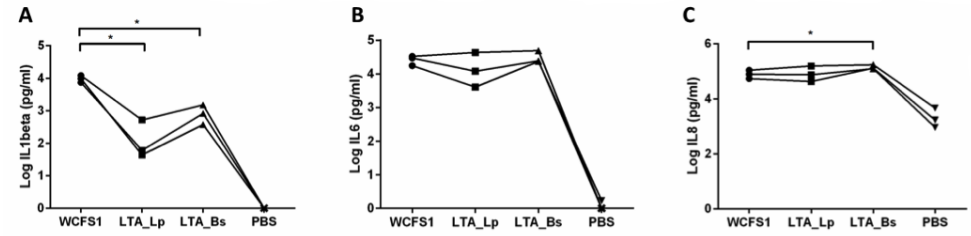

Figure S7. Immunomodulatory effect of cells and purified LTA from *L. plantarum* WCFS1 (LTA-Lp), and purified LTA from *B. subtilis* (LTA-Bs) in PBMC stimulation assays (n=3 donors), measuring inflammation-related cytokine secretions. The productions of pro-inflammatory cytokine IL1β (A), IL6 (B) and IL8 (C) are presented as Log values. The cytokine levels for individual donors stimulated with purified LTA were connected by a line to focus the read-outs on changes elicited by the origins of LTA. Significant differences between cytokine levels induced by the cells and purified LTAs (paired *t*-test) are indicated; *, $P \leq 0.05$.

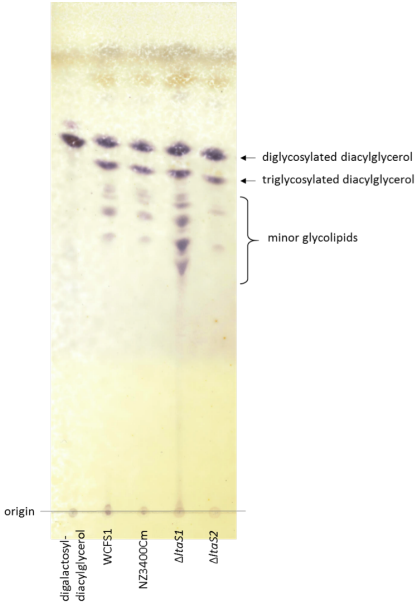

Figure S8. Total membrane lipids (1 mg each) isolated from *L. plantarum* WCFS1, NZ3400Cm, and *ltaS* mutants (Δ*ltaS1* and Δ*ltaS2*) were separated by thin layer chromatography (TCL) according to the method described previously [1]. Digalactosyl-diacylglycerol (30 µg) was used as a reference. Possible identity of the bands were indicated by the arrows on the right based on published LTA structure of *L. plantarum* [2].

Table S1. *ltaS* homologues found in lactobacilli strains and their similarity to LtaS of *Staphylococcus aureus* and *L. plantarum* WCFS1.

Bacterial strains	*ltaS* homologues	Identity to *S. aureus* LtaS (%)	Identity to *ltaS1* (%)	Identity to *ltaS2* (%)
L. plantarum WCFS1	*ltaS1 (lp_1283)*	46	100	54
	ltaS2 (lp_2580)	45	53	100
L. acidophilus NCFM	*LBA0447*	44	60	47
	LBA0750	40	45	52
L. amylovorus GRL 1112	*LA2_02370*	45	60	48
	LA2_03975	40	46	53
L. brevis ATCC 367	*LVIS_1546*	44	72	50
	LVIS_1812	43	50	65
L. casei ATCC 334	*LSEI_0868*	46	64	50
	LSEI_1123	42	47	55
L. casei BL23	*YfnI (LCABL_09330)*	46	64	50
	LCABL_12830	42	47	55
L. crispatus ST1	*LCRIS_00447*	45	60	48
	LCRIS_00753	41	46	54
L. delbrueckii subsp. *bulgaricus* ATCC 11842	*Ldb0690*	40	47	54
	Ldb1835	45	57	47
L. delbrueckii subsp. *bulgaricus* ATCC BAA-365	*LBUL_0623*	40	47	53
	LBUL_1707	45	57	50
L. delbrueckii subsp. *bulgaricus* ND02	*LDBND_0620*	40	47	53
	LDBND_1710	45	57	47
L. fermentum CECT 5716	*LC40_1044*	44	63	48
	LC40_1079	49	55	71
L. fermentum IFO 3956	*LAF_1649*	44	63	48
	LAF_1697	45	51	67
L. gasseri ATCC 33323	*LGAS_1259*	40	47	52
	LGAS_1586	46	61	48
L. helveticus DPC 4571	*lhv_0470*	44	60	48
L. helveticus CNRZ32	*lhe_0753*	40	44	53
	lhe_1606	44	60	48
L. jensenii 1153	*LBJG_00011*	44	60	48
	LBJG_00646	41	48	53
L. johnsonii ATCC 33200	*FC22_GL000409*	45	61	48
	FC22_GL001103	40	47	52
L. johnsonii FI9785	*FI9785_1288*	40	47	52
	FI9785_1550	46	61	48
L. johnsonii NCC 533	*LJ_1768*	46	61	48
	LJ_0920	40	47	49
	LJ_0921	42	53	65
	LJ_0922	39	47	46
L. paracasei subsp. *paracasei* 8700:2	*LBPG_02966*	46	64	50
	LBPG_03057	42	47	55
L. paracasei subsp. *paracasei* ATCC 25302	*HMPREF0530_2689*	43	47	55
	HMPREF0530_3015	46	64	50
L. reuteri 100-23	*Lreu23DRAFT_4562*	46	64	49
	Lreu23DRAFT_4733	45	50	65
L. reuteri JCM 1112	*LAR_1513*	46	64	49

	LAR_1723	45	50	65
L. rhamnosus GG	*LGG_00830*	46	64	50
	LGG_01082	42	47	56
L. rhamnosus HN001	*LRH_03392*	46	64	50
	LRH_04568	42	46	56
L. rhamnosus Lc 705	*LC705_00824*	46	64	50
	LC705_01159	42	47	56
L. rhamnosus LMS2-1	*HMPREF0539_0057*	46	64	50
	HMPREF0539_1187	42	47	56
L. sakei subsp. *sakei* 23K	*LCA_0465*	44	65	50
	LCA_1207	43	52	59
L. salivarius CECT 5713	*HN6_00326*	45	68	49
	HN6_00432	44	49	59
L. salivarius UCC118	*LSL_0394*	45	68	49
	LSL_0465	44	49	60

3

References

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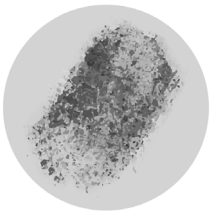
Chapter 4

Lipoproteins contribute to the anti-inflammatory capacity of *Lactobacillus plantarum* WCFS1

I-Chiao Lee, Iris I. van Swam, Sjef Boeren, Jacques Vervoort, Marjolein Meijerink, Nico Taverne, Marjo Starrenburg, Peter A. Bron, and Michiel Kleerebezem



Abstract

Bacterial lipoproteins are well-recognized microorganism-associated molecular patterns, which interact with Toll-like receptor (TLR) 2, an important pattern recognition receptor of the host innate immune system. Lipoproteins are conjugated with two- and/or three-acyl chains (di- or tri-acyl), which is essential for appropriate anchoring in the cell membrane as well as for the interaction with TLR2. Lipoproteins have mostly been studied in pathogens and have established roles in various biological processes, such as nutrient import, cell wall cross-linking and remodeling, and host-cell interaction. By contrast, information on the role of lipoproteins in the physiology and host interaction of probiotic bacteria is scarce. By deletion of *lgt*, encoding prolipoprotein diacylglyceryltransferase, responsible for lipidation of lipoprotein precursors, we studied the roles of the collective group of lipoproteins in the physiology of the probiotic model strain *Lactobacillus plantarum* WCFS1 by transcriptome and proteome analysis. To investigate the consequences of the *lgt* mutation in host-cell interaction, the capacity of mutant and wild-type bacteria to stimulate TLR2 signaling and inflammatory responses was compared using (reporter-) cell based models. These experiments exemplified the critical contribution of the acyl chains of lipoproteins in immunomodulation. To the best of our knowledge, this is the first study that investigated collective lipoprotein functions in a model strain for probiotic lactobacilli, and we show that the presence of lipoproteins in *L. plantarum* WCFS1 are critical drivers of anti-inflammatory host responses towards this strain.

Introduction

Bacterial lipoproteins are proteins that are post-translationally modified by acyl-conjugation, which anchors the protein on the extracellular face of the cytoplasmic membrane. These lipoproteins contain a typical N-terminal signal sequence that ends with the conserved [L/V/I]-[A/S/T]-[G/A]-C motif that is designated "lipobox" [1]. After export across the cell membrane, these lipoprotein precursors undergo their lipid modification, which is catalyzed by three conserved enzymes, following a mechanism that was first established in *Escherichia coli* [2]. Initially, the prolipoprotein diacylglyceryltransferase (Lgt) transfers a di-acylglyceryl moiety onto the indispensable cysteine residue in the lipobox [2], which is targeted by the lipoprotein signal peptidase (Lsp) that cleaves of the signal sequence directly N-terminally of the lipid-modified cysteine residue. In the third step, lipoprotein *N*-acyl transferase (Lnt) adds a third acyl chain to the free amino group of the lipidated cysteine (Figure 1). In Gram-negative bacteria, the third step is essential for the release and transport of lipoproteins from the cytoplasmic membrane to the outer membrane, but the *E. coli*-type Lnt enzyme appears to be absent in low-GC-content Gram-positive bacteria of the Firmicutes phylum [3, 4]. However, tri-acylated lipoproteins have recently reported in *Staphylococcus aureus*, suggesting the presence of an unidentified *N*-acyltransferase in some Firmicutes, and which has low sequence similarity with Lnt of Gram-negative bacteria, prohibiting its recognition as its functional equivalent [5, 6].

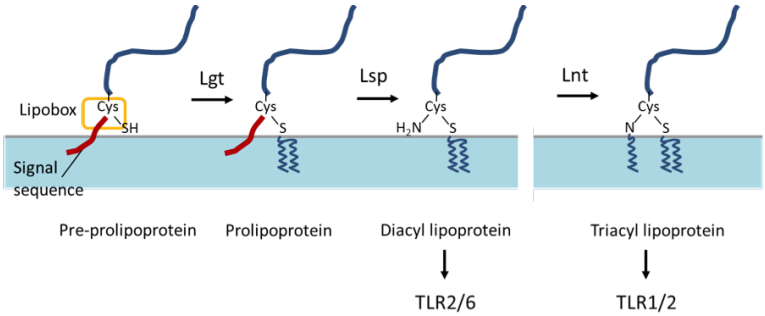


Figure 1. Schematic representation of bacterial lipoprotein biogenesis. After export across the cell membrane, pre-prolipoproteins undergo lipid modification by prolipoprotein diacylglyceryl-transferase (Lgt) which transfers a di-acylglyceryl moiety onto the cysteine residue in the lipobox, and results in prolipoproteins. Subsequently, lipoprotein signal peptidase (Lsp) cleaves of signal peptide at the direct N-terminal of the lipid-modified cysteine residue and results in diacyl lipoproteins. In some bacteria, lipoprotein N-acyl transferase (Lnt) adds a third acyl chain to the free amino group of the lipidated cysteine. The di- and tri-acyl lipoproteins produced by bacteria are differentially recognized by distinct TLR-2 heterodimers TLR2/6 and TLR1/2, respectively.

Lipoproteins are involved in various biological functions. Many lipoproteins function as substrate binding proteins (SBPs) of ATP-binding cassette (ABC) transporters involved in import of a variety of substrates, such as sugars, metal ions, amino acids, oligopeptides, and nucleotides [4]. SBPs provide high affinity substrate binding and delivery to the membrane permease components [7], which is important for nutrient uptake and may also play a role in environmental sensing [8-10]. Besides SBPs, lipoproteins in various Gram-positive bacteria also are predicted to function as enzymes, which are involved in modulation of two-component signal transduction systems, cell envelope stability, adhesion, protein secretion and folding, and electron transfer processes at the cell membrane [4, 11]. Several studies also evaluated the relation between lipoproteins and virulence, mainly by deleting essential genes in lipoprotein biogenesis like *lgt* or *lsp*, which

typically led to reduced adhesion and internalization and/or to attenuation of virulence in animal infection models [12].

By contrast, the role that lipoproteins play in the physiology and host interaction of probiotic bacteria has not been reported in much detail. Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host [13]. Interaction between probiotics and the host is proposed to play a key role in this beneficial relationship [14-16]. Bacterial lipoproteins are well-recognized microorganism-associated molecular patterns (MAMPs), which interact with Toll-like receptor (TLR) 2, an important pattern recognition receptor (PRR) of the host innate immune system [17, 18]. Moreover, the di- and tri-acyl lipoproteins produced by bacteria are differentially recognized by distinct TLR2 heterodimers TLR2/6 and TLR1/2, respectively [19]. Initially, it was thought that TLR2 heterodimers with TLR1 or TLR6 do not contribute to divergent immune responses but merely expand the repertoire of bacterial ligand recognition [20]. However, recent evidence illustrated that both the strength of signaling activation [21], and the downstream interaction with intracellular adaptors [22] are different for TLR1/2 and TLR2/6, suggesting their distinct role in innate signaling.

Deletion of *lgt* provides a means to study the general role of all lipoproteins in bacterial physiology and immunomodulation. Here we describe the impact of *lgt* mutation in the probiotic model strain *Lactobacillus plantarum* WCFS1 [23]. We explored the impacts of Lgt deficiency on the genome-wide gene expression pattern and on the secreted proteome. Furthermore, the capacity of the *L. plantarum lgt* mutant to stimulate TLR2 signaling and inflammatory responses was compared to those of the wild-type, illustrating the contribution of the lipoprotein acyl chains to immunomodulation. Although it has previously been established that lipoproteins are among the main TLR2 signaling ligands, to the best of our knowledge, this is the first study that explored the role of lipoproteins in immunomodulation by a model species of the probiotic lactobacilli.

Materials and methods

Bacterial strains and culture conditions. Bacterial strains used in this work are listed in Table 1. *Lactobacillus plantarum* WCFS1 and its derivatives were grown at 37°C in MRS broth (Difco, West Molesey, United Kingdom) without aeration. *Escherichia coli* strain TOP10 (Invitrogen, Bleiswijk, The Netherlands) was used as an intermediate cloning host, and was grown at 37°C in TY broth [24] with aeration [25]. Solid media were prepared by addition of 1.5% (w/v) agar to the broths. Antibiotics were added where appropriate and concentrations used for *L. plantarum* and *E. coli* strains were 10 μg/ml chloramphenicol (Cm), and 30 and 200 μg/ml erythromycin (Ery), respectively.

DNA manipulations. Plasmids and primers used are listed in Table 1. Standard procedures were used for DNA manipulations in *E. coli* [25]. Plasmid DNA was isolated from *E. coli* using a JETSTAR kit (Genomed GmbH, Bad Oberhausen, Germany). *L. plantarum* DNA was isolated as described previously [26]. PCR amplifications were performed using hot-start KOD polymerase (Novagen, Madison, USA). Amplicons were purified using Wizard®SV Gel and PCR Clean-Up System (Promega, Leiden, The Netherlands). Restriction endonucleases (Fermentas GmbH, St. Leon-Rot, Germany), MSB® Spin PCRapace (Invitek GmbH, Berlin, Germany), PCR Master

Mix (Promega) and T4 DNA ligase (Invitrogen) were used as specified by the manufacturers.

Construction of *lgt* deletion strain. The *lgt* deletion mutant was constructed as described previously [27], using a double crossing-over strategy to replace the *lgt* gene by a chloramphenicol resistance cassette (*lox66*-P_{32}*cat*-*lox71*)[27]. In this study, a derivative of the mutagenesis vector pNZ5319 [27], designated pNZ5319TAG was used to introduce a unique 42-nucleotide tag into chromosome during gene deletion, which can be used for mutant tracking purposes in mixed populations (not relevant for the study presented here). The upstream and downstream flanking regions of *lgt* (*lp_0755*) gene were amplified by PCR using the primer pairs lgt-up-F/R and lgt-down-F/R primers, respectively (Table 1). The amplicons generated were joined by a second PCR to *lox*66-P_{32}*cat*-*lox*71-tag by a splicing by overlap extension strategy [28], using lgt-up-F/lgt-down-R primers. The resulting PCR products were digested with SwaI and Ecl136II, and cloned into similarly digested pNZ5319TAG. The obtained mutagenesis plasmids were transformed into *L. plantarum* WCFS1 as described previously [26]. The resulting transformants were assessed for a double cross over integration event by selecting for Cm resistance and Ery sensitivity. The selected colonies were further confirmed by PCR using targets-out-F/R primers (Table 1). A single colony

Table 1. Bacterial strains, plasmids, and primers used in this study

Strains	**Characteristics**[a]	**Reference**
L. plantarum		
WCFS1	Single-colony isolate of *L. plantarum* NCIMB8826. Isolate from human saliva, UK.	(23)
NZ3400Cm	Cm^r; WCFS1 derivative; chromosomal integration of *cat* cassette into H-locus	(37)
NZ3565Cm (Δ*lgt*)	Cm^r; derivative of WCFS1 containing a *lox66*-P_{32}-*cat*-*lox71*-tag6.6 replacement of *lgt (lp_0755)* (*lgt*::*lox66*-P_{32}-*cat*-*lox71*-tag6.6)	This work
E. coli		
TOP 10	Cloning host; F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
Plasmids	**Descriptions**[a]	**Reference**
pNZ5319	Cm^r Ery^r; Mutagenesis vector for gene replacements in *L. plantarum*	(27)
pNZ3565	Cm^r Ery^r; pNZ5319 derivative containing homologous regions up- and downstream of *lgt* (*lp_0755*)	This work
Primers	**Sequence**[b]	**Reference**
is128 tag-lox66-F3	5'-AAATCTACCGTTCGTATAATGTATG-3'	(51)
is129 tag-lox71-R3	5'-CTCATGCCCGGGCTGTAACCG-3'	(51)
IS169	5'-TTATCATATCCCGAGGACCG-3'	(52)
87	5'-GCCGACTGTACTTTCGGATCC-3'	(52)
CreF	5'-CGATACCGTTTACGAAATTGG-3	(52)
CreR	5'-CTTGCTCATAAGTAACGGTAC-3'	(52)
EryintF	5'-TCAAATACAGCTTTTAGAACTGG-3	(52)
EryintR	5'-ATCACAAACAGAATGATGTACC-3'	(52)
lgt-up-F	5'-TTTGGCAGGAAGTGTAACCG-3'	This work
lgt-up-R	5'-GCATACATTATACGAACGGTAGATTTATTCACGCTACTGC-CATCTCC-3'	This work
lgt-down-F	5'-CGGTTACAGCCCGGGCATGAGGCAGAAAATAAGTAGATTA-GAGG-3'	This work
lgt-down-R	5'-AATCTCAGGTTTCCCCTCGC-3'	This work
lgt-out-F	5'-AAGTGTGGCCGCTTGAAAGGG-3'	This work
lgt-out-R	5'-AACATTTCTTTAGGCATCGCC-3'	This work

[a] Cm^r, chloramphenicol resistant; Ery^r, erythromycin resistant

[b] Underlined nucleotides indicate parts of the primers that are complementary to the is128-lox66-F3 and is129-lox71-R3 primers

displaying the anticipated antibiotic resistance phenotype and genotype was selected, yielding NZ3565Cm (*L. plantarum* WCFS1 Δ*lgt*).

Isolation of released proteins and SDS-PAGE. For the isolation of proteins released into the culture supernatants, *L. plantarum* WCFS1 and its Δ*lgt* derivative were grown overnight to an OD_{600} of approximately 5 in 100 mL of 2 x CDM. The culture supernatants were filtered through a hydrophilic polyvinylidene fluoride (PVDF) filter (0.22 µm pore size, 25 diameter; Millex Millipore, USA) to remove any remaining bacterial cells, and proteins were precipitated by adding trichloroacetic acid (TCA) to a final concentration of 16%, followed by an overnight incubation at 4°C. The precipitated proteins were pelleted by centrifugation at 16000×g for 15 min. The protein pellets were washed with 200 µl acetone and then air-dried at 50°C. Dried protein pellets were solubilized in NuPAGE loading buffer and dithiothreitol (DTT) reducing agent (both from Invitrogen). The samples of released proteins were visualized by SDS-PAGE using the NuPAGE® electrophoresis system with NuPAGE®Novex® 4-12% Bis-Tris gels with MOPS SDS running buffer (Invitrogen), followed by Coomassie brilliant blue staining using standard procedures [25] and overnight destaining in Milli-Q water.

Sample preparation for mass spectrometry. For in-gel trypsin digestion, the protein-containing SDS-PAGE gel was reduced with 10 mM dithiotreitol (DTT) in 50 mM ammonium bicarbonate (ABC) for 1 h at 60°C, followed by alkylation with 20 mM iodoacetamide in 100 mM Tris buffer (pH 8.0) in the dark for 1 h at room temperature. After thorough washing in Milli-Q water, the gel lane of each sample was divided into 5 slices that were individually cut into small pieces (ca. 1 mm^3). The gel pieces were transferred to protein LoBind tubes (Eppendorf, Hamburg, Germany) for all following procedures to minimize protein loss. Sample were freeze-thawed to increase enzyme accessibility of the gel pieces, and incubated in ABC buffer containing 5 ng/µL Bovine Sequencing Grade Trypsin (Roche) for 2 h at 45°C. The solution was sonicated briefly (1 sec) and was adjusted to an approximate pH of 2 with 10% trifluoroacetic acid (TFA).

The trypsin-digested samples were further cleaned up to remove any gel residues using C18 microcolumns as described previously [29]. In short, C18 microcolumns were prepared in 200-µL Eppendorf tips by placing a small piece (ca. 1 mm in diameter) of a C18 Empore disk and then applying 4 µL of 50% slurry of Lichroprep C18 column material in methanol. The microcolumns were washed twice with 200 µl methanol and subsequently equilibrated with 100 µl of 1ml/l formic acid (HCOOH). The samples were applied to the microcolumns and washed with 1ml/l HCOOH. Samples were eluted using 50 µl of 50% acetonitrile/30% 1 ml/l HCOOH into clean LoBind tubes. The sample volume was then reduced in a vacuum concentrator (Eppendorf Vacufuge) at 45°C for 20 to 30 minutes until a volume below 20 µl was reached.

The liquid-chromatography tandem mass spectrometry (LC-MS/MS) analysis was performed on a Proxeon EASY-nLC system (Thermo Scientific) coupled with a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific). The chromatographic separation was performed on a combination of a Prontosil 300-5-C18H pre-concentration column with a Prontosil 300-3-C18H analytical column (Bischoff Chromatography, Leonberg, Germany)[30]. For peptide identification, the protein reference database of *Lactobacillus plantarum* (strain ATCC BAA-793 / NCIMB 8826 / WCFS1) for peptides and proteins identification downloaded from Uniprot (http://www.uniprot.org/ accessed July 2014) was used. A set of 31 protein sequences of common contaminants was added including Trypsin (P00760, bovine), Trypsin (P00761, porcine), Keratin K22E (P35908,

human), Keratin K1C9 (P35527, human), Keratin K2C1 (P04264, human), and Keratin K1C1 (P35527, human). Label-free quantitation (LFQ) of detected proteins was calculated by MaxQuant algorithm to compare quantity cross samples. Relative abundances were calculated by the ratio of LFQ of detected peptides in wild type and *lgt* mutant and presented in Log_{10} value.

RNA isolation and microarray analysis. RNA isolation, labeling and hybridization were performed according to previously described methods [31, 32]. Briefly, *L. plantarum* NZ3400Cm and its *lgt* deletion derivative, NZ3565Cm, were grown in MRS with chloramphenicol and cells were harvested by centrifugation, following quenching and cell disruption by bead beating. RNA was purified using the High Pure Isolation Kit (Roche Diagnostics, Mannheim, Germany) including 1 h treatment with DNaseI (Roche Diagnostics). Five microgram of total RNA was used for cDNA synthesis. Cyanine-3 (Cy3) and cyanine-5 (Cy5) cDNA labeling was performed as described previously [31], using the CyScribe Post-Labeling and Purification kits according to the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, UK). Cy-dye-labeled cDNAs (0.5 μg each) were hybridized to *L. plantarum* WCFS1 printed-oligonucleotide DNA microarrays (Agilent Technologies, Amstelveen, the Netherlands). Hybridization and scanning procedures were performed as previously described [31]. The data were normalized using the Lowess normalization as available in MicroPrep [33]. CyberT [34] was used to compare the transcriptomes of NZ3400Cm and NZ3565Cm, resulting in a gene expression ratio and Benjamini and Hochberg's False Discovery Rate (FDR) [35] for each gene. The expression of a gene was considered significantly altered when the FDR-adjusted p value was < 0.05. The level of gene expression was also estimated on the basis of probe signals for a correlation analysis of transcriptional results and proteome detection.

Toll-like receptor (TLR) assay. Human embryonic kidney (HEK)-293 TLR reporter cell lines expressing human TLR1/2, TLR2/6, or TLR4, harboring pNIFTY, a NF-κB luciferase reporter construct (Invivogen, Toulouse, France) [36], were used. The HEK-293 reporter cell lines were seeded at 6×10^4 cells/well in 96-well plates and incubated overnight under standard culture conditions. Cells were then stimulated with late-stationary bacterial cultures of the *L. plantarum* NZ3400Cm, a *L. plantarum* WCFS1 derivative with a chromosomal integration of the *cat* cassette in a neutral chromosomal locus [37], and *lgt* deletion strain (NZ3565Cm) at a multiplicity of infection (MOI) of 1:10, HEK cell to bacteria. The TLR1/2 agonist Pam3CSK4 (5 μg/mL, Invivogen) and TLR2/6 agonist Pam2CSK4 (5 μg/mL, Invivogen) were used as positive controls and PBS served as the negative control.

Peripheral blood mononuclear cells (PBMC) assay. The assay was performed as described previously [38] and was approved by Wageningen University Ethical Committee and was performed according to the principles of the Declaration of Helsinki. Peripheral blood of healthy donors was from the Sanquin Blood Bank, Nijmegen, The Netherlands. PBMCs were separated from the blood using Ficoll-Paque Plus gradient centrifugation according to the manufacturer's description (Amersham biosciences, Uppsala, Sweden). The mononuclear cells were collected, washed in Iscove's Modified Dulbecco's Medium (IMDM) + glutamax (Invitrogen, Breda, The Netherlands) and adjusted to 1×10^6 cells/ml in IMDM + glutamax supplemented with penicillin (100 U/ml) (Invitrogen), streptomycin (100 μg/ml) (Invitrogen), and 1% human AB serum (Lonza, Basel, Switzerland). PBMCs (1×10^6 cells/well) were seeded a night prior to the experiment in 48-well tissue culture plates and incubate at 37°C in 5% CO_2. Bacteria from late-stationary phase were added to PBMCs at a MOI of 1:10 (PBMC to bacteria) PBMCs from 3 different donors were used

in the assay. Following 24 hr incubation at 37°C in 5% CO2, culture supernatants were collected and stored at -20°C prior to cytokine analysis. Cytokines were measured using a FACS CantoII flow cytometer (BD Biosciences, New Jersey, USA) and BD Cytometric Bead Array Flexsets (BD Biosciences) for interleukin (IL)10 and IL12p70, TNFα, IL6, IL1β, and IL8 according to the manufacturer's procedures. Concentrations of cytokines were calculated based on the standard curves in the BD Biosciences FCAP software.

Statistical analysis. The TLR and PBMC assays were performed in triplicate. One-way ANOVA followed by Tukey's multiple comparison correction was used to compare TLR2 activations between strains. The paired *t*-test was used to determine the Log values of PBMCs cytokine production after stimulated with wild-type verse mutant strains for individual donors. GraphPad Prism 5 software (GraphPad Software, San Diego, CA) was used for all determinations, and a *P* value <0.05 was considered significant.

Results

Impacts of lgt *deletion on* L. plantarum *transcriptome*

Prolipoprotein diacylglyceryl transferase (Lgt) is the key enzyme for lipidation in lipoprotein biosynthesis, where it catalyzes the transfer of a diacylglyceryl moiety onto a conserved cysteine in the lipobox of prolipoproteins [4]. The gene annotated to encode this function (*lgt*; *lp_0755*) in the *L. plantarum* WCFS1 genome [23] was mutated by double cross-over gene replacement of the *lgt* coding region by a chloramphenicol acetyltransferase (*cat*) cassette [27], resulting in an *lgt* deficient derivative of strain WCFS1, designated NZ3565Cm (Δ*lgt*). This *lgt*-mutant strain enables the study of the generic impact of lipoproteins on physiological and immunomodulatory properties of this model probiotic-bacterium.

Under laboratory conditions, the growth and cell-morphology of the *lgt* deletion mutant were undistinguishable from those of the wild-type strain (data not shown). This is in agreement with earlier observations that suggested that although Lgt is essential in Gram-negative bacteria, it appears to be dispensable in Gram-positive bacteria grown under laboratory conditions [4]. To further evaluate the impact on *L. plantarum* physiology, global transcriptional profiles of the *lgt* deletion strain were generated and compared to those of the wild-type strain. The transcriptome analysis confirmed the abolishment of *lgt* expression in the mutant strain (Table S1). Besides this clear effect, the *lgt* deletion only affected the expression of a small group of genes; 6 genes were down-regulated and 43 genes were up-regulated compared to the wild-type strain. These genes included a variety of functional categories, which were dominated by diverse metabolic pathways, but also included genes involved in cell envelope architecture, some ribosomal proteins, and several membrane-transport functions (Table S1). Furthermore, the gene encoding sucrose-6-phosphate hydrolase (lp_0187, *scrB*) was up-regulated almost 40-fold in *lgt* mutant (Table S1). This gene is involved in galactose metabolism, suggestive of possible impacts of *lgt* deletion in sugar metabolism. However, other genes in the same pathway do not appear to be concurrently up-regulated. Another gene, lp_2001, encoding a small membrane protein (70 a.a.) with unknown function, is down-regulated about 1000-fold. Notably, the expression of all lipoprotein encoding genes appeared to be unaltered in the *lgt* deletion strain relative to the wild-type.

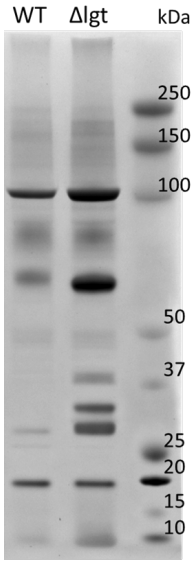


Figure 2. Secreted proteins extracted from *L. plantarum* WCFS1 (WT) and its *lgt* deletion derivative (Δlgt). Proteins were separated by SDS-PAGE and visualized by Coomassie blue staining. On the right side, the protein size marker (kDa), Precision Plus Protein™ Dual Color Standards (Bio-Rad).

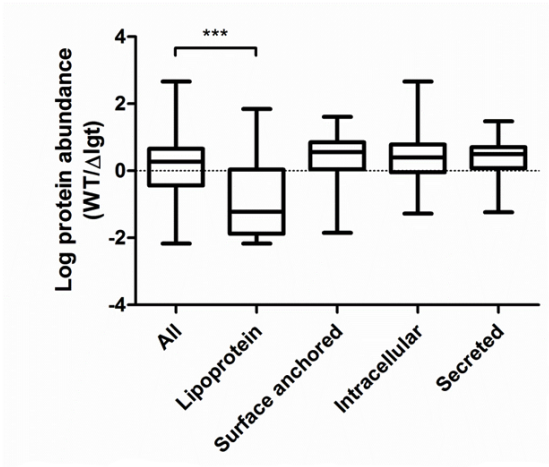


Figure 3. Relative abundance of secreted proteins in *L. plantarum* WCFS1 wild type (WT) and the *lgt* deletion strain (Δ*lgt*). The relative abundance is defined as the ratios in the label free quantitation (LFQ, Log10 value) of detected proteins in wild type and the deletion strain in tandem mass spectrometry (MS/MS) analysis. The abundance was compared with all proteins detected in both samples, or within specific protein groups. The ratios from specific proteins groups were compared against all proteins to test for significant differences using one-way ANOVA followed by Tukey's multiple comparison correction and significant differences are indicated; ***, $P \leq 0.001$.

Lgt is important for membrane anchoring of lipoproteins

We investigated the impact of *lgt* deletion on the membrane-anchoring of lipoproteins, by comparing the supernatants of wild type and the mutant using SDS-PAGE. The protein-pattern observed revealed a clear difference between the supernatant of the wild type strain and *lgt* mutant, which released much more proteins into its culture medium as compared to its parental strain (Figure 2). The SDS-PAGE gel containing supernatant proteins were subjected to in-gel trypsin digestion and were subsequently analyzed by tandem mass spectrometry (MS/MS) to identify the released proteins. There are 7 and 9 proteins that are exclusively detected in the supernatant of wild type and *lgt* mutant cultures, respectively (Table S2). The protein abundances of these proteins were set to detection limit to enable the calculation of the relative protein abundance of the proteins detected in both the wild type and *lgt* mutant culture supernatants. The overall protein-abundance in the culture supernatant of the wild type and its *lgt* derivative appeared to be similar, but the relative abundance of predicted lipoproteins was significantly more abundant in the supernatant of the *lgt* deficient strain compared to other classes of secretome proteins (Figure 3 and S1). Many of the lipoproteins found in higher abundance in the supernatant of *lgt* mutant belong to the predicted substrate binding proteins of ATP-binding cassette (ABC) transporters associated with various substrates, including iron, phosphate, amino acids, maltose and maltodextrin (Table S2). The proteome analysis detected 38 out of the 47 predicted lipoproteins encoded by the WCFS1 genome [39], thereby broadly representing this group of proteins. We compared the gene expression level of the lipoprotein encoding genes with the lipoproteins that were detected in

the culture supernatant of *lgt* strain. Most of the higher expressed lipoprotein encoding genes were detected in the proteomic analysis. However, the highest expressed lipoprotein encoding gene (*lp_1452*) was not detected in the proteome analysis, and also two lipoprotein encoding genes that were among the lowest in terms of transcript detection (*lp_0200* and *lp_0201*) were among the detected lipoproteins in the proteome analysis (Table S3). These findings illustrate that there is a relatively poor relationship between the transcript level of a specific gene and the detection of the protein it encodes in the supernatant fraction of these cells. The overall result of these analyses confirms the importance of Lgt in appropriate anchoring of lipoproteins in the cytoplasmic membrane.

