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Characterization of *Lactobacillus* pili and the niche-adaptation factors of intestinal *Lactobacillus ruminis*

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ACADEMIC DISSERTATION

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List of original publications

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I. Johanna Rintahaka*, **Xia Yu***, Ravi Kant, Airi Palva and Ingemar von Ossowski. (2014) Phenotypical analysis of the *Lactobacillus rhamnosus* GG fimbrial *spaFED* operon: surface expression and functional characterization of recombinant SpaFED pili in *Lactococcus lactis*. PLOS One 9(11): e113922.

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Abbreviations

APC	antigen-presenting cell
CRISPR	clustered regularly interspaced short palindromic repeat
DC	dendritic cell
ECM	extracellular matrix
EM	electron microscopy
EPS	exopolysaccharide
ETEC	enterotoxigenic *Escherichia coli*
Foxp3	forkhead box p3
GALT	gut-associated lymphoid tissue
GIT	gastrointestinal tract
GM-CSF	granulocyte/macrophage colony-stimulating factor
HEK	human embryonic kidney
HMW	high-molecular-weight
IBD	inflammatory bowel disease
IBS	Irritable bowel syndrome
IEC	intestinal epithelial cell
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
ILF	isolated lymphoid follicle
IL	interleukin
LAB	lactic acid bacteria
LP	lamina propria
LPS	lipopolysaccharide
MabA	modulator of adhesion and biofilm protein A
MAPK	mitogen-activated protein kinase
MHC I/II	major histocompatibility complex I/II
MLN	mesenteric lymph node
moDC	monocyte-derived dendritic cell
MRS	de Man-Rogosa-Sharpe
NF-κB	nuclear factor-κB
NK cell	natural killer cell
PP	Peyer's patch
PRR	pattern recognition receptor
ROS	reactive oxygen species
SCFA	short chain fatty acid
sIgA	secretory immunoglobulin A
T6SS	type VI secretion system
TCR	T cell receptor
TEM	transmission electron microscopy
TEER	trans-epithelial electrical resistance
Th cell	helper T cell
TJ	tight junction
TLR	toll-like receptor
TNF	tumor necrosis factor
Treg cell	regulatory T cell
ZO	zonula occludens

Abstract

The mammalian gut microbiota is composed of autochthonous species that permanently colonize the host intestine, and of allochthonous species that are only transiently able to occupy the intestinal environment. In this thesis research, *Lactobacillus ruminis* and *Lactobacillus rhamnosus* GG were investigated as paradigms for each type of microbe–host interaction, with special emphasis on the *in vitro* characterization of their adaptation factors in the host gastrointestinal tract (GIT).

As one of the best-characterized probiotics, *L. rhamnosus* GG is of great interest to researchers and the probiotic industry. According to previous genome analyses, *L. rhamnosus* GG has two pilus operons: *spa*CBA encoding the well-studied SpaCBA pili and *spaFED* putatively encoding SpaFED pili. However, the expression of SpaFED pili in *L. rhamnosus* GG under laboratory conditions has not thus far been reported. Nevertheless, *in vivo* expression of *spaFED* cannot be ruled out, either. Therefore, in this study, aimed at exploring the role of SpaFED pili in host adhesion and immunomodulatory effects, a nisin-induced expression system was used for the generation of SpaFED or SpaF-deleted pili in *Lactococcus lactis* NZ9000. The results revealed that SpaFED pili were essential in mediating lactococcal adhesion to intestinal cell lines (Caco-2 and HT-29), to certain extracellular matrix (ECM) proteins, and to porcine mucins, the tip pilin SpaF playing a central role as a focal adhesion factor. With regard to immunomodulation, SpaFED pili appeared to dampen the immune responses, which was largely attributed to the SpaF pilin, in human embryonic kidney (HEK) 293-blue cells expressing toll-like receptor (TLR) 2, and in Caco-2 cells. However, while encountering human peripheral blood monocyte-derived dendritic cells (moDCs), neither immune response enhancement nor immune dampening by SpaFED pili was observed.

For the autochthonous *L. ruminis* species, the mammalian GIT is so far the only identified niche. Consistent with genomic analyses, transmission electron microscopy (TEM) demonstrated that pili in *L. ruminis* ATCC 25644 of human origin consisted of the tip (LrpC), basal (LrpB), and backbone (LrpA) pilins, assembled in a pattern similar to that in SpaCBA and SpaFED pili of *L. rhamnosus* GG. Analogously to dissecting the characteristics of SpaFED pili in *L. rhamnosus* GG, recombinant *L. lactis* strains were constructed, producing either LrpCBA or LrpC-lacking pili of *L. ruminis*. Recombinant LrpCBA pili mediated lactococcal adherence to ECM proteins and intestinal epithelial cells, and also dampened TLR2-dependent NF-κB signaling and IL-8 production in HEK cells, whereas *L. ruminis* itself induced evidently elevated immune responses. When incubated with Caco-2 cells, *L. ruminis* and recombinant lactococcal constructs expressing pili with and without LrpC pilin lowered IL-8 production. Biofilm formation in *L. ruminis* was not attributed to LrpCBA pili. Subsequently, a novel *L. ruminis* strain (GRL1172) was isolated from porcine feces and demonstrated to be flagellated and piliated. To expand our knowledge of the autochthony-promoting properties of this gut-dwelling species, we analyzed the abilities of this new isolate and three other *L. ruminis* strains (a human isolate, ATCC 25644, and two bovine isolates, ATCC 27780 and ATCC27781) to adhere to host cells and extracellular matrix proteins, to inhibit pathogen binding and growth, to maintain epithelial barrier functions, and to modulate immune responses *in vitro*. The results indicated that the strains shared several characteristics, such as binding to ECM proteins and HT-29 cells, inhibition of pathogen adhesion and growth, maintenance of epithelial barrier functions in epithelial cells, and activation of innate immune responses to various extents.

In conclusion, this thesis study demonstrated the adhesiveness of SpaFED and LrpCBA pili, which may respectively promote the gut retention of *L. rhamnosus* GG (in addition to the SpaCBA pili) and of *L. ruminis*. Moreover, pilus-mediated dampening of innate immune responses might be a strategy for these two gut bacterial species to induce immune tolerance in the host. Additionally, *L. ruminis* inhibited enteropathogen adhesion and growth, as well as maintained intestinal barrier function, which could be regarded as beneficial to the host and which may in turn favor the persistent residence of *L. ruminis*.

1. Introduction

L. rhamnosus GG, originally isolated from the GIT of a healthy person (Goldin and Gorbach, 1984), has been extensively used in the food and pharmacy industries, and its consumption has been reported to be associated with several health-promoting effects, such as enhancing the treatment and prevention of atopic eczema and diarrhea (Kalliomäki et al., 2001; Kalliomäki et al., 2003; Guarino et al., 2009; Hojsak, 2017). Even so, the molecular mechanisms underlying these health benefits are not completely understood. *L. rhamnosus* GG, demonstrated to be a piliated bacterium, has two pilus gene clusters in its genome, one including *spa*CBA and the other including *spa*FED (Kankainen et al., 2009). SpaCBA pili promote strong adhesion to human intestinal epithelial cells and display immunomodulatory effects (Lebeer et al., 2012). They are recognized by TLR2, resulting in the activation of the TLR2-dependent NF-κB signaling pathway leading to the production of pro- and anti-inflammatory cytokines (von Ossowski et al., 2013). In contrast to the well-studied SpaCBA pili, the characteristics of SpaFED pili are largely unknown. SpaFED pili appear not to be produced by *L. rhamnosus* GG under laboratory conditions (Reunanen et al., 2012). However, SpaFED pili may be expressed in the host GIT, in a similar way as the tight adherence pili IV (TadIV) of *Bifidobacterium* spp., which are only expressed in the murine GIT *in vivo* and which play a crucial role in the bacterial colonization of the host (Motherway et al., 2011). The tip pilin SpaF is the only subunit of SpaFED pili responsible for mucus binding, as demonstrated by a study using recombinant SpaFED pilin proteins produced in *E. coli* (von Ossowski et al., 2010). However, little other functional data is presently available regarding *spa*FED-encoded pili. Therefore, one aim of this study was to explore the adhesive properties and immunomodulatory functions of SpaFED pili to better understand the function of *L. rhamnosus* GG pili.

L. ruminis is an autochthonous gut-resident bacterial species in humans as well as many animals (Sharpe et al., 1973; Al Jassim, 2003; Yun et al., 2005; O'Donnell et al., 2015). It is the only motile autochthonous species of the genus *Lactobacillus*, which has so far been recorded in the mammalian gut (Neville et al., 2012). In comparisons between flagellated strains and their isogenic mutants lacking flagella, it has been found that flagella are involved in bacterial binding to mucus (Troge et al., 2012) and the intestinal epithelium (Mahajan et al., 2009), and they are thus considered as early colonization factors (Rossez et al., 2015). Therefore, being flagellated could be related to the persistent gut colonization of *L. ruminis*. Additionally, *L. ruminis* exhibits immunomodulatory effects via its flagella (Neville et al., 2012) or other unknown factors (Taweechotipatr et al., 2009). Importantly, it can hinder the growth of enteropathogens such as vancomycin-resistant enterococci *in vitro* (Yun et al., 2005) and rotavirus *in vitro* and *in vivo* (Kang et al., 2015). However, despite many interesting effects, *L. ruminis* has surprisingly been poorly explored functionally, and is thus a much-overlooked and less characterized gut commensal bacterial species. In a recent study, a cluster of pilus-related genes, similar to those of *L. rhamnosus* GG, were identified in two *L. ruminis* strains, ATCC 25644 and ATCC 27782 (Forde et al., 2011), but to date there has been no report proving pilus gene expression in *L. ruminis* either *in vitro* or *in vivo*, or demonstrating the functional characteristics of *L. ruminis* pili. Analogously to the description of SpaCBA pili in *L. rhamnosus* GG (von Ossowski et al., 2013), one aim of this study was to investigate the phenotypic traits, including the adhesive properties and immunomodulatory effects, of *L. ruminis* pili.

The genomes of a limited number of *L. ruminis* strains isolated from humans, bovines, equines, and pigs have so far been sequenced (Forde et al., 2011; Lee et al., 2011; O'Donnell et al., 2015), and certain functional traits (e.g., fermentation patterns, survival capabilities and motility) of different host-sourced *L. ruminis* are known to be strain-specific (O'Donnell et al., 2011; Neville et al., 2012; O'Donnell et al., 2015). One aim of this work was to broaden knowledge of the characteristics of *L. ruminis* species. To do this, a novel *L. ruminis* strain was isolated from porcine feces, and its phenotypic characteristics

were dissected in parallel with those of one human and two bovine *L. ruminis* strains. In addition to elucidating the adhesive and immunomodulatory properties, another aim of this study was to analyze the effect of *L. ruminis* cells on epithelial barrier function, as many autochthonous or probiotic lactobacilli have been shown to maintain the integrity of the intestinal barrier both *in vitro* and *in vivo*, and some strains are even able to prevent barrier damage caused by enteropathogens (Roselli et al., 2007; Johnson-Henry et al., 2008; Karczewski et al., 2010; Liu et al., 2015; Yu et al., 2015). Moreover, one further aim of this study was to analyze the interactions between *L. ruminis* and certain enteric pathogens. It has been reported that both strongly (Coconnier et al., 1993; Abedi et al., 2013; Hynönen et al., 2014) and poorly (Tuomola et al., 1999; Gueimonde et al., 2006) adhesive lactobacilli can inhibit pathogen adhesion to host intestinal epithelial cells or mucus. As *L. ruminis* is an autochthonous member of the gut microbiota, this pathogen inhibition trait could be beneficial for its lifelong colonization of the GIT.

2. Literature review

2.1 Gut defense systems and homeostasis in mammals

The mucosal surface of the GIT is constantly exposed to foreign antigens, such as ingested food, drugs, and a complex microbial community called the gut microbiota. Although facing variable conditions, the mammalian gut is in a stable, healthy, and functional state under most circumstances. This is attributed to the intestinal epithelial barrier and the intestinal microbiota, as well as to several immunological mechanisms that lead to both the tolerance of harmless antigens (e.g. gut microbiota) and immunological defense reactions against pathogens.

2.1.1 Gut mucosal immunity

The gut mucosal surface is protected by gut-associated lymphoid tissue (GALT), which is the largest immune organ of the body and is composed of secondary lymphoid organs and lymphocytes, as presented in Figure 1. Specifically, Peyer's patches (PPs), the inductive sites for the intestinal immune response in the small intestine, are composed of lymphoid follicles and covered by follicle-associated epithelium containing specialized intestinal epithelial cells (IECs), called M cells (Nagler-Anderson, 2001; Jung et al., 2010). M cells deliver antigens from the lumen to antigen-presenting cells such as DCs in PPs. Isolated lymphoid follicles (ILFs) are intestinal lymphoid tissues that work as inductive sites similar to PPs. The lamina propria (LP), the thin connective tissue layer located beneath the epithelium, acts as an immune effector site and has abundant immune cells (Nagler-Anderson, 2001; Artis, 2008). Mesenteric lymph nodes (MLNs), another type of immune inductive site, collect lymphatic drainage (containing, for example, food antigens and gut-resident microbes) from the LP, PPs, and the intestinal mucosa (Macpherson and Smith, 2006; Round and Mazmanian, 2009). Effector T cells are also found between the intestinal epithelial cells, where they are referred to as intraepithelial lymphocytes (IELs) (Murphy et al., 2008). In response to infection, IELs rapidly release cytokines and chemokines, thus eliminating the pathogens (Hayday et al., 2001).

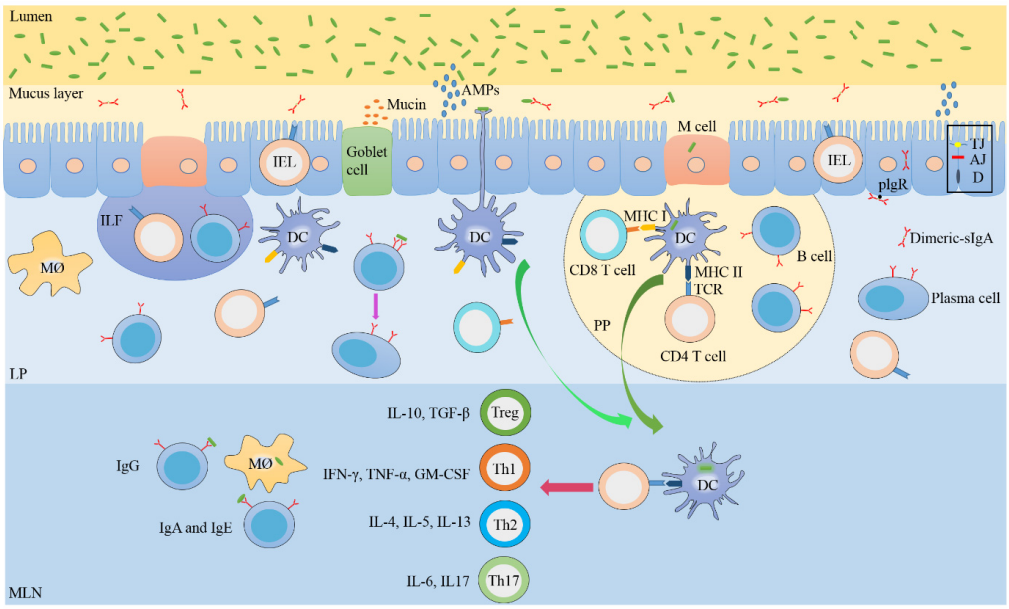


Figure 1. Schematic model of the epithelial barrier and gut-associated lymphoid tissue. LP, lamina propria; MLN, mesenteric lymph node; PP, Peyer's patch; ILF, isolated lymphoid follicle; IEL, intraepithelial lymphocyte; MHC I/II, major histocompatibility complex I/II; DC, dendritic cell; MØ, macrophage; TCR, T-cell receptor; AMPs; antimicrobial peptides; dimeric-sIgA, dimeric secretory

immunoglobulin A; pIgR, polymeric immunoglobulin receptor; Treg, regulatory T cell; Th, helper T cell; TJ, tight junction; AJ, adherens junction; D, desmosome. DCs harboring bacterial antigens from PPs and the LP migrate to the MLN and activate naïve T cells (CD8 T cells and CD4 T cells). B cells are activated with or without the help of Th cells. Data from Turner, 2009; Murphy et al., 2008; and Peterson and Artis, 2014.

The immune system consists of innate and adaptive immunity. Innate immunity is the first line of immune defense, composed of the physical (e.g., skin, epithelia, and mucus) and chemical barriers (e.g., acidity of the stomach, lysozyme, and antimicrobial peptides secreted by host cells), innate immune cells, and the complement system (Murphy et al., 2008). It distinguishes self from non-self, and is immediately activated upon the recognition of foreign antigens through a limited number of pattern recognition receptors (PRRs). In contrast, adaptive immunity takes several days to develop after pathogenic encounter, and thus generates immunological memory and long-lasting protection, dependent on a broad range of rearranged receptors on the surface of B and T lymphocytes (Akira et al., 2006).

Innate immunity

Cells of intestinal innate immunity include macrophages, DCs, granulocytes (neutrophils, basophils, and eosinophils), mast cells, and natural killer (NK) cells. Among these, phagocytes such as macrophages, immature DCs, and neutrophils work as the first-line defense to clear foreign antigens. They detect bacterial components via their surface receptors, followed by rapidly and efficiently engulfing and degrading the microbes that breach the intestinal epithelium (Murphy et al., 2008). After encountering antigens, macrophages residing in lymphoid and non-lymphoid tissues (Geissmann et al., 2010) also release cytokines and chemokines that induce inflammation and recruit other immune cells to the infection sites, thus killing the invaders (Galli et al., 2011). DCs, rich in LP and Peyer's patches, are divided into two groups: conventional DCs, which are specialized antigen-presenting cells (APCs), and plasmacytoid DCs, which produce virus-induced interferon, but are not as crucial as conventional DCs in antigen presentation owing to their inadequate production of major histocompatibility complex (MHC) II and costimulatory molecules (Murphy et al., 2008; Reizis et al., 2011).

Innate immune responses are activated through the recognition of microbe-associated molecular patterns (MAMPs) by PRRs expressed on immune cells and intestinal epithelial cells (IECs). PRRs are able to detect a wide range of MAMPs shared by both the gut microbiota and the pathogens, such as flagellin, lipopolysaccharides (LPS), and pili. TLRs recognizing various bacterial components are signaling receptors and among the best characterized PRRs (Eisenbarth and Flavell, 2009). Activation of any TLR is able to provoke NF-κB signaling, dependent on adaptor proteins that contribute to the signal transduction, such as myeloid differentiation primary response gene 88 (MyD88), TIRAP, and TRIF (Kawai and Akira, 2007). In the cytoplasm of inactivated cells, NF-κB is bound to its inhibitor, IκB (Figure 2). Upon stimulation, IκB is phosphorylated by its kinase, IKK, resulting in the translocation of NF-κB to the nucleus, which then induces the transcription of various cytokine and chemokine genes (Tak and Firestein, 2001), and the expression of costimulatory molecules on cells such as DCs (Kawai and Akira, 2007).

An important part of innate immunity is the generation of reactive oxygen species (ROS), in which polymorphonuclear leukocytes (e.g. neutrophils) and mononuclear phagocytes (e.g. macrophages) are involved (Fang, 2004). Upon bacterial stimulus, ROS production is immediately induced via the NADPH phagocyte oxidase-dependent pathway, working as a host defense (Fang, 2004). The roles of ROS are versatile and not restricted to the innate immune response: not only do they directly target and disrupt microbial DNA, RNA, membranes, and proteins (Cabiscol et al., 2010), but they also regulate host immunity. ROS are able to modulate various immune cells, including the induction of maturation

of DCs, apoptosis in NK cells, and differentiation of B cells (Hansson et al., 1996; Yang et al., 2013). Additionally, ROS help polymorphonuclear leukocytes to migrate and prolong their stay at the infection sites, leading to bacterial killing (Nathan and Cunningham-Bussel, 2013). Moreover, ROS play a role in signaling that leads to the activation of NF-κB and mitogen-activated protein kinases pathways (Hancock et al., 2001; Son et al., 2011).

