In vitro characterization of commercial and potential probiotic lactic acid bacteria: Interactions with human cells

In vitro karakterisering av kommersielle og potensielle probiotiske melkesyrebakterier: Interaksjon med humane celler

Philosophiae Doctor (PhD) Thesis

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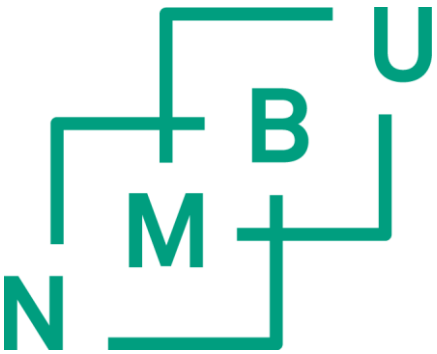

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Ås, October 2014

Hanne Jensen

ABSTRACT

Lactic acid bacteria (LAB), especially bacteria belonging to the genus *Lactobacillus*, are recognized as common inhabitants of the human gastrointestinal tract and have received considerable attention in the last decades due to their postulated health-promoting effects. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" by FAO/WHO. The main aim of this work was to increase the current understanding of how probiotic bacteria and LAB interact with cells of human origin.

Characterization of 18 LAB strains in typical probiotic screening assays revealed that *L. reuteri* strains tolerate gastric and small intestinal conditions very well, have a high adhesion to cell lines of intestinal origin, and can possibly strengthen the epithelial barrier *in vitro* over 24 h, thus revealing some interesting characteristics compared to the other strains investigated. Based on these results, strains were selected for further studies to better understand the mechanisms behind the observed diversity. When further investigating the excellent adhesion of *L. reuteri* we found that the protein encoded by *hmpref0536_10633* plays a critical role in binding of *L. reuteri* ATCC PTA 6475 to Caco-2 cells and mucus *in vitro*, and we propose that this LPxTG motif containing protein should be referred to as cell and mucus binding protein A (CmbA). Furthermore, *L. reuteri* ATCC PTA 6475 and DSM 20016 induced the highest cytokine secretion from THP-1 cells and the highest activation of NF-κB in the U937-3xkB-LUC cell line, whereas other known probiotic bacteria such as *L. plantarum* 299v and *L. rhamnosus* GG had little effect. The *L. reuteri* LPxTG protein Hmpref0536_10802 appears to be of importance for the secretion of IL-8 and TNF-α from THP-1 cells. In a Caco-2 model of the intestinal epithelial barrier *in vitro* we found no further beneficial effect of the selected probiotic bacteria and LAB compared to the control. In general, well documented commercially available strains, such as *L. rhamnosus* GG and *L. plantarum* 299v, performed relatively poor compared to other LAB in the typical probiotic screening assays used in this work. However, the ultimate performance criterion of a probiotic strain is the ability to confer health benefits in the host. Nevertheless, *in vitro* methods are highly important to increase our current understanding of how probiotic bacteria and LAB interact with cells of human origin. The involvements of CmbA in adhesion to intestinal epithelial cells and mucus and Hmpref0536_10802 in immune stimulation of THP-1 cells are two novel contributes to the puzzle that one day will help us to fully understand the interaction between LAB and cells of human origin.

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SAMMENDRAG

Melkesyrebakterier (MSB), særlig bakterier tilhørende slekten *Lactobacillus*, er en naturlig del av vår tarmflora og har fått stor oppmerksomhet de siste tiårene på grunn av deres påståtte helsebringende effekt. Probiotika er definert som "levende mikroorganismer som når de gis i tilstrekkelig mengde gir en helsegevinst for verten" av FAO/WHO. Målet for arbeidet i denne avhandlingen var å øke vår forståelse omkring samspillet mellom probiotiske bakterier/MSB og humane celler.

Karakterisering av 18 MSB i typiske probiotiske testmetoder viste at stammer av *L. reuteri* har en god toleranse for mage- og tynntarmssafter, har en sterk evne til å feste seg til tarmcellelinjer, og kan muligens styrke tarmbarrieren *in vitro* over en periode på 24 timer. *L. reuteri* utpekte seg således med noen interessante egenskaper sammenliknet med de andre MSB i testen. Basert på dette valgte vi ut noen MSB for videre studier. Da vi undersøkte hva som kan være årsaken til *L. reuteri*s gode bindeevne fant vi at proteinet som er kodet av *hmpref0536_10633* var veldig viktig for *L. reuteri* ATCC PTA 6475s bindeevne til Caco-2-celler og mucus *in vitro*. Dette nye LPxTG proteinet kalte vi «cell and mucus binding protein A» (CmbA). Det viste seg også at *L. reuteri* ATCC PTA 6475 og DSM 20016 var de to stammene som førte til den største cytokin sekresjonen fra THP-1-celler og den sterkeste aktiveringen av NF-κB i U937-3xkB-LUC-cellelinjen. Andre kjente probiotiske bakterier som *L. plantarum* 299v og *L. rhamnosus*s GG hadde liten effekt på THP-1- og U937-3xkB-LUC-celler. LPxTG proteinet Hmpref0536_10802 fra *L. reuteri* ATCC PTA 6475 viste seg å være av betydning for produksjon av IL-8 og TNF-α i THP-1 celler. I et Caco-2-modellsystem for tarmbarrieren fant vi ingen gunstig virkning av de utvalgte probiotiske bakterier og MSB sammenlignet med kontrollen. *In vitro* test systemer som benyttes i dette arbeidet brukes ofte i utvelgelse av probiotiske kandidater, men det er virkningen i menneske som er den ultimate testen for probiotisk effekt. Denne avhandlingen viser noen interessante eksempler på dette ettersom veldokumenterte kommersielle probiotiske stammer, som for eksempel *L. rhamnosus* GG og *L. plantarum* 299v, kom dårlig ut sammenlignet med andre MSB i våre tester. På den andre siden er bruk av *in vitro* metoder svært viktig for å øke vår forståelse av hvordan probiotiske bakterier og MSB kommuniserer med humane celler. Våre to funn om at CmbA er viktig for feste til tarmceller og mucus, og at Hmpref0536_10802 er viktig for immunstimulering av THP-1 celler, er to brikker i det store puslespillet som en dag vil hjelpe oss til å forstå samspillet mellom MSB og humane celler.

LIST OF PAPERS

List of papers included in the thesis

Paper I

Jensen H, Grimmer S, Naterstad K, Axelsson L. ***In vitro*** **testing of commercial and potential probiotic lactic acid bacteria.** *Int J Food Microbiol*. 2012, 153(1-2):216-22

Paper II

Jensen H, Roos S, Jonsson H, Rud I, Grimmer S, van Pijkeren JP, Britton RA, Axelsson L. **Role of *Lactobacillus reuteri* cell and mucus-binding protein A (CmbA) in adhesion to intestinal epithelial cells and mucus *in vitro*.** *Microbiology*. 2014, 60(Pt 4):671-81

Paper III

Jensen H, Drømtorp SM, Axelsson L, Grimmer S. **Immunomodulation of monocytes by probiotic and selected lactic acid bacteria.** *Probiotics & Antimicro. Prot.* 2014, DOI 10.1007/s12602-014-9174-2

Paper IV

Jensen H, Grimmer S, Axelsson L. **No effect of commercial probiotic and selected lactic acid bacteria in a model system of the intestinal epithelial barrier *in vitro*.** (Manuscript)

List of related papers by the author, not included in the thesis

Christoffersen TE, Jensen H, Kleiveland CR, Dørum G, Jacobsen M, Lea T. ***In vitro*** **comparison of commensal, probiotic and pathogenic strains of *Enterococcus faecalis*.** Br J Nutr. 2012. 14;108(11):2043-53

ABBREVIATIONS

CmbA	cell and mucus binding protein A
CPS	capsular polysaccharides
DC	dendritic cell
GALT	gut associated lymphoid tissue
GIT	gastrointestinal tract
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
Ig	immunoglobulin
IL	interleukin
LAB	lactic acid bacteria
LPS	lipopolysaccharide
LTA	lipoteichoic acid
M cell	microfold cell
MAMP	microbe-associated molecular pattern
MAPK	mitogen-activated protein kinases
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	natural killer cell
NLR	nucleotide-binding oligomerization domain receptors (NOD-like receptors)
PAMP	pathogen-associated molecular patterns
PP	Peyer's patches
PRR	pattern recognition receptors
SDP	sortase dependent protein
SrtA	sortase A
TER	transepithelial electrical resistance
TJ	tight junction
TLR	Toll-like receptors
WTA	wall teichoic acid
ZO-1	zonula occludens 1

INTRODUCTION

Lactic acid bacteria

Lactic acid bacteria (LAB) have traditionally been associated with food because of their preservative actions due to acidification, and/or enhancement of flavor, texture and nutrition [1]. Their natural habitats are diverse, varying from food, plants and sewage, to the oral, genital and gastrointestinal tract (GIT) of humans and animals [1]. Gene gain through horizontal gene transfer or gene duplication is likely to have contributed to the evolution of LAB and their adaption to various environmental niches [2]. LAB is a group of gram-positive bacteria united by morphological, metabolic and physiological characteristics. They have a low G+C (guanine plus cytosine) content [2], are nonsporulating, nonrespiring but aerotolerant cocci or rods, and produce lactic acid as one of the main fermentation products of carbohydrates [3]. LAB belong to the phylum *Firmicutes*, class *Bacilli* and order *Lactobacillales*. Families of LAB include *Aerococcaceae*, *Carnobacteriacea*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae*, and *Streptococcaceae* (http://www.uniprot.org/taxonomy/186826). LAB are generally considered beneficial microorganisms, with some strains even being health promoting (probiotic) bacteria [3].

The *Lactobacillus* genus

Lactobacillus is a large genus within the LAB with its 163 species (September, 2014. http://www.bacterio.net/lactobacillus.html#r). Closely related *Lactobacillus* species can have different morphology and metabolism. As a consequence the taxonomy of this species is difficult [4]. Using modern, molecular-based techniques, the fastest way to identify lactobacilli to species level is by sequencing of the 16S rRNA gene. For some species (e.g. for distinguishing between *L. plantarum* and *L. pentosus*) it may be necessary to complement this analysis with sequencing of one or more housekeeping genes, e.g. *recA* (recombinase A), *dnaK* (heat shock protein HSP70), *rpoA* (RNA polymerase alpha subunit) or *pheS* (phenylalanyl-tRNA synthase alpha subunit) [5, 6]. Among the lactobacilli there are both aero-tolerant and anaerobe species and strains. However, all are classically regarded as strictly fermentative. Lactobacilli can be divided into three groups based on their fermentation characteristics: (1) the obligately homofermentative lactobacilli which ferment hexoses almost exclusively to lactic acid by glycolysis (i.e. Embden–Meyerhof–Parnas pathway) while pentoses and gluconate are not fermented as they lack phosphoketolase; (2) the facultatively heterofermentative lactobacilli that degrade hexoses to lactic acid by

glycolysis and are also able to degrade pentoses and often gluconate as they possess both fructose-bisphosphate aldolase and phosphoketolase; and (3) the obligately heterofermentative which degrade hexoses and pentoses by the phosphogluconate pathway producing acetate, ethanol, CO_2 and lactate as end products [7, 8]. In general, the genus has complex nutritional requirements, including need for carbohydrates, amino acids, peptides, fatty acid esters, salts, derivatives of nucleic acids and vitamins. Stored food rich in carbohydrates provides an ideal substrate for lactobacilli, thus they were among the first microorganisms used for fermentation of food [9]. Many *Lactobacillus* species are highly specialized and only found in a limited number of niches. Examples are *L. delbrueckii*, specifically subsp. *bulgaricus*, which is highly adapted to the dairy environment and applied in the production of yoghurt, species such as *L. acidophilus*, *L. johnsonii* and *L. reuteri* are typical inhabitants of the GIT and frequently used in probiotic products, whereas *L. iners* is a predominant member of the vaginal microbiota [10]. Other lactobacilli such as *L. plantarum* and *L. rhamnosus* are more versatile and can be found in many different ecological niches such as vegetables, meat, fish, and dairy products as well as in the GIT [10].

Health-promoting effects of lactic acid bacteria

The first historical evidence for consumption of dairy products dates back to ancient Egypt as early as 7000 BC [11]. Many thousands of years later, in 1908, Élie Metchnikoff introduced the "probiotic concept". In his work "The Prolongation of Life: Optimistic Studies" he proposed that consumption of certain LAB could promote health benefits for the consumer [12]. During the 1950s and 1960s and the booming era of the antibiotics, the concept laid more or less dormant, except in Japan where some product development occurred. Renewed interest for probiotics was gained in the 1980s and Roy Fuller summed up research in the area by defining it as 'live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance' [13]. Since then there has been many definitions of probiotics. Today, probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" by the Food and Agriculture Organization/World Health Organization (FAO/WHO). Probiotic organisms used in food must be capable of surviving passage through the gut, they must be able to proliferate and colonize the digestive tract, be safe and effective, and maintain their effectiveness and potency for the duration of the shelf-life of the product [14]. Furthermore, probiotic bacteria should be isolated from the same species

as the host, have a demonstrable beneficial effect on the host, non-pathogenic, non-toxic and free of side effects [15]. Most probiotics commercially available today belong to the genera *Lactobacillus* and *Bifidobacterium*.

Characteristics of some *Lactobacillus* species relevant for this study

Lactobacillus plantarum

L. plantarum is highly versatile and found in a variety of nutrient-rich habitats such as dairy products, vegetables, meat, fish, the human oral cavity and GIT. Its ability to adapt to different environments is reflected by the large genome size and the redundant gene repertoire [1, 2]. *L. plantarum* is a facultatively heterofermentative LAB. It is often one of the dominant species in foods such as sauerkraut, pickles, olives, sourdough and kimchi. In many of these fermentations *L. plantarum* species dominates in the later stages of fermentation, presumably due to its high acid tolerance [10]. However, *L. plantarum* can also be involved in spoilage of foods, such as meat, wine and orange juice [16].

L. plantarum WCFS1, a single colony isolate of *L. plantarum* NCIMB 8826 isolated from human saliva, was the first *Lactobacillus* to be sequenced [17], and has the largest known genome of the lactobacilli (3.31 Mb).

Within the species, *L. plantarum* 299v (DSM 9843) [18] is marketed as a human probiotic and a number of clinical intervention studies have been published (as reviewed by [19, 20])

Lactobacillus sakei

L. sakei is a facultatively heterofermentative LAB primary associated with fermented meat and fish. Its evolutionary ability to use meat as a growth substrate has given *L. sakei* metabolic capacities that differentiate it from other LAB [1, 21]. *L. sakei* is an industrially important species widely used in production of fermented sausages [22]. Although its main habitat is meat, the species has been isolated from fermented vegetal products and fish [23]. Some *L. sakei* have been reported as part of the spoilage flora in vacuum packed meat and fish [24, 25]. The complete genome sequence of *L. sakei* strain 23K (1884 kb) was published in 2005 by Chaillou et al. [21].

L. sakei has been observed as a transient member of the human GIT [26-28]. Although not common worldwide, *L. sakei* probio 65 isolated from Kimchi is marketed as probiotic (http://probionic.koreasme.com/en/sub3.html), and have shown some interesting results in animal and clinical studies with regards to atopic dermatitis [29-31].

Lactobacillus reuteri

L. reuteri belongs to the obligately heterofermentative LAB and the main end-products during fermentation of glucose are lactic acid, ethanol/acetic acid and CO_2. *L. reuteri* was previously misclassified as *L. fermentum* until Kandler et al. [32] proposed *L. reuteri* as a new species in 1980. Strains of *L. reuteri* have been isolated from the GIT, mother's milk and vagina of humans and animals [33, 34]. However, the primary habitat of the species appears to be the GIT, and the species has been designated a universal entero-*Lactobacillus* [35] and a vertebrate symbiont of the GIT [34]. The ability of strains of *L. reuteri* to produce potent antibacterial compounds in addition to bacteriocins is unique among the LAB [36]. Strains of *L. reuteri* can produce the antimicrobial substances reuterin (3-hydroxypropionaldehyde) [37, 38], reutericin [39] and reutericyclin [40, 41].

L. reuteri DSM 17938, a derivative of a strain isolated from human breast milk [42], is marketed as a probiotic for humans. Several clinical studies indicate positive effect on infant colic, necrotizing enterocolitis in neonates, diarrhea and respiratory tract infections [43-46]. *L. reuteri* ATCC PTA 6475 is a candidate probiotic known for its anti-inflammatory properties [47-50]. Furthermore, this strain has shown promising effects in animal studies [51-53].

Lactobacillus rhamnosus

L. rhamnosus is a versatile species which can be found in many different ecological niches. It belongs to the facultatively heterofermentative LAB. *L. rhamnosus* GG is a human probiotic strain with thorough clinical documentation [54, 55], and probably the world's most researched probiotic strain. *L. rhamnosus* GG (ATCC 53103) was originally isolated from fecal samples of a healthy adult by Sherwood Gorbach and Barry Goldwin (thus the name GG), and selected as a probiotic strain based on acid and bile tolerance, adhesion to human small intestinal and buccal cells, and vigorous growth *in vitro* [56]. The strain is known to have a pili-mediated adhesion [57-60], lipotechoic acid (LTA) as a key immune effector molecule, and the secreted proteins p75 and p40 (later renamed Major Secreted Protein Msp1 and Msp2 [61]) as probiotic effector molecules [62]. The beneficial effects of *L. rhamnosus* GG have been studied extensively in clinical trials and human intervention studies and the reader is referred to [55, 62, 63] for reviews.

The human gastrointestinal tract: the place where bacteria interact with the host

The human digestive tract, also known as the alimentary canal, runs from the mouth to the anus via regions each specialized for a particular stage in the digestive process. The anatomically distinct regions include; the oral cavity, pharynx, esophagus, stomach, small intestine (duodenum, jejunum and ileum), and large intestine (ascending, transverse and descending colon, and rectum). The stomach and the intestines constitute the GIT. This is the place where bacteria come in direct contact with the host, and the following sections will describe important factors for this interaction.

The human microbiota

Within the digestive tract approximately 10^{14} microorganism peacefully coexist with the host, making it one of the most densely populated habitats known [64, 65], outnumbering the amount of somatic cells by a factor of ten [66]. The human microbiota is important for degrading and fermenting complex dietary and host derived macromolecules to make them accessible to the host, to synthesize essential amino acids, vitamins, and short chain fatty acids [11]. The GIT segments have different physicochemical conditions (e.g. low pH in the stomach, bile and gall salts in the duodenum, and digestive enzymes in the small intestine) which affect the community composition and microbial densities [11] (Fig. 1). Furthermore, the microbiota present in the intestinal lumen differs significantly from the microbiota attached to and embedded in the mucus layer [67].

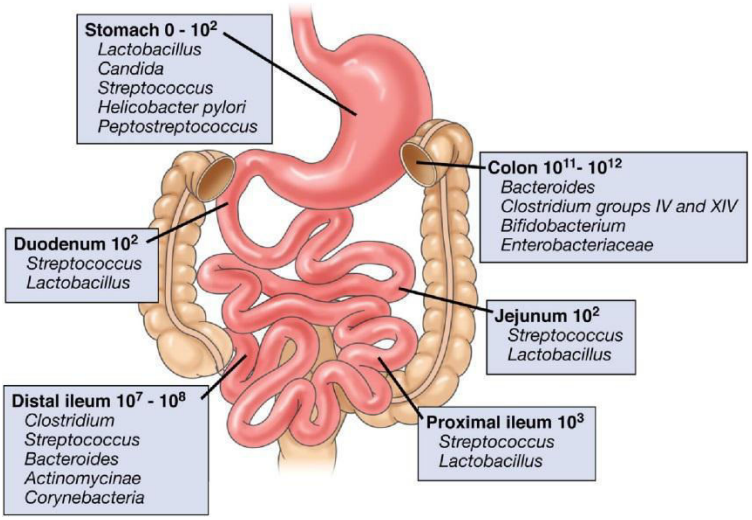

Figure 1. Microbial species, distribution and numbers in the gastrointestinal tract. Composition and luminal concentrations of dominant microbial species in various regions of the GIT. Reprinted with permission from Sartor [68].

The European MetaHit consortium study has revealed that the human microbiota profiles can be grouped in three major bacterial enterotypes dominated by *Bacteroides*, *Prevotella* and *Ruminococcus* [69]. The traditional view is that the GIT is sterile at birth and that colonization begins immediately after birth (vaginal and skin bacteria). However, recently the dogma of a sterile existence in utero has been challenged by new findings which indicate that microbial contact begins prior to birth. Traces of microbes such as microbial DNA and cell structures from intestinal bacteria have been detected in placenta, amniotic fluid and fetal membranes [70-73]. Furthermore, microbial DNA has also been found in the meconium of healthy term neonates [74, 75]. During the first year of life the microflora is relatively simple and varies widely between individuals. For a review on the gut microbiota from infant to elderly the reader is referred to O'Toole el al. [76].

The intestine

Intestinal epithelial cells

The luminal intestinal microbiota is separated from the host tissues by an epithelial monolayer forming a barrier between the intestinal lumen and the lamina propria. Furthermore, the epithelial layer is covered by a protective layer of mucus. The small intestine is organized with crypts and villi to increase the absorptive surface area, whereas in the colon there are no villi and the surface is flat. Pluripotent stem cells resides in the crypts, and new cells migrate upwards and differentiate into one of the three different cell lineages; (1) enterocytes which absorb nutrients (small intestine) and water (colon); (2) enteroendocrine cells which secrete enteric hormones; and (3) goblet cells which produce mucus [77]. When intestinal cells reach the tip of the villus they undergo spontaneous apoptosis and are shed of into the gut lumen [78]. The entire epithelium of the intestine is renewed approximately every fifth day [77].

The mucus layer

Goblet cells of the intestine produce mucins that that forms the mucus layer. The mouse colonic mucus consists of two layers extending 150 μm above the epithelial surface. This layer is divided into an outer loosely packed layer and a firmly packed inner layer, and the presence of bacteria is restricted to the outer layer, whereas the inner layer is essential free of bacteria [79]. In humans, the mucus layer is also divided in the two layers as described above, and the thickness varies from 50 to 800 μm [80]. It is not known whether the mucus in the small intestine also consists of two layers and if it entirely covers the large surface area of the villi in the small intestine. Furthermore, there are large variations in the density

and composition of bacteria in the colon and small intestine, suggesting that microbe–epithelial interactions will be different in each location [77]. The main structural components of mucus layer are mucins which are large, heavily and diversely O-linked glycoproteins. In the small intestine and colon, the mucus layer mainly consists of the secreted MUC2, whereas MUC1, MUC5AC and MUC6 are the main mucins in the stomach. The secreted mucins form extended net-like structures forming the intestinal mucus layers. For a review the reader is referred to [81, 82].

The gut associated lymphoid tissue

The gut associated lymphoid tissue (GALT) is the largest immune organ in our body and the major site of defense against potentially infectious agents and foreign antigens. Peyer's patches (PP) are organized lymphoid nodules of the ileum. PP are covered by an epithelium that contains specialized microfold cells (M cells) which sample antigen directly from the lumen and deliver it to antigen-presenting cells resulting in immunity and secretion of IgA. Humans secrete several grams of IgA into the intestinal lumen each day, and this exerts considerable immune pressure on the luminal microbiota [83]. Dendritic cells (DC) play a key role in the induction of tolerance and immunity. They are specialized antigen presenting cells that can extend dendrites through the epithelial tight junctions (TJs) and sample antigens from the intestinal lumen. DC mainly resides in the PP, but can also sample luminal antigen in the lamina propria. Furthermore, in the lamina propria also macrophages and B cells are specialized antigen presenting cells that patrol mucosal tissues and receive antigens from the periphery [11]. Antigen presenting cells that capture antigens and later display them on their surface can promote the development of naive T cells into defense directed T helper cells or tolerance associated regulatory T cells (Treg). This activation is dependent on many factors including the class of antigen presenting cell, as well as the type and strength of the stimuli [11].

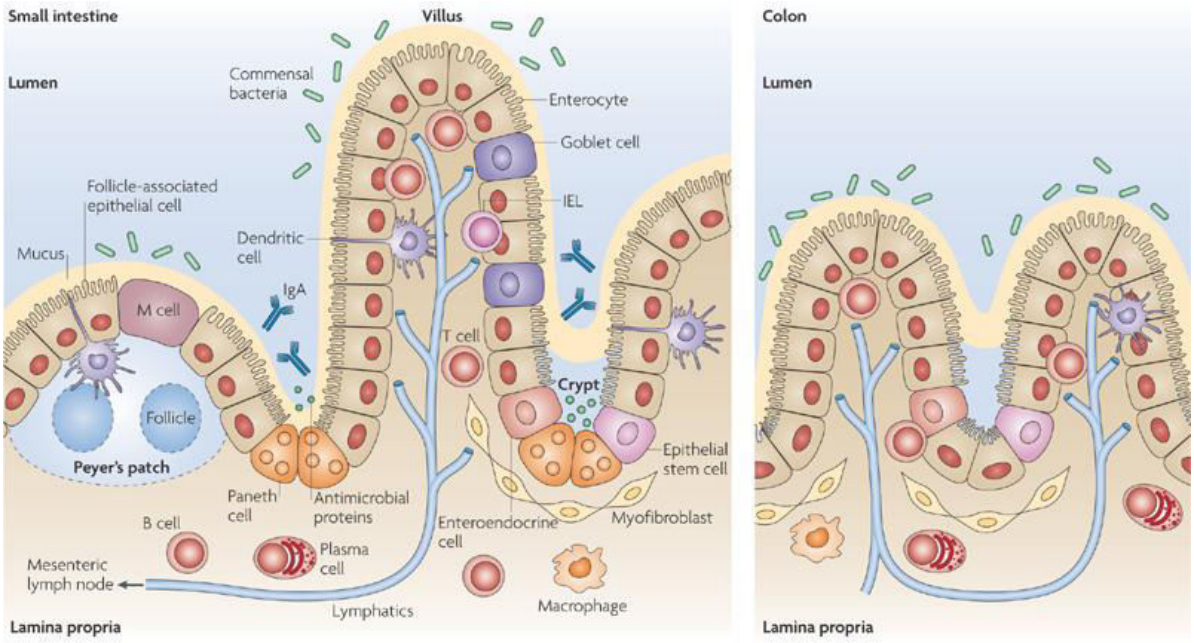

Figure 2. Cells of the intestine, the mucus layer and the gut associated lymphoid tissue. A single layer of intestinal epithelial cells and the mucus layer provide a physical barrier that separates the bacteria in the intestinal lumen from the underlying lamina propria. Cells of the epithelial layer are enterocytes, mucus producing goblet cell, hormone producing enteroendocrine cells, paneth cells and stem cells. Beneath the intestinal epithelial barrier, the lamina propria is made up of stromal cells (myofibroblasts), B cells, T cells, macrophages and DC. The latter have projections that sample content of the intestinal lumen. The small intestine has regions of specialized epithelium termed follicle-associated epithelium and microfold (M) cells that overlie the Peyer's patches and sample the intestinal lumen. Reprinted with permission from Abreu [84]

Host recognition of bacteria

An important fraction of probiotic effector molecules resides in the bacterial cell envelope (Fig. 3). This part of the bacterium is the first to interact with intestinal host cells. Examples of lactobacilli cell surface molecules that have the potential to be recognized by human cells are peptidoglycan, lipoteichoic acid (LTA), wall teichoic acid (WTA), capsular polysaccharides (CPS) and extracellular (glyco)proteins. Many of these have been proven important as effector molecules for probiotic effects [85-87]. Furthermore, probiotic metabolites and genomic DNA can also be recognized by the host [88, 89]. All of these structures mentioned above are conserved microbial molecular structures commonly referred to as microbe-associated molecular patterns (MAMPs).

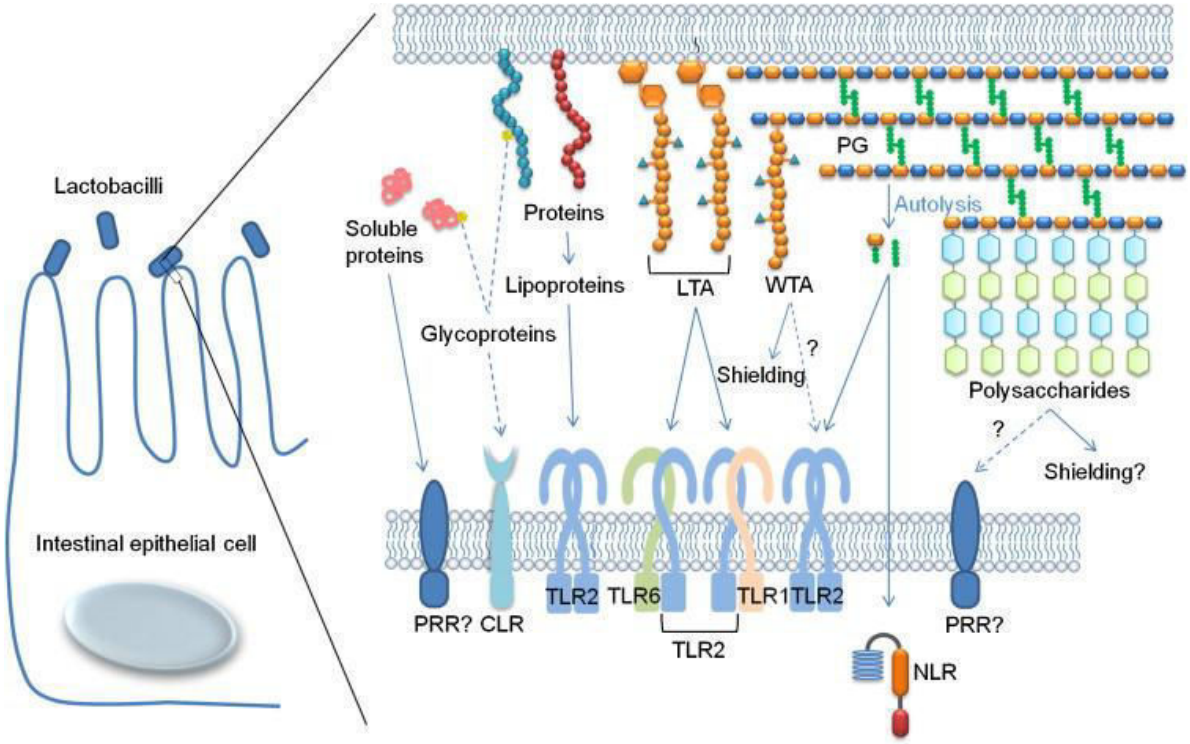

Figure 3. The lactobacilli cell envelope and interaction with receptors on a human intestinal epithelial cell. Peptidoglycan, polysaccharides, LTA , WTA, surface proteins, glycoproteins, lipoproteins and secreted proteins from the lactobacilli have the potential to be recognized by Toll like receptors (TLRs), NOD-like receptors (NLRs), and C-type Lectin Receptors on intestinal epithelial cells of the host. Reprinted with permission from Lee et al. [86]

LAB and probiotic interactions with the human host are based on the ability of human cells to recognize specific bacterial components or products. Lactobacilli or molecules of lactobacilli can induce responses in the host by binding to pattern recognition receptors (PRR) expressed on immune cells intestinal epithelial cells (IECs) among others. PRR recognize and bind MAMPs which leads to activation of intracellular pathways such as mitogen-activated protein kinases (MAPK) and NF-κB [90] (Fig 4). PRR can be divided in several families; e.g. the Toll-like receptors (TLRs), the retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), the nucleotide oligomerization domain-like (NOD) receptors (NLRs), and the C-type Lectin Receptors (CLRs), of which the TLR family is the best characterized. Each PRR recognizes a specific molecular pattern and can be expressed on the cell surface, in intracellular compartments or in the cytosol. For a review the reader is referred to [83, 91, 92].

