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Insights into the molecular bases of the interaction of *Bifidobacterium bifidum* PRL2010 with the human host and with other human gut commensals.

Ph.D. Thesis

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" It always seems impossible until it's done"

- Nelson Mandela

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Summary

Members of the *Bifidobacterium* genus are of common inhabitants of the gastro-intestinal tract (GIT) of mammals. Bifidobacteria are high G+C Gram-positive obligate anaerobes belonging to the *Actinobacteria* phylum. Of the 47 bifidobacterial so far identified taxa, 11 have been isolated from human GIT. Bifidobacteria are among the first colonizers of the sterile GITs of newborns and they predominate in breast-fed infants until weaning. After this phase *Bacteroides* and other groups become dominant. In fact, bifidobacteria are not numerically dominant in the complex adult intestinal microflora, but they are considered as key commensals because they promote a healthy status in the host. For this reason bifidobacteria are often used as healthy components in functional foods. Recently numerous molecular approaches have emerged for the identification and characterization of bifidobacterial strains. In this context, genomic-based efforts, i.e., probiogenomics, have helped us to understand the mechanism mediating the host-microbiota interaction and in which way the bifidobacteria adapt themselves to the gastro-intestinal tract of the host.

In this thesis were investigated the molecular bases sustaining the interaction of bifidobacteria with the host as well as with the other commensals members of the human GIT through *in vitro* and *in vivo* tests.

Sommario

I bifidobatteri sono tra i microrganismi maggiormente presenti nel tratto gastro-intestinale (GIT) dei mammiferi. Sono batteri anaerobi obbligati appartenenti al phylum degli *Actinobacteria*, Gram-positivi ad alto contenuto di G+C nel genoma. Sono state identificate 47 taxa di bifidobatteri, 11 dei quali isolati dal GIT umano. Sono i primi colonizzatori del tratto gastro-intestinale dei neonati e predominano in quelli allattati al seno, invece dopo lo svezzamento sono in quantità minore rispetto a *Bacteroides* ed altri gruppi microbici. Infatti, i bifidobatteri anche se non sono numericamente dominanti nella complessa microflora intestinale degli adulti, sono considerati dei commensali importanti nel promuovere lo stato di salute dell'ospite e per questa ragione sono spesso usati come componenti salutari negli alimenti funzionali.

Recentemente sono emersi numerosi approcci molecolari per l'identificazione e la caratterizzazione di ceppi di bifidobatteri. Studi basati sulla genomica (probiogenomica) hanno permesso di comprendere i meccanismi che mediano l'interazione tra microrganismo e ospite e in che modo i bifidobatteri si adattano alla nicchia ecologica umana.

In questa tesi sono stati studiati i meccanismi molecolari responsabili dell'interazione dei bifidobatteri con l'ospite e con gli altri commensali del GIT umano, utilizzando sia test *in vitro* che *in vivo*.

1. Introduction

1.1 Gastro-intestinal microbiota

The human body surfaces are home to complex microbial communities leading to an over-representation of microbial cells respect to mammalian's cells. In fact, the health human organism can be said to be composed of over 10^{14} cells, of which only about 10% are animal cells (somatic and germ cells) (Gill, Pop et al. 2006). The vast majority of the microbial cells in that mass reside in the gastrointestinal tract (GIT) (10 to 100 trillion) (Gill, Pop et al. 2006). The human GIT is an extremely complex microbial ecosystem, this genome contains at least 100 times as many genes as our own genome (Gill, Pop et al. 2006). The microbial component (also termed the microbiota) largely influence the human health (Kurokawa, Itoh et al. 2007). Our gut microbiota can be pictured as a microbial organ placed within a host organ, which is composed of different cell lineages with a capacity to communicate with one another and the host; it consumes, stores, and redistributes energy; it mediates physiologically important chemical transformations; and it can maintain and repair itself through self-replication (Backhed, Ley et al. 2005). Our relationship with members of this microbiota is often described as commensal relationships (one partner benefits and the other is apparently unaffected) as opposed to mutualistic interactions (both partners experience increased fitness) (Backhed, Ley et al. 2005).

However, our distal intestine is an anaerobic bioreactor that harbors the large majority of our GIT microorganisms. In this ecosystem bacteria synthesize essential amino acids and vitamins and they are involved in the breakdown of indigestible components of the human diet, like polysaccharides that are recalcitrant to digestion by mammalian's enzymes, including plant-derived pectin, cellulose, hemicellulose, and resistant starches (Backhed, Ley et al. 2005). Some gut microbial members are permanently established (mucosa adherent components), whereas others may represent transient gut microbiota members (Eckburg, Bik et al. 2005, Ley, Peterson et al. 2006, Turroni, Marchesi et al. 2009). Residents gut microbiota members are also known to constitute an autochthonous microbiota, often establishing symbiotic relationships with the human organism, while travelers members of the gut microbiota are forming an allochthonous

microbiota that ingested through the diet and/or they are derived from environmental contaminations (Turroni, Ribbera et al. 2008). The adult human GIT contains members of all tree domains of life: Archaea, Eukarya and Bacteria. The main bacterial divisions that dominate the GIT encompass *Firmicutes* (46-60%), *Proteobacteria* (10-30%), *Bacteroidetes* and *Actinobacteria* (8-28%) (Turroni, Ribbera et al. 2008). The majority of GIT microbiota resides in colon, where their abundance reach 10^{12} cells/g of luminal content (Isolauri, Salminen et al. 2004). Bacterial populations residing in the upper compartments of the human GIT (mouth, esophagus, stomach) are less variable and numerous. Despite the high species richness and inter-individual variability of the intestinal microbiota, a limited number of bacterial phylotypes (groups of bacterial DNA sequences sharing more than an arbitrarily chosen level of similarity) is more prevalent amongst individuals and might therefore represent a shared phylogenetic core (Tap, Mondot et al. 2009, Qin, Li et al. 2010). However, an accurate estimation of the size of the phylogenetic core remains still undefined (Gerritsen, Smidt et al. 2011). About 70% of GIT microbiota is considered to be variable among individuals as well as within the identical host. The GIT microbiota diversity result as a consequence of strong host selection and host-microbe coevolution (Backhed, Ley et al. 2005). The structure and composition of the gut microbiota reflect natural selection (Backhed, Ley et al. 2005). At the microbial level it is due to the combined action of various mechanisms, e.g. horizontal gene transfer (HGT), gene duplication, gene decay and genome rearrangements (Snel, Bork et al. 2002, Kunin and Ouzounis 2003, Ventura, Canchaya et al. 2007). HGT represents the principal genetic mechanism, implemented through the activities of bacteriophage, IS elements, transposons, conjugative plasmids, and DNA-mediated transformation (Ley, Peterson et al. 2006). Furthermore, observed vertical genome evolution can be attributed to mutations, deletions and chromosomal rearrangements (Giraud, Matic et al. 2001). On the other hand, composition of microbiota in various regions of GIT differs due to variable environmental condition (Backhed, Ding et al. 2004). As above mentioned it is well established that the gut microbiota composition is highly variable between

individuals, and it is also subject to considerable change throughout the lifetime of an individual. External factors shaping the human GIT microbiota include antibiotic therapy and diet (Palmer, Bik et al. 2007). Recent studies into the impact of antibiotics on the microbiota have focused on the emergence of resistant strains and their influence on the microbial community. Results have shown that short and long-term antibiotic therapy affect diversity and biomass of the intestinal microbiota, with microbial composition resilience remaining deficient for long time after the treatment (Perez-Cobas, Artacho et al. 2013). Moreover, the availability of carbohydrates that escape metabolism and adsorption in the small intestine (e.g., plant polysaccharides) has a major influence on the microbiota that ultimately becomes established in the colon (Kaplan and Hutkins 2000).