Acyl chains of lipoproteins are important for TLR1/2 signaling capacity of L. plantarum *WCFS1*

The human innate immune system has been reported to recognize bacterial lipoproteins by TLR1/2 and TLR2/6 heterodimers that recognize tri- and di-acylated lipoproteins, respectively [1]. The impact of *lgt* deletion in *L. plantarum* on TLR2 heterodimer signaling was investigated using established HEK-293 reporter cell lines that express human TLR1/2 or TLR2/6 heterodimers and contain a NF-κB promoter controlled luciferase gene [37]. The synthetic agonists Pam3CSK4 and Pam2CSK4 were used as positive controls for TLR1/2 and TLR2/6 activation, respectively. The wild-type strain *L. plantarum* NZ3400Cm [37] stimulated both TLR1/2 and TLR2/6 signaling at a moderate level (Figure 4A and B, respectively). The *lgt* deletion strain, Δ*lgt* (NZ3565Cm), stimulated significantly lower TLR1/2 signaling as compared to the wild-type strain (Figure 4A), whereas its capacity to stimulate TLR2/6 signaling appeared to be unaffected as compared to the wild-type strain (Figure 4B). The observation that the *lgt* mutant of *L. plantarum* WCFS1 affected

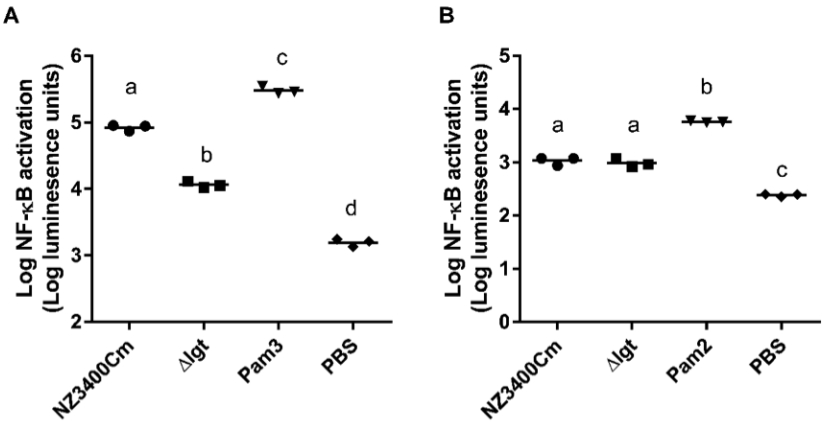


Figure 4. TLR1_2 and TLR2_6 signaling capacities of NZ3400Cm, a *L. plantarum* WCFS1 derivative with a chromosomal integration of the *cat* cassette in a neutral chromosomal locus [37] and the *lgt* deletion mutant, NZ3565Cm (Δ*lgt*). TLR1_2 (A) and TLR2_6 (B) activation were determined using TLR-expressing HEK cell lines, containing a NF-κB responsive luciferase reporter system. Measurements were performed in triplicate and are presented as Log luminescence units, and individually displayed (n=3), while the bar indicates the median. PBS serves as a negative control, while Pam3CysSK4 (Pam3) and Pam2CysSK4 (Pam2) are the positive stimulus of TLR1_2 (A) and TLR2_6 (B) activation, respectively. Data comparison of the wild-type and the deletion derivative was tested for significant differences using one-way ANOVA followed by Tukey's multiple comparison correction and samples with significant different NF-κB activation are indicated with different letters.

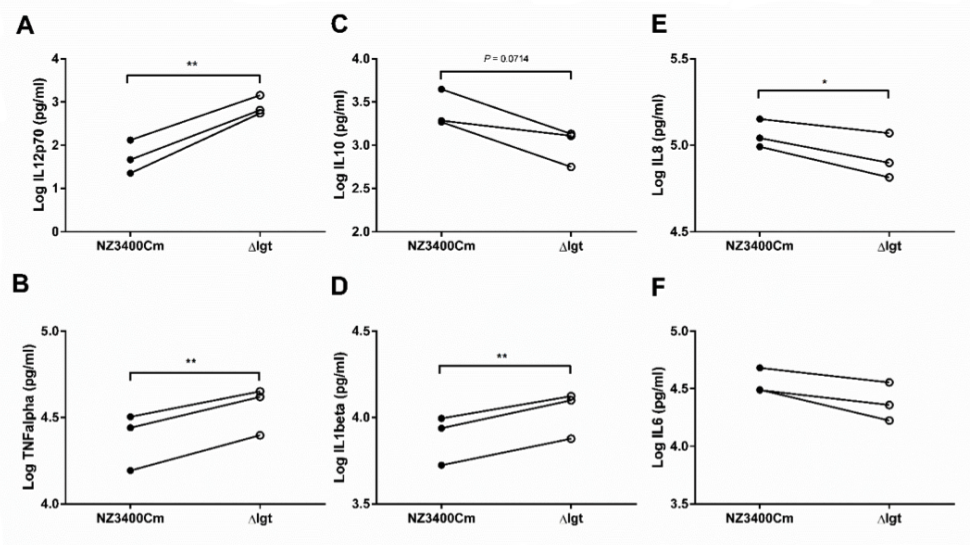


Figure 5. Immunomodulatory effect of NZ3400Cm, a *L. plantarum* WCFS1 derivative with a chromosomal integration of the *cat* cassette in a neutral chromosomal locus [37], and the *lgt* deletion strain NZ3565Cm (Δ*lgt*). Cytokine production was determined in human PBMCs (n=3 donors) after 24 h co-incubation with the bacterial cells. The IL12 (A), TNFα (B), IL10 (C), IL1β (D), IL8 (E), and IL6 (F) cytokine production levels are presented as Log10 values. The cytokine levels for individual donors stimulated with the strains were connected by a line to focus the read-outs on changes elicited by the deletion. Significant differences between cytokine levels induced by wild-type strains and their corresponding mutants (paired *t*-test) are indicated; *, $P \leq 0.05$; **, $P \leq 0.01$; the *P* value of the difference of IL10 production by the NZ3400Cm and Δ*lgt* strains is indicated in the corresponding panel C.

TLR1/2 signaling and not TLR2/6 signaling, suggests that tri-acylated lipoproteins are dominant in this strain. However, there is no homologue genes of E. coli-type *lnt*, which encodes *N*-acyl transferase responsible for the addition of the third acyl chain [2], found in the WCFS1 genome. This could suggest a presence of an unidentified *N*-acyltransferase in *L. plantarum*.

Lipid moiety of lipoproteins is important for anti-inflammatory properties of L. plantarum *WCFS1*

Although many studies have shown that Gram-positive pathogens that lack Lgt activity display attenuated immune activation or virulence, little is known about the effect of this phenotype in probiotic bacteria. We explored the impact of *lgt* deletion on general immune responses using cytokine production by human peripheral blood mononuclear cells (PBMCs). The *lgt* deletion mutant stimulated a more pro-inflammatory responses in PBMCs as compared to the wild type strain (NZ3400Cm), including a higher production of the pro-inflammatory cytokines, IL12, TNFα, IL1β, and IL8 (Figure 5ABDE, respectively). Moreover, the *lgt* mutant strain induces significantly lower levels of production of the anti-inflammatory cytokine IL10 relative to the wild-type (Figure 5C). As a consequence, the IL10/IL12 ratio, which has been reported as an indicator for *in vivo* performance in a mouse colitis model, is significantly lower in *lgt* mutant than the wild type (Figure S2), implying a more pro-inflammatory properties in the mutant. These results illustrate the importance of Lgt and lipoproteins in the overall immunomodulatory properties associated with *L. plantarum* WCFS1, and in particular exemplify the contribution of lipoproteins in the anti-inflammatory properties in *L. plantarum* WCFS1.

Discussion

Lgt is an essential enzymes in Gram-negative bacteria [4]. Since many lipoproteins in Gram-negative bacteria are localized at the outer membrane, defects in lipoprotein biosynthesis will cause mislocalization and/or accumulation of the precursors in the periplasmic space, which has been reported to be lethal to the cells [3, 40]. In contrast, these enzymes appear dispensable in all tested Gram-positive bacteria to date [4]. The *lgt* deletion derivative of *L. plantarum* not only displayed normal morphology and growth, it also affected the expression of only a relatively small amount of genes (Table S1). The smaller impact of *lgt* deletion in Gram-positive bacteria may be explained by the different impact of the loss of Lgt function on the subcellular location of lipoproteins, where in Gram-negative bacteria these proteins accumulate and possibly clog-up the periplasm, whereas in Gram-positive bacteria they tend to remain functionally localized in the appropriate location or are released into the medium. The former explanation (retained functional localization) is supported by the observation that an *lgt* mutation is not lethal in *Bacillus subtilis*, although at least the lipoprotein PrsA fulfills an essential role in this bacterial species [41]. The *lgt* deletion led to mislocalization and release of a range of lipoproteins into the culture supernatant in *L. plantarum* WCFS1, affecting the subcellular location of many substrate binding proteins (SBPs) associated with ABC transporters annotated to be involved in the import of amino acids, oligopeptides, maltose and maltodextrin. Such mislocalization of these SBPs could reduce the efficiency of transport of the corresponding substrates, which may be reflected in changes in the corresponding metabolic processes, and thereby explain some of the transcriptome adjustments observed in the *lgt* mutant strain. However, we did not observe differences in growth characteristics between the wild type strain and its *lgt* derivative when cultured in minimal medium with maltose as a sole carbon source (Figure S3). This lack of consequence in growth characteristics of the *lgt* mutation, may be explained by a certain proportion of SBPs that remains associated with the corresponding transporters or by the elevated expression of the transport functions, which may compensate for the erroneous localization of the corresponding SBP function. The latter explanation appears to be supported by the up-regulation of expression of some transporter encoding genes in the *lgt* mutant (Table S1). Nevertheless, the *lgt* encoded function is dispensable for the laboratory-growth of *L. plantarum* WCFS1 and deletion of the *lgt* gene has a minimal impact on bacterial physiology under the conditions tested.

Our data showed that no clear correlation between transcriptional expression level and proteomic detection of lipoproteins in the supernatants. This result could imply that the anchoring of lipoproteins is affected by more than Lgt alone. It is not clear whether Lsp is able to efficiently cleave all the non-lipid modified precursor lipoproteins in *lgt* mutant. Studies have shown that differently processed lipoprotein precursors (with uncleaved signal peptides or cleaved by Lsp or other peptidases) can be detected in an Lgt deficient background [4]. Failure to cleave off the signal peptide may retain lipoprotein precursors anchored in the cell membrane. Alternatively, non-lipidated lipoprotein precursors could also get trapped in the cell envelope which prevents their release into the cell's environment. In addition, gene transcript levels may not predict protein level accurately, since protein abundance is controlled by multiple mechanisms, including transcript and protein stability, the translation efficiency of the transcript and the regulation thereof [42]. Irrespective of the relatively poor relationship between transcript and protein detection levels, our data indicate that the *lgt* deletion affected the biogenesis and cell membrane anchoring of at least the majority of lipoproteins.

TLR2/6 and TLR1/2 heterodimers recognize di- or tri-acylated lipoproteins, respectively. Crystal structure analyses revealed that TLR1 has a hydrophobic pocket that enables the binding of the third acyl chain, which is lacking in TLR6 that can only accommodate di-acylated lipoproteins [43, 44]. The third acyl chain has been shown to be essential for biosynthesis and biogenesis of lipoproteins in Gram-negative bacteria that are ending up on the outer membrane of these bacteria [3], and appropriate subcellular localization (i.e., outer membrane biogenesis) of lipoproteins is essential in these bacteria. Moreover, since no orthologue genes of *lnt*, the gene responsible for transferring a third acyl chain on the N-terminal cysteine of lipoproteins, has been recognized in the genomes of low-GC-content Gram-positive bacteria, such as species belonging to the genera of *Bacillus*, *Lactobacillus*, *Listeria*, *Staphylococcus*, and *Streptococcus*, it has been assumed that these Gram-positive bacteria have di-acyl lipoproteins [45]. However, recent studies revealed that staphylococcal lipoproteins are tri-acylated, suggesting the presence of an unidentified *N*-acyltransferase in some Gram-positive bacteria, and which has insufficient similarity with *E. coli*-type Lnt to be recognized as its functional equivalent [5, 6]. Similarly, although the *L. plantarum* genome lacks an Lnt homologue, the removal of acylation of its lipoproteins by *lgt* deletion significantly reduced the TLR1/2 signaling capacity but did not affect TLR2/6 signaling, implying that lipoproteins in this bacterial strain are mainly tri-acylated. Future biochemical analysis is still required to verify the lipoprotein structures in this strain. The genome of WCFS1 contains three genes that are annotated to encode acyltransferases, *lp_0856*, *lp_0925*, and *lp_1181*, while also the membrane protein encoded by *lp_1916* also contains a conserved domain of the acyltransferase family. One of these genes is likely to encode the enzyme responsible for tri-acylation of lipoproteins in *L. plantarum* WCFS1, but such function remains to be established. Overall, the potentially important role of lipoproteins in probiotic function and in particular in immunomodulation is supported by the work presented here, and this role deserves further refined elucidation in terms of structure function correlation in the context of host-cell signaling by lipoproteins.

Given that lipoproteins are a ligand of TLR2, an important PRR of the innate immune system, many studies have addressed the effect of *lgt* or *lsp* deletions on immune responses to, and virulence of Gram-positive pathogens [4]. Although the majority of these studies reported that *lgt* or *lsp* deletion leads to attenuation in immune activation and/or reduced virulence of Gram-positive pathogens *in vitro* and *in vivo*, some conflicting results have also been reported. For example, *lgt* and *lsp* deletion derivatives of *Streptococcus equi* [46] and *Streptococcus suis* [47], did not display attenuation in their natural hosts (pony and pig, respectively). Moreover, although a *Listeria monocytogenes lgt* mutant fails to activate TLR2 signaling it is significantly less virulent in a mouse infection model [48], whereas *lgt* mutants of *Streptococcus agalactiae* [49] and *Staphylococcus aureus* [50] are hypervirulent in mouse infection models, and this phenotype is thought to be related to the loss of TLR2 activation by these mutant strains. These results imply that a subtle and strain-specific balance between escaping protective immune defense related to loss of TLR2 activation and attenuated virulence by the loss of lipoprotein acylation in *lgt* mutants. Although they are recognized as important signaling molecules, the role of lipoproteins in the immunomodulatory effect of probiotics has rarely been studied. Our results show that the *L. plantarum lgt* deletion derivative elicited more pro-inflammatory responses in PBMCs compared to its parental strain, suggesting that lipoproteins are important mediators of immune system recognition for probiotics and may drive a more anti-inflammatory response to such probiotic bacteria, a hallmark of tolerance. However, the exact mechanism (and diversity) behind lipoprotein mediated immune responses toward probiotics remains to be elucidated. Further studies focusing on purified lipoproteins could

enable the controlled attenuation of pro-inflammatory immune responses in PBMCs, and may also be used to decipher their mechanistic interplay with pro-inflammatory pathways elicited by lipoprotein deficient strains. Moreover, structure-function studies of lipoproteins from pathogens and probiotics may unravel some key determinants involved in immune system recognition and activation, which may enable the host cells to distinguish harmful and beneficial bacteria.

Acknowledgements

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Supplemental Material

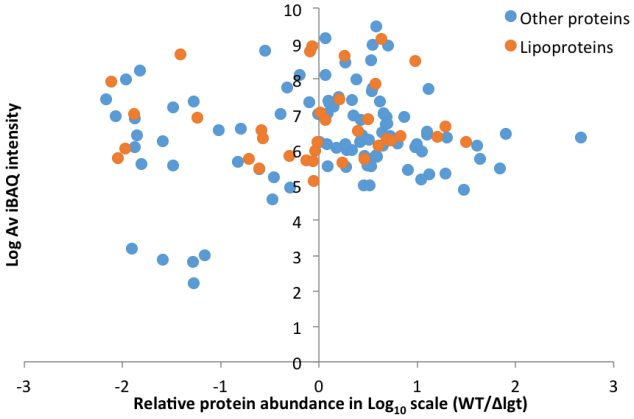

Figure S1. Relative abundance of secreted proteins in *L. plantarum* WCFS1 wild type (WT) and the *lgt* deletion strain (Δlgt). The relative abundance was the ratio taken from the label free quantitation (LFQ, Log10 value) of detected proteins in wild type and the deletion strain in tandem mass spectrometry (MS/MS) analysis. The Av iBAQ intensity represents average Intensity-based absolute quantification of the protein detected in WT and Δlgt. The red dots represent lipoproteins, whereas blue dots represent all other type of proteins.

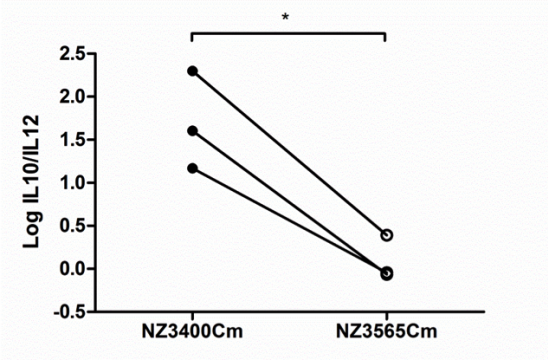

Figure S2. IL10/IL12 cytokine ratio elicited by *L. plantarum* NZ3400Cm and the *lgt* deletion strain NZ3565Cm (Δ*lgt*) following co-incubation with PBMC. The cytokine levels for individual donors stimulated with the strains were connected by a line to focus the read-outs on intrapersonal changes elicited by the deletion. The differences of IL10/IL12 ratio between NZ3400Cm and NZ3565Cm were statistically analyzed using paired t test; *, $P \leq 0.05$.

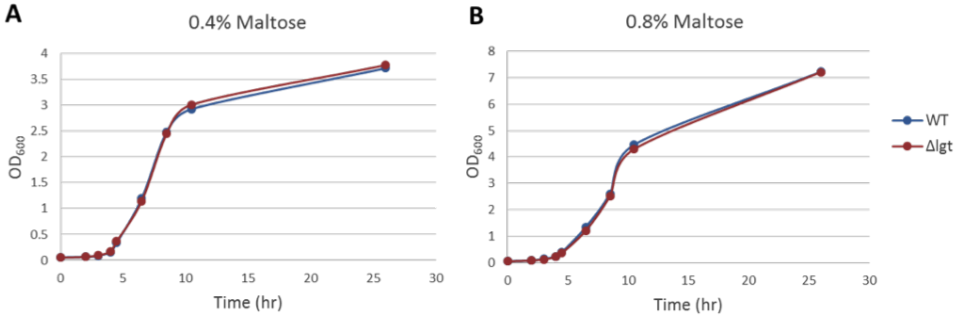

Figure S3. Comparison of the growth curves of L. plantarum NZ3400Cm and the *lgt* deletion strain (Δ*lgt*). Bacteria were cultured in chemical defined medium with 0.4% (A) or 0.8% (B) maltose as a sole carbon source at 37° C. The growth was monitored for 26 hour by measuring the absorbance at 600 nm (OD_{600}).

Table S1. Genes with altered expression in Δ*lgt* mutant as compared to wild type *L. plantarum* WCFS1[a]

Gene ID	Function	Subcellular localization	WT/Δlgt Ratio	FDR[a]
Up-regulated genes				
lp_0187	sucrose-6-phosphate hydrolase	Intracellular	3.96E+01	3.64E-04
lp_0247	PTS system, cellobiose-specific EIIC component	Membrane protein	1.23E+01	8.99E-03
lp_0757	UTP-glucose-1-phosphate uridylyltransferase	Secretory	9.39E+00	2.21E-02
lp_0756	glycerol-3-phosphate dehydrogenase (NAD(P)+)	Intracellular	8.50E+00	1.40E-02
lp_1879	DNA-binding protein	Intracellular	6.08E+00	9.66E-03
lp_0409	bacteriocin immunity protein PlnM	Membrane protein	6.04E+00	4.21E-02
lp_0230	PTS system, mannitol-specific EIICB component	Membrane protein	5.76E+00	8.62E-03
lp_2126	30S ribosomal protein S20	Intracellular	4.99E+00	9.71E-03
lp_0242	nucleoside-diphosphate kinase	Intracellular	4.75E+00	1.20E-02
lp_2968	Nitroreductase	Intracellular	4.45E+00	1.18E-02
lp_3529	transcription antiterminator, BlgB family	Intracellular	4.21E+00	1.38E-02
lp_2363	H(+)-transporting two-sector ATPase, epsilonsubunit	Intracellular	4.14E+00	1.10E-02
lp_3316	hypothetical protein	Intracellular	3.75E+00	3.21E-02
lp_1112	fumarate hydratase	Intracellular	3.64E+00	9.86E-03
lp_0418	two-component response regulator PlnD,repressor	Intracellular	3.26E+00	1.22E-02
lp_2230	hypothetical protein	Intracellular	2.95E+00	2.95E-02
lp_1360	MarR family transcriptional regulator	Intracellular	2.95E+00	3.30E-02
lp_2781	PTS system, cellobiose-specific EIIB component	Secretory	2.92E+00	1.18E-02
lp_1982	N-acetylmuramoyl-L-alanine amidase	N-terminally anchored	2.82E+00	1.14E-02
lp_1406	D-alanine-poly(phosphoribitol) ligase, subunit2	Intracellular	2.78E+00	2.53E-02
lp_0709	UDP-glucose 4-epimerase	Intracellular	2.73E+00	3.55E-02
lp_3243	hypothetical protein	Intracellular	2.70E+00	9.33E-03
lp_2125	30S ribosomal protein S15	Intracellular	2.64E+00	2.18E-02
lp_0899	membrane protein	Membrane protein	2.62E+00	2.92E-02
lp_2483	hypothetical protein	Intracellular	2.61E+00	3.91E-02
lp_0262	trehalose operon transcriptional repressor,GntRfamily	Intracellular	2.58E+00	3.60E-02
lp_1101	L-lactate dehydrogenase	Intracellular	2.48E+00	3.57E-02
lp_2516	hypothetical protein	Intracellular	2.47E+00	1.74E-02
lp_1637	RNA binding protein	Intracellular	2.44E+00	2.12E-02
lp_1535a	50S ribosomal protein L32	Intracellular	2.37E+00	1.68E-02

lp_0286	PTS system, cellobiose-specific EIIC component	Membrane protein	2.34E+00	3.53E-02
lp_0164	membrane protein	Membrane protein	2.32E+00	3.85E-02
lp_0362	acetyl-CoA carboxylase, biotin carboxyl carrierprotein	Intracellular	2.27E+00	3.42E-02
lp_0838	membrane protein	Membrane protein	2.23E+00	3.32E-02
lp_1624	50S ribosomal protein L28	Intracellular	2.22E+00	2.40E-02
lp_3128	DNA-binding ferritin-like protein, DPS family	Intracellular	2.17E+00	3.54E-02
lp_2652	nucleotide-binding protein, universal stressprotein UspA family	Intracellular	2.16E+00	3.08E-02
lp_2114	NTP pyrophosphohydrolase	Intracellular	2.13E+00	4.54E-02
lp_1415	hypothetical protein	Intracellular	2.07E+00	3.40E-02
lp_1864	carboxy-terminal proteinase, S41family,peptidoglycan-bound	N-terminally anchored	2.03E+00	2.18E-02
lp_3184	branched-chain amino acid transport protein	Membrane protein	2.00E+00	4.47E-02
lp_0543	RNA binding protein, contains ribosomal proteinS1 domain	Intracellular	1.99E+00	3.48E-02
lp_1241	hypothetical protein	Intracellular	1.91E+00	3.39E-02
Down-regulated genes				
lp_0533	transport protein, QueT family	Membrane protein	5.27E-01	3.43E-02
lp_1181	acyltransferase/acetyltransferase	Membrane protein	4.65E-01	4.44E-02
lp_0169	dihydroxyacetone phosphotransferase, ADP-binding subunit	Intracellular	4.18E-01	1.35E-02
lp_1521	alcohol dehydrogenase	Intracellular	1.61E-01	9.24E-03
lp_2537	homoserine O-succinyltransferase	Intracellular	1.46E-02	2.23E-03
lp_0755	prolipoprotein diacylglyceryl transferase (*lgt*)	Membrane protein	3.16E-03	8.51E-07
lp_2001	membrane protein	Membrane protein	9.90E-04	2.40E-04

[a] FDR, False discovery rate; only genes with fdr < 0.05 are considered altered.

Table S2. Proteins detected in mass spectrometry with different abundances between wild type *L. plantarum* WCFS1 and Δ*lgt* mutant

Gene ID	Relative abundance (WT/ Δlgt)[a]	Function	LocateP Subcellular Localization Prediction	Mol. weight [kDa]
Only detected in Δ*lgt*				
lp_0200	-	ABC transporter, substrate binding protein	Lipid anchored	61.0
lp_0929	-	alkaline shock protein	Intracellular	15.8
lp_0937	-	membrane alanine aminopeptidase (aminopeptidase N)	Intracellular	93.9
lp_1539	-	lipoprotein precursor	Lipid anchored	21.1
lp_2974	-	ABC transporter, substrate binding protein	Lipid anchored	33.8
lp_3018	-	ABC transporter substrate binding protein	Lipid anchored	32.9
lp_3302	-	ABC transporter substrate binding protein	Lipid anchored	33.9
lp_3352	-	small heat shock protein	Intracellular	16.7
lp_3642	-	ABC transporter, substrate binding protein, mannose related oligosaccharides	Lipid anchored	54.1
At least 2x higher in Δ*lgt*				
lp_0018	0.013	ABC transporter, substrate binding protein, oligopeptide	Lipid anchored	59.7
lp_0175	0.053	ABC transporter, substrate binding protein, maltodextrin	Lipid anchored	45.7
lp_0200	0.026	ABC transporter, substrate binding protein	Lipid anchored	61.0
lp_0201	0.008	ABC transporter, substrate binding proteins	Lipid anchored	61.1
lp_0733	0.033	phosphate ABC transporter, substrate binding protein	Lipid anchored	31.5
lp_0783	0.096	ABC transporter, substrate binding protein	Lipid anchored	61.1
lp_1010	0.281	serine-type D-Ala-D-Ala carboxypeptidase	Lipid anchored	33.0
lp_1070	0.013	lipoprotein precursor, FMN-binding protein	Lipid anchored	43.4
lp_1146	0.262	lipoprotein, pheromone precursor (putative)	Lipid anchored	108.0
lp_1452	0.015	peptidylprolyl isomerase	Lipid anchored	32.6
lp_1473	0.011	iron chelatin ABC transporter, substrate binding protein (putative)	Lipid anchored	21.1
lp_2312	0.195	ABC transporter, substrate binding protein, histidine	Lipid anchored	31.3
lp_2350	0.008	ABC transporter, substrate binding protein, D-Methionine -like precursor	Lipid anchored	29.9
lp_2397	0.497	prophage P2a protein 59; extracellular polysaccharide deacetylase, lipid-anchored (putative)	Lipid anchored	31.6
lp_2450	0.013	prophage P2a protein 7; extracellular protein with lipoprotein anchor	Lipid anchored	12.0
lp_2519	0.009	cell surface hydrolase, DUF915 family, membrane-bound (putative)	Lipid anchored	31.7
lp_2906	0.407	DNA-entry nuclease	Lipid anchored	34.3
lp_2974	0.016	ABC transporter, substrate binding protein	Lipid anchored	33.8

lp_2985	0.026	branched-chain amino acid ABC transporter, substrate binding protein	Lipid anchored	41.9
lp_3018	0.013	ABC transporter substrate binding protein	Lipid anchored	32.9
lp_3103	0.033	iron chelatin ABC transporter, substrate binding protein	Lipid anchored	34.1
lp_3209	0.011	ABC transporter, substrate binding protein, cystine	Lipid anchored	29.0
lp_3214	0.007	ABC transporter, substrate binding protein, cystathionine (putative)	Lipid anchored	29.3
lp_3302	0.068	ABC transporter substrate binding protein	Lipid anchored	33.9
lp_3642	0.053	ABC transporter, substrate binding protein, mannose related oligosaccharides	Lipid anchored	54.1
lp_0076	0.246	elongation factor G	Intracellular	77.0
lp_0764	0.336	Phosphoglucomutase	Intracellular	63.5
lp_0929	0.053	alkaline shock protein	Intracellular	93.9
lp_1046	0.472	ribosomal protein L24	Intracellular	11.4
lp_1879	0.246	DNA-binding protein	Intracellular	62.9
lp_3352	0.350	small heat shock protein	Intracellular	16.7
lp_1229	0.039	mannose-specific adhesion	LPxTG Cell-wall anchored	60.3
lp_0295	0.160	transport protein, MMPL family	Multi-transmembrane	143.3
lp_0925	0.149	Acyltransferase	Multi-transmembrane	15.8
lp_1000	0.014	transcriptional attenuator, cell envelope-related, LytR family	N-terminally anchored	28.2
lp_1097	0.269	manganese/zinc ABC transporter, substrate binding protein	Secretory(released)	42.6
lp_1278	0.058	serine-type D-Ala-D-Ala carboxypeptidase	Secretory(released)	38.6
Only detected in WT				
lp_0542	-	septum formation initiator	N-terminally anchored	15.2
lp_0575	-	mannose PTS, EIIAB	Intracellular	35.3
lp_1043	-	ribosomal protein L29	Intracellular	7.5
lp_1050	-	ribosomal protein S8	Intracellular	14.6
lp_1097	-	manganese/zinc ABC transporter, substrate binding protein	Secretory(released)	34.6
lp_3614	-	Oxidoreductase	Intracellular	33.4
lp_3686	-	ABC transporter, substrate binding protein	Lipid anchored	60.5
At least 2x higher in WT				
lp_2145	3.775	extracellular protein, DC-SIGN ligand	C-terminally anchored	45.8
lp_3678	3.872	cell surface protein precursor, DUF916 family	C-terminally anchored	39.6
lp_0355	3.309	multicopper oxidase (putative)	Intracellular	56.8
lp_0575	13.299	mannose PTS, EIIAB	Intracellular	35.3
lp_0578	2.867	non-ribosomal peptide synthetase NpsA	Intracellular	602.6
lp_0728	13.425	GroEL chaperonin	Intracellular	57.4
lp_0937	3.414	membrane alanine aminopeptidase (aminopeptidase N)	Intracellular	128.2

lp_1043	79.973	ribosomal protein L29	Intracellular	7.5
lp_1050	19.487	ribosomal protein S8	Intracellular	14.6
lp_1053	5.335	ribosomal protein S5	Intracellular	17.3
lp_1054	2.682	ribosomal protein L30	Intracellular	6.7
lp_1635	5.452	signal recognition particle protein Ffh	Intracellular	236.1
lp_1897	6.808	pyruvate kinase	Intracellular	43.3
lp_2055	3.094	ribosomal protein S2	Intracellular	30.2
lp_2118	12.661	trigger factor; peptidylprolyl isomerase	Intracellular	49.4
lp_2417	2.485	prophage P2a protein 40; major capsid protein	Intracellular	38.1
lp_2793	3.150	Unknown	Intracellular	76.3
lp_2988	7.437	extracellular zinc metalloproteinase, M10 family (putative)	Intracellular	28.1
lp_3170	2.172	phosphoglycerate mutase family protein	Intracellular	26.1
lp_3614	462.380	Oxidoreductase	Intracellular	33.4
lp_0625	2.397	prophage P1 protein 2, mitogenic factor, cell surface lipoprotein	Lipid anchored	41.8
lp_0689	2.187	cell surface protein, lipoprotein precursor	Lipid anchored	23.1
lp_1072	43.973	thiamin biosynthesis lipoprotein ApbE	Lipid anchored	34.6
lp_1609	4.847	glycine betaine/carnitine/choline ABC transporter, substrate binding protein	Lipid anchored	53.9
lp_3686	69.772	ABC transporter, substrate binding protein	Lipid anchored	60.5
lp_0197	3.233	cell surface protein precursor	LPxTG Cell-wall anchored	105.2
lp_0800	10.941	cell surface protein precursor	LPxTG Cell-wall anchored	226.4
lp_0923	3.653	cell surface protein precursor	LPxTG Cell-wall anchored	83.3
lp_0946	2.707	mucus-binding protein (putative)	LPxTG Cell-wall anchored	37.7
lp_1643	4.321	mucus binding protein precursor	LPxTG Cell-wall anchored	22.2
lp_2925	9.621	cell surface protein precursor	LPxTG Cell-wall anchored	89.5
lp_2940	11.259	cell surface protein precursor	LPxTG Cell-wall anchored	44.3
lp_3114	8.059	mucus-binding protein (putative)	LPxTG Cell-wall anchored	214.8
lp_0802	4.387	polar amino acid ABC transporter, substrate binding and permease protein	Multi-transmembrane	52.3
lp_0856	3.466	acyltransferase (putative)	Multi-transmembrane	74.4
lp_2914	2.922	integral membrane protein	Multi-transmembrane	66.8
lp_0043	2.774	serine protease HtrA	N-terminally anchored	43.1
lp_0542	40.886	septum formation initiator	N-terminally anchored	15.2
lp_0593	2.916	transcriptional attenuator, cell envelope-related, LytR family	N-terminally anchored	43.6
lp_0600	4.196	extracellular zinc metalloproteinase, M10 family (putative)	N-terminally anchored	26.5
lp_0628	4.558	prophage P1 protein 5, superinfection exclusion (cell surface N-anchored)	N-terminally anchored	37.9
lp_0688	2.626	DNA entry nuclease	N-terminally anchored	36.2

lp_0746	20.051	phosphate ABC transporter, substrate binding protein	N-terminally anchored	31.5
lp_1568	4.064	transpeptidase (penicillin binding protein 2B)	N-terminally anchored	34.8
lp_1751	31.422	transpeptidase-transglycosylase (penicillin binding protein 1A)	N-terminally anchored	50.0
lp_2737	16.042	cell surface hydrolase, DUF915 family, membrane-bound (putative)	N-terminally anchored	29.7
lp_2809	9.599	extracellular protein (putative)	N-terminally anchored	23.2
lp_2812	4.905	extracellular protein, membrane-anchored (putative)	N-terminally anchored	28.5
lp_2845	13.153	extracellular transglycosylase (putative)	N-terminally anchored	30.7
lp_3014	3.529	extracellular transglycosylase (putative)	N-terminally anchored	21.3
lp_3093	4.562	muramidase (putative)	N-terminally anchored	89.6
lp_3411	9.962	extracellular protein, DUF1002 family	N-terminally anchored	34.2
lp_3421	3.818	extracellular protein, gamma-D-glutamate-meso-diaminopimelate muropeptidase (putative)	N-terminally anchored	36.8
lp_0092	29.929	ABC transporter, substrate binding protein, oligopeptide	Secretory(released)	59.8
lp_0141	6.321	extracellular protein	Secretory(released)	28.6
lp_0302	3.404	extracellular transglycosylase (putative)	Secretory(released)	26.3
lp_0304	5.058	extracellular transglycosylase (putative)	Secretory(released)	22.1
lp_0924	4.442	extracellular protein	Secretory(released)	68.6
lp_1697	19.569	adherence protein, chitin-binding domain	Secretory(released)	31.6
lp_2541	2.890	ABC transporter, substrate binding protein	Secretory(released)	35.0
lp_2978	2.253	extracellular protein	Secretory(released)	25.5
lp_3189	3.826	serine-type D-Ala-D-Ala carboxypeptidase	Secretory(released)	47.0
lp_3412	4.978	extracellular protein	Secretory(released)	118.7
lp_3414	3.493	extracellular protein	Secretory(released)	27.4
lp_3676	12.665	extracellular protein	Secretory(released)	56.8
lp_2960	4.815	lipase/esterase, subfamily of SGNH-hydrolases	transmembrane	25.5

[a] FDR Relative abundances were calculated by the ratio of Label-free quantitation (LFQ) of detected peptides in wild type and *lgt* mutant.