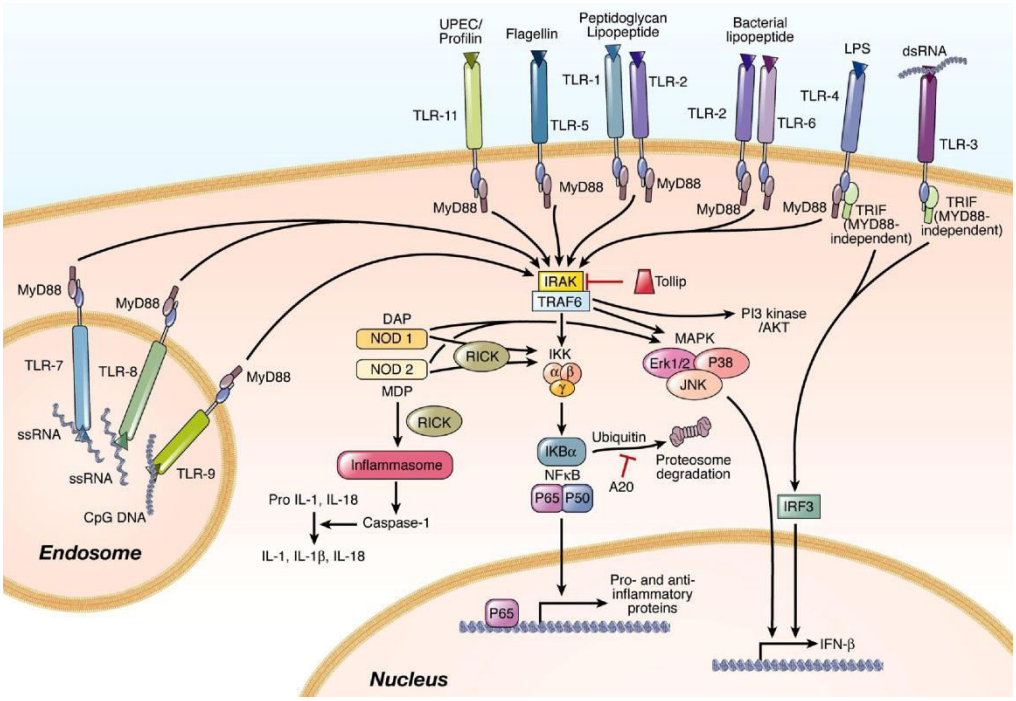

Figure 4: Microbe associated molecular patterns, pattern recognition receptors) and activation of cellular pathways. Recognition of bacterial ligands by membrane-bound TLR and NLR, signaling through conserved pathways such as NF-κB and MAPK signal transduction pathways. Reprinted with permission from [68].

Probiotics mechanisms of action

Mechanisms by which probiotics mediate their health benefits on the host can be divided into three categories; (1) certain probiotics have antimicrobial activity and can exclude or inhibit pathogens; (2) probiotic bacteria can enhance the intestinal epithelial barrier; and (3) probiotic bacteria can modulate the host immune response [11, 93, 94] (Fig. 5). Furthermore, there is evidence that the gut microbiota and probiotics can communicate intra- and interspecies with chemical signals called autoinducers, and with the human host by hormones and hormone-like chemicals [95-97]. This form of communication is probably of great importance for probiotic properties, but details remains to be elucidated.

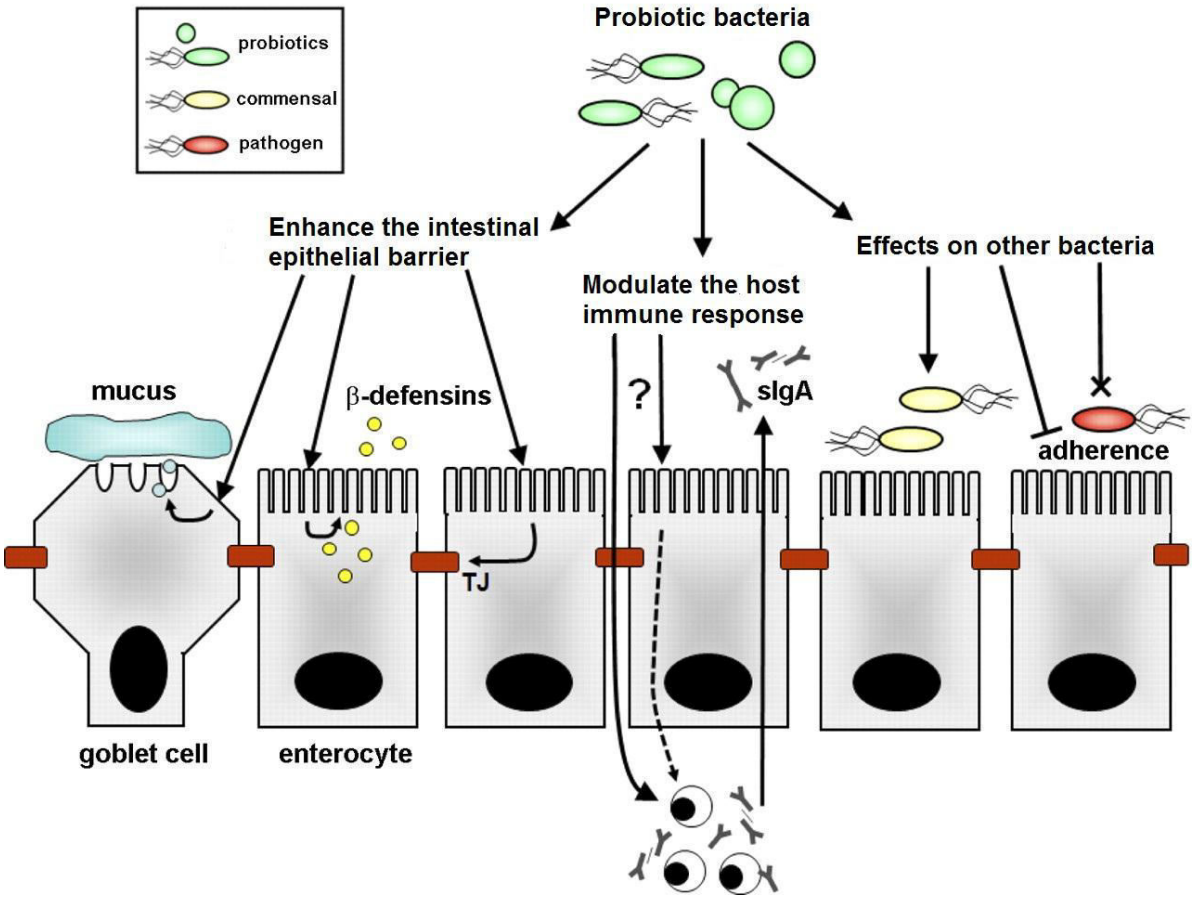

Figure 5. Mechanisms of action of probiotic bacteria. Proposed mechanisms of action of probiotic bacteria can be divided in three categories: (1) Probiotic bacteria can have an effect on other bacteria and thereby exclude or inhibit pathogens (decrease luminal pH, antimicrobial peptides, inhibit bacterial invasion, block bacterial adhesion to epithelial cells); (2) probiotic bacteria can enhance the intestinal epithelial barrier (increase mucus production, defensin production, enhancement of TJ proteins and prevention of apoptosis); and (3) probiotic bacteria can modulate the host immune response (effects on epithelial cells, DC, monocytes/macrophages, and lymphocytes). (Modified from [80]).

Despite intense research the overall evidence for effects of probiotics is limited. Accurate comparison of the results from clinical studies is difficult due to the diversity in study design and the probiotic strains used. At this stage, it is impossible to draw general conclusions on the health impact of probiotics, and large meta-analyses conclude that further studies are needed to draw strong conclusions. As a consequence the European Food Safety Agency (EFSA) has deemed the health claims of probiotics as insufficient [98]. Despite this, the global market of probiotic ingredients, supplements, and foods reached nearly $23.1 billion in 2012 and is expected to grow to nearly $36.7 billion in 2018

according to BCC research (http://www.bccresearch.com/market-research/food-and-beverage/probiotics-market-fod035d.html?tab=toc).

Even though EFSA has deemed all probiotic health claims as insufficient at present, there are a vast number of animal and clinical studies that report beneficial effects. Most probiotic effects that have been demonstrated appear to be highly strain-specific [85]. Furthermore, the microbiota of the host will impact the fate of the probiotic administered, and thus probiotic effects can even be "host-specific". It is difficult to measure health benefits in already healthy individuals. As a result, most probiotic effects described today are in compromised or diseased populations. Systematic review and meta-analysis suggests that probiotics are both safe and effective for preventing *Clostridium difficile*-associated diarrhea [99], antibiotic-associated diarrhea [100], and may be effective in treating persistent diarrhea in children [101]. Furthermore, used alongside rehydration therapy, probiotics appear to be safe and have clear beneficial effects in shortening the duration and reducing stool frequency in acute infectious diarrhea [102]. Another systematic review indicates that probiotics may be more beneficial than placebo for preventing acute upper respiratory tract infections and reducing antibiotic use [103]. For preterm infants, enteral probiotics supplementation significantly reduced the incidence of severe necrotizing enterocolitis and mortality [104]. Regarding IBD, there is insufficient evidence to make conclusions about the efficacy of probiotics for maintenance of remission in ulcerative colitis [105] and no evidence to suggest that probiotics are beneficial for the maintenance of remission in Crohn's disease [106]. Finally, probiotics are not an effective treatment for eczema, and probiotic treatment carries a small risk of adverse events [107]. One important thing to keep in mind is that this area of research is relatively new, as the first probiotic intervention trial dates back to 1997 [108]. In the coming years, many of these mechanisms will be further clarified.

Bacterial adhesion to intestinal epithelial cells and mucus

Bacterial adherence to IECs and/or mucus is frequently considered to be a desirable feature for a probiotic strain as it can promote the gut residence time, pathogen exclusion, and interaction with host epithelial and immune cells [93]. Probiotic bacteria are generally not long term inhabitants of the GIT [109], instead they divide very slowly in the intestine while remaining metabolically active [110]. The process of adhesion appears to be multifactorial as adhesion can not be attributed to one component [111], and includes passive forces, electrostatic interactions, hydrophobic, steric forces, and specific bacterial structures such as

external appendages [112]. Despite new sophisticated methodologies, bacterial adhesion capacity is most commonly studied *in vitro* with epithelial cell lines or immobilized intestinal mucus or extracellular matrix molecules as model systems.

Adhesins of lactobacilli

Adhesins of lactobacilli can be classified according to their targets in the intestinal mucosa (e.g. mucus components, extracellular matrix proteins), according to their localization on the bacterial surface (e.g. surface layer proteins), or according to how they are anchored to the bacterial surface (e.g. sortase-dependent proteins (SDPs)) [113].

Adlerberth et al. [114] were one of the first to show carbohydrate specificity for the binding of *L. plantarum* strains when demonstrating a mannose-specific adherence mechanism to a human intestinal cell line. Roos and Jonsson ([115]) described an extracellular mucus binding protein (Mub) for *L. reuteri* 1063 responsible for the adhesion to intestinal mucus. Later, Pretzer et al. [116] identified the mannose-specific adhesin (*msa* gene) of *L. plantarum* WCFS1 that contains a domain similar to the Mub domain identified by Roos and Jonsson [115]. Msa is responsible for the mannose-specific adherence mechanism previously mentioned (see above). Mannose is a constituent of the mucin glycosylation moieties. Many other LAB have Mub domains with sequences similar to the Mub domains in *L. reuteri* and *L. plantarum*, indicating potential mucus binding proteins [117]. Furthermore, Mub-domain containing proteins are the most abundant in lactobacilli of the GIT, thus supporting the hypothesis that the domain is involved in bacterial adherence to the intestinal mucus [117]. In addition to specific bacterial adhesins, other cell surface molecules, such as S-layer proteins, LTA and exopolysaccharides [93], and extracellular appendages, such as flagella, fimbriae and pili [81], can also contribute to adhesion to host epithelial cells and mucus. The reader is referred to Sánchez et al. [118], Vélez et al. [113] or Juge [81] for review on adherence factors.

Sortase dependent proteins (SDPs)

SDPs are a group of surface-associated proteins in gram-positive bacteria. Many of them have been shown to impact the adhesive ability of several lactobacilli. SDPs have a common molecular structure that includes an N-terminal signal peptide, often with an YSIRK-G/S motif that promotes secretion [119] and directs the protein to a specific surface localization [120], a C-terminal LPxTG motif, followed by a C-terminal transmembrane helix and a positively charged tail [93, 121]. Sortase A (SrtA) cleaves the LPxTG motif between the threonine and glycine residues, and covalently links the threonin carboxyl group to amino

groups provided by the cell wall cross-bridges of peptidoglycan precursors [122]. Thus, a SDP is linked to the cell wall and displayed on the bacterial surface. Examples of SDPs adhesins of lactobacilli are Msa in *L. plantarum* WCFS1 [116], Mub in *L. reuteri* 1063 [115] and *Lactobacillus* epithelium adhesin of *L. crispatus* ST1 [123].

The intestinal epithelial barrier

The intestinal epithelial barrier consists of a monolayer of epithelial cells, epithelial cell-cell connections that seals adjacent epithelial cells together, a mucus layer, antimicrobial peptides, and secretory IgA [80]. This barrier needs to be tight enough to protect the lamina propria from the microbial content of the intestinal lumen while at the same time being permeable enough for uptake of nutrients and water. Uptake of small molecules such as short-chain fatty acids, amino acids, electrolytes and sugars are transported-mediated through cells (transcellular transport), while medium sized (< 600 Da in vivo and 10kDa in cell lines) hydrophilic compounds are transferred between cells (the paracellular route). Protein-sized molecules are normally restricted from the paracellular route [124].

Enterocyte cell-cell connections are essential for the intestine barrier function and the intercellular junctional complexes consist of TJs, adherence junctions, gap junctions and desmosomes [80], of which TJs are the best characterized (see González-Mariscal [125] for review). The TJ transmembrane proteins occludins and claudins link enterocytes together trough their extracellular loops, and intracellular zonula occludens (ZO) scaffolding proteins link the transmembrane proteins to the actomyosin cytoskeleton and several cytoplasmic regulatory proteins [80, 126]. TJs are not as tight as the name implies, they are dynamic and functionally responsive to a variety of stimuli, thus regulating the permeability of the cell layer. The permeability of the epithelial barrier is regulated by phosphorylation of TJ proteins and crosstalk with cellular signalling pathways (see González-Mariscal [127] for review). Claudins seems to be the major determinants of intestinal permeability [80, 126]. Claudin-1, -3, -5, -8, -11, -14 and -19 are typical barrier builders which tighten the epithelial barrier, whereas claudin-2 and -10 are mediators of paracellular permeability [128]. The distribution of claudins along the intestine reflects the barrier properties. The colon has the highest distribution of “tightening” claudins (-1, -3, -4, -5 and -8), followed by the duodenum which also has a strong expression of claudin-2. The jejenum and the ileum have a lower expression of tightening claudins and a higher expression of paracellular permeability mediators (Claudin-2) [128]. The effect of occludins seems to be secondary as occludin deficient mice have close to normal intestinal barrier function [126].

Effector molecules for strengthening the intestinal epithelial barrier

Impairment of the intestinal epithelial barrier is associated with the pathogenesis of various gastrointestinal diseases, such as inflammatory bowel disease (IBD), celiac disease, gastrointestinal infections, diarrhea, and critical illness [129]. Recent studies show promising results of probiotic therapy by improving the epithelial barrier [129]. Possible ways of strengthening the intestinal epithelial barrier include mechanisms such as induction of mucin secretion, enhancement of TJ function, upregulation of cytoproptective heat shock proteins, and prevention of apoptosis of epithelial cells [93]. Intestinal permeability can be modulated directly by bacteria by release of metabolites (e.g. acetate and butyrate), cellular structural component or through the secretion of soluble peptides and toxins. Bacteria can also alter the intestinal permeability indirectly by interactions with the host immune cells and subsequent release of cytokines which can both enhance or reduce the barrier function [130]. The bacterial components responsible for strengthening the epithelial barrier include cell surface factors, secreted proteins, soluble proteins, and bacterial DNA [93]. Most studies preformed focus on probiotics ability to protect against intestinal barrier dysfunctions caused by invasive pathogens, pro-inflammatory cytokines or other barrier disruptors. The mechanisms behind these protective effects have not been conclusively demonstrated, but may involve TLR2 signalling which transiently increase TER and elevate ZO-1 and ZO-2 *in vitro* [83]. Only a few probiotic effector molecules which strengthen the epithelial barrier have been described. Examples are the secreted proteins p40 and p75 from *L. rhamnosus* GG [131, 132] and a large but unidentified proteinaceous soluble factors (>50 kDa) from the probiotic mixture VSL#3 [133].

Immune modulation

Immune modulation is one of the key mechanisms of probiotic bacteria. Together the IECs, DCs and macrophages continuously sense the environment and coordinate different defenses for the protection of mucosal tissues. A detailed description of immune responses is beyond the scope of this work. Briefly, innate defenses (the evolutionary older part of the immune system, includes PRR, MAMPs and PAMPs) include the production of antimicrobial compounds (defensins and nitric oxide, etc.) and the secretion of chemokines such as IL-8 that recruit neutrophils (i.e. phagocytes) that are capable of ingesting microorganisms or particles. On the other hand, adaptive immune responses against commensal, probiotic, and pathogenic bacteria are mediated in the GALT and create highly specific, very powerful responses to particular antigens.

Possible beneficial effects of probiotic bacteria on the host immune system include preventing upper respiratory tract infections, preventing atopic eczema, allergic rhinoconjunctivitis, and asthma, as well as treating acute IBD and maintenance therapy of IBD.

Allergy is a hypersensitivity reaction mediated by specific antibody-mediated or cell-mediated immunologic mechanisms and clinically manifested as atopic eczema, allergic rhinoconjunctivitis, or asthma. Most clinical studies on allergy have been performed with *L. rhamnosus* GG [90]. At present, no general recommendations for the use of probiotics in clinical practice with allergy can be given [134]. There are a few clinical trials with outstanding findings but also some studies reporting negative results [107, 134].

It is commonly accepted that environmental, genetic, immunological, and microbial factors all can contribute to the development of IBD. The etiology of IBD remains unclear, but it is believed to be the result of complex aberrant immune responses to undetermined environmental factors (most likely intestinal microorganisms) in the GIT of genetically susceptible hosts [135]. For treatment of active ulcerative colitis and maintenance therapy, the clinical evidence of efficacy is strongest for VSL#3 and *Escherichia coli* Nissle 1917 [135]. However, taken together there is insufficient evidence to make conclusions about the efficacy of probiotics for maintenance of remission in ulcerative colitis [105]. The results from clinical trials in the treatment of active Crohn's disease and the maintenance of its remission are disappointing, and at present there is no evidence to suggest that probiotics are beneficial in Crohn's disease [106, 135].

The results from clinical studies of probiotics for preventing acute upper respiratory tract infections are more positive. A large systematic review indicates that probiotics may be more beneficial than placebo for preventing acute upper respiratory tract infections and reducing antibiotic use [103].

The following sections will give an introduction to cytokines and pathways important for probiotic stimulation of the host immune system.

The NF-κB pathway

The NF-κB pathway is one key signaling channel for activation of immune responses secondary to a variety of stimuli. This pathway represents an important point of communication between probiotics and beneficial microbes and cells of the host [136]. NF-κB is a nuclear transcription factor. In its inactive state NF-κB is located in cytosol as a protein complex. Upon receptor signaling (TLR and NLR) NF-κB is liberated and

translocate to the nucleus where it acts as a transcription factor of specific genes [90]. NF-κB is known for its dualistic function, and is important for intestinal immune homeostasis [137]. In the intestinal epithelia, intrinsic NF-κB signaling prevents apoptosis of IECs avoiding breaches of the epithelial barrier, whereas excessive NF-κB activation of IECs promotes detrimental intestinal inflammation [138]. Traditionally, most studies related to probiotic or beneficial bacteria investigate the ability to prevent NF-κB activation and influence the downstream cytokine secretion. However, not all probiotic bacteria inhibit NF-κB activation. Some stimulate NF-κB and cause increased cytokine secretion [136].

Cytokines

Cytokines are small, soluble secreted peptides or proteins that affect the growth or function of cells. Cytokines include interleukins (IL), interferons, chemokines, colony-stimulating factor and many growth factors [139]. Cytokines do not have an effect on their own, but act by binding to specific surface receptors in the membrane of cells. Most commonly, cytokines are autocrine (act on the same cell that produce them) or paracrine (act on cells near by), however cytokines may also have systemic effects (e.g. TNF-α, IL-1β and IL-6) [140]. Basal production of cytokines is usually low or absent, their production is regulated by various inducing stimuli [140]. Cytokines are most often secreted by immune cells and act on immune cells, thus orchestrating most aspects of the immune system. However, cytokines can also be secreted by somatic cells and somatic cells can respond to cytokines.

Interleukin-8 (CXCL-8)

IL-8 (CXCL-8) is a chemokine which primarily attracts phagocytes such as neutrophiles and macrophages. IL-8 is secreted by leucocytic cells such as monocytes, macrophages, neutrophils, T-cells and natural killer (NK) cells, and somatic cells like endothelial cells, fibroblasts and epithelial cells [141]. Secretion of IL-8 is activated by pro-inflammatory cytokines (e.g. IL-1, TNF-α), bacterial (e.g. LPS) or viral products via the transcriptional factors NF-κB and AP-1 [141]. Recruitment of neutrophils and monocytes to the site of infection is the main effect of IL-8. However, IL-8 also plays an important role in activation of neutrophils by increasing degranulation, oxidative burst and intracellular calcium concentrations as well as enhancing the killing of intracellular pathogens [141]. In the gut, IL-8 is known to play an important role in mucosal healing [142]. IL-8 also enhances the migration of IEC *in vitro* [143].

TNF-α

TNF-α is a pro-inflammatory cytokine with a variety of biological effects. Local production of TNF-α plays an important role in containment and elimination of local infections. Due to its serious systemic effects, the expression of TNF-α is tightly regulated on the transcriptional, translational and post-translational level [144]. Monocytes, macrophages, T cells, B cells, NK cells and some non-immune cells can all release TNF-α in response to stimuli [144]. The effect of TNF-α on macrophages include increased production of cytokines, enhanced phagocytosis and anti-microbial response [145]. Endothelial cells upregulate leucocyte adhesion molecules in response to TNF-α and thereby contribute to leucocyte recruitment [145]. TNF-α is also involved in cell proliferation, differentiation and apoptosis. Furthermore, TNF-α is also an important cytokine in chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease [145].

Interleukin-12

IL-12 is a pro-inflammatory cytokine. Earlier, it was simultaneously described as CLT maturation factor (CLMF), a T cell stimulating factor (TSF) and a NK cell stimulatory factor (NKSF) [146]. Thus, the main action of IL-12 is to activate NK cells and to induce the differentiation from naïve cells to T_{H1} cells. IL-12 is mainly produced by antigen presenting cells (DC, macrophages). Production of IL-12 is strictly regulated which is consistent with the crucial role of IL-12 in regulating nearly every aspect of the immune response [146]. The production of IL-12 is negatively regulated by a set of powerful inhibitors (e.g. IL-10) [146].

Interleukin-10

IL-10 is an anti-inflammatory cytokine which is a key regulator of immune responses. It was originally described as cytokine synthesis inhibitory factor (CSIF) because of its ability to turn off cytokine production in T cells [147]. IL-10 is produced mainly by leukocytes including T cells, B cells, monocytes, macrophages and DC, as well as some epithelial cells [148]. For antigen presenting cells such as DC and macrophages, the production of IL-10 is induced by recognition of MAMPs or pathogen-associated molecular patterns (PAMPs) by cell surface of cytoplasmic PRR. Secretion of IL-10 can result in numerous effects. In sum, the main effect of IL-10 is to suppress multiple immune responses through individual actions on T cells, B cells, antigen presenting cells, and other cell types, and to skew the immune response from T_{H1} to T_{H2} [149]. IL-10 is produced at high levels relatively late in the immune response compared to some of the pro-inflammatory cytokines [150]. In

humans, IL-10 and its receptor play a critical role in controlling immune responses in the intestinal mucosa [148].

The ability to induce secretion of IL-10 is frequently considered as an important characteristic of probiotic bacteria with beneficial effects on the immune system. The ratio of IL-10 and IL-12 is often used *in vitro* to select potential probiotic strains [151-153]. Bacteria with a high IL-10/IL-12 ratio is regarded as anti-inflammatory bacteria.

Probiotic effector molecules for immunomodulation

Most studies with regard to immunomodulatory effects of LAB and probiotic bacteria do not describe the bacterial molecule(s) responsible for the observed effects. However, some lactobacilli surface proteins that stimulate host signalling pathways and the immune system have been identified. Examples are p75 (Msp1) and p40 (Msp2) of *L. rhamnosus* [132], p75 and p40 homolog proteins of *L. casei* [132, 154], STp of *L. plantarum* BMCM12 [155], Surface layer protein A (SlpA) of *L. acidophilus* NCFM [156], Msa of *L. plantarum* WCFS1 [157], and GroEL (Hsp60 class) of *L. johnsonii* La1 (NCC533) [158].

Survival through the GIT

According to the FAO/WHO definition, probiotic bacteria should be viable at the time of ingestion, capable of surviving passage through the digestive tract, and have the ability to proliferate in the gut [14]. The human stomach secrets approximately 2.5 litres of gastric juice every day, giving a gastric pH of 1.5 during fasting and 3 to 5 during food intake [159]. It is essential for the bacteria to have protection systems to withstand the low pH in the human stomach [159]. Native resistance to gastric acid is a rare probiotic property [159]. It is accepted that a combination of strategies which results in removal of protons (H+), alkalization of the external environment, changes in composition of the cell envelope, production of general shock proteins and chaperones, expression of transcriptional regulators, and responses to change in cell density contribute to survival [159]. In the small intestine, exposure to bile is another serious challenge for bacterial cells. Approximately 1 litre of bile is secreted into the small intestine every day [160]. The principal function of bile is to aid in fat absorption during digestion. Another important function is to prevent bacteria in the small intestinal from overgrowing and becoming a health issue for the host [161]. Bile acids are surfactants which can disrupt the plasma membrane of bacterial cells. The primary effect of bile on bacterial cells is on the cell membrane, where bile affects phospholipids and proteins, disrupting cellular homeostasis. Furthermore, bile can disturb macromolecule stability on bacterial cells, induce secondary structure formation in RNA, induce DNA

damage, activate enzymes involved in DNA repair, induce conformational change in proteins resulting in misfolding or denaturation, and cause oxidative stress through generation of oxygen free radicals [160]. Tolerance to gastric acid and bile have become important selection criteria for new probiotic strains [14], and assays for resistance to gastric acidity and bile acids are among the main currently used *in vitro* test for the study of probiotic strains [162].

Live vs dead probiotic bacteria

Based on numerous studies it is clear that both live and dead probiotic bacteria can generate a wide range of biological responses [88]. However, according to the FAO/WHO definition, probiotic bacteria are live bacteria [14]. Live cells in a probiotic product will indeed lose viability and the actual product will contain varying populations of dead cells. The population of dead cells might be even larger than that of live cells but this is frequently not known. Thus, there is always a possibility that an unknown amount of dead cells are being administered with the live cells [88]. Furthermore, many of the live ingested probiotic bacteria will not survive the harsh conditions of the stomach and the intestine [159, 160]. Therefore, some of the benefits derived from consumption of probiotics are likely to derive from the presence of metabolites and/or dead probiotic cells in the GIT. Recently, The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic was published [163], and the experts conclude that "the development of metabolic by-products, dead microorganisms, or other microbial based, nonviable products has potential; however, these do not fall under the probiotic construct". They also concluded to retain the FAO/WHO definition for probiotics, with a minor grammatical correction as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [163]. The definition is clear, a probiotic bacterium is a live bacterium. However, bacterial metabolic by products, dead bacteria and other nonviable products can indeed have beneficial effects; they should simply not be classified as probiotics. The reader is referred to Gobbetti et al. [164], Taverniti et al. [89] or Adams [88] for reviews on the live/dead discussion

OBJECTIVES OF THIS STUDY

The main aim of the study was to increase the current understanding of how probiotic bacteria and LAB interact with cells of human origin. The study comprises work on strain characteristics and possible mechanisms of action. Use of *in vitro* methods to study strain characteristics of LAB in cell lines of human origin were a major part of the project. Furthermore, the use of knock-out mutants was central in investigating the mechanisms of action in the interaction between LAB and human cells. Specifically the aims were to:

- Investigate the diversity of 18 selected commercial and potential probiotic LAB using common *in vitro* screening assays such as transit tolerance in the upper human gastrointestinal tract, adhesion capacity to human intestinal cell lines and effect on epithelial barrier function.

- Elucidate the role of sortase (SrtA), four putative sortase-dependent proteins (SDPs), and one C-terminal membrane anchored cell surface protein of *L. reuteri* ATCC PTA 6475 in adhesion to Caco-2 cells and mucus *in vitro*.

- Elucidate immune stimulating properties of commercially available and potential probiotic LAB and putative surface proteins of *L. reuteri* ATCC PTA 6475 *in vitro* using cytokine secretion from the monocytic cell line THP-1 and NF-κB activation in the monocytic cell line U937-3xkB-LUC.

- Study the role of commercial and potential probiotic LAB on the intestinal epithelial barrier *in vitro* using a model system of Caco-2 cells and measuring transepithelial electrical resistance (TER), paracellular transport of FITC-Dextran, and qRT-PCT of the tight junction genes *zo-1* and *claudin-1*.

MAIN RESULTS AND DISCUSSION

LAB have traditionally been associated with food because of their preservative actions and/or enhancement of flavor, texture and nutrition [1]. However, their natural habitats are diverse, varying from food, sewage and plants, to the oral, genital and GIT of humans and animals [1]. Some LAB, especially bacteria belonging to the genus *Lactobacillus* and *Bifidobacterium*, have received considerable attention in the last decades due to their postulated health-promoting effects (probiotic bacteria). Probiotics have antimicrobial activity and can exclude or inhibit pathogens, enhance the intestinal epithelial barrier and modulate the host immune response [11, 93, 94]. Despite intense research in recent years much is unknown about the mediators and mechanisms responsible for their beneficial effect. The main aim of this study was to increase the current understanding of how probiotic bacteria and LAB interact with cells of human origin. Strain characteristics of 18 LAB and performance in typical probiotic *in vitro* screening assays such as transit tolerance in the upper human GIT, adhesion capacity to human intestinal cell lines and effect on epithelial barrier function is described in paper I. The results in this paper gave us a base for selecting interesting strains for further studies of adhesion to intestinal cells, immune stimulatory properties and effect on the intestinal epithelial barrier *in vitro*. Immune stimulatory properties of seven LAB was evaluated in paper III using cytokine secretion from the monocytic cell line THP-1 and NF-κB activation in the monocytic cell line U937-3xkB-LUC. Paper IV describes the effect of six LAB on the epithelial barrier *in vitro* using polarized monolayers of Caco-2 cells. Transepithelial electrical resistance (TER), paracellular permeability of fluorescein isothiocyanate–dextran (FITC-Dextran), and quantitative real-time reverse transcription PCR (qRT-PCR) of the TJ genes *zo-1* and *claudin-1* was evaluated. Paper II and III use mutagenesis of the genes encoding SrtA, four putative SDPs, and one C-terminal membrane anchored cell surface protein of *L. reuteri* ATCC PTA 6475 to investigate the mechanism of action for *L. reuteri* adhesion to Caco-2 cells and immune stimulation of THP-1 and U937-3xkB-LUC cells. Two of the genes investigated proved to be of importance for adhesion and immune stimulation of cells of human origin.

The objective of paper I was to study the diversity of selected commercial and potential probiotic bacteria in various *in vitro* models. The 18 selected bacteria include commercially available probiotics, starter cultures, and potential probiotic LAB isolated from humans or food. Both species and strain specific effects were observed, demonstrating a vast diversity among the strains investigated. Viable counts after simulated gastric transit

tolerance showed that *L. reuteri* strains and *P. pentosaceus* tolerate gastric juice well, with no reduction of viability. *L. plantarum* strains, *L. gasseri* and *L. rhamnosus* GG revealed a moderate 1 to 2 log reduction in viability, whereas *L. pentosus*, *L. farciminis* and *L. sakei* strains lost viability over 180 min. For *L. reuteri* DSM 20016, ClpL chaperone and a putative cell wall-altering esterase (lr1516) seem important for survival at low pH [165]. All strains tested tolerate the simulated small intestinal juice well. The bacterial adhesion to the human intestinal cell lines Caco-2, HT-29 and LS 174T revealed major species and strain differences. *L. plantarum* MF1298 and *L. reuteri* DSM 20016, mm4-1a (ATCC PTA 6475) and fj1 (ATCC PTA 5289) revealed a significant higher adhesion compared to the other strains tested. *L. reuteri* are known to have a good adhesion to intestinal cell lines [166] and mucus [167]. *L. reuteri* DSM 17938 revealed low adhesion to the intestinal cell lines compared to the other *L. reuteri*. The *L. reuteri* strains with a high adhesion are all clustered in one phylogenetic group, lineage II, of *L. reuteri*, which essentially contains strains of human origin, whereas *L. reuteri* DSM 17938 are in lineage VI which contain strains of multiple species [33]. MacKenzie et al. [167] have reported corresponding strain and host specific adhesion of *L. reuteri*. The MUB protein first identified by Roos and Jonsson [115] is not present in DSM 20016, mm4-1a (ATCC PTA 6475) or fj1 (ATCC PTA 5289) [167]. Thus, other surface proteins are likely to be responsible for the adhesion. All strains, both living and UV-inactivated, had little effect on the epithelial barrier function *in vitro*. However, living *L. reuteri* strains revealed some increase of the TER from 6 to 24 h. Overall, *L. reuteri* strains revealed some interesting characteristics compared to the other strains investigated.

