Molecular analysis of candidate probiotic effector molecules of *Lactobacillus plantarum*

Daniela Maria Remus

Thesis committee

Promoter

Prof. dr. Michiel Kleerebezem
Professor of Bacterial Metagenomics
Wageningen University

Co-promoter

Dr. Peter A. Bron
Senior Scientist, NIZO food research BV, Ede

Other members

Prof. dr. Tjakko Abee, Wageningen University
Prof. dr. Pascal Hols, University of Lovain (Lovain la Neuve), Belgium
Dr. Philippe Langella, National Institute of Agricultural Research (INRA), Paris, France
Prof. dr. Roland J. Siezen, Radboud University, Nijmegen

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Daniela Maria Remus

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Daniela Maria Remus
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Summary

Lactobacilli occupy diverse natural habitats, including dairy products and the mammalian gastrointestinal (GI) tract, and several *Lactobacillus* strains are marketed as probiotics that can interact with host cells in the GI tract by which they are proposed to beneficially influence the health status of their consumers. The discovery of probiotic effector molecules is instrumental to understand the exact modes of probiotic action, which is required for their controlled, safe, and purpose-directed application. This thesis focuses on the molecular characterization of effector molecules of *L. plantarum* WCFS1, a model organism for probiotic lactobacilli. The molecular mechanism underlying the previously established growth phase-dependent capability of *L. plantarum* to modulate NF-κB associated pathways in the mucosa of healthy human volunteers was studied by a combined approach of genome-wide transcriptomics and cell surface proteomics. The impact of cell surface trypsinization samples on NF-κB promoter activation was assessed using a dedicated NF-κB reporter derivative of the Caco-2 intestinal epithelial cell (IEC) line. Surface proteome fractions derived from late stationary, but not from mid-logarithmic phase bacterial cells were found to be effective attenuators of NF-κB activation. One of the surface proteins that was found to be up-regulated in the late stationary phase, i.e. StsP, a sortase-dependent protein (SDP), was previously shown to be induced in the intestinal tract of mice and humans. Increased StsP expression was engineered *in vitro* and was shown to elicit strong NF-κB attenuation in IECs, providing evidence for the role of this cell surface protein in host cell signaling. SDPs were further studied in a sortase-deficient *L. plantarum* strain (*srtA*), in which SrtA-deficiency led to decreased amounts but not complete lack of specific SDPs. In addition, SDPs could readily be removed form the cell surface of the SrtA-deficient strain using LiCl-based protein extraction, which was not observed with the wild-type strain. The LiCl extraction impacted strongly on the pro-inflammatory signaling properties of the SrtA-deficient but not of the wild-type strain, supporting a role of one or more SDPs in attenuation of host immune responses. Next to proteins, the role of bacterial surface glycans in host interaction was studied in capsular polysaccharide (CPS)-deficient mutants, revealing that reduced levels of cell surface glycans increase

host cell recognition via Toll-like receptor 2. The results presented in this thesis highlight the communication strategies employed by *L. plantarum* by correlating specific host responses to bacterial effectors that were modulated based on their expression, sub-cellular location, and/or exposure on the bacterial cell surface.

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Chapter 1

General Introduction

An intimate tête-à-tête - How probiotic lactobacilli communicate with the host

Daniela M. Remus

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Abstract

Pharmaceutical agents are routinely used in the treatment of gastrointestinal disorders and their role as modulators of host cell responses is well characterized. In contrast, the understanding of the molecular mechanisms, which determine the role of probiotics, i.e. health-promoting bacteria, as host cell modulators is still in its infancy. Both *in vitro* and *in vivo* studies are just starting to reveal the capability of probiotic lactobacilli to modulate host cell-signaling networks and the associated influences on downstream regulatory pathways, including modulation of mucosal cytokine profiles that dictate host immune functions. The communication between probiotic lactobacilli and intestinal host cells is multifactorial and involves an integrative repertoire of receptors on the host side that recognize multiple effector molecules on the bacterial side, of which most have been found to be cell wall- or cell surface-associated compounds and proteins. This chapter describes the discovery of these bacterial effector molecules and their role in strain- and species-specific modulation of host signaling pathways. Unraveling the mechanisms responsible for probiotic–host interactions will progress this research field towards molecular science and will provide markers for probiotic product quality control as well as host- response efficacy. These developments can ultimately lead to a more dedicated, personalized application of probiotics with strong molecular and scientific support for health promotion.

Introduction

The first historical evidence for the consumption of fermented dairy products by humans can be traced back to ancient Egypt as early as 7000 BC (1). Thousands of years later, in 1908, Elie Metchnikoff introduced the idea that fermentation of dairy products brings about more than just extended shelf life and enhanced taste. His work entitled “The prolongation of life” proposed that these products also promote beneficial health effects upon the consumer, which was associated with the consumption of certain lactic acid bacteria (LAB) (2). These findings set the scene for the concept of “probiotic bacteria”. The definition of the term probiotics underwent several changes until the Food and Agriculture Organization of the United Nations and the World Health Organization proposed the currently widely accepted definition that probiotics are “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host (3). Remarkably, more than a century after the observations by Metchnikoff, LAB, and more specifically certain *Lactobacillus* species, still represent a substantial proportion of the probiotics marketed today (4).

The beneficial effects of probiotic lactobacilli have been exemplified by a manifold collection of *in vitro* and *in vivo* studies, which revealed a variety of host effects and generated hypotheses of the mechanisms by which lactobacilli influence their hosts (5). The beneficial strategies of lactobacilli include the reduction of infection risks by strengthening the barrier function of the intestinal epithelium via stimulation of mucin secretion (6) or enhancement of tight-junction functioning (7, 8). Alternatively, probiotics may directly affect the clearance of pathogens by competitive exclusion (9, 10), for example by synthesizing antimicrobial substances such as bacteriocins (11) or lactic acid (12, 13). In addition, the modulation of the host immune response has been proposed as one of the prominent modes of probiotic action accomplished by lactobacilli (5, 14).

The tightening of legislation in the European Union for probiotic health claims has led to continued industrial interest to unravel the molecular mechanisms responsible for the observed beneficial probiotic traits. This chapter will discuss the scientific progress that has been made in unraveling the mechanisms involved in the molecular probiotic-host crosstalk. These advances will pave

the way into the next generation of probiotic applications and might stimulate the use of probiotics to support conventional pharmacological therapies or targeted modulation of health parameters in specific subjects (15).

The gastrointestinal tract – microbes meet immune system

The human gastrointestinal (GI) tract is colonized by a myriad of microbial cells termed the microbiota, and represents one of the most densely populated microbial habitats known in biology (Fig. 1). This is exemplified by the fact that the number of bacterial cells in the intestine vastly outnumbers the amount of human somatic cells (16). The process of intestinal colonization is initiated right after birth and after a period of high community composition dynamics during infant years, the microbiota reaches a more stable and personal community (17, 18). The human GI tract encompasses several anatomically distinct regions; the stomach, the small intestine (subdivided into duodenum, jejunum, and ileum), and the large intestine (ascending, transversing and descending colon, and rectum). Many of these segments provide distinct physicochemical conditions, for example, a low pH in the stomach and the presence of bile salts in the duodenum (19-21), which affect microbiota densities (Fig. 1) and the community composition along the length of the GI tract. The GI physiology is profoundly influenced by the residing microbiota. It degrades and ferments complex dietary and host-derived macromolecules such as polysaccharides to make their nutritional value accessible to the host (22) and synthesizes compounds important for the human physiology like essential amino acids, vitamins (23), and short chain fatty acids (24).

The luminal intestinal microbiota is separated from host tissues by an epithelial monolayer that forms the barrier between the intestinal lumen and the lamina propria, which underlies the epithelium and contains various immune cells (25). The microbiota continuously interacts with the GI mucosa and functions as main stimulus for the intestinal mucosal immune system, shaping and modulating its development and activity (26, 27). The GI epithelium includes several cell types, including Goblet and Paneth cells, whose activities influence the local microbiota communities. Goblet cells secrete large and highly variable glycoproteins termed mucins with a molecular weight ranging from one- to several-million Daltons (28). Mucins

form a protective layer against luminal antigens like bacteria (29) and limit lumen-content transit through the mucosal surface. Paneth cells on the other hand secrete a variety of antimicrobial substances, digestive enzymes, and growth factors (30). In addition, the epithelium contains endocrine cells that secrete peptide hormones and regulate glucose homeostasis, food intake, and gastric emptying (31). However, the most abundant cell types in the GI epithelial layer are the enterocytes, hyperpolarized cells interconnected by junctional complexes, mainly tight junctions. The primary role of enterocytes is related to the main function of the intestinal mucosa, i.e. nutrient absorption and metabolism. In addition, enterocytes take up and process luminal antigens (28) and thereby play a pivotal immunosensory role as they capture and transduce GI luminal signals and respond via secretion of chemokines and cytokines that alert and direct innate and adaptive immunity (32). Besides enterocytes, several other cell types play prominent roles in GI antigen trapping and immune response modulation including microfold-

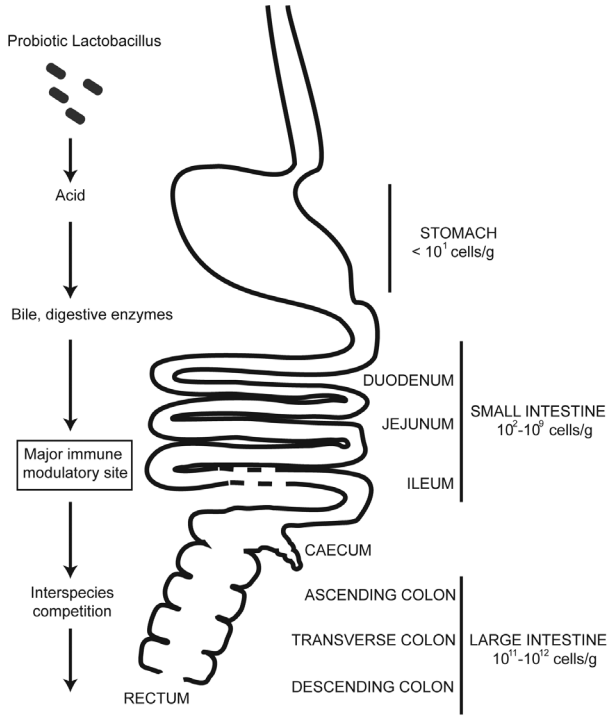

Fig. 1. Schematic representation of the different compartments of the human gastrointestinal (GI) tract divided into stomach, small intestine, and large intestine. The variable physiological conditions affect the microbiota densities along the GI tract. The small intestine possesses a predominant "immune modulatory potential" as it contains the highest number of immune cells of the human body combined with a relatively low number of bacteria, thereby representing an important interface for probiotic interactions.

containing M-cells, dendritic cells (DCs), macrophages, and B-cells (Fig. 2). M-cells are follicle-associated epithelium (FAE) cells, which directly overlay the gut-associated lymphoid tissue (GALT) with its vast number of lymphoid follicles, and are concentrated in the Peyer's patches in the ileum. Peyer's patches form regions where dedicated luminal antigen sampling via M-cell transephithelial transport takes place, which is essential for downstream immunological responses (33-36). DCs, macrophages, and B-cells are specialized antigen presenting cells (APCs) that patrol mucosal tissues and receive antigens from the periphery (32). APCs that captured antigens are able to promote the development of naive T cells into defense directed T helper cells of the classes 1, 2, and 17 (T_H1, T_H2, T_H17) and tolerance associated regulatory T (T_{reg}) cells. This activation is dependent on many factors including the class of APC as well as the type and strength of the sensed stimuli (37-39). Upon differentiation, T cells secrete distinct signaling proteins termed cytokines. T_H1 cells mainly secrete interferon (IFN)-γ and to a lesser extend interleukin (IL)-12, T_H2 cells secrete predominantly IL-4 but also IL-5 (40), and T_H17 cells secrete IL-17, IL-17F, and IL-22 (38). T_{reg} cells secrete IL-10 and transforming growth factor (TGF)-β (41). T-cell differentiation and cytokine profiles determine the outcome of the cellular immune response, either anti-inflammatory or regulatory/pro-inflammatory whereby a balanced ratio of these cytokines controls immune homeostasis. As the microbiota acts as main stimulus for the GI immunosensory cells, it plays a prominent role in determining cellular host responses and is capable of regulating and sustaining immune homeostasis.

The potential of the various types of immunosensory cells described above to recognize, capture, and process antigens such as bacteria is based on their pattern recognition receptors (PRRs). PRRs mediate innate immune recognition (42) and generally recognize molecular structures shared by a variety of bacterial species, which are designated microbe-associated molecular patterns (MAMPs) (43, 44). The best-characterized PRR families to date are the Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors (NLRs), and C-type lectin receptors (CLRs). Individual receptors either expressed at the cell surface or in intracellular membrane vesicles,

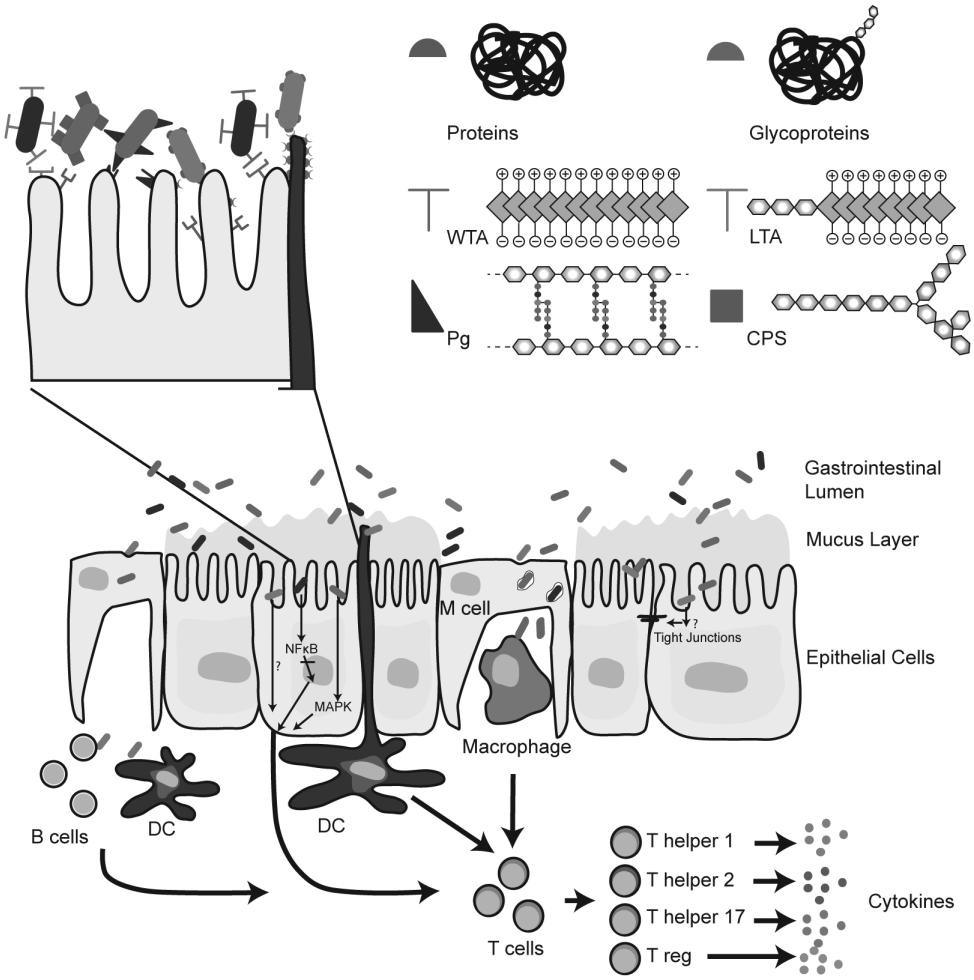

Fig. 2. Bacterial host interaction within the GI mucosa. *Lactobacillus* strains and species (indicated as different colored bacterial cells) are able to interact with human epithelial and immune cells via binding to specific pattern recognition receptors (PRRs). The PRRs recognize specific microbe-associated molecular patterns (MAMPs), including capsular polysaccharides (CPS), wall- and lipoteichoic acids (WTAs and LTAs, respectively), peptidoglycan (PG), proteins and glycoproteins. Depending on the species and strain, bacterial recognition is based on a different set of MAMPs and PRRs. The host-microbe interaction can influence tight junction protein expression and promote the differentiation of naive T cells into T_H1, T_H2, T_H17 cells, as well as regulatory T cells (Treg). These T-cell differentiations and their corresponding cytokine secretion determine the outcome of the cellular immune response towards either pro-or anti-inflammatory pathways, whereby a balanced ratio of these cytokines promotes immune homeostasis. Figure adapted from (4, 86).

recognize specific classes of MAMPs such as lipopolysaccharides, lipoteichoic acids, flagellin, lipoproteins, lipopeptides, as well as *meso*-diaminopimelic acid, and muramyl dipeptide fragments of peptidoglycan (25, 45-47). Upon antigen recognition, PRRs trigger complex, and highly integrated cellular signaling cascades that feed, in some cases through one or two adaptor proteins called MyD88 and Trif (48), into key regulator pathways like nuclear factor kappa B (NF-κB), or mitogen-activated protein (MAP) kinases (49).

A balanced relationship between microbiota and the mucosal immune system is one of the key factors to sustain GI homeostasis, which is emphasized by the loss of this balance in the context of certain GI diseases including inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis (50). These disorders are characterized by deregulation of the relationship between the intestinal immune system and commensal microbiota members that may be driven by misleading cytokine profiles (18). Evidence concerning the importance of the microbiota for the development and activity of the GI immune system and simultaneously the relevance of intact and protective intestinal barriers led to an increased attention of probiotic applications to support intestinal health. The interaction between probiotic bacteria and the host intestinal epithelial and immune cells may stimulate host cellular networks to sustain or strengthen mucosal homeostasis and prevent excessive inflammatory responses (51). It seems likely that the quantitative impact of probiotics on immunomodulation may be of prominent significance especially in the small intestine, where a substantial proportion of the immunomodulatory capacity of the body is located, whilst the endogenous microbial communities are relatively small (Fig. 1) (52, 53), allowing a drastic, temporal modulation of its composition by consumption of probiotic products. The following parts of this chapter will focus on the mode of probiotic action accomplished by selected lactobacilli, highlighting microbial molecules that directly impact on host cellular responses and thereby determine probiotic functionality.

Lactobacilli stimulate intestinal host cell responses

The intestinal crosstalk between lactobacilli and host cells has mainly been studied in *in vitro* model set-ups that allow high throughput screening. These

models simulate GI tract conditions in simplified, well controlled, and easily accessible systems (54). Intact bacteria and bacteria-derived molecules such as their metabolites and proteins are used as stimuli for different types of human cell lines including epithelial cell lines such as Caco-2, HT-29, and T84, or immune cell lines like peripheral blood mononuclear cells (PBMCs), DCs, or macrophages. The human colon adenocarcinoma cell lines Caco-2 and T84 originate from a tumor of the human colon epithelium (colonocytes). Caco-2 cells display characteristics of ileal epithelial cells and therefore represent small intestine-type enterocytes (55, 56), whereas the T84 cell line represents mucin secreting colon cells (57). HT-29 cells are considered to be representative for colon enterocytes, which can secrete mucins (58-60) and lack connecting structures such as tight junctions (61). These cell lines are commonly applied to assess the impact of lactobacilli on host cellular responses including intestinal barrier integrity and immunomodulation.

Specific *Lactobacillus* species and strains affected epithelial integrity of Caco-2, HT-29, and T84 cell monolayers as measured by trans-epithelial electrical resistance, TEER (62, 63). It has been proposed that barrier function is enhanced by up-regulation of tight junction proteins. For example, *L. acidophilus* ATCC4356 elicited increased TEER across Caco-2 cell monolayers, which was accompanied by the up-regulation of several tight junction related genes (64). Additionally, TEER enhancement in Caco-2 and HT-29 cells by *L. plantarum* MB452 was proposed to depend on modulation of cytoskeleton and tight junctional protein phosphorylation (65). Moreover, a recent crossover study in healthy human volunteers revealed that mucosal exposure to *L. plantarum* WCFS1 elicited a re-organization of tight-junction protein conformation in duodenal epithelial cells *in vivo* (8). Subsequent *in vitro* experiments employing Caco-2 cells showed that the latter effect depended on TLR-2 mediated signaling, thereby providing a first step towards the identification of the host-signaling pathway involved in this effect.

Most of the available immunomodulatory activities of *Lactobacillus* species and strains have been assessed by *in vitro* measurements of cytokine secretion in epithelial and immune cells. Among those, IL-10 is a marker for an anti-inflammatory response, while IL-12 and tumor necrosis factor (TNF)-α are determined as markers for a typical pro-inflammatory response.

Lactobacilli were shown to influence cell responses of Caco-2 (66), HT-29 (67, 68), and also of specialized immune cells including PBMCs, DCs, and macrophages in a species- and strain- dependent manner (69-73). To date, only a limited number of studies succeeded to associate the observed *in vitro* effects to the underlying molecular mechanisms and/or the microbial molecules that determine the cytokine profiles, which will be discussed below. The cellular host response towards lactobacilli has been assessed in several studies that focused on altered expression of pathways centered on regulator molecules such as NF-κB or MAPKs. Some lactobacilli inhibit NF-κB activation, for example, *L. rhamnosus* GG decreased IκB degradation and decreased TNF-α -induced IL-8 production in Caco-2 cells (66). *L. bulgaricus* LB10 reduced TNF-α-induced IL-8 secretion in HT-29 cells via reducing nuclear translocation of p65 (74). In contrast, *L. crispatus* induced TNF-α and IL-1β production via NF-κB activation in the macrophage cell line THP-1 (75), and *L. rhamnosus* GG inhibited pro-apoptotic p38 MAPK activation in TNF-α, IL-1 and IFN-γ stimulated young adult mouse colon explants (YAMC) and HT-29 cells (76). These studies showed that lactobacilli affected NF-κB and MAPKs related pathways to alter downstream cytokine secretions and immune responses in intestinal epithelial an immune cells in a strain-and species-dependent manner (77). Despite the high scientific value of these *in vitro* model-based findings, their extrapolation to the *in vivo* situation is far from straightforward since cell line-based observations fail to accurately reflect the intestinal tissue complexity including extensive communication between different cell types and the impact of the resident microbiota. Moreover, *in vitro* studies generally do not consider the fact of microbial adaptation to the GI tract conditions, an aspect, which has been assessed in several studies. As an example, the gene expression profiles of *L. plantarum* during mouse (78-80) and human GI tract passage (81) have been studied. Next to the identification of candidate bacterial effector molecules that are expressed under intestinal conditions, including genes encoding the sortase dependent proteins Lp_0800 and Lp_2940 (78, 79, 81), these studies revealed that *L. plantarum* gene expression not only altered dependent on the conditions encountered in the mouse or human GI tract, but was also influenced by the diet consumed by the host (80,

81). These observations emphasize the requirement for *in vivo* studies to accurately reflect microbial impact on host responses, which have only been recently published. In fact, an *in vivo* duodenal mucosal-tissue transcriptome study highlighted the NF-κB pathway modulatory capacities of *L. plantarum* WCFS1 in healthy human volunteers (82) that were shown to depend on the growth phase at which the *L. plantarum* cells were harvested prior to consumption. Only stationary phase *L. plantarum* cells induced NF-κB-dependent pathways that included both NF-κB-activation as well as NF-κB-attenuation factors like A20 and IκB. Overall these responses did not lead to any detectable immune cascade activation, supporting the typical adjuvanticity or tolerance stimulation effects associated with *L. plantarum* consumption (83, 84). In contrast, mid-logarithmic phase *L. plantarum* cells did not affect the NF-κB pathways significantly, but led to expression of genes participating in positive regulation of host cell proliferation. A follow up study that employed an identical intervention and sampling regime exemplified the differential modulation of host cellular pathways by specific commercially available probiotic lactobacilli (85). *L. acidophilus* Lafti L10 predominantly stimulated and regulated immune response pathways associated with innate and T_H1 related responses, while *L. casei* CRL-431 predominantly affected pathways associated with cell proliferation and metabolism. *L. rhamnosus* GG consumption led to modulation of signaling cascades related to wound repair, healing and ion-homeostasis (85). This study clearly established the differential signaling capacities of specific *Lactobacillus* species, which is likely to be caused by the distinct repertoire of MAMPs or effector molecules, which are expressed in a growth phase- and strain-specific manner. To this end, the first bacterial effector molecules have been identified that start to shed light on the molecular crosstalk between microbes and their hosts, which will be discussed below.

Molecular analysis of conserved microbial molecules as probiotic effector molecules

The availability of several complete lactobacilli genome sequences (21) has paved the way for comparative genomics and genetic engineering to identify bacterial molecules that determine probiotic function. To date,

several MAMPs have been indentified that can be connected to specific host responses (86). These effector molecules are in many cases associated with the bacterial cell wall (21) (Fig. 2). Similar to other Gram-positive microbes, the cell walls of LAB predominantly consist of a thick layer of peptidoglycan (PG), an organized polymer of repeating pentapeptide cross-linked *N*-acetylglucosamine and *N*-acetylmuramic disaccharide units (87, 88). PG is essential for maintaining the cellular shape and integrity and was shown to modulate immune responses (89-91). PG is recognized by TLR-2, which was postulated to preferably recognize diaminopimelic acid (DAP)-containing peptidoglycan of Gram-negative bacteria and to a lesser extend lysine-containing PG motifs of Gram-positive bacteria (89). In addition, PG fragments of lactobacilli like *L. johnsonii* JCM 2012 and *L. plantarum* ATCC 14917 were demonstrated to suppress IL-12 production in macrophages via mechanisms that involved TLR-2, but also NOD receptors that recognize DAP (NOD1)- or lysine (NOD2)- containing PG-derived muropeptides (92). In addition, purified PG from *L. salivarius* Ls33 exhibited an IL-10 associated anti-inflammatory potential, altered DC and T-cell regulatory functions in a NOD2-dependent manner, and protected mice from trinitrobenzene sulfonic acid (TNBS) induced colitis (93). In comparison, the non-protective *L. acidophilus* NCFM strain was lacking an additional muropeptide in the PG structure that was linked to the protective effect exerted by *L. salivarius* Ls33. Hence, this elegant study clearly established the role of PG components to differentially modulate host responses and represents an initial step towards understanding strain specificity of immune modulation on the molecular level. However the effect on signaling capacity by other modifications of the basic peptidoglycan polymer including amidation, acetylation, and glycosylation remain largely unexplored in probiotic lactobacilli, while research in human pathogens has shown that such modifications impact on host invasion and virulence efficacy (94) and might be crucial for modulation of host cell responses by probiotics as well. Nevertheless, these studies indicate the involvement of specific PRRs in PG recognition, i.e. the integration of a combined signaling of PG via TLR-2 and specific NLRs allows the differential recognition and cognate response towards PG sensing that discriminate between lysine, DAP, and PG pentapeptide cross bridges (95-97). Besides

PG, the LAB cell wall also contains teichoic acids (TAs), which are highly variable, phosphate-rich glycopolymers that are either covalently linked to peptidoglycan (wall teichoic acids, WTAs), or anchored in the cytoplasmic membrane (lipoteichoic acids, LTAs) (98). TAs are important for bacterial cell division and morphogenesis (99), but have also been highlighted for their significant role in bacterial recognition by host cells and processes like adherence and immunomodulation (100). LTAs appeared to play a prominent role in determination of the pro-inflammatory potential of certain *Lactobacillus* strains, which was not only supported by analysis of intact bacterial cells but also by analysis of purified LTA molecules. For example, LTAs isolated from *L. fermentum* YIT 0159 or *L. casei* YIT 9029 induced pro-inflammatory TNF-α secretion from macrophages in a TLR-2-, dose-, and strain-dependent manner (101). Moreover, purified LTAs of *L. plantarum* NCIMB8826, strongly induced TLR-2-dependent pro-inflammatory responses *in vitro* (102). Importantly, a *dlt* mutant derivative of this strain, which was deficient in D-alanylation of its LTA backbone, elicited a significantly reduced pro-inflammatory response *in vitro* compared to the parental strain. This effect was extrapolated to show that the *dlt* mutant, in contrast to the wild-type strain, protected mice from TNBS induced colitis (102). Similar results were obtained in studies using a *L. rhamnosus* GG derivative unable to D-alanylate its LTAs (103) and a *L. acidophilus* NCFM *ltaS* gene deletion mutant entirely deficient in LTA biosynthesis (104, 105). Taken together, these targeted mutagenesis studies established that alteration of the native LTA structure or its entire depletion significantly affected the signaling capacity of *Lactobacillus* strains via TLR-2-dependent pathways and commonly result in more anti-inflammatory responses, thereby creating a direct link between *Lactobacillus* molecules and their host side receptor counterpart. The role of *Lactobacillus* WTAs as an immune modulatory component has not been studied so extensively. However WTAs of *L. casei* Shirota and *L. plantarum* ATCC 14917 were recently demonstrated to act synergistically with LTAs to balance IL-10 and IL-12 production in mouse derived macrophages, which involved TLR-2-signaling (106). Specific lactobacilli possess the genetic capacity to synthesize multiple WTA variants (107), which today provides a unique opportunity to manipulate the molecular structure of these MAMPs

in an isogenic background. This could lead to more detailed analysis of the WTA molecular structures and their role as immune signaling regulators.
In some cases, the cell envelope the lactobacilli is decorated with capsular polysaccharides (CPS). These complex, surface located, high molecular weight hetero- or homo-polysaccharides (108) show high diversity in terms of their biochemistry as well as their production levels in individual bacterial species and strains. Specific CPS molecules derived from commensal intestinal microorganisms have been shown to be potent immunomodulating molecules (109). In analogy, the isolated CPS of *Lactobacillus casei* strain Shirota was shown to suppress pro-inflammatory immune responses in macrophages (110). Alternatively, surface polysaccharides may also act as shielding molecules that reduce exposure of other microbial cell surface compounds. For example, an *L. rhamnosus* GG derivative deficient in biosynthesis of a galactose-rich polysaccharide displayed increased adherence and biofilm formation capacity, which is likely to be associated with reduced shielding of certain adhesion molecules (111). In addition, an *in vitro* assay revealed that an EPS-deficient derivative displayed an increased sensitivity to the antimicrobial peptide LL-37 when compared to the parental strain, suggesting a role for extracellular polysaccharides in protection against host defense activity as well (112). The valuable information obtained from the first described CPS-deficient mutant in a *Lactobacillus* species strengthens the requirement for the construction of additional CPS-deficient strains to allow comparative analyses. To this end, the recent construction of an *L. plantarum* WCFS1 CPS-negative strain (113) might shed light on the strain specificity of the phenomena observed in *L. rhamnosus* GG.
Overall, the cell wall localized MAMPs described above are conserved in the majority of lactobacilli. Nevertheless, substantial differences in immunomodulatory capacities of these species and strains have been observed, which may depend on (subtle) differences in the chemical structures of these MAMPs, for example, D-alanylation or glycosylation ratios (102), chain length variation (114), sub-cellular localization (113), or strain-specific variation in the pentapeptide cross-linkages within the PG network (86). These studies clearly demonstrated that host responses are in parts determined by specific cell wall molecules and that host pathway modulation

can depend on biochemical modifications of certain molecules or their degree of interaction. Another fact that needs to be considered is the role of strain-specific proteinaceous compounds that may also antagonistically or synergistically affect the signaling capacities of the cell wall molecules described above.

Molecular analysis of strain-specific proteinaceous molecules as probiotic effector molecules

The cell surface of lactobacilli is decorated with proteins of which the composition and expression may be strain-specific. Some of these proteins have been recognized as probiotic effector molecules that influence host cell functions. For instance, combining the information available from comparative genome hybridization (CGH) experiments with phenotypic information (115) led to the *in vitro* identification of the mannose-specific adhesion (Msa) in *L. plantarum* WCFS1 (116). Msa was shown to elicit specific innate immune responses in pig mucosal tissues *in vivo* (117). Recent studies utilizing the same genotype-phenotype matching strategy also correlated specific immune cell responses to molecular variations between closely related strains of *L. plantarum*. Human blood-derived DCs were co-cultured with 42 different *L. plantarum* strains, revealing variable and strain-dependent IL-10 (up to 39-fold) and IL-12 (up to 600-fold) secretion levels (70). Analogously, co-culturing of the same strains with human PBMCs revealed similar variations of strain-specific IL-10 and IL-12 secretion levels, albeit with a lower overall fold-difference between individual strains (IL-10; 14-fold and IL-12; 16-fold) (71). *In silico* genotype-phenotype matching employing CGH profiles of the 42 *L. plantarum* strains enabled the correlation of IL-10 production capacity in PBMCs and DCs to the strain's capacity to synthesize and transport bacteriocins (70, 71). This correlation was verified by construction of several bacteriocin-gene deficient derivatives of the *L. plantarum* WCFS1 model strain, which were subsequently shown to indeed elicit altered cytokine profiles in either immune cell type compared to the wild-type (70, 71). These findings expand the role of bacteriocins in probiotics from the typical pathogen inhibition, as was illustrated elegantly for the anti-listerial salivaricin Abp118 of *L. salivarius* UCC118 (11), towards additional roles of these

molecules in directing host communication. Next to the bacteriocin genes, one of these studies (70) identified a regulator encoding gene to be involved in immunomodulation in DCs. This regulator was proposed to control the expression of a downstream gene putatively involved in glycosylation of TAs, thereby providing a link to its possible mechanism of modulation of immune cell recognition via TLR-2.