Table S3. The transcriptional levels of predicted lipoprotein genes in *L. plantarum* WCFS1 wild type or in *lgt* mutant

Gene ID[a]	Gene Name	Function	Expression level[b] in WT	Expression level[b] in Δ*lgt*
lp_0201	dacB	serine-type D-Ala-D-Ala carboxypeptidase	1.12E-06	2.48E-07
lp_0200	pstF	phosphate ABC transporter, substrate binding protein	7.64E-07	3.24E-07
lp_2985	-	lipoprotein precursor	1.47E-06	1.54E-06
lp_3642	-	sugar ABC transporter, substrate binding protein	5.62E-06	2.62E-06
lp_0733	-	ABC transporter substrate binding protein	1.37E-06	3.80E-06
lp_0715	-	ABC transporter substrate binding protein	1.53E-05	4.25E-06
lp_2974	-	cell surface hydrolase (putative)	7.65E-06	4.27E-06
lp_3214	-	amino acid ABC transporter, substrate binding protein	5.87E-06	8.57E-06
lp_0018	-	lipoprotein precursor, peptide binding protein OppA homolog	1.11E-05	9.61E-06
lp_2098	-	lipoprotein precursor (putative)	9.52E-06	1.31E-05
lp_3686	-	lipoprotein precursor, peptide binding protein OppA homolog	1.03E-05	1.36E-05
lp_1915	prsA	peptidylprolyl isomerase	1.67E-05	1.55E-05
lp_0182	-	prophage Lp1 protein 2	1.72E-05	1.76E-05
lp_1261	-	lipoprotein precursor	2.12E-05	2.00E-05
lp_3018	-	ABC transporter, substrate binding protein	1.72E-05	2.15E-05
lp_1935	fecB	iron chelatin ABC transporter, substrate binding protein (putative)	2.12E-05	2.38E-05
lp_1995	opuC	glycine betaine/carnitine/choline ABC transporter, substrate binding protein	3.56E-05	3.02E-05
lp_3302	-	amino acid ABC transporter, substrate binding protein	3.47E-05	3.14E-05
lp_0689	endA	DNA-entry nuclease	2.85E-05	3.44E-05
lp_0988	-	lipoprotein precursor (putative)	3.73E-05	3.71E-05
lp_3103	livA	branched-chain amino acid ABC transporter, substrate binding protein	1.68E-05	3.80E-05
lp_0175	-	mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase	2.42E-05	4.18E-05
lp_0932	-	lipoprotein precursor, peptide binding protein OppA homolog	7.69E-05	5.56E-05
lp_1812	-	lipoprotein precursor (putative)	4.12E-05	5.69E-05
lp_2393	-	hypothetical protein	8.88E-05	8.31E-05
lp_1072	-	lipoprotein precursor, peptide binding protein OppA homolog	1.19E-04	1.06E-04
lp_2397	-	lipoprotein precursor	7.53E-05	1.22E-04
lp_2519	-	amino acid ABC transporter, substrate binding protein	1.06E-04	1.26E-04
lp_1473	apbE1	thiamin biosynthesis lipoprotein ApbE	8.04E-05	1.32E-04
lp_2934	-	prophage Lp2 protein 59; xylanase/chitin deacetylase (putative)	1.20E-04	1.38E-04
lp_2906	-	lipoprotein precursor	1.68E-04	1.40E-04

lp_1369	-	lipoprotein precursor	6.97E-05	1.40E-04
lp_1539	-	lipoprotein precursor	1.48E-04	1.56E-04
lp_2312	-	lipoprotein precursor	2.73E-04	2.02E-04
lp_1070	phnD	phosphonates ABC transporter, substrate binding protein (putative)	1.85E-04	2.58E-04
lp_1010	-	prophage Lp1 protein 66, lipoprotein precursor	3.18E-04	2.71E-04
lp_0625	-	prophage Lp2 protein 7	2.42E-04	2.80E-04
lp_2350	-	cell surface hydrolase (putative)	3.49E-04	2.93E-04
lp_0473	-	lipoprotein precursor	2.68E-04	2.93E-04
lp_3193	fhuD	ferrichrome ABC transporter, substrate binding lipo protein	2.36E-04	3.03E-04
lp_2450	glnH2	glutamine ABC transporter, substrate binding protein	3.22E-04	3.12E-04
lp_0881	-	lipoprotein precursor, peptide binding protein OppA homolog	4.70E-04	3.62E-04
lp_3209	prtM2	peptidylprolyl isomerase	6.75E-04	4.88E-04
lp_1609	oppA	oligopeptide ABC transporter, substrate binding protein	5.82E-04	5.69E-04
lp_0783	malE	maltose/maltodextrin ABC transporter, substrate binding protein	1.27E-03	5.73E-04
lp_1146	glnH1	glutamine ABC transporter, substrate binding protein	8.46E-04	6.75E-04
lp_1452	-	lipoprotein precursor	7.19E-04	7.45E-04

[a]The encoding genes of predicted lipoproteins that did not detected in supernatants by proteomic analysis were labeled red.

[b]The expression levels were estimated based on average probe signals detected in microarray analysis. The list was arranged in an order of increasing transcription levels based on average probe signals in the *lgt* mutant.

4

Chapter 5

GtfA and GtfB are both required for protein *O*-glycosylation in *Lactobacillus plantarum*

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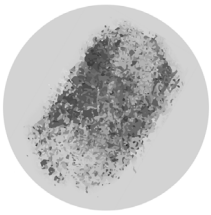

Abstract

Acm2, the major autolysin of *Lactobacillus plantarum* WCFS1, was recently found to be *O*-glycosylated with *N*-acetylhexosamine, likely *N*-acetylglucosamine (Glc*N*Ac). Here, we set out to identify the glycosylation machinery by employing a comparative genomics approach to identify Gtf1 homologues, which are involved in fimbriae-associated protein 1 (Fap1) glycosylation in *Streptococcus parasanguinis*. This *in silico* approach resulted in the identification of 6 candidate *L. plantarum* WCFS1 genes with significant homology to Gtf1, namely *tagE1* to *tagE6*. These candidate genes were targeted by systematic gene deletion, followed by assessment of the consequences on glycosylation of Acm2. We observed a changed mobility of Acm2 on SDS-PAGE in the *tagE5E6* deletion strain, while deletion of other *tagE*s resulted in Acm2 mobility comparable to the wild type. Subsequent mass spectrometry analysis of excised and in-gel digested Acm2 confirmed the loss of glycosylation on Acm2 in the *tagE5E6* deletion mutant, whereas a lectin blot using Glc*N*Ac-specific succinylated wheat germ agglutinin (sWGA) revealed that besides Acm2, *tagE5E6* deletion also abolished all-but-one other sWGA-reactive, protease-sensitive signals. Only complementation of both tagE5 and tagE6 restored those sWGA-lectin signals, establishing that TagE5 and TagE6 are both required for the glycosylation of Acm2 as well as the vast majority of other sWGA-reactive proteins. Finally, sWGA-lectin blotting experiments using a panel of 8 other *L. plantarum* strains revealed that protein glycosylation is a common feature in *L. plantarum* strains. With the establishment of these enzymes as protein glycosyltransferases, we propose to rename TagE5 and TagE6 to GtfA and GtfB, respectively.

Introduction

Probiotics, of which the majority belong to the genera *Lactobacillus* and *Bifidobacterium* [1-3], have been defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [4]. One mechanism by which these health benefits are mediated is through molecular interactions between probiotic bacteria and host cells, in which bacterial surface molecules appear to play a pivotal role [1-3]. These surface effector molecules include canonical polymers such as wall- and lipo-teichoic acid, peptidoglycan, and capsular polysaccharides, but also proteinaceous molecules [2, 5-7].

Many proteinaceous molecules have established functions associated with adhesion to intestinal mucus, such as the mucin-binding proteins (Mub) of *Lactobacillus acidophilus* NCFM [8] and *Lactobacillus reuteri* 1063 [9], a mucus adhesion promoting protein (MapA) of *L. reuteri* 104R [10], and the mannose-specific adhesin (Msa) of *Lactobacillus plantarum* WCFS1 [11]. Examples of proteins involved in adhesion to epithelial cells include the surface layer proteins of *Lactobacillus brevis* ATCC 8287 [12], *Lactobacillus crispatus* JCM 5810 [13] and *Lactobacillus helveticus* R0052 [14]. In addition to their role in the adhesive capacity to mucus and epithelial cells, some *Lactobacillus* surface proteins are able to bind with extracellular matrix (ECM), which is a complex structure surrounding epithelial cells and is composed of various proteins including laminin, collagen and fibronectin. Reported examples include the collagen-binding protein of *L. reuteri* NCIB11951 [15] and fibronectin-binding protein A of *L. acidophilus* NCFM [8].

Other surface proteins impact on probiotic-host interactions via their immunomodulating capacity, for example Msp1 and Msp2, two peptidoglycan hydrolases of *Lactobacillus rhamnosus* GG, which promote epithelial homeostasis [16, 17]. Recombinant Msp2 was also shown to prevent and ameliorate experimental colitis in mice by an epidermal growth factor receptor-dependent mechanism [18]. Furthermore, surface layer protein A (SlpA) of *L. acidophilus* NCFM was documented to be recognized by the dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) receptor and as a consequence modulates human DCs and T cell functions, leading to regulatory T cell differentiation through increased IL-10 and reduced IL-12p70 production [19]. A serine and threonine rich peptide (STp) harbored by protein D1 that is secreted by *Lactobacillus plantarum* BMCM12 represents another example of a proteinaceous effector molecule, as it was recently demonstrated to stimulate regulatory responses in human intestinal DCs [20].

The most common modification found in proteinaceous molecules is glycosylation, in which glycans can be attached to the amide nitrogen of asparagine, i.e. *N*-glycosylation, or to the hydroxyl oxygen of serine or threonine, i.e. *O*-glycosylation [21]. Although protein glycosylation was initially studied exclusively in eukaryotes, bacterial protein glycosylation has recently received increasing attention and it is now clear that bacteria can also modify proteins with diverse *N*-linked and *O*-linked glycan moieties [22-26]. So far, most studies on bacterial protein glycosylation focused on pathogenic organisms [23, 27-29], resulting in the identification of general glycosylation pathways [26], including an *N*-glycosylation pathway in *Campylobacter jejuni* [30, 31] and *O*-linked glycosylation systems in *Neisseria gonorrhoeae* [32, 33]. Specific machineries responsible for *O*-glycosylation of abundant surface proteins such as flagellin and pilin have also been described in various pathogenic bacteria [34-36]. Moreover, fimbriae-associated protein 1 (Fap1), a serine-rich adhesin of *Streptococcus parasanguinis*, has been demonstrated to be heavily

glycosylated with *N*-acetylglucosamine (Glc*N*Ac) and glucose [37, 38]. This glycosylation requires the concerted activity of two putative glycosyltransferases; Gtf1 and Gtf2 [37]. More recent studies pinpointed that protein glycosylation also occurs in certain human intestine commensals, including several *Bacteroides* species [39, 40], and probiotic species such as *L. plantarum* WCFS1 [41, 42] and *L. rhamnosus* GG [43]. More specifically, Msp1 of *L. rhamnosus* GG is *O*-glycosylated at serine residues 106 and 107 and its glycan moieties are recognized by the Concanavalin A (ConA) lectin, which is specific for mannose and/or glucose moieties [43]. Similarly, the major autolysin of *L. plantarum* WCFS1, Acm2, was shown to be *O*-glycosylated in its N-terminal alanine, serine and threonine rich region (AST domain), which could be selectively detected by using the Glc*N*Ac-specific biotinylated succinylated wheat germ agglutinin (sWGA) lectin [41, 42]. Intriguingly, AST domains are present in several other protein encoded in the *L. plantarum* WCFS1 genome, including several other peptidoglycan hydrolases [41] and lp_2145 [44], suggesting that these proteins could also be subjected to glycosylation [41]. Indeed, a recent study found 10 novel glycoproteins in *L. plantarum* WCFS1, including 2 AST domain-containing peptidoglycan hydrolases (Lp_2162 and Lp_3421), 4 cytoplasmic proteins (DnaK, ELp_2152, FtsY and FtsK1), and the secreted proteins Lp_2260 and Lp_1643 [45].

To date, no protein glycosylation machinery has been described for *Lactobacillus* species [41, 43]. Here, we employed a comparative genomics approach to identify Gtf1 homologues in the genome of *L. plantarum* WCFS1, resulting in the identification of 6 candidate genes (previously annotated as poly(glycerol-phosphate) α-glucosyltransferases, i.e. *tagE1* to *tagE6*) that might encode protein glycosyltransferases [46]. These candidate genes were targeted by a gene deletion and complementation approach, after which we assessed the consequences of these genetic modifications for the presence of glycan moieties in proteins by employing the Glc*N*Ac-specific lectin sWGA in blotting experiments. Moreover, we specifically assessed the impact of *tagE5E6* deletion on the previously established glycosylation of Acm2 [41, 42] by mass-spectrometry analysis (MS). These experiments revealed that TagE5 and TagE6 are both required for the glycosylation of proteins, including Acm2, in *L. plantarum* WCFS1. Moreover, expansion of our lectin-blotting experiments to a panel of other *L. plantarum* strains revealed protein glycosylation is widespread in this species. To the best of our knowledge, these results represent the first example of a protein glycosylation machinery in a *Lactobacillus* species.

Materials and Methods

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Lactobacillus plantarum* strains were grown at 37°C in MRS broth (Difco, West Molesey, United Kingdom) without aeration. *Escherichia coli* strain TOP10 (Invitrogen, Bleiswijk, The Netherlands) was grown at 37°C in TY broth [47] with aeration [48]. Solid media were prepared by adding 1.5% (w/v) agar to the broths. Where appropriate, antibiotics were added for *L. plantarum* and *E. coli* at 10 μg/ml chloramphenicol, and 30 and 200 μg/ml erythromycin, respectively.

DNA manipulations. Primers used are listed in Table 2 and were synthesized by Sigma-Aldrich (Zwijndrecht, The Netherlands). Standard procedures were used for DNA manipulations in *E.*

Table 1. Bacterial strains and plasmids used in this study

Strains	Characteristics[a]	Reference[b]
L. plantarum		
WCFS1	Single-colony isolate of *L. plantarum* NCIMB8826	[46]
ATCC 14917	Isolate from pickled cabbage	ATCC
ATCC 8014	Isolate from maize ensilage	ATCC
CIP104440	Isolate from human stool	CIP
CIP104450	Isolate from human stool	CIP
NC8	Isolate from grass silage	[76]
NCIMB 12120	Origin from Ogi, Nigeria	NCIMB
LP80	Isolate from silage	[77]
LP85-2	Origin from silage, France	[57]
Δ*acm2*	NZ3557Cm; Cm^r; derivative of WCFS1 containing a *lox66*-P_{32}-*cat*-*lox71*-tagH9 replacement of *acm2* (*acm2*::*lox66*-P_{32}-*cat*-*lox71*-tagH9)	[41]
Δ*tagE1*	NZ3540Cm; Cm^r; derivative of WCFS1 containing a *lox66*-P_{32}-*cat*-*lox71*-tag8.5 replacement of *tagE1* (*tagE1*:: *lox66*-P_{32}-*cat*-*lox71*-tag8.5)	this work
Δ*tagE2E3*	NZ3541Cm; Cm^r; derivative of WCFS1 containing a *lox66*-P_{32}-*cat*-*lox71*-tagF10 replacement of *tagE2E3* (*tagE2E3*::*lox66*-P_{32}-*cat*-*lox71*-tagF10)	this work
Δ*tagE4*	NZ3542Cm; Cm^r; derivative of WCFS1 containing a *lox66*-P_{32}-*cat*-*lox71*-tagG1 replacement of *tagE4* (*tagE4*:: *lox66*-P_{32}-*cat*-*lox71*-tagG1)	this work
Δ*tagE5E6*	NZ3543Cm; Cm^r; derivative of WCFS1 containing a *lox66*-P_{32}-*cat*-*lox71*-tagG7 replacement of *tagE5E6* (*tagE5E6*::*lox66*-P_{32}-*cat*-*lox71*-tagG7)	this work
tagE5E6 complementation	NZ8204CmEm; Cm^{r}Em^r; derivative of NZ3543^{Cm} containing chromosomally integrated pNZ8204 at $tRNA^{Ser}$ site	this work
tagE6 complementation	NZ8205CmEm; Cm^{r}Em^r; derivative of NZ3543^{Cm} containing chromosomally integrated pNZ8205 at $tRNA^{Ser}$ site	this work
tagE5 complementation	NZ8206CmEm; Cm^{r}Em^r; derivative of NZ3543^{Cm} containing chromosomally integrated pNZ8206 at $tRNA^{Ser}$ site	this work
E. coli		
TOP 10	Cloning host; F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
Plasmids		
pNZ5319	Cm^r Em^r; Mutagenesis vector for gene replacements in *L. plantarum*	[50]
pNZ3540	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of *tagE1*	this work
pNZ3541	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of *tagE2-E3*	this work
pNZ3542	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of *tagE4*	this work
pNZ3543	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of *tagE5E6*	this work
pMEC10	Em^r; Integration plasmid	[78]
pNZ8204	Em^r; pMEC10 derivative harboring *tagE5E6*	this work
pNZ8205	Em^r; pMEC10 derivative harboring *tagE6*	this work
pNZ8206	Em^r; pMEC10 derivative harboring *tagE5*	this work

[a] Cm^r , chloramphenicol resistant; Em^r, erythromycin resistant.

[b] ATCC, American Type Culture Collection, Manassas, Va.; CIP, Collection de l'Institut Pasteur, Paris, France.; NCIMB, National Collections of Industrial, Food and marine Bacteria, Aberdeen, Scotland.

coli [48]. Plasmid DNA was isolated from *E. coli* using a JETSTAR kit (Genomed GmbH, Bad Oberhausen, Germany). *L. plantarum* DNA was isolated and transformed as described previously [49]. PCR amplifications were performed using hot-start KOD polymerase (Novagen, Madison, USA). Amplicons were purified using Wizard®SV Gel and PCR Clean-Up System (Promega, Leiden, The Netherlands). Restriction endonucleases (Fermentas GmbH, St. Leon-Rot, Germany), MSB® Spin PCRapace (Invitek GmbH, Berlin, Germany), PCR Master Mix (Promega) and T4 DNA ligase (Invitrogen) were used as specified by the manufacturers.

Table 2. Primers used in this study

Primers	Sequence[a]	Reference
is128 tag-lox66-F3	5'-AAATCTACCGTTCGTATAATGTATG-3'	[79]
is129 tag-lox71-R3	5'-CTCATGCCCGGGCTGTAACCG-3'	[79]
87	5'-GCCGACTGTACTTTCGGATCC-3'	[50]
CreF	5'-CGATACCGTTTACGAAATTGG-3'	[50]
CreR	5'-CTTGCTCATAAGTAACGGTAC-3'	[50]
EryintF	5'-TCAAATACAGCTTTTAGAACTGG-3'	[50]
EryintR	5'-ATCACAAACAGAATGATGTACC-3'	[50]
tagE1-Up-F	5'-GCCGCAACAACCATACTGGG-3'	this work
tagE1-Up-R	5'-GCATACATTATACGAACGGTAGATTTAAAATAATACAT-CACCTAGCCG-3'	this work
tagE1-Down-F	5'-CGGTTACAGCCCGGGCATGAGATAGCAGCACTTTA-AGAACTGG-3'	this work
tagE1-Down-R	5'-GCGATTACATCGCCTTGGCG-3'	this work
tagE1-out-F	5'-GCTAGTCATGTCACGGATGC-3'	this work
tagE1-out-R	5'-TCACTCACAATAAATTCCCCC-3'	this work
tagE2E3-Up-F	5'-GCAATTACAATGTTGTGCGGC-3'	this work
tagE2E3-Up-R	5'-GCATACATTATACGAACGGTAGATTTGAAGTAAAACATA-CAGTCACCC-3'	this work
tagE2E3-Down-F	5'-CGGTTACAGCCCGGGCATGAGACGGCTTAAGTAGATTT-GACGG-3'	this work
tagE2E3-Down-R	5'-AAGTGCGCGTTTTAGTACGC-3'	this work
tagE2E3-out-F	5'-TACGGTTATTTTCCGGCTCG-3'	this work
tagE2E3-out-R	5'-ATCGGTGGCCTTTTACTTGG-3'	this work
tagE4-Up-F	5'-CGTATCGATTGTTGACAGCG-3'	this work
tagE4-Up-R	5'-GCATACATTATACGAACGGTAGATTTATCGGCTAAACAAC-CACATGC-3'	this work
tagE4-Down-F	5'-CGGTTACAGCCCGGGCATGAGGAAATACATTTGCTACGC-CCC-3'	this work
tagE4-Down-R	5'-CGAAGTGACGACTGCAAACG-3'	this work
tagE4-out-F	5'-CTTTCGTAGCCAAAATCGACG-3'	this work
tagE4-out-R	5'-CAAGAACAAGTCACAGCCGC-3'	this work
tagE5E6-Up-F	5'-ATTGGAAACGTTCTGTGCGG-3'	this work
tagE5E6-Up-R	5'-GCATACATTATACGAACGGTAGATTTGTTGTTCAGTGAATAT-CAAAAATGG-3'	this work
tagE5E6-Down-F	5'-CGGTTACAGCCCGGGCATGAGATAATACATTATTACTC-GCTCCC-3'	this work
tagE5E6-Down-R	5'-AGTTGTTGATGAACTGCTGC-3'	this work
tagE5E6-out-F	5'-AAATAATAGTTAGGGGTGAACAC-3'	this work
tagE5E6-out-R	5'-CTTCAGCACTACTTGATGTGC-3'	this work
tRNA	5'-GCGAACCGGCTAATACCGGC-3'	[80]
IC013	5'-AGCTAACAGACCGGTAGCTGCCAATGAAG-3'	this work
IC014	5'-AACCAGAGCTCCTGGCTGCTACGTGAACCTAATTCC-3'	this work
IC015	5'-TTTCCGAGCTCGCGTTACTAGTTTAGCCGGTGCCTG-3'	this work
IC016	5'-TATTGGTTCACAAAAAAATTCATTATTACTCGCTCCCTTA-CACGA-3'	this work
IC017	5'-CGTGTAAGGGAGCGAGTAATAATGAATTTTTTTGTGAAC-CAATATT-3'	this work
IC021	5'-ACGCCACATGCAGTCGATCC-3'	this work
IS169	5'-TTATCATATCCCGAGGACCG-3'	[81]
IS247	5'-AGATTGTACTGAGAGTGCACC-3'	this work
IS260	5'-GTTGAAAGAACCTGTACTCTCC-3'	this work

[a] Underlined nucleotides indicate parts of the primers that are complementary to the is128-lox66-F3 and is129-lox71-R3 primers.

Construction of *tagE* deletion mutants. The *tagE* deletion mutants were constructed as described previously [50], using a double crossing-over strategy to replace the target *tagE* genes by a chloramphenicol resistance cassette (*lox66*-P_{32}*cat*-*lox71*) [50]. In this study, a derivative of the mutagenesis vector pNZ5319 [50], designated pNZ5319TAG (Bron *et al.*, unpublished data) was used to introduce a unique DNA-tag into the chromosome during gene deletion, which can be used for mutant tracking purposes in mixed populations (not relevant for the study presented here). The upstream and downstream flanking regions of each *tagE* gene set (*tagE1*, *tagE2E3*, *tagE4* and *tagE5E6*) were amplified by PCR using tagEs-Up-F/R and tagEs-Down-F/R primers, respectively (Table 2). Each amplicon generated was subsequently joined by a second PCR to tag-*lox*66-P_{32}*cat*-*lox*71 by a splicing by overlap extension strategy [51], using tagEs-Up-F/tagEs-Down-R primer pairs (Table 2). The resulting PCR products were digested with *Swa*I and *Ecl*136II, and cloned into similarly digested pNZ5319TAG. The obtained mutagenesis plasmids were transformed into *L. plantarum* WCFS1 as described previously [49]. The resulting integrants were assessed for a double cross over integration event by using tagEs-out-F/R primers (Table 2). For each of the mutant constructions a single colony displaying the anticipated genotype was selected, yielding the mutants NZ3540Cm (Δ*tagE1*), NZ3541Cm (Δ*tagE2E3*), NZ3542Cm (Δ*tagE4*), and NZ3543Cm (Δ*tagE5E6*).

Complementation of Δ*tagE5E6*. The Genomic organization of *tagE5 and tagE6* (*lp_2843-2844*) is shown in figure 1A. The *tagE5E6* genes and the individual *tagE6* gene of *L. plantarum* WCFS1 were amplified including their native promoter (P_{tagE6}, upstream of *tagE6*) using primers IC013/IC014 and IC013/IC015, respectively. Since *tagE5* is also transcribed from the *tagE6* promoter, the P_{tagE6} promoter and *tagE5* were joint by a splicing by overlap extension strategy [51]. The promoter was amplified by using primers IC013/IC016, while primer IC016 containing the initial 23 bp nucleotides of *tagE5*. The *tagE5* gene was amplified using primer IC017 which contains the terminal 21 nucleotides of the promoter region, and primer IC014. These two PCR

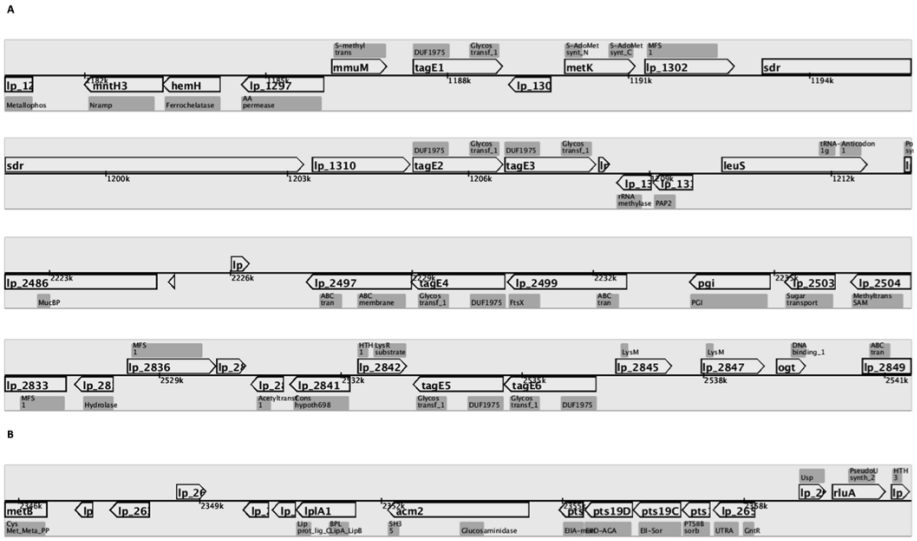

Figure 1. Genetic organization views of (A) *tagE1-E6* and their neighboring genes, (B) *acm2* generating by Microbial Genomic context Viewer (MGcV) [82]. The annotated Pfam domains of each gene was shown in grey shades.

products were mixed in a molar ratio of 1:1 and amplified using primers IC013/IC014 to join the promoter and *tagE5*. A *Sac*I site was introduced by primer IC014 and IC015 downstream of *tagE6* or *tagE5*, respectively. pMEC10 was digested by *Sac*I and *Sfo*I whereas PCR products of *tagE5E6*, *tagE6* and *tagE5* were digested with *Sac*I. Digested fragments were ligated using T4 DNA ligase. Subsequently, the ligation mixtures were transformed into *E. coli* TOP10; positive clones were selected by colony PCR [52] using primers IC013/IC015 for *tagE6*, IC014/IC017 for *tagE5* and IS260/IS247 for *tagE5E6*. Resulting plasmids were designated pNZ8204, pNZ8205 and pNZ8206 for the complementation plasmid of *tagE5E6*, *tagE6* and *tagE5*, respectively. Integrity of nucleotide sequences for each construct was confirmed by sequence analysis. Subsequently, the complementation plasmids were introduced into the Δ*tagE5E6* strain by electroporation as described previously [49]. Transformants were screened for chloramphenicol- and erythromycin-resistance, followed by PCR amplifications to confirm the chromosomal integration of introduced plasmid using primers tRNA/IC021 for NZ8204 and NZ8205, and tRNA/IC020 for NZ8206.

Preparation of surface proteins, whole cell extracts and proteinase K treatment. Overnight cultures of *L. plantarum* strains were diluted in fresh MRS broth to an optical density at 600 nm (OD_{600}) of 0.1. After 5 hours of incubation at 37°C (OD_{600} of approximately 1.0), the exact OD_{600} of the cultures were determined and cells were harvested by centrifugation at 3000 × g for 10 min at 4°C. For surface protein isolation, a procedure adapted from Fredriksen *et al*. [41] was used. Briefly, harvested cells were washed once with PBS to remove residual medium and resuspended in 1 ml cold PBS. Surface proteins were extracted by a gentle agitation at 600 rpm for 30 min using an eppendorf thermomixer (Eppendorf, Hamburg, Germany). The supernatants were collected after centrifugation at 5,000 × *g* for 10 min. The surface proteins were precipitated from supernatants by adding trichloroacetic acid (TCA) to a final concentration of 16% and an overnight incubation at 4°C, followed by centrifugation at 16,000 × *g* for 15 min. The precipitated proteins were washed with 200 µl acetone and then air-dried with open lids at 50°C. Dried protein pellets were solubilized in NuPAGE loading buffer and reducing agent (both from Invitrogen). The NuPAGE buffer volumes were normalized by OD_{600} measurement of original cultures to ensure the samples represent the surface proteins from similar amounts of cells and these samples were subsequently used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Coomassie brilliant blue staining [48].

For whole cell extract samples, harvested cells were washed once 50 mM Sodium-phosphate buffer, pH 7 to remove residual medium and subsequently resuspended in 50 mM Sodium-phosphate buffer, pH 7 to a final OD_{600} equivalent of 2.5. Cell suspensions of 1 ml were added to a screw-cap 2 ml tube containing 1 g zirconium beads. Cells were disrupted by three rounds of bead-beating (30 s at speed 4.0) using a Fastprep cell disrupter (QBiogene Inc., Cedex, France), interspaced with cooling intervals on ice. The tubes were left for 5 min to allow zirconium bead sedimentation. The resulting supernatants were collected as whole cell extracts and used in sWGA lectin blot experiments.

For proteinase K treatment, the whole cell extract samples were treated with proteinase K (QIAGEN GmbH, Hilden, Germany; final concentration of 50 µg/ml) for 10, 30, or 60 min at 37°C.

SDS-PAGE and lectin blot analyses. SDS-PAGE and wet blotting were performed using

the NuPAGE® electrophoresis system (Invitrogen) and XCell II™ Blot Module (Invitrogen), respectively, as described in the user manuals. Whole cell extracts were mixed with NuPAGE sample buffer and were separated under denaturing condition on NuPAGE®Novex® 4-12% Bis-Tris gels with MOPS SDS running buffer (Invitrogen).

For visualization of surface proteins by Coomassie brilliant blue, the standard procedure was used [48]. For lectin blotting, the gels were transferred to nitrocellulose membranes (Thermo Scientific, Bremen, Germany) using wet blotting method described in the NuPAGE manual (Invitrogen). The membranes were blocked with 3% bovine serum albumin (BSA) in PBS containing 0.05% Tween20 (PBST) for 1 hour at room temperature. The membranes were then incubated with a biotinylated succinylated wheat germ agglutinin (sWGA; Vector Labs, Burlingame, USA, final concentration of 14.3 μg/ml), *Dolichos biflorus* lectin (Sigma-Aldrich, Zwijndrecht, The Netherlands, final concentration of 14.3 μg/ml) or *Lens culinaris* lectin (EY Labs Inc., San Mateo, USA, final concentration of 5 μg/ml) in the blocking solution, followed by incubation with 0.1 nl/ml (1:10000 dilution) of streptavidin poly-horse radish peroxidase (poly-HRP; ImmunoTools GmbH, Friesoythe, Germany). In between the incubations, the membranes were washed three times with PBST for 15 min. Precision Plus Protein™ Dual Color Standards (Bio-Rad, Richmond, USA) was used as a reference of molecular size. RNase B (New England BioLabs, Ipswich, USA) was used as a positive control for sWGA blotting. After the membranes were washed, they were developed by using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) and Kodak BioMax Light film (Kodak, Rochester, USA).

Mass spectrometry. The protein bands apparent around 100kDa were excised from a Commassie blue stained gel (see above), followed by characterization of the glycosylation pattern using the same method as described by Rolain *et al*. [42]. Briefly, the protein was in-gel digested with trypsin (Promega) for 16 h at 37°C. Digested peptides were recovered and vacuum dried (Speedvac SC200, Savant). Peptides were then dissolved in 0.025% (v/v) trifluoroacetic acid (TFA) and 5% (v/v) acetonitrile (ACN) and desalted using a C18 Pep Map 100 pre-column (10 mm, 5 μm i.d., 100 Å), and subsequently subjected to reverse phase chromatography using Ultimate 3000 chromatography chain (LC Packings) with a C18 Pep Map 100 analytical column (150 mm, 3 μm i.d., 100 Å). Peptides were back-flushed onto the analytical column with a flow rate of 300 nl/min using a 180 min linear gradient from 8 to 76% (v/v) ACN in water containing 0.1% (v/v) TFA in 4% ACN-0.1% TFA and 0.085% (v/v) TFA in 80% ACN-0.1% TFA. The eluted peptides were mixed with a-cyano-4-hydrocinnamic acid (4 mg/ml in 70% ACN-0.1% TFA) and spotted directly onto a MALDI target using a Probot system (LC Packings). The spotted plates was analyzed in reflector mode on an Applied Biosystems 4800 MALDI TOF/TOF Analyzer using a 200 Hz solid state laser operating at 355 nm. MS spectra were obtained using a laser intensity of 3600 and 2000 laser shots per spot in the *m/z* range of 800 to 4000, while MS/MS spectra were obtained by automatic selection of the 20 most intense precursor ions per spot using a laser intensity of 4000 and 2000 laser shots per precursor. Collision induced dissociation was performed with an energy of 1 kV with air gas at a pressure of 1 x 10^6 Torr. Data were collected using the Applied Biosystems 4000 Series ExplorerTM Software. LC-MS/MS data were processed using the Applied Biosystems GPS ExplorerTM 3.6 Software.

For peptide identification, a local database containing Acm2 sequence was used with the tolerance was set to 200 ppm on the precursors and 0.3 Da on the fragments. One trypsin miscleavage was

authorized. For modifications, methionine oxidation and Hex*N*Ac glycosylation (203.08 Da) on Ser, Thr and Asn were selected. Hex*N*Ac-modified peptides were checked by manual de novo sequencing on the MS/MS fragmentation spectra. The data presented for WT were combined of 2 independent analyses, while for tagE5E6 deletion mutant were combined of 3 independent analyses.