The results from paper I gave us a base for selecting interesting strains for further studies. *L. reuteri* ATCC PTA 6475, DSM 20016 and DSM 17938, *L. plantarum* MF1298, NC8 and 299v and *L. rhamnosus* GG were selected. Of these strains, *L. reuteri* DSM 17938, *L. plantarum* 229v and *L. rhamnosus* GG are commercial, well documented, probiotic strains. *L. reuteri* ATCC PTA 6475 (designated 6475 in the following) and *L. plantarum* MF1298 are candidate probiotics. *L. reuteri* DSM 20016 and *L. plantarum* NC8 are plasmid-free strains and as such used as laboratory “work-horses”, representative for the species. The DSM 20016 strain is also the designated type strain of *L. reuteri*. See Table 1 in Paper I for references to all strains.

SDPs are a group of surface-associated proteins in Gram-positive bacteria, many of which have been shown to impact the adhesive ability of lactobacilli. In paper II we elucidated the role of SrtA, four putative SDPs, and one C-terminal membrane anchored cell

surface protein of *L. reuteri* 6475 in adhesion to Caco-2 cells and mucus *in vitro*. The characterization of the functionality of these proteins includes adhesion to Caco-2 and mucus *in vitro*, mutagenesis of specific genes, and complementation of mutants. *L. reuteri* 6475 *srtA*$^-$ revealed a significantly lower adhesion to Caco-2 cells and mucus compared with the wild type indicating involvement of SDPs in adhesion. Evaluation of the bacterial adhesion revealed that of the five putative surface protein mutants tested, only a null mutation in the *hmpref0536_10633* gene, encoding a putative SDP with an LPxTG motif, resulted in a significant loss of adhesion to both Caco-2 cells and mucus. Complementation with the functional gene on a plasmid restored adhesion to Caco-2 cells. Furthermore, overexpression of *hmpref0536_10633* in strain 6475 resulted in an increased adhesion to Caco-2 cells and mucus compared with the wild type strain. Our findings demonstrate that the protein encoded by *hmpref0536_10633* plays a critical role in binding of *L. reuteri* 6475 to Caco-2 cells and mucus *in vitro*, and propose that this protein should be referred to as cell and mucus binding protein A (CmbA). Furthermore, we made a surprising observation regarding the *cmbA* gene of strain 6475. The annotated *cmbA* gene is reported to be 3093 bp and holds three identical tandem repeat regions of 288 bp each. The cloning of *cmbA* resulted in a gene of 2517 bp, ~0.6 kbp shorter than expected from the annotated genome sequence. Sequencing of the cloned *cmbA* revealed that it only had one of the tandem repeat regions. Several control PCRs with different primer pairs were performed, apparently verifying that the chromosomal *cmbA* in our culture of strain 6475 was shorter and only had one such repeat region. It was not known, at that time, whether this represented a sequencing error and/or artefact in the reported 6475 genome sequence, or if variants of the gene exist. From other genome sequences, e.g. the sequence of the highly related JCM 1112 (DSM 20016) strain, gene variation could be suspected since the *cmbA* gene of this strain is annotated as containing 5 repeats. Shortly after the publication of paper II, Etzold et al. [168] published the X-ray crystal structure of a peptide corresponding to a single *cmbA* repeat region. The peptide has a divergent Ig-like β-sandwich fold, sharing structural homology with the Ig-like interrepeat domain of internalins of the food borne pathogen *Listeria monocytogenes.* Interestingly, they found an intra- and inter-strain genetic variation at the *cmbA* locus with 1–5 repeats in strain ATCC PTA 5289, 1–9 repeats in strains ATCC PTA 6475 and CF4-6g, 1–10 repeats in DSM 20016 and 1–14 repeats in strain LMS11-3. They also detected different-sized CmbA proteins produced in the same culture, suggesting that the gene undergoes very rapid variation in one and the same culture. Etzold et al. [168] speculate that an increased number of repeats may correlate with increased binding to

mucus. However, the complementation and overexpression studies performed in paper II show that CmbA containing a single repeat region is sufficient to mediate good adhesion to IECs *in vitro*. The role of the number of repeats and how the variation is regulated remains to be investigated.

Probiotic bacteria can modulate the host immune response. Most studies with regard to immunomodulatory effects of LAB and probiotic bacteria do not describe the bacterial molecule(s) responsible for the observed effects. In paper III we use cytokine secretion from the monocytic cell line THP-1 and NF-κB activation in the monocytic cell line U937-3xkB-LUC to elucidate immune stimulating abilities of 7 live and UV-inactivated LAB. We found that *L. reuteri* 6475 and DSM 20016 induced the highest cytokine secretion from THP-1 cells and the highest activation of NF-κB in the U937-3xkB-LUC cell line, whereas other known probiotic bacteria such as *L. plantarum* 299v and *L. rhamnosus* GG had little effect. These results are in accordance with Paper I where we observed that *L. reuteri* strains differ significantly from other LAB strains in typical probiotic assays. Furthermore, also in this study the genetic differences in *L. reuteri* were obvious as *L. reuteri* DSM 17938 (lineage VI) induced a lower cytokine secretion and NF-κB activation compared to strain 6475 and DSM 20016 (lineage II). Live and UV-inactivated preparations resulted in significant different responses for two of the strains investigated. Furthermore, live preparations were stronger NF-κB activators compared to UV-inactivated preparations. Notably, *L. plantarum* MF1298 which has been shown to possess several *in vitro* probiotic characteristics in other studies [169, 170], was a strong cytokine inducer only when UV-inactivated. These results add to the complexity in the interaction between LAB and human cells and suggest the possible involvement of secreted pro- and anti-inflammatory mediators of LAB. The initial observation that *L. reuteri* strains were more potent stimulators than other LAB in this study prompted us to make use of *L. reuteri* ATCC PTA 6475 mutants (ref. paper II above) to investigate the possible involvement of SDPs in stimulation of THP-1 and U937-3xkB-LUC cells. We found that the *L. reuteri* LPxTG protein Hmpref0536_10802 is of importance for the induction of IL-8 and TNF-α in THP-1 cells. Its homologue in strain DSM 20016 is annotated as an amidase. The same mutant strain revealed a reduced adhesion to mucus only in Paper II, an observation which further strengthens the interest in Hmpref0536_10802 as an effector molecule of *L. reuteri* 6475. *L. reuteri* 6475 is known for its anti-inflammatory properties [47, 48, 50]. *In vitro*, bacteria with high IL10/IL12 ratio are characterized as anti-inflammatory. The THP-1 cells used in this study did not secrete IL-10 upon LAB stimulation. However, as IL-10 is produced at high levels relatively late in the immune

response compared to some of the pro-inflammatory cytokines (e.g. IL-12) [150] it might be that the THP-1 cells would have produced IL-10 with longer incubation time. *L. reuteri* strains were also the strongest NF-κB activators. This might be a beneficial property as NF-κB is important for intestinal immune homeostasis [137] and intrinsic NF-κB signaling prevents apoptosis IECs [138]. However, the aim of Paper III was not to characterize anti-inflammatory and pro-inflammatory strains, but rather to elucidate immune stimulatory properties of LAB.

Disruption of the intestinal epithelial barrier function and elevated permeability to luminal toxins, allergens and pathogens is now recognized as having a role in various gastrointestinal disorders [130, 171], and recent studies show promising results of probiotic therapy by improving the epithelial barrier [129]. In paper IV we investigate the effect of 6 selected commercial probiotic bacteria and LAB on the epithelial barrier *in vitro*. This includes functional assessment of polarized Caco-2 monolayer by measuring TER and paracellular permeability of fluorescein isothiocyanate–dextran (FITC-Dextran), and quantitative real-time reverse transcription PCR (qRT-PCR) of the TJ genes *zo-1* and *claudin-1*. N-(3-oxododecanoyl)-L-homoserine lactone (3O–C_{12}–HSL, hereafter HSL) from *Pseudomonas aeruginosa* was used to disrupt the Caco-2 barrier and create a model of "leaky gut". The aims were; (1) to evaluate if prophylactic incubation with LAB could prevent or reduce HSL damage of the Caco-2 monolayer, and (2) to evaluate if incubation with LAB could aid in regeneration of the epithelial barrier. Several other *in vitro* studies have shown the ability of LAB to prevent disruption of the intestinal epithelial barrier [131, 172-174]. Surprisingly, we found no beneficial effect of commercially probiotic bacteria and selected LAB in ability to prevent or regenerate HSL induced damage of Caco-2 monolayers compared to the control. Furthermore, after 1.5 and 4 h we found no difference in gene expression of the TJ genes *claudin-1* and *zo-1* as a result of LAB treatment. In Paper I we showed that *L. reuteri* strains revealed some increase of the TER from 6 to 24 h. However, after such a long incubation period there can be many other factors than the bacteria itself which are responsible for TER (pH etc.). Thus, 6 h was set as the maximum incubation time in Paper IV. As for the gene expression, it could be that the timing in our model could be more optimized. However, the development of the model was thorough, and special care was taken to control variable parameters.

The use of *in vitro* methods to study strain characteristics of LAB in cell lines of human origin has been a major part of this project. During my PhD I have often been asked questions like "which is the best probiotic?" and "can any of these strains be used as

probiotics?". It is important to emphasize that the ultimate performance criterion of a probiotic strain is the ability to confer health benefits in the host. Exogenously applied lactobacilli are generally only able to temporally colonise the GIT. A healthy gut microbiota is quite stable and will to some extent show resistance to colonization of new species [93]. Furthermore, the host age, genetics and environmental factors such as the endogenous microbiota, will impact whether individuals are responders or non-responders to a probiotic strain. This work demonstrated that *in vitro* characteristics of a probiotic strain can not directly be extrapolated to humans as well documented commercially available strains, such as *L. rhamnosus* GG and *L. plantarum* 299v, perform relatively poor compared in several assays.

Common for all *in vitro* assays are that the conditions will be quite different from the conditions in the human host. Some examples of this with specific reference to this work are; (1) in the human gut, food matrix protects the bacteria from the deleterious effect of gastric and small intestinal secretes [160, 175]; (2) the host defense systems, competition with the resident microbiota, mucosal shedding, and peristaltic flow are likely to modify the bacterial adhesion [93]; (3) the Caco-2 model of intestinal epithelial barrier do not produce mucin and there is an absence of physical parameters such as intestinal motility and transit time [176]; and (4) no *in vitro* models of immune modulation will be able to capture all the complex interactions and signalling cascades following bacterial stimulation. Nevertheless, typical probiotic *in vitro* assays like the ones used in this study is commonly used as screening assays to select new potential probiotic bacteria.

A detailed knowledge of the bacterial physiology and molecules that play a role in the interaction with the host is of crucial importance for a better understanding of potential health benefits of LAB. In this work, we regard the *in vitro* methods as important tools to investigate the characteristics and diversity of LAB. Such *in vitro* methods are highly important to increase our current understanding of how probiotic bacteria and LAB interact with IECs of human origin. Our findings that CmbA is highly important in adhesion to IECs and mucus, and that Hmpref0536_10802 is of importance in immune stimulation of THP-1 cells are two novel contributes to the puzzle that one day will help us to fully understand the interaction between LAB and cells of human origin.

CONCLUDING REMARKS AND FURTHER WORK

This work demonstrates that the *in vitro* characteristics of a probiotic strain can not directly be extrapolated to humans as well documented commercially available strains, such as *L. rhamnosus* GG and *L. plantarum* 299v, perform relatively poor compared to other LAB in several typical probiotic screening assays. The results show that the LAB tested have a large diversity. Overall, *L. reuteri* strains revealed some interesting characteristics compared to the other strains. By use of mutagenesis in *L. reuteri* 6475 we were able to identify two novel effector molecules of the strain. The protein encoded by *hmpref0536_10633*, which we proposed to be referred to as cell and mucus binding protein A (CmbA), plays a critical role in binding of *L. reuteri* 6475 to Caco-2 cells and mucus *in vitro*. Furthermore, the LPxTG protein Hmpref0536_10802 of *L. reuteri* 6475 is of importance for the induction of IL-8 and TNF-α in THP-1 cells, and may also play some role in the binding to mucus.

A selection of methods were used to study the strain characteristics of selected LAB and probiotic bacteria, including adhesion to intestinal epithelial cell lines, tolerance to simulated gastric and intestinal juices, effect on the intestinal epithelial barrier *in vitro*, and immunostimulation of monocytic cell lines. However, it is obviously that the results described herein can not be directly extrapolated to the situations in humans. It would therefor be of great interest to investigate the role of *L. reuteri* 6475 CmbA and Hmpref0536_10802 in humans. Furthermore, more *in vitro* studies to further characterize CmbA and Hmpref0536_10802 and their precise mechanism of action are needed. It can be speculated that different regions of CmbA are responsible for the adhesion to IECs and mucus as the adhesion to mucus was not fully restored upon complementation with functional *cmbA*. By introducing random mutations in the different regions of CmbA perhaps more can be learned about the part of the protein which is important for adhesion. Moreover, it would also be of great interest to try to characterize the CmbA "receptors" on IECs and mucus. In this work, the cloning of *cmbA* resulted in a gene ~0.6 kbp shorter than expected from the annotated genome sequence. Later, Etzold [168] showed that *L. reuteri* 6475 displays an intra-strain genetic variation at the *cmbA* locus. Preliminary results in our laboratory suggest that the growth conditions of the strain may be of importance for the number of repeats (e.g., growth on agar vs. broth). Further investigations should aim to understand how expression of the tandem repeats is regulated and the practical role of expressing 1 or 9 repeats.

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In vitro testing of commercial and potential probiotic lactic acid bacteria

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ABSTRACT

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host. The objective of this study was to investigate the diversity of selected commercial and potential probiotic lactic acid bacteria using common in vitro screening assays such as transit tolerance in the upper human gastrointestinal tract, adhesion capacity to human intestinal cell lines and effect on epithelial barrier function. The selected bacteria include strains of *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus farciminis*, *Lactobacillus sakei*, *Lactobacillus gasseri*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri* and *Pediococcus pentosaceus*.

Viable counts after simulated gastric transit tolerance showed that *L. reuteri* strains and *P. pentosaceus* tolerate gastric juice well, with no reduction of viability, whereas *L. pentosus*, *L. farciminis* and *L. sakei* strains lost viability over 180 min. All strains tested tolerate the simulated small intestinal juice well. The bacterial adhesion capacity to human intestinal cells revealed major species and strain differences. Overall, *L. plantarum* MF1298 and three *L. reuteri* strains had a significant higher adhesion capacity compared to the other strains tested. All strains, both living and UV-inactivated, had little effect on the epithelial barrier function. However, living *L. reuteri* strains revealed a tendency to increase the transepithelial electrical resistance (TER) from 6 to 24 h.

This work demonstrates the diversity of 18 potential probiotic bacteria, with major species and strain specific effects in the in vitro screening assays applied. Overall, *L. reuteri* strains reveal some interesting characteristics compared to the other strains investigated.

1. Introduction

Lactic acid bacteria (LAB) are characterise by their production of lactic acid and are predominant participants in many industrial and artisanal plant, meat and dairy fermentations. Furthermore, LAB are indigenous habitants of the human gastro intestinal tract (GIT), and are thought to be among the dominant colonists of the small intestine (Marco et al., 2006).

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" by the Food and Agriculture Organization/World Health Organization (FAO/WHO). Most probiotics commercially available today belong to the genera *Lactobacillus* and *Bifidobacterium*. Several mechanisms by which probiotics mediate their health benefits on the host have been suggested, and can be divided into three categories; (i) certain probiotics have antimicrobial activity and can exclude or inhibit pathogens; (ii) probiotic bacteria can enhance the intestinal epithelial barrier; and (iii) probiotic bacteria are believed to modulate the host immune response (Ezendam and Loveren, 2006; Lebeer et al., 2008; Lebeer et al. 2010; Marco et al., 2006). The mechanisms of health promoting effects of probiotic bacteria have proven difficult to elucidate in detail, and traditionally most attention has been given to their antipathogenic properties (Lebeer et al., 2008).

To perform their effect in the intestine, probiotic bacteria should be capable of surviving passage through the GIT. Thus, it is essential for the bacteria to have protection systems to withstand the low pH in the stomach, digestive enzymes and bile of the small intestine. Approximately 2.5 l of gastric juice (Cotter and Hill, 2003) and 1 l of bile (Begley et al., 2005) are secreted into the human digestive tract every day. Tolerance to gastric acid and bile has thus become important selection criterion for probiotic strains.

In addition to survive the harsh environment in the GIT, bacterial adherence to intestinal epithelial cells and/or mucus is frequently considered to be a desirable feature of a probiotic strain, as it can promote the gut residence time, pathogen exclusion, and interaction with host epithelial and immune cells (Collado et al., 2009; Lebeer et al., 2008; Marco et al., 2006). The mechanisms of adhesion is not fully understood, however bacterial cell-surface associated proteins

Abbreviations: LAB, lactic acid bacteria; TER, transepithelial electrical resistance; GIT, gastrointestinal tract.

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with mucus- and intestinal cell binding properties have been identified and characterised in probiotic strains (Sánchez et al., 2008; Vélez et al., 2007). The process of adhesion appears to be multifactorial as adhesion cannot be attributed to one component (Izquierdo et al., 2009) and includes electrostatic interactions, hydrophobic interactions, and specific bacterial structures such as external appendages (Servin and Coconnier, 2003). Despite new sophisticated methodologies, bacterial adhesion capacity is most commonly studied in vitro with epithelial cell lines, immobilized intestinal mucus or extracellular matrix molecules.

Another proposed mechanism of action of probiotic LAB is the ability to strengthen the epithelial barrier (Lebeer et al., 2008). Impairment of the intestinal epithelial barrier is associated with the pathogenesis of various gastrointestinal diseases, and recent studies show promising results of probiotic therapy by improving the epithelial barrier (Mennigen and Bruewer, 2009). The intestinal epithelial barrier consists of a monolayer of epithelial cells, epithelial cell–cell connections that seal adjacent epithelial cells together, a mucus layer, antimicrobial peptides, and secretory IgA (Ohland and Macnaughton, 2010). Enterocyte cell–cell connections are essential for the intestinal barrier function and the intercellular junctional complexes consist of tight junctions, adherence junctions, gap junctions and desmosomes (Ohland and Macnaughton, 2010). Tight junctions are dynamic and functionally responsive to a variety of stimuli, thus regulating the permeability of the cell layer (González-Mariscal et al., 2008). The bacterial components responsible for strengthening the epithelial barrier include cell surface factors, secreted proteins, soluble proteins, and bacterial DNA (Lebeer et al., 2008). In vitro, bacterial effect on the epithelial barrier can be monitored by measuring transepithelial electrical resistance (TER) of polarized cell monolayers.

The objective of this work was to study the diversity of selected commercial and potential probiotic bacteria in various in vitro models. The selected bacteria include commercially available probiotics, starter cultures, and potential probiotic LAB isolated from humans or food.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The origin of *Lactobacillus* and *Pediococcus* strains that were used is shown in Table 1. Strains were maintained at −80 °C in 20% (v/v) glycerol. Before experiments, strains were grown anaerobically on Man Rogosa Sharpe (MRS) agar (Oxoid, Hampshire, UK) for 48 h at 30 °C (*Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactobacillus farciminis* and *Pediococcus pentosaceus* strains) or 37 °C (*Lactobacillus rhamnosus*, *Lactobacillus gasseri*, and *Lactobacillus reuteri* strains), scraped from MRS agar in Dulbecco's Phosphate Buffered Saline (DPBS) (SigmaAldrich, St. Louis, MO), and diluted to a final concentration of 10^8 cfu/ml by measuring the optical density at 600 nm. If necessary, the suspension was concentrated to 10^9 cfu/ml.

2.2. Cell cultures

The commonly used human colorectal adenocarcinoma cell lines Caco-2 and HT-29, and the mucin producing cell line LS 174T were used. All three cell lines were originally obtained from the American Type Tissue Collection (Rockville, MD). Caco-2 were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 20% heat inactivated foetal bovine serum (FCS), 1% nonessential amino acids, 100 U/ml Penicillin and 100 μg/ml Streptomycin. HT-29 and LS 174T were grown in DMEM supplemented with 10% FCS, 1% nonessential amino acids, 100 U/ml Penicillin and 100 μg/ml Streptomycin. All solutions were obtained from Invitrogen (Carlsbad, CA). The cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, and subcultivated at 70 to 80% confluence.

Table 1
Species, strain identity, and origin.

Species	Strain identity	Origin
Lactobacillus plantarum	WCFS1	Single colony variant of NCIMB 8826 (Kleerebezem et al., 2003)
Lactobacillus plantarum	NC8	Grass silage (Shrago et al., 1986)
Lactobacillus plantarum	299v (DSM 9843)	Sourdough. ProViva brand of probiotic products. (Johansson et al., 1993)
Lactobacillus plantarum	MF1298	Norwegian mutton salami (Klingberg et al., 2005)
Lactobacillus plantarum	AD2	Rye, sourdough (Skrede et al., 2001)
Lactobacillus pentosus	MF 1300	Norwegian mutton salami (Klingberg et al., 2005)
Lactobacillus sakei	Lb790	Meat (Schillinger and Lucke, 1989)
Lactobacillus sakei	23K	Sausage (Berthier et al., 1996; Dudez et al., 2002)
Lactobacillus sakei	MF1053	Fermented fish (Norwegian "rakfisk") (McLeod et al., 2008)
Lactobacillus sakei	LS 25	Commercial starter culture for salami sausage (Hagen et al., 2000)
Lactobacillus rhamnosus	GG (ATCC 53103)	Human intestine (Silva et al., 1987). Commercially available probiotic bacteria.
Lactobacillus gasseri	ATCC 33323	Type strain. Human intestine (Lauer and Kandler, 1980)
Lactobacillus farciminis	MF1318 (DC11)	Fermented sausage (Klingberg et al., 2005)
Pediococcus pentosaceus	Q3	Rye
Lactobacillus reuteri	DSM20016	Type strain. Human intestine (Kandler et al., 1980)
Lactobacillus reuteri	DSM 17938	Plasmid cured variant of ATCC 55730. Human breast milk. (Rosander et al., 2008). Commercially available probiotic bacteria.
Lactobacillus reuteri	mm4-1a (ATCC PTA 6475)	Human breast milk (Oh et al., 2009)
Lactobacillus reuteri	fj1 (ATCC PTA 5289)	Human oral cavity. (Oh et al., 2009). Commercially available probiotic bacteria.

DSM, Deutsche Sammlung von Mikroorganismen; ATCC, American Type Culture Collection.

2.3. In vitro transit tolerance in the upper gastrointestinal tract

Transit tolerance in the upper human GIT was assessed in an in vitro model simulating gastric and small intestinal juices. The method was adapted after Charteris et al. (1998) and Huang and Adams (2004). Simulated gastric juice was prepared by dissolving pepsin from porcine gastric mucosa in 0.5% w/v sterile saline to a final concentration of 2 g/l and adjusting the pH to 3. Simulated small intestinal juice was prepared by dissolving pancreatin from porcine pancreas, 250 mg/l, and porcine bile extract, 0.45%, in 0.5% w/v sterile saline and adjusting the pH to 7.5. All chemicals were obtained from SigmaAldrich (St. Louis, MO). The solutions were prepared fresh the same day as the experiment.

Bacterial tolerance to simulated digestive juices were tested by mixing 15 μl 10^8 cfu/ml bacterial suspension with 1000 μl gastric or small intestinal juice and 485 μl 0.5% w/v sterile saline in a 2 ml micro tube. The average final pH of the simulated mixtures was 3.12 and 7.46 for the pH 3.0 gastric- and pH 7.5 small intestinal juices, respectively. The mixture was vortexed at maximum settings for 10 s

and incubated at 37 °C. When testing gastric tolerance, 100 μl was removed after 90 and 180 min for determination of viable counts. Tolerance to small intestinal juice was determined by removing 100 μl after 120 and 240 min. To determine the number of viable counts, serial dilutions were plated on MRS agar using WASP spiral plater (Don Whitley Scientific, West Yorkshire, UK) and incubated aerobically at 30 or 37 °C for 48 h before enumeration of colony forming units on a colony counter (ProtoCOL2, Synbiosis, Cambridge, UK). The number of colony forming units was expressed as log values, and tolerance over time was compared for the strains investigated. Experiments were performed with duplicate determinations and repeated three times.

2.4. Bacterial adhesion assay

Bacterial adhesion capacity to the human colon adenocarcinoma cell lines Caco-2, HT-29 and LS 174T was investigated. The cell lines were subcultivated in 12 well culture plates and grown at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air until confluence (normally 2 to 3 days). Before experiments, the cell layers were washed with DPBS to remove antibiotics from the original cell media and fresh cell media without antibiotics were added to the cells. Bacteria were added to a final concentration of 10^6 cfu ($\pm$0.5 log) in 1 ml cell media per well. After 1 h incubation, the cell layers were washed three times with DPBS to remove non-adherent bacteria, before the cell layers were lysed by addition of 0.1% Triton-X100 in DPBS (Sigma-Aldrich, St. Louis, MO). The remaining suspensions with viable adhered bacteria were diluted and plated onto MRS agar by a WASP spiral plater. The number of colony forming units was counted with a colony counter after aerobic incubation for 48 h at 30 or 37 °C. Bacterial adhesion capacity was calculated as percent of adhered bacteria in relation to the total number of bacteria added. Experiments were performed with triplicate determinations, and repeated three times.

2.5. Transepithelial electrical resistance as measurements of epithelial barrier function

Bacterial effect on the epithelial barrier was evaluated by measurement of TER using the Millicell Electrical Resistance System (Millipore, Bedford, MA). To obtain polarized monolayers, Caco-2 cells were seeded onto cell culture inserts (0.4 μm pore size, 12 mm diameter, polyethylene terephthalate, Millipore Bedford, MA) at a concentration of 3×10^5 cells per filter. The filters were maintained with a volume of 1 ml in the apical compartment and 2 ml in the basolateral compartment, and cell media was changed three times per week. For TER experiments the cells were grown in Minimum Essential Medium (MEM) containing GlutaMAX™, Earle's salts and 25 mM HEPES buffer (Invitrogen, Carlsbad, CA) supplemented with 20% heat inactivated FCS, 1% nonessential amino acids, 100 units/ml Penicillin and 100 μg/ml Streptomycin. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Functional polarity was developed after 14 days, and confirmed by scanning electron microscopy before each experiment.

Both living and UV inactivated bacteria were tested. Strains were inactivated by UV-light for 20 min, and only confirmed inactivated suspensions were used. The day before experiment, the filters were washed with DPBS to remove traces of antibiotics and the cell media was changed to the original media without antibiotics. TER was measured before the addition of bacteria ($t=0$), and after 1, 2, 4, 6 and 24 h incubation. Bacterial effect on TER was tested with 10^8 cfu per well in 1 ml, and DPBS was used as control. Ratio of TER (TER_{tx}/TER_{t0}) was calculated and bacterial effect on the epithelial barrier over time was compared for the strains investigated. Experiments were performed with duplicate determinations and repeated three times.

2.6. Statistics

Results are expressed as the mean and standard error mean (SEM) of three experiments with duplicate or triplicate determinations. The statistical analyses were preformed in GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). The results from in vitro transit tolerance in the upper GIT were analysed using a two-tailed paired *t*-test. Bacterial adhesion capacities were analysed using one-way analysis of variance (ANOVA) with Tukey's Test as a post-hoc test. TER results, as measurements of epithelial barrier function, were analysed using ANOVA with Dunnett's Multiple Comparison Test as a post-hoc test. Differences were considered statistically significant at $p<0.05$.

Table 2
Effect of simulated gastric and small intestinal juice on the viability of eighteen potential probiotic lactic acid bacteria.

	Viable counts (log cfu/ml) during simulated gastric transit tolerance			Viable counts (log cfu/ml) during simulated small intestinal transit tolerance		
	0 min	90 min	180 min	0 min	120 min	240 min
WCFS1	6.39 ± 0.01	5.40 ± 0.06**	4.43 ± 0.15**	6.27 ± 0.02	6.27 ± 0.02	6.23 ± 0.02
NC8	6.32 ± 0.04	5.99 ± 0.04*	4.00 ± 0.16**	6.37 ± 0.03	6.24 ± 0.05	6.27 ± 0.08
299v	6.46 ± 0.04	5.18 ± 0.09***	2.56 ± 0.23***	6.47 ± 0.02	6.15 ± 0.09	6.15 ± 0.13
MF1298	6.32 ± 0.02	6.30 ± 0.04	4.97 ± 0.17*	6.37 ± 0.02	6.33 ± 0.03**	6.45 ± 0.11
AD2	6.33 ± 0.00	5.41 ± 0.34	5.06 ± 0.28*	6.34 ± 0.02	6.21 ± 0.04	6.24 ± 0.06
MF 1300	6.25 ± 0.09	5.01 ± 0.03***	<1.20	6.22 ± 0.04	5.97 ± 0.07*	5.83 ± 0.06
Lb790	5.85 ± 0.08	<1.20	<1.20	5.77 ± 0.03	5.15 ± 0.02**	5.10 ± 0.06**
23K	5.93 ± 0.14	<1.20	<1.20	6. 00 ± 0.05	6.05 ± 0.07	6.02 ± 0.06
MF1053	5.63 ± 0.17	<1.20	<1.20	6.00 ± 0.05	5.80 ± 0.19	5.87 ± 0.11
LS 25	6.19 ± 0.06	<1.20	<1.20	6.17 ± 0.06	6.02 ± 0.11	6.11 ± 0.01
GG	6.22 ± 0.05	5.86 ± 0.45	5.06 ± 0.12*	6.36 ± 0.05	5.99 ± 0.22	5.37 ± 0.16**
ATCC 33323	6.11 ± 0.05	6.11 ± 0.06	4.94 ± 0.25**	6.34 ± 0.04	6.15 ± 0.02*	6.18 ± 0.02*
MF1318	5.83 ± 0.06	1.39 ± 0.69***	<1.20	6.11 ± 0.13	5.53 ± 0.11*	5.52 ± 0.10*
Q3	5.87 ± 0.10	5.82 ± 0.09	5.69 ± 0.01	5.88 ± 0.04	5.84 ± 0.03	5.86 ± 0.03
DSM20016	5.82 ± 0.08	5.82 ± 0.37	5.70 ± 0.42	6.16 ± 0.04	6.11 ± 0.10	6.13 ± 0.02
DSM17938	5.94 ± 0.07	5.53 ± 0.35	5.36 ± 0.40	6.17 ± 0.05	6.01 ± 0.02	5.97 ± 0.04
mm4-1a	5.82 ± 0.02	5.77 ± 0.39	6.01 ± 0.01**	6.12 ± 0.05	6.19 ± 0.02	6.15 ± 0.06
fj1	6.02 ± 0.05	6.24 ± 0.14	6.20 ± 0.09	6.31 ± 0.01	6.26 ± 0.02	6.25 ± 0.04

Results are shown as mean ± SEM. Viable counts (log cfu/ml) of each strain at, 90, 180, 120 and 240 min were compared with that at 0 min (paired *t*-test, two tailed), n = 3.
** $p<0.01$.
* $p<0.05$.
*** $p<0.001$.

3. Results

3.1. In vitro transit tolerance in the upper gastrointestinal tract

When exposed to simulated gastric juice of pH 3, the LAB investigated form several group-levels of tolerance (Table 2); *L. reuteri* strains DSM 20016, mm4-1a and fj1 and *P. pentosaceus* Q3 retained the same level of viability for up to 180 min; *L. reuteri* DSM 17938 showed a minor 0.5-log reduction in viability after 180 min; *L. plantarum* strains WCFS1, NC8, MF1298, AD2, *L. rhamnosus* GG, and *L. gasseri* ATCC 33323 revealed a moderate 1 to 2-log reduction in viability after 180 min; *L. plantarum* 299v showed a major 4-log reduction in viability; whereas *L. pentosus*, *L. farciminis* and all *L. sakei* strains had a total loss of viability after 180 min in simulated gastric juice.

All strains tested tolerated the simulated small intestinal juice with pancreatin and bile well, with none of the strains exceeding 1-log reduction in viability (Table 2). The strains that retained the same level of viability throughout the 240 min test period include *L. plantarum* WCFS1, NC8, MF1298, and AD2, *L. sakei* 23K and LS 25, *P. pentosaceus* Q3, and *L. reuteri* mm41-a and fj1. Strains with a minimal 0.1 to 0.5-log reduction include *L. plantarum* 299v, *L. pentosus* MF1300, *L. sakei* MF1053, *L. gasseri* ATCC 33323, and *L. reuteri* DSM20016 and DSM 17938. Whereas *L. sakei* Lb790, *L. rhamnosus* GG, and *L. farciminis* MF1318 had a minor 0.5 to 1-log reduction in viability.

Reductions >0.5 log cfu were statistically significant at $p<0.05$ (Table 2).