Adaptive immunity

APCs that have taken up antigens either stay in PPs and LPs or migrate to MLNs, as shown in Figure 1, and then present antigens to naïve lymphocytes (Iwasaki and Medzhitov, 2010; Perez-Lopez et al., 2016). Both DCs and macrophages work as APCs, but macrophages are not as efficient as DCs (Gordon and Taylor, 2005). The naïve T lymphocytes are divided into CD4 and CD8 T cells, and their activation is dependent on the antigens and co-stimulatory molecules on APCs. Naïve CD8 T cells detect antigen peptides through MHC I molecules on APCs, resulting in cytotoxic T cells that aim at killing infected cells; in contrast, sensing the antigen peptides through MHC II molecules on APCs, CD4 T cells differentiate into several subtypes of effector T helper (Th) cells, including the best-studied ones, Th1, Th2, Th17, and regulatory T (Treg) cells (Murphy et al., 2008). Th1 cells, secreting interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and granulocyte/macrophage colony-stimulating factor (GM-CSF), attack intracellular foreign antigens carried by the macrophages and help B cells to differentiate into plasma cells that mainly produce immunoglobulin (Ig) G; Th2 cells, releasing IL-4, IL-5, and IL-13, primarily mediate plasma cells to produce IgE and secretory IgA (sIgA); Th17 cells produce IL-17, which preserves the integrity of the mucosal barrier and combats pathogens; Treg cells, producing cytokines such as IL-10 and transforming growth factor-β, establish immune tolerance to self-antigens and inhibit redundant immune responses (Murphy et al., 2008; Sakaguchi et al., 2008; Gensollen et al., 2016).

After encountering the antigens, naïve B cell are activated with or without the assistance of Th cells. When activated in a T cell-independent way, the humoral immune response is less robust. For example, bacterial polysaccharides attach to B-cell receptors and thus induce the development of plasma cells, but fail to generate memory B cells in most cases (Pollard et al., 2009). The antigens (invaded bacteria, viruses, and their products) bound to antibodies produced by plasma cells are detected and eliminated by phagocytes or through complement activation (Murphy et al., 2008).

Effector T cells and primed B cells in MLNs enter the bloodstream, but exhibit gut-homing capacities, i.e. they return to the LP through the circulation (Brandtzaeg and Johansen, 2005). In the LP, dimeric sIgA antibodies produced by plasma cells are attached to the polymeric immunoglobulin receptors (pIgRs) on the basolateral side of IECs and transported across the epithelium to the lumen, where they bind to foreign antigens and limit the penetration of luminal bacteria, thus fortifying the epithelial barrier (Hooper and Gordon, 2001; Macpherson and Harris, 2004; Perez-Lopez et al., 2016).

Tolerance

The intestinal immune system develops tolerance to harmless antigens, such as diet and host gut microbiota (Mowat, 2003). Defect in immune tolerance to the gut microbiota is considered as an important cause of inflammatory bowel disease (IBD) (Round and Mazmanian, 2010). The underlying mechanisms for the immune tolerance are associated with innate and adaptive immunity.

In the presence of the gut microbiota and under normal healthy conditions, DCs, via surface receptors, can capture some of the invading gut-resident bacteria through M cells or directly access the intestinal lumen via the protruding dendrites (Macpherson and Uhr, 2004; Kelsall, 2008). Subsequently, they migrate to and are confined in the MLNs, which primes the local mucosal immune responses but avoids systemic immunity (Macpherson and Uhr, 2004).

The location of PRRs on IECs is of great importance in gut homeostasis (Artis, 2008). Certain TLRs (TLR3, TLR7, and TLR8) and nucleotide-binding oligomerization domain-like receptors are respectively found in the endosome and cytoplasm of IECs (Peterson and Artis, 2014), which keeps them at a distance from the gut microbiota residing in the lumen and mucus layer, largely limiting direct contact with these microbes and thus preventing the activation of immune responses by IECs. The locations of TLRs on IECs are illustrated in Figure 2. Moreover, TLR5 is considered to be located on the basolateral side of IECs. It detects basolaterally translocated flagellins in IECs, thus activating proinflammatory immune responses (Gewirtz et al., 2001a). As an example, the enteropathogen *Salmonella* typhimurium (but not intestinal *E. coli*) has the potential to transfer flagellins to the basolateral side without bacterial invasion (Gewirtz et al., 2001a). Furthermore, after encountering the microbial DNA, basolateral TLR9 triggers NF-κB signaling; in contrast, recognition of microbial ligands by apical TLR9 not only inhibits NF-κB pathway, but also induces tolerance that inhibits inflammatory signaling (e.g., NF-κB activation and IL-8 production) activated by the subsequent stimulation of other TLRs (Lee et al., 2006).

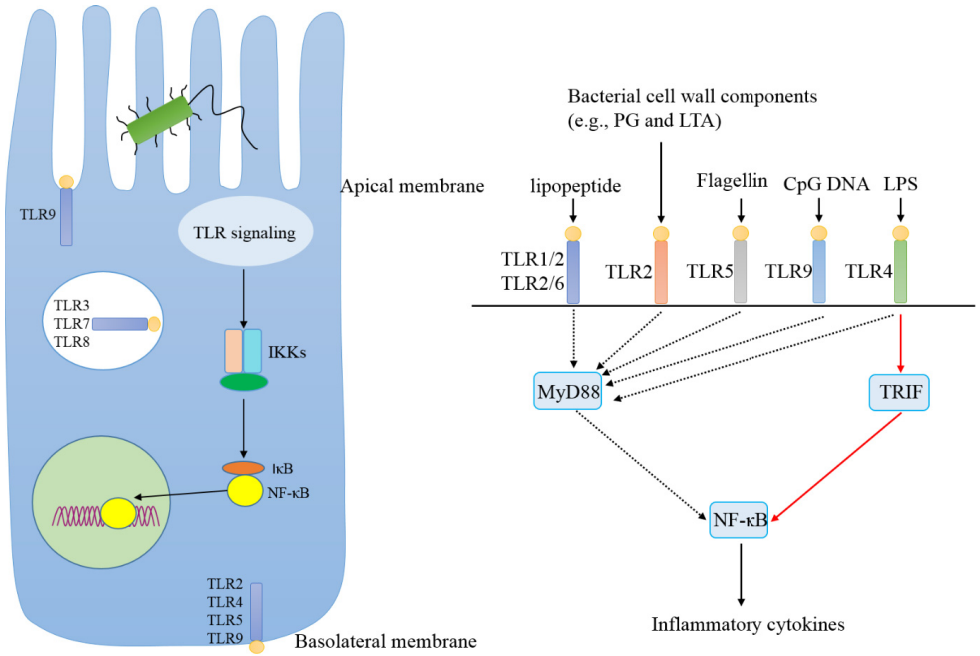


Figure 2. **Toll-like receptors of intestinal epithelial cells and their bacterial ligands**. IκB, inhibitor of NF-κB; IKKs, kinases of IκB; PG, peptidoglycan; LTA, lipoteichoic acid; LPS, lipopolysaccharide; MyD88 and TRIF, signaling adaptor proteins; CpG, a cytosine nucleotide linked to a guanine nucleotide by a phosphate. Adapted from Peterson and Artis, 2014; and Kawai and Akira, 2010.

The presentation of gut microbe-derived antigens by retinoic-acid-receptor-related orphan receptor-γt positive (RORγt^{+}) innate lymphoid cells through MHC II limits the T-cell response primed by the gut microbes and can also directly cause the death of these activated T cells, thereby contributing to the maintenance of immune tolerance towards the microbiota (Hepworth et al., 2013; Hepworth et al., 2015). Furthermore, gut sIgA antibodies can mediate tolerance to gut microbes. Secreted IgA adheres to the surface of the bacterial antigens to avert gut-associated bacteria–host interaction, which would otherwise lead to unnecessary immune responses (Honda and Littman, 2016). For instance, the gut symbiont bacterium *Bacteroides thetaiotaomicron* induces robust pro-inflammatory responses in

germfree mice lacking secreted IgA (Peterson et al., 2007). Moreover, intestinal Treg cells expressing transcription factor forkhead box P3 (Foxp3) are largely implicated in tolerance towards the gut microbiota (Bilate and Lafaille, 2012; Cebula et al., 2013). Intracellular Foxp3 mediates the development and function (e.g. suppression of immune response) of Treg (Fontenot et al., 2003). Tolerogenic $Foxp3^+$ Treg cells are more abundant in the intestinal LP than in other parts of body (Atarashi et al., 2011). It has been reported that the gut microbiota can induce the development of $Foxp3^+$ Treg cells (Round and Mazmanian, 2010; Atarashi et al., 2011) and shape the diverse TCR repertoire on them, which is distinct from that on other intestinal T-cell subsets (Lathrop et al., 2011). Recognition of antigens derived from the gut microbiota by TCRs on $Foxp3^+$ Treg and other types of Treg cells can lead to the secretion of anti-inflammatory cytokines (e.g. IL-10 and transforming growth factor-β), which also contributes to immune tolerance (Sakaguchi et al., 2008).

2.1.2 Intestinal epithelial barrier

In addition to the immunological defense systems, the cell composition and structure of the epithelial layer are of paramount importance for gut functions and homeostasis. The intestinal epithelium is made up of a single layer of epithelial cells of several types. Enterocytes, the major class of IECs, are in charge of absorption, while specialized and secretory IECs promote digestive or barrier functions (Peterson and Artis, 2014). Specifically, enteroendocrine cells secret a variety of hormones that assist in digestion. Goblet cells synthesize trefoil peptides and mucins (e.g., MUC1 and MUC2) that form a mucus layer located directly above the epithelial layer (Figure 1), thereby protecting the gut by reducing the contact between IECs and the gut microbiota. For instance, mucin MUC2-lacking mice spontaneously suffer from colonic inflammation (Van der Sluis et al., 2006). Paneth cells, another type of specialized IECs, are capable of secreting many antimicrobial peptides (e.g., defensins), which break down important bacterial surface components (e.g., surface membranes and Gram-positive cell wall peptidoglycans) in order to minimize the bacterial number at the intestinal epithelial surface (Tlaskalová-Hogenová et al., 2011; Peterson and Artis, 2014). The adjacent epithelial cells are connected by junctions, including tight junctions (TJs), adherens junctions, and desmosomes (Figure 1), thus forming a well-sealed epithelium (Turner, 2009). Among them, the TJ is the most decisive factor in controlling mucosal permeability. It is mainly composed of various proteins, including transmembrane (e.g., claudin family and occludin) and peripheral membrane proteins (e.g. zonula occludens, ZO) (Turner, 2009). Collectively, these structural characteristics lead to intact barriers that prevent the invasion of gut-resident microbes and pathogens, and, together with the immunological defense systems and the normal gut microbiota (see below), lay the foundation for maintaining intestinal immune homeostasis.

2.2 Gut microbiota

The microbiota residing in the mammalian gut lumen and mucosa represents greater diversity and larger number of microbes (bacteria, viruses, and parasites) than the microbiota in other niches of the body. Human gut microbiota contains about 100 trillion organisms, which is approximately equal to the total number of human cells in the body (Sender et al., 2016). Collectively, 99.1% of the human gut microbiome (collective genome of the gut microbiota) is of bacterial origin (Qin et al., 2010), and it is estimated that 100–1000 bacterial species reside in the human gut (Browne et al., 2016). Along the GIT, the concentration of oxygen continuously declines, while the diversity and abundance of intestinal bacteria gradually rise in both the human and animal gut, as illustrated in Figure 3. For instance, the upper bowel in humans, from the stomach to the jejunum, possesses fewer bacteria, ranging from 10^1 to 10^7 organisms per ml of intestinal content, while the distal small intestinal tract and colon harbor 10^{10} to 10^{13} per ml (Dethlefsen et al., 2006; O'Hara and Shanahan, 2006; Sekirov et al., 2010; Zoetendal et al., 2012). Most gut bacteria are considered uncultivable in laboratories (Walker et al., 2014). However,

a recent study challenged such a notion, demonstrating that a large proportion of human gut bacteria are cultivable by using a novel workflow that combines metagenomics and culture (Browne et al., 2016). With more cultivable bacteria, it is possible to characterize the gut microbiota at the species level, further expanding our knowledge of this "newly considered organ".

As previously mentioned, the gut microbiota consists of autochthonous (indigenous) microbes that persistently colonize the GIT and allochthonous (transient) members that pass through the GIT like hitchhikers (Ley et al., 2006a). Autochthonous microbes are thought to be in close contact with the mucosa, while allochthonous microbes mainly reside in the intestinal lumen (Nava and Stappenbeck, 2011). In general, both autochthonous and allochthonous gut microbes may represent different types of symbiotic relationships with the host, including mutualism, commensalism, or parasitism (Walter et al., 2011). In particular, mutual benefit between the host and the microbes is considered to be the main status (Macpherson and Harris, 2004). The GIT provides a nutrient-rich niche for the gut microbiota with steady growth conditions under most circumstances, while in return the gut microbiota assists the GIT in fulfilling its functions.

There has been increasing interest in studying the structure and composition of the gut microbiota, especially that of humans. Fecal samples, which resemble the bacterial composition of the distal colon, have frequently been used in most of the studies, but lumen contents and mucosal samples from different segments of the GIT have also been investigated. Generally, the gut microbiota is divided into the mucosal and luminal one, and these two microbiotas differ in microbial populations and functions (Eckburg et al., 2005). For example, in humans, the anaerobe/aerobe ratio in the luminal microbiota is higher than that in the mucosal microbiota (O'Hara and Shanahan, 2006). Since the mucosal microbiota is closer to the intestinal epithelium, it might be more important in mediating mucosal immune responses, while the luminal microbiota might be more involved in digestion (Van den Abbeele et al., 2011).

2.2.1 Structure and composition of the gut microbiota of humans and pigs

Humans and pigs share many similarities in the anatomy and physiology of the GIT, which is made up of the stomach, small intestine (duodenum, jejunum, and ileum) and large intestine (cecum, colon, and rectum), as shown in Figure 3 (Pond and Houpt, 1978; Pryde et al., 1999; Loh et al., 2006). Nevertheless, the lengths of the small intestine and colon of adult pigs are about 15–22 m and 4–6 m, respectively (Mochizuki and Makita, 1998), while the corresponding values are approximately 6 m and 1.5 m in adult humans. With regard to physiological functions, the stomach mainly initiates the breakdown of proteins and fats. It produces hydrochloric acid, which makes the stomach acidic and also assists in the conversion of saliva-sourced nitrite to nitric oxide, eliminating swallowed pathogens (Benjamin et al., 1994). The small intestine, with bile acids secreted in the duodenum and digestive enzymes present along the whole length, is primarily in charge of the digestion and absorption of ingested food, including carbohydrates, proteins and fat, as well as the absorption of most vitamins, iron, calcium, and water (Ganong and Ganong, 1995). The large intestine is responsible for final water absorption and the digestion of indigestible substances through microbial fermentation (Ganong and Ganong, 1995; Bäckhed et al., 2005).

Human gut microbiota

The advent of modern technologies, such as 16S ribosomal RNA gene-based sequencing, metagenomics, and transcriptomics, has enabled the detection of a much wider range of bacterial identities at the species level and facilitated the study of the impact of human gut microbiota on host health. Irrespective of differences in age and geographic location, it has been reported that the human gut microbiota is dominated by four phyla: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (De Filippo et

al., 2010; Li et al., 2014; Odamaki et al., 2016). However, accumulated evidence has shown that the composition of the gut microbiota changes from birth to later life.

The delivery mode, feeding methods, and antibiotics greatly affect the gut microbiota composition in infants (Penders et al., 2006; Dominguez-Bello et al., 2010). Generally, the infant gut microbiota has smaller bacterial loads (Mariat et al., 2009) and larger variation between individuals (Yatsunenko et al., 2012) in comparison to the microbiota of older age groups; in babies fed with either breast milk or formula, it is dominated by the genus *Bifidobacterium*. With the introduction of solid foods, the bacterial diversity increases and the composition is modified: bifidobacteria and members of the *Enterobacteriaceae* family decrease, while *Lachnospiraceae* and *Ruminococcaceae* increase (Fallani et al., 2010; Bergström et al., 2014). An adult-like gut microbiota is developed within the first three years (Yatsunenko et al., 2012; Bergström et al., 2014). Based on the analysis of fecal samples or colonic mucosal tissue from healthy adults, the gut microbiota in healthy human adults mainly consists of the two most abundant phyla, Firmicutes and Bacteroidetes (Eckburg et al., 2005; Qin et al., 2010), in which most of the members are anaerobic, and which include the genera *Eubacterium*, *Clostridium*, *Ruminococcus*, *Faecalibacterium*, and *Bacteroides* (Palmer et al., 2007). Studies on the bacterial composition of the small intestine in healthy adults are considerably limited compared to those of the colon. In general, the small intestine has a lower bacterial diversity than the colon, as revealed by 16S rRNA gene sequencing and metagenomics (Booijink et al., 2010; Zoetendal et al., 2012). Ileostomy effluent samples from one healthy adult revealed that the small intestine was dominated by *Clostridium*, *Streptococcus*, *E. coli*, and high G+C Gram-positive bacteria (Zoetendal et al., 2012). Subsequently, another study found that ileostomy samples from four healthy adults were dominated by the genera *Streptococcus* and *Veillonella* (den Bogert et al., 2013). The gut microbiota in elderly people also shows changes compared to younger adults. Typically, Bacteroidetes is more abundant and the most dominant, while members of Firmicutes are decreased (Claesson et al., 2011). A higher abundance of *Bacteroides*, *Eubacterium*, and *Clostridiaceae* has also been observed in elderly Japanese over 70 years old (Odamaki et al., 2016). In addition to age, many other factors are also associated with the individual variability in bacterial composition, such as diet and antibiotics. However, the gut microbiota within an individual is relatively stable upon perturbations throughout life (Lozupone et al., 2012).

Pig gut microbiota

The bacterial composition of the pig gut microbiota resembles that in humans. Firmicutes and Bacteroidetes are also predominant in pigs at any age (Lamendella et al., 2011; Looft et al., 2012; Kim and Isaacson, 2015); however, differences between these two mammals still exist (Figure 3). For instance, bifidobacteria either cannot be found or are detected at low levels in the pig gut (Leser et al., 2002; Dowd et al., 2008; Petri et al., 2010; Zhao et al., 2015), whereas they are common colonizers in the human gut (Turroni et al., 2008). In contrast, the genus *Lactobacillus* is abundant in the pig gut microbiota (Dowd et al., 2008; Niu et al., 2015), while lactobacilli are subdominant in the human gut, comprising maximally about 1% of the total bacteria (Guarner and Malagelada, 2003; Mueller et al., 2006). In studies that have used strict anaerobic culture methods, the majority of pig gut bacteria have been identified as Gram positive and have mainly included lactic acid bacteria (LAB, mostly consisting lactobacilli and streptococci), eubacteria, and clostridia (Salanitro et al., 1977; Russell, 1979; Robinson et al., 1984; Pryde et al., 1999). Subsequently, comparative 16S DNA sequencing analysis has not only confirmed the above findings, but also revealed that the genera *Bacteroides* and *Prevotella* dominate in the less-abundant Gram-negative group (Leser et al., 2002). In the GIT of newborn and suckling piglets, the first dominant colonizers include clostridia and lactobacilli (Konstantinov et al., 2006; Petri et al., 2010). From weaning to maturity, these bacteria are still predominant, even though the relative proportions show fluctuations (Konstantinov et al., 2006). Similarly to the human gut microbiota, the bacterial number in the lumen content of pigs is much higher than that of the intestinal wall, based on

direct microscopic counts and viable colony counts (Allison et al., 1979; Russell, 1979; Pryde et al., 1999). Recently, a broader view of the ecology of the pig gut microbiota in different regions of the GIT was presented. It was found by pyrosequencing that the ileum with digesta from weaned pigs (about 21 days old) mainly contains *Clostridium* spp., *Lactobacillus* spp., *Streptococcus* spp., and *Sarcina* spp. (Dowd et al., 2008). Later, Isaacson and Kim (2012) revealed the bacterial composition in the jejunum, which was dominated by the phylum Firmicutes (90%). In contrast, Zhao et al. demonstrated that the main phyla in the jejunum of adult pigs include Proteobacteria (79.5%), Firmicutes (18.7%), and Bacteroidetes (1%) (Zhao et al., 2015). Numerous studies have shown that in the ileum, Firmicutes and Proteobacteria are the major phyla (Dowd et al., 2008; Isaacson and Kim, 2012; Looft et al., 2014; Zhao et al., 2015), with *Streptococcus*, *Clostridium*, *Anaerobacter*, and *Lactobacillus* being the most important at the genus level. Two studies demonstrated that the major phyla in the cecum and colon/mid-colon were Firmicutes and Bacteroidetes (Isaacson and Kim, 2012; Looft et al., 2014), whereas in another study, Firmicutes and Proteobacteria were the dominant phyla (Zhao et al., 2015). Considering the differences in pig breeds, diet, growth conditions, and other factors, such a discrepancy is conceivable.