Results

Comparative genomics and mutagenesis of candidate protein glycosyl tranferases

Acm2 of *Lactobacillus plantarum* WCFS1 was previously established to be *O*-glycosylated with *N*-acetylhexosamines (Hex*N*Ac), most likely Glc*N*Ac, at multiple positions in its AST domain [41, 42]. Glycosylation with Glc*N*Ac was also found in flagellin of *Listeria monocytogenes* [35] and Fap1 of *S. parasanguinis* [37, 38]. The glycosylation with Glc*N*Ac moieties in the latter species requires two genetically coupled functions, Gtf1 and Gtf2 [37]. Bu *et al*. suggested that Gtf1 catalyzes Glc*N*Ac glycosylation via its C-terminal glycosyltransferase domain, while Gtf2 might act as a chaperon to maintain correct folding of Gtf1 and to promote efficient glycosylation [37, 53]. Based on these previous findings, we performed a BlastP analysis [54, 55] using the Gtf1 sequence to identify candidate protein-glycosyltransferases in the *L. plantarum* WCFS1 genome. Six genes (*tagE1* to *tagE6*), which are annotated as poly(glycerol-phosphate) α-glucosyltransferases, and consequently are thought to be involved in teichoic acid glycosylation [46], appeared the closest homologues of the Gtf1 protein and all share more than 20% sequence identity with Gtf1. Two pairs of *tagE* genes are genetically coupled in the *L. plantarum* chromosome (*tagE2-tagE3* and *tagE5-tagE6)* (Figure 1). Therefore, all 6 *tagE* genes identified were targeted by gene deletion, with the notion that the genetically coupled *tagE* pairs were deleted jointly. This genetic engineering approach yielded four *L. plantarum* WCFS1 derivatives, NZ3540Cm (Δ*tagE1*), NZ3541Cm (Δ*tagE2E3*), NZ3542Cm (Δ*tagE4*), and NZ3543Cm (Δ*tagE5E6*).

Deletion of tagE5 and tagE6 abolishes protein glycosylation in L. plantarum *WCFS1*

Surface proteins derived from the *tagE* deletion mutants, as well as the wild type and *acm2* deletion mutant [41] were analyzed by SDS-PAGE. One protein band detected in the wild type extract appeared to be absent in the sample derived from the *acm2* deletion derivative, suggesting this protein band represents Acm2 (Figure 2A). To substantiate this suggestion, the band representing Acm2 was excised, in-gel digested with trypsin, and the resulting peptides were extracted and subjected to liquid chromatography coupled to mass spectrometry analysis (LC-MS/MS), which generated MS-spectra that represented 75% and 69% coverage of the mature Acm2 protein sequence and its N-terminal glycosylated AST domain, respectively (Figure S1A). Importantly, the MS spectra detected 5 different glycopeptides (designated glyco I, II, IV, V and VI, according to nomenclature introduced by Rolain *et al.* [42]; Table 3) that appeared all located in the AST domain and to be one-, two-, or three-fold substituted with a molecule of an approximate mass of 203 Da, which corresponds to the previously suggested glycosylation with Glc*N*Ac [41, 42, 45] (Table 3 and Table S1). This observation is in agreement with the apparent molecular weight of wild type Acm2 that was estimated to be approximately 100 kDa (Figure 2), which is higher than

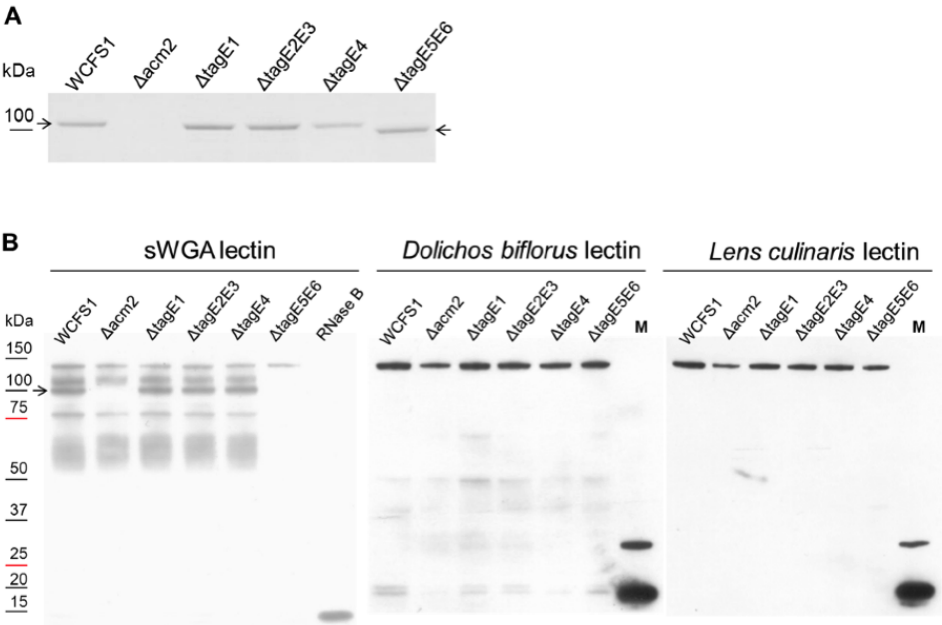

Figure 2. (A) Coomassie brilliant blue stained SDS-PAGE of surface proteins extracted from the *tagE* deletion mutants as well as from wild type and *acm2* deletion mutant to detect Acm2 (indicated by the arrow). (B) Succinylated wheat germ agglutinin (sWGA), *Dolichos biflorus* lectin, and *Lens culinaris* lectin blots of whole cell extracts derived from the *tagE* deletion mutants, *Lactobacillus plantarum* WCFS1 (wild type) and the *acm2* deletion mutant to assess glycan moieties. On the left side of the blot the protein size (kDa) are indicated based on the Precision Plus Protein™ Dual Color Standards (Bio-Rad) molecular marker (data not shown). The arrow indicates Acm2.

the predicted molecular weight of 78.9 kDa on basis of the mature protein sequence. Moreover, we were able to identified some of the glycosylated residues (Table 3 and Table S1), but not all. These glycosylated residues are all also found in the study of Rolain *et al.* [42]. Interestingly, different glycosylated forms were found in peptide Glyco II (one or two Hex*N*Ac) and Glyco VI (one, two or three Hex*N*Ac), which might imply a dynamic level of glycosylation in Acm2. In the Δ*tagE1*, Δ*tagE2E3*, and Δ*tagE4* mutants, the mobility of the Acm2 protein appeared to be unaffected as compared to the wild-type strain. By contrast, the Acm2 protein present in the wild type was absent in the Δ*tagE5E6* deletion strain but a protein band of higher mobility (lower apparent molecular weight) appeared in the gel (Figure 2A). These observations provide a first clue that TagE5 and/or TagE6 are involved in the glycosylation of Acm2. Indeed, the loss of glycosylation of Acm2 in the Δ*tagE5E6* deletion strain could also be confirmed by LC-MS/MS, as the Acm2 protein band extracted from gel was used to generate MS-spectra that enabled 52% and 59% coverage of the mature Acm2 protein sequence and its AST domain, respectively (Figure S1B). Notably, 6 distinct peptides of the AST domain that contained proposed Hex*N*Ac glycosylations [42] (designated glyco II to VII; Table 3) that were detected in the wild type Acm2 protein spectra (glyco II, IV, V and VI in this work and glyco VII in the work of Rolain et al. [42]) were also detected in the Acm2 protein spectra derived from the *tagE5E6* deletion strain, albeit, in the latter strain these peptides consistently lacked the substitutions seen in the wild type (Table

Table 3. Numbers of HexNAc on trypsinized Acm2 peptides isolated from wild-type (WT) or TagE5E6 deletion mutant (ΔtagE5E6)

			WT			ΔtagE5E6		
Name[a]	Peptide sequence[b]	Calculated $[M+H]^{+}$[c]	Number of HexNAc[d]	Observed *m/z* WT	Δm (Da) Wt[e]	Number of HexNAc	Observed *m/z* WT	Δm (Da) ΔtagE5E6[e]
Glyco I	GNSAASAASQQVTLSAGSQTETTA-AGATDQSVASDGAK	3495.62	2	3901.25	405.63	ND	/	/
Glyco II	TDDQAESTSTTTATTSATSR	2030.89	0	2031.62	0.73	0	2031.67	0.78
Glyco II	TDDQAESTSTTTATTSATSR	2030.89	1	2234.68	203.79			
Glyco II	TDDQAESTSTTTATTSATSR	2030.89	2	2437.74	406.85			
Glyco III	ADSTGPQSQSSASEAAK	1620.72	ND	/	/	0	1621.55	0.83
Glyco IV	DNAATSATADSTTSAVDQLDK	2080.94	2	2487.75	406.81	ND	/	/
Glyco V	ASAATSQASHSTTNETAK	1761.81	2	2168.75	406.94	0	1762.63	0.82
Glyco VI	ASAAASQDSHVTTDQSSVTVTSEVAK	2576.22	0	2576.86	0.64	0	2576.9	0.68
Glyco VI	ASAAASQDSHVTTDQSSVTVTSEVAK	2576.22	1	2779.89	203.67			
Glyco VI	ASAAASQDSHVTTDQSSVTVTSEVAK	2576.22	2	2982.94	406.72			
Glyco VI	ASAAASQDSHVTTDQSSVTVTSEVAK	2576.22	3	3186	609.78			

[a] Glycopeptide number as reported in JBC

[b] The glycosylated amino-acids that could be identified are displayed in grey

[c] Calculated [M+H]+ values correspond to non-glycosylated peptides

[d] Number of HexNAc detected or not (ND, not detected) of peptides by LC MSMS from secreted Acm2 digested by trypsin

[e] Δm, difference between calculated and observed m/z values

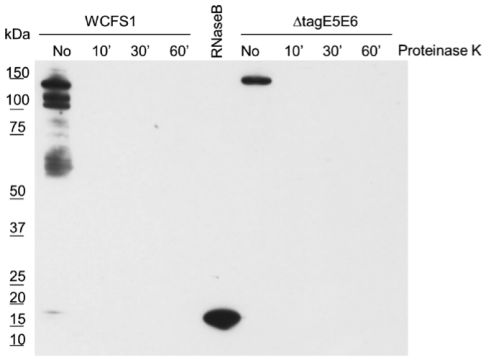

Figure 3. The sWGA blot of whole cell extracts derived from wild type and *tagE5E6* deletion mutants with or without proteinase K treatment for 10, 30, or 60 min. On the left side of the blot the protein size (kDa) are indicated based on the Precision Plus Protein™ Dual Color Standards (Bio-Rad) molecular marker.

3 and Table S1). In addition, three peptides (glyco III, IV, and V) were exclusively detected in their non-glycosylated form in Acm2 isolated from the Δ*tagE5E6* mutant (Table 3). These data reveal that the Hex*N*Ac-glycosylated peptides derived from Acm2 are only detected in their non-glycosylated form in the Δ*tagE5E6* mutant (Table 3), supporting the role of TagE5E6 in the Acm2 glycosylation that is observed in the wild type strain.

To further investigate this, we employed a lectin-based detection of glycan moieties using biotinylated sWGA, specific for Glc*N*Ac, in a Western-blot like set up. This approach showed that an sWGA-recognized protein of approximately 100 kDa derived from the wild type was absent in the *acm2* deletion strain, reconfirming the glycosylation of Acm2. Moreover, the glycans linked to these proteins only react with the Glc*N*Ac-specific lectin sWGA but not with *Dolichos biflorus* lectin (specific for α-Gal*N*Ac) nor with *Lens culinaris* lectin (specific for α-mannose) (Figure 2B), implying the glycan is most likely Glc*N*Ac. Interestingly, the sWGA blot revealed signals other than Acm2 that appeared to be glycosylated that were detected in both wild type- and *acm2* mutant-derived whole cell extracts. All these signals were lost when sWGA blotting experiments were performed using samples that were proteinase K treated, indicating that all glycan signals in the lectin blotting experiment were derived from proteinaceous molecules (Figure 3). In addition, the sWGA blot revealed that deletion of *tagE1*, *tagE2E3*, or *tagE4* did not affect protein glycosylation, since these mutants displayed the same banding pattern as was observed for the wild type strain. By contrast, deletion of *tagE5* and *tagE6* abolished almost all detectable sWGA-specific signals, including that of Acm2, indicating that TagE5 and TagE6 play a critical role in the glycosylation of Acm2 and the additional proteins detected. Intriguingly, a single band of an apparent molecular weight of approximately 125kDa appeared not only unaffected by the Δ*tagE5E6* mutation but also recognized by *Dolichos biflorus* and *Lens culinaris* lectins (Figure 2B), implying that another, TagE5/E6-independent mechanism of glycosylation may be active for the glycosylation of this particular protein. Taken together, these results evidence the essential role of TagE5 and/or TagE6 for protein glycosylation in *L. plantarum* WCFS1.

Both TagE5 and TagE6 are required for protein glycosylation in L. plantarum *WCFS1*

To investigate whether TagE5, TagE6 or their concerted action is required for protein glycosylation in *L. plantarum* WCFS1, the Δ*tagE5E6* mutant was complemented with *tagE5, tagE6* or both genes.

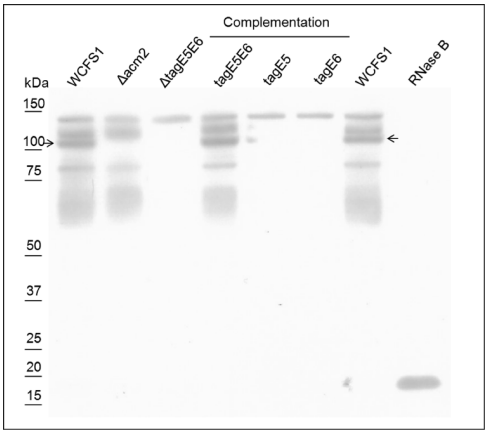

Figure 4. The sWGA blot of wild type, *acm2* and *tagE5E6* deletion mutants, and a panel of complemented mutants. On the left side of the blot the protein size (kDa) are indicated based on the Precision Plus Protein™ Dual Color Standards (Bio-Rad) molecular marker (data not shown). The arrow indicates Acm2.

Complementations were achieved by integrating a single copy of the original gene(s) at a specific chromosomal site located downstream of the $tRNA^{Ser}$ locus, under control of the native *tagE6*-promoter. Whole cell extracts from the different complemented Δ*tagE5E6* derivatives were analyzed by SDS-PAGE and sWGA blotting, and compared to extracts derived from the wild type, as well as the Δ*acm2* and Δ*tagE5E6* mutants. Complementation with either *tagE5* or *tagE6* did not restore protein glycosylation and generated the same banding patterns as observed for the Δ*tagE5E6* strain (Figure 4). However, complementation with the complete locus, encompassing both *tagE5* and *tagE6*, restored not only the glycosylation of Acm2 but also of all other proteins that were detected in the wild type banding pattern. These results indicate that glycosylation of proteins in *L. plantarum* WCFS1 requires both TagE5 and TagE6 activities.

5

Protein glycosylation is a common feature in L. plantarum *strains*

Using previously generated comparative genome hybridization (CGH) data for 42 *L. plantarum* strains [56, 57], we concluded that the 6 *tagE* genes recognized in the genome of *L. plantarum* WCFS1 appear to be conserved among all these 42 strains, with the notable exception of strain NCIMB12120 that appeared to lack genes that hybridize to the *L. plantarum* WCFS1 *tagE4*, *tagE5*, and *tagE6* probes. To evaluate glycosylation of proteins in other *L. plantarum* strains, 9 of the 42 mentioned strains were selected, including NCIMB12120 and LP85-2 from the subspecies *argentoratensis*, for analysis of whole cell extracts by SDS-PAGE and Glc*N*Ac-specific sWGA blotting. Notably, these 9 strains were selected to maximize the coverage of the phylogenetic tree based on the whole genome comparative genome hybridization datasets [56], as well as to include strains isolated from diverse niches (Table 1). All selected strains, including NCIMB12120, displayed sWGA-recognized glycosylated proteins that displayed similar banding pattern on SDS-PAGE gels (Figure 5). This result strongly suggests that glycosylation of proteins is a common feature in the species *L. plantarum*.

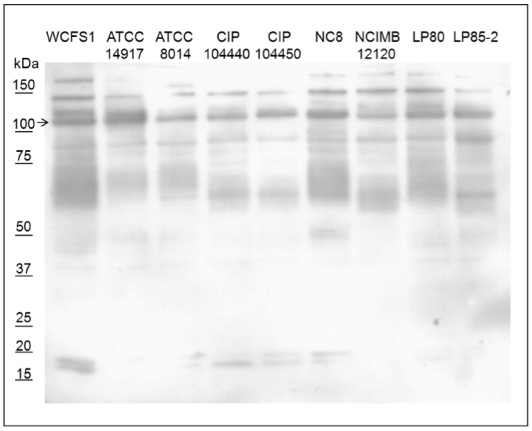

Figure 5. Assessment of 9 *Lactobacillus plantarum* strains for protein glycosylation, using whole cell extract SDS PAGE and sWGA lectin-based detection. On the left side of the blot the protein size (kDa) are indicated based on the Precision Plus Protein™ Dual Color Standards (Bio-Rad) molecular marker (data not shown). The arrow indicates Acm2 of strain WCFS1.

Discussion

Existing information on the protein glycosyltransferase Gtf1 in *S. parasanguinis* [37, 38, 41], enabled us to employ a comparative genomics approach, resulting in the identification of the 6 TagE orthologues as candidate protein glycosyltransferases in *L. plantarum* WCFS1. All 6 TagE proteins contain a GT1_gtfA_like domain designated cd04949 [58] at their C-terminal ends [46]. This domain is named after *gtfA* in *Streptococcus gordonii*, where it plays a role in the *O*-linked glycosylation and this family containing this domain is most closely related to GT1 family of glycosyltransferases [58]. Limiting the amount of glycosyltransferases (50 annotated in the *L. plantarum* WCFS1 genome [46]) to the six TagE glycosyltransferases as most likely candidates for protein glycosylation, enabled us to perform a systematic gene deletion and complementation strategy, followed by assessment of the consequences for protein glycosylation. This approach revealed that the concerted activity of TagE5 and TagE6 is required for the previously established glycosylation of Acm2 [41, 42], as well as other proteins. To the best of our knowledge, these results represent the first example of a protein glycosylation machinery in a *Lactobacillus* species. The TagE proteins of *L. plantarum* WCFS1 are annotated according to their originally predicted function in teichoic acid glycosylation (poly[glycerol-phosphate]-α-glucosyltransferases). The glucose substitution levels in lipoteichoic acids (LTA) of *L. plantarum* WCFS1 are very low [59, 60], while glucose is a backbone constituent into the repeating unit of wall teichoic acids (WTA) that does not have additional glucose substitutions in this strain [60, 61]. Neither LTA nor WTA of *L. plantarum* WCFS1 contains *N*-acetylhexosamine [59, 61], the glycan transferred by TagE5 and TagE6. Since the glucose substitution level is very low in LTA from *L. plantarum* WCFS1, we have isolated deacylated and dealanylated LTA (dd-LTA) to obtain better nuclear magnetic resonance (NMR) spectral resolution for signals from anomeric protons of sugar residues. The 1-D ^{1}H NMR spectra revealed that dd-LTA isolated from Δ*tagE5E6* mutants has the same level of glycosylation as LTA isolated from WT (Figure S2A). Moreover, 2-D NMR spectra also showed that the glycosylation position of LTA is unaltered in the *tagE5E6* deletion mutant (Figure S2B). Therefore, with the establishment of TagE5 and TagE6 as dedicated protein glycosyltransferases we propose to rename these enzymes (and genes) to GtfA (*gtfA*) and GtfB (*gtfB*), respectively.

Currently established bacterial *O*-linked glycosylation pathways employ either block or sequential transfer pathways for the addition of sugars to proteins [26]. The block transfer pathway is exemplified by the glycosylation of *Neisseria* spp. pilin. This pathway assembles an oligosaccharide using nucleotide-activated sugars on a lipid anchor at the cytoplasmic side of the inner membrane. The assembled oligosaccharide is subsequently translocated across the inner membrane by a flippase to the periplasm, where the lipid-linked oligosaccharide is transferred to Ser/Thr residues of proteins [26, 33]. On the other hand, the sequential transfer pathway, for example employed in flagellar glycosylation of *Campylobacter jejuni,* transfers nucleotide-activated sugars individually onto Ser/Thr residues of proteins at the cytoplasm-inner membrane interface [26]. Acm2 of *L. plantarum* WCFS1 undergoes cytoplasmic *O*-glycosylation with single *N*-acetylhexosamine moieties, likely Glc*N*Ac, at multiple sites of its AST domain [41, 42]. The fact that this glycosylation occurs in the cytoplasm might imply that the machinery responsible for Acm2 glycosylation is more similar to the sequential transfer pathway. Moreover, the glycosylation nature of Acm2 is similar to the glycosylation found in flagellin from *L. monocytogenes*, which is glycosylated with single Glc*N*Ac at 3 to 6 sites [35], and in Fap1 fimbrial adhesin from *S. parasanguinis*, of which all the oligosaccharides are primed with Glc*N*Ac [37]. Interestingly, since the glycosyltransferases responsible for their glycosylations (GmaR for listerial flagellin [62] and Gtf1/Gtf2 for Fap1 [37, 53]) are predicted to be cytoplasmic proteins, the glycosylation of flagellin from *L. monocytogenes* and Fap1 from *S. parasanguinis* are also thought to occur in the cytoplasm. Notably, another example of a *Lactobacillus* glycoprotein, Msp1 of *L. rhamnosus* GG, was found to be glycosylated in the supernatant but not in the cytosolic fraction [43], hinting towards species-specific *O*-glycosylation pathways in *Lactobacillus* species.

We have successfully identified the glycosyltransferases GtfA/B for the glycosylation of Acm2 based on the similar glycan moieties found in Fap1. However, the protein property and function is completely different between Acm2 and Fap1. Fap1 belongs to serine-rich repeat proteins (SRRPs), which are a family of surface exposed adhesion-mediated proteins predominately found within the oral *Streptococcus* species [63]. Currently, seven SRRPs have been researched, including Fap1 of *S. parasanguinis*, Has and GspB of *S. gordonii*, PsrP of *Streptococcus pneumonia*, Srr-1 and Srr-2 of *Streptococcus agalactiae*, and SraP of *Staphylococcus aureus* [53]. Each SRRP locus locates at a close proximity with a highly conserved core region, consisting of accessory secretory components and two essential glycosyltransferases [38]. In this work, we found GtfA and GtfB are required for the glycosylation of Acm2 as well as other unidentified proteins other than SRRPs family. Moreover, *acm2* (*lp_2645*) locates in a distinct region of the chromosome, not linked to the *gtfA/B* (*lp_2843/lp_2844*) genes (Figure 1), which is also distinct from *Streptococcus* SRRPs. Recently, the two glycosyltransferases, Gtf1 and Gtf2, of *S. parasanguinis* have been investigated and it was found that the glycosylation of Fap1 requires the glycosyltransferase activity from Gtf1 together with the chaperon function of Gtf2 to maintain the correct folding of Gtf1 [53]. However, GtfA and GtfB in *L. plantarum* are both homologs of Gtf1 in *S. parasanguinis* and display much lower similarity with the chaperon Gtf2. Although we have not experimentally excluded the possibility that the co-expression of GtfA or GtfB of *L. plantarum* WCFS1 is required for the correct folding of GtfB or GtfA, respectively, it does not seem likely that either GtfA or GtfB acts as a chaperon.

Comparative genome hybridization (CGH) data suggests that 6 orthologous of *tagE* genes are typically present in *L. plantarum* strains [56], with the notable exception of strain NCIMB12120 that appears to lack *tagE4* and *gtfA-gtfB*. However, this strain still contains sWGA recognized,

glycosylated proteins, similar to the other 7 strains tested (Figure 5). NCIMB12120 belongs to a subspecies (*argentoratensis*) different from the reference strain WCFS1. Strains in this subspecies commonly have a smaller genome size [64] and appear to lack homologues of approximately 20% of the genes present in WCFS1 [57]. Despite the apparent absence of *tagE4* and *gtfA-gtfB* in NCIMB12120, the glycosylation of proteins apparently still occurs, suggesting that this strain (subspecies) encodes genes with the same function that are of low homology and therefore were missed in the CGH analysis. Taken together, our data suggests that glycosylation as well as the presence of *tagE* genes are common features in *L. plantarum* strains. Moreover, the sequence and length of Acm2 is highly similar in all sequenced *L. plantarum* strains, e.g. WCFS1 [46], ST-III [65] JDM1 [66] and NC8 [67] (785 residues) and ATCC14917 (781 residues). This suggests that the Acm2 protein of different *L. plantarum* strains may all have similar sizes as well as similar degrees of glycosylation and are represented by the universal abundant protein band around 100kDa (Figure 5).

Other *Lactobacillus* species also harbor genetically coupled *gtfA-gtfB* homologues, for example, *tagE2-tagE3* of *Lactobacillus casei* BL23, *lsei_0891-lsei_0892* of *L. casei* ATCC334 [68] and *yohH-yohJ* of *L. rhamnosus* GG [69]. However, the genomes of other species, including *Lactobacillus acidophilus* NCFM [70], do not appear to contain *gtfA-gtfB* homologues, while *Lactobacillus johnsonii* NCC533 [71] and *Lactobacillus delbrueckii* supsp. *bulgaricus* ND02 [72] harbor a single gene displaying similarity with *gtfA-gtfB*. Although we successfully identified the role of GtfA-GtfB in glycosylation of proteins based on their sequence homology with Gtf1 of *S. parasanguinis*, sequence similarity alone did not provide a direct identification of this specific glycosyltransferase function, since all 6 TagE proteins display a similar degree of sequence homology with Gtf1. The role of the other 4 TagE glycosyltransferases in *L. plantarum* WCFS1 is currently unestablished but might involve the transfer of other glycan moieties to proteins or *N*-glycosylation. Indeed, among recently found glycoproteins in *L. plantarum* WCFS1, glycosylation of hexoses was also found in Lp_2162, Lp_3421 and DnaK, besides the Hex*N*Ac substitutions already established for Acm2 [45]. Moreover, some lectin-based studies suggested the presence of glycoproteins modified with glycans other than Glc*N*Ac, such as glycoproteins of *L. acidophilus* JCM1132^{T} (recognized by β-galctoside-specific lectin)[73], SlpA of *L. acidophilus* NCFM (recognized by fucose- and mannose-specific lectins)[19, 74], and Msp1 of *L. rhamnosus* GG (recognized by glucose- and mannose-specific ConA lectin)[43]. Furthermore, many *Lactobacillus* genomes encode the genes to produce multiple nucleotide-activated sugars, including UDP-glucose, UDP-galactose, sialic acid and dTDP-rhamnose [6], suggesting the potential capacity to glycosylate proteins with diverse sugar moieties. Alternatively, WTA of *L. plantarum* WCFS1 contains glucose in its backbone [61] and biosynthesis of this structure could require the activity of specific TagE proteins, as predicted by the current annotation.

We have conclusively shown that protein glycosylation is a common feature in *L. plantarum* strains and is not targeting a single protein, but modifies a much broader range of proteinaceous compounds. One important question remains unanswered: what is the biological role of protein glycosylation in lactobacilli? Earlier studies in pathogens showed that glycoproteins are often involved in adherence, pathogenicity, flagella assembly and protein stability [23]. A more recent example illustrated that the glycans attached on surface layer proteins of *Tannerella forsythia*, which is implicated in periodontitis, modulate the function of DCs and suppress T-helper 17 responses [75]. To this end, it is intriguing that glycosylation of Msp1 of *L. rhamnosus* GG is not essential for

its peptidoglycan hydrolyase activity [17, 43], neither for activating Akt signaling in Caco-2 cells [43], but does influence Msp1 protein stability and protein localization [43]. Moreover, Lebeer *et al*. suggested the possibility of an indirect modulating role of the Msp1 glycan moieties in Akt activation via shielding bacteria and host interaction [43]. Furthermore, the ConA and *Aleuria aurantia* (AAL)-reactive glycans on SlpA of *L. acidophilus* NCFM is essential for the modulation of T cell function and led to more IL-4 production [19]. Importantly, it was recently established that *O*-glycosylation of Acm2 in *L. plantarum* functions as a major negative modulator of Acm2 peptidoglycan hydrolase activity [42], which is the first evidence that glycosylation regulates the bacterial enzyme activity. In fact, we observed different glycoforms of GlycoII and GlycoVI (Table 3), which might imply a kinetic modulation of Acm2 hydrolase activity via *O*-glycosylation [42]. Our future work will focus on recognizing the biological roles of glycosylation of other proteins in *L. plantarum*, especially in relation to its possible consequences for host-microbe interactions in the gastrointestinal tract.

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Supplemental Material

Materials and methods

Bacterial strains and growth conditions. *Lactobacillus plantarum* WCFS1 (wild-type) and *tagE* deletion mutants (see Table 1) were cultured in 20 L MRS broth (Merck, Darmstadt, Germany) at 37°C for 20 hours using a Techfors-S bioreactor (Infors, Bottmingen, Switzerland) at a constant pH of 6.8 by titration of 6M NaOH with a built-in peristaltic pump and stirrer at 100 rpm. The cells were collected by centrifugation using an AVANTI J-25 centrifuge (Beckman-Coulter, Brea, USA) at 9000 × g at room temperature and were subsequently washed once with 200 ml of phosphate buffer saline.

Preparation of lipoteichoic acid. Lipoteichoic acid (LTA) of the cells cultured was isolated and purified by butanol extraction, hydrophobic interaction chromatography (HIC), and anion-exchange chromatography as described by Morath *et al*. [1]. Briefly, the cells were disrupted by a French press cell (SLM Instruments Inc., Urbana, USA) and supernatants after centrifugation (23000 × g for 30 min at 4°C) were collected by pipetting. Subsequently, the supernatants were extracted with the same volume of butanol for 30 min at room temperature and the water phase was collected and lyophilized after centrifugation at 23000 × g for 30 min at room temperature. The lyophilized fraction was then separated on a Octyl-Sepharose 4 FastFlow column (GE Healthcare, Little Chalfont, UK) with a stepwise elution of 13-31% 1-propanol in 100 mM sodium acetate buffer (pH 4.7). LTA in the fractions was detected by ashing of organic phosphate [2] as described by Allen R. J. [3]. For 2D NMR analysis Δ*tagE5E6*-derived LTA was subsequently purified on a DEAE-Sepharose 4 FastFlow column (GE Healthcare) with a linear gradient elution of 0-1.0 M sodium chloride in sodium acetate buffer by using an AKTA FPLC system (GE Healthcare). LTA in the separated fractions were collected by lyophilisation after dialysis against water.

Preparation of deacylated and dealanylated LTA. To improve NMR spectral resolution for signals from anomeric protons of sugar residues, deacylated and dealanylated LTA (dd-LTA) were prepared as described by Simpson *et al*. [4]. Briefly, approximately 10 mg of the isolates of the HIC analyses were dissolved in 250 µl water, mixed with the same volume of 30% ammonium hydroxide, and incubated overnight at room temperature. The mixtures were extracted with the same volume of chloroform-methanol (1:1, v/v) once, followed by two chloroform extractions. dd-LTA in the solution was collected by lyophilisation.

NMR spectroscopic analysis of LTA from Δ*tagE5E6* mutant. LTA from HIC fractions and their dd-LTA were dissolved in 400 µl of D_2O and used for measurement of 1D NMR spectra. Purified LTA from Δ*tagE5E6* was also used for measurement of 2D NMR spectra. The 1D (^{1}H and ^{13}C) and 2D NMR spectra (COSY, H-H correlation spectroscopy; TOCSY, H-H totally correlated spectroscopy; HSQC, H-C hetero-nuclear single quantum coherence; HMBC, H-C hetero-nuclear multiple-bond connectivity) were recorded on an AVANCE III 500 MHz NMR spectrometer (Bruker Daltonics, Bremen, Germany) at 333 K with proton and carbon frequencies of 500.13 MHz and 125.77 MHz. The 2D NMR spectra were acquired with 512 increments of 8 scans for COSY, 512 increments of 16 scans for TOCSY and HSQC, and 1024 increments of 96 scans for HMBC, respectively. Sodium 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (Sigma-Aldrich, Zwijndrecht, The Netherlands) was used as an internal reference for chemical shift (δ_H 0.00 and δ_C 0.00).

