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Bifidobacterium - Human Host Interaction: Role of Human Plasminogen

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TABLE OF CONTENTS

Abstract

Bifidobacterium is an important genus of the human gastrointestinal microbiota, affecting several host physiological features. Despite the numerous *Bifidobacterium* related health-promoting activities, there is still a dearth of information about the molecular mechanisms at the basis of the interaction between this microorganism and the host. Bacterial surface associated proteins may play an important role in this interaction because of their ability to intervene with host molecules, as recently reported for the host protein plasminogen. Plasminogen is the zymogen of the trypsin-like serine protease plasmin, an enzyme with a broad substrate specificity.

Aim of this thesis is to deepen the knowledge about the interaction between *Bifidobacterium* and the human plasminogen system and its role in the *Bifidobacterium*-host interaction process. As a bifidobacterial model, *B. animalis* subsp. *lactis* BI07 has been used because of its large usage in dairy and pharmaceutical preparations.

We started from the molecular characterization of the interaction between plasminogen and one bifidobacterial plasminogen receptor, DnaK, a cell wall protein showing high affinity for plasminogen, and went on with the study of the impact of intestinal environmental factors, such as bile salts and inflammation, on the plasminogen-mediated *Bifidobacterium*-host interaction. According to our *in vitro* findings, by enhancing the activation of the bifidobacterial bound plasminogen to plasmin, the host inflammatory response results in the decrease of the bifidobacterial adhesion to the host enterocytes, favouring bacterial migration to the luminal compartment. Conversely, in the absence of inflammation, plasminogen acts as a molecular bridge between host enterocytes and bifidobacteria, enhancing *Bifidobacterium* adhesion. Furthermore, adaptation to physiological concentrations of bile salts enhances the capability of this microorganism to interact with the host plasminogen system.

The host plasminogen system thus represents an important and flexible tool used by bifidobacteria in the cross-talk with the host.

INTRODUCTION

The Gut Microbiota

A huge amount and a wide range of microorganisms populate the human body. It has been estimated that the components of this human-associated microbiota outnumber human somatic and germ cells by a factor of ten, leading to reconsider human beings as "supraorganisms". This microbial community contains members of the three domains of life, *Eukarya*, *Bacteria* and *Archaea*, with their relative viruses. Among the different regions of the human body where these microbial communities are present, the gastrointestinal tract (GIT) represents the most densely populated habitat (Gill *et al.*, 2006; Turnbaugh *et al.*, 2007; Ley *et al.*, 2008; Neish, 2009).

The human gut microbiota

The GIT is a complex ecosystem composed of several host cells, lining the whole structure, and the intestinal microbiota, dominated by bacteria. Different kinds of eukaryotic cells constitute the intestine: absorptive enterocytes, mucus-secreting goblet cells, antimicrobial peptides-producing Paneth cells and neuroendocrine enterochromaffin cells (Daneman and Rescigno, 2009). In addition to their physiological role in digestion and absorption, these cells and their products constitute the intestinal epithelial barrier that, together with immune cells, interact with microorganisms and components present in the gut (Rescigno, 2011).

The human intestine harbours a highly complex microbial ecosystem, populated with as many as 100 trillion cells belonging to more than 1000 different species (Ley *et al.*, 2006a; Rajilić-Stojanović *et al.*, 2007; Qin *et al.*, 2010) (Fig. 1). It has been estimated that the collection of all the microbial genomes present in the gut, the microbiome, contains more than 100 times the number of genes in the human genome (Bäckhed *et al.*, 2005).

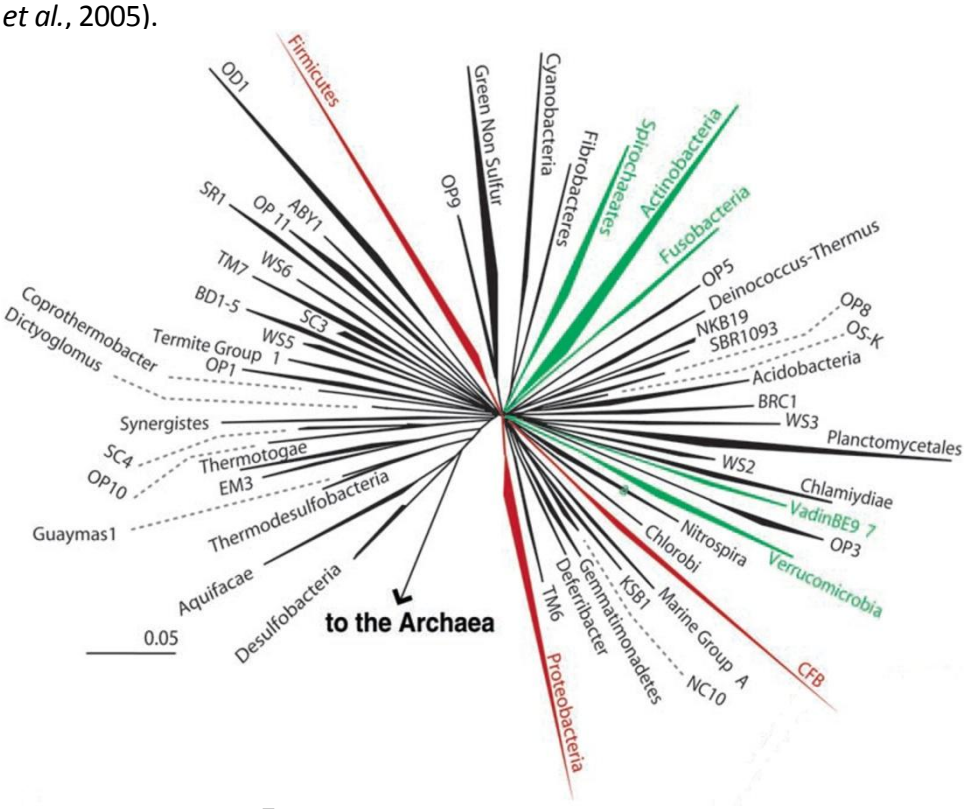


Fig. 1. Representation of the diversity of bacteria in the human intestine. Phylogenetic tree of the domain *Bacteria* based on 8903 representative sequences of the 16S rRNA gene. Wedges represent divisions (superkingdoms): those numerically abundant in the human gut are indicated in red, rare divisions are green, and undetected are black. Wedge length is a measure of evolutionary distance from the common ancestor. Scale bar indicates the degree of diversity (evolutionary distance) within a division, in terms of the fraction of the 16S rRNA gene nucleotides that differ between member sequences. CFB, *Cytophaga-Flavobacterium-Bacteroides* (Bäckhed *et al.*, 2005).

Space and time greatly influence the arrangement of the microbiota in the human gut. On the "biogeography" point of view, microbiota composition changes along the length of the whole GIT and across the mucosal barrier, from the epithelial surface through the mucus layer to the lumen.
Despite its low pH, the stomach possesses a low density rich microbiota, containing up to a few hundreds members. Components of gastric microbiota belong primarily to the *Firmicutes*, *Actinobacteria*, *Bacteroides*, *Proteobacteria* and *Fusobacteria* phyla, with less abundant members from TM7 phylum, *Deferribacteres*, *Deinococcus*/*Thermus* and others (O'May *et al.*, 2005; Bik *et al.*, 2006; Andersson *et al.*, 2008). Further downstream, the small intestine presents an associated microbiota similar to that of the stomach (Hayashi *et al.*, 2005). Along the length of the GIT, moving from the stomach towards distally intestinal regions, a shift in community structure has been reported: facultative anaerobes are replaced by obligate anaerobes (Wang *et al.*, 2003; Hayashi *et al.*, 2005). The lower GIT contains the highest density of microorganisms, with an estimated concentration of 10^{11}-10^{12} bacterial cells per g of gut content, one of the densest community known (Whitman *et al.*, 1998). The colon microbiota is predominated by two bacterial phyla, *Bacteroides* and *Firmicutes*, which represent together up to 90% of the total microbiota in the distal gut; *Actinobacteria* and to a lesser extent *Proteobacteria* and *Fusobacteria* have been detected as subdominant phyla (Fig. 2). Despite this small amount of divisions, hundreds of species and thousands of strains inhabit the distal gut, leading to a significant variability among individuals (Bäckhed *et al.*, 2005; Eckburg *et al.*, 2005; Ley *et al.*, 2006a, 2006b, 2008; Dethlefsen *et al.*, 2008; Turnbaugh *et al.*, 2009; Qin *et al.*, 2010).
Microbiota composition differs across the transversal axis of the gut, from the epithelium to the lumen. Factors such as host defence molecules in the mucus layer or the oxygen gradient through this layer have a significant impact on the amount and the composition of the microbiota from the epithelium to the lumen (Van den Abbeele *et al.*, 2011). For the same reason, the microbiota associated with the intestinal mucosa has a different composition from the faecal microbiota of the same individual (Momozawa *et al.*, 2011).
The microbial community reveals a high inter-individual variability due to host genetics, age, diet, microbial phylogeny, environment and health status. These factors have an important impact on the composition and variability of the microbiota, and every individual has its own unique microbiota, characterized by a specific combination of species and strains, like a fingerprint (Zoetendal *et al.*, 1998, 2001; Bäckhed *et al.*, 2005; Ley *et al.*, 2008; Benson *et al.*, 2010). Despite inter-individual composition variation, microbial function in healthy individual is maintained, suggesting the existence of a core microbiome in terms of functional genes (Tap *et al.*, 2009; Turnbaugh *et al.*, 2009; Qin *et al.*, 2010).
Bacteria are the major components of the gut microbiota, but other microorganisms inhabit this ecosystem. The two predominant *Archaea* found in the gut microbiota are *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* (Scanlan *et al.*, 2008b; Dridi *et al.*, 2009). Among the eukaryotic members of the intestinal microbiota, culture-dependent approaches have detected *Candida* and *Saccharomyces* spp., but yeasts become less represented with culture-independent

analyses, that have revealed the predominance of species belonging to the *Blastocystis* genus (Ott *et al.*, 2008; Scanlan and Marchesi, 2008). The viral component of the human gut microbiota has received limited attention, and recent studies have disclosed interesting insights, pointing out the enormous amount of estimated viruses present and their great diversity (Breitbart *et al.*, 2003, 2008; Zhang *et al.*, 2006; Reyes *et al.*, 2010).

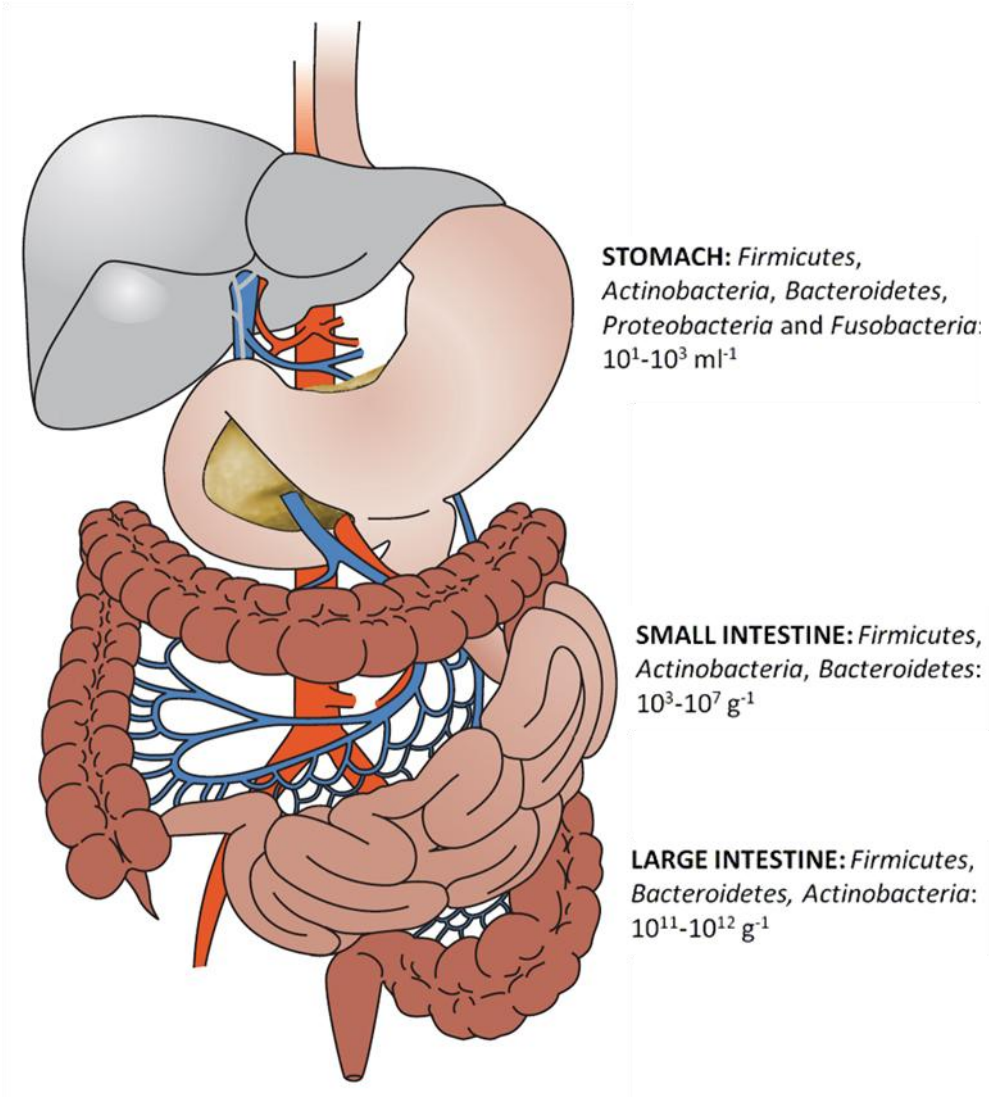


Fig. 2. Composition and luminal concentrations of dominant bacterial phyla along the human gastrointestinal tract (O'Hara and Shanahan, 2006; Marchesi, 2011).

Colonization and ageing

Microbial colonization of the gut starts immediately after birth. Newborns acquire their microbial community coming in contact with bacteria from environmental and maternal origin (Palmer *et al.*, 2007; O'Toole and Claesson, 2010). The initial lower gut community includes enterococci, streptococci, staphylococci, *Bacteroides* members, bifidobacteria, clostridia (Mackie *et al.*, 1999; Favier *et al.*, 2002; Park *et al.*, 2005a; Palmer *et al.*, 2007). It is thought that the first aerobes and facultative anaerobes that colonize the gut reduce the redox potential of the newborns intestine, creating favourable environmental conditions for the later colonization, growth and establishment of obligate anaerobes (Stark and Lee, 1982). Many factors influence the early colonization process: composition of the maternal microbiota, vaginal or caesarean delivery, full or preterm gestation, breast or formula feeding, environmental exposure and clinical intervention, like antibiotics (Favier *et al.*, 2003; Schwiertz *et al.*, 2003; Palmer *et al.*, 2007; Biasucci *et al.*, 2010; Dominguez-Bello *et al.*, 2010; Vaishampayan *et al.*, 2010; Morowitz *et al.*, 2011).

From the second year of life, the composition of gut microbiota acquires a more definite and stable profile, converging to a generalized adult distal gut community (Palmer *et al.*, 2007). During adulthood, a relative stability characterizes the gut microbiota, which displays limited compositional fluctuations over extended periods; factors that can impact the microbiota composition are antibiotic treatments, some pathological conditions (for instance inflammatory bowel diseases or obesity), changes in diet and lifestyle (Frank *et al.*, 2007; Dethlefsen *et al.*, 2008; Spor *et al.*, 2011).

The ageing process involves changes in the composition and diversity of the gut microbiota, such as reduction of the *Bifidobacterium* presence (Woodmansey, 2007). Recently, the gut microbiota of elderly and centenarians has been studied with novel molecular techniques in order to shed some light on the changes that take place during the last part of life (Rajilić-Stojanović *et al.*, 2009; Biagi *et al.*, 2010; Claesson *et al.*, 2011).

The remarkable temporal stability of the adult gut microbiota could be explained by the resilience characteristic of this community: the microbial components of an individual microbiota have coevolved in that peculiar environment, occupying specific niches within the community and maintaining composition and function of the microbial association. This relationship established among all the "authoctonous" members of the microbiota allows the microbial community to recover to a similar composition as before the disturbance within a timeframe of few weeks (Van den Abbeele *et al.*, 2011). Another linked aspect is the colonization resistance: once the gut microbiota has been established, its members occupy all the available niches in the gut, precluding exogenous microorganisms to colonize the intestine (Guarner and Malagelada, 2003; Sansonetti and Medzhitov, 2009).

Analysis of the microbiota

The composition of the human intestinal microbiota has been studied until recently by using traditional culturing techniques, such as anaerobic cultures (Moore and Holdeman, 1974). These methods provide only a partial vision of the biodiversity of the human gut microbiota because just a minor fraction (20-40%) of this community can be cultured (Langendijk *et al.*, 1995; Suau *et al.*, 1999; Hayashi *et al.*, 2003; Eckburg *et al.*, 2005). Moreover, culture-based methods are laborious, time-consuming and more inclined to statistical and methodological errors. The development of culture-independent methods based on molecular techniques allowed faster and more detailed analysis of the composition of this complex microbial community (Furrie, 2006).

The most commonly employed strategies for uncovering the composition of complex microbial ecosystems utilize the 16S ribosomal RNA (16S rRNA) gene sequence. This gene consists of approximately 1500 nucleotides and presents regions conserved among all the bacteria, interspersed with 9 hyper-variable regions (V1 to V9), in which the sequence can be genus or species specific (Tannock, 1999).

One molecular method commonly used for the analysis of microorganisms in complex environmental samples is the FISH technique, Fluorescence *In Situ* Hybridization. This strategy relies on fluorescent oligonucleotide probes designed on 16S rRNA gene sequences specific for bacterial species or genus, allowing bacteria visualization and quantification through microscopy or flow cytometry (Zoetendal *et al.*, 2004).

A PCR based method widely used to characterize the composition of complex bacterial communities is the quantitative real time-PCR. Genus or species specific primers designed on the 16S rRNA gene sequence are used to allow the quantification of different bacterial populations present in the ecosystem (Zoetendal *et al.*, 2004).

Fingerprinting techniques allow semi-quantitative analysis of microbial communities by separating fragments of the 16S rRNA gene and detecting variations in the sequence among members of communities. The most important fingerprinting techniques are Denaturing/Temperature Gradient Gel Electrophoresis (D/TGGE), Single Strand Conformational Polymorphism (SSCP) and Terminal-Restriction Fragment Length Polymorphism (T-RFLP).

Microarray technology has been developed to study bacterial diversity in several complex ecosystems, among which the intestinal microbiota (Vaughan *et al.*, 2000; Zoetendal *et al.*, 2004). Diversity DNA microarrays are usually based on the sequence of the 16S rRNA gene and allow the identification of bacteria in complex microbial communities. The huge amount of different specific probes present on the microarrays can give more precise information on the composition of the microbiota (Palmer *et al.*, 2006, 2007; Paliy *et al.*, 2009; Rajilić-Stojanović *et al.*, 2009).

In recent times, next generation sequencing technologies have been applied for the deep analysis of the microbiota (Mardis, 2008; Shendure and Ji, 2008). Pyrosequencing is one of the main method: the massive sequencing of vast numbers of 16S rRNA genes from the complex gut

microbiota can further characterize in depth the intestinal ecosystem (Ronaghi *et al.*, 1998; Margulies *et al.*, 2005; Andersson *et al.* 2008; Dethlefsen *et al.,* 2008; Claesson *et al.*, 2009).
The last frontier of microbial ecology is represented by metagenomic analysis techniques (Gill *et al.*, 2006). These culture-independent methods are based on the analysis of the total genomic DNA present in a microbial community (metagenome) by sequencing or functional analysis. Together with other meta-"omic" approaches, such as metatranscriptomics, metaproteomics and metabonomics, these techniques provide further insights on the activity and functionality of the gut microbiota (Zoetendal *et al.*, 2008).

Microbiota activities and interactions with the host

Through millennia, the host and the microbiota microorganisms have evolved an intricate network of reciprocal beneficial relationships, leading to the establishment of a balanced mutualistic symbiosis. It is thought that microorganisms that constitute the intestinal microbiota have been selected by the host because they have to face many specific host factors, such as physical conditions along the GIT (pH, temperature, retention time), diet types and the variety of defense systems present in this habitat (innate and adaptive immune system). The host environment has shaped the community structure of the microbiota, selecting for those microbial communities adapted to that habitat; and microorganisms have evolved specific functions useful for the host that the host hasn't evolved by itself: this has led to the establishment of a functional stable and coevolved cooperation (Ley *et al.*, 2006a, 2008; O'Hara and Shanahan, 2006; Blaser and Kirschner, 2007; Muegge *et al.*, 2011; Spor *et al.*, 2011; Van den Abbeele *et al.*, 2011).
Microorganisms of the human gut microbiota provide the host with many metabolic activities that the host does not possess by itself, contributing to its nutrition. They possess a large metabolic degradation capacity of host indigestible substrates, such as complex plant-derived polysaccharides; they can degrade host mucin and influence bile salts and cholesterol metabolism, with an important impact on host energy harvest. Short chain fatty acids (SCFAs) are the end-products of several microbial fermentations in the gastrointestinal ecosystem with important roles in energy and health-promoting effects to the host. Some essential vitamins, amino acids and several bioactive compounds are synthesized by microorganisms residing in the colon, that can also favour calcium, magnesium and iron uptake (Tannock, 1997; Hooper *et al.*, 2001, 2002; Guarner and Malagelada, 2003; Macfarlane and Macfarlane, 2003; Flint *et al.*, 2008; Cani and Delzenne, 2011; Kau *et al.*, 2011).
The intestinal microbiota plays also an important role as a barrier against invaders of the GIT. Competition for adhesion sites and production of antimicrobial peptides and SCFAs protect the gut mucosa from pathogen or opportunistic microorganisms (Liévin-Le Moal and Servin, 2006; Sansonetti and Medzhitov, 2009; Fukuda *et al.*, 2011; Ashida *et al.*, 2012).
Moreover, the enormous microbial amount of the gut microbiota plays an essential role in the training and modulation of the gastrointestinal immune system. The direct or indirect interaction

between gut microorganisms and the epithelium is crucial for an appropriate shaping of the host innate and adaptive immune system, since the beginning of life. Innate and adaptive immune system actively monitors bacterial interactions with the intestinal mucosa interface, activating responses to limit bacterial contact with the mucosal surface. On the other hand, the resident gut microbiota has evolved traits in order to be ignored, or at least tolerated, by the gut immune system and stably inhabits that environment. Immunological host response and symbiont microorganisms modulation allow thus the establishment of an homeostatic balance, characterized by host tolerance towards gut microbiota members (Duerkop *et al.*, 2009; Round and Mazmanian, 2009; Sansonetti and Medzhitov, 2009; Garrett *et al.*, 2010; Hooper and Macpherson, 2010; Van den Abbeele *et al.*, 2011) (Fig. 3).

Gut microbiota and disease

Individual microbial composition feels the effects of pathological conditions, antibiotic treatments, stress, diet changes and intestinal physiological alteration (Macfarlane and Cummings, 2002). The unnatural shift in the community composition of a "healthy" microbiota leads to an altered microbial colonization, named dysbiosis, with negative effects on the host health, such as a higher susceptibility to enteropathogens infections. Dysbiosis can also affect the immune system: recent studies have revealed altered immune responses in subjects with an unbalanced gut microbial composition (Round and Mazmanian, 2009; Lee and Mazmanian, 2010).

In recent years, several studies have highlighted connections between intestinal microbiota composition and some pathologies, not only gastrointestinal but also systemic diseases: irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), colorectal cancer (CRC), allergies, obesity, insulin resistance, cardiovascular disease (Seksik *et al.*, 2003; Noverr and Huffnagle, 2005; Scanlan *et al.*, 2006, 2008a; Frank *et al.*, 2007; Kassinen *et al.*, 2007; O'Mahony *et al.*, 2009; Cani and Delzenne, 2011; Sobhani *et al.*, 2011; Wang *et al.*, 2011b). It's important to note, however, that it is really difficult to determine if the detected microbial variations are one of the causes or a consequence of the disease (Zoetendal *et al.*, 2006).

IBD, with the two main forms Crohn's disease and ulcerative colitis, and IBS are chronically relapsing gut diseases with a high incidence especially in developed countries. Shared characteristics are alterations in immune responses, altered microbiota composition, impaired intestinal barrier functions. Numerous studies have been carried out to unravel the intricate interconnections of gut microbial composition and inflammation and the underlying molecular mechanisms, and a recent review has focused on the possible role of bacterial proteases in the pathogenesis of these gastrointestinal disorders (Steck *et al.*, 2011).

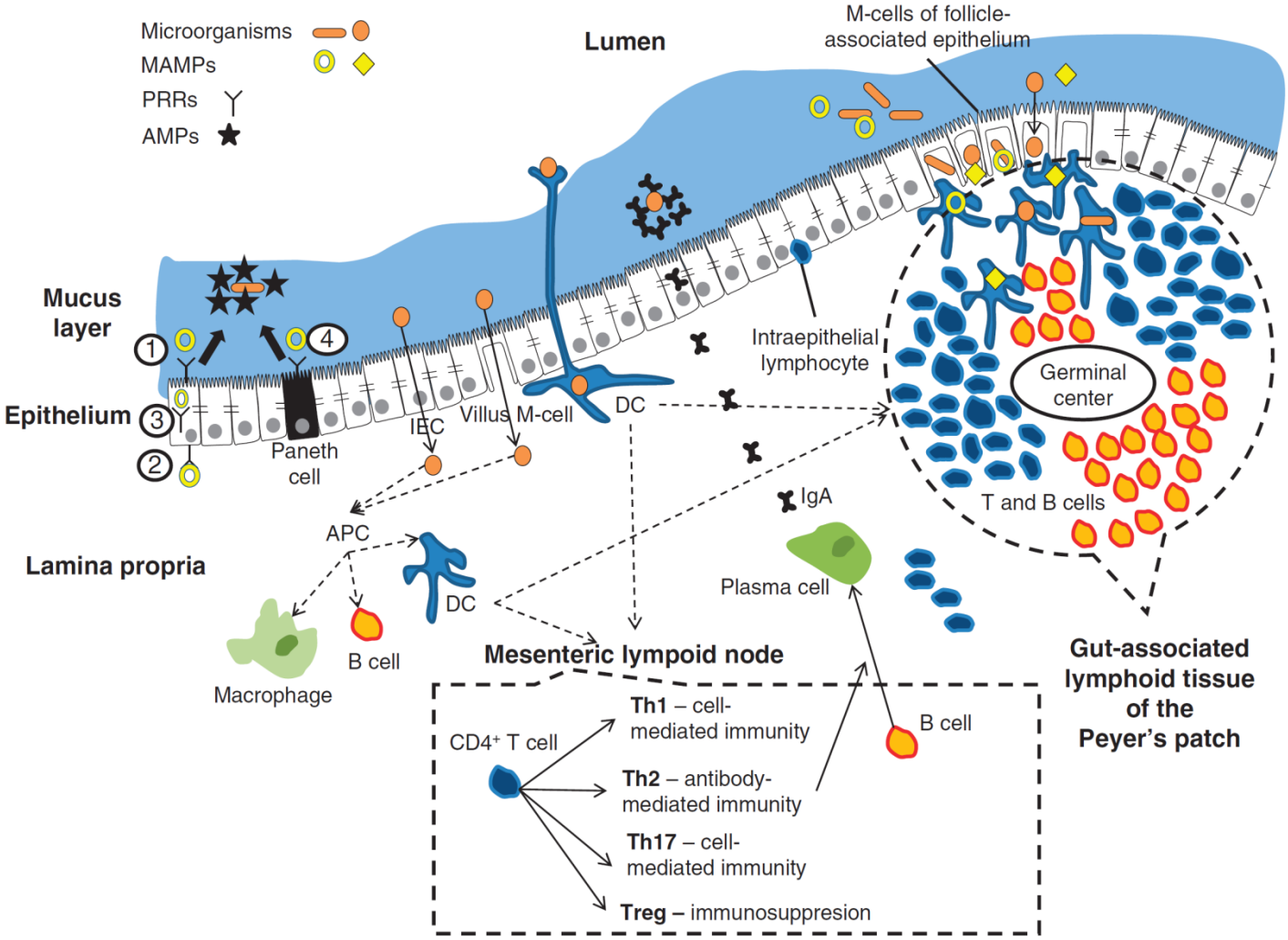


Fig. 3. Host immune system and microorganisms at the intestinal epithelial surface. A constant signalling between microorganisms and the host takes place at the epithelial surface of the intestinal mucosa. Intestinal epithelial cells (IECs) and dendritic cells (DCs) extending beyond the epithelium are essential for the initial recognition of microorganisms. Innate immunity pattern recognition receptors (PRRs) detect microorganisms-associated molecular patterns (MAMPs), allowing the host to distinguish between a microbial friend or foe and to initiate expression of anti-microbial peptides (AMPs). An important feature to distinguish between a microbial friend or foe is the strategic localization of PRRs and essential co-receptors: polarized (1–2), intracytosolic (3) or on specialized cells (4). Microorganisms can cross the epithelial barrier through IECs, DCs, villus M-cells and M-cells of the follicle-associated epithelium overlying the gut-associated lymphoid tissue of the Peyer's patches. After or during this transport, antigens are engulfed in antigen presenting cells (APCs), that can be DCs, macrophages or B-cells. DCs travel from the epithelium through the lamina propria towards mesenteric lymphoid nodes, where they activate cells of the adaptive immune system. They are the only cells that can immediately bind with naïve T-cells, and through cytokine release, influence their maturation to Th1, Th2, Th17 or Treg. Th2 cells interact with B-cells to become plasma cells that secrete IgA antibodies and coat luminal microorganisms in order to prevent them from breaching the epithelium. Alternatively, DCs can also locally activate adaptive immune cells in the gut associated lymphoid tissue of the Peyer's patches (Van den Abbeele *et al.*, 2011).

Several clinical trials have demonstrated the impact of probiotics consumption on the altered gut microbial compositions characteristic of gastrointestinal disorders, suggesting a way to prevent and treat intestinal disorders (Chermesh and Eliakim, 2006; Jonkers and Stockbrügger, 2007; Imaoka *et al.*, 2008). Probiotics have been defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2006). General characteristics of probiotic bacteria are effectiveness, safety and the capacity to go through the gastric barrier without their impairment from the action of acids, bile or proteolytic enzymes. Mechanisms by which probiotics may exert benefits to the host include modulation of the host immune system and pathogen prevention by binding site competition as well as antimicrobial compounds production (Fooks and Gibson, 2002; Vanderpool *et al.*, 2008). The gut microbiota can be modulated also by the use of "prebiotics", usually oligosaccharides that act as specific substrate for probiotic bacteria (such as members of *Bifidobacterium* and *Lactobacillus* genera) favouring their growth; generally, prebiotic substrates are not degraded by the host (Hamilton-Miller, 2004; Ouwehand *et al.*, 2005; Hord, 2008).

Bifidobacterium

Bifidobacterium is an important genus of the human intestinal microbiota. The bifidobacteria amount in the human gut seems to be dependent on various factors, for instance age and diet. Bifidobacteria are among the first colonizers of the GIT of newborns and represent the dominant genus of healthy breast-fed infants. During the first year of life, the intestinal microbiota undergoes several changes and bifidobacteria become less abundant, reaching an estimate 3% of the gut microbiota composition in the adults (Favier *et al.*, 2002; Penders *et al.*, 2006; Zoetendal *et al.*, 2006; Kurokawa *et al.*, 2007; Turroni *et al.*, 2008, 2009; Ventura *et al.*, 2009a; Roger *et al.*, 2010).
Bifidobacteria are Gram-positive, non-motile, non-spore-forming, non-gas-producing anaerobic bacteria (Scardovi, 1986; Ventura *et al.*, 2004b; Klijn *et al.*, 2005). Their morphology is variable and usually referred as bifid or irregular V- or Y-shaped rods, similar to branches (Fig. 4). The reason of this peculiar irregular shape is not yet clear, but different studies have pointed out that the absence of some nutrients in growth media can affect the shape (Glick *et al.*, 1960; Kojima *et al.*, 1968; Kojima *et al.*, 1970; Husain *et al.*, 1972).

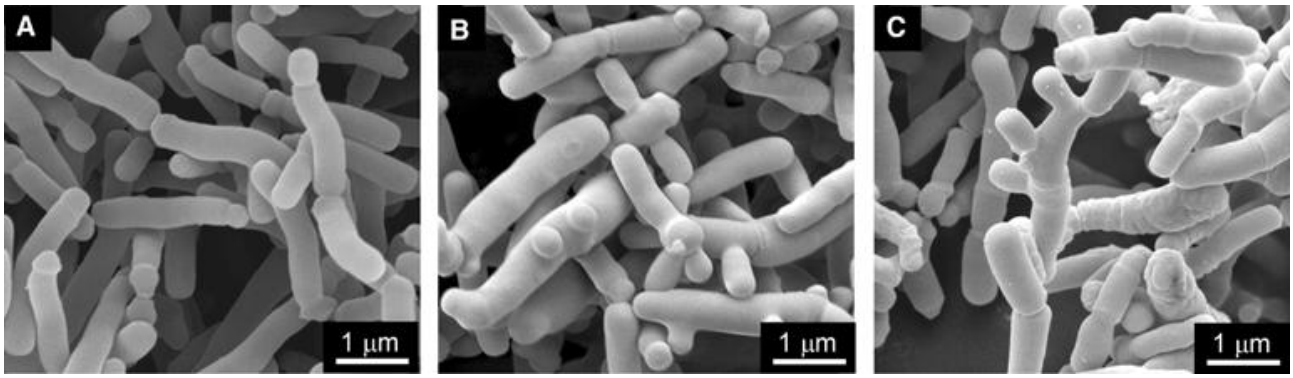


Fig. 4. Scanning electron micrographs of *B. longum* NCC2705 cells. Bacteria have been grown in MRS medium under anaerobic conditions. During the exponential phase typical rod shapes are observed (A), while bifid or branched shaped forms are recognized in transition (B) and stationary (C) phases (Klijn *et al.*, 2005).

Bifidobacteria were first isolated from the faeces of breast-fed infants by Henri Tissier in 1899, during his PhD project, and named *Bacillus bifidus* (Tissier, 1900). In 1924 Orla-Jensen proposed the novel genus *Bifidobacterium* (Orla-Jensen, 1924), but it has been recognized as an independent genus only in 1973 (Poupard *et al.*, 1973; Bergey *et al.*, 1974; Biavati *et al.*, 2000).
Two different approaches have been developed to isolate, detect and identify bifidobacteria: traditional culturing methods and molecular analysis. Culturing methods rely on the use of selective media. Selectivity can be achieved by means of antibiotics, such as neomycin, polymyxin B, nalidixic acid, mupirocin, or other inhibitory agents, like lithium chloride and propionate.

Several molecular methods are culture-free and allow *in situ* analysis, avoiding the limitations of culturing (Mohania *et al.*, 2008; Lee and O'Sullivan, 2010; Ashraf and Shah, 2011).
Bifidobacteria are present in several ecological niches: different animal intestines (humans, mice, rabbit, cow, chicken, insects), oral cavity, sewage, food (Ventura *et al.*, 2007b). The presence of bifidobacteria in the last two habitats is liable to be a consequence of contamination from the intestinal environment. Some bifidobacterial species are broadly distributed in the intestine of a wide variety of animals, whereas other species show a high ecological adaptation to a specific animal gut (Lamendella *et al.*, 2008).

Taxonomy

The genus *Bifidobacterium* is a member of the *Bifidobacteriaceae* family and *Bifidobacteriales* order and belongs to the *Actinobacteria* phylum (Dworkin *et al.*, 2006). Members of this phylum show a great variety of morphologies and physiological and metabolic characteristics, in addition to the extreme variety of their ecological niche: they range from plant to soil to GIT.
Actinobacteria is one of the largest phyla in the *Bacteria* domain, characterized by Gram-positive microorganisms with high G+C DNA content, ranging between 51% to 70% (Dworkin *et al.*, 2006; Ventura *et al.*, 2007b). Two families belong to this order, *Bifidobacteriales* and *Incertae*. Based on the most recent "Taxomonic Outline of Bacteria and Archea" (TOBA 7.7), *Bifidobacteriaceae* family can be divided into five genera: *Bifidobacterium*, *Aeriscardovia*, *Gardnerella*, *Parascardovia*, *Scardovia* (Garrity *et al.*, 2007).
At present 39 species belong to the genus *Bifidobacterium*, isolated from the intestine of humans, mammals and insects, and also from raw milk, dental caries and sewage.
Comparative analysis of the 16S rRNA gene sequences and the sequence analysis of other housekeeping genes (*recA*, *tufA*, *atpD*, *groEL*, *dnaK*, *grpE*, *clpP*, *hrcA*) allowed great advances in bifidobacterial taxonomy (Kullen *et al.*, 1997; Jian *et al.*, 2001; Ventura *et al.*, 2004b, 2005b; Zomer *et al.*, 2009). However, phylogenetic analysis based on a single molecular marker could not adequately reconstruct evolutionary relationships among bacteria, because of horizontal gene transfer phenomena, variable mutation or recombination rates (Ventura *et al.*, 2007a). For this reason, analysis based on a set of combined alignments of conserved orthologous genes (supertrees - Bininda-Emonds, 2004) can produce a more robust evolutionary representation of bacterial phylogeny (Brown, 2001). The application of this approach allowed the clustering of the bifidobacterial species into six different phylogenetic groups: *B. adolescentis*, *B. asteroides*, *B. boum*, *B. longum*, *B. pullorum*, *B. pseudolongum* (Ventura *et al.*, 2006a) (Fig. 5). Recently, the availability of several bifidobacterial genome sequences allows the application of a whole-genome phylogenomic approach, that will offer more insights into the classification of the *Bifidobacterium* genus (Lee and O'Sullivan, 2010).

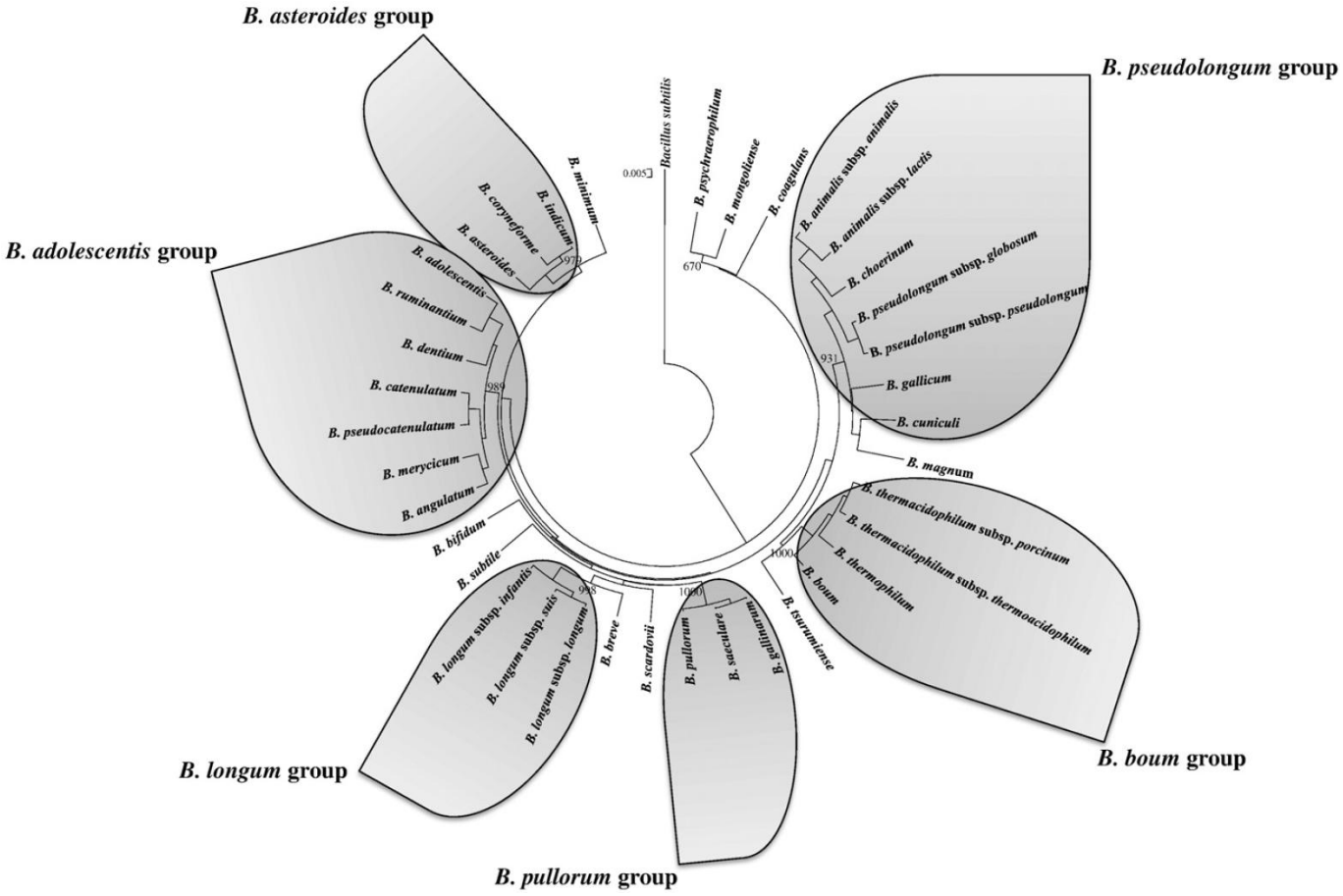


Fig. 5. Phylogenetic tree of members of the *Bifidobacterium* genus as based on a comparative sequence analysis of 1500 nucleotides of their 16S rRNA gene. The different phylogenetic clusters are highlighted (Turroni *et al.*, 2011).

The complete genome of 21 bifidobacterial strains has been sequenced to date and made publicly available in the GenBank database (Table 1). The genome size of bifidobacteria ranges from about 2.0 to 2.8 Mb, with a G+C content between 54 and 67% (Lee and O'Sullivan, 2010).

Table 1 - Sequenced *Bifidobacterium* genomes

Species	Genome size (bp)	% GC	genes	proteins	rRNA operons	Source	Accession number	Institution	References
B. adolescentis ATCC15703	2,089,645	59.2	1,702	1,632	5	Human GIT	NC_008618.1	Gifu University, Japan	Published in database only (2006)
B. animalis subsp. *lactis* AD011	1,933,695	60.5	1,603	1,527	2	Infant faeces	NC_011835.1	Korea Research Institute of Bioscience and Biotechnology, Republic of Korea	Kim *et al.*, 2009
B. animalis subsp. *lactis* BB-12	1,942,198	60.5	1,706	1,642	4	Fermented milk	CP001853.1	Chr Hansen A/S, Denmark	Garrigues *et al.*, 2010

B. animalis subsp. *lactis* BI04	1,938,709	60.5	1,631	1,567	4	Adult faeces	NC_012814.1	Danisco/Penn State University, USA	Barrangou *et al.*, 2009
B. animalis subsp. *lactis* BLC1	1,943,990	60.5	1,622	1,549	4	Functional foods	CP003039.1	Alimentary Pharmabiotic Centre, National University of Ireland, Ireland.	Bottacini *et al.*, 2011
B. animalis subsp. *lactis* CNCM I-2494	1,943,113	60.5	1,724	1,660	4	Fermented dairy product	CP002915.1	Danone Research, France	Chervaux *et al.*, 2011
B. animalis subsp. *lactis* DSM10140	1,938,483	60.5	1,629	1,566	4	French yogurt	NC_012815.1	Danisco/Penn State University, USA	Barrangou *et al.*, 2009
B. animalis subsp. *lactis* V9	1,944,050	60.5	1,636	1,572	4	Healthy child faeces	CP001892.1	Inner Mongolia Agricultural University, China	Sun *et al.*, 2010
B. bifidum PRL2010	2,214,656	62.7	1,791	1,706	3	Human GIT	NC_014638.1	University of Parma, Italy	Turroni *et al*., 2010a
B. bifidum S17	2,186,882	62.8	1,845	1,783	3	Breast-fed infant faeces	NC_014616.1	University of Ulm, Germany	Zhurina *et al.*, 2011
B. breve ACS-071-V-Sch8b	2,327,492	58.7	2,011	1,826	3	Human vagina	CP002743.1	The J. Craig Venter Institute, USA	GenBank direct submission (2011)
B. breve UCC2003	2,422,684	58.7	1,985	1,854	2	Infant nursing stool	CP000303.1	Alimentary Pharmabiotic Centre, National University of Ireland, Ireland	O'Connell Motherway *et al.*, 2011
B. dentium Bd1	2,636,367	58.5	2,197	2,129	4	Dental caries	NC_013714.1	University of Parma, Italy University College of Cork, Ireland	Ventura *et al.*, 2009c
B. longum subsp. *infantis* ATCC15697	2,832,748	59.9	2,588	2,416	4	Infant GIT	NC_011593.1	University of California Davis, USA	Sela *et al.*, 2008
B. longum subsp. *infantis* 157F	2,400,312	60.1	2,062	1,991	4	Infant faeces	NC_015052.1	University of Tokyo, Japan	Fukuda *et al.*, 2011
B. longum subsp. *longum* BBMN68	2,265,943	59.9	1,878	1,806	3	Healthy centenarian faeces	NC_014656.1	China Agricultural University, China	Hao *et al.*, 2011
B. longum subsp. *longum* DJO10A	2,375,792	60.1	2,061	1,989	4	Human GIT	NC_010816.1	University of Minnesota, USA	Lee *et al.*, 2008
B. longum subsp. *longum* JCM 1217	2,385,164	60.3	2,009	1,924	4	Human GIT	NC_015067.1	University of Tokyo, Japan	Fukuda *et al.*, 2011
B. longum subsp. *longum* JDM301	2,477,838	59.8	2,035	1,958	3	Human faeces	NC_014169.1	Shanghai Jiao Tong University School of Medicine, China	Wei *et al.*, 2010
B. longum subsp. *longum* KACC 91563	2,385,301	59.8	2,036	1,971	3	Neonates faeces	CP002794.1	National Institute of Animal Science, Republic of Korea	Ham *et al.*, 2011
B. longum subsp. *longum* NCC2705	2,256,640	60.1	1,798	1,727	4	Human GIT	NC_004307.2	Nestle Research Centre, Switzerland	Schell *et al.*, 2002

The number of rRNA operons among *Bifidobacterium* species varies from 2 to 5, with a variability both among species and strains (Table 1). This variability might represent diverse ecological strategies adopted by different strains (Klappenbach *et al.*, 2000).

Physiology

Human bifidobacterial strains grow at an optimal temperature of 36-38°C, but animal strains grow at a higher temperature, approximately 41-43°C. The extreme temperatures are reached by *B. thermacidophilum*, which has been reported to growth at temperatures as high as 49.5°C, and *B. psychraerophilum*, that can grow at 8°C (Gavini *et al.*, 1991; Dong *et al.*, 2000; Simpson *et al.*, 2004). Similarly to other organisms, bifidobacteria possess specific molecular mechanisms to cope with heat stress. They possess several molecular chaperones belonging to HSP60 (GroEL/GroES complex), HSP70 (DnaK, GrpE, DnaJ), HSP100 (ClpBCX) and small heat shock proteins (sHSPs) families to face heat stress, as well as the more general SOS response has been demonstrated to be involved (Ventura *et al.*, 2006b, 2007c; Zomer *et al.*, 2009).

Bifidobacteria are generally described as strict anaerobes, although some of them can tolerate limited amounts of oxygen. The sensitivity to oxygen is variable, with the fermented-milk adapted *B. animalis* subsp. *lactis* the most aerotolerant (de Vries and Stouthamer, 1969; Scardovi, 1986; Shimamura *et al.*, 1992; Meile *et al.*, 1997; Simpson *et al.*, 2005; Kawasaki *et al.*, 2006). The capability to tolerate and survive in the presence of oxygen depends on the activity of some enzymes capable to detoxify and remove reactive oxygen species (ROS). Genomic and functional analysis of bifidobacteria revealed the presence of specific enzymes involved in these activities, in particular NADH oxidase, peroxyredoxin family alkyl hydroperoxide reductase gene analogues, thioredoxin reductase gene analogues and oxidative damage repair-related genes (Shimamura *et al.*, 1992; Shin and Park, 1997; Talwalkar and Kailasapathy, 2003; Lee and O'Sullivan, 2010). A recent study has provided more insights on the mechanisms of oxygen tolerance in *B. animalis* subsp. *lactis*. Ruiz *et al.* (2012a) have reported that oxygen induces an overproduction of several glycolytic proteins and enzymes involved in redox reactions, the higher expression of specific oxidases and a higher membrane F_1F_0-type ATPase activity, all of these counteracting oxygen induced damages and maintaining the correct redox status of the cells.

The optimum pH for growth of several bifidobacterial strains is between 6.5 and 7, but they can survive at lower pH, suggesting the presence of efficient acid tolerance mechanisms (Waddington *et al.*, 2010). The F_1F_0-type ATPase system is involved in acid tolerance by pumping protons out of the cell. This system is encoded by the *atp* operon in bifidobacteria and it is supposed to be essential for their response to acid stress (Matsumoto *et al.*, 2004; Ventura *et al.*, 2004a). Recently, an alternative mechanism for acid tolerance has been revealed in *B. dentium*: it possesses the glutamate-dependent acid resistance system 2, which encodes a glutamate decarboxylase and a glutamate/gamma-aminobutyrate antiporter (Ventura *et al.*, 2009c).

The capability to adapt and tolerate bile salts and acidic stress is a characteristic shared by several members of the *Bifidobacterium* genus, although high variability has been detected among strains (Sánchez *et al.*, 2007a; Waddington *et al.*, 2010). Adaptation and tolerance to physiological concentrations of bile salts (typically below 5 mM) is an essential factor for bacterial survival and colonization of the human GIT (Ridlon *et al.*, 2006; Sánchez *et al.*, 2007b). Bile salts are detergent-like compounds secreted into the intestine during digestion for the emulsification and absorption of fats, but they exert also a strong antimicrobial activity (Bernstein *et al.*, 1999b). The ability to tolerate these compounds gives bifidobacteria a selective advantage in the gut ecosystem. Bile salts resistance in bifidobacteria has been extensively studied, and these efforts have led to the identification in two *Bifidobacterium* species of a putative multidrug resistance (MDR) system, named *betA* (*b*ile *e*fflux *t*ransporter), whose expression is induced by bile (Gueimonde *et al.*, 2009). It's worth noting that bile salts have a profound effect on the global protein expression in bifidobacteria (Sánchez *et al.*, 2007b; Ruiz *et al.*, 2009).

Metabolism

The genome analysis of bifidobacteria has improved the comprehension of the biosynthetic and metabolic capabilities of these microorganisms, starting from the bioinformatics analysis of the first bifidobacterial genome completely sequenced (Schell *et al.*, 2002). These investigations have confirmed previous findings on the nutritional demands and biosynthetic features of *Bifidobacterium* (Hassinen *et al.*, 1951; Matteuzzi *et al.*, 1978; Deguchi *et al.*, 1985), and added more information on the metabolic potential of this genus (Schell *et al.*, 2002).

Bifidobacterial genomes harbour genes for the synthesis of at least 19 amino acids from ammonia and also from different intermediates (Lee and O'Sullivan, 2010). They can produce a variety of B vitamins: thiamine (B1), nicotinic acid (B3) and folic acid (B9), while some strain differences have been reported for the biosynthesis of riboflavin (B2) and pyridoxine (B6), as well as for cobalamin (B12); bifidobacteria have an absolute requirement for pantothenate (B5) and biotin (B7) (Hassinen *et al.*, 1951; Gyllenberg and Carlberg, 1958; Tamura, 1983; Deguchi *et al.*, 1985). Bifidobacterial genomes encode all the genes required for the biosynthesis of purine and pyrimidine nucleotides (Lee and O'Sullivan, 2010).

Bifidobacteria can use a wide variety of different carbohydrates as carbon source, and these metabolic activities may vary considerably among bifidobacterial strains (Pokusaeva *et al.*, 2011). Hexose such as glucose and fructose are metabolized for energy production via the "bifid shunt", a metabolic pathway involving the key enzyme fructose-6-phosphate phosphoketolase (F6PPK) (Scardovi and Trovatelli, 1965; de Vries *et al.*, 1967; de Vries and Stouthamer, 1967, 1968; Scardovi, 1986; Bezkorovainy and Miller-Catchpole, 1989). Bifidobacterial genomes contain genes for the utilization of a broad spectrum of substrates, such as glucose, fructose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, arabinose, xylose, ribose, sucrose, lactose, cellobiose, melibiose, gentobiose, maltose, isomaltose, raffinose and mannose (de Vries and Stouthamer,

1968; Pokusaeva *et al.*, 2011). It has been reported the preference of bifidobacteria for utilizing di- and oligosaccharides: this could represent an evolutionary adaptation to an environment poor in monosaccharides but rich in complex sugars (Amaretti *et al.*, 2006; Vernazza *et al.*, 2006). Carbohydrate transportation occurs via permeases belonging to four classes: ATP-binding cassette (ABC)-type transport systems, a glucose-specific phosphotransferase transport system (PTS), MFS (major facilitator superfamily) systems, and the glycoside-pentoside-hexuronide (GPH) cation symporter family (Parche *et al.*, 2007) (Fig. 6). All these systems present different level of sugar specificity. Bifidobacterial genomes possess a large array of genes for the utilization of complex carbohydrates. Host-indigestible complex polysaccharides are highly available nutrients in the lower gut and the capability to digest these molecules provides bifidobacteria with an ecological advantage in that specific niche (Hooper *et al.*, 2002; Schell *et al.*, 2002; Klijn *et al.*, 2005; Parche *et al.*, 2007; Turroni *et al.*, 2008). Among bifidobacterial strains a great variability in carbohydrate and alcohol utilization has been reported. Several glycosyl hydrolases are still uncharacterized, but physiological data show that bifidobacteria can metabolize a wide spectrum of substrates: galactooligosaccharides (GOS), xylooligosaccharides (XOS), pectin, fructooligosaccharides (FOS), plant oligosaccharides and mucin (Kaplan and Hutkins, 2000; Van Laere *et al.*, 2000; Slováková *et al.*, 2002; Palframan *et al.*, 2003; Ruas-Madiedo *et al.*, 2008; Turroni *et al.*, 2010a; Pokusaeva *et al.*, 2011). Glycosyl hydrolases have also been found extracellularly, probably for digestion of plant cell wall material and extracellular polysaccharides to smaller sugars (Schell *et al.*, 2002; Pokusaeva *et al.*, 2011).

Recently, the capability of one bifidobacterial species to metabolize human intestinal mucus has been demonstrated, providing some insights on the protein expression and metabolic changes of this microorganism in response to different environmental conditions (Ruiz *et al.*, 2011).

Genes coding for proteins involved in complex polysaccharide utilization are organized in clusters, and their expression may be regulated by LacI-type repressors, suggesting a substrate-dependent repression (Rodionov *et al.*, 2001; Schell *et al.*, 2002; Rodionov, 2007; Lee *et al.*, 2008; Sela *et al.*, 2008; Nentwich *et al.*, 2009). This negative regulation of gene expression could facilitate a rapid response to fluctuation in nutrient availability and variety. Some bifidobacterial strains have the capability to degrade several host-derived glycans, among which syalic acid containing carbohydrates present in mucin, glycosphingolipids and human milk (Human Milk Oligosaccharides, HMOs), highlighting the strong symbiotic relationship between the host and this microbial group (Hoskins *et al.*, 1985; Corfield *et al.*, 1992; Wang *et al.*, 2001). Polysaccharides metabolism specialization is strongly linked with the host type. *B. longum* subsp. *longum* possesses a large arsenal of enzymes specialized to degrade plant-derived dietary fibers, such as xylan, arabinan and arabinofuran, highly present in the intestine of an adult human host. On the contrary, *B. longum* subsp. *infantis* has genes predicted to be involved in HMOs degradation and this is in accordance to the presence of this subspecies mainly in breast-fed infant gut. In addition to specific carbohydrate degradation, *B. lonugm* subsp. *infantis* also contains a complete urease gene cluster that enables it to utilize urea present in human milk for its nitrogen needs, differently

from the other species that lack this cluster (Lee and O'Sullivan, 2010). It could be speculated that the genome of these two microorganisms encodes peculiar metabolic features that have been tailored to the nutrient availability of that specific gut environment.