3.2. Bacterial adhesion capacity

The adhesion capacity of the 18 strains tested was highly variable (<1% to 25%), depending on the bacterial strain and the human cell line tested (Fig. 1A–C). In general, the three *L. reuteri* strains DSM 20016, mm4-1a, and fj1 possessed a high adhesion capacity (11 to 26%) compared to the other strains investigated. Furthermore, *L. plantarum* MF1298 revealed an intermediate adhesion capacity (6.5 to 8.9%) to all cell lines tested. *L. rhamnosus* GG adhered quite low to Caco-2 and HT-29 cells (2.7 and 1.5% respectively), and moderately (4.5%) to LS 174T cells. *P. pentosaceus* Q3 adhered quite well to HT-29 (6.2%) compared to Caco-2 and LS 174T cells. For the highly adherent strains (*L. plantarum* MF1298, *L. reuteri* DSM 20016, mm4-1a and fj1) Caco-2 cells (Fig. 1A) appeared to be the most favourable cell type for bacterial adhesion followed by LS 174T (Fig. 1C) and HT-29 cells (Fig. 1B). The statistical analysis of bacterial adhesion capacities to Caco-2, HT-29 and LS 174T are given in Supplementary Table A2 A–C.

3.3. Epithelial barrier function

TER of polarized Caco-2 cells was used as an indicator of the intestinal epithelial barrier function, and the effect of both living (Fig. 2A–C) and UV-inactivated bacteria (results not shown) was tested. 15 of the 18 living bacteria tested, as well as the control (DPBS), induced an increase in TER after 1 h incubation. *L. reuteri* fj1 was the only bacteria that induced a drop in TER after 1 h (Fig. 2C). Following 2 to 6 h incubation there were no major differences between the strains in their effect on the Caco-2 monolayer. When comparing the ratio of TER after 6 to 24 h incubation, the four *L. reuteri* strains DSM 20016, DSM 17938, mm4-1a, and fj1 were the only strains that showed a tendency to increase the ratio of TER over that time period (Fig. 2C). Whereas some strains, especially *L. rhamnosus* GG, revealed a tendency to decrease the ratio of TER over the same time period (Fig. 2 C). However, no statistically significant differences were detected when the 18 strains were compared to the control (ANOVA with Dunnet's post-hoc test). Complete data (mean ± SEM) for the effect of living bacteria are given in Supplementary Table A1. The UV-inactivated bacteria revealed no major change in the ratio TER during 24 h incubation (results not shown).

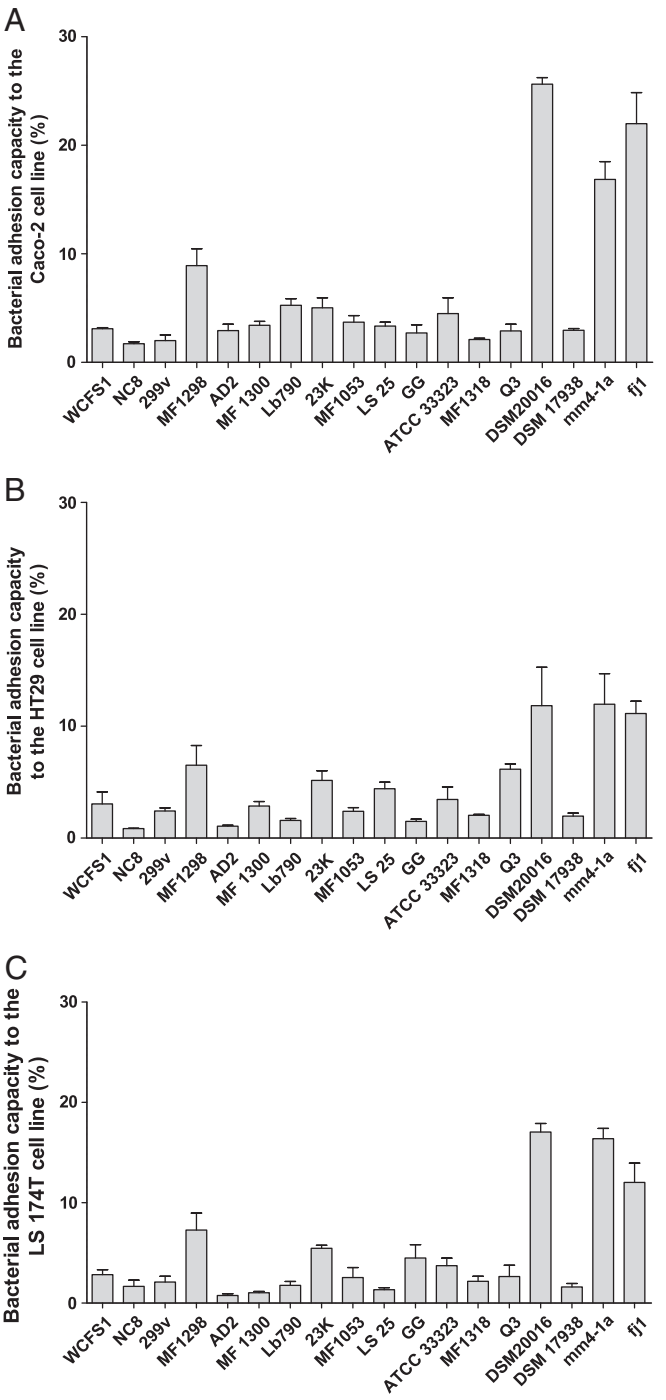

Fig. 1. The bacterial adhesion capacity of selected strains to Caco-2 (A), HT-29 (B), and LS 174T (C) cells. Adhesion capacity is calculated as the percentage of adhered bacteria in relation to the total number of bacteria added ($\log 6 \pm 0.5$). The results are expressed as mean ± SEM, n = 3.

4. Discussion

In this study, commercially available probiotic LAB, starter cultures, as well as potential probiotic LAB isolated from the human intestine and food were characterised. Both species and strain specific effects were seen, demonstrating a vast diversity among the strains investigated.

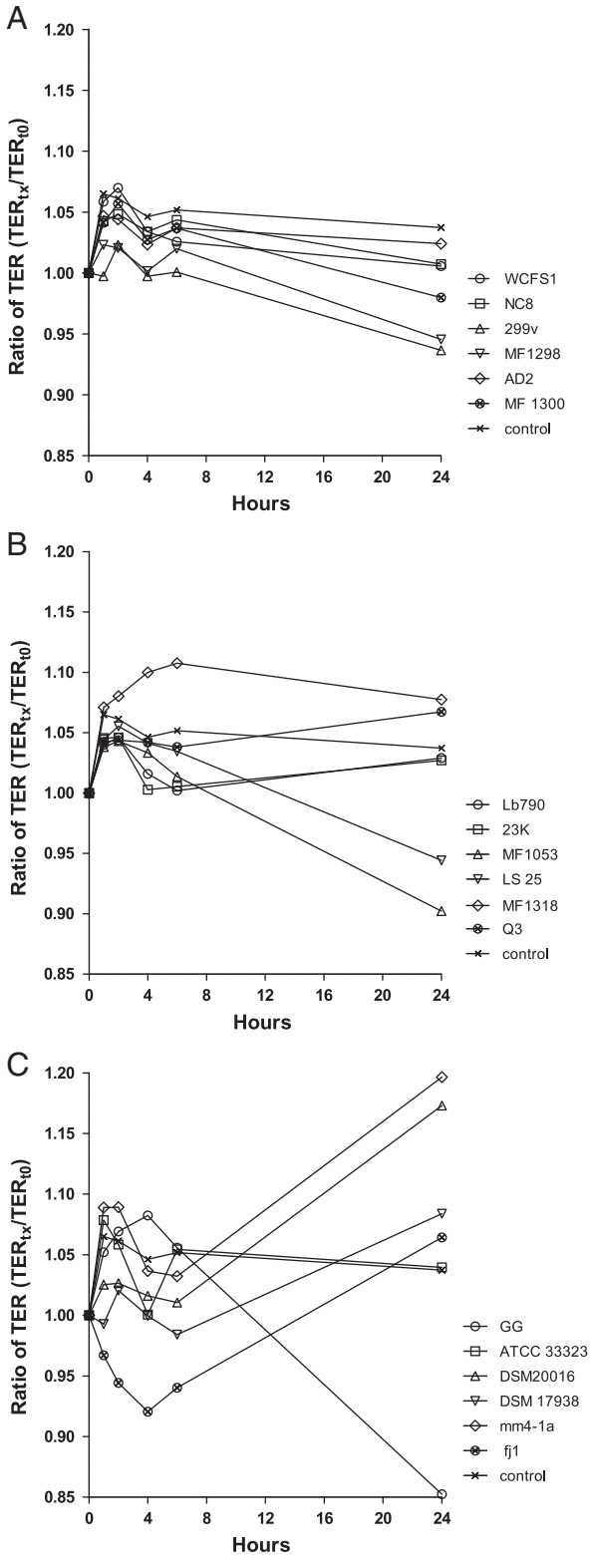

Fig. 2. TER of polarized Caco-2 monolayers exposed to *L. plantarum* and *L. pentosus* strains (A), *L. sakei*, *L. farciminis* and *P. pentosaceus* strains (B), or *L. rhamnosus*, *L. gasseri* and *L. reuteri* strains (C) at a concentration of 10^8 cfu/ml, or without bacteria (DPBS). TER is expressed as the ratio of TER at time t in relation to the initial value (t0) for each strain. The results are expressed as mean, n = 3. No statistical significant differences were observed between the stains and the control (ANOVA with Dunnett's Multiple Comparison Test). Complete data set with mean ± SEM can be found in Supplementary Table A1.

4.1. In vitro transit tolerance in the upper gastrointestinal tract

Native resistance to gastric acid is a rare probiotic property, and it is essential for bacteria to have protection systems for low pH (Cotter and Hill, 2003). The excellent ability to remain viable in simulated gastric juice and the good bile tolerance of *L. reuteri* strains observed in this study are in accordance with previous results (Rosander et al., 2008; Wall et al., 2007; Whitehead et al., 2008). For *L. reuteri* DSM 20016, ClpL chaperone and a putative cell wall-altering esterase (lr1516) seem important for survival at low pH (Wall et al., 2007). Furthermore, we found that *L. rhamnosus* GG revealed the same tolerance to simulated gastric and small intestinal juice over time as previously described (Charteris et al., 1998). *L. plantarum* MF1298, *L farciminis* MF 1318 and *L. pentosus* MF1300 have previously been tested as candidates of probiotic meat starter cultures (Klingberg et al., 2005a), and the results for MF 1298 and MF 1318 are in accordance with the previous report, whereas strain MF 1300 was more sensitive to low pH in the current study. It is not surprising that all *L. sakei* strains lost viability in simulated gastric juice, as the species is known to be sensitive to low pH (Haller et al., 2001).

All strains tested tolerate simulated small intestinal juice for 240 min. Samples collected after 1 min incubation (results not shown) revealed severe stress, i.e. strains appear as viable not culturable cells, for *L. pentosus* MF1300, *L. rhamnosus* GG, *L. farciminis* MF1318, and *L. sakei* strains. Following 1 min exposure, the strains adapted and returned to normal within 120 min. Incubation with pancreatin alone revealed no such stress after 1 min (results not shown). Thus, it is likely that bile is the agent that causes stress. A similar immediate stress response of bile has previously been shown for *Enterococcus faecalis* (Flahaut et al., 1996). The outcome of bile exposure will depend upon the conditions a bacterial cell face prior to entry to the small intestine, and exposure to one type of stress may protect against other types of stress (Begley et al., 2005). The method used in this study does not give any information about the outcome of bile exposure after exposure to gastric acid. The in vitro conditions that the bacteria are exposed to in this study are quite different from the in vivo situation. In the human gut, food matrix will protect the bacteria from the deleterious effect of gastric and small intestinal secretions (Begley et al., 2005; Huang and Adams, 2004). However, the in vitro tolerance assay provides important information about species and strain differences.

4.2. Bacterial adhesion capacity

The bacterial adhesion capacity of the 18 potential probiotic bacteria revealed major species and strain differences, with adhesion capacities varying from <1 to 25%. It is commonly accepted that the adhesion properties and mechanism for *Lactobacillus* are strain and matrix dependent (Adlerberth et al., 1996; Laparra and Sanz, 2009; Tallon et al., 2007). The epithelial cells of the intestine are covered by a protective layer of mucus, which offers attachment sites for gut bacteria. Extracellular mucus binding proteins such as MUB in *L. reuteri* 1063 (Roos and Jonsson, 2002) and the mannose-specific adhesin (Msa) of *L. plantarum* WCFS1 (Pretzer et al., 2005) are examples of important adhesion factors. Many other LAB have similar domains, indicating potential mucus binding proteins (Boekhorst et al., 2006). Furthermore, other cell surface molecules such as S-layer proteins, lipoteichoic acid, and exopolysaccharides also contribute to specific and/or nonspecific adhesion to host epithelial cells (Lebeer et al., 2008).

L. reuteri strains are known to have a good adhesion capacity to intestinal cell lines (Wang et al., 2008) and mucus (MacKenzie et al., 2010). The four *L. reuteri* strains in our study revealed major strain specific abilities to adhere to human intestinal cells; strain DSM 20016, mm4-1a, and fj1 had a very high adhesion capacity, whereas DSM 17938 had a poor adhesion capacity. Corresponding strain and

host specific adhesion capacities of *L. reuteri* have been reported (MacKenzie et al., 2010). The difference in adhesion capacity that we observed with *L. reuteri* strains can most likely be explained at the gene level, as DSM 20016, mm4-1a, and fj1 are clustered in one phylogenetic group of *L. reuteri*, and strain DSM 17938 in another (Oh et al., 2009). The MUB protein first identified by Roos and Jonsson (2002) is not present in DSM 20016, mm4-1a or fj1 (MacKenzie et al., 2010). Thus, other similar proteins are likely to be responsible for the adhesion. The intermediate adhesion capacity of *L. plantarum* MF1298 to Caco-2 cells is in accordance with previous reports (Klingberg et al., 2005a). Surprisingly, *L. rhamnosus* GG which is known to have a good adhesion capacity to epithelial cells and mucus (Collado et al., 2007; Tuomola et al., 1999; Tuomola and Salminen, 1998; Xu et al., 2009) adhered quite poorly to Caco-2 and HT-29 cells but revealed a somewhat better adhesion capacity to the mucin producing cell line LS 174T. Recently, SPaCBA pili in *L. rhamnosus* GG was shown to mediate adhesion to mucus (Kankainen et al., 2009), and that might explain why strain GG revealed a better adhesion capacity to LS 174T in our study.

In vitro results for bacterial adherence capacity to epithelial cells lines are difficult to extrapolate to the situation in the human GIT, where the host defence systems, competition with the resident microbiota, mucosal shedding, and peristaltic flow are likely to modify the bacterial adhesion (Lebeer et al., 2008). However, in vitro experiments are essential to understand the mechanisms of adhesion and provide important information regarding species and strain differences.

4.3. Epithelial barrier function

Measuring TER of polarized cell monolayers is commonly used as a screening assay to test for probiotic effects (Johnson-Henry et al., 2008; Klingberg et al., 2005b; Otte and Podolsky, 2004). In the current study, the four *L. reuteri* strains (living only) were the only bacteria that revealed a tendency to increase the ratio of TER from 6 to 24 h incubation. Whereas some strains, especially *L. rhamnosus* GG, revealed a tendency to decrease the ratio of TER over the same time period. However, none of these changes was statistically significant. Special precaution, such as using a cell media with low glucose levels and monitoring pH during the experiment, was taken to avoid large pH drops. No major changes in pH was observed during the experiments, and cannot be the reason for the increase/decrease in ratio of TER from 6 to 24 h. *L. plantarum* MF1298 has previously been shown to induce a dose dependent increase in TER (Klingberg et al., 2005b). This effect was not reproducible in our cell system.

TER is closely related to junctional strands of zonula occludens between epithelial cells (Claude, 1978; Claude and Goodenough, 1973), and the number of cell–cell connections per area (size of cells) (Claude and Goodenough, 1973). Caco-2 cells form less permeable tight junctions than those found in the human intestinal epithelium (Artursson et al., 2001; Lennernäs et al., 1996; Press and Di Grandi, 2008). Furthermore, the tightness of the monolayer resembles colonic rather than small intestinal tissue, the monolayer holds solely absorptive enterocytes, does not produce mucin, and lacks intestinal motility (Gan and Thakker, 1997; Hilgendorf et al., 2000). Our TER values were on average 2500 $\Omega\,cm^2$ when the Caco-2 monolayers were fully differentiated, much higher than commonly reported, and might explain why we did not observe more strain specific effects on the epithelial barrier in vitro.

5. Conclusion

In vitro assessments of potential probiotic bacteria have traditionally paid special attention to tolerance to the hostile environment of the stomach and small intestine, and ability to adhere to intestinal surfaces. However, it is important to point out that the ultimate performance criterion of a probiotic strain is the ability to confer health benefits in the host. The current study shows some interesting examples of this, since well documented commercially available strains, such as *L. rhamnosus* GG and *L. plantarum* 299v, performed relatively poor compared to other strains in our assays. Furthermore, bacterial tolerance to gastric and intestinal juice in vitro is not necessarily representative of in vivo behaviour (Morelli, 2007), and adhesion properties may vary with adhesion matrix and bacterial lot numbers (Tuomola et al., 2001).

In our study, the in vitro methods were selected to investigate the diversity of potential probiotic LAB, not as a screening test for probiotic activity. Our findings revealed that *L. reuteri* strains tolerate gastric and small intestinal conditions very well, have a high adhesion capacity, and can possibly strengthen the epithelial barrier in vitro over 24 h, thus revealing some interesting characteristics compared to the other strains investigated. Furthermore, *L. plantarum* strains tolerate gastric and small intestinal conditions better than *L. sakei* strains.

Further investigations will aim to better understand the mechanisms behind the observed diversity.

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Table A1. TER of polarized Caco-2 monolayers exposed to eighteen potential probiotic lactic acid bacteria at a concentration of 10^8 cfu/ml, or without bacteria (DPBS)

	Ratio of TER (TER_{tx}/TER_{t0})				
Strain	**1 h**	**2 h**	**4 h**	**6 h**	**24 h**
WCFS1	1.06 ± 0.04	1.07 ± 0.03	1.03 ± 0.03	1.03 ± 0.05	1.01 ± 0.05
NC8	1.04 ± 0.04	1.05 ± 0.03	1.03 ± 0.05	1.04 ± 0.06	1.01 ± 0.03
299v	1.00 ± 0.03	1.02 ± 0.02	1.00 ± 0.02	1.00 ± 0.05	0.94 ± 0.04
MF1298	1.02 ± 0.04	1.02 ± 0.02	1.00 ± 0.03	1.02 ± 0.05	0.95 ± 0.03
AD2	1.05 ± 0.06	1.04 ± 0.03	1.02 ± 0.04	1.04 ± 0.07	1.02 ± 0.03
MF 1300	1.04 ± 0.04	1.06 ± 0.02	1.03 ± 0.03	1.04 ± 0.05	0.98 ± 0.02
Lb790	1.04 ± 0.04	1.05 ± 0.04	1.02 ± 0.05	1.00 ± 0.06	1.03 ± 0.02
23K	1.05 ± 0.04	1.05 ± 0.04	1.00 ± 0.04	1.01 ± 0.08	1.03 ± 0.05
MF1053	1.04 ± 0.04	1.04 ± 0.05	1.03 ± 0.04	1.01 ± 0.06	0.90 ± 0.05
LS 25	1.04 ± 0.04	1.06 ± 0.06	1.04 ± 0.04	1.03 ± 0.05	0.94 ± 0.01
GG	1.05 ± 0.05	1.07 ± 0.08	1.08 ± 0.05	1.06 ± 0.08	0.85 ± 0.06
ATCC 33323	1.08 ± 0.05	1.06 ± 0.06	1.00 ± 0.04	1.05 ± 0.05	1.04 ± 0.06
MF1318	1.07 ± 0.04	1.08 ± 0.06	1.10 ± 0.06	1.11 ± 0.08	1.08 ± 0.06
Q3	1.04 ± 0.05	1.04 ± 0.07	1.04 ± 0.04	1.04 ± 0.06	1.07 ± 0.06
DSM20016	1.03 ± 0.05	1.03 ± 0.06	1.02 ± 0.04	1.01 ± 0.06	1.17 ± 0.08
DSM17938	0.99 ± 0.02	1.02 ± 0.08	1.00 ± 0.04	0.98 ± 0.06	1.08 ± 0.04
mm4-1a	1.09 ± 0.07	1.09 ± 0.07	1.04 ± 0.07	1.03 ± 0.07	1.20 ± 0.10
fj1	0.97 ± 0.02	0.94 ± 0.01	0.92 ± 0.02	0.94 ± 0.02	1.06 ± 0.01
control	1.06 ± 0.05	1.06 ± 0.05	1.05 ± 0.07	1.05 ± 0.06	1.04 ± 0.06

The results are expressed as ratio of TER (TER_{tx}/TER_{t0}) for each strain. Mean ± SEM, n = 3. No statistical significant differences were observed between the strains and the control (ANOVA with Dunnett's Multiple Comparison Test).

Table A2 A: Statistical analysis of bacterial adhesion capacity to Caco-2 cells

	WCFS 1	NC8	299v	MF 1298	AD2	MF 1300	Lb 790	23K	MF 1053	LS 25	GG	ATCC 33323	MF 1318	Q3	DSM 20016	DSM 17938	mm4-1a	fj1
WCFS1		ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	***	***
NC8	ns		ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	***	***
299v	ns	ns		**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	***	***
MF1298	*	**	**		*	*	ns	ns	ns	*	*	ns	**	*	***	*	***	***
AD2	ns	ns	ns	*		ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	***	***
MF 1300	ns	ns	ns	*	ns		ns	ns	ns	ns	ns	ns	ns	ns	***	ns	***	***
Lb790	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	***	ns	***	***
23K	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	***	ns	***	***
MF1053	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	***	ns	***	***
LS 25	ns	ns	ns	*	ns	ns	ns	ns	ns		ns	ns	ns	ns	***	ns	***	***
GG	ns	ns	ns	*	ns	ns	ns	ns	ns	ns		ns	ns	ns	***	ns	***	***
ATCC 33323	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	***	ns	***	***
MF1318	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	ns	ns		ns	***	ns	***	***
Q3	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns		***	ns	***	***
DSM20016	***	***	***	***	***	***	***	***	***	***	***	***	***	***		***	***	ns
DSM 17938	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***		***	***
mm4-1a	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***		ns
fj1	***	***	***	***	***	***	***	***	***	***	***	***	***	***	ns	***	ns	

The data were analysed with one way ANOVA and Tukey's post hoc test, n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant.

Table A2 B: Statistical analysis of bacterial adhesion capacity to HT-29 cells

	WCFS 1	NC8	299v	MF 1298	AD2	MF 1300	Lb 790	23K	MF 1053	LS 25	GG	ATCC 33323	MF 1318	Q3	DSM 20016	DSM 17938	mm4-1a	fj1
WCFS1		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	ns	**	**
NC8	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	***	***
299v	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	***	**
MF1298	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
AD2	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	***	***
MF 1300	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	**	ns	**	**
Lb790	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	***	ns	***	***
23K	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	*	ns	*	ns
MF1053	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	***	ns	***	**
LS 25	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	*	ns	*	*
GG	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	***	ns	***	***
ATCC 33323	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	**	ns	**	*
MF1318	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	***	ns	***	**
Q3	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns
DSM20016	**	***	***	ns	***	**	***	*	***	*	***	**	***	ns		***	ns	ns
DSM 17938	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***		***	***
mm4-1a	**	***	***	ns	***	**	***	*	***	*	***	**	***	ns	ns	***		ns
fj1	**	***	**	ns	***	**	***	ns	**	*	***	*	**	ns	ns	***	ns	

The data were analysed with one way ANOVA and Tukey's post hoc test, n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant.

Table A2 C: Statistical analysis of bacterial adhesion capacity to LS 174T cells

	WCFS 1	NC8	299v	MF 1298	AD2	MF 1300	Lb 790	23K	MF 1053	LS 25	GG	ATCC 33323	MF 1318	Q3	DSM 20016	DSM 17938	mm4-1a	fj1
WCFS1		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	***	***
NC8	ns		ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	***	***
299v	ns	ns		*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	***	***
MF1298	ns	**	*		**	**	*	ns	ns	**	ns	ns	*	ns	***	**	***	ns
AD2	ns	ns	ns	**		ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	***	***
MF 1300	ns	ns	ns	**	ns		ns	ns	ns	ns	ns	ns	ns	ns	***	ns	***	***
Lb790	ns	ns	ns	*	ns	ns		ns	ns	ns	ns	ns	ns	ns	**	ns	***	***
23K	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	***	ns	***	**
MF1053	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	ns	***	ns	***	***
LS 25	ns	ns	ns	**	ns	ns	ns	ns	ns		ns	ns	ns	ns	***	ns	***	***
GG	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	ns	***	ns	***	***
ATCC 33323	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		ns	ns	***	ns	***	***
MF1318	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns		ns	***	ns	***	***
Q3	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		***	ns	***	***
DSM20016	***	***	***	***	***	***	***	***	***	***	***	***	***	***		***	ns	*
DSM 17938	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***		***	***
mm4-1a	***	***	***	***	***	***	***	***	***	***	***	***	***	***	ns	***		ns
fj1	***	***	***	ns	***	***	***	**	***	***	***	***	***	***	*	***	ns	

The data were analysed with one way ANOVA and Tukey's post hoc test, n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant.

Paper II

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Role of *Lactobacillus reuteri* cell and mucus-binding protein A (CmbA) in adhesion to intestinal epithelial cells and mucus *in vitro*

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Lactobacillus reuteri, a symbiotic inhabitant of the gastrointestinal tract in humans and animals, is marketed as a probiotic. The ability to adhere to intestinal epithelial cells and mucus is an interesting property with regard to probiotic features such as colonization of the gastrointestinal tract and interaction with the host. Here, we present a study performed to elucidate the role of sortase (SrtA), four putative sortase-dependent proteins (SDPs), and one C-terminal membrane-anchored cell surface protein of *Lactobacillus reuteri* ATCC PTA 6475 in adhesion to Caco-2 cells and mucus *in vitro*. This included mutagenesis of the genes encoding these proteins and complementation of mutants. A null mutation in *hmpref0536_10255* encoding *srtA* resulted in significantly reduced adhesion to Caco-2 cells and mucus, indicating involvement of SDPs in adhesion. Evaluation of the bacterial adhesion revealed that of the five putative surface protein mutants tested, only a null mutation in the *hmpref0536_10633* gene, encoding a putative SDP with an LPxTG motif, resulted in a significant loss of adhesion to both Caco-2 cells and mucus. Complementation with the functional gene on a plasmid restored adhesion to Caco-2 cells. However, complete restoration of adhesion to mucus was not achieved. Overexpression of *hmpref0536_10633* in strain ATCC PTA 6475 resulted in an increased adhesion to Caco-2 cells and mucus compared with the WT strain. We conclude from these results that, among the putative surface proteins tested, the protein encoded by *hmpref0536_10633* plays a critical role in binding of *Lactobacillus reuteri* ATCC PTA 6475 to Caco-2 cells and mucus. Based on this, we propose that this LPxTG motif containing protein should be referred to as cell and mucus binding protein A (CmbA).

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INTRODUCTION

Strains of *Lactobacillus reuteri* have been isolated from the gastrointestinal tract, mother's milk and vagina of humans and animals (Oh *et al.*, 2010; Walter *et al.*, 2011). However, the primary habitat of the species appears to be the gastrointestinal tract, and the species has been designated a universal entero-*Lactobacillus* (Casas & Dobrogosz, 2000) and a vertebrate symbiont of the gastrointestinal tract (Walter *et al.*, 2011). *Lactobacillus reuteri* is marketed as a probiotic for humans and several clinical studies indicate positive effects (Weizman *et al.*, 2005; Agustina *et al.*, 2012; Hunter *et al.*, 2012; Szajewska *et al.*, 2013). *Lactobacillus reuteri* ATCC PTA 6475, isolated from human mother's milk, is a candidate probiotic with anti-inflammatory properties (Lin *et al.*, 2008; Jones *et al.*, 2011; Thomas *et al.*, 2012), which has shown promising effects in animal studies

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Abbreviations: IEC; intestinal epithelial cell, SDP; sortase-dependent protein.

One supplementary figure and three supplementary tables are available with the online version of this paper.

(Eaton *et al.*, 2011; Preidis *et al.*, 2012; McCabe *et al.*, 2013).

Bacterial adherence to intestinal epithelial cells (IECs) and/or mucus is frequently considered to be a desirable feature for a probiotic strain as it can promote the gut residence time and interaction with host epithelial and immune cells (Lebeer *et al.*, 2008; Kleerebezem *et al.*, 2010; Juge, 2012). In general, adhesins of lactobacilli can be classified according to their targets in the intestine (e.g. mucus components, extracellular matrix proteins), according to their localization on the bacterial surface (e.g. surface layer proteins) or according to how they are anchored to the bacterial surface [e.g. sortase-dependent proteins (SDPs)] (Vélez *et al.*, 2007). The mechanisms of adhesion are not fully understood; however, several reports have shed light on the mechanisms for a variety of *Lactobacillus* species (Roos *et al.*, 1996; Rojas *et al.*, 2002; Roos & Jonsson, 2002; Granato *et al.*, 2004; Buck *et al.*, 2005, 2009; Pretzer *et al.*, 2005; Bergonzelli *et al.*, 2006; van Pijkeren *et al.*, 2006; Kankainen *et al.*, 2009; Vélez *et al.*, 2010; Sánchez *et al.*, 2011; von Ossowski *et al.*, 2011). In addition to specific bacterial adhesins, other cell surface molecules, such as S-layer proteins, lipoteichoic acid and exopolysaccharides (Lebeer *et al.*, 2008), and extracellular appendages, such as flagella, fimbriae and pili (Juge, 2012), can also contribute to adhesion to host epithelial cells and mucus. See Vélez *et al.* (2007), Juge (2012) or Lebeer *et al.* (2008) for comprehensive reviews on adherence factors.

Several adhesins of *Lactobacillus reuteri* have been described. The first *Lactobacillus reuteri* surface protein involved in adhesion to be described was CnBP of *Lactobacillus reuteri* NCIB 11951 (Aleljung *et al.*, 1994; Roos *et al.*, 1996), which binds to collagen type I. Later, the adhesion-promoting protein MapA of *Lactobacillus reuteri* 140R (Rojas *et al.*, 2002) was described. This protein binds to both mucus (Rojas *et al.*, 2002) and Caco-2 cells (Miyoshi *et al.*, 2006), and two receptor-like molecules for MapA have been identified on Caco-2 cells (Miyoshi *et al.*, 2006). CnBP and MapA are considered homologues in light of their similarity at the amino acid level (94 %). Roos & Jonsson (2002) were the first to describe a mucus-binding protein of *Lactobacillus reuteri* when they described an extracellular mucus-binding protein (Mub) in *Lactobacillus reuteri* 1063. The crystal structure of the Mub protein has been determined, which revealed an unexpected immunoglobulin binding activity (MacKenzie *et al.*, 2009). Other surface proteins of *Lactobacillus reuteri* that contribute to adhesion include a high-molecular-mass surface protein (Lsp) and methionine sulfoxide reductase B (MsrB), which both contribute to adherence and ecological performance of *Lactobacillus reuteri* 100-23 in the murine gut (Walter *et al.*, 2005).

SDPs are a group of surface-associated proteins in Gram-positive bacteria, many of which have been shown to impact the adhesive ability of several lactobacilli. SDPs have a common molecular structure that includes an N-terminal signal peptide, often with an YSIRK-G/S motif that promotes secretion (Bae & Schneewind, 2003) and directs the protein to a specific surface localization (DeDent *et al.*, 2008), a C-terminal LPxTG motif, followed by a C-terminal transmembrane helix and a positively charged tail (Lebeer *et al.*, 2008; Call & Klaenhammer, 2013). Examples are the mannose-specific adhesin (Msa) in *Lactobacillus plantarum* WCFS1 (Pretzer *et al.*, 2005), Mub in *Lactobacillus reuteri* ATCC 53608 (strain 1063) (Roos & Jonsson, 2002) and *Lactobacillus* epithelium adhesin of *Lactobacillus crispatus* ST1 (Edelman *et al.*, 2012). Sortase A (SrtA) cleaves the LPxTG motif between the threonine and glycine residues, and covalently links the threonine carboxyl group to amino groups provided by the cell wall cross-bridges of peptidoglycan precursors (Marraffini *et al.*, 2006). Thus, a SDP is linked to the cell wall and displayed on the bacterial surface.

Lactobacillus reuteri ATCC PTA 6475 has a single gene encoding SrtA, five putative SDPs and one putative C-terminal membrane-anchored cell surface protein with similarities to SDPs but lacking the LPxTG motif. Among the human *Lactobacillus reuteri* strains, ATCC PTA 6475 is highly adherent to mucus (MacKenzie *et al.*, 2010) and various intestinal human cell lines (Wang *et al.*, 2008; Jensen *et al.*, 2012). Here, we present a study performed to elucidate whether SrtA and five of the six above-mentioned putative surface proteins of *Lactobacillus reuteri* ATCC PTA 6475 play a role in the ability of the strain to adhere to IECs and mucus. The characterization of the functionality of these proteins includes adhesion to IECs and mucus *in vitro*, mutagenesis of specific genes, and complementation of mutants. We found that the putative SDP encoded by the gene *hmpref0563_10633* plays a significant role in the ability to adhere to IECs and mucus, and propose that this protein should be referred to as cell and mucus-binding protein A (CmbA).