The recognition of certain lactobacilli by DCs has been proposed to involve DC-SIGN, the DC-specific intercellular adhesion molecule 3-grabbing non-integrin receptor. It was shown that strains of *L. reuteri* and *L. casei* were recognized by DC-SIGN, which elicited specific maturation patterns of immature DCs, which were subsequently shown to stimulate the differentiation of T_{reg} cells from naive T-cell populations (72). Surface layer protein (Slp) A of *L. acidophilus* NCFM was shown to be recognized by this receptor, thereby eliciting specific cytokine productions and immune responses in immature human DCs, whereas deletion of the SlpA-encoding gene led to significant reduced DC-SIGN-binding and altered cytokine patterns (118). Notably, purified SlpA was recognized by fucose- and mannose-specific lectins, which further supports its role as ligand of the DC-SIGN receptor that is known to recognize mannose- and fucose-containing structures (118, 119). Slps are not only found in strains of *L. acidophilus*, but have also been identified in several other lactobacilli such as *L. helveticus*, *L. brevis*, and *L. acidophilus* (21). Moreover, the Slp of *L. helveticus* was demonstrated to be involved in competitive exclusion of intestinal pathogens, suggesting additional roles for Slps (120). However, lactobacilli that lack surface layer proteins might still be recognized by DC-SIGN or other C-type lectin receptors, as they might synthesize glycosylated proteins. *L. plantarum* WCFS1, for example, expresses several glycoproteins as found via protein fractionation and gel based detection methods (Fig. 3A). One of the candidate glycoproteins of *L. plantarum* WCFS1 (Lp_2145) has a large extracellular exposed amino acid stretch rich in serine and threonine residues, which may serve as target residues for O-glycosylation (121), possibly involving the activity of the genetically linked glycosyltransferase (Lp_2142) (Fig. 3B). The smearing appearance of Lp_2145 on a protein gel supports its glycosylation by an endogenous protein modification pathway in *L. plantarum* WCFS1, especially

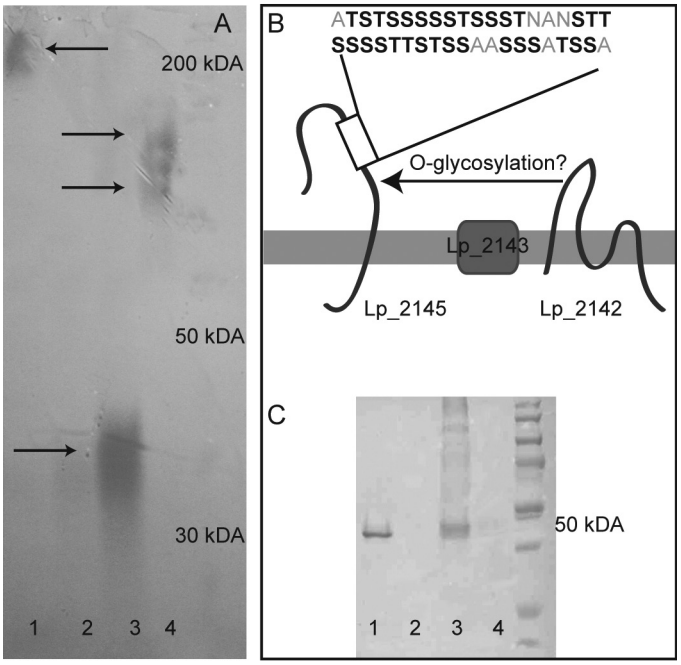

Fig. 3. A: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, containing whole proteome based sub-fractions of L. plantarum WCFS1. Glycoproteins were detected by Periodic acid-Schiff (PAS) staining, which revealed the presence of glycoproteins in the cytosol (lane 3), the envelope fraction (lane 1), and in the secreted protein fraction (lane 4). **B:** the Lp_2145 protein has a large extracellular exposed amino acid stretch rich in serine and threonine, which might be target for glycosylation by the action of a genetically linked glycosyltransferase (Lp_2142). C: Western blot detection of Lp_2145 by anti-His4-HRP conjugated antibody. Lp_2145-His6 protein overexpression was performed using the nisin controlled gene expression (NICE) system (122). Lp_2145 synthesis was nisin-induced in L. lactis MG1363 using 25 ng/L of culture (lane 1) and in L. plantarum WCFS1 using 250 ng/L (lane 3). Samples loaded in lanes 2 and 4 were derived from cultures not induced with nisin.

since heterologous expression (122) of the same protein in *Lactococcus lactis* MG1363 led to a non-smearing, distinct protein band on the gel of the anticipated molecular weight of the unmodified protein (Fig. 3C). More research on probiotic bacterial glycoproteins will be required to elucidate their mechanism of biosynthesis as well as their putative role in modulation of host cell responses. Other proteins, such as the secreted proteins p40 and p75 of *L. rhamnosus* GG were shown to modulate specific host cellular pathways, to stimulate Akt and inhibit apoptosis in TNF-induced mouse colon epithelial cells and cultured mouse colon explants (123). Homologues of genes that encode for p40 and p75 were also found in the genomes of *L.*

casei, L. paracasei and *L. rhamnosus.* Recently, the *L. casei* BL23 proteins were demonstrated to play similar roles in probiotic functionality (60).
Several proteins have been highlighted for their capability to beneficially modulate host cellular functions. These proteins appeared to be strain specific and part of a complex network, which involves other proteins, conserved cell wall polymers, and posttranslational events.

How molecular science may change the probiotic field

The probiotic concept captures the potential of certain bacteria to beneficially influence human health. It is now well established that within the GI system, probiotics are capable to interact with the host's epithelial- and immune cells and thereby impact epithelial barrier and immune functions. Severe epithelial integrity dysfunction including high-level permeability of the intestinal epithelium and consequently significant passage of antigens into the underlying lamina propria is associated with digestive disorders such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and celiac disease (124, 125). The alteration of tight junction proteins is nowadays considered as one potential mechanism underlying the development of such diseases (126). The probiotic role of lactobacilli in maintaining intestinal epithelial integrity is mainly investigated *in vitro*, either to study their preventative, indirect effect to subsequent challenge by bacterial pathogens (65, 127) and molecules such as proinflammatory cytokines (128) or, although to a lesser extent, to determine their direct impact on tight junction protein expression (64). Unfortunately, *in vivo* studies that address the impact of lactobacilli on junctional complexes are sparse but include a study conducted in mice suffering from colitis (129) and a recent study conducted in healthy humans (8). These studies propose that probiotic lactobacilli may either directly or indirectly provide protective effects on epithelial barrier function. However, further elucidation of the precise mechanisms and bacterial effector molecules involved is required to fully understand these beneficial effects and to exploit them to their full extend.
Specific MAMPs have been identified in the last few years that could be linked to certain immune modulatory capacities of lactobacilli *in vitro* using host cell line-based assays (70, 71, 92, 110, 118) as well as *in vivo* by

using mice colitis models (93, 102). Importantly, several of these studies pinpointed that especially LTAs contribute to the induction of proinflammatory cytokine profiles in a species-specific manner (102-105). The observed species-specificity likely lies in the fact that LTAs contain variable degrees of substitutions, which highlights the requirement for more structural studies on these MAMPs, which have only recently been initiated (130, 131). Detailed characterization of the full repertoire of MAMPs in their context of an intact bacterial cell and their associated signaling capacity via specific PRRs could enable purpose-directed, molecular-based probiotic applications. Ultimately, these efforts could close the gap between pharmaceuticals and probiotics and their modulation of human health on a complementary systemic and local basis, respectively.

Next to the initiation of bacterial effector molecule identification and characterization, reciprocal efforts have been launched for the host side. This equally important contribution embodies diverse host cellular receptors (25) but is further complicated by varying host dietary regimes (80) and genetic factors. Considering the latter, it is intriguing that a recent metagenomics approach revealed the functional composition of the human intestinal microbiome, enabling the discovery of three host-microbial symbiotic states termed enterotypes (132, 133). These observations position healthy consumers at different base-line microbiota functionalities and could explain the fact that non-responsiveness and interpersonal differential responses have been reported in probiotic studies (85). Overall, these studies direct us towards the concept of a more personalized application of probiotics to improve their efficacy depending on the host genetic background. Another factor contributing to the observed interpersonal differential responses can be found in the occurrence of single nucleotide polymorphisms (SNPs) within TLR-encoding genes (134), which can affect ligand recognition efficiency by the host cells (135). These intertwined aspects of probiotic communication with host cells cannot be addressed appropriately *in vitro*, where the use of different cell lines complicates comparative analysis and extrapolation of results obtained *in vitro* towards *in vivo* efficacy in humans is far from trivial and presents a major challenge for the future. Nevertheless, the general steps that are typically taken towards understanding probiotic function include *in*

vitro screening of selected bacteria or bacterial derived molecules, which in some cases are subsequently investigated *in vivo* in different animal models and in sporadic cases examined in humans (136). Despite some successful examples of this trial and error approach regarding *in vitro* to *in vivo* translation (137), it seems more straightforward to immediately assess probiotic efficacy *in vivo*, despite the fact that these studies are not suitable for high throughput screening approaches. To this end, some studies that were conducted in healthy humans investigated the impact of lactobacilli on intestinal tissue responses (82, 85, 138), which represents an elegant approach for the selection of specific probiotic strains for dedicated applications. Moreover, this might be an appropriate strategy to obtain molecular science-based leads in the relevant host system (human individuals) and to prevent the challenges of reproducing *in vitro* observations *in vivo*.

Outline of the thesis

The work presented in this thesis focuses on the molecular characterization of effector molecules of *L. plantarum* WCFS1, a model strain widely used to unravel the underlying mechanism of host-microbe interactions in the context of probiotic function.

Chapter 2 refers to a previous study, which demonstrated that *L. plantarum* WCFS1 modulates NF-κB related pathways in humans, notably only if harvested from the late stationary but not from the mid-logarithmic growth phase. A growth phase-dependent transcriptome profile was combined with the characterization of the cell-surface proteome using cell surface trypsinization and shotgun proteomics of bacteria derived from the mid-logarithmic and late stationary growth phase. The generated peptide samples were tested for their NF-κB modulatory capacities using an intestinal epithelial NF-κB reporter cell line, which revealed that bacterial cell surface peptides from the late stationary but not from the mid-logarithmic growth phase are potent NF-κB attenuating molecules.

Chapter 3 focuses on the role of sortase A (SrtA) of *L. plantarum* WCFS1. Cell surface trypsinization and shotgun proteomics revealed that the isogenic

SrtA-deficient strain displayed significantly reduced levels of sortase-depended proteins (SDP) on the cell surface. Nonetheless, gastrointestinal persistence of *L. plantarum* in mice, and cytokine secretion patterns induced in monocyte derived immature dendritic cells (iDCs) were not affected by the *srt*A mutation. By a LiCl treatment, several SDPs could be extracted from the cell surface of the *srt*A knockout but not from the wild-type strain, which caused increased proinflammatory cytokine production in iDCs elicited by the *srt*A gene deletion mutant. This data suggests a role of SDP in attenuation of immune system stimulation.

Chapter 4 focuses on Lp_0800 (here renamed StsP), a high molecular weight, SDP of *L. plantarum*, which was previously shown to be induced in the intestine of mice and humans. By chromosomal promoter replacement, StsP was expressed *in vitro* in a wild-type and *srtA* mutant background. Comparative proteomics allowed the confirmation of the predicted sortase-dependent surface anchoring of StsP. Tryptic surface peptide fractions of the StsP-producing strain as well as gel-purified StsP peptides were found to be strong NF-κB attenuators in intestinal epithelial cells.

Chapter 5 describes 4 capsular polysaccharide (*cps*) gene clusters encoded by *L. plantarum*, which are predicted to be involved in the biosynthesis and transport of cell envelope associated polysaccharides. Gene cluster deletion mutants, either lacking individual or combinations of *cps* clusters, were constructed. Genome-wide transcriptome profiles of the wild-type strain were compared with those of the *cps* mutant strains, revealing pleiotrophic effects of some of the *cps* mutations, especially in functions related to amino acid metabolism and transport, but no compensatory effects on other *cps* cluster expression levels. Moreover, the impact of *cps* cluster deletion on the amount of CPS produced, its molar mass and monosaccharide composition were evaluated in the mutant strains, revealing an alteration of surface glycans in each of the *cps* mutant strains. Interestingly some *cps* mutants displayed an increased capability to activate TLR-2 signaling, demonstrating that the modulation of the cell surface by reduced levels of polysaccharides may enhance host cell recognition.

Chapter 6 discusses the major finding of the thesis connected to recent scientific insights on host-microbe interactions in the context of health and disease.

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Chapter 2

Late stationary phase surface peptides of *Lactobacillus plantarum* attenuate NF-κB activation

Daniela M. Remus, Fabrizia Fusetti, Jurgen Karczewski, Roger S. Bongers, Irene Konings, Bert Poolman, Maria L. Marco, Paul de Vos, Jerry M. Wells, Michiel Wels, Peter A. Bron, and Michiel Kleerebezem

Abstract

L. plantarum WCFS1 was previously shown to modulate human mucosal nuclear factor-κB (NF-κB) related pathways *in vivo* in a growth phase-determined manner. Here, we characterize the growth phase-specific transcriptome patterns in *L. plantarum* WCFS1 focusing on genes predicted to encode cell surface associated proteins, which we parallel with the characterization of the cell surface proteome of bacteria derived from the mid-logarithmic and late stationary growth phase using cell surface trypsinization and shotgun proteomics. Overall, 75 % of surface proteome-encoding genes, which were among the highly expressed genes, could also be detected in the trypsin-assessed proteome. Differential expression of 21 extracellular proteins was detected when comparing the two growth phases, whereas 17 and 33 proteins were exclusively present in the late stationary and mid-logarithmic growth phase, respectively. Using a NF-κB reporter assay in Caco-2 intestinal epithelial cells, we demonstrated that only late stationary phase surface-accessible peptides were capable of attenuating NF-κB activation elicited in response to flagellin and the *L. plantarum* cell envelope. Our work provides new insights into the induction of tolerance-related responses in the intestinal mucosa by *L. plantarum* WCFS1 and identifies late stationary phase peptides as mediators for NF-κB attenuation.

Introduction

Lactobacilli colonize diverse natural habitats including dairy products, plants, and the mammalian vaginal, oral, and gastrointestinal (GI) tract (1). Selected *Lactobacillus* strains are marketed as probiotics, defined as living organisms that, when administered in adequate amounts, confer a health benefit to the host (2, 3). The cellular probiotic-host interactions are ultimately mediated by probiotic "effector molecules" (4, 5), of which most that were identified to date have been shown to be associated with the bacterial cell surface. Examples include bacterial cell wall compounds such as lipoproteins, lipoteichoic acids, peptidoglycan, and cell wall bound polysaccharides (4, 6). In addition to these more conserved components of the Gram-positive cell wall, strain-specific surface proteins have been identified that play a role in host cell adhesion and intestinal persistence including Lp_2940 (7, 8) and Msa of *L. plantarum* WCFS1 (9, 10), MapA and Mub of the *L. reuteri* strains 104R and 1063, respectively (11, 12), and the pilin protein SpaC of *L. rhamnosus* GG (13). The surface layer protein SlpA of *L. acidophilus* NCFM was shown to modulate the immune response of dendritic cells (14), and the surface-associated proteins p40 and p75 of *L. rhamnosus* GG affect specific host cell pathways (15). Besides these genuine bacterial surface associated proteins, so-called 'moon-lightning' proteins, i.e. cytoplasmic proteins that despite the absence of secretion signals are localized on the bacterial cell surface (16), have been identified as probiotic effector proteins. For example, the GroEL chaperone (17) and elongation factor Tu (18) of *L. johnsonii* La1 were implicated in cell attachment and immune modulation, while GAPDH of *L. plantarum* LA 318 was documented to be essential for bacterial adherence to human colonic mucin (19).

In the current post-genomic era, whole-genome sequencing projects have been initiated for more than 100 *Lactobacillus* strains of a variety of species (20, 21). The complete *L. plantarum* WCFS1 genome was the first to be publically accessible (22), and subsequent experimental and *in silico* analyses with this strain have generated genome-based metabolic- (23) as well as gene regulation- models (24) and elucidated bacterial transcriptional responses to stresses relevant for intestinal passage and industrial processing (25-27). The parental strain of *L. plantarum* WCFS1 was demonstrated to display

relatively high survival and persistence rates in the human GI tract (28) and to bind to human mucosal cells (29). Consequently, it has been employed extensively as a model organism to study the molecular basis of host-microbe interactions (4). This has led, among others, to the identification of *L. plantarum* genes specifically induced during GI tract passage in mice as well as their expression levels at different intestinal sites (7, 30). These findings were subsequently complemented by the assessment of global transcription profiles of *L. plantarum* residing in the caecum of mice (31) as well as in the human intestine (32). Recent work has shown that *L. plantarum* WCFS1 modulates cellular functions of human dendritic cells (33) and peripheral blood mononuclear cells (34) *in vitro* as well as human tight junction protein complexes *in vivo* (35). The host response to *L. plantarum* was investigated in healthy human volunteers where a nutrigenomic approach allowed the characterization of the mucosal transcriptome responses in the small intestine upon *L. plantarum* consumption (36). This study highlighted the activation of gene regulatory networks associated with the canonical pathway of nuclear factor (NF)-κB. Notably, the modulation of these NF-κB associated responses was strictly dependent of the growth phase from which the bacteria were harvested prior to consumption, i.e. only stationary phase bacteria elicited significant NF-κB responses (by activating and attenuating its associated genes), while mid-logarithmic phase bacteria did not affect these pathways (36). In this study, the distinctive, growth phase dependent effects were tentatively assigned to postulated changes in the bacterial cellular “make-up”, drawing our attention to growth phase specific expression of microbial (surface) compounds.

To address the question of which molecules are responsible for the growth phase-depended NF-κB modulation *in vivo*, we assessed the growth-dependent transcriptional response of *L. plantarum*, focusing on expression patterns of genes predicted to encode surface associated proteins. In parallel, we characterized the *L. plantarum* cell surface proteome composition by trypsinization of intact bacterial cells (37, 38) harvested from the mid-logarithmic and late stationary growth phase, coupled to peptide analysis by linear ion trap-Orbitrap hybrid mass spectrometry. Subsequently, the cell surface peptides obtained from the mid-logarithmic and late stationary growth

phase were used in an NF-κB reporter assay in intestinal epithelial cells to assess the impact of different peptide fractions on NF-κB signaling. We found that only late stationary phase peptides attenuated NF-κB signaling, which could confidently be assigned to the surface proteome derived peptides on basis of the observation that cytosolic peptides from the same cells activated rather than attenuated NF-κB signaling.

Materials and Methods

Bacterial strains and culture conditions

L. plantarum WCFS1 (22) was grown in 2 × chemical defined media (CDM) (23) at pH 5.9 containing 1.5 % (wt/vol) glucose. For the surface proteome profiling and DNA microarray analyses, overnight-grown cultures were diluted to an initial optical density at 600 nm (OD_{600}) of 0.16 and inoculated into a Sixfors fermentor system with six individual 500-mL vessels (Infors, Bottmingen, Switzerland). Fermentations were performed at 37 ^{o}C, constantly agitated at 150 rpm, and maintained at pH 5.9 by the temporal addition of 2.5 M NaOH. For surface proteome analysis, samples were taken at OD_{600} = 1.0 and after 24 h (late stationary phase of growth). For transcriptome analyses, samples were collected at different stages of logarithmic and stationary growth phases (Fig. 1).

Growth phase-dependent transcriptome profiling of *L. plantarum*

Total *L. plantarum* RNA was isolated from cultures harvested at different growth stages using standard protocols (31, 39). In short, following methanol quenching (40), cells were harvested by centrifugation (6000 × g, 20 min, 4^{o}C), resuspended in 400 µL ice-cold CDM medium and transferred to tubes containing 500 µL phenol/chloroform solution (4:1 [v/v]), 30 µL 10 % sodium dodecyl sulfate, 30 µL 3 M sodium acetate (pH 5.2), and 0.5 g zirconium beads. Cells were disrupted by bead beating using a Savant FastPrep FP120 instrument (Qbiogen Inc., Illkirch, France) and RNA was collected from the aqueous phase followed by further purification using the High Pure Isolation Kit (Roche Diagnostics, Germany). RNA concentration and purity were determined using A_{260} and A_{280} measurements using an ND-1000 spectrometer (NanoDrop Technologies Inc., Wilmington, United States),

and RNA quality was verified with a 2100 Bioanalyser (Agilent Technologies, Amstelveen, the Netherlands). Only Samples displaying a 23S/16S RNA ratio equal or superior to 1.6 were used for labeling.

Transcriptome analysis and interpretation

3 µg RNA was used for cDNA synthesis. Cyanine-3 (Cy3) and cyanine-5 (Cy5) cDNA labeling was performed as described previously (33) using the CyScribe Post-Labeling and Purification kits according to the manufacturer's instructions (Amersham Biosciences, Bucking-Hamshire, UK). Cydye-labeled cDNAs (0.5 mg each) were hybridized to *L. plantarum* WCFS1 printed-oligonucleotide DNA microarrays (Agilent Technologies, Amstelveen, the Netherlands). The array design and transcriptome data used in this study were deposited under platform GPL13984 and accession numbers GSE31000 and GSE31076 in NCBI's Gene Expression Omnibus (GEO) (41, 42) at http://www.ncbi.nlm.nih.gov/geo/. Hybridization and scanning procedures were performed as previously described (33). As fluorescence intensities can differ between individual array slides, slide scanning was carried out at several photo multiplier tube (pmt) values, and the optimal scan of each individual microarray was selected on the basis of signal distribution (combination of a low number of saturated spots and a low number of low signal spots). The data were normalized using the Lowess normalization as available in MicroPrep (43). Median intensities of different probes per gene are given as absolute gene expression intensities.

The gene expression intensities were compared and clustered using Short Time-series Expression Miner, STEM (version 1.3.6, http://www.cs.cmu.edu/jernst/stem) (44). The STEM Clustering Algorithm was applied with a maximum number of model profiles of 50 and a maximum unit change in model profile between time points of 2. Gene expression microarray data were visualized using the Biological Networks Gene Ontology, BiNGO (http://www.gnu.org/) for assessing functional gene main and subclasses [gathered from (22)] significantly [Hypergeometric test, corrected for multiple testing using False Discovery Rate (45), ($p < 0.05$)] overrepresented within the tested dataset (46). The multi experiment viewer, MEV (http://www.tm4.org) was used to visualize gene expression levels across several time points (47).

Cell surface trypsinization of *L. plantarum* cells and surface proteome profiling

The protocol for the surface trypsinization was adapted from Severin *et al*. (48). Cell cultures were harvested from bioreactors (200 mL at OD_{600} = 1 and 50 mL after overnight growth, which was then diluted to obtain 200 ml concentrated equivalent to OD_{600} = 1), and pelleted by centrifugation (6000 × g, 20 min, 4°C). Pellets were washed twice with 20 mL isotonic 20 mM Tris/HCl pH 7.6 buffer containing 150 mM NaCl, 1 M xylose, 20 mM $CaCl_2$ and 5 mM DTT and subsequently resuspended in 5 mL of the same buffer, followed by the addition of 15 µg mass-spectrometry grade trypsin (Sigma T6567, Sigma-Aldrich, Missouri, USA). Samples were incubated for 10 min at 25 °C, aliquoted into Eppendorf tubes and centrifuged (20000 x g, room temperature, 1 min). The shaved peptide-containing supernatants were collected and filtered through a cellulose-acetate filter (0.2 µm pore size, 25 mm diameter; Sigma-Aldrich Missouri, USA). To further complete protein digestion, 5 µg of trypsin was added and samples were incubated overnight (4 rpm, 25 °C). Trypsin was inactivated by addition of trifluoroacetic acid (TFA) to a final concentration of 0.1 % (v/v). Samples were freeze-dried and stored at -20 °C prior to mass-spectrometry analysis.

Mass-spectrometry-based surface peptide identification and data interpretation

Prior to chromatographic separation, shaved peptides mixtures were cleaned up on reverse phase C18 TopTips (GlyGen Inc., Columbia, USA) and eluted with 80 % methanol in 5 % formic acid. For 2-dimensional chromatography the shaved peptides were separated on a 50 x 1.0 mm silica-based polysulfoethyl aspartamide strong cation exchange (SCX) column (column volume 0.039 ml) (PolyLC Inc., Columbia USA) mounted on an Ettan MDLC system (GE Healthcare, Buckinghamshire, United Kingdom) run at a flow rate of 50 µL/min. Solutions A (10 mM triethylammonium phosphate, pH 2.7, 25 % acetonitrile) and B (10 mM triethylammonium phosphate, pH 2.7, 25 % acetonitrile, 500 mM KCl) were used as the mobile phase. Peptides were eluted with a 3-step gradient: 1) 0 to 5 % B in 5 column volumes (CV); 2) followed by 12 to 30 % B in 10 CV; and 3) 24-60 % B in 5 CV. Fractions

were collected at 2 minute intervals. Eluted peptides were vacuum dried and resuspended in 50 µL of 5 % formic acid.

Subsequent peptide separation by reverse phase chromatography was carried out on C18 capillary column (ID 75 µm x 150 mm, 3 µm particle size, Dr. Maisch, Ammerbuch-Entringen, Germany) packed in- house and mounted on a Proxeon Easy-LC system (Proxeon Biosystems, Odense, Denmark), in line with a trapping pre-column (ReproSil-Pur C18-AQ, ID 100 µm x 20 mm, Proxeon). Solutions of 0.1 % formic acid in water and a 0.1 % formic acid in 100 % acetonitrile were used as the mobile phase. Peptides were eluted during a 90 min 4-35 % acetonitrile gradient at a flow rate of 250 nL/min.

Eluted peptides were analyzed using a linear ion trap-Orbitrap hybrid mass spectrometer (LTQ-Orbitrap, Thermo Fisher Scientific, Bremen, Germany). MS scans were acquired in Fourier transformed mode in a range from 300 to 2000 m/z, with a resolution of 60,000. The real time lock-mass option was used for internal calibration. A maximum of 7 ions per scan were submitted to MS/MS fragmentation (35 % normalized collision energy), spectra were detected in the linear ion trap.

The MS raw data were submitted to Mascot (version 2.1, Matrix Science, London, UK) using the Proteome Discoverer 1.0 analysis platform (Thermo Fisher Scientific, Bremen, Germany) and searched against the *L. plantarum* proteome (3063 entries), combined with reversed entries for all protein sequences to assess false positive discovery rates. Sequences of porcine trypsin (NCBI accession: P00761) and human keratins (P35908, P35527, P13645, NP_006112) were added to the database. Peptide tolerance was set to 10 ppm and 0.8 Da for intact peptides and fragment ions respectively; the option semi-trypsin was chosen to allow for 2 uncut trypsin cleavage sites. Oxidation of methionine residues and deamidation of asparagine and glutamine were specified as variable modifications. The MS/MS-based peptide and protein identifications were further validated with the program Scaffold v3.0 (Proteome Software Inc., Portland, USA). Protein identifications based on at least 1 unique peptide identified by MS/MS with a confidence of identification probability higher than 95 %, were accepted. Peptide identifications were accepted if they could be established at greater than 95

% probability as specified by the Peptide Prophet algorithm (49). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principle of parsimony (50). The false positive discovery rate was calculated by dividing 2 times the number of proteins identified in the reversed database by the sum of all 828 proteins identified in forward and reversed versions of the database. In all measured samples, less than 1 % hits from the reversed database were detected.
To estimate the relative abundance of each predicted surface protein, cell surface trypsinization was performed on three independent cell cultures at both mid-logarithmic and late stationary phase. Peptides were separated by reverse phase chromatography using a 140 min long 2-35 % acetonitrile gradient and analyzed by LC-ESI-MS as described above. Spectral count normalization was applied as described previously (51, 52). The Normalized Spectral Abundance Factor (NSAF) was calculated independently for each of the three replicate samples. Significant differences in NSAF values between time points were determined by a two-tailed t-test and p-values of < 0.05 were considered significant.

Isolation of cytoplasmic proteins

Mid-logarithmic and late stationary phase *L. plantarum* cells were harvested as described above. Cells of both cultures were diluted to OD_{600} = 4 using PBS buffer, pH 7.5. Samples (10 mL each) were passed through a french pressure cell at 1150 psi (SLM Instruments Inc., Urbana, USA). To remove intact cells and cell debris, the samples were centrifuged (6000 × g, 15 min, 4 °C), and subsequently ultracentrifuged (200000 × g, 20 hours, 4 °C). The final supernatant was collected, 20 µg trypsin (Sigma T6567, Sigma-Aldrich, Missouri, USA) was added and samples were incubated over night at 25 °C. Trypsin was inactivated by addition of TFA to a final concentration of 0.1 % (v/v). Samples were freeze-dried and stored at -20 °C prior use.

Preparation of protein free cell envelopes of *L. plantarum*

Protein-free *L. plantarum* derived cell envelopes were prepared and used as stimuli for NFκB activation. *L. plantarum* WCFS1 was grown in 2 × CDM (23) containing 1.5 % (wt/vol) glucose. After overnight culturing at 37 °C, cells

were harvested from the late stationary growth phase. Cells were washed three times in PBS buffer, pH 7.5, subsequently diluted to OD_{600} = 2.5 of which 2 mL were used for bead beating. Samples were centrifuged (5 min, at 2500 × g, room temperature) to remove intact cells and the supernatant was collected and again bead beaten. The cell pellet was collected by centrifugation (5 min, 12000 × g, room temperature), washed 3 times with PBS buffer, pH 7.5 and incubated with DNAse (500 ng/mL) and RNAse (1 µg/mL) for 3 h at 30 °C. Cell pellets were obtained by centrifugation (5 min, 12000 × g, room temperature) and washed 5 times with PBS buffer, pH 7.5, afterwards resuspended in 2 mL of PBS buffer pH 7.5 and ProteinaseK was added (1 µg/mL). The sample was incubated for 24 h at 30 °C applying constant rotation (20 rpm). After incubation, the sample was centrifuged (5 min, 12000 × g, room temperature) and washed 10 times using PBS buffer pH 7.5. The dry weight of the protein free cell envelope pellets were determined before being dissolved in PBS buffer pH 7.5.

Caco-2 NF-κB-GFP reporter assay

Tryptic peptide fractions obtained from surface shaving of whole cells or isolated cytosolic fractions of cells from mid-logarithmic and late late stationary growth phases were purified on reverse phase C18 TopTips (Poly LC Inc., Columbia, USA) as described above and subsequently evaluated using the NF-κB-promoter activation assay in Caco-2 cells (supplemental material). Caco-2 cells were seeded into black 384- wells plates with a clear bottom and cultured for 2 weeks to allow them to fully differentiate into mature villus-like enterocytes. Mid-logarithmic and late stationary growth phase peptide fractions were added to the wells with and without the simultaneous addition of flagellin and the cell envelope material isolated from *L. plantarum.* As controls, no peptides (negative control), flagellin or the cell envelope fraction (positive controls) were added. All sample conditions were tested in triplicates. Additionally, late- stationary phase peptide fractions obtained from SCX-based chromatographic fractionation were added to the wells with and without the simultaneous addition of flagellin and incubated as described above. After incubation, cells were washed once with PBS and fixed using 4 % paraformaldehyde. Nuclei were stained for 20 min using 1:500 dilution of

DRAQ5 (Enzo Life Sciences BVBA, Zandhoven, Belgium). Confocal images were obtained using a Zeiss LSM 510 system consisting of a Zeiss Axioskop with a Zeiss LD Plan Neofluar 40 × NA 0.6 objective. GFP fluorescence intensities of single cells were analysed with CellProfiler 2.0 (Whitehead Institute for Biomedical Research, Cambridge, USA). GFP fluorescence intensities per cell were measured and one-way analysis of variance (one-way ANOVA) was used to determine statistical differences between samples; $p < 0.05$ was considered significant.

Results

Growth-phase dependent gene expression analysis in *L. plantarum*

DNA microarray analyses were used to compare global growth-phase dependent gene expression of *L. plantarum* WCFS1 during mid-logarithmic (OD_{600} = 1.0) to transition (OD_{600} = 5.85), early- and late stationary phase (OD_{600} = 5.6-4.6) (Fig. 1). Absolute gene expression levels of all genes

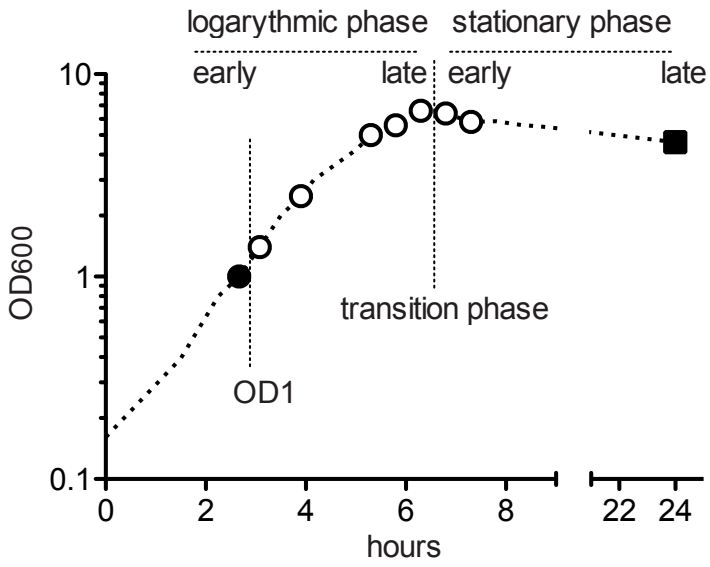


Fig. 1. Growth of *Lactobacillus plantarum* WCFS1 in 2 x chemical defined medium (CDM) containing 1.5 % glucose (wt/vol). Growth conditions were kept at pH 5.9, constant agitation (150 rpm) and 37 degrees Celsius. Open circles (○) show time points at which samples were taken for transcriptome analysis, to access the changes of gene expression between mid-logarithmic and late stationary phase of growth. Black dot (●) represents harvesting time point for proteome profiling and black square (■) represents harvesting time point for proteome and transcriptome profiling. These time points were previously chosen for evaluating the growth phase specific modulation of NK-κB by *L. plantarum* in vivo (36).

were clustered by STEM and visualized using BiNGO and MEV. In addition, predicted extracellular genes (55) were clustered by STEM to identify transcriptional responses of genes that were anticipated to contribute to specific host-cell responses.

A total of 75.4 % of the annotated *L. plantarum* WCFS1 genes were clustered by STEM into eight significant profiles (data not shown). The two clusters, which contained the highest number of genes, were selected for BiNGO

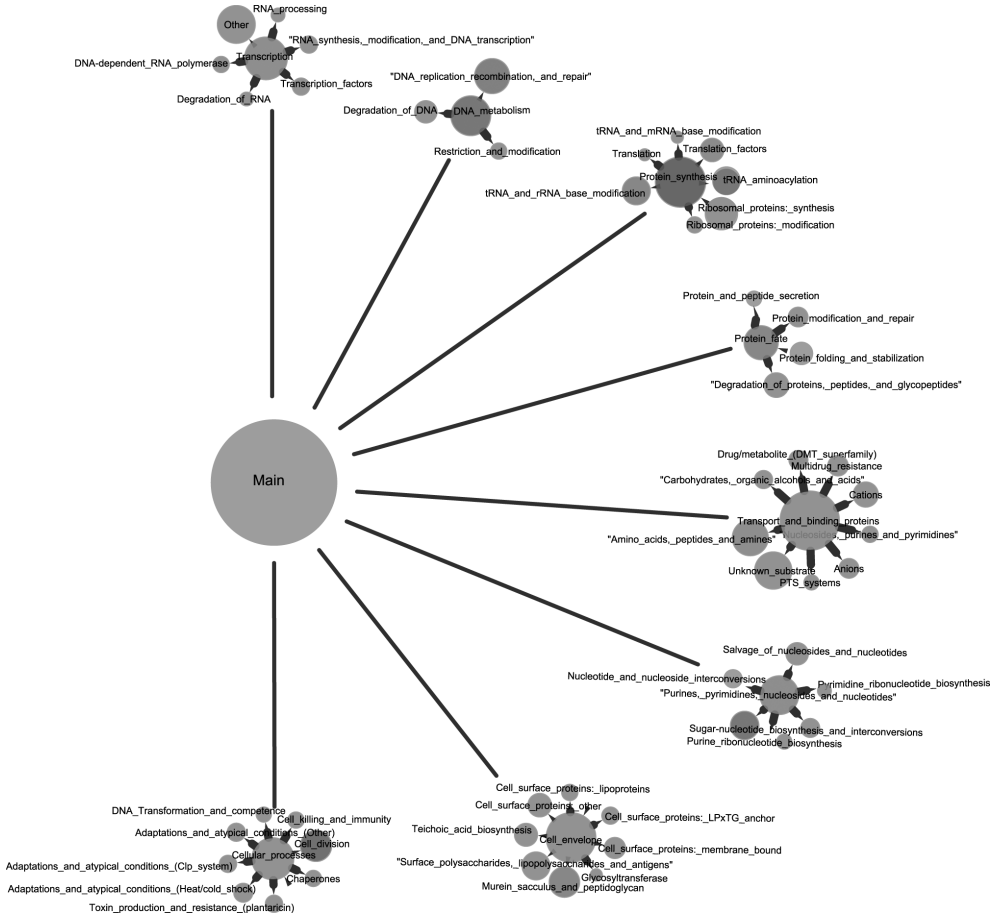
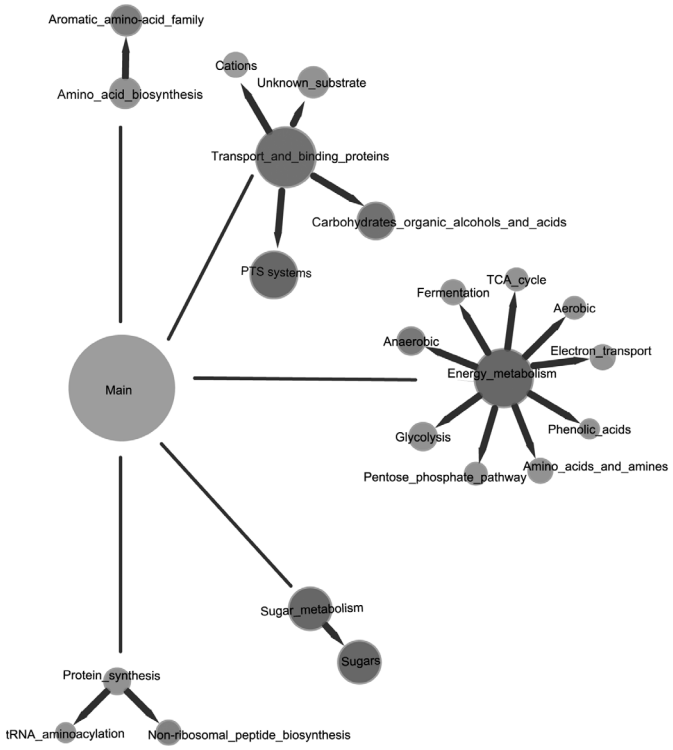


Fig. 2. Bingo images presenting significantly overrepresented gene main and subclasses. The area of a node is proportional to the number of genes in the test set annotated to the corresponding gene category.