An interesting metabolite produced by a bifidobacterial strain is lantibiotic, encoded by a characteristic operon (Lee *et al.*, 2008). Lantibiotics are a broad-spectrum class of bacteriocins, small antimicrobial peptides which inhibit the same or closely related species, giving that microorganism a competitive advantage in the ecosystem (Klaenhammer, 1993; Chen and Hoover, 2003). Bioinformatics analysis of the available genomes has shown the presence of homologues remnants of the lantibiotic operon in different species, hints of a diffuse production in bifidobacteria.

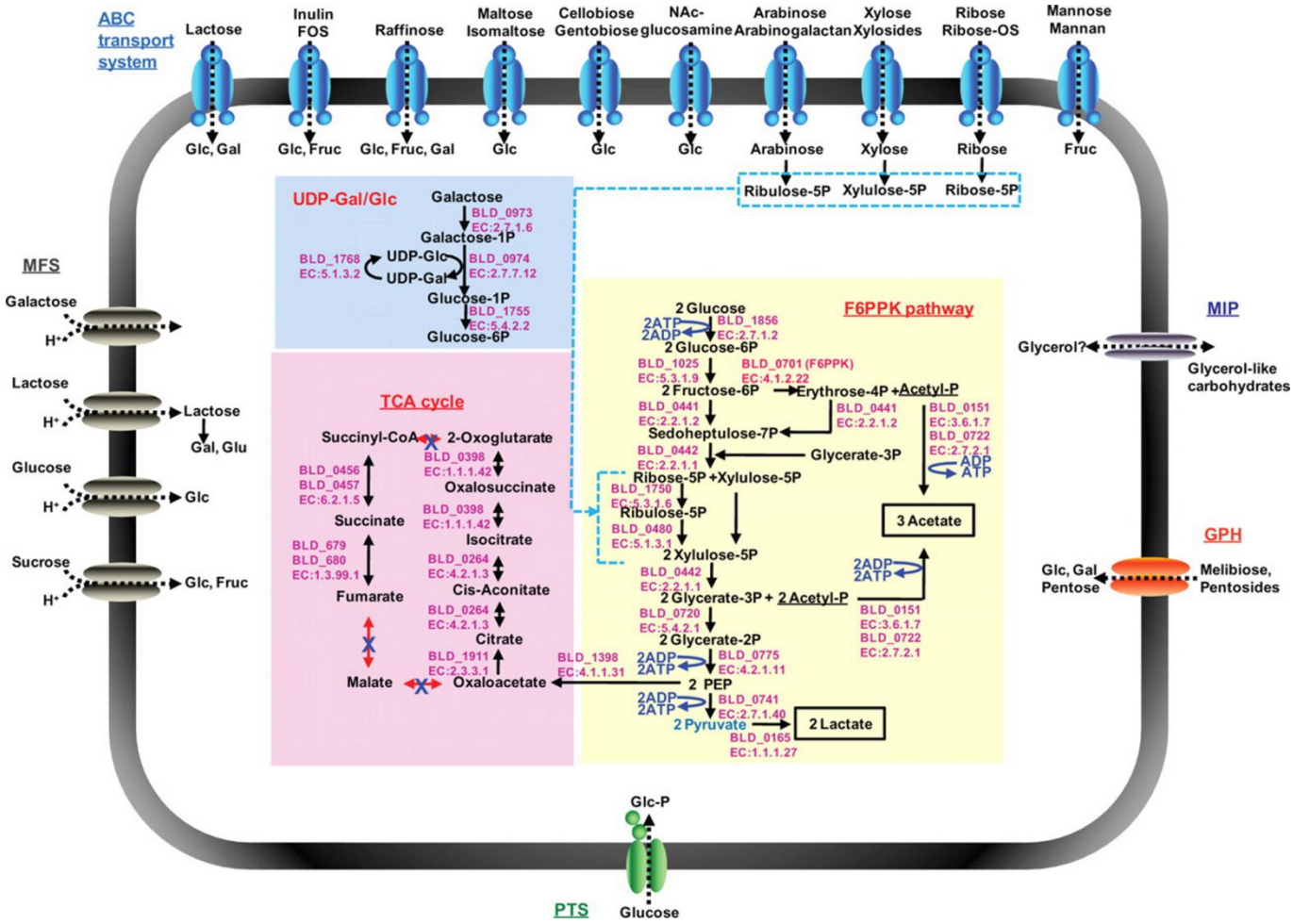


Fig. 6. Overview of predicted carbohydrate uptake and metabolism systems in bifidobacteria. The fructose-6-phosphate phosphoketolase (F6PPK) pathway, partial tricarboxylic acid (TCA) cycle, and UDP-glucose/galactose system (UDP-Gal/Glc) are indicated with different background colors (light yellow for F6PPK, light purple for the partial TCA cycle, and sky blue for the UDP-Glc/Gal system). Blue, ATP-binding cassette (ABC)-type transport systems; green, phosphotransferase transport system (PTS); grey, major facilitator superfamily (MFS); dark purple, major intrinsic protein (MIP) family; orange, glycoside-pentoside-hexuronide (GPH) cation symporter family. Genes encoding predicted metabolic enzymes from *B. longum* subsp. *longum* DJO10A are indicated (Lee and O'Sullivan, 2010).

Interaction with the host

The molecular basis of the interaction between members of the *Bifidobacterium* genus and the host GIT remains mostly unclear. Bioinformatics analysis of bifidobacterial genomes has revealed a wealth of features linked to the metabolic adaptation of bifidobacteria to the gut environment. But it has also uncovered many aspects involved in the relationship with the host.

Bifidobacteria are predicted to encode cell wall associated structures that may play an important role in the establishment and maintaining of the interaction with the host. The bioinformatics analysis of the first sequenced bifidobacterial genome (*B. longum* subsp. *longum* NCC2705) allowed the identification of more than 200 extracellular proteins, including 59 surface-associated lipoproteins and 26 solute-binding proteins of ABC transport systems (Schell *et al.*, 2002). Comparative functional genome analyses have disclosed components of the bifidobacterial protein export systems: they contain Sec-dependent pathways, type I and II signal peptidases and components of signal recognition particle (SPR)-dependent pathways (Lee and O'Sullivan, 2010).

Several surface associated proteins are covalently linked to the peptidoglycan layer by sortase transpeptidases. These enzymes recognize the LPXTG motif, a signature that target the protein to the cell wall (Navarre and Schneewind, 1999). The majority of LPXTG-proteins encoded by bifidobacterial genomes are unknown, but they are potential cell surface proteins that can be involved in cell or mucus attachment (Lee and O'Sullivan, 2010). Indeed, recent studies have revealed that cell surface components play an important role in the adhesion of bifidobacteria. Structures present on the cell wall of bifidobacteria have been demonstrated to mediate adhesion to intestinal epithelial cells and this ability is strain dependent and influenced by environmental conditions, such as pH, sugar availability and bile salts (Riedel *et al.*, 2006; Guglielmetti *et al.*, 2009). Recent genomic analyses have revealed the presence of homologues of *tad* genes in bifidobacteria. *tad* (*t*ight *ad*herence) loci are involved in pilus construction and supposed to be important for adhesion to cell surface and mucus (Kachlany *et al.*, 2001; Lee and O'Sullivan, 2010). The capability of bifidobacteria to construct pili may play important roles in their attachment to mucus and intestinal epithelial cells, as recently reported for the strain *B. breve* UCC2003: the identified *tad* locus results essential for efficient host colonization of the murine intestine (O'Connell Motherway *et al.*, 2011).

Different kinds of secreted proteins have been found in *Bifidobacterium* (Sánchez *et al.*, 2008). The majority of secreted proteins are enzymes involved in different pathways, such as cell wall biosynthesis or carbohydrate degradation. Other secreted proteins may have a possible role in the interaction with the host. Recently, a serine protease inhibitor (serpin) has been characterized in different strains of *Bifidobacterium* (Ivanov *et al.*, 2006; Turroni *et al.*, 2010b). This molecule is able to inhibit several host proteases, among which neutrophil elastase that is released during the inflammatory response; its inhibition by bifidobacterial serpin could in part explain some of the immunomodulatory properties of this microorganism (Burg and Pillinger, 2001; Reeves *et al.*, 2002). The expression of serpins could also be viewed as an ecological advantage in the highly

proteolytic gut ecosystem, where the protection against exogenous proteolysis may have an important impact on the interaction of these microorganisms with the host.
An interesting study has revealed another bifidobacterial component that can have a role in the immunomodulation: the *B. animalis* subsp. *lactis* pentapeptide CHWPR has been shown to influence the expression of genes involved in inflammation and oncogenesis, with a deep impact on the GIT physiology (Mitsuma *et al.*, 2008).
Another important actor that may have a role in GIT-bifidobacteria interaction is exopolysaccharide (EPS). Bifidobacteria contain clusters carrying genes predicted to be involved in EPS biosynthesis and extracellular polysaccharides from several strains have been characterized (Abbad Andaloussi *et al.*, 1995; Ruas-Madiedo *et al.*, 2007; Audy *et al.*, 2010; Lee and O'Sullivan, 2010). Bifidobacterial EPS can have a great variety of roles: adhesion to host cells, self-protection against toxic compounds such as bile salts, fermentable carbohydrate source for other gut microorganisms, counteraction of bacterial toxin cytotoxic effects on eukaryotic cells (Pérez *et al.*, 1998; Ruas-Madiedo *et al.*, 2006, 2009, 2010; Salazar *et al.*, 2008).
Colonization of the GIT with different microbial species determines a diverse host response. An interesting study has investigated the consequences of co-colonization of mice with two components of the human gut microbiota, *Bacteroides thetaiotaomicron* and *Bifidobacterium longum* subsp. *longum*. It has been evidenced a differential immune host response towards the two bacteria, suggesting that host responses may affect bifidobacteria survival and have an impact on the composition of the gut microbiota. (Sonnenburg *et al.*, 2006).

Mobile genetic elements, prophages, restriction-modification systems, plasmids

Transposable elements are mobile genetic units that shift from one position to another by a process of transposition; they can be grouped in two classes: insertion sequences (IS) and transposons. Eight IS families have been found in bifidobacterial genomes. *B. longum* subsp. *infantis* contains the greatest number and variability of IS elements, displaying all the eight families, while *B. dentium* possess the smallest set of these elements (Lee and O'Sullivan, 2010). IS elements are often involved in genome rearrangement or deletion events, supporting the role of these elements in genome plasticity and rapid genome adaptation to novel environments (Darling *et al.*, 2008; Lee *et al.*, 2008; Bickhart *et al.*, 2009; Ooka *et al.*, 2009). Only one transposon has been identified among bifidobacteria, transposon Tn*5432*, present in several strains of *B. thermophilum* (van Hoek *et al.*, 2008).
Prophage-like elements have been reported in the genome of different *Bifidobacterium* species (Ventura *et al.*, 2009b). They share nucleotide and organizational homology with double-stranded DNA bacteriophages infecting high G+C Gram-positive bacteria, suggesting a common phage evolution within the ecosystem. A recent comparative analysis of these elements has highlighted that bifidobacteria are under an intense selective pressure from (pro)phages, with a deep impact on bifidobacterial population composition in the gut (Ventura *et al.*, 2009b). An interesting

characteristic of these prophage-like elements is their integration site: they commonly integrate into a $tRNA^{met}$ gene, and this integration site is well conserved among bifidobacteria (Ventura *et al.*, 2005a, 2009b). Genomic analysis allowed the detection of CRISPR structures in several bifidobacteria (CRISPRs: clustered regularly interspaced short palindrome repeats) (Lee and O'Sullivan, 2010). CRISPRs have been demonstrated to be involved in acquiring resistance against infecting bacteriophages, protecting the microorganism from the infection (Barrangou *et al.*, 2007; Deveau *et al.*, 2008). The presence of these structures in bifidobacteria can be due to the need of protection from bacteriophages present in the complex intestinal environment (Ventura *et al.*, 2009b).

Genes for restriction-modification systems coding for methyltransferases and restriction enzymes have been found in bifidobacterial genomes (Schell *et al.*, 2002; Lee *et al.*, 2008; O'Connell Motherway *et al.*, 2009; Yasui *et al.*, 2009). These systems have an essential role in protection of methylated host DNA against invasion of unmethylated foreign DNA (Price and Bickle, 1986). These protection systems represent the major obstacle that inhibits the introduction of foreign DNA into bifidobacteria; recently, some studies have demonstrated the possibility to overcome this problem by the methylation of the shuttle vector and the removal of restriction sites (O'Connell Motherway *et al.*, 2009; Yasui *et al.*, 2009; Kim *et al.*, 2010).

Plasmids are not frequently found among members of the genus *Bifidobacterium*, and approximately 20% of the isolated bifidobacterial strains contains detectable plasmids (Sgorbati *et al.*, 1982). The majority of the currently sequenced bifidobacterial plasmids have been isolated from *B. longum* strains, but they are present also in other species: *B. pseudolongum*, *B. indicum*, *B. asteroides*, *B. breve*, *B. bifidum*, *B. catenulatum* and *B. pseudocatenulatum* (Sgorbati *et al.*, 1986a, 1986b; Iwata and Morishita, 1989; Gibbs *et al.*, 2006; Alvarez-Martín *et al.*, 2007; Shkoporov *et al.*, 2008). These plasmids vary in size from 1.8 kb to 10.2 kb and the majority of them replicate via a rolling circle mechanism, whereas the others have theta replication features (Corneau *et al.*, 2004; Lee and O'Sullivan, 2006, 2010; Alvarez-Martín *et al.*, 2007; Moon *et al.*, 2009). On the basis of a comparative analysis of the bifidobacterial plasmid replication proteins, these plasmids can be clustered into five different groups (Corneau *et al.*, 2004; Guglielmetti *et al.*, 2007). The G+C content of the sequenced bifidobacterial plasmids is quite different from that of the host chromosome, suggesting a recent and non-bifidobacterial origin of these extrachromosomal elements (Lee and O'Sullivan, 2010). All of the sequenced plasmids of *Bifidobacterium* are cryptic, but one study has reported a possible function for an unsequenced plasmid: its presence in a *B. bifidum* strain is associated with the production of bifidocin B (Yildirim *et al.*, 1999). The presence of native bifidobacterial plasmids have provided the basis for the construction of cloning and expression vector systems. The main problems encountered in the bifidobacterial genetic engineering are the low transformation efficiencies, segregational instability and limited host-range of these plasmids (Argnani *et al.*, 1996; Rossi *et al.*, 1998; Fu *et al.*, 2005; Lee and O'Sullivan, 2006; Alvarez-Martín *et al.*, 2007). Transformation efficiencies have been improved by methylating plasmids, in order to overcome bifidobacterial restriction systems (O'Connell Motherway *et al.*,

2009; Yasui *et al.*, 2009). These vectors have been successfully used in promoter-screening assays, gene expression and protein secretion (Moon *et al.*, 2005; Park *et al.*, 2005b, 2008; Klijn *et al.*, 2006; Rhim *et al.*, 2006; Cronin *et al.*, 2008). Recent studies have revealed an interesting medical application of these engineered bifidobacterial plasmids. Anaerobic bacteria have been demonstrated to localize in hypoxic regions, such as tumour areas (Nakamura *et al.*, 2002; Vassaux *et al.*, 2006), allowing an accurate targeting of these regions. Researchers have demonstrated the possibility to target bifidobacteria carrying the gene of interest, for example cytosine deaminase, precisely in the tumour region (Yazawa *et al.*, 2000, 2001; Li *et al.*, 2003; Cronin *et al.*, 2010). This opens novel scenarios for the development of the bifidobacterial genetic engineering.

Plasminogen/Plasmin System

The plasminogen/plasmin system is a proteolytic system that has a crucial role in several extracellular proteolytic processes present in mammals. It is involved in fibrinolysis, homeostasis and degradation of the extracellular matrix (ECM) and basement membrane, cellular migration, wound healing, tissue remodelling and inflammatory processes (Marsh, 1981; Dano *et al.*, 1985; Saksela and Rifkin, 1988) (Fig. 7).

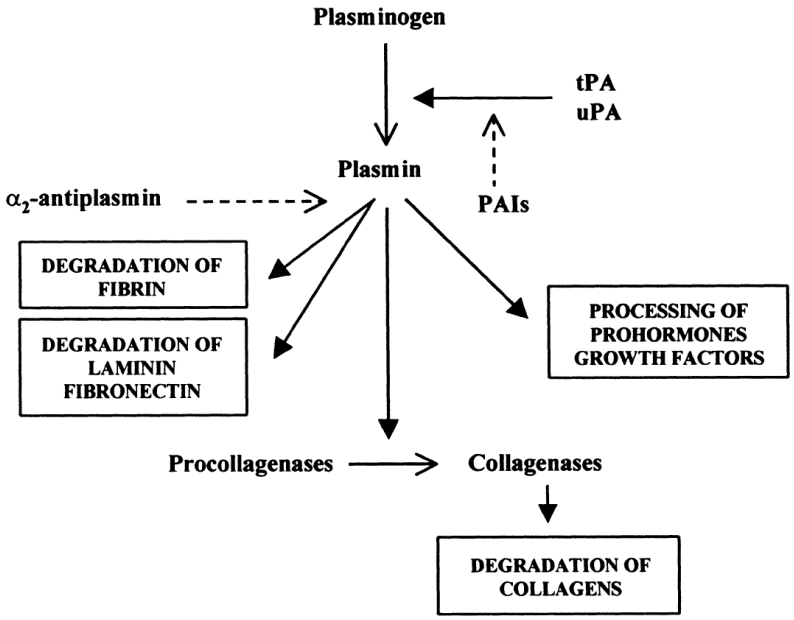


Fig. 7. The mammalian plasminogen system. The proenzyme plasminogen (Plg) is converted to the active protease plasmin by tissue-type Plg activator (tPA) or urokinase (uPA). Plasmin degrades fibrin and various ECM components, such as laminin and fibronectin, and also activates procollagenases to active collagen-degrading enzymes. Dashed arrows indicate inhibition of the enzymes: Plg activator inhibitors (PAIs) inhibit Plg activation, and α_2-antiplasmin is the main inhibitor of plasmin (Lähteenmäki *et al.*, 2001).

Key actors: plasminogen and plasmin

The key protein is plasminogen (Plg), the proenzyme form of the serine-protease plasmin. Plg is a single chain glycoprotein with a molecular mass of approximately 92 kDa; the proenzyme circulates in human plasma at concentrations around 200 µg/ml (ca. 2 µM; Miyashita *et al.*, 1988), but it is also present in interstitial fluids and milk (Myöhänen and Vaheri, 2004; Wang *et al.*, 2006). Plg is mainly synthesized by hepatocytes, but other tissue sources have been identified, including the intestine (Zhang *et al.*, 2002). Human Plg is synthesized as a 810 amino acids (aa) protein; the cleavage of the 19 aa signal peptide leads to the mature protein (791 aa) with an N-terminal glutamic acid residue (Glu-Plg) (Fig. 8). Glu-Plg possesses a phosphorylation site [Ser_{578} (Wang *et al.*, 1997)] and two glycosylation sites [Thr_{346}, Asn_{289} (Hayes and Castellino, 1979a, 1979b, 1979c)], but two Plg glycoforms can be distinguished: type I, glycosylated at both sites, and type II, with only O-glycosylation. Besides Glu-Plg, other Plg isoforms are present originating from enzymatic hydrolysis of specific peptide bonds: Lys-Plg [84 kDa, 713-714 aa (Mori *et al.*, 1995)] and Val-Plg or mini-Plg [38 kDa, 344 aa (Folkman, 1995)]. Plasmin formation from these truncated forms is faster respect to Glu-Plg.

The conversion of Plg into the active enzyme plasmin involves the proteolytic cleavage of the Arg_{561}-Val_{562} peptide bond, resulting in an N-terminal heavy chain and a C-terminal light chain, linked by two disulphide bonds (Cys_{548}-Cys_{666}; Cys_{558}-Cys_{566}). The heavy chain possesses the N-terminal activation peptide (residues 1-77, approx. 8kDa), followed by 5 consecutive kringle domains (K_1-K_5); the light chain harbours the serine-protease domain (25 kDa) containing the catalytic triad His_{603}-Asp_{646}-Ser_{741} (Vassalli *et al.*, 1991; Ponting *et al.*, 1992).

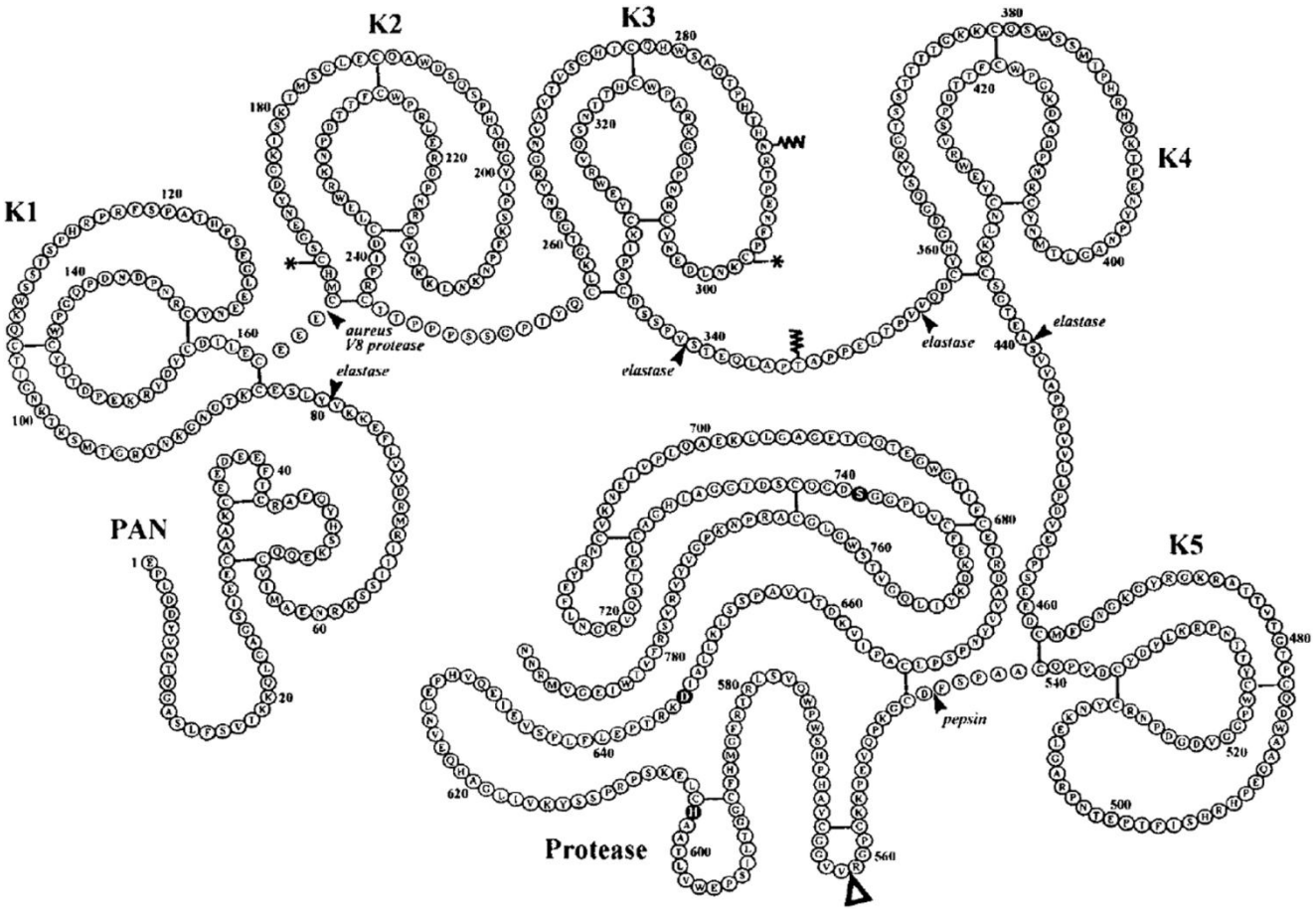


Fig. 8. Structure of human plasminogen. Plasminogen is the zymogen form of plasmin, a fibrinolytic serine protease. It consists of an N-terminal activation peptide (PAN), five conserved kringle domains (K1–K5), as well as the protease domain. The triangle indicates where plasminogen activators (urokinase [uPA] and tissue-type plasminogen activator [tPA]), cleave plasminogen to yield the active serine protease plasmin (Soff, 2000).

Kringle domains are structures composed of around 80 amino acidic residues (65 kDa), folded in a triple loop stabilized by 3 disulphide bridges (Sottrup-Jensen *et al.*, 1978). These domains mediate inter-molecular interactions, regulating the activity of proteins containing these domains. Many molecules possess kringle-like structures: prothrombin, Plg activators tissue-type Plg activator (tPA) and urokinase (uPA), angiostatin [it has the first four kringles of Plg (O'Reilly *et al.*, 1994)], apolipoprotein A (it contains more than 40 kringles), hepatocyte growth factor (HGF) and HGF-like proteins, defensins, coagulation factor XII (Ponting *et al.*, 1992; Bork *et al.*, 1996). Several studies

show that kringles interact with ligands by means of lysine binding sites [LBS (Ponting *et al.*, 1992)]. Regading Plg/plasmin, kringles 1, 4 and 5 exhibit the highest affinity to lysine containing ligands, whereas kringle 2 possesses the weakest affinity (Marti *et al.*, 1997).
The main target of plasmin proteolytic activity is fibrin, a protein derived from fibrinogen that constitutes clot structure. Besides this molecule, plasmin recognizes many substrates, among which there are vitronectin, fibronectn and laminin, the most important components of basement membrane and ECM (Plow *et al.*, 1995). Plasmin can also activate other proteolytic enzymes, such as matrix metalloproteinases and macrophage latent elastases, that are responsible of the degradation of collagen, elastin and proteoglycans (Murphy *et al.*, 1999).

Regulating the plasminogen/plasmin system: activators, inhibitors, receptors

The Plg/plasmin proteolytic system is tightly regulated in human tissues because Plg is highly abundant in the human organism and plasmin has a wide substrate spectrum.
Plasmin activation is mediated by Plasminogen Activators (PAs). PAs physiologically present in mammals are tPA and uPA (Castellino and Powell, 1981): both are secreted serin-proteases activated by proteolytic cleavage. tPA is mostly synthesized by vascular endothelial cells and contains a finger domain that binds fibrin (homologous to the domain present in type-1 fibronectin), a growth factor like domain (GFD), two kringle domains and the catalytic domain; this activator possesses high affinity to Plg only in the presence of fibrin, allowing plasmin formation directly on the clot. uPA has a structure similar to tPA: it possesses GDF domain, one kringle and the protease domain. Urokinase acts in different situations and cellular localizations: it binds to a specific receptor present on the cellular membrane (uPA receptor, uPAR) and this binding enhances uPA activity and protects it from PA inhibitors and plasmin. Because of these different binding properties, these two activators act in different processes: uPA is mainly involved in Plg activation during eukaryotic cell migration processes, whereas tPA has a primary role in fibrinolysis (Lijnen and Collen, 1995; Plow *et al.*, 1999).
Besides activators, several specific inhibitors, belonging to the serpin group, regulate the Plg/plasmin system. These molecules form a stable complex in the active site, blocking the catalytic activity of the enzyme. The main plasmin inhibitor is α_2-antiplasmin (α_2-AP): it binds kringle domains and blocks the active site, inhibiting efficiently soluble plasmin. Another important inhibitor highly present in human plasma is α_2-macroglobulin (α_2-M), which is less active respect to α_2-AP (Miyashita *et al.*, 1988). PA inhibitors (PAI1-3) negatively regulate plasmin activity in an indirect way, blocking PAs activity (Saksela and Rifkin, 1988; Rijken, 1995).
Other actors involved in the regulation of the Plg/plasmin system are Plg receptors: these molecules are characterized by low Plg affinity, high density and ubiquitous distribution. Indeed, these receptors are displayed on the surface of several mammals cells and recognize Plg kringle domains (Hajjar, 1991; Miles *et al.*, 1991; Redlitz and Plow, 1995). Plg receptors can be proteins with C-terminal lysine residues (α-enolase) or lysine-rich proteins (anfoterin), and even non

proteic receptors (gangliosides and glycosaminoglycans); the molecular identity of these receptors is specific to the cell type (Plow *et al.*, 1995; Miles *et al.*, 2005). Plg receptors exert a key control on plasmin activity, localizing proteolytic activity on the cell surface, enhancing Plg activation and plasmin activity and protecting cell-bound plasmin from inactivation. Plg binding to the receptor alters its conformation, facilitating its activation by PAs and protecting the active protease from α_2-AP inhibitor (Wiman *et al.*, 1979; Mangel *et al.*, 1990). Modulation of Plg receptors is a further aspect of plasmin activity regulation: fluctuation in receptor presence on the cell surface may act as a control mechanism of many cellular Plg/plasmin-dependent responses (Herren *et al.*, 2003).

Plasminogen in the gut

Gut epithelium constitutes an efficient selective barrier between lumen and the underlying tissues. The epithelial cells form a tight monolayer since junctional complexes allow adhesion between cells and between cells and basement membrane. However, enterocytes are continuously replaced by a rapid cell turnover that starts from stem cells located at the crypts basis: adhesion sites must be disrupted and reorganized in a controlled manner and this process involves the Plg/plasmin system (Gibson *et al.*, 1998). The uPA-uPAR system allows the cell surface localization of the proteolytic activity of plasmin, thus enabling intercellular junctions disruption by diminishing cell-cell and cell-basement membrane adhesion and favouring the turnover process. Components of the Plg/plasmin system have been individuated in different compartments of the gut. Zhang *et al.* (2002) have shown that Plg and tPA genes are expressed at low level in the gut; colon epithelial cells secrete uPA and express uPAR (Gibson *et al.*, 1991). uPA is highly expressed on the epithelial surface of the colon and the upper part of villi in the small intestine, in differentiated cells involved in absorption processes. These cells are weakly bound to the basement membrane and subjected to a continuous turnover; after apoptosis the cells are dispersed in the intestinal lumen or phagocytized by subepithelial macrophages. There are evidences that this system has an important role in the cell turnover of the epithelium present in other districts in addition to gut, such as skin and cornea (Lazarus and Jensen, 1991; Morimoto *et al.*, 1993).

Plg/plasmin system is involved not only in physiological but also in pathological processes. Plg dependent extracellular proteolysis plays a crucial role in tumor cell invasiveness. Controlled degradation of components of basement membrane and ECM allows tumor cells to invade contiguous tissues and generate metastases. This process is tightly regulated by cytokines and growth factors present in the surrounding environment and requires the overexpression of proteolytic systems components, one of which is the Plg/plasmin system (Berger, 2002; Saucy *et al.*, 2011). Tumor cells display plasmin and uPA receptors on the plasma membrane, focusing the proteolytic activity on their surface; moreover, they produce PAI-1 and PAI-2 in order to prevent excessive ECM degradation and facilitating adhesion/detachment processes and migration through this matrix (Tang and Wei, 2008). Besides cancer, inflammatory processes deeply affect

the host Plg/plasmin system equilibrium. In particular, pro-inflammatory cytokines elicit the up-regulation of Plg activator uPA, shifting the Plg/plasmin system balance towards active plasmin in inflamed tissues. The increased availability of active plasmin enhances the release of proinflammatory mediators and the activation of pro-forms of matrix metalloproteases (MMPs), thus amplifying the inflammatory reaction (Mondino and Blasi, 2004; Okumura *et al.*, 2008).

Microorganisms and Human Plasminogen Interaction

A great variety of eukaryotic and prokaryotic organisms interact with components of the Plg/plasmin system. Most of these organisms are pathogens, taking advantage of the proteolytic activity of this system in order to facilitate invasion and establishment in the host. Table 2 shows a list of these organisms, both pathogens and commensals.

Table 2 - Plg interaction with prokaryotic and eukaryotic organisms

	Organisms	**References**
Bacteria	*Aeromonas hydrophila*	Sha *et al.*, 2009
	Bacillus anthracis	Agarwal *et al.*, 2008; Matta *et al.*, 2010
	Bacteroides fragilis	Sijbrandi *et al.*, 2005, 2008; Ferreira Ede *et al.*, 2009
	Bifidobacterium animalis subsp. *lactis*; *B. longum*; *B. bifidum*; *B. breve*	Candela *et al.*, 2007, 2009
	Borrelia burgdorferi; *B. coriaceae*; *B. garinii*; *B. parkeri*; *B. anserina*; *B. turicatae*; *B. hermsii*; *B. crocidurae*	Fuchs *et al.*, 1994; Klempner *et al.*, 1996; Hu *et al.*, 1997; Nordstrand *et al.*, 2001; Floden *et al.*, 2011
	Branhamella catarrhalis	Ullberg *et al.*, 1990
	Escherichia coli	Kukkonen *et al.*, 1998; Lähteenmäki *et al.*, 2001
	Francisella tularensis	Clinton *et al.*, 2010
	Fusobacterium nucleatum subsp. *nucleatum*; subsp. *vincentii*; subsp. *polymorphum*	Darenfed *et al.*, 1999
	Haemophilus influenzae	Ullberg *et al.*, 1990; Barthel *et al.*, 2011
	Helicobacter pylori	Dubreuil *et al.*, 2002; Jönsson *et al.*, 2004
	Lactobacillus crispatus; *L. johnsonii*	Antikainen *et al.*, 2007a, 2007b; Hurmalainen *et al.*, 2007
	Legionella pneumophila	Vranckx *et al.*, 2007
	Leptospira interrogans; *L. borgpetersenii*; *L. kirshneri*; *L. santarosai*; *L. biflexa*	Vieira *et al.*, 2009, 2010; Oliveira *et al.*, 2011
	Listeria monocytogenes	Schaumburg *et al.*, 2004
	Mycobacterium tuberculosis	Monroy *et al.*, 2000; Xolalpa *et al.*, 2007
	Mycoplasma fermentans; *M. gallisepticum*	Yavlovich *et al.*, 2001, 2007; Chen *et al.*, 2011
	Neisseria meningitidis; *N. gonorrhoeae*	Ullberg *et al.*, 1992; Knaust *et al.*, 2007
	Peptostreptococcus micros	Grenier and Bouclin, 2006
	Porphyromonas gingivalis	Grenier, 1996
	Proteus mirabilis	Ullberg *et al.*, 1990; D'Alessandro *et al.*, 2011
	Pseudomonas aeruginosa	Ullberg *et al.*, 1990; da Silva *et al.*, 2004; Kunert *et al.*, 2007
	Salmonella enterica	Kukkonen *et al.*, 1998; Lähteenmäki *et al.*, 2001; Kukkonen and Korhonen, 2004
	Staphylococcus aureus	Lähteenmäki *et al.*, 2001; Mölkänen *et al.*, 2002

	group A, C and G streptococci; *Streptococcus suis*; *S. mutans*; *S. pneumoniae*; *S. agalactiae*; *S. anginosus*, *S. oralis*; *S. mitis*; *S. salivarius*; *S. canis*	Pancholi and Fischetti, 1998; Lähteenmäki *et al.*, 2001; Jobin *et al.*, 2004; Urdaneta *et al.*, 2004; Bergmann and Hammerschmidt, 2007; Magalhães *et al.*, 2007; Kinnby *et al.*, 2008; Itzek *et al.*, 2010; Fulde *et al.*, 2011
	Treponema denticola	Fenno *et al.*, 2000
	Yersinia pestis	Sodeinde *et al.*, 1992; Lathem *et al.*, 2007
Yeasts/moulds	*Aspergillus fumigatus*	Behnsen *et al.*, 2008; Zaas *et al.*, 2008
	Candida albicans	Crowe *et al.*, 2003; Jong *et al.*, 2003; Poltermann *et al.*, 2007; Luo *et al.*, 2009
	Cryptococcus neoformans	Stie *et al.*, 2009
	Paracoccidioies brasiliensis	Barbosa *et al.*, 2006; Nogueira *et al.*, 2010
	Pneumocystis jiroveci (*carinii*)	Fox and Smulian, 2001
Protozoa	*Leishmania mexicana*	Calcagno *et al.*, 2002; Vanegas *et al.*, 2007
	Plasmodium falciparum; *P. berghei*	Ghosh *et al.*, 2011
	Trichomonas vaginalis	Mundodi *et al.*, 2008
	Trypanosoma cruzi	Almeida *et al.*, 2004; Rojas *et al.*, 2008
Worms	*Clonorchis sinensis*	Wang *et al.*, 2011a
	Dirofilaria immitis	González-Miguel *et al.*, 2012
	Echinostoma caproni	Marcilla *et al.*, 2007
	Fasciola hepatica	Bernal *et al.*, 2004
	Onchocerca volvulus	Jolodar *et al.*, 2003
	Schistosoma bovis	Ramajo-Hernández *et al.*, 2007; de la Torre-Escudero *et al.*, 2010

Bacterial pathogens and the plasminogen/plasmin system

Several pathogenic bacteria have developed different strategies in order to make use of the high Plg concentration in body fluids and the broad spectrum of plasmin substrates during the host colonization process (Sun *et al*., 2004; Bergmann *et al.*, 2005; Lähteenmäki *et al.*, 2005; Bergmann and Hammerschmidt, 2007) (Fig. 9). Few bacterial species directly activate Plg by producing PAs, but a considerably higher amount of pathogens have been found to bind Plg on their surface *via* Plg surface receptors, favouring plasmin conversion by host PAs. In this way, microorganisms acquire a broad spectrum surface associated proteolytic activity: this characteristic favours migration through tissue physical barriers and responds to the microorganism nutritional demands during the invasion and colonization processes. Indeed, *in vitro* studies have shown that plasmin immobilized on different pathogens ([e.g.] *Yersinia pestis*, *Borrelia burgdorferi*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Salmonella enterica*) degrades mammals ECM, allowing them to penetrate through basement membrane and epithelial cell monolayers, a process described as "bacterial metastasis" (Lähteenmäki *et al.*, 2005). Nevertheless, direct involvement of Plg activation in pathogenesis *in vivo* has been reported only for some of these microorganisms

(*Yersinia pestis*, *Borrelia burgdorferi* and species belonging to group A streptococci, or GAS) (Lähteenmäki *et al.*, 2005; Bergmann and Hammerschmidt, 2007).

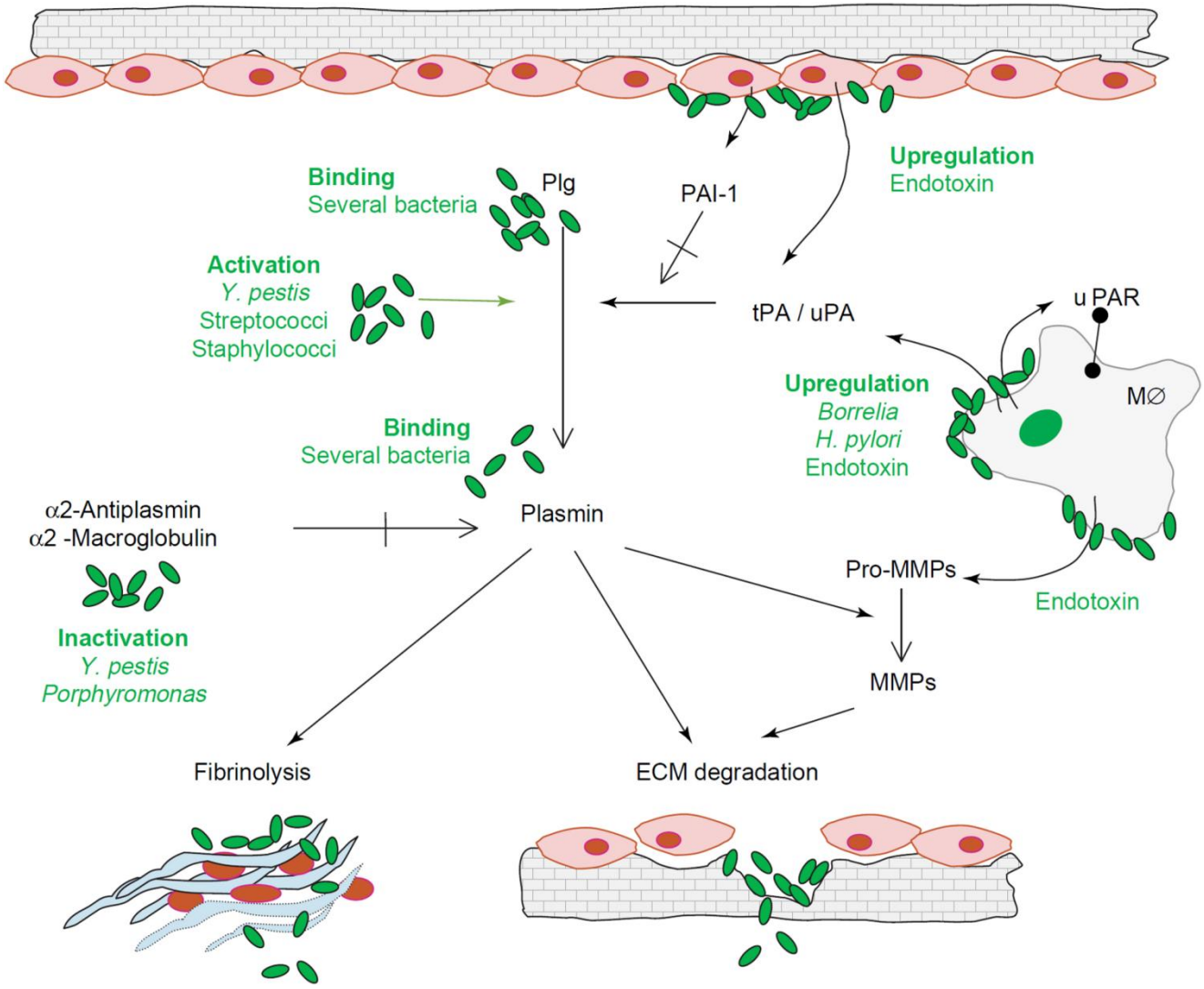


Fig. 9. Overview of the mammalian plasminogen (Plg) system and its control and how pathogenic bacteria engage the system. tPA: tissue-type plasminogen activator; uPA: urokinase; PAI-1: plasminogen activator inhibitor 1; uPAR: uPA receptor; ECM: extracellular matrix; Pro-MMPs: precursors of matrix metalloproteases; MMPs: matrix metalloproteases; MΦ: macrophages (Lähteenmäki *et al.*, 2005).

Bacterial plasminogen activators

Bacterial proteins that function as PAs can be divided in two groups: enzymes that resemble mammalian PAs in functions, such as Pla in *Yersinia pestis* or *Salmonella enterica* PgtE, and proteins that are not enzyme themselves but form 1:1 complexes with Plg and plasmin, as streptokinase (SK) in *Streptococcus* (A, C and G groups) and staphylokinase (SAK) in *Staphylococcus aureus* (Lähteenmäki *et al.*, 2001).

The surface protease Pla of the plague bacterium *Y. pestis* is a highly efficient Plg activator. Pla cleaves Plg at the same peptide bond as the host PAs tPA and uPA; it also acts as adhesin, with affinity for ECM and basement membrane laminin. Studies with mice infected with Pla-knock out *Y. pestis* strains have shown the importance of this protein in the pathogen migration from the primary intradermal infection site into blood circulation. Pla belongs to the family of omptins, a class of surface adhesins/proteases found in the enterobacterial outer membrane; these proteins share a common β-barrel fold (Sodeinde *et al.*, 1992; Lathem *et al.*, 2007). *Salmonella enterica* PgtE omptin activates Plg *in vitro*, but the role of Plg activation in salmonellosis pathogenesis remains unclear (Kukkonen and Korhonen, 2004). Another omptin with a Plg activation function is Lpa, found on the outer membrane of the pathogen *Legionella pneumophila* (Vranckx *et al.*, 2007). The periodontal pathogen *Porphyromonas gingivalis* possesses an 80-kDa trypsin-like protease that activates Plg and inactivates plasmin inhibitors α_2-AP and α_2-M, leading to uncontrolled plasmin activation (Grenier, 1996).

SK and SAK proteins are secreted bacterial PAs that form a 1:1 complex with Plg, leading to changes in conformation that promote Plg activation to plasmin. There are some differences in Plg activation mediated by these two proteins. SK acts directly as a PA: the formation of the SK-Plg complex induces a conformational change in Plg structure, leading to its conversion to plasmin. Differently from SK, SAK-Plg complex is enzymatically inactive and requires conversion of Plg to plasmin; only the SAK-plasmin complex acts as a PA and activates preferably fibrin-bound Plg (Lähteenmäki *et al.*, 2001). SK has been largely studied in several GAS species; Sun *et al*. (2004) have underlined the importance of plasmin in the pathogensis of *Streptococcus* infections. In fact, without plasmin activity, local microvascular occlusion caused by fibrin clots formation represents a host defence mechanism against GAS. SK activated plasmin mediates fibrin degradation and allows invasive microorganisms to cross this barrier, favouring bacterial dissemination.

Bacterial plasminogen receptors and "moonlighting proteins"

Bacterial Plg receptors (PlgR) are surface structures used by bacteria to immobilize Plg and favour its conversion to plasmin by PAs. This system allows microorganisms to acquire a strong proteolytic activity localized on their external surface, cell wall or outer membrane. The main PlgRs in Gram-negative bacteria are fimbriae and flagella, filamentous appendages present on the surface of these organisms (Lähteenmäki *et al.*, 2001). In addition, some Gram-negative microorganisms harbour on their outer membrane proteins that bind host Plg. Two Plg binding proteins have been identified in the spirochete *Borrelia burgdorferi*, the etiological agent of Lyme disease: the outer surface protein A (OspA) and a 70 kDa surface protein that shows homology to the periplasmic ABC transpoter proteins of other bacterial pathogens (Fuchs *et al.*, 1994; Hu *et al.*, 1997). No endogenous PAs have been found, but bacterial bound Plg is activated to plasmin by human uPA, promoting ECM components degradation and bacterial migration through endothelial monolayers (Coleman and Benach, 1999). It has been demonstrated that *B. burgdorferi* cells and

purified OspA stimulate pro-uPA production in human monocytes, besides the expression increase and release of uPAR from monocytes (Coleman *et al.*, 2001; Coleman and Benach, 2003). Recent studies have demonstrated the presence of two Plg receptors on the surface of the pathogenic bacteria *Helicobacter pylori*: PgbA and PgbB, characterized by a high lysine content (Jönsson *et al.*, 2004). The Gram-negative bacterium *Bacteroides fragilis*, a common member of the human microbiota but also an opportunistic pathogen, can bind Plg molecules on its surface. This binding depends on the presence of specific proteins in the outer membrane, such as Pbp (putative Plg binding protein). *B. fragilis* is not capable to activate Plg to plasmin, and the role of Pbp could be only sequestering Plg and keeping it locally on the bacterial surface in an inactive form (Sijbrandi *et al.*, 2005, 2008).

In Gram-positive bacteria, Plg binding proteins have been identified on their cell wall. PlgR activity has been detected on several pathogens, and most of these molecules have other important functions, such as adhesion, movement, enzymatic activity, nutrient uptake, or interaction with immune system. Moreover, a single bacterial species can express different PlgR kinds (Lähteenmäki *et al.*, 2001).

GAS express different surface Plg binding proteins; one with the highest affinity is the Plasminogen binding group A streptococcal M-protein, PAM. M-proteins are antiphagocytic fibrillar, helical surface proteins containing numerous repeated regions (Berge and Sjobring, 1993). Plg interaction is mediated by two 13-aa repeated sequences (a1 and a2) containing a central lysine residue, localized to the N-terminal portion on the exposed surface of PAM. The substitution of the lysine residue with alanine residue in the a1 sequence has been demonstrated to decrease Plg binding of 80% (Ringdahl and Sjobring, 2000). Recently, other surface proteins that bind Plg with high affinity have been identified in *Streptococcus*: PAM-related protein (Prp), belonging to the M-protein family (Sanderson-Smith *et al.*, 2007), and choline-binding protein E (CBPE), member of the surface exposed choline-binding protein family (Attali *et al.*, 2008b).

Recently, some cytoplasmic proteins have been identified as bacterial PlgRs. These proteins have essential roles in bacterial metabolism and growth and are usually localized in the cytoplasm, but they have been found to be exposed on the bacterial cell surface, even if they lack any exporting and anchoring signal. These molecules have been described as “moonlighting proteins” because of their ability to have more than one job or, in a scientific language, to harbour more than one biological function, and this multitasking characteristic is embedded within a single polypeptide chain (Jeffery, 1999, 2009). The secretion and anchoring mechanisms of these moonlighting proteins are still unclear. Moonlighting proteins perform multiple independent and often unrelated activities without partitioning these functions into different domains. In a recent review, it is stated that the term “moonlighting” can describe two different phenomena. The first is the presence of a second, third, etc. “biochemical” site(s) on the protein enabling additional biological activities; but these new functions may be exerted only when the moonlighting protein is in a location different from the one normally occupied. Thus, beside a biochemical moonlighting there is also a “geographical” moonlighting, and both may be required to the exhibition of a true

moonlighting activity (Henderson and Martin, 2011). The presence of moonlighting functions in several proteins is not typical of bacteria, but it is a widespread phenomenon present in all the kingdoms of life (Huberts and van der Klei, 2010). Focusing on bacteria, many of the principal moonlighting proteins can be gathered in two main groups: metabolic enzymes, among which components of the glycolytic pathway are the most represented, and housekeeping proteins, specifically molecular chaperones such as heat shock proteins (HSPs).

Some glycolytic enzymes have been found interacting in a specific manner with Plg in different microorganisms; among these proteins, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and α-enolase have been the most investigated.

GAPDH has been the first PlgR identified on GAS capable of binding Glu-Plg. Site-specific mutagenesis studies have demonstrated the importance of the C-terminal lysine residue in the interaction between GAPDH and Plg: in fact, leucine substitution of this residue leads to a Plg binding reduction. Nonetheless, cells bearing a mutation in this protein show a Plg binding similar to non mutant cells, indicating the existence of other surface PlgRs on these microorganisms (Winram and Lottenberg, 1998). GAPDH has been found in the cell wall protein fraction of *Listeria monocytogenes* and could represent another virulence factor of this pathogenic bacterium (Schaumburg *et al.*, 2004). Plg binding of this enzyme has also been found for different *Escherichia coli* pathogenic strains (Egea *et al.*, 2007).

α-enolase is an essential enzyme in glycolysis: it catalyzes the conversion of 2-phospho-D-glycerate (2-PGE) to phosphoenolpyruvate (PEP). In eukaryotic organisms the active enzyme forms a dimer, while in bacteria it is found as an octamer (Brown *et al.*, 1998; Ehinger *et al.*, 2004). α-enolase cell surface localization is very common in several prokaryotic and eukaryotic organisms (Pancholi, 2001). This enzyme is the main PlgR present on the surface of several *Streptococcus* species. Two lysine residues localized on the C-terminal of *Streptococcus pneumoniae* α-enolase (binding site 1, BS1) are important in the interaction with Plg/plasmin (Bergmann *et al.*, 2001), but removal of these two amino acids by carbossipeptidase B treatment, or their substitution with two leucine residues, partly decreases Plg binding. A second Plg binding site (BS2) has been therefore identified on the α-enolase of *S. pneumoniae* (amino acidic sequence: ^{248}FYD**KE**R**K**VY^{256}) (Bergmann *et al.*, 2003) and the crystallographic structure resolution of this protein has shown its localization on a surface exposed loop (Ehinger *et al.*, 2004). α-enolase surface localization and its Plg binding activity have been demonstrated for several prokaryotic and eukaryotic organisms, both commensals and pathogens (Table 3).

Table 3 - α-enolase - Plg interaction

	Organisms	**References**
Bacteria	*Aeromonas hydrophila*	Sha *et al.*, 2009
	Bacillus anthracis	Agarwal *et al.*, 2008
	Bifidobacterium animalis subsp. *lactis*, *B. longum*; *B. bifidum*; *B. breve*	Candela *et al.*, 2007, 2009
	Borrelia burgdorferi	Floden *et al.*, 2011; Nogueira *et al.*, 2012; Toledo *et al.*, 2012
	Lactobacillus crispatus; *L. johnsonii*	Antikainen *et al.*, 2007b
	Listeria monocytogenes	Schaumburg *et al.*, 2004
	Mycoplasma fermentans; *M. gallisepticum*	Yavlovich *et al.*, 2007; Chen *et al.*, 2011
	Neisseria meningitidis	Knaust *et al.*, 2007
	Staphylococcus aureus	Mölkänen *et al.*, 2002
	group A streptococci; *Streptococcus pneumoniae*; *S. mutans; S. suis*; *S. oralis*; *S. mitis*; *S. salivarius*	Pancholi and Fischetti, 1998; Bergmann *et al.*, 2001; Ge *et al.*, 2004; Jones and Holt, 2007; Esgleas *et al.*, 2008; Itzek *et al.*, 2010
Yeasts	*Candida albicans*	Jong *et al.*, 2003
	Paracoccidioies brasiliensis	Nogueira *et al.*, 2010
	Pneumocystis jiroveci (carinii)	Fox and Smulian, 2001
Protozoa	*Leishmania mexicana*	Vanegas *et al.*, 2007
	Plasmodium falciparum; *P. berghei*	Ghosh *et al.*, 2011
	Trichomonas vaginalis	Mundodi *et al.*, 2008
Worms	*Clonorchis sinensis*	Wang *et al.*, 2011a
	Echinostoma caproni	Marcilla *et al.*, 2007
	Fasciola hepatica	Bernal *et al.*, 2004
	Onchocerca volvulus	Jolodar *et al.*, 2003
	Schistosoma bovis	Ramajo-Hernández *et al.*, 2007; de la Torre-Escudero *et al.*, 2010

Plg binding and adhesion to ECM proteins have been usually regarded as virulence factors, involved in bacterial invasion and colonization processes. However, a study of Antikainen *et al.* (2007b) has pointed out that α-enolase molecules localized both on the cell wall of different commensal species of *Lactobacillus* (*L. crispatus* ST1, *L. johnsonii* F133) and on the surface of pathogenic bacteria *Streptococcus pneumoniae* TIGR4, *S. pyogenes* IH32030 and *Staphylococcus aureus* 8325-4 possess an equivalent Plg binding and ECM proteins adhesion activity. But, differently from *Lactobacillus*, *Streptococcus* and *Staphylococcus* express their own Plg activators: the interaction with Plg/plasmin system might therefore represent an ecologic strategy common among bacteria that inhabit different niches of the same ecosystem, leading to a different interaction outcome (commensalism or pathogenesis).

Another important moonlighting protein belonging to the HSP group is the molecular chaperone DnaK, a member of the HSP70 family. Molecular chaperones form complexes whose components

are highly conserved and present both in eukaryotes and prokaryotes. These protein complexes are involved in protein folding processes: folding and assembling of newly synthesized proteins, refolding of unfolded or aggregated proteins, cellular protein transport. Several molecular chaperone complexes have been identified; one of the most important is the HSP70 complex, homologue to the DnaK complex present in bacteria. This chaperone system allows protein folding through ATP-dependent binding and release cycles of hydrophobic segments of an unfolded polypeptide chain. DnaK is one component of this complex and shows a highly conserved structure: an N-terminal ATPase domain and a C-terminal substrate binding domain (Hartl, 1996; Bukau and Horwich, 1998; Mayer and Bukau, 2005). DnaK has been found on the surface of three pathogenic species (*Listeria monocytogenes*, *Neisseria meningitidis*, *Mycobacterium tuberculosis*) and its interaction with Plg/plasmin system has been demonstrated (Schaumburg *et al.*, 2004; Knaust *et al.*, 2007; Xolalpa *et al.*, 2007).