METHODS

Bacterial strains and growth conditions. The strains used in this study are shown in Table 1. Strains were maintained at –80 °C in 20 % (v/v) glycerol. *Lactobacillus reuteri* strains were grown at 37 °C in Man–Rogosa–Sharpe (MRS) broth or on MRS agar. *Lactococcus lactis* MG1363 was grown at 30 °C in M17 supplemented with glucose [0.5 % (w/v)]. Strains harbouring the pSIP411 vector or its derivatives were cultured in the presence of 10 μg erythromycin ml^{-1} (Sigma-Aldrich). All culture media were from Oxoid.

Cell culture. The human colorectal adenocarcinoma cell line Caco-2 (HTB-37) was obtained from the American Type Culture Collection. Caco-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 20 % heat-inactivated FBS, 1 % non-essential amino acids, 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1}. All solutions were obtained from Invitrogen. The cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 and subcultivated at 70–90 % confluence.

Sequence analysis of *Lactobacillus reuteri* ATCC PTA 6475. In order to identify genes encoding cell wall anchor domain proteins (Marraffini *et al.*, 2006), the genome sequence and identified proteins of strain ATCC PTA 6475, hereafter called 6475 (previously named

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
Strains		
Lactobacillus reuteri		
6475	ATCC PTA 6475 (earlier designated MM4-1A); WT, host strain; human breast milk (a kind gift from BioGaia AB, Stockholm, Sweden)	Oh *et al.* (2010)
6475 *cmbA*$^-$	Derivative of 6475; nonsense mutation in *cmbA* (*hmpref0536_10633*) encoding a hypothetical LPxTG motif containing protein*	This work
6475 *10146*$^-$	Derivative of 6475; nonsense mutation in *hmpref0536_10146* encoding a hypothetical LPxTG motif containing protein*	This work
6475 *11993*$^-$	Derivative of 6475; nonsense mutation in *hmpref0536_11993* encoding a putative C-terminal membrane-anchored cell surface protein*	This work
6475 *10802*$^-$	Derivative of 6475; nonsense mutation in *hmpref0536_10802* encoding a LPxTG motif containing amidase*	This work
6475 *10154*$^-$	Derivative of 6475; nonsense mutation in *hmpref0536_10154* encoding a LPxTG motif containing Ser/Thr protein phosphatase*	This work
6475 *srtA*$^-$	Derivative of 6475; nonsense mutation in *srtA* (*hmpref0536_10255*) encoding sortase A*	This work
6475(pSIPΔ)	Derivative of 6475 containing pSIPΔ	This work
6475(pSIP-*cmbA*)	Derivative of 6475 containing pSIP-*cmbA*	This work
6475 *cmbA*$^-$(pSIPΔ)	Derivative of 6475 *cmbA*$^-$ containing pSIPΔ	This work
6475 *cmbA*$^-$(pSIP-*cmbA*)	Derivative of 6475 *cmbA*$^-$ containing pSIP-*cmbA*	This work
6475 *srtA*$^-$(pSIPΔ)	Derivative of 6475 *srtA*$^-$ containing pSIPΔ	This work
6475 *srtA*$^-$(pSIP-*srtA*)	Derivative of 6475 *srtA*$^-$ containing pSIP-*srtA*	This work
Lactococcus lactis		
MG1363	Intermediate cloning host	Gasson (1983)
Plasmids		
pSIP411	Em^r; SppIP-based expression vector with P_{sppQ}::*gusA*	Sørvig *et al.* (2005)
pSIPΔ	Em^r; pSIP411 derivative without *gusA*	This work
pSIP-*cmbA*	Em^r; pSIP411 derivative containing *cmbA* under control of P_{sppQ}	This work
pSIP-*srtA*	Em^r; pSIP411 derivative containing *srtA* under control of P_{sppQ}	This work

*Details given in Table S1.

MM4-1A; GenBank accession number ACGX02000000, sequences ACGX02000001–ACGX02000007), were reanalysed after the preliminary analysis made by Saulnier *et al.* (2011). The sorting motif LPxTG was searched for manually in the protein sequences, and YSIRK-G/S signal sequences (pfam04650), cell wall anchor domains (TIGR01167) and other protein domains were searched for in GenBank and with BLASTP at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). Secretion signal peptides were predicted with SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP) (Petersen *et al.*, 2011) and transmembrane helices were predicted with TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM). Repeats in the protein sequences were identified using RADAR (http://www.ebi.ac.uk/Tools/pfa/radar).

Construction of *Lactobacillus reuteri* mutants. Mutagenesis in *Lactobacillus reuteri* 6475 was performed using ssDNA recombineering as described previously (van Pijkeren & Britton, 2012). Defined nonsense mutations were established within the *Lactobacillus reuteri* 6475 genes *hmpref0536_10633*, *hmpref0536_10255*, *hmpref0536_10146*, *hmpref0536_11993*, *hmpref0536_10154* and *hmpref0536_10802* (Table S1, available in the online Supplementary Material); hereafter referred to as *cmbA*, *srtA*, *10146*, *11993*, *10154* and *10802*, respectively.

Reagents and enzymes. Total DNA was isolated using the DNeasy Tissue kit (Qiagen) and the Bacterial Genomic DNA Purification kit (EdgeBio). Plasmid DNA was isolated using the QIAprep Miniprep kit (Qiagen). Lysozyme (20 mg ml^{-1}) and mutanolysin (40 U ml^{-1}) (Sigma-Aldrich) were used in all lysis steps. Restriction endonucleases *Nco*I, *Xba*I and *Xho*I, and T4 DNA ligase were obtained from New England Biolabs. Phusion High-Fidelity DNA Polymerase (Finnzymes/Thermo Fisher Scientific) and Maxima Hot Start polymerase (Thermo Fisher Scientific) were used for PCR. PCR fragments required for cloning were recovered from 0.7 % agarose gels using the QIAquick Gel Extraction kit (Qiagen). All kits were used according to the manufacturers' instructions.

Cloning of *Lactobacillus reuteri* ATCC PTA 6475 *cmbA* and *srtA*. Plasmid pSIP411 (Sørvig *et al.*, 2005), known to yield inducible and titratable gene expression in *Lactobacillus reuteri* 6475 (van Pijkeren & Britton, 2012) through the P_{sppQ} (previously P_{orfX}) promoter, was used as cloning vector. Plasmid constructions were performed with *Lactococcus lactis* MG1363 as an intermediate host. *Lactococcus lactis* MG1363 and *Lactobacillus reuteri* were transformed by electroporation using the Gene Pulser system (Bio-Rad) as described previously (Holo & Nes, 1989; Ahrné *et al.*, 1992), except that 0.5 % sucrose in 10 % glycerol was used as electroporation buffer for *Lactobacillus reuteri*. The *Lactobacillus reuteri* 6475 *cmbA* gene contains an internal *Nco*I site. In order to clone the gene at the *Nco*I/*Xba*I sites in pSIP411 (at the ATG start codon), replacing the reporter gene *gusA*, the internal *Nco*I site was removed and the fourth base of the gene was changed from C to G to create an *Nco*I site at the 5′ end of the gene. This was accomplished by fusion PCR (Horton & Pease, 1991), where the complete coding region of *cmbA* was amplified in two parts and assembled, including the base changes, before ligation

into the pSIP411 vector at the *Nco*I and *Xba*I sites (primers used for fusion PCR are listed in Table S2). The base changes resulted in codon changes at the positions corresponding to aa 2 (CTA to GTA) and aa 407 (TCC to TCG) in *cmbA*. The former results in an amino acid residue change (Leu to Val), which was anticipated not to affect the function of the protein. Cloning of the *Lactobacillus reuteri* 6475 *srtA* gene was performed using synthetically manufactured DNA (GenScript). The synthetic fragment consisted of the P_{sppQ} promoter and ribosome binding site, as present in pSIP411, and the *srtA* gene with the following base changes: A instead of G at position 1 (changing the start codon from GTG to ATG to ensure expression in the pSIP system) and T instead of C at positions 202 and 405 (removing internal *Bgl*II and *Nco*I sites to minimize problems in future cloning in the pSIP plasmids). None of the codon changes resulted in an amino acid residue change in the corresponding protein. The synthetic fragment was flanked by *Bgl*II and *Xho*I sites, which were used for cloning in pSIP411, replacing the P_{sppQ}::*gusA* region. pSIP411 with the *gusA* reporter gene deleted, designated pSIPΔ, was also used as a control. Sanger sequencing of the final constructions was performed to ensure correct sequences. Molecular cloning and gel electrophoresis procedures were performed using standard procedures. All primers used in this study are listed in Table S2.

Adhesion assays. The initial adhesion assays (for strain 6475 and mutants) were performed as follows. Before adhesion experiments, strains were taken from frozen stock and grown in MRS broth at 37 °C. The strains were subcultured once before experiments. On the day of experiment, the strains were inoculated to OD_{600} 0.1 in MRS broth and grown at 37 °C. At OD_{600} 1.0±0.1, the cells were harvested by centrifugation at 4000 r.p.m. for 10 min, washed in Dulbecco's PBS (DPBS) and resuspended in 1 vol. DPBS (Sigma-Aldrich). In the complementation/overexpression adhesion assays, where the pSIP-inducible gene expression system (Sørvig *et al.*, 2005) was used, the strains were inoculated to OD_{600} 0.1 in two separate tubes for each strain. At OD_{600} 0.3, SppIP-inducing peptide (Molecular Biology Unit, University of Newcastle, UK) was added to one set of cultures. For *Lactobacillus reuteri* 6475, 6475 *cmbA*$^-$ and their derivative strains, 50 ng SppIP ml^{-1} was used. For 6475 *srtA*$^-$ and derivative strains, 1 ng SppIP ml^{-1} was used due to severe growth inhibition at higher concentrations for the strain containing pSIP-*srtA*. At 1.5 h after induction (OD_{600} 1.0±0.1), both induced and non-induced cultures were harvested as described above. *Lactobacillus reuteri* adhesion to Caco-2 cells was tested as described previously (Jensen *et al.*, 2012). Briefly, *Lactobacillus reuteri* strains were added (~5×10^6 c.f.u.) to confluent cell layers in 1 ml antibiotic-free cell media per well. After 1 h incubation, the cell layer was washed to remove non-adherent bacteria and lysed by addition of 0.1 % Triton X-100 (Sigma-Aldrich) in DPBS. The remaining suspensions with viable adhered bacteria were plated onto MRS agar and the number of c.f.u. was counted after 48 h incubation. Adhesion to Caco-2 cells was calculated as per cent of adhered bacteria in relation to the total number of bacteria added. Experiments were performed with triplicate determinations and repeated three to six times. The bacterial adhesion to mucus for *Lactobacillus reuteri* strains (Table 1) was tested as described previously (Roos & Jonsson, 2002). Briefly, mucus from pig small intestine (obtained from slaughterhouse material at Uppsala, Sweden) was prepared and coated in microtitre wells (MaxiSorp; Nunc). Strains were prepared as described above and washed once in PBS pH 6.0 (PBS) supplemented with 0.05 % Tween 20 (PBST) and resuspended in an equal volume of the same buffer. An aliquot of 100 µl bacterial suspension was added to each well and incubated overnight at 4 °C. The wells were washed three times with PBST, the buffer was poured off and OD_{405} was measured using a plate reader after the wells had dried. Wells coated with BSA were used as control. Experiments were performed with triplicate determinations and repeated three times.

Statistics. ANOVA of the bacterial adhesion to Caco-2 cells was performed in Minitab version 16 (Minitab) using the General Linear Model and Tukey's or Dunnet's post-hoc test. Differences were considered statistically significant at $P\leqslant0.05$. Illustrations were created in Prism version 5.0 (GraphPad).

RESULTS

Sequence analysis of *Lactobacillus reuteri* ATCC PTA 6475

The sequences of the proteins encoded by the draft genome sequence of *Lactobacillus reuteri* ATCC PTA 6475 were analysed, and one sortase and five cell wall anchor domain proteins with LPxTG motifs were identified. One protein with a similar architecture, but lacking the actual LPxTG motif, thus predicted to be anchored to the cell envelope by a C-terminal membrane anchor, was also found. Furthermore, two putative pseudogenes encoding domains related to cell wall anchoring were identified. The genetic loci in strain 6475, the corresponding loci in the fully sequenced and highly related strain JCM 1112 (Walter *et al.*, 2011), the identity of the proteins, and some of their features are shown in Table 2.

Adhesion of *Lactobacillus reuteri* mutants to Caco-2 cells and mucus

Bacterial adhesion to the human colorectal cell line Caco-2 and mucus was investigated initially for *Lactobacillus reuteri* 6475, the *srtA* mutant and the five mutants for genes encoding putative surface proteins (Table 1). The growth of the strains was followed as a part of the Caco-2 cell adhesion assay (see Methods). The growth rates were very similar (results not shown). *Lactobacillus reuteri* 6475 *srtA*$^-$ revealed a significantly lower adhesion to Caco-2 cells and mucus compared with 6475 ($P=0.0057$ and $P=0.00017$, respectively) (Fig. 1), indicating involvement of SDPs in adhesion. Of the LPxTG protein mutants, *Lactobacillus reuteri* 6475 *cmbA*$^-$ revealed a significant loss of adhesion to Caco-2 cells, whereas the other mutants did not show significantly reduced adhesion compared with 6475 (1.3 vs 4.8 %, $P<0.0001$) (Fig. 1a). *Lactobacillus reuteri* 6475 *cmbA*$^-$ also showed a total loss of adhesion to mucus ($P<0.0001$). *Lactobacillus reuteri* 6475 *10802*$^-$ had a significant loss of adhesion to mucus compared with 6475 ($P=0.0161$), whereas the other mutants did not show significantly reduced adhesion to mucus (Fig. 1b). Only *Lactobacillus reuteri* 6475 *srtA*$^-$ and 6475 *cmbA*$^-$ thus revealed significantly reduced adhesion to both Caco-2 cells and mucus, and only those strains were selected for complementation.

Cloning of *cmbA*

The annotated *hmpref0536_10633* (*cmbA*) gene of strain 6475 is reported to be 3093 bp (Fig. S1). However, the cloning procedure yielded a gene of 2517 bp. Sequencing of

Table 2. Genes encoding cell wall anchor domain proteins and related proteins in *Lactobacillus reuteri* 6475

Locus tag HMPREF0536	Homologue in strain JCM 1112	Annotation/features/comments	Size (aa)	Mutant analysed
10255	LAR_0227	Sortase	234	Yes
10146	LAR_0989	LPxTG protein, Pilus_PilP and Rib regions	630	Yes
10154	LAR_0983	LPxTG protein, 5′-nucleotidase/2′,3′-cyclic phosphodiesterase	752	Yes
10633	LAR_0958	LPxTG protein, YSIRK-G/S signal sequence	1030	Yes
10706	LAR_0903	LPxTG protein, a second putative start 87 bp downstream (size 272 aa)	301	NA
10802	LAR_0813	LPxTG protein, amidase, a second putative start 25 bp downstream (size 637 aa)	645	Yes
11242-11241	LAR_1193-1192	11242: YSIRK-G/S signal sequence; 11241: LPxTG motif; ORFs separated by frame shift, putative pseudogene	328*	NA
12042	LAR_0089	YSIRK-G/S signal sequence, truncated, no LPxTG motif, putative pseudogene	607*	NA
11993	LAR_0044	C-terminal membrane-anchored cell surface protein	951	Yes

NA, Not available.
*Truncated genes.

the cloned gene revealed that the difference was due to a part where the annotated gene shows the presence of three identical tandem repeat regions of 288 bp (Fig. S1), whereas the cloned gene only has one such region. Several control PCRs with different primer pairs were performed, verifying that the *cmbA* gene in our culture of strain 6475 was ~0.6 kbp shorter than expected from the annotated genome sequence (not shown). Whether this represents a sequencing error and/or artefact in the reported 6475 genome sequence, or that variants of the gene exist, is not known at present.

Complementation of mutants: adhesion to Caco-2

Based on the initial adhesion experiments (Fig. 1), *Lactobacillus reuteri* 6475 *cmbA*$^-$ and 6475 *srtA*$^-$ were complemented with the corresponding functional gene using the pSIP411 vector and its inducible gene expression system. Gene expression in complemented strains was first validated for *cmbA* using quantitative real-time PCR. This showed that *cmbA* expression increased in the complemented strain ~400-fold during induction compared with the WT expression from the chromosome (Table S3 and accompanying text). Together with controls, the adhesion of the complemented strains to Caco-2 cells was tested (Fig. 2). Similar to the initial experiments with the original mutants (see above), the growth was monitored for the different variant strains as a part of the adhesion assay. The growth rates of the strains again appeared similar (not shown). After SppIP induction of *cmbA* expression in *Lactobacillus reuteri* 6475 *cmbA*$^-$(pSIP-*cmbA*) the adhesion was restored to that of *Lactobacillus reuteri* 6475 (4.7 vs 5.2 %), significantly higher than the corresponding non-induced strain (4.7 vs 0.9 %, P=0.0017) (Fig. 2). Furthermore, SppIP induction of vector *cmbA* in *Lactobacillus reuteri* 6475(pSIP-*cmbA*), i.e. overexpression of *cmbA* in the WT strain, resulted in a significantly higher adhesion compared with the corresponding non-induced strain (8.5 vs 3.6 %, P=0.0001) and the WT strain (8.5 vs 5.2 %, P=0.0095) (Fig. 2). The SppIP-induced *Lactobacillus reuteri* 6475 *srtA*$^-$(pSIP-*srtA*) did not show a higher adhesion than the corresponding non-induced strain (2.7 vs 3.0 %). However, both these strains showed a somewhat higher adhesion than *Lactobacillus reuteri* 6475 *srtA*$^-$ and 6475 *srtA*$^-$(pSIPΔ) (~1.7 %) (Fig. 2), although the difference was not statistically significant. The presence of the pSIPΔ vector alone did not influence the adhesion with statistical significance. However, non-induced *Lactobacillus reuteri* 6475(pSIPΔ) and 6475(pSIP-*cmbA*) did show a somewhat poorer adhesion compared with *Lactobacillus reuteri* 6475 (3.8 and 3.6 vs 5.2 %, respectively) (Fig. 2). Furthermore, the induction peptide SppIP (1 or 50 ng ml^{-1}) did not influence the growth of the strains (results not shown) or the adhesion to Caco-2 cells (Fig. 2).

Complementation of mutants: adhesion to mucus

Bacterial adhesion to immobilized mucus was investigated for *Lactobacillus reuteri* 6475, 6475 *cmbA*$^-$, 6475 *srtA*$^-$ and their derivative strains (Fig. 3). SppIP induction of vector *cmbA* in 6475(pSIP-*cmbA*) resulted in a significantly higher adhesion compared with both the corresponding non-induced strain (P<0.0001) and 6475 (P<0.0001) (Fig. 3). On the contrary, SppIP induction of vector *cmbA* in 6475 *cmbA*$^-$(pSIP-*cmbA*), i.e. complementation of the *cmbA* mutant, did not restore fully the adhesion to that of 6475. A clear trend towards increased adhesion for the complemented strain compared with 6475 *cmbA*$^-$ was observed (Fig. 3), although the difference was not statistically significant in the ANOVA. After complementation with vector *srtA* in *Lactobacillus reuteri* 6475 *srtA*$^-$, the adhesion was restored to that of 6475 for both SppIP-induced and non-induced cultures, and was significantly higher than 6475 *srtA*$^-$ (P<0.0001 for both SppIP-induced and non-induced cultures). Although showing clearly higher adhesion than

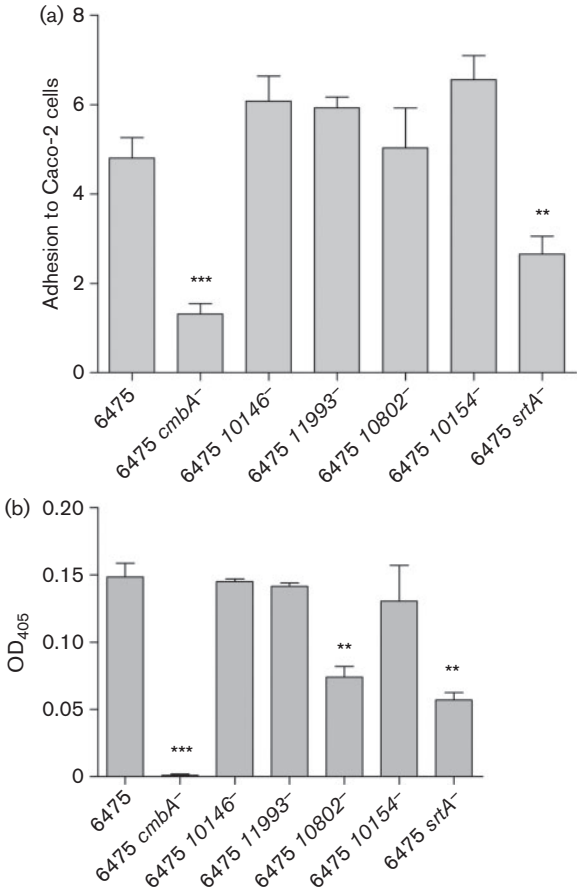

Fig. 1. (a) Adhesion of *Lactobacillus reuteri* 6475 and mutant strains to Caco-2 cells. *Lactobacillus reuteri* strains were added (~5×10^6 c.f.u.) to confluent cell layers in 1 ml antibiotic-free cell media per well. After 1 h incubation, the cell layer was washed to remove non-adherent bacteria and lysed by addition of 0.1 % Triton X-100 (Sigma-Aldrich) in DPBS. The remaining suspensions with viable adhered bacteria were plated onto MRS agar and the number of c.f.u. counted after 48 h incubation. Adhesion to Caco-2 cells was calculated as per cent of adhered bacteria in relation to the total number of bacteria added. At least three independent biological replicates were performed with each strain in triplicate. (b) Adhesion of *Lactobacillus reuteri* 6475 and mutant strains to mucus. Mucus from pig small intestine was prepared and coated in microtitre wells. An aliquot of 100 µl bacterial suspension was added to each well and incubated overnight at 4 °C. The wells were washed three times with PBST, the buffer was poured off and OD_{405} was measured using a plate reader after the wells had dried. At least three independent biological replicates were performed with each strain in triplicate. All results are expressed as means; error bars, SEM. *Lactobacillus reuteri* mutants were compared with *Lactobacillus reuteri* 6475 (ANOVA, General Linear Model, Dunnet's post-hoc test), $*P\leqslant0.05$, $**P\leqslant0.01$, $***P\leqslant0.001$.

the mutant, non-induced *Lactobacillus reuteri* 6475(pSIP-*cmbA*) revealed, for unknown reasons, a significantly lower adhesion compared with non-induced 6745 ($P=0.0002$), whereas *Lactobacillus reuteri* 6475 harbouring pSIPΔ did not show reduced adhesion under these conditions (Fig. 3).

DISCUSSION

In this study, we addressed the effect of inactivating sortase (SrtA), four putative SDPs and one C-terminal membrane-anchored cell surface protein of *Lactobacillus reuteri* ATCC PTA 6475 on adhesion to Caco-2 cells and mucus. The putative SDP, encoded by the gene *hmpref0536_10633*, proved to be highly important for adhesion to both Caco-2 cells and mucus *in vitro* as shown by various mutant and complemented strains (Figs 1, 2 and 3). We therefore propose the protein encoded by this gene to be named and referred to as cell and mucus binding protein A (CmbA).

According to the genome sequence of *Lactobacillus reuteri* 6475, CmbA encodes a polypeptide of 1030 aa containing an N-terminal YSIRK-G/S type signal peptide and a C-terminal LPxTG motif followed by a hydrophobic region predicted to be a transmembrane helix and a positively charged tail (Fig. S1). The LPxTG motif is recognized by SrtA (Marraffini *et al.*, 2006; Spirig *et al.*, 2011; Call & Klaenhammer, 2013), which in turn is responsible for anchoring of the protein to the cell wall. When using the C-terminal 42 aa of the predicted protein (including the LPxTG motif, the hydrophobic region and the positively charged tail) as the search string in a Pfam search (http://pfam.sanger.ac.uk), a very significant similarity for this region was obtained with the protein family 'Gram positive anchor' (Pfam: PF00746). CmbA thus has all the features that define SDPs (Schneewind *et al.*, 1992; Navarre & Schneewind, 1994, 1999; Bae & Schneewind, 2003; van Pijkeren *et al.*, 2006; Schneewind & Missiakas, 2012; Call & Klaenhammer, 2013; Remus *et al.*, 2013). CmbA is not classified as a 'MucBP' protein (Pfam: PF06458); however, BLASTP searches revealed that the best similarity scores were obtained with mucus binding proteins and LPxTG proteins in other *Lactobacillus* species, especially those of the so-called '*acidophilus* complex' (Kullen *et al.*, 2000), e.g. *Lactobacillus gasseri* MV-22 mucus binding protein (GenBank accession number ZP_07711536), *Lactobacillus crispatus* ST1 mucus binding protein (GenBank accession number YP_003602126), *Lactobacillus johnsonii* NCC 533 MucBP region protein (GenBank accession number NP_964406) and *Lactobacillus acidophilus* NCFM mucus binding protein (GenBank accession number YP_194552). The homologies to these proteins were in the range of 30–35 % identity (45–55 % similarity considering conservative amino acid changes). Among analysed and sequenced *Lactobacillus reuteri* strains, CmbA is unique to *Lactobacillus reuteri* 6475, JCM 1112 (DSM 20016), ATCC PTA 4659 (MM2-3) and ATCC PTA 5289 (FJ1). These strains are almost identical at the genome level with only a maximum of nine SNP differences between them (Walter *et al.*, 2011) and are all clustered in one phylogenetic group, lineage II, of *Lactobacillus reuteri* (Oh *et al.*, 2010), which essentially contains *Lactobacillus reuteri* strains of human origin. In

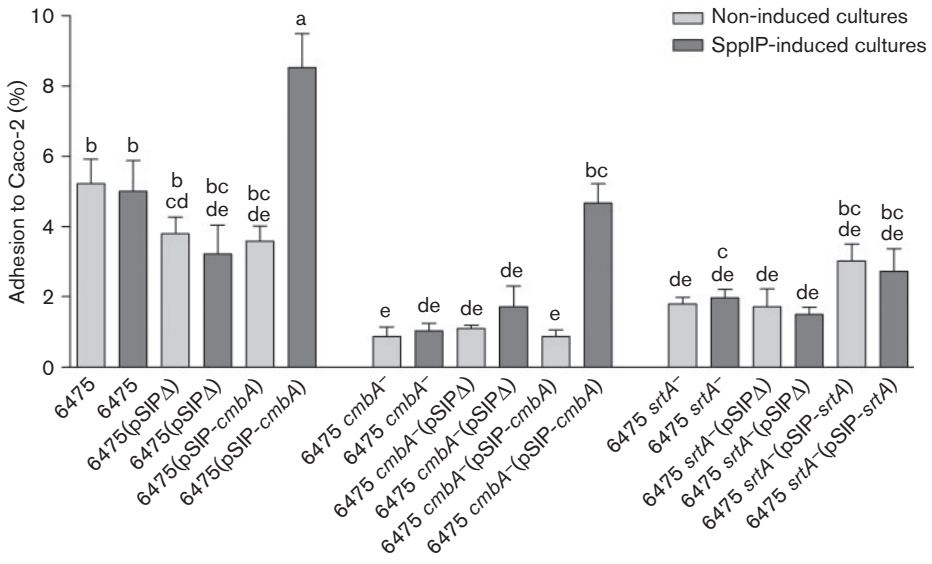

Fig. 2. Adhesion of *Lactobacillus reuteri* strains to Caco-2 cells with and without SppIP induction. *Lactobacillus reuteri* strains, either induced or non-induced with SppIP, were added (~5×10^6 c.f.u.) to confluent cell layers in 1 ml antibiotic-free cell media per well. After 1 h incubation, the cell layer was washed to remove non-adherent bacteria and lysed by addition of 0.1 % Triton X-100 (Sigma-Aldrich) in DPBS. The remaining suspensions with viable adhered bacteria were plated onto MRS agar and the number of c.f.u. was counted after 48 h incubation. Adhesion to Caco-2 cells was calculated as per cent of adhered bacteria in relation to the total number of bacteria added. At least three independent biological replicates were performed with each strain in triplicate. The results are expressed as means; error bars, SEM. Letters above columns refer to the ANOVA: means that do not share a letter are significantly different (ANOVA, General Linear Model, Tukey's post-hoc test accounting for the interaction between strains and level of induction), $P\leqslant0.05$.

addition, and notably, the *cmbA* gene is differently reported in GenBank for some of these strains with regard to the tandem repeat region (Fig. S1): the JCM 1112 sequence (GenBank accession number NC_010609.1) contains five repeats, the 6475 draft sequence (GenBank accession number ACGX02000000) reports three (with a comment: 'unresolved tandem repeat'), and the ATCC PTA 4659 draft sequence (GenBank accession number ACLB01000000) has the repeat region as a separate contig. The cloning of *cmbA* from the 6475 strain in our collection yielded a gene with only one of these repeat regions. This might indicate that variants of the gene exist. Whether this is the case and if this has any bearing on the function of CmbA remains to be investigated. Worthy of note is that repeat regions, with variable numbers in different strains, have been found in other adhesins from lactobacilli (Boekhorst *et al.*, 2006; Gross *et al.*, 2010). Human *Lactobacillus reuteri* strains are also found in another phylogenetic group (lineage VI). One representative of this latter group is the commercial strain DSM 17938, which lacks CmbA. This strain has been shown to adhere significantly less to IECs than the strongly adhering strains 6475, DSM 20016 and ATCC PTA 5289 (FJ1) in the same assay system as used here (Jensen *et al.*, 2012). One might therefore speculate that CmbA has a specific interaction with structures on human IECs that renders the strains possessing the protein to be highly adherent.

The *cmbA* mutant showed a significant reduction in adhesion to Caco-2 cells and a total loss of adhesion to mucus. This effect was reversible for adhesion to Caco-2 cells upon complementation with *cmbA* expressed from a vector. In addition, overexpression of *cmbA* in the WT strain [i.e. strain 6475(pSIP-*cmbA*)] did increase adhesion to both Caco-2 cells and mucus. In light of this, it was somewhat surprising that the adhesion to mucus was poorly restored with complemented *cmbA*. It should be noted, however, that there was a relatively clear trend towards increased adhesion for the complemented strain compared with the mutant. The lack of statistical significance in the ANOVA may in part be a result of uncertainties in the measurements at these low levels of adhesion (in the case of the *cmbA* mutant, no adhesion could be measured). Lack of full complementation cannot be copy number or pSIP system related, as such effects would also have been evident in studies of the same culture in adhesion to Caco-2 cells. The effect must therefore be specific for the mucus binding property of CmbA. Some possibilities exist for obtaining poor complementation, e.g. improper folding of the overexpressed protein that specifically affects mucus binding or improper co-expression of CmbA in relation to additional unidentified factors specifically involved in adhesion to mucus. This remains to be investigated, and has to await a more thorough characterization of CmbA and possible interactions with

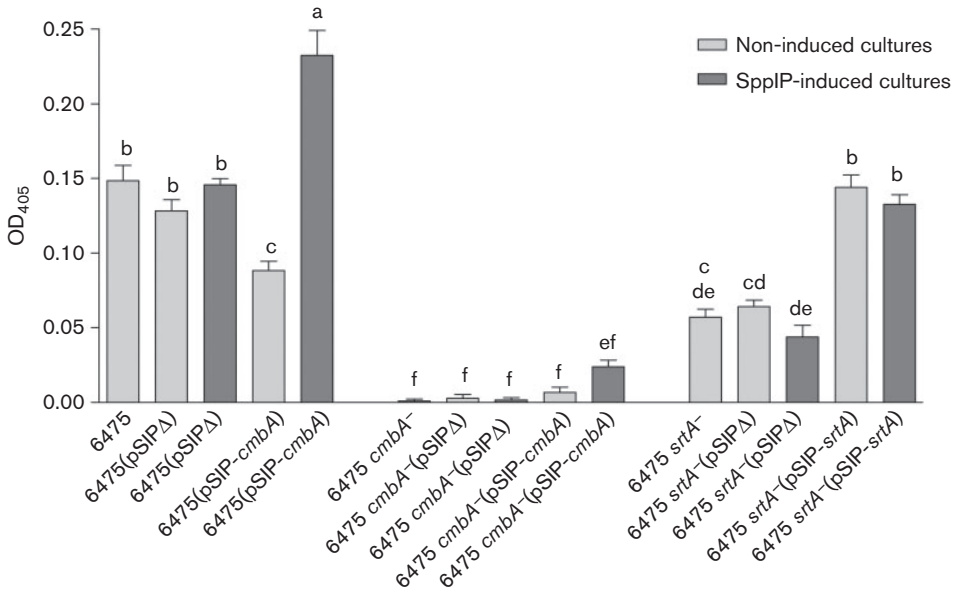

Fig. 3. Adhesion of *Lactobacillus reuteri* strains to mucus with and without SppIP induction. Mucus from pig small intestine was prepared and coated in microtitre wells. An aliquot of 100 µl bacterial suspension, either induced or non-induced with SppIP, was added to each well and incubated overnight at 4 °C. The wells were washed three times with PBST, the buffer was poured off and OD_{405} was measured using a plate reader after the wells had dried. At least three independent biological replicates were performed with each strain in triplicate. The results are expressed as means; error bars, SEM. Letters above columns refer to the ANOVA: means that do not share a letter are significantly different (ANOVA, General Linear Model, Tukey's post-hoc test), $P \leqslant 0.05$.

other proteins. Other adhesion factors to IECs and mucus have been described previously for *Lactobacillus reuteri* (Miyoshi *et al.*, 2006; MacKenzie *et al.*, 2010), and the present study also indicated that the putative SDP encoded by the gene *hmpref0536_10802* was involved in mucus binding (Fig. 1b), but not in IEC binding. Nevertheless, the results indicate a significant role of CmbA in the adhesive properties of *Lactobacillus reuteri* 6475.