Table S1. List of detected Acm2 peptides

Lactobacillus plantarum WCFS1 WT

Start - End	Observed m/z	Mr (expected)	Mr (calculated)	ΔMr (Da)	Miss	Sequence[a]	Ions score*
42 - 79	3902.26	3901.25	3901.78	-0.53	0	K.GNSAASAASQQVTL-SAGSQTETTAAGATD-QSVASDGAK.T; **2 HexNAc**	10
80 - 99	2031.62	2030.61	2030.89	-0.28	0	K.TDDQAEST-STTTATTSATSR.V	112
80 - 99	2234.68	2233.68	2233.97	-0.29	0	K.TDDQAEST-STTTATTSATSR.V; 1 **HexNAc**	46
80 - 99	2437.74	2436.73	2437.05	-0.32	0	K.TDDQAEST-STTTATTSATSR.V; **2 HexNAc**	92
128 - 148	2487.75	2486.74	2487.10	-0.36	0	K.DNAATSATADSTTSAVD-QLDK.T; **2 HexNAc**	44
152 - 169	2168.75	2167.74	2167.97	-0.23	0	K.ASAATSQASHSTTNE-TAK.A; **2 HexNAc**	17
170 - 195	2576.86	2575.85	2576.22	-0.37	0	K.ASAAASQDSHVTTD-QSSVTVTSEVAK.S	17
170 - 195	2779.89	2778.89	2779.30	-0.41	0	K.ASAAASQDSHVTTD-QSSVTVTSEVAK.S; 1 **HexNAc**	20
170 - 195	2982.91	2981.90	2982.38	-0.48	0	K.ASAAASQDSHVTTD-QSSVTVTSEVAK.S; **2 HexNAc**	38
170 - 195	2982.94	2981.93	2982.38	-0.44	0	K.ASAAASQDSHVTTD-QSSVTVTSEVAK.S; **2 HexNAc**	50
170 - 195	3185.99	3184.98	3185.46	-0.48	0	K.ASAAASQDSHVTTD-QSSVTVTSEVAK.S; **3 HexNAc**	19
170 - 195	3186.00	3184.99	3185.46	-0.47	0	K.ASAAASQDSHVTTD-QSSVTVTSEVAK.S; **3 HexNAc**	29
219 - 236	1821.68	1820.67	1820.88	-0.20	0	K.IETAVAADAVQSSAM-MAR.S	31
240 - 252	1495.59	1494.58	1494.78	-0.20	0	R.AMTSQEIFLSQIK.A	55
240 - 252	1511.58	1510.57	1510.77	-0.20	0	R.AMTSQEIFLSQIK.A Oxidation (M)	34
240 - 252	1511.58	1510.58	1510.77	-0.19	0	R.AMTSQEIFLSQIK.A Oxidation (M)	38
253 - 261	903.36	902.36	902.46	-0.11	0	K.AGAISGWNK.Y	73
262 - 295	3589.36	3588.35	3588.86	-0.51	0	K.YQVLPSVTAAQAILES-GWGQSQLATQGNNLF-GIK.G	34
296 - 323	3284.08	3283.07	3283.52	-0.45	0	K.GSYQGQSIY-FPTQEWNGSQYITIQDA-FR.K	20
296 - 323	3285.07	3284.06	3283.52	0.54	0	K.GSYQGQSIY-FPTQEWNGSQYITIQDA-FR.K	11
324 - 344	2385.87	2384.86	2385.19	-0.32	1	R.KYPNWSASVEDHGA-FLVVNPR.Y	13
325 - 344	2257.78	2256.77	2257.09	-0.32	0	K.YPNWSASVEDHGAFLV-VNPR.Y	39
345 - 355	1300.50	1299.50	1299.65	-0.15	0	R.YSNLIGVTDYR.R	76
345 - 355	1300.60	1299.59	1299.65	-0.05	0	R.YSNLIGVTDYR.R	18
357 - 384	3016.11	3015.10	3015.54	-0.44	0	R.VASLLQQDGYATAPTY-ASSLISIIEYNK.L	57

385 - 420	3860.19	3859.18	3859.73	-0.54	0	K.LHEWDQEALSGQA-SGGNDNNQVQPDQD-VTPTSGTHK.F	41
424 - 452	2966.97	2965.97	2965.41	0.56	0	K.TTTIHNAPDATSAVVG-TYNAGETVNYNGK.L	33
453 - 462	1130.51	1129.50	1129.62	-0.13	0	K.LTVGNATWLR.Y	41
463 - 471	1046.37	1045.36	1045.48	-0.12	0	R.YQSYSGVSR.Y	44
472 - 497	2804.90	2803.90	2804.29	-0.40	0	R.YVMISQTTTNDNNN-QATVTPASGSYK.F	49
472 - 497	2820.90	2819.89	2820.29	-0.40	0	R.YVMISQTTTNDNNN-QATVTPASGSYK.F Oxida-tion (M)	13
511 - 529	2034.68	2033.68	2033.97	-0.29	0	K.TAQVVGTYNAGET-VYYNGK.I	147
530 - 539	1105.49	1104.48	1104.59	-0.11	0	K.ITTGGTTWLR.Y	15
540 - 573	3551.18	3550.18	3550.67	-0.49	0	R.YLSYSGAQHYVAMS-GDEVGSVAKPDV-VATSGSYR.F	13
574 - 581	909.40	908.39	908.53	-0.14	1	R.FTKTTAIK.S	51
606 - 615	1175.48	1174.48	1174.61	-0.13	0	K.VTTNGQTWLR.Y	26
606 - 615	1175.54	1174.54	1174.61	-0.07	0	K.VTTNGQTWLR.Y	18
659 - 682	2417.77	2416.76	2417.11	-0.35	0	K.NTPAGNAPSVGTYSAG-DTVYYNAK.V	32
683 - 692	1145.52	1144.52	1144.60	-0.08	0	K.VTANGQTWLR.Y	18
683 - 692	1145.52	1144.52	1144.60	-0.08	0	K.VTANGQTWLR.Y	18
683 - 692	1145.53	1144.53	1144.60	-0.07	0	K.VTANGQTWLR.Y	17
683 - 692	1145.54	1144.53	1144.60	-0.07	0	K.VTANGQTWLR.Y	18
728 - 735	951.41	950.40	950.52	-0.12	0	R.FVTTTNIR.T	45
742 - 769	3124.00	3122.99	3122.46	0.53	0	R.ASVVGEYNPGETVYYN-GTVQAEGYTWLR.Y	10
742 - 769	3124.07	3123.06	3122.46	0.60	0	R.ASVVGEYNPGETVYYN-GTVQAEGYTWLR.Y	13
774 - 782	933.36	932.36	932.47	-0.12	0	R.SGATHYVAK.L	32
774 - 785	1232.54	1231.53	1231.62	-0.09	1	R.SGATHYVAKLEG.-	34

[a]The number of HexNAc were all confirmed by manual de novo sequencing on the MS-MS fragmentation spectra and the glycosylated amino-acids that could be identified are bold and underlined.

Lactobacillus plantarum WCFS1 tagE5E6 deletion mutant

Start - End	Observed m/z	Mr (expected)	Mr (calculated)	ΔMr (Da)	Miss	Sequence	Ions score
80 - 99	2031.67	2030.67	2030.89	-0.22	0	K.TDDQAEST-STTTATTSATSR.V	55
80 - 99	2031.82	2030.81	2030.89	-0.07	0	K.TDDQAEST-STTTATTSATSR.V	146
111 - 127	1621.55	1620.54	1620.72	-0.18	0	K.ADSTGPQSQSSASE-AAK.D	72
128 - 148	2081.81	2080.81	2080.94	-0.13	0	K.DNAATSATADSTTSAVD-QLDK.T	103
152 - 169	1762.63	1761.62	1761.81	-0.19	0	K.ASAATSQASHSTTNE-TAK.A	82
152 - 169	1762.78	1761.77	1761.81	-0.04	0	K.ASAATSQASHSTTNE-TAK.A	124
170 - 195	2576.90	2575.89	2576.22	-0.33	0	K.ASAAASQDSHVTTD-QSSVTVTSEVAK.S	78
170 - 195	2577.08	2576.07	2576.22	-0.15	0	K.ASAAASQDSHVTTD-QSSVTVTSEVAK.S	28
219 - 236	1821.83	1820.82	1820.88	-0.05	0	K.IETAVAADAVQSSAM-MAR.S	64
240 - 252	1495.61	1494.60	1494.78	-0.17	0	R.AMTSQEIFLSQIK.A	28
240 - 252	1495.71	1494.71	1494.78	-0.07	0	R.AMTSQEIFLSQIK.A	92
240 - 252	1511.73	1510.73	1510.77	-0.04	0	R.AMTSQEIFLSQIK.A Oxidation (M)	43

253 - 261	903.41	902.40	902.46	-0.06	0	K.AGAISGWNK.Y	65
253 - 261	903.43	902.43	902.46	-0.03	0	K.AGAISGWNK.Y	58
262 - 295	3589.56	3588.55	3588.86	-0.31	0	K.YQVLPSVTAAQAILES-GWGQSQLATQGNNLF-GIK.G	27
296 - 323	3285.01	3284.01	3283.52	0.49	0	K.GSYQGQSIY-FPTQEWNGSQYITIQDA-FR.K	13
324 - 344	2386.06	2385.06	2385.19	-0.13	1	R.KYPNWSASVEDHGA-FLVVNPR.Y	25
325 - 344	2257.98	2256.98	2257.09	-0.12	0	K.YPNWSASVEDHGAFLV-VNPR.Y	13
345 - 355	1300.50	1299.50	1299.65	-0.15	0	R.YSNLIGVTDYR.R	65
345 - 355	1300.66	1299.65	1299.65	0.00	0	R.YSNLIGVTDYR.R	72
345 - 356	1456.72	1455.71	1455.75	-0.04	1	R.YSNLIGVTDYRR.V	15
357 - 384	3016.32	3015.31	3015.54	-0.23	0	R.VASLLQQDGYATAPTY-ASSLISIIEYNK.L	62
453 - 462	1130.64	1129.64	1129.62	0.01	0	K.LTVGNATWLR.Y	42
463 - 471	1046.39	1045.38	1045.48	-0.10	0	R.YQSYSGVSR.Y	41
463 - 471	1046.47	1045.46	1045.48	-0.02	0	R.YQSYSGVSR.Y	58
511 - 529	2034.72	2033.71	2033.97	-0.26	0	K.TAQVVGTYNAGET-VYYNGK.I	75
530 - 539	1105.59	1104.58	1104.59	-0.01	0	K.ITTGGTTWLR.Y	45
574 - 581	909.42	908.41	908.53	-0.12	1	R.FTKTTAIK.S	43
606 - 615	1175.50	1174.49	1174.61	-0.12	0	K.VTTNGQTWLR.Y	18
606 - 615	1175.58	1174.57	1174.61	-0.04	0	K.VTTNGQTWLR.Y	27
651 - 658	909.48	908.47	908.50	-0.02	0	R.FTQTTAIK.N	55
659 - 682	2418.02	2417.02	2417.11	-0.10	0	K.NTPAGNAPSVGTYSAG-DTVYYNAK.V	26
683 - 692	1145.49	1144.49	1144.60	-0.11	0	K.VTANGQTWLR.Y	29
683 - 692	1145.58	1144.57	1144.60	-0.03	0	K.VTANGQTWLR.Y	31
728 - 735	951.44	950.44	950.52	-0.08	0	R.FVTTTNIR.T	45
728 - 735	951.50	950.49	950.52	-0.03	0	R.FVTTTNIR.T	41
774 - 782	933.40	932.39	932.47	-0.08	0	R.SGATHYVAK.L	28

* Ions score cutt off ≥ 10

Supplemental figures

A.

Protein View: Acm2 isolated from wild type

Sequence coverage of mature Acm2 (753 aa): **75%**

Sequence coverage of Acm2 AST domain (211 aa): **69%**

1 MKIGMTKKVVTSLLLSTALL PMLSGKADTA SANQKPAAAT KGNSAASAAS
51 QQVTLSAGSQ TETTAAGATD QSVASDGAKT DDQAESTSTT TATTSATSRV
101 TVRAASQAAK ADSTGPQSQS SASEAAKDNA ATSATADSTT SAVDQLDKTA
151 KASAATSQAS HSTTNETAKA SAAASQDSHV TTDQSSVTVT SEVAKSAASS
201 AAPKQATEQA VAAKISPKIE TAVAADAVQS SAMMARSTRA MTSQEIFLSQ
251 IKAGAISGWN KYQVLPSVTA AQAILESGWG QSQLATQGNN LFGIKGSYQG
301 QSIYFPTQEW NGSQYITIQD AFRKYPNWSA SVEDHGAFLV VNPRYSNLIG
351 VTDYRRVASL LQQDGYATAP TYASSLISII EYNKLHEWDQ EALSGQASGG
401 NDNNQVQPDQ DVTPTSGTHK FTK**TTTIHNA PDATSAVVGT YNAGETVNYN**
451 GKLTVGNATW LRYQSYSGVS RYVMISQTTT NDNNNQATVT PASGSYKFTA
501 KTNIRSAASK **TAQVVGTYNA GETVYYNGKI TTGGTTWLRY LSYSGAQHYV**
551 AMSGDEVGSV AKPDVVATSG SYRFTKTTAI KSSPATSATT VGSYNAGDTV
601 YYNGK**VTTNG QTWLR**YMSYS GAQHYVQISG ESTSTNVDKP QVTPQSGSYR
651 FTQTTAIK**NT PAGNAPSVGT YSAGDTVYYN AKVTANGQTW LR**YLSYSGAQ
701 HYVAISGNAA TGNTTSKPVT NSQGAFR**FVT TTNIR**TAPST R**ASVVGEYNP**
751 GETVYYNGTV QAEGYTWLRY LSR**SGATHYV AKLEG**

B.

Protein View: Acm2 isolated from **Δ***tagE5E6*

Sequence coverage of mature Acm2 (753 aa): **52%**

Sequence coverage of Acm2 AST domain (211 aa): **59%**

1 MKIGMTKKVVTSLLLSTALL PMLSGKADTA SANQKPAAAT KGNSAASAAS
51 QQVTLSAGSQ TETTAAGATD QSVASDGAK**T DDQAESTSTT TATTSATSRV**
101 TVRAASQAAK **ADSTGPQSQS SASEAAKDNA ATSATADSTT SAVDQLDK**TA
151 K**ASAATSQAS HSTTNETAKA SAAASQDSHV TTDQSSVTVT SEVAK**SAASS
201 AAPKQATEQA VAAKISPK**IE TAVAADAVQS SAMMAR**STR**A MTSQEIFLSQ**
251 IKAGAISGWN KYQVLPSVTA AQAILESGWG QSQLATQGNN LFGIKGSYQG
301 QSIYFPTQEW NGSQYITIQD AFRKYPNWSA SVEDHGAFLV VNPRYSNLIG
351 VTDYRRVASL LQQDGYATAP TYASSLISII EYNKLHEWDQ EALSGQASGG
401 NDNNQVQPDQ DVTPTSGTHK FTK**TTTIHNA PDATSAVVGT YNAGETVNYN**
451 GK**LTVGNATW LRYQSYSGVS R**YVMISQTTT NDNNNQATVT PASGSYKFTA
501 KTNIRSAASK **TAQVVGTYNA GETVYYNGKI TTGGTTWLR**Y LSYSGAQHYV
551 AMSGDEVGSV AKPDVVATSG SYR**FTKTTAI K**SSPATSATT VGSYNAGDTV
601 YYNGK**VTTNG QTWLR**YMSYS GAQHYVQISG ESTSTNVDKP QVTPQSGSYR
651 FTQTTAIKNT PAGNAPSVGT YSAGDTVYYN AKVTANGQTW LRYLSYSGAQ
701 HYVAISGNAA TGNTTSKPVT NSQGAFR**FVT TTNIR**TAPST RASVVGEYNP
751 GETVYYNGTV QAEGYTWLRY LSR**SGATHYV AKLEG**

Figure S1. Protein view (Mascot, Matrix Science) and coverage of the LC/MS spectra of Acm2 isolated from (A) *Lactobacillus plantarum* WCFS1 and (B) the *tagE5E6* deletion mutant strain. Detected peptides are shown in red. The predicted signal peptide and the AST domain are presented in bold and underlined, respectively [5].

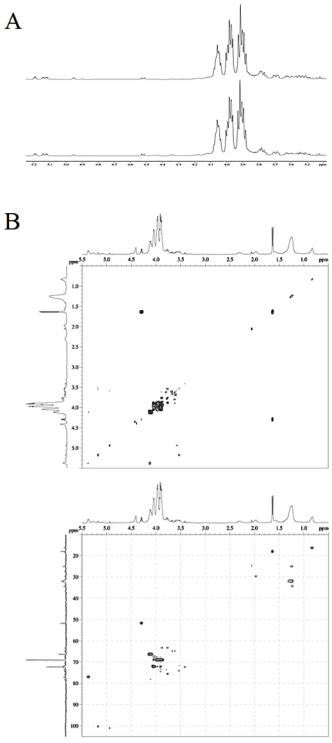

Figure S2. The nuclear magnetic resonance (NMR) analysis of purified LTA and deacylated dealanylated LTA (dd-LTA) from wild-type and Δ*tagE5E6* mutant. (A) Comparison of ^{1}H NMR spectra of dd-LTA from wild-type (upper panel) and Δ*tagE5E6* (lower panel). (B) COSY (upper panel) and HSQC (lower panel) spectra of purified LTA from Δ*tagE5E6*.

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5

Chapter 6

Strain-specific features of extracellular polysaccharides and their impact on host interactions of *Lactobacillus plantarum*

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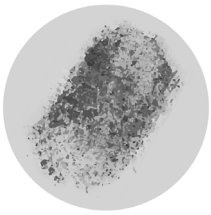

Abstract

Lactobacilli are found in diverse environments and are widely applied as probiotic, health-promoting food supplements. Polysaccharides are ubiquitously present on the cell surface of lactobacilli and are considered to contribute to species- and strain-specific probiotic effects that are typically observed. The two *L. plantarum* strains SF2A35B and Lp90 have obvious ropy phenotypes, implying high extracellular polysaccharide (EPS) production levels. In this work, we set out to identify the genes involved in EPS production in these *L. plantarum* strains and demonstrate their role in EPS production by gene deletion analysis. A model *L. plantarum* strain WCFS1 and its previously constructed derivative that produced reduced levels of EPS were included as a reference. The constructed EPS-reduced derivatives were analyzed for the abundance and sugar composition of their EPS, revealing *cps2*-like gene clusters in SF2A35B and Lp90 responsible for major EPS productions. Moreover, these mutant strains were tested for phenotypic characteristics that are of relevance for their capacity to interact with the host epithelium in the intestinal tract, including bacterial surface properties as well as survival under the stress conditions encountered in the gastrointestinal tract (acid and bile stress). In addition, TLR2 signaling and immunomodulatory capacities of the EPS negative derivatives and their respective wild-type strains were compared, revealing strain-specific impacts of EPS on immunomodulatory properties. Taken together, these experiments illustrate the importance of EPS in *L. plantarum* strains as a strain-specific determinant in host interaction.

Introduction

Lactobacilli are lactic acid bacteria (LAB) found in diverse environments ranging from fermented food to the human gastrointestinal tract, and are widely applied in the food industry as well as in probiotic products [1]. Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [2]. The beneficial effects attributed to probiotic bacteria are often considered species- and strain-specific and have been proposed to at least involve cell-surface components, including peptidoglycan, teichoic acids, (glyco)proteins and polysaccharides [3].

Polysaccharides are ubiquitously present on the *Lactobacillus* cell surface [4], and have been assigned different nomenclature, including wall polysaccharide (WPS), extracellular polysaccharide (EPS), and capsular polysaccharide (CPS), which refers to the mechanism by which the polysaccharide molecule is connected to the cell. However, the distinction between these terms remains a matter of debate because in many cases the mechanism(s) involved in (covalent) cell-wall linkage are unknown, and growth conditions and physiology status of the bacteria can greatly influence the localization of surface polysaccharides [4-6]. Therefore, in this work we use the term extracellular polysaccharides (EPS) to consider WPS, EPS and CPS combined, irrespective of the linkage type to the cell wall. The EPS are mostly heteropolysaccharides formed with repeating oligosaccharide units that consist of considerably variable sugar compositions, sugar-linkages and -branching, and non-sugar modifications [7, 8], but commonly contain D-glucose, D-galactose and/or L-rhamnose, and in some cases *N*-acetylglucosamine, *N*-acetylgalacotsamine or glucuronic acid [3]. The high EPS diversity is reflected by the variability of the glycosyltransferases in the gene clusters involved in EPS biosynthesis [9, 10]. The complexity of EPS structure is exemplified further by the observation that one bacterial genome could possess multiple polysaccharide biosynthesis encoding gene clusters. As an example, the *Lactobacillus plantarum* WCFS1 genome encodes 4 CPS biosynthesis gene clusters [11, 12].

EPS of lactobacilli have been reported to be involved in various biological functions, such as phage absorption [13, 14], adhesion to human cells or to other bacteria [15, 16], and immunomodulation [17, 18]. Various studies have employed gene deletion mutation of EPS associated genes as a strategy to investigate the biological roles of EPS. For example, the deletion of the entire EPS gene cluster in *Lactobacillus johnsonii* NCC533 resulted in loss of bacterial (fuzzy) encapsulation and slightly increased gut persistence in a murine model [19]. In addition, deletion of the priming glycosyltransferase encoding gene, *welE*, in the EPS biosynthesis gene cluster of *Lactobacillus rhamnosus* GG strongly reduced the strain's capacity to produce high-molecular-weight, galactose-rich polysaccharides while small glucose-rich EPS remains, and resulted in enhanced adhesion to mucus and an epithelial cell line [16]. However, the enhanced adhesion did not translate to a better gastrointestinal persistence of the *welE* deletion mutant. A further study found that EPS plays a protective role against intestinal antimicrobial and complement factors [20]. In *Lactobacillus casei* Shirota, deletion of part of the WPS encoding gene cluster led to a reduction of high molecule weight polysaccharides associated to the bacterial cell surface, and the thermally inactivated mutant strain induced higher levels of TNFα, interleukin-12 (IL12), IL10, and IL6 production in mouse macrophage and spleen cell lines *in vitro*. Notably, whereas the wild-type *L. casei* Shirota strain has been reported to suppress lipopolysaccharide-induced IL6 production in mouse macrophage-like cells, its WPS deletion derivative lacked this suppressive ability [17]. Mutation analysis of the 4 *cps*

gene clusters in *L. plantarum* WCFS1 revealed pronounced differential impacts of the mutation of the different clusters, illustrated by the differential impact of these mutations on the transcriptome profile in the mutant strain, and on the surface glycan composition. Moreover, the differential characteristics of the individual, and combined *cps* gene cluster mutants was also observed in their capacity to stimulate TLR2 activation, where *cps1* and *cps3* deletions did not impact on TLR2 activation while *cps2* and *cps4* deletions led to mild but significant TLR2 activation, but mutation of 3 or all 4 *cps* gene clusters induced a strong increase of the TLR2 signaling capacity of the strain that also lost most of its surface glycan structure production capacity [12].

The work related to the species *L. plantarum* was performed using the type strain WCFS1, which does not produce large amounts of EPS and does not have a ropy phenotype. We found two *L. plantarum* strains SF2A35B and Lp90, by contrast, have an obvious ropy phenotype, implying much higher EPS production levels as compared to strain WCFS1. In this work we set out to identify the genes involved in EPS production in these *L. plantarum* strains and demonstrate their role in EPS production by mutation analysis. Based on the comparison between the genome of SF2A35B and Lp90 and other sequenced *L. plantarum* strains, both of the ropy strains SF2A35B and Lp90 possesses its own unique polysaccharide gene clusters. The gene clusters were deleted and the resulting mutants were analyzed for the abundance and sugar composition of their EPS. Moreover, these mutant strains were tested for phenotype characteristics that are of relevance for their capacity to interact with the host intestinal tract, including bacterial surface properties and survival under acid and bile stresses. In addition, TLR2 and immunomodulatory capacities of the wild-type and respective mutant strains were compared, illustrating strain specific and varying impacts of the removal of the EPS in individual strains of the *L. plantarum* species.

Materials and methods

Bacterial strains and culture conditions. Bacterial strains used in this work are listed in Table 1. *Lactobacillus plantarum* strains were grown at 37°C in MRS broth (Difco, West Molesey, United Kingdom) without aeration. *Escherichia coli* strain TOP10 (Invitrogen, Bleiswijk, The Netherlands) was used as an intermediate cloning host, and was grown at 37°C in TY broth [21] with aeration [22]. Solid media were prepared by adding 1.5% (w/v) agar to the broths. Antibiotics were added where appropriate and concentrations used for *L. plantarum* and *E. coli* strains were 10 μg/ml chloramphenicol (Cm), and 30 and 200 μg/ml erythromycin (Ery), respectively.

DNA manipulations. Plasmids and primers used are listed in Table 2. Standard procedures were used for DNA manipulations in *E. coli* [22]. Plasmid DNA was isolated from *E. coli* using a JETSTAR kit (Genomed GmbH, Bad Oberhausen, Germany). *L. plantarum* DNA was isolated and transformed as described previously [23]. PCR amplifications were performed using hot-start KOD polymerase (Novagen, Madison, USA). Amplicons were purified using Wizard®SV Gel and PCR Clean-Up System (Promega, Leiden, The Netherlands). Restriction endonucleases (Fermentas GmbH, St. Leon-Rot, Germany), MSB® Spin PCRapace (Invitek GmbH, Berlin, Germany), PCR Master Mix (Promega) and T4 DNA ligase (Invitrogen) were used as specified by the manufacturers.

Construction of *cps* deletion mutants. The *cps* deletion mutants were constructed as

Table 1. Bacterial strains and plasmids used in this study

Strains	Characteristics[a]	Reference
L. plantarum		
WCFS1	Single-colony isolate of *L. plantarum* NCIMB8826. Originally isolated from human saliva, UK.	[11]
SF2A35B[b]	Isolate from sour cassava, South America; synonym NIZO1839	[57]
Lp90	Isolate from a red must, Italy	[58]
NZ3550Cm	Cm^r; derivative of WCFS1 containing a *lox66*-P_{32}-*cat*-*lox71*-tag2.2 replacement of *cps1A-3J* (*cps1A-3J*::*lox66*-P_{32}-*cat*-*lox71*-tag2.2)	[59]
NZ3561BCm	Cm^r; derivative of SF2A35B containing a *lox66*-P_{32}-*cat*-*lox71*-tag9.3 replacement of *lpSF_839-853* (*lpSF_839-853*:: *lox66*-P_{32}-*cat*-*lox71*-tag9.3)	this work
NZ8220Cm	Cm^r; derivative of Lp90 containing a *lox66*-P_{32}-*cat*-*lox71*-tag10.3 replacement of *lp90_1067-1077* (*lp90_1067-1077*::*lox66*-P_{32}-*cat*-*lox71*-tag10.3)	this work
E. coli		
TOP 10	Cloning host; F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen

[a] Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant

[b] Putative subspecies *argentoratensis*

described previously [24], using a double crossing-over strategy to replace the target *cps* gene cluster by a chloramphenicol resistance cassette (*lox66*-P_{32}*cat*-*lox71*)[24]. In this study, a derivative of the mutagenesis vector pNZ5319 [24], designated pNZ5319TAG was used to introduce a unique 42-nucleotide tag into chromosome during gene deletion, which can be employed for mutant tracking purposes in mixed populations (not relevant for the study presented here). The upstream and downstream flanking regions of each *cps* gene cluster (*lpSF_837-853* in SF2A35B strain, and *lp90_1067-1077* in Lp90 strain) were amplified by PCR using targets-Up-F/R and targets-Down-F/R primers, respectively (Table 2). Each amplicon generated was subsequently joined by a second PCR to *lox*66-P_{32}*cat*-*lox*71-tag by a splicing by overlap extension strategy [25], using targets-Up-F/targets-Down-R primer pairs (Table 2). The resulting PCR products were digested with *Swa*I and *Ecl*136II, and cloned into similarly digested pNZ5319TAG. The obtained mutagenesis plasmids were transformed into *L. plantarum* strains as described previously [23]. The resulting transformants were assessed for a double cross over integration event by selecting individual colonies that displayed a Cm resistant and Ery sensitive phenotype. The selected colonies were further confirmed by PCR using targets-out-F/R primers (Table 2). For each of the mutant constructions a single colony displaying the anticipated phenotype and genotype was selected, yielding the mutants NZ3561BCm (Δ*lpSF_837-853*), and NZ8220Cm (Δ*lp90_1067-1077*).

Transmission electron microscopy (TEM). The production of EPS in WCFS1, SF2A35B and Lp90 was analyzed by TEM. A half milliliter of overnight cultures of *L. plantarum* strains were centrifuged at 12,000 x g for 2 min at 20 °C and resuspended in 100 µl of PBS at pH 7.0 before the analysis. For the analysis, electron microscopy grids commercially copper-coated Formvar 300 holes and carbon (Electron Microscopy Sciences, Hatfield PA) were used. Immediately before use, the grids were subjected to an ion discharge for one minute to make the carbon film more hydrophilic thus favoring adsorption of the sample. Subsequently, 10 µl of sample was added on the grid for 1 min. The excess solution was removed using filter paper, followed by negative staining by 2% uranyl acetate for 40 sec. Finally, excess staining agent was removed and the samples were observed by transmission electron microscope JEOL JEM1011 stabilized at 100 kV.

Surface polysaccharide isolation and sugar composition determination. EPS was

Table 2. Plasmids and primers used in this study

Plasmids	Description[a]	Reference
pNZ5319	Cm^r Em^r; Mutagenesis vector for gene replacements in *L. plantarum*	[24]
pNZ3561	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of *lpSF_839-853*	this work
pNZ8220	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of *lp90_1067-1077*	this work
Primers	**Sequence[b]**	**Reference**
is128 tag-lox66-F3	5'-AAATCTACCGTTCGTATAATGTATG-3'	[60]
is129 tag-lox71-R3	5'-CTCATGCCCGGGCTGTAACCG-3'	[60]
IS169	5'-TTATCATATCCCGAGGACCG-3'	[31]
87	5'-GCCGACTGTACTTTCGGATCC-3'	[24]
CreF	5'-CGATACCGTTTACGAAATTGG-3'	[24]
CreR	5'-CTTGCTCATAAGTAACGGTAC-3'	[24]
EryintF	5'-TCAAATACAGCTTTTAGAACTGG-3'	[24]
EryintR	5'-ATCACAAACAGAATGATGTACC-3'	[24]
lpSF-Up-F	5'-AAATCACCGACCACGTAAGG-3'	this work
lpSF-Up-R	5'-GCATACATTATACGAACGGTAGATTTTGAAGTATGCAAATGTT-TCGGTTTTGATGTC-3'	this work
lpSF-Down-F	5'-CGGTTACAGCCCGGGCATGAGTGGCATAATGAAGTTCCTT-TCAGATTTCACAAAGTCC-3'	this work
lpSF-Down-R	5'-GACATGGCAATATTCCACATGAG-3'	this work
lpSF-out-F	5'-GTCCGTACAACCATCTTACC-3'	this work
lpSF-out-R	5'-GCGACAACAAGGTCAAAACC-3'	this work
lp90-Up-F	5'-AGTATCGGGTGCGACCGATG-3'	this work
lp90-Up-R	5'-GCATACATTATACGAACGGTAGATTTTGCTTGATCCATCAT-TCACTCTCC-3'	this work
lp90-Down-F	5'-CGGTTACAGCCCGGGCATGAGTGCACAGTGTTTCCGACT-GAG-3'	this work
lp90-Down-R	5'-GCTATCGCCGCTTTACATGC-3'	this work
lp90-out-F	5'-GCCATAGCTGTACGCTAAAAGG-3'	this work
lp90-out-R	5'-CGGCTTACCATATCTCATCG-3'	this work

[a] Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant

[b] Underlined nucleotides indicate parts of the primers that are complementary to the is128-lox66-F3 and is129-lox71-R3 primers.

isolated and analyzed according to previously described methods [12]. *L. plantarum* strains and their mutant derivatives were grown in 50 ml 2 × CDM until late stationary phase. After growth, cultures were incubated at 55°C for 1 h, followed by pelleting of the bacterial cells (6000 × g, 15 min, room temperature). The supernatants were supplemented with erythromycin and lincomycin (10 μg/mL each) to avoid growth during dialysis, which was performed in dialysis tubes (molecular weight cutoff of 12–14000 Da, Fisher Scientific, Landsmeer, The Netherlands) incubated overnight against running tap water, followed by dialysis for 1 h against deionized water. The dialyzed samples were freeze-dried, weighted, and stored at −20°C until further analysis.

Abundance and sugar compositions of EPS were analyzed as described previously [12]. Freeze-dried samples were dissolved in eluent (100 mM $NaNO_3$ + 0.02% NaN_3), and then were separated by Size Exclusion Chromatography (SEC) with refractive index (dRI), UV (280 nm), viscosity and MALLS detection (ViscoStar, Wyatt Technologies, Santa Barbara, USA), using columns of TSK gel PWXL Guard, TSK gel G6000 PWXL, and TSK gel G5000 PWXL. During SEC, polysaccharide peaks were collected and hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120°C for 75min, then dried and dissolved in water. The quantitative monosaccharide composition of the polysaccharide fractions was analyzed using High Performance Anion Exchange Chromatography with Pulsed

Amperometric Detection (HPAEC-PAD) with PA-1 column. The monosaccharides were eluted isocratically with 16 mM sodium hydroxide followed by the elution of the acid monosaccharides starting at 20 min with a linear gradient to 200 mM sodium hydroxide + 500 mM sodium acetate in 20 min. Data analysis was performed with Dionex Chromeleon software version 6.80. Quantitative analyses were carried out using standard solutions of the monosaccharides (rhamnose, galactosamine, glucosamine, galactose, glucose, mannose, xylose, galacturonic acid, and glucuronic acid) (Sigma-Aldrich, St. Louis, USA).

Zeta potential and hydrophobicity. Overnight cultures were washed twice with 10 ml PBS and bacteria were resuspended in PBS at an optical density at 600 nm (OD_{600}) of 1. Zeta potential was measured at 20°C using ZetaSizer cuvettes DST1070 (Malvern Instruments, Malvern, UK) in a Zetasizer nano series (Malvern Instruments).

Surface hydrophobicity was determined using microbial adhesion to solvents (MATS) method [26]. Briefly, overnight cultures of *L. plantarum* strains were harvested by centrifugation 5,000 x g, 10 min, washed twice and resuspended to OD_{600} of 1 (A_0) in PBS. 5 ml of bacterial suspension was mixed by 2 min vortexing with 2 ml petroleum ether (the solvent) in a 10 ml glass tube. The tubes were incubated statically for 15 min at room temperature to allow phase separation of the mixture. The aqueous phase was collected and its OD_{600} was measured (A_1). The cell surface hydrophobicity (CSH) was presented as the percentage of microbial retained in the solvent, and calculated as $(1 - A_1/ A_0) \times 100$.

***In vitro* Caco-2 adhesion assay.** For adhesion tests, Caco-2 cells were seeded in 96-well tissue culture plates (Falcon Microtest, Becton Dickinson, NJ, USA) at a concentration of 1.6×10^4 cells per well and cultured for 12-15 days, as previously described [27]. The *L. plantarum* cells were harvested at late stationary phase, pelleted by centrifugation, and resuspended in antibiotic-free DMEM. The obtained monolayers (about 5.0×10^4 cells/well as counted in a Bürker chamber) of differentiated cells that mimic small intestine mature enterocytes [28, 29] were overlaid with stationary phase cells of *L. plantarum* (OD_{600} of 5.0), at a multiplicity of exposure (MOE) of 1:1000, Caco-2 cells to bacteria.

After 1 h of incubation at 37 °C under 5% CO_2 atmosphere, adhesion determination-wells were washed three times with PBS, pH 7.4 to remove unbound bacteria. No washing was performed on control wells, with the aim to recover both adherent and not adherent bacteria. Caco-2 cells and adherent bacteria were then detached by trypsin-EDTA 0.05% (GIBCO) for 10 min at 37 °C and resuspended in sterile PBS (GIBCO). Serial dilutions of samples were plated onto MRS agar plates to determine the number of cell-bound bacteria (viable counts) expressed as CFUs, which was corrected by the measurement of total bacterial load obtained from the unwashed control wells (i.e. both adherent and not adherent bacteria). All adhesion experiments were performed in triplicate.

Biofilm formation. The ability of *L. plantarum* strains to adhere to a glass surface, thereby forming a biofilm, was assessed according to [30], with minor modifications. Briefly, 5 ml of MRS broth were inoculated with 2% (v/v) of overnight cultures of *L. plantarum* strains and incubated for 1, 2 and 7 days at 37 °C, in an orbital shaker at 200 rpm. Residues were washed twice with distilled water, air-dried and then it was stained with crystal violet solution (5 g/L, 0.5% w/v). The biofilm ring was solubilized with acetic acid (30% v/v) and optical density was measured at

570 nm. Each experiment was performed in triplicate.

***In vitro* GI tract survival assay.** The assay was performed as described previously [31]. Briefly, stationary-phase bacterial cultures were harvested, and treated 60 min in stomach-like environment at pH 2.4, followed by neutralization and 60 min in intestine-like condition. Samples were collected before treatments, and after stomach-like and intestine-like conditions to determine colony forming unit (CFU) counting using spot-plating [32]. The survival results were presented as relative survival, which is the log-scale CFU per volume, divided (normalized) by the log-scale CFU at the start of the experiment.