A: Expression profile that was characterized by a decline of expression during transition and stationary phase of growth. Significantly overrepresented within this profile are main gene classes associated with protein synthesis, protein fate, transcription, and DNA and nucleotide metabolism, as well as functional subclasses like cell division and biosynthesis and degradation of murein sacculus and peptidoglycan.

analysis to identify overrepresented functional gene classes. The majority of significantly clustered genes (46 %) displayed an expression profile that was characterized by a consistent decline of expression during transition from mid-logarithmic towards stationary phase of growth. The main functional classes significantly overrepresented within this gene-profile were associated with protein synthesis, protein fate, transcription, as well as DNA and nucleotide metabolism. Moreover, functional subclasses including cell division and degradation of murein sacculus and peptidoglycan were significantly overrepresented in this profile while their corresponding main classes were not overrepresented (Fig. 2A). These classes reflect the cellular response to the decline of growth in stationary phase and involve factors related to



B: Expression profile that was characterized by an expression increase until late logarithmic phase. Overrepresented gene main classes within this expression profile include sugar metabolism, genes for transport and binding functions (PTS system), and central intermediary metabolism (Biosynthesis and degradation of polysaccharides).

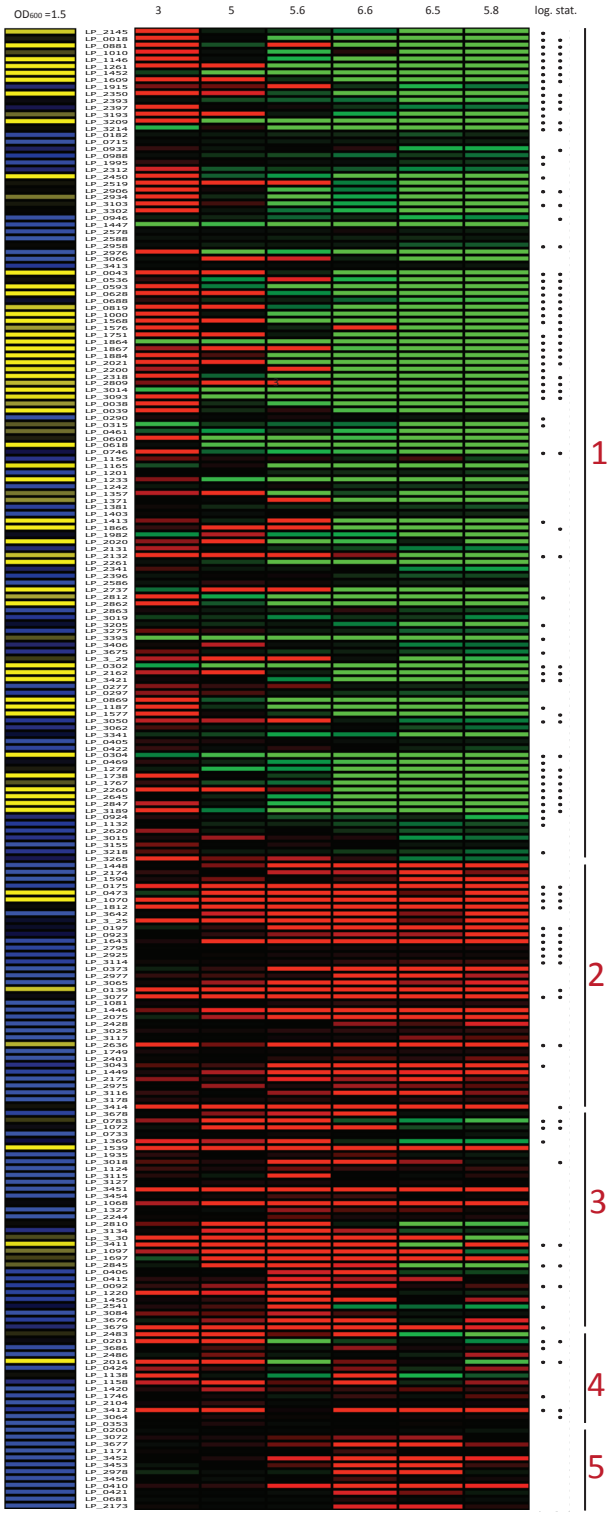
the growth and synthesis of macromolecular biomass components such as ribosomes, DNA, and cell wall.

A second cluster containing 6 % of all annotated genes, displayed a consistent increase of expression over time with maximum expression levels reached between transition and early-stationary phase, followed by a decline of gene expression towards late stationary growth phase. Overrepresented main functional classes among these genes included energy metabolism and genes for transport and binding functions including genes specifying components of the phosphotransferase (PTS) systems (Fig. 2B). These responses correlate with the emergence of carbon starvation at the end of logarithmic growth. Overall, these findings are in agreement with known physiological changes occurring in bacteria during the transition from logarithmic- to stationary phase of growth.

Growth specific regulation of genes encoding extracellular proteins

Genes predicted to encode extracellular proteins encompass 236 of all annotated *L. plantarum* WCFS1 genes (7.7 %) and are predicted to be entirely or largely located outside of the cell (55). Based on sequence analysis, these proteins are annotated as N-terminally- (110 proteins), C-terminally- (7 proteins), or lipid-anchored (46 proteins) to the cell membrane, released (43 proteins), or sortase-dependent cell wall-anchored containing an LPXTG motif (32 proteins). A total of 215 of the 236 (91 %) extracellular genes were grouped into 5 clusters by STEM according to their expression levels in the cultures. Two clusters contained genes with decreasing expression levels between mid-logarithmic to stationary phase of growth (Fig. 3). Among these genes, 55 % were characterized by a consistent decline of expression in subsequent growth phases (cluster 1). Notably, most genes encoding membrane anchored proteins or with murein sacculus- and peptidoglycan-associated functions (*lp_3014* and *lp_2645*) were found within this cluster. Another proportion of genes (17 %) displayed increasing expression levels

Fig. 3. Heatmap of STEM generated clusters (A-E) of the surface proteome. Yellow to blue color (high to low) code represents absolute gene expression levels at OD600 = 1.5. Red to green (= high to low) color code represents gene expression levels ranging from late-logarithmic over transition into stationary phase relatively to OD600 = 1.5 to follow gene expression trends over time. Asterisks represent protein detection in mid-logarithmic and late stationary phase, respectively.


OD600 =1.5
3
5
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1
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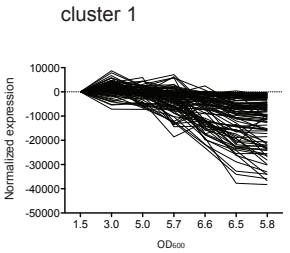
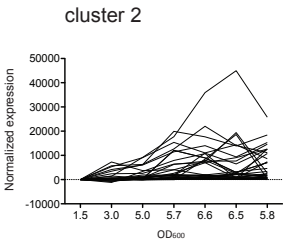
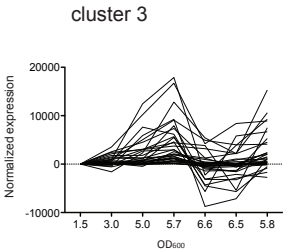
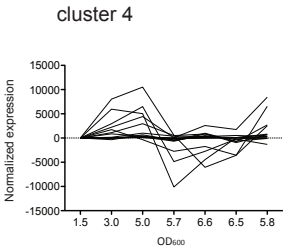
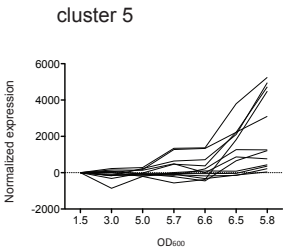

cluster 1
Normalized expression
OD600


cluster 2
Normalized expression
OD600


cluster 3
Normalized expression
OD600


cluster 4
Normalized expression
OD600


cluster 5
Normalized expression
OD600

during the transition from mid-logarithmic to stationary phase, followed by a decline of gene expression towards late stationary phase (cluster 2) (Fig. 3) and mainly include genes encoding for LPXTG anchored proteins (*lp_2925*). Two profiles encompassing 15 % and 7 % of the genes were defined by growth phase-dependent fluctuation of expression (cluster 3 and 4) and both contained genes with transport- and ligand binding-associated functions (*lp_0783* and *lp_3686*) while another profile (6 %; cluster 5) showed a consistent increase of expression during mid-logarithmic until stationary growth phase and contained genes encoding secreted (released) proteins (*lp_2173*). Taken together these data demonstrate a dynamic and significant modulation of exoproteome encoding gene expression in different growth phases.

Mass-spectrometry- based identification of surface proteins of *L. plantarum*

A comprehensive mass-spectrometry-based proteomic analysis was carried out to identify surface proteins expressed by *L. plantarum.* To achieve a high level of surface proteome coverage, tryptic peptides were obtained by cell surface trypsinization followed by a MuDPIT (Multidimensional Protein Identification Technology) approach (56) and combined with high resolution LC-ESI-MS/MS. In total, 124 extracellular proteins were identified covering 53 % of the predicted extracellular proteome. These proteins include 34 lipid-anchored, 52 N-terminally-anchored, 13 LPXTG cell wall-anchored, one C-terminally-anchored and 25 secreted (released) proteins. Overall (combining 1D- and 2D-LC-MS/MS), 33 extracellular proteins were exclusively detected in the mid-logarithmic phase, whereas 17 proteins were exclusively detected in the late stationary phase (table S2, S3). Protein quantification by applying the Normalized Spectral Abundance Factor (NSAF) based on the three biological replicates of the 1D-LC-MS/MS experiment revealed 21 extracellular proteins detected in significant different quantities ($p < 0.05$) in mid-logarithmic and late stationary growth phase (Fig. 4). In fact, 13 proteins were detected in higher amounts in the late stationary phase and 8 proteins were found in higher amounts in samples from the mid-logarithmic phase.
Overall, 39 % of the total number of MS/MS spectra identified in the mid-

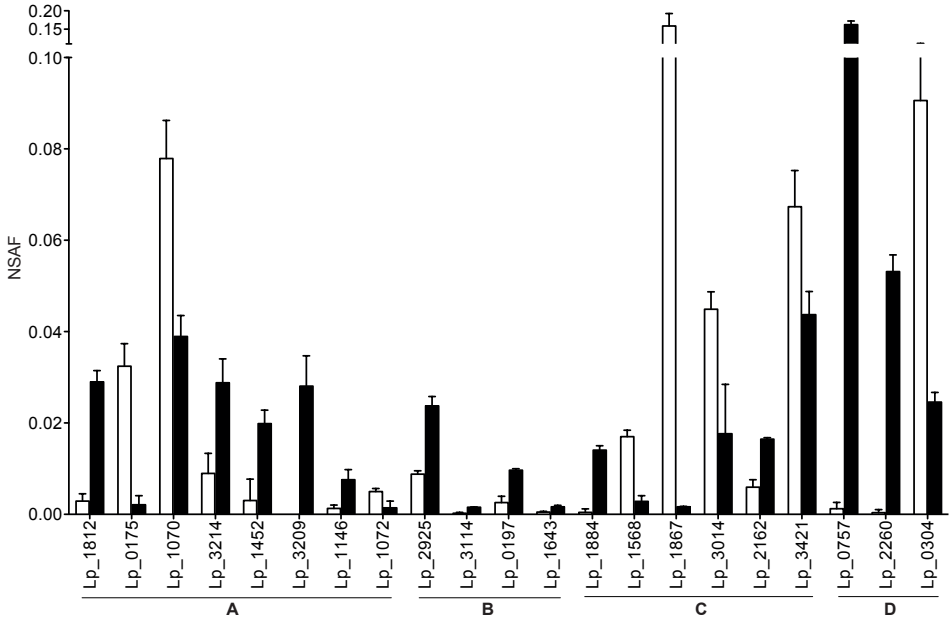


Fig. 4. NSAF values of proteins that were detected in significantly different quantities ($p < 0.05$) between mid-logarithmic and late stationary growth phase. The averages and standard deviations of all protein sorted from lowest to highest p-values within each class of a sub-cellular protein location (A = lipid anchored, B = LPXTG cell wall anchored, C = n-terminally anchored and D = released) is shown. White columns represent NSAF values obtained at mid-logarithmic growth phase; black columns represent NSAF values obtained in the late stationary growth phase.

logarithmic phase were assigned to extracellular proteins in addition to 10 %, which was assigned to multi-transmembrane proteins. The percentage of spectra assigned to extracellular and multi-transmembrane proteins decreased to 10 % and 3 % respectively in samples harvested at late stationary phase. The presence of peptides derived from cytoplasmic proteins in all samples is a common phenomenon particularly observed when applying highly sensitive chromatography methods such as LC-MS/Ms approaches (57). However, this can only in part be explained by cellular autolytic processes, since cytosolic proteins are also found in the mid-logarithmic samples where the autolysis process is considered to be a minor event (58).

Linking transcriptome and surface proteome in mid-logarithmically growing cells

To verify the consistency between gene transcription levels and protein detection by mass-spectrometry in mid-logarithmically growing cells, *L. plantarum* cultures were grown in bioreactors until OD_{600} = 1.0 using standard conditions. The transcriptional response was assessed by DNA microarrays. Subsequently, extracellular gene expression data was correlated to the extracellular proteome identified by mass-spectrometry. Overall, genes that fell within the top-20 % quantile of gene expression were more consistently detected at the protein level, demonstrating a clear correlation of gene expression and protein detection (Fig. 5). In fact, proteins coded by 75 % of the highest expressed gene sets were consistently detected (Fig. 5A). Moreover, different correlation patterns were observed depending on the subcellular protein locations (Fig. 5 B-E). For example, in the top-20 % quantile of gene expression values the secreted (released) and also LPXTG anchored proteins displayed higher detection rates than the lipid anchored or N-terminally anchored proteins due to their complete secretion and easy

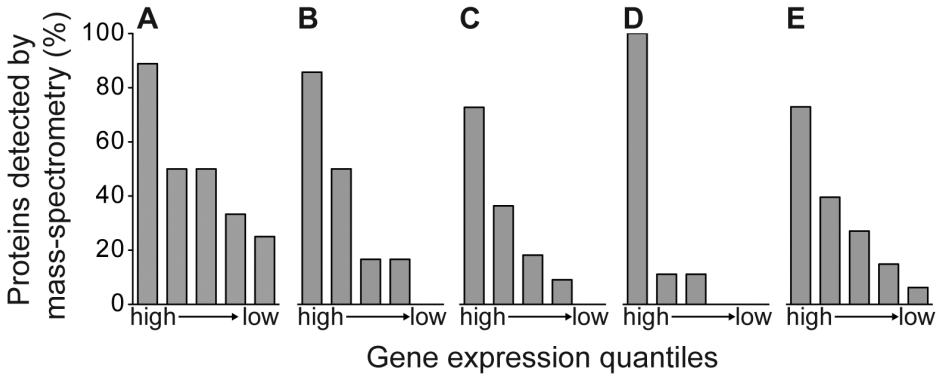


Fig. 5. Expression quantiles (20 % increments), showing dependency of protein detection by mass-spectrometry on gene expression levels at mid-logarithmic growth phase. *L. plantarum* cultures were grown in fermentors using standard conditions, harvested at OD_{600} = 1, microarray analysis were performed and extracellular gene expression data were extracted. Each bar (1-5) represents a 20 % quantile and the height of each bar is the percentage of proteins, which was detected by mass-spectrometry in that quantile of expression. Higher percentages of proteins could be detected in 20 % quantiles representing genes with higher transcription levels, demonstrating a clear correlation of gene expression and mass-spectrometry detection whereas differential correlation pattern were observed dependent on subcellular protein locations (A = lipid anchored, B = LPXTG cell wall anchored, C = n-terminally anchored and D = released, E = predicted extracellular)

accessibility. Some genes were highly expressed but the encoded proteins were not detected by mass-spectrometry. This discrepancy was most likely due to the small size of some proteins (Lp_3677), making them inaccessible to trypsin or to the lack of trypsin cleavage sites (Lp_1539). Similarly, only membrane anchored proteins with large soluble domains were accessible for trypsin digestion in whole cells which may explain why some of the proteins in this class were not detected despite the high level of transcription of the corresponding genes.

Differential modulation of the NF-κB pathway by peptide fractions obtained from mid-logarithmic and late stationary growth phase *L. plantarum* cells

The capacity of *L. plantarum* to modulate NF-κB regulated cascades in human duodenal mucosa tissues depends on the growth phase at which *L. plantarum* was harvested prior to consumption (36). To evaluate whether the cell surface peptides were responsible for the observed differential tissue response to *L. plantarum in vitro,* peptide fractions from mid-logarithmic and late stationary growth phase were tested for their ability to regulate GFP expression in a Caco-2 NF-κB reporter cell line (Fig. 6.1).

Incubation of the NF-κB reporter cell line with mid-logarithmic phase peptides did not affect the background level of NF-κB activity in these cells (Fig. 6.2). Incubation with late stationary phase peptides on the other hand, resulted in a 3-fold ($p < 0.05$) decrease of GFP intensity per cell in comparison to the control cells, indicating that these peptides were able to attenuate the basal level of NF-κB activity (Fig. 6.2, A). In contrast, cytosolic peptides of both, mid-logarithmic and late stationary growth phases strongly activated NF-κB (Fig. 6.2, A). The differential attenuation effect of *L. plantarum* surface peptides on NF-κB activity was further investigated by assessing ability of mid-logarithmic and late stationary peptides to attenuate NF-κB activation in response to flagellin and *L. plantarum*-derived cell envelopes (Fig. 6.2 B, C). Flagellin induced the expression of GFP in the reporter cells by a factor of 30 in comparison to the control cells, whereas this was approximately 3 fold ($p < 0.05$) for the cell envelope (CE) fraction. Strikingly, incubation of reporter cells with flagellin and the late stationary phase peptides decreased GFP

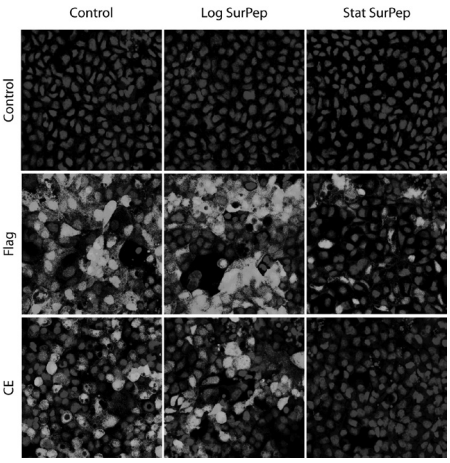


Fig. 6.1. Confocal microscopy images of confluent monolayers of the NF-κB-GFP intestinal epithelial cell line.
First row: Epithelial cells were incubated without (Control), or with addition of *L. plantarum* peptides obtained from mid-logarithmic (Log SurPep), or late stationary (Stat SurPep) growth phase. Second row: Cells were incubated with flagellin (Flag) alone, or with addition of Log SurPep, or Stat SurPep. Third row: Cells were incubated with protein-free cell envelopes (CE) alone, or with addition of Log SurPep, or Stat SurPep. Flagellin and cell envelopes stimulated reporter cells, whereas flagellin induced GFP much stronger than the cell envelopes. Co-stimulation, using peptides and flagellin or cell envelopes, respectively revealed high GFP expression when using Log SurPep but a clear decrease in GFP expression by Stat SurPep**.**

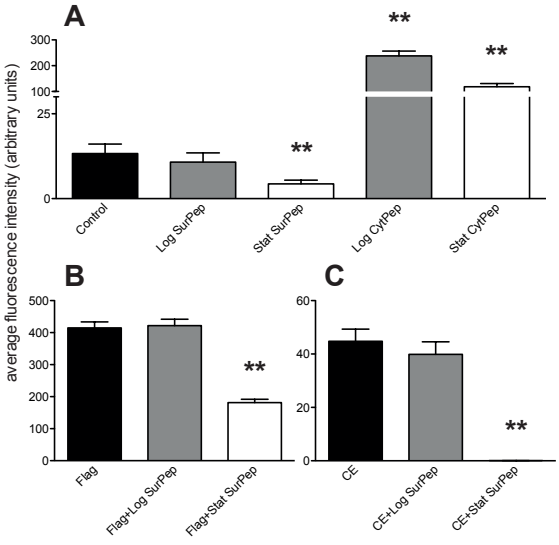


Fig. 6.2: Mean GFP fluorescence intensities of NF-κB-GFP Caco-2 cells. Error bars represent standard error of measurements.
A: GFP expression was not induced by mid-logarithmic phase peptide fractions (Log SurPep) alone. Incubation of cells with late stationary phase peptides (Stat SurPep) resulted in a 3-fold decrease of GFP intensity per cell in comparison to cells incubated with mid-logarithmic peptides or to the control, suggesting an attenuation of NF-κB by Stat SurPep, which led to lower background fluorescence. Cytosolic peptides of mid-logarithmic (Log CytPep) and late stationary phases (Stat CytPep) strongly activated NF-κB (factor 18 and 10, respectively). **B:** GFP expression in flagellin (Flag) and protein free cell envelopes (CE)-stimulated reporter cells with and without addition of mid-logarithmic (Log SurPep) and stationary phase (Stat SurPep) peptides. Both, Flag and CE induced NF-κB activation (30 fold and 3 fold, respectively). Stat SurPep, but not Log SurPep attenuated NF-κB by a factor of 2.3 in flagellin-induced cells, and entirely in cell envelope-activated cells. ** depict significant differences as determined by one-way ANOVA ($p < 0.05$)

expression by a factor of 2.3. Moreover, GFP expression was completely abrogated when CE was added together with the late stationary phase peptides ($p < 0.05$). In contrast, the mid-logarithmic phase peptides had no attenuating effect on flagellin and CE-induced expression of the GFP reporter. (Fig. 6). These results indicate that only late stationary phase peptides specifically attenuate NF-κB induction both in unstimulated cells (basal NF-κB activity) and stimulated cells.

Discussion

Lactobacilli are widely used in food fermentation processes and as probiotics, i.e. health promoting microorganisms. To understand the molecular mechanism behind the growth phase-dependent capacity of *L. plantarum* WCFS1 to modulate NF-κB-related pathways (36), we characterized its cell surface proteome.

Several proteomics-based studies have addressed the molecular mechanisms behind the interactions between lactobacilli and their environments, which include adaption to fermentation and GI tract conditions (59-63) and lactobacillus-host interactions within the framework of probiotic function (64, 65). Generally, these studies employ gel-based approaches, which despite their obvious advantages have the drawback of low recovery and resolution of membrane proteins and limited visualization of low abundant and high molecular weight proteins (66). The study presented here employs a gel-free method which was shown to lead to higher detection rates of cell surface proteins compared to gel-based approaches (67). The combination of cell surface trypsinization with a MuDPIT shotgun proteomics strategy (56) led to the identification of 124 surface proteins, representing 53 % of the predicted surface proteome of *L. plantarum* WCFS1 (55). Proteins that were not detected were most probably too small, not accessible to trypsin proteolysis or not expressed under the applied conditions. The latter explanation was supported by the transcriptional data obtained. Although the correlation of short-lived mRNA abundances generally fails to truly predict corresponding long-lived protein amounts (68-71), a correlation between gene expression levels and corresponding detection rates by MS at mid-logarithmic growth phase was observed. We found that higher gene expression levels led to

higher protein detection rates, and that 75 % of all extracellular proteins were detected if their corresponding gene expression levels fell within the top-20 % quantile of gene expression. As a result of the low overall cellular metabolic activity in stationary phase, the transcriptional values clearly decreased for most (extracellular) genes. Nevertheless, several proteins remained stable and detectable throughout growth, indicating that their mRNA levels are unlikely to reliably represent their abundances at the protein level (67, 72, 73). Consequently proteome-transcriptome correlation analysis was not carried out at late stationary growth phase.

Our work conclusively demonstrates that cytosolic peptides derived from either mid-logarithmic or late stationary phase bacterial cells activate NF-κB, which is in clear contrast to the surface-associated peptides derived from stationary phase bacterial cells, which attenuate NF-κB activation. The late stationary phase peptides attenuated basal levels of NF-κB reporter activity in unstimulated epithelial cells as well as in flagellin- and cell envelope-stimulated cells. To further pinpoint the peptides involved in the observed attenuation effect, we pooled SCX chromatography fractions that were used for the MuDPIT experiment into 10 fractions. Several of these fractions (2, 3, 5 and 7) were characterized by a clear attenuation effect of the flagellin-induced NF-κB activation. Notably, fraction 7 showed the strongest attenuation capacity (Fig. 7). Furthermore, this fraction contains, among others, tryptic peptides derived from the Lp_0800 protein (chapter 4), which has been reported to be constitutively upregulated in the GI-tract in mouse and human studies (7, 30, 32), and which was found to be more abundant in the late stationary phase, which could have let to the NF-κB-attenuating potential of the sample.

The combination of peptide generation, separation and automated microscopy analysis is a powerful tool to pinpoint effector peptides and allows high-throughput screening of peptides derived from putative probiotic organism. This approach can be easily extended by e.g. the screening of knockout libraries to pinpoint the impact of specific proteins/peptides, which seems a relevant step towards identifying putative probiotic effector proteins. The fact that peptides and not intact proteins are potent NF-κB- attenuating molecules bears great potential, as e.g. secondary and tertiary structures of

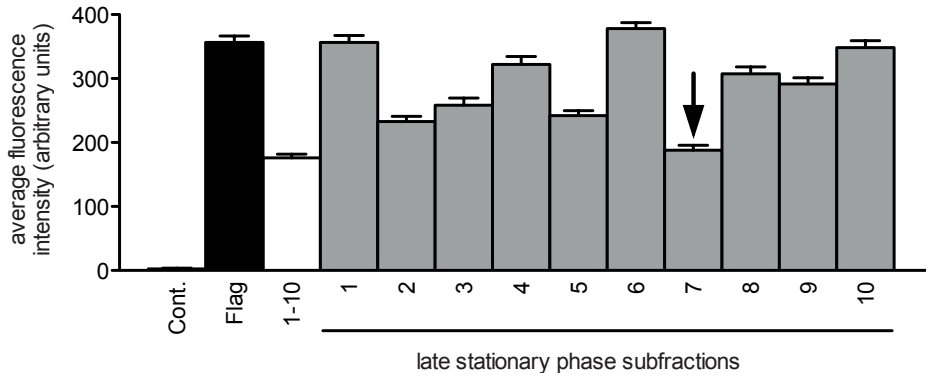


Figure 7: Mean GFP fluorescence intensities of NF-κB-GFP Caco-2 cells after incubation with strong cation exchange sub-fractions of late stationary phase peptides. Error bars represent standard error of measurements. Several sub-fractions showed an attenuation effect, suggesting the presence of several attenuator peptides throughout those fractions. The attenuation effect of sub-fraction 7 appeared to be not significantly different from the effect observed by the whole pool (sub-fractions 1-10), as determined by one-way analysis of variance (one-way ANOVA, $p < 0.05$).

proteins can be ignored.

The here presented *in vitro* approach directly targets an *in vivo* observed effect of *L. plantarum* WCFS1 (36) that emphasized that only late stationary phase derived *L. plantarum* cells were able to modulate NF-κB signaling. We could identify late stationary phase peptides as NF-κB-attenuation molecules, and future work will focus on the selection of specific target proteins/peptides that will be either synthesized or over expressed to connect specific peptides to the NF-κB-attenuation effect.

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Chapter 2

Supplemental Material
Late stationary phase surface peptides of *Lactobacillus plantarum* attenuate NF-κB activation

Daniela M. Remus, Fabrizia Fusetti, Jurgen Karczewski, Roger S. Bongers, Irene Konings, Bert Poolman, Maria L. Marco, Paul de Vos, Jerry M. Wells, Michiel Wels, Peter A. Bron, and Michiel Kleerebezem

Material and Methods

Construction of pLenti-NF-κB-GFP

pLenti-NFκB-GFP was constructed using a SOEing PCR strategy (1) from pNifty2-luciferase (InivoGen, San Diego, USA) and the IκB-GFP plasmid (2). The NF-κB binding consensus was amplified by PCR using primer pL_F and pL_R. The GFP gene was amplified by forward primer GFP_F and GFP_R (Table S1). The fragments were connected via PCR using primer pL_F and pL_R. This product was purified and ligated into pLenti6-V5-TOPO (Invitrogen, San Diego, USA). The CMV promotor from the pLenti6/V5-D-TOPO-NFkB-GFP construct was removed by digestion with *Cla*I and *Bam*HI followed by a T4 DNA polymerase reaction to generate a blunt-ended vector backbone.

Construction of the NFκB-GFP Caco-2 reporter cell-line

The NFκB-GFP Caco-2 reporter cell-line was genereated using lentiviral transduction of the NFκB-GFP construct using the ViraPower protocol (Invitrogen, San Diego, USA). Briefly, human embryonic kidney (HEK)293FT (Invitrogen, San Diego, USA) were cultured according manufacturer's descriptions and seeded into 6-wells plates. At 90-95 % confluency, HEK293 cells were transfected with 10 µg pLenti-NFκB-GFP and ViroPower helperplasmid mix using lipofectamine 2000 in OptiMem I serum-reduced medium. Supernatant of transfected HEK293 cells was collected during 48 hrs, applied through a 0.45 µm filter, and stored at 4 °C. Caco-2 cells were cultured as described before (3) and grown in 6 wells plates to 70 % confluency. Lentiviral particles were added to the Caco-2 cells and incubated overnight. 10 µg/mL Polybrene® was added to enhance the efficiency of uptake of the lentiviral particles. After transduction, Caco-2 cells were grown on blasticin selection and checked for GFP fluorescence. After successful transduction and selection, Caco-2 cells were seeded in cell culture petridishes and single cell colonies were picked using a Zeiss inverted microscope with fluorescence illumination and GFP filtersets (Chroma Technology, Bellows Falls, USA). Several clones of the NF-κB-GFP Caco-2 reporter cell-line were examined for responsiveness to typical NF-κB inducing cytokines as TNF-α and Toll-like receptor (TLR) ligands Pam3CSK and flagellin.

Table S1. Primers used for the construction of pLenti-NF-κB-GFP

Primer name	Primer sequence
pL_F	5'-caccggatctgcgatcgctgaattctggg-3'
pL_R	5'-ccttgctcaccatggctctgtctcagg-3'
GFP_F	5'-cctgagacagagccatggtgagcaagg-3'
GFP_R	5'-cggcatggacgagctgtacaagtaagaattc-3'

Table S2. *L. plantarum* proteins found exclusively in late stationary growth phase

Protein ID	Protein name	Function/gene product
Lp_0139	Lai	linoleic acid isomerase
Lp_0932	Lp_0932	lipoprotein precursor
Lp_0946	Lp_0946	mucus-binding protein (putative)
Lp_1576	Lp_1576	rhodanese family protein
Lp_1577	Lp_1577	unknown
Lp_1866	Lp_1866	extracellular protein (putative)
Lp_3018	Lp_3018	ABC transporter substrate binding protein
Lp_3064	Lp_3064	extracellular protein

Lp_3173	Lp_3173	cell surface protein, membrane-anchored (putative)
Lp_3414	Lp_3414	extracellular protein

Table S3. *L. plantarum* proteins found exclusively in mid-logarithmic growth phase

Protein ID	Protein name	Function/gene product
Lp_0290	Lp_0290	transcriptional attenuator, cell envelope-related, LytR family
Lp_0315	PotD	spermidine/putrescine ABC transporter, substrate binding protein
Lp_0469	Bla1	beta-lactamase
Lp_0988	Lp_0988	lipoprotein precursor
Lp_1132	Lp_1132	unknown, N-terminal fragment (pseudogene)
Lp_1187	Lp_1187	glycosylhydrolase (putative)
Lp_1256	Lp_1256	extracellular protein
Lp_1369	Lp_1369	lipoprotein precursor
Lp_1413	Pbp2A	transpeptidase-transglycosylase (penicillin binding protein 2A)
Lp_1746	Lp_1746	D-Methionine ABC transporter, substrate binding protein (putative)
Lp_1915	Lp_1915	lipoprotein precursor
Lp_1995	Lp_1995	lipoprotein precursor (putative)
Lp_2145	Lp_2145	extracellular protein
Lp_2200	Pbp2B	transpeptidase, penicillin binding protein 2B
Lp_2450	Lp_2450	prophage P2a protein 7; extracellular protein with lipoprotein anchor
Lp_2541	Lp_2541	ABC transporter, substrate binding protein
Lp_2796		cell surface protein precursor
Lp_2812		extracellular protein, membrane-anchored (putative)
Lp_3043	Zmp4	extracellular zinc metalloproteinase
Lp_3059	AapA	adherence-associated protein
Lp_3193	PrtM2	peptidylprolyl isomerase
Lp_3205	Lp_3205	cell surface hydrolase, DUF915 family, membrane-bound (putative)
Lp_3218	Lp_3218	extracellular protein, membrane-anchored (putative)
Lp_3393	Lp_3393	cell surface hydrolase, membrane-bound
Lp_3675	Sip3	signal peptidase I
Lp_3679	Lp_3679	extracellular protein
Lp_3686	Lp_3686	ABC transporter, substrate binding protein

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Chapter 3

The impact of *Lactobacillus plantarum* sortase on target-protein sorting, gastrointestinal persistence, and *in vitro* immunomodulation

Daniela M. Remus, Roger S. Bongers, Marjolein Meijerink, Fabrizia Fusetti, Bert Poolman, Paul de Vos, Jerry M. Wells, Michiel Kleerebezem, and Peter A. Bron

Abstract

Sortases are transpeptidases that couple surface proteins to the peptidoglycan of Gram-positive bacteria, and several sortase-dependent proteins (SDPs) have been demonstrated to be crucial for the interactions of pathogenic and non-pathogenic bacteria with their hosts. Here, we studied the role of sortase A (SrtA) in *L. plantarum* WCFS1, a model organism for probiotic lactobacilli. An isogenic *srtA* deletion derivative was constructed, which did not show residual SrtA activity. DNA microarray-based transcriptome analysis revealed that the *srtA* deletion had only minor impact on the full-genome transcriptome of *L. plantarum*, while the expression of SDP-encoding genes remained completely unaffected. Mass-spectrometry analysis of the bacterial cell surface proteome, which was assessed by trypsinization of intact bacterial cells and by LiCl protein extraction, revealed that SrtA is required for the appropriate subcellular location of specific SDPs and for their covalent coupling to the cell envelope, respectively. We further found that SrtA-deficiency did not affect the persistence of *L. plantarum* in the gastrointestinal tract of mice. In addition, an *in vitro* immature dendritic cell (iDC) assay revealed that the removal of surface proteins by LiCl impacted strongly on the pro-inflammatory signaling properties of the SrtA-deficient strain but not of the wild-type, which suggest a role of SDPs in host immune response modulation.