The *srtA* mutant showed a significantly reduced adhesion compared with *Lactobacillus reuteri* 6475, but a somewhat higher adhesion than the *cmbA* mutant. This supports the strong *in silico* evidence that CmbA is an SDP, and is in line with previous studies on SDPs and sortase-deficient mutants. SDPs may still be found as surface proteins in *srtA* mutants (Bierne *et al.*, 2002; Nobbs *et al.*, 2007; Remus *et al.*, 2013), although significantly decreased in abundance and possibly displayed in a non-optimal fashion. The reason for the increased adhesion for the *srtA* mutant compared with the *cmbA* mutant may thus be explained by the physical properties of the C-terminal end of the CmbA protein and the fact that *cmbA* is still expressed in the *srtA* mutant. In the *srtA* mutant, the protein will be exported through the Sec machinery by way of the signal sequence (Ton-That *et al.*, 2004). It is, however, no longer cleaved at the LPxTG site and therefore not covalently bound to the peptidoglycan, but the C-terminal transmembrane helix and the positively charged tail may anchor some of the expressed protein to the membrane. Thus, it is likely that whilst CmbA is not coupled to the peptidoglycan in the absence of SrtA, and therefore not optimally displayed, it remains surface associated in the cytoplasmic membrane by the C-terminal anchor and promotes some adhesion of *Lactobacillus reuteri* to IECs and mucus. The adhesion of the *srtA* mutant was essentially restored by introducing pSIP-*srtA* without induction. This was particularly evident with regard to mucus adhesion. Induction with 1 ng SppIP ml^{-1} did not increase adhesion further. This level of SppIP is well below the saturation level, but still promotes measurable and higher expression in *Lactobacillus reuteri* compared with the non-induced state (unpublished observations). Higher induction levels were tested, but this resulted in severe growth inhibition of the strain. This may indicate that *srtA* expression in the WT is at a relatively low level due to possible toxic effects of high SrtA concentrations, consistent with the protein being membrane located. The GTG start codon, used in the native *srtA* gene, but changed to ATG in the cloned version, is generally also an indication of downregulation of expression (Vellanoweth & Rabinowitz, 1992; O'Donnell & Janssen, 2001). The P_{sppQ} promoter in the pSIP411 vector was shown previously to be not inactive completely in the non-induced state, i.e. a very minor, but still detectable, degree of gene expression occurred also without the presence of the induction peptide SppIP (Sørvig *et al.*, 2005). This minor expression of *srtA*, together with a more

effective translational start, could thus be enough to complement the mutant. Due to the sensitivity of *srtA* expression in *Lactobacillus reuteri* 6475 as described above, overexpression of *srtA* in the WT strain was anticipated to also give severe growth inhibition and therefore not tested.

Of the five putative surface protein mutants tested, only a null mutation in the *hmpref0536_10633* gene (*cmbA*), encoding a putative surface protein with an LPxTG motif, had a significant loss of adhesion to both Caco-2 cells and mucus. The *hmpref0536_10802* gene might play a role in adhesion of *Lactobacillus reuteri* 6475 to mucus, although the effect of inactivation of this gene was not as large as inactivation of *cmbA*. None of the other putative cell wall/membrane-anchored proteins investigated in the present study appeared to be important for adhesion to Caco-2 cells or mucus. Whether these proteins are expressed and, if so, their role in the surface properties of *Lactobacillus reuteri* 6475 remain to be investigated. The automatic bioinformatic analysis initially did not designate *hmpref0536_10706* as SDP encoding (due to a possible start codon 87 bp downstream, leading to a protein without a signal sequence; Table 2). A mutant for this remaining putative SDP gene was therefore not available for this study. This SDP also remains to be evaluated for any role in the adhesive properties of strain 6475.

The importance of CmbA in adhesion to both IECs and mucus adds to the complexity of the interactions that mediate the adhesion of gut bacteria to the intestine. As the mucus layer of the intestine is renewed continuously, it would probably be advantageous for probiotic bacteria to have the ability to bind to various intestinal surfaces as this would allow for prolonged time in the intestine for interactions with IECs and immune cells. The importance of CmbA in colonization of *Lactobacillus reuteri* 6475 in the intestine *in vivo* will have to be validated in future studies, but as this strain of *Lactobacillus reuteri* most probably does not bind to intestinal surfaces of mice (Oh *et al.*, 2010; Frese *et al.*, 2011; Walter *et al.*, 2011), such studies also require the development of suitable models.

In conclusion, we have identified a novel cell and mucus-binding protein, CmbA, of *Lactobacillus reuteri* 6475. Other surface proteins of *Lactobacillus reuteri* 6475 may contribute to the adhesion properties of the strain. However, since the *cmbA* mutant had a very significant loss of adhesion to Caco-2 cells and a total loss of adhesion to mucus, effects that were partially reversible by complementation of mutants, CmbA is clearly a highly important protein for the adhesive properties of *Lactobacillus reuteri* 6475.

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SUPPLEMENTARY MATERIAL

The role of *Lactobacillus reuteri* Cell and Mucus Binding protein A (CmbA) in adhesion to intestinal epithelial cells and mucus *in vitro*

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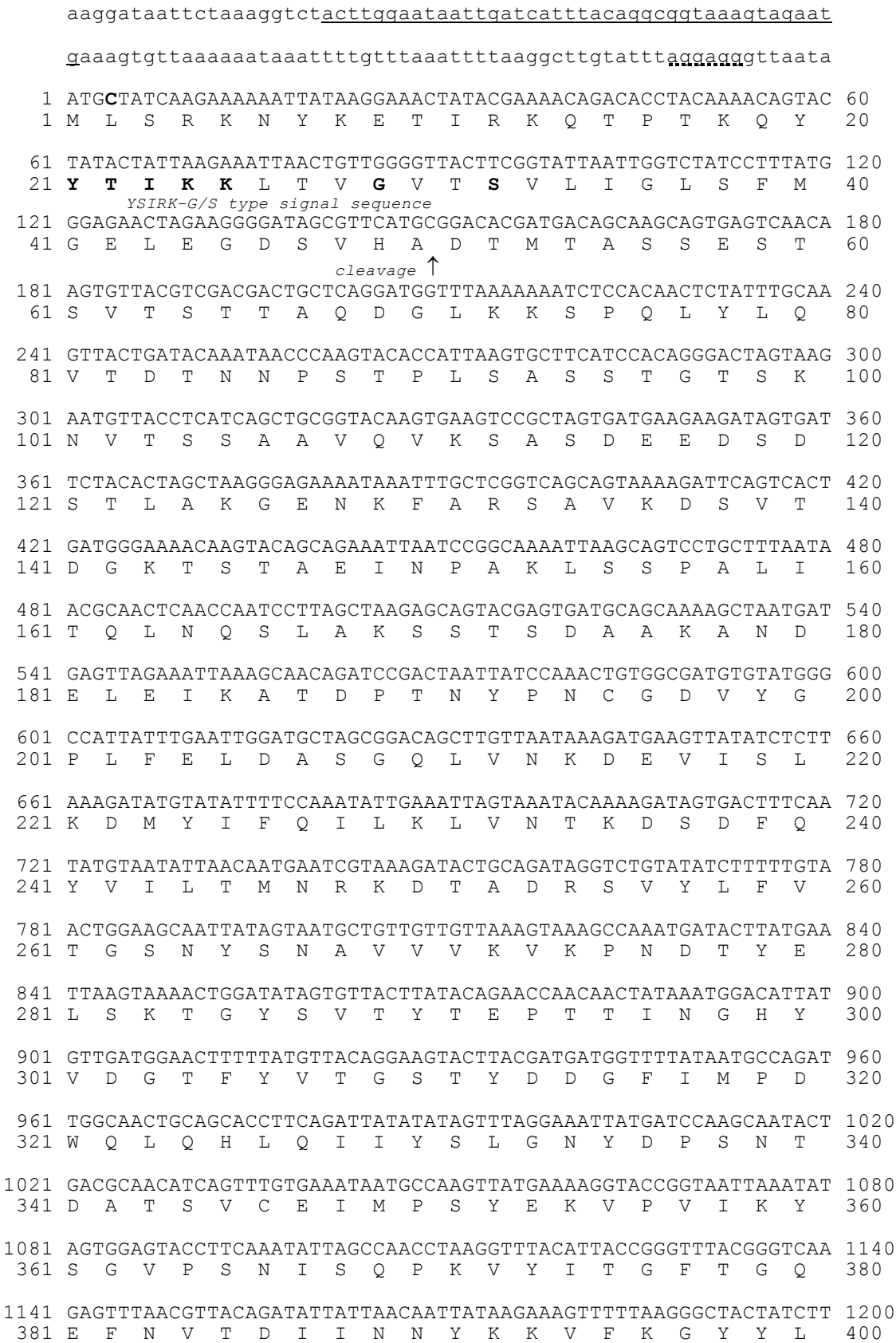
Supplementary material, Figure S1



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1261 TTAAAGACATATTATGATAATGATGGTAATGTTGACTTTAAGGGCTTGTATCATCAAATT 1320
 421 L  K  T  Y  Y  D  N  D  G  N  V  D  F  K  G  L  Y  H  Q  I   440

1321 GATGATCAGGGAACAATGAGTGTGAGTGTTCTTAATGCAGATAATAAAACAATTGTTGGA 1380
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2161 GGTAAGTACACACTTAGTTTGAATACGACCGGAGAAGCAGCCTTACGTAAGGCTAACCCG 2220
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2341 GAGCCAACGACAGTTCAGTGGAGTGGCTTGAAAGGACTGAACACTTCAACCTTAACGTCC 2400
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```

Fig S1. Nucleotide sequence and the deduced amino acid sequence of *cmbA* based on the draft genome sequence of *L. reuteri* ATCC PTA 6475 (GenBank: ACGX02000000). Features of the sequence are indicated as follows: underlined nucleotide sequence: putative promoter sequence (last nucleotide, position -60, predicted transcription start) and a predicted transcription termination sequence; dotted underlined nucleotide sequence: consensus Shine Dalgarno sequence; nucleotides in bold at positions 4 and 1221: these Cs were changed to Gs in the cloned gene to introduce and remove *Nco*I sites, respectively (see main text for details); amino acid sequence in bold: YSIRK-G/S like motif in signal sequence; underlined amino acid sequence: the cell wall anchoring motif LPXTG; dotted underlined amino acid sequence: membrane spanning domain; double underlined amino acid sequence: positively charged tail (membrane anchoring). The signal sequence and the tandem repeats are indicated under the amino acid sequence. Greyed out region represents the sequence missing in the cloned *cmbA* obtained in this study.

(Promoter predicted by: Neural Network Promoter Prediction,

http://www.fruitfly.org/seq_tools/promoter.html; Transcription terminator predicted by:

ARNold, http://rna.igmors.u-psud.fr/toolbox/arnold/; Signal sequence predicted by: SignalP

4.1 Server, http://www.cbs.dtu.dk/services/SignalP/)

Supplementary material, Table S1

Mutagenesis scheme for putative surface protein genes and sortase gene in *L. reuteri* 6475. All mutations were done by creating a stop codon early in the genes (leading to truncated proteins of a size 15% or less of the full proteins). The sites for the mutations were generally chosen based on the ease of creating a suitable restriction site used in the screening. Mutants were verified with sequencing. For details of the procedure, see van Pijkeren & Britton (2012).

Table S1. Mutation of putative surface protein genes and sortase gene in *L. reuteri* 6475.

Locus tag HMPREF0536	**Designation**	**Amino acid change**	**Recombineering oligo**[a] (5' -> 3')	**Screen oligo#1** (5' -> 3')	**Screen oligo#2** (5' -> 3')	**Screen oligo#3** (5' -> 3')
10633	*cmbA*	(I35X), (G36I)	tactattaagaaattaactgttggggt tacttcggtatta**TGAAT**tctatcctt tatgggagaactagaaggggatagcgt tcatgcgga	gcaaactcaaaatat gaagaagctatagaa	ctagttctcccataa aggatagaattca	NA[b]
10255	*srtA*	(V26X)	tccctactaagtaagacttaatctgtt ggttaaaaattaa**CTA**aactgatacca gtaacagtacaacaaccgctgtccacc gt	ttgtactgttactgg tatcagtttag	actttacaaaatcag aaaacatttgcgt	atcgattaataccat tggagcaattac
10146	*10146*	(A88X), (D89I)	tctttcgtttgtttaacttgattctta ttagaatcgacattttga**ATTCA**gtct gccaaattttgattagcaatattaaga tcattattt	aacgaatgggattaa agattagtttcaatg	attcttattagaatc gacattttgaattca	NA
11993	*11993*	(Y51X), (A52I)	gattgcaattgtgcaagttgctgatca gcagttgcccgaacttg**AATTC**aagta acttggtcattacttacttgtgcatta ttttgacta	gttgcaagagatgct tcagcattttt	caagtaagtaatgac caagttacttgaatt	cgtgggaacaggttt gaaaaaatttaaatt
10154	*10154*	(Y70X), (Q71A), (N72Y)	ccatgcaggtcattgattcctaaaatc tgaactggaatgtcat**AAGCT**tatttg gctaggtcagaccaatcagtagtcgtt tgcggagta	tgggatattaacggt gagtggtaaat	taaaatctgaactgg aatgtcataagct	agtatggttgcattc caaatgggatt
10802	*10802*	(D73E), (Q74A), (Q75X)	atttttgatatgtatcattaactaac tgttgactatcgattt**AAGCT**tcttta acttgttgtgcagttacaagagctgat tgattttca	acaagacaaaatgga ttgctatgtgg	ttcttaaggaaccac cagcatcattt	NA

[a] mutated bases are in bold uppercase; [b] NA, not applicable

Supplementary material, Table S2

Table S2. Primer/probes used in this study

Name	Direction	Oligonucleotide primer sequence
Primers used for cloning of cmbA		
cmbA-f1	Forward start	5'-ATGCACCATGGTATCAAGAAAAAATTATAAGGAAAC-3'
cmbA-f2	Forward middle	5'-ATCTTCAAAATCCTAATGTGGCGTCGATGGGAACTCTTTCCCAATTTG-3'
cmbA-r1	Reverse middle	5'-GACGCCACATTAGGATTTTGA-3'
cmbA-r2	Reverse end	5'-ATGCATCTAGACTAATCATGTTTACGCTTC-3'
Primers used for sequencing		
cmbA-seqf1	Forward (1)	5'-CTCCACAACTCTATTTGCAA-3'
cmbA-seqf2	Forward (2)	5'-GTGACTTTCAATATGTAATATTAA-3'
cmbA-seqf3	Forward (3)	5'-GTCGATGGGAACTCTTTCC- 3'
cmbA-seqf4	Forward (4)	5'-GGGCTATGATAAGAAAGTCTA-3'
cmbA-seqr3	Reverse	5'CGTTCCAGGTTGACCATCA-3'
Sip3	pSIP411 forward	5'-GTCTAAGGAATTGTCAGATAGGC-3'
Sip16	pSIP411 reverse	5'-ATTAGTCTCGGACATTCTGC-3'
Primer/Probe used for real-time PCR		
CmbA (lar_0958)	Forward	5'-ATCCAAACTGTGGCGATGTG-3'
CmbA (lar_0958)	Reverse	5'-AAGCTGTCCGCTAGCATCCA-3'
CmbA (lar_0958)	Probe[1]	6FAM-ATGGGCCATTATTTG-MGBNFQ
SecY (lr_0469)	Forward	5'-CCGCGTTTTGTTGAATGGA-3'
SecY (lr_0469)	Reverse	5'-TCGGGTTGCTTGATTAAGTTTTC-3'
SecY (lr_0469)	Probe[1]	6FAM-TAAACAAGGAGAAGTAGGACGG-MGBNFQ

[1]Taq probes, 6-FAM (6-carboxyfluorescein), MGB (6-carboxytetramethylrhodamine).

Supplementary material, Table S3

Table S3. Expression of *cmbA* (qRT-PCR)

Strain	**Fold change[a] Mean**	**Fold change[a] SD**
6475(pSIP-*cmbA*) vs 6475(pSIPΔ)[b]	355.9	88.2
6475 *cmbA*$^-$(pSIP-*cmbA*) vs 6475 *cmbA*$^-$(pSIPΔ)[b]	456.2	179.2
6475 *cmbA*$^-$(pSIP-*cmbA*) vs 6475(pSIP-*cmbA*)[b]	0.9	0.1

[a]Fold change in *cmbA* expression was calculated by the $\Delta\Delta C_T$ method ($2^{-\Delta\Delta CT}$). The presented values are mean±SD from two independent experiments.
[b]Comparison of SppIP induced cultures

Quantitative real-time reverse transcription PCR of *cmbA* expression

cmbA gene expression was determined by quantitative real-time reverse transcription PCR (qRT-PCR) in samples from Caco-2 adhesion assay to determine chromosomal and vector expression of *cmbA*. At the start of the Caco-2 adhesion experiments, an aliquot of the bacteria culture was mixed with RNA protect Bacteria Reagent (Qiagen) and frozen at -80 °C until RNA isolation. Purification and extraction of total RNA was done using the RNeasy Mini kit (Qiagen) according to manufacturer's instructions. NanoDrop was used to test the quality of RNA, and only samples with satisfactory quality were used for qRT-PCR. The qRT-PCR was performed as described previously (Rode *et al.*, 2007) using primers and Taqman® probes designed with Primer Express® Software v3.0 (Applied Biosystem) (primer and probes are listed in Table S1). The target site of the *cmbA* primer and probe set was upstream of the *cmbA* nonsense mutation. Relative gene expression was calculated by the ΔC_T method, using *secY* as the endogenous reference gene (Wall *et al.*, 2007). There was no difference in the amplification efficiencies of *cmbA* and *secY*. Fold change in gene expression was calculated using the $\Delta\Delta C_T$ method ($2^{-\Delta\Delta CT}$) (Livak & Schmittgen, 2001).

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Paper III

Immonumodulation of monocytes by probiotic and selected lactic acid bacteria

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Abbreviations: LAB, lactic acid bacteria; *Lact., Lactobacillus*; GIT, gastrointestinal tract; PPR, pattern recognition receptors; MAMPs, microbe associated molecular patterns; IL, interleukins; PBMC, peripheral blood mononuclear cells; PP, peyer's patches; IECs, intestinal epithelial cells; SDPs, sortase dependent proteins

Abstract

Some lactic acid bacteria (LAB), especially bacteria belonging to the genus *Lactobacillus*, are recognized as common inhabitants of the human gastrointestinal tract and have received considerable attention in the last decades due to their postulated health-promoting effects. LAB and probiotic bacteria can modulate the host immune response. However, much is unknown about the mediators and mechanisms responsible for their immunological effect. Here we present a study using cytokine secretion from the monocytic cell line THP-1 and NF-κB activation in the monocytic cell line U937-3xkB-LUC to elucidate immune stimulating abilities of LAB *in vitro*. In this study we investigate both commercially available and potential probiotic LAB strains, and the role of putative surface proteins of *Lactobacillus (Lact.) reuteri* using mutants. *Lact. reuteri* strains induced the highest cytokine secretion and the highest NF-κB activation, whereas *Lact. plantarum* strains and *Lact. rhamnosus* GG were low inducers/activators. One of the putative *Lact. reuteri* surface proteins, Hmpref0536_10802, appeared to be of importance for the stimulation of THP-1 cells and the activation of NF-κB in U937-3xkB-LUC cells. Live and UV-inactivated preparations resulted in different responses for two of the strains investigated. Our results add to the complexity in the interaction between LAB and human cells and suggest the possible involvement of secreted pro- and anti-inflammatory mediators of LAB. It is likely that it is the sum of bacterial surface proteins and bacterial metabolites and/or secreted proteins that induce cytokine secretion in THP-1 cells and activates NF-κB in U937-3xkB-LUC cells in this study.

Introduction

The human gut microbiota consists of over 1000 species [1] which are essential for the development and maintenance of a healthy and well-functioning immune system that can differentiate between pathogens and commensals. Some lactic acid bacteria (LAB), especially bacteria belonging to the genus *Lactobacillus*, are recognized as common inhabitants of the gastrointestinal tract (GIT) and have received considerable attention in the last decades due to their postulated health-promoting effects. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" by the Food and Agriculture Organization/World Health Organization (FAO/WHO). Most probiotics today belong to the genera *Lactobacillus* and *Bifidobacterium*. Probiotic bacteria can modulate the host immune response [2-5]. However, much is unknown about the mediators and mechanisms responsible for their immunological effect.

Cells of the human host can recognize probiotic bacteria. Specialized receptors on human cells (pattern recognition receptors (PPR)) recognize conserved bacterial molecular structures (microbe associated molecular patterns (MAMPs)). One result is signaling to induce production of cytokines, chemokines and other factors [6]. An important fraction of probiotic effector molecules resides in the bacterial cell envelope. This part of the bacteria is the first to interact with intestinal cells of the host. Examples of lactobacilli cell surface molecules that have the potential to be recognized by human cells are peptidoglycan (PG), lipoteichoic acid (LTA), wall teichoic acid (WTA), capsular polysaccharides (CPS) and extracellular (glyco)proteins. Many of these have been proven important as effector molecules for immune modulation by lactobacilli [7-9]. Furthermore, probiotic metabolites and genomic DNA is also known to modulate the host immune response [10,11].

NF-κB is a nuclear transcription factor. The NF-κB pathway is one key signaling channel for activation of immune responses following a variety of stimuli [12]. Cytokines are small, soluble secreted peptides or proteins that affect the growth or function of cells. Cytokines include interleukins (IL), interferons, chemokines, colony-stimulating factor and many growth factors. Cytokines do not have an effect on their own, but act after binding to specific surface receptors in the membrane of cells [13]. IL-8 (CXCL-8) is a chemokine which primarily attracts phagocytes such as neutrophiles and macrophages. Secretion of IL-8 is activated by pro-inflammatory cytokines (e.g. IL-1, TNF-α), bacterial (e.g. LPS) or viral products via the transcriptional factors NF-κB and AP-1 [14]. TNF-α is a pro-inflammatory

cytokine with a variety of biological effects. Local production of TNF-α plays an important role in the control and elimination of local infections. Due to its serious systemic effects, the expression of TNF-α is tightly regulated on the transcriptional, translational and post-translational level [15].

At present, there is an ongoing debate whether probiotic bacteria need to be alive to have a beneficial effect [16,17,10], as they pose a risk for the immunocompromised population. The identification of probiotic effector molecules with immunomodulatory properties is highly important for their application as bioactives that can be administered in a non-viable form. Furthermore, probiotic effector molecules allow prediction of what part of the population that would benefit of treatment with a specific strain [7].

Here we present a study using cytokine secretion from the monocytic cell line THP-1 and NF-κB activation in the monocytic cell line U937-3xkB-LUC in response to direct interactions with selected LAB. In this study we investigate both commercially available and potential probiotic LAB strains, and the role of putative surface proteins of *Lactobacillus (Lact.) reuteri* using mutants. Furthermore, we compare the effect of both live and UV-inactivated LAB, illustrating the importance of investigating both and demonstrating the complexity of LAB-host interaction.

Materials and methods

Bacterial culture and preparation of UV-inactivated bacteria

The *Lactobacillus* strains used in this study are shown in Table 1. Strains were maintained at −80 °C in 20% (v/v) glycerol. Before the experiments, strains were grown anaerobically on Man Rogosa Sharpe (MRS) agar (Oxoid, Hampshire, UK) for 48 h at 37 °C, scraped from MRS agar in Dulbecco's Phosphate Buffered Saline (DPBS) (SigmaAldrich, St. Louis, MO), and adjusted to a final concentration of 10^8 cfu/ml by measuring the optical density at 600 nm.

For preparation of UV-inactivated bacteria the strains were grown and adjusted as described above and concentrated to 10^9 cfu/ml. Strains were inactivated by UV-light for 20 min, and frozen −80 °C for later use. The number of bacterial cells/ml was determined by using a flow cytometer (guava easyCyte™, Millipore, Billerica, MA) after UV-inactivation. Only confirmed inactivated suspensions (no growth on MRS agar) were used.

Table 1. Species, strain identity, and origin of *Lactobacillus* used in this study

Strains	Relevant characteristics	Reference
L. reuteri		
DSM 20016	Type strain. Human intestine.	[56]
DSM 17938	Plasmid cured variant of ATCC 55730.Human breast milk.	[57]
ATCC PTA 6475	Earlier designated MM4-1A. Wild-type, host strain. Human breast milk.	[25]
6475 *cmbA*$^-$	Derivative of ATCC PTA 6475. Nonsense mutation in *cmbA*.	[31]
6475 *10146*$^-$	Derivative of ATCC PTA 6475. Nonsense mutation in *hmpref0536_10146*.	[31]
6475 *11993*$^-$	Derivative of ATCC PTA 6475. Nonsense mutation in *hmpref0536_11993*.	[31]
6475 *10802*$^-$	Derivative of ATCC PTA 6475. Nonsense mutation in *hmpref0536_10802*.	[31]
6475 *10154*$^-$	Derivative of ATCC PTA 6475. Nonsense mutation in *hmpref0536_10154*.	[31]
6475 *srtA*$^-$	Derivative of ATCC PTA 6475. Nonsense mutation in *srtA*.	[31]
L. plantarum		
NC8	Grass silage.	[58]
299v (DSM9843)	Sourdough. ProViva brand of probiotic products.	[59]
MF1298	Norwegian mutton salami.	[26]
L. rhamnosus		
GG (ATCC53103)	Human intestine. Commercially available probiotic bacteria.	[60]

Cell culture

The human monocytic leukemia cell line THP-1 (TIB-202™, ATCC®) was grown in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS), 150 µg/ml gentamicin, 2 mM L-glutamine and 0.5 mM 2-mercaptoethanol. The U937-3xkB-LUC cell line [18], a human monocytic cell line stably transfected with a luciferase reporter that contains 3 NF-κB binding sites, was maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin, 50 mg/mL streptomycin, and 75 µg/mL hygromycin. All solutions were obtained from Invitrogen (Carlsbad, CA). Both cell lines were routinely maintained at 37 °C in a humidified atmosphere of 5% CO_2, and subcultivated three times per week. After 20 (U937-3xkB-LUC) and 25 (THP-1) passages the cells were replaced by frozen stock.

Bacterial stimulation of THP-1 cells

24 h before experiments, THP-1 cells were plated out in 12-well tissue culture plates at the concentration 3×10^5 cells/ml. The following day, THP-1 cells and bacterial cells were mixed in the culture plates as follows: Approximately 5.7×10^5 THP-1 were stimulated with 1.5×10^8 live or UV-inactivated *Lact. plantarum* NC8, MF1298, and 299v, *Lact. rhamnosus* GG, *Lact. reuteri* DSM20016, DSM17938, and ATCC PTA 6475 (hereafter designated as 6475), and

mutant strains of *Lact. reuteri* 6475 in a total volume of 2 ml/well (a THP1-bacterial cell ratio of approximately 1:250) for 6 h in humidified atmosphere of 5% CO_2 at 37 °C. After 6 h the supernatants were harvested and stored at -20 °C until cytokine assay by sandwich ELISA. Experiments were performed with duplicate determinations and repeated three times. Gentamicin (150 µg/ml, as in the maintenance cell media) was used to prevent overgrowth of LAB during the 6 h incubation. HPLC measurements after co-culture revealed that the gentamicin dose applied effectively reduced overgrowth but did not affect the metabolism of the LAB severely.

Cytokine measurement

THP-1 secretion of the cytokines IL-8, IL-10 and TNF-α were analyzed by sandwich ELISA using commercially available capture and detection antibodies from BD-Pharmingen (Oxford, UK) as previously described [19]. The detection limits for the cytokines were 2 pg/ml for IL-8, and 4.1 pg/ml for IL-10 and TNF-α.

NF-κB assay

On the day before the experiments, U937-3xkB-LUC cells were sub-cultured at a density of 3×10^5 cells/ml. The following day, the cell media was changed to RPMI-1640 with 2 % FBS, 2 mM L-glutamine, 50 U/mL penicillin and 50 mg/mL streptomycin, and the cells were seeded out in white 96 well plates at a density of 1×10^5 cells/ml, 100 µl/well. U937-3xkB-LUC cells were incubated with all bacteria, both live and UV-inactivated, at a concentration of 1×10^5, 1×10^6, 1×10^7 and 1×10^8 cfu/ml for 6.5 h at 37 °C and 5 % CO_2 to measure induction of NF-κB activity. DPBS was used as a control. The NF-κB activity was determined by measuring the luciferase activity after addition of Bright-Glo Reagent (Promega, Madison, WI) as described by the manufacturer. Luminescence was detected for 1 min using Glomax96 Microplate Luminometer (Promega). Bacterial induction of NF-κB activity was calculated as percentage of control. Experiments were performed with triplicate determinations, and repeated three times. Cell viability was determined by the trypan blue exclusion assay after 6.5 h incubation with bacteria.

Statistical analysis

Results are expressed as the mean ± standard error mean (SEM) of three experiments with duplicate/triplicate determinations. Analysis of variance (ANOVA) with Tukey's post-hoc test was performed in Minitab version 16 (Minitab Ltd., Coventry, UK). Differences were

considered statistically significant at P≤0.05. Illustrations were created in Prism version 5.0 (GraphPad Software, La Jolla, CA).

Results

Cytokine production by THP-1 cell line

IL-8, IL-10 and TNF-α secretion from THP-1 cells was measured in cell supernatants by ELISA after 6 h co-incubation with live and UV-inactivated LAB. All strains were efficient inducers of the cytokines IL-8 and TNF-α, and some more efficient than others. On the contrary, IL-10 secretions after stimulation with both live and UV-inactivated LAB were minor (mostly below the detection limit (4 pg/ml) (results not shown)). THP-1 cells were stained with trypan blue and investigated under microscope at the end of experiments. No unusual cell features were observed (results not shown).

Lact. reuteri DSM 20016 and 6475, both live and UV-inactivated, induced significantly higher cytokine levels than *Lact. reuteri* DSM 17938, *Lact. plantarum* 299v, NC8 and *Lact. rhamnosus* GG (Fig 1a-b, 2a-b).

The cytokine secretion after stimulation with UV-inactivated bacteria (1b, 1d, 2b, 2d) was for most strains similar to the cytokine secretion after stimulation with live bacteria (1a, 1c, 2a, 2c). However, deviations were observed for *Lact. plantarum* MF1298 and *Lact. reuteri* 6475 10802$^-$. The IL-8 secretion after stimulation with live *Lact. plantarum* MF1298 similar to the other *Lact. plantarum* strains (Fig 1a), whereas the UV-inactivated MF1298 induced a significant higher cytokine secretion compared to the other *Lact. plantarum* strains (1b) and a 10 fold higher IL-8 secretion compared to the live MF1298 (Fig 1a, 1b). Furthermore, the TNF-α secretion after stimulation with UV-inactivated MF1298 (Fig 2b) was 1.6 fold higher compared to TNF-α secretion after stimulation with live MF1298 (Fig 2a). *Lact. reuteri* 6475 10802$^-$ induced the same level of IL-8 and TNF-α as wild-type 6475 and other mutant strains after incubation with live bacteria (Fig 1c, 2c), whereas the UV-inactivated 6475 10802$^-$ induced significantly lower IL-8 and TNF-α levels compared to the wild type 6475 and other mutant strains (Fig 1d, 2d). The IL-8 secretion induced by UV-inactivated 6475 10802$^-$ was 4 fold reduced compared to live 6475 10802$^-$ (Fig 1c-d), and the TNF-α secretion induced by UV-inactivated 6475 10802$^-$ was 10 fold reduced compared to the live strain (Fig 2c-d).