Toll-like receptor (TLR) assay. The assay was performed as described previously [12]. Briefly, human embryonic kidney (HEK)-293 TLR reporter cell lines expressing human TLR1/2, TLR2/2, TLR2/6 or TLR4, and pNIFTY, an NF-κB luciferase reporter construct (Invivogen, Toulouse, France) [33], were used. The HEK-293 reporter cell lines were seeded at 6×10^4 cells per well in 96-well plates and incubated overnight under standard culture conditions. Cells were then stimulated with 2 independently grown bacterial cultures of the *L. plantarum* wild-type strains (WCFS1, SF2A35B, and Lp90) or their *cps* deletion mutants (NZ3550Cm, NZ3561BCm, and NZ8220Cm) at a MOE of 1:10, HEK cell to bacteria, while the TLR1_2 agonist Pam3CSK4 (5 μg/mL, Invivogen) and TLR2_6 agonist Pam2CSK4 (5 μg/mL, Invivogen) was used as positive control and PBS served as the negative control. Following a 6 hr incubation period, the medium was replaced with Bright-Glo™ (Promega Benelux BV, Leiden, The Netherlands), the plate was vortexed for 5 min, and the luminescence was measured using a Spectramax M5 (Molecular Devices, Sunnyvale, USA).

Peripheral blood mononuclear cells (PBMC) assay. The assay was performed as described previously [34] and was approved by Wageningen University Ethical Committee and was performed according to the principles of the Declaration of Helsinki. Peripheral blood of healthy donors was from the Sanquin Blood Bank, Nijmegen, The Netherlands. PBMCs were separated from the blood using Ficoll-Paque Plus gradient centrifugation according to the manufacturer's description (Amersham biosciences, Uppsala, Sweden). The mononuclear cells were collected, washed in Iscove's Modified Dulbecco's Medium (IMDM) + glutamax (Invitrogen, Breda, The Netherlands) and adjusted to 1×10^6 cells/ml in IMDM + glutamax supplemented with penicillin (100 U/ml) (Invitrogen), streptomycin (100 μg/ml) (Invitrogen), and 1% human AB serum (Lonza, Basel, Switzerland). PBMCs (1×10^6 cells/well) were seeded a night prior to the experiment in 48-well tissue culture plates and incubate at 37°C in 5% CO_2. Bacteria from late-stationary phase were added to PBMCs at a MOE of 1:10, PBMC to bacteria. PBMCs from 3 different donors were used in the assay. Following 24 hr incubation at 37°C in 5% CO2, culture supernatants were collected and stored at -20°C until cytokine analysis. Cytokines were measured using a FACS CantoII flow cytometer (BD Biosciences, New Jersey, USA) and BD Cytometric Bead Array Flexsets (BD Biosciences) for interleukin (IL)10 and IL12p70 (henceforth referred to as IL12), TNFα, IL6, IL1β, and IL8 according to the manufacturer's procedures. Concentrations of cytokines were calculated based on the standard curves in the BD Biosciences FCAP software.

Statistical analysis. All analyses were performed in triplicate except zeta potential measurements were done in quadruple. The One-way ANOVA followed by Tukey's multiple comparison test was used to compare the means of adhesion, biofilm formation, and TLR2 activations between strains. The cytokine productions in PBMC assays were transformed to Log values to compare the

stimulation between wild-type and mutant strains using the paired *t* test with respects to individual donors. The differences in surface properties among strains were determined by Kruskal-Wallis analysis of variance (ANOVA) on Ranks and Dunn's test for multiple comparisons. GraphPad Prism 5 software (GraphPad Software, San Diego, CA) was used for all determinations, and a *P* value of <0.05 was considered significant.

Result

Identification of genes responsible for the ropy phenotype of SF2A35B and Lp90

Under normal laboratory conditions of growth, the strains SF2A35B and Lp90 display an obvious ropy-phenotype in comparison to the model strain WCFS1 (Figure S1), which implies a substantially higher level of surface polysaccharide production in these strains as compared to WCFS1. To explore the gene cluster(s) that is (are) responsible for this ropy phenotype, the genomes of both strains were sequenced [35] and the genes that could be related to capsular polysaccharide (CPS) biosynthesis were annotated in detail. The genomes of the SF2A35B and Lp90 strains encode 3 and 4 recognizable and apparently complete *cps* clusters, respectively. Comparison of the genomes of the ropy strains with that of strain WCFS1, indicates that the polysaccharide synthesis cluster assigned *cps* cluster 4 in strain WCFS1 is most conserved among the *L. plantarum* strains (data not shown), and particularly the gene cluster assigned *cps* cluster 2 in strain WCFS1 appears to be variable, and contains genes that are present only in the ropy strains SF2A35B and Lp90 (Figure 1). In addition, strain SF2A35B appears to lack the entire *cps* cluster 1 (nomenclature according to strain WCFS1 genome) but contains 5 unique genes within its *cps* cluster 2, which were assigned the gene identifiers *lpSF_846*, *lpSF_847*, and *lpSF_849-851*. While both LpSF_847 and LpSF_850

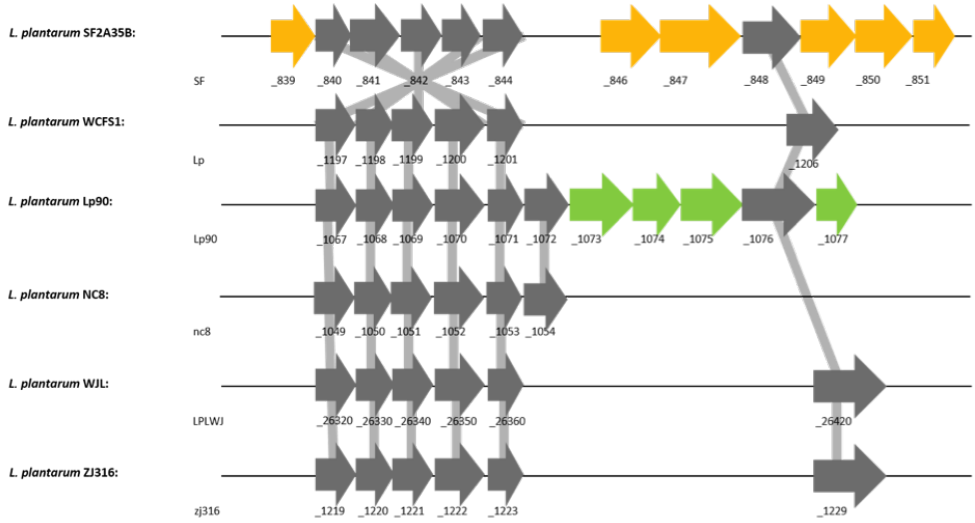

Figure 1. Genetic organization of *cps2* gene cluster of *L. plantarum* WCFS1 across other strains of this species for which the genome sequence was published [11, 35, 61-63]. The grey arrows indicate conserved genes, while orange and green colored arrows indicate different sets of genes uniquely found in the SF2A35B and Lp90 strains, respectively. Grey colored connecting blocks indicate regions of high sequence conservation identified in the genomes of the indicated strains

are hypothetical proteins with unknown function, the other 3 proteins have functions related to polysaccharide biosynthesis, including a putative polysaccharide polymerase protein (LpSF_846), and two glycosyltransferases (LpSF_849 and LpSF_851). Analogously, also the *cps* cluster 2 of strain Lp90 encompassed 4 genes that are specific for that strain, i.e., *lp90_1073-1075* and *lp90_1077*. Lp90_1073 is annotated as a hypothetical membrane protein. Moreover, Lp90_1074 is a family 2 glycosyltransferase, Lp90_1075 is a polysaccharide pyruvyl transferase, and Lp90_1077 is a putative mannosyltransferase, and thus are possible involved in polysaccharide biosynthesis.

Thereby, the *cps* 2 cluster assigned in strain WCFS1 appears to be highly diverse among different strains of the species *L. plantarum*, which is corroborated by the observation that the genes specifically present in the *cps* clusters of strains SF2A35B and Lp90 are not orthologs of each other and each represent the typical *cps* gene cluster mosaic-like reorganizations.

Deletion of the SF2A35B and Lp90 unique cps *clusters abolishes the ropy phenotype*

In order to investigate whether the unique *cps* cluster 2 associated gene sets are responsible for the ropy phenotype in strains SF2A35B and Lp90, the *cps* clusters containing these genes were deleted using a double cross-over gene-replacement strategy, resulting in the strains NZ3561BCm (Δ*lpSF_837-853* derivative of SF2A35B) and NZ8220Cm (Δ*lp90_1067-1077* derivative of Lp90). Both *cps* cluster mutations led to a loss of the ropy phenotype. Moreover, transmission electron micrographs clearly established that the deletion mutants constructed produce much less surface-associated polysaccharide-like structures (grey net-like substances) around the cells as compared to their parental strains (Figure 2). Taken together these data illustrate that the unique *cps* clusters found in SF2A35B and Lp90 are responsible for the ropy phenotype and encode for surface polysaccharide production, both of which are much less apparent in *cps* cluster mutant derivatives.

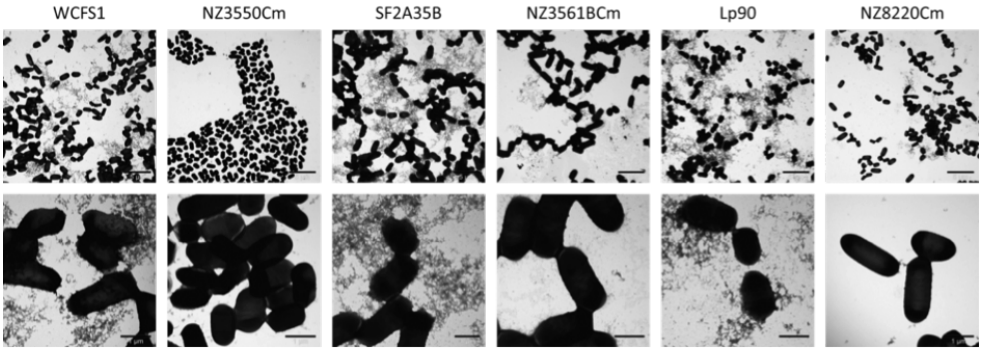

Figure 2. TEM analysis of *L. plantarum* strains and their deletion mutant derivatives. *L. plantarum* strains were negatively stained with 2% uranyl acetate to visualize surface polysaccharides that are visible as the grey, net-like substances surrounding the cells, and were directly observed using TEM without section. The upper panel shows the overview (scale bar is 5 μm) and the lower panel shows a higher magnification (scale bar is 1 μm).

Abundance and sugar compositions of EPS diverse in three L. plantarum and their cps deletion mutants produces much less EPS

The ropy strains, SF2A35B and Lp90, and their *cps2*-like gene cluster deletion mutants, NZ3561BCm and NZ8220Cm, were further studied for the effect of EPS in relation to probiotic properties. The model strain *L. plantarum* WCFS1 and its *cps* deletion mutant NZ3550Cm (Δ*cps1A-3J*) were also

Table 3. The sugar composition of surface polysaccharides isolated from *L. plantarum* strains and their *cps* cluster deletion derivatives

Sugar (% of total sugars)	***L. plantarum* strains**					
	WCFS1	NZ3550Cm	SF2A35B	NZ3561BCm	Lp90	NZ8220Cm
Glucose	65.8	69.2	2.0	n.d.	3.9	n.d.
Glucosamine	21.7	24.7	0.1	n.d.	24.5	n.d.
Galactose	12.5	6.1	66.6	n.d.	22.2	n.d.
Galactosamine	n.d.	n.d.	31.3	n.d.	24.4	n.d.
Rhamnose	n.d.	n.d.	n.d.	n.d.	25.0	n.d.
Mw (kDa)	5652	3606.1	506.2	n.d.	280.6	n.d.
Total polysaccharide isolated (mg/L)	4.0	7.0	59.5	1.1	85.7	2.6

*n.d; 'not detected'

included as references. First, to confirm the reduction in EPS production in the deletion mutants, the polysaccharides were isolated for quantification and determination of the sugar compositions by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). SF2A35B and Lp90 both produce high amounts of EPS, which are approximately 15- and 20-fold higher than EPS isolated from WCFS1 strain, respectively (Table 3). Besides a large difference in abundance, the sugar compositions of EPS also diverse in 3 *L. plantarum*. The EPS from WCFS1 composed of high percentages of glucose while the EPS from SF2A35B composed of high percentages of galactose and galactosamine but low in glucose and glucosamine. The EPS from Lp90 consists of similar percentages of glucosamine, galactose, galactosamine and rhamnose but with a low percentage of glucose (Table 3). Moreover, the EPS isolated from WCFS1 has a molecular weight larger than 5500 kDa; the EPS from SF2A35B is about 500 kDa, while EPS of Lp90 is about 280 kDa, the smallest in molecular weight (Table 3).

The *cps* deletion mutants of the ropy strains produces much less polysaccharides around the cells. The deletion of the *cps2*-like cluster in SF2A35B and Lp90 resulted in more than 95% reduction in total amount of EPS, and yielding an overall amount of EPS that is comparable to that isolated from the non-ropy WCFS1. The reduction of EPS production in the mutants was confirmed by microscopy observations that establish much less surface associated polymer structures in the mutants as compared to their cognate wild-types. Unfortunately, the EPS levels in *cps* deletion mutants of SF2A35B and Lp90 appeared too low to determine the sugar compositions. The CPS-negative derivative of WCFS1 produced a similar amount of EPS as the wild type; and the sugar composition percentages are also comparable between the two strains. However, the molecular weight is 60%-reduced in *cps* mutant, suggesting the impact of *cps* deletion in polysaccharide structures (Table 3).

These results clearly establish that *cps* 2-like cluster in both SF2A35B and Lp90 plays a dominant role in EPS production of both strains, and these clusters support a substantially higher polysaccharide production as compared to its related gene cluster in strain WCFS1 (*cps2*-gene cluster). Variations in abundance, glycan composition, and molecular weight support the typical mosaic-like genetic organization of the *cps* clusters, which are apparently subject to high frequency evolutionary adaptation [36].

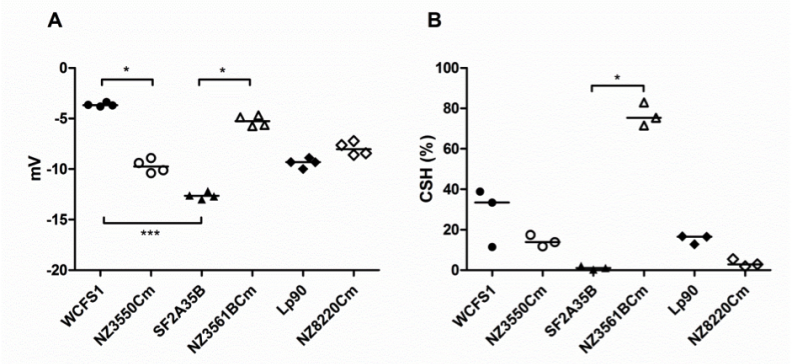

Figure 3. (A) Zeta potential and (B) cell surface hydrophobicity (CSH) of *L. plantarum* strains and their deletion mutant derivatives. All individual data points are shown (n = 4 for zeta potential and n = 3 for hydrophobicity measurements) as well as the median (bar). Closed symbols represent wild-type strains, while open symbols represent their *cps* deletion derivatives. Statistical significance of observed differences was determined by applying Kruskal-Wallis test; $P \leq 0.05$ (overall ANOVA); ***, $P \leq 0.001$; *, $P \leq 0.05$.

Impact of the cps2*-like gene cluster on surface physicochemical properties*

Surface physicochemical properties are important for bacterial physiology and interaction with their environment [37]. The influence of deletion of the *cps2*-like gene cluster in terms of surface charge and hydrophobicity were investigated. The surface charges were assessed by zeta potential measurement, which determines the mobility of cells in an electric field which is determined by cell-surface charge. SF2A35B strain is the most negatively-charged among the wild-type strains tested, whereas WCFS1 and Lp90 have moderate negative surface charge (Figure 3A). Comparative analysis of the *cps*-deletion mutants and their corresponding wild-type strains, revealed quite distinct consequences in the 3 strains. Deletion of *cps*-2 associated functions in SF2A35B led to a significant reduction of negative cell-surface charge. Conversely, deletion of the *cps*1-3 clusters in WCFS1 led to an increased negative surface charge, whereas the mutation of *cps*-2 associated functions in Lp90 did not significantly affect the surface charge in this strain (Figure 3A).

Cell surface hydrophobicity is another physicochemical feature of the cell surface that can be readily determined. Although the hydrophobicity of the different *L. plantarum* strains were somewhat variable, there was no significant difference observed between the 3 wild type strains tested (Figure 3B). Mutation of the *cps-2* like clusters in the 3 genetic backgrounds only elicited a significant affect in surface hydrophobicity in strain SF2A35B where the deletion of the *cps-2* associated genes led to a strong increase in surface hydrophobicity (Figure 3B). Notably, the analogous mutations in the WCFS1 and Lp90 genetic background did not appear to influence the surface hydrophobicity in a significant manner.

Taken together, these measurements underpin the impact of EPS on physicochemical surface properties of bacteria. However, the impact of *cps*-mutation on surface charge and hydrophobicity appears to strongly vary between strains.

Impact of the cps2*-like gene cluster on adhesion and biofilm formation*

Adhesion and colonization are considered important aspects of probiotics to deliver their health beneficial functions, such as pathogen exclusion and immunomodulation [38, 39]. We further investigated the impact of *cps* deletion on adhesion and biofilm formation of the same set of *L.*

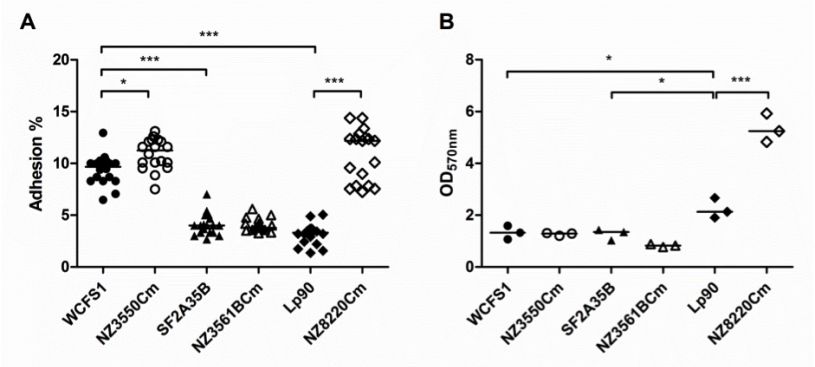

Figure 4. (A) adhesion to Caco-2 cells and (B) biofilm formation on day 7 of *L. plantarum* strains and their *cps* mutant derivatives. Adhesion to Caco-2 of *L. plantarum* strains was performed in triplicate and done by applying washed *L. plantarum* cells to Caco-2 at MOE 1:1000, following by plating to determine the CFU of adherent and non-adherent cells. Biofilm formation was monitored for 7 days and performed in triplicates. The biofilm was washed, air-dried, and then stained with a crystal violet solution. The quantification of the biofilm formation was done by solubilizing the biofilm in acetic acid and measuring the optical density at 570 nm. All data points are shown and bar indicates median. Closed symbols represent wild-type strains, while open symbols represent cps deletion mutants. Statistically significant differences between wild-type and corresponding *cps* deletion mutant, as well as between the three wild-type strains, in adhesion and biofilm formation were determined by one-way ANOVA followed by Tukey's multiple comparison; $P \leq 0.001$ (overall ANOVA); ***, $P \leq 0.001$; **, $P \leq 0.01$; * $P \leq 0.05$.

plantarum strains. The adhesive capacity was tested *in vitro* using human intestinal cell line Caco-2. Among the 3 tested *L. plantarum* strains, WCFS1 has higher adhesion to the intestinal cells while SF2A35B and Lp90 strains have similar level of adhesion, which is about 2-fold lower than that of WCFS1 (Figure 4A). The impact of the *cps-2* like gene cluster on adhesion was investigated by comparing the *cps* mutant with the corresponding parental strains. The *cps* mutant of Lp90, NZ8820Cm strain has improved adhesion to Caco-2 comparing to Lp90 wild-type strain (Figure 4A), suggesting the EPS hinders the adhesion of Lp90 to Caco-2 cells. On the other hand, the deletion of *cps* clusters WCFS1 and SF2A35B strains have no significant influence on adhesion (Figure 4A), which again demonstrates the impact of the *cps* gene clusters on adhesion follows a strain-dependent manner. The biofilm formation was monitored for 7 days. The *cps* deletion mutant of Lp90, NZ8220Cm strain, has a clear increases in biofilm formation (Figure 4B); the difference is largest during the first 2 days (Figure S2). In cases of the strain WCFS1 and SF2A35B, the *cps* deletion appears to only have a transient effect on biofilm formation, illustrated by the enhanced biofilm formation only on day 2 and day 1, respectively (Figure S2), and followed by decreased biofilms of the mutant. Nevertheless, the biofilm formation in these cases mostly appeared unaffected. Overall, the *cps* deletion has the clearly the greatest impact on adhesion and biofilm formation in the Lp90 background, and impacts much less profoundly on these characteristics of the other two strains.

Reduction of EPS does not alter gastrointestinal survival

The capability to survive digestive tract passage is an important characteristic for probiotic bacteria [31, 40]. To test whether the reduction in EPS impacts on the survival of the SF2A35B and Lp90 strains under gastrointestinal (GI) conditions, an *in vitro* GI survival assay was performed, which was shown to qualitatively reflect the actual *in vivo* persistence in the human intestine [31]. This assay monitors the relative survival of bacteria under conditions that mimic those encountered

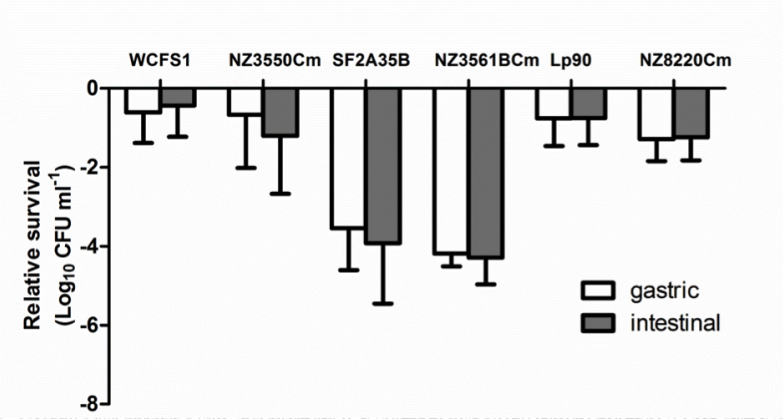

Figure 5. Relative survival of *L. plantarum* strains and the deletion mutant derivatives during an *in vitro* gastrointestinal assay. The survival was measured in 3 independent assays, each using triplicate enumerations for CFU. Data shown are means ± standard deviations. Statistical differences were determine by one-way ANOVA followed by Tukey's multiple comparison correction.

in the stomach, including acid pH and enzyme exposure, followed by those resembling the small intestine, i.e., neutral pH, exposure to pancreatic digestive enzymes, and bile [31]. Comparative evaluation of survival characteristics of the strains revealed no difference in GI survival between *cps* deletion mutants and their parental wild-type (Figure 5), suggesting the reduction of EPS does not alter the survival under *in vitro* GI stresses. However, the GI survival is significantly different for the 3 wild-type *L. plantarum* strains (Figure 5). Strain SF2A35B (and its corresponding *cps* mutant) display the poorest survival among the three strains, and is approximately a 1000-fold more sensitive for the stomach mimicking conditions as compared to WCFS1, which corroborates previous results reported by van Bokhorst-van de Veen *et al*. [41]. The Lp90 strain displays approximately equal survival characteristics as compared to strain WCFS1 (Figure 5) and thereby classifies among the better surviving strains of *L. plantarum* [41]. Next to the strain specific survival

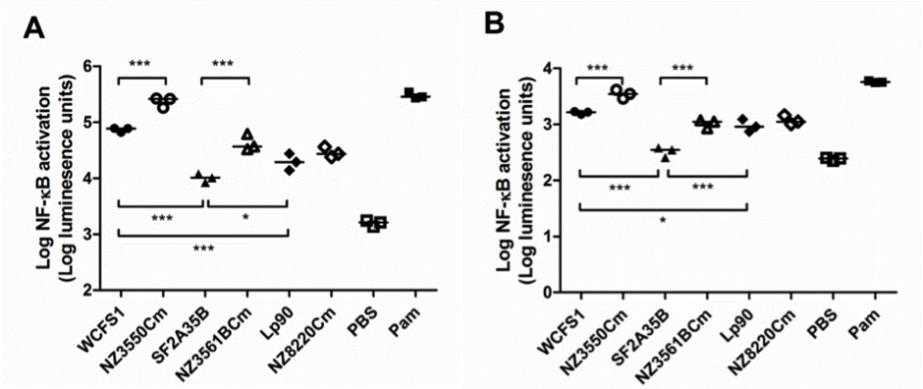

Figure 6. Effects of *L. plantarum* strains and the deletion mutant derivatives on TLR1_2 (A) and TLR2_6 (B) signaling using TLR-expressing HEK cell lines, containing a NF-κB responsive luciferase reporter system. Measurements were performed in triplicate and are presented as Log luminescence units, and individually displayed (n=3) and the bar indicates the median. PBS serves as negative control, and Pam3CysSK4 and Pam2CysSK4 are the positive stimulant of TLR1_2 and TLR2_6 activation, respectively. Data comparison of the wild-type strains and their corresponding *cps* deletion derivatives as well as the 3 wild-type strains were tested for significant differences using one-way ANOVA followed by Tukey's multiple comparison correction and significant differences are indicated; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.

capacities, these experiments clearly establish that the intrinsic capacity to produce EPS does not contribute to survival under GI-tract mimicking conditions.

Deletion of cps gene clusters alters TLR2 signaling in WCFS1 and SF2A35B strains

The effect of *cps* gene clusters deletion of WCFS1, SF2A35B, and Lp90 strains on host cell signaling was investigated using an established TLR2 signaling assay [12] that employs HEK-293 reporter cell lines. HEK-293 reporter cells expressing TLR4 were employed as a negative control to illustrate the signaling is TLR2 specific (Figure S3B). The signaling of TLR1_2, TLR2_6 (Figure 6A and 6B, respectively), and TLR2 (Figure S3A) were determined and displayed as Log values of bioluminescence units, and the experiments were performed in triplicate. The results obtained from the 3 TLR reporter cell lines are consistent. The wild-type *L. plantarum* strains elicited different TLR2 mediated signaling intensities, and strain SF2A35B showed consistently the lowest signaling among the three strains tested (Figure 6). The *cps* deletion derivatives of WCFS1 and SF2A35B displayed approximately 3-fold increased TLR1_2 and TLR2_6 signaling as compared to their respective wild-type strains (Figure 6), implying that removal of EPS led to more effective exposure of microorganism-associated molecular patterns (MAMPs) molecules that are recognized by TLR2. Notably, deletion of *cps2*-like gene cluster in the SF2A35B strain's background (strain NZ3561BCm) led to levels of TLR2 signaling are resembling those of the WCFS1 strain (Figure 6). In contrast, *cps* deletion in the Lp90 strain did not affect TLR2 mediated signaling and both wild type and *cps* derivative strains stimulate a moderate TLR2 signaling as compared to the other strains (Figure 6). The relative NF-κB pathway activation measured in the three TLR2 expressing reporter cell lines (TLR1_2, TLR2_6, and TLR2) were very similar, suggesting that the WCFS1 and SF2A35B strains possess MAMPs, which can interact with the 3 receptors. These MAMPs could be undistinguishably recognized by TLR2, TLR1_2 and TLR2_6 or the TLR2-, TLR1_2-, and TLR2_6-specific MAMPs are present in a similar amount in WCFS1 and the SF2A35B strain (Figure 6 and S3A). Taken together, although both SF2A35B and Lp90 produce large amounts of EPS, their influence on human TLR2 signaling is not the same in these two strains, suggesting that the possible TLR2-ligand shielding effect of EPS is not generic and appears strain- or polysaccharide-specific.

The cps *gene cluster deletions alter cytokine productions of PBMC*

We further explored the impact of *cps* gene cluster deletions on general immune responses using cytokine production by human peripheral blood mononuclear cells (PBMCs) following stimulation with the different bacterial strains. These experiments clearly illustrate the strain-dependent impact of EPS removal on the immunomodulation of different *L. plantarum* strains: in strain WCFS1, EPS removal results in slightly more anti-inflammatory cytokine profiles, by reducing the stimulation of IL12 and/or by elevating the induction of IL10 in some donors (Donor 2, Figure 7A); in SF2A35B, EPS removal increased the general immune-stimulations regardless of pro- or anti-inflammatory responses (Figure 7B); in Lp90, the EPS has the least pronounced influence on immunomodulatory properties of the strain (Figure 7C). Taken together, besides the role of EPS in modulation of TLR-signaling capacity, these surface molecules also impact on the bacterial capacity to elicit a general immune response. Moreover, analogous to what was observed for TLR-signaling the impact of EPS interference by *cps* mutation appears to be strain specific, and may play a prominent role in strain-specific immunomodulatory properties.

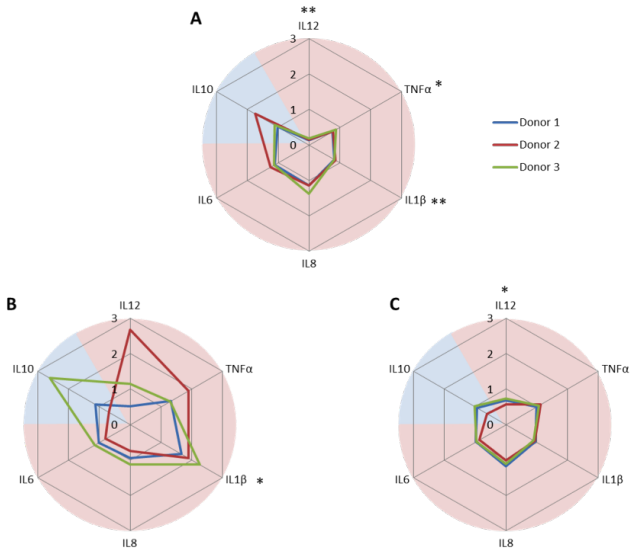

Figure 7. Comparison of immunomodulatory effect of *L. plantarum* strains and their *cps* deletion derivatives, (A) NZ3550Cm/WCFS1, (B) NZ3561BCm/SF2A35B, and (C) NZ8220Cm/Lp90, in the production of inflammation-related cytokines by PBMC (n=3 donors). The results from donor 1, 2, and 3 were shown as blue-, red-, and green-line, respectively. The productions of pro-inflammatory cytokines (shaded red) IL12p70, TNFα, IL1β, IL8 and IL6 and anti-inflammatory cytokine IL10 (shaded blue) are presented as fold changes in cytokines productions stimulated by the cps deletion mutants verse parental wild type *L. plantarum* strains. Significant differences between cytokine levels induced by wild-type strains and mutants (paired t-test) are indicated; *, $P \leq 0.05$; **, $P \leq 0.01$.

Discussion

The protective nature as well as the relevance for virulence made EPS a target for extensive researches in pathogenic bacteria. EPS can form a physical barrier for pathogens to obstruct antimicrobial substances [42, 43]. The capsule-forming polysaccharides in pathogenic bacteria are often immune-inert and provide a strategy for the bacteria to escape from the host immune defenses [42]; therefore, EPS is often a determinant of pathogenicity. Furthermore, the EPS exhibits large diversity, even within in one species, which is the basis for the determination of serogroups/serotypes [44]. For example, over 90 serotypes of *Streptococcus pneumoniae* have been identified based on the CPS structures [44, 45]. For some species, such as *Vibrio cholera* and *Escherichia coli*, only certain serotypes are disease-causing [44]. Overall, although the production of EPS is not essential under laboratory settings, EPS of pathogens is a key determinant for surviving in natural habitats as well as for different life-styles, i.e. (lack of) virulence. [42, 44].

In contrast to pathogens, EPS has been studied to a much less extent in probiotic bacteria such as lactobacilli. Although EPS production, composition, the genes involved in EPS biosynthesis, and the biological functions of EPS have been reported, so far less attention has been paid to EPS as a determinant for strain-specific features of probiotics. The probiotic function is conveyed through probiotic-host interactions, which are affected by many factors including bacterial surface physicochemical (charge and hydrophobicity), adhesion, and immunomodulatory properties. The surface charges of Gram-positive bacteria arise from various components, e.g., ionized phosphate,

amino, and carboxylate groups of surface polymers; mainly teichoic acids, but also proteins and polysaccharides [46]. Notably, our sugar composition analysis is not designed to detect the presence of charged glycans, such as glucuronic and galacturonic acids, in EPS isolated from the 3 *L. plantarum* strains (Table 3), suggesting the effect of EPS on surface charges may result in more exposure of other charged molecules, most likely teichoic acids, on the bacterial cell surface, which may be reflected in the increased zeta potential measurement observed in the mutant relative to the parental strain (Figure 3A). Additionally, the deletion of the *cps* gene clusters in strain WCFS1 has previously been shown to have global effects on gene expression in this strain, including altered expression levels of genes encoding extracellular proteins and teichoic acid decoration [12]. These transcriptional changes may alter surface molecules and thereby contribute to the changes in surface charges.

Adhesion is an important feature for appropriate persistence the in human gut and delivery of probiotics to exert their health-promoting effects [5, 47]. There is conflicting data in literature whether there is a correlation between ability to adhere to host mucosal tissues and surface hydrophobicity [48, 49] or not [50, 51]. In our experiments we could not correlate hydrophobicity and cell-adhesion, e.g., *cps* deletion in SF2A35B increased hydrophobicity but did not affect adhesion to Caco-2 (Figure 3B and 4A). Moreover, *cps* deletion in strain Lp90 did not significantly impact on hydrophobicity and if anything appeared to slightly reduce hydrophobicity in this strain background, whereas it drastically increased the capacity to adhere to Caco2 cells (Figure 3B and 4A). The latter finding may imply that adhesion to Caco-2 of Lp90 is mediated by specific ligand receptor interaction rather than by generic hydrophobic interactions, and that the ligand-receptor interactions are shielded or prevented by the large amounts of surface polysaccharides produced in this strain. To confirm these more specific interactions between this bacterial strain and these epithelial cells actually occur would require further study, but may reveal specific adhesion factors that are of interest in the field of host-microbe interactions.