Interaction of *Bifidobacterium* with human plasminogen

The molecular mechanisms implicated in the interaction between *Bifidobacterium* and the host gut remain largely unclear, but recently some studies have pointed out that these microorganisms can interact with the human Plg. Indeed, these studies have shown that PlgRs are present on the surface of human GIT symbiont bacteria, such as members of *Bifidobacterium* genus. Candela *et al.* (2007) have demonstrated that four strains of three different species of *Bifidobacterium* (*B. animalis* subsp. *lactis*, *B. longum* and *B. bifidum*) possess a dose-dependent Plg binding activity. A proteomic analysis of the cell wall protein fraction of *B. animalis* subsp. *lactis* BI07, a probiotic strain used in the pharmaceutical preparation VSL#3, allowed the identification of five putative Plg binding proteins: DnaK, glutamine synthetase, α-enolase, bile salt hydrolase and phosphoglycerate mutase. All these proteins, except glutamine synthetase, have a C-terminal lysine residue, probably involved in Plg interaction. Like other Gram-positive bacteria, some of these *B. animalis* subsp. *lactis* BI07 PlgRs are moonlighting proteins. A deep analysis of the extracellular proteome of the probiotic strain *B. animalis* subsp. *lactis* BB-12 allowed the identification of four proteins (choloylglycine hydrolase, glutamine synthetase, enolase, DnaK) with high homology to the PlgRs exposed on the cell wall of *B. animalis* subsp. *lactis* BI07. It could be speculated that these proteins interact with Plg, considering the high homology level between these molecules and the fact that another strain of the *B. animalis* subsp. *lactis* taxon binds Plg. The possible role of these molecules in Plg binding highlights the importance of the Plg/plasmin system in the bacteria-host interaction and colonization (Gilad *et al.*, 2011).

As reported above, α-enolase plays an important role in Plg binding in numerous microorganisms. The Plg binding activity of *B. animalis* subsp. *lactis* BI07 α-enolase has been deeply characterized, allowing the identification of two binding sites: a C-terminal lysine residue (BS1) and an internal binding site (BS2). Like *Streptococcus pneumoniae* α-enolase, Plg binding mainly depends on the internal binding site (Candela *et al.*, 2009).

Enzymatic activity assays have shown that Plg bound on the *B. animalis* subsp. *lactis* BI07 outer surface is activated to plasmin by host PAs: in this way, the microorganism acquires a surface-associated proteolytic activity useful for ECM components degradation (Candela *et al.*, 2008b). It is worth noting that *B. animalis* subsp. *lactis* BI07 does not express endogenous PAs and it does not possess any surface proteolytic activity in the absence of Plg.

In the human GIT, pathogen and symbiont bacteria compete each other to colonize the same ecological niche. As reported by Ochman and Moran (2001), the biological process of host colonization is very similar for pathogens and symbionts, independently from the final outcome. It might be hypothesized that these microorganisms share some molecular mechanisms useful to establish and maintain their relationship with the host: the interaction with the host Plg/plasmin system can be one of these strategies. The result of the interaction (pathogenesis or commensalism) shall therefore be determined by other microbial characteristics, such as pathogenicity islands. In addition, an important aspect to consider is the effect of the microenvironment on the biology of the microorganisms. Different environmental conditions affect protein expression and the global response of the microorganism to that specific situation, shaping the outcome of the interaction with the host. As an example, in the GIT the presence of bile salts can have an important impact on the interaction between bacteria and the host. Recent studies have highlighted the capability of different *Bifidobacterium* species to adapt to bile salts, leading to a large-scale change in protein expression profile (Sánchez *et al.*, 2007b; Ruiz *et al.*, 2009). This change could affect the mode of colonization of *Bifidobacterium*, with consequences for the microorganism-host interaction.

The host colonization and the establishment of a microorganism in a particular niche are extremely intricate processes, involving the host genetics, the microorganism genetics and its capability to respond and adapt to the different environmental conditions. The final outcome derives from the combination of all these factors, and both pathogens and commensals face the same difficulties: it could be supposed that they often follow the same path and use similar instruments to find their way and survive in that specific ecosystem.

PROJECT OUTLINE

Among the components of the human gastrointestinal microbiota, the genus *Bifidobacterium* represents one of the main health promoting groups, affecting several host physiological features (Lee and O'Sullivan, 2010; Cronin *et al.*, 2011). Despite the presence of bifidobacteria in the human gut has been related to numerous health-promoting activities, there is a lack of information about the molecular mechanisms at the basis of the interaction between these probiotic microorganisms and the host. Proteins present on the microorganism surface may play an important role in the interaction with the host since they can intervene with host molecules and components present in the host environment. Recently, the interaction of bifidobacterial surface proteins and a host protein present in the gastrointestinal environment, plasminogen (Plg), has been reported (Candela *et al.,* 2007). Plg is the monomeric proenzyme of plasmin, a trypsin-like serine protease with a broad substrate specificity.

The studies presented here aim to shed some light on the dynamics of interaction between *Bifidobacterium* and the human Plg/plasmin system, looking on this host system as a valuable tool used by the microorganism for the interaction with the host, following the track of similar studies carried out on pathogens. The studies reported in this thesis start from the molecular analysis of the specific Plg binding of one bifidobacterial receptor, going on with the study of the biological significance of this interaction, examining the impact of environmental variables on the Plg binding to bifidobacterial cells and the role of Plg/plasmin system in the interplay between bacteria and host cells.

Bifidobacteria possess different Plg receptors on their cell surface, and one of these, α-enolase, a Plg receptor widely shared among bacteria, has been characterized (Candela *et al.*, 2007, 2009). DnaK represents another important Plg receptor, conserved among bifidobacteria. Here, the molecular and biochemical characterization of the bifidobacterial surface Plg receptor DnaK has been undertaken, with particular attention on the structure of this protein and its binding to human Plg.

The second aspect taken into consideration has been the environment and its variables. Factors present in the gut environment can have an important influence on the interactions that take place in the gut ecosystem. Bile salts are among the major challenging factors because of their bacterial toxicity (Begley *et al.*, 2005). In recent times, the adaptation of bifidobacterial species to bile salts has been evaluated, revealing many changes in the cell wall proteome (Ruiz *et al.*, 2009), that can likely affect the modality of interaction with the host. Considering the impact of bile salts adaptation on the *Bifidobacterium* extracellular proteome, the effect of bile salts on the Plg binding activity of *Bifidobacterium* has been evaluated.

The role of Plg/plasmin system in the biology of interaction between *Bifidobacterium* and human intestinal epithelial cells is another important aspect studied to elucidate the importance of this host system in the cross-talk between this probiotic microorganism and the host. To this aim, *Bifidobacterium* Plg-mediated early interaction with human enterocytes has been investigated, using HT29 cell line as model system for the human intestinal epithelium, being a mucus-

producing cell line derived from human colorectal adenocarcinoma (Fogh and Trempe, 1975; Rousset, 1986).

Gut environmental conditions can affect the host Plg/plasmin system equilibrium; in particular, inflammatory processes involve an up-regulation of the Plg activators expression, leading to a clear increase of plasmin availability in inflamed tissues (Mondino and Blasi, 2004; Killeen *et al.*, 2009). Taking into account changes associated with gut inflammatory processes, the impact of inflammation on the *Bifidobacterium*-Plg-host enterocytes early interaction has been evaluated, mimicking this condition stimulating HT29 cells with the pro-inflammatory cytokine TNF-α, an important mediator of gut inflammation (Ma *et al.*, 2004; Yan *et al.*, 2008).

Components of the Plg/plasmin system are present in the gastrointestinal environment (Zhang *et al.*, 2002), but until now the relevance of the interaction between *Bifidobacterium* and this system in a physiological ecosystem has not yet been elucidated. To this purpose, the capability of bifidobacteria to recruit components of the Plg/plasmin system present at physiological concentrations has been evaluated in a physiological human intestinal ecosystem, represented by crude faecal extracts. These extracts has been chosen as a model of the human gastrointestinal environment because they represent the only possibility for a non-invasive sampling of the human gastrointestinal tract content and reflect the entire content of the full length of the colon-rectum (Ang *et al.*, 2010).

The comprehension of the molecular mechanisms underlying the cross-talk between bifidobacteria and the human host and the modulation of this interaction under different environmental conditions can be important to better understand the probiotic properties of this bacterial group.

The research project presented here led to the publication of three research articles: “DnaK from *Bifidobacterium animalis* subsp. *lactis* is a surface-exposed human plasminogen receptor upregulated in response to bile salts” (Candela *et al.*, 2010, Microbiology, 156:1609-1618); “Relevance of *Bifidobacterium animalis* subsp. *lactis* plasminogen binding activity in the human gastrointestinal microenvironment” (Candela *et al.*, 2011, Applied and Environmental Microbiology, 77:7072-7076); “Tumor Necrosis Factor Alpha modulates the dynamics of the plasminogen-mediated early interaction between *Bifidobacterium animalis* subsp. *lactis* and human enterocytes” (Centanni *et al.*, 2012, Applied and Environmental Microbiology, 78:2465-2469).

CHAPTER I

MOLECULAR CHARACTERIZATION OF THE BIFIDOBACTERIAL SURFACE PLASMINOGEN RECEPTOR DNAK

Introduction

Due to its intrinsic proteolytic potential, host Plg/plasmin system represents a valuable tool used by several microorganisms to interact with the host. Plg molecule possesses an N-terminal pre-activation peptide, five consecutive kringle domains and a serine-protease domain (Ponting *et al.*, 1992). The capability to intervene with this system is a host-colonization tactic present both in pathogens and commensals of the human GIT, as *Lactobacillus* and *Bifidobacterium* (Parkkinen and Korhonen, 1989; Schaumburg *et al.*, 2004; Lähteenmäki *et al.*, 2005; Sijbrandi *et al.*, 2005; Bergmann and Hammerschmidt, 2007; Hurmalainen *et al.*, 2007; Candela *et al.*, 2008b). The recruitment of host Plg on the microorganism cell surface, and its following conversion to the active enzyme plasmin, allows the microorganism to acquire a surface-associated and host-derived proteolytic activity, functional to the migration across tissue barriers and to the nutritional demands during the colonization process (Lähteenmäki *et al.*, 2005).

Binding of host Plg on the microorganism depends on the presence of Plg binding proteins on the microorganism outer surface. Lysine residues present on these receptors mediate the interaction with Plg molecules, specifically with Plg kringle domains; these lysine residues have been located in the C-terminal portion of the proteins or/and in lysine enriched internal Plg binding motive(s) (Bergmann and Hammerschmidt, 2007). One of the most investigated Plg receptor, found in bacteria but also in yeasts, protozoa and worms, is α-enolase (Fox and Smulian, 2001; Jolodar *et al.*, 2003; Jong *et al.*, 2003; Bernal *et al.*, 2004; Marcilla *et al.*, 2007; Nogueira *et al.*, 2010; Avilán *et al.*, 2011; Wang *et al.*, 2011a). α-enolase is a glycolytic cytoplasmic enzyme that has also been found on the cell surface of several prokaryotic and eukaryotic organisms (Pancholi, 2001); in this different localization it displays an additional function different from the one exerted in the cytoplasm, a "moonlighting" function (Jeffery, 1999). Several studies have been performed to elucidate molecular and biochemical characteristics of the α-enolase-human Plg interaction in numerous streptococcal species. In *Streptococcus pneumoniae* surface α-enolase binds human Plg with high affinity and presents two Plg binding sites, a C-terminal binding site constituted by two lysine residues, and an internal binding site, with the amino acid sequence ^{248}FYD**KE**R**K**VY^{256} (Bergmann *et al.*, 2001, 2003). This internal binding site is the major determinant of host Plg binding because of its localization on the outer surface of the octameric molecule, as revealed by the crystallographic resolution of the protein structure (Ehinger *et al.*, 2004). A recent molecular and biochemical study has underlined the importance for Plg binding of lysine residues present in a similar internal binding site also in GAS α-enolase, probably acting in concert with C-terminal lysine residues (Cork *et al.*, 2009). Recently, α-enolase has been found as a Plg receptor also in commensal bacteria. Antikainen and co-workers showed that α-enolase molecules localized both on the cell surface of different commensal *Lactobacillus* species and on the surface of *Streptococcus pneumoniae*, *S. pyogenes* and *Staphylococcus aureus* possess an equivalent Plg binding activity (Antikainen *et al.*, 2007b).

Different bifidobacterial species have shown Plg binding activity. It has been demonstrated that *Bifidobacterium longum*, *B. bifidum*, *B. breve* and *B. animalis* subsp. *lactis* possess Plg receptors on their cell wall (Candela *et al.*, 2007, 2009). *B. animalis* subsp. *lactis,* initially isolated from fermented milk (Meile *et al.*, 1997; Lee and O'Sullivan, 2010), is usually present in the gut of healthy infants and adults (Wall *et al.*, 2008; Turroni *et al.*, 2009) and is one of the most common probiotic bifidobacterial species utilized in commercial dairy products and pharmaceutical preparations in Europe and North America, combining health-promoting activities with technological properties (Barrangou *et al.*, 2009). One widely used *B. animalis* subsp. *lactis* strain, BI07, has been chosen as a bifidobacterial model and the proteomic analysis of its cell wall fraction allowed the identification of five putative human Plg receptors: DnaK, glutamine synthetase, α-enolase, bile salt hydrolase (BSH) and phosphoglycerate mutase (Candela *et al.*, 2007). The complete inhibition of Plg binding to *B. animalis* subsp. *lactis* BI07 cells in the presence of the lysine analogue ε-aminocaproic acid (EACA) suggested that lysine residues present on these surface Plg receptors are involved in Plg binding, a common trait of Plg receptors. Analogously to other Gram-positive bacteria (Lähteenmäki *et al.*, 2001), the Plg binding proteins found on the surface of *B. animalis* subsp.*lactis* BI07 are "moonlighting" proteins, highly conserved cytoplasmic proteins that, displayed on the bacterial cell surface, acquire a different secondary function. The analysis of the cell wall fractions of other bifidobacterial strains in order to screen common conserved putative surface Plg receptors allowed the identification of two major Plg binding proteins: α-enolase and DnaK (Candela *et al.*, 2009).

B. animalis subsp. *lactis* BI07 α-enolase is localized both in the cytoplasm and in the cell wall, and its Plg binding capability has been demonstrated. It shows a high affinity for human Plg, with an equilibrium dissociation constant in the nanomolar range. The construction of a homology model of bifidobacterial α-enolase allowed the identification of two putative Plg binding sites: a C-terminal binding site, constituted by a unique lysine residue, and an internal binding site, homologous to the internal binding site of pneumococcal α-enolase, with amino acid sequence ^{248}FYN**KE**TG**K**Y^{256}. Mutagenesis and biochemical analysis demonstrated that the internal binding site is essential for human Plg binding, while the C-terminal lysine is only marginally involved in this interaction (Candela *et al.*, 2009).

DnaK represents another Plg receptors conserved among bifidobacteria, but it has been also detected on the surface of pathogenic bacteria, such as *Listeria monocytogenes*, *Neisseria meningitidis* and *Mycobacterium tuberculosis*, where it shows a human Plg binding activity (Schaumburg *et al.*, 2004; Knaust *et al.*, 2007; Xolalpa *et al.*, 2007). Here the Plg binding function of *B. animalis* subsp. *lactis* BI07 DnaK has been investigated from the molecular and biochemical point of view, with an attempt to identify the determinants of this interaction.

Materials and methods

Bacterial strains, media and growth conditions

Bifidobacterium animalis subsp. *lactis* BI07, isolated from dairy products, was cultured in de Man-Rogosa-Sharpe (MRS) medium (Difco) supplemented with 0.05% (wt/vol) L-cysteine at 37°C in anaerobiosis. The anaerobic condition was obtained in a jar by using Anaerocult A (Merck). Bifidobacterial cells were grown for 18 h until they reached the stationary phase. *Escherichia coli* OneShot TOP10, BL21Star(DE3) and XL10-Gold strains were cultured in Luria-Bertani (LB) medium at 37°C with shaking. MagicMedia (Invitrogen) was used for recombinant protein expression. 50 µg/ml kanamycin were added as selective agent when appropriate.

Extraction of bifidobacterial cytoplasmic and cell wall proteins

Cytoplasmic proteins were extracted as reported by Candela *et al.* (2009). In brief, after washing in 50 mM Tris-HCl (pH 7.6), stationary phase *B. animalis* subsp. *lactis* BI07 cells were resuspended in 500 µl of TE buffer (50 mM Tris-HCl [pH 7.6], 5 mM EDTA) and 50 µl of Complete Protease Inhibitors Solution (Roche) were added. The suspension was sonicated to disrupt bacterial cells and centrifuged for 10 min at 14000 rpm at 4°C to precipitate debris. The supernatant was collected and centrifuged for 2 h at 45000 rpm at 4°C by using a Beckman Ultracentrifuge L7-55. The supernatant, containing cytoplasmic proteins, was stored at -20°C.
Cell wall proteins were extracted as reported by Hardie and Williams (1998). Stationary phase cells of *B. animalis* subsp. *lactis* BI07 were collected, washed in 50 mM Tris-HCl (pH 7.6) and resuspended in 2 ml of protoplast buffer (50 mM Tris-HCl [pH 7.6], 1 M sucrose, 1.4 mM phenylmethylsulfonyl fluoride, 15 mg/ml lysozyme). After an incubation for 90 min at 37°C, the suspension was centrifuged for 3 min at 4000 rpm at 4°C and the supernatant, containing the cell wall proteins, was collected and stored at -20°C. In order to remove sucrose excess, cell wall proteins were precipitated with 15% trichloroacetic acid (TCA), centrifuged for 10 min at 13000 rpm at 4°C, and resuspended in acetone. After 5 min incubation on ice, proteins were centrifuged for 10 min at 13000 rpm at 4°C and acetone was completely removed.

Electron microscopy and immunoblot analysis

Pre-embedding immunogold experiments were performed in order to visualize DnaK on the cell surface of *B. animalis* subsp. *lactis* BI07 cells, using whole bifidobacterial cells. Stationary phase cells were washed with PBS and adjusted to the concentration of 1×10^9 CFU/ml. Bacterial cells were resuspended in 100 µl of rabbit polyclonal anti-meningococcal DnaK antiserum (Knaust *et al.*, 2007) diluted 1:100 in PBS-1% bovine serum albumin (BSA) and incubated for 1 h at 25°C under constant agitation. Bacteria were washed twice with 1 ml PBS-1%BSA and resuspended in 25 µl of

10 nm gold particles conjugated anti-rabbit IgG (AuroProbe, GE Healthcare) diluted 1:5 in PBS-1% BSA, incubating for 30 min at 25°C under agitation. Bacteria cells were collected, washed twice in PBS-1% BSA and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 4 h at 4°C. Transmission electron microscopy (TEM) processing was carried out as reported by Candela *et al.* (2007). Glutaraldehyde-fixed bacteria were washed with 0.15 M cacodylate buffer and postfixed for 1 h at 4°C with 1% OsO_4. After washing, bacteria were dehydrated in a graded alcohol series and embedded in araldite (Fluka, Sigma-Aldrich). Ultrathin sections, obtained with a Reichert OMu3 ultramicrotome, were counterstained with uranyl acetate and lead citrate and examined with a Philips 400T transmission electron microscope. Electronic microscopic experiments were repeated four times.

Western blotting analysis was performed for both cytoplasmic and cell wall proteins of *B. animalis* subsp. *lactis* BI07. After mixing samples with loading buffer (0.5 M Tris-HCl [pH 6.8], 4% (wt/vol) SDS, 20% glycerol, 0.1% bromophenol blue, 5% β-mercaptoethanol) and boiling for 5 min, 10 µg of proteins were resolved by SDS-PAGE in 12% polyacrylamide gel using a SE 250 mini vertical electrophoresis unit (GE Healthcare) for 2.5 h at 160 V. Proteins were blotted onto a nitrocellulose membrane (BioRad) using the Minitrans-Blot Electrophoretic Cell (BioRad) at 100 V, 90 mA, at 4°C overnight. After protein transfer, the membrane was blocked in a solution of 4% skim milk (Fluka, Sigma-Aldrich) in TBS-T (20 mM Tris-HCl [pH 7.6], 0.5 M NaCl, 0.15% Tween 20) for 1 h at 25°C and subsequently incubated with cross reactive anti-meningococcal DnaK antiserum (Knaust *et al.*, 2007) for 1 h at 25°C under constant agitation. The membrane was then washed three times in TBS-T and incubated with the horseradish peroxidase conjugated anti-rabbit IgG (GE Healthcare) at 25°C for 1 h. After three TBS-T washings, the membrane was incubated with ECL Plus (GE Healthcare) and the chemiluminescent signal detected by using a PhosphorImager Storm system (GE Healthcare).

Construction of a *B. animalis* subsp. *lactis* BI07 genomic library and screening for the *dnaK* gene

The restriction enzyme *Mbo* I was employed to partially digest 300 µg of *B. animalis* subsp. *lactis* BI07 chromosomal DNA. DNA fragments of about 20 kb were isolated by ultracentrifugation at 26000 rpm for 24 h at 18°C in a sucrose gradient 10%-40%, purified by precipitation with ethanol and ligated to *Bam*H I digested Lambda EMBL3 vectors (Stratagene). Lambda EMBL3/*Bam*H I Gigapack III Gold Cloning kit (Stratagene) was used to package recombinant lambda phages and the library was amplified and titered following the kit instructions. In order to screen for the *dnaK* gene in the library, a specific probe was obtained amplifying the *B. longum* NCC2705 chromosomal DNA with the primers set L-DnaK (5'-TTGGCACGTGCAGTTG-3') and R-DnaK (5'-TCACTTGTTGTCCTTGTCG-3'), designed on the basis of the genome sequence of *B. longum* NCC2705. The *dnaK* gene targeting DNA probe was labelled with digoxygenin by using the DIG-DNA Labelling and Detection Kit (Roche). For *dnaK* gene detection, phage library was plated on 120 mm NZY agar plates using *E. coli* XL1-Blue MRA P2 as host strain. Plates were blotted onto

nylon membranes (Hybond N+, GE Healthcare). Neutralization, denaturation, crosslinking, membranes hybridization with the DIG-labelled DNA probe and detection were carried out as described in the DIG-DNA Labelling and Detection kit manual (Roche). Plaques corresponding to the positive signals in the original plates were cut out and resuspended in SM buffer to isolate the recombinant phage population. Phage DNA was extracted with the Qiagen Lambda mini kit (Qiagen), positive clones were sequenced and the *B. animalis* subsp. *lactis* BI07 *dnaK* gene sequence was obtained.

Cloning, expression and purification of *B. animalis* subsp. *lactis* BI07 His_6-DnaK

Genomic DNA was extracted from 1 ml of a *B. animalis* subsp. *lactis* BI07 overnight culture by using DNeasy Blood and Tissue kit (Qiagen) following the kit manual instructions. The genomic DNA was used as template in a PCR reaction for the amplification of the *dnaK* gene using the primer set DnaK-TOPO-L (5' -CACCATGGGACGCGCAGTTGGT- 3') and DnaK-TOPO-R (5'-TTACTTGTTGTCCTTGTCGTCGTC- 3'), designed on the basis of the sequence of the phage clone containing bifidobacterial *dnaK* gene. The CACC sequence at the 5' terminus of DnaK-TOPO-L primer is essential for the PCR product directional cloning in the expression vector pET200/D-TOPO (Invitrogen). Amplification reaction was carried out in a Biometra Thermal Cycler II thermocycler (Biometra), using Platinum *Pfx* DNA polymerase (Invitrogen). The PCR reaction was conducted in a final volume of 50 µl, containing 0.3 µl 50 µM primer DnaK-TOPO-L, 0.3 µl 50 µM primer DnaK-TOPO-R, 6 µl 2.5 mM dNTPs, 1 µl 50 mM $MgSO_4$, 10 µl 10X *Pfx* Amplification buffer, 10 µl 10X PCR_x Enhancer Solution, 0.4 µl Platinum *Pfx* DNA polymerase, 5 µl *B. animalis* subsp. *lactis* BI07 genomic DNA 8 ng/µl, 17 µl H_2O. The amplification reaction was carried out in a thermocycler with a first denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, elongation at 68°C for 1 min 30 s, with a final elongation step at 68°C for 10 min. Amplification product (1866 nt) was visualized on 1% agarose gel electrophoresis, using λDNA/*Eco*RI+*Hin*dIII (MBI Fermentas) as molecular weight marker, then purified with QIAquick Gel Extraction Kit (Qiagen) and quantified with Qubit fluorometer (Invitrogen) and the sequence was controlled using a PRISM377 sequencer (ABI-Perkin Elmer). The PCR product was cloned in the expression vector pET200/D-TOPO (Invitrogen) to obtain the construct pDKwt using the Champion pET Directional TOPO Expression Kit (Invitrogen). *E. coli* OneShot TOP10 and *E. coli* BL21Star(DE3) (Invitrogen) were utilized for *B. animalis* subsp. *lactis* BI07 *dnaK* gene cloning and expression, respectively. The His_6-tagged fusion DnaK was purified by affinity chromatography under native conditions on Ni-nitrilotriacetic acid resin according to the ProBond™ Purification System manual (Invitrogen). The purified bifidobacterial DnaK protein was dialyzed at 4°C using Spectra/Por membranes 6000-8000 Da (Spectrum Laboratories Inc) and 20 mM Tris-HCl, 120 mM NaCl as dialysis buffer. His_6-tagged recombinant protein His_6-DnaK expression was verified by immunoblot analysis with polyclonal anti-meningococcla DnaK antiserum (Knaust *et al.*, 2007) and anti HisTag antibody (Sigma).

Site-directed mutagenesis for construction of DnaK$^{\Delta Lys621}$

As several Plg binding proteins, *B. animalis* subsp. *lactis* BI07 DnaK possesses a C-terminal lysine residue potentially involved in Plg binding. To investigate the contribution of this specific residue in Plg interaction a DnaK mutant protein was constructed. To this aim, plasmid holding the mutant *dnaK* gene deleted of the nucleotides encoding the C-terminal lysine residue (pDKbs1) was created by using QuikChange Multi Site Directed Mutagenesis kit (Stratagene). To replace the C-terminal lysine residue with a stop codon the primer DnaKmutBS1 (5'-GACGACGACAAGGACAACTAATAAAAGGGCGAGCTC-3') was designed. The reaction mix was constituted by 1.8 µl (100 ng) 50 µM 1:10 DnaKmutBS1 primer, 1 µl dNTP mix, 2.5 µl 10X reaction buffer, 1 µl QuikChange Multi enzyme blend, 1 µl pDKwt template, H_2O to 25 µl. Mutagenesis reaction was carried out in a thermocycler with an initial denaturation step at 95°C for 1 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min and 65°C for 15 min 10 s. The DNA template was removed by *Dpn*I treatment at 37°C for 2 h. The reaction was used to transform *E. coli* XL10-Gold strain and several clones were sequenced to isolate the mutant. The mutant protein His_6-DnaK$^{\Delta Lys621}$ was expressed and purified as described previously for the wild type recombinant DnaK protein.

In silico modelling of *B. animalis* subsp. *lactis* BI07 DnaK

A homology model of *B. animalis* subsp. *lactis* BI07 DnaK was constructed in order to characterize possible internal putative Plg binding site(s), as frequently found for other bacterial Plg binding proteins. The homology model was calculated as reported by Zambelli *et al.* (2009). The amino acidic sequence of *B. animalis* subsp. *lactis* BI07 DnaK (Appendix 1) was used to search for possible structural templates by using the bioinformatic tool BLAST (Altschul *et al.*, 1990, 1997). Four DnaK and three Hsc70 structures with a high identity score were recovered, including full-length proteins and isolated domains (Table 4).

In order to identify a conserved motif, other nine DnaK sequences reported to possess a Plg binding activity were also included in the considered dataset. Multiple sequence alignment was carried out by using ClustalW (Thompson *et al.*, 1994). Secondary structure prediction for all sequences was performed using the program JPred (Cuff *et al.*, 1998) and the alignment was manually optimized basing on the secondary structure information. Fifty model structures were calculated with the program MODELLER 9v6 (Martí-Renom *et al.*, 2000) using the structural templates indicated above. The best model was selected on the basis of the lowest value of the MODELLER objective function. Structure validation was performed with PROCHECK software (Laskowski *et al.*, 1993). The last 35 residues of the protein were not modelled due to the absence of structural data covering such region. The program UCSF Chimera (Pettersen *et al.*, 2004) was used for protein visualization. The analysis of protein-protein interaction sites was carried out by using a combination of the predictions obtained from the following tools: SPPIDER predictor

(Porollo and Meller, 2007), ProMate (Neuvirth *et al.*, 2004), cons-PPISP (Zhou and Shan, 2001), PPI-Pred (Bradford and Westhead, 2005), PINUP (Liang *et al.*, 2006).

Table 4 - DnaK structural templates

ID	Chain	Source	Description	Plg binding activity	Identity (%)	Length (aa)	Resolution (Å)
/	/	*Bifidobacterium animalis* subsp. *lactis* BI07	DnaK	yes	/	621	/
ABF57997	/	*Bifidobacterium bifidum*	DnaK	yes	92	623	/
YP_910410	/	*Bifidobacterium adolescentis*	Chaperone DnaK (Hsp70)	/	93	626	/
ZP_00121343	/	*Bifidobacterium longum* NCC2705	Molecular chaperone	yes	92	626	/
ABF57996	/	*Bifidobacterium breve*	DnaK	yes	89	626	/
NP_283534	/	*Neisseria meningitidis* Z2491	Molecular chaperone DnaK	yes	55	642	/
NP_273598	/	*Neisseria meningitidis* MC58	Molecular chaperone DnaK	yes	55	642	/
NP_214864	/	*Mycobacterium tuberculosis* H37Rv	Molecular chaperone DnaK	yes	68	625	/
YP_014090	/	*Listeria monocytogenes* str. 4b F2365	Chaperone protein DnaK	yes	60	613	/
NP_464998	/	*Listeria monocytogenes* EGD-e	Molecular chaperone DnaK	yes	59	613	/
1YUW	A	*Bos taurus*	Hsc70 (E213A/D214A mutant)	/	50	554	2.60
1DKG	D	*Bos taurus*	DnaK (ATPase domain)	/	54	383	2.80
1HPM	A	*Bos taurus*	Hsc70 (ATPase fragment)	/	46	386	1.70
2E8A	A	*Homo sapiens*	Hsc70 (ATPase fragment)	/	46	391	1.77
1DKZ	A	*Escherichia coli*	DnaK (substrate binding domain)	/	55	219	2.00
1BPR	A	*Escherichia coli*	DnaK (substrate binding domain)	/	60	191	NMR
1Q5L	A	*Escherichia coli*	DnaK (peptide binding domain)	/	69	125	NMR

Plasminogen binding analysis by solid phase binding assay

Plg binding activity of recombinant bifidobacterial DnaK or mutant DnaK$^{\Delta Lys621}$ was evaluated by means of a solid phase Plg binding assay, performed as previously described by Sanderson-Smith *et al.* (2006, 2007). Human Plg (Sigma-Aldrich) was biotinylated by using EZ-Link Micro Sulfo-NHS-SS Biotinylation Kit (Pierce). Ninety-six-well microtiter plates (OptiPlate-96, Perkin Elmer) were coated with 150 nM recombinant DnaK or DnaK$^{\Delta Lys621}$ (50 µl in 0.1 M $NaHCO_3$) and incubated overnight at 4°C. After a washing step with PBS, plates were blocked with 200 µl of in PBS-2% bovine serum albumin (BSA) for 1 h at 37°C. Plates were washed three times and increasing concentration of biotinylated human Plg in PBS (2, 6, 18, 36, 55, 110, 166, 250 nM) were added to the plates, in the presence or absence of 50-fold molar excess of unlabelled Plg, incubating the plates for 2 h at 25°C. After three washings in PBS, 50 µl ExtrAvidin HRP conjugated (Sigma-Aldrich) diluted 1:5000 in PBS-1% BSA were added to each well and incubated for 2 h at 25°C. Plates were

washed four times with PBS and 0.05% Tween 20 and 100 µl of Chemiluminescent Peroxidase Substrate for ELISA (Sigma-Aldrich) were added to each well, reading luminescence by using Victor^{3}V 1420 Multilabel Counter scanner (Perkin Elmer) and the software Wallac 1420 WorkStation. Wells coated with BSA were used as negative control for Plg binding. For both the recombinant proteins, Plg binding assays were performed three times and for a given Plg concentration each measure was repeated in triplicate. The experiments were repeated in the presence of 0.5 M of the lysine analogue ε-aminocaproic acid (EACA) (Sigma-Aldrich) in order to evaluate the involvement of lysine residues in the DnaK-Plg binding. Data were normalized against the highest and lowest luminescence values, and non-linear regression analysis was performed with Graph Pad Prism software, version 5.0 (Graph Pad software). One- and two-site binding analysis were carried out for equilibrium dissociation constant (K_D) calculation and the best-fit curve was selected.

Impact of DnaK and enolase on *B. animalis* subsp. *lactis* BI07 cells plasminogen binding activity

The role of DnaK and enolase in the interaction between human Plg and whole *B. animalis* subsp. *lactis* BI07 cells was evaluated in a Plg binding assay with bifidobacterial cells pretreated with cross reactive anti-meningococcal DnaK antiserum (Knaust *et al.*, 2007) or anti-pneumococcal enolase antiserum (Bergmann *et al.*, 2003). Stationary phase *B. animalis* subsp. *lactis* BI07 cells were resuspended in PBS at a concentration of 1 x 10^9 CFU/ml and incubated for 1 h at 25°C under agitation with anti-meningococcal DnaK antiserum diluted 1:250 or anti-pneumococcal enolase antiserum diluted 1:100 in PBS-1% BSA. Bacterial cells were washed with PBS-1% BSA, resuspended in PBS and incubated with 100 µg of human Plg (Sigma-Aldrich) for 30 min at 37°C under agitation. After three washings with PBS to remove unbound Plg, bacterial cells were resuspended in 20 µl NH_4Cl 2% for MALDI-TOF analysis of Plg binding. One µl of bacterial suspension was spotted, airdryed and then overlaid with 1.0 µl of matrix solution (12.5 mg/ml ferulic acid in a mixture of formic acid/acetonitrile/water 17:33:50). The resulting droplet was left to crystallize by airdrying and then analyzed in a Voyager DE Pro MALDI-TOF mass spectrometer (Applied Biosystems) operating in linear positive ion mode in the range of 20000-100000 Da and equipped with a pulsed N_2 laser at 337 nm. Default operating conditions were as follow: accelerating voltage 25000 V, grid voltage 22500 V, extraction delay time 750 ns. All acquisitions were generated automatically on the instrument software (Voyager^{TM}, Biospectrometry Workstation) using a random search pattern mode and based on overaging 1600 shots from 20 non-overlapping positions (80 shots/position). A standard bovine serum albumin (BSA) solution (67 kDa) was used for data external calibration. Two-hundred ng of human Plg (Sigma-Aldrich) were used as analytical standard.

Results

Surface localization of *B. animalis* subsp. *lactis* BI07 DnaK protein

The immunoblot analysis using a cross reactive anti-meningococcal DnaK antiserum (anti-DnaK) (Knaust *et al.*, 2007) demonstrated the presence of the DnaK protein both in the cytoplasm and in the cell wall fraction of *B. animalis* subsp. *lactis* BI07 (Fig. 10).

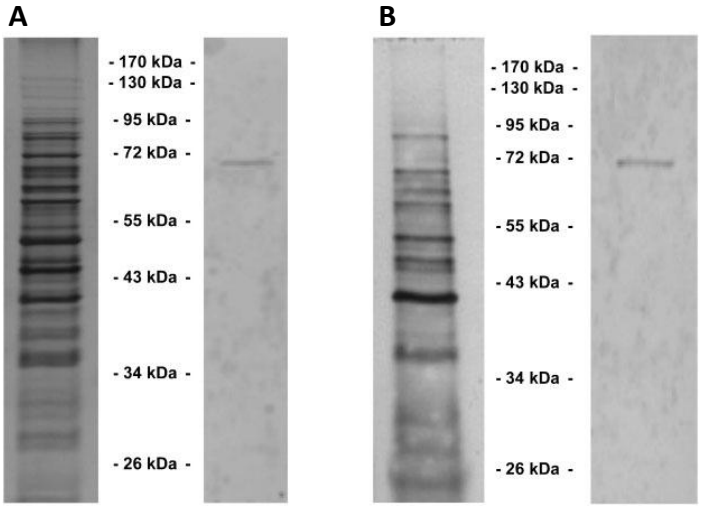
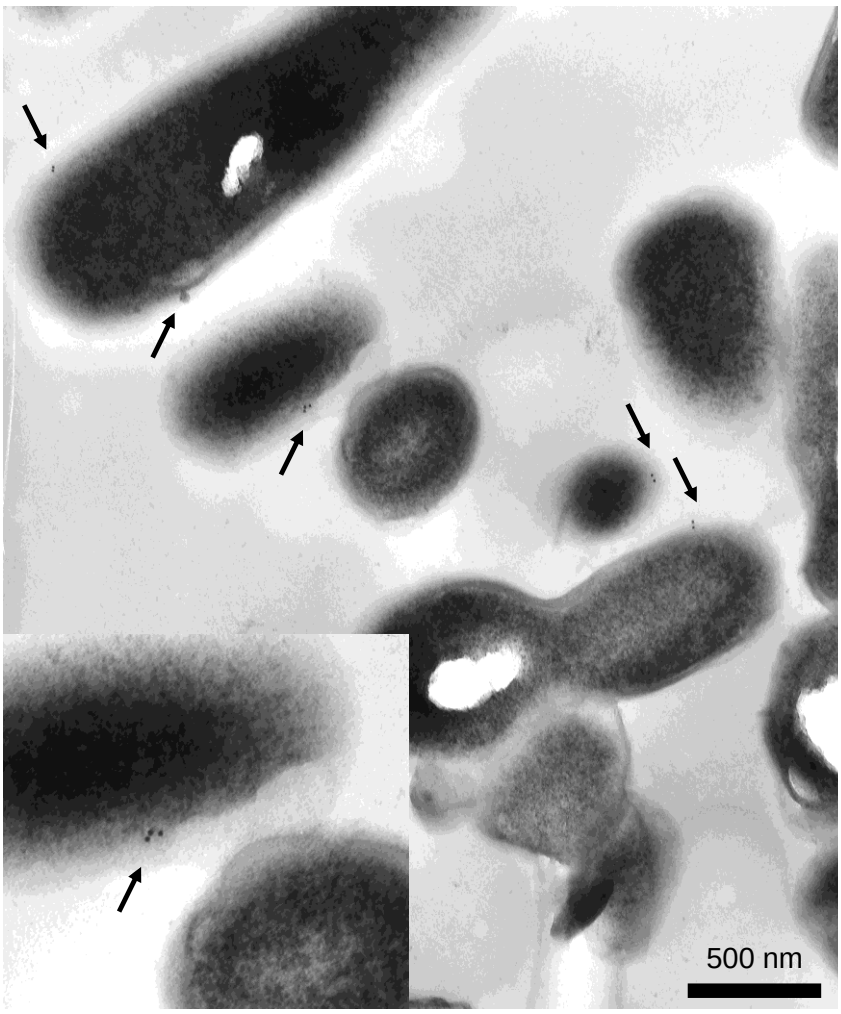


Fig. 10. (right) Immunoblot analysis (right panel) using anti-DnaK polyclonal antiserum of cytoplasmic (A) and cell wall (B) protein fractions of *B. animalis* subsp. *lactis* BI07 resolved by SDS-PAGE (left panel).

Fig. 11. (below) Immunoelectron microscopic localization of DnaK on the cell surface of *B. animalis* subsp. *lactis* BI07. DnaK was detected on the bacterial surface by anti-DnaK antiserum and secondary antibody coupled to 10 nm gold particles in pre-embedding experiments. An ultrathin section shows DnaK (black dots, arrowed) localized directly in the cell-wall region.



The visualization of the DnaK protein on the bifidobacterial cell surface was performed by using immunoelectron microscopy. *B. animalis* subsp. *lactis* BI07 cells were incubated with the anti-DnaK antiserum under pre-embedding conditions and subsequently with the secondary antibody conjugated with 10 nm gold particles. Ultrathin sections analysis at a magnification of X 22000 demonstrated the presence of DnaK directly on the bacterial cell wall region, as indicated by the black dots pointed by arrows (Fig. 11). Secondary antibody unspecific binding was not detected (data not shown).

Analysis of *B. animalis* subsp. *lactis* BI07 *dnaK* gene

A phage library of the *B. animalis* subsp. *lactis* BI07 genome was constructed and, using a digoxigenin-labelled DNA probe designed on the *B. longum* NCC2705 *dnaK* gene, it was screened for the *B. animalis* subsp. *lactis* BI07 *dnaK* gene. The nucleotide sequence of *B. animalis* subsp. *lactis* BI07 *dnaK* gene (GenBank acc. no.: AB514431, Appendix 1) showed an identity of 87% with the *dnaK* gene of *B. longum* NCC2705 (GenBank acc. no.: AE014295) and 100% with the one of *B. animalis* subsp. *lactis* DSM10140 (GenBank acc. no.: CP001606).

Analysis of *B. animalis* subsp. *lactis* BI07 DnaK - human plasminogen binding

With the purpose to analyze the specific human Plg binding activity of the *B. animalis* subsp. *lactis* BI07 DnaK protein, a recombinant His_6-tagged DnaK protein was expressed, purified and used in a solid phase Plg binding assay, as reported by Sanderson-Smith and co-workers (Sanderson-Smith *et al.*, 2007). A dose-dependent binding between biotinylated human Plg and immobilized recombinant DnaK was demonstrated and a saturation binding was achieved in the presence of a Plg concentration of 150 nM (Fig. 12A). In the presence of 50-fold molar excess of unlabelled Plg non-specific binding was determined. The subtraction of non-specific binding from the total binding obtained at each concentration of biotinylated human Plg allowed the calculation of Plg specific binding to DnaK. BSA-coated wells were used as negative control. The best-fit non linear regression analysis permitted the calculation of an equilibrium dissociation constant (K_D) for the interaction with Plg of about 11 nM. The essential role of DnaK lysine residues in the interaction with Plg was proved in an solid phase Plg binding assay in the presence of 0.5 M EACA as lysine competitor, that completely inhibited Plg binding. The C-terminal lysine residues have been indicated as main Plg binding sites for numerous Plg receptors (Lähteenmäki *et al.*, 2001; Bergmann and Hammerschmidt, 2007). To assess the involvement of the C-terminal lysine residue of *B. animalis* subsp. *lactis* BI07 DnaK in Plg binding, the codon encoding the C-terminal lysine residue at position 621 was deleted. The recombinant His_6-tagged $DnaK^{\Delta Lys621}$ was expressed, purified, immobilized and its Plg binding activity was assessed in a saturation binding analysis, performing a solid phase Plg binding assay (Fig. 12B). *B. animalis* subsp. *lactis* BI07 $DnaK^{\Delta Lys621}$ displayed a dose-dependent and saturable Plg binding activity, with a K_D value of about 20 nM, reflecting an affinity only slightly lower with respect to wild type DnaK protein.

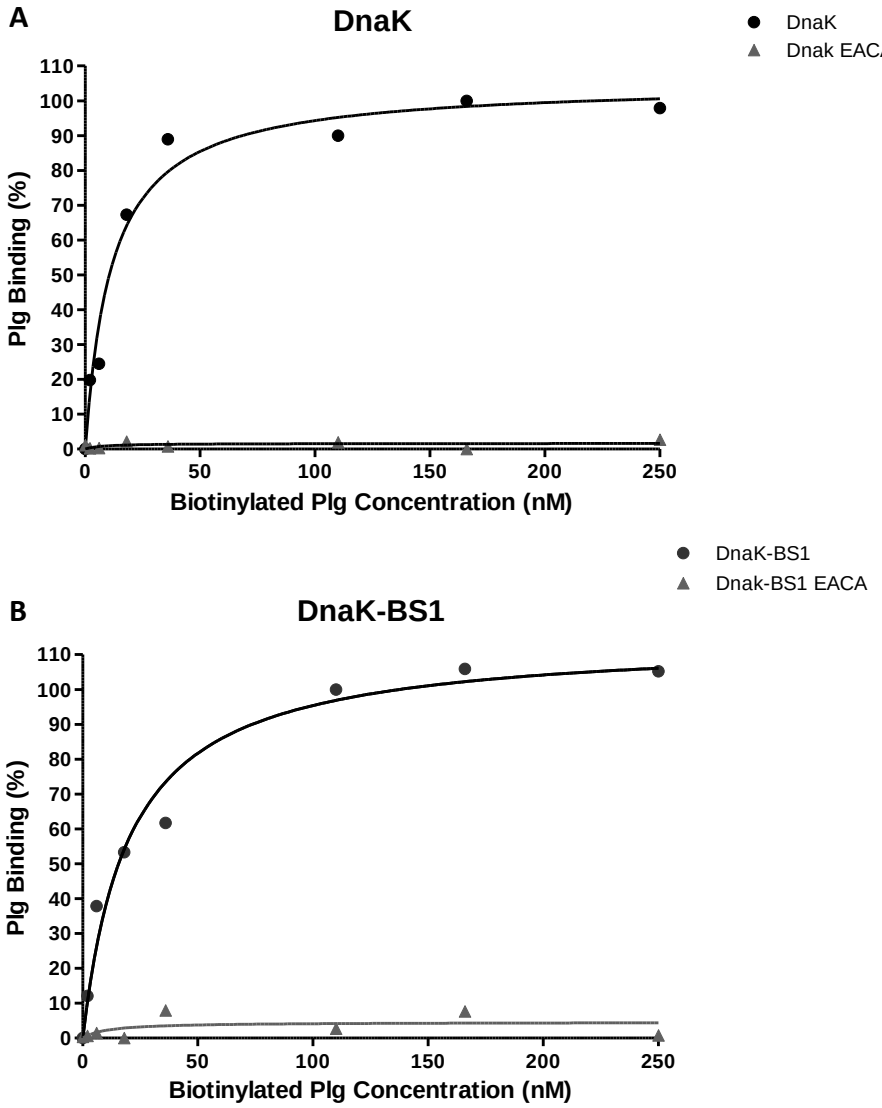


Fig. 12. Saturation binding analysis of biotinylated plasminogen to immobilized recombinant *B. animalis* subsp. *lactis* BI07 His-tagged DnaK (A) and DnaK$^{\Delta Lys621}$, here indicated as DnaK-BS1 (B). The black dots represent the specific binding. Specific Plg binding was calculated at each concentration of biotinylated Plg by subtracting non-specific binding obtained in the presence of 50-fold molar excess of unlabelled Plg from total binding. One-site hyperbolic binding function was fitted to the data and the K_D determined. The experiment was repeated in the presence of the lysine analogue EACA 0.5 M (grey triangles).

In silico modelling of *B. animalis* subsp. *lactis* BI07 DnaK and prediction of putative plasminogen binding site(s)

In order to determine the presence of putative Plg binding site(s) in the *B. animalis* subsp. *lactis* BI07 DnaK, a protein homology model was constructed. As template, the coordinates of seven homologous proteins (or protein domains) of known structure were used; Fig. 13 shows the multiple alignment of the amino acid sequence of *B. animalis* subsp. *lactis* BI07 DnaK with other nine bacterial DnaK proteins interacting with Plg and the sequence of seven template structures. The Ramachandran plot analysis of the calculated structure model of *B. animalis* subsp. *lactis* BI07 DnaK revealed that 95% residues reside in the most favoured and in the allowed regions, indicating an high quality model. The global architecture of the structure model (Fig. 14) resembles the structural organization of HSP70 family proteins, with a Nucleotide Binding Domain (NBD) from residue 1 to 366, followed by a flexible linker characterized by the conserved motif DVLL[IL]DVTP (residues 367-375) and a C-terminal Substrate Binding Domain (SBD), spanning residues 376 to 586.The NBD and the SBD domains interact by two α-helices and a β-strand formed by residues 124-138, 487-501 and 189-195, respectively. *B. animalis* subsp. *lactis* BI07 DnaK model structure shows

a high lysine content, with 45 lysine residues accounting for 7.7% of total residues, and most of them (41) are found uniformly distributed on the surface of the protein model structure (Fig. 15). The C-terminal region of SBD domain, from residue 503 to 586, folded as helices presents an elongated topology, where 12 exposed lysine residues are placed very closed to each other.

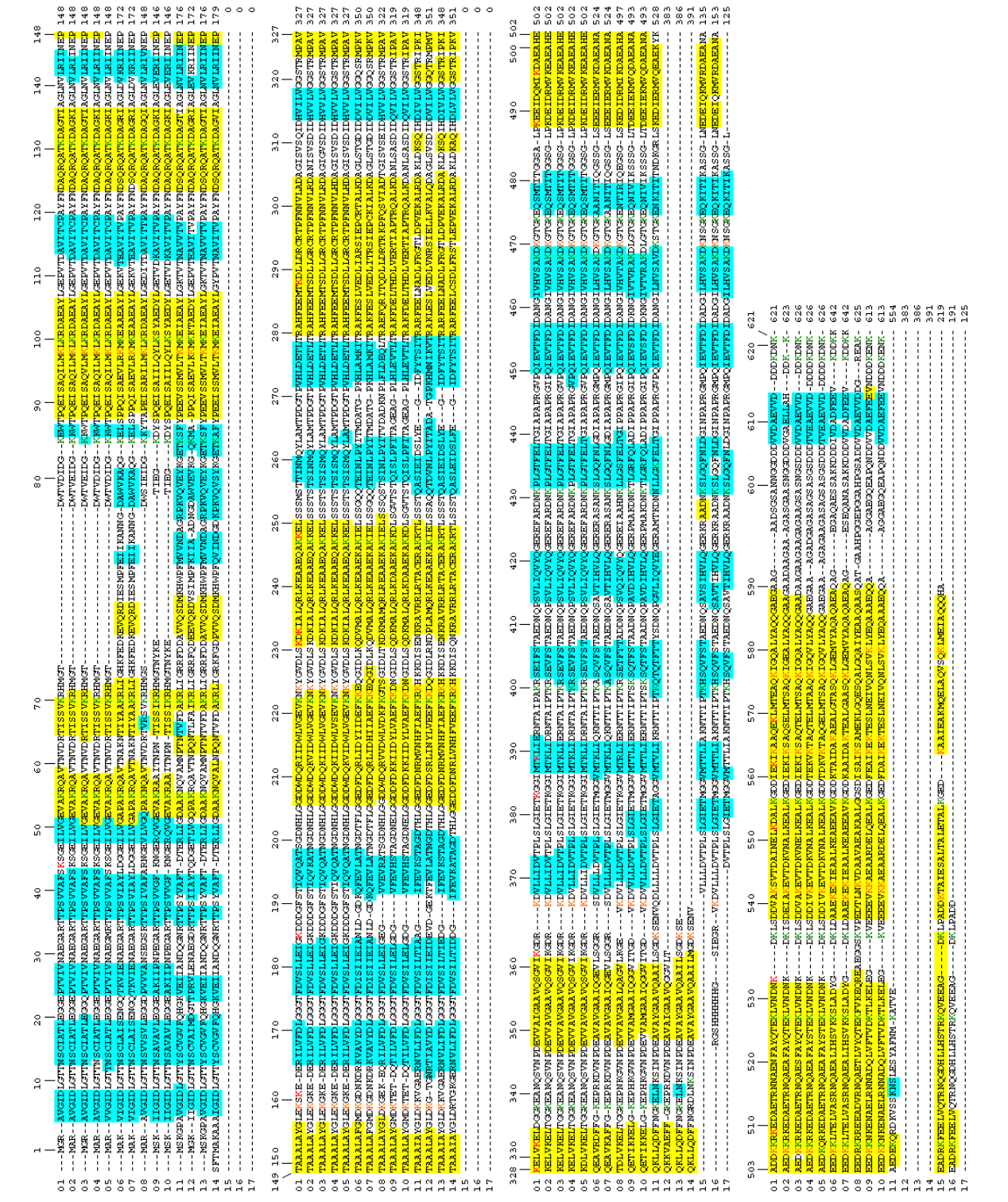


Fig. 13. Multiple sequence alignment of the DnaK sequences of *B. animalis* subsp. *lactis* BI07 (01), *B. bifidum* ID: ABF57997 (02), *B. adolescentis* ID: YP_910410 (03), *B. longum* ID: ZP_00121343 (04), *B. breve* ID: ABF57996 (05), *N. meningitidis* ID: NP_283534 (06), *N. meningitidis* ID: NP_273598 (07), *L. monocytogenes* ID: YP_014090 (09), *L. monocytogenes* ID: NP_464998 (10), *Bos taurus* ID: 1YUW (11), *Bos taurus* ID: 1DKG (12), *Bos taurus* ID: 1HPM (13), *Homo sapiens* ID: 2E84 (14), *E. coli* ID: 1DKZ (15), *E. coli* ID: 1BPR (16), *E. coli* ID: 1Q5L (17). The secondary structure indication (α-helix, *yellow*; β-strand, *cyan*) are derived from JPred prediction for *B. animalis* subsp. *lactis* BI07 DnaK and from the PDB structure for all the remaining sequences.

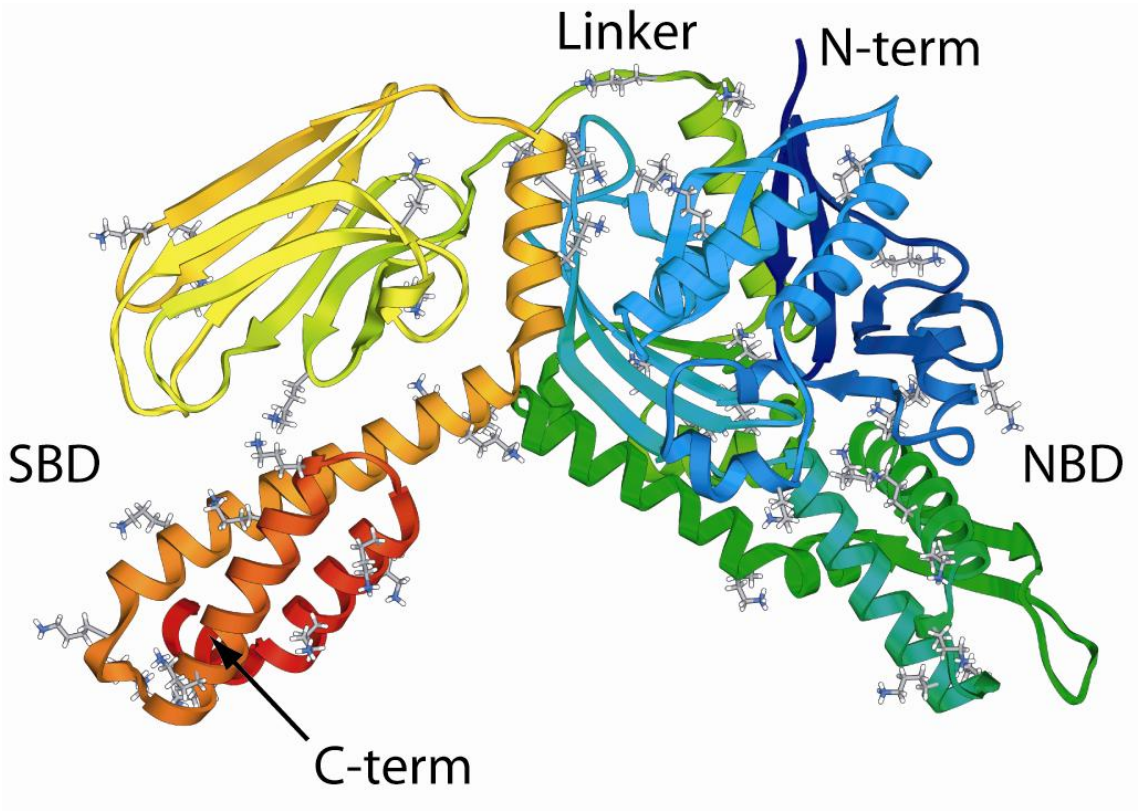


Fig. 14. Ribbon diagram of the model structure of *B. animalis* subsp. *lactis* BI07 DnaK coloured from *blue* in the proximity of the N-terminus to *red* at the C-terminus. Lysine residues are reported as "sticks and balls" and atoms are coloured according to atom type (carbon, *grey*; nitrogen, *blue*; oxygen, *red*; hydrogen, *white*).