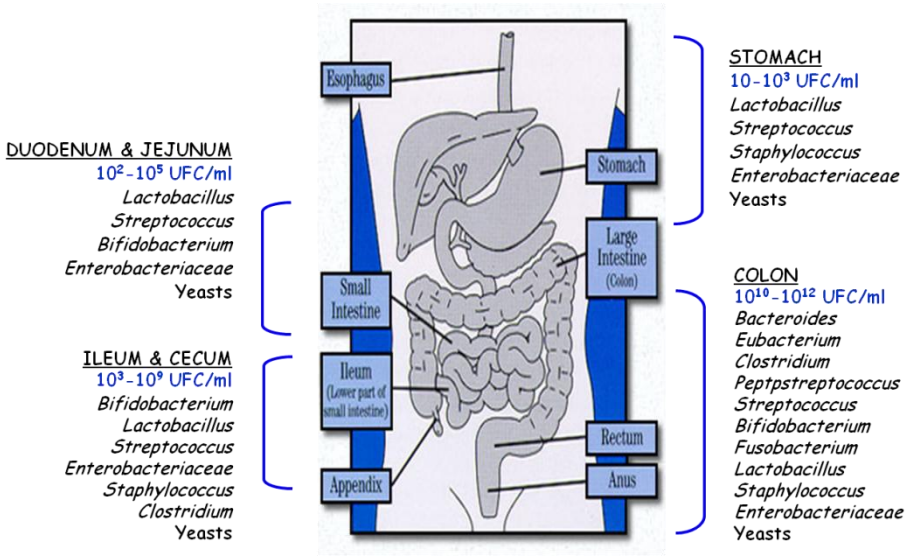

Fig. 1.1: Bacterial species present in the different regions of human gastrointestinal tract.

1.2 Bifidobacteria

The genus *Bifidobacterium*, which is a member of the *Bifidobacteriaceae* family and subsequently belongs to the *Actinobacteria* phylum (Stackebrandt and Tindall 2000). Bifidobacteria are characterized to include Gram-positive microorganisms with a high G+C DNA content. They were first isolated from faeces of a breast-fed infant by Tissier in 1899, and then named *Bacillus bifidus* (Tissier 1900). However, because of their morphological and physiological features, which are similar to those of lactobacilli, they were classified as members of the genus *Lactobacillus* during a large part of the 20th century and only recently they have been recognized constituting a different genus. Currently, the genus *Bifidobacterium* is comprised of 47 different taxa recognized, all of which have been isolated from the GIT contents of mammals, birds, or insects (Ventura, Canchaya et al. 2007, Ventura, Turroni et al. 2009, Ventura M 2014). Furthermore, the genus *Bifidobacterium* includes three subspecies lineages: i) *Bifidobacterium animalis* subsp. *animalis* and *Bifidobacterium animalis* subsp. *lactis*; ii) *Bifidobacterium pseudolongum* subsp. *globosum* and *Bifidobacterium pseudolongum* subsp. *pseudolongum*; iii) *Bifidobacterium longum* subsp. *infantis*, *Bifidobacterium longum* subsp. *longum* and *Bifidobacterium longum* subsp. *suis* species. Recently, three new species have been isolated from the bumblebee digestive tracts, i.e., *Bifidobacterium actinocoloniiforme*, *Bifidobacterium bohemicum* and *Bifidobacterium bombi* (Killer, Kopecny et al. 2009, Killer, Kopecny et al. 2010). The last species characterized are *Bifidobacterium kashiwanohense*, isolated from healthy infant faeces (Morita, Nakano et al. 2011);

Bifidobacterium reuteri, *Bifidobacterium callitrichos*, *Bifidobacterium saguini*, *Bifidobacterium stellenboschense* and *Bifidobacterium biavatii* were isolated from faeces of common marmoset (Callithrix jacchus) and red-handed tamarin (Saguinus midas) (Endo, Futagawa-Endo et al. 2012). *Bifidobacterium moukalabense* isolated from the faeces of wild west lowland gorilla (Gorilla gorilla gorilla) in Gabon (Tsuchida, Takahashi et al. 2013). In addition, two bifidobacterial taxa, i.e., *Bifidobacterium crudilactis* and *Bifidobacterium mongoliense*, were

isolated from raw milk cheeses (St Marcellin, Vercors area, France) (Delcenserie, Taminiau et al. 2013). Taking their different ecological niches into account and combining this information with a comparative analysis of their 16S rRNA sequences, as well as with other housekeeping genes (*clp*C, *dna*J, *xfp*, *dna*B, *rpo*C and *pur*F), the various *Bifidobacterium* taxa can be clustered into six different phylogenetic groups named *Bifidobacterium adolescentis*-, *Bifidobacterium asteroides*-, *Bifidobacterium boum*-, *Bifidobacterium longum*-, *Bifidobacterium pullorum*-, *and Bifidobacterium pseudolongum*-phylogenetic group (Ventura, Canchaya et al. 2007).

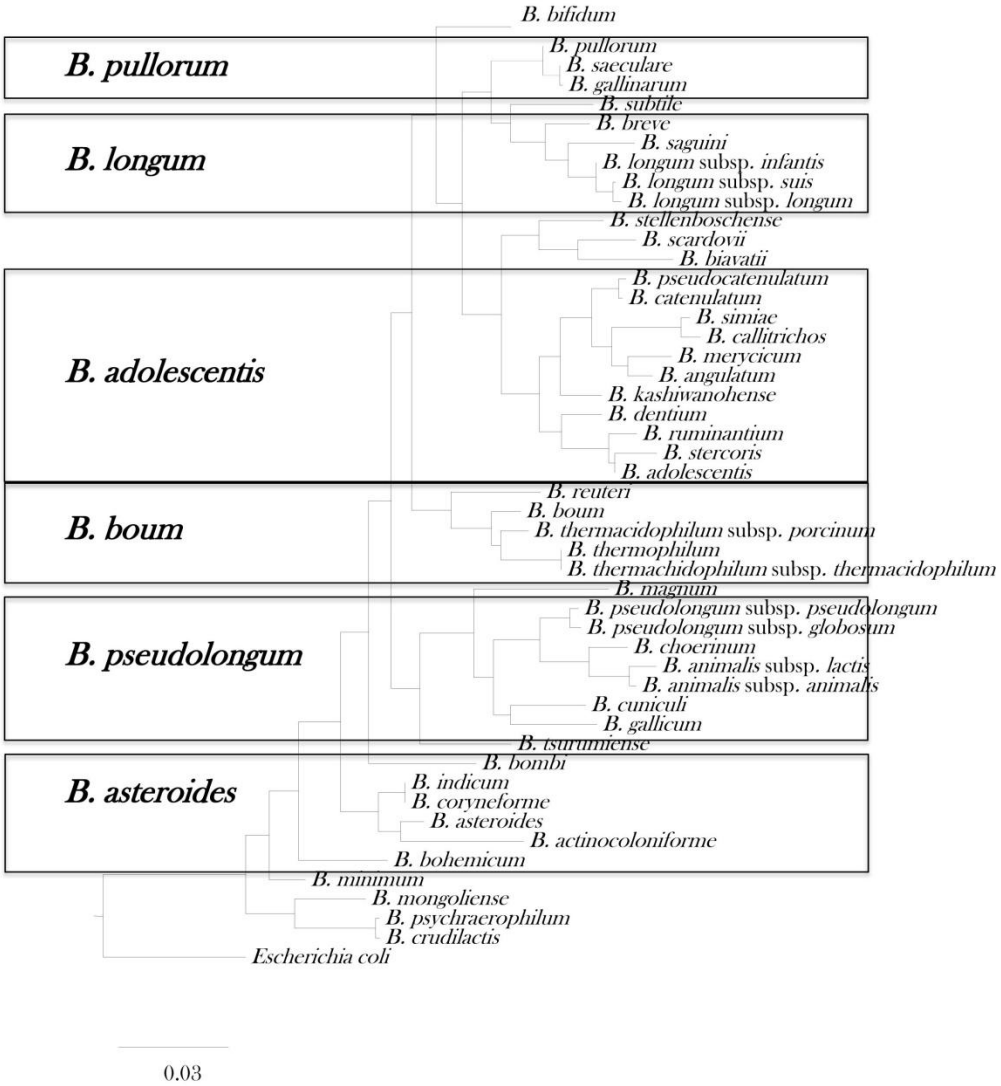

Fig. 1.2: Phylogenetic tree of the genus *Bifidobacterium* based on 16S rRNA gene sequences.