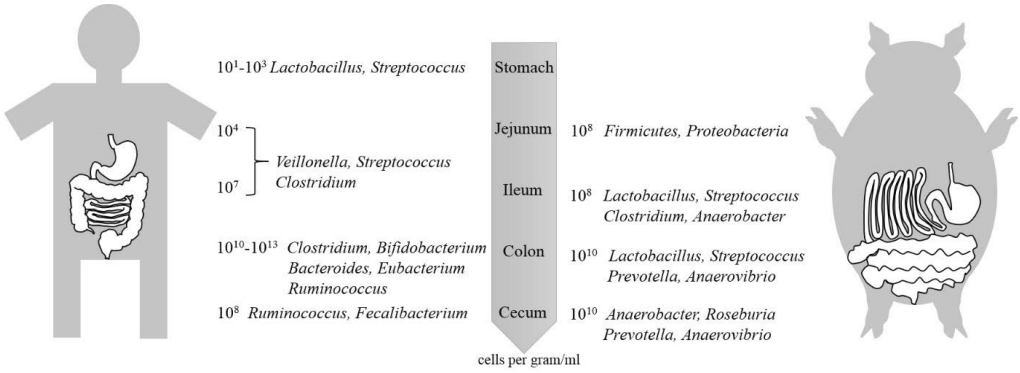


Figure 3. The gastrointestinal tract and the dominant phyla or genera in different regions of the GIT in adult humans and pigs. Data of human studies from Dethlefsen et al., 2006; O'Hara and Shanahan, 2006; Sekirov et al., 2010; and Zoetendal et al., 2012. Data of pig studies from Allison et al., 1979; Pryde et al., 1999; Konstantinov et al., 2006; Loh et al., 2006; Looft et al., 2014; and Zhao et al., 2015.

2.2.2 Role of the gut microbiota

The gut microbiota has highly evolved to adapt to the host. Therefore, in healthy individuals, the intestinal immune system exhibits tolerance to the resident microbes, while it protects the host from intestinal pathogen infection. It is now evident that the interactions between the intestinal immune system and the gut microbiota play a vital role in the maintenance of gut homeostasis. Meanwhile, the gut microbiota has a role in the modulation of host metabolism and the control of invading pathogens. However, it is also implicated in host diseases.

2.2.2.1 Structural and developmental effects of the intestine

Structural development of the GIT

In the absence of the gut microbiota, germfree mice generally have a smaller intestinal surface area (Gordon and Bruckner-Kardoss, 1961), longer and thinner villi, and a lower vessel density in the villi (Sommer and Bäckhed, 2013). However, introducing the normal gut microbiota to germfree mice resulted in the villi becoming shorter and wider, and thus more capable of efficiently inhibiting microbial invasion (Sommer and Bäckhed, 2013). Additionally, the structural development of the

intestinal immune system in germfree mice shows multiple defects, thus highlighting the important role of the gut microbiota in its development. As an example, without gut microbiota, ILFs are few and undeveloped, resulting in a lack of B cells (Bouskra et al., 2008; Hooper et al., 2012). However, the number of ILFs was increased by the colonization of germfree mice with Gram-negative gut bacteria (e.g., *Bacteroides distasonis* and *E. coli*) or the introduction of peptidoglycan isolated from *E. coli* (Bouskra et al., 2008). Moreover, as compared with colonized animals, germfree animals possess fewer and smaller PPs and MLNs, a thinner LP, and fewer plasma cells in germinal centers (Round and Mazmanian, 2009). Collectively, the lack of the gut microbiota results in the impaired development of GALTs, therefore leading to deficiencies in immune responses, as demonstrated by the observation that germfree mice exhibit an increased susceptibility to infections caused by enteropathogens (Round and Mazmanian, 2009).

Enhancement of the intestinal epithelial barrier

Mounting evidence has shown that the gut microbiota is capable of fortifying the physical and chemical intestinal barriers: the mucus layer, the epithelial layer, and secreted antimicrobial peptides (e.g. α-defensins). In antibiotic-treated mice, the expression of MUC2 mucin, which is the major component of the mucus layer, was reduced in goblet cells, resulting in a thinner inner mucus layer, which in turn caused more severe enteric pathogen *Citrobacter rodentium*-derived colitis, compared to untreated mice (Wlodarska et al., 2011). Additionally, in germfree mice, the colonization of *Bacteroides thetaiotaomicron* (a member of the gut microbiota) strengthened the intestinal barrier functions through the upregulation of expression of Sprr2a protein, which contributed to the formation of desmosomes (Hooper et al., 2001). Moreover, gut commensal *E. coli* strains (e.g., *E. coli* Nissle 1917 and ECOR63) and their extracellular products enhanced the epithelial barrier function via the upregulation of ZO-1 and claudin-14, and the downregulation of claudin-2 involved in epithelial pore formation, thus fortifying TJs (Ukena et al., 2007; Alvarez et al., 2016). In addition to the presence of bacterial cells, bacterial metabolites also fortify the epithelial barrier. As an example, short-chain fatty acids (SCFAs) produced by gut-resident bacteria increase MUC2 expression in IECs and modulate the location of ZO-1 and occludin, which thus increases the trans-epithelial electrical resistance (TEER, a parameter to assess the epithelial barrier integrity) (Willemsen et al., 2003; Peng et al., 2009). Importantly, the gut microbiota has been shown to protect the intestinal epithelia from radiation-induced injury (Rakoff-Nahoum et al., 2004) and to stimulate epithelial proliferation in the mouse small intestine (Hörmann et al., 2014). Additionally, the gut microbiota enhances the barrier function by inducing the production of antimicrobial molecules (Kamada et al., 2013). The introduction of gut-resident bacteria to germfree mice strongly triggers the coordinated expression of antimicrobial lectin REGIIIγ in Paneth cells, which binds to the peptidoglycan of luminal bacteria and thus restricts bacterial invasion (Cash et al., 2006).

2.2.2.2 Immunological effects

The mucus layer minimizes direct contact with the gut microbiota, and the intestinal epithelium reduces the interactions between the gut microbiota and the intestinal immune system. However, some bacteria in the gut lumen, as well as bacterial products and structural components, have the potential to translocate through mucus and attach to or enter the IECs, thus activating inflammatory innate immune responses. In a healthy host, the intestinal immune system is tolerant to the gut microbiota, but initiates inflammatory immune responses in order to eliminate invading pathogens. The gut microbiota has the potential to modulate mucosal immunity by contributing to both immunological defense and tolerance. Indeed, mono-colonization of germfree mice with 53 individual bacterial species from the human gut microbiota covering 5 dominant phyla (Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria) revealed that the majority of the tested bacteria had strain-specific immunomodulatory properties (Geva-Zatorsky et al., 2017).

Effects on innate immunity. Recognition of the gut-resident microbes by TLRs at the mucosal site is crucial for maintaining gut immune homeostasis. It has been demonstrated that antibiotic-treated mice lose the ability to produce cytokines via the MyD88-dependent pathway, whereas conventional mice yield a pronounced level of IL-6 that could protect the host from colonic injury caused by dextran sulfate sodium (Rakoff-Nahoum et al., 2004). Similarly, a recent study proved that, under steady-state conditions, sensing of microbial signaling from the gut microbiota by intestinal macrophages can activate the MyD88-dependent pathway and evoke IL-1β secretion, leading to the constant production of growth factor GM-CSF (also termed CSF2), which promotes intestinal homeostasis by regulating the number and function of macrophages and DCs (Mortha et al., 2014). In contrast, encountering of antibiotic-treated microbiota by macrophages resulted in the dysfunction of DCs (Mortha et al., 2014).

Moreover, the gut microbiota enhances ROS generation and thus mediates innate immune responses. IECs contacted by gut commensal bacteria immediately produce ROS, which results in IκB degradation (but not dissociation from NF-κB) through cullin-dependent signaling, thus leading to the suppression of the NF-κB signaling pathway (Kumar et al., 2007). SCFAs produced by gut bacteria also stimulate ROS production. Butyrate produced by commensal bacteria degraded IκB, resulting in the inhibition of NF-κB activation (Kumar et al., 2009). Notably, SCFAs are also able to regulate the expression of cytokines and chemokines in various leukocytes and then exhibit anti-inflammatory effects (Vinolo et al., 2011b). For example, butyrate impairs the production of pro-inflammatory cytokines (IL-12 and TNF-α), but upregulates anti-inflammatory IL-10 secretion, and is also able to inhibit NF-κB activation (Segain et al., 2000; Säemann et al., 2000; Vinolo et al., 2011a). A recent study has demonstrated that other metabolites, including palmitoleic acid and tryptophan, have an inhibitory effect on the production of pro-inflammatory cytokines induced by certain pathogens *ex vivo* (Schirmer et al., 2016), suggesting that these metabolites may be used to treat diseases caused by excessive pro-inflammatory cytokine production, such as autoimmune disease.

Effects on adaptive immunity. The gut microbiota is associated with T-cell differentiation and pro/anti-inflammatory cytokine production. Th17 cells are deficient in the small intestinal LP of germfree mice; however, upon colonization with a normal mouse gut microbiota or segmented filamentous bacteria (most closely related to *Clostridia*), they are upregulated, which promotes the secretion of various cytokines protecting the host from pathogen infections (Ivanov et al., 2009; Atarashi et al., 2015). In mice with *Citrobacter rodentium* infection, SCFAs promote the differentiation of naïve T cell into Th1 or Th17 in order to fight pathogens (Park et al., 2015).

Meanwhile, the gut microbiota is also able to modulate Treg differentiation. Oral inoculation of a mixture of 17 clostridial strains isolated from human feces largely enriched Tregs and IL-10 in the colon LP of germfree mice, and provided protection against colitis (Atarashi et al., 2013). The three dominant SCFAs, butyrate, propionate, and acetate (Macfarlane and Macfarlane, 2003), also promote the generation of colonic Tregs and further dramatically increase the expression of Foxp3 and IL-10, which suppresses the excessive immune responses and protects the host against colitis (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013).

Furthermore, the gut microbiota plays an essential role in sIgA production. Plasma cells in the intestine of germfree animals are drastically decreased (Honda and Littman, 2016), and the produced sIgA fails to bind the bacterial components (Macpherson et al., 2000), while the introduction of a normal gut microbiota immediately promotes sIgA secretion. It has also been reported that sIgA diversity is enhanced by the increased complexity of the gut microbiota, as germfree mice colonized with the complex gut microbiota from specific-pathogen-free mice generate more diverse IgA repertoires than those colonized with single strains of gut microbes (e.g., segmented filamentous bacteria or *Clostridium* strains) (Lindner et al., 2015), therefore binding to a larger extent of antigens.

2.2.2.3 Protective effects against gastrointestinal pathogens

The gut microbiota located in the mucus layer and close to the intestinal epithelia potentially acts as a microbial barrier to protect the host from pathogen infection, highlighted by the observation that the susceptibility to intestinal pathogens is largely increased in germfree and antibiotic treated-mice (Lawley et al., 2009; Fukuda et al., 2011). Protective effects against pathogens conferred by the gut microbiota are generally grouped into two types, direct and indirect inhibition, which are both based on multiple mechanisms (Buffie and Pamer, 2013).

Direct inhibition of pathogens

First of all, the ability of the gut microbiota to outcompete pathogens for common and essential nutrients might lead to the starvation or exclusion of pathogens (Kamada et al., 2012; Kamada et al., 2013). As an example, a commensal microbe, *Escherichia coli* Nissle 1917, outcompeted *Salmonella* typhimurium for iron and thus reduced *Salmonella* colonization of the GIT of chronically and acutely infected mice (Deriu et al., 2013). In addition, the gut microbes may inhibit pathogen colonization by occupying common binding sites. For instance, the adhesive *L. crispatus* JCM 8779 isolated from human feces strongly reduced the adherence of *Enterococcus faecalis* TT1 to Caco-2 cells (Todoriki et al., 2001). Furthermore, acidic conditions (e.g. lactic acid) and certain metabolites, such as SCFAs and antimicrobial molecules produced by the gut microbiota, have a pivotal role in pathogen inhibition. The growth of *Escherichia coli* O157:H7 is markedly inhibited by acetate, butyrate, and propionate produced by gut anaerobes (Shin et al., 2002; Fukuda et al., 2011). Butyrate also reduces the invasion of IECs by *Salmonella enterica* by down-regulating the expression of several virulence genes (Gantois et al., 2006). Bacteriocins are antimicrobial peptides secreted by various gut-resident bacteria that can inhibit bacterial growth. For instance, bacteriocins produced by *Bacillus thuringiensis* effectively kill clinical *Clostridium difficile* strains *ex vivo* (Rea et al., 2010). Recently, 74 bacteriocin gene clusters were found in the human gut microbiome (Walsh et al., 2015), suggesting that the human gut microbiota has the potential to inhibit intestinal pathogen growth. Notably, recent studies have demonstrated that antimicrobial proteins with antagonistic effects against competitors and enteric pathogens are secreted through the type VI secretion systems (T6SSs) of gut-dwelling bacteria in a cell-to-cell contact manner (Russell et al., 2014; Hecht et al., 2016). T6SSs are frequently found in the genomes of many gut-resident bacteria, especially in members of the gut-dominant phylum Bacteroidetes (Chatzidaki-Livanis et al., 2016). For instance, non-pathogenic and enteropathogenic *Bacteroides fragilis* strains are ubiquitous in the GIT, and a recent *in vivo* study revealed that non-pathogenic *B. fragilis* utilizes T6SSs to provide colonization resistance against enteropathogenic *B. fragilis* and to suppress enteropathogenic *B. fragilis*-induced colitis (Hecht et al., 2016).

Indirect inhibition of pathogens

The gut microbiota can also indirectly inhibit pathogen colonization based on its ability to strengthen the epithelial barrier function and to evoke mucosal immune responses. The underlying mechanisms include the promotion of antimicrobial peptide expression and sIgA production, effects on T-cell differentiation, and pro/anti-inflammatory cytokine secretion in the host, as discussed in sections 2.2.2.1 and 2.2.2.2. Furthermore, another indirect inhibition mechanism of pathogens is associated with the production of secondary bile acids, which are transformed from bile acids by the gut microbiota (Rolhion and Chassaing, 2016). Secondary bile acids have antimicrobial effects, as demonstrated by the observation that their production was decreased in antibiotic-treated mice, resulting in the colonization of pathogenic *Clostridium difficile* (Theriot and Young, 2015; Theriot et al., 2016). In another example, *Clostridium scindens*, a member of the gut microbiota that participates in secondary bile acid synthesis, inhibits *Clostridium difficile* growth in a manner dependent on secondary bile acids (Buffie et al., 2015).

2.2.2.4 Nutritional effects

In accordance with the variation in bacterial composition between the small intestinal and colon microbiota, it has been reported that the bacteria in the small intestine are capable of the rapid uptake and degradation of simple carbohydrates, while colon bacteria specifically ferment complex carbohydrates (Zoetendal et al., 2012). The gut microbiota also enhances nutrient absorption, as demonstrated by the observation that the mRNA levels of genes involved in lipid absorption are elevated after introducing a gut-resident bacterial species, *Bacteroides thetaiotaomicron*, to germfree mice (Hooper et al., 2001).

In the colon, the gut microbiota ferments indigestible carbohydrates, such as starch and fiber, and many of the resulting metabolites, such as SCFAs, serve as energy sources for the host (Macfarlane and Macfarlane, 2003; Qin et al., 2010). Specifically, as the main SCFA, most acetate is absorbed by liver cells and used as the substrate for cholesterogenesis and lipogenesis (den Besten et al., 2013; Canfora et al., 2015); propionate appears to be the precursor of gluconeogenesis, while butyrate is a substrate for lipogenesis (Wong et al., 2006; Canfora et al., 2015) and the preferred energy source for colon epithelial cells (Donohoe et al., 2011). Additionally, SCFAs have other nutritional functions in the body, as they may, for instance, promote adipocyte differentiation, insulin sensitization, and blood glucose level control (Canfora et al., 2015). Collectively, the potential of bacterial metabolites to modulate the host metabolism may affect the ability of the host to maintain blood glucose levels and lipid content, as well as body energy homeostasis. An imbalanced gut microbiota produces imbalanced metabolites that may lead to host diseases. The gut microbiota is also involved in drug detoxification (Claus et al., 2011) and the beneficial transformation of bioactive compounds (O'keefe, 2016), whereas it is additionally implicated in some detrimental activities as well, such as the generation of carcinogens (Hope et al., 2005) and the activation of genotoxicants (Blaut et al., 2006).

In addition to SCFAs, certain gut bacteria are suppliers of amino acids and vitamins, including vitamins of the B group (e.g., folate and riboflavin) and vitamin K (Hill, 1997; Qin et al., 2010). Vitamin production by the gut microbiota compensates for the biosynthesis deficiency of many essential vitamins in humans (Hill, 1997; LeBlanc et al., 2013). Compared to conventionally reared animals, germfree animals require vitamin K and more B-group vitamins in their diet (Sumi et al., 1977; Wostmann, 1981), indirectly suggesting that bacterially synthesized vitamins can be assimilated by the host. A study performed in humans directly demonstrated that folate produced by small intestinal bacteria was absorbed and used by the human body (Camilo et al., 1996).

2.2.2.5 Pathogenic effects

An increasing number of studies have demonstrated that the gut microbiota is associated with various disorders of metabolism, the GIT, heart and brain. These disorders include type 1 (Vatanen et al., 2016) and 2 diabetes (Mardinoglu et al., 2016), obesity (Ley et al., 2006b), liver diseases through the gut–liver axis, alcoholic (Yan et al., 2011) and nonalcoholic fatty liver disease (Abu-Shanab and Quigley, 2010)), irritable bowel syndrome (IBS) (Kassinen et al., 2007), IBD (Morgan et al., 2012), heart failure (Kamo et al., 2017), asthma (Abrahamsson et al., 2014), allergy (West et al., 2015), GI and non-GI cancers (Yoshimoto et al., 2013; Louis et al., 2014), and even anxiety and depression (Foster and Neufeld, 2013), autism (Strati et al., 2017), multiple sclerosis (Berer et al., 2011), and Parkinson's disease (Scheperjans et al., 2015) through the gut–brain axis. An imbalanced gut microbiota has been associated with the pathogenesis of these diseases, but whether the dysbiosis is a causative contributor or a consequence of these disorders remains in most cases unknown. Dysbiosis is characterized by the reduction in commensals and diversity, and the expansion of pathobionts, i.e. bacteria that live as commensals in the GIT under normal circumstances, but which have the potential to cause diseases (Levy et al., 2017). For instance, a reduced abundance of Firmicutes (e.g. *Faecalibacterium prausnitzii*)

and a relative enrichment in *Enterobacteriaceae* (e.g. *E. coli*) was observed in patients suffering from IBD, both in Crohn's disease affecting the whole GIT, and in ulcerative colitis only affecting the colon (Baumgart and Sandborn, 2007; Kostic et al., 2014; Matsuoka and Kanai, 2015). Treatments, such as fecal microbiota transplantation and probiotics, aiming at normalizing the gut microbiota, have been employed with promising outcomes to ameliorate IBD (Matsuoka and Kanai, 2015).

2.3 Lactic acid bacteria

LAB, also termed as the order *Lactobacillales*, are Gram-positive, nonsporulating cocci or rods, which are listed under the phylum Firmicutes (Von Wright and Axelsson, 2011). They inhabit a variety of niches, including the environment, plants, fermented food, skin, the oral cavity, and the GIT of mammals. Since ancient times, LAB have been extensively used in food processing, such as food fermentation. Meanwhile, they are generally considered as safe bacteria with various health-promoting properties. Among LAB, *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus* are the most important genera (Makarova et al., 2006). Based on their fermentation characteristics, LAB are divided into homofermentative LAB, which produce lactic acid as the main end-product, and heterofermentative LAB, which produce lactic acid as well as carbon dioxide, acetic acid, and ethanol (Kandler, 1983). In the human GIT, LAB account for a small part of the gut microbiota (Douillard and de Vos, 2014). For instance, in the study of Rossi et al. (2016), their abundance was 0.08–0.3% of the total bacteria in human fecal samples. Nevertheless, they provide many beneficial effects to the host. Lactic acid and other metabolic products (e.g. bacteriocins) have antimicrobial effects that are able to inhibit infection by various pathogens (Dicks and Botes, 2009). LAB also synthesize important vitamins in the GIT, such as folate and riboflavin (LeBlanc et al., 2013). Importantly, LAB possess immunomodulatory properties that strengthen host immunity (Perdigon et al., 2002; Dicks and Botes, 2009).