Introduction

In Gram-positive bacteria, the covalent coupling of proteins to the cell wall peptidoglycan, also known as cell wall sorting, occurs via a conserved mechanism that involves the action of membrane anchored transpeptidases termed sortases (1). Based on primary sequences and substrate specificities, sortases are grouped into four classes (A-D) of which sortase A (SrtA) is referred to as the ''housekeeping sortase'' (2, 3). The role of SrtA in surface protein sorting was initially described in *Staphylococcus aureus* (4, 5), where it was found to recognize proteins bearing the conserved C-terminal cell wall sorting signal LPXTG (6). SrtA cleaves the LPXTG consensus sequence between the threonine and glycine residue and subsequently joins the carboxyl group of threonine to the amino group of the pentaglycine branched peptidoglycan intermediate lipid II (7). Next to the LPXTG motif, sortase-dependent proteins (SDPs) additionally harbor a C-terminal stop-transfer membrane anchoring domain (1) that consists of a hydrophobic trans-membrane domain followed by positively charged amino acid residues (5, 8, 9), which ensures membrane anchoring of the precursor protein and thereby prevents complete secretion of SDPs prior to peptidoglycan coupling (2).

Several studies focused on the role of sortases in the context of pathogenesis, revealing that sortases assemble virulence factors on bacterial cell surfaces, which is crucial for adhesion and pathogenicity processes (10-15), biofilm formation (16, 17), and iron uptake (18, 19). A limited number of studies focused on sortases in (probiotic) lactobacilli and its role in the interaction with host cells. The deletion of the sortase encoding gene or the SDP LspA reduced adherence of *L. salivarius* UCC118 to epithelial cell lines (20), whereas gastrointestinal (GI) persistence of *L. johnsonii* NCC533 in mice was not affected by sortase deletion (21). The Mub protein of *L. acidophilus* NCFM mediates adhesion to epithelial cell lines and mucin (22), and the pilin protein SpaC of *L. rhamnosus* GG facilitates bacterial binding to host cell mucus (23). The mannose specific adhesin (Msa) of *L. plantarum* WCFS1 and the genetically closely related strain 299v (24) were identified as key-proteins for mannose-specific adhesion, a phenomenon that appeared completely sortase-dependent (25, 26). In addition, Msa of the latter strain was shown to modulate innate immune response pathways in a small

intestinal segment perfusion pig-model (27). Moreover, *L. plantarum* WCFS1 genes, which were shown to be specifically upregulated during mice GI tract passage, included the SDPs Lp_0800 and Lp_2940 (28, 29), suggesting a particular role for these proteins *in vivo*. This notion was further supported by the finding that an Lp_2940-deficient strain was severely compromised in the mouse GI-tract (30).

L. plantarum WCFS1 is a model organism for probiotic lactobacilli, and it is predicted that its genome encodes a single sortase (*srtA*) (31, 32) and 27 SDPs, of which the majority harbors a modified variant of the SrtA consensus sequence, i.e. LPQTXE (33). Here, we studied the role of SrtA in *L. plantarum* WCFS1 and assessed the consequences of *srtA* gene deletion on bacterial cell surface protein sorting by using trypsinization of intact bacterial cells and LiCl extraction of cell surface proteins. Mass-spectrometry analysis of the surface proteome samples derived from the wild-type and a *srtA*-deficient derivative clearly revealed that SrtA is required for the appropriate subcellular location and for the cell envelope coupling of specific SDPs. In addition, an *in vivo* persistence assay in mice was conducted, demonstrating that *srtA*-deficiency did not affect *L. plantarum* GI-robustness, while an immature dendritic cell (iDC) assay revealed that LiCl-based removal of surface proteins influenced the pro-inflammatory signaling properties of the SrtA-deficient but not of the wild-type strain.

Material and Methods

Bacterial strains and culture conditions

The bacterial strains, plasmids, and primers that were used in this study are listed in table S1. *Escherichia coli* strains MC1061 (34) and TOP-10 (Invitrogen, Carlsbad, USA that were used as intermediate cloning hosts for the construction of pNZ7111 and pNZ7102 or pNZ7114, respectively, were grown aerobically in TY medium (35). *L. plantarum* WCFS1 (32), WCFS1-R (28), NZ7104 (25), NZ7102 and NZ7114 (see below) were cultivated without agitation in Mann-Rogosa Sharpe (MRS; Merck, Darmstadt, Germany) or 2 times concentrated chemical defined media (2 × CDM) (36) supplemented with 1.5 % (wt/vol) glucose. All bacteria were grown at 37 °C and if appropriate, the media were supplemented with antibiotics; for *E. coli* ampicillin (50 μg/mL),

and chloramphenicol (10 μg/mL); for *L. plantarum* chloramphenicol (10 μg/mL), erythromycin (10 or 30 μg/mL for selection or replica-plating, respectively) or rifampicin (50 μg/mL).

DNA manipulation techniques

Plasmid DNA was isolated from *E. coli* using Jetstar columns as recommended by the manufacturer (Genomed GmbH, Bad Oberhausen, Germany). For DNA manipulations in *E. coli*, standard procedures were used (35). Restriction endonucleases (Invitrogen, Carlsbad, USA), T4 DNA ligase (Boehringer, Mannheim, Germany), Pfx- (Promega, Leiden, The Netherlands) and KOD (Toyobo, Osaka, Japan) DNA polymerases were used as specified by the manufacturers. Primers were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Chromosomal DNA isolation, preparation of electrocompetent cells, and DNA transformation of *L. plantarum* were performed as described previously (37, 38).

Construction of NZ7102 and NZ7114

The *L. plantarum* sortase-encoding *srtA* gene (*lp_0514*) deletion was performed according to previously described methods (39). Briefly, 1.0 kb of the 5'- and 3'- flanking regions of *srtA* were amplified using chromosomal DNA of *L. plantarum* WCFS1 as template and the primer pairs lp_0513F/ lp_0513R, and lp_0515F/lp_0515R, respectively. In addition, the *loxP-cat-loxP* fragment was amplified using the primers PmlI-loxF and EclI-loxR and pNZ5319 as template. The three amplicons were joined by PCR using primers lp_0513F and lp_0515R, and the generated 3.0 kb DNA fusion fragment was cloned into PmeI-Ecl136II digested pNZ5319 (39) yielding pNZ7114. This plasmid was integrated into the *L. plantarum* WCFS1 chromosome through a double-cross over event, replacing the *srtA* gene by the chloramphenicol acetyltransferase gene (P_{32}-*cat*) cassette, which was subsequently removed by the temporal expression of the Cre recombinase yielding NZ7114.

For the *in vivo* persistence assay, a rifampicin-chloramphenicol resistant control derivative of *L. plantarum* WCFS1-R (28) was constructed that allows selective plating after GI passage in conventional mice. The pNZ7101 (30) derived chloramphenicol cassette was integrated in the intergenic region of

the convergently oriented genes *lp_2681* and *lp_2683*. A 2.1 kb fragment of the *lp_2681-lp_2683* locus was amplified by PCR using primers HF and HR and chromosomal DNA of *L. plantarum* WCFS1 as template and cloned into pGEMt (Promega, Madison, USA), yielding pNZ7111. The P32-*cat* chloramphenicol resistance cassette of pNZ7101 was amplified by PCR using the primers P32catF and P32catR. The resulting 1.1 kb amplicon was digested with *Pac*I and cloned into similarly digested pNZ7111. The resulting plasmid pNZ7102 was integrated into the *lp_2681-lp_2683* intergenic region of *L. plantarum* WCFS1-R (28) using a double crossing over strategy (39). A colony, which displayed the correct phenotype (chloramphenicol resistance and erythromycin sensitivity) and genotype was designated NZ7102. The anticipated genetic organization of the modified *lp_2681-lp_2683* locus was confirmed using the primer pairs SCO_2681 plus catR and SCO_2683 plus catF. A colony, which displayed the correct phenotype (chloramphenicol resistant and erythromycin sensitive) and genotype was designated NZ7102.

Assessment of *in vitro* SrtA activity in *L. plantarum*

The protocol for *in vitro* assessment of SrtA activity was adapted from Ton-That *et al*. (40). *L. plantarum* WCFS1 and NZ7114 were grown in 2 × CDM until late stationary phase. Cells were harvested by centrifugation (6000 × g, 20 minutes, 4 °C), resuspended in PBS (pH 7.5) and disrupted by bead-beating using zirconium beads (3 × 30 seconds at 4 m/s) using a Savant FastPrep FP120 instrument (Qbiogen Inc., Illkirch, France). Samples were centrifuged (2500 × g, 10 minutes, room temperature) to remove intact cells and beads. The supernatants were collected, centrifuged (12000 × g, 10 minutes, 4 °C), and the pelleted cell-envelopes were resuspended in 50 mM Tris-HCl (pH 7.5) to an OD_{600} of 2.0. The sortase substrate peptide Dabcyl-GTLPQTDEQE-Edans (EZbiolab, Carmel, USA) was dissolved in Tris-HCl (pH 7.5) and added to the reaction to a final concentration of 10 mM. The strong nucleophile hydroxylamine (NH_2OH), which was previously demonstrated to enhance sortase activity *in vitro* (40), was added to a final concentration of 0.2 M. All samples were incubated at 37 °C for 1 hour, followed by centrifugation (12000 × g, 5 minutes, 4 °C) to remove the cell envelope material. The supernatants were analyzed in a fluorometer using

350 nm excitation followed by assessment of emission at 495 nm.

Transcriptome analysis and data interpretation

L. plantarum and NZ7114 were grown in MRS and harvested at OD_{600} = 1.0. RNA was isolated according to previously described methods (41, 42). In short, following methanol quenching (43), cells were harvested by centrifugation (6000 × g, 20 min, 4°C), resuspended in 400 µL ice-cold CDM medium and transferred to tubes containing 500 µL phenol/chloroform solution (4:1 [v/v]), 30 µL 10 % sodium dodecyl sulfate, 30 µL 3 M sodium acetate (pH 5.2), and 0.5 g zirconium beads. Cells were disrupted by bead beating using a Savant FastPrep FP120 instrument (Qbiogen Inc., Illkirch, France) and RNA was purified from the aqueous phase using the High Pure Isolation Kit (Roche Diagnostics, Germany). RNA concentration and purity were determined using A_{260} and A_{280} measurements, using a ND-1000 spectrometer (NanoDrop Technologies Inc., Wilmington, United States), and RNA quality was verified with a 2100 Bioanalyser (Agilent Technologies, Amstelveen, the Netherlands). Samples displaying a 23S/16S RNA ratio equal or superior to 1.6 were used for labeling.

3 µg RNA was used for cDNA synthesis. Cyanine-3 (Cy3) and cyanine-5 (Cy5) cDNA labeling was performed as described previously (44), 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). The array design and transcriptome data were deposited under platform GPL13984 and accession number GSE34999 in NCBI's Gene Expression Omnibus (GEO) (45, 46). Hybridization and scanning procedures were performed as previously described (44). Slide scanning was carried out at several photo multiplier tube (pmt) values, and the optimal scan of each individual microarray was selected on the basis of signal distribution (combination of a low number of saturated spots and a low number of low signal spots). The data were normalized using the Lowess normalization as available in MicroPrep (47). For statistical significance, False Discovery Rate (FDR) was used (48) with a FDR-adjusted p-value cutoff of 0.05 employed

for genes showing at least 2-fold altered expression levels.

Surface proteome profiling of *L. plantarum*

The protocol for the surface trypsinization was adapted from Severin *et al*. (49). *L. plantarum* and NZ7114 were grown in 2 × CDM using a Sixfors fermentor system (Infors, Bottmingen, Switzerland) under constant agitation (150 rpm). The pH was maintained at 5.9 by the titration of 2.5 M NaOH. Cell cultures were harvested from fermentors (200 mL at OD_{600} = 1.0 and 50 mL after overnight growth, which was subsequently diluted to obtain 200 mL concentrated equivalent to OD_{600} = 1.0) and pelleted by centrifugation (6000 × g, 20 min, 4 °C). Pellets were washed twice in 20 mL 20 mM Tris/HCl pH 7.6 buffer containing 150 mM NaCl, 1 M xylose, 20 mM $CaCl_2$ and 5 mM DTT and were subsequently resuspended in 5 mL of the same buffer, followed by the addition of 15 µg mass-spectrometry grade trypsin (Sigma T6567, Sigma-Aldrich, Missouri, USA). Samples were incubated for 10 min at 25 °C, aliquoted into Eppendorf tubes, and centrifuged (20000 x g, room temperature, 1 min). The tryptic peptide-containing supernatants were collected and filtered through a cellulose-acetate filter (0.2 µm pore size, 25 mm diameter; Sigma-Aldrich Missouri, USA). To further complete protein digestion, 5 µg of trypsin was added and the samples were incubated overnight (4 rpm, 25 °C). Trypsin was inactivated by the addition of trifluoroacetic acid (TFA) to a final concentration of 0.1 % (v/v). The samples were freeze-dried and stored at -20 °C prior to mass-spectrometry analysis.

Extraction of bacterial cell surface associated proteins with LiCl

To extract non-covalently bound surface proteins, 100 mL of overnight grown 2 x CDM cultures of *L. plantarum* and NZ7114 were harvested by centrifugation (6000 x g, 20 minutes, 4 °C). Cell pellets were washed 3 times in PBS pH 7.5 and subsequently incubated in 2 mL 1.5 M LiCl (20 minutes on ice) followed by centrifugation (12000 x g, 20 minutes, 4 °C). The supernatants were precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 10 % and samples were incubated for 1 hour on ice followed by centrifugation (12000 x g, 20 minutes, 4 °C). The protein pellets were washed 3 times with acetone and air-dried. For trypsin digestion, the

protein pellet was dissolved in 6 M urea and subsequently diluted in 100 mM triethylammonium bicarbonate buffer to a final concentration of 1 M urea (final sample volume of 30 µL). For reduction and alkylation, Tris [2-carboxyethyl] phosphine (TCEP) was added to a final concentration of 50 mM (incubation for 20 minutes at 60 °C) followed by the addition of iodoacetamide (IAA) to a final concentration of 500 mM prior to incubation for 1 hour at room temperature. Subsequently, 500 ng sequencing grade trypsin (Promega, Leiden, The Netherlands) was added and samples were incubated overnight at 37 °C. Trypsin was inactivated by addition of TFA to a final concentration of 0.1 % (v/v) prior to mass-spectrometry analysis.

Mass-spectrometry based surface proteome profiling

Peptides (either derived from surface trypsinization or LiCl-based protein extraction) were separated by reverse phase chromatography on a C18 capillary column (ID 75 µm x 150 mm, 3 µm particle size, Dr. Maisch, Ammerbuch-Entringen, Germany) packed in-house and mounted on a Proxeon Easy-LC system (Proxeon Biosystems, Odense, Denmark), in line with a trapping pre-column (ReproSil-Pur C18-AQ, ID 100 µm x 20 mm, Proxeon). Solutions of 0.1 % formic acid in water and 0.1 % formic acid in 100 % acetonitrile were used as mobile phases. Peptides were eluted over 140 minutes using a 4-35 % acetonitrile gradient at a flow rate of 250 nL/min. Eluted peptides were analyzed using a linear ion trap-Orbitrap hybrid mass spectrometer (LTQ-Orbitrap, Thermo Fisher Scientific, Bremen, Germany). The MS raw data were submitted to Mascot (version 2.1, Matrix Science, London, UK) using the Proteome Discoverer 1.1 analysis platform (Thermo Fisher Scientific, Bremen, Germany) and searched against the *L. plantarum* proteome (3063 entries) (32)i, combined with reversed entries for all protein sequences to assess false discovery rates. Peptide tolerance was set to 10 ppm and 0.8 Da for intact peptides and fragment ions, respectively. The option semi-trypsin was chosen allowing for up to 2 undigested cleavage sites. Oxidation of methionine residues and deamidation of asparagine and glutamine were specified as variable modifications. The MS/MS based peptide and protein identifications were further validated with the program Scaffold v3.0 (Proteome Software Inc., Portland, USA). Protein identifications

based on at least 1 unique peptide identified by MS/MS with a confidence of identification probability higher than 95 %, were accepted. Peptide identifications were accepted if they could be established at greater than 95 % probability as specified by the Peptide Prophet algorithm (50). Significant changes in protein levels were calculated based on the spectral counts of each protein, which were normalized to the sum of all spectra detected in the individual sample.

***In vivo* gastrointestinal persistence analysis**

The *in vivo* persistence of NZ7102 (chloramphenicol-rifampicin resistant control strain) and the rifampicin resistant SrtA negative derivative NZ7104 (25) were investigated in a mouse model system. Animal experiments were performed at the Institute Pasteur (Lille, France) according to guidelines N° 86/609/CEE of the French government. Seven weeks-old female Balb/c mice were purchased from Iffa Credo (Saint-Germain-sur-l'Arbresle) and had *ad libitum* access to tap water and standard mice chow during the experiment. Overnight MRS cultures of both strains were mixed, pelleted (6000 x g, 10 minutes, room temperature), and resuspended at 10^{10} colony forming units (CFUs)/mL. Two mice received a 100 mL dose of the bacterial mixed suspension by intragastric administration at 2 consecutive days (days -1 and 0). Individual fecal samples were collected daily during 5 days following the last administration (days 1-3, and 5) and resuspended in MRS followed by homogenization. Serial dilutions were plated on MRS plates containing 5 μg/mL chloramphenicol and 50 μg/mL rifampicin and incubated for 72 hours at 37 °C. An average of 36 full grown colonies were randomly picked per time point per mouse and were identified as NZ7102 or NZ7104 using a a 3-primer colony-PCR reaction (univHTP, NZ7102HTP and NZ7104HTP, table S1) designed to result in a 0.5 or 0.8 kb amplicon using NZ7102 or NZ7104 cells as template, respectively. The ratio of the NZ7102 and NZ7104 populations per time point was used as an indication for their relative *in vivo* persistence.

Immunological cell assays

After overnight growth in 2 x CDM (36), *L. plantarum* and NZ7114 were

harvested by centrifugation (6000 x g, 20 minutes, 4 °C) and either washed 3 times in PBS pH 7.5 or 1.5 M LiCl for surface protein extraction followed by 5 times washing of the cells in PBS (pH 7.5). Bacterial cells were applied to human monocyte derived immature dendritic cells (iDCs) obtained from 3 independent buffy coats (Sanquin Blood bank, Nijmegen, Netherlands). The procedures of monocyte isolation and iDC differentiation was recently described in detail (44). In short, human monocytes were isolated from blood using a combination of Ficoll density centrifugation and cell separation using CD14-specific antibody coated magnetic microbeads (Miltenyi Biotec B.V., Leiden, The Netherlands). The purity and viability of isolated CD14+ cell fraction was greater than 90 % and 95 %, respectively. To generate immature DCs (iDCs), the purified CD14+ cells were cultured for 6 days in RPMI 1640 medium (Invitrogen, Carlsbad, USA), supplemented with 100 units/mL penicillin G (Invitrogen, Carlsbad, USA), 100 µg/mL streptomycin (Invitrogen, Carlsbad, USA), IL-4 (R&D systems, Inc., Minneapolis, USA) and granulocyte-macrophage colony-stimulating-factor (GM-CSF) (R&D systems, Inc., Minneapolis, USA). GM-CSF and IL-4 were added to differentiate the monocytes into DCs. At day 6 the iDCs (1×10^6 /mL) were stimulated by the addition of *L. plantarum* strains at a 1:10 iDC to bacterial cell ratio. After 48 hours, supernatants were analyzed for IL-10 and IL12p70 using a cytometric bead-based immunoassay (51) according to the manufacturer's protocol (BD biosciences, Breda, The Netherlands). The flow cytometric data were analyzed using the BD FCAP software.

Results

The *srtA* gene encodes the sole sortase of *L. plantarum* WCFS1 and its deletion has only minor impact on the bacterial transcriptome

In contrast to other lactic acid bacteria such as *Lactococcus lactis* subsp. *lactis* IL1403 and *L. johnsonii* NCC533 that encode multiple sortase copies (33), the *L. plantarum* WCFS1 genome encodes a single sortase gene, i.e. *srtA* (31, 32). To verify this *in silico* prediction, cell envelopes of *L. plantarum* WCFS1 and its isogenic *srtA* deletion strain NZ7114 were incubated with a chemically synthesized dabcyl-GTLPQTDEQE-edans fluorophore peptide that encompasses the conserved LPQTDE sorting signal found in most SDPs

of *L. plantarum* (33, 52). Sortase activity can be monitored in this assay by an increase in fluorescence due to the sortase-dependent peptide cleavage, which separates the edans fluorophore from the dabcyl quencher (40). The addition of the fluorophore peptide to the wild-type cell envelopes led to a clear increased fluorescence value, whereas the value obtained with the srtA mutant envelope did not exceed background fluorescence as measured for samples to which either no cell envelopes or no fluorescent peptide were added (Fig. 1). These measurements demonstrate that *srtA* encodes the sole sortase of *L. plantarum* WCFS1 and confirms earlier *in silico* predictions.

To evaluate the consequences of *srtA* deletion on the genome wide transcriptome, we performed DNA microarray based transcriptome analyses, which revealed that only 20 genes displayed significantly altered transcription levels in the NZ7114 mutant compared to the wild-type (table S2). Remarkably, these include the upregulation (up to 62-fold) of all 11 genes encoded in the capsular polysaccharide cluster 2 (*cps2A-K)*. Notably, none of the differentially regulated genes encodes a SDP. In addition, we found that *srtA* deletion did not affect growth rate, the final OD_{600} reached, or cell shape as investigated by light microscopy (data not shown). Taken together, our data show that the absence of sortase had only minor consequences for the *L. plantarum in vitro* physiology.

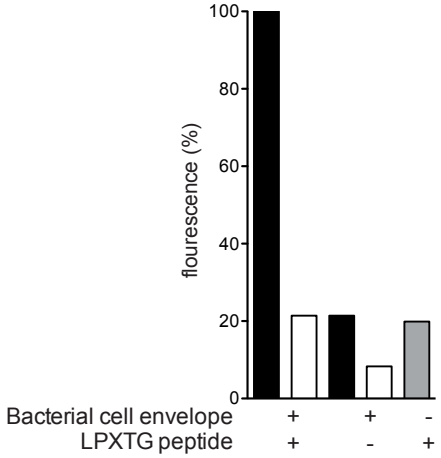

Fig. 1. The dabcyl-GTLPQTDEQE-edans fluorophore peptide was incubated with cell envelopes of *L. plantarum* WCFS1 (black bars) or its sortase-deficient derivative NZ7114 (white bars), or without addition of cell envelope material (grey bar). Cleavage of the LPQTDE motif was monitored as an increase in fluorescence, as presented relative to the highest value obtained.

Sortase is essential for appropriate subcellular localization of SDPs

Wild-type and NZ7114 cells were grown until late stationary phase and exposed to surface trypsinization, followed by MS/MS-based protein identification. By this approach, a total of 8 SDPs could be identified (Fig. 2). Overall, SDP were represented by an average of 2 % and 0.06 % of the assigned spectra in the samples derived from the wild-type and NZ7114, respectively. The subcellular localization of 2 SDPs appeared to be unaffected by the absence of sortase as illustrated by the fact that Lp_2940 and Lp_0197 abundancy was not significantly different in the wild-type and NZ7114 surface proteome samples. In contrast, the remaining 6 SDPs were found to be significantly more abundant in the surface proteome fractions of the wild-type as compared to NZ7114 (Lp_1229 [Msa], Lp_1643, Lp_2925, and Lp_3114), or were exclusively detected in the wild-type surface proteome (LP_0923 and Lp_0946) (Fig. 2). These experiments demonstrate that, although the consequences of SrtA deficiency were different for individual SDPs, sorting deficiency reduced the cell envelope-abundance of the majority of the detected SDPs.

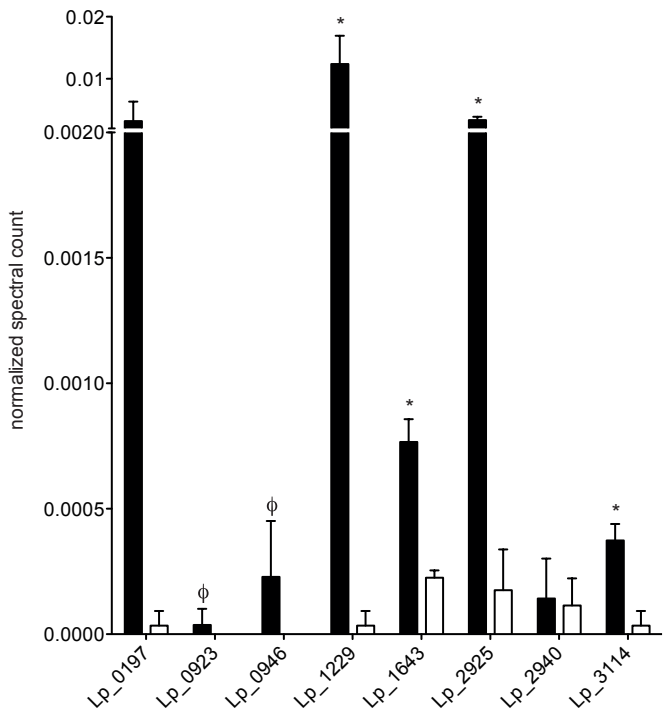

Fig. 2. Normalized spectral count of proteins that were detected by surface trypsinization of intact bacterial cells of *L. plantarum* WCFS1 (black bars) and its sortase deficient derivative NZ7114 (white bars). Data are presented as the mean +/− standard deviation based on 3 biological replicates. Asterisks indicate significant differences based on a 2-tailed t-test ($p < 0.05$), whereas ϕ depicts proteins that were exclusively found in the wild-type surface proteome.

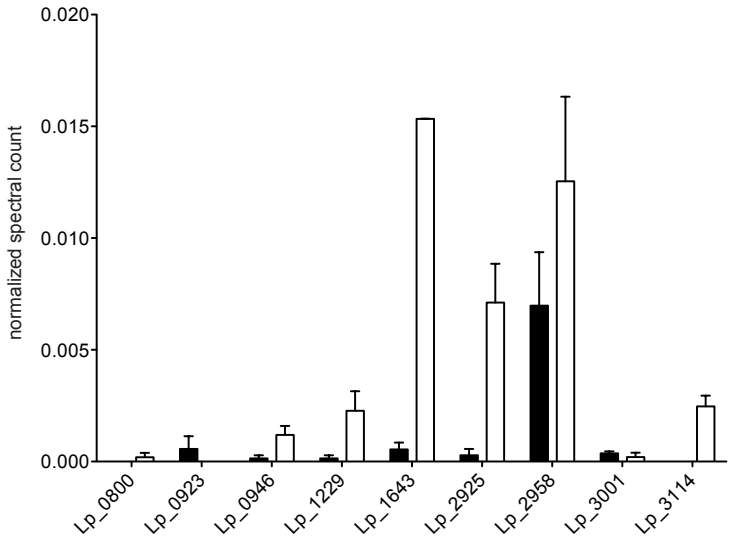

Fig. 3. Normalized spectral count of proteins that were detected by LiCl surface protein extraction of intact bacterial cells of *L. plantarum* WCFS1 (black bars) and its sortase deficient derivative NZ7114 (white bars). Data are presented as the mean +/− the range between 2 biological replicates.

Sortase is essential for covalent coupling of SDPs to peptidoglycan

Loosely associated proteins were extracted from wild-type and NZ7114 cells by LiCl. Overall, SDPs were represented by an average of 0.9 % and 4.1 % of the assigned spectra in the samples derived from the wild-type and NZ7114, respectively. In the LiCl extracted fractions, 9 SDPs were identified, of which 7 were exclusively or more abundantly found in the NZ7114-derived samples (Fig. 3). These included Lp_0946, Lp_1229, Lp_1643, Lp_2925, Lp_2958, which were detected in higher amounts and Lp_0800 and Lp_3114, which were exclusively detected in samples derived from NZ7114. In contrast, the abundance of Lp_3001 remained unaffected by the *srtA* deletion. Lp_0923 was only detected in the LiCl extraction derived from the wild-type, but not in that of the NZ7114. These data suggest that the cell-surface binding of SDPs is non-covalent in NZ7114, which generally allows SDPs to be more readily extracted by LiCl incubation compared to the wild-type strain.

Sortase deletion does not impact on gastrointestinal persistence of *L. plantarum*

The experiments above clearly established that several SDPs are not covalently coupled to peptidoglycan in the SrtA-deficient strain NZ7114. GI survival and persistence is considered an important trait of probiotic strains (53), and we therefore determined the effect of hampered SDP sorting on this trait. Notably, we used an earlier described sortase mutant in this experiment (NZ7104) (25), since this strain is chloramphenicol and rifampicine resistant, allowing selective plating of murine fecal samples. The relative survival and persistence in the mouse GI tract of this mutant was compared to *L. plantarum* NZ7102 (chloramphenicol and rifampicin resistant strain representative for the wild-type) in a competitive colonization experiment. NZ7102 and NZ7104 were administered to mice in equal amounts by gastric intubation at days -1 and 0, and the population ratio was determined on days 1-3 and 5 by antibiotic-based selective plating from fecal pellets, followed by colony identification by a discriminative 3-primer PCR. The PCR reactions on the input samples (days -1 and 0) indicated that both strains were administered in approximately equal amounts (ratios between $\log_2$ 1 and $\log_2$ -1, data not shown), establishing the validity of the 3-primer PCR approach, whilst pinpointing that ratio changes larger than 2-fold up or down can be monitored with this approach. Considering these sensitivity limits, NZ7102 and NZ7104 were detected at indistinguishable levels in samples derived from fecal pellets at all time points after administration (Fig. 4). In addition, NZ7104 could readily be detected 5 days after the last administration. These

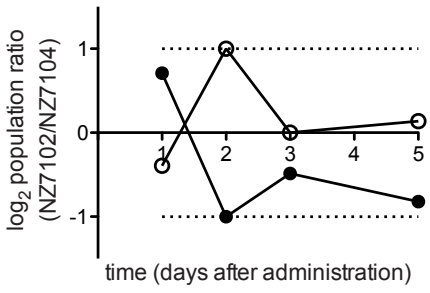

Fig. 4. Log-normalization of population ratios of *L. plantarum* NZ7102 and NZ7104 after GI passage in two mice (filled and open circles, repectively) as assessed by antibiotic-based selective plating from fecal samples followed by genotype identification based on a discriminative 3-primer colony PCR.

observations indicate that the SrtA-deficiency and the associated altered subcellular localization for SDPs do not affect GI survival and/or persistence of *L. plantarum*.

The presence of SDPs affects immune responses in human immature dendritic cells

Modulation of immune responses has been proposed as one of the prominent actions by which probiotics can beneficially affect their hosts (54, 55). To investigate the role of SDPs in immunomodulation, human monocyte-derived iDCs were co-cultured with *L. plantarum* WCFS1 or NZ7114 cells that were washed with PBS (control) or 1.5 M LiCl (removal of non-covalently bound proteins). When washed with PBS, both strains elicited similar amounts of IL-10 and IL-12p70 from iDCs (Fig. 5). Similarly, IL-10 levels detected when employing LiCl-washed WCFS1 or NZ7114 cells were similar to the cells treated with PBS. However, while IL-12p70 levels induced by LiCl–treated and control-treated wild-type cells were similar, IL-12p70 levels significantly

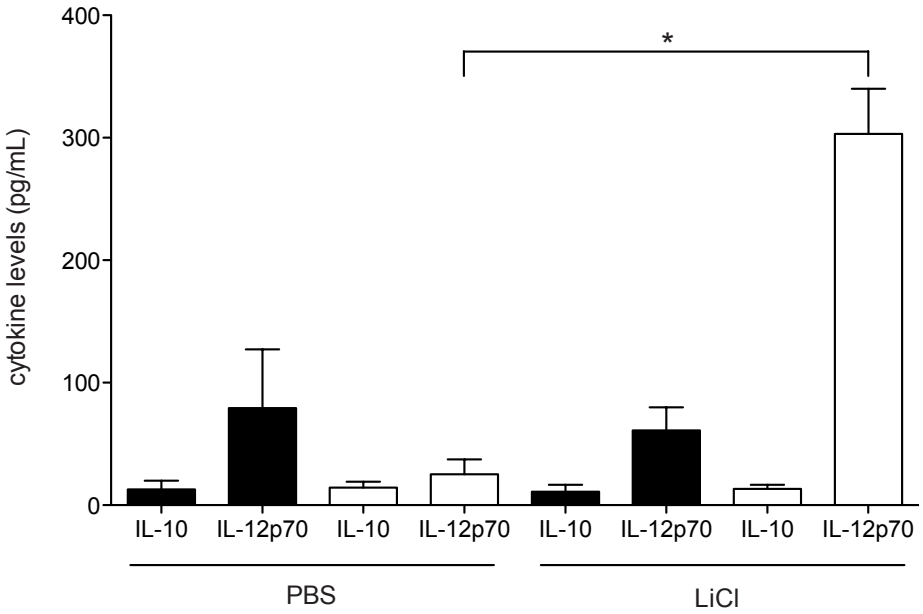

Fig. 5. IL-10 and IL-12p70 secretion pattern by monocyte derived immature dendritic cells after stimulation with *L. plantarum* WCFS1 (black bars) or its sortase deficient derivative NZ7114 (white bars). Data are presented as the mean +/- standard deviation based on three donors. The asterisk indicates significant differences based on a 2-tailed t-test ($p < 0.05$).

increased (12-fold) after co-culture with NZ7114 (Fig. 5). These data suggest a potential role of SDPs in attenuation of pro-inflammatory responses, either directly or indirectly by shielding the exposure of pro-inflammatory compounds present in the cell envelope, such as teichoic acids and/or peptidoglycan.