1.2.1 Bifidobacteria and gut microbiota development

The neonatal GIT is sterile, and its colonization occurs within the first few days of life (Guarner and Malagelada 2003). The development of the intestinal microbiota of infant is combined with maternal vaginal microbiota, but only within the first day after birth (Turroni, Ribbera et al. 2008). Intestinal microbiota composition of infants delivered through Caesarean section is therefore different from that of infants born vaginally (F. Guarner 2003). Thus, initial bacterial colonization is dependent on the microbial communities to which newborns are exposed. In vaginal deliveries, facultative anaerobes including members of the *Streptococcus* and *Escherichia* genera dominate during the first days of postnatal life (Bik, Eckburg et al. 2006, Turroni, Ribbera et al. 2008). The early gut microbiota of infants delivered by caesarean section are dominated by *Klebsiella*, *Enterobacter*, and *Clostridium*, in contrast to vaginally delivered infants, which possess a microbiota with a high proportion of bifidobacteria (Hayashi, Sakamoto et al. 2002). Maternal milk may also serve as an inoculum for breast-feeding infants, and is presumed to play a crucial role in the establishment of a bifidobacteria-abundant infant gut microbiota before the weaning period (Palmer, Bik et al. 2007, Zilberstein, Quintanilha et al. 2007, Turroni, Ribbera et al. 2008). A consistent correlation has been observed between gut microbiota composition and type of feeding (i.e., human milk vs formula milk), with a higher abundance of bifidobacteria in breastfed infants as compared to those that are bottle-fed (Roediger 1980). In the case of breast-fed infants 60-90% of the bacteria belong to genus *Bifidobacterium*, and at lesser extends *Bacteroides* and *Lactobacillus* (below 1%).

The selective growth of bifidobacteria observed in breast-fed newborns has been attributed to oligosaccharides contained in human milk named Human Milk Oligosaccharides (HMOs) (see below) (Knol, Scholtens et al. 2005, Ward, Ninonuevo et al. 2006, Wada, Ando et al. 2008, Turroni, Foroni et al. 2011, Kim, An et al. 2013).

Instead in the adult human gut microbiota members of the genera *Bifidobacterium*, *Bacteroides, Clostridium and Eubacterium* are barely detected (Backhed, Ley et al. 2005), in fact *Lactobacillus, Escherichia, Enterobacter, Streptococcus or Klebsiella* dominate.

1.2.2 Bifidobacterial ecology

Bifidobacteria are generally host-animal-specific microorganisms and can be separated as "human group", "animal group" and others (Ventura, van Sinderen et al. 2004). Many subspecies of the genus *Bifidobacterium* have been isolated from the GIT of humans (Trovatelli, Crociani et al. 1974, Lauer 1990, Hoyles, Inganas et al. 2002) and other animals (Scardovi, Trovatelli et al. 1979, Watabe, Benno et al. 1983, Biavati and Mattarelli 1991, Biavati, Mattarelli et al. 1991, Zhu, Li et al. 2003), milk products (Watanabe, Makino et al. 2009), blood and food (Ventura, Canchaya et al. 2007). Three species of bifidobacteria were also found in honeybees (Scardovi and Trovatelli 1969). In addition, Mrazek et al (Mrazek, Strosova et al. 2008) detected bifidobacteria through molecular techniques in other social insects, including wasps (*Vespula vulgaris*), hornets (*Vespa crabro*) and cockroaches (*Nauphoeta cinerea*) (Killer, Kopecny et al. 2010), which was found to be distinct from the bifidobacterial populations identified in the hindgut of honeybees (Killer, Kopecny et al. 2011).

1.3 Probiogenomic

Despite its importance, the precise composition and activities of the human GIT microbiota is still not well defined. For this reason, an accurate analysis of the microbial consortium residing within these human body compartments is an essential step to understand how the various elements of the GIT microbiota interact and consequently affect the health status of the host (Neish 2009). Research in microbiology has remarkably changed during the last decades, largely due to the availability of novel whole-genome sequencing approaches. In fact, the decoding of the genome sequences of more than 4,000 bacteria, as currently present in the NCBI database (www.ncbi.nlm.nih.gov) has greatly advanced our understanding of bacterial biology. The initial microbial genomics efforts were mainly directed toward decoding the genomes of pathogenic bacteria because of their impact on human well-being. The obtained genomic data have opened new avenues of research and even sparked the origin of a new genomics-based discipline, called pathogenomics, which aims to understand the genetic basis of bacterial pathogenesis (Pallen and

Wren 2007). Recently, genome sequencing has also directed its interest toward food-related bacteria, intestinal commensals and probiotic bacteria. In 2009, a correspondingly novel discipline designated as probiogenomics was defined, which aims to provide insights into the diversity and evolution of commensal/probiotic bacteria and to reveal the molecular basis for their health-promoting activities (Ventura, O'Flaherty et al. 2009). The public availability of full genome sequence data has significantly expanded our understanding of the biology of these microorganisms and has generated an enormous amount of information on metabolic capabilities, genetics and phylogeny of these bacteria.

1.3.1 Probiotics

Probiotics are non-pathogenic live microorganisms that are administered to alter the intestinal microbiota and confer a beneficial effect on health (Shanahan 2005). The term derived from Greek and meaning "for life", emerged from observations early in the 19th century by Elie Metchnikoff, known as the "father of probiotics" who hypothesized that the long and healthy lives of Bulgarian peasants were rooted in their consumption of fermented milks containing beneficial *Lactobacillus*. In 2001, the Food and Agriculture Organization (FAO) established the definition for probiotics as "Live microorganisms which when administered in adequate amount confer a health benefit on the host" (FAO/WHO, 2002). This definition implies that safety and efficacy must be demonstrated for each probiotic strain. No specific criteria for selecting new probiotics have been so far proposed, however general criteria that must be taken into account involve the capacity to adhere to the intestinal mucosa, antimicrobial production and the ability to tolerate acid and bile stress (Collins, Thornton et al. 1998). There are accumulating evidences underpinning the capacity of probiotic strains to exert one or more of the following positive activities such as anti-inflammatory immune-modulation, reduction of atopic disease symptoms, beneficially influencing the composition and activity of intestinal microbiota, prevention or suppression of bacterial infections, reduction of lactose intolerance, reduction of intestinal inflammation, production of specific short chain fatty acids, conjugated linoleic acids and

vitamins and alleviation of constipation (Goldin 1998, Ouwehand, Lagstrom et al. 2002, Saxelin, Tynkkynen et al. 2005). Although there are suggestive evidences for each of these functional claims, the molecular mechanisms behind such probiotic activities remain largely unknown (Turroni, Ventura et al. 2014). The decoding of microbial genome sequences, i.e., microbial genomics, offers the possibility of accelerating research into the molecular mechanisms followed by probiotic bacteria to establish themselves within the human body as well as to interact with the human host (Ventura, Turroni et al. 2012). Lactobacilli and bifidobacteria have traditionally been considered the most common probiotics candidates, together with nonpathogenic *Escherichia coli*, *Saccharomyces boulardii* (Kanauchi, Andoh et al. 2013).