2.3.1 The genus *Lactobacillus*

Lactobacillus is the largest and highly heterogeneous genus of LAB (Claesson et al., 2007), with currently more than 220 species identified from various origins (http://www.bacterio.net, last searched September 2017). Members of the genus *Lactobacillus* are rod-shaped, anaerobic or aerotolerant bacteria, classified as obligately homofermentative, or facultatively/obligately heterofermentative lactobacilli (Hammes and Vogel, 1995). *Lactobacillus* species have a long history of utilization in food production and preservation. Due to their beneficial effects, *Lactobacillus* strains have been consumed as probiotic supplements by humans and animals more frequently than other LAB (Ouwehand et al., 2002). Additionally, some genetically modified lactobacilli have proven to be effective delivery vectors of foreign (immunogenic or therapeutic) antigens in mucosal vaccination (Wyszyńska et al., 2015). Furthermore, a recent study has revealed that the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas adaptive immune systems are broadly present in lactobacilli (Sun et al., 2015). CRISPR-Cas systems contribute to resistance to foreign genetic material, such as bacteriophage DNA (Horvath and Barrangou, 2010), consequently increasing the survival rates of lactobacilli in competitive environments, such as the GIT.

In the GIT, lactobacilli are either permanent colonizers or transient passengers. Thus far, it has been demonstrated that in the *Lactobacillus* genus, *L. ruminis*, *L. salivarius*, *L. gasseri*, and *L. reuteri* are truly autochthonous species (Tannock et al., 2000; Reuter, 2001), which have adapted to the host GIT environment and are thus able to colonize certain sites lifelong. In contrast, several well-studied species, including *L. acidophilus* and one of the best characterized health-promoting microbes, *L. rhamnosus* GG, originate from food products and have been referred to as allochthonous strains (Walter, 2008).

2.3.1.1 Probiotic lactobacilli

As defined by the World Health Organization (WHO), probiotics are live microorganisms that confer a health benefit on the host when administered in adequate amounts. Possible probiotic actions are illustrated in Figure 4. Probiotics are versatile with regard to their functions. They have been proposed, for example, to cure or prevent diarrhea (Isolauri et al., 1991; Szajewska and Mrukowicz, 2001; Dendukuri et al., 2005), control IBS and IBD (Kruis et al., 1997; Niedzielin et al., 2001; Whelan and Quigley, 2013), prevent necrotizing enterocolitis in babies (Lin et al., 2005; AlFaleh and Anabrees, 2014) and atopic dermatitis in infants and young children (Kalliomäki et al., 2001; Kalliomäki et al., 2003; Pelucchi et al., 2012), reduce cholesterol levels (Ooi and Liong, 2010), ameliorate constipation (Chmielewska and Szajewska, 2010), and relieve allergies and asthma (Majamaa and Isolauri, 1997; West et al., 2016).

Therefore, searching for probiotic candidates is still of great interest nowadays. The screening process is based on a few established selection criteria. First and foremost, probiotics must be safe to the host. Another important prerequisite is that, in order to maintain the viability of the bacteria, candidate probiotics should be able to withstand the harsh conditions encountered during industrial processing as well as in the GIT (e.g. the acidity in the stomach). Furthermore, an adhesive property is a priority for a candidate probiotic in order to increase retention in the GIT and to interact with the host. Targets used when testing adherence *in vitro* usually comprise extracellular matrix (ECM) proteins, intestinal mucus, intestinal epithelial cells (e.g., Caco-2 and HT-29), and host tissues (Ouwehand and Salminen, 2003). Additionally, based on adhesion, candidate probiotics should also have potential to inhibit the adhesion of gastrointestinal pathogens to host tissues, and thus provide beneficial effects (Collado et al., 2007). The commonly employed *in vitro* inhibition assay types, based on the same substrates as the adhesion tests, are the following: competition (probiotics and pathogens are added simultaneously), competitive exclusion (probiotics are added before pathogens), and displacement (pathogens are added before probiotics) (Lee et al., 2003; Collado et al., 2007; Hynönen et al., 2014). The following mechanisms have been suggested to underlie inhibition: i) competition for common adhesion sites on the host intestinal mucosa, ii) bacterial coaggregation, and iii) the secretion of inhibitory proteins (Lebeer et al., 2008; Hynönen et al., 2014). Lastly, still another practical requirement is that preferably it should be possible to consider candidate probiotics as immune boosters that have the potential to modulate immune responses (Delcenserie et al., 2008).

In addition to *Lactobacillus*, *Bifidobacterium* and other LAB strains such as *Streptococcus thermophilus* (Saavedra et al., 1994), *Streptococcus salivarius* K12 (Burton et al., 2006), and *Enterococcus faecium* (Hlivak et al., 2005) are commonly consumed as probiotics. Commercial probiotic products can contain single bacterial strains or mixtures of several species representing several genera (Table 1). Examples of the proposed health-promoting properties of lactobacilli currently used as probiotics in Finland are listed in Table 1.

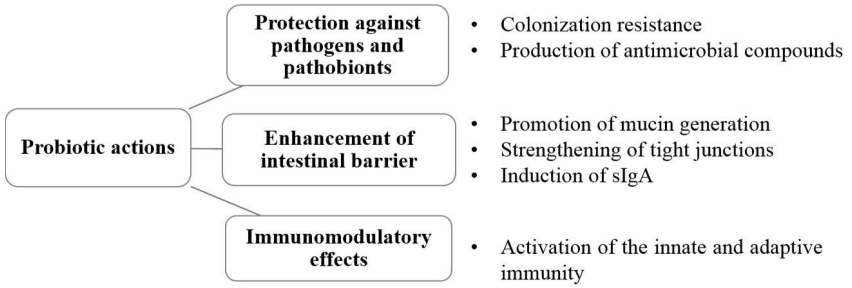


Figure 4. **Proposed beneficial actions of *Lactobacillus* probiotics**. Adapted from Lebeer et al., 2008.

Table 1. Selected *Lactobacillus*-containing probiotics marketed in Finland for children and adults

Products	*Lactobacillus* species included	Examples of effects proven by *in vitro* and *in vivo* studies
Monostrain: **Rela** Bacterial mixture: **Lacto seven**, **Puhdas+ vahva maitohappobakteeri** ("clean + strong lactic acid bacterium"), and **YA: Maitohappobakteeri+*S. Boulardii***	*L. reuteri*	Inhibition of the growth of enteric bacterial pathogens *in vitro* (Spinler et al., 2008) Increase in the number of B and CD4+ T lymphocytes in the GIT (Valeur et al., 2004) Suppression of *Helicobacter pylori* infection in children (Lionetti et al., 2006) Prevention of antibiotic-associated diarrhea (Cimperman et al., 2011) Amelioration of infantile colic (Savino et al., 2007)
Monostrain: **Gefilus** and **Floridral** Bacterial mixture: **Lacto seven**, **Idoform**, **Puhdas+ vahva maitohappobakteeri**, **YA: Maitohappobakteeri+*S. Boulardii***, **Probiootti junior**, **Probiootti comp**, **Probiootti plus**, and **Probiotika**	*L. rhamnosus*	Summarized in chapter 2.3.1.3.2
Bacterial mixture: **Lacto seven**, **Acidophilus Forte**, **Ratiopharm**: **maitohappobakteeri+B/BCD**, **Replete intensive**, **Acidophilus**, **Vivomixx**, **Probiootti strong**, **Probiootti comp**, **Probiootti plus**, and **Probiotika**	*L. acidophilus*	Reduction of adherence to tissue culture cells and modification of virulence of *E. coli* O157: H7 *in vitro* and *in vivo* (Medellin-Pena and Griffiths, 2009) Amelioration of symptoms of collagenous colitis (Wildt et al., 2006) Modulation of immature DCs and T-cell functions *in vitro* (Konstantinov et al., 2008) Improvement in insulin sensitivity in type 2 diabetes *in vivo* (Andreasen et al., 2010) Improvement in stool frequency and immunity (Ouwehand et al., 2008) Relief of bloating symptoms in patients with functional bowel disorders (Ringel et al., 2011)
Bacterial mixture: **Vivomixx**, **Probiootti comp**, and **Probiootti plus**	*L. paracasei*	Modulation of the immune system *in vivo* (Roessler et al., 2008) Amelioration of constipation (Valerio et al., 2010)
Monostrain: **Lactophilus** Bacterial mixture: **Lacto seven**, **Probiotika**, **Puhdas+ vahva maitohappobakteeri**	*L. casei*	Promotion of Treg development and IL-10 production *in vitro* (Smits et al., 2005) Reduction of antibiotic- and *Clostridium difficile*-associated diarrhea (Gao et al., 2010)
Monostrain: **Probi: Mage** Bacterial mixture: **Lacto seven**, **Duolac**, **Vivomixx**, and **Probiotika**	*L. plantarum*	Fortification of the intestinal barrier by enhancement of the expression of tight junction proteins *in vivo* (Karczewski et al., 2010) Enhancement of systemic immunity in the elderly (Mane et al., 2011)
Bacterial mixture: **Replete intensive**	*L. salivarius*	Amelioration of the symptoms of atopic dermatitis in children (Niccoli et al., 2014) Antimicrobial effects against pathogens from infected pancreatic necrosis *in vitro* (Ridwan et al., 2008)

Notably, in contrast to the probiotic side, there is evidence showing the pathogenic potential of lactobacilli, as they may sometimes cause diseases in compromised individuals. *Lactobacillus* sepsis has been found in children supplemented with *L. rhamnosus* GG to treat and prevent short gut syndrome (Kunz et al., 2004), necrotizing enterocolitis (Dani et al., 2016), or antibiotic-associated diarrhea (Land et al., 2005; Dani et al., 2016). Furthermore, a premature infant developed sepsis that was caused by *L. acidophilus* infection obtained through an inserted intravenous central catheter (Thompson et al., 2001). Nevertheless, in total only a few severe infection cases caused by *Lactobacillus* in high-risk patients have been reported. Therefore, collectively, *Lactobacillus* are generally considered safe.

2.3.1.2 Selected *Lactobacillus* surface structures

As in other Gram-positive bacteria, the cell wall of *Lactobacillus* consists of a thick and multilayered peptidoglycan, and membrane-bound and cell wall-bound components. A schematic illustration of the surface structures of *Lactobacillus* is presented in Figure 5. Specifically, lipoproteins, lipoteichoic acids (LTA), and flagella are anchored to the cell membrane, while teichoic acids, pili, and S-layers, as well as other cell wall polysaccharides including exopolysaccharides (EPS), are either covalently or non-covalently anchored to the peptidoglycan (Desvaux et al., 2006; Lebeer et al., 2008; Schneewind and Missiakas, 2012). Structural variations in cell surface composition exist in different species/strains. Notably, certain surface components, such as S-layers, pili, and flagella, are strain/species-specific and only present in some lactobacilli. *Lactobacillus* surface structures have versatile functions that are associated with immunomodulatory, adhesive, and protective properties, as well as with motility, and they fundamentally contribute to the reported health-promoting effects.

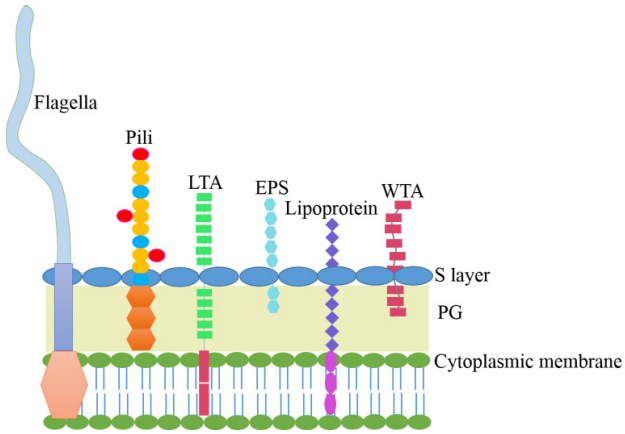


Figure 5. **Schematic presentation of the surface structures of *Lactobacillus***. LTA, lipoteichoic acid; EPS, exopolysaccharide; WTA, wall teichoic acid; PG, peptidoglycan. Pili, EPS, WTA, and the S-layer are anchored to the PG. Flagella, LTA, and lipoproteins are anchored to the cytoplasmic membrane. Data from Desvaux et al., 2006; Lebeer et al., 2008; and Schneewind and Missiakas, 2012.

2.3.1.2.1 Pili

Pili, also known as fimbriae, are hair-like surface appendages, protruding from the body of certain Gram-positive and Gram-negative bacteria, and implicated in adhesion, biofilm formation, and conjugation (Christie, 2001; Proft and Baker, 2009). In Gram-positive bacteria, pili are very thin (a few nanometers across) but long, being a few micrometers in length (Kang and Baker, 2012). In 1968, pili in a Gram-positive bacterium were first observed in *Corynebacterium renale* by electron microscopy (EM) (Yanagawa et al., 1968). Subsequently, an increasing number of piliated pathogenic and non-pathogenic Gram-positive bacteria have been detected by EM, such as *Streptococcus* (Lauer et al., 2005), *Actinomyces* (Mishra et al., 2007), and even probiotic bifidobacteria (Motherway et al., 2011). In older studies, several *Lactobacillus* strains were demonstrated to

have pili on the surface by EM, including *Lactobacillus fermentum* (Barrow et al., 1980) and vaginal isolates (*L. acidophilus*, *L. jensenii*, *L. fermentum*, *L. rhamnosus*, and *L. casei*) (McGroarty, 1994). Recently, pili have been observed on *Lactobacillus johnsonii* (Pridmore et al., 2004) and *L. rhamnosus* GG (Lebeer et al., 2009). However, only a few pilus gene clusters have been found in lactobacilli. One pioneering study demonstrated that the genome sequence of *L. johnsonii* NCC 533 has a fimbrial operon (Pridmore et al., 2004). Thereafter, a comparative genomic analysis revealed the presence of *spaCBA* and *spaFED* gene clusters in *L. rhamnosus* GG, each cluster being composed of three pilin genes and a sortase-specific gene (Kankainen et al., 2009). Subsequently, these two pilus gene clusters have also been identified in 36 strains of the species *L. paracasei* (Smokvina et al., 2013). Recently, a comparative genomic study revealed that 51 LAB strains in *L.casei*/*rhamnosus*, *L. ruminis*, *Lactobacillus brevis*/*parabrevis*, and *Lactobacillus composti* clades were predicted to have altogether 67 pilus gene clusters; of these 51 strains, 42 strains possessed one pilus cluster (Sun et al., 2015).

Structurally, typical pilus shafts in *Lactobacillus* are composed of the major pilin protein (SpaA) building up the pilus shaft, and one or two minor pilins (SpaB and SpaC) that are located at the pilus base and tip, respectively. In contrast to Gram-negative bacteria, these pilins are covalently connected to each other, and the pilus shaft is covalently linked to the bacterial cell wall. Each pilin harbors an LPXTG sorting motif, specific for sortase recognition, at the carboxyl terminus (Kang and Baker, 2012). Sortases are membrane-associated transpeptidases and, of the several classes of sortases, those of classes A and C mediate pilus assembly (Hendrickx et al., 2011). Additionally, many pilins possess the YPKN pilin motif and the E box, which are essential for pilus assembly (Mandlik et al., 2008b).

Until 2003, the mechanism of pilus assembly had only been uncovered in the pathogen *Corynebacterium diphteriae* (Ton-That and Schneewind, 2003), thereafter being used as a paradigm of pilus assembly in Gram-positive bacteria. Pilins are exported across the bacterial membrane via the Sec-dependent pathway, while the hydrophobic domain of each pilin is anchored to the membrane (Danne and Dramsi, 2012). Typically, pilus polymerization takes place when the pilus-specific sortase C (also called pilin-specific sortase) cleaves the LPXTG motif of the tip and backbone pilins, forming an acyl-enzyme intermediate that continues to recruit backbone pilins, until the basal pilin connects to the pilin shaft by polymerization of sortase A, which attaches the pili to the cell wall (Danne and Dramsi, 2012).

Gram-positive pili are involved in various activities. In Gram-positive bacteria, pilins not only work as adhesins that facilitate bacterial colonization in the host, but also contribute to biofilm development (Mandlik et al., 2008b). Importantly, Gram-positive pili interact with host immunity (Danne and Dramsi, 2012). However, to date, the characteristics of *Lactobacillus* pili have only been studied in *L. rhamnosus* GG, discussed in chapter 2.3.1.3.1

2.3.1.2.2 Flagella

Flagella, projecting out from the body of prokaryotic cells, are essential organelles that mediate motility. From the bacterial perspective, flagellar clockwise and anticlockwise rotation generates the tumbling and running (swimming or swarming) movements, respectively (Korani et al., 2009). Flagellar organization on both Gram-positive and negative bacteria can be grouped into four patterns, including monotrichous (one polar flagellum), amphitrichous (two polar flagella at opposite sites), lophotrichous (a few polar flagella at one site), and peritrichous (many flagella around the bacterial body) (Leifson, 1951). Given the significant sequence conservation of genes associated with flagellar assembly, both Gram-positive and negative bacteria appear to generate flagella similarly (Beatson et al., 2006). The flagellar structure is composed of several parts: the basal body, the motor, the switch, the hook working as a universal joint, the helical filament, capping proteins, and junction proteins located between the hook and the filament (Macnab, 2003). The rod of the basal body, which is embedded in the cell wall, markedly differs between Gram-positive and negative bacteria (Altegoer et al., 2014). Flagellin, also known as FliC, constitutes the flagellar filament (Ramos et al., 2004). There are several

domains in the flagellin, including the D0 and D1 domains highly conserved in all bacteria, and variable domains that differ not only between Gram-positive and negative bacteria, but also within Gram-positive or negative bacteria (Smith et al., 2003; Beatson et al., 2006; Altegoer et al., 2014). After being recognized by TLR5 on the host cells, flagellins of Gram-positive and negative bacteria activate either the NF-κB or the p38 MAPK signaling pathway; the choice appears to be dependent on the TLR5 binding site of the D1 domain (Hayashi et al., 2001; Smith et al., 2003; Yu et al., 2003; Verma et al., 2005).

Motility provides bacteria with tremendous benefits, as demonstrated by strains not belonging to the genus *Lactobacillus*. Motile bacteria are able to reach favorable niches for colonization and nutrient intake, and escape toxic substances (Ottemann and Miller, 1997). Flagella may also contribute to the pathogenicity of some pathogenic bacteria by propelling them to the targeted location of the host. For instance, the gastric pathogen *Helicobacter pylori* uses such motility to colonize the stomach, leading to disease, whereas flagellated but non-motile mutants are less able to access infection sites, being thus less virulent (Ottemann and Lowenthal, 2002). Additionally, flagella facilitate biofilm formation in both Gram-positive and Gram-negative bacteria (O'Toole et al., 2000; Lemon et al., 2007; Houry et al., 2010). A biofilm is a collection of bacteria growing on a surface and frequently embedded in an extracellular polymeric matrix. It protects the bacteria against the harsh environment and harmful agents, e.g. antibiotics (Costerton et al., 1999; O'Toole et al., 2000). When coated by a biofilm, some pathogens, such as *Pseudomonas aeruginosa*, can then cause chronic or even life-threatening infections, such as airway infections in cystic fibrosis patients (Parsek and Singh, 2003). Moreover, flagella are involved in adhesion, as demonstrated by the observation that an *E. coli* mutant without flagella was less adhesive on different surfaces compared to the wild type (Friedlander et al., 2013).

Generally, most LAB are aflagellated. Of some strains in the genera *Lactobacillus*, *Enterococcus* (Langston et al., 1960), *Sporolactobacillus* (Kitahara and Suzuki, 1963), and *Carnobacterium* (Nicholson et al., 2015), flagella have occasionally been visualized. Flagellated lactobacilli demonstrated by EM to date are summarized in Table 2. Among them, most strains originate from the environment. Other motile lactobacilli have been reported without EM verification of flagella, including the species *Lactobacillus nagelii* (Neville et al., 2012), *Lactobacillus vini* (Rodas et al., 2006), *Lactobacillus uvarum* (Mañes-Lázaro et al., 2008), *Lactobacillus oeni* (Mañes-Lázaro et al., 2009a), and *Lactobacillus aquaticus* (Mañes-Lázaro et al., 2009b).