Discussion

Sortases mediate the covalent anchoring of proteins to the cell wall peptidoglycan of Gram-positive bacteria (1) and have been shown to hereby facilitate the interactions between bacteria and their environments. Deletion of *srtA* in *L. plantarum* WCFS1 resulted in complete abolishment of sortase activity, while it elicited only minor transcriptome consequences under laboratory growth conditions. Importantly, although 6 (out of 8) SDPs could be detected by surface trypsinization in both the parent and *srtA* mutant strain, *srtA* deletion led to a significant decrease in the relative abundance of the SDPs. This observation indicates that while SDPs may not be coupled covalently to the peptidoglycan in the absence of sortase activity, they remain surface localized possibly by stop-transfer mediated anchoring in the cytoplasmic membrane (56). However, as shown by LiCl extraction, the majority of SDPs were loosely associated to the cell surface suggesting that the hydrophobic C-terminal stop-transfer domain does not allow robust membrane anchoring which consequently results in the release of the SDPs. In *L. plantarum* WCFS1, the absence of SrtA did not affect intestinal persistence in mice, a result that corroborates earlier results obtained for *L. johnsonii* NCC533 and its sortase-deficient derivative (21). At a first glance, our result appears to contradict the observation that persistence of a *L. plantarum* WCFS1 mutant that lacked the SDP Lp_29*40* was clearly compromised in the mouse intestinal tract (30). However, our surface proteome analyses revealed that *srtA* mutation did not affect surface-exposure of Lp_2940, which could explain the unaffected intestinal persistence phenotype in strain NZ7104. In addition, Lp_2940 was not extracted by LiCl from cells of the *srtA* deletion mutant, which suggests that this protein is still anchored within the cell envelope. Contrary to this, previous work has shown that deletion of either *msa* or *srtA* resulted in a complete loss of *L. plantarum* mannose-specific adherence (25). Analogously, surface trypsinization revealed that

Msa surface exposure is severely compromised in the SrtA-deficient strain, which explains the impact of the *srtA* mutation on mannose specific adhesion capacity (25). These results clearly show that the consequences of SrtA-deficiency may vary for different SDPs. Such variations may depend on folding and/or stability characteristics of the SDP protein when its biogenesis is impaired by the lack of sortase activity, or could relate to differences in the capacity of the C-terminal stop-transfer sequences of the different SDPs to anchor them robustly in the cytoplasmic membrane and thereby sustain their surface exposure and function.

Co-culturing of human monocyte derived iDCs with *L. plantarum* WCFS1 or its SrtA-deficient derivative revealed that LiCl extraction of bacterial cells impacted strongly on the pro-inflammatory signaling properties of the SrtA-deficient strain while the wild-type remained unaffected. More specifically, the LiCl-treated cells of the *srtA* mutant strain strongly stimulated secretion of the pro-inflammatory cytokine IL-12p70. This may be directly caused by the lower abundance of one or more SDPs in these cells, or may also be due to a loss of “shielding” of cell-signaling compounds by SDP proteins (57). The observation that the abundance of SDPs on the cell surface of *srtA* mutant cells is generally much lower as compared to their levels encountered in wild-type cells may suggest that these proteins have an active role in immunomodulation rather than through shielding of other cell envelope components. However, the induction of the expression of the *cps2* gene cluster in the *srtA* mutant may lead to a higher level of cell-surface glycan production, which in its turn might contribute to shielding of immunomodulatory cell envelope components and/or altered levels of phagocytosis due to changes in surface properties (chapter 5). Overall, the experiments presented here illustrate the complexity encountered when aiming to specify causal relationship between specific bacterial or probiotic molecules and certain immune responses. Moreover, this work demonstrates the importance of assessment of the full repertoire of potential immune signaling components that reside within bacterial cell envelopes, which includes the sortase dependent surface proteome, but also encompasses the more extensively studied structural cell envelope polymers like peptidoglycan (58, 59), lipoteichoic acid (60-62), and cell surface polysaccharides (chapter 5).

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Chapter 3

Supplemental Material

The impact of *Lactobacillus plantarum* sortase on target-protein sorting, gastrointestinal persistence, and *in vitro* immunomodulation

Daniela M. Remus, Roger S. Bongers, Marjolein Meijerink, Fabrizia Fusetti, Bert Poolman, Paul de Vos, Jerry M. Wells, Michiel Kleerebezem, and Peter A. Bron

Table S1: Strains, plasmids and primers used in this study

Strains		**Reference**
E.coli		
MC1061	Cloning host	(1)
TOP10	Cloning host	Invitrogen, Carlsbad, USA
L.plantarum		
WCFS1	Single-colony isolate of *L. plantarum* NCIMB8826	(2, 3)
WCFS1-R	Rif^R, derivative of *L. plantarum* WCFS1	(4)
NZ7102	Rif^R, Cm^R, *L. plantarum* WCFS1-R derivative containing *cat* marker in intergenic region of lp_2681-lp 2683	This work
NZ7104	Rif^R, Cm^R, *L. plantarum* WCFS1-R derivative, *srtA :: cat*	(5)
NZ7114	*srtA::cat* derivative of WCFS1	This work
Plasmids		
pNZ7101	Cm^r, Em^r vector for construction of *L. plantarum* gene replacement mutants	(4)
pGEMt	Amp^r cloning vector	Promega, Madison, USA
pNZ7111	Amp^r, pGEMt derivative containing 2.1 kb fragment of the *L. plantarum* lp_2681 and lp_2683 locus	This work
pNZ7102	Amp^r, Cm^r, pNZ7111 derivative containing P32-*cat* originating from pNZ7101	This work
pNZ7114	Cm^r, Em^r; pNZ5319 derivative containing 5'- and 3' flanking regions of *srtA* (*lp_0514*)	This work
pNZ5319	Cm^r, Em^r; chromosomal integration vector	(6)
Primers		
HF	5'-ACGGCTACTTTTAACGAAAATCAGATTTATC-3'	
HR	5 '-CGTCAGACTGAGCAGCAACCAACGGCCGTTTATCC-3'	
P32catF	5'-ATGCTTAATTAAGATGAGTACGGTCAAGTATG-3'	
P32catR	5'-ATGCTTAATTAAGGCAACAGTTTAACGATTAC-3'	
SCO_2681	5'-CTGATCAGGAAACGGTGACG-3'	
catR	5'-CTCTTCAATTGTCTAAATC-3'	

SCO_2683	5’-GCTTGGAAACGGGCTGTCCG-3’
catF	5’-CAGATAGGCCTAATGACTGG-3’
univHTP	5’-GCCGACTGTACTTTCGGATC-3’
NZ7102HTP	5’-TTAGTTGTTCAGATTCCAGGC-3’
NZ7104HTP	5’-GCTATCATCAATAGACCCCC-3’
lp_0513F	5’-CTGGCGTTGAAATTCAGGTC-3’
lp_0513R	5’-CGAACGGTAGATTTAAATTGTTTGGACTTCATTAATCCCGCCTCC-3’
PmlI-loxF	5’-AAACAATTTAAATCTACCGTTCG-3’
EclI-loxR	5’-CTCATGCCCGGGCTGTAC-3’
lp_0515F	5’-GTACAGCCCGGGCATGAGTATTAACAATTATTACATTCTAGGGG-3’
lp_0515R	5’-TGACAAAAGCGGCCATCACG-3’

Table S2: Significant fold-changes of gene expression in response to *srtA* gene deletion.

Gene ID	Gene name	Function	fold change*
lp_0076	*fusA1*	elongation factor G	-100
lp_0077	*lp_0077*	unknown	-19
lp_0078	*lp_0078*	transport protein	-10
lp_0080	*lp_0080*	acetyltransferase, GNAT family (putative)	-13
lp_0915	*lp_0915*	phage protein	82
lp_1196	*lp_1196*	integrase/recombinase	-23
lp_1197	*cps2A*	polysaccharide biosynthesis protein, chain length regulator (putative)	62
lp_1198	*cps2B*	polysaccharide biosynthesis protein; regulator	22
lp_1199	*cps2C*	polysaccharide biosynthesis protein; phosphatase (putative)	23
lp_1200	*cps2D*	UDP N-acetyl glucosamine 4-epimerase, NAD dependent	12
lp_1201	*cps2E*	priming glycosyltransferase	16
lp_1202	*cps2F*	glycosyltransferase	13
lp_1203	*cps2G*	polysaccharide biosynthesis protein	10
lp_1204	*cps2H*	polysaccharide polymerase	12
lp_1205	*cps2I*	oligosaccharide transporter (flippase)	6
lp_1206	*cps2J*	glycosyltransferase	3
lp_1207	*cps2K*	polysaccharide biosynthesis protein (putative)	3
lp_1386	*lp_1386*	cation efflux protein (putative)	-2
lp_3444	*lp_3444*	transcription regulator, Crp family	3
lp_3634	*lp_3634*	alpha-1,2-mannosidase (putative)	7

*For statistical significance, FDR (7) was used with an adjusted p-value cutoff of 0.05 for genes showing at least 2-fold altered expression levels.

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Chapter 4

The gastrointestinally induced surface protein StsP of *Lactobacillus plantarum* attenuates NF-κB responses in intestinal epithelial cells

Daniela M. Remus, Jurgen Karczewski, Fabrizia Fusetti, Bert Poolman, Paul de Vos, Jerry M. Wells, Peter A. Bron, and Michiel Kleerebezem

Abstract

The transcription of *lp_0800* (here renamed *stsP*), which encodes a serine- and threonine-rich, high molecular weight, sortase-dependent cell surface protein of *Lactobacillus plantarum*, was previously shown to be induced in the intestine of mice and humans, which suggests a role for StsP in the intestinal lifestyle of this lactic acid bacterium. To enable investigation of the StsP protein *in vitro*, the *in vivo* induced endogenous *stsP* promoter was replaced by a constitutive promoter in *L. plantarum* WCFS1 and a sortase-deficient derivative. Trypsinization of intact bacterial cells followed by gel-free proteomics as well as gel-based analysis of subcellular protein fractions confirmed the expression, biogenesis, and sortase-dependent cell envelope anchoring of StsP. Moreover, StsP-expressing *L. plantarum* WCFS1 cells attenuated NF-κB responses in an intestinal epithelial NF-κB reporter cell line while this effect was not observed with the wild-type strain. Furthermore, increased NF-κB attenuation was observed with tryptic surface peptides derived from the StsP-expressing strain as compared to peptides derived from wild-type cells, and peptides from gel-purified StsP virtually eliminated flagellin-induced NF-κB activation. Taken together, these findings highlight the role of StsP in attenuation of NF-κB signaling in the gastrointestinal tract, thereby providing a possible molecular explanation of the previously determined activation of tolerance associated pathways in the mucosa of healthy human volunteers upon consumption of *L. plantarum* WCFS1 (1).

Introduction

The mammalian gastrointestinal (GI) tract contains a vast, complex and dynamic community of microbes that intimately interacts with the intestinal mucosa, thereby profoundly influencing host physiology and metabolism (2-4). Functions of the intestinal microbiota include the promotion of mucosal immune system development (5, 6), intestinal epithelial cell maturation (4), protection against pathogenic infection (7, 8), degradation of otherwise indigestible macromolecules (9), and synthesis of essential nutrients (10, 11).

The molecular communication between microbiota and host tissues involves microbe associated molecular pattern (MAMPs), which are recognized by host pattern recognition receptors (PRRs) that are expressed by cells of the intestinal epithelium (12). Although the beneficial, protective, and homeostatic role of the microbiota has been extensively studied (13), information concerning the underlying molecular mechanisms is sparse. In this context, the application of probiotics, i.e. health-promoting microorganisms (14), and their MAMPs have proven to be a suitable tool to assess the molecular basis of host-microbe interactions in the intestine (15). Moreover, these dietary microbes offer an attractive possibility to investigate host cell responses *in vivo* (1, 16, 17) and to temporarily manipulate the intestinal microbiota (18-20), of which the latter is probably most relevant in the small intestine, that combines large numbers of immune system related cells with a relatively small-sized microbiota (21, 22).

The probiotic cell surface is the first site of contact between microbe and host, and several cell surface-related MAMPs have been identified, which include the conserved polymers commonly present in the bacterial cell envelope such as teichoic acid and peptidoglycan. In addition, several proteins of specific probiotics, mainly lactobacilli, have been implicated in a range of host side effects (23, 24). Examples include the p40 and p75 proteins of *L. rhamnosus* GG that stimulate intestinal epithelial integrity by activation of protein kinase C and the MAP kinase pathways (25). Additionally, administration of purified p40 reduced intestinal epithelial apoptosis and disruption of barrier function in the colon epithelium in an EGFR-dependent manner (26). Similarly, a role for homologues of these proteins could be established in *L. casei* BL23

(27). Moreover, *L. acidophilus* NCFM was found to bind to the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) receptor on dendritic cells (DCs) through SlpA, thereby modulating DC maturation and cognating T cell functions (28). This limited amount of *in vitro* studies focusing on specific extracellular proteins has been complemented by generic, *in situ* gene expression studies, which led to the identification of genes of *Lactobacillus reuteri* 100-23, which were specifically induced in the gastrointestinal tract (29). Similarly, several genes of *L. plantarum* WCFS1 (30, 31) and the genetically closely related strain 299v (32) were identified as "gut-inducible", among which *lp_0800* (here renamed *stsP*) was found in various *in situ* expression studies reported to date. An initial recombinase-based *in vivo* expression technology (R-IVET) screening study revealed induction of *stsP* transcription in the intestine of conventionally raised mice (33), which was confirmed by a quantitative real-time RT-PCR approach additionally revealing that *stsP* was particularly up-regulated in the murine small intestine (34). *In vivo* transcriptome analyses consequently confirmed the induction of *stsP* in the intestine of germ-free mice fed a Western-style diet and in the intestine of humans (35). The *stsP* gene encodes a high molecular weight protein (2139 amino acids) containing a C-terminal LPQTG sequence motif predicting its sortase A-dependent (36) cell wall coupling (37).

Here, we expressed the StsP protein *in vitro* by chromosomal promoter replacement in the genetic background of *L. plantarum* WCFS1 and its sortase-deficient derivative. Proteomics approaches confirmed StsP expression and sortase A dependent cell-surface coupling. The role of StsP in host-cell communication was studied using a NF-κB intestinal epithelial reporter cell line. Bacterial cells that express StsP attenuated flagellin-induced NF-κB activation, which was not observed with wild-type cells. In addition, peptides obtained by trypsinization of StsP-expressing cells attenuated NF-κB activation much stronger than peptides from the wild-type strain. Moreover, tryptic peptides derived from gel-purified StsP could virtually silence the NF-κB activation, thereby specifically assigning these NF-κB attenuating effects to StsP.

Material and Methods

Bacterial strains and culture conditions

Escherichia coli TOP-10 (Invitrogen, Carlsbad, USA) was used as intermediate cloning host and grown aerobically in TY medium (38). For genetic manipulation, *L. plantarum* WCFS1 (30, 31) and its derivatives (table S1) were cultivated in Mann-Rogusa Sharpe (MRS; Merck, Darmstadt, Germany) without agitation. For all proteomic- and functional assays, the *Lactobacillus* strains were grown in 2-fold concentrated (2 ×) chemical defined media (CDM) (39) supplemented with 1.5 % (wt/vol) glucose. All bacteria were grown at 37 °C and if appropriate, the media were supplemented with antibiotics; for *E. coli* chloramphenicol at 10 μg/mL, for *L. plantarum* chloramphenicol at 10 μg/mL for plasmid selection, and 10 μg/mL chloramphenicol and/or 30 μg/mL erythromycin for replica plating.

DNA manipulation techniques

Plasmid DNA was isolated from *E. coli* using Jetstar columns following the manufacturer's instructions (Genomed GmbH, Bad Oberhausen, Germany). Restriction endonucleases (Fermentas, St. Leon-Rot, Germany), KOD DNA polymerase (Toyobo, Osaka, Japan) and T4 DNA ligase (Invitrogen, Carlsbad CA, USA) were used as recommended by the manufacturers. Primers were obtained from Invitrogen (Carlsbad CA, USA), and DNA sequencing reactions were performed at BaseClear (Leiden, The Netherlands). Chromosomal DNA isolation, preparation of electro-competent cells and DNA transformation of *L. plantarum* were performed as described before (40, 41).

Construction of StsP expressing strains

Plasmids, primers, and strains used in this study are listed in table S1. In order to study the role of StsP, we aimed to enhance its *in vitro* expression by replacement of the native, *in vivo* induced *stsP* promoter by the promoter sequence of *lp_2145* (P_{2145}), which was chosen based on its constitutive, intermediate level of expression (Bron *et al*., unpublished data available at http://www.cmbi.ru.nl/fermdb). To obtain a mutagenesis construct for the intended promoter replacement upstream of *stsP*, a 1252 bp DNA fragment was synthesized (Gene-art, Regensburg, Germany) that encompasses 187

bp at its 5'-end that corresponds with the *lp_2145* promoter (P_{2145}), followed by 1065 bp of the 5'-coding-region of *stsP*, in which a hexahistidine (His6)-tag sequence was introduced (see supplemental material). At the N-terminus, 10 codons were optimized according to the *L. plantarum* codon preference (see supplementary material). The synthetic fragment was PvuI-digested and cloned into PvuI-Ecl136II digested pNZ5319 (42), yielding pNZ3801. The upstream flanking region of *stsP* was amplified using primers LF_8f and LF_8r and *L. plantarum* WCFS1 chromosomal DNA as a template. The resulting 1.0 kb amplicon was digested with XhoI and cloned into XhoI-SwaI digested pNZ3801, and the resulting plasmid was designated pNZ3808. This plasmid was integrated into the chromosome of *L. plantarum* WCFS1 and its sortase-deficient derivative NZ7114 (chapter 3) by double-crossover (42), to obtain the StsP-expressing strains NZ3808 and NZ3814 in which the *stsP* gene is transcribed from the constitutive *lp_2145* promoter. The anticipated genetic context of the *stsP* locus was confirmed by PCR.

Cell surface trypsinization of intact *L. plantarum* cells

The protocol for surface trypsinization was adapted from Severin *et al*. (43). All cultures were grown in fermentors (Infors, Bottmingen, Switzerland), and cells were harvested at mid-logarithmic (200 ml at OD_{600} of 1.0) and late stationary phase of growth (50 ml at OD_{600} of approximately 4.5) by centrifugation (6000 × g, 20 min, 4 °C). Cell pellets were washed twice with 20 mL isotonic 20 mM Tris/HCl pH 7.6 buffer containing 150 mM NaCl, 1 M xylose, 20 mM $CaCl_2$, and 5 mM DTT and subsequently resuspended in 5 mL of the same buffer followed by the addition of 15 µg mass-spectrometry grade trypsin (Sigma T6567, Sigma-Aldrich, Missouri, USA). Samples were incubated for 10 min at 25 °C, aliquoted into Eppendorf tubes and centrifuged (20000 × *g*, room temperature, 1 min). The shaved peptide-containing supernatants were collected and filtered through a cellulose-acetate filter (0.2 µm pore size, 25 mm diameter; Sigma-Aldrich, Missouri, USA). 5 µg of trypsin was added and samples were further incubated overnight (4 rpm, 25 °C). Trypsin was inactivated by addition of trifluoroacetic acid (TFA) to a final concentration of 0.1 % (v/v). Samples were freeze-dried and stored at -20 °C prior to mass-spectrometry analysis.

Bacterial cell fractionation

For isolation of bacterial cell envelopes, 100 mL bacterial cultures (of *L. plantarum* WCFS1 and its derivatives) were grown overnight (OD_{600} approximately 4.5) in 2 × CDM without antibiotics. Cells were harvested by centrifugation (6000 × g, 20 min, 4 °C) and subsequently washed once with PBS pH 7.5. For isolation of released proteins, culture supernatants were filtered through an cellulose-acetate filter (0.2 µm pore size, 25 mm diameter; Sigma-Aldrich, Missouri, USA), and proteins were precipitated by the addition of trichloroacetic acid (TCA) to a final concentration of 10 %. After 2 hours incubation on ice, samples were centrifuged (12000 × g, 45 minutes, 4 °C). Protein pellets were washed 3 times with acetone, air-dried and stored at -20 °C. Loosely associated surface proteins were isolated by a LiCl extraction procedure. Bacteria cell pellets (from 100 mL overnight culture) were resuspended in 2 mL 1.5 M LiCl and incubated for 20 minutes on ice, followed by centrifugation (12000 × g, 20 minutes, 4 °C) to remove intact cells. The proteins in the supernatant were precipitated by TCA and protein pellets were washed with acetone and stored (see above).

For isolation of the cell envelope material, bacterial samples were disrupted by 3-times passage through a french pressure cell at 1150 psi (SLM Instruments Inc., Urbana, USA). Remaining intact bacterial cells were removed from the lysates by low-rate pre-spinning (3500 × g, 10 min, 4 °C), and the cleared lysates (supernatants) were ultracentrifuged (50000 × g, 20 h, 4 °C) to harvest the cell envelope fractions. Cell envelope pellets were washed 3 times in icecold PBS pH 7.5 and stored at -20 °C.

SDS PAGE and in-gel tryptic digestion

Protein samples were separated under denaturing conditions using Novex 4–12 % Bis-Tris gels (Invitrogen, Carlsbad, USA) followed by Coomassie brilliant blue staining. In gel tryptic digestions were performed as previously described (44). Briefly, gel bands were excised and destained using 50 % acetonitrile in 50 mM NH_4HCO_3. Subsequently, gel slices were dehydrated by addition of 100 % acetonitrile followed by rehydration in a trypsin solution containing 10 ng/µL trypsin (Sigma-Aldrich, St. Louis, USA or Promega, Madison, USA) in 40 mM NH_4HCO_3 and 10 % acetonitrile. The samples were

incubated for 2 h at 37 °C. Subsequently, 10 µL of 25 mM NH_4HCO_3 was added and samples were incubated overnight at 37 °C. The tryptic peptides were extracted with 30 µL of 20 %, 50 %, and 70 % acetonitrile in 1 % TFA, subsequently pooled, concentrated in a speed-vac (Savant, Midland, USA).

Mass-spectrometry-based surface peptide identification and data interpretation

Prior to chromatographic separation, peptides obtained by cell surface trypsinization were purified on reverse phase C18 TopTips (GlyGen Inc., Columbia, USA) and eluted with 80 % methanol in 5 % formic acid. Peptide samples (obtained by cell surface trypsinization or derived from gels) were separated by reverse phase chromatography on an in-house packed C18 capillary column (ID 75 µm x 150 mm, 3 µm particle size, Dr. Maisch, Ammerbuch-Entringen, Germany) mounted on a Proxeon Easy-LC system (Proxeon Biosystems, Odense, Denmark) in line with a trapping pre-column (ReproSil-Pur C18-AQ, ID 100 µm x 20 mm, Proxeon). Solutions of 0.1 % formic acid in water and a 0.1 % formic acid in 100 % acetonitrile were used as mobile phases. Peptides were eluted over 140 minutes (surface tryptic peptides) or 45 minutes (gel isolated tryptic peptides) using a 4-35 % acetonitrile gradient at a flow rate of 250 nL/min. Eluted peptides were analyzed using a linear ion trap-Orbitrap hybrid mass spectrometer (LTQ-Orbitrap, Thermo Fisher Scientific, Bremen, Germany). The MS raw data were submitted to Mascot (version 2.1, Matrix Science, London, UK) using the Proteome Discoverer 1.0 analysis platform (Thermo Fisher Scientific, Bremen, Germany) and searched against the *L. plantarum* proteome database (3063 entries), while false positive discovery rates were assessed by searching against a database containing reversed sequence entries for all proteins in the *L. plantarum* proteome. Peptide tolerance was set to 10 ppm and 0.8 Da for intact peptides and fragment ions respectively; the option semi-trypsin was chosen for protease specificity, allowing for up to 2 missed cleavages. Oxidation of methionine residues and deamidation of asparagine and glutamine were specified as variable modifications. The MS/MS-based peptide and protein identifications were further validated with the Scaffold v3.0 software (Proteome Software Inc., Portland, USA). Peptide identifications

were accepted if they could be established at greater than 95.0 % probability as specified by the Peptide Prophet algorithm (45). Protein identifications were accepted if they could be established at greater than 99.0% probability, as judged by the Protein Prophet algorithm (46) based on at least 2 unique peptides. The Normalized Spectral Abundance Factor (NSAF) (47) was used to estimate StsP abundances in different samples.

Caco-2 NF-κB-GFP reporter assay

Tryptic peptides obtained from cell surface trypsinization or derived from gels were evaluated using NF-κB-promoter activation assays. Caco-2 cells were seeded into black 384- wells plates with a clear bottom and cultured for 2 weeks. Peptides were added to the wells with or without the simultaneous addition of 1 µg/mL flagellin. As controls, no peptides (negative control) or flagellin (positive control) were added. All samples were incubated for 24 h. Intact bacterial cells were added to the medium of the reporter cells. For this, *L. plantarum* WCFS1 and its derivatives were grown overnight in 2 × CDM (OD_{600} approximately 4.5). The bacteria were supplemented with chloramphenicol to a final concentration of 20 µg/mL, and incubated for 15 min. Subsequently, cells were harvested by centrifugation (6000 × g, 20 min, 4 °C), washed 3x in PBS pH 7.5 and diluted to apply a 1:100 human-to-bacterial cell ratio per well. Samples were incubated for 4.5 h. All sample conditions were tested in duplicate wells (3 readings/well). After incubation, the cells were fixed using 4 % paraformaldehyde. Nuclei were stained for 20 min using a 1:500 dilution of DRAQ5 (Enzo Life Sciences BVBA, Zandhoven, Belgium). Confocal images were obtained using a Zeiss LSM 510 system consisting of a Zeiss Axioskop with a Zeiss LD Plan Neofluar × 50 NA 0.6 objective. GFP fluorescence intensities per cell were analysed with CellProfiler 2.0 (Whitehead Institute for Biomedical Research, Cambridge, USA) a 2-tailed t-test was used to determine statistical differences between samples; p values < 0.05 were considered significant.

Results

Promoter replacement to induce StsP expression *in vitro*

As previously shown, expression of *lp_0800* was induced in the intestinal tract of conventional and germ-free mice, as well as in humans (33-35). The *lp_0800*-encoded protein is rich in serines and threonines and we propose to rename it StsP, for serine and threonine rich surface protein. Notably, the N-terminal signal peptide sequence of StsP shares homology with those of the LPxTG anchored surface proteins GspB of *Streptococcus gordonii* (48) and Fab1 of *Streptococcus parasanguis* (48, 49), which were shown to be heavily glycosylated. Moreover, the signal peptide is followed by an alanine, serine, threonine (AST)-rich domain (Fig. 1), which was previously shown to be a target site for O-glycosylation in Acm2 of *L. plantarum* WCFS1 (50). StsP harbors two bacterial Ig-like domains (group 3), which are characteristic elements for bacterial surface proteins that have been reported to facilitate binding to host cells (51) and their extracellular matrix proteins (52, 53). Moreover, the StsP sequence encompasses two DUF285 domains of unknown function (Fig. 1), which was originally identified in mycoplasma lipoproteins but is also found in proteins of *Listeria*, *Enterococcus* and *Heliobacter* (54). Additionally, full-length homologues of StsP were found to be encoded by the majority of *L. plantarum* strains (55). In conclusion, the fact that the expression of *stsP* appears to be specific for the gastrointestinal tract, combined with the presence of several domains that may be involved in microbe-host communication, triggered our interest to study this protein.

To this end, *in vitro* expression of StsP was accomplished by replacement

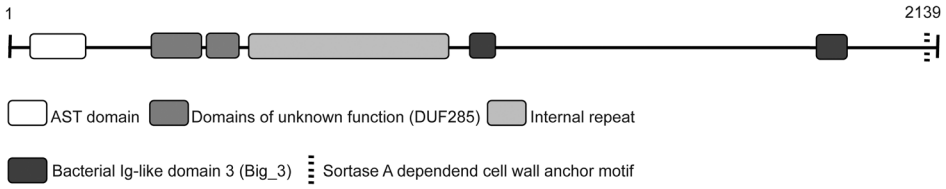

Fig. 1. The StsP protein comprises 2139 amino acids and harbors two DUF285 domains, two Big_3 domains, an internal repeat, an N-terminal alanine, serine, threonine-rich (AST) domain, and a C-terminal cell wall anchor motif.

of the native, *in vivo* induced *stsP* promoter by the promoter sequence of *lp_2145* (P_{2145}).

By using sequencing analysis, two mutant variants were found, one of which contained the His6-tag while a second variant was lacking the His6-tag. The latter likely occurred due to a crossing-over event at the 5' side of the hexahistidine tag sequence. For the experiments presented in this chapter, the non-tagged mutant variant was chosen. After initial confirmation of enhanced *stsP* transcription by quantitative reverse transcription (qRT)-PCR (data not shown), bacterial cell surface trypsinization was used to investigate StsP protein expression during different stages of *in vitro* growth. As anticipated on basis of its specific *in vivo* induction in the intestinal tract, the StsP protein was not readily detected in the samples obtained from the wild-type strain harvested at mid-logarithmic and late stationary phase of growth. Notably, a previous study that employed an in-depth (2-dimensional chromatography coupled to MS-MS) proteomics approach revealed that StsP could be detected in minute amounts in logarithmic phase derived *L. plantarum* WCFS1 cells, whereas it appeared somewhat more dominant in stationary phase derived cells (chapter 2). However, StsP could be readily detected in all samples derived from the *stsP*-expressing strain NZ3808 (data not shown). Taken together, these data demonstrate that the promoter replacement strategy employed results in enhanced expression of StsP in both logarithmically growing and stationary phase cells.

StsP is coupled to peptidoglycan in a sortase dependent manner

The StsP gene is predicted to encode a surface localized protein that is covalently anchored to peptidoglycan in a sortase-dependent manner (37). As surface trypsinization revealed StsP protein expression as well as its surface localization, its covalent coupling to the bacterial cell envelope was investigated in subcellular fractions of *L. plantarum* NZ3808 and its sortase-deficient (NZ3814) derivative that also expresses StsP under the control of P_{2145}, which were analysed by SDS-PAGE and mass-spectrometry. Mass spectrometry analysis of LiCl-extracts of whole cells revealed the presence of StsP in all samples of both NZ3808 and NZ3814. However, StsP was detected at higher relative abundance (average of 96 assigned spectra,

corresponding to NSAF of 0.05) in samples derived from NZ3814 (Δ*srtA*) as compared to those derived from NZ3808 (average of 28 assigned spectra corresponding to an average NSAF of 0.02). The covalent coupling of StsP was further assessed by isolation of cell envelopes of LiCl-washed NZ3814 and NZ3808 cells followed by SDS-PAGE gel elecrophoresis (Fig. 2). Notably, the StsP protein band was readily detected at its predicted molecular weight (226 kDa) in the NZ3808-derived samples, but not in the samples derived from NZ3814 (Δ*srtA*) or the parental strain WCFS1. Taken together, these data underpin that StsP is exposed at the bacterial cell-surface, and support that a substantial fraction of the total amount of protein is covalently anchored to the cell surface in a sortase-dependent manner.

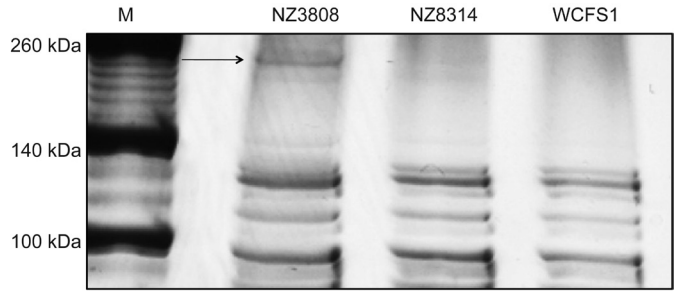

Fig. 2. SDS-PAGE analysis of cell envelope fractions of NZ3808 (lane 2), NZ3814 (lane 3) and WCFS1 (lane 4). M indicates protein molecular weight marker, and proteins were visualized by silver staining. The arrow indicates the StsP protein band close to the expected molecular weight of 226 kDa.

Involvement of StsP in NF-κB pathway modulation

To investigate the impact of StsP on the NF-κB modulatory potential of *L. plantarum*, intact bacterial cells of the wild-type and NZ3808 were incubated with NF-κB reporter cells. In this cell-line NF-κB activation can be monitored by increased GFP expression (chapter 2). Stationary phase derived *L. plantarum* WCFS1 cells elicited a 4-fold increased GFP expression in this reporter cell line, as compared with control reporter cells that were not incubated with bacteria (Fig. 3), which reiterates that *L. plantarum* is capable of modulating NF-κB signaling (1). In contrast, NZ3808 cells were completely unable to elicit NF-κB activation. Previously, it was established

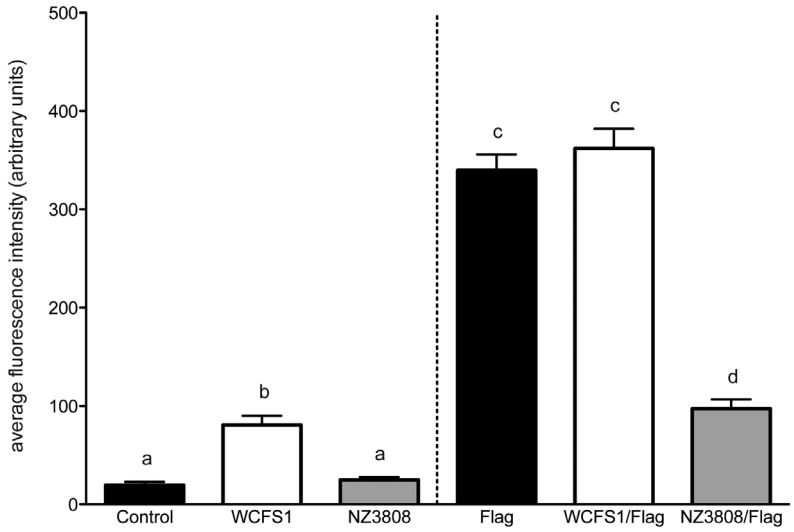

Fig. 3. NF-κB activity modulation by late-stationary phase intact bacterial cells of *L. plantarum* WCFS1 (white) and NZ3808 (grey) under non-stimulating and flagellin-induced (Flag, 1mg/mL) conditions (black). Quantified GFP fluorescence intensities in NF-κB reporter cells expressed as mean fluorescence level per single cell. Error bars represent standard error of measurements (n ~ 600 cells). Distinctive characters above the bars indicate significant differences between a and b as well as between c and d, as determined by a 2-tailed t-test ($p < 0.05$).

that NF-κB responses in this reporter cell line can be triggered to high levels by stimulation with purified flagellin (chapter 2). The flagellin-induced NF-κB activity remained unaffected by the addition of wild-type *L. plantarum* WCFS1 cells, whereas the addition of NZ3808 cells strongly attenuated the flagellin-induced NF-κB activation. These results suggest a prominent role for StsP in the interaction of *L. plantarum* WCFS1 with the NF-κB signaling pathway in intestinal epithelial cells.