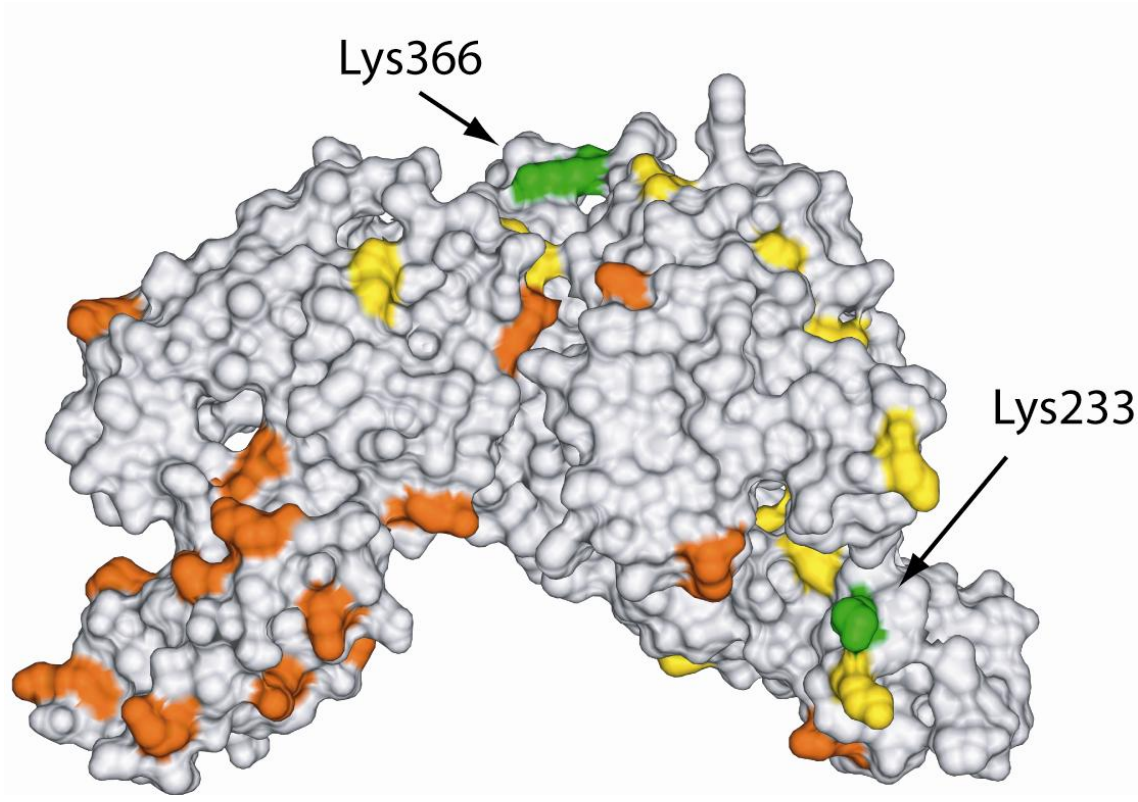


Fig. 15. Solvent excluded surface of the model structure of *B. animalis* subsp. *lactis* BI07 DnaK reported in the same orientation as in Fig. 14. Lysine residues are coloured according to the predicted interaction attitude (low, *orange*; medium, *yellow*; strong, *green*).

This DnaK structural model has been utilized to locate putative protein-protein interaction sites using several predictor tools, such as SPPIDER, ProMate, cons-PPISP, PPI-Pred, PINUP. As a result of this investigation, the best score lysine residues evidenced are Lys-233 and Lys-366, both located in the NBD domain and in regions predicted to be favourable to protein-protein interaction (Fig. 16). Additionally, both Lys-233 and Lys-366 are present in protruding edges of the protein surface, close to another lysine residue, Lys-231 and Lys-362, respectively, similar to the enolase Plg binding site. On the contrary, a low probability of protein-protein interaction is associated with the lysine-rich C-terminal region of SBD domain. Lys-618 and Lys-621 where not included in the present protein model structure because of the absence of structural data for this region in the template structure, and with this model it is not possible to predict the Plg binding capability of these two residues.

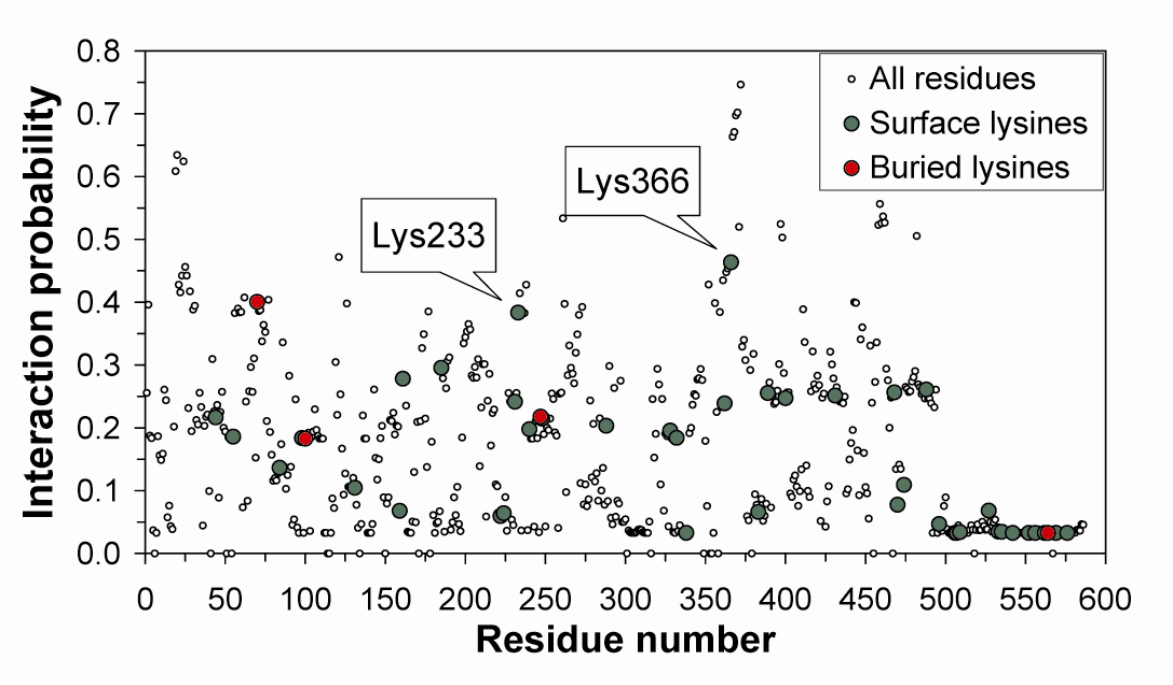


Fig. 16. Protein-protein interaction attitude prediction calculated normalizing the results from SPPIDER, ProMate, cons-PPISP, PPI-Pred, and PINUP servers.

Role of surface enolase and DnaK in *B. animalis* subsp. *lactis* BI07 plasminogen binding

The role of surface DnaK and enolase as Plg receptors on *B. animalis* subsp. *lactis* BI07 cells was experimentally evaluated by analyzing the impact of anti-DnaK or anti-enolase antibodies treatment on the bifidobacterial Plg binding capability. MALDI-TOF-MS analysis was used to compare Plg binding activity of bifidobacterial cells untreated or pretreated with anti-DnaK or anti-pneumococcal enolase antisera (anti-Eno) (Candela *et al.*, 2009). As shown in Fig. 17, the anti-Eno treatment decreased the Plg binding activity of *B. animalis* subsp. *lactis* BI07 of about 30%, while the treatment with anti-DnaK antiserum greatly impaired this interaction, reducing *B. animalis* subsp. *lactis* BI07 binding activity of about 80%. No reduction in Plg binding was measured when using anti-rabbit IgG as a blocking antibody. Data obtained from this assay imply that enolase but mainly DnaK are important for the capture of Plg on the surface of *B. animalis* subsp. *lactis* BI07.

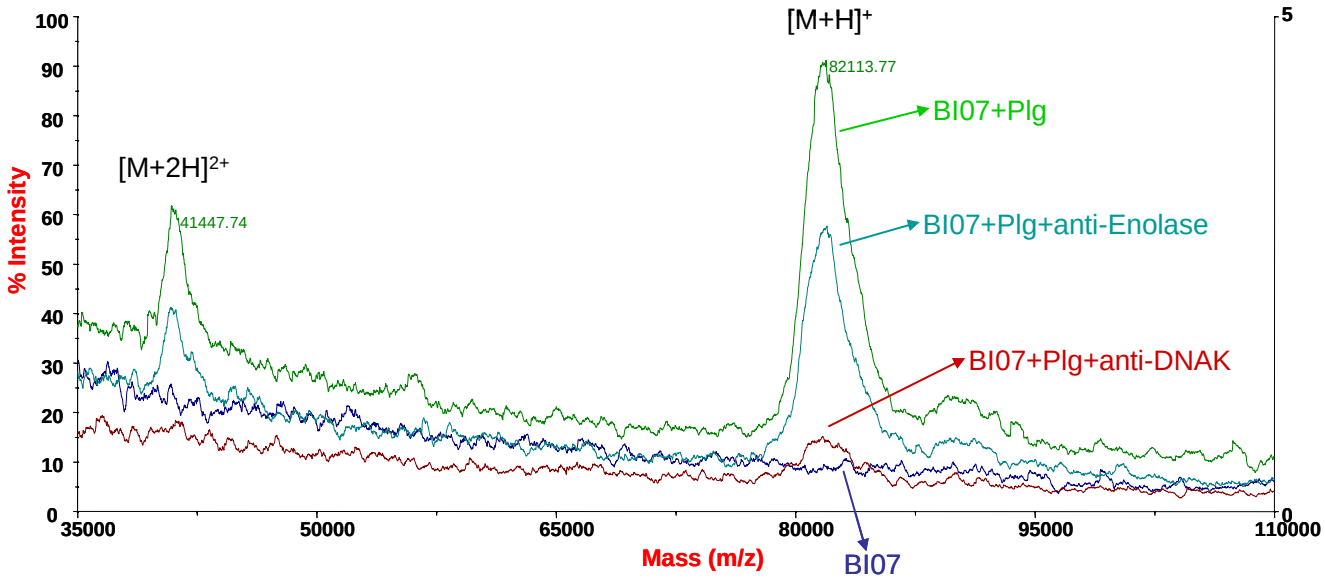


Fig. 17. MALDI-TOF analysis of Plg binding to *B. animalis* subsp. *lactis* BI07 cells untreated (BI07+Plg), and pre-treated with anti-DnaK (BI07+Plg+anti-DnaK) or anti-Eno (BI07+Plg+anti-Enolase). As negative control, *B. animalis* subsp. *lactis* BI07 cells not incubated with Plg (BI07) are shown. Plg $[M+H]^+$ signal at 81 kDa, $[M+2H]^{2+}$ at 40 kDa.

Discussion

This work has demonstrated the importance of surface DnaK in the interaction between *B. animalis* subsp. *lactis* BI07 and human Plg. A highly conserved 67 kDa cytoplasmic protein, DnaK belongs to the heat shock protein 70 family (HSP70), chaperone proteins with important roles in protein folding and transport (Bukau and Horwich, 1998).

B. animalis subsp. *lactis* BI07 DnaK has been characterized in order to achieve a comprehensive view of this protein in the context of the interaction with human Plg. Immunoelectron microscopy allowed the visualization of DnaK protein on the cell wall of *B. animalis* subsp. *lactis* BI07. Despite its surface localization, DnaK lacks any of the predicted protein sorting and bacterial cell wall anchoring signals, similarly to the surface located *B. animalis* subsp. *lactis* BI07 enolase, as reported by Candela and co-workers (Candela *et al.*, 2009). The secretion and surface localization of these cytoplasmic proteins, known as moonlighting proteins for their ability to carry out a second different function, are still unknown. Some hints come from a recent finding for *Streptococcus pneumoniae*: Claverys and Havarstein (2007) reported bacterial scavenging of intracellular proteins released through allolysis as a possible mechanism for surface localization of cytoplasmic proteins. DnaK protein has been found in the surface associated proteome of several bacteria, the pathogens *Neisseria meningitidis*, *Lysteria monocytogenes* and *Mycobacterium tuberculosis* (Schaumburg *et al.*, 2004; Knaust *et al.*, 2007; Xolalpa *et al.*, 2007) and the human gut microbiota probiotic member *Lactobacillus salivarius* (Kelly *et al.*, 2005), but the role of DnaK has Plg receptor has been reported only for *N. meningitidis*, *L. monocytogenes* and *M. tuberculosis*. The affinity of the interaction between human Plg and *B. animalis* subsp. *lactis* BI07 DnaK has been investigated through a saturation binding analysis using a purified recombinant His_6-tagged DnaK protein. It has been found that *B. animalis* subsp. *lactis* BI07 DnaK possesses a high affinity for human Plg, with a K_D of about 11 nM, a value higher respect to that reported for *B. animalis* subsp. *lactis* BI07 enolase (K_D = 42.8 nM), but close to the affinity values for human Plg of enolases from *Streptococcus pyogenes*, *S. pneumoniae* and *S. suis* (1-4 nM, 0.55 nM and 14 nM, respectively) (Pancholi and Fischetti, 1998; Bergmann *et al.*, 2003; Esgleas *et al.*, 2008). Similarly to other Plg receptors, lysine residues were essential for DnaK-Plg interaction, as indicated by the complete inhibition of Plg binding in the presence of the lysine analogue EACA. The best fit non linear regression analysis of data recovered in the saturation binding assay indicated that *B. animalis* subsp. *lactis* BI07 DnaK possesses exclusively one binding site for human Plg. Generally, the C-terminal lysine residue has been pointed out as the major binding site for Plg interaction for numerous proteins, but the deletion of this residue (Lys-621) in *B. animalis* subsp. *lactis* BI07 DnaK only slightly affected the interaction with Plg, with a K_D of about 20 nM. *In silico* analysis were performed to construct an homology model of the *B. animalis* subsp. *lactis* BI07 DnaK protein in order to try to uncover putative internal Plg binding sites or motifs. From the analysis of the DnaK homology model, two internal lysine-rich motifs (Lys-321-Lys-233 and Lys-362-Lys-366) have been

found that have high probability of interaction with Plg. According to this prediction, these lysine residues could represent possible candidates for bifidobacterial DnaK internal Plg binding site(s). These data are in agreement with findings described for *N. meningitidis* DnaK by Knaust *et al.* (2007), that reported the main role of internal Plg binding motifs rather than C-terminal lysine residues for DnaK-Plg binding. Internal Plg binding sites have been identified in several bacterial Plg receptors: enolases from *S. pneumoniae*, *Aeromonas hydrophila* and *B. animalis* subsp. *lactis*, and PAM, a Plg binding group A streptococcal M-type surface protein (Bergmann *et al.*, 2005; Candela *et al.*, 2009; Sha *et al.*, 2009). The homology search within the *B. animalis* subsp. *lactis* BI07 DnaK aminoacid sequence did not show a significant similarity with internal Plg binding sites of enolases from *B. animalis* subsp. *lactis* BI07 and *S. pneumoniae* (Bergmann *et al.*, 2003; Candela *et al.*, 2009).

After the molecular characterization of DnaK, the importance of the specific surface DnaK and α-enolase proteins on the bacterial Plg binding capability has been investigated. Blocking the surface DnaK with cross reactive DnaK-specific antiserum greatly impaired the human Plg binding activity of *B. animalis* subsp. *lactis* BI07 cells, with a Plg binding reduction of about 80%. The same treatment with anti-Eno antiserum to block surface enolase decreased Plg binding to *B. animalis* subsp. *lactis* BI07 cells only of the 30%. This difference could be partially explained by the higher Plg affinity of DnaK compared to enolase, but another important aspect to bear in mind that can have an important impact in receptor blocking is the different efficacy of the antisera in blocking the respective antigens. It's worth noting that several other variables may impact the contribution of a single receptor to Plg capture on the bacterial cell surface, such as Plg receptor availability on the bacterial cell surface and the binding epitope number (Knaust *et al.*, 2007).

DnaK importance as a Plg receptor for *B. animalis* subsp. *lactis* BI07 has been demonstrated throughout this chapter. Further studies of this bifidobacterial receptor can be carried out in order to better characterize the mechanism of Plg binding, both in *B. animalis* subsp. *lactis* BI07 and in other bifidobacterial strains.

CHAPTER II

BILE SALTS EFFECTS ON *BIFIDOBACTERIUM ANIMALIS* SUBSP. *LACTIS* BI07 PLASMINOGEN BINDING

Introduction

The gut environment represents a highly challenging place for commensal microorganisms. Several biological barriers threaten bacterial survival: pH variations, different oxygen levels, nutrient limitations, elevated osmolarity (Chowdhuri *et al.*, 1996). Besides this, bacteria have to face numerous toxic compounds of intestinal origin, among which bile components are the most important. Major constituents of bile are bile salts, detergent-like compounds synthesized from cholesterol by hepatocytes and stored in the gallbladder as amino acid conjugates, usually glycine- or taurine-conjugates. Bile is secreted into the intestine during digestion, playing a major role in the emulsification, solubilisation and absorption of fats. Besides this physiological role, bile possesses strong antimicrobial properties (Begley *et al.*, 2005). The main antibacterial characteristic affects bacterial membrane integrity: due to their amphipathic nature, bile salts cause membrane damages disrupting the lipid bilayer structure. In addition, they induce protein misfolding and oxidative damage to DNA (Bernstein *et al.*, 1999a, 1999b; Leverrier *et al.*, 2003). The capability to adapt and tolerate bile salts, present usually at concentrations below 5 mM (Hofmann, 1999), is an essential feature of intestinal microorganisms for the colonization of the human gastrointestinal tract (Begley *et al.*, 2005; Ridlon *et al.*, 2006). Bile-modifying enzymes and bile transporters have been described in intestinal bacteria (Piddock, 2006; Kim and Lee, 2008), but the mechanisms used by these bacteria to cope with bile stress are still poorly understood. Recent studies have begun to reveal some molecular mechanisms of the adaptation and tolerance to bile salts of *Bifidobacterium*. Changes in the expression profile after bile salts exposure of two *Bifidobacterium* species, *B. longum* and *B. animalis* subsp. *lactis*, have been investigated recently (Sánchez *et al.*, 2005, 2007b). It has been reported that bifidobacterial responses to bile stress involve the up-regulation of general stress response chaperones and oxidative stress target proteins, expression changes of several proteins and enzymes involved in carbohydrate metabolism leading to changes in the manner of energy production, up-regulation of proteins involved in transcription, translation and metabolism of amino acids and nucleotides. Besides these wide-range responses that impact on general metabolic pathways, recent studies have elucidated some specific molecular mechanisms directly involved in bile resistance/tolerance. It has been shown that *B. longum* NCC2705 possesses a bile efflux transporter whose expression is induced by bile at concentrations similar to those present in the colon (Gueimonde *et al.*, 2009). More recently, six bile-induced putative transport systems have been described in another bifidobacterial species, *B. breve* UCC2003 (Ruiz *et al.*, 2012b), each one with different substrate specificities and induction profiles leading to a complementary activity among them. Counteracting bile toxicity by direct bile transport outside the cell, these systems could play a significant role in bifidobacteria resistance and tolerance to bile, underlining the importance of these transport systems for bifidobacterial survival in and colonization of the gut environment. Most of the bacterial factors involved in the colonization process and interaction with the host are

localized on the microorganism cell surface, and global expression profile changes due to adaptation responses can affect these extracellular components. Recently, the effect of bile on the *B. longum* cell wall proteome have been investigated with the attempt to evaluate a possible impact of bile on the interaction between *Bifidobacterium* and the host (Ruiz *et al*., 2009). Ruiz and co-workers have reported the up-regulation of several cell-wall proteins in response to bile and, among them, they have identified two surface Plg receptors, enolase and glutamine synthetase, suggesting that the adaptation of *B. longum* to bile environment can prompt the expression of bacterial factors useful for facilitating the colonization of the human gut. Here the effect of the adaptation to a bile environment on the Plg binding capability of *B. animalis* subsp. *lactis* BI07 has been assessed, with a particular interest on the Plg binding proteins present on the bifidobacterial cell surface.

Materials and methods

Culturing of *Bifidobacterium animalis* subsp. *lactis* BI07

In this study the strain *Bifidobacterium animalis* subsp. *lactis* BI07 was used, originally isolated from dairy products. Bacteria were cultured in de Man-Rogosa-Sharpe (MRS) medium (Difco) supplemented with 0.05% (wt/vol) L-cysteine at 37°C in an anaerobic atmosphere, obtained in a jar by using Anaerocult A (Merck). Bifidobacteria cells were grown for 18 h until they reached the stationary phase. When necessary, bile salts (oxgall, Difco) were added to a final concentration of 1.2 or 3 g/l. Oxgall was used because of the difficulties in recovering human bile salts. Differently from human bile, oxgall possesses higher bile salt and phospholipid concentrations and lower phospholipid/bile salt molar ratios, while the ratio of glycine- to taurine-conjugated bile acids is similar (Hafkenscheid and Hectors, 1975; Coleman *et al.*, 1979). For growth curve study, liquid cultures were performed in 100 ml MRS medium supplemented with 0.05% L-cysteine in the absence and in the presence of oxgall. The 100 ml liquid cultures were inoculated at 1% (vol/vol) with fresh overnight cultures, incubated in anaerobiosis and monitored spectrophotometrically at 600 nm.

Extraction of bifidobacterial cell wall proteins

Bifidobacterial cell wall protein fraction was extracted as reported by Hardie and Williams (1998). Stationary phase cells of *B. animalis* subsp. *lactis* BI07 were collected, washed in 50 mM Tris-HCl (pH 7.6) and resuspended in 2 ml of protoplast buffer (50 mM Tris-HCl [pH 7.6], 1 M sucrose, 1.4 mM phenylmethylsulfonyl fluoride, 15 mg/ml lysozyme). The suspension was incubated at 37°C for 90 min, then centrifuged for 3 min at 4000 rpm at 4°C and the supernatant, containing the cell wall proteins, was collected and stored at -20°C. Proteins were subsequently precipitated with 15% trichloroacetic acid (TCA) and acetone in order to remove sucrose excess.

Two-dimensional gel electrophoresis and comparative analysis of protein expression

Proteomic analysis was carried out as reported by Candela *et al.* (2007). *B. animalis* subsp. *lactis* BI07 cell wall proteins were solubilised in IEF solution containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 0.005% (vol/vol) 2-mercaptoethanol for two-dimensional polyacrylamide gel electrophoresis (2DE) analysis. The estimate of total protein concentration was carried out with PlusOne 2D Quant kit (GE Healthcare). Isoelectric focusing was performed using Immobiline DryStrips with a linear gradient between 4 and 7 (in 7 cm) on an IPGphor system (GE Healthcare). The rehydration of 40 µg of cell wall proteins was carried out for 12 h in 125 µl of buffer A (8 M urea, 2% [wt/vol] CHAPS, 2% [vol/vol] Ampholine pH 4.0 to 6.5 [GE Healthcare], 10 mM dithiothreitol, 0.8% bromophenol blue).

Proteins were focused for a total of 10 kV · h. Reduced and alkylated IPG strips (Görg *et al.*, 1988) were loaded onto 12% acrylamide separating gels and proteins were resolved by SDS-PAGE at 160 V for 2.5 h using a SE 250 mini vertical electrophoresis unit (GE Healthcare). Spots were visualized by silver staining. GS-800 imaging densitometer (Bio-Rad) was used for the digital acquisition of gels protein patterns. Spot detection, matching and the analysis of differentially expressed proteins were performed by PDQuest v. 8.0.1 software (Bio-Rad). For each conditions, two biological and three technical replicates were carried out to constitute one replicate group with averaged normalized intensities for each spot.

Comparative analysis was performed between cell wall fractions from stationary-phase bacterial cells grown in MRS medium with and without 1.2 g/L oxgall. Differentially expressed proteins were considered proteins showing at least two-fold enhanced or decreased expression levels. The identification of plasminogen (Plg) binding proteins was carried out comparing the two dimensional gels with the reference map shown by Candela *et al.* (2007).

MALDI-TOF-MS analysis of plasminogen binding to *B. animalis* subsp. *lactis* BI07

A MALDI-TOF-MS based approach was used to evaluate Plg binding to *B. animalis* subsp. *lactis* BI07 cells, as reported by Candela and co-workers (Candela *et al.*, 2008a). In brief, 1×10^9 CFU of stationary-phase *B. animalis* subsp. *lactis* BI07 cells grown in MRS medium in the presence or absence of 1.2 g/l bile salts (oxgall, Difco) were incubated with 0, 10, 20, 50, 100 and 200 µg of human Plg (Sigma-Aldrich). After three washings in PBS to remove unbound Plg, bifidobacterial cells were resuspended in 20 µl NH_4Cl 2% for MALDI-TOF analysis in order to detect bacteria surface bound Plg. One µl of each bacterial suspension was spotted, airdryed and then overlaid with 1.0 µl of matrix solution (12.5 mg/ml ferulic acid in a mixture of formic acid/acetonitrile/water 17:33:50). The resulting droplet was left to crystallize by airdrying and then analyzed in a Voyager DE Pro MALDI-TOF mass spectrometer (Applied Biosystems) operating in linear positive ion mode in the range of 20000-100000 Da and equipped with a pulsed N_2 laser at 337 nm. Default operating conditions were as follow: accelerating voltage 25000 V, grid voltage 22500 V, extraction delay time 750 ns. All acquisitions were generated automatically on the instrument software (Voyager™, Biospectrometry Workstation) using a random search pattern mode and based on overaging 1600 shots from 20 non-overlapping positions (80 shots/position). A standard bovine serum albumin (BSA) solution (67 kDa) was used for data external calibration. Two-hundred ng of human Plg (Sigma-Aldrich) were used as analytical standard. Three biological and three technical replicates were performed for each Plg concentration.

Plasmin activity assay

The assay was carried out as previously reported by Candela *et al.* (2008b), with minor modifications. After a washing step in PBS, 1×10^9 CFU/ml of stationary-phase *B. animalis* subsp. *lactis* BI07 cells grown in MRS with or without 1.2 g/l bile salts (oxgall, Difco) were incubated with 20 µg/ml human Plg (Sigma-Aldrich) for 30 min at 37°C. Bacteria were washed twice in PBS and resuspended in 50 mM Tris-HCl (pH 7.5). One hundred µl of bacterial suspension were added per well in a 96-well microtitre plate (Perkin Elmer) and 0.06 KIU of urokinase (uPA, Calbiochem) were added to activate Plg. A volume of 30 µl of the plasmin chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (0.54 mM; Sigma-Aldrich) was added to each well and absorbance at 405 nm was measured immediately after the addition of the chromogenic substrate and every 7 min during 1 h incubation at 37°C by using the Victor^{3}V multilabel plate reader (Perkin Elmer). As negative control, *B. animalis* subsp. *lactis* BI07 cells not treated with Plg were used. Further control for the kinetic experiment were carried out with bifidobacteria incubated with Plg in the presence of 0.1 M of the lysine analogue ε-aminocaproic acid (EACA), and in the absence of uPA. Spontaneous hydrolysis of D-Val-Leu-Lys 4-nitroanilide dihydrochloride was checked with the chromogenic substrate alone and in the presence of uPA. For each condition, two experimental replicates and ten technical replicates were performed.

Results

B. animalis subsp. *lactis* BI07 growth in the presence of bile salts

The ability of *B. animalis* subsp. *lactis* BI07 to grow in the presence of bile salts was tested. *B. animalis* subsp. *lactis* BI07 was grown in batch cultures in the absence and in the presence of bile salts at concentrations of 1.2 and 3 g/l. As reported for *B. longum* NCIMB 8809 (Sánchez *et al.*, 2005), during the exponential phase bacterial growth rates were very similar for the three different conditions (Fig. 18). Differently, the CFU/ml reached at the stationary phase was lower in the presence of bile salts, being 1.2×10^9 in the absence of bile salts, 5×10^8 and 1×10^8 in the presence of 1.2 and 3 g/l of bile salts, respectively.

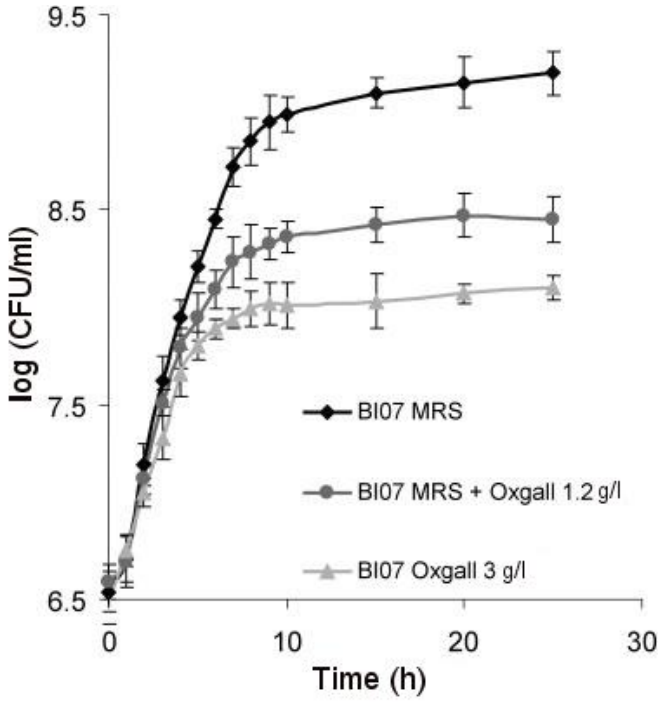


Fig. 18. Growth curves of *B. animalis* subsp. *lactis* BI07 in MRS medium with 0 (black), 1.2 (dark grey) and 3 g/l bile salts (light grey). For all the experimental conditions growth curves were performed in three biological replicates and values represent the mean ± SD.

Analysis of bifidobacterial cell wall protein expression profile in the presence and in the absence of bile salts

The impact on the cell wall proteome of long-term exposure of *B. animalis* subsp. *lactis* BI07 to bile salts was investigated, with particular attention on the expression of surface Plg receptors. To this aim, a comparative proteomic approach was carried out. Cell wall proteomes of *B. animalis* subsp. *lactis* BI07 grown in media supplemented or not with 1.2 g/l bile salts were compared by using two-dimensional gel electrophoresis, followed by software analysis to evaluate expression profile changes (Fig. 19). Bile salts adaptation involved several physiological modifications that affect cell wall proteome, as indicated in Fig. 19; Plg binding proteins numbering follows the one

reported by Candela *et al.* (2007). Interestingly, after bile salts exposure, three Plg binding proteins were up-regulated: DnaK, enolase and phosphoglycerate mutase showed a 2.8-, 2.3- and 10-fold up-regulation, respectively. The exposure to bile salts induced a slight down-regulation of the putative Plg binding protein bile salt hydrolase (BSH), by about 1.7-fold, as well as for the as yet unidentified Plg binding proteins no. 2 and no. 8.

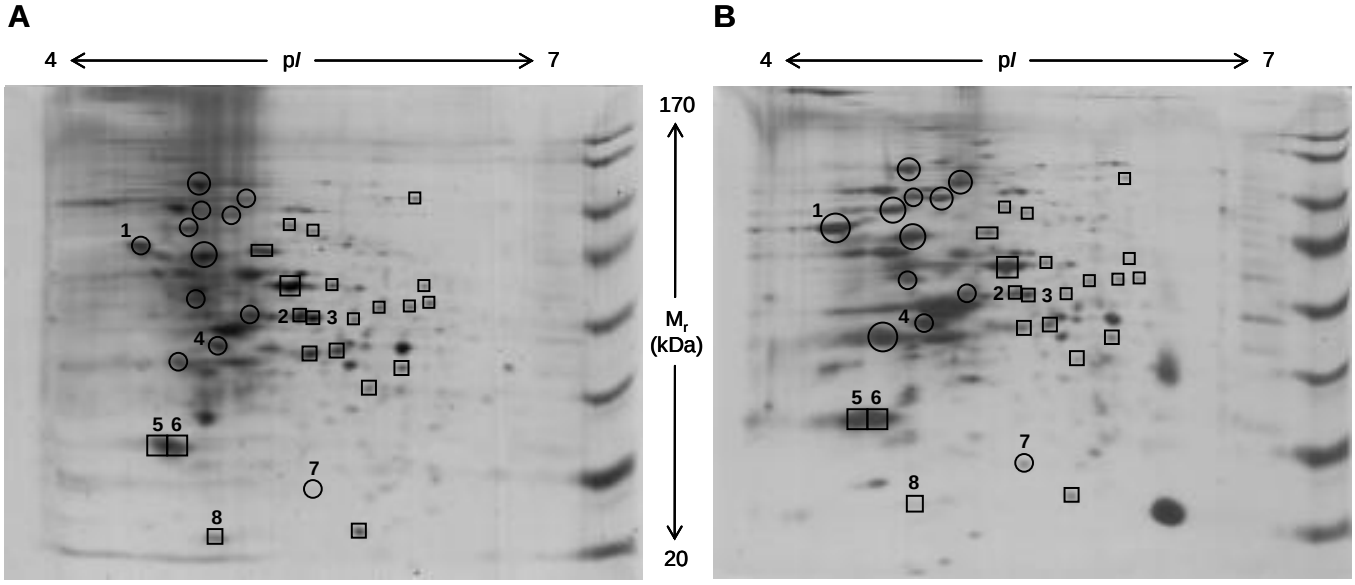


Fig. 19. Cell-wall proteome of stationary-phase *B. animalis* subsp. *lactis* BI07 cells cultured with (B) and without (A) bile salts at 1.2 g/l. Cell-wall proteins up- and downregulated as a consequence of bile salts adaptation are marked with circles and squares, respectively. Plg-binding proteins are numbered: DnaK (1), unidentified (2), glutamine synthetase (3), enolase (4), BSH (5, 6), phosphoglycerate mutase (7), unidentified (8).

B. animalis subsp. *lactis* BI07 - human plasminogen binding capability in the presence of bile salts

Physiological changes resulting from the *B. animalis* subsp. *lactis* BI07 long-term exposure to bile salts can have an important impact on the capability of this microorganism to bind human Plg. In order to elucidate if bile salts adaptation affects *Bifidobacterium* Plg binding activity, stationary-phase *B. animalis* subsp. *lactis* BI07 cells grown with or without 1.2 g/l of bile salts were incubated with increasing concentrations of human Plg and the bacterial-bound Plg was measured by MALDI-TOF-MS analysis (Fig. 20). In both conditions *B. animalis* subsp. *lactis* BI07 displayed a dose-dependent Plg binding activity, but the capability to recruit Plg on the cell surface was 10 times more high in bacteria grown in the presence of bile salts.

Plasmin activity assay was carried out to compare the capacity of *B. animalis* subsp. *lactis* BI07 grown in the absence or presence of 1.2 g/l of bile salts to acquire a surface-associated plasmin activity after incubation with human Plg and the addition of host PA. Stationary-phase cells were incubated with 20 µg/ml Plg and the cell-bound Plg was activated to plasmin by the addition of uPA. The evaluation of acquired bacterial surface-associated plasmin activity was carried out in kinetic experiments, measuring the hydrolysis of the plasmin-specific chromogenic substrate

D-Val-Leu-Lys 4-nitroanilide dihydrochloride every 7 minutes over a period of 1 h (Fig. 21). The plasmin activity associated with microorganisms grown in the presence of bile salts was greater compared to bifidobacteria grown in standard conditions, as indicated by the increased rate of chromogenic substrate hydrolysis. The inhibition of plasmin formation on the *B. animalis* subsp. *lactis* BI07 cell surface by treatment with the lysine analogue EACA underlined the crucial role of lysine residues in Plg recruitment on bacterial cell surface.

Fig. 20. MALDI-TOF analysis of the plasminogen binding activity of stationary-phase cells of *B. animalis* subsp. *lactis* BI07 grown in the presence (OX) or in the absence (MRS) of 1.2 g bile salts/l. The bars represent the response, expressed as Plg amplitude/reference signal amplitude (RS amplitude), with the standard deviation, of *B. animalis* subsp. *lactis* BI07 cells incubated with different concentrations of Plg (10, 20, 50, 100, 200 µg/ml). Reference signal: 23 000 Da.

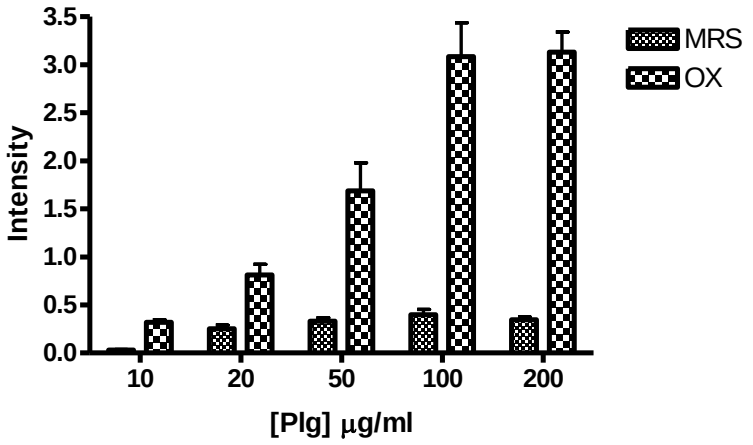
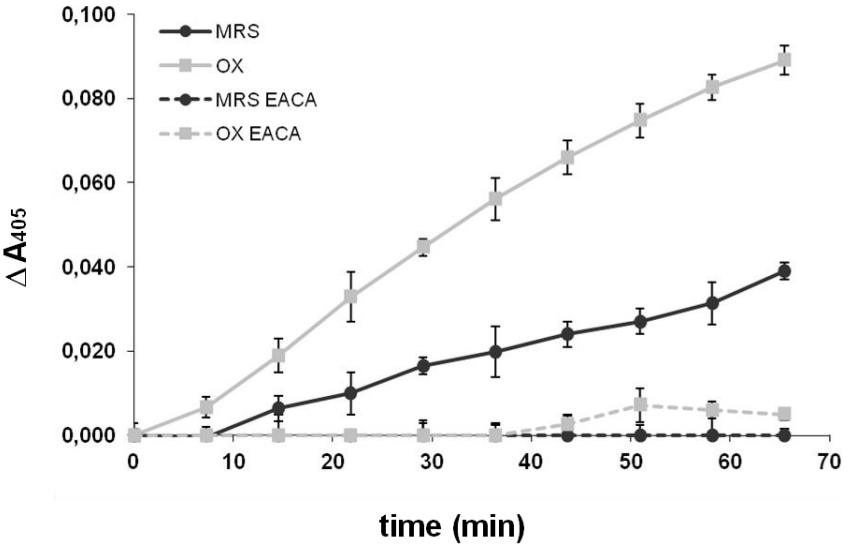


Fig. 21. Plasmin activity of *B. animalis* subsp. *lactis* BI07 cells grown in the presence (OX) or in the absence (MRS) of 1.2 g bile salts/l. Plg-pretreated bacterial cells were incubated with uPA and then with the plasmin-specific chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride. The kinetics of plasmin formation was evaluated by measuring the increase in A_{405} at intervals of 7 min during 1 h incubation. Bacterial cells untreated with Plg were used as negative control. Experiments were repeated by incubating the bacteria with Plg in the presence of 0.1 M EACA. Values represent the mean ± SEM of 20 measurements.



Discussion

B. animalis subsp. *lactis* BI07 adaptation to bile salts induces a global physiological response affecting several cell pathways and compartments. Long term exposure of *B. animalis* subsp. *lactis* BI07 to bile salts has an important impact on the cells growth: even if the growth rate during the exponential phase is similar, the bacterial concentration reached at the stationary phase is less in the presence of increasing concentrations of bile salts, probably because bacterial metabolism is more focused on the specific responses needed to cope with the stressor present in the environment. A similar growth profile has been reported for another bifidobacterial strain, *B. longum* NCIMB 8809 (Sánchez *et al.*, 2005), which has been grown in medium containing similar bile salts concentrations, described as "sublethal" because bacteria can still grow even if to a lesser amount.

Physiological adaptation to bile salts leads to several changes in the bifidobacterial cell wall proteome, as previously reported for *B. longum* (Ruiz *et al.*, 2009). Similarly to *B. longum*, *B. animalis* subsp. *lactis* BI07 exposure to a bile environment determines an up-regulation of three surface Plg binding proteins, DnaK, α-enolase and phosphoglycerate mutase. The up-regulation of DnaK has been reported as a common response to bile salts in several bifidobacterial species (Schmidt and Zink, 2000; Savijoki *et al.*, 2005; Sánchez *et al.*, 2007b). To investigate if the changes resulting from bile salts adaptation could have a role on the interaction between *B. animalis* subsp. *lactis* BI07 and human Plg, Plg binding activity and Plg-dependent plasmin activity of *B. animalis* subsp. *lactis* BI07 grown in the presence or absence of 1.2 g/l bile salts were compared. Bifidobacterial cells grown with bile salts were about 10 times more efficient in Plg binding respect to cells grown in standard medium and the addition of host uPA led to a significant higher rate of surface-associated plasmin activity. As demonstrated in this work, bile salts enhance *B. animalis* subsp. *lactis* BI07 capability to interact with the host Plg system, illustrating the potential impact of one gut environment variable on the interaction process between *Bifidobacterium* and the host. The increase in Plg surface recruitment can be partially explained by the up-regulation of surface Plg receptors, but other mechanisms can contribute, such as different Plg receptor accessibility due to changes in the bifidobacterial cell-surface structure in response to bile adaptation (Ruiz *et al.*, 2007), that can influence bacterial affinity for human Plg (Stie *et al.*, 2009).

Bifidobacterium capability to interact with host Plg system has been regarded as one of the possible mechanisms that facilitate colonization of the human gut ecosystem (Candela *et al.*, 2008b). The increased ability of *Bifidobacterium* to interact with this system as a consequence of adaptation to a gut environmental factor may favour its colonization capacity in the gastrointestinal ecosystem.

CHAPTER III

PLASMINOGEN ROLE IN THE EARLY INTERACTION BETWEEN *BIFIDOBACTERIUM* AND HUMAN ENTEROCYTES IN NORMAL AND INFLAMED CONDITIONS

Introduction

The capacity of bifidobacteria to intervene with the host Plg/plasmin system has been considered as an important actor in the interaction between *Bifidobacterium* and human intestinal epithelial cells (Sánchez *et al.*, 2008; Gilad *et al.*, 2011). The modality of interaction with host Plg/plasmin system components is similar to the one characteristic of several enteropathogens, such as *Salmonella enterica*, *Listeria monocytogenes* and *Escherichia coli* (Parkkinen and Korhonen, 1989; Schaumburg *et al.*, 2004; Lähteenmäki *et al.*, 2005; Sijbrandi *et al.*, 2005; Sun, 2005; Sebbane *et al.*, 2006). The role of Plg system in bacterial pathogenicity has been widely elucidated and the capability of microorganisms to intervene with this host system has been regarded as a paradigm of pathogenicity (Parkkinen and Korhonen, 1989; Steinert *et al.*, 2000; Schaumburg *et al.*, 2004; Lähteenmäki *et al.*, 2005; Sijbrandi *et al.*, 2005; Sebbane *et al.*, 2006; Bergmann and Hammerschmidt, 2007). Indeed, thank to the broad spectrum specificity of the enzymatic activity of plasmin, the acquisition of a Plg-dependent surface-bound proteolytic activity facilitates bacterial pathogens transmigration through tissue barriers and dissemination in the host tissues, a process named “bacterial metastasis” in analogy to the role of Plg in tumour cell invasion (Ertongur *et al.*, 2004; Setyono-Han *et al.*, 2005). Since several *Bifidobacterium* strains, such as the common human gut inhabitant *B. animalis* subsp. *lactis* (Turroni *et al.*, 2009), possess a probiotic and harmless nature, the capacity of these microorganisms to interact with host Plg/plasmin system should have a different aim than bacterial invasion, but the significance of this interaction for bifidobacterial ecology is still poorly understood.

The impact of the gut environmental status on the intestinal microbiota composition can be very deep. Inflammatory bowel disease (IBD) constitutes a group of disorders characterized by chronic and relapsing inflammation in the gastrointestinal tract frequent in Western countries (Loftus, 2004). Gut microbiota of IBD patients is characterized by a considerable depletion of health-promoting groups, as members of *Clostridium leptum* group, *Clostridium* cluster XIVa, *Bacteroidetes* and *Bifidobacterium* (Frank *et al.*, 2007; Peterson *et al.*, 2008; Sokol *et al.*, 2008, 2009). Several studies have demonstrated that intestinal inflammatory processes alter the microbial ecology of the human gut, but the molecular mechanisms involved remain almost unclear (Lupp *et al.*, 2007; Rawls, 2007; Stecher *et al.*, 2007; Walker *et al.*, 2011). It has been reported that gut inflammatory processes affect the host Plg/plasmin system equilibrium; in particular, inflammation involves an NF-κB dependent up-regulation of Plg activator uPA, leading to a clear increase of active plasmin availability in inflamed tissues (Mondino and Blasi, 2004; Okumura *et al.*, 2008; Killeen *et al.*, 2009). If the host Plg/plasmin system may have a role in the interplay between *Bifidobacterium* and the human host, inflammation dependent fluctuations of the components of the Plg/plasmin system could modulate the dynamics of interaction with the host intestinal epithelium, with consequences on the bifidobacterial ecology in the gut ecosystem.

Here the role of human Plg in the early *Bifidobacterium*-enterocyte interaction process has been investigated, evaluating this Plg-mediated interaction in two different environmental conditions, in the absence and in the presence of inflammation. As a bifidobacterial model, *B. animalis* subsp. *lactis* BI07 has been chosen. The HT29 cell line has been selected as human enterocyte model. This cell line derives from human colonic adenocarcinoma and, under standard culture conditions, shows a polarized organization similar to that found in a normal intestinal epithelial tissue, with mucus-secreting cells allowing the formation of a mucus layer on the apical side of the cell monolayer (Fogh and Trempe, 1975; Rousset, 1986). To mimic inflammatory conditions, the pro-inflammatory cytokine TNF-α has been used to stimulate HT29 cell monolayers. TNF-α has been selected because of the central role played by this cytokine in intestinal inflammation, as reported by several studies (Ma *et al.*, 2004; Yan *et al.*, 2008).

Materials and methods

Bacteria, media and growth conditions

Bifidobacterium animalis subsp. *lactis* BI07 was grown in de Man-Rogosa-Sharpe (MRS) medium (Difco) supplemented with 0.05% (wt/vol) L-cysteine at 37°C in anaerobic conditions, obtained in a jar by using Anaerocult A (Merck). Bifidobacterial cells were grown for 18 h until they reached the stationary phase. The enteropathogen *Salmonella enterica* serovar Typhimurium was cultured in brain-heart infusion (BHI) broth (Difco) in aerobiosis for 12 h at 30°C with shaking.

Eukaryotic cell culture

Human colonic epithelial HT29 cell line (German Collection of Microorganisms and Cell Cultures [DSMZ], Germany) was cultured in Dulbecco's modified Eagle's minimal essential medium with 4.5 g/l glucose (DMEM, PAA Laboratories) supplemented with 10% heat-inactivated foetal calf serum (FCS, PAA Laboratories), 1% L-glutamine (PAN Biotech GmbH) and 1% penicillin-streptomycin (100 U/ml penicillin, 100 µg/ml streptomycin) (PAN Biotech GmbH). Cells were routinely propagated in 60.1-cm^2 petri dishes (TPP) at 37°C in 5% CO_2 in a humidified atmosphere until they reached 90% confluence. For adhesion and intracellular invasion assays, 2.5×10^5 HT29 cells were seeded per well in 24-well tissue culture plates (TPP) and 12 mm-diameter glass coverslips, allowing them to grow to confluent monolayers. For transmigration assay, 1×10^5 HT29 cells were layered on 3 µm-pore size transwell inserts (Falcon - Becton Dickinson) in 24-well tissue culture plates and grown to confluent and fully differentiated monolayers. The tightness of the cell layers was verified as described by Attali *et al.* (2008a). Briefly, 200 µl of complete medium were added in the upper compartment, followed by incubation at 37°C and 5% CO_2 for 16 h. The absence of medium in the lower compartment was the proof of complete tightness of the cell layer. Twenty-four h before each assay (adhesion, invasion, transmigration), the cell medium was replaced with Interaction Medium (IM) (DMEM, 25 mM HEPES, 1 g/l glucose [Gibco], 1% FCS); to induce a pro-inflammatory response (O'Hara *et al.*, 2006), 2 ng/ml human recombinant TNF-α (Thermo Scientific) were added when necessary.

Antiserum development against *B. animalis* subsp. *lactis* BI07

Polyclonal antibodies against *B. animalis* subsp. *lactis* BI07 were generated in Balb/C mice according to standard protocols. In brief, 1×10^8 heat-inactivated *B. animalis* subsp. *lactis* BI07 cells were mixed with Freund's incomplete adjuvant and the mixture was intraperitoneally injected in 12-week-old female Balb/C mice (Jackson Laboratories). After 10 days, the antigen injection was repeated and blood was recovered after control of antibody titer after 21 days. Serum samples from non-immunized mice were used as control.

Bacterial cells incubation with plasminogen, uPA, aprotinin and anti-human plasminogen antibodies

For Plg pre-treatment, stationary phase *B. animalis* subsp. *lactis* BI07 cells were collected by centrifugation at 6000 rpm for 10 min, washed in IM and resuspended in IM at the concentration of 1×10^8 CFU/ml before the incubation with 100 µg/ml human plasminogen (Plg, Sigma-Aldrich) for 30 min at 37°C. Bacteria were washed with IM to remove unbound Plg. For uPA pre-incubation, Plg pre-treated bacterial cells were incubated with 1 µg/ml human urokinase (uPA, Sigma-Aldrich) for 30 min at 37°C, then washed with IM. Whenever required, 8 U/ml aprotinin (Sigma-Aldrich) were added to the cell monolayers 10 min before bacterial cell addition in order to inhibit plasmin activity (Bergmann *et al.*, 2005; Attali *et al.*, 2008a). To prevent the Plg-mediated adhesion to epithelial cells, Plg pre-treated *B. animalis* subsp. *lactis* BI07 cells were incubated with 5 µg/ml of polyclonal goat anti-human Plg IgG (Kordia) for 1 h at 37°C. As negative control, the incubation of Plg-untreated *B. animalis* subsp. *lactis* BI07 cells with polyclonal goat anti-human Plg IgG was carried out.

Adhesion and intracellular invasion assays, immunofluorescence microscopy and quantitative PCR

The adherence of *B. animalis* subsp. *lactis* BI07 to HT29 cell monolayers was determined by quantitative PCR (qPCR), as reported by Candela *et al.* (2005). In brief, confluent HT29 cell monolayers grown in 24-well tissue culture plates were incubated with 5×10^7 bifidobacterial CFU in IM for 1 h at 37°C and 5% CO_2. Bacterial cells in the supernatants were collected by centrifugation at 6000 rpm for 10 min and resuspended in 400 µl of PBS for subsequent qPCR quantification. After three washings with PBS, 200 µl of trypsin 0.05%/EDTA 0.02% solution (PAN Biotech GmbH) were added to each well and incubated for 10 min at 37°C. Wells were rinsed with 200 µl of PBS and bacteria adherent to HT29 cells were quantify by qPCR. Before quantification, aliquots of cell suspension were treated with 1 mg/ml trypsin inhibitor (Type I-S: from soybean, Sigma-Aldrich) in H_2O for 10 min at 25°C. Quantitative PCR reactions were performed in a LightCycler instrument (Roche), with SYBR Green I as fluorophore and the *Bifidobacterium* genus-specific primer set Bif164 (5'-CATCCGGCATTACCACCC-3') and Bif662 (5'-CCACCGTTACACCGGGAA-3') (Kok *et al.*, 1996; Vitali *et al.*, 2003). Amplification was carried out in a final volume of 20 µl containing 2 µl of cell suspension, following the instruction of the LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche) kit manual. The experimental protocol consisted of a starting denaturation step at 95°C for 10 min, followed by amplification consisting of 40 cycles of 4 steps each at the temperature transition time of 20°C/s: denaturation at 95°C for 15 s, annealing at 60°C for 25 s, extension at 72°C for 30 s, and fluorescence acquisition at 90°C for 5 s, then by melting curve analysis: heating at 20°C/s to 95°C, cooling at 20°C/s to 60°C with 15 s hold and then heating 0.2°C/s until 99°C. Serial dilutions of *B. animalis* subsp. *lactis* BI07 suspension in PBS, ranging from 1×10^6 to 1×10^3 CFU/ml, were amplified and used as internal standards for quantification. To

assess whether Plg secreted by HT29 cells contributes to the *B. animalis* subsp. *lactis* BI07 adhesion to enterocytes, the same adhesion assay was performed with HT29 cell monolayers pre-incubated with 5 µg/ml of polyclonal goat anti-human Plg IgG for 1 h at 37°C in 5% CO_2. For each experimental condition, six independent replica experiments were carried out.

Immunofluorescent studies of bifidobacterial adherence and internalization were carried out as recently described (Bergmann *et al.*, 2009), with some modifications. Confluent HT29 cell monolayers grown on 12 mm-diameter glass coverslips in 24-well tissue culture plates were incubated with 5×10^7 bifidobacterial CFU in IM per well for 1 h at 37°C and 5% CO_2. Non-adherent bacteria were removed by rinsing the HT29 cell layers three times with PBS. After washings, cells were fixed on glass coverslips with 1% paraformaldehyde at 4°C overnight. The fixed samples were blocked with PBS-10% FCS and then incubated for 1 h with mouse anti-bifidobacterial antiserum diluted 1/100 in PBS-10% FCS, followed by 30 min of incubation with Alexa-Fluor 488-labelled goat anti-mouse IgG (green, dilution 1/200; MoBiTec) at 25°C at dark. After permeabilization of the cells with 0.1% Triton X-100 for 5 min, HT29 cells were stained with Alexa-Fluor 568-labelled phalloidin (red; Molecular Probes) diluted 1/200 in PBS-10% FCS and incubated for 45 min at 25°C at dark. To differentiate extracellular from intracellular bacteria, fixed samples were stained with mouse anti-bifidobacterial antiserum in combination with a secondary Alexa-Fluor 488-labelled goat anti-mouse IgG (green, dilution 1/200) at 25°C at dark. After several washings with PBS, samples were permeabilized with 0.1% Triton X-100 for 5 min and then incubated with mouse anti-bifidobacterial antiserum, followed by 30 min of incubation at 25°C at dark with Alexa-Fluor 568-labelled goat anti-mouse IgG (red, MoBiTec), diluted 1/300 in PBS-10% FCS. This procedure results in Alexa-Fluor 568-labelled intracellular bacteria (red) and Alexa-Fluor 488/568-labelled extracellular bacteria (green/yellow). Glass coverslips were mounted in ProLongGold (Invitrogen) and incubated overnight before observation (fluorescence microscope Axiophot, Zeiss). The Axio Vision Rel 4.7.2 software was used for image acquisition and ImageJ software to process images for contrast and brightness. Pictures of the same area were taken for each sample under both fluorescent light and phase contrast. HT29 cells and bacteria were counted in at least 8 different microscopic views for each coverslip. For each experimental condition, three independent adhesion experiments were performed. After 1 h incubation in IM in 5% CO_2 atmosphere, *B. animalis* subsp. *lactis* BI07 survival was checked by plating bacterial suspension on MRS agar plates.

Intracellular invasion assays of *Salmonella enterica* serovar Typhimurium were performed as described by Hess *et al.* (2002). Briefly, 1×10^8 CFU/ml *S.* Typhimurium suspension in IM were added to confluent HT29 cell monolayers grown in 24-well tissue culture plates and incubated for 1 h at 37°C in 5% CO_2. After five washing steps in PBS, 1 ml of IM containing 50 µg/ml gentamicin sulphate (Cambrex/BioWhittaker) was added each well and plates were incubated for 2.5 h at 37°C in 5% CO_2. HT29 cell monolayers were washed five times with PBS and lysed with 1% Triton X-100 for 5 min at 37°C. Intracellular bacteria were quantified by counting after culture on BHI agar plates. Invasion experiments were repeated six times.

Transmigration assay

The assay was carried out following the protocols described in Hess *et al.* (2002) and Attali *et al.* (2008a). A volume of 200 µl of a bacterial suspension containing 1×10^8 CFU/ml *B. animalis* subsp. *lactis* BI07 or *S.* Typhimurium in IM was added to the upper compartment of a transwell insert with membrane pore size of 3 µm (Falcon - Becton Dickinson) and incubated for 1 h at 37°C in 5% CO_2. The lower compartment contained 600 µl of IM. To quantify bacterial transmigration by colony counting, the basal chamber was sampled, serially diluted and plated on the appropriate agar medium. The assay was done twice for each experimental condition.