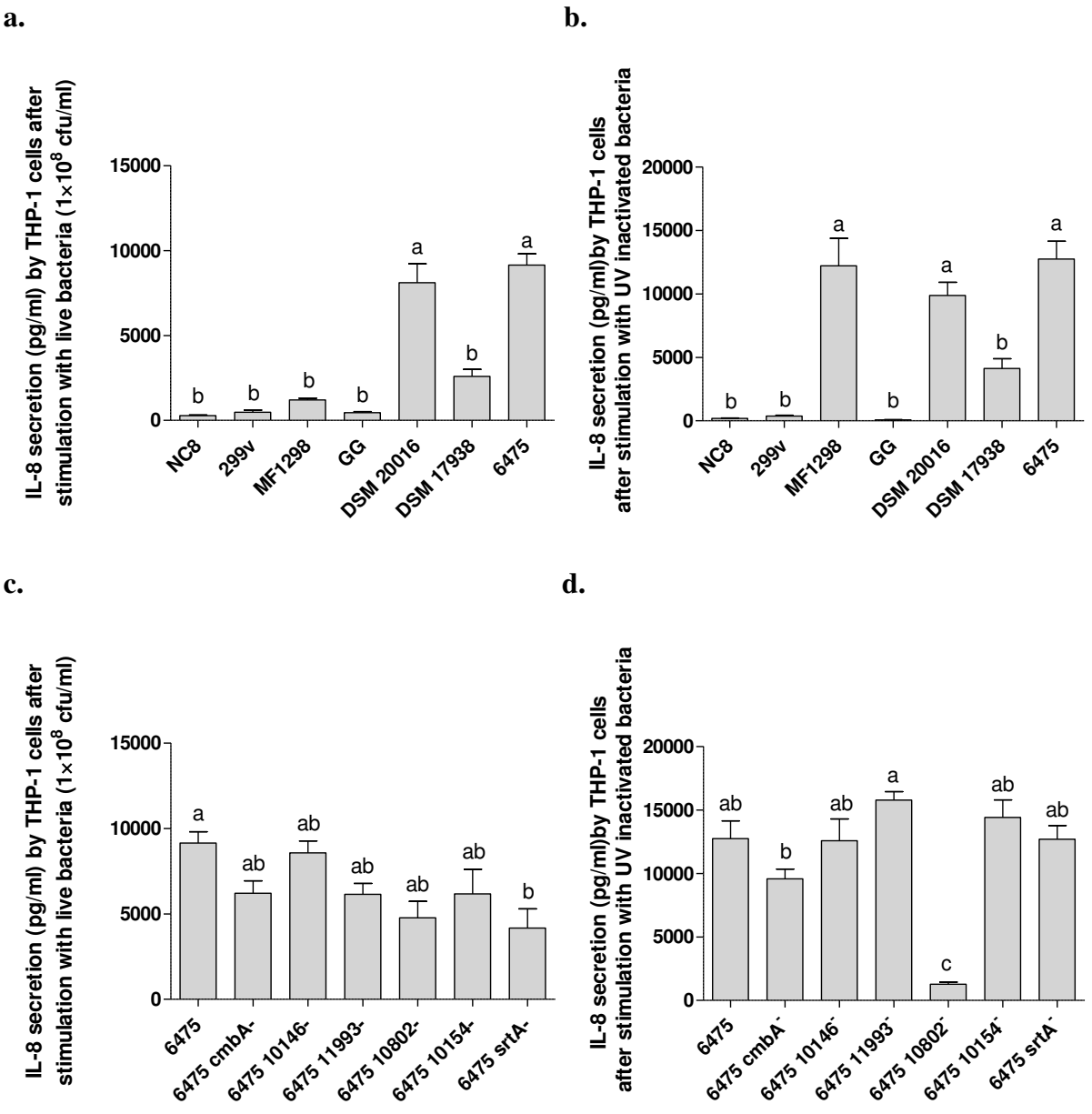

Figure 1. IL-8 secretion from THP-1 cells after 6 h co-culture with live LAB strains (a), UV-inactivated LAB strains (b), live mutant strains of *Lact. reuteri* (c) and UV-inactivated mutant strains of *Lact. reuteri* (d). IL-8 was measured by sandwich ELISA using commercially available antibodies (BD-Pharmingen). The results are expressed as mean ± SEM, n=3. Letters above bars refer to the ANOVA: means that do not share a letter are significantly different (ANOVA, Tukey's post-hoc test), $P \leq 0.05$.

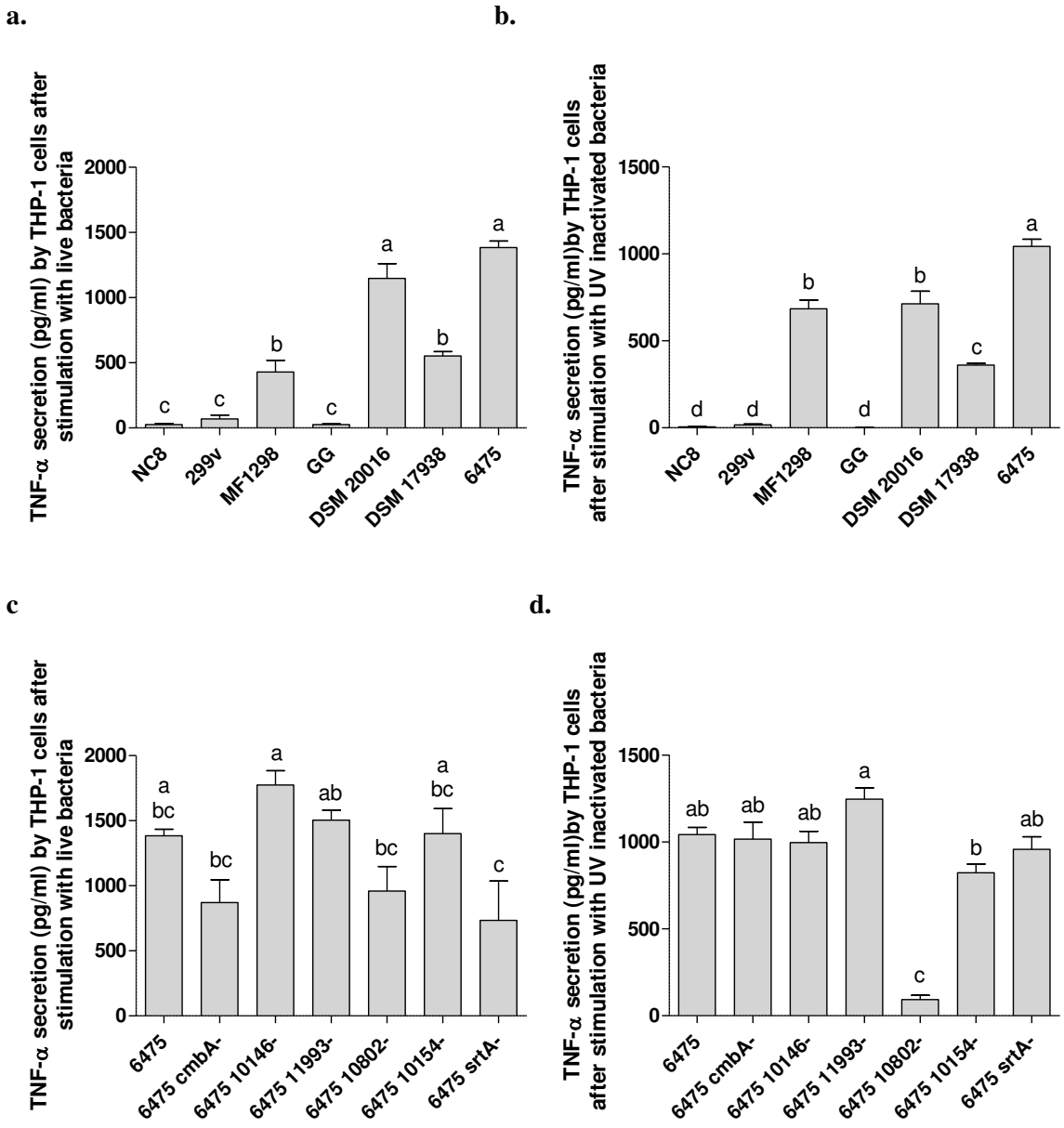

Figure 2. TNF-α secretion from THP-1 cells after 6 h co-culture with live LAB strains (a), UV-inactivated LAB strains (b), live mutant strains of *Lact. reuteri* (c) and UV-inactivated mutant strains of *Lact. reuteri* (d). TNF-α was measured by sandwich ELISA using commercially available antibodies (BD-Pharmingen). The results are expressed as mean ± SEM, n=3. Letters above bars refer to the ANOVA: means that do not share a letter are significantly different (ANOVA, Tukey's post-hoc test), $P \leq 0.05$.

Lact. reuteri 6475 10802$^-$ and srtA$^-$ revealed a significantly reduced cytokine induction compared to the wild type strain 6475. The IL-8 and TNF-α secretion after stimulation with UV-inactivated *Lact. reuteri* 6475 10802$^-$ was significantly reduced compared to the wild type 6475 (1280 pg/ml vs 12746 pg/ml and 93 pg/ml vs. 1044 pg/ml respectively) (Fig 1d, 2d).

However, there were no significant differences between live *Lact. reuteri* 6475 10802$^-$ and the wild type strain with regard to IL-8 and TNF-α secretion (Fig 1c, 2c). Incubation with live *Lact. reuteri* srtA$^-$ resulted in significantly lower IL-8 concentration in supernatants compared to 6475 (4173 pg/ml vs. 9140 pg/ml) (Fig 1c), an effect which was not observed for TNF-α (2c) or after stimulation with UV-inactivated 6475 srtA$^-$ (Fig 1d, 2d).

Activation of NF-κB in U937-3xkB-LUC cell line

NF-κB activation was tested after 6.5 h co-culture with U937-3xkB-LUC cells and live and UV-inactivated LAB. When the strains were tested at low concentrations (1×10^5 and 1×10^6 cfu/ml) no major differences in their ability to induce NF-κB were observed (results not shown), and at a high concentration, 1×10^8 cfu/ml, the U937-3xkB-LUC cells died (results not shown). When the strains were tested at 1×10^7 cfu/ml, a clear effect on the NF-κB activation was observed.

Live LAB revealed a high ability to activate NF-κB. In general, *Lact. reuteri* strains were the strongest activators followed by *Lact. rhamnosus* GG, *Lact. plantarum* 299v, MF1298 and NC8. *Lact. reuteri* 6475 and DSM 20016 induced a significantly higher NF-κB activation compared to *Lact. plantarum* 299v, NC8, MF1298 and *Lact. rhamnosus* GG (Fig 3a). There were no major differences in NF-κB activation after incubation with UV-inactivated LAB (113% to 186%) (Fig 3b). However, *Lact. reuteri* 6475 induced a significantly higher NF-κB activation compared to *Lact. plantarum* 299v, NC8 and *Lact. rhamnosus* GG (Fig 3b). UV-inactivated LAB (Fig 3b, 3d) generally induced a lower NF-κB activation than live LAB (Fig 3a, 3c). For all *Lact. plantarum* strains investigated there were no major difference between UV-inactivated and live strains in their ability to activate NF-κB (Fig 3a-b). Whereas, especially live *Lact. reuteri* strains and to some extent live *Lact. rhamnosus* GG induced a much higher NF-κB activation compared to the UV-inactivated strains (Fig 3a-b).

There were no significant differences between *Lact. reuteri* 6475 and its mutant strains (live and UV-inactivated) in their ability to induce NF-κB activation in U937-3xkB-LUC cells (Fig 3c-d). Although not statistically significant, *Lact. reuteri* 6457 10802$^-$ induced the lowest NF-κB activation of the 6575 strains (112% vs. 186% (UV-inactivated) and 261% vs. 419% (live), 6457 10802$^-$ vs. wild type) (Fig 3 c-d).

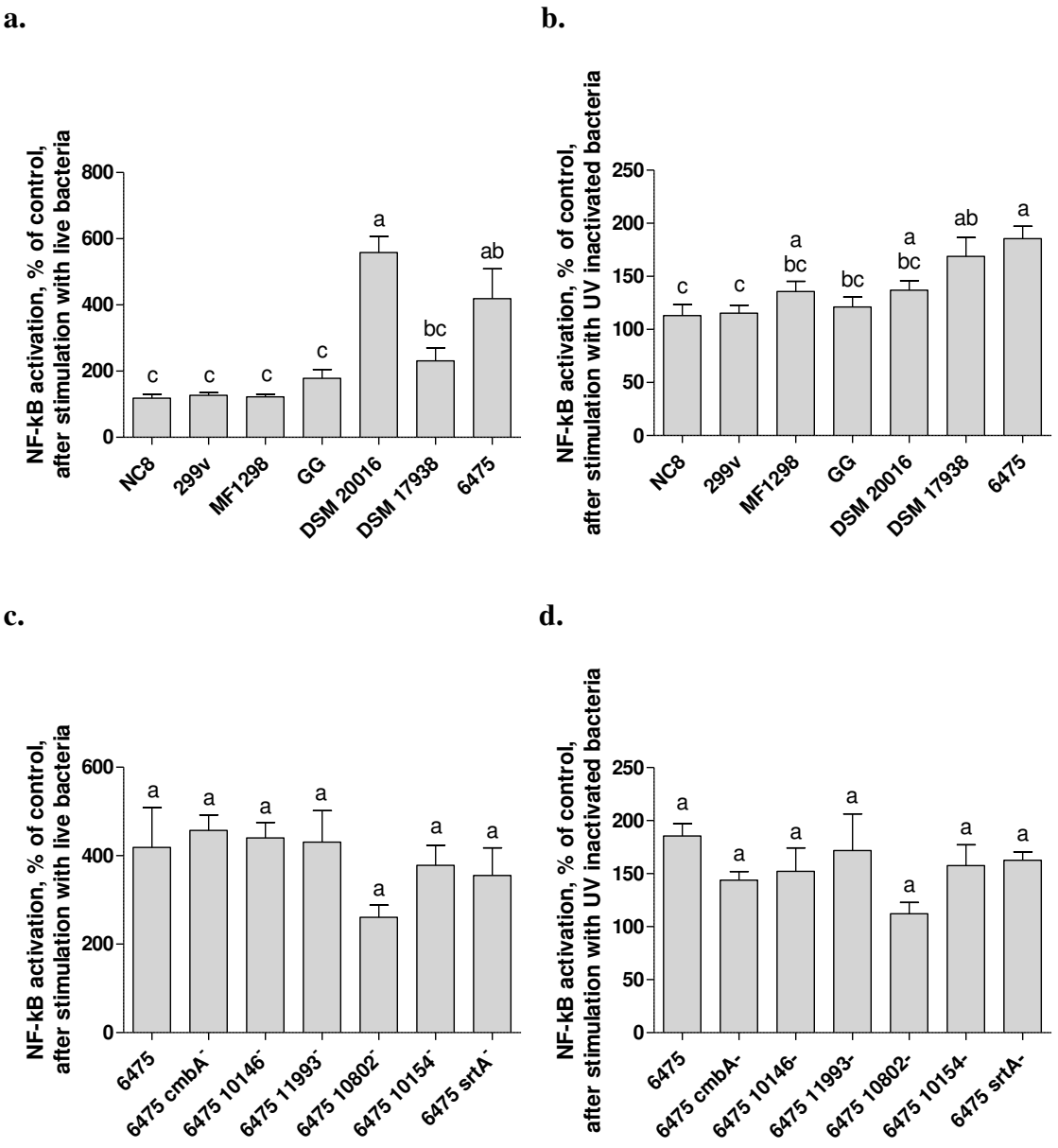

Figure 3. Effect of *Lactobacillus* strains on NF-κB activity. U937-3xκB-LUC cells were incubated with live LAB strains (a), UV-inactivated LAB strains (b), live mutant strains of *Lact. reuteri* (c) and UV-inactivated mutant strains of *Lact. reuteri* (d) for 6.5 h before luciferase activity was measured (Promega). The results are expressed as mean ± SEM, n=3. Letters above bars refer to the ANOVA: means that do not share a letter are significantly different (ANOVA, Tukey's post-hoc test), $P \leq 0.05$.

Discussion

Many studies describe immune stimulatory, or modulatory, properties of probiotic bacteria both *in vitro*, in animal studies and in clinical trials. However, despite intense research the overall evidence for immune effects of probiotics is limited [20,21]. Furthermore, most

probiotic effects that have been demonstrated appear to be species-specific or even strain-specific [7]. In the literature, peripheral blood mononuclear cells (PBMC) are commonly used to screen for probiotic anti-inflammatory properties. However, the results using PBMC will differ from donor to donor. Thus, we selected two monocytic cell lines for testing the selected LAB and probiotic bacteria. In humans, the situation is more complex. Peyer's patches (PP) are organized lymphoid nodules of the ileum. PP are covered by a epithelium that contains specialized microfold cells (M cells) which sample antigen directly from the lumen and deliver it to antigen-presenting cells resulting in immunity and secretion of IgA [6]. Cells such as dendritic cells (DC), macrophages and B cells are specialized antigen presenting cells that patrol mucosal tissues and receive antigens from the periphery [22]. Furthermore, probiotics or molecules of probiotics can induce immune responses in the host by binding to PRR expressed on immune cells intestinal epithelial cells [23]. Nevertheless, *in vitro* studies using cell lines are highly important to understand more about the complex interaction between LAB/probiotic bacteria and the host.

We found that *Lact. reuteri* 6475 and DSM 20016 induced the highest cytokine secretion from THP-1 cells and the strongest activation of NF-κB in the U937-3xkB-LUC cell line, whereas other known probiotic bacteria such as *Lact. plantarum* 299v and *Lact. rhamnosus* GG had little effect. Furthermore, we found that the *Lact. reuteri* protein Hmpref0536_10802 is of importance for the induction of IL-8 and TNF-α in THP-1 cells.

Immunomodulatory effects of selected lactic acid bacteria

Basal cytokine secretion from THP-1 cells is low, often below the detection limit. Hence, all LAB investigated in this study are potent stimulators, with *Lact. reuteri* DSM 20016 and 6475 strains being the strongest cytokine inducers and *Lact. plantarum* 299v and NC8 and *Lact. rhamnosus* GG being the lowest stimulators. These results are in accordance with our previous study investigating 18 LAB strains, where we observed that *Lact. reuteri* strains differ significantly from other LAB strains in typical probiotic assays [24]. Of the *Lact. reuteri* strains in this study, DSM 17938 in general induced a significant lower cytokine secretion from THP-1 cells compared to the other *Lact. reuteri* strains. This can probably be explained at the genome level as DSM 20016 and 6475 are clustered in one phylogenetic group (lineage II) of *Lact. reuteri*, and strain DSM 17938 in another (lineage VI) [25]. Live *Lact. plantarum* MF1298 induced high cytokine secretion from THP-1 cells in this study, and differ from the other *Lact. plantarum* strains investigated. Notably, *Lact. plantarum* MF1298

has been shown to possess several *in vitro* probiotic characteristics in other studies [24,26]. However, in a small, randomised controlled clinical trial, this strain revealed unfavorable effects in subjects with irritable bowel syndrome [27].

Activation of NF-κB activation in U937-3xkB-LUC cell line

The NF-κB pathway is one key signaling channel for activation of immune responses secondary to a variety of stimuli. This pathway represents an important point of communication between probiotics and beneficial microbes and cells of the host [28]. NF-κB is known for its dualistic function, and is important for intestinal immune homeostasis [29]. In the intestinal epithelia, intrinsic NF-κB signaling prevents apoptosis of intestinal epithelial cells (IECs) avoiding breaches of the epithelial barrier, whereas excessive NF-κB activation of IECs promotes detrimental intestinal inflammation [30].

We found that live *Lact. reuteri* strains were the strongest NF-κB activators. The high level of NF-κB activation observed for live *Lact. reuteri* was not observed after incubation with UV-inactivated *Lact. reuteri*. The reason for this is not known. It could be that some metabolite or secreted protein from live *Lact. reuteri* is important for the activation of NF-κB. *Lact. plantarum* NC8, MF1298 and the well-known probiotic strains *Lact. plantarum* 299v and *Lact. rhamnosus* GG did not induce *NF*-κB in our assay.

The importance of *Lactobacillus reuteri* Hmpref0536_10802 and SrtA

The initial observation that certain *Lact. reuteri* strains generally were more immunostimulatory than other LAB used in this study, prompted us to investigate *Lact. reuteri* strain 6475 more closely and take advantage of the availability of mutants of this strain where the genes of putative sortase dependent, surface associated proteins were inactivated [31]. Sortase dependent proteins (SDPs) are suggested to play a crucial role in *Lactobacillus*-host interactions [2], and many have been shown to impact the adhesive ability of various *Lactobacillus* strains [31-34]. SDPs have a common molecular structure that includes an N-terminal signal peptide, often with an YSIRK-G/S motif that promotes secretion [35] and directs the protein to a specific surface localisation [36], a C-terminal LPxTG motif, followed by a C-terminal transmembrane helix and a positively charged tail [2,37]. The LPxTG motif is recognized by sortase (SrtA) [37-39], which in turn is responsible for anchoring of the protein to the cell wall. LPxTG proteins are likely to also be important for stimulation of immune

cells of the host. The mannose-specific adhesin (Msa) of *Lact. plantarum* WCFS1 is one example of such a protein [40].

In its UV-inactivated form the mutant strain *Lact. reuteri* 6475 10802$^-$ induced significantly lower cytokine secretion from THP-1 cells compared to the 6475 wild type. According to the genome sequence of *Lact. reuteri* 6475, *hmpref0536_10802* encodes a polypeptide of 645 amino acids containing N-terminal Gamma-glutamyl cyclotransferase (GGCT)-like domains (also called AIG2-like family). Its homologue in strain DSM 20016 is annotated as an amidase. It has all the signatures of an SDP (a signal sequence, a C-terminal LPxTG motif followed by a hydrophobic region predicted to be a transmembrane helix and a positively charged tail), and is therefore most likely displayed on the surface of the bacterial cells. The lower cytokine secretion induced by the mutant indicates that the protein Hmpref0536_10802 is of importance for the direct interaction and the following cytokine secretion from THP-1 cells. The precise function of the protein is not known, but we have previously shown that *Lact. reuteri* 6475 10802$^-$ has a reduced ability to adhere to porcine mucus, but not to an intestinal cell line, compared to the wild type [31]. *Lact. reuteri* 6475 is a candidate probiotic known for its anti-inflammatory properties. Mechanisms such as activation of c-Jun and the transcription factor AP-1 [41] and the ability to convert L-histidine into the immunoregulatory signal histamine [42] are of importance for the anti-inflammatory effects of *Lact. reuteri* 6475. Furthermore, the genes cyclopropane fatty acid synthase (*cfa*) [43] and *Lact. reuteri*-specific immunoregulatory (*rsiR*) gene [44] has been implicated in the anti-inflammatory properties of *Lact. reuteri* 6475. In addition to these immunomodulatory properties *in vitro*, *Lact. reuteri* 6475 has also shown promising effects in animal studies [45-47].

In this study also live *Lact. reuteri* srtA$^-$ induced a significantly reduced IL-8 secretion from THP-1 cells compared to the wild type. The *srtA* mutant represents the sum of ineffective anchoring of SDPs proteins, and it is impossible to say if one (e.g Hmpref0536_10802) or more of these proteins are responsible for the observed effect. Previous studies have shown that SDPs can still be found (significantly decreased in abundance) as surface proteins in *srtA* mutants [48,49]. Thus, it is likely that while LPxTG proteins are not covalently coupled to the peptidoglycan in the absence of SrtA they remain surface associated in the cytoplasmic membrane by the C-terminal hydrophobic domain and the positively charged tail and can still induce cytokine secretion in THP-1 cells.

Live and UV-inactivated lactic acid bacteria induce different responses *in vitro*

Immunomodulatory studies use live bacteria, inactivated bacteria (heat, irradiation etc.), cell surface components, genomic DNA or cell-free supernatants. There are a limited number of studies which investigate the effect of both live LAB, inactivated LAB and cell supernatants like in this study [50-53]. The majority of the strains investigated in this study induced the same level of cytokines when tested live and as UV-inactivated. However, UV-inactivated *Lact. plantarum* MF1298 induced a high cytokine secretion from THP-1 whereas live MF1298 induced a low cytokine secretion. Furthermore, UV-inactivated *Lact. reuteri* 6475 10802$^-$ induced a low cytokine secretion from THP-1 cells and live 6475 10802$^-$ induced a high cytokine secretion. We observed no effect of cell-free spent media or cell-free media conditioned with UV-inactivated strains for 6 h (preliminary unpublished results). In addition, separating THP-1 cells and LAB with a porous filter membrane gave no detectible cytokine secretion from the THP-1 cells (preliminary unpublished results). This indicates that the direct contact between bacteria and THP-1 cells is highly important for the observed effects. The reason why *Lact. plantarum* MF1298 and *Lact. reuteri* 6475 10802$^-$ induce different responses is unknown. Plausible explanations can be that the live bacteria produce metabolites or secrete proteins which reduce (*Lact. plantarum* MF1298) or increase (*Lact. reuteri* 6475 10802$^-$) the cytokine secretion from THP-1 cells, or that some compound on the live bacteria masks the cell surface molecule that interacts with the THP-1 cell (*Lact. plantarum* MF1298). This study is one of few which compare the immunomodulatory properties of both live and UV-inactivated LAB, and gives an excellent example of the importance of investigating both.

It is clear that both live and dead probiotic bacteria can generate a wide range of biological responses [10]. However, according to the WHO definition, probiotic bacteria are live bacteria. Live cells in a probiotic product will indeed lose viability and the actual product will contain varying populations of dead cells. The population of dead cells might be even larger than that of live cells but this is frequently not known. Furthermore, many of the live ingested probiotic bacteria will not survive the harsh conditions of the stomach and the intestine [54,55]. Therefore, some of the benefits derived from consumption of probiotics are more likely to be due to the presence of metabolites or of dead probiotic cells in the gastrointestinal tract. See Gobbetti et al. [17], Taverniti et al. [11] or Adams [10] for review on the live/dead probiotic discussion.

Conclusion

We found that *Lact. reuteri* DSM 20016 and the probiotic candidate strain 6475 induced the highest cytokine secretion and the highest NF-κB activation. Well known probiotic strains such as *Lact. plantarum* 299v and *Lact. rhamnosus* GG had little effect on cytokine secretion from THP-1 cells and activation of NF-κB in the U937-3xkB-LUC cell line. The putative *Lact. reuteri* LPxTG protein Hmpref0536_10802 appeared to be of importance for the stimulation of THP-1 cells and possibly the activation of NF-κB in U937-3xkB-LUC cells. Furthermore, live and UV-inactivated preparations resulted in different responses for two of the strains investigated. This further adds to the complexity in the interaction between LAB and human cells and suggests the possible involvement of secreted pro and anti-inflammatory mediators of LAB.

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Paper IV

No effect of commercial probiotic and selected lactic acid bacteria in a model system of the intestinal epithelial barrier *in vitro*

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Abstract

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host. Several mechanisms by which probiotics mediate their health benefits have been suggested, and enhancement of the intestinal epithelial barrier is one of them. The intestinal epithelia form a selective permeable barrier between the intestinal lumen and gut content and the submucosa. Disruption of the barrier function and elevated permeability to luminal toxins, allergens and pathogens is now recognized as having a role in various gastrointestinal disorders such as Chron's disease, irritable bowel syndrome (IBS), coeliac disease and type-1 diabetes. In this study we investigate the effect of selected commercial probiotic bacteria and lactic acid bacteria (LAB) on the epithelial barrier *in vitro*. The investigation includes functional assessment of polarized Caco-2 monolayer by measuring transepithelial electrical resistance (TER) and paracellular permeability of fluorescein isothiocyanate–dextran (FITC-Dextran), and quantitative real-time reverse transcription PCR (qRT-PCR) of the tight junction genes *zo-1* and *claudin-1*. The aim was to test if pre-incubation with LAB could prevent or reduce induced damage of the Caco-2 monolayer, and if incubation with LAB could aid in restoring the epithelial barrier after disruption. We found no further beneficial effect of the probiotic bacteria and LAB in this *in vitro* model of the intestinal barrier compared to the control.

Introduction

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host [1]. Most probiotics commercially available today belong to the genera *Lactobacillus* and *Bifidobacterium*. Several mechanisms by which probiotics mediate their health benefits have been suggested, and enhancement of the intestinal epithelial barrier is one of them [2].

The intestinal epithelial barrier consists of a monolayer of epithelial cells, a mucus layer, antimicrobial peptides, and secretory IgA [3]. Enterocyte cell-cell connections are essential for this barrier witch forms a selective permeable barrier between the intestinal lumen and gut content and the submucosa. The intracellular junctional complexes consist of tight junctions (TJ), adherence junctions (AJ), gap junctions and desmosomes [4, 5]. The TJ transmembrane proteins occludins and claudins link enterocytes together through their extracellular loops, and intracellular zonula occludens (ZO) scaffolding proteins link the transmembrane proteins to the actomyosin cytoskeleton and several cytoplasmic regulatory proteins [3]. In TJ, claudins seems to be the major determinants of intestinal permeability [6]. Some claudins (e.g. claudin-1) are barrier builders whereas others (e.g. claudin-2 and -10) are mediators of paracellular permeability, and the distribution of claudins along the intestine reflects the barrier properties/physiological function [7].

Intestinal permeability can be modulated directly by bacteria by release of metabolites (e.g. acetate and butyrate), cellular structural component or through the secretion of soluble peptides and toxins. Bacteria can also alter the intestinal permeability indirectly by interactions with the host immune cells and subsequent release of cytokine which can both enhance or reduce the barrier function [8]. Furthermore, the epithelial barrier is regulated by phosphorylation of TJ proteins and crosstalk with cellular signaling pathways (see González-Mariscal [9] for review). Disruption of the barrier function and elevated permeability to luminal toxins, allergens and pathogens (also called “leaky gut”) is now recognized as having a role in various gastrointestinal disorders such as Chron’s disease, irritable bowel syndrome (IBS), coeliac disease and type-1 diabetes [8, 10].

The aim of the present study was to investigate the effect of selected commercial probiotic bacteria and lactic acid bacteria (LAB) on the epithelial barrier *in vitro*. The investigation includes functional assessment of polarized Caco-2 monolayer by measuring transepithelial electrical resistance (TER) and paracellular permeability of fluorescein

isothiocyanate–dextran (FITC-Dextran), and characterization of TJ gene expression by quantitative real-time reverse transcription PCR (qRT-PCR).

Materials and methods

Bacterial culture and preparation of UV inactivated bacteria

The origin of *Lactobacillus* strains that were used is shown in Table 1. Strains were maintained at −80 °C in 20% (v/v) glycerol. Before experiments, strains were grown anaerobically on Man Rogosa Sharpe (MRS) agar (Oxoid, Hampshire, UK) for 48 h at 37 °C, scraped from MRS agar in Dulbecco's Phosphate Buffered Saline (DPBS) (SigmaAldrich, St. Louis, MO), and adjusted to a final concentration of 10^8 cfu/ml by measuring the optical density at 600 nm.

Table 1. Species, strain identity, and origin

Species	Strain idenity	Origin
Lactobacillus plantarum	NC8	Grass silage [11]
Lactobacillus plantarum	299v (DSM 9843)	Sourdough. ProViva brand of probiotic products. [12]
Lactobacillus plantarum	MF1298	Norwegian mutton salami [13]
Lactobacillus rhamnosus	GG (ATCC 53103)	Human intestine [14]. Commersial availible probiotic bacteria.
Lactobacillus reuteri	DSM 20016	Type strain. Human intestine [15]
Lactobacillus reuteri	DSM 17938	Plasmid cured variant of ATCC 55730. Human breast milk. [16]. Commersial availible probiotic bacteria.

DSM, Deutsche Sammlung von Mikroorganismen; ATCC, American Type Culture Collection

Cell culture

The commonly used human colorectal adenocarcinoma cell line Caco-2, a generous gift from Prof. Kirsten Sandvig, The Norwegian Radium Hospital, was used. Caco-2 cells were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% heat inactivated foetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin. All solutions were obtained from Invitrogen (Carlsbad, CA). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and subcultivated at 70 to 80% confluence.

To obtain polarized monolayers, Caco-2 cells were seeded onto cell culture inserts (0.4 μm pore size, 12 mm diameter, polyethylene terephthalate, Millipore Bedford, MA) at a concentration of 3×10^5 cells per filter. The cell culture inserts were maintained with a volume of 1 ml in the apical compartment and 2 ml in the basolateral compartment, and cell media was changed three times per week. DMEM in the maintenance media was replaced with low glucose DMEM (1 g/l), to prevent overgrowth of bacteria during experiments. Functional polarity was developed after 14 days, and confirmed by scanning electron microscopy before each experiment. TER values of fully differentiated monolayers were on average 400 Ω cm^2.

Transepithelial electrical resistance (TER)

TER was measured on polarized monolayers of Caco-2 using the Millicell Electrical Resistance System. (Millipore, Bedford, MA). Only inserts with electrical resistance over 400 Ω were used in experiments.