Table 2. Flagellated *Lactobacillus* strains, demonstrated by electron microscopy

Strains	Origin	Flagella type	References
L. ruminis			
ATCC27780	Bovine rumen	Peritrichous	Sharpe et al., 1973
ATCC27781	Bovine rumen	Monotrichous	O'Donnell et al., 2015
ATCC27782	Bovine rumen	Peritrichous	Neville et al., 2012
DPC 6830 and 6831	Porcine feces	Peritrichous	O'Donnell et al., 2015
DPC 6832, 6833, 6834, 6835 and 6836	Equine feces	Peritrichous	O'Donnell et al., 2015
L. mali JCM 1153 JCM 1161 JCM 3821 JCM 3822	Fermented cider and wine must	Peritrichous	Kaneuchi et al., 1988
L. agilis BKN88	A variant of chick strain *L. agilis* JCM 1048	Peritrichous	Kajikawa et al., 2016
L. sucicola JCM 15457	Oak tree	Peritrichous	Irisawa and Okada, 2009
L. satsumensis DSM 16230	Mashes of shochu, a Japanese distilled alcoholic beverage	Peritrichous	Endo and Okada, 2005
L. ghanensis DSM 18630	Ghanaian fermenting cocoa	Peritrichous	Nielsen et al., 2007
L. capillatus DSM 19910	Brine of stinky tofu, a Chinese traditional snack	Peritrichous	Chao et al., 2008
L. curvatus NRIC 0822	Kabura-zushi, a Japanese fermented food	Peritrichous	Cousin et al., 2015

The characteristics of *Lactobacillus* flagella are poorly known, with only a few published studies mainly focusing on immunomodulatory effects. In one study, flagellins isolated from *L. ruminis* ATCC 27782, *L. nagelii* DSM 13675, *Lactobacillus mali* DSM 20444, and *Lactobacillus ghanensis* L489 induced IL-8 production in HT-29 cells via the TLR5-dependent signaling pathway (Neville et al., 2012). In another study, depolymerized flagellar filaments from *Lactobacillus agilis* BKN88 stimulated TLR5-modulated IL-8 production in Caco-2 cells to a degree that was much lower than that induced by the same amount of *Salmonella* flagellins, whereas whole *L. agilis* BKN88 cells did not have immune stimulating effects (Kajikawa et al., 2016).

2.3.1.3 *Lactobacillus rhamnosus* GG

L. rhamnosus GG is one of the best-studied lactobacilli, with many proven beneficial effects, and has been used as a probiotic for almost three decades. It was originally isolated from a stool sample of a healthy human in the 1980s, with desirable characteristics including resistance to gastric acid and bile, adherence to human IECs, the production of an antimicrobial agent, and an appreciable growth rate (Goldin and Gorbach, 1984). Since then, it has been attracting increasing attention. It has been used in dairy products since 1990 (Salminen et al., 2002) and has rapidly become a popular probiotic in the food industry and pharmacies worldwide. After discontinuing the consumption, probiotic *L. rhamnosus* GG can be identified in most stool samples for 4 days and in 33% of the samples up to 7 days; however, even though its existence is prolonged in human colonic mucosae, it ultimately disappears (Goldin et al., 1992; Alander et al., 1999). Therefore, it has been regarded as an allochthonous member of the gut microbiota. However, EPS (Lebeer et al., 2011) and mucus-specific adhesins, such as pili, the mucus-binding factor (von Ossowski et al., 2011), and the modulator of adhesion and biofilm (MabA) protein (Velez et al., 2010), appear to prolong the gastrointestinal retention time of *L. rhamnosus* GG.

2.3.1.3.1 *L. rhamnosus* GG pili

The presence of pili in *L. rhamnosus* GG was first observed in 2009: a comparative genomic analysis revealed two pilus gene clusters (*spaCBA* and *spaFED*) in the genome (Kankainen et al., 2009). SpaCBA and SpaFED pili in *L. rhamnosus* GG are each composed of three different pilin subunit types. SpaA (31 kDa) or SpaE (45 kDa) builds up the backbone of the pilus fiber, SpaB (21 kDa) or SpaD (51 kDa) forms the pilus base, and SpaC (91 kDa) or SpaF (104 kDa) is positioned at the pilus tip (Kankainen et al., 2009; von Ossowski et al., 2010). Each pilin except for SpaE has an LPXTG-like motif and an E-box, and an YPKN pilin motif was found in SpaA, SpaB, and SpaD (Kankainen et al., 2009). For the SpaCBA pili, the ratio of each pilin (SpaA:SpaB:SpaC) is 5:2:1, as analyzed by mass spectrometry (Tripathi et al., 2013). Reunanen et al. (2012) clearly demonstrated in their study that there were 10 to 50 pili per *L. rhamnosus* GG cell, the length of a pilus was up to 1 μm, and SpaC was located at the tip and along the pilus shaft. Subsequently, the existence of SpaCBA pili was further confirmed by immuno-TEM with SpaA and SpaB antisera. A comparative analysis of the *L. rhamnosus* pan-genome revealed that all 13 tested strains possessed the *spaFED* operon, which was categorized as belonging to the core genome, while only the genomes of four strains (including *L. rhamnosus* GG) in *L. rhamnosus* species harbored the *spaCBA* loci (Kant et al., 2014). However, thus far, native expression of SpaFED pili has not been demonstrated in any *L. rhamnosus* strain *in vitro*.

Functional properties of SpaCBA pili

The SpaCBA pili have been well characterized. The major feature is adhesiveness, such as binding to the human intestinal epithelial cell line Caco-2 and human intestinal mucus (Lebeer et al., 2012; von Ossowski et al., 2013), which probably facilitates *L. rhamnosus* GG residence in the GIT. The SpaC pilin subunit exhibits a strong binding capacity to human intestinal mucus; in contrast, this binding ability of *L. rhamnosus* GG is almost completely abolished when *L. rhamnosus* GG is pretreated with SpaC antiserum or when it carries an inactivated *spaC* gene (Kankainen et al., 2009). Furthermore, the SpaC pilin subunit adheres to Caco-2 cells, porcine type II mucin, type I collagen, and murine intestinal epithelia (Lebeer et al., 2012; Tripathi et al., 2013;

Ardita et al., 2014). A subsequent study showed that, in addition to SpaC, the recombinant SpaB pilin protein expressed in *E. coli* also binds significantly to human intestinal mucus; however, the mucus-binding property of *L. rhamnosus* GG pretreated with SpaB antiserum was not affected, indicating that the binding of SpaB to mucus was mediated by non-specific electrostatic interactions rather than by the presence of specific binding sites (von Ossowski et al., 2010). The recombinantly expressed SpaA pilin subunit lacked the capacity to adhere to human intestinal mucus (von Ossowski et al., 2010). Taken together, the SpaC pilin subunit was an indispensable and dominant adhesive factor in *L. rhamnosus* GG. Nevertheless, it is worthwhile to mention that in addition to SpaC, other adhesins such as MabA and mucus binding factor also contribute to the adhesion of *L. rhamnosus* GG to mucus (Velez et al., 2010; von Ossowski et al., 2011). Notably, SpaCBA pili also take part in biofilm formation in a SpaC-dependent way; one possible mechanism is that the SpaC–SpaC interactions (homophilic adhesion) lead to bacterial aggregation, which contributes to biofilm formation (Lebeer et al., 2012; Tripathi et al., 2013).

In addition to adhesion, another important feature of SpaCBA pili is their immunomodulatory effect. A mutant lacking SpaCBA pili induces an elevated level of proinflammatory cytokine IL-8 mRNA expression in Caco-2 cells as compared to the wild type *L. rhamnosus* GG (Lebeer et al., 2012). Conversely, a recombinant lactococcal strain producing SpaCBA pili activates TLR2-dependent signaling, which leads to NF-κB induction and IL-8 production in HEK293 cells, and upregulates the production of pro- and anti-inflammatory cytokines (TNF-α, IL-6, IL-10, and IL-12) in moDCs (von Ossowski et al., 2013). The dampening and boosting immunological effects of SpaCBA pili may somehow be balanced within the gut and thus promote the maintenance of gut immune homeostasis.

Functional properties of SpaFED pili

In comparison to SpaCBA pili, studies on the function of SpaFED pili were sparse before this thesis work, probably due to the fact that the native expression of SpaFED in *L. rhamnosus* GG or other lactobacilli has not yet been observed under laboratory conditions. However, the existence of SpaFED pili in *L. rhamnosus* GG in the GIT cannot be excluded, either. Of the three pilin subunits of *L. rhamnosus* GG that have been recombinantly expressed in *E. coli*, only SpaF was shown to have the potential to adhere to human intestinal mucus, at a level similar to SpaC (von Ossowski et al., 2010).

2.3.1.3.2 *L. rhamnosus* GG contributes to gastrointestinal well-being

The consumption of probiotics is a safe and effective way to generate beneficial effects in the host intestine. Numerous studies have demonstrated that *L. rhamnosus* GG exhibits health-promoting effects, such as effects on dental health (Näse et al., 2001), allergic diseases (Peldan et al., 2017), and especially on the maintenance of gastrointestinal health, including the fortification of the intestinal barrier and combatting of GIT disorders.

First of all, *L. rhamnosus* GG is able to promote the intestinal barrier function in many pathological states. For example, alcohol treatment of Caco-2 cells dislocates and decreases the expression of tight junction proteins (e.g., ZO-1, claudin-1 and occludin), whereas such pathogenic effects are completely prevented in Caco-2 cells that are pre-treated with the *L. rhamnosus* GG culture supernatant (Wang et al., 2011). In another example, *L. rhamnosus* GG culture supernatant elevates the production of mucin, IgA, and ZO-1 in the neonatal rat intestine, through which intestinal damage caused by *E. coli* K1 is prevented (He et al., 2017). Other animal studies have further strengthened this notion by showing that *L. rhamnosus* GG decreases gut permeability caused by alcohol consumption, thus preventing endotoxemia and ameliorating liver damage in a rat model (Forsyth et al., 2009; Wang et al., 2011).

Furthermore, *L. rhamnosus* GG has anti-inflammatory activities *in vitro* and *in vivo*. *In vitro* studies performed on human intestinal epithelial cells have indicated that *L. rhamnosus* GG suppresses IL-8 production induced by TNF-α and *E. coli* K88, in part as a result of the blockage of NF-κB nuclear translocation and/or the inhibition of pathogen adhesion (Zhang et al., 2005; Roselli et al., 2006; Donato et al., 2010). In a mouse model,

L. rhamnosus GG has been demonstrated to upregulate the mRNA of IL-10R2 (a subunit of IL-10 receptor), thereby attenuating the concentrations of pro-inflammatory cytokines (e.g., TNF-α and macrophage inflammatory protein (MIP)-2) in the immature colon and protecting mice from intestinal damage induced by inflammatory factors (e.g., platelet activating factor and LPS) (Mirpuri et al., 2012). Additionally, mice infected with *Pseudomonas aeruginosa* showed elevated mRNA levels of pro-inflammatory cytokines, including IL-6, IL-1β, and TNF-α, whereas *L. rhamnosus* GG treatment significantly down-regulated these levels in the colon (Khailova et al., 2016).

L. rhamnosus GG has been shown to modulate the composition of the gut microbiota. A 16S rRNA gene sequencing analysis revealed that *L. rhamnosus* GG suppressed the pathogenic expansion of the members of Proteobacteria and Actinobacteria in mice fed with alcohol, thereby preventing epithelial barrier disruption, endotoxemia, and liver damage (Bull-Otterson et al., 2013). Additionally, human intervention trials have further proved that *L. rhamnosus* GG is able to modulate the gut microbiota composition. When infants with cow's milk allergy were treated with extensively hydrolyzed casein formula and *L. rhamnosus* GG, they acquired tolerance towards cow's milk, whereas control infants who were only supplemented with extensively hydrolyzed casein formula remained allergic (Canani et al., 2015). In most tolerant infants, the amount of fecal butyrate was significantly increased, which was associated with significantly increased gut butyrate-producing bacteria, such as the genera *Blautia* and *Roseburia* (Canani et al., 2015). In another study applying a metagenomics approach, *L. rhamnosus* GG was shown to alter and protect the gut microbiota against antibiotic disturbance in children (Korpela et al., 2016): long-term *L. rhamnosus* GG consumption increased the quantity of species related to *Lactobacillus*, *Lactococcus*, *Prevotella*, and *Ruminococcus*, but decreased the amount of species related to *E. coli*, *Eubacterium cylindroides*, and *Clostrium ramosum*. Penicillin treatment enriched potential pathogens, including *Clostridium* cluster I and relatives of *Clostridium difficile*; nevertheless, such pathological effects were normalized by *L. rhamnosus* GG (Korpela et al., 2016).

Based on effects such as those described above, *L. rhamnosus* GG has been widely used to prevent and treat GIT disorders in humans, with promising outcomes. Early in 1987, *L. rhamnosus* GG showed the potential to prevent the relapse of *Clostridium difficile* colitis (Gorbach et al., 1987). Later, *L. rhamnosus* GG also effectively prevented the relapse of ulcerative colitis (Zocco et al., 2006). In placebo-controlled trials, the administration of *L. rhamnosus* GG has prevented and enhanced the treatment of antibiotic- and healthcare-associated diarrhea (e.g. rotavirus gastroenteritis) (Isolauri et al., 1991; Kaila et al., 1995; Arvola et al., 1999), reduced nosocomial gastrointestinal and respiratory tract infections (Hojsak et al., 2010), and alleviated the symptoms of IBS (Pedersen et al., 2014).

2.3.1.4 *Lactobacillus ruminis*

L. ruminis belongs to the *L. salivarius* clade (Sharpe et al., 1973). Members of this clade have usually originated from food and the environment, as well as the mammalian GIT, and several species in this clade can be regarded as candidate probiotics due to their beneficial properties, such as immunomodulatory effects and pathogen inhibition (Neville and O'Toole, 2010). Of the 25 species in this clade (Neville and O'Toole, 2010), *L. ruminis* is one of the poorly characterized members. It was first isolated in 1960 from the stool sample of a healthy human (Lerche and Reuter, 1960), and later from the rumen contents of a cow (Sharpe et al., 1973), the large intestinal contents of pigs (Al Jassim, 2003), horse feces (Willing et al., 2009), and the bovine uterus (Gärtner et al., 2015). An increasing number of new isolates have been identified in different mammalian hosts, including humans (Yun et al., 2005; O'Donnell et al., 2015). Due to its consistent identification in the mammalian intestine and feces, *L. ruminis* has been considered as one of the few autochthonous *Lactobacillus* species in the GIT (Tannock et al., 2000; Reuter, 2001). It has evolved to adapt to the GIT environment, as it has, for instance, the potential for anaerobic respiration (Kant et al., 2017).

The autochthony of *L. ruminis* could be explained by its stress survival, carbohydrate utilization, and intestinal colonization. A recent study demonstrated that 16 *L. ruminis* strains of human, porcine, bovine, and equine origin are resistant to porcine bile, and most strains are able to grow in simulated gastric fluid with a low pH and digestive enzymes (O'Donnell et al., 2015), indicating that *L. ruminis* is able to survive in the intestine. Moreover, *L. ruminis* exhibits metabolic versatility. Thereby, it can obtain energy via various sources and may have growth benefits over some other gut microbes in the nutritionally competitive intestine. *L. ruminis* strains show abilities to metabolize diverse carbohydrates to varying degrees, including monosaccharides and non-digestible oligosaccharides, which are rich in the colon (O'Donnell et al., 2011; O'Donnell et al., 2015). A recent pan-genome study provided additional data about the above findings. In this study, the analysis of the core genome of 9 *L. ruminis* strains revealed that it can synthesize indispensable enzymes for fermentation via the phosphoketolase pathway, suggesting that *L. ruminis* has the potential to utilize hexose and pentose carbohydrates (Kant et al., 2017). Therefore, it was concluded that *L. ruminis* may use both homofermetative and heterofermentative pathways to widen its repertoire of energy sources (Kant et al., 2017). Additionally, in de Man-Rogosa-Sharpe (MRS) medium supplemented with glucose, lactose, or sucrose, 16 *L. ruminis* strains of different origins had varying abilities to produce EPS (O'Donnell et al., 2015). Specifically, 3 human, 1 porcine, and 2 bovine strains produced EPS under these three conditions, whereas 4 strains, including one bovine isolate (ATCC 27782), did not synthesize EPS at all (O'Donnell et al., 2015), even though a gene cluster for EPS production was detected in the genome of this bovine isolate genome (Forde et al., 2011). However, the possibility to synthesize EPS *in vivo* or with other carbon sources could not be ruled out, as in the species *L. salivarius* (Raftis et al., 2011), EPS production largely relies on the growth conditions, including carbohydrate availability. Furthermore, the genome of this bovine strain contained genes encoding a bacteriocin, as well as accessory proteins and proteins related to bacteriocin immunity and regulation, whereas bacteriocin activity has not been reported in this strain so far (Forde et al., 2011). In contrast, the human strain ATCC 25644 lacks genes for bacteriocin peptides and the transport apparatus (Forde et al., 2011).

Microbial surface structures are pivotal in mediating microbe–host interactions and essential for the microbe to prevail in an ecological niche. Genes encoding sortase A and C, and LPXTG motif-containing proteins including sortase-dependent pili were identified in the genomes of *L. ruminis* ATCC 25644 isolated from a human, and in ATCC 27782 of bovine origin; the transcription of the pilus genes was remarkably up-regulated in the human strain compared to that in the bovine strain, when they were anaerobically cultivated in MRS for 15 h (Forde et al., 2011). Additionally, 7 other *L. ruminis* strains with human, bovine, porcine, and equine origins were predicted to have pilus genes and to express sortase-dependent surface proteins containing an intact LPXTG domain, indicating that these strains have the potential to express pili (Kant et al., 2017).

In addition to pili, flagella also probably contribute to *L. ruminis* autochthony. With the potential to display flagella, *L. ruminis* could reach nutrient-rich locations and epithelial cells by penetrating the mucus layer, thus outcompeting other gut microbes and prolonging the gut retention time via adhesion by pili or other surface proteins. An early study by Sharpe et al. (1973) clearly demonstrated that certain *L. ruminis* strains were flagellated, as examined by EM. Subsequently, motility genes involved in flagellar assembly and chemotaxis have been found in the genomes of many *L. ruminis* strains, including isolates of human, bovine, porcine, and equine origin (Forde et al., 2011; Neville et al., 2012; Lawley et al., 2013; Kant et al., 2017). Human *L. ruminis* was observed to be motile as early as in the 1960s (Lerche and Reuter, 1960), but motility has not subsequently been detected, despite a complete set of flagellar biogenesis genes being found in the genomes of some strains, including ATCC 25644 (Forde et al., 2011; Neville et al., 2012). In contrast, other mammalian-sourced *L. ruminis* strains are motile (Neville et al., 2012; O'Donnell et al., 2015). Of the 16 mammalian strains studied by O'Donnell et al. (2015), 10 were able to swarm on solid agar plates; among them, porcine and equine isolates possessed more flagella on a single bacterium, and were thereby more motile than bovine isolates (O'Donnell et al., 2015). Nevertheless, some bacterial cells of the human non-motile strain ATCC 25644 are able to perform a tumbling movement after passing through the murine GIT (Neville et al., 2012). Importantly,

flagella are typical MAMPs that can be recognized by PRRs of the host cells and thus can induce inflammatory cytokine production. As an example, the recombinantly expressed flagella of ATCC 25644 produced in *E. coli*, flagella purified from ATCC 27782, and also flagellated ATCC 27782 bacterial cells promote IL-8 secretion through the TLR5 signaling pathway in various cell lines (Neville et al., 2012).

Considering the probiotic potential of *L. ruminis*, its putative immunomodulatory and anti-pathogen properties are of interest. *L. ruminis* has been reported to promote the production of TNF *in vitro* (Taweechotipatr et al., 2009), and activate the NF-κB pathway in the human monocyte cell line THP-1 (Taweechotipatr et al., 2009). In addition, *L. ruminis* SPM0211 inhibited the growth of antibiotic-resistant pathogens (vancomycin intermediate-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus*) in a co-culture-based assay (Yun et al., 2005). In a later study, *L. ruminis* SPM0211 was found to inhibit the growth of rotavirus in Caco-2 cells and in the GIT of neonatal mice, in which significantly upregulated IFN-α/β levels were detected (Kang et al., 2015). Considering the probiotic potential, however, several strains, especially human isolates, are not applicable due to their susceptibility to an acidic pH (O'Donnell et al., 2015).

3. Aims of the study

Members of the gut microbiota live permanently (autochthonous species) or survive for a certain period of time (allochthonous species) in the harsh GIT conditions. *L. ruminis*, a motile species and one of the few autochthonous *Lactobacillus* in the mammalian gut, is poorly investigated. In contrast, *L. rhamnosus* GG, an allochthonous bacterium, is a popular and broadly used probiotic. The SpaCBA pili of *L. rhamnosus* GG have been investigated thoroughly. However, the properties of its SpaFED pili are largely unknown. Therefore, we chose these two species, representing different types of gut residence, and phenotypically characterized their pili. Subsequently, we also analyzed other niche-adaptive factors of *L. ruminis* (e.g. pathogen inhibition) and other interactions of *L. ruminis* with the host. The specific aims of this study were:

- To phenotypically characterize the SpaFED pili of *L. rhamnosus* GG, recombinantly expressed in *Lactococcus lactis* (I);

- To phenotypically characterize the LrpCBA pili of *L. ruminis*, recombinantly expressed in *Lactococcus lactis* (II);

- To investigate the adhesive, pathogen inhibitory and immunomodulatory functions of four *L. ruminis* strains of different mammalian origin (III).