1.4 Genomics of bifidobacteria

Currently, the genus *Bifidobacterium* includes 47 taxa (Turroni, van Sinderen et al. 2011) which 31 completely sequenced bifidobacterial genomes were publicly available (Schell, Karmirantzou et al. 2002, Lee, Karamychev et al. 2008, Sela, Chapman et al. 2008, Barrangou, Briczinski et al. 2009, Kim, Jeong et al. 2009, Ventura, Turroni et al. 2009, Turroni, Bottacini et al. 2010, Hao, Huang et al. 2011, Zhurina, Zomer et al. 2011, Bottacini, Milani et al. 2012) with genome sequences of additional 35 strains still unfinished (NCBI source). Notably, for a small number of cases such as *B. bifidum, B. longum* subsp *longum*, (Schell, Karmirantzou et al. 2002, Lee, Karamychev et al. 2008) *B. animalis* subsp. *lactis* (Barrangou, Briczinski et al. 2009, Kim, Jeong et al. 2009) two or more genome sequences are publicly available. Genomics data has significantly enhanced and will continue to improve our knowledge on the functionality of various *Bifidobacterium* taxa. Furthermore, the acquired genomic information data has also provided clues as to how bifidobacteria have adapted to the GIT environment and how they interact with their host. Comparative genome investigations of bifidobacterial strains completely sequenced, have highlighted that bifidobacterial pan-genome consists of more than 5,000 genes (Bottacini, Medini et al. 2010, Ventura, Turroni et al. 2014). In addition, it was identified a set of genes shared by all sequenced *Bifidobacterium* species, which represents a presumed core genome, including 967 genes encoding for housekeeping functions (replication, transcription, translation, cell envelope biogenesis and signal transduction) and involved in carbohydrate metabolism (Bottacini, Medini et al. 2010). Furthermore, the above mentioned comparative genome analysis allowed the identification of the so called Truly Unique Genes (TUG), including genes present only in a reference bifidobacterial genome and absent in any other genome ranging from 21 to 230. Most of these unique genes have unknown functions but probably are important for bifidobacterial persistence in the GIT such as the ability of these bacteria to interact with their natural environmental and other microorganism (Bottacini, Medini et al. 2010).

1.5 Genetically manipulation of the genus *Bifidobacterium*

In order to exploit the molecular strategy used by bifidobacteria to colonize and survive within the human body, valuable methods to genetically manipulate their genomes are needed. However, studies focusing on bifidobacteria at the molecular level would be hampered by the absence of an efficient transformation system (Rossi, Brigidi et al. 1996). Although the electroporation as an effective technique for introducing DNA into many types of eukaryotic and prokaryotic cells, has proven to be widely applicable to genetically transform bacterial strains from several genera of lactic acid bacteria, like *Lactococcus, Pediococcus, Lactobacillus, Enterococcus* (Luchansky, Muriana et al. 1988, Cruz-Rodz and Gilmore 1990), corynebacteria and brevibacteria (Bonnassie, Burini et al. 1990, Na, Shen et al. 1995), the large majority of *Bifidobacterium* strains, have largely proved to be recalcitrant to acquire alien DNA (Argnani, Leer et al. 1996). In this context, bifidobacterial display variable efficiencies of transformation between strains, ranging between $2x10^2$ and $7x10^4$ /μg DNA (Matsumura, Takeuchi et al. 1997). Low transformation efficiency is a bottleneck for gene manipulation (Sakaguchi, He et al. 2012). The observed differences in transformation efficiency among different strains of bifidobacteria may be attributed, at least in part, to restriction/modification (R/M) systems, which are ubiquitous among prokaryotes and generally comprise a restriction endonuclease (REase) and a cognate methyltransferase (MTase) (Tock and Dryden 2005). R/M systems are believed to serve primarily as defensive instruments that protect prokaryotic cells against invading DNA such as promiscuous plasmids or infecting bacteriophage (Tock and Dryden 2005). The contribution of R/M systems in reducing or abolishing transformation within bifidobacteria, also reduced the chances to create gene knockout mutants. Generally, the homologous recombination events occur with a very low frequency and a very high transformation efficiency (over 10^5 CFU/μg plasmid DNA) is generally required in order to achieve successfully recombination events (Suzuki and Yasui 2011). Recently, to the best of our knowledge, the first reliable system for creating

insertion mutation in a member of the genus *Bifidobacterium*, was obtained for *Bifidobacterium breve* UCC2003 (O'Connell Motherway, O'Driscoll et al. 2009).

1.6 Bifidobacteria and utilization of host glycans

The genomic adaptation is obvious in many bifidobacterial genomes where over 8% of annotated genes encode enzymes involved in carbohydrate metabolism which may be derived from the diet but also from the host (Turroni, Foroni et al. 2011). As expected, the genomes of gut commensals also carry more genes involved in mucin degradation than genomes of microorganisms from other environments (Walker, Cerdeno-Tarraga et al. 2006). Mucins are such host-produced glycoproteins secreted by goblet cells in the intestine, and form the main glycoprotein component of the mucus layer covering the intestinal mucosa (Ruas-Madiedo, Gueimonde et al. 2008). Given the diversity and complexity of mucin structures found within the gut (Podolsky 1985, Jensen, Kolarich et al. 2010), specific strategies for deconstructing these molecules must be inherent features in the genomes of mucin using bacteria. Recently, the genome sequence of *Bifidobacterium bifidum* PRL2010 was fully decoded (Turroni, Bottacini et al. 2010) revealing novel insights into the metabolic strategies followed by this strain to metabolize mucin-derived carbohydrates. *In silico* analyses of its chromosome together with functional genome approaches revealed the existence of a gene set involved in mucin metabolism.

The main monosaccharide constituents of mucin-derived glycoproteins are N-acetylglucosamine, N-acetylgalactosamine, fucose, and galactose, and these glycoproteins are sometimes decorated with sialic acid and sulfate groups (Forstner 1995). These investigations suggested the existence of specific *B. bifidum* PRL2010 enzymatic pathways for example by the activity of enzymes that remove sialic acid and fucose moieties from galactoN-biose (GNB) and its extended derivatives present in various mucin O-glycans (Podolsky 1985, Lloyd, Burchell et al. 1996, Wada, Ando et al. 2009). Another example of host-glycans is represented by HMOs (Sela, Chapman et al. 2008). HMOs are present at 10 to 20 g/liter in human milk (Kunz, Rudloff et al. 2000, Urashima, Saito et al. 2001) and are characterized by their complex structures (Kobata and Ginsburg 1972,

Haeuw-Fievre, Wieruszeski et al. 1993). More than 130 types of HMOs have been isolated and lacto-N-tetraose (Gal1,3GlcNAc1,3Gal1,4Glc), lacto N-fucopentaose (Fuc1,2Gal1,3GlcNAc1,3Gal1,4Glc), lacto-N-difucohexaose (Fuc1,2Gal1,3 [Fuc1,4] GlcNAc1, 3Gal1,4Glc) and 2-fucosyllactose (Fuc1,2Gal1,4Glc) are known to be abundant, especially in colostrums (Erney, Malone et al. 2000, Urashima, Saito et al. 2001, Asakuma, Urashima et al. 2008). LoCascio et al. and Ward et al. (Ward, Ninonuevo et al. 2006, LoCascio, Ninonuevo et al. 2007) demonstrated the ability of bifidobacteria to assimilate HMOs as the sole carbon source by conducting growth experiments and mass spectrometric analyses (Wada, Ando et al. 2008). Notably, *Bifidobacterium longum* subsp. *infantis* ATCC15697, which was isolated from infant feces, was demonstrated to possess a 43 kb gene cluster responsible for transport and utilization of HMOs (Sela, Chapman et al. 2008). Notably the proteome of this strain encompasses four glycosyl hydrolases that were predicted to cleave HMOs such as sialidase, a fucosidase, an N-acetyl-hexosaminidase and a galactosidase (Sela, Chapman et al. 2008).

In contrast, the genomes of *B. animalis* subsp. *lactis* strains and *B. breve* UCC2003, do not encompass the genetic loci predicted to be involved in the synthesis of the enzymatic arsenal needed for HMO degradation. However, they are predicted to encode the enzymes necessary for the breakdown of complex plant-derived oligosaccharides, which is a clear indication of plant-niche-specific adaptation (Ryan, Fitzgerald et al. 2005, Barrangou, Briczinski et al. 2009, Pokusaeva, O'Connell-Motherway et al. 2009, Pokusaeva, Fitzgerald et al. 2011).