N-(3-oxododecanoyl)-L-homoserine lactone (3O–C_{12}–HSL, hereafter HSL) from *Pseudomonas aeruginosa* has been shown to disturb the integrity of the Caco-2 epithelial barrier by activation of p38 and p42/44 MAPK pathways [17], and was used to impair the Caco-2 monolayer in these experiments.

TER was evaluated in two experimental setups (illustrated in Figure 1). In setup one the aim was to test if incubation with LAB can prevent or reduce HSL damage of the cell layer. The day before experiment the filters were washed twice with DPBS to remove antibiotics from the cells and the filters were placed in new 12 well plates. Fresh media without antibiotics were added to the filters, 900 μl on the apical side and 2 ml on the basolateral side. LAB were adjusted to 10^8 cfu/ml as described above and diluted to 10^6 cfu/ml. Caco-2 filters were co-incubated with bacteria, 10^5 cfu/ml, for approximately 16 h (overnight) at 37 °C and 5% CO_2. The following day the filters were washed three times with DPBS to remove all bacteria and 1 ml fresh media without antibiotics were added to the apical compartment. TER was measured three times for each filter before 1 μl HSL (100mM) was added to the apical side (with the exception of the DPBS control). TER was measured after 1, 2, 4 and 6 h incubation. Due to bacterial removal/degradation of HSL, LAB and HSL could not be present together in our *in vitro* model.

In setup two the aim was to investigate if LAB aid in regeneration of the epithelial barrier after HSL disruption of the cell layer. The day before experiment the cells were washed as described for setup one. On the day of experiment, bacteria was adjusted to 10^8 cfu/ml as described above, centrifuged at 3000 rpm for 10 min and resuspended in equal

volume of cell media without antibiotics. The filters were washed twice in DPBS and fresh media without antibiotics was added to the wells, 1 ml on the apical side and 2 ml on the basolateral side. TER was measured three places on each filter immediately before the start of the experiment (0 h). 1 µl HSL (100 mM) was added to each well on the apical side and the plates were incubated for 30 min before new TER measurements. Following this, all filters, except the HSL control, were washed three times with DPBS to remove HSL. The filters were transferred to new 12 well plates and fresh cell media was added to the filters, 0.5 ml on the apical side and 2 ml on the basolateral side. Following this, 0.5 ml of the 10^8 cfu/ml cultures in cell media was added to the apical side (resulting in a test concentration of 5×10^7 cfu/ml). TER was measured 1, 2, 4 and 6 h after the addition of bacteria. pH was measured in all filters at the end of the experiment.

Ratio of TER (TER_{tx}/TER_{t0}) was calculated for each strain. Experiments were conducted with duplicate determinations, and repeated three to four times.

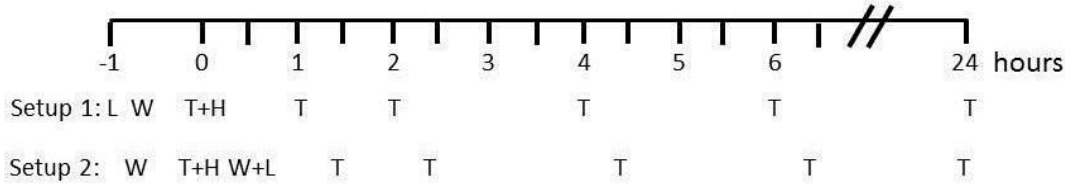

Figure 1. Schematic illustration of setup 1 and 2 in TER experiments. L indicates *Lactobacillus*; W indicates DPBS wash; T indicates TER; H indicates HSL

Paracellular permeability

Preparation of polarized monolayers, test setup and aim was the same as for TER setup one described above. The Caco-2 filters were treated as in TER setup one the day before experiment. On the day of experiment, the filters were washed three times to remove bacteria. The filters were transferred to new 12 well plates and cell media without phenol red, antibiotics and serum was added to all filters, 920 µl to the apical side and 1 ml on the basolateral side. 80 µl FITC–Dextran (25 µg/ml) with an average molecular weight of 3000 to 5000 Da (FD4, SigmaAldrich) was added to each well on the apical side. Finally, 1 µl of HSL (100 mM) was added to all wells on the apical side except for the cell media control wells. After 2, 4 and 6 hours the filters were transferred to new 12 well plates with 1 ml media on the basolateral side, this to prevent contamination of FITC–Dextran over time. At the end of

the experiment, FITC–Dextran concentrations in all basolateral samples were determined using a flourometer (FLUOstar OPTIMA, BMG LABTECH, Ortenberg, Germany). Experiments were conducted with duplicate determinations on the same day as TER experiments and repeated three to four times.

Quantitative real-time reverse transcription PCR of tight junction gene expression

Polarized filters were prepared as described above, and the filters were treated as in TER setup two. 1.5 and 4 h after incubation with LAB the filters were washed three times with DPBS. Following this, 150 µl DPBS and 750 µl RNA protect Cell Reagent (Qiagen) were added to the filters. After approximately 5 min at room temperature the Caco-2 cells were mixed with a pipette to detach all cells form the filters, transferred to micro tubes and frozen at -80 °C until RNA isolation. Experiments were conducted with duplicate determinations and repeated three times (separate from TER and paracellular transport assay). Purification and extraction of total RNA was done using the RNeasy Plus Mini Kit and QIAshredder columns (Qiagen N.V., Venlo, The Netherlands) according to manufacturer's instructions. NanoDrop (NanoDrop Technologies, Inc., Wilmington, DE) was used to test the RNA concentration. Selected samples were analysed using a Bioanalyzer system (Agilent Technologies, Inc., Santa Clara, CA) to confirm satisfactory quality for qRT-PCR. The qRT-PCR was performed as described previously [18] using TaqMan Gene Expression Assays from Life Technologies Ltd (Paisley, UK) (Assay ID: Hs01551861_m1 (ZO-1), Hs00221623_m1 (claudin-1), Hs99999901_s1 (18S)). Relative gene expression was calculated by the ΔC_T method, using 18S as the endogenous reference gene. There was no difference in the amplification efficiencies.

Statistical analysis

Results are expressed as the mean and standard error mean (SEM) of three to four experiments with duplicate determinations. The statistical analyses were preformed in GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). TER and paracellular transport data were analyzed for each time point using ANOVA with Tukey's post-hoc test. Differences were considered statistically significant at $p \leq 0.05$.

Results

Bacterial effect on the transepithelial electrical resistance of polarized Caco-2 monolayers

TER of polarized Caco-2 cells was used as an indicator of the intestinal epithelial barrier function, and the effect of selected LAB was tested in two experimental setups.

In TER setup one the aim was to test whether prophylactic incubation with LAB could prevent or reduce HSL damage of the cell layer. The control, DPBS, was not treated with HSL in the experiment. None of the LAB tested succeeded in preventing HSL damage in the Caco-2 monolayer. After addition of HSL, TER values in all monolayers decreased rapidly. The variance, both within and between experiments, was high. *L. reuteri* DSM 17938 had the lowest reduction in TER over 6 h, *L. reuteri* DSM 20016 and *L. plantarum* strains revealed an intermediate drop in TER over 6 h, whereas *L. rhamnosus* GG had the highest drop in TER over 6 h (Supplementary material, Table S1). However, there were no significant differences between the strains (Figure 2).

In TER setup two the aim was to test if LAB aid in regeneration of the epithelial barrier after HSL disruption of the Caco-2 monolayer. The control was treated with HSL before removal after 0.5 h as for all LAB strains. Addition of HSL to the Caco-2 monolayer induced a rapid decrease in TER. The drop in TER values continued up to 4 h, 3.5 h after removal of HSL and addition of LAB or DPBS (Figure 3 and Supplementary material, Table S2). *L. plantarum* 299v and *L. rhamnosus* GG were the strains that revealed the best regeneration of TER (from lowest to highest TER value after removal of HSL, Supplementary material, Table S2). However, none of the LAB strains tested were more efficient compared to the control (DPBS) in regeneration of TER with any statistical significance (ANOVA) (Figure 3).

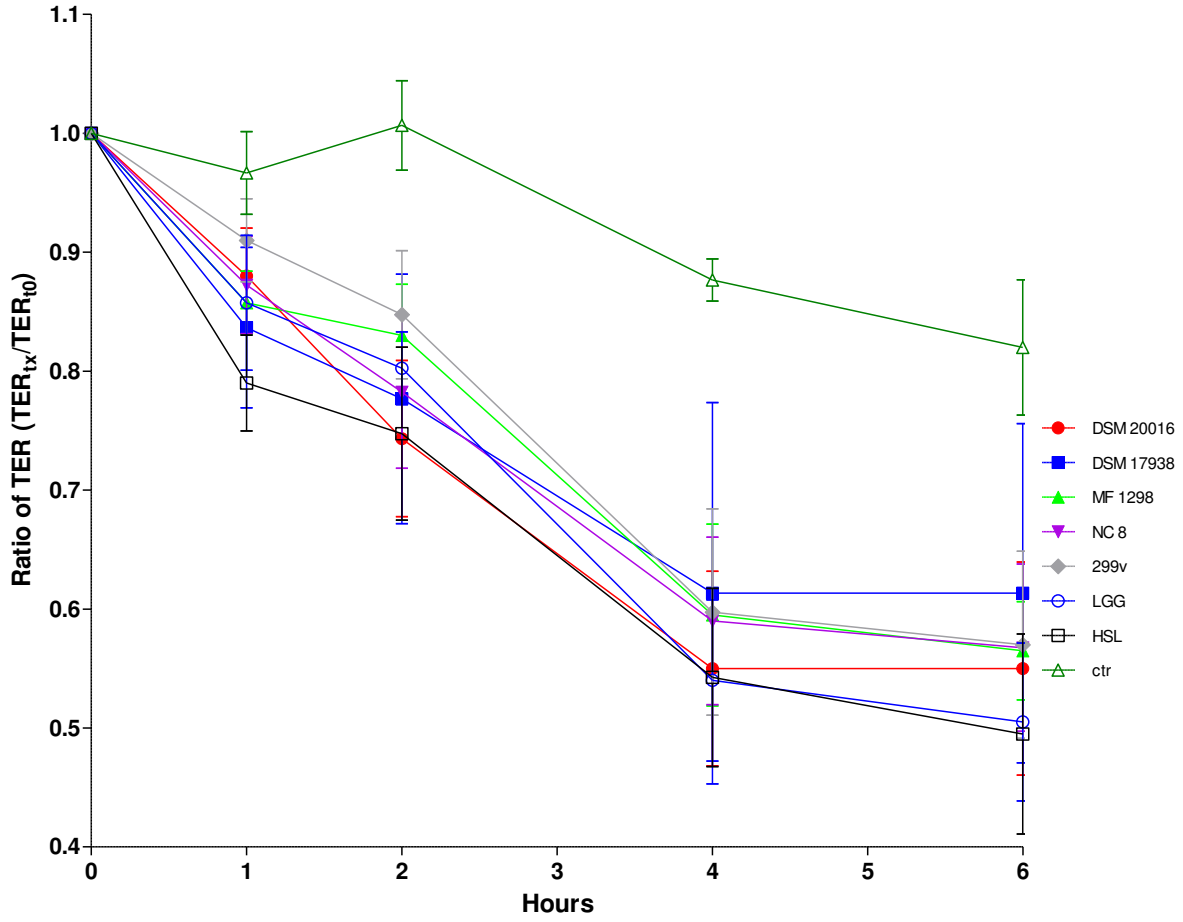

Figure 2. TER of polarized Caco-2 monolayers in experimental setup one. Caco-2 cells were incubated with *L. plantarum* NC8, 299v, MF1298, *L. reuteri* DSM 20016, DSM 17938, and *L. rhamnosus* GG (10^5 cfu/ml) over night before addition of HSL the following morning. TER is expressed as the ratio of TER at time t in relation to the initial value (t0) for each strain. The results are expressed as mean ± SEM, n=3-4. No statistical significant differences were observed between the stains and the control at the given time points (ANOVA with Tukey's post hoc test).

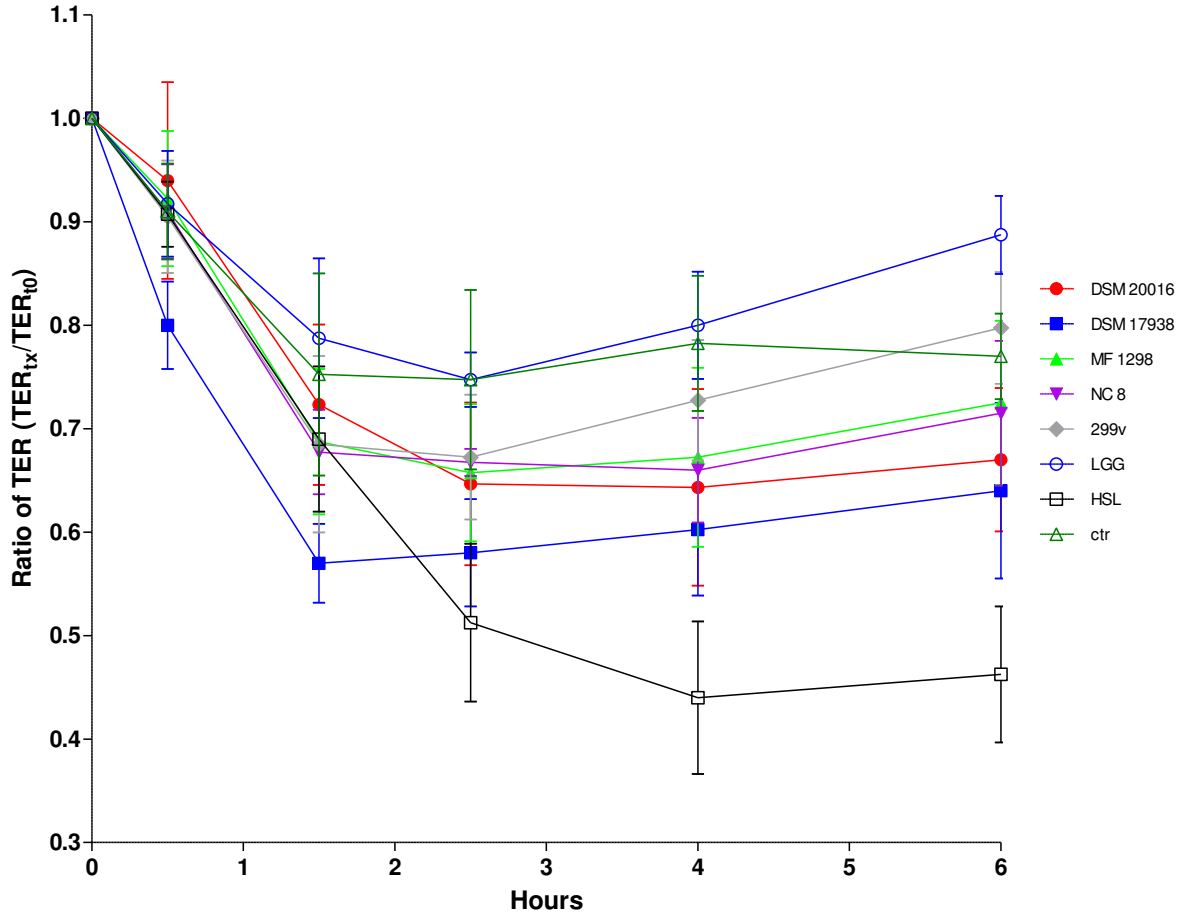

Figure 3. TER of polarized Caco-2 monolayers in experimental setup two. Caco-2 cells were incubated with HSL for 30 min before wash and addition of *L. plantarum* NC8, 299v, MF1298, *L. reuteri* DSM 20016, DSM 17938, and *L. rhamnosus* GG and DPBS (5×10^7 cfu/ml). TER is expressed as the ratio of TER at time t in relation to the initial value (t0) for each strain. The results are expressed as mean ± SEM, n=3-4. No statistical significant differences were observed between the stains and the control at the given time points (ANOVA with Tukey's post hoc test).

Bacterial effect on paracellular transport over polarized Caco-2 monolayers

The aim was to test whether prophylactic incubation with LAB could prevent or reduce HSL damage of the Caco-2 monolayer. The control, DPBS, was not treated with HSL in the experiment. None of the LAB tested succeeded in preventing HSL damage of the Caco-2 monolayer (Figure 4). After addition of HSL, FITC–Dextran values in the basolateral compartment increased slowly, reaching a maximum after 6h for all strains (Figure 4, Supplementary material Table S3). *L reuteri* DSM 20016 revealed the lowest passage of FITC–Dextran over 6 h (28 ng/ml), whereas *L. rhamnosus* GG and *L. reuteri* DSM 17938 filters revealed a passage of FITC–Dextran close to the HSL filter over 6 h (38 and 37 ng/ml

vs 40 ng/ml) (Supplementary material, Table S3). However, the differences were not statistically significant (Figure 4).

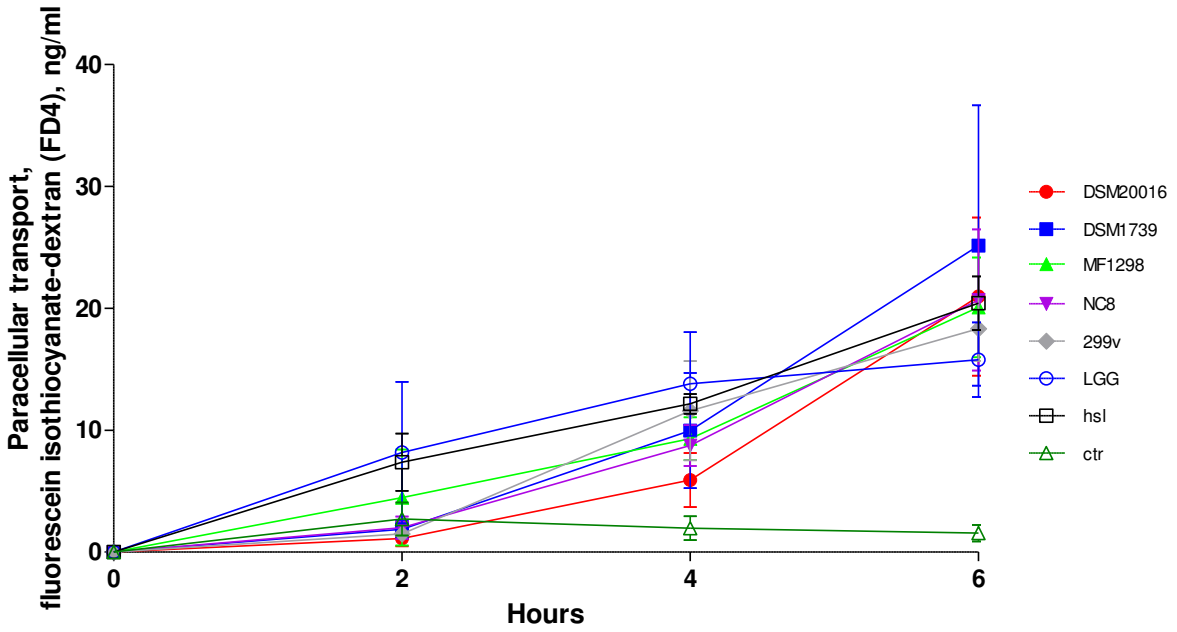

Figure 4. Paracellular transport of FITC–Dextran over polarized Caco-2 monolayers (experimental setup one). Caco-2 cells were incubated with *L. plantarum* NC8, 299v, MF1298, *L. reuteri* DSM 20016, DSM 17938, and *L. rhamnosus* GG (10^5 cfu/ml) over night before addition of HSL the following morning. FITC–Dextran (ng/ml) was measured in samples from the basolateral compartment. The results are expressed as mean ± SEM, n=3-4. No statistical significant differences were observed between the stains and the control at the given time points (ANOVA with Tukey's post hoc test).

Quantitative real-time reverse transcription PCR of TJ genes

The aim with qRT-PCR was to measure changes in tight junction gene expression as a result of LAB regeneration of the epithelial barrier after HSL disruption of the Caco-2 monolayer. The selected LAB strains showed no different effect on the expression of *claudin-1* and *zo-1* compared to the DPBS control and HSL treated Caco-2 cells after 1.5 h and 4 h (supplementary material, Table S4).

Discussion

Measuring TER and paracellular transport of FITC–Dextran over polarized cell monolayers are two methods to investigate the permeability (through the paracellular pathway) of the epithelial barrier *in vitro* [9]. These methods are commonly used as screening assays to test for probiotic effects [19-21]. The LAB tested represents well documented commercial

probiotic strains (*L. rhamnosus* GG [22], *L. plantarum 299v* [23], *L. reuteri* DSM 17938 [24]), candidate probiotics (*L. plantarum* MF1298 [13, 25]), and reference strains (*L. plantarum* NC8, *L. reuteri* DSM 20016). The latter two are naturally plasmid-free strains, often used as model strains in various studies on LAB [26, 27]. These six strains were chosen from a somewhat larger selection of LAB strains used in a previous study [28], based on their diversity in typical probiotic screening assays. In this study the aims were; (1) to evaluate if prophylactic incubation with LAB could prevent or reduce HSL damage of the Caco-2 monolayer, and (2) to evaluate if incubation with LAB could aid in regeneration of the epithelial barrier. DPBS and HSL were used as controls. We found no beneficial effect of commercially probiotic bacteria and selected LAB in ability to prevent or regenerate HSL induced damage of Caco-2 monolayers compared to the control. Furthermore, after 1.5 and 4 h we found no difference in gene expression of the tight junction genes *claudin-1* and *zo-1* as a result of LAB treatment.

Another study has shown that *L. casei* GG treatment of Caco-2 monolayers prevent translocation of *E. coli* C25, an effect that was not abolished by removing pre-incubated *L. casei* GG prior to addition of *E. coli* C25. The prevention of translocation was accomplished without a *L. rhamnosus* GG induced rise in TER [29]. Therefore, we hypothesized that pre-incubation with LAB could have a preventive effect on the HSL damage of the Caco-2 monolayer. However, both the commercial probiotic bacteria and the selected LAB tested had no preventive effect on the Caco-2 cell layer as shown by TER measurements and the paracellular transport of FITC-Dextran.

Miyauchi et al. [30] demonstrated that *L. salivarus* strains are divergent in their capacity for barrier protection, some strains prevented H_2O_2 induced reduction in barrier function (TER) and disruption of tight junction proteins, whereas other strains had no such effect. Furthermore, Seth et al. [31] found that the secreted proteins p40 and p75 from *L. rhamnosus* GG attenuate H_2O_2 induced disruption of tight junction proteins and adherence junction proteins in Caco-2 monolayers through activation of PKC and MAP kinase. Karczewski et al. [32] fond that *L. plantarum* WCFS1 up regulate TJ proteins via Toll like Receptor (TLR) 2 in humans and in Caco-2 cells. After 6 h co-culture of *L. plantarum* WCFS1 and Caco-2 polarized monolayers there was no change in TER or paracellular permeability. However, they observed changes in the cellular distribution of ZO-1 that significantly increased the resistance to phorbol 12,13-dibutyrate (PDBu) disruption of the Caco-2 monolayer [32]. Anderson et al. [33] found that *L. plantarum* MB452 enhanced the expression of 19 genes involved in the tight junction signaling pathway in healthy cells,

among them *occluding*, *zo-1*, *zo-2* and *cingulin*. In this study, we found no effect of commercial probiotic bacteria and selected LAB in ability to prevent or reduce the HSL induced damage in the Caco-2 monolayer. Furthermore, we found no difference in the gene expression of the TJ genes *claudin-1* and *zo-1* after treatment with LAB.

The reason why we find no effect of the selected bacteria in this *in vitro* model is most likely because they have no further beneficial effect compared to the DPBS control. The development of the model was thorough, and most parameters are under control. However, there are some limitations of the model which will be discussed in the next section. Furthermore, it could be that the timing in our model could be optimized more. There might be more visible differences in regeneration of the epithelial barrier after 12 h or 24 h. However, after such a long incubation period there can be many other factors than the bacteria itself which are responsible for TER (pH etc.). Thus, 6 h was set as the maximum time. As for the gene expression, *claudin-1* had the highest gene expression at 1.5 h whereas the expression of *zo-1* was the highest at 4 h. It might be that sampling before 1.5 h would the best for *claudin-1* and sampling after 4 h the best for *zo-1*.

Limitations of the Caco-2 model

When Caco-2 cells are grown as a monolayer on a permeable support, the monolayer differentiates and become polarized enterocytes with expression of microvilli resembling enterocytes of the small intestine. These polarized cells are bound together by TJ and AJ proteins, making the Caco-2 cells a good *in vitro* model for the intestinal epithelial barrier. However, the Caco-2 cell line is a heterogeneous cell line and culture related conditions can have major effect on the characteristics of the cell line (see Sambuy et al. [34] for a detailed review). Furthermore, there are several factors that can be varied in the Caco-2 model of intestinal epithelial barrier which can result in differences in the model; (1) the source and passage number of Caco-2 cells, (2) incubation media used for growth and maintenance, (3) diameter and pore size of the permeable support, (4) the buffering system used under experiments, (5) variability in time and temperature and/or (5) the use of mixing or stirring apparatus during the experiment [34-36].

The TER values of differentiated Caco-2 monolayers are also important for the model. Press and Di Gandi [37] report that TER values below 150 Ω cm2 usually indicate leaky monolayers assumed to have leakage due to imperfect occluding junctions or holes in the monolayer, and that TER values greater than 750 Ω cm2 often lead to large variance in triplicate data points possibly due to cell clumping on the support membrane. Previous studies

have shown that TER measurements are temperature dependent [38]. Ideally, TER measurements should be performed at a constant temperature of 37 °C to mimic the human physiological situation. This is not easily obtained and requires an electrode which is fixed in the incubator or a slide warmer [38]. With the Millicell Electrical Resistance System used in this study, it is impossible to keep the temperature of the cell culture plate constant. However, special caution was taken to always treat one strain similar (same position in the culture plate, timed addition of bacteria, wash and TER measurement etc.) and ratio of TER (TER_{tx}/TER_{t0}) was calculated, eliminating the temperature issues the best possible way.

Mukherjee et al. [39] conclude that TER is not a reliable measurement of the Caco-2 monolayer integrity as 100 % recovery of TER values was observed within 24 h after restoration of normal growth conditions, whereas toxicity tests such as MTT and modified trypan blue exclusion assay revealed considerable toxicity of the Caco-2 cells. Most studies use long incubation periods (up to 24 h) to measure TER effects of polarized monolayers [19, 28, 40, 41]. After initial pilot studies we concluded that such long incubation time is not desirable as different growth of strains, pH effects, metabolites and other factors can influence the results greatly (unpublished results). Thus, 6 h was set as the maximum co-incubation time.

There are several other limitation with the Caco-2 model: (1) the tightness of the monolayer resembles more colonic than small intestinal tissue, with poor permeability for compounds with paracellular transport; (2) it consists of solely absorptive enterocytes, whereas intestinal epithelium is a mixture of different cell types; (3) Caco-2 cells do not produce mucin; (4) permeabilities for compounds that are transported via carrier-mediated absorption are lower in the Caco-2 system compared to human intestine; (5) absence of physical parameters such as intestinal motility and transit time [42, 43]. However, such limitations will always be present in *in vitro* assays. In future studies it would be interesting to evaluate the effect of the strain investigated on polarized mucus-secreting cell line (e.g. LS 174T or HT29 MTX).

Conclusion

The aim of the present study was to test if pre-incubation with LAB could prevent or reduce HSL damage of the Caco-2 monolayer, and if incubation with LAB could aid in restoring the epithelial barrier after HSL disruption. TER, paracellular transport of FITC-Dextran, and qRT-PCT of the tight junction genes *zo-1* and *claudin-1* was evaluated. We found no

beneficial effect of the probiotic bacteria and LAB in this *in vitro* model of the intestinal barrier compared to the DPBS control.

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.

Supplementary material

Table S1: Details from TER setup one

	DSM 20016		DSM 17938		MF 1298		NC 8		299v		LGG		HSL		ctr	
	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM
0 h	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
1 h	0.88	0.07	0.84	0.12	0.86	0.05	0.87	0.08	0.91	0.07	0.86	0.11	0.79	0.08	0.97	0.06
2 h	0.74	0.11	0.78	0.18	0.83	0.09	0.78	0.13	0.85	0.11	0.80	0.06	0.75	0.15	1.01	0.07
4 h	0.55	0.14	0.61	0.28	0.60	0.15	0.59	0.14	0.60	0.17	0.54	0.14	0.54	0.15	0.88	0.03
6 h	0.55	0.16	0.61	0.25	0.57	0.08	0.57	0.14	0.57	0.16	0.51	0.13	0.50	0.17	0.82	0.10
Difference, max to min	0.45		0.39		0.44		0.43		0.43		0.50		0.51		0.18	

L. reuteri DSM 20016, *L. reuteri* DSM 17938, *L. plantarum* MF1298, *L. plantarum* NC8, *L. plantarum* 299v, *L. rhamnosus* GG
Orange color indicate the lowest TER value measured, green color indicate the initial TER value.
TER are given as ratio of TER (TERtx/TERt0).

Table S2: Details from TER setup two

	DSM 20016		DSM 17938		MF 1298		NC 8		299v		LGG		HSL		ctr	
	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM
0 h	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0
0.5 h	0.94	0.10	0.80	0.04	0.92	0.07	0.91	0.05	0.91	0.05	0.92	0.05	0.91	0.03	0.91	0.05
1.5 h	0.72	0.08	0.57	0.04	0.69	0.07	0.68	0.04	0.69	0.09	0.79	0.08	0.69	0.07	0.75	0.10
2.5 h	0.65	0.08	0.58	0.05	0.66	0.07	0.67	0.01	0.67	0.06	0.75	0.03	0.51	0.08	0.75	0.09
4 h	0.64	0.09	0.60	0.06	0.67	0.09	0.66	0.05	0.73	0.06	0.80	0.05	0.44	0.07	0.78	0.07
6 h	0.67	0.07	0.64	0.08	0.73	0.08	0.72	0.07	0.80	0.05	0.89	0.04	0.46	0.07	0.77	0.04
Difference, min to max after HSL	0.03		0.07		0.07		0.05		0.13		0.14				0.03	

L. reuteri DSM 20016, *L. reuteri* DSM 17938, *L. plantarum* MF1298, *L. plantarum* NC8, *L. plantarum* 299v, *L. rhamnosus* GG
Orange color indicate the lowest TER value measured, green color indicate the highest TER value after HSL.
TER are given as ratio of TER (TERtx/TERt0).

Table S3. Details from Paracellular Transport Assay

	DSM20016		DSM1739		MF1298		NC8		299v		LGG		HSL		ctr	
	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM
0 h	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2 h	1.13	0.65	1.88	0.70	4.48	3.94	2.00	0.92	1.49	0.36	8.17	5.80	7.37	2.35	2.71	1.35
4 h	5.92	2.22	9.98	4.72	9.31	1.76	8.75	1.67	11.60	4.08	13.80	4.24	12.16	0.80	1.95	0.98
6 h	20.97	6.49	25.16	11.50	20.08	4.09	20.68	5.79	18.33	2.64	15.78	3.06	20.42	2.20	1.56	0.68
Total paracellular transport 0-6 h	28.01		37.02		33.87		31.43		31.42		37.76		39.96		6.22	

L. reuteri DSM 20016, *L. reuteri* DSM 17938, *L. plantarum* MF1298, *L. plantarum* NC8, *L. plantarum* 299v, *L. rhamnosus* GG

Paracellular transport is measured as ng/ml of fluorescein isothiocyanate–dextran (FITC–Dextran, FD4) in the basolateral compartment of polarized Caco-2 monolayers.

Table S4. Details from qRT-PCR of the TJ genes *claudin-1* and *zo-1*

	claudin-1				zo-1			
	1.5 h		4 h		1.5 h		4 h	
Strain/treatment	AVG	SD	AVG	SD	AVG	SD	AVG	SD
L. reuteri DSM 20016	3.27	1.41	3.22	2.34	5.81	0.71	4.02	1.06
L. reuteri DSM 17938	2.76	1.31	3.17	3.50	5.38	1.03	4.84	2.15
L. plantarum MF1298	3.05	1.38	2.31	3.72	5.45	0.83	3.65	2.78
L. plantarum NC8	3.13	1.16	1.69	0.58	6.39	1.93	3.44	1.06
L. plantarum 299v	3.20	1.20	3.46	2.39	5.34	0.42	5.25	1.98
L. rhamnosus GG	2.71	1.31	3.82	3.36	5.42	1.16	5.15	1.70
HSL, DPBS	2.68	2.08	4.39	5.64	5.33	1.22	5.76	4.71
DPBS	3.06	2.28	3.97	3.15	5.52	1.50	6.87	4.04
HSL	2.96	1.42	3.77	3.25	5.85	1.00	5.49	2.39

Relative gene expression was calculated by the ΔCT method, using 18S as the endogenous reference gene.

TaqMan Gene Expression Assays were from Life Technologies Ltd (Paisley, UK) (Assay ID: Hs01551861_m1 (ZO-1), Hs00221623_m1 (claudin-1), Hs99999901_s1 (18S)).