Immunomodulation is one important mechanism underlying the proposed health-beneficial effects of probiotic bacteria [52, 53]. EPS have been suggested to affect the immune responses of lactobacilli. For example, the EPS-deficient mutant of *L. casei* Shirota stimulates more pro-inflammatory cytokine production in mouse macrophages and spleen cells than the parental strain [17]. Ghadimi *et al*. also suggested that the presence of genes to synthetize polysaccharide might potentially modulate the immunomodulatory property of *Lactobacillus fermentum* strains toward Th1-inducing cytokine secretion in PBMCs, while the strain lacks the genes led to Th1/Th2-suppressing cytokine productions [18]. Interestingly, *L. plantarum* strain SF2A35B was shown previously to elicit very limited immune responses in PBMCs as well as blood-monocyte derived dendritic cells [34, 54]. The observation that this phenotypic trait is combined with the strain's ropy phenotype, could imply that the high level polysaccharide production by this strain impacts on its immunomodulatory capacity, e.g., by shielding specific cell envelope associated signaling molecules thereby suppressing its immunomodulatory stimulation. The shielding effect of EPS has been reported also in *L. plantarum* WCFS1 [12] and *L. rhamnosus* GG [20]; we confirmed this observation in WCFS1 with the *cps* deletion mutant, NZ3550Cm, which stimulates higher TLR2 signaling than wild type strain (Figure 7). The data presented here appear to be at least partially in agreement with this proposed role of EPS, in the sense that the SF2A35B strain stimulates low TLR2 signaling (Figure 6) and induces the production of low amounts of IL10 and IL12 in PBMCs as compared to the WCFS1 strain (Figure S5A and B, respectively) [34]. One of these

earlier studies also reported induced low amounts of IL10 and IL12 in PBMCs stimulated with SF2A35B compared to those stimulated by other *L. plantarum* strains, including WCFS1 [34], which has been suggested as an indicative marker for the prediction of *in vivo* inflammation-suppressive effects of different probiotics in a mouse colitis model [55]. However, the PBMC stimulation data obtained here do not corroborate this finding, since the IL10/IL12 ratio elicited in PBMCs was not significantly different after stimulation with either WCFS1 or SF2A35B, which is in agreement with a previous study [34]. In addition, the higher TLR2-activating NZ3550Cm, however, stimulated significantly lower productions of IL12, TNFα and IL1β in the PBMC assay as compared to WCFS1 (Figure 7 and S3ABD). These observations clearly establish that although the simplified assay models like the HEK-TLR reporter cell systems may provide very relevant information related to receptor specific signaling capacities, their predictive value in relation to immune-cell stimulation and cytokine production profiles elicited in immune cells is quite limited. The complexity of immune cell modulation, which is driven by a plethora of different input signals that are translated by highly integrative signal transduction pathways into cellular response patterns are not appropriately understood to adequately predict the role and hierarchical importance of a single receptor stimulation.

We demonstrated in this work that EPS is an important surface molecule influencing in a strain-specific manner on many aspects important for probiotic-host interaction, including surface properties, adhesion and biofilm formation, and immunomodulation. The strain-dependent effects of EPS are in good agreement with the previously reported notion that structural differences in EPS are a key determinant of strain specificity in pathogens [56]. Some of the measurements appear to be in agreement with the postulated role of surface polysaccharide as a shielding layer for cells, but such shielding function may not be valid for all strains, and specific bacterial adhesins and other surface molecules may influence the shielding role of EPS. Moreover, EPS of bacteria may by themselves have specific (yet undefined) interactions with host cells, for example through interaction with C-type lectin receptors (CLRs), which is a class of pattern recognition receptors (PRRs) that recognize specific glycan moieties by means of their lectin domains. Although the human genome encodes many CLRs, only very limited information is available for their ligand recognition specificity or their precise role in signaling and immunomodulation. Overall, what is becoming clear is that EPS, in combination with other cell envelope components, can contribute to the strain-specific interaction with the host mucosal surfaces, which may likely be underlying probiotic mechanisms of action. Further studies on the diversity and complexity in the structure of EPS as well as on the identification specific lectin receptors of immune cells, which interact with polysaccharides, are essential to understand this strain-dependence at the molecular level.

Acknowledgement

The authors thank Michiel Wels and Luigi Orrù for the annotation of the *cps* gene clusters of SF2A35B and Lp90 strains respectively, and Mariya Tarazanova for technical assistance for zeta potential and hydrophobicity measurements.

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Supplemental Material

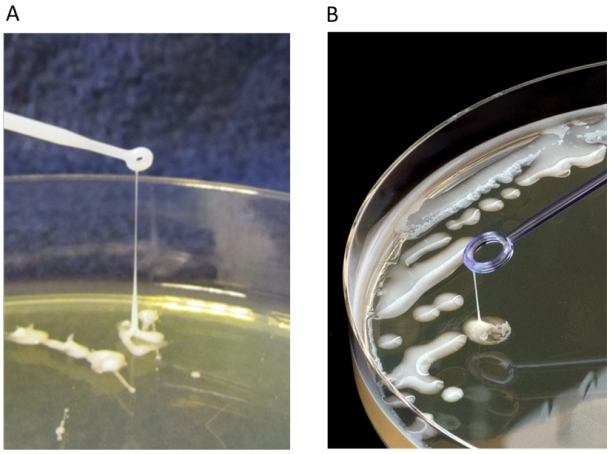


Figure S1. Ropy phenotype of (A) SF2A35B and (B) Lp90 strains. The strains were cultured on MRS agar plate.

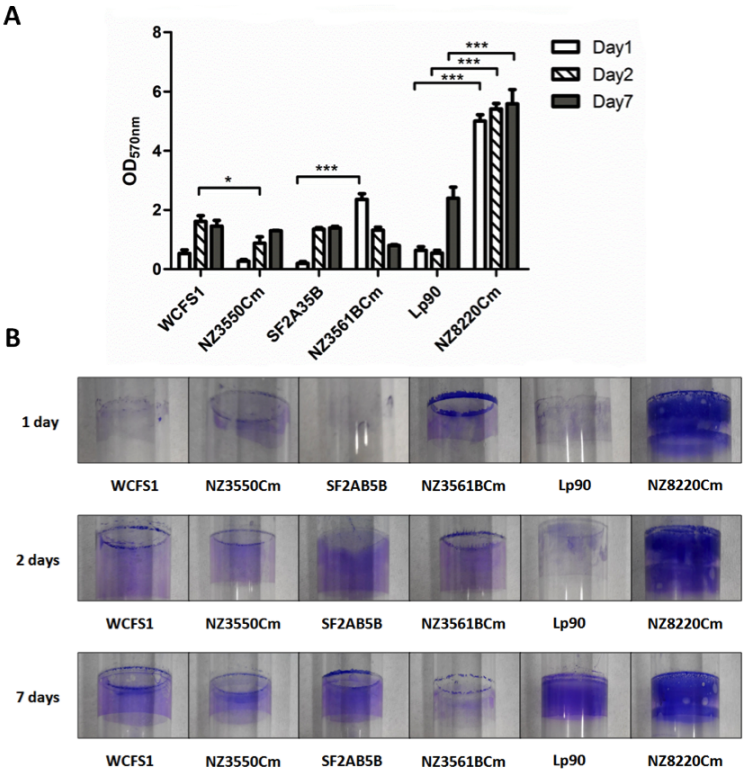


Figure S2. Biofilm formation of *L. plantarum* strains and their *cps* mutant derivatives, showing in (A) OD values and in (B) pictures of biofilm. Biofilm formation was measured on day 1, day 2 and day 7 in triplicates. In panel (A), the biofilm formation was shown as mean ± SD, with open, hatched, and black bars represent day 1, day 2, and day 7 OD measurements, respectively. Statistically significant differences between wild-type and corresponding *cps* deletion mutant, as well as between the three wild-type strains, were determined by one-way ANOVA followed by Tukey's multiple comparison; $P \leq 0.001$ (overall ANOVA); ***, $P \leq 0.001$; * $P \leq 0.05$.

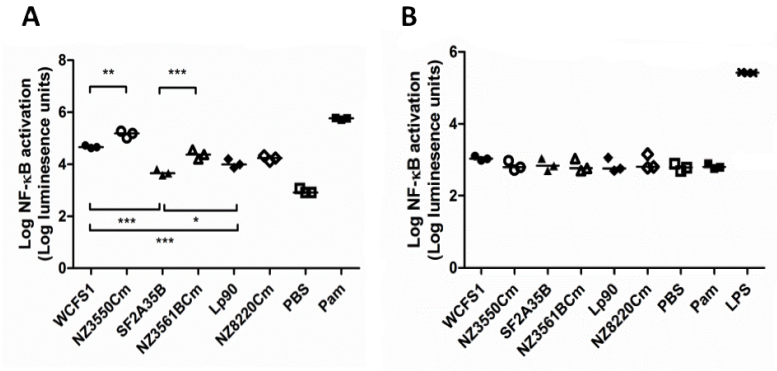


Figure S3. Effects of *L. plantarum* strains and the deletion mutant derivatives on host TLR signaling presented as Log luminescence units using (A) TLR2-expressing and (B) TLR4-expression HEK reporter cell lines. All data points are shown and bar indicates median (n=3). PBS serves as negative control while Pam3CysSK4 and LPS are the positive stimulants of TLR2 and TLR4 activation, respectively. Differences between wild-type and corresponding cps deletion mutant as well as between 3 wild-type strains were compared. The statistical differences were determine by one-way ANOVA followed by Tukey's multiple comparison. $P \leq 0.05$ (overall ANOVA). ***, $P \leq 0.001$; *, $P \leq 0.05$.

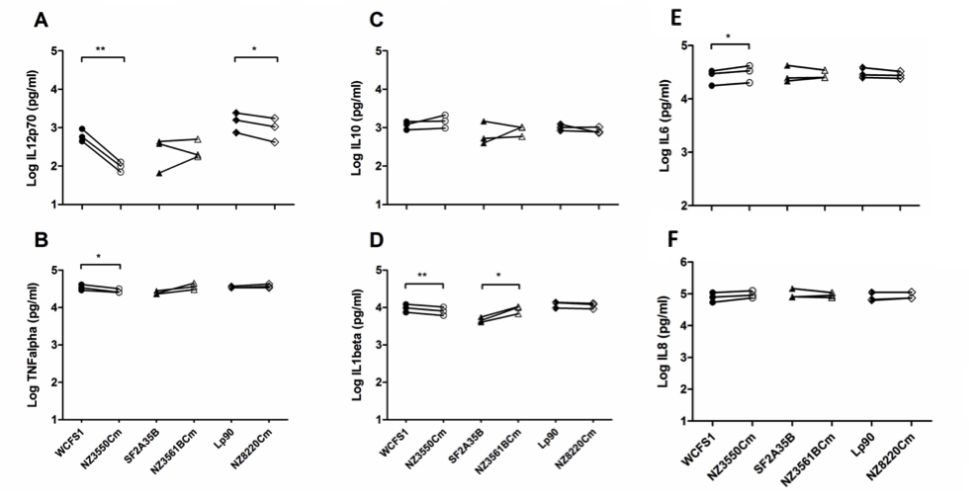


Figure S4. Immunomodulatory effect of *L. plantarum* strains and the deletion mutant derivatives on the cytokine production of human PBMCs (n=3 donors). The (A) IL12, (B) TNFα, (C) IL10, (D) IL1β, (E) IL6, and (F) IL8 cytokine productions presented as Log pg/ml values. The cytokine levels for individual donors stimulated with wild-type and corresponding mutant bacteria were connected by a line to focus the read-outs on changes elicited by the *cps* mutation. Significant differences between cytokine levels induced by wild-type strains and their corresponding mutants (paired t-test) are indicated; **, $P \leq 0.01$; *, $P \leq 0.05$.

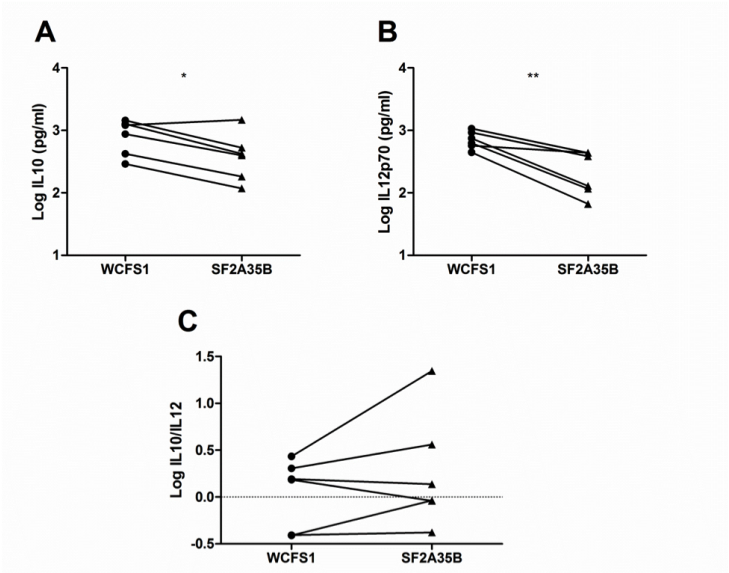
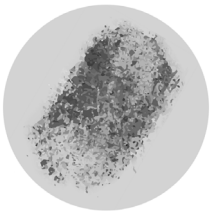


Figure S5. Comparison of (A) IL10 and (B) IL12 production of PBMCs (n = 6 donors) stimulated by *L. plantarum* WCFS1 and SF2A35B strains and (C) IL10/IL12 was also compared of the same strains. The cytokine levels for individual donors stimulated with WCFS1 and SF2A35B were connected by a line to focus the read-outs on changes elicited by the strains. The differences between wild-type and corresponding mutant were statistically analyzed by using paired t test. **, $P \leq 0.01$; *, $P \leq 0.05$.

Chapter 7

General Discussion

I-Chiao Lee



This thesis focuses on potential effector molecules, including lipoteichoic acids (LTA), lipoproteins, glycoproteins, and extracellular polysaccharides (EPS), in the probiotic model strain *Lactobacillus plantarum* WCFS1, and their role as first line interactors with host cells. Genetic engineering strategies were employed to delete or modify these molecules and study the consequences of these modulations on the biochemical properties of the molecules themselves, and on general bacterial physiology, as well as their impact on TLR2-signaling in reporter cell lines, and immunomodulation in peripheral blood mononuclear cells (PBMCs). This chapter will discuss the main findings presented in the thesis and position them in the context of host-bacteria interaction and species/strain specificity.

Host receptors interact with bacterial molecules

The gastrointestinal (GI) mucosa is an important site where bacteria encounter the host and vice versa. The intestinal epithelial cells (IECs) and dendritic cells (DCs) in the mucosa are among the major players in the interaction with bacteria. Host cells express pattern recognition receptors (PRRs) that recognize ligands of microbial origin, named microorganism-associated molecular patterns (MAMPs). These ligands can be considered to be conserved among many microorganisms and are often bacterial surface molecules [1].

Toll-like receptors

Toll-like receptors (TLRs) are transmembrane receptors, which are integral membrane proteins located in the cytoplasmic or intracellular membranes. They consist of an leucine-rich repeat (LRR) domain and an Toll/IL-1 receptor (TIR) domain [2]. There are 10 members of TLR family known

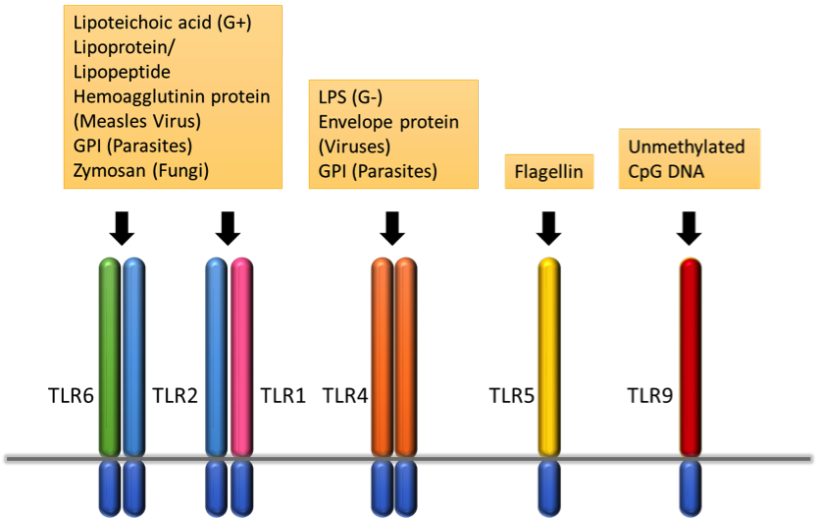


Figure 1. Ligand specificity of TLRs. Toll-like receptors (TLRs) recognize various microorganism components. TLR2 is able to form heterodimers with TLR6 or TLR1 and binds to diverse molecules including lipoteichoic acids from Gram-positive bacteria (G+), lipoproteins, and zymosan from Yeast. TLR4 recognizes lipopolysaccharide (LPS) of Gram-negative bacteria (G-) and viral proteins. TLR5 is specific for bacterial flagellin while TLR9 recognizes unmethylated CpG motif in bacterial and viral DNA. GPI, glycosylphosphatidylinositol.

in human [3]. The sequence variation in LRR domains determines the ligand specify of TLRs. For example, TLR4 recognizes lipopolysaccharides of Gram-negative bacteria, while TLR9 recognizes unmethylated CpG from bacterial and viral DNA (Figure 1) [2]. Upon ligand recognition, TLRs can assemble into a homodimer or heterodimer configuration, allowing the intracellular TIR domains to interact with cytoplasmic adaptors, including myeloid differentiation primary response protein (MyD88) and TIR domain-containing adaptor protein (TIRAP), to initiate signaling [3]. TLR1-5 and 9 can all be expressed by IECs in human small intestine and colon but at varying levels and polarized distribution in the apical and basolateral side of the epithelial cell layer [4]. TLR1, 2, 4, and 5 have been shown to be expressed in immature DCs but their expression decreases upon DC maturation, whereas TLR3 was found to be only expressed in matured DCs [5, 6]. Besides mucosal tissues, TLRs are also expressed in monocytes, mast cells, and PBMCs [3].

In this thesis, we focus on TLR2 signaling because it has been reported to recognize various microbial molecules, including LTA, and lipoproteins/lipopeptides (Figure 1). TLR2 is able to form heterodimers with TLR1 or TLR6 that recognize tri- or di-acyl lipoproteins/ lipopeptides, respectively. The minimal structure required for TLR2 recognition was determined to be a Cys-Ser/Thr/Gly/Ala lipopeptide, containing at least one-ester-bound fatty acid acyl chain with an optimal length of C16 [7-9]. The structural analysis of TLR2/6-Pam_2CSK_4 (a synthetic TLR2/6 lipopeptide agonists; Figure 2A) has shown that the cysteine contributes strongly to the hydrophobic interaction with TLR2/6 dimer, while the hydroxyl side chain of the second serine forms a hydrogen bond with F325 residue of TLR2 [10]. LTA, in comparison to lipopeptides, does not consist of amino acids but contains a glycan backbone. The structural analysis of the TLR2-LTA interaction, using LTA derived from *Streptococcus pneumoniae*, has shown that the hydrophilic oxygen atoms in the LTA glycan backbone repel the hydrophobic residues of the TLR2 binding pocket, and fail to form hydrogen bonds with TLR2. This structural difference hinders the heterodimerization of TLR2 and as a consequence prevents downstream signaling [10]. In chapter 3, we conclude that the purified LTAs from *L. plantarum* WCFS1 and *Bacillus subtilis* 168 (LTA-Lp and LTA-Bs, respectively) have distinct TLR2 signaling capacity in TLR2-reporter HEK 293 cell lines, where LTA-Bs is a potent TLR2 activator and LTA-Lp barely signals via TLR2. The structural difference of the LTA glycan backbone (LTA-Lp is Glu1→6Gal1→2Glu and LTA-Bs is Glu1→6Glu) may

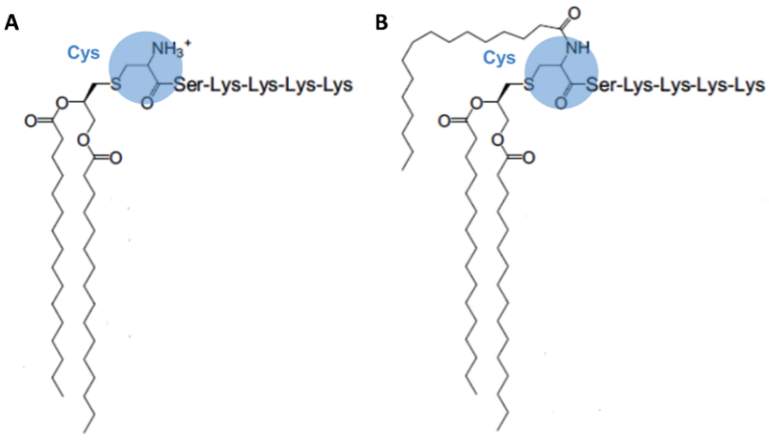


Figure 2. The structures of synthetic lipopeptides Pam_2CSK_4 (A) and Pam_3CSK_4 (B); figure adapted from Kang *et al.* [10].

be an important contributor to the difference in activation of TLR2 signaling. Moreover, previous work has shown that LTA isolated from *L. plantarum* NCIMB8826 (the parental strain of WCFS1, which is a single colony isolate of NCIMB8826; [11]) induces Tumor necrosis factor (TNF)-α production in mouse bone marrow cells, in a TLR2-dependent manner [12]. Analogously, we observed that LTA-Lp induces pro-inflammatory cytokine production in PBMCs (Chapter 3). These observations suggest that co-receptors are required for TLR2 signaling by LTA from *L. plantarum*, which are absent in HEK cells. Potential co-receptors are CD14 and CD36 given that blocking these receptors inhibits *Staphylococcus aureus* LTA-induced TNF-α production from human monocytes [13]. Another argument concerning the TLR2 signaling capacity of LTA is the possible contamination of LTA preparations with lipoproteins. Our LTA purification procedure employed three sequential steps, i.e., 1-butanol extraction, hydrophobic interaction chromatography, and anion exchange chromatography, to minimize contamination and obtain pure LTA. However, when applied in a high concentration (10 μg/ml), the purified LTA-Lp could induce a low level of TLR1_2 activation, but failed to induce any TLR2_6 activation. The data presented in chapter 4 imply that the lipoproteins of WCFS1 may be tri-acylated and signal via TLR1/2 heterodimers, and not via TLR2/6 heterodimers, and that TLR1/2 signaling can be abolished by removal of lipid moiety of lipoproteins (i.e., by *lgt* mutation). These observations may suggest that TLR1/2 signaling observed with high dose LTA-Lp may be due to trace contamination of the LTA fractions by lipoproteins. In conclusion, LTA of *Lactobacillus plantarum* WCFS1 is a much weaker inducer of TLR2 (hetero-) dimerization and signaling than lipoproteins/lipopeptides of the same strain. The glycan backbone of LTA could play a determining role in TLR2 signaling, which may depend on the presence and activity of other co-receptors, such as CD14 and CD36. A different degree of lipoprotein contaminations in purified LTA preparations (in different laboratories) further confounds the multifactorial process of LTA-induced TLR2 signaling, resulting in inconsistent observations in the studies that evaluate TLR2 signaling by LTA.

C-type lectin receptors

Another important PRR family is the C-type lectin receptors (CLRs). CLRs contain at least one carbohydrate recognition domain, which determines their glycan-ligand specificity [14]. Based on studies with pathogens, CLRs play an important role in immune system modulation. They recognize diverse glycans and activate varying downstream signaling pathways, many of which cross-talk with TLR signaling pathways [14]. A well-studied example in DCs and macrophages is the cell-type specific intracellular adhesion molecule-3 grabbing non-integrin (DC-SIGN), which recognizes (polymeric) mannose and fucose glycans. DC-SIGN plays an important role in DCs maturation, and mediates DCs migration and T cell activation [15]. For example, *Lactobacillus casei* NIZO B255 and *Lactobacillus reuteri* ASM20016 have been shown to induce regulatory T cell development through modulating DCs maturation via DC-SIGN interaction [16]. Subsequent work showed that surface layer protein A (SlpA) of *Lactobacillus acidophilus* NCFM binds to DC-SIGN and modulates DCs functions and thereby stimulates regulatory differentiation in naive T-cells [17]. We have detected components, likely to be proteins, in the secreted fraction of *L. plantarum* WCFS1 that are recognized by Concanavalin A (ConA), a mannose- and glucose-specific lectin (Figure 3B), which may also be recognized by DC-SIGN and could fulfill a similar role as SlpA of *L. acidophilus*. Interestingly, these ConA-recognized glycan-conjugate components are more abundantly present in the supernatants of *L. plantarum* WCFS1 derivatives that lack a functional LTA synthase gene (*ltaS*), in particular a high molecular weight (MW) band (>250 kDa)

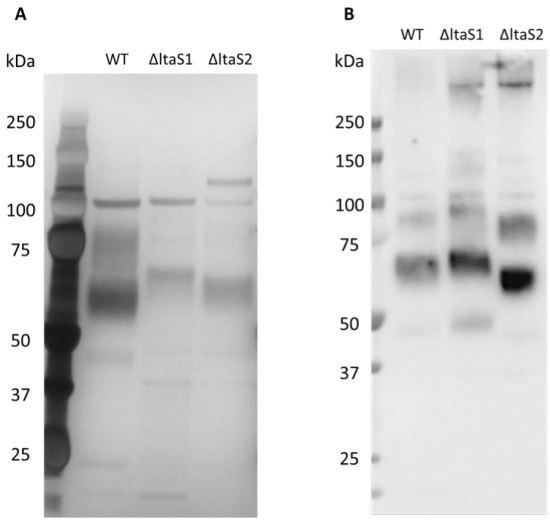


Figure 3. Secreted proteins extracted from *ltaS* deletion mutants (ΔltaS1 and ΔltaS2) as well as from wild type (WT) visualized by (A) silver stain stained or by (B) concanavalin A lectin in SDS-PAGE. On the right side, the protein size (kDa) are indicated based on the Precision Plus Protein™ Dual Color Standards (Bio-Rad).

is uniquely present in supernatants of the mutants (Figure 3B). Based on its MW, this band is likely to represent Sdr (*lp_1303a*), a 3,378-residue transmembrane protein containing a Ser-Asp-repeat domain of more than 1,600 residues [11], which possibly is selectively released from the cell membrane due to cell envelope instability caused by LTA-deficiency in the mutants. Intriguingly, the expression of *sdr* is down-regulated in both *ltaS* mutants, suggesting that erroneous subcellular localization (i.e., secretion versus membrane anchored) elicits a downregulating feed-back mechanism (chapter 3). Extracellular proteins containing a similar serine-repeat domain have been found in other Gram-positive bacteria, and the serine residues have been proposed to be subjected to *O*-glycosylation by adjacent glycosyltransferase genes [18]. In *L. plantarum* WCFS1, *sdr* is genetically linked to 3 glycosyltransferase encoding genes, *tagE1-3*, which are annotated as poly(glycerol-phosphate) α-glucosyltransferases. In chapter 5, we identified the first protein glycosyltransferases GtfA and GtfB in *L. plantarum*, which were originally annotated as TagE5 and TagE6 and were predicted to play a role in lipoteichoic acid glycosylation. Moreover, our work illustrated that protein glycosylation is a conserved feature in the species *L. plantarum* (chapter 5), revealing very limited variation in glyco-protein patterns produced by different strains. The finding that the *tagE*-annotated genes may be involved in protein glycosylation could support the postulated role of TagE1-3 in protein glycosylation rather than LTA glycosylation. Although our primary result shows no changes in the pattern of ConA-reactive components in the whole cell extracts of *tagE1*, *tagE2E3*, and *tagE4* deletion mutants compared to *L. plantarum* WCFS1 wild type (Figure 4), future investigation using Sdr-containing envelope fraction is still required to investigate the possible role of TagE1-3 in protein glycosylation. In addition, evaluating the possible role of the secreted fraction of the *ltaS1* and/or *ltaS2* strains in DC-SIGN modulation using monocyte-derived immature dendritic cells in naive T-cell differentiation assays *in vitro* are important to evaluate the postulated role of the *L. plantarum* Sdr as DC-SIGN ligand. Moreover, deletion of the *sdr* gene in both wild-type and *ltaS* mutant backgrounds of *L. plantarum* may further establish this role.

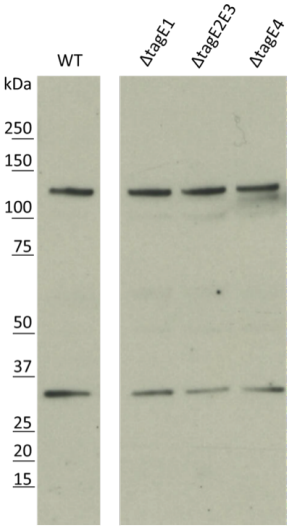


Figure 4. Concanavalin A lectin blots of whole cell extracts derived from *L. plantarum* WCFS1 wild type, Δ*tagE1*, Δ*tagE2E3*, and Δ*tagE4* strains. On the left side of the blot the protein sizes (kDa) are indicated based on the Precision Plus Protein™ Dual Color Standards (Bio-Rad) molecular marker.

Next to glycan moieties on proteins, also extracellular polysaccharides (EPS) can interact with CLRs. Generally, EPS seems to play an indirect role in host interaction and immunomodulation by shielding other surface molecules. The shielding effect is supported by the enhanced TLR2 signaling activation upon deleting gene clusters responsible for surface polysaccharide biosynthesis in *L. plantarum* WCFS1 [19] and SF2A35B (chapter 6). In *L. rhamnosus* GG, deletion of high-MW, galactose-rich EPS led to an increased adhesive capacity to intestinal epithelial cells, which implies shielding of surface adhesins by the EPS [20]. In addition, this galactose-rich EPS of *L. rhamnosus* GG has a protective effect against host immune defense molecules, such as LL-37 antimicrobial peptide and complement factors [21]. On the other hand, recent studies have evidenced a direct role in host signaling of EPS. EPS isolated from *L. acidophilus* NCFM was shown to stimulate gene expressions of interleukin-1α (IL-1α), chemokine C-C motif 2 (CCL2), TNF-α, and pentraxin 3 (PTX3) *in vitro* (Caco-2 cell) and *in vivo* (mice cecum and colon) [22]. High-MW cell wall polysaccharides of *L. casei* Shirota has been proposed to elicit a suppressive effect on macrophage activation and on lipopolysaccharide (LPS)-induced production of IL6 in mouse macrophage-like cells [23, 24]. Another bacterial carbohydrate, polysaccharide A (PSA) from the human commensal *Bacteroides fragilis*, was shown to modulate cytokine production of DCs and active $CD4^+$ T cells in a TLR2-dependent manner [25], which could involve CLRs by their interaction with PSA-derived carbohydrate ligands. Moreover, the acidic EPS fraction isolated from *L. plantarum* N14 [26] and EPS isolated from *Lactobacillus delbrueckii* TUA4408L [27] have been shown to mediate the inhibitory effect on pro-inflammatory responses in porcine intestinal epithelial (PIE) cells upon enterotoxigenic *Escherichia coli* (ETEC). Besides acting as a solitary immune modulator, EPS isolated from *L. rhamnosus* LOCK 0900 has the ability to tune the immune response elicited by *L. plantarum* WCFS1 in mouse bone marrow-derived dendritic cells (BM-DCs) but does not induce cytokine production when applied alone [28]. Different fractions of the EPS from *L. rhamnosus* LOCK 0900 show distinct immunomodulatory capacities: a branched heteropolysaccharide with high-MW of 830 kDa enhanced anti-inflammatory IL10 production induced by *L. plantarum* WCFS1 while another low-MW fraction polymer of 18 kDa enhanced the production of IL-12p70 [28]. This data suggests an interplay of EPS-recognizing CLRs with other PRRs stimulated by *L. plantarum* cells and this interplay can result in distinct immune responses.

We and others have shown that the EPS produced by different strains is highly diverse in quantity, glycan-composition and structure, i.e. glycan bonding types (chapter 6), which in combination with glycosylated protein moieties exposed on the bacterial cell surface and their collective role in glycan-mediated modulation of CLR signaling and host-cell responses are important targets for future research. Immune cells that express a large number of CLRs, such as DCs and macrophages [14], can be suitable to study the immunomodulatory effect of EPS. In addition, *in vitro* reporter systems, analogous to the TLR-transfected HEK NF-κB reporter cell lines [29], are useful to screen potential CLR ligands. However, the downstream signaling cascades of CLRs are largely unknown [14, 30]. Analysis of the gene expression modulated in these immune cells upon contact with EPS may be useful to identify the CLR(s) involved in their recognition and possible downstream signaling pathways elicited by EPS immunomodulation.

Structural and immunomodulatory diversity of surface molecules

Cell envelope molecules of Gram-positive bacteria, including peptidoglycan, LTA, proteins, and polysaccharides, play important roles in bacterial physiology and bacterial interaction with the environment. In this section, we compared the common and distinct features of these molecules in pathogenic and probiotic bacteria, especially focusing on LTA, lipoproteins, and polysaccharides.

Lipoteichoic acids

Structural analyses of LTA revealed a substantial degree of structural diversity of these molecules in different bacteria, allowing the grouping of LTAs into 5 types based on the structure [31, 32]. Most LTAs that have been studied to date belong to type-I, including the LTA from *S. aureus*, *B. subtilis*, and *L. plantarum*. Type-I LTA are composed of an unbranched 1–3 linked glycerol-phosphate (GroP) backbone decorated with D-alanyl and glycosyl-substitutions to a variable degree, which is anchored to the cell membrane by a glycolipid. Within this type, the structure of LTA may still vary in backbone chain length, degree and glycan-specificity of its substitutions, and the chemical structure of the anchoring glycolipid moiety [32-34]. More specifically, type-I LTAs were found to be glycosylated with *N*-acetylglucosamine (GlcNAc) or glucose, and the glycolipid glycan structure could range from mono- to tetra-saccharides. The type-II to type-V LTAs have more complex and sugar-containing repeating units instead of the 'simple' GroP repeating unit in the type-I backbone. Type-II LTA, found in *Lactococcus garvieae* strain Kiel 4217 [35], and Type III LTA, found in *Clostridium innocuum* [31], have a proposed backbone composition consisting of α-Gal(1–6)-α-Gal(1–3)-GroP and α-Gal(1–3)-GroP–repeating units, respectively. The backbone of type-V LTA, found in *Peptostreptococcus anaerobius* [36] and *Clostridium difficile* [37], are proposed to encompass α-D-GlcNAc(1–3)-α-D-GlcNAc repeating units linked through phosphodiester bridges. The type-IV LTA, found in *S. pneumoniae* [38]; [39], have an α-1-4-linked pseudopentasaccharide repeating units consisting of 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose (AATGal), glucose, ribitol-phosphate, and two GlcNAc residues. These structural variations are likely to have profound effects on the signaling capacities of the different LTA molecules, placing the canonical assumption that LTA is recognized by TLR-2 complexes in the mammalian cell into a more refined context. This is further illustrated by a study that compared type-IV LTA from *S. pneumoniae* and type-I LTA from *S. aureus*, and concluded that type-IV LTA displays more potent pro-inflammatory properties in human mononuclear cells as compared to

type-I LTA [38]. Importantly, this study isolated LTA from *lgt* deletion derivatives that produce no lipoproteins to avoid lipoprotein contamination of the purified LTA [38]. The LTA from the Δ*lgt* derivative of *S. aureus* did not activate human mononuclear cells, which is in apparent contradiction with previous studies that reported on pro-inflammatory properties of LTA from *S. aureus* [40, 41]. Modifications of LTA other than their complete deletion (*ltaS* mutation) have also been employed to evaluate the host-modulation by LTA. In particular the use of *dlt* mutants, which produce modified LTA that lacks D-alanyl substitutions, has shown that the D-alanyl depletion of LTA in *L. plantarum* NCIMB8826 enhances the strain's anti-inflammatory properties in a mouse colitis model [12] and reduces visceral pain perception in a rat colorectal distension model [42]. In contrast, the *dlt* mutant of *Lactobacillus rhamnosus* GG did not display enhanced anti-inflammatory properties *in vitro* [43], but still suppressed disease symptoms in a mouse colitis model [44].