Evaluation of plasminogen activation by HT29 cells

This assay was carried out as described by Attali *et al.* (2008a) with slight modifications. In brief, HT29 cells were grown to confluent monolayers on 24-well tissue culture plates. Twenty-four h before the assay, the medium was replaced with DMEM 1 g/l glucose without phenol red (Sigma-Aldrich) supplemented with 1% FCS in the presence or absence of 2 ng/ml TNF-α. After medium removal, 200 µl of 100 µg/ml human Plg in PBS were added to each well and incubated for 1 h at 37°C. Positive and negative controls were carried out without cells using 200 µl of 100 µg/ml human Plg in PBS in the presence or absence of 0.5 µg uPA, respectively. After Plg incubation, 100 µl of the plasmin chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (0.54 mM; Sigma-Aldrich) were added to each well and incubated for 30 min at 37°C. Subsequently, 150 µl of the supernatant were transferred to transparent 96-well microtiter plates (Nunc) and absorbance at 405 nm was measured using the Victor^{3}V multilabel plate reader (Perkin Elmer). The assays were performed two times independently, repeating each experimental condition four times.

Statistical analysis

Data were expressed as the mean ± s.e.m. . The statistical significance of between-group comparisons was determined with Mann-Whitney U test, and the probability value $P < 0.001$ was considered statistically significant.

Results

Plasminogen role in the interaction between *B. animalis* subsp. *lactis* BI07 and HT29 cells

At first, the contribution of endogenous Plg secreted by HT29 cells on the adhesion of *B. animalis* subsp. *lactis* BI07 to the enterocyte surface was evaluated. To this aim, the adhesion of *B. animalis* subsp. *lactis* BI07 cells to HT29 cell monolayers untreated or pre-treated with polyclonal anti-human Plg IgG was compared by qPCR. In accordance to our data, the anti-human Plg antiserum pre-treatment resulted in a slight decrease of bifidobacterial adhesion to the enterocyte surface (-16%), although this decrease was not significant ($P > 0.05$). These data pointed out that the endogenously produced Plg of HT29 cells exerted only a marginal contribution to the *B. animalis* subsp. *lactis* BI07 adhesion to HT29 cells. The role of Plg in the *B. animalis* subsp. *lactis* BI07-enterocyte interaction process was therefore evaluated by pre-incubating bifidobacterial cells with 100 µg/ml human Plg, as reported by Attali *et al.* (2008a), and then bacterial adhesion to HT29 cells was assessed with a conventional adhesion assay. In particular, *B. animalis* subsp. *lactis* BI07 cells were pre-incubated with human Plg alone or Plg + uPA, and the interaction with the enterocyte cell line HT29 was compared with the one shown by untreated bifidobacterial cells. Adherence of bifidobacteria was quantify by qPCR (Table 5). *B. animalis* subsp. *lactis* BI07 adhesion to HT29 cell monolayers was significantly enhanced by human Plg pre-incubation (+225%, $P < 0.001$); specifically, Plg pre-treatment increased bifidobacterial adhesion from 18.87 ± 1.20 to 61.08 ± 2.85 bacteria/HT29 cell. Plg contribution to the bifidobacterial adhesion to HT29 enterocyte cell surface was completely abolished when Plg pre-treated *B. animalis* subsp. *lactis* BI07 cells were incubated with polyclonal anti-human Plg IgG (Table 5). Interestingly, the addition of the Plg activator uPA lowered the adhesion of *B. animalis* subsp. *lactis* BI07 pre-treated with Plg to 28.87 ± 1.93 bacteria/HT29, a drop of 50% with respect to the value obtained without uPA ($P < 0.001$). In order to determine whether the acquisition of a cell surface plasmin activity was involved in the reduction of *B. animalis* subsp. *lactis* BI07 adhesion to HT29 cells, the assay was repeated in the presence of the plasmin inhibitor aprotinin (Table 5). Aprotinin addition determined the complete recovery of the Plg-dependent HT29 cell adhesion of *B. animalis* subsp. *lactis* BI07 cells pre-incubated with Plg + uPA. Aprotinin did not exert any impact on the *B. animalis* subsp. *lactis* BI07 adhesion to HT29 cell monolayers in the absence of Plg pre-treatment (Table 5). These data demonstrated that the uPA-dependent acquisition of bacterial cell surface plasmin activity was enough to reduce the contribution of human Plg to *B. animalis* subsp. *lactis* BI07 adhesion to the HT29 cell surface. The visualization of bifidobacterial adherence to HT29 cell monolayers by immunofluorescence microscopy confirmed the increase in amount of attached bacteria after Plg pre-incubation and the impact of uPA and aprotinin on bifidobacterial adhesion (Fig. 22).

Table 5 - qPCR quantification of the plasminogen-mediated adhesion of *B. animalis* subsp. *lactis* BI07 to untreated and TNF-α pre-treated HT29 cells.

	No. of bacteria / HT29 cell (mean ± SD)	
Experimental condition(s)[a]	**No treatment**	**+ TNF-α**
BI07	18.87 ± 1.20	26.65 ± 2.02
BI07 + Plg	61.08 ± 2.85	41.09 ± 2.74
BI07 + Plg + anti-Plg	19.40 ± 1.24	-
BI07 + Plg + uPA	28.87 ± 1.93	36.57 ± 2.27
BI07 + Plg + uPA + Aprotinin	81.15 ± 3.58	79.81 ± 3.52
BI07 + Plg + Aprotinin	-	75.96 ± 5.67
BI07 + Aprotinin	20.23 ± 2.21	23.55 ± 1.55

[a]: Untreated and TNF-α pre-treated (+ TNF-α) confluent HT29 cell monolayers were incubated with 5 x 10^7 CFU of: untreated *B. lactis* BI07 cells (BI07); Plg pre-treated *B. lactis* BI07 cells (BI07 + Plg); Plg pre-treated *B. lactis* BI07 cells incubated with anti-Plg IgG (BI07 + Plg + anti-Plg); Plg and uPA pre-treated *B. lactis* BI07 cells (BI07 + Plg + uPA); Plg and uPA pre-treated *B. lactis* BI07 cells in the presence of aprotinin (BI07 + Plg + uPA + Aprotinin); Plg pre-treated *B. lactis* BI07 cells in the presence of aprotinin (BI07 + Plg + Aprotinin); *B. lactis* BI07 cells in the presence of aprotinin (BI07 + Aprotinin).

Enterocyte internalization and transmigration of *B. animalis* subsp. *lactis* BI07 untreated, pre-treated with Plg or with Plg and uPA was studied. HT29 cells internalization was evaluated by double immunofluorescence microscopy, enabling a differential staining of intracellular (red) and adherent (green/yellow) bacteria. Six independent interaction experiments were performed for each experimental condition. As shown in Fig. 23, microscopic analysis of the whole HT29 cell monolayer did not reveal any bifidobacterial internalization by the HT29 cell line in any of the tested conditions. As a control, the enterocyte internalization of the pathogen *S. enterica* serovar Typhimurium was investigated using a gentamicin protection assay (Hess *et al.*, 2002). Accordingly to data reported by Hess and co-workers (Hess *et al.*, 2002), after 1 h of incubation with 1 × 10^8 CFU of *S. enterica*, a HT29 cell internalization value of 9.32 ± 1.58 × 10^4 CFU of this microorganism was determined. In accordance to the results obtained by qPCR, in double immunofluorescence microscopy experiments Plg pre-treatment increased the adhesion of *B. animalis* subsp. *lactis* BI07 to HT29 cell monolayers (Fig. 23, +Plg). With the purpose of analyzing the transmigration of *B. animalis* subsp. *lactis* BI07 through HT29 cell monolayers, a transwell system-based transmigration assay was carried out. Bacterial cells were added to the upper compartment of the transwell inserts containing the HT29 cell monolayers and, after 1 h of co-incubation, bacterial transmigration was quantified by plating the medium from the lower compartment. Interestingly, neither untreated nor Plg pre-treated nor Plg and uPA pre-treated *B. animalis* subsp. *lactis* BI07 cells displayed transmigration through HT29 cell monolayers (data not shown). As a control, the transmigration of 2 × 10^7 CFU of *S. enterica* serovar Typhimurium bacteria through HT29 cell

monolayers was evaluated. Accordingly to data reported by Hess *et al.* (2002), $7.84 \pm 1.03 \times 10^5$ bacteria penetrated from the apical to the basal compartment of HT29 cell monolayers. Taken together these data demonstrated that Plg recruited on the *B. animalis* subsp. *lactis* BI07 cell surface enhances bacterial adhesion to the enterocytes, whereas the activation of bacterial bound Plg to plasmin by host uPA results in a significant decrease of the Plg-mediated adhesion process. The acquisition of a cell surface plasmin activity does not result in *B. animalis* subsp. *lactis* BI07 invasion of host intestinal epithelial cells or transmigration through enterocyte monolayers.

TNF-α impact on the plasminogen-mediated interaction between *B. animalis* subsp. *lactis* BI07 and HT29 cells

HT29 cell monolayers were stimulated with the pro-inflammatory cytokine TNF-α to mimic an inflammatory condition and the interaction between these layers and untreated, Plg or Plg + uPA pre-treated *B. animalis* subsp. *lactis* BI07 cells was studied by qPCR and immunofluorescence microscopy analysis, as previously described. In TNF-α incubated HT29 cell monolayers, only a minor increase of Plg pre-treated *B. animalis* subsp. *lactis* BI07 adhesion to enterocytes was detected. While untreated bifidobacterial cells showed an adhesion value of 26.65 ± 2.02 bacteria/HT29 cell, Plg pre-treatment resulted in a bacterial adhesion value of 41.09 ± 2.74 bacteria/HT29 cell (Table 5). Furthermore, after HT29 monolayers pre-incubation with TNF-α, comparable adhesion values were shown by Plg pre-treated and Plg + uPA pre-treated *B. animalis* subsp. *lactis* BI07 cells (Table 5). These data suggested that HT29 cell response to TNF-α stimulation significantly affects Plg-mediated contribution to *B. animalis* subsp. *lactis* BI07 adhesion to enterocyte surface. Plg-dependent *B. animalis* subsp. *lactis* BI07 adherence to TNF-α incubated HT29 cell monolayers was efficiently recovered with the addition of aprotinin, both for Plg and Plg + uPA pre-treated bifidobacterial cells (Table 5). Similarly to data obtained in non-inflamed conditions, aprotinin did not exert any effect on the *B. animalis* subsp. *lactis* BI07 adhesion to TNF-α stimulated HT29 cells in the absence of pre-treatment with Plg. Quantitative PCR data of bifidobacterial adherence were confirmed by the immunofluorescence microscopy visualization of *B. animalis* subsp. *lactis* BI07 adhesion to HT29 cell monolayers (Fig. 24). After bifidobacterial adhesion in inflamed conditions, the analysis of bacterial internalization and transmigration was carried out. Similarly to the non inflamed status, bacterial internalization (Fig. 25) and transmigration (data not shown) were not found after the co-incubation of untreated, Plg or Plg + uPA pre-treated *B. animalis* subsp. *lactis* BI07 cells with TNF-α stimulated HT29 cell monolayers.

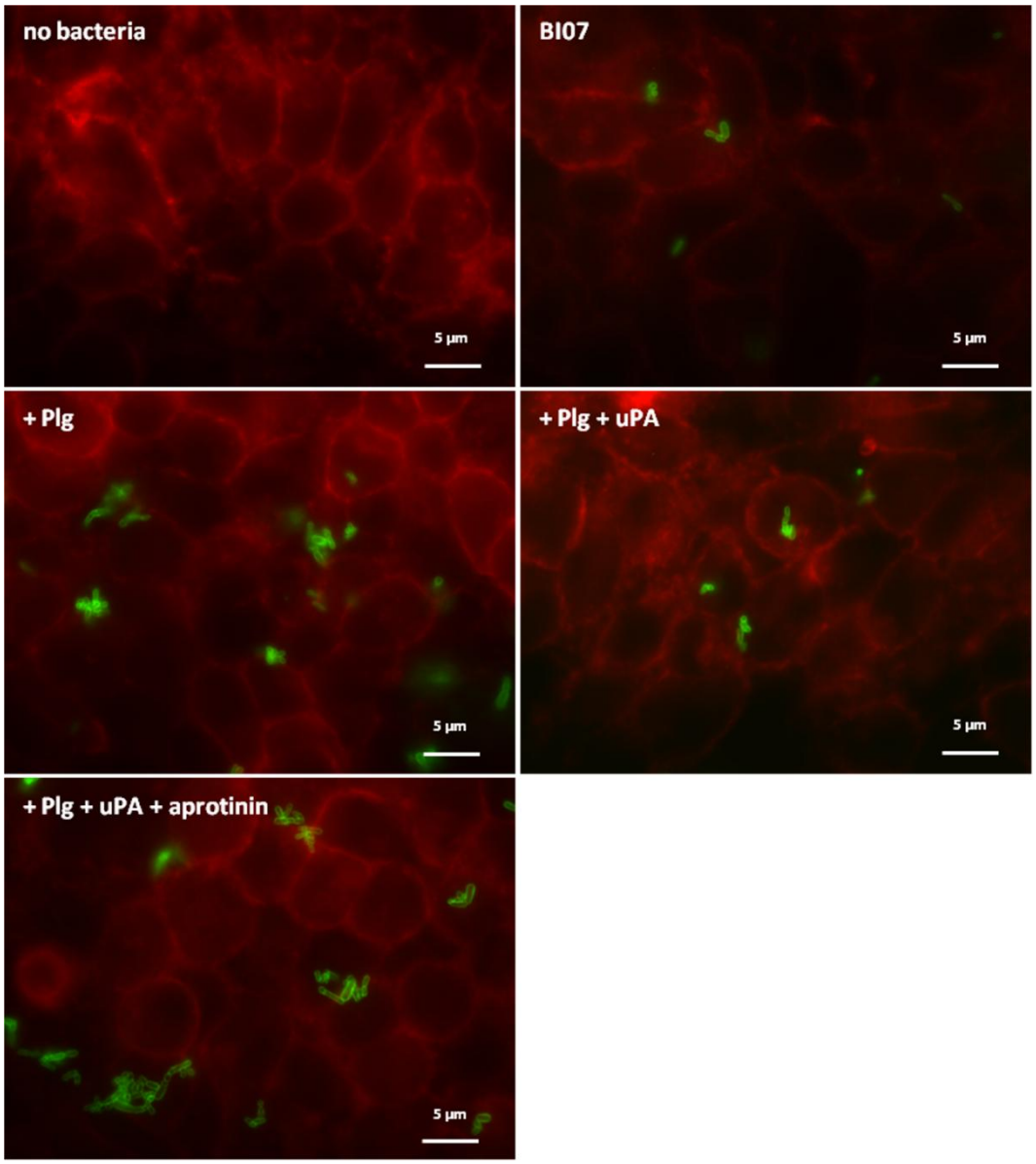


Fig. 22. Immunofluorescence microscopy visualization of *B. animalis* subsp. *lactis* BI07 adhesion to HT29 cells. Confluent HT29 cell monolayers were incubated with 5 x 10^7 CFU of *B. animalis* subsp. *lactis* BI07 untreated (BI07), pre-treated with Plg (+Plg), pre-treated with Plg and uPA (+Plg+uPA) and with Plg and uPA in the presence of aprotinin (+Plg+uPA+aprotinin). Adherent bacterial cells were visualized (magnification: X 100) by staining with mouse anti-bifidobacterial antiserum and Alexa-Fluor 488-conjugated anti-mouse antibody (green). HT29 cells were labelled with Alexa-Fluor 568-conjugated phalloidin (red). Assays were repeated three times, and a representative experiment is shown for each experimental condition.

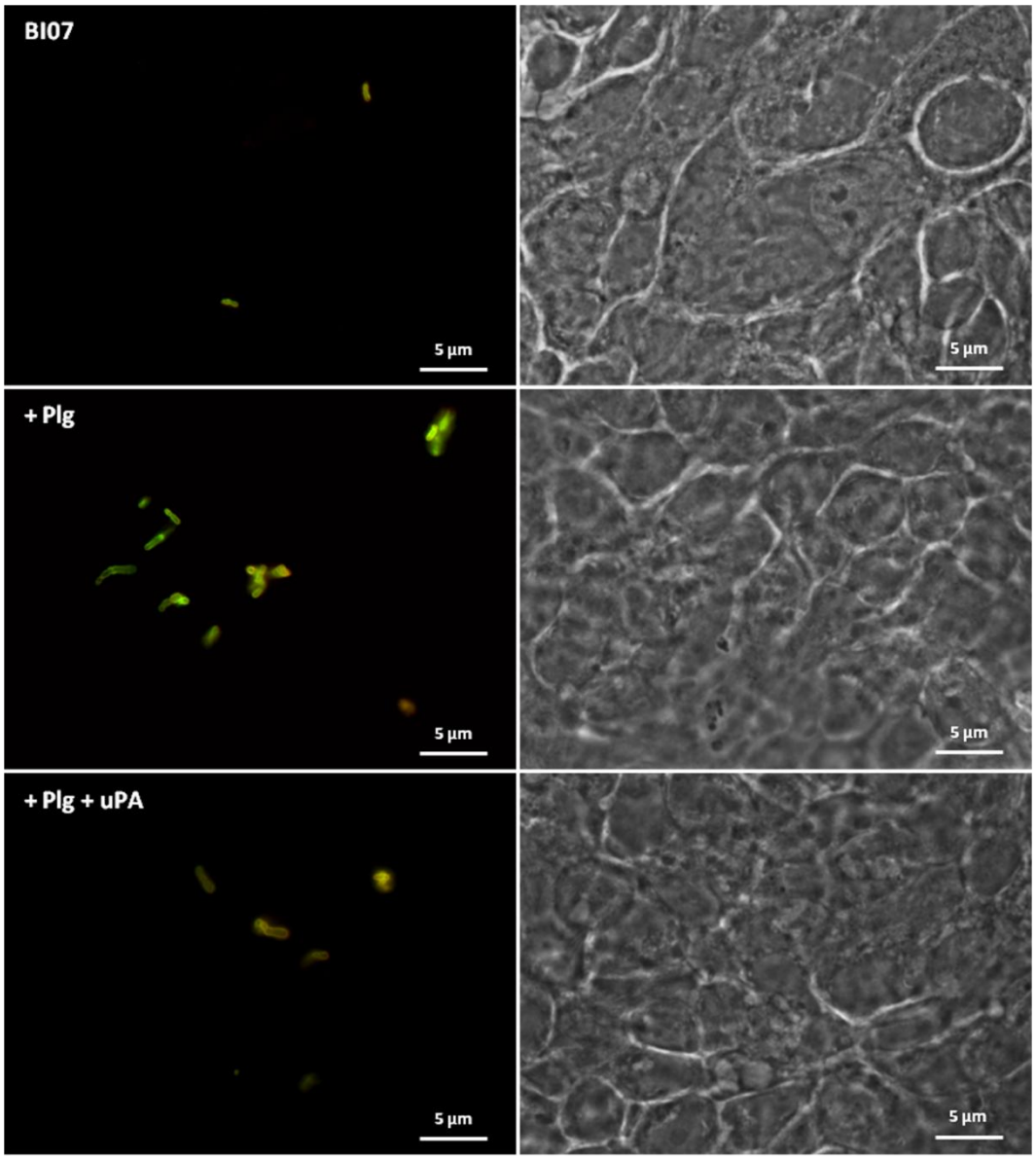


Fig. 23. Intracellular invasion assay of *B. animalis* subsp. *lactis* BI07 into HT29 cells. HT29 cell monolayers grown to confluence were incubated with 5 x 10^7 CFU of *B. animalis* subsp. *lactis* BI07 untreated (BI07), pre-treated with Plg (+Plg) and pre-treated with Plg and uPA (+Plg+uPA). Intracellular and adherent bacteria were differentiated by double immunofluorescence microscopy. Adherent *B. animalis* subsp. *lactis* BI07 bacteria were stained with mouse anti-bifidobacterial antiserum followed by a secondary Alexa-Fluor 488-conjugated anti-mouse antibody (green). After HT29 cell permeabilization, internalized bacteria were stained with mouse anti-bifidobacterial antiserum and a secondary Alexa-Fluor 568-conjugated anti-mouse antibody (red). The corresponding phase contrast images are shown in the right panel (magnification: X 100). For each experimental condition, the assay was repeated three times. A representative experiment is shown.

In order to investigate whether stimulation with TNF-α cytokine favoured migration of Plg pre-treated *B. animalis* subsp. *lactis* BI07 to the lumen compartment, quantification of bifidobacterial cells present in the supernatant of TNF-α incubated and non-incubated HT29 cell monolayers was performed. qPCR analysis allowed the detection of $1.91 \pm 0.55 \times 10^7$ bifidobacterial cells in the supernatant of TNF-α stimulated HT29 cells, a value two times higher than the amount of bifidobacteria detected in the supernatant from untreated HT29 cell monolayers ($9.22 \pm 1.78 \times 10^6$ bifidobacterial cells). Together, these data suggest that enterocyte inflammatory response triggers the activation of the *B. animalis* subsp. *lactis* BI07 cell surface bound Plg to plasmin, lowering its adhesion to HT29 cell surface and shifting bacterial migration to the enterocyte supernatant.

Evaluation of plasminogen activation by HT29 cells pre-incubated or not with TNF-α

To determine if a TNF-α mediated pro-inflammatory response enhanced Plg activation by HT29 cells, a plasmin activity assay was carried out. Untreated and TNF-α pre-treated HT29 cell monolayers were incubated with human Plg and activation to the proteolytic form plasmin was assessed by measuring the hydrolysis of the plasmin-specific chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride. TNF-α stimulated HT29 cells displayed a two-fold increase of Plg activation compared to untreated enterocytes ($P < 0.001$), proving the impact of a TNF-α pro-inflammatory response on the HT29 cell Plg activators system.

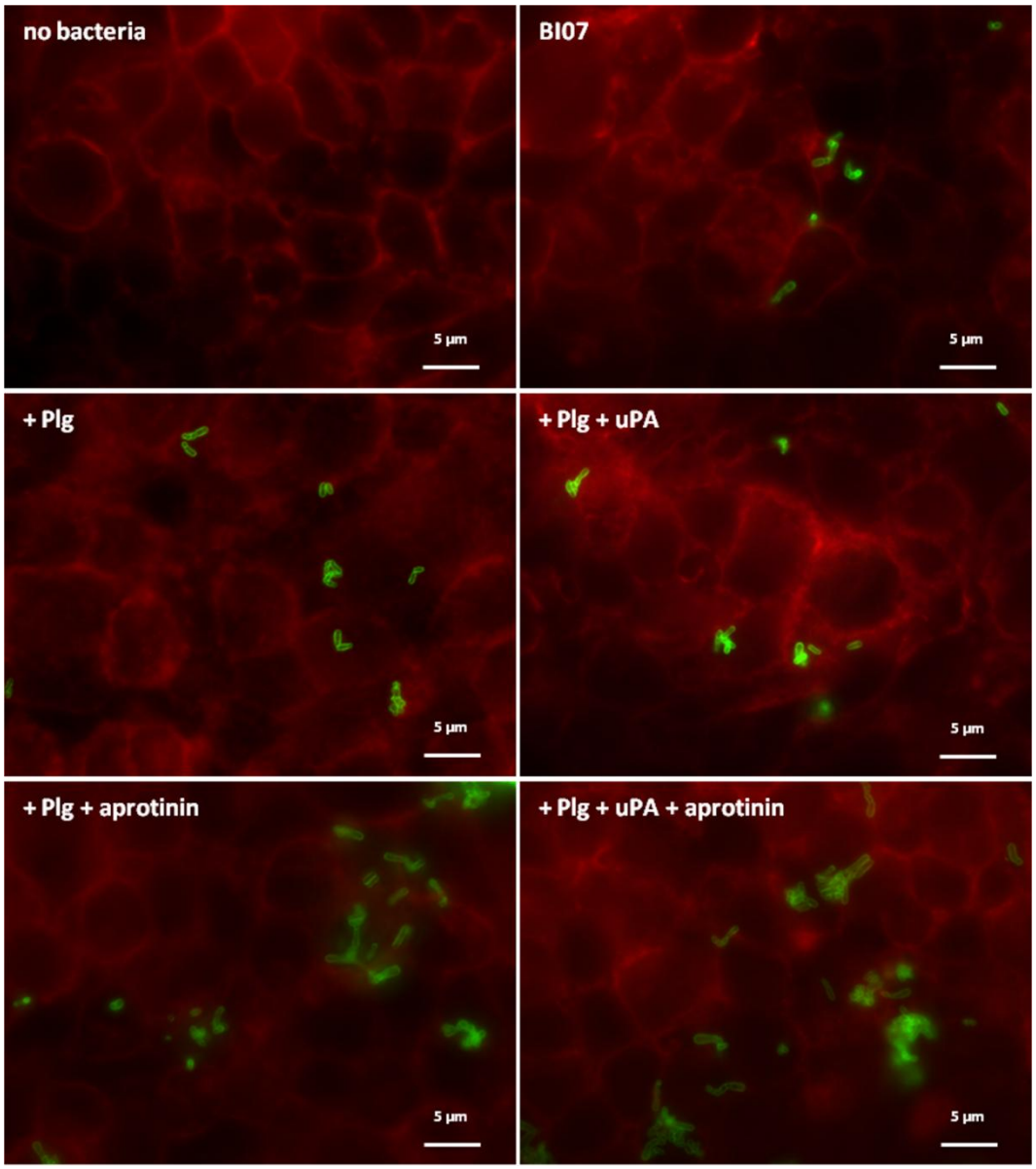


Fig. 24. Immunofluorescence microscopy visualization of *B. animalis* subsp. *lactis* BI07 adhesion to TNF-α stimulated HT29 cells. Confluent HT29 cell monolayers were pre-treated with TNF-α and incubated with 5 x 10^7 CFU of *B. animalis* subsp. *lactis* BI07 untreated (BI07), pre-treated with Plg (+Plg), pre-treated with Plg and uPA (+Plg+uPA), and with Plg or Plg and uPA in the presence of aprotinin (+Plg+aprotinin; +Plg+uPA+aprotinin). Adherent bacterial cells were visualized (magnification: X 100) by staining with mouse anti-bifidobacterial antiserum and Alexa-Fluor 488-conjugated anti-mouse antibody (green). HT29 cells were labelled with Alexa-Fluor 568-conjugated phalloidin (red). Assays were repeated three times, and a representative experiment is shown for each experimental condition.

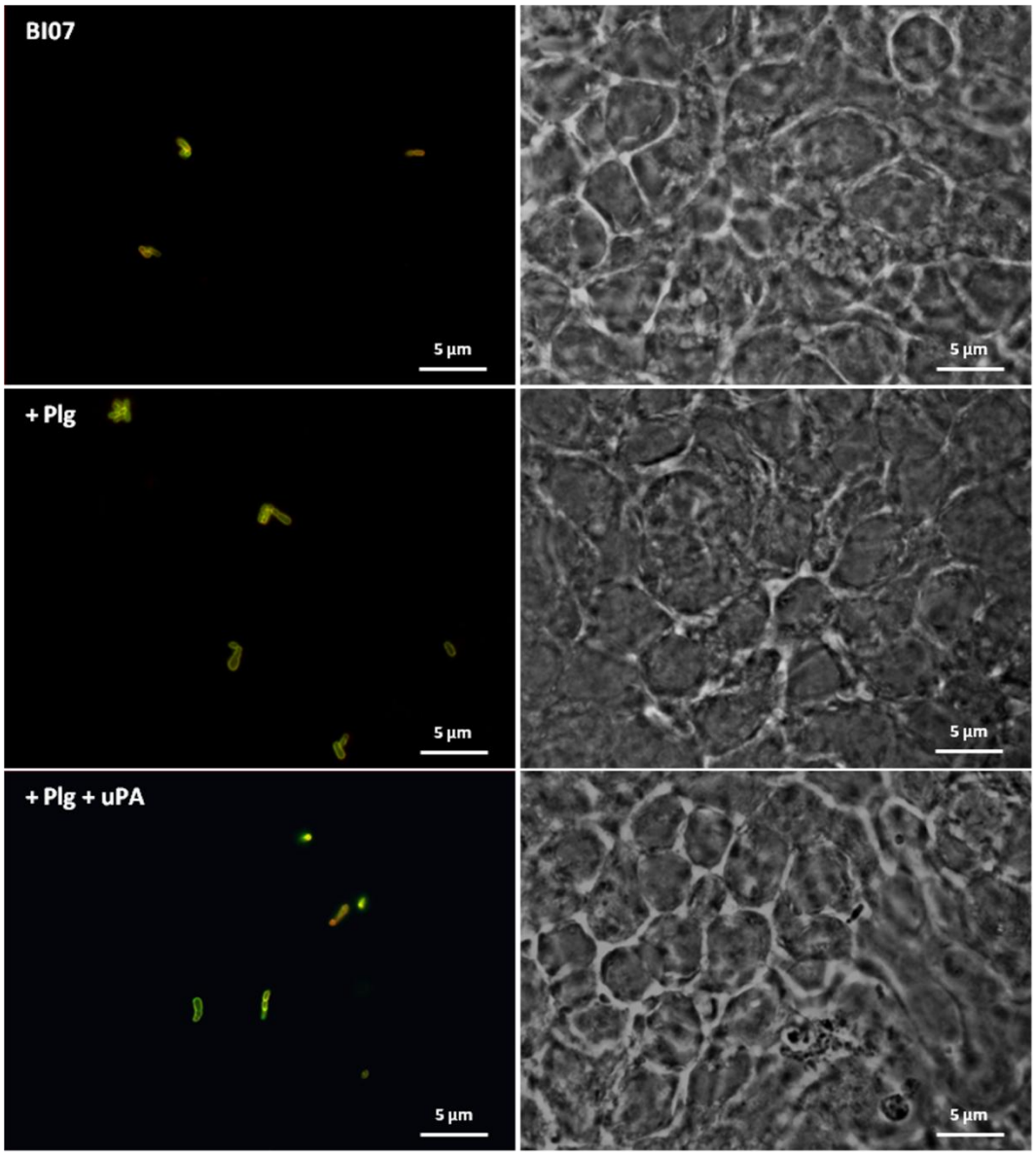


Fig. 25. Intracellular invasion assay of *B. animalis* subsp. *lactis* BI07 into TNF-α pre-treated HT29 cells. TNF-α pre-treated confluent HT29 cell monolayers were incubated with 5 x 10^7 CFU of *B. animalis* subsp. *lactis* BI07 untreated (BI07), pre-treated with Plg (+Plg) and pre-treated with Plg and uPA (+Plg+uPA). Intracellular and adherent bacteria were differentiated by double immunofluorescence microscopy. Adherent *B. animalis* subsp. *lactis* BI07 bacteria were stained with mouse anti-bifidobacterial antiserum followed by a secondary Alexa-Fluor 488-conjugated anti-mouse antibody (green). After a permeabilization step, invading bacteria were stained with mouse anti-bifidobacterial antiserum and a secondary Alexa-Fluor 568-conjugated anti-mouse antibody (red). The corresponding phase contrast images are shown in the right panel (magnification: X 100). For each experimental condition, the assay was repeated three times and a representative experiment is shown.

Discussion

The host Plg system can represent an important tool used by *Bifidobacterium* in the dynamics of cross-talk with the human host. To elucidate the significance of this host system in the starting events of the interaction process between bifidobacteria and human intestinal epithelial cells, the role of human Plg in the *Bifidobacterium animalis* subsp. *lactis* BI07-HT29 cells early interaction has been studied. As reported in this chapter, human Plg recruited on *B. animalis* subsp. *lactis* BI07 cell surface significantly enhanced the adherence of this microorganism to the host enterocytes. Bacterial bound Plg can thus act as a "molecular bridge" between bifidobacterial and human enterocytes receptors, increasing bacterial adhesion to the host intestinal epithelium, as suggested by Pancholi and co-workers for streptococcal adhesion to pharingeal cells (Pancholi *et al.*, 2003). In the presence of host uPA, *B. animalis* subsp. *lactis* BI07 acquired a surface-associated proteolytic activity that caused a reduction in bacterial adherence to enterocytes surface and supported the migration of bacterial cells to the enterocyte supernatant compartment. *B. animalis* subsp. *lactis* BI07 acquisition of surface-associated proteolytic activity did not result in enterocyte internalization or bacterial transmigration through enterocyte monolayers, demonstrating the strict commensal nature of this health-promoting species. Thus, even if the modality of interaction with host Plg system components is similar both for the probiotic microorganism *B. animalis* subsp. *lactis* BI07 and pathogenic bacteria (Parkkinen and Korhonen, 1989; Schaumburg *et al.*, 2004; Lähteenmäki *et al.*, 2005; Sijbrandi *et al.*, 2005; Sun, 2005; Sebbane *et al.*, 2006), these findings demonstrate that the outcomes of this interaction with regard to host colonization are different. Cell surface bound Plg increases bacterial adhesion to host epithelium both for pathogens and *B. animalis* subsp. *lactis* BI07 (Pancholi *et al.*, 2003; Attali *et al.*, 2008a), but activation of this surface associated Plg to the active form plasmin by host PAs determines a different phenotype of interaction with the host. Indeed, *B. animalis* subsp. *lactis* BI07 acquisition of cell surface-bound plasmin activity does not determine pericellular invasion, a common behaviour for pathogenic bacteria, but favours bifidobacterial migration to the luminal compartment. The capability to interact with host Plg system cannot be considered a virulence factor by itself, as this characteristics is shared among symbionts and pathogens (Antikainen *et al.*, 2007b; Kinnby *et al.*, 2008). This property could instead be viewed as an ancestral mechanism of interaction between bacteria and the host, evolved before the radiation of pathogens from commensal microorganisms (Ochman *et al.*, 2000; Blaser and Kirschner, 2007). The ability to intervene with this host system becomes a fundamental tool for host invasion only in association with other pathogenicity attributes, as it happens for pathogens (Sun *et al.*, 2004).

The outcome of the interaction *Bifidobacterium* - host Plg system - intestinal epithelium can be modulated by variables present in the intestinal environment, such as the occurrence of intestinal mucosa inflammation. To this aim, this interaction has been studied in a simplified model of inflamed intestinal epithelium, HT29 cells stimulated with the pro-inflammatory cytokine TNF-α

(Ma *et al.*, 2004; Yan *et al.*, 2008). Mimicking the situation in an inflamed human intestinal tract (Mondino and Blasi, 2004; Okumura *et al.*, 2008), accordingly to our data TNF-α mediated inflammation of HT29 cells enhanced the ability of these cells to activate human Plg, most likely by up-regulation of uPA activity. In a recent study, NF-κB activation mediates up-regulation of uPA activity in response to pro-inflammatory stimuli in human colorectal cell lines (Killeen *et al.*, 2009); thus, in HT29 cell line model, the observed TNF-α-dependent increase of Plg activation capacity might be dependent on the activation of NF-κB pathway via TNF-α (Vallee *et al.*, 2004). Our data showed that TNF-α modulates the dynamics of the Plg-mediated interaction between *B. animalis* subsp. *lactis* BI07 and HT29 enterocytes: the TNF-α mediated inflammatory response enhances HT29 cell capacity to activate human Plg, promoting the activation of the *B. animalis* subsp. *lactis* BI07 cell surface associated Plg to plasmin. This reduces Plg-dependent contribution to the bacterial adhesion and favours bacterial migration to the lumen compartment. Confirming these findings, the presence of the plasmin inhibitor aprotinin was sufficient to restore the Plg-mediated adhesion of *B. animalis* subsp. *lactis* BI07 to inflamed HT29 cells. Similarly to the results described for non-inflamed HT29 cells, no evidence of intracellular invasion or transmigration were observed even in TNF-α stimulated enterocytes, further confirming the commensal nature of *B. animalis* subsp. *lactis* BI07.

These data suggest that the phenotype of interaction of *B. animalis* subsp. *lactis* BI07 with the host Plg/plasmin system depends on the physiological status of the intestinal epithelium: in non-inflamed regions of the gut epithelium, Plg increases *B. animalis* subsp. *lactis* BI07 adherence, while the presence of an inflammation-dependent epithelial response would activate bacterial surface associated Plg to plasmin, favouring bifidobacteria shifting to the luminal compartment. It can be hypothesized that this peculiar dynamics of interaction between *B. animalis* subsp. *lactis* BI07 and the host Plg system could be relevant in the microecology of *Bifidobacterium* in the human gut. In non-inflamed intestinal tissue sites, the suitable ecological niches for *B. animalis* subsp. *lactis* BI07, this microorganism can make use of the available Plg to enhance adhesion to the gut mucosa. In case of inflamed gut regions, the acquisition of surface-bound plasmin activity allows the microorganism to shift to the luminal compartment, going away from the inflamed sites. Since the host Plg/plasmin system components fluctuate in response to the inflammatory conditions of the gut environment, this system can be used by bifidobacteria as a "sensor system" to detect the inflammatory status of the surrounding gut microenvironment. As bifidobacteria are unable to face an inflamed environment (Stecher and Hardt, 2008), the host Plg system may be used to sense and escape gut inflammation, allowing *B. animalis* subsp. *lactis* BI07 to get away from inflamed regions. The model of interaction described here might be one of the mechanisms involved in the reduction of the relative abundance of *Bifidobacterium* observed in IBD patients (Sokol *et al.*, 2009; Veiga *et al.*, 2010). The differences in the phenotype of interaction with the host Plg system between *B. animalis* subsp. *lactis* BI07 and enteropathogens could become relevant in the context of inflammation-dependent dysbiosis in the human gut. It is broadly known that enteropathogens, such as *S. enterica* or *Campylobacter jejuni*, take advantage of host

inflammatory response to overcome symbiont microbiota members, as bifidobacteria (Lupp *et al.*, 2007; Rawls, 2007; Stecher *et al.*, 2007; Stecher and Hardt, 2008). Enteropathogens utilize the inflammation-dependent increase in plasmin availability to invade host tissue (Parkkinen and Korhonen, 1989; Schaumburg *et al.*, 2004; Lähteenmäki *et al.*, 2005; Sijbrandi *et al.*, 2005; Sun, 2005; Sebbane *et al.*, 2006), a behaviour completely different from bifidobacteria, that use surface-bound plasmin to escape from inflamed sites. The opposite behaviour of symbionts and pathogens with respect to plasmin role in the interaction process with the host may support the shift of balance from protective microbiota to pathogens reported in inflamed gastrointestinal sites. Nevertheless, this hypothesis needs to be demonstrated by *in vivo* studies designed to verify host Plg system role in the inflammation-dependent dysbiosis in the human gut.

CHAPTER IV

BIFIDOBACTERIUM ANIMALIS SUBSP. *LACTIS* B107 - PLASMINOGEN INTERACTION IN A HUMAN GUT MICROENVIRONMENT

Introduction

The capability to interact with the host Plg/plasmin system has been considered as a possible novel component in the molecular cross-talk between bifidobacteria and the host (Sánchez *et al.*, 2008; Gilad *et al.*, 2011), but until now plasmin(ogen) binding to *Bifidobacterium* has been studied only in phosphate-buffered saline (PBS) suspensions, an *in vitro* and simplified environment. In order to determine whether this interaction has some significant relevance in the complex human gastrointestinal environmental niche, the capability of the probiotic strain *Bifidobacterium animalis* subsp. *lactis* BI07 (Turroni *et al.*, 2010c) to recruit Plg from crude protein extracts from human faeces has been investigated. Faecal samples have been chosen as an *ex vivo* model of the human gastrointestinal ecosystem for several reasons: they represent a non-invasive sampling of the gastrointestinal tract environment, and crude faecal protein extracts reflects the entire content of the full length of the colon-rectum, containing proteins and peptides present due to leakage, secretion or esfoliation (Ang *et al.*, 2010).

Materials and methods

Samples collection and crude faecal extracts preparation

Faecal samples were collected from 10 healthy Italian subjects aged ≥ 30 years. None of the individuals had a history of gastrointestinal disorders at the time of sampling. No dietary restrictions was prescribed for at least 4 weeks before sampling, with the exception of functional foods, probiotics and antibiotics. From each enrolled subject an informed consent was obtained. Crude faecal protein extracts were obtained following the procedure described by Ivanov *et al.* (2006), with minor modifications. In brief, faecal samples were diluted 1:1 (wt/vol) in ice-cold PBS (Phosphate-Buffered Saline) in a stomacher bag and homogenized for 2 min at high speed in a stomacher (Seward), until a uniform consistency was achieved. Aliquots (10 ml) of each homogenized sample were collected in tubes and Complete Protease Inhibitors Solution (Roche) was added. 3-mm glass beads (BioSpec Products) were added before treating the samples with FastPrep (MP Biomedicals) at 5.5 ms for 1 min 3 times. Glass beads and debris were removed by centrifugation at 12000 rpm for 10 min. Protein concentration in supernatants was measured by NanoDrop (NanoDrop Technologies).

Bifidobacterium strains and growth conditions

For this study, five bifidobacterial strains were selected. *Bifidobacterium animalis* subsp. *lactis* BI07 was originally isolated from fermented milk, whereas *B. longum* ATCC 15707, *B. breve* ATCC 15700, *B. bifidum* DSM20456 and *B. adolescentis* ATCC 15703 were isolated from human intestine (Meile *et al.*, 1997; Ventura *et al.*, 2007d; Lee and O'Sullivan, 2010). Bacteria were cultured in de Man-Rogosa-Sharpe (MRS) medium (Difco) supplemented with 0.05% (wt/vol) L-cysteine at 37°C in anaerobic conditions, obtained in a jar by using Anaerocult A (Merck). Bifidobacteria cells were grown for 18 h until they reached the stationary phase.

Bifidobacterium interaction with human faecal extracts

Stationary-phase bifidobacterial cells were resuspended at a concentration of 1×10^9 CFU/ml in 10 ml of crude faecal protein extracts from subjects 1 to 10, or in 10 ml PBS as a negative control. After incubation at 37°C for 1 h, bacterial cells were recovered by centrifugation at 6000 rpm for 10 min at 4°C and washed in 50 mM Tris-HCl (pH 7.6). In order to detect faecal proteins captured on the bifidobacterial cell surface, the bacterial cell wall protein fractions were purified as reported by Hardie and Williams (1998). Bifidobacterial cells were resuspended in 500 µl of protoplast buffer (50 mM Tris-HCl [pH 7.6], 1 M sucrose, 1.4 mM phenylmethylsulfonyl fluoride, 15 mg/ml lysozyme) and incubated for 90 min at 37°C. The suspension was then centrifuged for 3 min at 4000 rpm at 4°C and the supernatant, containing the cell wall proteins, was collected and

stored at -20°C. In order to remove sucrose excess, cell wall proteins were precipitated with 15% trichloroacetic acid (TCA), centrifuged for 10 min at 13000 rpm at 4°C, and resuspended in acetone. After 5 min incubation on ice, proteins were centrifuged for 10 min at 13000 rpm at 4°C and acetone was completely removed.

Immunoblot analysis

To detect Plg presence, Western blotting analysis was performed for both crude faecal protein extracts and bifidobacterial cell wall protein fractions after incubation with faecal extracts. As positive controls, human Plg (Sigma-Aldrich) and Plg activated to plasmin by urokinase (uPA, Calbiochem) were used. Aliquots of 10 µg of proteins were mixed with loading buffer (0.5 M Tris-HCl [pH 6.8], 4% (wt/vol) SDS, 20% glycerol, 0.1% bromophenol blue, 5% β-mercaptoethanol), boiled for 5 min and loaded onto 12% SDS-PAGE gels and resolved for 2.5 h at 160 V using a SE 250 mini vertical electrophoresis unit (GE Healthcare). Proteins were then blotted onto a nitrocellulose membrane (Bio-Rad) using the Minitrans-Blot Electrophoretic Cell (BioRad) at 100 V, 90 mA, at 4°C overnight. After transfer, the membrane was incubated for 1 h at 25°C in a blocking solution (4% skim milk (Fluka, Sigma-Aldrich) dissolved in TBS-T [20 mM Tris-HCl [pH 7.6], 0.5 M NaCl, 0.15% Tween 20]), followed by an incubation with goat polyclonal anti-human Plg IgG antibodies (Kordia) for 1 h at 25°C under constant agitation. The membrane was then washed four times in TBS (20 mM Tris-HCl [pH 7.6], 0.5 M NaCl) and incubated with the horseradish peroxidase conjugated anti-goat IgG (Sigma-Aldrich) at 25°C for 1 h. After four TBS washings, the membrane was incubated with ECL Plus (GE Healthcare) and the chemiluminescent signal was detected by using a PhosphorImager Storm system (GE Healthcare).

Two dimensional gel electrophoresis, comparative proteomic analysis and protein identification

In order to investigate if bifidobacteria can interact with other proteins present in human faeces, the cell wall protein fraction of *B. animalis* subsp. *lactis* BI07 incubated with crude faecal extract from subject 1 or with PBS, and the faecal proteins from subject 1 were analyzed by comparative proteomics, following the procedure reported by Candela *et al.* (2007). Proteins were solubilised in IEF solution containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 0.005% (vol/vol) 2-mercaptoethanol for two-dimensional polyacrylamide gel electrophoresis (2DE) analysis. The estimate of total protein concentration was carried out with PlusOne 2D Quant kit (GE Healthcare). Isoelectric focusing was performed using Immobiline DryStrips with a linear gradient between 4 and 7 (in 7 cm) on an IPGphor system (GE Healthcare). The rehydration of 40 µg of cell wall proteins was carried out for 12 h in 125 µl of buffer A (8 M urea, 2% [wt/vol] CHAPS, 2% [vol/vol] Ampholine pH 4.0 to 6.5 [GE Healthcare], 10 mM dithiothreitol, 0.8% bromophenol blue). Proteins were focused for a total of 10 kV · h. Reduced and alkylated IPG strips (Görg *et al.*, 1988) were loaded onto 12% acrylamide separating

gels and proteins were resolved by SDS-PAGE at 160 V for 2.5 h using a SE 250 mini vertical electrophoresis unit (GE Healthcare). Spots were visualized by silver staining. GS-800 imaging densitometer (Bio-Rad) was used for the digital acquisition of gels protein patterns. Comparison among gel in order to detect protein spots differently present on the gels was carried out with PDQuest v. 8.0.1 software (Bio-Rad). The selected protein spots were excised from the acrylamide gel and subjected to in-gel tryptic digestion and extraction of peptides (Shevchenko *et al.*, 1996); TipZip (Millipore) was used to purify the extracted peptides. A MALDI-TOF-MS approach was employed to generate peptide mass fingerprinting maps of the tryptic peptides, using a Voyager-DE Pro Biospectrometry work station (Applied Biosistems). All spectra were obtained in reflectron mode, with accelerating voltage of 20 kV and 40-ns delayed extraction. Internal calibration with peptides derived from trypsin auto-proteolysis was carried out. Peptide fingerprints obtained were scanned with Aldente (Gasteiger *et al.*, 2005; http://www.expasy.org/aldente/) and ProFound (http://prowl.rockefeller.edu) database searching algorithms for protein spot identification, considering positive proteins with a minimum of four matching peptides.

Results

Plasminogen detection in human crude faecal extracts

To evaluate the presence of plasmin(ogen) in human faeces, an immunoblot analysis with polyclonal anti-human Plg IgG antibodies was carried out with crude faecal extracts from subject 1 to 10. As reported in Fig. 26A, with the exception of subject 7, all the faecal proteomes showed bands attributable to plasmin heavy chain A and light chain B isoforms. Moreover, subject 1, 3 and 8 displayed bands corresponding to Plg isoforms. These data prove the presence of Plg and its derivatives in the healthy human gastrointestinal tract, each subject with its own peculiar band pattern.

Bifidobacterium capability to recruit plasminogen present in human faecal extracts

The capability of *Bifidobacterium* to recruit Plg and derivatives present in human crude faecal protein extracts was investigated. At first, the ability of one strain, *B. animalis* subsp. *lactis* BI07, to capture on its surface Plg and derived molecules from the 10 different faecal extract samples was tested. Stationary-phase *B. animalis* subsp. *lactis* BI07 cells were incubated with crude faecal extracts from subjects 1 to 10. Bifidobacterial cell wall protein fractions were purified and examined by immunoblot analysis with anti-human Plg IgG antibodies in order to detect Plg captured on *B. animalis* subsp. *lactis* BI07 cell surface. As negative control bifidobacterial cells were incubated with PBS under the same experimental conditions, and as positive control faecal extracts were incubated without bacterial cells. The cell wall protein fractions from *B. animalis* subsp. *lactis* BI07 incubated with the faecal extracts showed a Plg- and plasmin-related band pattern corresponding to the one detected in the respective crude faecal fractions (Fig. 26B). Negative controls did not exhibit any Plg- and plasmin-related band pattern (data not shown). These data prove the capacity of this bifidobacterial strain to recruit Plg and plasmin isoforms present at physiological concentrations in crude protein extracts from human faeces.

The next step was to investigate whether the capability to capture Plg and derivatives from crude faecal extracts was a common trait among different bifidobacteria. To this aim, four human *Bifidobacterium* strains belonging to as many species were chosen: *B. longum* ATCC 15707, *B. breve* ATCC 15700, *B. bifidum* DSM20456 and *B. adolescentis* ATCC 15703. These bifidobacterial cells were incubated with faecal extracts obtained from subject 1 under the same experimental conditions, and the purified cell wall fractions were analyzed by immunoblot analysis to detect Plg and plasmin isoforms presence. As shown in Fig. 27, all the bifidobacterial strains studied were able to recruit Plg and its derivatives present in human faeces, with *B. animalis* subsp. *lactis* BI07 and *B. bifidum* DSM20456 displaying the highest efficiencies in Plg/plasmin recruitment.

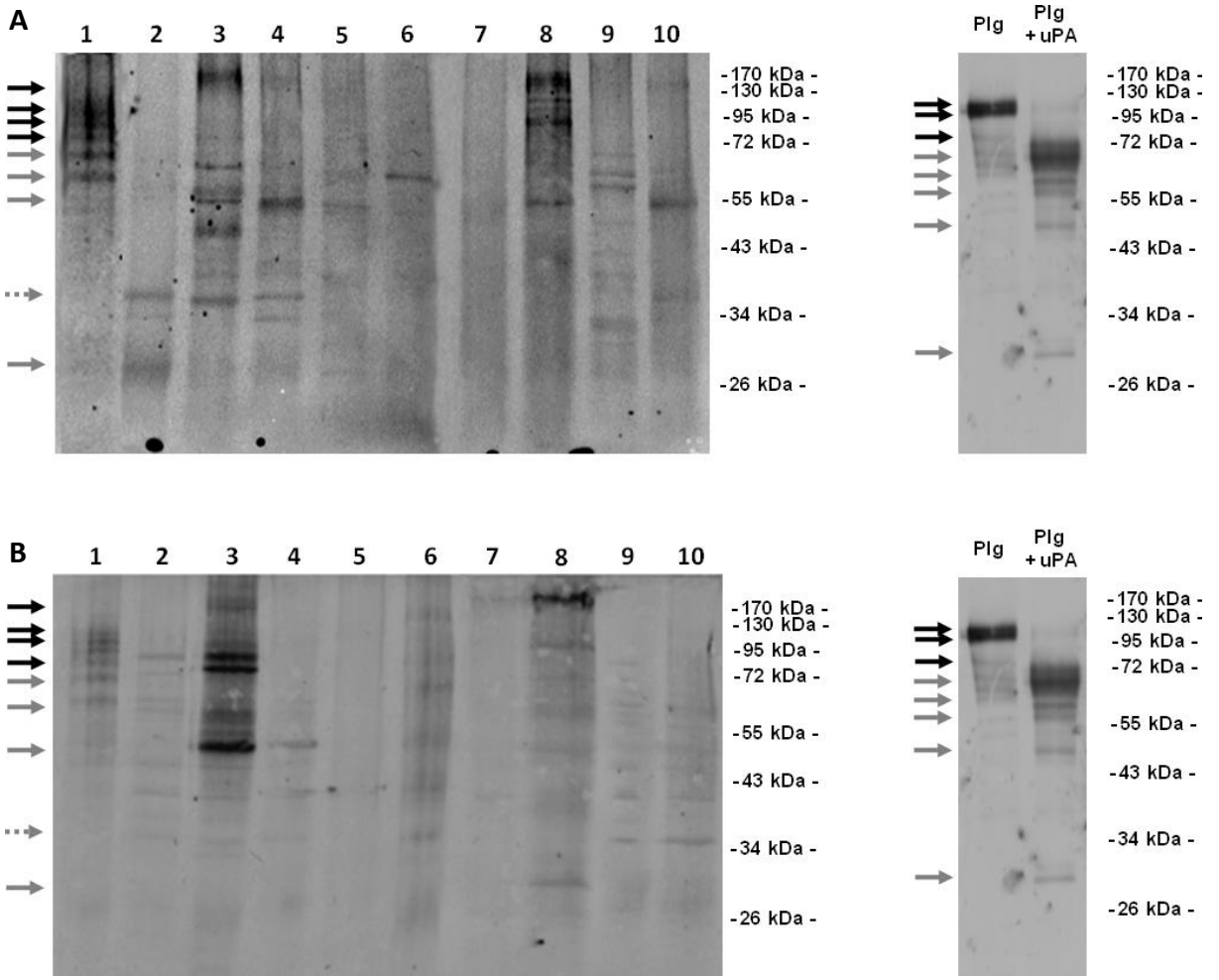


Fig. 26. (A) Immunoblot analysis of crude faecal extracts from subjects 1 to 10 carried out by using polyclonal anti-Plg IgG antibodies. As positive controls, 5 µg (each) of Plg and Plg activated to plasmin by a preincubation with 0.06 kallikrein inhibitor units (KIU) uPA was loaded. Black arrows indicate protein bands attributable to plasminogen isoforms (~90 to 100 kDa). Grey arrows indicate plasmin heavy chain A (~65 kDa), plasmin heavy chain A short form (~57 kDa), and plasmin light chain B (~25 kDa) isoforms. Dotted arrow indicates angiostatin (~38 kDa), which corresponds to the first four kringle domains of Plg isoforms. (B) Immunoblot analysis with polyclonal anti-Plg IgG antibodies of cell wall proteins purified from *B. animalis* subsp. *lactis* BI07 cells incubated with faecal extracts from subjects 1 to 10. Arrows indicate Plg and plasmin isoforms as reported above.

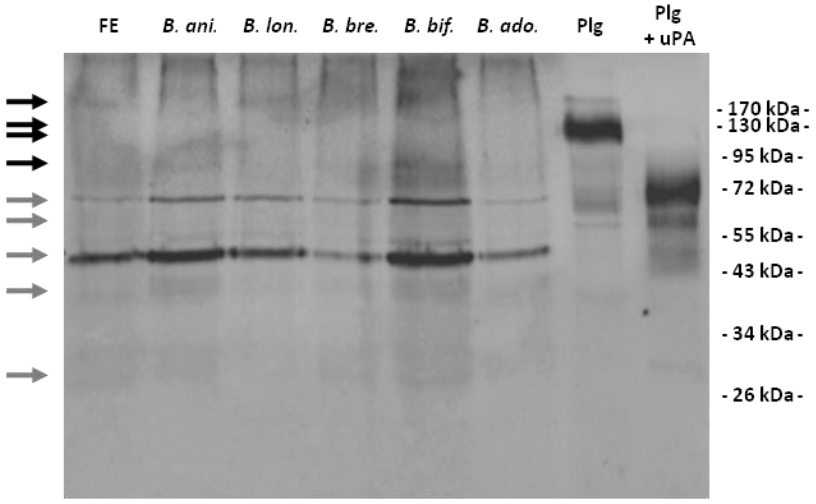


Fig. 27. Ten to the 9 CFU/ml (each) of *B. animalis* subsp. *lactis* BI07 (*B. ani.*), *B. longum* ATCC 15707 (*B. lon.*), *B. breve* ATCC 15700 (*B. bre.*), *B. bifidum* DSM20456 (*B. bif.*), and *B. adolescentis* ATCC 15703 (*B. ado.*) were incubated in parallel with crude extracts from human faeces (FE). Bacterial cell wall proteins were purified, and plasmin(ogen) was detected by immunoblot analysis with polyclonal anti-Plg IgG antibodies. Black arrows indicate protein bands attributable to plasminogen isoforms (~90 to 100 kDa). Grey arrows indicate plasmin heavy chain A (~65 kDa), plasmin heavy chain A short form (~57 kDa), and plasmin light chain B (~25 kDa) isoforms.