1.7 Microbial molecules mediating host interaction

The adhesion of pathogenic and commensal bacteria to the host cells and tissues is considered as an important step in the initiation of disease or mediating beneficial effects to the host, respectively (Scott and Zahner 2006, Linke-Winnebeck, Paterson et al. 2013). The adhesion to GIT mucosa is considered as an essential colonization step for gut commensals as well as by pathogenic bacteria (Scott and Zahner 2006). Human gut commensals and pathogenic bacteria may share few common mechanisms of adhesion to epithelial tissues, and thus commensal bacteria may compete with pathogens by occupying adhesion sites (Lebeer, Vanderleyden et al. 2010, Ashida, Ogawa et al. 2012). Furthermore, adhering commensals have close contact with the host epithelium and are proposed to prime the immune system and to affect gut maturation as well as to enhance the epithelial integrity (Ewaschuk, Diaz et al. 2008, Lebeer, Vanderleyden et al. 2010, Maynard, Elson et al. 2012, Kainulainen, Reunanen et al. 2013). So far, little is known about the genetic basis of interactions between gut commensals such as bifidobacteria and the intestinal host mucosa. Valuable candidates modulating the microbe-host interaction are including capsular polysaccharides (EPS), serpin, teichoic acids, pili sortase-dependent and Type IVb pili (for details see below).

1.7.1 Capsular polysaccharides

Human gut commensals are known to synthesize cell envelope-associated structures, which are claimed to sustain a pivotal role in sustaining microbe-host interactions. Many sequenced genomes of bifidobacteria are predicted to encode an extracellular polysaccharide (EPS) and such an extracellular structure may be important in bacterial colonization or adherence to host cells, while it could also contribute to resistance to stomach acids and bile salts (Perez, Minnaard et al. 1998, Ventura, Canchaya et al. 2007). Exopolysaccharides (EPS) are carbohydrate polymers present as an extra-cellular layer covering the surface of Gram-positive as well as Gram-negative bacteria. It seems that these polymers could play a relevant role in the cross-talk between the

EPS-producing bacteria and the gut environment of the host (Hidalgo-Cantabrana, Sanchez et al. 2014).

1.7.2 Serine protease inhibitors

Serine protease inhibitor, also known as serpin, represents a large class of polypeptide serine protease inhibitors that are involved in regulation of a wide spectrum of protease-mediated processes (Silverman, Bird et al. 2001, Gettins 2002). Bifidobacteria such as *B. breve* UCC2003 as well as *Bifidobacterium breve* 210B (Alvarez-Martin, Motherway et al. 2012) are predicted to produce serpins, which are aimed to protect their surface-exposed and extracellular proteins (Turroni, Foroni et al. 2010). Their production is enhanced when bacterial cultures are exposed to various host-derived proteases (Turroni, Foroni et al. 2010, Alvarez-Martin, O'Connell Motherway et al. 2012). This finding is highly relevant since many of these proteases are normally found in the human gut and thus the presence of a protease inhibitor may provide an ecological advantage to bifidobacteria since serpin activity may protect them against these host proteases.

1.7.3 Teichoic acids

Gram-positive bacteria have been shown to modify their cell envelopes in multiple ways to prevent the access and damage by antimicrobial molecules (Xia, Kohler et al. 2010). In addition to covalent modification of peptidoglycan (PG) and phospholipids, the production of protective capsule and slime polymers, and the secretion of proteinaceous evasins (Foster 2005, Kraus and Peschel 2008), most Gram-positive bacteria have teichoic acids (TAs) or related glycopolymers that play crucial roles in bacterial survival under disadvantageous conditions and during gut colonization and infection (Weidenmaier, Kokai-Kun et al. 2004, Weidenmaier, Kokai-Kun et al. 2004, Dubail, Bigot et al. 2006, Weidenmaier and Peschel 2008, D'Elia, Henderson et al. 2009, Kohler, Weidenmaier et al. 2009). The known TA functions are: i) protection against harmful molecules and environmental stresses, ii) control of enzyme activities and cation concentrations in the cell envelope, and iii) binding to receptors and surfaces (Xia, Kohler et al. 2010) TAs are

usually constitutively produced and either connected to PG (wall teichoic acids, WTA) or to the cytoplasmic membrane (lipoteichoic acids, LTA). LTA polymers are attached to the cytoplasmic membrane via a glycolipid anchor, which is a diglucosyl diacylglycerol in *Sthaphylococus aureus* and most other staphylococcal species (Wicken and Knox 1975). As a likely consequence of the unique biosynthetic pathway, the structures of LTA are usually less diverse than those of WTA (Fischer 1994). At the 2-hydroxyl group of the glycerol, *S. aureus* LTA is substituted with D-alany ester or a-GlcNAc (Fischer 1994). Very little is known about the molecular structure of the components of the cell wall of bifidobacteria. *Bifidobacterium bifidum* subspecies *pennsylvanicum* was the first example among Gram-positive bacteria with an unusual lipoteichoic acid, it contains L-alanine in place of the D-alanine found in lipoteichoic acids (Fischer 1987). In a recent work it was suggested the therapeutic efficacy of lipoteichoic acid of *Bifidobacterium* because it could enhance antitumor effect (Xie, Wang et al. 2012).

1.7.4 Pili

Pili (Latin for "hairs") or fimbriae (Latin for "threads") are long proteinaceous structures, first observed in Gram-negative bacteria in the early 1950s, by electron microscopy
(Houwink and van 1950). These polymers are composed of non-covalently associated protein subunits called pilins or fimbrins. In Gram-negative bacteria, four types of pili have been characterized in detail over the last decades: the type I and type P of *Escherichia coli* (chaperone/usher pathway); the type III of *Yersinia, Shigella and Salmonella* (secretion needle); the type IV of *Neisseria*; and curli of *E. coli* and *Salmonella*. (Danne and Dramsi 2012). These various pili have been implicated in many functions such as adhesion to host cells, biofilm formation, DNA uptake, immune evasion, etc. (Fronzes, Remaut et al. 2008). In Gram-positive bacteria, pili were first observed in *Corynebacterium renale* in 1968 (Yanagawa, Otsuki et al. 1968, Yanagawa and Otsuki 1970). This remarkable observation remained forgotten for years until the first molecular characterization of pilus biogenesis in *Corynebacterium diphtheria* by Ton-That and Schneewind (Ton-That and Schneewind 2003, Ton-That, Marraffini et al. 2004).

Pili were subsequently found in the three major streptococcal pathogens (*Streptococcus agalactiae*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae*) and later on in many other Gram-positive bacteria (Telford, Barocchi et al. 2006, Mandlik, Swierczynski et al. 2008, Hendrickx, Budzik et al. 2011, Kreikemeyer, Gamez et al. 2011, Kang and Baker 2012). These pili are very different from those described in Gram-negative species. They are made of covalently linked pilins, forming a thin string of beads of approx. 3 nm of diameter and of various lengths (0.1- 5 µm). Pili are encoded within pathogenicity islands known as pilus islands, which have greatly facilitated their discovery in the available genome sequences. In Gram-positive bacteria, surface proteins are anchored to the cell wall by sortases. These enzymes covalently link proteins via a transpeptidation mechanism, to assemble pili. In *Actinomyces* the *fim*QP and the *fim*AB-loci encode fimbrial structures composed of a shaft protein, being either FimA or FimP, and a tip protein, represented by either FimB or FimQ, respectively (Yeung and Ragsdale 1997). Fimbriae have never been described in bifidobacteria, but these appendages could mediate microbial adhesion and colonization of epithelial, mucosal, or other host cell surfaces (Schell, Karmirantzou et al. 2002). The genome sequence of *B. longum* NCC2705 has revealed the presence of a gene with the 30% of identity to fimbriae structures of *Actinomyces naeslundii*, but it remains to be determined if they act like fimbriae, and if they possibly contribute to attachment or retention in the GIT (Schell, Karmirantzou et al. 2002).