To verify that the surface exposure of StsP is involved in the observed NF-κB attenuation, surface peptide fractions were generated by whole-cell trypsin shaving of *L. plantarum* WCFS1 and NZ3808 and the relative attenuating capacity of these peptide fractions was determined using the flagellin-induced NF-κB reporter cell line (Fig. 4). A minor degree of NF-κB attenuation was observed with surface-peptides derived from mid-logarithmic wild-type

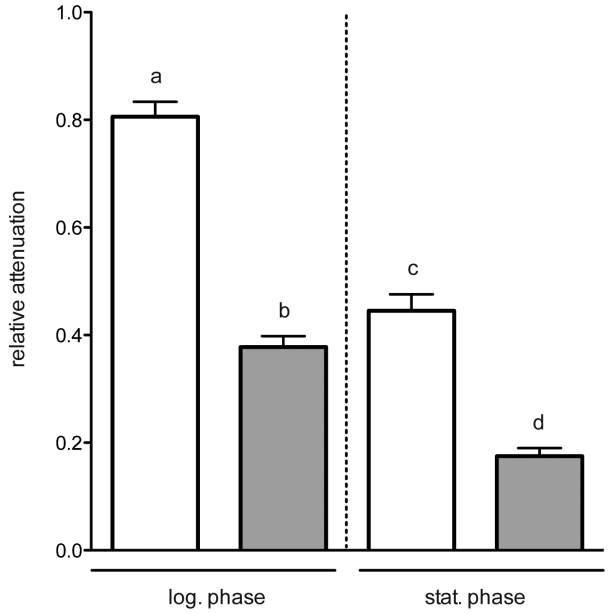

Fig. 4. NF-κB attenuation by surface peptides obtained by surface trypsinization of *L. plantarum* WCFS1 (white) and NZ3808 (grey) cells in flagellin-induced NF-κB reporter cells (relative to flagellin induced NF-κB activation). Peptides were obtained from cells harvested at mid-logarithmic (log.) and late stationary (stat.) growth phase. Error bars represent standard error of measurements (n ~ 600 cells). Distinctive characters above the bars indicate significant differences between a and b as well as between c and d, as determined by a 2-tailed t-test ($p < 0.05$).

L. plantarum cells, whereas late stationary phase derived peptides elicited a much stronger NF-κB attenuation (Fig. 4), which corroborates previous findings (chapter 2). Notably, the surface peptides derived from either mid-logarithmic or late stationary phase cells of the StsP-expressing NZ3808 strain were highly effective in the attenuation of flagellin-induced NF-κB activity (Fig. 4), which corroborates the proposed role of StsP in NF-κB modulation.

To further pinpoint the attenuation of NF-κB activation to the StsP protein, StsP derived peptides were purified from gel. Mass spectrometry analysis of the gel-excised StsP–protein band revealed that 53 spectra could be assigned to StsP whereas additional 35 spectra were assigned to 7 other proteins. Application of this peptide sample to flagellin-induced NF-κB reporter cells elicited complete attenuation of the NF-κB response in comparison to the control (Fig. 5). Taken together, StsP appears to effectively mediate NF-κB attenuation, which can be elicited by either the entire protein (as observed with whole cells), but also by StsP-derived peptides obtained by trypsinization of whole cells or gel-extracted protein bands.

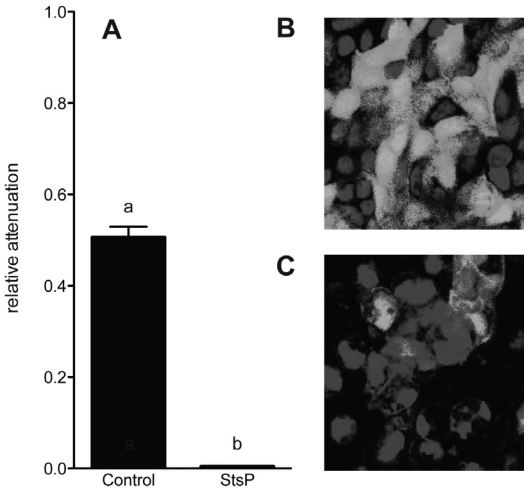

Fig. 5. Panel A: NF-κB attenuation in NF-κB reporter cells by peptides generated from gel-purified StsP (relative to flagellin induced activation) and control material obtained from a protein-free SDS-PAGE gel piece. Error bars represent standard error of measurements (n ~ 600 cells). Panels B and C: Confocal microscopy images of NF-κB reporter cells. Reporter cells were incubated with flagellin, and either the control sample, i.e. a trypsin digested protein free SDS-PAGE gel piece (B) or gel- isolated StsP tryptic peptides (C) were added. Distinctive characters above the bars indicate significant differences between a and b, as determined by a 2-tailed t-test ($p < 0.05$).

Discussion

The close proximity of bacteria and intestinal epithelial cells facilitates intimate and dynamic interactions, supporting the significant cross-talk between bacteria and host-mucosa that has been reported (56-58). However, our knowledge of the bacterial molecules involved in microbial signaling in the intestine and the consequences for mucosal and systemic physiology remains fragmented. This knowledge is currently biased towards the role of conserved bacterial glycopolymers, such as lipoteichoic acid and peptidoglycan, whereas the possible role of bacterial proteinaceous molecules in mucosal signaling remains largely unknown (23). Based on its consistent *in situ* induction in the intestinal tract of mice and men (33-35), *stsP* was targeted by genetic engineering to establish its expression under *in vitro* conditions. The expression of the protein and also its predicted subcellular location could be confirmed, and the data clearly support the predicted StsP anchoring to the cell envelope by sortase.

Intriguingly, the StsP signal peptide shares characteristics with signal peptides associated with generally large, glycosylated proteins of Firmicutes (48, 49). Additionally, StsP is rich in threonine and serine residues that could be targeted by O-glycosylation. Remarkably, recently glycosylation was observed for the serine-threonine rich major autolysin of *L. plantarum* WCFS1, which was found to be O-glycosylated at an N-terminal alanine,

serine and threonine rich (AST) domain (50). Analogously, the AST domain of the Msp1/p75 protein of *Lactobacillus rhamnosus* GG has been demonstrated to be glycosylated (59). Intriguingly, fragments of this protein were shown to stimulate Akt activation in intestinal epithelial cells (60), although recent studies suggest that this may be independent of the glycan conjugation (59). However, preliminary experiments that employed different glycoprotein staining methodologies, including the Periodic acid-Schiff (PAS) and Pro-Q® Emerald 300 glycoprotein stain (Invitrogen, Carlsbad, USA) failed to detect StsP glycosylation (data not shown).

With respect to its role in host cell signaling, our data conclusively show that StsP expressing cells are potent attenuators of (flagellin-induced) NF-κB activation in intestinal epithelial cells. In addition, the NF-κB response attenuation could with great likelihood be assigned to peptides derived from gel-purified StsP protein, supporting the role of StsP or peptides derived from StsP in attenuation of the NF-κB signaling cascades in intestinal epithelial cells. The observed attenuation effects likely did not occur via inhibition of flagellin-mediated TLR-5 signalling, as the NF-κB attenuation was also observed under non-stimulating conditions.

Intestinal epithelial cells function as main barrier between microbes and the sub-epithelial tissues e.g. the lamina propria and its endogenous immune cells (12). In the intestinal epithelium, sophisticated molecular mechanisms ensure the maintenance of mucosal immune homeostasis including a prominent role of tolerance-like responses towards the commensal microorganisms that inhabit the intestinal tract lumen (3, 4). The consequences of deterioration of homeostasis or an imbalance of tolerance and inflammatory responses may lead to excessive immune responses towards gut-commensals, which has been proposed to be causal in the development of inflammatory bowel diseases such as Ulcerative colitis and Crohn's disease (61, 62). It is hypothesized that the NF-κB pathway activity and modulation in intestinal epithelial cells contribute to maintenance of intestinal homeostasis (63) and adequate cellular protection (64, 65) whereby NF-κB activity in epithelial cells is proposed to play a pivotal role in the establishment and maintenance of mutualism (66). Intriguingly, NF-κB attenuation in epithelial cells is considered an important mechanism in the modulation of innate immune

responses by bacterial and viral pathogens, which can induce apoptosis, and increase viral or bacterial progeny (67, 68). However, several studies have highlighted the capability of non-pathogenic bacteria (including probiotics) to also attenuate NF-κB responses (69-73), which similarly may be essential for their establishment as commensal bacteria.

The expression of StsP is specifically induced when *L. plantarum* resides in the intestinal tract (33-35), suggesting that its expression is triggered by physicochemical conditions encountered in the intestinal tract or by the interaction with mucosal tissues. The notion that StsP plays a prominent role in attenuating the activation of immune-associated signaling pathways suggests that StsP is one of the molecules that enable *L. plantarum* to persist and survive in the intestinal habitat by the active prevention of NF-κB activation and the cognate triggering of intestinal defense mechanisms. NF-κB-pathway modulation in the small intestine of healthy human volunteers upon the consumption of *L. plantarum* was previously established *in vivo* (1). This study highlighted both the transcriptional activation of components of the NF-κB complex as well as factors involved in attenuation of NF-κB activation in the mucosal tissues of the participating volunteers. We propose that StsP plays a prominent role in the latter NF-κB associated responses *in vivo*. Notably, the *in vivo* modulation of the NF-κB response in humans depended on the growth phase from which the *L. plantarum* bacteria were harvested (1), and was only observed when stationary phase derived bacteria were consumed. This notion is in good agreement with our previous study that revealed expression of StsP particularly in the stationary phase of growth (chapter 2), further supporting the proposed role of StsP in NF-κB attenuation. Taken together, our observations open avenues to identify and synthesize specific StsP-derived peptides and test these as bio-actives for NF-κB modulation.

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Chapter 4

Supplemental Material

The gastrointestinally induced surface protein StsP of *Lactobacillus plantarum* attenuates NF-κB responses in intestinal epithelial cells

Daniela M. Remus, Jurgen Karczewski, Fabrizia Fusetti, Bert Poolman, Paul de Vos, Jerry M. Wells, Peter A. Bron, and Michiel Kleerebezem

Table S1: Strains, plasmids and primers used in this study.

Material	Relevant features	Reference
Strains		
E.coli		
TOP10	Cloning host	Invitrogen, Carlsbad, USA
L.plantarum		
WCFS1	Single-colony isolate of *L. plantarum* NCIMB8826	(1, 2)
NZ3808	Cm^R; WCFS1 derivative; chromosomal replacement of the *stsP* promoter by the *lp_2145* promoter	this work
NZ7114	Cm^R; *srtA::cat* derivative of WCFS1	chapter 3
NZ3814	Cm^R; NZ7114 derivative; chromosomal replacement of the *stsP* promoter by the *lp_2145* promoter	this work
Plasmids		
pNZ5319	Cm^r Em^r; chromosomal integration vector	(3)
pNZ3801	Cm^r Em^r; pNZ5319 derivative containing the promoter replacement (synthetic) fragment	this work
pNZ3808	Cm^r Em^r; pNZ5319 derivative containing 5' flanking region of StsP and promoter replacement fragment	this work
Primers		
LF_8f	5'-ATGGCTCGAGAACACGGTAAGCACCCAGAT-3'	
LF_8r	5'-CAAGAATCCATGCAATAAATCATATC-3'	

Synthetic fragment consisting of *lp_2145* promoter sequence (italic) followed by the stsP gene sequence. Bold characters indicate optimized codons, underlined ATG indicates start of translation and underlined sequence depicts his-tag sequence.

ACCCGGGAGTCGTTATCTCCTCCTGAATGTGATTTACTGT
ATTCAGGAGGAGATGGTTCATTTAGATGGAAATAATGATT
GATAAATTGTTATCCAGAGTATAGTATTAGTTTATAGGGT
AAAAAGAGTCTGATGGTCTTCGTTACCCGTTAAAATGGTA
*TTGAACTTGGGAGGTGTGATCATCGTG*ATGAAT**CGG**TTT**A**
TTACG**AGTAAACAA**CATTATAAAATGTATAAA**AAAGGTCG**
GTTTTGG**GTT**TTTGCTGGTATTACCGTGGCTACCTTTACT
CTGAATCCACTGATAAGTCGGGCAGATACGGAAACGACGA
CCGCAGCCACCGCAGCTACGACCACTGCTGGGGCATCCTC
ACATCATCACCATCACCATTCATCCAACTCACAGGTTTTA
AGGACTACTACGACTAGTACAACAGGGGCAACGACTCAGT
CTAGTGCGACGGCGATTAATGCGGCAACAACAAATACTAG
TGCTCAAAAAAAACAGGCGGTTAGTGGCACCACTACTGAC
TCAAAGGCTGAACAACCGGTTACGGCAGTAGGAGAAAATG
AAAACGCAACCAGTAATCTCAGTACCTCTGATTCGGCATC
AGCAAGCAGTCAGGCTAAAACTGGTTCTGGCAACAGTTTG
GACCAAACGTCAAACAGCTCAGTAAGTGTCGCATCGAGTT
CACAGAAAGTCACAACACAAAATTCTGATTATCAAAATGA
TCAAGGAACGGGCTCTGAGAGTGGCATTCAATCTAATGTG
ACGGACACGGTGGTGGCTGATGAATCACTACAAACTAATC
GTTCAAGTGTCGCATCGCCAAGTACTAGCACAATGGCCAG
CATTGGTGACTCAGACTCGAAAGATTCTAATGAAACTGAA
AAAGTTGTGGATTCAGAAACTAGTCCGATAGTTGTAACTG
CCACAACTAATACAATTACGACGACTAACGATAAAGTCCA
GTTAAATCGGGCACTATTAGCGCGAGCCGCCATACCCGCA
ATTGTACAGTCAGGGACGCTTGGAACTAGCCAATGGACGA
TGAATAGCGACGGTGTTGTTACAATTGGTGCAGGGGATTG
GAGTAATGTTGATGACGTGTCAGCCCTCTTTTATACATTG
GGTTCGACGGTGACCGGTGTCGTCATCGATGGTAAGGTTA
ATGCTGGTGAGGATCTCAGCTATCTGTTTTTTAAATCACC
AAACCTGGCGACGATTACTGGCTTTCAGAATATTGATACA
TCGATCGTCACG

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Chapter 5

Impact of 4 *Lactobacillus plantarum* capsular polysaccharide clusters on surface glycan composition and host cell signaling

Daniela M. Remus, Richard van Kranenburg, Iris van Swam, Nico Taverne, Roger Bongers, Jerry M. Wells, Michiel Wels, Peter A. Bron, and Michiel Kleerebezem

Abstract

The *Lactobacillus plantarum* WCFS1 genome encodes 4 capsular polysaccharide (*cps*) gene clusters. We constructed gene deletion mutants that lack individual (Δ*cps1A-I*, Δ*cps2A-J*, Δ*cps3A-J* and Δ*cps4A-J*) or combinations of (Δ*cps1A-3J* and Δ*cps1A-3I*, Δ*cps4A-J*) *cps* clusters and assessed the genome wide impact of these mutations by transcriptome analysis. This analysis revealed that *cps* cluster deletion influenced the expression of variable gene sets in the individual *cps* cluster mutants, whereas considerable numbers of up- and down-regulated genes were found to be shared mainly between *cps* cluster mutant 1 and 2 as well as between *cps* cluster mutant 3 and 4. In addition, the impact of these mutations was evaluated based on the characteristics of isolated surface polysaccharides. The composition of the overall cell envelope polysaccharide fraction was altered in each of the mutant strains, implying that all clusters are active in *L. plantarum*. The Δ*cps1A-I* strain produced surface polysaccharides in equal amounts as compared to the parental strain, although the polysaccharides were characterized by a reduced molar mass and the lack of rhamnose. The mutants that lacked functional copies of *cps2A-J*, *cps3A-J* or *cps4A-J* showed clearly decreased levels of surface polysaccharides, whereas the molar mass and the composition of produced polysaccharides was not affected by these cluster mutations. In the quadruple mutant, the amount of surface polysaccharides was strongly reduced and completely lacked rhamnose. The impact of the *cps* cluster mutations on toll-like receptor (TLR) signaling in host cells was evaluated using a TLR-2 reporter cell line. In comparison to the *L plantarum* wild-type like derivative, TLR-2 activation remained unaffected by the Δ*cps1A-I* and Δ*cps3A-J* mutants but slightly increased after stimulation by the Δ*cps2A-J* and Δ*cps4A-J* mutants. Notably the Δ*cps1A-3J* and Δ*cps1A-3J,* Δ*cps4A-J* mutants elicited the strongest responses and clearly activated TLR-2 signaling, which suggests that the reduction of surface polysaccharide leads to enhanced release and/or exposure of TLR-2-activating molecules.

Introduction

The Gram-positive bacterial cell envelope is a multilayered structure, which is mainly composed of peptidoglycan embedded with teichoic acids, proteins, and polysaccharides and which is essential to maintain cellular integrity and shape (1). The molecules of the cell envelope collectively decorate the bacterial surface in a strain- and species-specific manner and facilitate important bacterial processes such as stress- and environmental-adaption, surface-colonization, and –adhesion (2).

Lactic acid bacteria (LAB) are Gram-positive bacteria that can be encountered in a wide range of environmental niches, including the gastrointestinal (GI) tract of humans, plant materials, and dairy products. Some of these are of industrial relevance as LAB fermentation contributes to preservation of food raw materials, but also adds to their flavor and texture (3). Additionally, some LAB are marketed as health-promoting organisms or probiotics (4). Several LAB are able to synthesize extracellular polysaccharides (EPS), many of which consist of heteropolysaccharides built up from regular repeating oligosaccharide units that commonly contain the monosaccharides galactose, glucose, and in several cases also encompass rhamnose, *N*-acetyl-glucosamine, *N*-acetyl-galactosamine, mannose, and non-carbohydrate substitutions (5-8). The repeating units are synthesized in the cytoplasm and assembled on the lipid carrier undecaprenyl phosphate by sequential transfer of monosaccharides from nucleotide sugars by specific glycosyltransferases (GTs). Membrane associated and assembled repeating-unit oligosaccharides are thought to be translocated across the cytoplasmic membrane and polymerized to the eventual polysaccharide by a dedicated transport and polymerization machinery (9, 10). The overall capacity for this type of polysaccharide synthesis is encoded by monocistronically transcribed operons (11) that are readily detectable by their conserved structural composition (Wzy-dependent polymer gene cluster) found in many Gram-positive bacteria and are best documented for *Streptococcus pneumonia* (12, 13). The first genes in these clusters (*wzd*, *wze*, *wzh*) are generally involved in modulation of capsule synthesis and form a tyrosine kinase phosphoregulatory system that controls polymer length (13). These

regulatory gene-cassettes are followed by a variable number of genes encoding glycosyltransferases, which are involved in repeat unit synthesis on the cytoplasmic face of the cell membrane (13). Once synthesized the repeat unit is flipped across the membrane by a dedicated flippase (Wzx) and subsequently polymerized (Wzy) to form the extracellular polysaccharide (13).

EPS produced by LAB has received a lot of attention due to their role as thickening agents that are naturally produced during fermentation and influence the textural properties of fermented dairy and non-dairy products (9, 14-17). Besides the industrial relevance of polysaccharides produced by LAB, they may also play a role in the interaction between microbes and the host intestinal mucosa. While in pathogenic bacteria such as *Streptococcus pneumoniae*, polysaccharide capsules are extensively studied and were shown to play important roles in virulence by inhibiting opsonization and phagocytosis and also as important serotyping antigens for epidemiological studies (18, 19), the role of cell surface polysaccharides of commensal or probiotic bacteria is far less well understood. In *Lactobacillus rhamnosus* GG, biosynthesis of the high-molecular-weight, galactose-rich EPS molecules negatively affects the bacterial capacity to bind to intestinal epithelial cells, which may be due to in the shielding of adhesins (20). Additionally, surface polysaccharides may also contribute to protective shielding against intestinal innate immune factors such as the antimicrobial peptide LL-37 (21). A direct role in host signaling has been proposed for purified CPS of *Lactobacillus casei* Shirota that was shown to mediate the suppression of pro- inflammatory responses in macrophages (22). Moreover, several CPS related genes of *Lactobacillus plantarum* were up-regulated *in vivo* in the GI-tract of mice and humans (23), and a recent study tentatively correlated elevated expression of *cps* related genes to enhanced survival under GI-tract mimicking conditions in this species (van Bokhorst-van de Veen, unpublished data).

Here we characterize the 4 *cps* gene clusters encoded by *L. plantarum* WCFS1, an extensively studied model organism, aiming to identify bacterial molecules underlying microbe-host interactions in the context of probiotic function (24). We constructed *cps*-cluster deficient strains, including single (Δ*cps1A-I*, Δ*cps2A-J*, Δ*cps3A-J*, Δ*cps4A-J*), triple (Δ*cps1A-3J*), and

quadruple (Δ*cps1A-3J*, Δ*cps4A-J*) deletion mutants. Transcriptome profiling of all mutants was employed to investigate genome-wide transcriptional consequences of *cps* cluster deletion(s), while high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) enabled the evaluation of the amount, carbohydrate composition, and molar mass of CPS isolated from the mutants in comparison to the wild-type. Finally, the impact of the *cps* cluster mutations on toll-like receptor (TLR) signaling in host cells was evaluated using TLR reporter cell lines.

Materials and Methods

Bacterial culture conditions

Escherichia coli strain TOP-10 (Invitrogen, Carlsbad, USA), which was used as intermediate cloning host, was grown aerobically in TY medium. *L. plantarum* WCFS1 (25, 26) and its derivatives (Table S1) were either cultivated in Mann-Rogosa Sharpe (MRS; Merck, Darmstadt, Germany) or in 2-fold concentrated (2 ×) chemical defined media (CDM) (27) supplemented with 1.5 % (wt/vol) glucose without agitation. All bacteria were grown at 37 °C and when appropriate, the media were supplemented with antibiotics; for *E. coli* and *L. plantarum* chloramphenicol was added at 10 μg/mL, and during replica plating of *L. plantarum* chloramphenicol and erythromycin were added at final concentrations of 10 μg/mL and 30 μg/mL, respectively.

DNA manipulation techniques

Plasmid DNA was isolated from *E. coli* using Jetstar columns following the manufacturer's instructions (Genomed GmbH, Bad Oberhausen, Germany). Restriction endonucleases (Fermentas, St. Leon-Rot, Germany), KOD-DNA polymerase (Toyobo, Osaka, Japan) and T4 DNA ligase (Invitrogen, Carlsbad CA, USA) were used as recommended by the manufacturers. Primers were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands), and DNA sequencing reactions were performed at BaseClear (Leiden, The Netherlands). Chromosomal DNA isolation, preparation of electrocompetent cells and DNA transformation of *L. plantarum* were performed as described previously (28, 29).

Construction of *cps* cluster deletion mutants

Plasmids, primers and strains used in this study are listed in table S1. The *cps* deletion mutants were constructed according to previously described methods (30), by which the target *cps* clusters were replaced by a chloramphenicol acetyltransferase (*cat*) gene cassette. In this study a derivative of the commonly used mutagenesis vector pNZ5319 (30), designated pNZ5319TAG (Bron *et al*., unpublished data) was used that introduces a unique DNA-tag into the chromosome during gene deletion, which can be used for detection purposes (Bron *et al*., unpublished data). The 5'- and 3'- flanking regions of the individual *cps* gene clusters (*cps1A-I*, *cps2A-J*, *cps3A-J, and cps4A-J*) and of the *cps1A-3J*- and the H-locus- spanning regions were amplified by PCR. The amplicons representing the flanking regions of the target *cps* clusters and the H-locus were subsequently joined by a second PCR to the *tag-lox66*-F3/ *tag-lox71*-R3 or *tag-lox66*-F2/*tag-lox71-catR2* cassette, respectively. The resulting amplicons were cloned into SwaI-Ecl136II digested pNZ5319TAG. The obtained mutagenesis plasmids were integrated into the *L. plantarum* WCFS1 chromosome by double cross over replacement of the target gene (clusters) by the *cat* cassette, yielding the deletion mutant strains with the genotypes NZ3548Cm (Δ*cps1A-I*), NZ5333ACm (Δ*cps2A-J*), NZ3549Cm (Δ*cps3A-J*), and NZ3550Cm (Δ*cps1A-3J*), as well as the tagged strain NZ3400Cm (*lp_2681*-P_{32}-*cat-lp_2683*). For construction of the quadruple mutant (Δ*cps1A-3I*, Δ*cps4A-J*), the mutagenesis plasmid pNZ3550 (Table S1) was integrated into the Δ*cps*4A-J chromosome, in which the *cat* cassette was prior removed by the temporal expression of the Cre recombinase (30), yielding the deletion mutant strain NZ3534Cm (31). The anticipated genotype of all mutants was confirmed by PCR using primers flanking the sites of recombination.

RNA isolation

L. plantarum and its *cps*-cluster deficient derivatives were grown in 2 × CDM and RNA was isolated according to previous described methods (32, 33). In short, following methanol quenching (34), cells were harvested by centrifugation (6000 × g, 20 min, 4°C), resuspended in 400 µL ice-cold

CDM medium and transferred to tubes containing 500 µL phenol/chloroform solution (4:1 [v/v]), 30 µL 10 % sodium dodecyl sulfate, 30 µL 3 M sodium acetate (pH 5.2), and 0.5 g zirconium beads. Cells were disrupted by bead beating using a Savant FastPrep FP120 instrument (Qbiogen Inc., Illkirch, France), and RNA was purified from the aqueous phase using the High Pure Isolation Kit (Roche Diagnostics, Germany). RNA concentration and purity were determined using A260 and A280 measurements using a ND-1000 spectrometer (NanoDrop Technologies Inc., Wilmington, United States), and RNA quality was verified with a 2100 Bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands). Samples that displayed a 23S/16S RNA ratio equal or superior to 1.6 were used for labeling.

Transcriptome analysis and interpretation

3 µg RNA was used for cDNA synthesis. Cyanine-3 (Cy3) and cyanine-5 (Cy5) cDNA labeling was performed as described previously (35), 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). The array design and transcriptome data were deposited under platform GPL13984 and accession number GSE34690 in NCBI's Gene Expression Omnibus (GEO) (36, 37) at http://www.ncbi.nlm.nih.gov/geo/. Hybridization and scanning procedures were performed as previously described (35). Slide scanning was carried out at several photo multiplier tube (pmt) values, and the optimal scan of each individual microarray was selected on the basis of signal distribution (combination of a low number of saturated spots and a low number of low signal spots). The data were normalized using the Lowess normalization as available in MicroPrep (38)/. For statistical significance, Benjamini and Hochberg's False Discovery Rate (FDR) was used (39), with a FDR-adjusted p-value cutoff of 0.05, employed for genes showing at least 2-fold altered expression.

Surface polysaccharide isolation and determination

Surface polysaccharides were isolated and characterized according to

previously described methods (40, 41). *L. plantarum* WCFS1 and its mutant derivatives were grown in 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 x g, 15 min, room temperature). The supernatants were supplemented with erythromycin (30 μg/mL), transferred to dialysis tubes (molecular weight cutoff of 12-14000 Da, Fisher Scientific, Landsmeer, The Netherland) and dialyzed overnight against running tap water followed by dialysis for 4 h against deionized water. The dialyzed samples were freeze-dried and stored at -20 °C until further analysis.

Samples were dissolved in eluent (100 mM $NaNO_3$ + 0.02% NaN_3), and polysaccharides were separated by size exclusion chromatography (SEC), light scattering was measured at 632.8 nm, UV-adsorption of proteins was measured at 280 nm, viscosity was measured with a viscosity detector (ViscoStar, Wyatt Technologies, Santa Barbara, USA), and sample concentrations were measured by determining their refraction index. During SEC, polysaccharide peaks were collected and hydrolyzed with 2 M trifluoroacetic acid (TFA), 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) equipped with a gold electrode. 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).

TLR-2 signaling assay

Human embryonic kidney (HEK)-293 cells not expressing TLR receptors but harbouring pNIFTY, a NF-κB luciferase reporter construct (Invivogen, Toulouse, France) were used as the negative control in the NF-κB assays. HEK-293 cells (Invivogen, Toulouse, France) expressing human TLR-2 and

pNIFTY, a NF-κB luciferase reporter construct (Invivogen) were derived as previously described (42). The HEK-293 TLR-2 reporter cell line was seeded at 5 × 10^5 cells/cm^2 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 like derivative (NZ3400Cm) or *cps* deletion mutants (15 CFU/HEK-293 cell). After this incubation period, the medium was replaced with Bright-Glo™ (Promega Benelux BV, Leiden, The Netherlands), the plate was vortexed, and the luminescence was measured using a Spectramax M5 (Molecular Devices). As positive control, the TLR-2 agonist Pam3CysSK4 (5 µg/mL) was used, and as negative control, no bacterial cells were added to the HEK-293 cells.

Results and Discussion

CPS biosynthesis cluster organization in *L. plantarum* WCFS1

The *L. plantarum* WCFS1 genome contains two regions with capsular polysaccharide biosynthesis genes (Fig. 1A and B). One region of 49 kb contains three gene clusters separated by transposase genes and has been identified as a genomic life-style island with high variability between *L. plantarum* strains (43). Of these three gene clusters, *cps1* and *cps2* are unique to WCFS1 while gene cluster *cps3* is conserved in ST-II and ATCC 14917 but not in JDM1 (Fig. 1 and Table S2). A second region of 14 kb comprises the WCFS1 *cps4* gene cluster and is conserved in other *L. plantarum* strains (Table S2). Clusters 2 and 4 have a typical structure of a Wzy-dependent polymer gene cluster (Fig. 1; Table S2). The first three genes (*cps2ABC* and *cps4ABC*) are homologous to the typical components of the tyrosine kinase phosphoregulatory system involved in modulation of capsule synthesis, *wzd*, *wze*, and *wzh* (13). The fourth gene in both gene clusters is predicted to encode an UDP-*N*-acetylglucosamine 4-epimerase catalyzing the interconversion between UDP-*N*-acetyl-D-glucosamine and UDP-*N*-acetyl-D-galactosamine (*cps2D*, and *cps4D*). The fifth gene is predicted to encode the priming glycosyltransferase catalyzing the transfer of a sugar-1-phosphate from an UDP-sugar to the undecaprenyl-phosphate, the first step in the synthesis of the repeat unit (*cps2E* and *cps4E*). The *cps* clusters 2 and 4 contain three additional genes with high sequence similarity to (predicted)

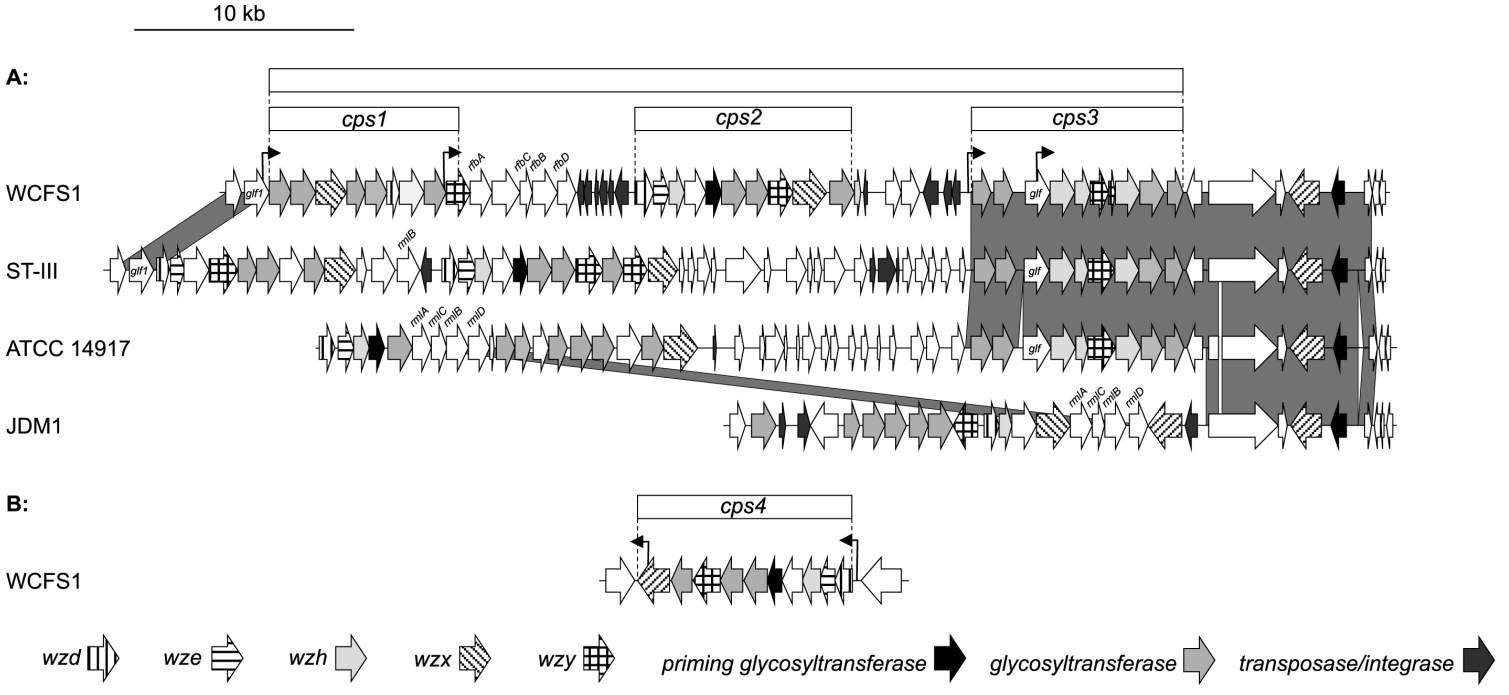

Fig. 1. (A) Genetic organization of the *L. plantarum* WCFS1 polysaccharide biosynthesis gene clusters 1, 2, and 3 (AL935263; lp_1176 through lp_1234) and comparison with the corresponding regions of *L. plantarum* strains ST-III (NC_014554; LPST_C0945 through LPST_C0997), JDM-1 (NC_012984; JDM1_1015 through JDM1_1041), and ATCC 14917 (ACGZ02000014; HMPREF0531_11685 through HMPREF0531_11729; ACGZ02000010; HMPREF0531_11316 through HMPREF0531_11319.
(B) Genetic organization of the *L. plantarum* WCFS1 polysaccharide biosynthesis gene cluster 4. The corresponding regions are conserved in *L. plantarum* ST-III, ATCC 14917, and JDM1 sharing 99%, 99%, and 97% nucleotide identity with WCFS 1, respectively. Arrows indicate predicted promoters with e-value $\leq 10^{-5}$ (48) Open bars indicate the regions deleted in the *cps* cluster deletion derivatives NZ3548Cm, NZ5333ACm, NZ3549Cm, NZ3534Cm, NZ3550Cm, and NZ3680Cm.

glycosyltransferase genes indicating that the encoded polysaccharides would be made up of quatro-saccharide repeat units (*cps2FGJ* and *cps4FGI*). The 3' region of both clusters also encodes homologs of the typical flippase (*cps2I* and *cps4J*) and polymerase (*cps2H* and *cps4H*) functions required for capsule synthesis (13).