Although some correlations have been made between LTA structure and its role in immunomodulation, the results are strongly confounded by large differences in LTA purification procedures that are bound to influence the level of lipoprotein contamination. Moreover, the large variety of *in vivo* and *in vitro* models used to test the role of LTA in host response modulation, as well as the immune response readouts employed enhanced the inconsistences in the results obtained. Therefore, it is of great importance to systemically perform structural and immunological comparison of the LTA obtained from different bacteria to pinpoint the structural determinants involved in their immunomodulation properties.

Lipoproteins

The first biochemical evidence of bacterial lipoproteins was reported by Hantke and Braun in *Escherichia coli*; an abundant lipoprotein Lpp, later named Braun's lipoprotein, contains an N-terminal *S*-glycerylcystein residue modified with 3 acyl chains (*N*-acyl-*S*-diacyl-glyceryl-cysteine; figure 5) [45]. The biosynthesis pathway of bacterial lipoproteins was also determined in *E. coli*, which consists of 3 steps catalyzed successively by 3 enzymes: prolipoprotein diacylglyceryltransferase (Lgt), lipoprotein signal peptidase (Lsp), and lipoprotein *N*-acyl transferase (Lnt) [46]. Further studies have identified other lipoproteins in Gram-negative bacteria as tri-acyl form, including *Brucella* spp. [peptidoglycan-linked lipoprotein (Pal)] [47], *Haemophilus influenza* type b strain Minn A [outer membrane protein (OMP) P6] [48], and *Porphyromonas gingivalis* (lipoprotein PG1828) [49]. Based on these studies as well as the presence of the three conserved enzymes responsible for tri-acyl lipoprotein biosynthesis (Lgt, Lsp, and Lnt), it is widely accepted that the tri-acylated lipoproteins are the common structure in Gram-negative bacteria [50]. Nevertheless, di-acylated lipoproteins in Gram-negative bacteria have also been reported. The cytochrome *c* subunit in the photosynthetic reaction center of *Blastochloris viridis* (formerly known as *Rhodopseudomonas viridis*) is a di-acyl lipoprotein (*S*-diacyl-glyceryl-cysteine; figure 5) [51]. Since no orthologous protein of the tri-acyl-catalizing *E. coli*-type *lnt* was found in the genomes of *Mycoplasma* species, in combination with the detection of di-acylated lipoproteins [52-54], led to the general assumption that Gram-positive bacteria possess only di-acylated lipoproteins [50]. Analogously, recent studies reported lipoproteins of *Listeria monocytogenes* to be di-acylated [55]. However, recent studies have also identified a tri-acylated SitC lipoprotein in four *S. aureus* strains and one *Staphylococcus epidermidis* strain [56, 57], although these species also do not encode an *E. coli*-type *lnt* orthologues in their genomes. Furthermore, three novel lipoprotein structures were identified in Gram-positive bacteria. First, the lyso type lipoproteins (*N*-acyl-*S*-monoacyl-glycerylcysteine;

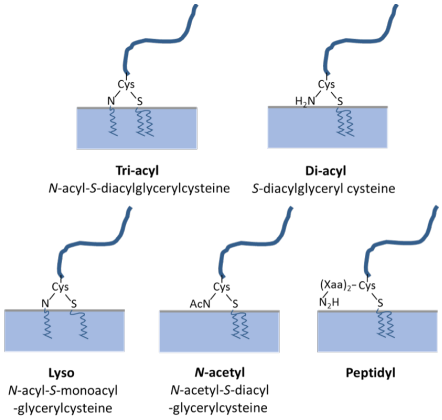


Figure 5. Different structural forms of bacterial lipoproteins.

figure 5) found in several Gram-positive bacteria, including *Enterococcus faecalis*, *Bacillus cereus*, *Streptococcus sanguinis*, and *Lactobacillus bulgaricus* [55]. In addition, *N*-acetyl type (*N*-acetyl-*S*-diacyl-glycerylcysteine, figure. 5) lipoproteins are found in food-associated Bacilli (*Bacillus subtilis* and *Bacillus icheniformis*) and in *Bacillus*-related extremophiles (*Oceanobacillus iheyensis* and *Geobacillus kaustophilus*) [55]. Finally, an unconventional di-acyl lipoprotein structure, termed peptidyl type (Figure 5), was found in two lipoproteins of *Mycoplasma fermentans*, of which two additional amino acid residues are attached in the *N*-terminal side of lipidated cysteine. Lipoprotein MBIO_0319 has additional alanyl-serine residues and lipoprotein MBIO_0661 has alanyl-glycine residues [55]. While two other lipoproteins, MBIO_0763 and MBIO_0869, in *M. fermentans* are di-acyl form, this conventional di-acyl structure is not detected in the MS analysis of peptidyl MBIO_0319 and MBIO_0869, suggesting the lipoprotein structures are specific for certain lipoproteins [55]. The authors speculate that peptidyl lipoproteins in *M. fermentans* may result from a unique specificity for cleavage sites of Lsp compared to that of other bacteria [55]. Importantly, Kurokawa *et al.* have reported that lipoproteins, including SitC, SA1659 and SA2079, of *S. aureus* switches to a di-acyl form instead of its tri-acyl form when the cells are cultured in more acidic (pH 6) conditions and reach a stationary-growth phase [58]. Additionally, high-temperature and high-salt conditions also enhance di-acyl lipoprotein accumulation in *S. aureus* [58]. This is the first evidence of structural alteration in bacterial lipoproteins upon environmental changes. Interestingly, since environmental conditions alter lipoproteins structures in *S. aureus* [58], it may be feasible to compare gene expression profiles in these conditions to accelerate the identification of a functional equivalent of Lnt in Gram-positive bacteria. Taken together, recent structural analyses have expanded the possible variations in bacterial lipoproteins.

Di- and tri-acyl lipoproteins are well-known TLR2 ligands and are recognized by TLR2/6 and TLR1/2 heterodimer, respectively [59]. The lyso-type lipoproteins were shown to have a different reactivity toward TLR2 heterodimers, where *B. cereus* OppA signals via both TLR2/6 and TLR1/2 while *E. faecalis* PrsA is only recognized by TLR2/6 heterodimer [55]. OppA and PrsA differ in their acyl chains and protein sequences [55], which may lead to this different reactivity toward TLR2 heterodimers. Further comparisons of other lyso-form lipoproteins and their TLR2 heterodimer selectivity are required to identify the determinative structures for TLR2 heterodimer recognition. On the other hand, acetyl-type lipoproteins are recognized, as

expected, via TLR2/6 [55]. The TLR2 signaling capacity of peptidyl-type lipoproteins has not yet been determined due to the difficulties to obtain sufficient amounts native lipoproteins [55]. Although signaling through TLR2/6 or TLR1/2 heterodimers was initially considered not to contribute to differential immune responses, but merely to expand the repertoire of bacterial ligand recognition [60], recent studies illustrated that the downstream signaling may depend on the type of TLR2 heterodimers. This is illustrated by the observation that in mouse bone marrow-derived macrophages (BMDMs), TLR2-mediated β-amyloid peptide (Aβ; the main component of amyloid deposits in the brain of Alzheimer's disease patients)-induced inflammation is enhanced by TLR1 but inhibited by TLR6 [61]. The authors suggested that the distinct responses between TLR1/2 and TLR2/6 might be caused by differences in Aβ recognition, in combination with differences in the cytoplasmic domains of TLR1 and TLR2 [61]. Moreover, distinct intracellular adaptors have been reported for TLR1/2 and TLR2/6 heterodimers. TLR2/6 is bridged by Toll/IL-1 receptor (TIR) domain-containing adapter protein (TIRAP), also known as MyD88adapter-like (Mal), to phosphoinositide 3-kinase (PI3K), whereas TLR1/2 depends on a different but unidentified adaptor [62]. However, the distinctive functioning of TLR1/2 and 2/6 heterodimers, is not yet resolved, because the application of inhibitory peptides that block TIRAP/Mal adaptor functioning, could block the cellular signaling of activated TLR1/2 but not of TLR2/6, which is in apparent contradiction to the reported adaptor dependency [63]. Irrespective of the unclarities that remain, these results support a distinctive role of TLR1/2 and TLR2/6 in immune signaling.

The environmental modulation of lipoprotein acylation in *S. aureus* may offer possibilities to decipher in detail the immune responses elicited by di-acyl or tri-acyl lipoproteins of this species. Our results suggest that the lipoproteins of *L. plantarum* WCFS1 are tri-acylated based on TLR2 heterodimers selectivity (chapter 4) and these results require further biochemical analysis to confirm the structure. We also searched for candidate genes that could encode the Lnt functional equivalent in strain WCFS1. Following identification of the Lnt-like function in Gram-positive bacteria, mutation of that function would facilitate the comparison of the immunomodulatory properties elicited by specific di- and tri-acyl lipoproteins derived from the same species and strain, which would be instrumental to determine whether TLR1/2 and TLR2/6 activation can lead to distinct immune responses in the host.

Extracellular polysaccharides

EPS have the most diverse structural properties among bacterial surface components. Most EPS molecules produced by lactic acid bacteria belong to the heteropolysaccharides with repeating oligosaccharides units that contain 3 to 8 monosaccharides, which commonly consist of D-glucose, D-galactose and/or L-rhamnose, and in some cases *N*-acetylglucosamine, *N*-acetylgalacotsamine or glucuronic acid [64], and are arranged in variable sugar-linkages and -branching, and non-glycan modifications [65, 66]. The EPS diversity is reflected by the variability of the glycosyltransferases in the gene clusters involved in EPS biosynthesis and is exemplified further by multiple polysaccharide biosynthesis encoding gene clusters [67, 68]. For example, the *L. plantarum* WCFS1 genome encodes 4 CPS biosynthesis gene clusters [11, 19]. We have illustrated that EPS production has variable and strain-specific impacts on surface properties and immunomodulation in individual *L. plantarum* strains (chapter 6). Moreover, recent findings have supported the direct contributions of EPS in host interaction (discussed above), strengthening the role of EPS as a strain-specificity determinant. Notably, two structurally distinct EPS molecules (polymer

L900/2 and L900/3) were isolated from *L. rhamnosus* LOCK 0900, which were shown to possess distinct immunomodulatory properties [28]. The high-MW (average 830 kDa) branched polymer L900/2 contains a heptasaccharide repeating unit, consisting of one D-galactose, four D-fucose, one D-mannose, and one D-glucose, and pyruvic acid. The low-MW polymer L900/3 (average 1.8 kDa) is contains a pentasaccharide repeating unit, consisting of two D-glucose, one D-galactose, one *N*-acetylmannosamine, and one *N*-acetylgalacotsamine and a phosphate group. Immunomodulation assays showed that L900/2 could enhance *L. plantarum* WCFS1-induced IL10 production in BM-DCs, while L900/3 stimulated the production of IL-12p70 [28]. These results imply the structures of EPS can determine their immunomodulatory properties. It will be interesting to investigate the immunomodulatory property of intact *L. rhamnosus* LOCK0900, which contains both polymers, in combination with *L. plantarum* WCFS1-induced cytokine productions in mouse BM-DCs. A recently-developed lectin microarray that applies fluorescent-stained bacteria onto lectins-coated glass slides can efficiently profile surface glycome of bacteria [69]. This method has been applied to *L. casei*/*L. paracasei* strains. A probiotic strain, *L. casei* Shirota (Yakult) bound dominantly to CSL, a rhamnose-specific lectin [70] although EPS of strain Shirota contains not only rhamnose, but also glucose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine [69, 71]. This observation could imply that within the EPS structures, the different glycan moieties may be differentially accessible. Furthermore, single-molecule atomic force microscopy (AFM), which can map on nanoscale distribution of single molecular recognition sites on biosurfaces [72], combined with fluorescence microscopy has been applied to image the distribution of WTA on the surface of *L. plantarum* [73]. This technology can deliver high resolution imaging views of probiotic surface properties, including glycan moieties detected by lectin-activated AFM tips. Emerging technologies like lectin microarrays and AFM in combination with immunomodulation assays in CLR-expressing cells, such as DCs and macrophages, can provide novel avenues in research aiming to unravel structure-function relationships of EPS in the context of immune system-interaction.

Concluding remarks and future perspectives

In this thesis, we have focused on the molecules on bacterial cell surface, including LTA, lipoproteins, glycoproteins and EPS, in the probiotic strain *L. plantarum* WCFS1. We have shown that these molecules not only play important roles in bacterial physiology, but also in the interaction with the host mucosa through pattern recognition receptors (PRRs) expressed by the host cells. Although these molecules are commonly present in Gram-positive bacteria, evidence is accumulating (see above) that substantial structural diversity of these canonical cell envelope compounds exists between species and strains of the same species. Another example of such diversity in *L. plantarum* can be found in a study that revealed that 6 distinct types of repeating units of wall teichoic acids (WTA) exists [74]. In addition, although *L. plantarum* WCFS1 only produces glycerol-type of WTA under laboratory conditions, the strain possesses the genetic capacity to synthesis ribitol-type WTA [75]. Interestingly, *ltaS* deletion led to elevated expression of the genes involved in this ribitol-type WTA accompanied with suppressed expression of the genes involved in glycerol-type WTA, which could imply that LTA synthesis influences WTA synthesis (chapter 3). On top of the

strain specific structural features of cell envelope components, the *S. aureus* example of growth-phase and culture-conditions dependent alterations in lipoprotein structures illustrates an even further dimension of diversity [58]. Intriguingly, an *in vivo* study revealed that consumption of *L. plantarum* WCFS1 from different growth-phase induced distinct gene expression profiles in human duodenal mucosa [76]. These results indicate that different physiological states of the bacteria can induce different responses in the host, which may be depending on differences in bacterial surface structures. Another important notion is that although *Lactobacillus salivarius* Ls33 and *L. acidophilus* NCFM possess the same peptide-bridging their peptidoglycan, these strains release different muropeptide fragments due to differences in their peptidoglycan modulating enzyme repertoire [77, 78]. *L. acidophilus* NCFM releases exclusively a muropeptide GlcNAc-MurNAc-L-Ala-D-Glu-L-Lys-D-Asn (M-tri-N), carrying an epsilon-linked D-Asn, whereas *Lactobacillus salivarius* Ls33 produces an additional muropeptide without D-Asn [GlcNAc-MurNAc-L-Ala-D-Glu-L-Lys (M-tri)] [77]. This Ls33-specific additional muropeptide is responsible for the protective effect of Ls33 in a mouse model of colitis, which is not seen with the NCFM strain [77]. This example further complicates the notion of variation between cell envelope structural properties of bacteria of a genus or species, it underpins that even with identical molecules to start with, the bacterial background and processing capacities may determine which structural properties are exposed to the environment and the host cell. Taken together, we are slowly obtaining a clearer picture of the way that surface molecules are influenced by genetic background, physiological status, environmental factors, and other biological processes to form a unique molecular signature associated with each strain that consequently elicit different responses when interacting with host cells.

How host cells perceive the molecular signature of specific bacteria brings the host-bacteria interaction to another multi-level of complexity. For example, intestinal epithelial cells express multiple PRRs, including TLR1 to 9, and multiple nucleotide oligomerization domain-like receptors (NLRs) and CLRs [79]. These PRRs share several key components in downstream signaling pathways, such as adaptor protein myeloid differentiation primary response 88 (MyD88), mitogen-activated protein kinases (MAPKs), and nuclear factor (NF)-κB [79]. How a cell integrates the multiple-stimuli presented by a complete bacterium, or even by several bacteria, into a decisive response of the downstream signaling pathways that determine cellular response is still largely unclear. Moreover, intestinal mucosa encompass a variety of different cells, including several epithelial cell lineages like enterocytes, Goblet cells, Paneth cells, and enteroendocrine cells, as well as several immune cell lineages like DCs, macrophages, T and B cells. These cells communicate via cytokines, chemokines, and other effectors to the overall response of the mucosal tissue [80, 81]. Moreover, the cellular and molecular make-up of the mucosa from different healthy human volunteers is quite variable, suggesting that several 'solutions' exist to accommodate health and homeostasis. This degree of individuality is also very much apparent in the analysis of transcriptional profiles of mucosal biopsies from individual volunteers and has led to the proposition of what was designated "the band-width of health" [80]. Nevertheless, dietary interventions have illustrated that conserved, and biologically coherent responses can be measured in mucosal transcriptomes upon the consumption of a probiotic product [76, 82]. However, whether these responses to probiotics are having a relevant impact on the health status of an individual is likely to depend very strongly on the baseline situation (i.e., the individuals starting situation / transcriptional profile). The basis for the observed cellular and molecular individuality can be multifactorial, and could include components of the host genotype, the dietary habits, or

the endogenous microbiota of the intestinal tract.

The complexity of host-microbe interactions indicated above, and our (very) limited understanding of the molecular details involved, imply that this domain requires substantially more research to unravel structure-function relations in bacterial molecules that are of importance to their role in host communication. It seems unavoidable that we abandon the relatively naïve view of conserved ligands of host receptors like LTA, LPS etc., which is denying the substantial chemical diversity hidden under those generic names. Molecular and structural details play a critical role in species and strain specific host-microbe interactions, and the future challenge is to determine which molecular signatures are more or less important in the hierarchy of host interactions and the responses that drive cellular and tissue responses, and finally influencing the function of the complete organism.

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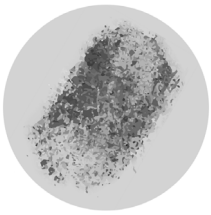
Summary / Samenvatting



Summary

Lactobacillus plantarum is found in various environmental habitats, including fermentation products and the mammalian gastrointestinal tract, and specific strains are marketed as probiotics, which are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'. Throughout the studies of the mechanisms underlying probiotic activity, it became apparent that the probiotic effects are often species and/or strain specific. This situation has led more researchers to focus on the molecular characteristics of probiotic strains intending to link specific molecular structures to specific probiotic functions, and thereby deduce the mechanisms of molecular communication of probiotics. This thesis focuses on potential cell envelope effector molecules involved in interaction with the mammalian host cells, including lipoteichoic acid (LTA), lipo- and glyco-proteins, and extracellular polysaccharides (EPS), of *L. plantarum* WCFS1, a model strain for probiotic lactobacilli with a well-annotated genome sequences and sophisticated genetic engineering tools. First, existing research regarding the potential roles in probiotic functionality of *Lactobacillus* surface molecules in terms of their biosynthesis pathways and structure variations as well as interaction with host Pattern Recognition Receptors (PRRs) and immunomodulatory properties of these molecules are summarized and compared to provide an overview of the state-of-the-art in probiotic effector molecule research. Subsequently, specific molecules that reside in the cell envelope of *L. plantarum* WCFS1 were study for their role in bacterial physiology, as well as their role as ligands in Toll-like receptor (TLR) 2 signaling and immunomodulatory properties using human-cell co-incubation models. Our results showed that the deficiency of LTA had a drastic impact on cell division, cell morphology and growth in *L. plantarum* WCFS1, while LTA-deficient cells also elicited more pro-inflammatory responses in PBMCs rather than the expected loss of pro-inflammatory capacity as was observed with similar mutants of *Lactobacillus acidophilus* NCFM. Further studies on the signaling capacity of the purified LTA from *L. plantarum* WCFS1 revealed that these molecules are poor TLR2 activators, which is in clear contrast to the highly potent TLR2 stimulatory capacity of LTA obtained from *Bacillus subtilis*, implying that structural differences of the LTA produced by different bacteria are prominent determinants of their TLR2 signaling capacity and immunomodulatory properties. Lipoproteins of *L. plantarum* WCFS1 were studied using a derivative strain that is deficient in prolipoprotein diacylglyceryltransferase (Lgt), which transfers acyl chain moieties onto lipoproteins. The lipid moiety was shown to be important for proper anchoring of lipoproteins and TLR1/2 signaling capacity, but did not affect TLR2/6 signaling, suggesting that lipoproteins of *L. plantarum* WCFS1 are predominantly (if not exclusively) triacylated. The Lgt deficient strain elicited more pro-inflammatory responses in PBMCs as compared to the wild type, indicating that the native lipoproteins could play a role in dampening inflammation upon host-probiotic interaction. In addition, we explored the protein glycosylation machinery in *L. plantarum* WCFS1, responsible for the glycosylation of the major autolysin (Acm2) of this bacterium, which was previously shown to be *O*-glycosylated with *N*-acetylhexosamine conjugates. Using sequence similarity searches in combination with a lectin-based glycan detection and mass spectrometry analysis, two glycosyltransferases, GtfA and GtfB (formerly annotated as TagE5 and TagE6, respectively), were shown to be required for the glycosylation of Acm2 and other unidentified *L. plantarum* WCFS1 glycosylated proteins. These results provide the first example of a general protein-glycosylation machinery in a *Lactobacillus* species. Finally, extracellular polysaccharides (EPS) in *L. plantarum* were studied in two strains that produce large amounts of EPS: *L. plantarum* SF2A35B and Lp90, in comparison

to the lowly producing model strain WCFS1. Based on genome sequence comparison, both of the high producer strains were found to possess strain-specific and unique polysaccharide gene clusters. These gene clusters were deleted and the mutants were shown to have lost the capacity to produce large amounts of EPS, and were studied in relation to their properties in host-bacteria interaction. The results illustrate strain-specific and variable impacts of the removal of the EPS in the background of individual *L. plantarum* strains, supporting the importance of EPS in *L. plantarum* strains as a strain-specific determinant in host interaction. Overall, this thesis showed that surface molecules not only play important roles in bacterial physiology, but also in the interaction with the host mucosa through pattern recognition receptors expressed by the host cells. With the growing amount of evidence of structural variations in surface molecules, which are influenced by genetic background, physiological status, environmental factors, and other biological processes, these molecules form a unique signature associated with each strain that as a consequence elicits a strain-specific response when interacting with host cells.

Samenvatting

Lactobacillus plantarum wordt aangetroffen in verschillende omgevingen, onder andere in fermentatie producten en in de darm van zoogdieren. Specifieke stammen van deze bacteriesoort worden verkocht als probiotica; levende micro-organismen die wanneer ze in voldoende hoeveelheid worden geconsumeerd een gezondheidsverbeterend effect geven in de gastheer. Tijdens het bestuderen van de mechanismes die ten grondslag liggen aan probiotische activiteit is het duidelijk geworden dat probiotische effecten vaak soort of zelfs stam specifiek zijn. Deze bevinding heeft onderzoekers geïnspireerd om probiotische stammen moleculair te karakteriseren om daarmee specifieke moleculen van de probiotica te koppelen aan probiotische functies, met als doel om vanuit die kennis het moleculaire mechanisme van communicatie met de gastheer bloot te leggen.

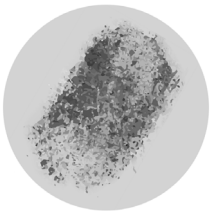
Dit proefschrift concentreert zich op celwand moleculen die een rol kunnen spelen in die moleculaire communicatie met de cellen van de gastheer, waarbij gekeken is naar lipoteichoinezuren (LTA), vetzuur- (lipoproteïnen) en suiker- (glycoproteïnen) geconjugeerde eiwitten en extracellulaire polysachariden (EPS) van de *Lactobacillus plantarum* stam WCFS1, een organisme dat fungeert als model voor probiotische lactobacillen. Voor deze bacterie zijn zowel een goed geannoteerde genoom sequentie als geavanceerde methoden voor genetische modificatie beschikbaar. Als eerste wordt een overzicht gegeven van het wereldwijd lopende onderzoek dat zich richt op de potentiele rol in probiotische functionaliteit van *Lactobacillus* celwand moleculen, hoe de biosynthese van deze moleculen verloopt en de structurele verschillen die zijn gevonden. Ook wordt beschreven hoe de verschillende celwand moleculen interacties kunnen aangaan met receptoren in gastheercellen die structurele patronen in dit soort moleculen van micro-organismen kunnen herkennen en welke immuun systeem modulaties door deze moleculen kunnen worden veroorzaakt. Hiermee wordt een overzicht gegeven van de status van het onderzoek dat zich richt op de moleculen die verantwoordelijk zijn voor probiotische effecten. Vervolgens worden verschillende moleculen die zich bevinden in de celwand van *L. plantarum* WCFS1 onderzocht om te bepalen welke rol ze spelen in de bacteriële fysiologie, maar ook welke rol ze spelen als liganden in de door Toll-like receptor (TLR) 2 gecontroleerde signalering en immuun modulatie,

waarbij gebruik is gemaakt van humane-cellen in co-incubatie modellen. De resultaten laten zien dat LTA deficiëntie drastische gevolgen heeft voor celdeling, morfologie en groei van *L. plantarum* WCFS1. LTA deficiënte bacteriën veroorzaken een meer pro-inflammatoire reactie in humane immuun cellen (PBMCs) in tegenstelling tot het verwachte verlies aan pro-inflammatoire reacties, zoals eerder is gezien met soortgelijke mutanten van *Lactobacillus acidophilus* NCFM. De hierop volgende studies richten zich op de signaal capaciteit van gezuiverd LTA van *L. plantarum* WCFS1, en tonen aan dat deze moleculen weinig TLR-2 activerende werking hebben, hetgeen duidelijk verschillend is ten opzichte van de sterke TLR-2 stimulerende rol die gemeten kan worden voor het gezuiverde LTA van *Bacillus subtilis*. Deze resultaten impliceren dat structurele verschillen in de LTA moleculen die gemaakt worden door verschillende bacteriën belangrijke determinanten zijn voor de TLR-2 activerende en immuun systeem modulerende rol van deze moleculen. Lipoproteïnen van *L. plantarum* WCFS1 zijn bestudeerd door gebruik te maken van een mutant die deficiënt is voor het enzym dat verantwoordelijk is voor de vetzuur koppeling aan lipoproteïnen, pro-lipoproteïne diacylglyceryltransferase (Lgt). De vetzuurkoppeling blijkt belangrijk te zijn voor de juiste membraan-verankering en de TLR1/2 signalerende werking van lipoproteïnen, maar heeft geen invloed op de TLR2/6 signalerende werking, hetgeen suggereert dat de lipoproteïnen van *L. plantarum* WCFS1 voornamelijk (zo niet volledig) drie vetzuur conjugaties bevatten. De Lgt deficiënte bacteriestam geeft versterkte pro-inflammatoire reacties in PBMCs vergeleken met de wild-type stam, wat aangeeft dat de oorspronkelijke lipoproteïnen een rol kunnen spelen in het dempen van inflammatoire reacties in bacterie gastheer interacties. Ook is de eiwit glycolsylering machinerie onderzocht in *L. plantarum* WCFS1, die betrokken is bij de koppeling van suikers aan het belangrijkste autolysine van deze bacterie (Acm2), waarvan in eerder werk is aangetoond dat het eiwit geconjugeerd is via *O*-glycosylering met N-acetylhexosamine. Door sequentie vergelijking gecombineerd met lectine gebaseerde glycoproteïnen detectie en massa spectroscopie analyse, zijn twee glycosyl-transferases, GtfA en GtfB (voorheen geannoteerd als TagE5 en TagE6, respectievelijk) geïdentificeerd die noodzakelijk zijn voor de glycosylering van Acm2 en andere niet geïdentificeerde glycoproteïnen in *L. plantarum* WCFS1. Deze resultaten leveren het eerste voorbeeld van een algemene machinerie betrokken bij eiwit glycosylering in een *Lactobacillus* soort. Tot slot, worden de extracellulaire polysachariden (EPS) in *L. plantarum* bestudeerd in twee stammen die grote hoeveelheden EPS produceren; *L. plantarum* SF2A35B en Lp90, en vergeleken met een stam die kleinere hoeveelheden EPS produceert, WCFS1. Op basis van genoom sequentie vergelijking van de beide stammen die veel EPS produceren werden stam-specifieke en unieke clusters van genen gevonden die coderen voor EPS productie. Deze clusters werden verwijderd en de mutant stammen bleken geen grote hoeveelheden EPS meer te produceren en de eigenschappen van deze stammen in communicatie met gastheercellen is bestudeerd. De resultaten geven aan dat de uitschakeling van EPS productie stam-specifieke en variabele consequenties heeft die afhankelijk zijn van de achtergrond van de individuele stam waarin dit wordt bestudeerd waarmee het belang van EPS als determinant van *L. plantarum* stam-specifieke gastheer interacties wordt onderstreept.

Resumerend, dit proefschrift toont aan dat oppervlakte moleculen van bacteriën niet alleen een belangrijke rol spelen in de bacteriële fysiologie, maar ook in de interactie met de mucosa van de gastheer doordat ze herkent worden als liganden door specifieke receptoren die door gastheercellen tot expressie worden gebracht. Het toenemende bewijs dat er structurele varianten bestaan van deze oppervlakte moleculen, die worden beïnvloed door de genetische achtergrond, de fysiologische status, verschillende omgevingsfactoren en andere biologische processen, geeft

aan dat deze moleculen een unieke, stam-specifieke structurele signatuur vormen die stam-specifieke reacties teweeg brengen in de interactie met gastheercellen.

Acknowledgement



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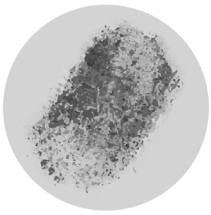
About the author



About the author

I-Chiao Lee was born in Taipei, Taiwan on 21st February 1983. After graduated from Taipei First Girls' High School in 2001, she started her study at National Taiwan University and obtained Bachelor of Science in Life sciences in 2005. In year 2006 to 2008, she worked as a research assistant in a laboratory led by Dr. Steve R. Roffler in Institute of Biomedical Sciences, Academia Sinica Taiwan and focused on improving surface protein expression stability and quantity, and on modifying enzyme specificity using directed molecular evolution techniques. In 2008, she pursued her scientific career by moving to the Netherlands and started her Master study in Biotechnology, specialized in molecular and cell biotechnology, at Wageningen University. During this study, she compared *in vitro* gastrointestinal tract survival of different probiotic strains and identified a regulator improving the survival of *Lactobacillus plantarum* WCFS1 under supervision of Dr. Hermien van Bokhorst-van de Veen, Dr. Peter A. Bron, and Prof. Dr. Michiel Kleerebezem at NIZO food research BV in Ede, The Netherlands. Her internship was done in Genetwister Technologies in Wageningen, focusing on identifying molecular markers for apple ripening under the supervision of Dr. Emilie Ferrier and Mark de Heer. After she has obtained the Master degree with "cum laude" in 2010, she started her Ph.D. project at Wageningen University under the supervision of Dr. Peter A. Bron and Prof. Dr. Michiel Kleerebezem in 2011. Her work on the molecular analysis of potential probiotic effector molecules of *Lactobacillus plantarum* was part of the Top Institute Food and Nutrition project "Gastrointestinal Health". Her work led to the discovery of bacterial molecules that influence host cellular signaling responses and contributes to an advanced understanding of probiotic-host interactions in the context of immunomodulation and probiotic strain specificity.

List of publications

Lee, I-C., van Swam, I.I., Tomita, S., Morsomme, P., Rolain, T., Hols, P., Kleerebezem, M., and Bron, P. A., GtfA and GtfB are both required for protein O-glycosylation in Lactobacillus plantarum. *J Bacteriol*, 2014. **196**(9): p. 1671-82

Lee, I-C., Tomita, S., Kleerebezem, M., and Bron, P.A., The quest for probiotic effector molecules—Unraveling strain specificity at the molecular level. *Pharmacol Res*, 2013. **69**(1): p. 61-74.

Tomita, S., **Lee, I-C**., van Swam, I.I., Boeren, S., Vervoort, J., Bron, P.A., and Kleerebezem, M., Characterisation of the transcriptional regulation of the *tarIJKL*-locus involved in ribitol-containing wall teichoic acid biosynthesis in *Lactobacillus plantarum*. *Microbiology*, 2015. Published Online: 17/12/2015.

Van Bokhorst-van de Veen, H., **Lee, I-C**., Marco, M.L., Wels, M., Bron, P.A., and Kleerebezem, M., Modulation of *Lactobacillus plantarum* gastrointestinal robustness by fermentation conditions enables identification of bacterial robustness markers. *PLoS One*, 2012. **7**(7): p. e39053.

Lin, Y.C., Chen, B.M., Lu, W.C., Su, C.I., Prijovich, Z.M., Chung, W.C., Wu, P.Y., Chen, K.C., **Lee, I-C**., Juan, T.Y., Roffler, S.R., The B7-1 cytoplasmic tail enhances intracellular transport and mammalian cell surface display of chimeric proteins in the absence of a linear ER export motif. *PloS One*, 2013. **8**(9): p. e75084.

Lee, I-C., Caggianiello, G., van Swam, I.I., Taverne, N., Meijerink, M., Bron, P.A., Spano, G. and Kleerebezem, M. Strain-specific features of extracellular polysaccharides and their impact on host interactions of *Lactobacillus plantarum*. *Submitted*.

Patent

Van Bokhorst-van de Veen, H., **Lee, I-C**., Wels, M., De Vos, P., Bron, P.A., Bongers, R.S., Wiersma, A., Kleerebezem, M., and Nauta, A. Probiotics with enhanced survival properties. Publication number: US20140106030 A1.

Overview of completed training activities

Discipline specific activities

Functional Metagenomics of the Intestinal Tract and Food-Related Microbes, ABS/VLAG, 2011

Genetics and physiology of food-associated microorganisms, VLAG, 2013

PhenoLink course, TIFN, 2013

ALW molecular genetics annual meeting, Lunteren, The Netherlands, 2011, 2012, and 2014

Gut day Symposium, Wageningen, The Netherlands, 2011

Symposium on Lactic Acid Bacteria, Egmond aan Zee, The Netherlands, 2011 and 2014

SFB 766 Bacterial cell envelope symposium, Tübingen, Germany, 2013

General courses

VLAG PhD week, 2011

Technique for writing and presenting scientific papers, Wageningen University, 2012

PhD competency assessment, Wageningen University, 2014

Project and Time management, Wageningen University, 2014

Information Literacy including EndNote Introduction, Wageningen University, 2014

Career orientation, Wageningen University, 2014

Intellectual property workshop, TIFN, 2012

Optional

Preparation PhD research proposal

Work discussion meetings, NIZO food research, 2011-2014

Work discussion meetings, Host-Microbe Interactomics, 2011-2014

Expert and work discussion meetings, TIFN, 2011-2014

WE-days, TIFN, 2012-2014

NIZO day, NIZO food research, 2011

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