4. Materials and methods

The plasmid, bacterial strains, cells and adhesion targets used in this study are listed in Table 3. The experimental methods are summarized in Table 4. The detailed culture conditions and methodological descriptions are presented in the articles.

Table 3. Plasmid, strains, cells, and proteins used in this work

	Plasmid, strain, cell, or protein	Origin and/or reference	Study
Plasmid	pKTH5080: a pNZ8032-derived expression vector containing a nisin-inducible promoter	von Ossowski et al., 2013	I, II
Lactic acid bacteria	*L. rhamnosus* GG	Human	I
	L. ruminis ATCC 25644	Human	II,III
	L. ruminis ATCC 27780	Bovine	III
	L. ruminis ATCC 27781	Bovine	III
	L. ruminis GRL1172	Pig (this study)	III
	GRS71: *Lactococcus lactis* NZ9000	De Ruyter et al., 1996	I, II
	GRS1052: *L. lactis* NZ9000 + pKTH5080	von Ossowski et al., 2013	I, II
	GRS1224: *L. lactis* NZ9000 + pKTH5080 with *lrpCBA* of *L. ruminis* ATCC 25644	This study	II
	GRS1225: *L. lactis* NZ9000 + pKTH5080 with *lrpΔCBA* of *L. ruminis* ATCC 25644	This study	II
	GRS1189: *L. lactis* NZ9000 + pKTH5080 with *spaFED* of *L. rhamnosus* GG	This study	I
	GRS1226: *L. lactis* NZ9000 + pKTH5080 with *spaΔFED* of *L. rhamnosus* GG	This study	I
	GRS1185: *L. lactis* NZ9000 + pKTH5080 with *spaCBA* of *L. rhamnosus* GG	von Ossowski et al., 2013	II
	GRS1211: *L. lactis* NZ9000 + pKTH5080 with *spaΔCBA* of *L. rhamnosus* GG	von Ossowski et al., 2013	II
Pathogens	*Escherichia coli* ETEC ($F4^+$)	Pig, Roselli et al., 2007	III
	E. coli ATCC 43894 (EHEC, O157)	Human	III
	E. coli ERF 2014 ($F18^+$, O141)	Pig, Hynönen et al., 2014	III
	Salmonella enterica serotype typhimurium ATCC 14028	Chicken	III
	Yersinia enterocolitica DSM 13030	Human	III
	Listeria monocytogenes ATCC 19117	Sheep	III

Table 3. Plasmid, strains, cells, and proteins used in this work (continued)

	Plasmid, strain, cell, or protein	Origin or reference	Study
Cells	HT-29	Human colorectal adenocarcinoma (ECACC)	I, II, III
	Caco-2	Human colon adenocarcinoma (ATCC)	I, II, III
	IPEC-1	Porcine small intestinal epithelium (DSMZ)	III
	moDCs	Human monocytes (Finnish Red Cross)	I, II
	HEK293-TLR2 cells	Human embryonic kidney, engineered (InvivoGen, USA)	I, II, III
	HEK293-TLR5 cells	Human embryonic kidney, engineered (InvivoGen)	I, II, III
Other substrates for adhesion assays	ECM proteins: fibronectin, collagen type I and IV	Human	I, II, III
	Type II mucin	Pig	I, II

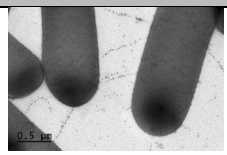
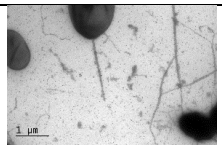
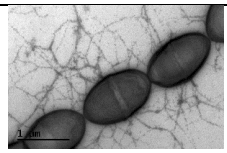
Table 4. Methods used in this study

Methods		Study
DNA manipulation and analysis	Construction of recombinant *L. lactis* strains	I, II
	Sequencing and sequence comparisons	III
Analysis of bacterial surface structures	Nisin-induced expression of pilus genes in recombinant *L. lactis*	I, II
	Western blotting	I, II
	Electron microscopy	I, II, III
	Biofilm formation assay	II
Adhesion assays	Adherence to intestinal epithelial cell lines	I, II, III
	Adherence to porcine mucin	I, II
	Adherence to extracellular matrix proteins	I, II, III
Pathogen inhibition assays	Inhibition of pathogen growth by *L. ruminis* supernatants	III
	Inhibition of pathogen adherence by *L. ruminis* to intestinal epithelial cells and ECM proteins	III
Barrier function analysis	Measurement of paracellular permeability and TEER	III
	Immunofluorescence assay of TJ proteins	III
Analysis of immunomodulatory effects	Stimulation of HEK-TLR2/TLR5 cells	I, II, III
	Measurement of IL-8 production in Caco-2 cells	I, II
	Measurement of cytokine production in moDCs	I, II

5. Results and discussion

The structural and functional traits of the studied *L. rhamnosus* GG SpaFED pili and *L. ruminis* LrpCBA pili are summarized in Table 5 and elaborated more closely in the later sections. The adhesiveness of SpaFED and LrpCBA pili most likely reflects the gut adaptation of these two bacterial species. Moreover, other features of *L. ruminis*, such as its ability to inhibit pathogens and to maintain barrier function in epithelial cells, suggest that this autochthonous species has a mutualistic relationship with the host. However, further *in vivo* studies are needed.

Table 5. Features of SpaFED pili in *L. rhamnosus* GG and LrpCBA pili in *L. ruminis*

	SpaFED pili of *L. rhamnosus* GG	**LrpCBA pili of *L. ruminis***
Native expression	Not yet detected	
Recombinant expression in *L. lactis*		
Pilin subunits	SpaD (major), SpaE (basal), and SpaF (tip, adhesin)	LrpA (major), LrpB (basal), and LrpC (tip, adhesin)
Pilus biogenesis	Sortase-dependent assembly	Sortase-dependent assembly
Adhesion to mucus	+	-
Adhesion to ECM proteins	fibronectin, collagen type I and IV	fibronectin, collagen type I and IV
Adhesion to IECs	HT-29, poorly to Caco-2	HT-29, Caco-2
Biofilm formation	No contribution	No contribution
Immunomodulation	Immune-dampening effect in HEK-TLR2 and Caco-2 cells	Immune-dampening effect in HEK-TLR2 and Caco-2 cells

5.1 *In silico* analysis of the *lrpCBA* operon (II)

To determine whether *L. ruminis* ATCC 25644 possesses pilus-encoding genes, its genome sequence data obtained from the NCBI database were analyzed. Scrutiny of the genome revealed that *L. ruminis* ATCC 25644 contains a pilus gene cluster (nucleotide position 23941 to 31363) that encodes three pilin subunits (LrpA, LrpB, and LrpC) and one sortase C class enzyme. Each pilus gene has the sequence for a ribosomal binding site and encodes an E-box domain (Figure 1 in II). Moreover, an N-terminal secretion signal and an LPXTG-like sortase recognition motif at the C-terminal domain were found in each predicted pilin protein, but only the predicted LrpA and LrpB proteins possessed a pilin motif. Additionally, a predicted collagen-binding domain was identified only in LrpC pilin, while no sequences encoding mucus-binding domains were found in the *L. ruminis* ATCC 25644 genome. BlastP analysis revealed that each pilin of *L. ruminis* ATCC 25644 shows the highest amino acid identity to the counterpart pilins from other *L. ruminis* strains. Sequence alignment of the *lrpC* genes of five *L. ruminis* strains from different origins, including ATCC 25644, revealed that these gene operons are highly similar with an identical ribosomal binding site and two consensus promoter elements. However, the similarities in amino acid sequences between *L. ruminis* ATCC 25644 and lactobacillar SpaCBA or SpaFED pilins were not very high. These results suggest that the *lrpCBA* pilus operon might be

specific to *L. ruminis* species, unlike *spaCBA* and *spaFED* operons, which can be found in many lactobacilli (Kankainen et al., 2009; Muñoz-Provencio et al., 2012; Smokvina et al., 2013).

5.2 Surface structures of *L. ruminis* revealed by Western blotting and TEM (II, III)

To detect native pilus expression on *L. ruminis*, Western blotting was first employed, as it is a widely used approach to detect bacterial pilin production. Each of the anti-pilin sera, including anti-LrpA, anti-LrpB, and anti-LrpC, detected ladder-like bands when membranes blotted with *L. ruminis* ATCC 25644 cells were probed, suggesting the presence of monomeric pilins as well as truncated and wall-attached LrpCBA pili of different sizes (Figure 3A in II). Indeed, as pili in Gram-positive bacteria are made of covalently linked adhesive pilin subunits, high-molecular-weight (HMW) bands are interpreted as pili of different lengths in Western blots (Reunanen et al., 2012). In contrast, no bands were observed when *L. rhamnosus* GG was used as the negative control.

Subsequently, immuno-TEM was applied to examine the surface display of LrpCBA pili. With single labeling, using antiserum against the backbone pilin (LrpA) of *L. ruminis* ATCC 25644, pilus shafts of different lengths were clearly observed (Figure 3B top left panel in II). These pili of different lengths were either broken or attached to the bacterial surface. Generally, most bacterial cells were piliated, while cells without pili were also observed. Notably, even though the LrpA antiserum was specifically generated against the ATCC 25644 LrpA pilin proteins, it could also be used to confirm the presence of pili in GRL1172 (our unpublished results). Thus, it is evident that the main pilus subunits of these two *L. ruminis* strains with different origins share structural similarity. In contrast, anti-LrpA antibodies did not detect *L. rhamnosus* GG pili (Figure 3B top right panel in II), demonstrating that the main subunit of *L. ruminis* pilus is distinct from that of *L. rhamnosus* GG.

To further determine the positions of other pilin subunits, double labeling was performed. When *L. ruminis* ATCC 25644 and porcine *L. ruminis* GRL1172 bacteria were treated with anti-LrpA and anti-LrpC antibodies, LrpC pilins were found at the pilus tip, as well as along the pilus shaft (Figure 3B bottom left panel in II and Figure 1B in III), which was reminiscent of the position of the tip pilin SpaC in SpaCBA pili of *L. rhamnosus* GG (Kankainen et al., 2009; Reunanen et al., 2012). It has been reported that the recognizable pilin motif is absent from the SpaC pilin, which makes it impossible to integrate it into the pilus backbone (Reunanen et al., 2012). However, its putative two E box motifs or other unidentified systems might account for its incorporation in the pilus fiber (Reunanen et al., 2012). In *L. ruminis* labeled with anti-LrpA and anti-LrpB antibodies, the pilus shaft was dominated by the LrpA pilin, while the basal pilin LrpB subunits were also present on the pilus backbone (Figure 3B bottom right panel in II). In conclusion, the TEM results of this study have thus revealed that *L. ruminis* is a piliated *Lactobacillus* species.

So far, *L. ruminis* has been considered as the only motile autochthonous *Lactobacillus* species in the human gut microbiota (Neville et al., 2012). A bioinformatics analysis identified genes encoding flagella in the genomes of ATCC 25644 and the bovine *L. ruminis* strain ATCC 27782, but the expression of flagella was only confirmed in the latter (Neville et al., 2012). Therefore, ATCC 25644 was regarded as aflagellated by these authors. Conversely, we found by EM that ATCC 25644 (Figure 6, our unpublished result) as well as GRL1172 (Figure 1A in III), when they were grown on MRS agar plates for 2 days, have flagella. ATCC 25644 possessed a single flagellum, while GRL1172 cells had one or several flagella. However, aflagellated cells of both strains were also present. Considering the harsh conditions of the GIT, being motile could confer many competitive advantages to autochthonous *L. ruminis*, as flagella are able to propel the bacteria towards nutrient-rich environments and other favorable colonization niches.

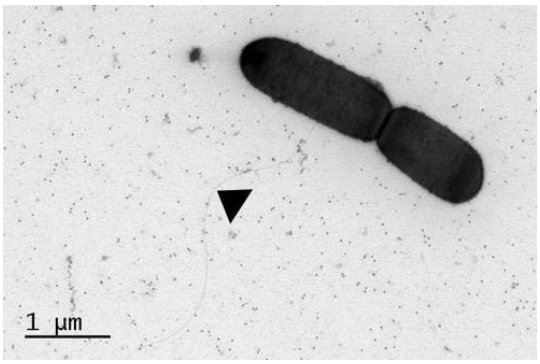


Figure 6. Visualization of flagella in *L. ruminis* ATCC 25644. Negative staining of *L. ruminis* cells. The black arrowhead indicates a flagellum.

5.3 Expression of *L. rhamnosus* GG SpaFED and *L. ruminis* LrpCBA pili in *Lactococcus lactis* (I, II)

To study the phenotypic characteristics of *L. rhamnosus* GG SpaFED and *L. ruminis* LrpCBA pili, the *spaFED* or *lrpCBA* operon was cloned into the nisin-dependent expression vector pKTH5080, and the recombinant plasmids were transformed into *Lactococcus lactis* NZ9000. The resulting recombinant strains were then named as GRS1189 or GRS1224, respectively. This cloning method has also been used to investigate other pilus types in Gram-positive bacteria (Buccato et al., 2006; Quigley et al., 2010; Oxaran et al., 2012), including *L. rhamnosus* GG SpaCBA pili (von Ossowski et al., 2013). To better understand the role of the tip pilin subunits, we also constructed recombinant lactococcal clones expressing SpaΔFED or LrpΔCBA pili without the tip pilin, termed as GRS1226 or GRS1225, respectively. To verify pilus expression in these recombinants, Western blotting was first conducted, followed by TEM. After nisin induction, the sonicated recombinant lactococcal cells were analyzed by immunoblotting with anti-pilin antibodies. Clear ladder-like protein bands appeared when sonicated GRS1189 and GRS1224 cells were immunoblotted with each of the SpaFED- (anti-SpaF, anti-SpaE and anti-SpaD) and LrpCBA-specific (anti-LrpC, anti-LrpB and anti-LrpA) antisera, respectively (Figure 4 in I and Figure 4B in II). Bands representing the monomeric pilins were obviously present: SpaD~51 kDa, SpaE~45 kDa, SpaF~104 kDa, LrpA~49 kDa, LrpB~39 kDa, LrpC~123 kDa. These results evidently demonstrated that SpaFED and LrpCBA pili can be expressed in lactococci. GRS1226 and GRS1225 were also proved to express backbone and basal pilins, but not tip pilins. As expected, the anti-pilin antibodies did not detect anything on polyvinylidene difluoride membranes blotted with sonicated *L. lactis* cells carrying the vector control without insert (GRS1052) (Figure 4 in I and Figure 4B in II).

We further applied TEM to visualize the recombinantly expressed SpaFED and LrpCBA pili in *L. lactis* in the same way as TEM was used to detect native LrpCBA pili in *L. ruminis* ATCC 25644. For *L. rhamnosus* GG SpaFED pili, nisin-induced GRS1189 (Figure 5A in I) and GRS1226 (Figure 5E in I) were single-labeled with anti-SpaD antibodies, and pilus structures were evidenced by black dots along the dense lines extending out from the cell wall surface. No pilus-like structures were detected in *Lactococcus lactis* NZ9000, used as the negative control (GRS71), when it was incubated with anti-SpaD, as shown in Figure 5B in I. Subsequently, double labeling was carried out using antibody pairs that detected either SpaD and SpaF pilins (Figure 5C and F in I) or SpaD and SpaE pilins (Figure 5D and G in I). In GRS1189, SpaF pilin was not only found at the pilus tip, but also along the shaft in a manner similar to the basal SpaE pilin. Such an assembly of minor pilins has also been observed in other pilus types in Gram-positive bacteria, such as in the SpaCBA pili in *Corynebacterium diphtheriae* (Mandlik et al., 2008a) and *L. rhamnosus* GG (Reunanen et al., 2012). In GRS1126, SpaF pilin was not present at the pilus tip as expected, but appeared on the pilus backbone. This result was not consistent with the Western blotting observation, where SpaF could not be detected in GRS1226 with anti-SpaF antibodies. This discrepancy suggests that anti-SpaF antibodies cross-react with the backbone pilin SpaD, and thus SpaF pilin, seemingly attaching to the pilus fiber, was a TEM artifact. It is worth

mentioning that pili in GRS1226 were much longer and occasionally more abundant per bacterium compared with those in GRS1189. One possible explanation is that the lack of the *spaF* gene results in the upregulation of the expression of the *spaD*, *spaE* and sortase-encoding genes.

Immuno-TEM was also applied to detect LrpCBA pili of *L. ruminis* on recombinant lactococci. After treatment with anti-LrpA antibodies, pili were clearly visible in GRS1224 and GRS1225 cells, whereas pili-like structures were not observed in GRS71, as expected (Figure 4C in II). Double labeling with anti-LrpA antibodies coupled with antibodies targeting LrpC or LrpB pilins revealed that pili were evidently exhibited in GRS1224 and GRS1225 (Figure 4C middle and bottom panels in II). As observed in the case of SpaF immunostaining of the deletion mutant GRS1226, LrpC subunits were observed on the pilus fiber of GRS1225 by TEM, although they were not detected by Western blotting with anti-LrpC antibodies. Then again, we considered such a phenomenon to be an artifact. Since there was a heavy background in some TEM images (Figure 4C in II), GRS1224 and GRS1225 were incubated with the corresponding pre-immune serum, and only a negligible background appeared (S4 Fig in II). These results indicated that the gold particles in the background (Figure 4C in II) were indeed due to specific antibody binding to pilus fragments. However, as the recombinant pili were longer than the native ones, they might have been fractured more easily. The broken pili were then recognized by specific antibodies, which seemed to be difficult to wash off, and this binding possibly formed the background.

5.4 Adhesion of *L. rhamnosus* GG SpaFED pili and *Lactobacillus ruminis* (I, II, III)

The intestinal barrier, including mucus and the epithelial layer, largely confines bacteria in the lumen and inhibits their invasion. The capacity of gut-resident microbes to adhere to host cells or tissues is regarded as niche-adaptation, as it facilitates host colonization either permanently or transiently. Mucin (or mucus), epithelial cells, and ECM proteins are usually used as substrates to assess bacterial adhesiveness.

5.4.1 Adhesion capacity of *L. rhamnosus* GG SpaFED pili (I)

As an allochthonous bacterium, *L. rhamnosus* GG utilizes its certain structural components, including EPS (Lebeer et al., 2011), mucus-binding factor (von Ossowski et al., 2011), MabA (Velez et al., 2010), and SpaCBA pili (von Ossowski et al., 2013), to extend its transient colonization of the GIT. As previously reported, the recombinantly expressed SpaF pilin subunit binds to human mucus as strongly as SpaC pilin protein (von Ossowski et al., 2010). To characterize the putative SpaFED pili, we tested their adhesion abilities and the contribution of SpaF subunits to adhesion using a lactococcal display system in *L. lactis*. Similarly to the recombinant SpaCBA pili, which adhered to human intestinal mucus (von Ossowski et al., 2013), *L. lactis* GRS1189 expressing SpaFED pili strongly bound to porcine mucin, whereas GRS1226 lacking the tip pilin SpaF showed a much weaker adhesion (Figure 6 in I). As the negative controls, GRS71 and GRS1052 showed no or negligible adherence to mucin. Thereby, the determining role of SpaF in mediating the mucin binding of SpaFED was confirmed.

ECM, composed of several proteins such as fibronectin and collagen, is located beneath epithelial cells. These ECM proteins are exposed in the damaged host mucosa and may thus promote microbial colonization (Štyriak et al., 2003). Pilus-mediated ECM adhesion has been reported in various Gram-positive bacteria (Telford et al., 2006; Hilleringmann et al., 2008). For instance, the *L. rhamnosus* GG pilus SpaC subunit binds to collagen (Tripathi et al., 2013). Accordingly, the adhesiveness of SpaF to ECM proteins was investigated using the lactococcal expression system. Obviously, GRS1189 adhered well to fibronectin as well as to type I and IV collagen (Figure 7 in I). The negative controls (GRS71 and GRS1052) and GRS1226 did not bind to any ECM proteins tested. The results indicated that the adhesion of SpaFED pili to ECM proteins is attributed to the SpaF pilin subunit.

We also investigated whether SpaFED pili could adhere to intestinal epithelial cells, which are often used as targets to assess the attachment and colonization of pathogenic and non-pathogenic bacteria (Štyriak et al.,

2003; Rendón et al., 2007; Chagnot et al., 2012). GRS1189 exhibited pronounced adherence to HT-29 cells, while a relatively low level of adherence to Caco-2 cells was observed (Figure 8 in I). However, these levels of GRS1189 were markedly higher than those of GRS71 and GRS1052. Once again, the importance of the SpaF subunit in adhesion was demonstrated, as GRS1226 showed an evident reduction in adherence to these two cell lines.