The organization of clusters 1 and 3 is different from that of clusters 2 and 4. Cluster 3 has no apparent homologues of the chain-length modulator genes *wzd*, *wze*, and *wzh*. It would be tempting to speculate that *cps3D*, *cps3E*, and *cps3H* serve such function, as their gene products show low sequence similarities to several polysaccharide biosynthesis proteins of unknown function and Cps3E shares distant homology (26 % identity) with the N-terminus of *Clavibacter michiganensis* subsp. *michiganensis* NCPPB 382 Wzc tyrosine kinase (functional homologues *wzd* and *wze*; NCBI accession number YP_001221462). The *cps3 wzy* homologue is split in two by a frame-shift caused by a single nucleotide insertion immediately upstream of the *cps3F* stop codon. It is unclear if a functional Wzy protein can be composed of Cps3F and Cps3G. The cluster contains three predicted glycosyltransferase genes but no priming glycosyltransferase gene. The presence of an acetyltransferase gene indicates acetylation of the repeat units. A set of genes encoding two transcriptional regulators and a mannose-specific adhesin protein separates the *cps3* gene cluster from a polysaccharide polymerase-like and priming glycosyltransferase gene. These genes could complete the polysaccharide synthesis machinery of *cps3*, which would encode a polysaccharide made up of acetylated quatro-saccharide repeat units. Notably, in *Lactobacillus rhamnosus*, exopolysaccharide gene clusters the priming glycosyltransferase genes are also separated from the body of the polysaccharide gene cluster (44). The *glf* gene is predicted to encode an UDP-galactopyranose mutase catalyzing the interconversion of UDP-galactopyranose and UDP-galacto-1,4-furanose. The *glf* gene of cluster 1 encodes a protein with 86 % identity to the protein encoded by the cluster 3 gene. The *cps1* cluster contains 5 predicted glycosyltransferase genes but like the *cps3* cluster, it appears to lack a priming glycosyltransferase gene. In addition, it also contains a predicted acetyltransferase gene, *wzx*, *wzy*, and *wzd* homologues but no *wze* and *wzh* homologues. Expression of the

cps1 cluster is predicted to produce a polysaccharide comprising acetylated hexa-saccharide repeat units providing the undecaprenylphosphate-sugars derived from other pathways can be used and Wze and Wzh are not required or can be used from other cps clusters.

Deletion of *cps* clusters impacts on the *L. plantarum* transcriptome

We previously generated global growth-phase dependent gene expression profiles of *L. plantarum* WCFS1 ranging from mid-logarithmic to late stationary growth phase (chapter 2). Analysis of the *cps* cluster-related gene expression patterns revealed that the *cps1A-I* cluster associated genes were highly expressed *in vitro*, whereas the genes of *cps* clusters *2A-K*, *3A-I* and *4A-J* were expressed at much lower levels (Fig. 2).

Global transcriptional profiles of the single *cps* cluster deletion strains were generated, which confirmed the complete abolishment of expression of the genes affected by the mutation. Next to this, these transcriptome analyses also revealed that *cps* cluster deletion affected variable gene sets in the individual *cps* cluster mutants as compared to the wild-type. Deletion of *cps* cluster 1 or 2 affected the largest group of genes (Table 1), whereas the transcriptome impact of deletion of cluster 4 was considerably smaller, and deletion of cluster 3 appeared the smallest (Table 1). Intriguingly, the transcriptome changes elicited upon deletion of *cps* clusters 1 or 2 as compared to the wild-type strain, shared a substantial number of down-regulated genes (87 shared repressed genes) associated with transport and metabolism of certain amino acids, including serine, glutamate, aspartate, asparagines, methionine, cysteine, and the ABC-transporter for the branch

Table 1. Transcriptional responses of *cps* mutants, Significantly regulated genes CPS mutant transcriptional responses, up- and down-regulated genes

	***L. plantarum cps* mutant strains**			
	NZ3548Cm Δ*cps1A-I*	NZ5333ACm Δ*cps2A-J*	NZ3549Cm Δ*cps3A-J*	NZ3534Cm Δ*cps4A-J*
Up-regulated	62	84	20	58
Down-regulated	124	158	20	49
Total	186	242	40	107

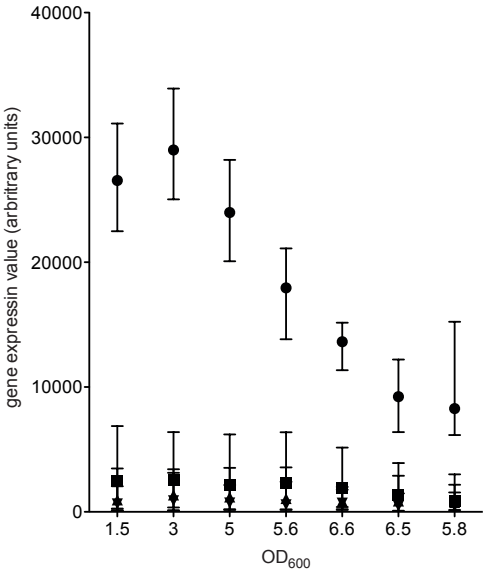

Fig. 2. Growth phase dependent (logarithmic to stationary phase) gene expression levels of individual *cps* gene clusters in *L. plantarum* WCFS1 (shown as average expression of *cps* genes of cluster 1 (●), 2 (■), 3 (▲) and 4(▼). Error bars represent the range of expression between the highest and lowest expressed gene in the different clusters.

chain amino acids (Table S3). Notably, genes that were consistently up-regulated upon deletion of cps clusters 1 and 2 (30 up-regulated genes shared, Table S3) included also some amino acid related functions, including a di- and tri-peptide transport system as well as specific glutamine and a facilitator-family branch-chain amino acid transport systems, but also several cell-envelope associated functions like some extracellular proteins, amino-sugar synthesis and teichoic acid decoration. Remarkably, of the 20 up-regulated genes observed in the *cps* cluster 3 deletion mutant relative to the wild-type strain, 11 were shared with the up-regulated gene set found in the *cps* cluster 4 deletion mutant (Table S3). These genes included metabolic functions associated with pyruvate discipation or its control like, L-lactate dehydrogenase (*ldhL2*), pyruvate dehydrogenase (*pdhA*, *pdhB*; also activated in the *cps* 2 deletion strain), NADH oxidase (*nox5*), but also several carbohydrate degradation and transport functions like an α-amylase (*malS*), and the genetically linked and correlated transport functions for maltodextrins (*mdxG*), as well as two 6-phospho-beta-glucosidase (*pbg5*, and *pbg4*), that appeared to be also induced in deletion mutants of *cps* clusters 2, and 1 and 2, respectively. Besides this universal induction of *pbg4* in all *cps* cluster deletion strains only two additional genes appeared to be consistently affected in all mutants, i.e., down-regulation of a prephenate dehydrogenase

(*tyrA*), and a sodium-coupled N-acetylneuramidate transporter (*lp_3563*). In addition, a FAD/FMN-containing dehydrogenase (*lp_0291*) appeared to be differentially affected in the 4 *cps* deletion strains, i.e., down-regulated upon deletion of *cps* cluster 1 or 2, but up-regulated when *cps* cluster 3 or 4 is deleted. Importantly, compensatory activation of one of the alternative *cps* clusters was not observed in any of the *cps* cluster deletion mutants. These results show that especially deletion of *cps* cluster 1 or 2 appears to have pleiotropic transcriptome consequences, which appear to be centered on metabolism and transport of various amino acids, but also affects several other functions. The observation that deletion of the capacity to produce CPS appeared to affect specific transport and metabolism functions, may suggest that the presence of polysaccharides in the cell envelope plays a prominent role in the access that the bacteria have to nutrients from the environment. Polysaccharides may function as macromolecules that sequester nutrients and thereby facilitate their import (45, 46), or alternatively they may form a capsular structure that surrounds the cell and inhibits transport of nutrients towards the membrane surface and thereby reduces transport efficiencies.

Individual *cps* clusters impact on *L. plantarum* surface glycans characteristics

Surface polysaccharides of *L. plantarum* WCFS1 and the *cps* cluster deletion mutants were isolated to assess their monosaccharide composition (Table 2). The results clearly demonstrated that the wild-type strain produces significant amounts of surface polysaccharides. Deletion of cluster 1 did not impact on the total amount of surface polysaccharides produced, which is remarkable in the light of the relatively high expression of the *cps1A-I* gene cluster in the wild-type (Fig. 2) and the lack of compensatory expression changes of clusters 2, 3, or 4 in the *cps1A-I* mutant. However, the deletion of cluster 1 led to a decreased molar mass of the isolated polysaccharides and influenced the monosaccharide composition, i.e. led to a reduced relative amount of galactose and to a complete lack of rhamnose. This observation establishes that the *cps1* cluster is functional and leads to production of a specific polysaccharide in *L. plantarum* WCFS1. In addition, the lack of rhamnose in the surface polysaccharide fraction obtained for strain

Table 2. CPS monosaccharide composition of *L. plantarum* WCFS1 and its *cps* cluster deletion mutant derivatives

	***L. plantarum* strains**					
Sugar (% of total sugars)	WCSF1	NZ3548Cm Δ*cps1A-I*	NZ5333ACm Δ*cps2A-J*	NZ3549Cm Δ*cps3A-J*	NZ3534Cm Δ*cps4A-J*	NZ3680Cm Δ*cps1A-3J*, Δ*cps4A-J*
Rhamnose	5.40	n.d.	3.08	3.81	4.29	n.d.
Glucosamine	3.28	5.04	3.28	3.45	2.95	3.93
Galactose	17.39	0.86	8.96	15.46	14.82	1.09
Glucose	27.98	23.81	29.16	25.28	27.03	22.22
Galacturonic-acid	45.66	69.79	55.52	52.00	50.92	72.23
Molar mass (kg/mol)	28.93	16.5	29.36	27.79	26.82	17.64
Polysaccharides isolated (mg / 500 mL)	15.76	15.31	5.99	7.84	6.44	1.91

NZ3548Cm (Δ*cps1A-I*) is in agreement with the annotation of *cps1H* as the only rhamnosyltransferase found in all *cps* clusters. As the expression of the *rfb* gene (genetically linked to the *cps1* cluster) did not decease in response to *cps1A-I* deletion but slightly increased, it is likely that indeed the deletion of *cps1H* causes the loss of rhamnose.

Despite the low expression levels of clusters 2, 3 and 4 (Fig. 2), deletion of each cluster reduced the production of surface polysaccharides indicating their respective contributions to the overall surface polysaccharides produced by the wild-type strain (Table 2). The polysaccharides isolated from these mutants showed similar molecular mass as the polysaccharides isolated from the wild-type strain, which indicates a particular role of the *cps* cluster 1 in polysaccharide chain length determination. Deletion of cluster 2 reduced the relative abundance of galactose in the surface polysaccharides, indicating the presence of this sugar in the *cps2*-encoded polysaccharide. In contrast, deletion of clusters 3 or 4 did not significantly affect the monosaccharide compositions of the polysaccharides produced. Notably, all polysaccharide molecules detected in the *cps2A-J*, *cps3A-J* and *cps4A-J* mutant strains contained rhamnose, whereby the presence of this monosaccharide in the surface polysaccharide fraction appears to be exclusively dependent on the *cps1A-I* cluster.

Deletion of all 4 *cps* clusters (Δ*cps1A-3J*, Δ*cps4A-J*) led to a substantial reduction (approximately 90 % in comparison to the wild-type) of the overall amount of surface polysaccharides isolated, which completely lacked rhamnose and showed a reduced relative amount of galactose, analogous to the Δ*cps1A-I* strain. As this mutant is expected to lack the genetic capacity to produce these typical wzy-dependent polysaccharides, the surface glycans isolated from this strain may derive from other surface polymers that are synthesized via other mechanisms. These remaining polysaccharides contain glucose, glucosamine, and galacturonic acid and might be components of teichoic acids, glycosylated proteins, and/or peptidoglycan. Remarkably, mutation of the individual *cps* clusters did not lead to an apparent phenotypic change in terms of growth or cell morphology, while the deletion of all four clusters (Δ*cps1A-3J*, Δ*cps4A-J*) caused aggregation of cells and rapid sedimentation (data not shown), which may be explained by increased cell

surface hydrophobicity due to reduced amounts of surface polysaccharides (47).

Deletion of *cps* clusters influences *L. plantarum* TLR-2 signaling capacity

The effects of the *cps* cluster deletion mutants on host cell signaling were examined in HEK-293 reporter cell lines stably expressing human TLR-2 and carrying a reporter plasmid containing firefly luciferase under the control of the human NF-κB promoter. HEK-293 cells do not normally produce Toll-like receptors (TLRs) and have been previously shown to be unresponsive to several MAMPs. HEK-293 cells transiently transfected with only the NF-κB reporter plasmid (pNiFTY) were unresponsive to TLR-2 agonists of *L. plantarum* demonstrating the requirement of hTLR-2 in this signaling pathway (not shown). *L plantarum* wild-type like derivative (NZ3400Cm) cells activated TLR-2 (Fig. 3). Overall, activation of TLR-2 remained unaffected by Δ*cps1A-I* and Δ*cps3A-J* but was slightly increased after stimulation by the Δ*cps2A-J* and Δ*cps4A-J* mutants, which are the strains that displayed the largest reduction in the amount of surface polysaccharides produced. Notably, exposure of TLR-2-expressing HEK-293 cells to Δ*cps1A-3J* and Δ*cps1A-3J,* Δ*cps4A-J* elicited the strongest responses and clearly activated TLR-2 signaling. Al-

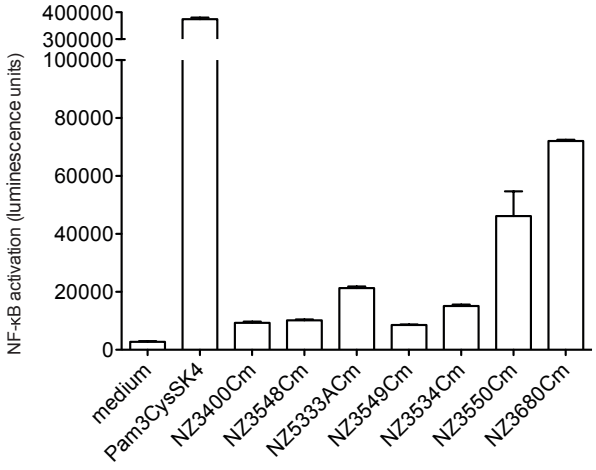

Fig. 3. Impact of *cps* cluster deletion on host cell signaling measured by a luminescence reporter in TLR-2-expressing HEK-293 cells.

though *cps4A-J* appears to be expressed at very low levels, its deletion in the Δ*cps1A-3J* background led to a substantial increase of the TLR-2 signaling capacity of the resulting strain, showing that deletion of all *cps* clusters and the concomitant severe reduction of surface polysaccharide production leads to enhanced release of TLR-2-activating MAMPs. These results suggest a role of surface polysaccharides in the shielding of other *L. plantarum* cell envelope MAMPs such as lipoproteins and teichoic acids that could activate TLR signaling, as proposed for *Lactobacillus rhamnosus* GG (20).

Concluding remarks

In *L. plantarum,* surface polysaccharide biosynthesis is encoded by 4 gene clusters, which independently contribute to the overall surface polysaccharides produced in this bacterium. Transcriptome analysis revealed that *cps* cluster deletion affected different sets of genes in the individual *cps* cluster mutants, whereas substantial numbers of regulated genes were shared between *cps* cluster mutant 1 and 2. Surface polysaccharide analysis revealed that the individual clusters influenced specific features of the polysaccharides produced, i.e. the amount, composition and molar mass. Although some of the individual mutants moderately affected TLR-2 receptor signaling, deletion of all clusters elicited a drastically increased TLR-2 activation. In conclusion, the *cps* cluster encoded surface polysaccharides contribute to the *L. plantarum* cell surface architecture, and probably reduce release and/or exposure of TLR-2-activating bacterial molecules.

Acknowledgements

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Chapter 5

Supplemental Material:
Impact of 4 *Lactobacillus plantarum* capsular polysaccharide clusters on surface glycan composition and host cell signaling

Daniela M. Remus, Richard van Kranenburg, Iris van Swam, Nico Taverne, Roger Bongers, Jerry M. Wells, Michiel Wels, Peter A. Bron, and Michiel Kleerebezem

Table S1: Primers[a], plasmids, and strains used in this study

Material	Relevant features	Reference
Strains		
E.coli		
TOP10	Cloning host	Invitrogen, Carlsbad, USA
L.plantarum		
WCFS1	Single-colony isolate of *L. plantarum* NCIMB8826	(1, 2)
NZ3548Cm	Cm^R; WCFS1 derivative; chromosomal replacement of the *cps1A-I* gene cluster	This work
NZ5333ACm	Cm^R; WCFS1 derivative; chromosomal replacement of the *cps2A-J* gene cluster	This work
NZ3549Cm	Cm^R; WCFS1 derivative; chromosomal replacement of the *cps3A-J* gene cluster	This work
NZ3534Cm	Cm^R; WCFS1 derivative; chromosomal replacement of the *cps4A-J* gene cluster	(3)
NZ3550Cm	Cm^R; WCFS1 derivative; chromosomal replacement of the *cps1A-3J* cluster	(3)
NZ3680Cm	Cm^R; WCFS1 derivative; chromosomal replacement of the *cps1A-3J*, *cps4A-J* cluster	(3)
NZ3400Cm	Cm^R; WCFS1 derivative; chromosomal integration of *cat* casette into H-locus	
Plasmids		
pNZ5319TAG	Cm^r, Em^r; 5319 chromosomal integration vector derivative containing a unique DNA tag	Bron et *al.* (unpublished)
pNZ3548	Cm^r, Em^r; pNZ5319TAG derivative containing 5' and 3' flanking region of the *cps1* gene cluster	This work
pNZ5333A	Cm^r, Em^r; pNZ5319TAG derivative containing 5' and 3' flanking region of the *cps2* gene cluster	This work
pNZ3549	Cm^r, Em^r; pNZ5319TAG derivative containing 5' and 3' flanking region of the *cps3* gene cluster	This work
pNZ3534	Cm^r, Em^r; pNZ5319TAG derivative containing 5' and 3' flanking region of the *cps4* gene cluster	(3)

pNZ3550	Cm^r,Em^r; pNZ5319TAG derivative containing 5' and 3' flanking region of the *cps1-3* gene clusters	(3)
pNZ3400	Cm^r, Em^r; pNZ5319TAG derivative containing 5' and 3' flanking region of the *lp_2681-lp_2683* (H-locus)-spanning region	This work
pNZ5348	Em^r; Cre expression plasmid	(4)

Primers	
cps1::cat (*lp_1177-1185*)	
ls199 lp1176F	GGAGTATGTTAATCAATTTGCCG
ls200 lp1176R	GCATACATTATACGAACGGTAGATTT CAAAGTCTCAACGCCCAACC
ls201 lp1186F	CGGTTACAGCCCGGGCATGAGCGT GGTTCCATGCTCGCAGCC
ls202 lp1186R	CGCCAAAACGTTCTGGATCC
cps2::cat (*lp_1197-1206*)	
is60 lp1197F	TAATCCCAATTGAAATCCAGCC
is63 lp1206R	ACAACTGCTTAATAGCCGGC
is130 lp1197R	GCATACATTATACGAACGGTAGATTT TTGATCCATCATTCACTCTCC
is131 lp1206F	CGGTTACAGCCCGGGCATGAGACA ATATTGAAACTGTCGTAAAG
cps3::cat (*lp_1215-1227*)	
ls205 lp1214F	CGTTGCACTTATTCATGGCGCG
ls206 lp1214R	GCATACATTATACGAACGGTAGATTT AATTTTTGTCATTAGCTTCCCCC
ls207 lp1228F	CGGTTACAGCCCGGGCATGAGGCC AAGCGGACCCATTTTTAACG
ls208 lp1228R	CTCTAATTATGAACATTGCACGC
cps4::cat (*lp_2108-2099*)	
is 66 lp2108F	AGATCTATCATCAGGACGGC
is132 lp2108R	GCATACATTATACGAACGGTAGATTT GTGCGAAAACCAATCTAGCCG
is133 lp2099F	CGGTTACAGCCCGGGCATGAGTATT ACTTGCTCCATTCCACACG

is69 lp2099R	TGATGAAGCTGCATCGACCC
cps1-3::cat (*lp_1117-1227*)	
Is199 lp1176F	GGAGTATGTTAATCAATTTGCCG
Is200 lp1176R	GCATACATTATACGAACGGTAGATTT CAAAGTCTCAACGCCCAACC
Is207 lp1228F	CGGTTACAGCCCGGGCATGAGGCC AAGCGGACCCATTTTTAACG
Is208 lp1228R	CTCTAATTATGAACATTGCACGC
lp_2681-P_{32}-*cat-lp_2683*	
H-2681F	CGACGTGGAAAAGGTCAAGGCTTTAC
H-2681R2	CATTAGTCTCGGACATTCTGCTCCC GGTGTATTTGCCGATGGGCAGCAACC
H-2683F2	CCGATCGCTACGAGAAGACGCACTAGT TTATTTTGATCTTCGGGATTGCG
H-2683R	CATCAGAGTGACGGAAGATGGGCTCG
tag-lox66-F2	CGGGAGCAGAATGTCCGAGACTAATG
tag-lox71-catR2	TAGTGCGTCTTCTCGTAGCGATCGG
is128 tag-lox66-F3	AAATCTACCGTTCGTATAATGTATGC
is129 tag-lox71-R3	CTCATGCCCGGGCTGTAACCG

[a] Underlined nucleotides indicate overlapping ends with tag-lox66-F3/tag-lox71-R3 or with tag-lox66-F2/tag-lox71-catR2 primer pairs

Table S2. CPS related genes of *L. plantarum* WCFS1 and their homologues in strains ATTC 14917, JDM1, and ST-III.

ORF	Gene name		Gene function	ATCC 14917[a]	JDM1[a]	ST-III[a]
lp_1175	*glpF4*		glycerol uptake facilitator protein	EFK29214.1|(100%)	ACT61904.1|(100%)	ADN98165.1|(100%)
lp_1176	*Glf*		UDP-galactopyranose mutase	EFK29215.1|(99%)	-	ADN98166.1|(98%)
lp_1177	*cps1A*		Glycosyltransferase	-	-	-
lp_1178	*cps1B*		glycosyltransferase, family 1 (GT1)	EFK29263.1|(38%)	ACT61913.1|(37%)	-
lp_1179	*cps1C*	*wzx*	oligosaccharide transporter (flippase)	EFK29225.1|(57%)	ACT61927.1|(54%)	ADN98174.1|(54%)
lp_1180	*cps1D*		Glycosyltransferase	-	-	ADN98170.1|(53%)
lp_1181	*cps1E*		acyltransferase/acetyltransferase	-	-	-
lp_1182	*cps1F*	*wzz* *wzd*	polysaccharide biosynthesis protein, chain length regulator	-	-	-
lp_1183	*cps1G*		glycosyltransferase, family 1 (GT1)	-	-	-
lp_1184	*cps1H*		glycosyltransferase, family 2 (GT2)	-	-	-
lp_1185	*cps1I*	*wzy*	polysaccharide polymerase	-	-	-
lp_1186	*rfbA*		glucose-1-phosphate thymidylyltransferase	-	ACT61919.1|(68%)	-
lp_1187			glycosyl hydrolase	-	-	-
lp_1188	*rfbC*		dTDP-4-dehydrorhamnose 3,5-epimerase	EFK29254.1|(99%)	ACT61920.1|(99%)	-
lp_1189	*rfbB*		dTDP-glucose 4,6-dehydratase	EFK29255.1|(100%)	ACT61921.1|(99%)	-
lp_1190	*rfbD*		dTDP-4-dehydrorhamnose reductase	EFK29256.1|(97%)	ACT61922.1|(98%)	-
lp_1191			transposase, fragment	-	-	-
lp_1192			transposase, fragment	-	-	ADO00259.1|(63%)
lp_1193			transposase, fragment	EFK29870.1|(59%)	-	-

lp_1194			transposase, fragment	-	-	-
lp_1195			transposase, fragment	-	-	-
lp_1196			integrase/recombinase	EFK29233.1|(95%)	ACT61924.1|(97%)	ADN98178.1|(89%)
lp_1197	*cps2A*	*wzd*	polysaccharide chain-length regulator	EFK29248.1|(96%)	ACT62653.1|(60%)	ADN98179.1|(93%)
lp_1198	*cps2B*	*wze*	polysaccharide chain-length regulator; tyrosine kinase	EFK29249.1|(97%)	ACT62652.1|(66%)	ADN98180.1|(94%)
lp_1199	*cps2C*	*wzh*	polysaccharide chain-length regulator; tyrosine phosphatase	EFK29250.1|(91%)	ACT62651.1|(57%)	ADN98181.1|(87%)
lp_1200	*cps2D*		UDP N-acetylglucosamine 4-epimerase, NAD dependent	EFK29472.1|(76%)	ADN98953.1|(76%)	ADN98182.1|(94%)
lp_1201	*cps2E*		priming glycosyltransferase, polyprenyl glycosylphosphotransferase	EFK29471.1|(75%)	ACT62648.1|(75%)	ADN98183.1|(92%)
lp_1202	*cps2F*		glycosyltransferase, family 1 (GT1)	-	ACT62647.1|(47%)	ADN98184.1|(49%)
lp_1203	*cps2G*		Glycosyltransferase	-	-	-
lp_1204	*cps2H*	*wzy*	polysaccharide polymerase	-	-	-
lp_1205	*cps2I*	*wzx*	oligosaccharide transporter (flippase)	-	-	-
lp_1206	*cps2J*		glycosyltransferase, family 1 (GT1)	-	-	-
lp_1207	*cps2K*		polysaccharide biosynthesis protein	EFK29257.1|(63%)	-	ADN98189.1|(73%)
lp_1208			transposase, fragment	-	-	-
lp_1210			hypothetical protein	-	-	-
lp_1211			hypothetical protein	EFK29321.1|(21%)	-	ADN98769.1|(21%)
lp_1212			transposase, fragment	-	-	-
lp_1213			transposase, fragment	-	-	-
lp_1214			transposase, fragment	-	-	-

lp_1215	*cps3A*		glycosyltransferase, family 2 (GT2)	EFK29283.1| (70%)	-	ADN98203.1| (79%)
lp_1216	*cps3B*		glycosyltransferase, family 2 (GT2)	EFK29284.1| (99%)	-	ADN98204.1|(81%)
lp_1219	*glf*		UDP-galactopyranose mutase	EFK29285.1|(99%)	-	ADN98205.1|(99%)
lp_1220	*cps3D*		polysaccharide biosynthesis protein	EFK29286.1|(99%)	-	ADN98206.1|(99%)
lp_1221	*cps3E*		polysaccharide biosynthesis protein; putative protein kinase	EFK29287.1|(99%)	-	ADN98207.1|(99%)
lp_1222	*cps3F*	*wzy*	polysaccharide biosynthesis membrane protein	EFK29288.1|(100%)	-	ADN98208.1|(100%)
lp_1224	*cps3G*	*wzy*	polysaccharide polymerase, partial	EFK29288.1|(99%)	-	ADN98208.1|(99%)
lp_1225	*cps3H*		polysaccharide biosynthesis protein	EFK29289.1|(100%)	-	ADN98209.1|(100%)
lp_1226	*cps3I*		O-acetyltransferase	EFK29290.1|(100%)	-	ADN98210.1|(100%)
lp_1227	*cps3J*		Glycosyltransferase	EFK29291.1|(99%)	-	ADN98211.1|(99%)
lp_1228			transcription regulator, AraC family	EFK29292.1|(100%)	-	ADN98212.1|(100%)
lp_1229			mannose-specific adhesin, LPXTG-motif cell wall anchor	EFK29624.1|(95%)	ACT61925.1|(99%)	ADN98213.1|(96%)
lp_1230			transcription regulator, MarR family	EFK29625.1|(100%)	ACT61926.1|(99%)	ADN98214.1|(100%)
lp_1231			oligosaccharide transporter (flippase)	EFK29626.1|(99%)	ACT61927.1|(99%)	ADN98215.1|(99%)
lp_1233			priming glycosyltransferase, undecaprenyl-phosphate beta-glucosephosphotransferase	EFK29627.1|(100%)	ACT61928.1|(100%)	ADN98216.1|(100%)
lp_1234			hypothetical protein	EFK29628.1|(100%)	ACT61929.1|(100%)	ADN98217.1|(100%)
lp_2109	*uvrC*		excinuclease ABC, subunit C	EFK29476.1|(100%)	ACT62654.1|(100%)	ADN98957.1|(99%)
lp_2108	*cps*4A	wzd	polysaccharide chain-length regulator	EFK29475.1|(98%)	ACT62653.1|(98%)	ADN98956.1|(98%)

lp_2107	*cps*4B	wze	polysaccharide chain-length regulator; tyrosine kinase	EFK29474.1|(99%)	ACT62652.1|(99%)	ADN98955.1|(99%)
lp_2106	*cps*4C	wzh	polysaccharide chain-length regulator; tyrosine phosphatase	EFK29473.1|(99%)	ACT62651.1|(99%)	ADN98954.1|(99%)
lp_2105	*cps*4D		UDP N-acetylglucosamine 4-epimerase, NAD dependent	EFK29472.1|(100%)	ACT62650.1|(99%) ACT62649.1|(99%)	ADN98953.1|(100%)
lp_2104	*cps*4E		polysaccharide biosynthesis polyprenyl glycosylphosphotransferase, priming glycosyltransferase	EFK29471.1|(99%)	ACT62648.1|(99%)	ADN98952.1|(99%)
lp_2103	*cps*4F		glycosyltransferase, family 1 (GT1)	EFK29470.1|(100%)	ACT62647.1|(95%)	ADN98951.1|(100%)
lp_2102	*cps*4G		glycosyltransferase, family 1 (GT1)	EFK29469.1|(97%)	ACT62646.1|(95%)	ADN98950.1|(100%)
lp_2101	*cps*4H	wzy	polysaccharide polymerase	EFK29468.1|(98%)	ACT62645.1|(97%)	ADN98949.1|(99%)
lp_2100	*cps*4I		glycosyltransferase, family 2 (GT2)	EFK29467.1|(99%)	ACT62644.1|(98%)	ADN98948.1|(99%)
lp_2099	*cps*4J	wzx	polysaccharide repeat unit transporter (flippase)	EFK29466.1|(99%)	ACT62643.1|(99%)	ADN98947.1|(99%)
lp_2098			Metallophosphoesteras, lipoprotein precursor	EFK29465.1|(99%)	ACT62642.1|(99%)	ADN98946.1|(99%)

[a] GenBank accession number [percentage identity with WCFS1 open reading frame (ORF)]

Table S3. Shared regulated genes between *cps* cluster mutants

Shared down-regulated genes between *cps* cluster mutants 1 and 2

Gene ID	Gene name	Product
lp_2532	*panE1*	2-dehydropantoate 2-reductase
lp_2788	*panE2*	2-dehydropantoate 2-reductase
lp_2035	*aroE*	3-phosphoshikimate 1-carboxyvinyltransferase
lp_1722	*lp_1722*	4-aminobutanoate transport protein
lp_1721	*gabT*	4-aminobutyrate aminotransferase
lp_0200	*lp_0200*	ABC transporter, substrate binding protein
lp_3214	*lp_3214*	ABC transporter, substrate binding protein, cystathionine (putative)
lp_0018	*lp_0018*	ABC transporter, substrate binding protein, oligopeptide
lp_0201	*lp_0201*	ABC transporter, substrate binding proteins
lp_2980	*lp_2980*	acetoin utilization protein (putative)
lp_2979	*lp_2979*	acetoin utilization protein (putative)
lp_0202	*lp_0202*	acetyltransferase, GNAT family (putative)
lp_1766	*lp_1766*	acetyltransferase, GNAT family, C-terminal fragment (putative)
lp_1765	*lp_1765*	acetyltransferase, GNAT family, N-terminal fragment (putative)
lp_3684	*lp_3684*	amidohydrolase family protein
lp_2920	*lp_2920*	amino acid or peptide transport protein, proline-containing (putative)
lp_3339	*lp_3339*	amino acid transport protein
lp_0339	*lp_0339*	aminotransferase
lp_0349	*amtB*	ammonium transport protein
lp_1652	*trpE*	anthranilate synthase, component I
lp_3666	*lp_3666*	aromatic compound hydratase/decarboxylase
lp_0956	*asnS1*	asparagine-tRNA ligase
lp_2830	*ansB*	aspartate ammonia-lyase
lp_0957	*asnA*	aspartate-ammonia ligase
lp_1374	*lp_1374*	bifunctional protein: homocysteine methyltransferase (cobalamin-dependent); methylenetetrahydrofolate reductase
lp_2981	*livE*	branched-chain amino acid ABC transporter, ATP-binding protein
lp_2982	*livD*	branched-chain amino acid ABC transporter, ATP-binding protein
lp_2983	*livC*	branched-chain amino acid ABC transporter, permease protein
lp_2984	*livB*	branched-chain amino acid ABC transporter, permease protein

lp_2985	*livA*	branched-chain amino acid ABC transporter, substrate binding protein
lp_2390	*bcaT*	branched-chain amino acid aminotransferase
lp_0433	*lp_0433*	carbon-nitrogen hydrolase family protein
lp_3151	*acm3-N*	cell wall hydrolase/muramidase, N-terminal fragment
lp_3517	*cblA3*	cystathionine beta-lyase
lp_2634	*cgs*	cysteine gamma synthase/O-succinylhomoserine (thiol)-lyase
lp_0203	*serA*	D-3-phosphoglycerate dehydrogenase
lp_1744	*lp_1744*	D-Methionine ABC transporter, ATP binding protein (putative)
lp_1745	*lp_1745*	D-Methionine ABC transporter, permease protein (putative)
lp_1746	*lp_1746*	D-Methionine ABC transporter, substrate binding protein (putative)
lp_2776	*dsdA*	D-serine ammonia-lyase (putative)
lp_0823	*lp_0823*	diguanylate cyclase/phosphodiesterase, EAL domain
lp_2953	*lp_2953*	esterase (putative)
lp_2915	*uvrA3*	excinuclease ABC, subunit A
lp_0291	*lp_0291*	FAD/FMN-containing dehydrogenase
lp_1581	*glnA*	glutamate--ammonia ligase
lp_0803	*glnQ1*	glutamine ABC transporter, ATP-binding protein
lp_1580	*glnR*	glutamine synthetase repressor, MerR family
lp_0822	*glmS1*	glutamine-fructose-6-phosphate transaminase (isomerizing)
lp_1324	*lp_1324*	glycerol-3-phosphate ABC transporter, ATP-binding protein (putative)
lp_1298	*metH*	homocysteine S-methyltransferase (cobalamin-dependent)
lp_1375	*metE*	homocysteine S-methyltransferase (cobalamin-independent)
lp_2535	*hom1*	homoserine dehydrogenase
lp_2537	*metA*	homoserine O-succinyltransferase
lp_1774	*lp_1774*	hydrolase (putative)
lp_0409	*plnM*	immunity protein PlnM
lp_2954	*lp_2954*	integral membrane protein
lp_0350	*hicD1*	L-2-hydroxyisocaproate dehydrogenase
lp_1082	*lp_1082*	malate / lactate dehydrogenase
lp_2848	*ogt*	methylated-DNA--[protein]-cysteine S-methyltransferase
lp_1764	*lp_1764*	muramoyl-tetrapeptide carboxypeptidase, S66 family (putative)
lp_1768	*lp_1768*	muramoyl-tetrapeptide carboxypeptidase, S66 family (putative)
lp_2531	*pts18CBA*	N-acetylglucosamine and glucose PTS, EIICBA
lp_3338	*nha2*	Na(+)/H(+) antiporter

lp_1747	*lp_1747*	nucleotide-binding protein, universal stress protein UspA family
lp_2536	*cysD*	O-acetylhomoserine sulfhydrylase
lp_0204	*serC*	phosphoserine aminotransferase
lp_0802	*lp_0802*	polar amino acid ABC transporter, substrate binding and permease protein
lp_1299	*tagE1*	poly(glycerol-phosphate) alpha-glucosyltransferase
lp_2034	*tyrA*	prephenate dehydrogenase
lp_2919	*pepR2*	prolyl aminopeptidase
lp_1979	*msrA3*	protein-methionine-S-oxide reductase
lp_2708	*pucR*	purine transport regulator
lp_1084	*aroD1*	shikimate 5-dehydrogenase
lp_3563	*lp_3563*	sodium-coupled N-acetylneuraminate transporter (putative)
lp_1083	*tkt2*	transketolase
lp_2789	*lp_2789*	transport protein
lp_3402	*lp_3402*	transport protein
lp_2768	*lp_2768*	transport protein
lp_0831	*lp_0831*	transport protein, C-terminal domain
lp_0830	*lp_0830*	transport protein, N-terminal domain
lp_2948	*lp_2948*	unknown
lp_2952	*lp_2952*	unknown
lp_3337	*lp_3337*	unknown
lp_0361	*lp_0361*	unknown
lp_1736	*lp_1736*	unknown
lp_p3_25		
lp_p3_21		