1.7.5 Type IVb pili

Recently, the Gram-positive pathogen *Mycobacterium tuberculosis* was found to express type IVb pili, which are encoded by the *tad* locus, similar to those specified by the Gram-negative tight adherence (tad) locus (Danelishvili, Yamazaki et al. 2010). Homologs of tad genes have been identified in the genomes of *Corynebacterium diphtheria*, *Thermobifida fusca*, and *Streptomyces coelicolor* (Tomich, Planet et al. 2007, Pelicic 2008). The Tad pili biosynthesis apparatus was first described in *Actinobacillus actinomycetemcomitans* and allows the production and assembly of pili, which in this bacterium mediate adhesion to surfaces and are essential for

colonization and pathogenesis (Kachlany, Levery et al. 2001, Schreiner, Sinatra et al. 2003). The Tad pilus is comprised of homopolymers of a single pilin subunit, although some pili possess an adhesive subunit at the pilus tip or can be decorated with pseudopilins along the pilus (Tomich, Planet et al. 2007, Pelicic 2008). In addition, clear homologs of the *tad* locus and the associated *tad*V gene encoding the prepilin peptidase are present in several *B. breve* strains examined by CGH (Comparative Genome Hybridization) as well as in all completed bifidobacterial genome sequences (Motherway, Zomer et al. 2011). Intriguingly, in *B. breve* UCC2003 a locus dedicated to the production of type IVB or Tad pili was among the most highly up-regulated genes during *in vivo* trials involving mouse models. Mutational analysis demonstrated that the UCC2003 *tad* gene cluster is crucial for efficient *in vivo* gut colonization in murine models, (Motherway, Zomer et al. 2011).

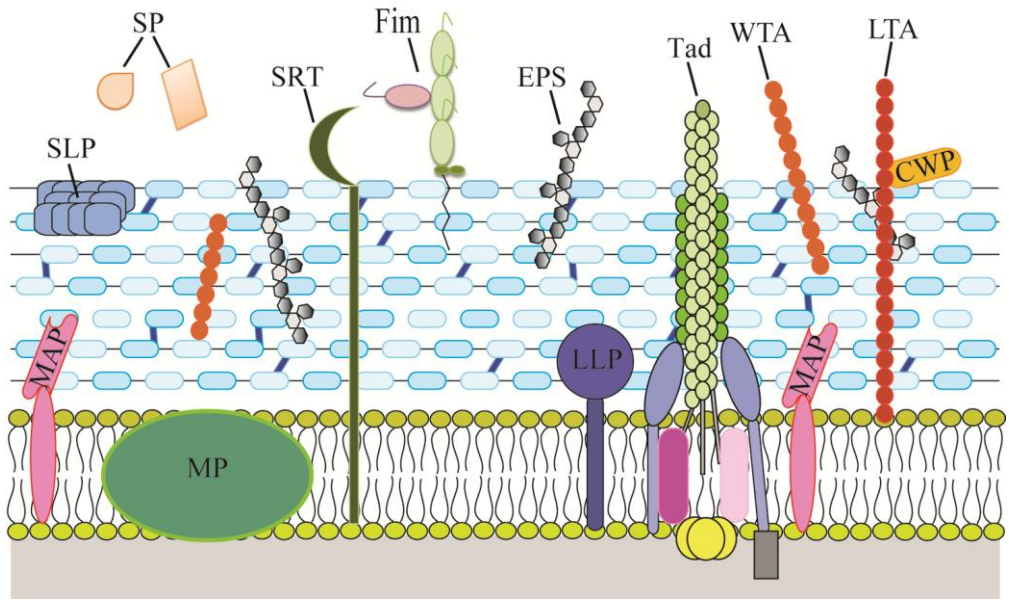

Fig 1.7: Schematic overview of the Gram-positive cell wall with main macromolecular structures implicated in host–microbe interaction. Wall and lipoteichoic acids (WTA and LTA), exopolysaccharide (EPS), as well as secreted proteins (SP), membrane proteins (MP), cell wall-associated proteins (CWP), lipoproteins (LPP), membrane-associated proteins (MAP), surface layer proteins (SLP), fimbrial proteins (Fim), tad proteins (TAD) and the sortase-dependent assembly apparatus (SRT) are indicated [This image has been taken by (Turroni, Ventura et al. 2014)].

1.8 Roles of pili in Gram-positive bacteria

To colonize host tissues, bacteria use a variety of surface exposed proteins, such as adhesins and proteases, as well as capsule and proteins interfering with innate immune clearance mechanisms. Several of these pilus-associated adhesin subunits are described as MSCRAMMs (microbial components recognizing adhesive matrix molecules), which mediate binding to host extracellular matrix (ECM) and are crucial for colonization and invasion. Pili contribute to initial steps of infection i.e. bacterial adhesion and colonization of host tissues (Kang and Baker 2012). They also play important roles in biofilm formation. Indeed, the SpaCBA pilus of *Lactobacillus rhamnosus* GG is involved in binding to human mucus via the adhesin SpaC. The presence of SpaC likely explains the ability of *L. rhamnosus* to persist longer in the human intestinal tract than strain LC705, which does not express pili during an intervention trial (Kankainen, Paulin et al. 2009, Lebeer, Vanderleyden et al. 2010). More recently, the SpaCBA pilus was shown critical for efficient adherence to the human intestinal epithelial cell line Caco-2 as well as for biofilm formation (Lebeer, Claes et al. 2012). As reported earlier for opportunistic bacteria, these data support the importance of pilus in host-colonization and persistence for commensal bacteria of the microbiota.

1.9 The impact of bifidobacteria on the resident members of the gut microbiota

To determine the molecular mechanisms driving the microbe-microbe as well as microbe-host interaction, Sonnenburg et al (Sonnenburg, Chen et al. 2006) have colonized germ-free mice with *Bacteroides thetaiotaomicron*, a prominent component of the adult human gut microbiota, and *Bifidobacterium longum* NCC2705, another common human gut commensal. Both bacterial genomes have been sequenced, so it has been possible to use a *B. thetaiotaomicron*/*B. longum* ''community'' GeneChip to simultaneously examine how each organism affects the other's transcriptome in a specified intestinal habitat of the host. This study highlighted how two resident human gut commensals influence the capacity of carbohydrate harvesting. In fact, whole genome transcriptional profiling of both bacterial species in their gut habitat revealed that the presence of *B. longum* subsp. *longum* NCC2705 elicits an expansion in the diversity of polysaccharides targeted for degradation by *B. thetaiotaomicron* (e.g., mannose- and xylose-containing glycans), and induces host genes involved in innate immunity. In fact, the presence of *B. longum* subsp. *longum* NCC2705 produced statistically significant changes in expression of 31 *B. thetaiotaomicron* glycoside hydrolases and two polysaccharide lyases when compared to *B. thetaiotaomicron* residing in the ceca of mono-associated mice (the vast majority of these genes (29/33) were up-regulated). Alternatively, co-colonization could result in changes in the environment, such as pH or osmolarity that render mannosidases expressed by NCC2705 more stable, or more efficient, resulting in their subsequent down-regulation. Each bacterial species, when present alone in the cecum, possesses a complement of glycoside hydrolases that is insufficient for efficient degradation of available xylose-containing glycans. However, when their repertoires of glycoside hydrolases are combined, complementary enzymes activities allow the two bacterial species to participate in a ''synergistic'' harvest of this class of glycans in a similar fashion to what previously described for other mixed microbial communities that degrade cellulose. This gnotobiotic mouse model provides a controlled case study of how a resident

symbiont and a probiotic species adapt their substrate utilization in response to one another, and illustrates both the generality and specificity of the relationship between a host, a component of its microbiota, and intentionally consumed microbial species. The ability of *B. longum* to repress host expression of antibacterial proteins may not only promote its own survival in the gut, but also influence the composition, structure, and function of its microbial community

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2. Aim of the Research

The human gastrointestinal (GIT) microbiota is a complex microbial environmental whose functions are believed to have a significant impact on human physiology. The *Bifidobacterium* genus is one of the most dominant members of the GIT of infants and bifidobacteria are frequently exploited as healthy components or probiotic ingredient in many functional foods. Despite the importance of bifidobacteria as inhabitants of human microbiota, only recently the probiogenomics studies (based on genetics, physiology and functional genomics) have highlighted the importance of these bacteria to adapt and interact with the host and with the other microorganisms of the GIT. The interaction between bacteria and the human affects the host in different ways. The aim of this PhD thesis is to investigate the molecular basis sustaining the interaction of the human commensal microorganism *Bifidobacterium bifidum* PRL2010 with the human host as well as with other human gut commensals.