B. animalis subsp. *lactis* BI07 interaction with other human faecal proteins

To investigate the capability of bifidobacteria to interact with other human faecal proteins besides plasmin(ogen), the same previously described interaction experiment was carried out using *B. animalis* subsp. *lactis* BI07 and faecal extract from subject 1 as models. Purified cell wall fractions from faecal extract-incubated *B. animalis* subsp. *lactis* BI07 cells (BI07-FE) and PBS-incubated *B. animalis* subsp. *lactis* BI07 cells (BI07-PBS) and the faecal protein extract from subject 1 (crude faecal proteins) were examined by comparative proteomics (Fig. 28). The comparative analysis allowed the detection of six protein spots specific for the human crude faecal protein fraction in the cell wall fraction from BI07-FE, revealing that *B. animalis* subsp. *lactis* BI07 is able to capture on its cell surface various components present in the human faeces. A MALDI-TOF-MS approach was used for the identification of these protein spots (Table 6). Two spots, 1 and 3, were identified as human Plg and spot 2 as plasmin heavy chain A, in agreement with Western blotting data. One spot, no. 4, was identified as human keratin 8 and spot 6 as an hypothetical protein from rice (*Oryza sativa*), whereas spot 5 remained unidentified.

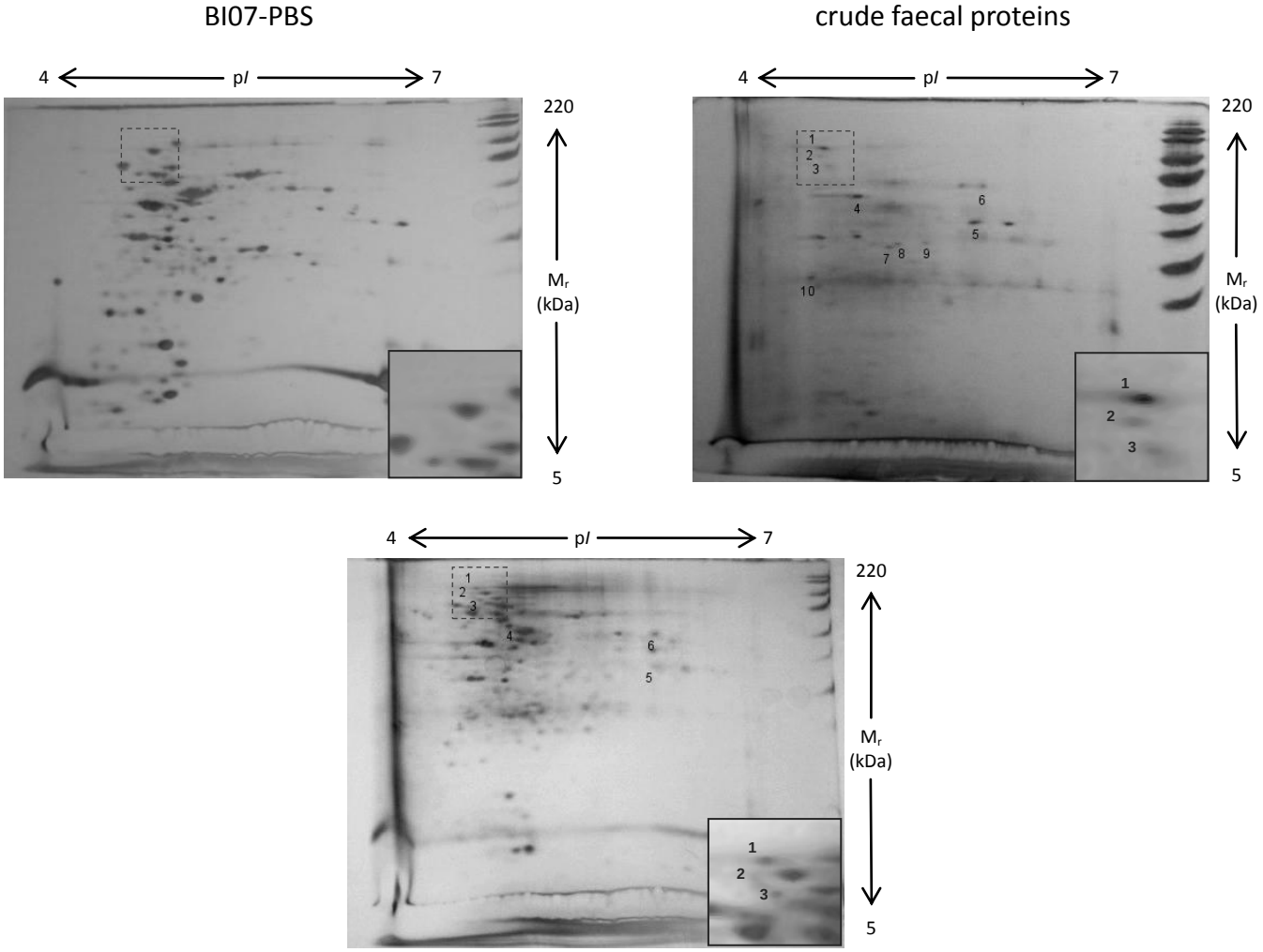


Fig. 28. Comparative proteomics of the cell wall fractions from BI07-PBS and BI07-FE and the crude faecal protein extract from subject 1. Numbered spots were analyzed by MALDI-TOF MS: protein spots 1 to 6 were specific for the crude faecal protein fraction and detected in the cell wall fraction from BI07-FE; protein spots 7 to 10 were detected only in the faecal protein fraction. The two-dimensional gel regions where plasminogen spots were identified are magnified in the insets.

Table 6 - Protein spots identification by MALDI-TOF MS

Spot ID[a]	Swiss Prot Accession No.	Protein Name	Source	Theoretical M_r/pI[b]	Experimental M_r/pI[b]	% sequence coverage
1	B2R7F8	cDNA, FLJ93426, highly similar to *Homo sapiens* plasminogen (PLG), mRNA	*Homo sapiens*	91/7.0	100/4.7	26
2	P00747	Plasmin heavy chain A	*Homo sapiens*	63/6.8	90/4.7	25
3	P00747	Plasminogen	*Homo sapiens*	88/7.1	80/4.7	25
4	P05787	Keratin, type II cytoskeletal 8	*Homo sapiens*	54/5.5	59/4.9	30
5	/	Unidentified	/	/	40/5.9	/
6	Q943E1	Putative uncharacterized protein	*Oryza sativa*	73/6.2	70/6.1	27
7	/	Unidentified	/	/	36/5.2	/
8	/	Unidentified	/	/	37/5.3	/
9	/	Unidentified	/	/	37/5.5	/
10	A1L302	LOC283685 protein	*Homo sapiens*	41/4.8	30/4.5	30

[a]: Spots 1 to 6 were specific for the human crude faecal protein fraction and detected in the cell wall fraction from BI07-FP; spots 7 to 10 were detected only in the faecal protein fraction.
[b]: M_r, molecular weight, in thousands.

Discussion

The capability of *Bifidobacterium* to recruit Plg molecules on its cell surface has been recently demonstrated (Candela *et al.*, 2007), but this interaction has been studied only in an *in vitro* and simplified environment, specifically in phosphate-buffered saline (PBS) suspensions. To investigate if *Bifidobacterium*-Plg binding possesses some relevance in the complex human gastrointestinal environment, the presence of plasmin(ogen) in human faeces and the ability of the probiotic strain *B. animalis* subsp. *lactis* BI07 to capture plasmin(ogen) present in faeces were evaluated.

The presence of human Plg and its derivatives in the human healthy gastrointestinal tract was confirmed, strengthening previous data that reported the presence of plasma proteins in faecal extracts (Oleksiewicz *et al.*, 2005; Ang *et al.*, 2010). Analysis of the cell wall protein fractions from *B. animalis* subsp. *lactis* BI07 cells incubated with human crude faecal extracts allowed the detection of Plg and plasmin isoforms in the bacterial cell envelope proteome after the interaction with faeces, with band patterns corresponding to those detected in the respective faecal extract. This finding demonstrated the capacity of this bifidobacterial strain to recruit plasmin(ogen) present at physiological concentrations in crude protein extracts from human faeces: up to now, this represents the first experimental evidence of a direct interaction between a bifidobacterial strain and a human protein present in a human gastrointestinal tract microenvironment, supporting the biological significance of *Bifidobacterium*-Plg binding in the human gut ecosystem. The capability to recruit plasmin(ogen) in a human physiological microenvironment is not specific of *B. animalis* subsp. *lactis* BI07 strain, but represents a common property shared among *Bifidobacterium* strains that generally inhabit the human gut, as demonstrated by the Western blot analysis on four different human bifidobacterial strains incubated with crude faecal extracts.

A comparative proteomic analysis was carried out to detect faecal proteins recruited on the cell wall of *B. animalis* subsp. *lactis* BI07. Apart from Plg and plasmin, bifidobacteria were capable to recruit human keratin and one hypothetical protein from *Oryza sativa*. Human faeces contains proteins of different origins, and plant proteins associated with food intake have already been detected in the human faecal metaproteome (Verberkmoes *et al.*, 2009).

The interaction with Plg system components can have an important impact on the dynamics of *Bifidobacterium*-host interaction *in vivo* in the human gut. Inflammatory processes in the gastrointestinal tract entail imbalances in the host Plg system (Mondino and Blasi, 2004) and fluctuations of the components of the Plg system dependent on the inflammatory status could influence the outcome of the interaction between *Bifidobacterium* and the host. Plg bound on the bifidobacterial cell surface can be activated by host Plg activators, endowing the microorganism with a host-derived surface-associated proteolytic activity useful in the host colonization process, as described by Candela and co-workers (Candela *et al.*, 2008b). Depending on the gut environment status, this surface-bound Plg can be more or less activated to plasmin, with consequences for the colonization process.

CONCLUSIONS

The red thread running throughout the years of the PhD period has been the study of the relationship between the host plasminogen/plasmin system and *Bifidobacterium*. *Bifidobacterium* is one important genus of the human gastrointestinal ecosystem, representing one of the main health promoting groups present in the human gut microbiota. During millennia, the intestinal microbiota, this huge and complex symbiotic microbial community that inhabits the human gastrointestinal tract (Turnbaugh *et al.*, 2007), evolved a strict inter-kingdom bionetwork that provides the human host with nutritional, metabolic and immunologic benefits (Neish, 2009). The magnitude of the interaction between gut microbiota microorganisms and the host is certainly huge, but until now only few molecules mediating the host-microbiota relationship have been described (Chow and Mazmanian, 2010). Since recent times, the capacity to interact with the host has been widely investigated in pathogens, which possess several mechanisms to intervene with and invade the host. Some microorganisms evolved different virulence strategies and molecular systems for the interaction with the host by their own, such as effector proteins delivered by type III secretion system (Sukhan, 2000; Parsot, 2005; Seveau *et al.*, 2007; Dussurget, 2008; Schroeder and Hilbi, 2008; Pulzova *et al.*, 2009; Ashida *et al.*, 2012), but others take advantage of systems already present in the host, as the Plg/plasmin system (Lähteenmäki *et al.*, 2005; Bergmann and Hammerschmidt, 2007). The host Plg/plasmin system has been increasingly recognized as an important molecular tool used by lactobacilli and bifidobacteria in the interplay with the host (Antikainen *et al.*, 2007a, 2007b; Candela *et al.*, 2007; Hurmalainen *et al.*, 2007). A recent study have identified bifidobacterial cell surface Plg receptors, some of which are shared among several bacteria, both pathogens and commensals (Lähteenmäki *et al.*, 2005; Antikainen *et al.*, 2007b; Candela *et al.*, 2007).

Molecular and biochemical characterization of one bifidobacterial surface Plg receptor, DnaK, has been carried out. The presence of DnaK on the cell surface of the probiotic strain *B. animalis* subsp. *lactis* BI07 has been demonstrated by means of an immunoelectron microscopy approach. The affinity of the interaction between DnaK and human Plg has been investigated through a saturation binding analysis: this study revealed that DnaK possesses a high affinity for human Plg, with a K_D in the nanomolar range (~ 11 nM). DnaK-Plg binding is dependent on lysine residues, but similarly to α-enolase, a *Bifidobacterium* surface Plg receptor already characterized (Candela *et al.*, 2009), the C-terminal lysine residue is involved in Plg binding to a minor extent, as the deletion of this residue in the DnaK protein only slightly affects the Plg binding affinity. Further studies are necessary to identify the DnaK residues responsible for the specific interaction with human Plg. Some residues have been identified as possible binding sites by means of bioinformatics studies, but the construction of mutant proteins appear essential to solve any doubt about this, or these, binding site(s).

An important aspect to take into account for a deeper characterization of the role of host Plg in the *Bifidobacterium* - host interplay is the environment where the interaction takes place. Among the components present in the gut environment, bile salts represent one of the major challenging factors because of their bacterial toxicity (Begley *et al.*, 2005). The adaptation of *Bifidobacterium*

to these components has been largely illustrated (Sánchez *et al.*, 2005, 2007b) and recent works have given more insights on the molecular and genetic mechanisms at the basis of this adaptation process (Gueimonde *et al.*, 2009; Ruiz *et al.*, 2012b). Adaptation to such a harsh environment involves several changes in the entire proteome; a recent study has reported the bile salts-dependent changes of the envelope proteome of one bifidobacterial species, *Bifidobacterium longum* (Ruiz *et al.*, 2009). With these premises, the impact of *B. animalis* subsp. *lactis* BI07 adaptation to physiological concentrations of bile salts on host Plg binding has been evaluated, considering the important impact that this environmental factor can assume in this interaction. The results have shown the up-regulation of important bifidobacterial surface Plg receptors, such as α-enolase and DnaK, and the enhancement of the capacity of this microorganism to intervene with the host Plg/plasmin system. Thus, the increased capability of *Bifidobacterium* to intervene with Plg/plasmin system as a consequence of adaptation to bile salts, present in the intestinal environment, may favour the colonization capability of this microorganism in the gut ecosystem.

The following aspect investigated has been the importance of this specific interaction between *Bifidobacterium* and the host Plg/plasmin system components present in a physiological ecosystem. Until now, the capability of bifidobacteria to interact with Plg/plasmin system components has been investigated only in a simplified environment, namely phosphate-buffered saline suspensions. To determine if this interaction has some relevance in the complex human gut environment, the capacity of *Bifidobacterium* to recruit components of the Plg/plasmin system from human crude faecal protein extract has been investigated. Crude protein extracts from human faeces represent a good model for the human gut environment, reflecting the entire content of the full length of the colon-rectum and being a simple non-invasive way of sampling of the gut environment (Ang *et al.*, 2010). The results of this study have shown the capability of different *Bifidobacterium* strains to capture on their cell surface host Plg and its derivatives present at physiological concentrations in these faecal extracts, supporting the significance of this interaction in the context of the human gastrointestinal ecosystem.

The last important point examined has been the role of human Plg in the biology of interaction between *Bifidobacterium* and host enterocytes, in order to elucidate the importance of this host system in the cross-talk between this probiotic microorganism and the human host. *B. animalis* subsp. *lactis* has been chosen as a bifidobacterial model, a subspecies frequently found in the gut of healthy adults and infants (Turroni *et al.*, 2009). HT29 cell line has been selected as a model system for the human intestinal epithelium: these cells derive from human colorectal adenocarcinoma and produce mucin, leading to the formation of a mucus layer on the surface of the cellular monolayer that mimics the mucus layer encountered by bacteria in the human colon (Fogh and Trempe, 1975; Rousset, 1986). The study illustrated in this thesis has demonstrated that Plg acts as a molecular bridge between bacteria and host cells, enhancing bifidobacterial adhesion to the enterocytes. In the presence of host Plg activators, the interaction phenotype greatly changes, with a marked decrease of bifidobacterial adhesion. However, the acquired cell surface proteolytic activity does not promote bacterial intracellular invasion of the enterocytes or

transmigration across the epithelial monolayers. Gut inflammation has been regarded as one of the principal condition that can have a great impact on the *Bifidobacterium*-host interaction: several studies have reported dysbiosis associated with inflammatory intestinal diseases, where a decrease in *Bifidobacterium* content has been illustrated (Frank *et al.*, 2007; Peterson *et al.*, 2008; Sokol *et al.*, 2008, 2009). Gut inflammation implies several host changes, and it has been reported that Plg/plasmin system components equilibrium is also affected by inflammatory processes: the balance is shifted towards an up-regulation of the plasminogen activators expression, leading to a marked increase of plasmin availability in inflamed tissues. Thus, inflammation can play a significant role in the Plg-mediated interaction between *Bifidobacterium* and the host gut. To elucidate the impact of inflammation on this interaction, *Bifidobacterium* - Plg - host enterocytes interaction has been studied in a model of inflamed intestinal epithelium, stimulating HT29 cells with the pro-inflammatory cytokine TNF-α (Ma *et al.*, 2004; Yan *et al.*, 2008). The TNF-α dependent increase of the enterocyte capability to activate Plg has led to a decrease in *B. animalis* subsp. *lactis* BI07 adhesion to the host enterocytes, showing a low adhesion phenotype comparable to the phenotype in the presence of Plg activators. Even in this case, the acquisition of a surface plasmin activity did not lead to bacteria internalization into enterocytes or transmigration across epithelial monolayers, but favours bacteria migration to the luminal compartment. *Bifidobacterium* shows a different behaviour depending on the inflammation level in that particular gut region, leaving the epithelium in case of inflammation; this could be one of the mechanisms used by *Bifidobacterium* that can explain its low presence in inflamed gut regions. This behaviour is completely different from that of pathogens, because pathogens use this surface associated proteolitic activity to invade tissues: the higher amount of plasmin present in an inflamed environment can improve the invasive characteristics of pathogenic bacteria.

The interaction between microorganisms and the host relies on the capacity of the microorganism to find a way to communicate with the host. In this context, the host plasminogen system can be viewed as an important and flexible tool used by bifidobacteria in the cross-talk with the host, allowing the modulation of the interaction with the host in response to different situations that this microorganism can encounter in the intestinal habitat. *Bifidobacterium* can use this host system present in the gut environment for its advantage: in case of favourable microenvironmental conditions, it allows the enhancement of bacterial adhesion, outcompeting other commensal or pathogenic bacteria in the presence of plasminogen, but plasminogen/plasmin system provides the microorganism with a system that allows to escape in case of “dangerous gut conditions”, for instance from an inflamed gut niche, since bifidobacteria cannot face inflammation. For *Bifidobacterium* this host system can be seen as a sensor of environmental gut conditions, but also a tool that allows the immotile microorganism to evade an unfavourable situation for its survival. The interaction with this system can thus enable bifidobacteria to better adapt to different human host gut niches.

Deepening the knowledge about the molecular mechanisms at the basis of the *Bifidobacterium* - host interplay is important to achieve a better comprehension of the symbiotic relationship the

human host shares with this probiotic microorganism. The studies illustrated here represent a first attempt to shed light on the molecular dynamics of the interaction between *Bifidobacterium* and the host, both in health and disease. Following this path, future researches should be performed on *in vivo* models to elucidate the impact of this host system in the ecology of *Bifidobacterium* in the intestinal tract. An interesting aspect to examine is the role of the host plasminogen system in the inflammation-dependent depletion of *Bifidobacterium* in the gastrointestinal tract. Moreover, the role of plasminogen in the biology of interaction of *Bifidobacterium* with the host should be studied in the presence of enteropathogens, as these microorganisms intervene with the host plasminogen/plasmin system with a completely different purpose, promoting their dissemination within the host tissues. The inflammation-dependent modulation of the host plasminogen system can impact the relationship bifidobacteria-enteropathogens-host. Favoring enteropathogen colonization, as well as the concomitant bifidobacterial migration to the luminal compartment, the inflammation-dependent increase of plasmin availability in the gastrointestinal could concur in establishing a pro-inflammatory bacterial community.

Considering the interaction with enteropathogens, the bifidobacterial capability to recruit plasminogen could be also viewed as a probiotic characteristic: acting as a plasmin scavenger, *Bifidobacterium* subtracts plasmin to enteropathogens, reducing their colonization of the gastrointestinal tract. This phenomenon would become relevant in the context of the inflammation-dependent dysbiosis in the human gastrointestinal tract, such as in inflammatory bowel disease (IBD). The awareness of the mechanisms behind probiotic properties of bifidobacteria could be important for a more rational use of probiotic preparations in the context of emerging gut inflammatory diseases, such as IBD, irritable bowel syndrome (IBS), food allergies, colorectal cancer (CRC) and metabolic syndrome.

These findings can open new outlooks to understand the dynamic of interaction of this probiotic bacterium with the human gut in different conditions. On a physiological level, the specific gut microenvironment, such as inflamed and not inflamed intestinal areas of IBD patients, has an important influence in the interaction between *Bifidobacterium* and the intestinal epithelium.

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ACKNOWLEDGEMENTS

Thank you all that made this true !

that made rich life very well.

I met lots of foreign friends

in a place of busy folk.

in the old Teutonic land,

Across Europe I was sent,

to go or for the purpose.

we have laughed, we have worked

with colleagues of all the sorts

I met science, challenge, help,

in the lab in which I worked

going on beyond the words.

with much care, with some stress,

and hard work has borne its fruit,

... and three years have passed away,

... and three years have passed away,
and hard work has borne its fruit,
with much care, with some stress,
going on beyond the snags.
In the lab in which I worked
I met science, challenge, help,
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we have laughed, we have worked
to go on for the purpose.
Across Europe I was sent,
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in a place of busy folk.
I met lots of foreign friends
that made rich life very well.
Thank you all that made this true !

APPENDICES

APPENDIX 1

Bifidobacterium animalis subsp. *lactis* BI07 *dnaK* gene

Length: 1866 bp - GenBank accession number: AB514431.1

```
   1    ATGGGACGCG CAGTTGGTAT TGATTTGGGT ACCACCAATT CCTGCATCGC AACGCTCGAG
  61    GGCGGTGAAC CGACCGTCAT CGTGAACGCT GAAGGTGCAC GCACCACACC GTCTGTGGTG
 121    GCATTCAGCA AGTCCGGCGA GATTCTCGTC GGCGAGGTTG CAAAGCGTCA GGCTGTGACC
 181    AACGTCGATC GCACGATCAG CTCCGTCAAG CGCCACATGG GCACCGACTG GACCGTTGAC
 241    ATCGACGGCA AGGAATGGAC TCCGCAGGAG ATTTCCGCAC AGATCCTCAT GAAGCTGAAG
 301    CGCGACGCCG AAGCTTACCT GGGCGAGCCG GTCACGGACG CCGTGATCAC CTGCCCTGCA
 361    TACTTCAACG ATGCACAGCG CCAGGCGACC AAGGACGCCG GCACAATCGC AGGCCTGAAC
 421    GTCCTGCGCA TCATCAACGA ACCGACCGCT GCAGCTCTGG CCTACGGTCT GGAGAAGAGC
 481    AAGGAAGACG AGCGCATTCT GGTCTTCGAT CTCGGCGGCG GCACCTTCGA TGTCTCCCTG
 541    CTGGAGATCG GCAAGGACGA CGACGGCTTC TCCACCATCC AGGTGCAGGC TACGTCGGGC
 601    GACAACCACC TCGGTGGCGA CGATTGGGAC CAGCGCATTA TCGACTGGCT CGTCGGCGAA
 661    GTGAAGAACA AGTACGGTGT TGACCTGAGC AAGGACAAGA TCGCCCTGCA GCGTCTGAAG
 721    GAAGCTGCCG AGCAGGCGAA GAAGGAGCTT TCCTCCTCGA TGTCCACCAC GATCAACATG
 781    CAGTACCTGG CCATGACCCC TGACGGGACC CCGGTGCACC TCGACGAGAC GCTCACCCGC
 841    GCCCACTTCG AGGAAATGAC CAAGGATCTG CTCGACCGCT GCCGCACGCC GTTCAACAAC
 901    GTGCTTGCAG ACGCCGGCAT CTCGGTCTCC CAGATCGACC ATGTGATTCT GGTCGGCGGC
 961    TCCACTCGTA TGCCTGCCGT GAAGGAGCTC GTGAAGGAGC TCGACGGCGG CAAGGAAGCC
1021    AACCAGTCCG TGAACCCGGA CGAAGTGGTG GCCATCGGCG CTGCCGTGCA GTCCGGCGTC
1081    ATCAAGGGCG ACCGCAAGGA CGTGCTGCTC ATCGACGTGA CGCCGCTGTC CCTCGGTATC
1141    GAGACCAAGG GCGGCATCAT GACGAAGCTC ATCGAGCGCA ACACCGCAAT CCCGGCGAAG
1201    CGTTCCGAGA TCTTCTCCAC CGCCGAAGAC AACCAGCCGT CCGTACTGAT TCAGGTCTAT
1261    CAGGGCGAGC GTGAATTCGC CCGCGACAAC AAGCCGCTGG GCACCTTCGA GCTGACCGGC
1321    ATCGCTCCGG CTCCTCGTGG CGTCCCGCAG ATCGAGGTCA CCTTCGACAT CGACGCCAAC
1381    GGCATCGTGC ACGTCTCCGC AAAGGACAAG GGCACGGGCA AGGAGCAGTC GATGACGATC
1441    ACCGGTGGCT CCGCACTGCC GAAGGAAGAG ATCGACCAGA TGATCAAGGA CGCCGAGGCC
1501    CACGAAGCGG ACGACAAGAA GCGTAAGGAA GACGCCGAGA CCCGCAACAA CGCCGAGAAC
1561    TTCGCATACC AGACCGAGAA GCTCGTCAAC GACAACAAGG ACAAGCTCTC CGATGACGTC
1621    GCGAAGTCCG TCACCGACGC GATTAACGAG CTCAAGGATG CCTTGAAGGG CGACGACATT
1681    GAGAAGATCA AGGCCGCCCA GGAGAAGCTG ATGACCGAGG CTCAGAAGAT CGGTCAGGCC
1741    CTCTACGCCC AGCAGGGTGC CGAAGGCGCT GCAGGCGCCG CCGACAGCGG CTCCGCGAAC
1801    AACGGTGGCG ACGATGACGT GGTCGACGCC GAGGTCGTGG ATGACGACGA CAAGGACAAC
1861    AAGTAA
```

Bifidobacterium animalis subsp. *lactis* BI07 DnaK protein

Length: 621 aa

```
   1    MGRAVGIDLG TTNSCIATLE GGEPTVIVNA EGARTTPSVV AFSKSGEILV GEVAKRQAVT
  61    NVDRTISSVK RHMGTDWTVD IDGKEWTPQE ISAQILMKLK RDAEAYLGEP VTDAVITCPA
 121    YFNDAQRQAT KDAGTIAGLN VLRIINEPTA AALAYGLEKS KEDERILVFD LGGGTFDVSL
 181    LEIGKDDDGF STIQVQATSG DNHLGGDDWD QRIIDWLVGE VKNKYGVDLS KDKIALQRLK
 241    EAAEQAKKEL SSSMSTTINM QYLAMTPDGT PVHLDETLTR AHFEEMTKDL LDRCRTPFNN
 301    VLADAGISVS QIDHVILVGG STRMPAVKEL VKELDGGKEA NQSVNPDEVV AIGAAVQSGV
 361    IKGDRKDVLL IDVTPLSLGI ETKGGIMTKL IERNTAIPAK RSEIFSTAED NQPSVLIQVY
 421    QGEREFARDN KPLGTFELTG IAPAPRGVPQ IEVTFDIDAN GIVHVSAKDK GTGKEQSMTI
 481    TGGSALPKEE IDQMIKDAEA HEADDKKRKE DAETRNNAEN FAYQTEKLVN DNKDKLSDDV
 541    AKSVTDAINE LKDALKGDDI EKIKAAQEKL MTEAQKIGQA LYAQQGAEGA AGAADSGSAN
 601    NGGDDDVVDA EVVDDDDKDN K
```

Appendix 2

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Bifidobacterial enolase, a cell surface receptor for human plasminogen involved in the interaction with the host

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The interaction with the host plasminogen/plasmin system represents a novel component in the molecular cross-talk between bifidobacteria and human host. Here, we demonstrated that the plasminogen-binding bifidobacterial species *B. longum*, *B. bifidum*, *B. breve* and *B. lactis* share the key glycolytic enzyme enolase as a surface receptor for human plasminogen. Enolase was visualized on the cell surface of the model strain *B. lactis* BI07. The His-tagged recombinant protein showed a high affinity for human plasminogen, with an equilibrium dissociation constant in the nanomolar range. By site-directed mutagenesis we demonstrated that the interaction between the *B. lactis* BI07 enolase and human plasminogen involves an internal plasminogen-binding site homologous to that of pneumococcal enolase. According to our data, the positively charged residues Lys-251 and Lys-255, as well as the negatively charged Glu-252, of the *B. lactis* BI07 enolase are crucial for plasminogen binding. Acting as a human plasminogen receptor, the bifidobacterial surface enolase is suggested to play an important role in the interaction process with the host.

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INTRODUCTION

Bifidobacteria represent one of the most important health-promoting groups of human intestinal microbiota (Schell *et al.*, 2002; Ventura *et al.*, 2009). Even though numerous health-promoting activities have been related to the presence of bifidobacteria in the human gastrointestinal tract (Guarner & Malagelada, 2003), knowledge of the mechanisms of interaction with the host is still in its infancy. The understanding of the *Bifidobacterium*–host interaction process, as well as its impact on human health, could be clarified by the identification and characterization of the bacterial proteins involved. In particular, representing the first line of contact with the intestinal epithelium, the proteins of the bacterial cell surface may play a critical role in the early interaction between microbes and the host (Klijn *et al.*, 2005). Recently, a proteomic approach identified five highly conserved cytoplasmic proteins in the cell wall fraction of *Bifidobacterium lactis* BI07 as putative human plasminogen (Plg) receptors: DnaK, glutamine synthetase, enolase, bile salt hydrolase and phosphoglycerate mutase (Candela *et al.*, 2007). Plg is the zymogen of plasmin, a trypsin-like serine protease with a broad substrate specificity. Plg is a single-chain glycoprotein with a molecular mass of 92 kDa and comprises an N-terminal pre-activation peptide (~8 kDa), five consecutive disulfide-bonded triple-loop kringle domains (K1–5), and a serine-protease domain containing the catalytic triad (Vassalli *et al.*, 1991). It is produced mainly by hepatocytes; however, other tissue sources for Plg synthesis have been identified, including the intestine (Zhang *et al.*, 2002). The active form of plasmin is involved in fibrinolysis (Collen &

Abbreviations: 2-PGE, 2-phosphoglycerate; EACA, ε-aminocaproic acid; PEP, phosphoenolpyruvate; Plg, plasminogen.

The GenBank/EMBL/DDBJ accession number for the enolase sequence of *B. lactis* BI07 is DQ117970.

Verstraete, 1975), homeostasis and degradation of the extracellular matrix and basement membrane (Saksela & Rifkin, 1988). The capability to intervene with the Plg/plasmin system is a strategy for host colonization shared by several pathogens and commensals of the human gastrointestinal tract (Parkkinen & Korhonen, 1989; Schaumburg *et al.*, 2004; Lähteenmäki *et al.*, 2005; Sijbrandi *et al.*, 2005; Bergmann & Hammerschmidt, 2007; Hurmalainen *et al.*, 2007; Candela *et al.*, 2008). With the recruitment of human Plg on the bacterial cell surface, and its subsequent conversion to plasmin, the micro-organism acquires a surface-associated and host-derived proteolytic activity, useful for facilitating the migration across physical and molecular barriers and for responding to the nutritional demands during the colonization process (Lähteenmäki *et al.*, 2005). Several bacterial receptors for human Plg have been characterized (Lähteenmäki *et al.*, 2001). Interestingly, most of them have other important functions besides Plg binding, such as adhesion, movement, enzymic activity or nutrient uptake. In particular, glycolytic enzymes including glyceraldehyde-3-phosphate dehydrogenase and enolase interact with Plg, as shown for different bacterial species (Bergmann & Hammerschmidt, 2007). In spite of the absence of peptides required for secretion and membrane anchorage, these key cytoplasmic enzymes are displayed on the bacterial cell surface, where they acquire a secondary 'moonlighting' function (Jeffery, 1999) that has been shown to be important in the bacteria–host interaction process.

A dose-dependent human Plg-binding activity was recently demonstrated in four bifidobacterial strains belonging to the species *B. lactis*, *B. bifidum* and *B. longum* (Candela *et al.*, 2007). The complete inhibition of Plg recruitment on the bifidobacterial cell surface in the presence of the lysine analogue ε-aminocaproic acid (EACA) suggested that the binding of human Plg to these bifidobacteria was strongly dependent on lysine residues of surface-exposed Plg receptors (Candela *et al.*, 2007). However, bifidobacterial receptors for human Plg have never been characterized. In an attempt to provide insights into the interaction between bifidobacteria and human-derived Plg, we assessed here the role of the bifidobacterial enolase as a surface-displayed Plg receptor in four bifidobacterial species with Plg-binding activity. The localization of enolase on the bacterial cell surface was demonstrated by immunoelectron microscopy in the model strain *B. lactis* BI07. The recombinant His-tagged enolase protein was purified and its Plg-binding activity was characterized with respect to the dissociation constant and the mechanism of Plg binding. By functioning as a surface receptor for human Plg, enolase may play an important role in the bifidobacterial–host interaction process.

METHODS

Bacterial strains, media, and growth conditions. Four bifidobacterial strains were studied. *B. bifidum* S16 and *B. longum* S123 were isolated from human faeces, whereas *B. breve* BBSF and *B. lactis* BI07 were isolated from dairy products. Bifidobacteria were cultured in MRS medium (Difco) supplemented with 0.05 % (w/v) L-cysteine at 37 °C in anaerobic conditions, obtained by using Anaerocult A (Merck) in a jar. The cultures were grown for 18 h until they reached the stationary phase. *Escherichia coli* strains OneShot TOP10, BL21Star(DE3) and XL10-Gold were cultured at 37 °C in Luria–Bertani (LB) medium with shaking. MagicMedia (Invitrogen) was used for recombinant protein expression. Kanamycin (50 μg ml^{-1}) was added as a selective agent when appropriate.

Extraction of bifidobacterial cytoplasmic proteins. Cytoplasmic proteins were extracted from 50 ml bacterial culture in the stationary growth phase. Cells were collected by centrifugation for 10 min at 3800 ***g***, 4 °C and then washed in 50 mM Tris/HCl (pH 7.6), resuspended in 500 μl TE buffer (50 mM Tris/HCl pH 7.6, 5 mM EDTA) and 50 μl Complete Protease Inhibitor Solution (Roche) were added. The suspension was sonicated for 8 min, power 30 W, pulse frequency 20 % in a Branson Sonifier 250 and centrifuged for 10 min at 22 000 ***g***, 4 °C. The supernatant was collected and centrifuged for 2 h at 176 000 ***g***, 4 °C by using a Beckman Ultracentrifuge L7-55. The supernatant, containing cytoplasmic proteins, was stored at −20 °C.

Resolution of *Bifidobacterium* cell wall proteins by 2D-electrophoresis and Plg overlay assay. Experiments were carried out as reported by Candela *et al.* (2007). Briefly, 40 μg bifidobacterial cell wall proteins was resolved by 2D-electrophoresis. Isoelectric focusing was carried out using Immobiline DryStrips with a linear pH gradient between 4 and 7 (7 cm) on an IPGphor system (GE Healthcare) and proteins were separated by SDS-PAGE at 160 V for 2.5 h. For the Plg overlay assay, the resolved proteins were blotted onto a nitrocellulose membrane (Pure nitrocellulose membrane, Bio-Rad) by using a Trans-Blot Electrophoretic Cell (Bio-Rad). After blocking, the membrane was incubated with 4 μg human Plg ml^{-1} (Sigma-Aldrich) in PBS for 1 h at 25 °C. Captured Plg was detected by incubating the membrane with goat anti-Plg IgG antibody (Kordia) and a peroxidase-conjugated anti-goat IgG (Sigma-Aldrich) as secondary antibody. To determine the role of the lysine-binding site(s) in Plg binding, the experiment was repeated in the presence of EACA. Primary and secondary antibody alone did not result in non-specific background binding (data not shown).

Protein identification using MALDI-TOF MS. The selected protein spots were excised from the acrylamide gel and subjected to in-gel tryptic digestion and extraction of peptides (Shevchenko *et al.*, 1996). The extracted peptides were purified with ZipTip (Millipore). Peptide mass fingerprinting maps of tryptic peptides were generated by MALDI-TOF MS with a Voyager-DE Pro Biospectrometry work station (Applied Biosystems), as reported by Candela *et al.* (2007). Aldente (http://www.expasy.org/tools/aldente) and ProFound (http://prowl.rockefeller.edu/prowl-cgi/profound.exe) database search algorithms were used for the identification of the proteins.

Electron microscopy and immunoblot analysis. In order to visualize bifidobacterial enolase on the *B. lactis* BI07 cell surface, we performed pre-embedding immunogold experiments using intact bacterial cells. Bifidobacterial cells recovered from a stationary-phase culture were washed in PBS and adjusted to a concentration of 1×10^9 c.f.u. ml^{-1}. Cells were resuspended in 100 μl rabbit polyclonal anti-pneumococcal enolase antiserum (Bergmann *et al.*, 2003) diluted 1 : 250 in PBS/1 % BSA, and incubated for 1 h at 25 °C under constant agitation. After two washes with 1 ml PBS/1 % BSA, bacteria were resuspended in 25 μl anti-rabbit IgG coupled to 10 nm gold particles (Auro Probe, GE Healthcare) diluted 1 : 5 in PBS/1 % BSA, and incubated for 30 min at 25 °C with constant agitation. Bacteria were then collected and washed twice in PBS/1 % BSA and fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer for 4 h at 4 °C.

Processing for transmission electron microscopy was carried out as reported by Candela *et al.* (2007). Electron microscopic experiments were repeated four times. For immunoblot analysis, *B. lactis* BI07 proteins (10 μg) were subjected to SDS-PAGE with 12 % polyacrylamide and blotted onto a nitrocellulose membrane by using a Minitrans-Blot Electrophoretic Cell (Bio-Rad). Post-transfer, the membrane was blocked in a solution of 4 % skim milk (Biolife) in TBS-T (0.15 % Tween 20 in TBS) and then incubated with rabbit polyclonal anti-streptococcal enolase antiserum (Bergmann *et al.*, 2003). Subsequently, the membrane was washed in TBS-T and incubated with the peroxidase-conjugated anti-rabbit IgG (GE Healthcare). After TBS-T washing, the membrane was incubated with ECL Plus (GE Healthcare), and the signal was detected by using a PhosphorImager Storm system (GE Healthcare).

Cloning, expression and purification of recombinant *B. lactis* BI07 enolase. In order to analyse the nucleotide sequence of the *B. lactis* BI07 enolase gene, chromosomal DNA was used as a template for PCR and the enolase gene was amplified with the primer pair L-FEno (5′-GAAACTCACGCCTTTACGGGCGTT-3′) and R-FEno (5′-TCAAGATACACAACCGTTTTAAGGAGT-3′), designed against the nucleotide regions downstream and upstream of the *B. longum* NCC2705 enolase gene (1299 bp, GeneID:1022550), respectively. The PCR product obtained was cloned into the pCRII-TOPO cloning vector (Invitrogen), following the protocol supplied by the manufacturer, and the DNA insert was sequenced using the primer set T7 and T7-reverse. For cloning and expression, the *B. lactis* BI07 enolase gene was amplified by PCR using the primer set EnoTOPO-L (5′-CACCATGGCAGTAATTGAAAGCGTGT-3′) and EnoTOPO-R (5′-TCACTTGGCCAGGTACTTCT-3′), and the PCR product was cloned into the expression vector pET200/D-TOPO (Invitrogen) to obtain the construct pENOwt. Cloning and expression of the *B. lactis* BI07 enolase gene were carried out in *E. coli* TOP-10 and *E. coli* BL21Star(DE3) (Invitrogen), respectively. The His-tagged fusion *B. lactis* BI07 enolase was purified by affinity chromatography under native conditions on Ni-NTA resin, according to the manufacturer's instructions (Invitrogen). The purified proteins were dialysed at 4 °C using Spectra/Por membranes 6000–8000 kDa (Spectrum Laboratories) and 20 mM Tris, 120 mM NaCl as dialysis buffer. The expression of the His-tagged recombinant protein His_6-enolase was verified by Western blot analysis with polyclonal anti-pneumococcal enolase antiserum (Bergmann *et al.*, 2001) and anti-HisTag (Sigma) antibody.

Site-directed mutagenesis. The QuikChange Multi Site-Directed Mutagenesis kit (Stratagene) was used to obtain a mutant plasmid library from pENOwt. Plasmids harbouring mutants of the *B. lactis* BI07 *enolase* gene, deleted of nucleotides encoding the C-terminal lysine (pENObs1), mutated in the internal Plg-binding site (pENObs2), or with both the deletion and the internal mutation (pENOdouble), were created. Primer ENOmutBS1 (5′-AAGAAGTACCTGGCCTGATGAAAGGGCGAGCTCAACG-3′) was designed to replace the C-terminal lysine with a stop codon. Primer ENOmutBS2 (5′-GAGTTCTACAACTTGGGGACCGGCTTGTACC-GCTTCGACGG-3′) was designed to replace Lys-251 and Lys-255 with leucine, and Glu-252 with glycine, as performed by Bergmann *et al.* (2003) on *Streptococcus pneumoniae* enolase. Equal amounts of each primer were added to a 50 μl PCR, together with 200 ng pENOwt as DNA template. The mutagenesis reaction was carried out in a thermocycler with a first denaturation step at 95 °C for 1 min, followed by 30 cycles at 95 °C for 1 min, 55 °C for 1 min and 65 °C for 14 min. The DNA template was removed by *Dpn*I treatment at 37 °C for 2 h. The mutant plasmid library was transformed into the *E. coli* XL10-Gold strain and a number of clones were sequenced to isolate the three mutants. Mutant proteins carrying the desired amino acid substitutions or deletion were expressed and purified as described above.

Enolase activity of bifidobacterial whole cells and purified *B. lactis* BI07 enolase protein. In order to determine the enolase activity of intact bifidobacterial cells, a direct enzyme assay was performed (Pancholi & Fischetti, 1998). Briefly, 1×10^9 bifidobacterial cells were washed three times in 100 mM HEPES buffer pH 7 and equal amounts were resuspended in reaction buffer [100 mM HEPES (pH 7), 10 mM $MgCl_2$, 7.7 mM KCl, 3 mM 2-phosphoglycerate (2-PGE)] or in control buffer [100 mM HEPES (pH 7), 10 mM $MgCl_2$, 7.7 mM KCl]. The bacterial suspensions were incubated for 3 min at 37 °C and subsequently centrifuged for 10 min at 19 000 ***g***, 4 °C. The supernatants were recovered and centrifuged for a further 10 min at 19 000 ***g***, 4 °C. After the second centrifugation step, the supernatants were recovered and phosphoenolpyruvate (PEP) concentration was determined by measuring the absorbance at 240 nm in a Jasco spectrophotometer (model 7800/V-520). The enzyme activity of the purified His-tagged enolase protein was determined in a single enzyme assay. Briefly, different amounts of purified recombinant His-tagged enolase protein were incubated for 3 min at 37 °C in reaction buffer containing 15 mM 2-PGE. The release of PEP was measured spectrophotometrically as described above. For kinetics studies, different amounts of 2-PGE (0.5–15 mM) were used in a single enzyme assay carried out with 5 μg purified His-tagged enolase protein. The K_m value was calculated from the Lineweaver–Burk plot.

Structural model of *B. lactis* BI07 enolase. The homology model of *B. lactis* BI07 enolase was calculated as reported by Zambelli *et al.* (2009). Using the tool BLAST (Altschul *et al.*, 1990, 1997), the amino acid sequence of *B. lactis* BI07 enolase (Swiss-Prot accession no. Q45RT9) was used to search for bacterial enolase structures with a high identity score. Four enolases with known protein structures were selected: *Strep. pneumoniae* enolase (PDB code 1W6T), *Enterococcus hirae* enolase (PDB code 1IYX), *Methanococcus jannaschii* enolase (PDB code 2PA6), and *Escherichia coli* enolase (PDB code 1E91). Multiple sequence alignment was carried out using CLUSTAL W (Thompson *et al.*, 1994). The prediction of the *B. lactis* BI07 enolase secondary structure was carried out using the JPRED tool (Cuff *et al.*, 1998) and the alignment was manually optimized based on the secondary structure information. Model structure was calculated with the program MODELLER 9v5 (Marti-Renom *et al.*, 2000), using the above indicated enolases as templates. The best model was selected on the basis of the lowest value of the MODELLER objective function. Structure validation was performed using PROCHECK (Laskowski *et al.*, 1993). The program UCSF Chimera (Pettersen *et al.*, 2004) was used for protein visualization.

Plg-binding analysis. Human Plg (Sigma-Aldrich) was biotinylated using the EZ-Link Micro Sulfo-NHS-SS Biotinylation kit (Pierce), following the manufacturer's instructions. The solid-phase Plg-binding assay was performed as previously described by Sanderson-Smith *et al.* (2006, 2007). Microtitre plates (96-well; OptiPlate-96, Perkin Elmer) were coated with 150 nM recombinant enolase (50 μl in 0.1 M $NaHCO_3$) and incubated at 4 °C overnight. After washing with PBS, the plates were blocked with 200 μl BSA 2 % in PBS for 1 h at 37 °C. After three PBS washes, increasing concentrations of biotinylated Plg in PBS (2, 6, 18, 36, 55, 110, 166 and 250 nM) were added to the plates, in the presence or absence of a 50-fold molar excess of unlabelled Plg. Plg was allowed to bind to immobilized proteins for 2 h at room temperature, then the plates were washed three times with PBS, and 50 μl ExtrAvidin horseradish-peroxidase-conjugated antibodies (Sigma-Aldrich), diluted 1 : 5000 in PBS/1 % BSA, was added to each well. Plates were incubated for 2 h at 25 °C and washed four times with PBS and 0.05 % Tween 20. One hundred microlitres of Chemiluminescent Peroxidase Substrate for ELISA (Sigma-Aldrich) was added to each well and luminescence was read using a Victor^{3}V 1420 Multilabel Counter scanner (Perkin Elmer) and the software Wallac 1420 WorkStation. As a negative control for Plg binding, BSA-coated plates were utilized. For each of the recombinant

proteins, Plg-binding experiments were repeated four times, whereas for a given Plg concentration, each measurement was repeated in triplicate. To assess the involvement of lysine residues in the enolase–Plg interaction, experiments were repeated in the presence of 0.5 M EACA. For analysis, data were normalized against the highest and lowest luminescence values, and nonlinear regression analysis was carried out using Graph Pad Prism (version 5.0, Graph Pad Software). For calculation of the equilibrium dissociation constant (K_D) one- and two-site binding analysis was performed and the best-fit curve was chosen.

RESULTS

Enolase is a conserved putative Plg receptor in *Bifidobacterium*

In order to screen the putative surface Plg receptors in *Bifidobacterium*, the cell wall fractions from the Plg-binding strains *B. longum* S123, *B. bifidum* S16 (Candela *et al.*, 2007) and *B. breve* BBSF (data not shown) were purified and a Plg overlay assay was carried out. To this end, cell wall proteins were resolved in a two-dimensional gel, immobilized on a nitrocellulose membrane, incubated with human Plg, and probed with anti-Plg antibody to identify bound Plg (Fig. 1). For each strain, the coordinates of the major putative Plg-binding proteins detected could be matched to a protein spot on the replica gel stained for proteins. Plg binding was completely inhibited in the presence of EACA (data not shown). Among the putative Plg-binding proteins, two proteins with an apparent molecular mass of 70 and 50 kDa and a pI of 4.5 and 4.7, respectively, were found to be conserved in the different bifidobacterial strains analysed. For each strain, the corresponding spots were excised from the gel and subjected to trypsin digestion and MALDI-TOF MS analysis for protein identification. The peptide fingerprints obtained were scanned with the searching tools Aldente and ProFound, and an unambiguous identification was obtained: the protein of 70 kDa and pI 4.5 was identified as DnaK and the protein of 50 kDa and pI 4.7 as enolase. DnaK and enolase had been previously identified as putative Plg receptors in a cell wall fraction of the Plg-binding strain *B. lactis* BI07 (Candela *et al.*, 2007). To investigate the role of bifidobacterial enolase as a receptor for human Plg, *B. lactis* BI07 was selected as model strain.

Localization of enolase protein on the *B. lactis* BI07 cell surface

At first, the distribution of enolase in cytoplasmic and cell wall fractions of *B. lactis* BI07 was evaluated by a Western blot experiment carried out with a cross-reactive polyclonal anti-pneumococcal enolase antiserum (anti-Eno) (Bergmann *et al.*, 2003). Enolase was detected in both the cytoplasmic and cell wall fractions of *B. lactis* BI07 (Fig. 2). In order to visualize the enolase protein on the *B. lactis* BI07 cell surface, an immunoelectron microscopy experiment was carried out. Intact *B. lactis* BI07 cells were incubated under pre-embedding labelling conditions with

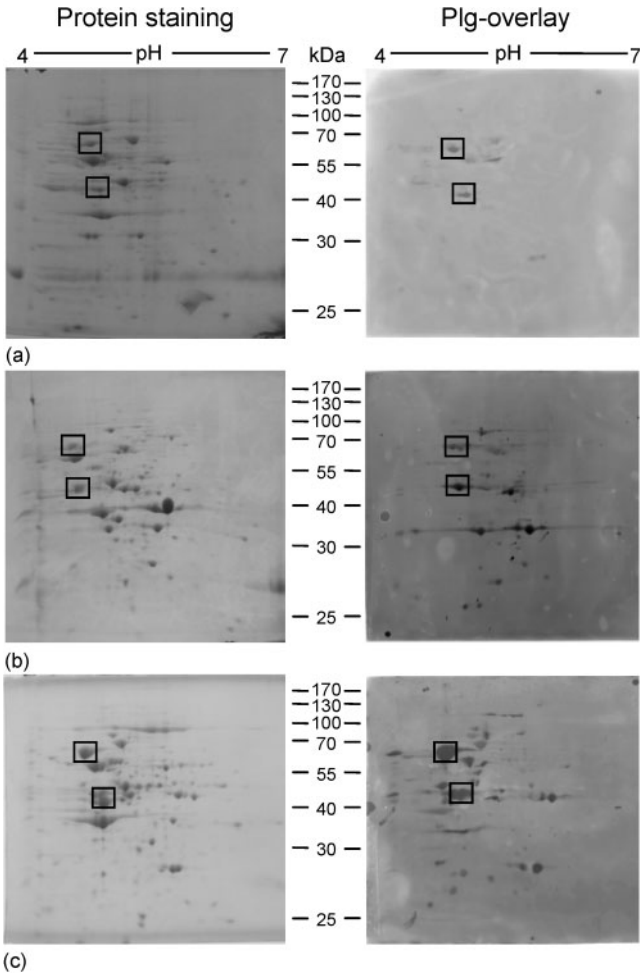

Fig. 1. Plg overlay assay carried out with the cell wall fraction of *B. longum* S123 (a), *B. bifidum* S16 (b) and *B. breve* BBSF (c). For each strain, the 2D-gel stained for proteins and the replicate 2D-gel used for Plg overlay are shown. Four micrograms Plg per ml was applied as the Plg overlay. Plg-binding proteins were detected with anti-Plg antibody and peroxidase-conjugated secondary antibody. The black squares indicate two proteins with an apparent molecular mass of 70 and 50 kDa and a pI of 4.5 and 4.7, which are conserved among the different bifidobacterial strains.

the anti-Eno antiserum followed by the secondary antibody labelled with 10 nm gold particles. Analysis of ultrathin sections at a magnification of $\times 22\,000$ (Fig. 3) detected the enolase (black dots) directly in the bacterial cell wall region. Non-specific binding of the secondary antibody was not detected (data not shown). The binding pattern of the anti-Eno antiserum suggests either that the distribution of enolase is in the form of a cluster, or that its epitopes are not uniformly exposed on the cell surface. The enzyme functionality of the surface enolase was evaluated by monitoring the conversion of 2-PGE to PEP, when supplied to viable bifidobacterial cells. Intact cells of *B. lactis* BI07 showed a dose-dependent enolase activity (data not shown).

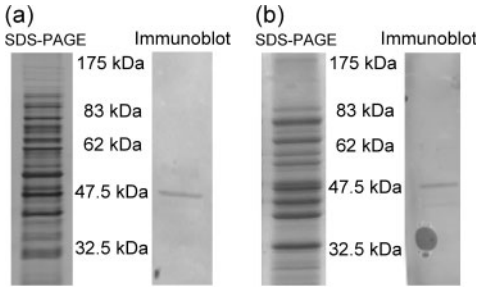

Fig. 2. Immunoblot analysis using anti-enolase antiserum of cytoplasmic (a) and cell wall (b) fractions of *B. lactis* BI07 resolved by SDS-PAGE.

Analysis of Plg binding to *B. lactis* BI07 enolase

In order to characterize the Plg-binding activity of *B. lactis* BI07 enolase, the gene was sequenced, cloned, and the recombinant His_6-tagged enolase protein was purified by affinity chromatography. The nucleotide sequence of *B. lactis* BI07 enolase (GenBank accession no. DQ117970) revealed 98 % identity to the enolase gene of *B. longum* NCC2705 (GenBank accession no. AE014295). In Fig. 4 we show the dose-dependent enzymic activity for the recombinant His_6-tagged *B. lactis* BI07 enolase. 2-PGE was converted to PEP with a specific activity of 1.35 μmol PEP synthesized per min per mg protein. These data indicate that *B. lactis* BI07 enolase was purified in the native 3D structure. To evaluate the specific Plg-binding activity of *B. lactis* BI07 enolase, a solid-phase Plg-binding assay (Sanderson-Smith *et al.*, 2007) was carried out. The saturation binding analysis of biotinylated Plg to immobilized recombinant *B. lactis* BI07 enolase is reported in Fig. 5. Non-specific binding was determined in the presence of a 50-fold molar excess of unlabelled Plg, and specific binding was calculated by subtracting non-specific binding from the total binding obtained at each concentration of biotinylated Plg. As a negative control, BSA-coated wells were utilized. According to our data, Plg binding to immobilized enolase was dose-dependent and saturable binding was achieved with 250 nM Plg. The best-fit nonlinear regression analysis allowed calculation of an equilibrium dissociation constant (K_D) for the interaction of enolase with Plg of 42 nM. The binding was completely inhibited in the presence of 0.5 M EACA, proving the crucial role of enolase lysine(s) in Plg binding.

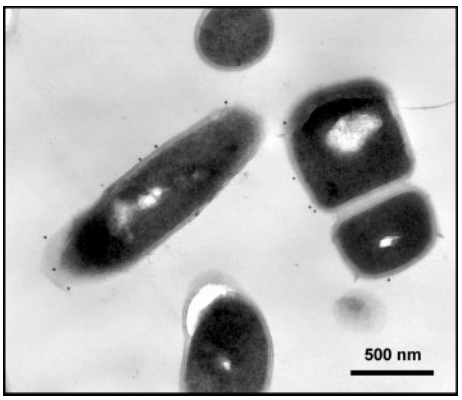

Fig. 3. Immunoelectron microscopic localization of enolase on the cell wall of *B. lactis* BI07. Enolase was detected on the bacterial surface by anti-enolase antiserum and a secondary antibody coupled to 10 nm gold particles in pre-embedding experiments. An ultrathin section of *B. lactis* BI07 cells at a magnification of ×22 000 shows enolase (black dots) localized directly in the cell wall region.

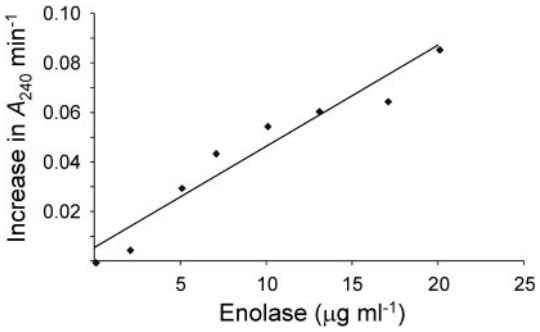

Fig. 4. Enzymic activity of the recombinant His_6-enolase of *B. lactis* BI07. The activity was determined using a direct enzyme assay: different amounts of the purified recombinant His_6-enolase were incubated with 15 mM 2-PGE. The release of PEP was evaluated by measuring the increase in absorbance at 240 nm.