The adhesiveness to intestinal components of SpaFED pili, especially the SpaF subunit, might offer *L. rhamnosus* GG a longer residence time in the gut, if SpaFED pili were expressed *in vivo*. Moreover, with ECM-adhesive factors such as SpaCBA (Lebeer et al., 2012; Tripathi et al., 2013) and SpaFED pili, *L. rhamnosus* GG might outcompete pathogens for binding sites in the ECM if the epithelial layer is broken, and thus prevent infection. However, such a hypothesis needs further confirmation and future work is warranted to identify appropriate conditions for SpaFED pilus expression.

5.4.2 Adhesion capacity of *Lactobacillus ruminis* (II, III)

In the stable GIT, autochthonous bacteria are supposed to live and proliferate in the intestinal lumen (van der Waaij et al., 2005). Nevertheless, the adherence of these bacteria to host intestinal substrates, such as mucins, other proteins, or epithelial cells, is usually assessed to explain their persistent presence in the gut (Vélez et al., 2007). Such adhesiveness of autochthonous bacteria conferred by surface structures such as pili denotes the potential to competitively inhibit or interfere with pathogen colonization.

Considering intestinal colonization, adherence to mucus is regarded as an advantage for both gut-resident bacteria and pathogens, as mucus forms the first layer of the intestinal barrier. However, according to a pan-genome analysis, *L. ruminis* genomes do not encode proteins that possess mucus-binding domains (Kant et al., 2017). In line with this prediction, our experiments revealed that *L. ruminis* ATCC 25644 cannot bind to mucus, and neither can the lactococcal recombinant constructs GRS1224 and GRS1225 expressing LrpCBA or LrpΔCBA pili (data not shown). Therefore, as an autochthonous bacterial species, *L. ruminis* must have evolved to adhere to other intestinal substrates in order to achieve its lifelong colonization in the GIT.

The *lrpC* gene, encoding the tip pilin LrpC in *L. ruminis* ATCC 25644, is predicted to encode a collagen-binding domain (S2 Fig in II). Therefore, we investigated the binding abilities of this *L. ruminis* strain to collagen as well as to fibronectin, and tested the effects of LrpCBA pili and the LrpC subunit on ECM protein binding by using the lactococcal clones GRS1224 and GRS1225. Evidently, in comparison with the controls GRS71 and GRS1052, *L. ruminis* pronouncedly adhered to collagen, but at a much higher level to type I than to type IV (Figure 5A and B in II). Similarly, nisin-induced GRS1224 markedly bound to type I collagen, while much less to type IV collagen, but still significantly better than the controls. Without the tip pilin LrpC, the adherence of GRS1225 to both collagen types was reduced compared to GRS1224, but was still obviously better than that of the controls.

Regarding fibronectin binding, the degree of adherence of each strain was analogous to that of type I collagen binding (Figure 5C in II). Specifically, *L. ruminis* itself was able to bind to fibronectin at a level similar to the binding to type I collagen. Again, GRS1224 strongly bound to fibronectin and a reduction in GRS1225 adherence was observed. The obtained results undoubtedly proved that LrpCBA pili contributed at least partly to the ECM-binding activity of *L. ruminis*, in which LrpC subunit has an indispensable role. Noticeably, *L. ruminis* adhered less readily to ECM proteins than GRS1224 and GRS1225, which might reflect that pili in each *L. ruminis* bacterium were less numerous, as observed by TEM.

We further tested whether other *L. ruminis* strains of different host origins share ECM-binding properties. In addition to ATCC 25644 used as a positive control, a newly isolated *L. ruminis* strain, GRL1172, and two bovine strains, ATCC 27780 and ATCC 27781, were assessed for their ECM-binding activities (Figure 7). All four strains had similar binding patterns, but with varying levels of efficiency: the binding level to fibronectin

was the highest for each strain, followed by type I collagen, with the lowest binding to type IV collagen (Figure 7). The adherence of all the strains to any ECM component was significantly higher than the binding to BSA. The binding to fibronectin of GRL1172 and ATCC 27781 was the highest, while GRL1172 and ATCC 25644 were the strongest binders to type I collagen. Among these strains, ATCC 27780 was the least adhesive strain to all tested ECM proteins. Since LrpCBA pili are essential in mediating the adherence of ATCC 25644 to ECM, we assume that the binding activities of the other three strains are also related to pili. In the genomes of both ATCC 27780 and ATCC 27781, at least one gene is predicted to encode a pilin protein that has a collagen-binding domain (Kant et al., 2017). Given that the predicted LrpC pilins of GRL1172 and ATCC 25644 share high similarity in their primary structures (data not shown), and that a collagen-binding domain has been identified in the LrpC pilin of ATCC 25644 (II), GRL1172 most likely also has a collagen-binding domain in its LrpC pilin. Moreover, the presence of pili in GRL1172 was demonstrated by TEM (Figure 1B in III) and pilus operons in the two bovine strains were verified by PCR (our unpublished data). For these three strains, unveiling the role of pili in adherence requires further investigation.

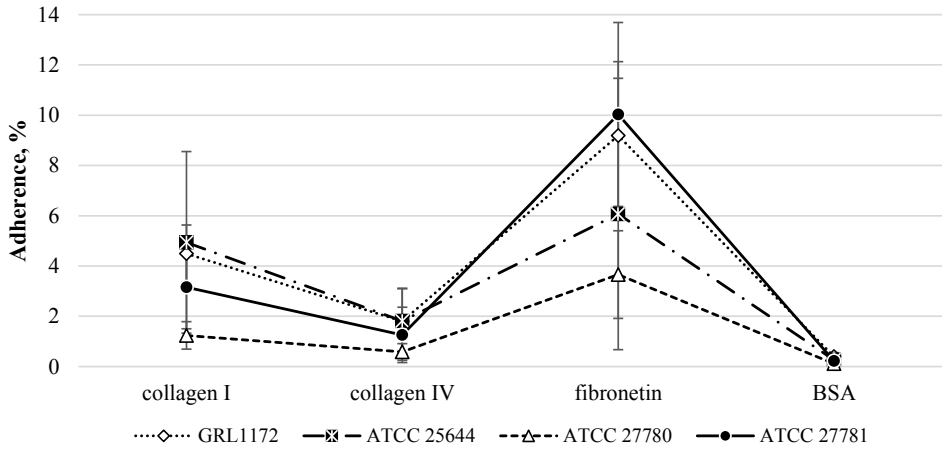


Figure 7. Adherence of *L. ruminis* to ECM proteins. Modified from Figure 2 in study III.

Since binding to epithelial cells could facilitate *L. ruminis* colonization and promote bacteria–host interactions, we tested whether *L. ruminis* and the recombinant constructs GRS1224 and GRS1225 bind to intestinal epithelial cells. ATCC 25644 adhered to Caco-2 and HT-29 cells at levels similar to the intrinsic binding of *L. lactis* strains GRS71 and GRS1052 (Figure 6 in II). However, GRS1224 efficiently bound to these two intestinal cell lines, indicating that LrpCBA pili can increase the adherence of *L. lactis*. The important role of the LrpC subunit in binding was again demonstrated, as lower adherence was detected for GRS1225 (Figure 6 in II). Similarly, all four *L. ruminis* strains bound weakly to Caco-2 (data not shown), but strongly to HT-29 (Figure 3 in III). Nevertheless, the porcine isolate GRL1172 could not bind to the porcine intestinal cell line IPEC-1, and neither could the other strains (data not shown). The pronounced adherence to HT-29 of human *L. ruminis* may reflect the frequent detection of this bacterial species in the human colon (Tannock et al., 2000; Reuter, 2001). As the non-human-originating *L. ruminis* strains were able to bind to human intestinal HT-29 cells and to human ECM proteins, host specificity of *L. ruminis* adherence was not seen in our current results. Host specificity is, however, difficult to investigate further due to the lack of available intestinal cells or proteins originating from animals.

5.5 Biofilm formation in *L. ruminis* (II)

Pili are able to participate in biofilm formation, as has been demonstrated with many Gram-positive bacteria (Mandlik et al., 2008b), including *L. rhamnosus* GG, in which SpaCBA pili play a focal role in facilitating biofilm formation (Lebeer et al., 2012). Accordingly, we tested whether the LrpCBA pili in *L. ruminis* possess such a feature *in vitro*. In the experimental set-up, *L. rhamnosus* GG, the SpaCBA-expressing strain GRS1185, and the SpaC-deleted GRS1211 construct (von Ossowski et al., 2013) served as controls. *L. rhamnosus* GG efficiently formed biofilms in polystyrene microplate wells, and SpaCBA pili appeared to partly contribute to this activity in a SpaC (tip pilin) dependent manner: the SpaC-deleted strain GRS1211 exhibited a substantial decrease in biofilm formation (Figure 7 in II). Similarly, *L. ruminis* ATCC 25644 had the capacity to form biofilms, whereas LrpCBA-piliated GRS1224 and GRS1225 failed to do so (Figure 7 in II). Therefore, we concluded that LrpCBA pili were not associated with biofilm formation in ATCC 25644. In the same way, the recombinant SpaFED pili did not participate in the biofilm formation of *L. rhamnosus* GG, either, as it was observed that the SpaFED-piliated strains GRS1189 and GRS1226 could not produce a biofilm. In fact, the obtained results are in line with the current knowledge that in *Lactobacillus* species, the involvement of pili in biofilm development is uncommon (Lebeer et al., 2012).

5.6 Pathogen inhibition by *L. ruminis* (III)

Members of the human gut microbiota exhibit pathogen inhibition by various mechanisms, including competition for common binding sites and nutrients, and the secretion of inhibitory factors (Todoriki et al., 2001; Kamada et al., 2013). As a gut-dwelling species, *L. ruminis* might have the potential to inhibit the adhesion and growth of enteropathogens. To prove this hypothesis, we first assessed the inhibitory effects of four *L. ruminis* strains on pathogen adhesion using three inhibition assays (competition, exclusion and displacement), with ECM proteins and intestinal epithelial cells as binding targets. We first found that *Yersinia enterocolitica* DSM 13030 can bind to fibronectin and type I collagen, and a F4-fimbriated ETEC strain was found to adhere to type I collagen. Thus, these two strains were used as target pathogens. We then assessed the ability of *L. ruminis* to inhibit their adherence to these ECM proteins. Competition and exclusion assays indicated that all the *L. ruminis* strains evidently inhibited the adhesion of both pathogens to varying degrees (Figure 8). Nevertheless, in exclusion assays, except for ATCC 25644, the inhibitory rates of *Yersinia* binding to type I collagen conferred by the strains decreased by about 15–20% when compared to the inhibition rates of fibronectin binding.

Subsequently, we investigated the inhibition abilities in intestinal epithelial cell models, including IPEC-1 and Caco-2, representing porcine and human small intestinal epithelial cells, respectively, even though all the *L. ruminis* strains poorly bound to these two cell lines. In line with previous studies demonstrating that poorly adhesive lactobacilli can still reduce pathogen adhesion (Tuomola et al., 1999; Gueimonde et al., 2006), all the *L. ruminis* strains clearly reduced the adherence of ETEC to IPEC-1 cells in competition and exclusion assays, while ATCC 27780 and ATCC 27781 showed slightly lower inhibitory rates in exclusion assays than in competition assays (Figure 5A and B in III). All the strains inhibited the adherence of *S.* typhimurium, *Yersinia*, and ETEC to Caco-2 cells at levels of around 20–50% in competition and 20–45% in exclusion assays (Figure 6A and B in III). Additionally, although the adhesion rates of ATCC 27780 to fibronectin and type I collagen were the lowest of all the *L. ruminis* strains, the inhibition of pathogen adherence to the same substrates by ATCC 27780 was as efficient as the inhibition caused by the other strains. Therefore, it appears that the inhibition of pathogen adherence is not associated with the adhesiveness of *L. ruminis*. Indeed, apart from competition for common binding sites, other factors, such as coaggregation with pathogens and the excretion of inhibitory compounds, can also inhibit pathogen adherence (Lebeer et al., 2008). However, evident coaggregation between *L. ruminis* and pathogens was not observed *in vitro* in the present study (results not shown).

Generally, studies on pathogen displacement by lactobacilli are scarce. The few studies available have shown that lactobacilli either fail to displace pre-adherent pathogens from host substances or generate much lower degrees of inhibition compared to competition or exclusion assays (Lee et al., 2003; Gueimonde et al., 2006). Accordingly, our results revealed that all the *L. ruminis* strains failed to displace previously adhered pathogens from fibronectin and type I collagen (Figure 8). Surprisingly, all the strains did, however, displace previously adhered *S.* typhimurium (inhibition of 20–30%), but not *Y. enterocolitica* or ETEC from Caco-2 cells (Figure 6C in III). Our results were in line with one previous study showing that the pathogen displacement efficiencies of lactobacilli are dependent on the pathogen strains used (Lee et al., 2003).

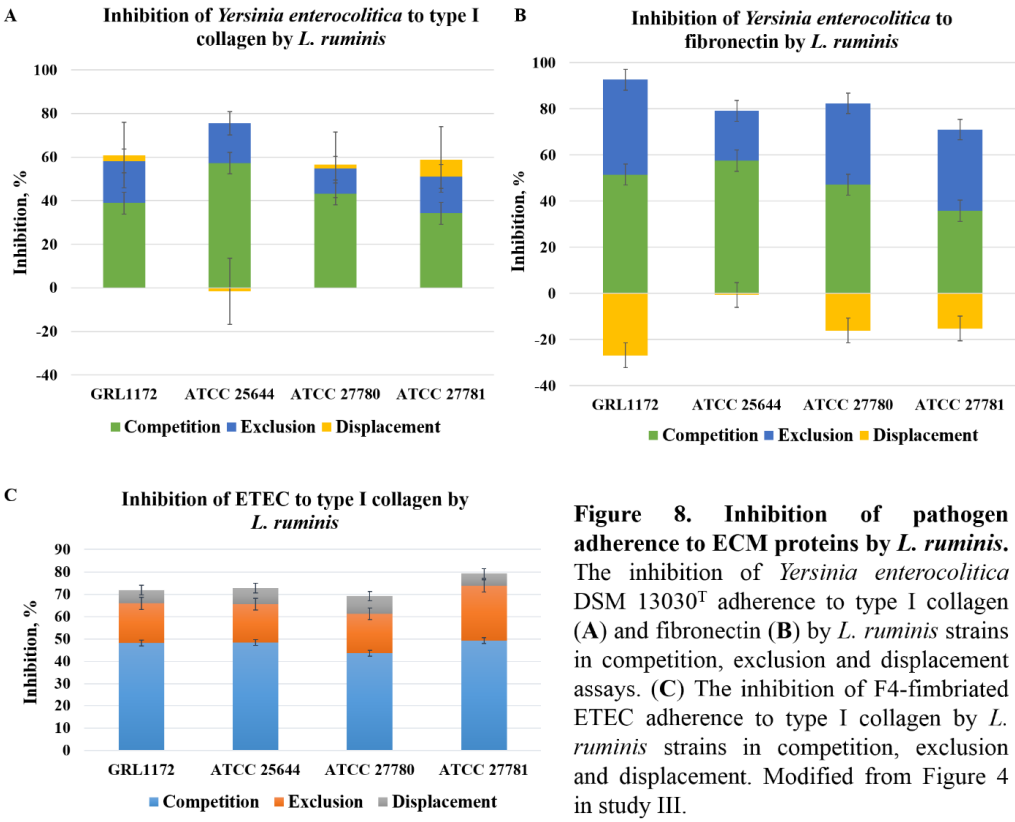


Figure 8. Inhibition of pathogen adherence to ECM proteins by *L. ruminis*. The inhibition of *Yersinia enterocolitica* DSM 13030^T adherence to type I collagen (**A**) and fibronectin (**B**) by *L. ruminis* strains in competition, exclusion and displacement assays. (**C**) The inhibition of F4-fimbriated ETEC adherence to type I collagen by *L. ruminis* strains in competition, exclusion and displacement. Modified from Figure 4 in study III.

The culture supernatants of the four *L. ruminis* strains inhibited the growth of six selected enteropathogens. The inhibition of ETEC growth was the strongest, with an approximately 1000-fold reduction (Figure 7 in III). The growth inhibition of other pathogens was decreased by about 10- to 100-fold. Notably, the growth inhibition was largely attributed to the pH of the supernatant, as the inhibition caused by the supernatant from ATCC 27780 with the lowest pH of 4.13 was the most efficient, while the supernatant from GRL1172 with the highest pH of 4.34 was the least efficient. Moreover, after adjusting the supernatants to the pH of plain MRS medium, a less than 10-fold reduction in pathogen growth was detected, confirming that the dominant contributors to growth inhibition were acids produced during fermentation. Lactic acid is apparently the principal factor, as *L. ruminis* is regarded as a homofermentative species (Kanlder and Weiss, 1986), although a recent publication suggested that it may also use heterofermentative pathways to widen its repertoire of energy sources (Kant et al., 2017). Apart from lowering the pH, lactic acid could also permeabilize the outer membrane of Gram-negative bacteria and thus improve the efficiency of other antimicrobial substances (Alakomi et al., 2000). In accordance with our results, many other lactobacilli originating from humans and

animals are able to inhibit the growth of intestinal pathogens due to the acidic environment they create (Annuk et al., 2003; Lähteinen et al., 2010). In addition to acids, other metabolites produced by lactobacilli, such as hydrogen peroxide, carbon dioxide, diacetyl, and bacteriocins, have antimicrobial effects (Ouwehand and Vesterlund, 2004). Genes encoding bacteriocins have been identified in the genomes of human *L. ruminis* ATCC 25644 and the bovine *L. ruminis* strain ATCC 27782 (Forde et al., 2011), but their presence in other *L. ruminis* strains and their expression *in vitro* require further investigation.

5.7 Immunomodulatory effects of *L. rhamnosus* GG and *L. ruminis* (I, II, III)

The host immune system mounts efficient protective immune responses against pathogens and exhibits tolerance to the diverse gut-dwelling microbes (Artis, 2008). For either immune tolerance or protective defense, the interactions between MAMPs and PRRs on epithelial or immune-related cells are of great importance. In *Lactobacillus*, cell surface components, including LTA, lipoproteins, lipopeptides, flagella (Wells, 2011), S-layers (Konstantinov et al., 2008; Hynönen et al., 2014), and SpaCBA pili in *L. rhamnosus* GG (Lebeer et al., 2012; von Ossowski et al., 2013), are able to activate innate immunity. In this study, we assessed the immunomodulatory effects of *L. rhamnosus* GG SpaFED pili and *L. ruminis* LrpCBA pili.

5.7.1 Immuno-characteristics of *L. rhamnosus* GG SpaFED pili (I)

Previously, it was reported that *L. rhamnosus* GG SpaCBA pili expressed in lactococci interact with the innate immune system (von Ossowski et al., 2013). SpaCBA pili, functioning as one type of MAMPs, were recognized by TLR2 and thus elicited NF-κB signaling as well as IL-8 production in HEK-TLR2 cells. Additionally, SpaCBA pili activated pro- and anti-inflammatory cytokine production in human-derived moDCs. Importantly, the tip pilin SpaC was essential in the above immune responses. To investigate whether *L. rhamnosus* GG SpaFED pili have similar effects, we tested the immuno-characteristics of GRS1189 and GRS1226 with the same methodology as used for SpaCBA pili. GRS71 and GRS1052, used as controls, induced marked NF-κB activation in HEK293-TLR2 cells, whereas GRS1189 evoked such a reaction at a level much lower than the controls (Figure 9 in I). However, GRS1226 lacking the tip pilin SpaF promoted NF-κB activation to a similar degree as the controls (Figure 9 in I). The results here suggested that the SpaFED pili appeared to hinder TLR2-dependent NF-κB signaling activated by *L. lactis*, in which the SpaF subunit appeared to have a determining role. To test whether such immune responses are activated by proteinaceous components, the above experiment was performed with the aforementioned lactococci that were treated by heat. After exposure to a protein-denaturing temperature, all the lactococcal strains almost failed to induce NF-κB activation (Figure 9 in I), suggesting that heat-labile proteins were implicated in the immunostimulating effect. Moreover, to determine whether such immune responses were triggered by direct contact between bacterial surface-associated proteins, Transwell cell culture membrane inserts with a 0.4-μm pore size were used to separate HEK-TLR2 cells from the lactococcal strains. Without cell-to-cell contact, NF-κB activation was eliminated, which also indicated that the proteinaceous stimuli were either attached to or released from the bacteria but could not go through the membrane (Figure 10 in I).