Chapter 3 describes the analysis of mupirocin resistance in the majority of bifidobacterial species to obtain a valid method to screen and isolate the bibifidobacteria. Furthermore, it was proposed a structural mechanism to explain mupirocin resistance in bifidobacteria.

Chapter 4 shows a physiological characterization of *B. bifidum* PRL2010. In particular some abilities of this commensal bacterium, i.e to resist to the harsh environmental conditions of the human gut, to adhere to intestinal cells and to prevent/reduce activity of pathogenic bacteria, are reported.

Chapter 5 proposes a novel method to transform and to genetically manipulate *B. bifidum* PRL2010.

In Chapter 6, provides molecular data about putative candidate bifidobacterial proteins, i.e. sortase-dependent pili, involved in the interaction with host.

Chapter 7 and Chapter 8 are focusing on the molecular characterization of sortase-dependent pili encoded by *B. bifidum* PRL2010. It was evaluated the effect of treatments simulating the passage of bifidobacterial cells through the GIT on the regulation of the expression of sortase-dependent pili. Furthermore, it was assessed how the interaction with the other gut commensals might affect the production of these extracellular proteins.

Finally, in Chapter 9 it is evaluated the ability of *B. bifidum* PRL2010 to survive into a food complex matrix such as kefir milk. Moreover, it was investigated through transcriptional analysis, the expression of sortase-dependent pili in this complex symbiotic ecosystem.

3. Insights into physiological and genetic mupirocin susceptibility in bifidobacteria

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Insights into Physiological and Genetic Mupirocin Susceptibility in Bifidobacteria▽

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Mupirocin is an antibiotic commonly used in selective media for the isolation of bifidobacteria. However, little is known about the genetic traits responsible for bifidobacterial resistance to mupirocin. Our investigation demonstrates that all of the bifidobacteria tested exhibit a phenotype of generally high resistance to this antibiotic. The genotypic reason for bifidobacterial mupirocin resistance was further characterized by sequencing of the isoleucyl-tRNA synthetase gene (*ileS*) coupled with three-dimensional modeling of the encoded protein and cloning of the *ileS* gene of *Bifidobacterium bifidum* PRL2010 in a mupirocin-sensitive *Escherichia coli* strain. These analyses revealed key amino acid residues of the IleS protein that apparently are crucial for conferring a mupirocin resistance phenotype to bifidobacteria.

Mupirocin is a narrow-spectrum antibiotic produced by *Pseudomonas fluorescens* (4, 17) and is active against certain Gram-negative and Gram-positive bacteria, including microorganisms that are used in fermented dairy products and functional foods, such as *Streptococcus* spp., *Lactococcus* spp., and *Lactobacillus* spp. (16). The antibacterial activity of mupirocin is due to competitive inhibition, as it competes with isoleucine as a substrate for isoleucyl-tRNA synthetase (6). The chemical structure of mupirocin resembles that of the isoleucyl-adenylate complex, and thus, the biochemical reason for its antagonistic activity corresponds to inhibition of the aminoacylation process in which isoleucyl-adenylate is synthesized.

Mupirocin is widely used as a selective agent for the isolation of bifidobacteria from complex ecosystems such as the human gut (16, 19). Bifidobacteria are Gram-positive microorganisms belonging to the *Actinobacteria* phylum that are natural inhabitants of the mammalian gastrointestinal tract (for a review, see reference 20). Bifidobacteria have recently generated growing scientific interest due to their presumed activity in maintaining gastrointestinal health and other beneficial or probiotic properties (13). Thus, significant efforts have been made to understand the genetic and ecological properties of this group of bacteria, including their susceptibility to different antibiotics (2, 9–11). So far, very little is known about the spectrum of bifidobacterial susceptibility to mupirocin, which will be of great interest in terms of developing a novel selective mupirocin-based medium for this group of microorganisms. Moreover, nothing is known about the molecular mechanisms responsible for the susceptibility/resistance of bifidobacteria to this antibiotic. Such knowledge may be useful for the development of genetic tools for bifidobacteria (e.g., gene knockout systems), while bifidobacterial mutants displaying higher/lower resistance to this antibiotic may also be used to monitor bifidobacterial colonization in clinical settings or *in vivo* trials.

Here, we provide an extensive analysis of mupirocin resistance in the majority of currently described bifidobacterial species, taking into consideration the genetic location of the mupirocin resistance determinant and linking the different levels of resistance identified in bifidobacteria to sequence variability of the isoleucyl-tRNA synthetase gene. Furthermore, we propose a structural mechanism to explain mupirocin resistance in bifidobacteria.

Identification of MICs. Bifidobacterial susceptibility to mupirocin was analyzed by MIC assays, which consisted of cultivating different bifidobacterial strains in the presence of various concentrations of this antibiotic ranging from 200 to 2,000 μg/ml. The bifidobacterial strains utilized were selected in order to represent the majority of the bifidobacterial species described so far (Fig. 1). Bifidobacteria were cultivated anaerobically using an anaerobic cabinet (Concept 400; Ruskin, West Yorkshire, United Kingdom) in de Man-Rogosa-Sharpe medium (Scharlau Chemie, Barcelona, Spain) supplemented with 0.05% (wt/vol) L-cysteine hydrochloride and incubated at 37°C for 16 h. The MIC for each strain employed in this study was determined (Fig. 1) according to a previously described method (11). All of the bifidobacterial species tested exhibited high mupirocin resistance (MIC values in excess of 2,000 μg/ml), with the exception of a small number of species, which were shown to be susceptible to this antibiotic at concentrations higher than 1,800 μg/ml (Fig. 1) and which were thus considered to exhibit reduced mupirocin resistance relative to the majority of the bifidobacterial strains tested. Such findings indicate that most, if not all, bifidobacteria exhibit stable resistance to mupirocin at moderate-to-high levels. Interestingly, for a small number of bifidobacterial species, the observed

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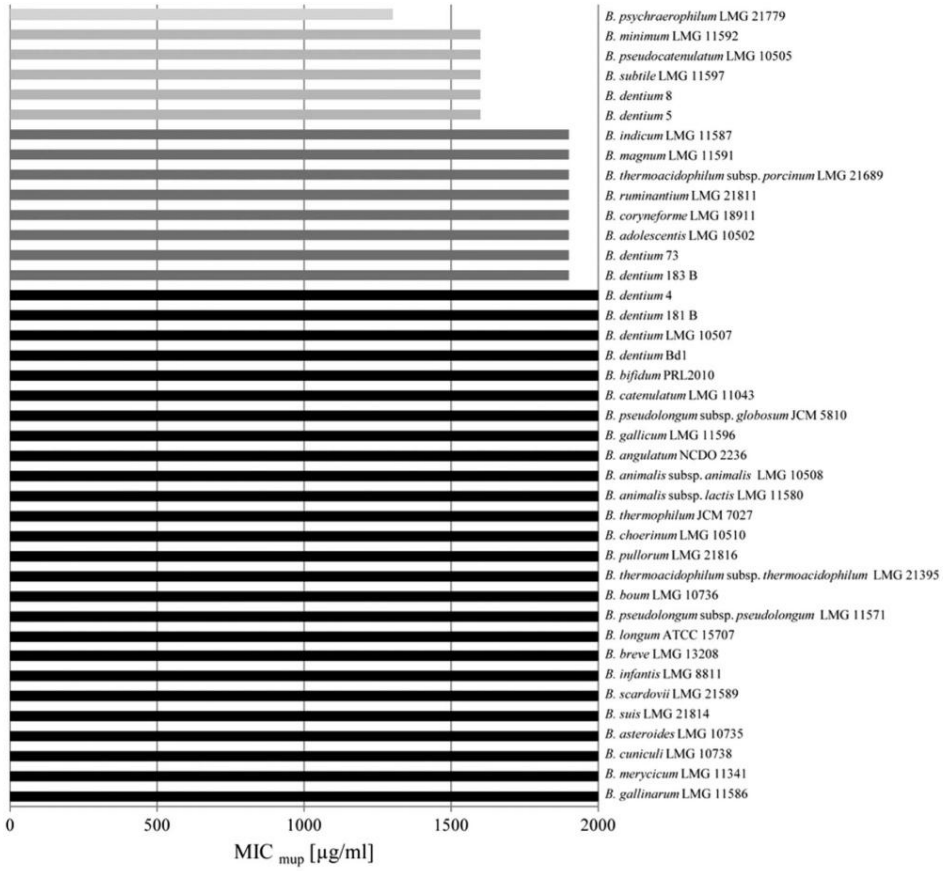