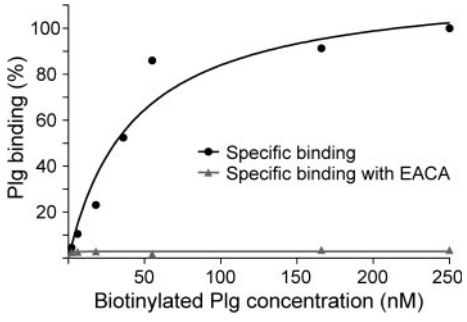

Fig. 5. Saturation binding analysis of biotinylated Plg to immobilized recombinant His-tagged *B. lactis* BI07 enolase. Specific Plg binding (•) was calculated at each concentration of biotinylated Plg by subtracting non-specific binding obtained in the presence of a 50-fold molar excess of unlabelled Plg from total binding. A one-site hyperbolic binding function was fitted to the data and the K_D determined. The experiment was repeated in the presence of the lysine analogue EACA (0.5 M, grey triangles).

Homology model of *B. lactis* BI07 enolase and identification of the putative Plg-binding sites

In an attempt to identify the putative Plg-binding site(s) of *B. lactis* BI07 enolase, a homology model of the protein was generated using the coordinates of four enolases of known structure: the enolases of *Strep. pneumoniae* (PDB code 1W6T) and *Enterococcus hirae* (PDB code 1IYX), which share 55 % identity with *B. lactis* BI07 enolase, and the enolases of *Escherichia coli* (PDB code 1E9I) and *M. jannaschii* (PDB code 2PA6), which show 52 % identity with *B. lactis* BI07 enolase (Fig. 6a). In Fig. 6(b), a model of the *B. lactis* BI07 enolase dimer is represented. The Ramachandran Plot analysis (Willard *et al.*, 2003) of the model for *B. lactis* BI07 enolase indicated a good stereochemical quality (data not shown). The monomer consists of two domains: the N-terminal domain contains a three-stranded antiparallel β-sheet followed by six α-helices, and the C-terminal domain is composed of an α/β-barrel structure. *B. lactis* BI07 enolase possesses a C-terminal lysine at amino acid position 432, which constitutes a homologue of the pneumococcal Plg-binding site BS1 (Bergmann *et al.*, 2003). In order to evaluate whether the surface loop L3 of *B. lactis* BI07 enolase contained a peptide region homologous to the internal Plg-binding site BS2, located on the surface-exposed loop L3 of *Strep. pneumoniae* enolase (residues 248–256; Ehinger *et al.*, 2004), the protein structures were superimposed. A 0.4 Å (0.04 nm) root-mean-square distance (r.m.s.d.) between the Cα atom positions of the *B. lactis* BI07 and pneumococcal enolases demonstrated an overall structural similarity between these two proteins. In particular, the enolases of *B. lactis* BI07 and *Strep. pneumoniae* shared a significant structural similarity in the loop L3 (r.m.s.d. of 0.64 Å, 0.064 nm) (Fig. 6c). Residues 248–256 within the L3 region of *B. lactis* BI07 enolase and the BS2 region of pneumococcal enolase displayed high structural homology.

Impact of BS1 and BS2 homologues on Plg binding to *B. lactis* BI07 enolase

In order to determine the role of the putative Plg-binding sites BS1 and BS2 of *B. lactis* BI07 enolase in Plg binding, three genetic mutants of the corresponding enolase (*eno*) gene were constructed: (i) the *eno*^{BS1} mutant gene, deleted of the BS1 pneumococcal homologue, obtained by the deletion of the codon encoding the C-terminal lysyl residue at position 432; (ii) the *eno*^{BS2} mutant gene, obtained by site-directed mutagenesis of the amino acids homologous to those that were previously demonstrated to be essential in Plg-binding to the BS2 site of the pneumococcal enolase (Bergmann *et al.*, 2003; Ehinger *et al.*, 2004), 251 (Lys→Leu), 252 (Glu→Gly) and 255 (Lys→Leu); and (iii) the *eno*^{double} mutant gene constructed by deleting the C-terminal lysine from the *eno*^{BS2} mutant. The recombinant His-tagged enolase^{BS1}, enolase^{BS2} and enolase^{double} proteins were purified and their specific Plg-binding activity was evaluated in a saturation binding analysis of biotinylated Plg to the immobilized recombinant proteins (Fig. 7). The interaction of enolase^{BS1} with Plg was dose-dependent and saturable, with a K_D of about 46.2 nM, a value comparable to that shown by the wild-type *B. lactis* BI07 enolase protein. In contrast, neither enolase^{BS2} nor enolase^{double} showed specific and saturable interaction with human Plg, proving the crucial role of the BS2 homologue in Plg binding to *B. lactis* BI07 enolase.

DISCUSSION

Plg-binding bifidobacterial strains belonging to the human-associated species *B. longum*, *B. bifidum* and *B. breve* (Ventura *et al.*, 2007), as well as the Plg-binding probiotic strain *B. lactis* BI07 (Candela *et al.*, 2007), share the enolase enzyme as one of their putative surface Plg receptors. Enolase is an essential glycolytic enzyme catalysing the formation of PEP from 2-PGE. Localized on the cell surface, it is one of the best-characterized human Plg receptors in prokaryotes and eukaryotes (Pancholi & Fischetti, 1998; Bergmann *et al.*, 2001; Crowe *et al.*, 2003; Schaumburg *et al.*, 2004; Lähteenmäki *et al.*, 2005; Antikainen *et al.*, 2007b; Knaust *et al.*, 2007). A single enolase gene is present in the published genomes of the bifidobacterial species *B. animalis* subsp. *lactis* (accession no. NC_011835), *B. longum* (accession no. NC_004307), *B. longum* subsp. *infantis* (accession no. NC_011593), *B. adolescentis* (accession no. NC_008618) and *B. bifidum* (accession no. NZ_ABQP00000000). Thus, analogously to *Streptococcus pyogenes*, *Strep. pneumoniae* and *Staphylococcus aureus* (Antikainen *et al.*, 2007a), the same enolase gene product is both an essential glycolytic enzyme in the cytoplasm and, localized on the cell surface, a receptor for human Plg. As reported for several prokaryotes and eukaryotes that display enolase on the cell surface (Pancholi, 2001), the bifidobacterial enolase lacks either predicted or detectable protein-sorting elements for secretion and anchorage onto the bacterial cell wall. The mechanisms of secretion and surface localization of this essential glycolytic enzyme are still under debate. Recently for *Strep. pneumoniae*, scavenging of cytoplasmic proteins released through allolysis was proposed to account for the presence of enolase on the cell surface (Claverys & Havarstein, 2007). On the other hand, according to Boël *et al.* (2004), the export of enolase may depend on the modification of the enzyme by covalent binding of 2-PGE to Lys-341. The role of enolase as a surface Plg receptor was further studied using *B. lactis* BI07 as a model strain. In a Western blot analysis we confirmed that *B. lactis* BI07 enolase is present in the cytoplasm and in the cell wall fractions. Moreover, providing direct evidence for the presence of enolase on the bifidobacterial cell surface, enolase was visualized on the cell surface of *B. lactis* BI07 by immunoelectron microscopy. Similarly to *Strep. pyogenes* (Pancholi & Fischetti, 1998), *Strep. pneumoniae* (Kolberg *et al.*, 2006) and *Strep. suis* (Esgleas *et al.*, 2008), the *B. lactis* BI07 enolase is displayed on the bacterial cell surface

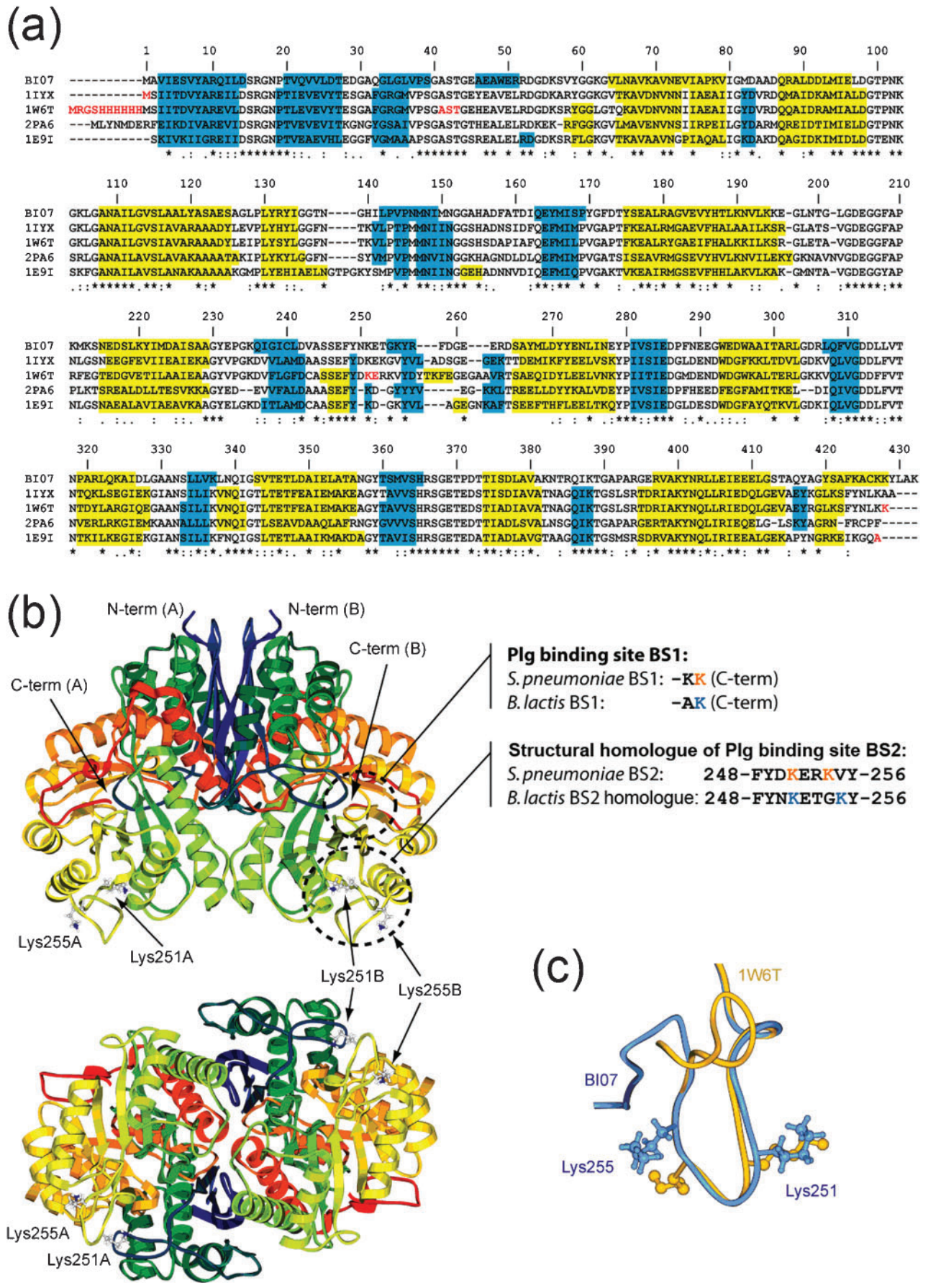
(a)
BI07
1IYX
1W6T
2PA6
1E9I
(b)
N-term (A)
N-term (B)
C-term (B)
C-term (A)
Lys255A
Lys251A
Lys251B
Lys255B
Plg binding site BS1:
S. pneumoniae BS1: -KK (C-term)
B. lactis BS1: -AK (C-term)
Structural homologue of Plg binding site BS2:
S. pneumoniae BS2: 248-FYDKERKVY-256
B. lactis BS2 homologue: 248-FYNKETGKY-256
(c)
1W6T
BI07
Lys255
Lys251

Fig. 6. (a) Multiple sequence alignment of the sequences of *Bifidobacterium lactis* BI07 (BI07), *Enterococcus hirae* (1IYX), *Streptococcus pneumoniae* (1W6T), *Methanococcus jannaschii* (2PA6) and *Escherichia coli* (1E91) enolases, with the secondary structure indication (α-helix, yellow; β-strand, cyan) derived from the JPRED prediction for *B. lactis* BI07 enolase and from the PDB structure for all the remaining sequences. (b) Ribbon scheme of dimeric *B. lactis* BI07 enolase model structure, derived using the sequence alignment reported in (a). In the bottom image the ribbons are rotated 90 ° around the long horizontal axis. Ribbons are coloured from blue, in the proximity of the N-terminus, to red at the C-terminus. (c) Detailed view of the superimposed L3 regions of *B. lactis* BI07 enolase (blue) and pneumococcal enolase (yellow). Lysines 251 and 255 and the pneumococcal homologue lysines 251 and 254, crucial for Plg binding, are represented in ball and stick notation. The molecular graphic images were produced using the UCSF Chimera package.

with relatively low abundance. The ability of intact *B. lactis* BI07 cells to convert 2-PGE to PEP in a direct enzyme assay demonstrated that the surface enolase still retains its functional enzyme activity. The His-tagged *B. lactis* BI07 enolase was purified and characterized with respect to its enzyme activity and, most importantly, its secondary 'moonlighting' function as a Plg receptor. In a direct enzyme assay, the K_m for 2-PGE was calculated to be 0.73 mM. This value is in the range reported for other bacterial enolases, including the *Strep. pyogenes* (1.492 mM) (Pancholi & Fischetti, 1998), *Strep. pneumoniae* (4.5 mM) (Bergmann *et al.*, 2001) and *Bacteroides fragilis* (0.210 mM) (Sijbrandi *et al.*, 2005) enolases. The affinity of *B. lactis* BI07 enolase for human Plg was calculated in a saturation binding analysis. The purified His-tagged *B. lactis* BI07 enolase showed a high affinity for human Plg, with a K_D value in the nanomolar range (42.8 nM). Compared to the eukaryotic enolase, which possesses a K_D value of about 0.1–2 μM (Redlitz *et al.*, 1995), the *B. lactis* BI07 enolase shows a significantly higher affinity for human Plg. On the other hand, in comparison with bacterial enolases from pathogenic micro-organisms, *B. lactis* BI07 enolase shows a slightly lower affinity to human Plg. *Strep. pyogenes*, *Strep. pneumonia*e and *Strep. suis* enolases demonstrated K_D values for human Plg of 1–4, 0.55 and 14 nM, respectively (Pancholi & Fischetti, 1998; Bergmann *et al.*, 2003; Esgleas *et al.*, 2008). The best-fit nonlinear regression of data obtained in the saturation binding analysis to Plg indicated that the *B. lactis* BI07 enolase possesses only one site of binding to human Plg. Our data are in general agreement with those reported for *Strep. pyogenes* (Derbise *et al.*, 2004) and *Strep. pneumoniae* enolases (Bergmann *et al.*, 2005). However, while in the case of *Strep. pyogenes* the C-terminal lysines have been indicated as the Plg binding site, for pneumococcal enolase Plg-binding mainly depends on the internal Plg-binding site BS2 (residues 248–256) located on the surface-exposed loop L3 (Bergmann *et al.*, 2003, 2005; Ehinger *et al.*, 2004), while the C-terminal lysine residue(s) (BS1) is either not or only marginally involved. In particular, within the Plg-binding site BS2 of pneumococcal enolase Lys-251, Lys-254 and Glu-252 are critical for Plg binding (Ehinger *et al.*, 2004). The homology model of the *B. lactis* BI07 enolase indicated that the protein possesses a structural homologue of both the internal Plg-binding site BS2 of pneumococcal enolase and the C-terminal lysine that constitutes the Plg-binding site BS1. Site-direct mutagenesis of Lys-251, Glu-252 and Lys-255 within the BS2 homologue of *B. lactis* BI07 enolase impaired its Plg-binding activity. In contrast, the deletion of the *B. lactis* BI07 enolase C-terminal lysine

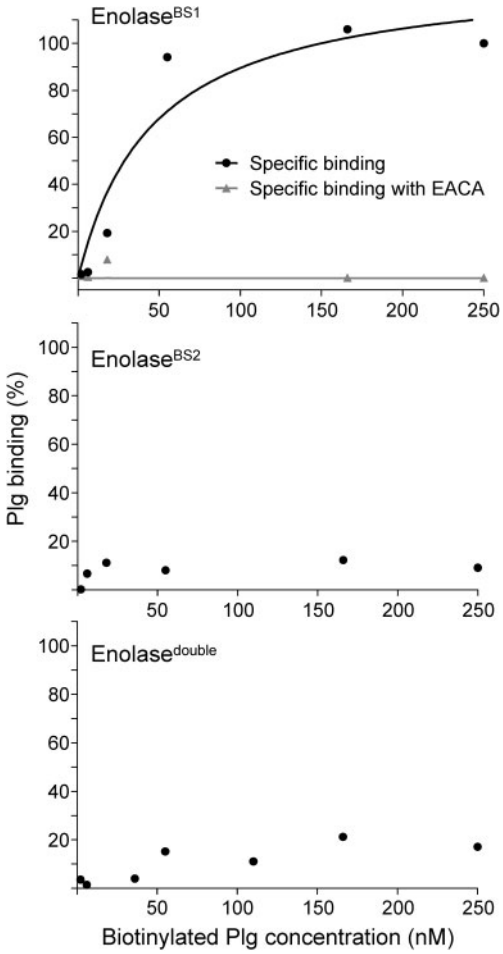

Fig. 7. Saturation binding analysis of biotinylated Plg to immobilized recombinant His-tagged *B. lactis* BI07 Enolase^{BS1}, Enolase^{BS2} and Enolase^{double} proteins. At each concentration of biotinylated Plg, specific binding (•) was calculated by subtracting the non-specific binding obtained in the presence of a 50-fold molar excess of unlabelled Plg from the total binding. For Enolase^{BS1}, a one-site hyperbolic binding function was fitted to the data and the K_D determined. The experiment was repeated in the presence of the lysine analogue EACA (0.5 M, grey triangles).

did not have any impact on Plg interaction. Taken together, our data demonstrate that the interaction between the *B. lactis* BI07 enolase and human Plg involves the internal Plg-binding site homologous to the BS2 site of pneumococcal enolase. In particular, as in the case of pneumococcal enolase (Ehinger *et al.*, 2004), the positively charged residues Lys-251 and Lys-255, and the negatively charged Glu-252, are vital for Plg interaction. Disruption of the enolase gene would provide *in vivo* proof of its role as a Plg receptor; however, the presence of only one bifidobacterial enolase gene, which is essential for bacterial survival, renders the construction of a viable enolase-defective *B. lactis* BI07 mutant impossible.

There is a growing number of reports concerning the expression of cytoplasmic housekeeping proteins on the bacterial cell surface of Gram-positive micro-organisms (Pancholi, 2001; Pancholi & Chhatwal, 2003; Schaumburg *et al.*, 2004; Sijbrandi *et al.*, 2005; Lee *et al.*, 2006; Antikainen *et al.*, 2007a; Knaust *et al.*, 2007). Although their mechanism of secretion still remains unclear, localized on the bacterial cell surface these multifunctional proteins acquire a 'moonlighting' function (Jeffery, 1999), with a role in the interaction process with the host. We believe that this is the first report of the localization of enolase on the cell surface of four species of *Bifidobacterium*, a health-promoting member of the human intestinal microbiota. Interestingly, the presence of surface enolase has been recently reported for two species belonging to the gut commensal genus *Lactobacillus* (Antikainen *et al.*, 2007a; Castaldo *et al.*, 2009). However, while the *Lactobacillus crispatus* surface enolase is a receptor for human Plg, the *Lactobacillus plantarum* surface enolase is a fibronectin-binding protein. Acting as a human Plg receptor, the bifidobacterial surface enolase may play a role in the interaction with the host. Although it will be necessary to screen for the presence of surface enolases in other bifidobacterial species, our findings raise the question of the impact of glycolytic enzymes in the biology of the *Bifidobacterium*–host interaction. In particular, concerning enolase, further studies are justified to investigate its capability to interact with extracellular matrix proteins such as laminin and fibronectin, as already reported for the enolases from *Lactobacillus*, *Streptococcus* and *Staphylococcus* (Antikainen *et al.*, 2007a).

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APPENDIX 3

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DnaK from *Bifidobacterium animalis* subsp. *lactis* is a surface-exposed human plasminogen receptor upregulated in response to bile salts

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Bifidobacterium animalis subsp. *lactis* lives in the gastrointestinal tract of most mammals, including humans. Recently, for the probiotic strain *B. animalis* subsp. *lactis* BI07, a dose-dependent plasminogen-binding activity was demonstrated and five putative plasminogen-binding proteins were identified. Here we investigated the role of surface DnaK as a *B. animalis* subsp. *lactis* BI07 plasminogen receptor. DnaK was visualized on the bacterial cell surface by transmission electron microscopy. The His-tagged recombinant DnaK protein showed a high affinity for human plasminogen, with an equilibrium dissociation constant in the nanomolar range. The capability to tolerate physiological concentrations of bile salts is a crucial feature for an intestinal symbiont micro-organism. By proteome analysis we demonstrated that the long-term exposure of *B. animalis* subsp. *lactis* BI07 to bile salts results in the upregulation of important surface plasminogen receptors such as DnaK and enolase. Moreover, adaptation of *B. animalis* subsp. *lactis* BI07 to physiological concentrations of bile salts significantly increased its capacity to interact with the host plasminogen system. By enhancing the bacterial capacity to interact with the host plasminogen, the gut bile environment may facilitate the colonization of the human host by *B. animalis* subsp. *lactis* BI07.

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INTRODUCTION

Human beings are in coevolution with a complex microbial community living in their gastrointestinal tract (GIT), the human intestinal microbiota (Gill *et al.*, 2006; Ley *et al.*, 2008). Active and metabolizing, the intestinal microbiota affects several host physiological features and has recently been considered as an integral component of the human physiology (Neish, 2009; Round & Mazmanian, 2009). Bifidobacteria are obligate anaerobes in the *Actinomycetales* branch of the high-G+C Gram-positive bacteria and can be found in the human GIT as both autochthonous and allochtonous residents (Klijn *et al.*, 2005). As inhabitants of the human gut they are only a minor component of the adult intestinal microbiota, but they largely predominate in the gastrointestinal ecosystem of breast-fed infants until weaning (Ventura *et al.*, 2009). The presence of bifidobacteria in the human GIT has been correlated with several health-promoting effects (Guarner & Malagelada, 2003); however, there is only fragmentary information about their ecology, physiology and interaction with the host. Originally isolated from fermented milk (Meile *et al.*, 1997), the species *Bifidobacterium animalis* subsp. *lactis* is commonly found in the guts of healthy adults and infants (Turroni *et al.*, 2009). The complete genome sequence of three *B. animalis* subsp. *lactis* strains has been recently determined, and important insights into its genetic basis for the interaction with the host and the adaptation to the human gut environment have been provided (Barrangou *et al.*, 2009). In particular, four genes with a possible role in the interaction with human epithelial cells have been identified, including two putative collagen-adhesion proteins, an elastin-binding protein and a fibronectin-binding protein. Another aspect of the *B. animalis* subsp. *lactis*–

Abbreviations: Anti-Eno, anti-pneumococcal enolase antiserum; BSH, bile salt hydrolase; EACA, ε-aminocaproic acid; GIT, gastrointestinal tract; PA, plasminogen activator(s); Plg, plasminogen; uPA, urokinase plasminogen activator.

host interaction was revealed by the dose-dependent human plasminogen (Plg)-binding activity demonstrated for the BI07 and DSM 10140 strains (Candela *et al.*, 2007).

Plg is the zymogen of plasmin, a trypsin-like serine protease with a broad substrate specificity. Plg is a single-chain glycoprotein with a molecular mass of 92 kDa and comprises an N-terminal pre-activation peptide (~8 kDa), five consecutive lysine-binding disulfide-bonded triple-loop kringle domains (K1–5), and a serine-protease domain containing the catalytic triad (Vassalli *et al.*, 1991). It is produced mainly by hepatocytes; however, other tissue sources for Plg synthesis have been identified, including the intestine (Zhang *et al.*, 2002). The active form, plasmin, is involved in fibrinolysis (Collen & Verstraete, 1975), homeostasis and degradation of the extracellular matrix and basement membrane (Saksela & Rifkin, 1988).

The capability to intervene with the Plg/plasmin system is a strategy for host colonization shared by several pathogens and commensals of the human GIT (Parkkinen & Korhonen, 1989; Schaumburg *et al.*, 2004; Lähteenmäki *et al.*, 2005; Sijbrandi *et al.*, 2005; Bergmann & Hammerschmidt, 2007; Hurmalainen *et al.*, 2007; Candela *et al.*, 2008b). The binding of Plg to the bacterial cell surface depends on the presence of surface Plg-binding proteins which can interact with Plg by means of a C-terminal lysine residue(s) or/and lysine-enriched internal Plg-binding motive(s) (Bergmann *et al.*, 2003, 2005; Bergmann & Hammerschmidt, 2007; Candela *et al.*, 2009; Sha *et al.*, 2009). With the recruitment of human Plg on the cell surface, and its subsequent conversion to plasmin, the micro-organism acquires a surface-associated and host-derived proteolytic activity useful for facilitating migration across physical and molecular barriers and for responding to the nutritional demands during the colonization process (Lähteenmäki *et al.*, 2005).

Similarly to the bifidobacterial species of human origin *Bifidobacterium bifidum* and *Bifidobacterium longum*, the probiotic species *B. animalis* subsp. *lactis* showed a dose-dependent binding activity to human Plg (Candela *et al.*, 2007). In the presence of host-derived Plg activators (PA), Plg captured on the *B. animalis* subsp. *lactis* cell surface was converted to plasmin, endowing the bacteria with a Plg-dependent surface-associated plasmin activity effective in the degradation of physiological host substrates (Candela *et al.*, 2008b). The complete inhibition of Plg binding to *B. animalis* subsp. *lactis* BI07 in the presence of the lysine analogue ε-aminocaproic acid (EACA) suggested that Plg binding is strongly dependent on lysine residues of surface Plg receptors. Five putative human Plg receptors were identified in the cell-wall fraction of *B. animalis* subsp. *lactis* strain BI07: DnaK, glutamine synthetase, enolase, bile salt hydrolase (BSH) and phosphoglycerate mutase (Candela *et al.*, 2007). Analogously to other Gram-positive bacteria (Lähteenmäki *et al.*, 2001), the *B. animalis* subsp. *lactis* Plg-binding proteins are highly conserved cytoplasmic proteins that, expressed on the bacterial cell wall, acquire a secondary 'moonlighting' function different from the one exerted in the cytoplasm. The glycolytic enzyme enolase of *B. animalis* subsp. *lactis* BI07 was characterized with respect to surface localization and Plg-binding activity. Proving its role as a bifidobacterial Plg receptor, *B. animalis* subsp. *lactis* BI07 enolase showed a high affinity for human Plg, with an equilibrium dissociation constant in the nanomolar range (Candela *et al.*, 2009).

Adaptation and tolerance to physiological concentrations of bile salts (usually below 5 mM) is a vital factor for bacterial colonization of the human gut ecosystem (Ridlon *et al.*, 2006; Sanchez *et al.*, 2007). Bile salts are detergent-like compounds that are secreted into the intestine during digestion. Besides their primary role in the emulsification and absorption of fats, bile salts also exert a strong antimicrobial activity by inducing membrane damage and causing oxidative stress to DNA (Bernstein *et al.*, 1999). The adaptation of *B. animalis* subsp. *lactis* to bile salts involves a global change in its protein expression profile (Sanchez *et al.*, 2007), and the issue of whether these changes could affect the manner of interaction with the host has been addressed. Very recently, with the attempt to specifically investigate if bile adaptation could influence the *Bifidobacterium*–host interaction, Ruiz *et al.* (2009) studied the effect of bile salts on the *B. longum* cell-wall proteome. The authors reported that the surface Plg receptors enolase and glutamine synthetase were upregulated in response to bile salts, suggesting that the adaptation of *B. longum* to the bile environment triggers the production of bacterial factors for intestinal colonization.

Here we assessed the role of DnaK as a surface Plg receptor in *B. animalis* subsp. *lactis* BI07 and also analysed the effect of the adaptation to the bile salts environment on the Plg-binding capacity of this bacterium. Demonstrating the influence of bile adaptation on the *B. animalis* subsp. *lactis*–host interaction process, the bile salts treatment induced the upregulation of surface Plg receptors and significantly increased the ability of the bacteria to interact with the host Plg system.

METHODS

Bacterial strains, media and growth conditions. *B. animalis* subsp. *lactis* BI07 was cultured in MRS medium (Difco) supplemented with 0.05 % (w/v) L-cysteine at 37 °C in anaerobic conditions, obtained by using Anaerocult A (Merck) in a jar. The bifidobacterial cells were grown for 18 h until they reached the stationary phase. Liquid MRS cultures were also performed with bile salts (oxgall; Difco) at the final concentrations of 1.2 and 3 g l^{-1}. Since human bile salts are difficult to retrieve, oxgall was used in this study. Oxgall has higher concentrations of bile salts and phospholipids and lower phospholipid/bile salts molar ratios than human bile (Coleman *et al.*, 1979), whereas the ratio of glycine- to taurine-conjugated bile acids is similar (Hafkenscheid & Hectors, 1975). *Escherichia coli* OneShot TOP10, BL21Star(DE3) and XL10-Gold strains were cultured at 37 °C in Luria–Bertani (LB) medium with shaking. MagicMedia (Invitrogen) was used for recombinant protein expression.

Kanamycin (50 μg ml^{-1}) was added as selective agent when appropriate.

Extraction of bifidobacterial cytoplasmic and cell-wall fractions. Cytoplasmic proteins were extracted as reported by Candela *et al.* (2009). Briefly, stationary-phase cells of *B. animalis* subsp. *lactis* BI07 were collected, washed in 50 mM Tris/HCl (pH 7.6), resuspended in 500 μl TE buffer [50 mM Tris/HCl (pH 7.6), 5 mM EDTA], and 50 μl Complete Protease Inhibitors Solution (Roche) was added. The suspension was sonicated and centrifuged for 10 min at 14 000 r.p.m. (22 000 ***g***) at 4 °C. The supernatant was collected and centrifuged for 2 h at 45 000 r.p.m. (176 000 ***g***) at 4 °C by using a Beckman Ultracentrifuge L7-55. The supernatant, containing cytoplasmic proteins, was stored at −20 °C. Cell-wall proteins were extracted as reported by Hardie & Williams (1998). Briefly, after washing in 50 mM Tris/HCl (pH 7.6), stationary-phase *B. animalis* subsp. *lactis* BI07 cells were resuspended in 2 ml protoplast buffer [50 mM Tris/HCl (pH 7.6), 1 M sucrose, 1.4 mM phenylmethylsulfonyl fluoride, 15 mg lysozyme ml^{-1}]. The suspension was incubated for 90 min at 37 °C and centrifuged for 3 min at 4000 r.p.m. (1667 ***g***) at 4 °C. The supernatant, containing the cell-wall proteins, was collected and stored at −20 °C.

Electron microscopy and immunoblot analysis. In order to visualize bifidobacterial DnaK on the *B. animalis* subsp. *lactis* BI07 cell surface, we performed pre-embedding immunogold experiments using whole bacterial cells. PBS-washed stationary-phase cells were adjusted to a concentration of 1×10^9 c.f.u. ml^{-1}, resuspended in 100 μl rabbit polyclonal anti-meningococcal DnaK antiserum (Knaust *et al.*, 2007) diluted 1 : 100 in PBS-1 % BSAe (BSA), and incubated for 1 h at 25 °C under constant agitation. After two washings with 1 ml PBS-1 % BSA, bacteria were resuspended in 25 μl anti-rabbit IgG coupled to 10 nm gold particles (Auro Probe, GE Healthcare) diluted 1 : 5 in PBS-1 % BSA and incubated for 30 min at 25 °C with constant agitation. Bacteria were then collected and washed twice in PBS-1 % BSA and fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer for 4 h at 4 °C. Transmission electron microscopy (TEM) processing was carried out as reported by Candela *et al.* (2007). Electron microscopic experiments were repeated four times. For immunoblot analysis, *B. animalis* subsp. *lactis* BI07 cytoplasmic or cell-wall proteins (10 μg) were subjected to SDS-PAGE with 12 % polyacrylamide and blotted onto a nitrocellulose membrane (Bio-Rad) by using the Minitrans-Blot Electrophoretic Cell (Bio-Rad). Post-transfer, the membrane was blocked in a solution of 4 % skim milk (Biolife) in TBS-T (0.15 % Tween 20 in TBS) and then incubated with anti-meningococcal DnaK antiserum (Knaust *et al.*, 2007). Subsequently, the membrane was washed in TBS-T and incubated with the peroxidase-conjugated anti-rabbit IgG (GE Healthcare). After TBS-T washing, the membrane was incubated with ECL Plus (GE Healthcare), and the signal was detected by using the PhosphorImager Storm system (GE Healthcare).

Construction of a *B. animalis* subsp. *lactis* BI07 genomic library and screening for the *dnaK* gene. Three hundred micrograms of *B. animalis* subsp. *lactis* BI07 chromosomal DNA was partially digested with the restriction enzyme *Mbo*I. DNA fragments of about 20 kb were isolated by ultracentrifugation at 26 000 r.p.m. for 24 h at 18 °C in a sucrose gradient (10–40 %), purified by precipitation with ethanol and ligated to *Bam*HI-digested Lambda EMBL3 vector (Stratagene). Recombinant lambda phages were packaged by using the Lambda EMBL3/*Bam*HI Gigapack III Gold Cloning kit (Stratagene). The library was amplified and titrated as described in the kit manual. A digoxigenin-labelled DNA probe targeting the *dnaK* gene was obtained by amplifying the chromosomal DNA of *B. longum* NCC 2705 with the primer set L-DnaK (5′-TTGGCACGTGCAGTTG-3′) and R-DnaKwt (5′-TCACTTGTTG-TCCTTGTCG-3′) (designed based on the genome sequence of *B. longum* NCC 2705) by using the DIG DNA Labelling and Detection kit (Roche). For screening, the phage library was plated on 120 mm NZY agar plates using *E. coli* XL1-Blue MRA P2 as host strain. Plates were blotted onto nylon membrane (Hybond-N+, GE Healthcare). Neutralization, denaturation, cross-linking, membrane hybridization with the DIG-labelled DNA probe, and detection were carried out as described by the DIG-DNA Labelling and Detection kit manual (Roche). Plaques corresponding to the positive signals in the original plates were cut out and resuspended in SM buffer to isolate the recombinant phage population. Phage DNA was extracted by using the Qiagen Lambda mini kit (Qiagen). Positive clones were sequenced and the sequence of the *B. animalis* subsp. *lactis* BI07 *dnaK* gene obtained.

Cloning, expression, purification of recombinant *B. animalis* subsp. *lactis* BI07 His_6-DnaK. *B. animalis* subsp. *lactis* BI07 chromosomal DNA was used as template in a PCR for the amplification of the *dnaK* gene using the primer set DnaK-TOPO-L (5′-CACCATGGGACGCGCAGTTGGT-3′) and DnaK-TOPO-R (5′-TTACTTGTTGTCCTTGTCGTCGTC-3′). The PCR product was cloned in the expression vector pET200/D-TOPO (Invitrogen) to obtain the construct pDKwt. Cloning and expression of *B. animalis* subsp. *lactis* BI07 *dnaK* gene were carried out in *E. coli* TOP-10 and *E. coli* BL21Star(DE3) (Invitrogen), respectively. The His-tagged fusion *B. animalis* subsp. *lactis* BI07 DnaK was purified by affinity chromatography under native conditions on Ni-nitrilotriacetic acid resin according to the manufacturer's protocol (Invitrogen). The purified protein was dialysed at 4 °C using Spectra/Por membranes 6000–8000 Da (Spectrum Laboratories) and 20 mM Tris, 120 mM NaCl as dialysis buffer. The expression of the His-tagged recombinant protein His_6-DnaK was verified by Western blot analysis with polyclonal anti-meningococcal DnaK antiserum (Knaust *et al.*, 2007) and anti-HisTag antibody (Sigma).

Plg-binding analysis by solid-phase binding assay. Human Plg (Sigma-Aldrich) was biotinylated by using the EZ-Link Micro Sulfo-NHS-SS Biotinylation kit (Pierce). The solid-phase Plg-binding assay was performed as previously described by Sanderson-Smith *et al.* (2006, 2007). Microtitre plates (96-well; OptiPlate-96, Perkin Elmer) were coated with 150 nM recombinant DnaK (50 μl in 0.1 M $NaHCO_3$) and incubated at 4 °C. After washing with PBS, plates were blocked with 200 μl BSA 2 % in PBS for 1 h at 37 °C. After three PBS washings, increasing concentrations of biotinylated Plg (2, 6, 18, 36, 55, 110, 166, 250 nM) in PBS were added to the plates, in the presence or absence of 50-fold molar excess of unlabelled Plg. Plg was allowed to bind to immobilized proteins for 2 h at 25 °C. Plates were washed three times in PBS and 50 μl ExtrAvidin HRP conjugated (Sigma-Aldrich) diluted 1 : 5000 in PBS-1 % BSA was added to each well. Plates were incubated for 2 h at 25 °C and washed four times with PBS and 0.05 % Tween 20. One hundred microlitres of Chemiluminescent Peroxidase Substrate for ELISA (Sigma-Aldrich) was added to each well and luminescence was read by using the Victor^{3}V 1420 Multilabel Counter scanner (Perkin Elmer) and the software Wallac 1420 WorkStation. As negative control for Plg binding, BSA-coated wells were utilized. Plg-binding experiments were repeated in three biological replicates where, for each Plg concentration, three technical replicates were performed. To assess the involvement of lysine residues in the DnaK–Plg interaction, experiments were repeated in the presence of the lysine analogue ε-aminocaproic acid (EACA) (0.5 M). Data were normalized against the highest and lowest luminescence value, and non-linear regression analysis was carried out by using GraphPad Prism (version 5.0). For calculation of the equilibrium dissociation constant (K_D), one- and two-site binding analyses were performed and the best-fit curve was chosen.

Two-dimensional gel electrophoresis and comparative analysis of protein expression. Experiments were carried out as

reported by Candela *et al.* (2007). Briefly, 40 μg samples of *B. animalis* subsp. *lactis* BI07 cell wall proteins were resolved by two-dimensional electrophoresis. Isoelectric focusing was carried out using Immobiline DryStrips with a linear pH gradient between 4 and 7 (7 cm) on an IPGphor system (GE Healthcare) and proteins were separated by SDS-PAGE at 160 V for 2.5 h. Spots were visualized by silver staining. Protein patterns in the gels were recorded as digitized images using a GS-800 imaging densitometer (Bio-Rad). Spot detection, matching and the examination of differentially expressed proteins were performed by PDQuest v. 8.0.1 software (Bio-Rad). Two biological and three technical replicates were made per condition and formed one replicate group with average normalized spot intensities. Comparison was carried out between cell-wall fractions from stationary-phase cells grown in MRS medium and in MRS in the presence of 1.2 g bile salts l^{-1} (oxgall; Difco). A threshold of at least two times enhanced or decreased expression level was chosen to consider the differentially expressed proteins. The Plg-binding proteins were identified by comparison of the two-dimensional gels with the reference map reported by Candela *et al.* (2007).

MALDI-TOF-MS analysis of Plg binding to *B. animalis* subsp. *lactis* BI07. Plg binding to *B. animalis* subsp. *lactis* BI07 cells was evaluated by using a MALDI-TOF-MS-based approach, as reported by Candela *et al.* (2008a). Stationary-phase *B. animalis* subsp. *lactis* BI07 cells (1×10^9 c.f.u.) grown in MRS with and without 1.2 g bile salts l^{-1} (oxgall; Difco) were incubated with 0, 10, 20, 50, 100 and 200 μg human Plg (Sigma-Aldrich). Subsequently, bifidobacteria were washed three times with PBS to remove unbound Plg. After washing, bacterial cells were resuspended in 20 μl 2 % NH_4Cl for MALDI-TOF analysis. In order to detect Plg recruited on the bacterial cell surface, 1.0 μl of each bacterial suspension was spotted, airdried, and then overlaid with 1.0 μl matrix solution (12.5 mg ferulic acid ml^{-1} in a mixture of formic acid/acetonitrile/water 17 : 33 : 50). The resulting droplet was left to crystallize by airdrying and analysed in a Voyager DE Pro MALDI-TOF mass spectrometer (Applied Biosystems) operating in linear positive ion mode in the range of 20 000–100 000 Da and equipped with a pulsed N_2 laser at 337 nm. Default operating conditions were as follows: accelerating voltage 25 000 V, grid voltage 22 500 V, extraction delay time 750 ns. All acquisitions were generated automatically on the instrument software (Voyager, Biospectrometry Workstation) using a random search pattern mode and based on overhanging 1600 shots from 20 non-overlapping positions (80 shots per position). Data were externally calibrated with a standard BSAe (BSA) solution (67 kDa). As analytical standard, 200 ng human Plg were spotted. For each Plg concentration three biological and three technical replicates were carried out.

In order to evaluate the impact of DnaK and enolase on *B. animalis* subsp. *lactis* BI07 Plg-binding activity, Plg-binding assay of whole *B. animalis* subsp. *lactis* BI07 cells was performed after pre-treatment of cells with cross-reactive anti-meningococcal DnaK antiserum (Knaust *et al.*, 2007) or anti-pneumococcal enolase antiserum (anti-Eno) (Bergmann *et al.*, 2003), respectively. To this aim, 1×10^9 c.f.u. stationary-phase *B. animalis* subsp. *lactis* BI07 cells were washed in PBS and incubated with anti-meningococcal DnaK antiserum diluted 1/250 or anti-Eno diluted 1/100 in PBS-1 % BSA, for 1 h at 25 °C under constant agitation. After two washings with PBS-1 % BSA, cells were resuspended in PBS and incubated with 100 μg human Plg (Sigma-Aldrich) for 30 min at 37 °C with constant agitation. Subsequently, bifidobacteria were washed three times with PBS to remove unbound Plg. After washing, bacterial cells were resuspended in 20 μl NH_4Cl 2 % for MALDI-TOF analysis of Plg binding, as previously described. For each experimental condition three biological and three technical replicates were carried out.

Plasmin activity assay. This was carried out as reported by Candela *et al.* (2008b). Briefly, stationary-phase *B. animalis* subsp. *lactis* BI07 cells grown in MRS with and without 1.2 g bile salts l^{-1} (oxgall; Difco) were washed with PBS, adjusted to a concentration of 1×10^9 c.f.u. ml^{-1} and incubated for 30 min at 37 °C with 20 μg Plg ml^{-1} (Sigma-Aldrich) in PBS. After two PBS washing steps, cells were resuspended in 50 mM Tris/HCl, pH 7.5 (TBS). A volume of 100 μl of the bacterial cell suspension was added per well of a 96-well microtitre plate. Plg was activated with 0.06 KIU urokinase PA (uPA) (Calbiochem), and 30 μl of plasmin-specific chromogenic substrate solution, containing 0.54 mM D-valyl-leucyl-lysine-*p*-nitroanilide dihydrochloride (S-2251) (Sigma-Aldrich), was added. Absorbance at 405 nm was measured by using a Multiskan Ascent V1.24 (Thermo Electron Corporation) immediately after the addition of S-2251 and at intervals of 7 min during 1 h incubation at 37 °C. As negative control the kinetic experiment was carried out with *B. animalis* subsp. *lactis* BI07 cells not incubated with Plg. Further controls were performed with *B. animalis* subsp. *lactis* BI07 cells incubated with Plg in the presence of 0.1 M EACA (Sigma-Aldrich) and in the absence of uPA. Controls for spontaneous hydrolysis of S-2251 were carried out with the chromogenic substrate alone and in the presence of PA. Experiments were repeated twice and 10 technical replicates were carried out for each condition

RESULTS

Localization of DnaK protein on the cell surface of *B. animalis* subsp. *lactis* BI07

The presence of the DnaK protein in the cytoplasmic and cell-wall fractions of *B. animalis* subsp. *lactis* BI07 was demonstrated by immunoblot analysis using a cross-reactive anti-meningococcal DnaK antiserum (anti-DnaK) (Knaust *et al.*, 2007) (Fig. 1). Immunoelectron microscopy was conducted to confirm the presence of DnaK on the surface of the bacteria. *B. animalis* subsp. *lactis* BI07 cells were incubated under pre-embedding labelling conditions with the anti-DnaK antiserum followed by the secondary antibody labelled with 10 nm gold particles. Analysis of ultrathin sections at a magnification of ×22 000 showed the presence of DnaK in the bacterial cell-wall region (black dots, arrowed in Fig. 2). No non-specific binding of the secondary antibody was detected (data not shown).

Analysis of Plg binding to *B. animalis* subsp. *lactis* BI07 DnaK

A genomic library of *B. animalis* subsp. *lactis* BI07 was constructed and screened for the *dnaK* gene by using a digoxigenin-labelled DNA probe targeting the *B. longum* NCC 2705 *dnaK* gene. The nucleotide sequence of *B. animalis* subsp. *lactis* BI07 *dnaK* (GenBank accession no. AB514431) showed 100 % identity with the *dnaK* gene of *B. animalis* subsp. *lactis* DSM 10140 (GenBank accession no. CP001606) and 87 % with that of *B. longum* NCC 2705 (GenBank accession no. AE014295). In order to study the specific Plg-binding activity of *B. animalis* subsp. *lactis* BI07 DnaK, a His_6-tagged DnaK protein was expressed and, after purification, a solid-phase Plg-binding assay was carried out (Sanderson-Smith *et al.*, 2007). The binding

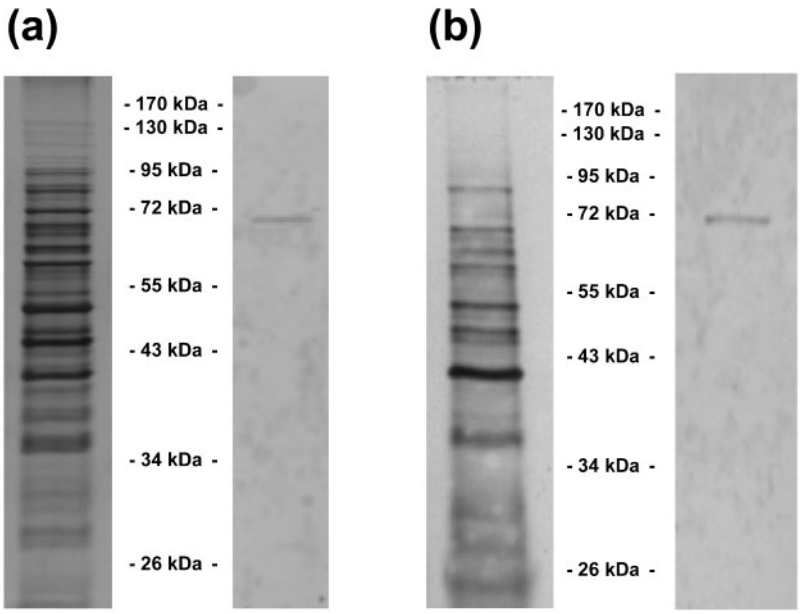

Fig. 1. Immunoblot analysis (right panels) using anti-DnaK antiserum of cytoplasmic (a) and cell wall (b) protein fractions of *B. animalis* subsp. *lactis* BI07 resolved by SDS-PAGE. (right panels).

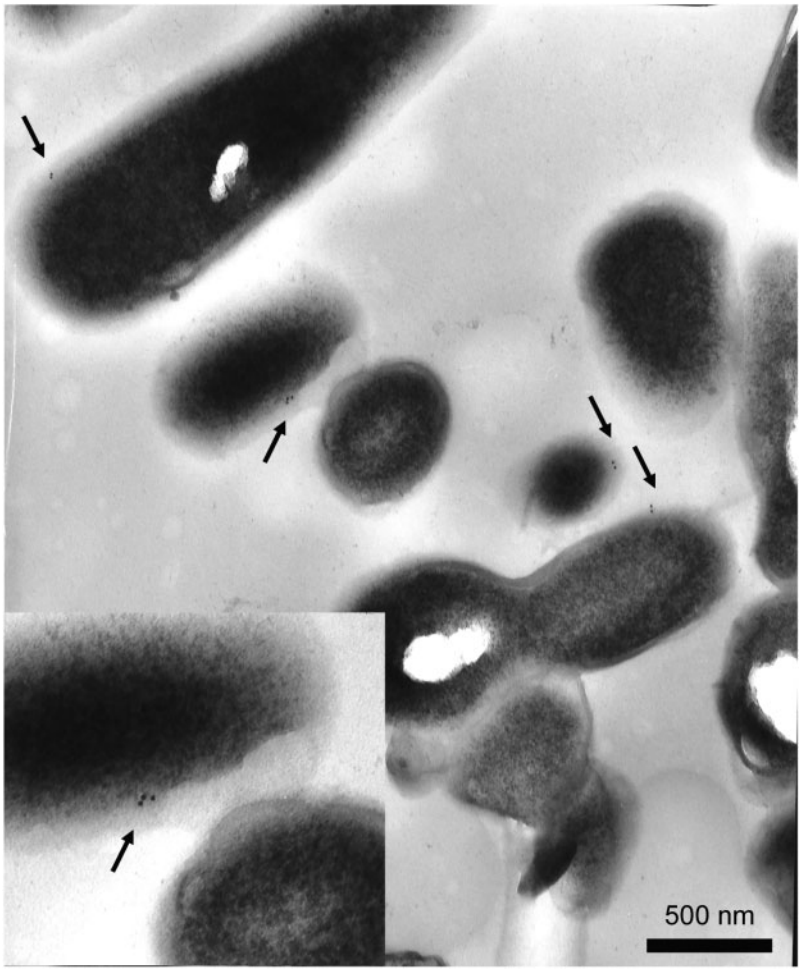

Fig. 2. Immunoelectron microscopic localization of DnaK on the cell surface of *B. animalis* subsp. *lactis* BI07. DnaK was detected on the bacterial surface by anti-DnaK antiserum and secondary antibody coupled to 10 nm gold particles in pre-embedding experiments. An ultrathin section shows DnaK (black dots, arrowed) localized directly in the cell-wall region.

assay demonstrated a dose-dependent interaction between biotinylated Plg and immobilized recombinant *B. animalis* subsp. *lactis* BI07 DnaK (Fig. 3). In the presence of 150 nM Plg a saturation binding was achieved. Non-specific binding was determined in the presence of 50-fold molar excess of unlabelled Plg, and specific binding was calculated by subtracting non-specific binding from the total binding obtained at each concentration of biotinylated Plg. As negative control BSA-coated wells were utilized. The best-fit non-linear regression analysis allowed calculation of an equilibrium dissociation constant (K_D) for the interaction with Plg of about 11 nM. The pivotal role of DnaK lysine residues in Plg binding was demonstrated with 0.5 M EACA as competitor, which completely inhibited Plg binding (Fig. 3).

Role of surface DnaK and enolase in Plg binding to *B. animalis* subsp. *lactis* BI07

In order to provide experimental evidence of the role of surface DnaK and enolase as Plg receptors, we evaluated the impact of pre-treatment with anti-DnaK or anti-enolase antibodies on *B. animalis* subsp. *lactis* BI07 Plg-binding activity. Bacterial cells were pre-treated with anti-DnaK or anti-Eno (Candela *et al.*, 2009) antisera and their Plg-binding activity was compared with that of untreated bacteria using MALDI-TOF analysis. As a control, *B. animalis* subsp. *lactis* BI07 cells not treated with Plg were utilized. The results showed that treatment with the anti-Eno antiserum decreased the Plg-binding activity by about 30 %, and treatment with the meningococcal anti-DnaK antiserum decreased it by about 80 % (Fig. 4). In contrast, no reduction in Plg binding was measured when using anti-rabbit IgG as blocking antibodies. These results suggest that both enolase and especially DnaK are of importance for the sequestration of plasminogen by *B. animalis* subsp. *lactis* B107.

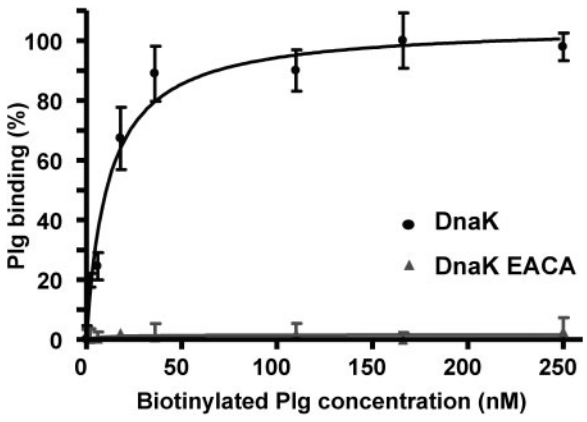

Fig. 3. Saturation binding analysis of biotinylated Plg to immobilized recombinant *B. animalis* subsp. *lactis* BI07 His-tagged DnaK. The black dots represent the specific binding. Specific Plg binding was calculated at each concentration of biotinylated Plg by subtracting non-specific binding obtained in the presence of 50-fold molar excess of unlabelled Plg from total binding. A one-site hyperbolic binding function was fitted to the data and the K_D determined. The experiment was repeated in the presence of the lysine analogue, EACA (0.5 M, grey triangles). Values represent the mean ± SD of 9 measurements.

Impact of bile salts exposure on the *B. animalis* subsp. *lactis* BI07–plasmin(ogen) interaction

First, the capability of *B. animalis* subsp. *lactis* BI07 to grow in the presence of bile salts was tested. To this end, *B. animalis* subsp. *lactis* BI07 was grown in batch cultures in the absence and in the presence of bile salts at concentrations of 1.2 and 3 g l^{-1} (Sanchez *et al.*, 2005). As reported for *B. longum* NCIMB 8809, growth rates were very similar during the exponential phase in each of the three different conditions (Fig. 5). In contrast, at the end of the stationary-phase the c.f.u. ml^{-1} were significantly lower in the presence of bile salts (1.2×10^9, 5×10^8 and 1×10^8 c.f.u. ml^{-1} for bile salts concentrations of 0, 1.2 and 3 g l^{-1}, respectively). In order to assess whether the physiological changes that are a consequence of the long-term exposure of *B. animalis* subsp. *lactis* BI07 to bile salts, can affect the expression of surface Plg receptors, a comparative proteomic approach was performed. The cell-wall proteomes of *B. animalis* subsp. *lactis* BI07 grown in media supplemented or not supplemented with bile salts at the concentration of 1.2 g l^{-1} were compared by two-dimensional gel electrophoresis (Fig. 6). Plg-binding proteins are numbered as reported by Candela *et al.* (2007). The expression profiles showed that exposure to bile salts induced a 2.8-, 2.3- and 10-fold upregulation of three Plg-binding proteins: DnaK, enolase and phosphoglycerate mutase, respectively. Surprisingly, the putative Plg-binding protein BSH was slightly downregulated (by about 1.7-fold) after bile exposure. A downregulation was also observed for the as yet unidentified Plg-binding proteins no. 2 and no. 8.