Shared up-regulated genes between *cps* cluster mutants 1 and 2

Gene ID	Gene name	Product
lp_2777	*pbg4*	6-phospho-beta-glucosidase
lp_2767	*lp_2767*	acetyltransferase (putative)
lp_1390	*lp_1390*	acetyltransferase, GNAT family (putative)
lp_3334	*adeC*	adenine deaminase
lp_3049	*lp_3049*	amino acid transport protein
lp_3278	*lp_3278*	amino acid transport protein
lp_0551	*dtpT*	di-/tripeptide transport protein
lp_1357	*lp_1357*	extracellular protein, membrane-anchored (putative)
lp_0053	*lp_0053*	fibronectin-binding protein (putative)
lp_0226	*gnp*	glucosamine-6-phosphate isomerase

lp_2110	*glnQ3*	glutamine ABC transporter, ATP-binding protein
lp_2111	*glnPH2*	glutamine ABC transporter, substrate binding and permease protein
lp_1175	*glpF4*	glycerol uptake facilitator protein
lp_2213	*brnT*	LIVCS family transporter, branched-chain amino acids, Leu & Ile & Val (promiscuous)
lp_3255	*lrgB*	murein hydrolase regulator (putative)
lp_0872	*gph1*	phosphohydrolase, possibly 2-phosphoglycolate phosphohydrolase; or inorganic pyrophosphatase
lp_2126	*rpsT*	ribosomal protein S20
lp_2503	*lp_2503*	sugar transport protein
lp_1372	*gtcA1*	teichoic acid glycosylation protein (putative)
lp_0347	*lp_0347*	transcription regulator, PadR family
lp_3358	*lp_3358*	transport protein
lp_2509	*lp_2509*	transport protein
lp_1863	*lp_1863*	transport protein
lp_0860	*lp_0860*	transposase, fragment
lp_3169	*lp_3169*	unknown
lp_0156	*lp_0156*	unknown
lp_3292	*lp_3292*	unknown
lp_0984	*lp_0984*	unknown
lp_p3_38		
lp_p3_37		

Shared up-regulated genes between *cps* cluster mutants 3 and 4

Gene ID	Gene name	Product
lp_2778	*pbg5*	6-phospho-beta-glucosidase
lp_2777	*pbg4*	6-phospho-beta-glucosidase
lp_0177	*mdxG*	ABC transporter, permease protein, maltodextrin
lp_0179	*malS*	alpha-amylase, maltodextrins and cyclomaltodextrins
lp_0291	*lp_0291*	FAD/FMN-containing dehydrogenase
lp_1101	*ldhL2*	L-lactate dehydrogenase
lp_3449	*nox5*	NADH oxidase
lp_2154	*pdhA*	pyruvate dehydrogenase complex, E1 component, alpha subunit
lp_2153	*pdhB*	pyruvate dehydrogenase complex, E1 component, beta subunit
lp_p3_38		
lp_p3_37		

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Chapter 6

General discussion

Daniela M. Remus

Abstract

The gastrointestinal (GI) microbiota shapes and directs host cell- and immune responses and plays a central role in host nutrition and health (1-3). Probiotic bacteria can interact with host cells in the (GI) tract by which they might beneficially influence the health status of their consumers (4). The cellular interactions between probiotic and host are determined by a repertoire of host cell receptors, which recognize microbes based on their microbial associated molecular patterns (MAMPs). The discovery of these microbial "effector molecules" is a prerequisite to understand the exact probiotic mode of action, which is required for their controlled, safe, purpose-directed, and person-specific applications (5, 6).

The work presented in this thesis focused on the molecular characterization of effector molecules of *L. plantarum* WCFS1 (7), a model organism for probiotic lactobacilli, which has been extensively used to unravel the intestinal microbe-host communication in the context of host-physiology modulation.

Lessons from *in vivo* studies - key to unravel microbial effector molecules

While *in vitro* studies on the interactions between microbes and their hosts are highly simplified, *in vivo* studies offer the possibility to study microbes in their real and generally multifactorial host environment. Techniques for determining microbial gene expression profiles *in vivo* have greatly contributed to our understanding of how microbes sense, adapt to, and survive within their hosts and have led to the identification of microbial genes specifically regulated in response to a host environment particularly in the context of pathogenesis (8, 9). Upon encountering their hosts, pathogens alter their gene expression, which was shown to not only depend on the pathogenic species and its host, but also on the respective step of infection process (10). Notably, the modulation of bacterial cell surface structures was shown to be crucial for bacterial virulence, e.g. to evade host defense mechanisms (11), which is reflected by the large number of cell envelope/ surface related genes that pathogenic bacteria modulate during infection. For example, during passage of the host stomach*, Campylobacter jejuni* was found to downregulate genes related to capsular polysaccharide (CPS) biosynthesis, which could imply reduced shielding of effector molecules such as adhesins, and thus initiate the bacteria-host contact (12). In addition, *C. jejuni* induces the expression of genes involved in flagellum biosynthesis to reach its target location within the gastrointestinal tract, but also attenuates their expression probably to evade host immune responses after successful colonization (13). *Listeria monocytogenes* activates the expression of genes encoding sortase, sortase dependent proteins and lipoproteins when it resides in the spleen of the host organism. Some of these listerial genes have been implicated in *Listeria* virulence (14). In this habitat, *Listeria* also induces the expression of *mprF* and *dlt*, which are involved in lysinylation of phospholipids and D-alanylation of lipoteichoic acid (LTA) respectively, which might lead to a reduced negative charge of the bacterial cell surface possibly increasing bacterial resistance against cationic host defense peptides (14). The generation of microbial gene expression profiles during infection and the identification of genes and their products that determine host contact, invasion, and immune evasion are crucial steps to understand the molecular

basis of pathogen-host interactions and opens the possibility to discover targets for antimicrobial therapy to control infectious diseases (15).
Although these studies have revealed much about the host-pathogen interplay, the mechanisms behind the establishment of homeostatic or beneficial host interaction with commensal or benign bacteria are far less well understood. In one study, germ-free mice were colonized with *Bacteroides thetaiotaomicron* with or without the probiotic strains *Bifidobacterium longum* and *L. casei* respectively (16). Whole genome transcriptional profiling revealed that when *B. thetaiotaomicron* encounters the individual probiotic strains, it expands the expression of genes involved in the acquisition and breakdown of polysaccharides (16), which highlighted the capability of microbes to adapt their gene expression to a specific host nutritional environment driven by factors that involve interspecies relationships within the gut ecosystem. Further valuable insights on the *in vivo* gene expression dynamics of non-pathogenic bacterial species derived from work conducted on lactobacilli. For example, *L. reuteri* 100-23 was found to upregulate three genes in the GI tract of conventional mice, two of which showed sequences homologies to genes encoding a xylose isomerase (*xylA*) and a peptide methionine sulfoxide reductase (*msrB*) that are likely involved in nutrient uptake and stress responses, respectively (17). In addition, *L. plantarum* WCFS1 and its genetically related strain *L. plantarum* 299v (18) were extensively studied *in vivo,* highlighting remarkable parallels between the communication strategies employed by *L. plantarum* and by pathogenic bacterial species especially related to the modulation of the bacterial cell surface architecture. Several *L. plantarum* genes that were found to be downregulated in the ceca of chow- and Western-diet fed germ free mice were related to D-alanylation of LTA (19). Notably, deficiency in effective D-alanylation of LTA was previously shown to enhance the anti-inflammatory capacity of *L. plantarum in vitro* and in a murine colitis model (20). In addition, the upregulation of *L. plantarum* genes involved in CPS biosynthesis has been observed in humans, chow-, and Western-diet fed germ free mice (21). In chapter 5, we found that removal of cell surface polysaccharides of *L. plantarum* WCFS1 (by deletion of individual and combinations of *cps* gene clusters) increased host cell recognition via Toll like receptor (TLR)-2 possibly due to the lack of shielding of bacterial TLR-2

ligands. The *in vivo* induction of several CPS related genes could therefore reflect a strategy by which *L. plantarum* avoids exposure of molecules (e.g. TAs and PG), which could be recognized by the host. It seems also possible that induced levels of CPS could protect the bacterium against the harsh intestinal conditions and therefore could enhance bacterial survival *in vivo*. Another study highlighted that *B. thetaiotaomicron* modulates its CPS related genes in germ-free mice reliant on the host diet, which was postulated to enhance host immune response evasion (22).

A resolvase-based *in vivo* expression technology (R-IVET) approach identified 72 *L. plantarum* genes significantly upregulated during the passage of *L. plantarum* through the gastrointestinal tract of conventional mice, including two genes encoding for the sortase depended proteins Lp_0800 (renamed to StsP; chapter 4), and Lp_2940 (23). As revealed by real-time reverse-transcription (RT)-PCR, *stsP* was induced at all locations along the length of the mice small intestine that were analyzed, whereas *lp_2940* was induced only in the small intestine and cecum (24). In a follow-up study, a *L. plantarum* mutant lacking *lp_2940* displayed reduced persistence or survival rates in the mouse GI-tract (25). Remarkably, this phenotype was not affected by the deletion of the sortase encoding gene (*srtA*, chapter 3), which is involved in the biogenesis of Lp_2940. The latter could be explained by the finding that the *srtA* deletion did not influence the cell surface location of Lp_2940 (chapter 3). Thus far, no function has been assigned to StsP, a sortase dependent- and high molecular weight cell surface protein. As shown in chapter 2, StsP is very low expressed *in vitro* (predominantly in the late-stationary phase) and to achieve increased StsP expression in *L. plantarum*, we replaced the (*in vivo* induced) endogenous *stsP* promoter by a constitutive promoter. We found that StsP expressing bacterial cells, bacterial cell surface enriched StsP-containing peptides, and gel purified StsP peptides attenuated NF-κB in intestinal epithelial cells (chapter 4). It was recently found that, next to pathogens, also commensal bacteria, including probiotics, are capable of modulating the NF-κB pathway (see below), mainly by attenuating its activation. Although one might speculate that inhibiting NF-κB mediated gene-transcription could have undesired consequences for the host, as e.g. NF-κB activation is crucial for the expression of cytokines and antimicrobial

factors (26), it seems rather unlikely that such NF-κB down-regulating factors interfere with adequate host responses to pathogenic bacteria that e.g. by influencing the intestinal barrier function and activating subepithelial host receptors generally elicit pronounced activation of host defense responses.
Using *L. plantarum* as a model organism *in vivo* led to the identification of several putative effector molecules, including sortase depended proteins (chapter 3 and 4) and capsular polysaccharides (chapter 5). The molecular characterization of these effector molecules has contributed to an advanced understanding of the *L. plantarum* intestinal lifestyle and of the communication strategies employed by this bacterium.

Impact of bacterial cell surface molecules on NF-κB signaling

In the gastrointestinal tract, an epithelial cell monolayer serves as main barrier to separate the intestinal microbiota from the host lamina propria, which includes the subepithelial immune cells (27). The expression of junctional complexes, a thick mucus layer, and the production of immunoglobulin A (IgA) are among the strategies by which the intestinal epithelium prevents colonization, penetration, and avoids excessive immune cell activation (28). The ability of the microbiota to affect host cell functions by interfering with cell signaling cascades such as the nuclear factor (NF)-κB pathways has been proposed as a further mechanism to sustain the intestinal microbiota-host mutualism and to prevent excessive activation of inflammatory responses (29). The mammalian NF-κB family of transcriptional regulators, i.e. NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and cRel (30) controls major cellular events ranging from cell proliferation, apoptosis to inflammation and was shown to be a key regulator of epithelial integrity and intestinal immune homeostasis (31). Under non-stimulating conditions, the NF-κB proteins are associated with inhibitory IκB kinase (IKK) proteins, which prevents NF-κB nuclear translocation (32). Blocking NF-κB activation is also achieved by several other inhibitor proteins such as the A20 deubiquitinase, which was found to negatively regulate inflammation and to be essential for maintaining immune homeostasis (33).
Recognition of bacterial cells or their MAMPs by host cell pattern recognition receptors (PRRs) leads, among others, to IκB phosphorylation, ubiquitination,

and proteolytic degradation. Consequently, NF-κB is liberated from the complex with IκB and can translocate into the nucleus where it binds to promoters containing κB recognition sites (34). Such regulatory sites are among others found in the majority of cytokines and chemokines encoding genes (35). Pathogenic bacteria have developed strategies to target this network at different regulatory levels, either inhibiting or activating its components. In most cases, pathogens prevent NF-κB activation to facilitate host invasion and circumvent host innate immune responses to establish infection (36). Pathogenic effector proteins, such as YopJ of *Yersinia pesti* (37) and SseL of *Salmonella typhimurium* (38) interfere with the NF-κB pathway at the IKK-level preventing its degradation, while others inhibit NF-κB signaling downstream of IKK, e.g. at the level of NF-κB- transcription, such as NleH1 of *Escherichia coli* (EHEC). NleH1 blocks phosphorylation of the ribosomal protein S3, a transcriptional co-factor, preventing its nuclear translocation and activation of RPS3-dependent genes (39). On the contrary, some pathogens activate NF-κB (40). For example, the LegK1 protein of *Legionella pneumophila* was shown to phosphorylate IκB provoking its ubiquitination and subsequent degradation (41). It can be hypothesized that activation of NF-κB is a strategy employed by *L. pneumophila* to block host cell apoptosis and to allow intracellular bacterial replication (42). Despite the fact that great progress is made in revealing the mode of action by which pathogens affect NF-κB activity, the molecular cell-to-cell communication strategies that have evolved between commensal bacterial and their hosts are just beginning to be understood. Nevertheless, some studies highlighted the potential of commensals and probiotics to influence host cell signaling pathways via NF-κB by impeding its activation, but failed to identify the underlying bacterial effector molecules. NF-κB activation is attenuated by the intestinal commensal *Bacteroides thetaiotaomicron* that enhances nuclear export of RelA (43), by nonvirulent *Salmonella* strains, which inhibit I-κB degradation (44), and by the probiotic strains *Lactobacillus casei* and *Lactobacillus rhamnosus*, through stabilization of I-κBa (45), or inducing the production of reactive oxygen species, and preventing Cul1 neddylation, respectively (46). Next to these *in vitro* studies, *L. plantarum* WCFS1 was shown to modulate NF-κB related pathways (by both activating complex

formation, but attenuating its complete activation) in healthy humans (47). Although it was found that *L. plantarum* cells harvested from the late stationary, and not of the mid-logarithmic phase of growth, significantly modulated the expression of genes associated with the NF-κB signaling cascade, the question "which microbial molecules determine the observed growth phase dependent effect on NF-κB modulation?" remained unanswered. Therefore, we studied the role of stationary-phase specific surface proteins as potential modulators of the NF-κB- cascade (chapter 2), and generated a comprehensive overview of the cell-surface proteome changes that occur in a growth phase dependent manner, analyzing both the transcription of the encoding genes as well as the proteins they encode. The cell surface proteins of *L. plantarum* WCFS1 were assessed by trypsinization of intact bacterial cells (chapter 3, 4). Besides other methods to selectively enrich for the cell surface proteome of gram-positive bacteria (48), this method, often referred to as "shaving", was effectively used in *Staphylococcus* (49, 50), *Streptococcus* (51), and *Bifidobacterium* (52). In the surface proteome study (chapter 2) of *L. plantarum* several extracellular proteins were exclusively present in either of the two growth phases, which were complemented by proteins that were detected in different quantities in mid-logarithmic and late stationary growth phase. Despite the observation that gene transcript levels correlated well with the corresponding protein detection during mid-logarithmic phase of growth (75 % coverage if genes felt in highest transcription quantile), growth phase dependent gene transcription trends in most cases failed to predict the relative abundance of the encoded proteins in stationary phase cells. For example, most proteins were found in both growth phases whereas most genes are silenced in stationary phase cells; i.e. not detected. These discrepancies are likely based on the fact that bacterial mRNAs have relatively short half-lives (53), while their corresponding proteins can be rather stable. In conclusion, growth phase related transcriptome profiles could be coupled to protein detection in logarithmically growing cells, but poorly predicted growth related changes in the surface proteome at late stationary phase, which highlights the requirement of complementary surface-proteome analysis.

We found that surface peptides from neither late stationary- or mid-logarithmic growth phase activated NF-κB in intestinal epithelial cells, but that peptides

derived from late stationary phase cells attenuated NF-κB activation. This attenuation was observed in unstimulated cells, but also in stimulated epithelial cells, independently of the mode of NF-κB activation employed, being either the TLR-5 ligand flagellin (54) or protein free *L. plantarum* cell envelops. Notably, this attenuation was not at all seen when surface shaved peptides obtained from mid-logarithmic bacteria were used. This study corresponds well with the *in vivo* observation of growth phase specific NF-κB modulation by *L. plantarum* (47) and suggests that proteins or peptides may act as effective NF-κB attenuating molecules. Moreover, these experiments highlighted that *L. plantarum* possesses both, NF-κB-attenuating- and activating molecules (peptides and protein-free cell envelope, respectively). One might speculate that attenuation of NF-κB, which is generally employed by pathogens to circumvent host inflammatory and innate immune responses (36), is one of the mechanism by which *L. plantarum* might avoid host defense mechanism. Remarkably, the *L. plantarum* protein StsP (chapter 4), which was upregulated under gastrointestinal conditions (21, 23, 24) was found to attenuate NF-κB, activation in intestinal epithelial cell, and therefore might be one of the crucial components to evade host innate responses *in vivo*. *In vivo* transcriptional responses to *L. plantarum* and a *stsP* deficient derivative as well as studies on intestinal persistence/survival efficacy might give further insights into the role of StsP in regulating intestinal tolerance.

L. plantarum possesses NF-κB activating and attenuating, pro- and anti-inflammatory signaling molecules (chapter 2, 3, 4), while their expression (chapter 2), and likely the degree of shielding (chapter 3, and 5), contribute to the overall host response. *In vivo* observation (chapter 2, and 4), that are consequently explained on the molecular level with purified bacterial cell material *in vitro* (proteins and peptides), turned out to be a successful strategy to link bacterial molecules to host responses. In the future, studies on the exact underlying receptor-ligand interactions (e.g. by the application of fluorescence resonance energy transfer, or FRET), and the identification of the exact components which are modulated within the NF-κB pathway [e.g. late stationary phase peptides may induce the NF-κB antagonist A20, which was also induced *in vivo* (47)], by host transcriptome analysis will contribute to a more complete understanding of communication between *L. plantarum*

and its host.

Microbial effect on the host – driven by communities, species, and molecules

Many years of co-evolution have shaped a mutualistic relationship between the microbiota that inhabit the gastrointestinal tract and the host species. The commensal microbiota contributes to the host nutrition/energy balance, confers colonization resistance against pathogens, and influences the structural and functional development of the mucosal immune system (55, 56).

Animal-studies that compared conventional with germ-free or bacteria-depleted conditions, have contributed to an increased appreciation about the importance of the intestinal microbiota on the host's physiology, in the context of both health and disease (57). For example, germ-free mice are defective in the development of the gut-associated lymphoid tissue (GALT), lack T cells such as T_H17, and FoxP3$^+$, show lower expression of TLRs in intestinal epithelial cells, and reduced numbers of Peyer's patches (58, 59). Additionally, in antibiotic treated mice, diminished MyD88-mediated expression of antimicrobial factors (e.g. RegIIIg and RegIIIb) led to an increase in permeability of the intestinal epithelial layer (60). Notably, the capability of the microbiota to elicit host innate defenses in epithelial cells, to produce short chain fatty acids, and degrade carbohydrates, follow the "concept of redundancy", which means that the effects employed by many different species (59, 61). While a complex intestinal microbiota was shown to be essential for the induction of oral tolerance to food antigens (62), other specific host effects were documented to be elicited specific microbial species or even by a single microbial effector molecule. For example, recent studies documented that segmented filamentous bacteria (SFB) promote the accumulation of T_H17 cells in the small intestine of mice, whereas mice with a SFB-deficient microbiota lack mucosal T_H17 cells (63, 64). Colonization of germ-free mice with a mix of 46 related *Clostridium* strains caused the release of transforming growth factor–β (TGF-β) by intestinal epithelial cells, and colonic T_{reg} cell accumulation, which was not achieved by other species including *B. fragilis*, *Lactobacillus*, or SFB (65). Colonization of germ-free

mice with *B. fragilis* led to increased IL-10–producing Foxp3$^+$ cells in the colon, which was mediated by TLR-2, and required the expression of a single bacterial molecule, the zwitterionic polysaccharide A (PSA) (66). PSA is one of eight distinct CPSs produced by *B. fragilis* (67), and has been implicated in a wide range of host effects (68), e.g. in the protection of mice from *Heliobacter hepaticus*- induced colitis, which was achieved by both, the oral administration of the PSA expressing *B. fragilis* strain, and purified PSA (69). Some bacteria express glycosylated proteins, using *N*-linked and/or O-linked glycosylation machineries (70). The glycosylated surface layer protein A of *L. acidophilus* NCFM mediates its binding to the dendritic cell C-type lectin receptor DC-SIGN, modulating DC- and T-cell functions (71). Another glycoprotein, the cell wall-associated peptidoglycan hydrolases (encoded by *p75*) of *L. rhamnosus* GG, stimulates Akt activation in intestinal epithelial cell signal transduction (72). Recently this protein was shown to contain an attached glycan, but this modification was not required for the Akt-signaling role of the protein (73). Several glycoproteins were identified in *L. plantarum* WCFS1 (chapter 1), one of which, the cell wall hydrolase/muramidase Acm2 was found to undergo cytoplasmic O-glycosylation at its N-terminal AST (alanine, serine, and threonine rich) domain (74). Mass-spectrometry analysis revealed the presence of 5 glycopeptides within the AST domain, all of which were detected in various glycoforms (74). Lectin blotting further revealed that Acm2 is glycosylated with *N*-acetylglucosamine (74). Whether Acm2 plays a role in the interaction of *L. plantarum* and its host is not yet known.

The work on PSA (69) and p40 (75) demonstrated the *in vivo* effects of single microbial molecules and has set the scene for future studies on probiotics and gut commensals to unravel the effectors underlying intestinal tolerance, which could potentially result in clinical applications. In the context of the latter, the application of purified molecules might not only be attractive as their synthesis could be rather cost-effective and fast, but also because they offer the possibility of well-controlled (e.g. dosage), specific, and targeted treatments. The latter could be achieved by e.g. the use of capsules or encapsulates that release their content at a desired location and which could be designed for specific personal requirements.

The future

The human gut microbiota encompasses more than 1000 species, and although it was recently classified into three general enterotypes (76, 77), its composition was shown to substantially differ between individuals (78), which could be dictated by factors such as host diet, genotype (79-81), and health status (82). The concept to use probiotics to modulate the intestinal microbiota and thereby introduce desired health effect faces a range of challenges. According to the proposed 'bandwidth of human health' (6), which is based on the assumption that the effects of a probiotic intervention depend on the consumer's initial state of intestinal homeostasis and health, it is likely that individuals benefit from different microbes and their effector molecules, which means that different strains need to be selected for different people. Accordingly, a previous study, which assessed *in vivo* mucosal responses of healthy humans to probiotic lactobacilli not only revealed that different stains induce specific host responses, but also highlighted the person-to-person variation in response transcriptomes (83). The establishment of host-specific genetic markers to classify individuals into distinct "types" of intestinal homeostasis and health, as well as the assessment of microbial marker genes that co-occur with a specific health or disease status will help to better select probiotics for the treatment and prevention of intestinal diseases. However, given the fact that the application of intact bacterial cells might include uncontrollable side effects (e.g. bacterial adaptation to a host environment; see above), the application of isolated effector molecules as health supporting supplements may provide much better controlled and therefore plausible health-stimulatory applications. Along this line, a recent functional metagenomics study identified bacterial genes that influence NF-κB signaling in intestinal epithelial cells (84). Specifically, two loci encoding an ATP-binding cassette transport system and a putative lipoprotein were identified as modulators for NF-κB (84). These molecules, next to p40 (75), p70 (72), PSA (69), and StsP (chapter 4), could be administered in pure form to elicit their specific effects intended for a specific host purpose/ physiological condition, and successfully replace the use of intact microbial cells.

Next to the use of functional metagenomics to discover microbial effector

molecules, metaproteomics appears to be powerful tool to reveal insights into the microbiota related proteome, including their putative effector molecules (85, 86). These studies should be extended in the future, and preferably include work on single probiotic strains or gut commensals (introduced into e.g. humans and/or mice) and their proteome adaption in response to the host, as well as on the modulation of posttranslational modification (PTMs) of proteins during host-interactions. Glycosylation of proteins can be considered as important signaling strategy employed by bacteria, which might only occur *in vivo* (87), a fact which highlights the requirement of proteome studies of microbes in response to their *in vivo* environment. Protein phosphorylation of commensals including probiotics is just beginning to be investigated. Recent work characterized the phosphoproteome of *L. rhamnosus* GG, and documented acid stress induced changes of the phosphorylation pattern of several proteins (88). Notably, protein phosphorylation was often found to control bacterial pathogenicity, e.g. by affecting the function of virulence factors, and of molecules involved in adhesion and modulation of host immune responses (89). As adaptations of phosphorylation machineries might not be reflected by a changed transcriptome, proteome studies including the analysis of phosphorylation but also other PTMs are of utmost importance for advanced understanding of the molecular basis underlying host-microbe interactions in the intestine.

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Appendices

Nederlandse samenvatting
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Overview of completed training activities

Nederlandse samenvatting

Lactobacillen komen in verschillende natuurlijke leefomgevingen voor, waaronder zuivelproducten en het humane maag-darmkanaal. Daarnaast zijn er specifieke *Lactobacillus* stammen op de markt als probiotica die een interactie aan kunnen gaan met gastheercellen in het maag-darmkanaal, waardoor ze een positieve invloed op de gezondheidsstatus van de consument hebben. De identificatie van probiotische effector moleculen is zeer belangrijk om beter te begrijpen hoe probiotica werken op het moleculaire niveau. Deze kennis is onmisbaar voor de gecontroleerde, veilige en specifieke toepassing van probiotica. Dit proefschrift concentreert zich daarom op de moleculaire karakterisatie van effector moleculen in een modelorganisme voor probiotische lactobacillen; *Lactobacillus plantarum* stam WCFS1. Het moleculaire mechanisme dat verantwoordelijk is voor de eerder geobserveerde groeifase afhankelijke capaciteit van *L. plantarum* om NF-kB geassocieerde routes te moduleren werd onderzocht met een gecombineerde aanpak van transcriptomics en celoppervlak proteomics. De invloed die monsters, verkregen door cel oppervlakte trypsinering, op NF-kB promoter activering hebben, werd bekeken met een toegewijd NF-kB reporter systeem in een Caco-2 darm epitheel cel (DEC) lijn. Fracties van het oppervlakte proteoom afkomstig van laat stationaire bacteriële cellen bleken NF-kB activering effectief te dempen, terwijl dit fenomeen niet werd gezien met monsters afkomstig van cellen die geoogst waren middenin de logaritmische groeifase. Een van de eiwitten die meer voorkwam in het monster uit de stationaire groeifase was StsP, een sortase afhankelijk eiwit (SAE) waarvan al was aangetoond dat de expressie geïnduceerd is in het maag-darmkanaal van zowel muizen als mensen. De StsP expressie werd vervolgens *in vitro* verhoogd met genetische technieken wat resulteerde in sterke demping van NF-kB in DECen, waarmee het bewijs geleverd is dat dit oppervlakte eiwit een rol speelt in de communicatie met gastheer cellen. SAEen werden vervolgens verder bestudeerd in een sortase deficiënte *L. plantarum* stam (*srtA*). De sortase deficiëntie in deze stam leidde tot verlaagde hoeveelheden maar geen totale afwezigheid van specifieke SAEen. Daarnaast konden SAEen gemakkelijk van het celoppervlak van de sortase deficiënte maar niet van de wildtype stam verwijdert worden en de SAE extractie had grote

gevolgen voor de pro-inflammatoire signaal eigenschappen van de sortase deficiënte stam. Deze gegevens zijn een belangrijke aanwijzing dat een of meer SAEen immuun reacties van de gastheer kunnen dempen. Naast eiwitten is de rol van capsulaire polysacchariden (CPSen) onderzocht door de wildtype stam en mutanten die geen CPS kunnen maken te vergelijken, waaruit bleek dat gereduceerde niveaus van deze cel oppervlakte suikers leidden to verhoogde herkenning van *L. plantarum* door de gastheer via Toll like receptor 2. De resultaten in dit proefschrift laten de communicatie strategieën van *L. plantarum* zien door bacteriële effector moleculen te manipuleren op het niveau van modulatie van expressie, subcellulaire lokalisatie en/of toegankelijkheid op de bacteriële cel en deze veranderingen vervolgens te correleren aan specifieke gasheer reacties.

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About the author

Daniela Maria Remus was born on December 3rd, 1980 in Cloppenburg, Germany. Upon graduation from business high school in 2001, she started her studies at the University of Bonn and graduated in 2007 passing her major and minors in molecular plant physiology, physics, and applied microbiology. During that time, she also joined the department of Molecular Physiology and Biotechnology of Plants led by Prof. Dr. Dorothea Bartels to study plant protein expression and phosphorylation in order to discover the molecular mechanism by which plants are able to survive dramatic water loss.

In 2007, she pursued her scientific career by moving to the Netherlands to start her Ph.D. project at Wageningen University under the supervision of Prof. Dr. Michiel Kleerebezem. Her work on the molecular analysis of candidate probiotic effector molecules of *Lactobacillus plantarum*, which is described in this book, was part of the Top Institute Food and Nutrition project "Fermentation Enhanced Probiotic Function". 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 gastrointestinal health and disease prevention.

Co-author affiliations

Daniela M. Remus[1,2,3], Fabrizia Fusetti[1,4], Jurgen Karczewski[1,5], Roger S. Bongers[1,2], Marjolein Meijerink[1,5], Irene Konings[1,5], Iris van Swam[1,2], Nico Taverne[1,5], Bert Poolman[4], Maria L. Marco[1,2,#], Paul de Vos[1,6], Jerry M. Wells[1,5], Michiel Wels[1,2], Richard van Kranenburg[7], Peter A. Bron[1,2,8] and Michiel Kleerebezem[1,2,3,5]

[1]TI Food & Nutrition, Nieuwe Kanaal 9A, 6709 PA Wageningen, The Netherlands

[2]NIZO food research, Kernhemseweg 2, 6718 ZB Ede, The Netherlands

[3]Laboratory for Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands

[4]Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, Netherlands Proteomics Centre & Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands

[5]Host-Microbe Interactomics Group, Wageningen University, De Elst 1, 6708 WD Wageningen, The Netherlands

[6]Pathology and Medical Biology, section Immunoendocrinology, University Medical Centre Groningen, Groningen, The Netherlands

[8]Kluyver Centre for Genomics of Industrial Fermentation/NCSB, P.O. Box 5057, 2600 GA Delft, The Netherlands

[7]Purac, P.O. Box 20, 4200 AA Gorinchem, The Netherlands

[#]Current address: Department of Food Science and Technology, University of California, One Shields Avenue, Davis, CA 95616, USA

Overview of completed training activities

Discipline specific activities

● *Cross-Talk Conference*, Wageningen, The Netherlands (2011)
● *10th Symposium on Lactic Acid Bacteria*, Egmond aan Zee, The Netherlands (2011)
● *4th FEMS Conference*, Geneva, Switzerland (2011)
● Courses in *NMR*, *X-Ray Crystallography* and *Electrochemistry of Proteins*, University of Lisbon, Faculty of Science and Technology Lisbon, Portugal (2010)
● *Micom 2010 - European Student Conference on Microbial Communication*, Jena, Germany (2010)
● *Summer Course Glycoscience*, Graduate School VLAG, Wageningen, The Netherlands (2010)
● Courses in *Mass-spectrometry of Proteins*, *Bioinformatics in Proteomics*, and *2-D Gel Electrophoresis*, University of York, Department of Biology and York Centre of Excellence in Mass Spectrometry, York, Great Britain (2009)
● *20th Joint Glycobiology Meeting*, Cologne, Germany (2009)
● *Glycobiology – A Sweet Science Coming of Age*, Amsterdam, The Netherlands (2009)
● *9th and 11th Annual Gut Day,* Wageningen and Vlaardingen, The Netherlands (2007/2009)
● *9th Symposium on Lactic Acid Bacteria*, Egmond aan Zee, The Netherlands (2007)

General activities

● *Preparation of a Grant Proposal*, University of Groningen and University of Amsterdam, Groningen/Amsterdam, The Netherlands (2011)
● *Improving Personal Effectiveness, Planning and Organization Skills*, Top Institute Food and Nutrition, Wageningen, The Netherlands (2009)
● *Scientific Networking*, Graduate School VLAG, Wageningen, The Netherlands (2008)
● *VLAG Ph.D. Week*, Graduate School VLAG, Wageningen, The Netherlands (2008)

Optionals

● *Molecular Genetics Annual Meeting*, Lunteren, The Netherlands (2009/2010)
● *TIFN Annual Conference*, Wageningen, The Netherlands (2010)
● *TIFN Project Meeting*, Wageningen, The Netherlands (2007-2011)
● *TIFN Program 3 Meeting*, Wageningen/Groningen, The Netherlands, Helsinki, Finland (2007-2011)
● *NIZO Lunch Meeting*, NIZO Food Research, Ede, The Netherlands (2007-2011)
● Preparation of a research proposal (2007)

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