Furthermore, the immune-dampening effect caused by SpaFED pili was also observed in intestinal Caco-2 cells. Compared to GRS1052, GRS1189 generated lower IL-8 production, whereas GRS1226 was as efficient as GRS1052 (Figure 11 in I). In contrast, as shown in Figure 12 in study I, GRS1189 no longer dampened the production of pro- and anti-inflammatory cytokines (TNF-α, IL-12 and IL-10) in moDCs, nor promoted these immune reactions like SpaCBA pili (von Ossowski et al., 2013). Since the moDCs were isolated from peripheral blood, we presumed that such immune responses might not fully reflect the bacteria–host cell interactions in the gut. If SpaFED pili are expressed *in vivo*, their immuno-dampening effects may represent adaptation to the host gut, counteracting the SpaCBA pili-induced immune responses, to achieve immune homeostasis.

5.7.2 Immuno-characteristics of *L. ruminis* (II, III)

Firstly, HEK-TLR2 cells were used to assess the immune stimulation profile of *L. ruminis* ATCC 25644 and its LrpCBA pili displayed on *L. lactis*. *L. ruminis* evoked pronounced levels of NF-κB activation and IL-8 production in HEK-TLR2 cells. This activation was slightly decreased when *L. ruminis* was treated with a protein-denaturing high temperature (Figure 8 in II), but largely and more pronouncedly decreased (though still measurable) when *L. ruminis* was segregated from HEK-TLR2 cells (Figure 9 in II). The results here indicated that the immunomodulatory effects of *L. ruminis* were partly related to its heat-labile proteins, which were either anchored to the cell surface or could not pass through the insert membrane if secreted or detached. However, GRS1224 and GRS1225 expressing LrpCBA or LrpΔCBA pili induced much lower immune responses than did *L. ruminis* and the controls, GRS71 and GRS1052 (Figure 8 in II). Similarly to *L. rhamnosus* GG SpaFED pili, we assumed that LrpCBA pili also dampened the immune responses elicited by *L. lactis*, but tip pilin LrpC did not have an essential role. When GRS71, GRS1052, GRS1224, and GRS1225 were treated with a high temperature and separated from HEK-TLR2 cells, these immune reactions were almost abolished (Figure 8 and 9 in II), which then again confirmed that the bacterial stimuli were heat-labile and were either attached to the cell surface or secreted but could not pass through the membrane.

Analogous experiments were performed with three other different-sourced *L. ruminis* strains, with the human strain ATCC 25644 used as a control. All the strains induced NF-κB activation and IL-8 production in HEK-TLR2 cells to varying degrees (Figure 10 in III and Table 6). Among them, the two bovine strains generated relatively low levels of these immune activities. After being inactivated by high temperature, all strains except the human isolate almost completely failed to evoke immune responses. Unanimously, all the strains partitioned from HEK-TLR2 cells by Transwell inserts no longer stimulated immune activities. However, we observed that the culture supernatants of the four *L. ruminis* strains retained the immunostimulatory effects (Figure 11 in III). Therefore, we assumed that some released cell surface components present in the spent culture supernatant, but not able to penetrate the Transwell insert membrane, such as LTA and lipoproteins, were involved, as demonstrated by other studies (Jones et al., 2005; Henneke et al., 2008). When the culture supernatant was treated with a high temperature, NF-κB activation was only partly affected, displaying a slight reduction, whereas IL-8 production was markedly reduced (Figure 11 in III). This suggested that some secreted or detached heat-resistant substances were associated with TLR2-dependent signaling.

Table 6. NF-κB signaling induced by *L. ruminis* in HEK-TLR2 and HEK-TLR5 cells

	Efficiency of induction*			
	HEK-TLR2		**HEK-TLR5**	
Strain or control		Heat treatment		Heat treatment
GRL1172	5.0±0.2***	1.3±0.1	12.2±2.6***	8.9±1.2
ATCC 25644	5.6±0.5***	2.0±0.2	6.2±1.8***	0.9±0.1
ATCC 27880	4.0±0.5***	1.6±0.0	11.6±2.4***	1.1±0.1
ATCC 27781	3.9±0.4***	1.0±0.1	12.2±2.4***	5.0±1.9
Medium	1	1	1	1

*Efficiencies are expressed as relative values of NF-κB activation compared to the induction caused by the growth medium. Pairwise comparisons between samples with or without heat treatment were shown as: ***P≤0.0001 (extremely significant).

In addition, to study TLR5 ligands in *L. ruminis*, we also tested whether *L. ruminis* could activate TLR5 signaling. *L. ruminis* is so far the only motile *Lactobacillus* species that is known to be an autochthonous

member in the mammalian gut microbiota (Neville et al., 2012). We utilized HEK-TLR5 cells to confirm the expression of flagella-encoding genes in this strain, as well as in the other three *L. ruminis* isolates, as flagellins appear to be the only ligands that induce TLR5 signaling (Hayashi et al., 2001). All four strains, after growing on MRS agar plates for 2 days (based on our preliminary experiments, flagella in *L. ruminis* are well expressed under these conditions), elicited conspicuous TLR5-dependent NF-κB activation (Figure 12A in III and Table 6), suggesting that in these strains, flagellar proteins were produced. Nevertheless, compared to the other three strains, ATCC 25644 induced considerably lower IL-8 production (Figure 12B in III). After high-temperature treatment, ATCC 25644 and ATCC 27780 totally failed to induce NF-κB signaling, but the other two *L. ruminis* strains still had immunostimulatory properties. Therefore, we speculated that the flagellins of GRL1172 and ATCC 27781 were more heat resistant, but the underlying mechanism is unknown. Separation from HEK-TLR5 cells did not affect NF-κB activation and IL-8 production induced by GRL1172 and ATCC 27781, while it lowered the NF-κB and IL-8-inducing activities of ATCC 25644 and ATCC 27780. It is reasonable to propose that flagellins or broken flagella can pass through the insert membrane with a pore size of 0.4 μm, since 0.2 μm pore-sized filters cannot prevent the passing of flagellins (Gewirtz et al., 2001b). It appeared that flagella in GRL1172 and ATCC 27781 were more likely to be detached, or these bacterial cells were more prone to be broken, thus resulting in more flagellins being released and correspondingly higher TLR5-dependent immune responses. Interestingly, GRL1172 and the two bovine strains elicited NF-κB activation to similar degrees, but varying IL-8 induction levels. Since flagellins are also able to promote the production of IL-8 through the TLR5-p38 MAPK signaling pathway, which is independent of the NF-κB route (Yu et al., 2003; Gewirtz et al., 2004), we assumed that these three strains had varying capabilities to activate the p38 route, thus resulting in varying IL-8 levels. It has also been reported that amino acid sequence variations in the structural domains of flagellin may account for differences in IL-8 secretion (Verma et al., 2005; Im et al., 2009); this finding may apply to *L. ruminis* flagellins as well.

Additionally, intestinal Caco-2 cells and moDCs were used to assess the immunomodulatory effects of *L. ruminis* ATCC 25644 and its LrpCBA pili. In Caco-2 cells, LrpCBA-expressing GRS1224 and GRS1225 constructs induced the production of IL-8 to a similar extent, but mildly lower compared to the controls GRS71 and GRS1052 (Figure 10 in II), indicating that LrpCBA pili have a dampening effect and the LrpC subunit is not the determining factor. Similar results were observed in HEK-TLR2 cells (Figures 8 and 9 in II). *L. ruminis* ATCC 25644 itself induced the lowest IL-8 secretion among all the strains, being at a level equivalent to the DMEM medium control (Figure 10 in II), indicating that other factors in *L. ruminis*, in addition to pili, dampen the IL-8 response. It is conceivable that host cells are tolerant to autochthonous *L. ruminis* cells in order to maintain gut homeostasis, which in return is also beneficial for the persistent residence of this bacterial species. Conversely, in HEK-TLR2 cells, *L. ruminis* ATCC 25644 efficiently evoked IL-8 secretion, and we speculate the following: Given that TLR2 is expressed on the basolateral membrane of IECs (Peterson and Artis, 2014), *L. ruminis* colonizing the mucosal layer under healthy conditions is not thought to induce TLR2-mediated pro-inflammatory responses through this receptor, which may partly explain the different immune behavior of *L. ruminis* in HEK-TLR2 (artificial cells overexpressing TLR2) and Caco-2 cells.

Subsequently, we noticed that all the lactococcal strains elicited almost similar degrees of TNF-α and IL-12 secretion in moDCs, which were lower than those induced by *L. ruminis* ATCC 25644 (S5 Figure in II). The result was in line with the observations with SpaFED pili in a similar experimental set-up (the first study in this work). The data here revealed that other MAMPs of *L. ruminis* rather than LrpCBA pili were engaged in the elevated production of inflammatory cytokines by in moDCs. Indigenous bacteria in the GIT are recognized by host innate cells (e.g., DCs) under normal circumstances, which activates the production of inflammatory cytokines *in vivo*, but still maintaining gut homeostasis (Rakoff-Nahoum et al., 2004). In this way, hypothetically, with the ability to stimulate moDCs, *L. ruminis* might contribute to gut homeostasis. However, the immune responses induced by indigenous bacteria are regulated and balanced, exhibiting an immune to tolerant state. Otherwise, this microbe–host interaction would result in detrimental effects.

5.8 Effects of *L. ruminis* on intestinal barrier function (III)

Intestinal epithelial cells are interconnected by junctions such as TJ protein complexes that are indispensable in the maintenance of barrier integrity. Confronting enteropathogens may result in changes in TJ protein distribution, thus leading to augmentation of permeability. It has been reported that several members of the gut microbiota of humans, pigs, and canines, such as *L. plantarum* (Qin et al., 2009), *L. acidophilus* (Kainulainen et al., 2015), and *L. amylovorus* (Roselli et al., 2016), are able to increase the TEER values and TJ protein expression, prevent pathogen-caused barrier damage, and thus preserve an intact epithelial layer. Therefore, to determine whether *L. ruminis* can affect intestinal barrier function *in vitro*, we exposed Caco-2 cells, grown on tissue-culture inserts, to four *L. ruminis* strains alone or with ETEC and measured changes in TEER values (Figure 9), dextran diffusion (Figure 8B in III), and the location of TJ proteins (Figure 9 in III). During the three-day measurement, TEER values were slightly decreased when Caco-2 cells were incubated with the cell culture medium alone, whereas the values dropped to about 15% of the original levels 24 h after the addition of ETEC (Figure 9). Conversely, all the four *L. ruminis* strains increased TEER values during the experimental period, though fluctuations were observed (Figure 9).

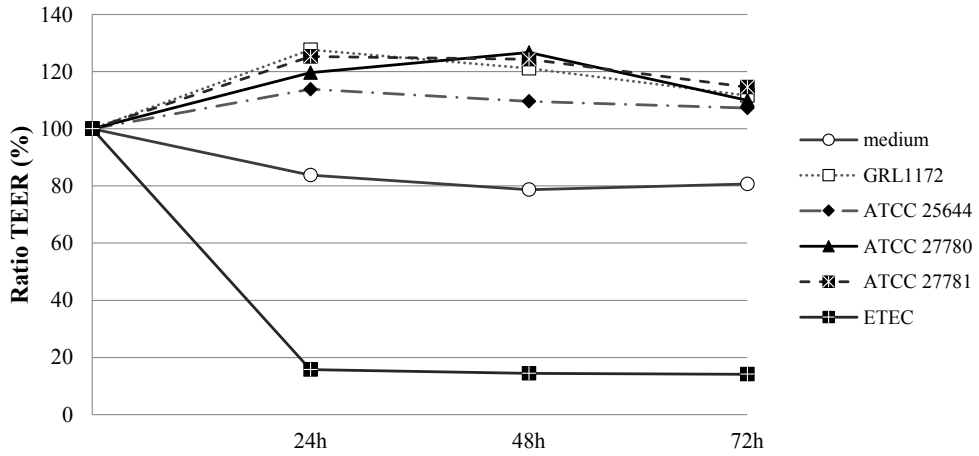


Figure 9. Changes of TEER values in Caco-2 induced by *L. ruminis* and ETEC. Modified from Figure 8A in study III.

Dextran, incubated on Caco-2 cells in the cell culture medium with or without *L. ruminis* cells, cannot diffuse through the epithelium. However, the addition of ETEC alone or with *L. ruminis* strains to the cell culture medium largely induced dextran diffusion. These results indicated that *L. ruminis* fortifies the barrier, but cannot inhibit barrier defects caused by ETEC, which was further confirmed by immunofluorescence assays locating TJ proteins. In these assays, clear and continuous lines were seen around each Caco-2 cell when they were exposed to the *L. ruminis* strains and the detection was performed with antibodies against TJ proteins (ZO-1, claudin-4, and occludin) (Figure 9A in III). In contrast, blurry cell boundaries, scattered fluorescent spots, or even visible holes appeared in Caco-2 cells incubated with ETEC alone or with ETEC and *L. ruminis* strains (Figure 9A and B in III), indicating that these TJ proteins were dislocated and the epithelial barrier was disrupted. Our results demonstrate that *L. ruminis*, as an autochthonous bacterial species in the gut, can maintain the epithelial barrier, which reflects the role of gut microbiota in maintaining host intestinal health.

6. Conclusions and future perspectives

The gut microbiota peacefully exists in the host GIT under healthy conditions, which is attributed to various niche-adaptation factors. In this study, *L. rhamnosus* GG and *L. ruminis*, respectively representing an allochthonous and an autochthonous member of the gut microbiota, were used as paradigms to investigate the gut-adaptive traits of intestinal bacteria.

In the first study, the *L. rhamnosus* GG SpaFED pili recombinantly expressed on *L. lactis* were characterized. SpaFED pili exhibited adhesive properties to mucin, ECM components, and intestinal epithelial cells (Caco-2 and HT-29), and the tip pilin SpaF was shown to be the essential adhesive factor. Therefore, in addition to the adhesive SpaCBA pili (von Ossowski et al., 2013), SpaFED pili might also contribute to the prolongation of the gut retention time of allochthonous *L. rhamnosus* GG. Moreover, SpaFED pili dampened the innate immune responses that were activated by *L. lactis* in HEK-TLR2 cells. This was completely opposite to the outcome seen with *L. rhamnosus* GG SpaCBA pili, which elevated NF-κB activation and IL-8 production (von Ossowski et al., 2013). In the gut environment, if SpaFED pili were expressed, their dampening effects might balance the immune responses induced by SpaCBA pili to maintain immune homeostasis. A pan-genomic study demonstrated the presence of the *spaFED* operon in all 13 of the *L. rhamnosus* strains studied, and it was thus considered as belonging to the core genome, whereas the *spaCBA* operon was only shared by four strains, and it was correspondingly excluded from the core genome (Kant et al., 2014). Additionally, the *spaFED* operon was also identified in the genomes of *L. casei* and *L. paracasei* (Muñoz-Provencio et al., 2012; Smokvina et al., 2013). Nevertheless, native SpaFED pili have not been proved to be present on any of these *Lactobacillus* strains under the experimental conditions used. However, we assume that *spaFED* operon might be expressed *in vivo* only in the host GIT, like the bifidobacterial Tad pilus genes (Motherway et al., 2011). To test this hypothesis, germfree mice could be fed with adequate numbers of *L. rhamnosus* GG cells, and intestinal extracts could be collected to determine SpaFED pilus expression by performing Western blotting or immuno-TEM at different time points after inoculation. If SpaFED pili were successfully detected by these proposed methods, derivatives of *L. rhamnosus* GG without SpaFED pili could be constructed to verify the observed properties of SpaFED pili *in vitro*, such as adhesiveness and immune dampening, to further investigate the functional characteristics of SpaFED pili *in vivo*.

In study II, a novel pilus type was identified in the gut-autochthonous species *L. ruminis*. LrpCBA pili in *L. ruminis* ATCC 25644, and especially the tip pilin LrpC, contributed to *L. ruminis* adherence to ECM proteins and intestinal epithelial cells, as demonstrated by the lactococcal expression system. However, neither *L. ruminis* nor *L. lactis* expressing LrpCBA pili bound to mucin. Presumably, *L. ruminis* can penetrate the mucus and reside closely to the epithelial cells. In addition to acting as an adhesive factor that may facilitate gut colonization, LrpCBA pili, when displayed on *L. lactis*, also had a dampening effect on *L. lactis*-induced NF-κB signaling in HEK-TLR2 cells and on IL-8 production in Caco-2 cells. These results suggest that LrpCBA pili might partly explain the immune tolerance of the host to *L. ruminis*, which thus indirectly contributes to the gut indigeneity of *L. ruminis*. Conversely, whole *L. ruminis* cells pronouncedly activated TLR2-dependent signaling in HEK-TLR2 cells, which indicated that other cell surface structures in *L. ruminis*, such as LTA and lipoproteins, are involved in immunomodulation. This, however, remains to be investigated. *L. ruminis* did not show any immunostimulatory effects when exposed to Caco-2 cells, thus suggesting that the host cells have evolved to tolerate the gut autochthonous species, which has also developed to not induce elimination by the host. However, *in vivo* studies are needed to characterize the roles of LrpCBA pili in the interactions between *L. ruminis* and the host GIT in more detail. To do this, the expression of LrpCBA pili *in vivo* could be investigated. Subsequently, the role of LrpCBA pili in *L. ruminis* gut colonization and immunomodulation could be evaluated by comparisons between *L. ruminis* and its mutants lacking LrpCBA pili or specific pilins. Meanwhile, other factors that contribute to the gut indigeneity of *L. ruminis* are worth exploring in the future.

In study III, a novel *L. ruminis* strain was isolated from porcine feces and was demonstrated by TEM to be piliated and flagellated. Some common features were observed in different *L. ruminis* strains by analyzing the functional characteristics of this new strain together with three other *L. ruminis* strains (one human and two bovine isolates). None of the strains could bind to mucin, but they bound to ECM components and to HT-29 cells, suggesting that *L. ruminis* may reside near the epithelial layer. Moreover, all the strains inhibited pathogen growth and adherence to host surfaces, including intestinal epithelial cells (Caco-2 and IPEC-1) and ECM proteins. Such properties can not only protect the host from pathogen infection, but also contribute to the long-term colonization of *L. ruminis*. Notably, even though the binding of these four *L. ruminis* strains to Caco-2 and IPEC-1 was poor, they conferred inhibition of pathogen colonization. On the grounds of this, it appears obvious that mechanisms other than competitive adherence are involved, which requires further investigation. Additionally, when incubated on Caco-2 cells, all the strains increased the TEER value, prevented dextran diffusion and maintained the location of TJ proteins, which supported the epithelial barrier functions of Caco-2 cells. This phenomenon can be regarded as an advantage to the host and it thus partly contributes to the gut indigeneity of *L. ruminis*. Furthermore, these strains evoked innate immune responses, including NF-κB activation and IL-8 production, in HEK-TLR2/TLR5 cells. The mucosal immune system exhibits tolerance to the indigenous microbes, and how the immunomodulatory *L. ruminis* and the host maintain the homeostasis therefore needs to be investigated in the future. Since flagellin, based on current knowledge, is the only known ligand for TLR5, the activation of TLR5 signaling thus indicates that in addition to the porcine isolate, the human and bovine *L. ruminis* are also flagellated. Of the gut indigenous lactobacilli, *L. ruminis* is currently considered as the only motile species (Neville et al., 2012). Flagella can propel *L. ruminis* through the mucus to reach nutrient-rich niches and facilitate colonization in the gut. However, *in vivo* studies are still required to determine whether the above traits can be detected in the host GIT and whether they contribute to the autochthony of *L. ruminis*, as well as to investigate the location of *L. ruminis* in the GIT.

Due to the potential gut autochthonicity-promoting factors (e.g. adhesiveness) and several interesting effects that *L. ruminis* may confer to the host, such as immunostimulation and pathogen inhibition, *L. ruminis* might be a candidate probiotic for humans and animals in the future. Even though some *L. ruminis* strains have been reported to be susceptible to gastric and bile acids (O'Donnell et al., 2015), they might survive in the GIT if delivered in enterocapsules or in other forms that delay their release in the GIT. The combination of several beneficial strains appears to be a trend for probiotic products, but whether *L. ruminis* has antagonistic effects on other probiotic strains should be explored if *L. ruminis* is to be considered as a probiotic in the future. Many *Lactococcus* and several *Lactobacillus* strains have been extensively studied as carriers for mucosal vaccination, due to features such as safety, adhesiveness, and abilities to boost immune responses (Wyszyńska et al., 2015), in a manner similar to adjuvants. Therefore, *L. ruminis* strains might also be considered as novel vaccine carriers, for the following reasons: (1) *L. ruminis* is a member of the genus *Lactobacillus* and an autochthonous species in the GIT, so it is regarded to be safe to the host; (2) since it is flagellated and piliated, *L. ruminis* could get closer to the intestinal epithelium, colonize it, and promote host–antigen interactions. These speculative applications are worth further investigation.

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