To investigate whether bile salts adaptation affects the Plg-binding capacity of *B. animalis* subsp. *lactis* BI07, we compared the Plg-binding activity of stationary-phase cells grown in the presence or in the absence of 1.2 g bile salts l^{-1}. *B. animalis* subsp. *lactis* BI07 cells were incubated with different concentrations of Plg and the bacterial-bound Plg was measured by MALDI-TOF (Fig. 7). Although *B. animalis* subsp. *lactis* BI07 showed a dose-dependent Plg-binding activity independently of bile salts supplementation, bacteria grown in the presence of bile salts were about 10 times more efficient in Plg binding. In a plasmin activity assay we compared the capability of *B. animalis* subsp. *lactis* BI07 cells grown with and without 1.2 g bile salts l^{-1} to acquire a Plg-dependent surface-associated plasmin activity after the addition of host PA. To this end, *B. animalis* subsp. *lactis* BI07 stationary-phase cells were incubated with 20 μg Plg ml^{-1} and the cell-captured Plg was then activated to plasmin by the addition of uPA. The

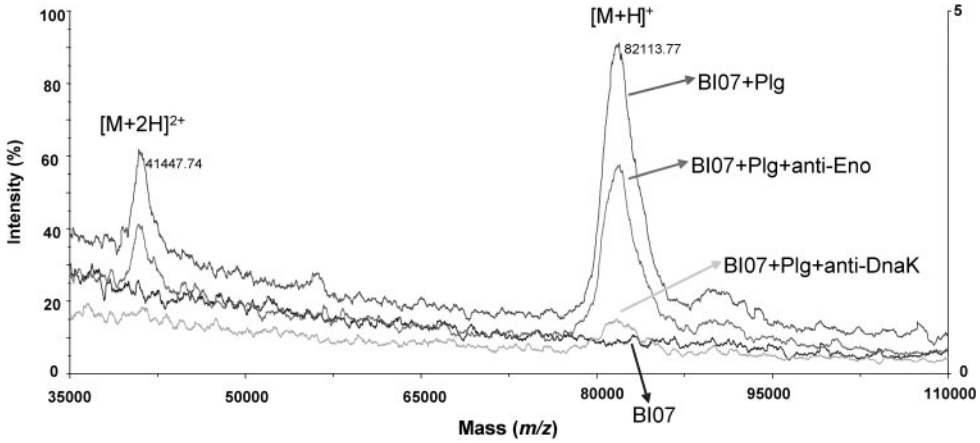

Fig. 4. MALDI-TOF analysis of Plg binding to *B. animalis* subsp. *lactis* BI07 cells untreated (BI07 + Plg), and pre-treated with anti-DnaK (BI07 + Plg + anti-DnaK) or anti-Eno (BI07 + Plg + anti-Eno). As negative control, *B. animalis* subsp. *lactis* BI07 cells not incubated with Plg (BI07) are shown. Plg $[M+H]^+$ signal at 81 kDa, $[M+2H]^{2+}$ at 40 kDa.

acquired bacterial surface-associated plasmin activity was evaluated in kinetic experiments by measuring the hydrolysis of the plasmin-specific chromogenic substrate S-2251 at intervals of 7 min over a period of 1 h (Fig. 8). The *B. animalis* subsp. *lactis* BI07 cells grown in the presence of bile salts showed a significant increase in the rate of S-2251 hydrolysis, demonstrating a higher rate of surface-associated Plg-derived plasmin activity compared with cells grown in standard conditions. As expected, the lysine analogue EACA was effective in inhibition of the plasmin formation of the *B. animalis* subsp. *lactis* BI07 cell surface, proving the essential role of the Plg lysine-binding sites for its recruitment to the bacterial cell surface (Fig. 8).

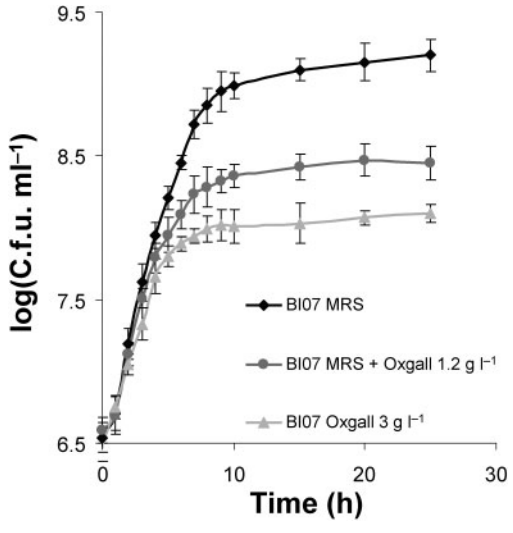

Fig. 5. Growth curves of *B. animalis* subsp. *lactis* BI07 in MRS with 0 (black), 1.2 (dark grey) and 3 g l^{-1} bile salts (light grey). For all the experimental conditions growth curves were performed in three biological replicates and values represent the mean ± SD.

DISCUSSION

In this work we demonstrate the importance of *B. animalis* subsp. *lactis* BI07 DnaK as a surface receptor for human Plg. DnaK is a highly conserved primary cytoplasmic protein of 67 kDa belonging to the heat-shock protein 70 family (HSP70) (Bukau & Horwich, 1998). HSP70s are chaperones with an important role in protein folding and transport. By immunoelectron microscopy we documented the presence of DnaK on the cell surface of *B. animalis* subsp. *lactis* BI07. Similarly to the surface-located *B. animalis* subsp. *lactis* BI07 enolase (Candela *et al.*, 2009), DnaK lacks predicted protein-sorting elements and anchorage on the bacterial cell wall. Mechanisms of secretion and surface localization of these cytoplasmic enzymes are still under debate. However, for *Streptococcus pneumoniae*, scavenging of cytoplasmic proteins released through allolysis was proposed as a possible mechanism for surface localization (Claverys & Havarstein, 2007). Strengthening our findings, DnaK had been previously shown to be present on the cell surface of several pathogens such as *Neisseria meningitidis*, *Listeria monocytogenes* and *Mycobacterium tuberculosis* (Schaumburg *et al.*, 2004; Knaust *et al.*, 2007; Xolalpa *et al.*, 2007), as well as the probiotic member of the human intestinal microbiota *Lactobacillus salivarius* (Kelly *et al.*, 2005). Although for *Lb. salivarius* no information had been provided concerning the DnaK function on the cell surface, in the case of *N. meningitidis*, *L. monocytogenes* and *M. tuberculosis* its role as receptor for human Plg was proved. The His-tagged *B. animalis* subsp. *lactis* BI07 DnaK protein was purified and its affinity for human Plg was evaluated in a saturation binding analysis. According to our data, the *B. animalis*

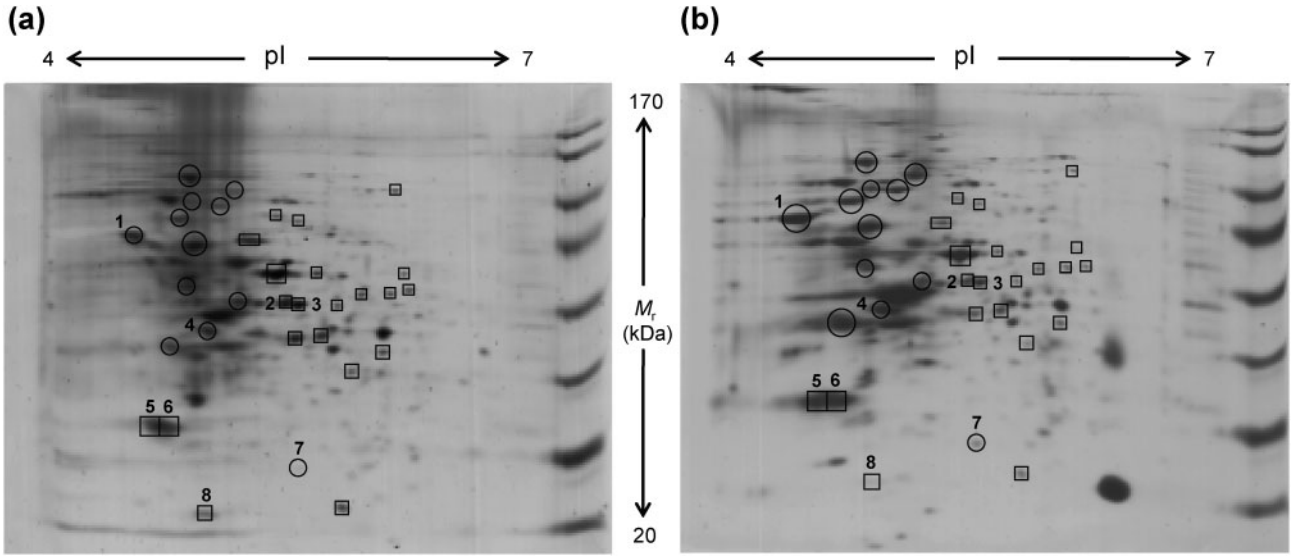

Fig. 6. Cell-wall proteome of stationary-phase *B. animalis* subsp. *lactis* BI07 cells cultured with (b) and without (a) bile salts at 1.2 g l^{-1}. Cell-wall proteins up- and downregulated as a consequence of bile salts adaptation are marked with circles and squares, respectively. Plg-binding proteins are numbered: DnaK (1), unidentified (2), glutamine synthetase (3), enolase (4), BSH (5, 6), phosphoglycerate mutase (7), unidentified (8).

subsp. *lactis* BI07 DnaK shows a high affinity for human Plg with a K_D of about 11 nM, a value significantly higher than that reported for *B. animalis* subsp. *lactis* BI07 enolase (K_D=42.8 nM) (Candela *et al.*, 2009). The complete inhibition of Plg binding in the presence of 0.5 M EACA indicates that DnaK lysine residue(s) are crucial for the Plg interaction. The pretreatment of *B. animalis* subsp. *lactis* BI07 cells with cross-reactive DnaK-specific antiserum had a detrimental impact on the bacterial Plg-binding capacity, demonstrating the importance of DnaK as a surface Plg receptor. Pre-treatment with anti-Eno also reduced Plg binding to *B. animalis* subsp. *lactis* BI07, but by only 30 %, compared to the 80 % reduction seen with anti-DnaK antiserum. While it would be tempting to speculate that this difference is due to the higher Plg affinity of DnaK compared to enolase, we can not exclude that it is the consequence of a different efficacy in blocking the respective antigens between the two antisera. Moreover, the contribution of a single Plg receptor to Plg recruitment on the bacterial cell surface can be influenced by other variables alongside the mere affinity for Plg, such as its availability on the cell surface and the number of binding epitopes (Knaust *et al.*, 2007).

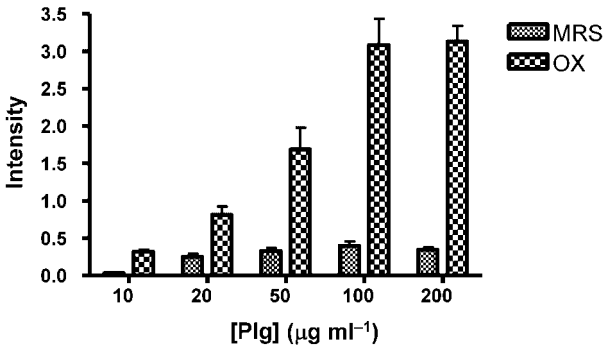

Fig. 7. MALDI-TOF analysis of the Plg-binding activity of stationary-phase cells of *B. animalis* subsp. *lactis* BI07 grown in the presence (OX) or in the absence (MRS) of 1.2 g bile salts l^{-1}. The bars represent the response, expressed as Plg amplitude/reference signal amplitude (RS amplitude), with the standard deviation, of *B. animalis* subsp. *lactis* BI07 cells incubated with different concentrations of Plg (10, 20, 50, 100, 200 μg ml^{-1}). Reference signal: 23 000 Da.

Adaptation of *B. animalis* subsp. *lactis* BI07 to bile salts induces a complex physiological response which influences the cell envelope proteome. Similarly to *B. longum* (Ruiz *et al.*, 2009), the long-term exposure of *B. animalis* subsp. *lactis* BI07 to bile salts results in the upregulation of the important surface Plg receptors DnaK and enolase. Supporting these findings, a recent study of the *B. animalis* subsp. *lactis* protein response to bile salts reported the upregulation of DnaK (Sanchez *et al.*, 2007). In order to investigate if the physiological changes as a consequence of bile salts response could have an impact on the *B. animalis* subsp. *lactis*–Plg interaction, we compared the Plg-binding activity and surface-associated Plg-dependent plasmin activity of *B. animalis* subsp. *lactis* BI07 cells grown with and without 1.2 g bile salts l^{-1}. According to our data, the *B. animalis* subsp. *lactis* BI07 cells grown in the presence of bile salts were about 10 times more efficient in Plg binding

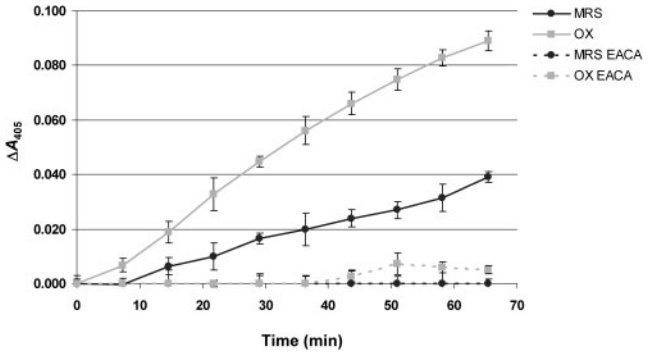

Fig. 8. Plasmin activity of *B. animalis* subsp. *lactis* BI07 cells grown in the presence (OX) or in the absence (MRS) of 1.2 g bile salts l^{-1}. Plg-pretreated bacterial cells were incubated with uPA and then with the plasmin-specific chromogenic substrate S-2251. The kinetics of plasmin formation was evaluated by measuring the increase in A_{405} at intervals of 7 min during 1 h incubation. Bacterial cells untreated with Plg were used as negative control. Experiments were repeated by incubating the bacteria with Plg in the presence of 0.1 M EACA. Values represent the mean ± SEM of 20 measurements.

compared to cells cultured in standard medium and, after the addition of host uPA, these bacteria exhibited a significantly higher rate of acquired Plg-dependent surface-associated plasmin activity. Taken together, these data prove that bile salts enhance the ability of *B. animalis* subsp. *lactis* BI07 to interact with the host Plg system, and show the potential impact of the GIT bile environment on the *B. animalis* subsp. *lactis*–host interaction process. Even if the upregulation of surface Plg receptors such as enolase and DnaK can partially explain this increase in Plg-binding activity, other mechanisms may be involved. For instance, affecting the accessibility of Plg receptors, changes in the *B. animalis* subsp. *lactis* cell-surface structure may occur in response to bile adaptation (Ruiz *et al.*, 2007) and may influence the bacterial affinity for human Plg (Stie *et al.*, 2009).

Besides the demonstration of the importance of DnaK as a surface Plg receptor, in this work we report for the first time that *B. animalis* subsp. *lactis* significantly increases its affinity for human Plg in response to physiological concentrations of bile salts. Since the ability of *Bifidobacterium* to interact with the plasmin(ogen) system of the human host may have a role in facilitating the colonization of the human GIT (Candela *et al.*, 2008b), our results provide evidence that the adaptation of *B. animalis* subsp. *lactis* to the gut bile environment may favour its capacity to colonize the human GIT. Further studies will be necessary to determine the mechanisms underlying the enhancement of *B. animalis* subsp. *lactis* BI07 Plg affinity in the presence of bile salts, as well as how bile influences the biology of the interaction process between *B. animalis* subsp. *lactis* BI07 and the host enterocytes. However, our findings provide some insights into the adaptation of *B. animalis* subsp. *lactis* to the human gastrointestinal environment. Moreover, the importance of DnaK as a *B. animalis* subsp. *lactis* Plg receptor justifies further studies to attempt to characterize the mechanisms of Plg binding in more detail.

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APPENDIX 4

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Relevance of *Bifidobacterium animalis* subsp. *lactis* Plasminogen Binding Activity in the Human Gastrointestinal Microenvironment▽

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Human plasmin(ogen) is regarded as a component of the molecular cross talk between the probiotic species *Bifidobacterium animalis* subsp. *lactis* and the human host. However, up to now, only *in vitro* studies have been reported. Here, we demonstrate that the probiotic strain *B. animalis* subsp. *lactis* BI07 is capable of recruiting plasmin(ogen) present at physiological concentrations in crude extracts from human feces. Our results provide evidence that supports the significance of the *B. lactis*-plasmin(ogen) interaction in the human gastrointestinal tract.

Bifidobacterium animalis subsp. *lactis* is a Gram-positive lactic acid bacterium belonging to the *Actinobacteria* phylum. Originally isolated from fermented milk (13, 14, 22), *B. animalis* subsp. *lactis* inhabits the gut of healthy adults and infants (20, 24). Merging health-promoting activities with technological properties, *B. animalis* subsp. *lactis* is one of the most common probiotic bifidobacterial species utilized in commercial dairy products in North America and Europe (3). However, despite its broad usage as a probiotic, few molecules that mediate the *B. animalis* subsp. *lactis*-host interaction have been described. We previously reported the capability of this probiotic microorganism to bind human plasmin(ogen) (5, 6, 7). According to our data, the probiotic strain *B. animalis* subsp. *lactis* BI07 (21) possesses a dose-dependent binding activity to human plasminogen (Plg) in concentrations ranging from 5 to 100 ng/μl (7). Plg (92 kDa) is the zymogen of the serine protease plasmin which is involved in fibrinolysis, homeostasis, and degradation of basement membranes and the extracellular matrix (17). Although it is produced mainly by hepatocytes, other tissue sources of Plg have been identified, including the intestine (25). Plg activation in human tissues is a process tightly regulated by the balance between plasminogen activators (PA), such as the urokinase-type activator (uPA) and the tissue-type activator (tPA), and their specific inhibitors, PAI-1 and PAI-2.

Plasmin captured on the bacterial cell surface has been reported to favor the bacterial dissemination in host tissues, and the capacity to intervene with the host Plg system has been traditionally regarded as a paradigm of bacterial pathogenicity (4, 12). However, Plg binding to commensal species of *Lactobacillus*, *Bifidobacterium*, and streptococci has been recently reported, raising a debate on the role of Plg binding in bacterial commensalism (2, 7, 11). In a previous *in vitro* study, we demonstrated that in the presence of Plg and host PA, *B. animalis* subsp. *lactis* acquires a surface-bound Plg-derived proteolytic activity effective in the degradation of physiological substrates (6). Thus, we hypothesized that the interaction with components of the host Plg system in the luminal compartment of the human gastrointestinal tract (GIT) would endow *B. animalis* subsp. *lactis* with a surface-associated plasmin activity useful to gain access to the mucus layer overlying enterocytes, the ideal habitat for commensal microorganisms (10, 19). However, even if the intervention with the host plasmin(ogen) system has been recently regarded as a component in the *B. animalis* subsp. *lactis*-host molecular cross talk (8, 18), plasmin(ogen) binding to *B. animalis* subsp. *lactis* has been studied only in phosphate-buffered saline (PBS) suspensions, and its relevance in the complex human gastrointestinal ecosystem remains an unanswered question. With the intent to shed light on this last issue, we evaluated the capability of the probiotic strain *B. animalis* subsp. *lactis* BI07 to recruit plasmin(ogen) present at physiological concentrations in human crude fecal extracts. Reflecting the entire content of the full length of the colon/rectum (1), fecal extracts represent the only possibility for a noninvasive sampling of the human GIT content. In our study, 10 healthy Italian individuals ≥30 years of age were enrolled. None of the subjects had a history of gastrointestinal disorders at the time of sampling. With the exception of antibiotics, probiotics, and functional foods, no dietary restriction was prescribed for at least 4 weeks prior to sampling. Informed consent was obtained from each enrolled subject. Fecal extracts were obtained by following the procedure reported by Ivanov et al. (9) with slight modifications. Briefly, 6 g of feces was resuspended in 10 ml of PBS with complete protease inhibitor (Roche). After the addition of 3-mm glass beads (BioSpec Products), samples were treated with FastPrep (MP Biomedicals) at 5.5 ms for 1 min 3 times. Debris and glass beads were removed by centrifugation at 12,000 rpm for 10

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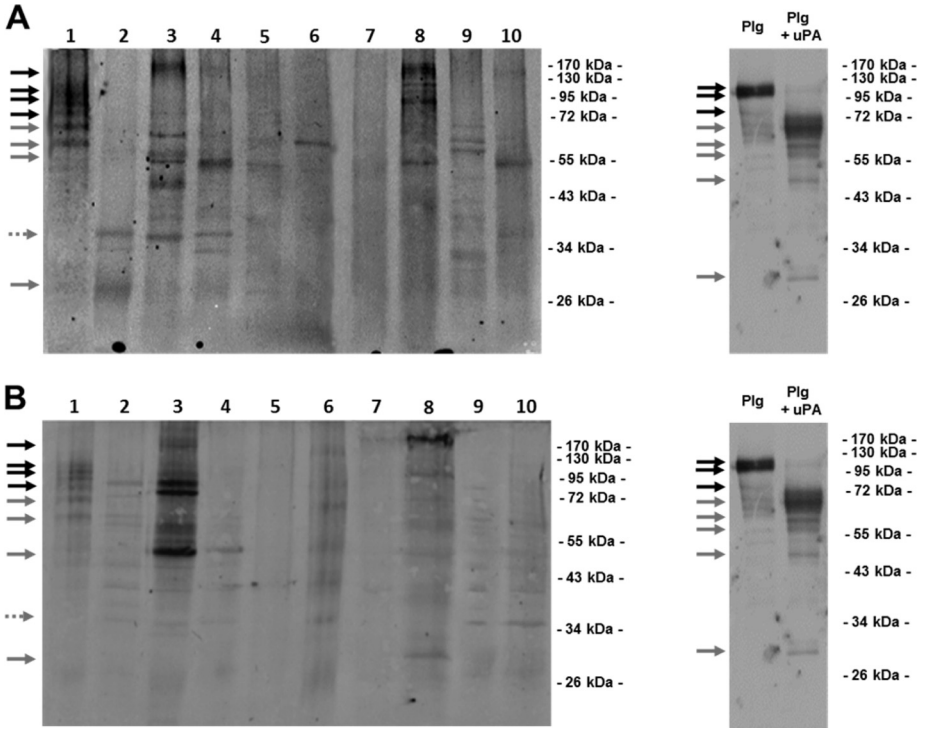

FIG. 1. (A) Immunoblot analysis of crude fecal extracts from subjects 1 to 10 carried out by using polyclonal anti-Plg IgG antibodies. As positive controls, 5 μg (each) of Plg and Plg activated to plasmin by a preincubation with 0.06 kallikrein inhibitor units (KIU) uPA was loaded. Black arrows indicate protein bands attributable to plasminogen isoforms (~90 to 100 kDa). Gray arrows indicate plasmin heavy chain A (~65-kDa), plasmin heavy chain A short form (~57-kDa), and plasmin light chain B (~25-kDa) isoforms. Dotted arrow indicates angiostatin (~38 kDa), which corresponds to the first four kringle domains of Plg isoforms. (B) Immunoblot analysis with polyclonal anti-Plg IgG antibodies of cell wall proteins purified from *B. animalis* subsp. *lactis* BI07 cells incubated with fecal extracts from subjects 1 to 10. Arrows indicate Plg and plasmin isoforms as reported above.

min. The protein concentration in supernatant was determined by NanoDrop (NanoDrop Technologies). The presence of human plasmin(ogen) in the crude fecal extracts was investigated by Western blotting. For each subject, 10 μg of fecal proteins was resolved by SDS-PAGE and Western blotted with polyclonal anti-human Plg IgG antibodies (Kordia) as reported by Candela et al. (7). As positive controls, Plg (Sigma) and Plg activated to plasmin by uPA (Calbiochem) were loaded. While the fecal extracts from subjects 1, 3, and 8 also showed bands attributable to Plg isoforms, all the fecal extracts analyzed, with

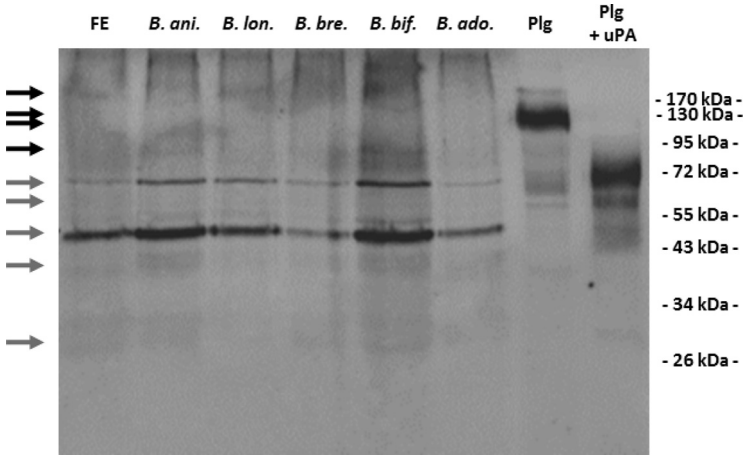

FIG. 2. Ten to 9 CFU/ml (each) of *B. animalis* subsp. *lactis* BI07 (*B. ani.*), *B. longum* ATCC 15707 (*B. lon.*), *B. breve* ATCC 15700 (*B. bre.*), *B. bifidum* DSM20456 (*B. bif.*), and *B. adolescentis* ATCC 15703 (*B. ado.*) was incubated in parallel with crude extracts from human feces (FE). Bacterial cell wall proteins were purified, and plasmin(ogen) was detected by immunoblot analysis with polyclonal anti-Plg IgG antibodies. Black arrows indicate protein bands attributable to plasminogen isoforms (~90 to 100 kDa). Gray arrows indicate plasmin heavy chain A (~65-kDa), plasmin heavy chain A short form (~57-kDa), and plasmin light chain B (~25-kDa) isoforms.

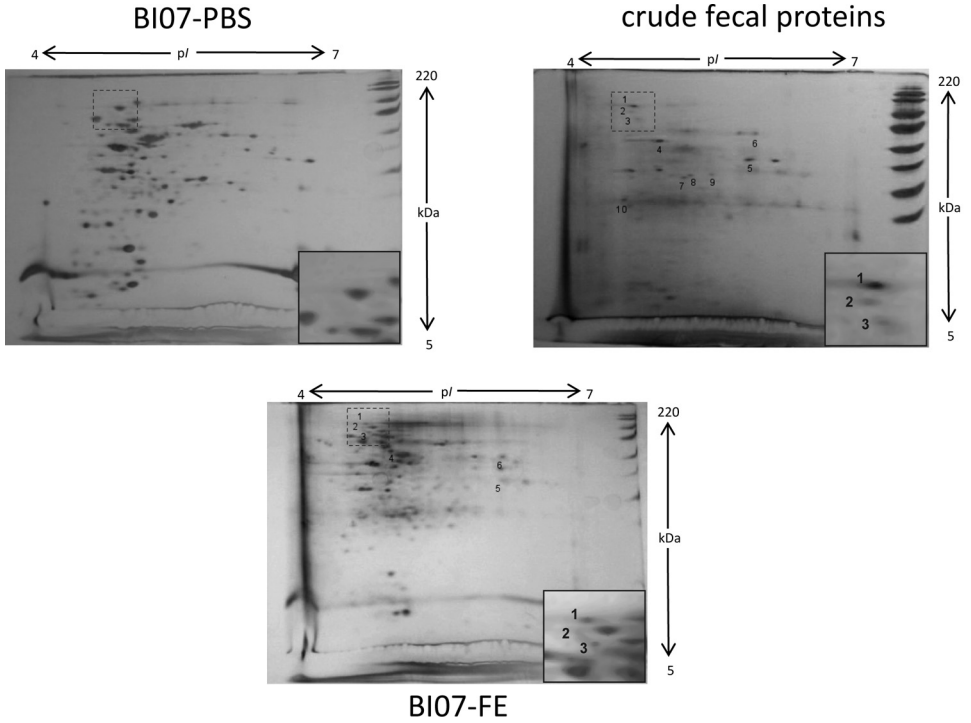

FIG. 3. Comparative proteomics of the cell wall fractions from BI07-PBS and BI07-FE and the crude fecal protein extract from subject 1. Numbered spots were analyzed by MALDI-TOF MS: protein spots 1 to 6 were specific for the crude fecal protein fraction and detected in the cell wall fraction from BI07-FE; protein spots 7 to 10 were detected only in the fecal protein fraction. The two-dimensional gel regions where plasminogen spots were identified are magnified in the insets.

the only exception being that of subject 7, showed bands attributable to plasmin heavy chain A and light chain B isoforms (Fig. 1A). These data confirm the presence of Plg and its derivatives in the healthy human GIT and strengthen previous data reporting the occurrence of plasma proteins in fecal extracts (1, 16). The capability of *B. animalis* subsp. *lactis* BI07 to recruit plasmin(ogen) and derivatives present at physiological concentrations in crude fecal extracts was next investigated. To this aim, stationary-phase *B. animalis* subsp. *lactis* BI07 cells (7) were resuspended at a concentration of 10^9 CFU/ml in 10 ml of crude fecal extracts from subjects 1 to 10. After 1 h of incubation at 37°C, bacterial cells were recovered by centrifugation and washed in 50 mM Tris-HCl (pH 7.6). In order to detect the Plg captured on the *B. animalis* subsp. *lactis* BI07 cell surface, the bacterial cell wall protein fractions were purified as previously described (7), resolved by SDS-PAGE, and Western blotted with anti-human Plg IgG antibodies (Kordia) as reported above. As negative controls, stationary-phase *B. animalis* subsp. *lactis* BI07 cells were incubated with PBS, and fecal proteins were incubated without bacterial cells. Interestingly, the cell wall fractions from *B. animalis* subsp. *lactis* BI07 cells incubated with the fecal extracts from subjects 1, 3, and 8 exhibited a Plg- and plasmin-related band pattern attributable to the one detected in the respective crude fecal fractions (Fig.

TABLE 1. Protein spot identification by MALDI-TOF MS

Spot ID[a]	Swiss-Prot accession no.	Protein name/description	Source	Theoretical M_r/pI[b]	Experimental M_r/pI[b]	% sequence coverage
1	B2R7F8	cDNA, FLJ93426, highly similar to *Homo sapiens* plasminogen (PLG), mRNA	*Homo sapiens*	91/7.0	100/4.7	26
2	P00747	Plasmin heavy chain A	*Homo sapiens*	63/6.8	90/4.7	25
3	P00747	Plasminogen	*Homo sapiens*	88/7.1	80/4.7	25
4	P05787	Keratin, type II cytoskeletal 8	*Homo sapiens*	54/5.5	59/4.9	30
5		Unidentified			40/5.9	
6	Q943E1	Putative uncharacterized protein	*Oryza sativa*	73/6.2	70/6.1	27
7		Unidentified			36/5.2	
8		Unidentified			37/5.3	
9		Unidentified			37/5.5	
10	A1L302	LOC283685 protein	*Homo sapiens*	41/4.8	30/4.5	30

[a] Spots 1 to 6 were specific for the human crude fecal protein fraction and detected in the cell wall fraction from BI07-FP; spots 7 to 10 were detected only in the fecal protein fraction.

[b] M_r, molecular weight, in thousands.

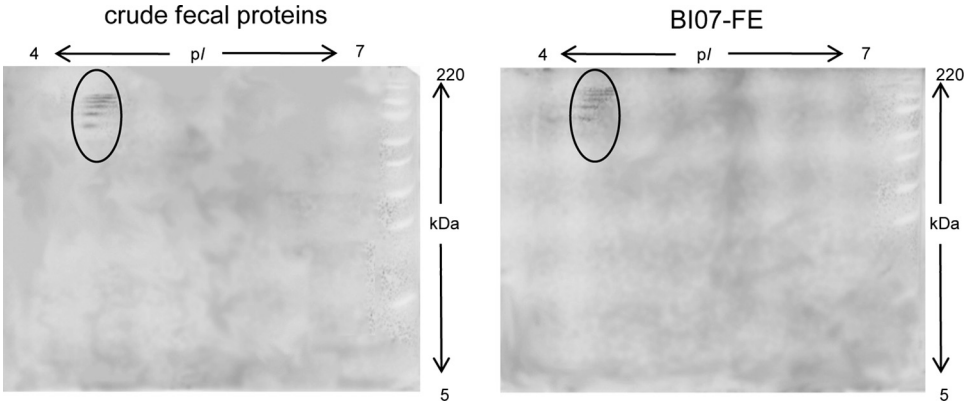

FIG. 4. Two-dimensional Western blot analysis of the crude fecal protein extract from subject 1 and the purified cell wall fraction from BI07-FE carried out with polyclonal anti-Plg IgG antibodies. Protein bands attributable to Plg and plasmin isoforms are circled.

1B). As expected, negative controls did not show any Plg- and plasmin-related bands (data not shown). These data demonstrate that *B. animalis* subsp. *lactis* BI07 is capable of recruiting Plg and plasmin isoforms present at physiological concentrations in crude extracts of human feces. As far as we know, this represents the first experimental evidence of a direct interaction between a probiotic bifidobacterial strain and a human protein in a human microenvironment. The capacity to interact with plasmin(ogen) present in crude extract from human feces was investigated in 4 human *Bifidobacterium* strains: *Bifidobacterium longum* ATCC 15707, *Bifidobacterium breve* ATCC 15700, *Bifidobacterium bifidum* DSM20456, and *Bifidobacterium adolescentis* ATCC 15703 (13) (Fig. 2). The interaction experiments were carried out as reported above. Interestingly, all the bifidobacterial strains studied were capable of recruiting plasmin(ogen) present in human feces. *B. animalis* subsp. *lactis* BI07 and *B. bifidum* DSM20456 showed the highest efficiencies in plasmin(ogen) recruitment.

With the intent to investigate whether *B. animalis* subsp. *lactis* BI07 is capable of interacting with other human fecal proteins besides plasmin(ogen), stationary-phase *B. animalis* subsp. *lactis* BI07 cells were incubated with the fecal extract from subject 1 as previously described. Subsequently, the cell wall protein fractions from fecal extract-incubated *B. animalis* subsp. *lactis* BI07 cells (BI07-FE) and PBS-incubated *B. animalis* subsp. *lactis* BI07 cells (BI07-PBS) and the fecal proteins from subject 1 (FP) were analyzed by comparative proteomics as reported by Candela et al. (5) (Fig. 3). Six protein spots specific for the human fecal protein fraction were detected in the cell wall fraction from BI07-FE, demonstrating that *B. animalis* subsp. *lactis* BI07 is capable of recruiting different components of the human fecal proteome to its cell surface. A tentative matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS)-based identification of spots 1 to 6 was performed as reported by Candela et al. (7) (Table 1). Confirming the data obtained by Western blotting, spots 1 and 3 were identified as human Plg and spot 2 as plasmin heavy chain A. These last data were also confirmed by two-dimensional Western blot analysis with anti-human Plg IgG antibodies (Kordia) (Fig. 4). Spot 4 was assigned to human keratin 8 and spot 6 to a hypothetical protein from *Oryza sativa*, whereas no identification was obtained for spot 5. Plant food-related proteins have already been identified as microbiota-associated proteins in the human fecal metaproteome (23). Our data provide the first experimental evidence of the *B. animalis* subsp. *lactis* plasmin(ogen) binding activity in the human GIT microenvironment. Allowing *B. animalis* subsp. *lactis* to acquire a host-derived surface-associated proteolytic activity, the interaction with components of the host Plg system can impact the dynamics of the *B. lactis*-host interaction process in the human GIT. Since gastrointestinal inflammatory processes involve imbalances of the host Plg system (15), it can be expected that the inflammation-dependent fluctuations of the components of the Plg system can modulate the biological outcome of the *B. lactis*-host interaction process. Studies in this direction should be carried out, leading to a more rational usage of this probiotic bifidobacterial species in inflammatory diseases.

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APPENDIX 5

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Tumor Necrosis Factor Alpha Modulates the Dynamics of the Plasminogen-Mediated Early Interaction between *Bifidobacterium animalis* subsp. *lactis* and Human Enterocytes

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The capacity to intervene with the host plasminogen system has recently been considered an important component in the interaction process between *Bifidobacterium animalis* subsp. *lactis* and the human host. However, its significance in the bifidobacterial microecology within the human gastrointestinal tract is still an open question. Here we demonstrate that human plasminogen favors the *B. animalis* subsp. *lactis* BI07 adhesion to HT29 cells. Prompting the HT29 cell capacity to activate plasminogen, tumor necrosis factor alpha (TNF-α) modulated the plasminogen-mediated bacterium-enterocyte interaction, reducing the bacterial adhesion to the enterocytes and enhancing migration to the luminal compartment.

Bifidobacterium is an important, occasionally dominant, genus of the human intestinal microbiota whose presence in the human gastrointestinal tract (GIT) has been associated with several health benefits (12, 17). Merging health-promoting activities and technological properties, *B. animalis* subsp. *lactis* is one of the most common probiotic species in North America and Europe (3). Because of the wide probiotic usage of this bacterium, different studies with the goal of better comprehending the molecular mechanisms involved in the bacterium-host interaction process have been recently carried out (3, 11). In this scenario, the capacity of this microorganism to intervene with the host plasmin(ogen) system has been recently regarded as a possible actor in the interplay with human enterocytes (11, 28). However, the role of the human plasminogen (Plg) system in the biology of the *Bifidobacterium*-host interaction process is still to be determined. The 92-kDa Plg is the monomeric proenzyme of the serine protease plasmin. Plg comprises an N-terminal ~8-kDa preactivation peptide, five consecutive disulfide-bonded kringle domains (65 kDa) that mediate binding to lysine-containing protein receptors, and a serine protease domain (25 kDa). Although Plg is produced mainly by hepatocytes, other tissue sources of Plg, including the intestine, have been identified (41). Plg is immobilized onto lysine-containing cell surface protein receptors such as fibrin as well as components of the extracellular matrix. Plg activation is a process tightly regulated by the balance between Plg activators (PAs), such as urokinase (uPA) and tissue-type (tPA) activators, and their specific inhibitors, PAI-1/2. Involved in fibrinolysis, enhancement of cell migration, and damages of tissue barriers, the Plg system has a key role in several human physiological and pathological processes (27).

We previously reported the capability of *B. animalis* subsp. *lactis* to bind human plasmin(ogen) (8). Plg binding to the bifidobacterial cell surface is mediated by five cell wall protein receptors: DnaK, glutamine synthetase, enolase, bile salt hydrolase, and phosphoglycerate mutase. According to Candela et al. (7), in the presence of Plg and host PAs, *B. animalis* subsp. *lactis* acquires a surface-bound Plg-derived proteolytic activity effective in the degradation of host physiological substrates. This modality of interaction with the components of the host Plg system resembles the one characteristic of several enteropathogens, such as *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* (16, 25, 29, 30, 32, 36). Different from the case for *Bifidobacterium*, the role of Plg in bacterial pathogens has been determined, and the bacterial capacity to intervene with the host Plg system has been traditionally considered a paradigm of pathogenicity (5, 16, 25, 29, 30, 32, 35). Facilitating the bacterial transmigration through epithelial monolayers, for pathogens, the acquisition of a Plg-dependent surface-associated plasmin activity is necessary for dissemination in the host tissues. This process has been defined as bacterial metastasis in analogy to the role of Plg in tumor cell invasion (10, 31). Originally isolated from fermented milk (19) and commonly found in the gut of healthy adults and infants (38), *B. animalis* subsp. *lactis* has been shown to possess a mutualistic nature, and its capacity to intervene with the host Plg system must serve a different purpose than the bacterial metastasis (7). Consequently, even if *B. animalis* subsp. *lactis* and pathogens show the same modality of interaction with the components of the host Plg system, the outcome of Plg-binding activity in the biology of interaction with the host must be different, and comprehension of its significance in bifidobacterial ecology is needed. In this work, we studied the role of the human Plg system in the early interaction process between *B. animalis* subsp. *lactis* BI07 and the human enterocyte line HT29 (26). Moreover, since gastrointestinal inflammatory processes involve unbalances of the components of the host Plg system (14, 20, 23), we investigated the impact of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) on the dynamics of the Plg-mediated *B. animalis* subsp. *lactis*

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BI07-HT29 cell early interaction. TNF-α was selected because this cytokine has been reported to play a central role in intestinal inflammation (18, 40).

HT29 cell culture conditions. HT29 cells were grown in Dulbecco's modified Eagle's minimal essential medium with 4.5 g/liter glucose (DMEM; PAA Laboratories) as reported by O'Hara et al. (22). For adhesion and intracellular invasion assays, 2.5×10^5 HT29 cells per well were seeded in 24-well tissue culture plates (TPP) and 12-mm-diameter glass coverslips and grown to confluent monolayers. For the transmigration assay, 1×10^5 cells were layered on 3-μm-pore size transwell inserts (Falcon; Becton Dickinson) in 24-well tissue culture plates and allowed to grow to confluent and fully differentiated monolayers. The tightness of the cell layers was verified as described by Attali et al. (2). Twenty-four hours before each assay, the cell medium was replaced with interaction medium (IM) (DMEM, 25 mM HEPES, 1 g/liter glucose [Gibco], 1% fetal calf serum [FCS]); when necessary, 2 ng/ml human recombinant TNF-α (Thermo Scientific) was added to induce a proinflammatory response (22).

Role of human Plg in the interaction process of *B. animalis* subsp. *lactis* BI07 and HT29 cells. To assess whether the HT29 cell endogenous Plg contributed to the early interaction between *B. animalis* subsp. *lactis* BI07 and HT29 cells, the impact of the enterocyte pretreatment with polyclonal goat anti-human Plg IgG (Kordia) (5 μg/ml) on *B. animalis* subsp. *lactis* BI07 adhesion was determined. *B. animalis* subsp. *lactis* BI07 adhesion to HT29 monolayers was measured by quantitative PCR (qPCR) as reported by Candela et al. (9). For each experimental condition, six independent replica experiments were performed. According to our data, the anti-human Plg pretreatment resulted in a slight, but not significant ($P > 0.05$), decrease of the bifidobacterial adhesion to the enterocyte surface (−16%). These data indicated that the endogenously produced Plg of HT29 cells exerted only a minimal contribution to the *B. animalis* subsp. *lactis* BI07-HT29 cell adhesion process. Consequently, in order to investigate the role of human Plg in the *B. animalis* subsp. *lactis* BI07-enterocyte interaction process, bacterial cells were preincubated with human Plg (Sigma) as reported by Attali et al. (2) and then subjected to a conventional HT29 cell adhesion assay (9). Thirty-minute preincubation at 37°C with 100 μg/ml human Plg significantly enhanced *B. animalis* subsp. *lactis* BI07 adhesion to HT29 cells (+225%; $P < 0.001$) (Table 1). The incubation of Plg-pretreated *B. animalis* subsp. *lactis* BI07 cells with 5 μg/ml anti-human Plg IgG resulted in the complete abolition of the Plg contribution to the bifidobacterial adhesion to the HT29 cell surface. These data demonstrated that human Plg captured on the *B. animalis* subsp. *lactis* BI07 cell surface significantly enhanced bacterial adhesion to the host enterocytes. As suggested by Pancholi et al. (24), the bacterium-bound Plg can act as a molecular bridge between bacterial and enterocyte receptors, enhancing bacterial adhesion to the host epithelium. Incubation of Plg-pretreated *B. animalis* subsp. *lactis* BI07 cells with 1 μg/ml uPA (Sigma) decreased the HT29 cell adhesion by ~50% with respect to the value obtained in the absence of uPA ($P < 0.001$) (Table 1). The addition of 8 U/ml of the plasmin inhibitor aprotinin (Sigma) (2) was effective in the complete recovery of the Plg-dependent enterocyte adhesion of *B. animalis* subsp. *lactis* BI07 cells preincubated with Plg plus uPA (Table 1), proving that the acquisition of a cell surface plasmin activity in the presence of uPA was sufficient to dampen the contribution of Plg to the *B. animalis* subsp. *lactis* BI07 adhesion to the enterocyte surface.

TABLE 1 qPCR quantification of the Plg-mediated adhesion of *B. animalis* subsp. *lactis* BI07 to untreated and TNF-α-pretreated HT29 cells

Experimental condition(s)[a]	No. of bacteria/HT29 cell (mean ± SD)	
	No treatment	+ TNF-α
BI07	18.87 ± 1.20	26.65 ± 2.02
BI07 + Plg	61.08 ± 2.85	41.09 ± 2.74
BI07 + Plg + anti-Plg	19.40 ± 1.24	ND[b]
BI07 + Plg + uPA	28.87 ± 1.93	36.57 ± 2.27
BI07 + Plg + uPA + aprotinin	81.15 ± 3.58	79.81 ± 3.52
BI07 + Plg + aprotinin	ND	75.96 ± 5.67
BI07 + aprotinin	20.23 ± 2.21	23.55 ± 1.55

[a] Untreated and TNF-α-pretreated (+ TNF-α) confluent HT29 cell monolayers were incubated with 5×10^7 CFU of untreated *B. animalis* subsp. *lactis* BI07 cells (BI07), Plg-pretreated BI07 cells (BI07 + Plg), Plg-pretreated BI07 cells incubated with anti-Plg IgG (BI07 + Plg + anti-Plg), Plg- and uPA-pretreated BI07 cells (BI07 + Plg + uPA), Plg- and uPA-pretreated BI07 cells in the presence of aprotinin (BI07 + Plg + uPA + aprotinin), Plg-pretreated BI07 cells in the presence of aprotinin (BI07 + Plg + aprotinin), and BI07 cells in the presence of aprotinin (BI07 + aprotinin).
[b] ND, not determined.

Next, we investigated HT29 cell internalization and transmigration of untreated, pretreated with Plg or with Plg and uPA *B. animalis* subsp. *lactis* BI07 bacteria. Enterocyte internalization was analyzed by double immunofluorescence microscopy, which enables a differential staining of intracellular and adherent bacterial cells, as reported by Bergmann et al. (4). For each experimental condition, three independent adhesion experiments were carried out. Antibodies against *B. animalis* subsp. *lactis* BI07 were generated in BALB/c mice according to standard protocols. Microscopic analysis of the whole HT29 cell layer revealed no bacterial internalization by the HT29 enterocyte cell line in any of the tested conditions (Fig. 1A). For comparison and as a control, the internalization of *Salmonella enterica* serovar Typhimurium by HT29 cells was determined using a gentamicin protection assay (13). *S.* Typhimurium, provided by A. Essig (Department of Medical Microbiology, University of Ulm, Germany), was cultured as reported by Candela et al. (9). In accordance with data reported by Hess et al. (13), we determined a HT29 cell internalization value of $(9.32 \pm 1.58) \times 10^4$ CFU of *S.* Typhimurium after 1 h of incubation with 1×10^8 CFU of this microorganism. In order to analyze the *B. animalis* subsp. *lactis* BI07 transmigration through HT29 monolayers, a transwell system-based transmigration assay was performed as reported by Attali et al. (2). Untreated, Plg-pretreated, and Plg- and uPA-pretreated *B. animalis* subsp. *lactis* BI07 cells showed no transmigration through the HT29 monolayers (data not shown). In a control experiment, the transmigration of *S.* Typhimurium bacteria across HT29 monolayers was determined. In accordance with the data reported by Hess et al. (13), $(7.84 \pm 1.03) \times 10^5$ bacteria penetrated from the apical compartment to the basal compartment of HT29 cells after 1 h of coincubation with 2×10^7 CFU. These last data proved that in *B. animalis* subsp. *lactis* BI07, the acquisition of a surface-associated plasmin activity did not result in enterocyte internalization or bacterial transmigration through enterocyte monolayers, supporting the strict commensal nature of this health-promoting species.

Even if the modality of interaction between *B. animalis* subsp.

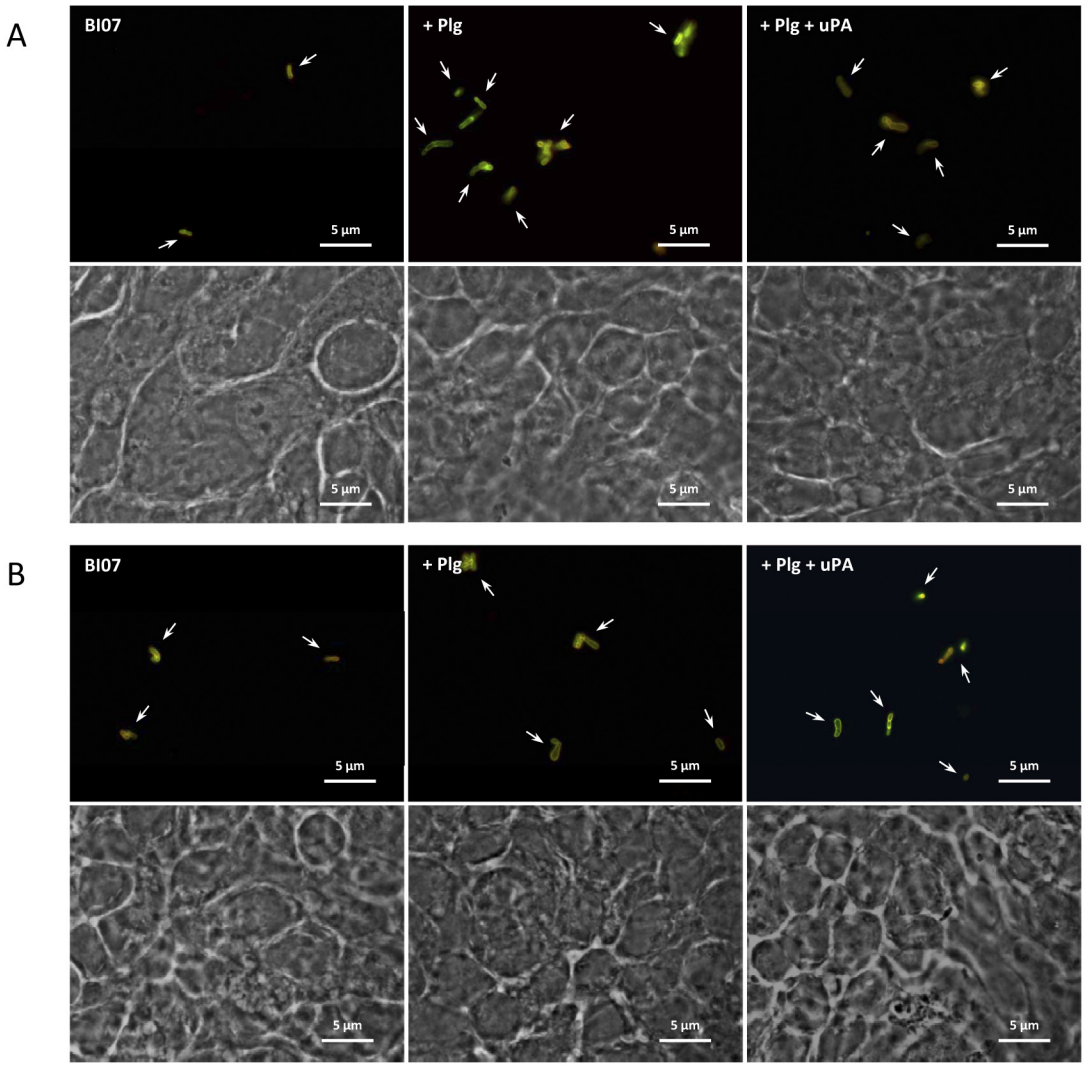

FIG 1 Intracellular invasion assays of *B. animalis* subsp. *lactis* BI07 in untreated (A) and TNF-α-pretreated (B) HT29 cells. HT29 cell monolayers grown to confluence were incubated with 5×10^7 CFU of *B. animalis* subsp. *lactis* BI07 untreated (BI07), pretreated with Plg (+Plg) and pretreated with Plg and uPA (+Plg+uPA). Intracellular and adherent bacteria were differentiated by double immunofluorescence microscopy. Adherent *B. animalis* subsp. *lactis* BI07 bacteria (indicated by arrows) were stained with mouse antibifidobacterial antiserum followed by a secondary Alexa Fluor 488-conjugated anti-mouse antibody (green). After HT29 cell permeabilization, internalized bacteria were stained with mouse antibifidobacterial antiserum and a secondary Alexa Fluor 568-conjugated anti-mouse antibody (red). HT29 cells are visualized in the corresponding phase-contrast images shown in the lower panel (magnification, ×100). For each experimental condition, the assay was repeated three times. Results of a representative experiment are shown.

lactis and the components of the host Plg system resembles the one described for several pathogens (16, 25, 29, 30, 32, 36), our data demonstrated that the consequences of this interaction with respect to host colonization are different. In fact, for both *B. animalis* subsp. *lactis* and pathogenic bacteria, cell-bound Plg enhances bacterial adhesion to the host epithelium (2, 24), but in the presence of host PAs, the phenotype of interaction with the host becomes totally different. Different from the case for pathogens, in *B. animalis* subsp. *lactis*, the acquisition of surface-associated plasmin activity does not result in pericellular invasion but rather supports bacterial migration to the luminal compartment. Thus, common to symbionts and pathogens (1, 15), the bacterial capacity to intervene with the host Plg system cannot be viewed as a determinant of pathogenicity by itself. Instead, it could be regarded as an ancestral mechanism of bacterium-host interaction evolved prior to the radiation of pathogens from commensals (6, 21). Acting in concert with other virulence attributes (2, 21), the capacity to intervene with the host Plg system appears to be a fundamental prerequisite for host invasion only for pathogens (37).

Impact of TNF-α on the Plg-mediated early interaction between *B. animalis* subsp. *lactis* BI07 and HT29 cells. To mimic an inflammatory status, HT29 cell layers were preincubated with the proinflammatory cytokine TNF-α, and their early interaction with Plg- or Plg- and uPA-pretreated and untreated *B. animalis* subsp. *lactis* BI07 cells was investigated by qPCR and immunomicroscopic analysis as reported above. Interestingly, we detected only a minor increase of *B. animalis* subsp. *lactis* BI07 adhesion in TNF-α-stimulated HT29 monolayers after Plg pretreatment (Table 1). Moreover, after the TNF-α preincubation of HT29 layers,

Plg-pretreated and Plg- and uPA-pretreated *B. animalis* subsp. *lactis* BI07 cells exhibited comparable adhesion values (Table 1). The addition of aprotinin effectively restored the Plg-dependent *B. animalis* subsp. *lactis* BI07 adhesion to TNF-α-stimulated HT29 layers for both Plg- and Plg- and uPA-pretreated *B. animalis* subsp. *lactis* BI07 cells (Table 1). Analogous to the data obtained with noninflamed HT29 cells, no bacterial internalization (Fig. 1B) or transmigration (data not shown) was detected when TNF-α-stimulated HT29 cells were incubated with Plg-pretreated, Plg- and uPA-pretreated, and untreated *B. animalis* subsp. *lactis* BI07 cells. Taken together, these data indicated that, by enhancing the enterocyte capacity to activate Plg, a TNF-α-mediated inflammatory response can modulate the dynamics of the Plg-mediated early interaction between *B. animalis* subsp. *lactis* and human enterocytes, lowering bacterial adhesion to the enterocyte surface and shifting migration to the luminal compartment. Confirming this hypothesis, in a conventional plasmin activity assay (2, 7), we demonstrated that the TNF-α pretreatment increased by 2-fold the HT29 cell capacity to activate Plg ($P < 0.001$).

Our data suggest that *B. animalis* subsp. *lactis* BI07 shows a different phenotype of interaction with the host Plg system depending on the inflammatory status of the host GIT epithelium. While Plg enhances *B. animalis* subsp. *lactis* adhesion in a noninflamed GIT epithelium, inflammation would result in the activation of bacterium-bound Plg and mediate the migration of the microorganisms to the luminal compartment. We hypothesize that this particular dynamic of interaction between *B. animalis* subsp. *lactis* and the components of the host Plg system could be of some relevance in the bifidobacterial microecology in the human GIT. In noninflamed regions of the human GIT, which represent the suitable ecological niche for *B. animalis* subsp. *lactis*, the microorganism can take advantage of the host Plg for the adhesion to the mucosal surface. On the other hand, in inflamed tissue sites, *B. animalis* subsp. *lactis* would acquire a surface-associated plasmin activity that, facilitating the bacterial migration to the luminal compartment, could function as a bacterial escape mechanism to circumvent the host inflammatory response. Unable to face inflammation (34), *B. animalis* subsp. *lactis* may thus utilize the host Plg system to sense and escape intestinal inflammation, abandoning inflamed gastrointestinal sites in favor of noninflamed ones. Our *in vitro* model of interaction could explain, at least in part, the observed decrease of the relative abundance of *Bifidobacterium* in inflammatory bowel disease (IBD) subjects (33, 39).

Conclusions. Investigating the role of Plg in the bifidobacterial biology in the human GIT, our experimental work represents a step forward in the comprehension of the factors that play a role in the dynamics of *Bifidobacterium*-host interaction in the human GIT. However, our *in vitro* data need to be confirmed by *in vivo* studies specifically designed to prove the role of the host Plg system in the inflammation-dependent bifidobacterial decrease in the human GIT. The achievement of this goal will allow a better understanding of the biology of this health-promoting microbiota component in the human GIT.

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