FIG. 1. MIC values identified in the different bifidobacterial species.

level of mupirocin susceptibility was shown to vary at the intraspecies level. This occurred within the species *Bifidobacterium dentium*, which includes strains that are moderately resistant to mupirocin (e.g., strains 5 and 8), as well as strains that are highly resistant to this antibiotic (e.g., strains 181 and 4) (Fig. 1). This suggests that moderate or high resistance to mupirocin is a strain-dependent feature rather than a species-dependent characteristic.

Such different levels of mupirocin susceptibility shown by bifidobacteria resemble those described for *Staphylococcus aureus* (3). In the latter case, the resistance of *S. aureus* to high levels of mupirocin (above 2,000 μg/ml) was due to the presence of an additional isoleucyl-tRNA synthetase-encoding gene, called *mupA* or *ileS2*, specifying a mupirocin-resistant isoleucyl-tRNA synthetase, which is similar to eukaryotic IleS (5). In contrast, bioinformatic screening for the *ileS* genes in currently available complete bifidobacterial genome sequences (*B. dentium* Bd1 [21], *B. longum* subsp. *longum* NCC2705 [15], *B. longum* subsp. *longum* DJO10A [8], and *B. animalis* subsp. *lactis* DSM10140 [1]), as well as incomplete bifidobacterial genome sequences (*B. dentium* ATCC 27678, *B. longum* subsp. *infantis* CCUG 52486, *B. bifidum* PRL2010, *B. bifidum* NCIMB 41171, *B. adolescentis* L2-32, and *B. catenulatum* DSM 16992 [NCBI sources]), showed that such bifidobacteria harbor a single chromosomal copy of *ileS* in their genomes.

Introduction of the *ileS* gene of *B. bifidum* PRL2010 into *E. coli*. In order to verify if the *ileS* gene product would be responsible for conveying resistance to mupirocin in bifidobacteria as it does in other bacteria (7), the coding region of the *ileS* gene of *B. bifidum* PRL2010 (here $ileS_{bifidum}$) was amplified by PCR using primers *ileS*1 (5′-GGCCAAGCTTGGTGAGCGAAACCACCAATTCC-3′) and *ileS*2 (5′-GGCGCTGCAGTCACGCCTTGGCGACCTCCAC-3′), including the HindIII and PstI restriction sites (underlined), respectively, cloned into the pUC19 vector under the control of the *lac* promoter, and subsequently electroporated into *E. coli* strain DH10B. The presence of recombinant plasmid pUC19-$ileS_{bifidum}$ was confirmed for transformants by PCR and sequencing of the PCR amplicons obtained. *E. coli* DH10B is susceptible to a low level of mupirocin (MIC of 15 μg/ml), but it was converted to

mupirocin resistance (MIC of 120 μg/ml) by transformation with pUC19-*ileS*$_{bifidum}$. Such results were also confirmed by the overlay disc diffusion test (data not shown). Although pUC19 is a high-copy-number plasmid, it has been previously demonstrated that overexpression of the *E. coli* mupirocin-sensitive *ileS* gene through cloning in pUC19 did not increase mupirocin resistance in *E. coli* (22), suggesting that high levels of a sensitive IleS protein do not provoke resistance to mupirocin. These results therefore clearly indicate that the *ileS*$_{bifidum}$ gene represents the genetic determinant of the mupirocin resistance displayed by bifidobacteria.

Structural analysis of the IleS proteins encoded by mupirocin-resistant bifidobacteria. As previously mentioned, it is well known that the protein encoded by *ileS* represents the target site of mupirocin (for reviews, see references 14 and 23). Multiple-sequence alignment of the *ileS* protein products from susceptible microorganisms (e.g., *Lactobacillus acidophilus*, *Enterococcus* spp., *Streptococcus* spp., *E. coli*, *Staphylococcus aureus*, and *Thermus thermophilus*) and mupirocin-resistant microorganisms such as *Bifidobacterium* spp. and *Pseudomonas* spp. highlights the presence of amino acid residues that may be responsible for structural differences in the IleS protein (Fig. 2) and consequently for the mupirocin resistance/susceptibility phenotype. BLASTP analysis of the IleS protein sequence encoded by *B. bifidum* PRL2010 against the Protein Data Bank (PDB) database allowed the identification of a reliable structural template (IleS from *Thermus thermophilus*) (12). No other IleS structural model phylogenetically close to bifidobacteria was found in the PDB database. Although the phylogenetic distance between *B. bifidum* and *T. thermophilus* is considerable, the level of sequence identity between the IleS protein of *B. bifidum* PRL2010 and the template is still significantly high (E value of $2e^{-170}$). Based on the structural analysis of *T. thermophilus* IleS (12), the predicted three-dimensional (3D) structure of IleS of *B. bifidum* PRL2010 (SWISS-MODEL at http://swissmodel.expasy.org/) contains a reactive site which is presumed to act as the binding site of Ile-AMP during the aminoacylation process. Employing the online tool First Glance in Jmol version 1.45, we further analyzed the IleS Ile-AMP binding site sequence from the mupirocin-sensitive microorganism *T. thermophilus*, which highlighted 18 residues that are believed to be involved in specific interactions with the Ile-AMP ligand. A further comparative analysis including a larger data set of IleS sequences from different microorganisms exhibiting various levels of susceptibility to mupirocin highlighted a number of amino acid residues that may be responsible for the mupirocin resistance displayed by bifidobacterial strains. In order to probe the molecular effects of changes in amino acid residues (mutations) in the IleS sequences of both mupirocin-resistant and mupirocin-susceptible bacteria upon the interaction of IleS with mupirocin, we used the publicly available 3D structure of IleS from *T. thermophilus* (PDB code 1GAX [12]) as a template for the prediction of the structural model of IleS of *B. bifidum* PRL2010 (Fig. 3). Notably, the susceptibility of these bacteria to mupirocin is totally different (sensitive versus resistant) and thus the in-depth analysis of the 3D structures of IleS from *T. thermophilus* and *B. bifidum* PRL2010 was used to study the molecular effects of amino acid substitutions at various positions within different IleS sequences (RG1 and RG2). Notably, the RG1 and RG2 sequences partially overlap those regions believed to be involved in specific interaction with the Ile-AMP ligand.

The binding site investigation showed that the lack of a hydrophobic interaction at the apolar mupirocin long tail can cause a decrease in binding energy and the release of this molecule from the active site in RG1. In fact, the hydrophobic valine residue of *T. thermophilus* is replaced in bifidobacteria with a tyrosine's hydrocarbon benzene ring, which is predicted to cause reduced stability of the ligand in the active site. Interestingly, in the mupirocin producer *P. fluorescens*, a tyrosine residue is present in one of the products of the two *ileS* genes carried on its genome, which confers self-immunity (18, 22). Notably, all organisms possessing high resistance to mupirocin contain a tyrosine residue or an alanine residue in the RG1 region of their IleS sequence, e.g., IleS2 of *S. aureus*.

Sequence analyses of *ileS* genes in bifidobacteria. A set of 34 bifidobacterial strains was subjected to PCR amplification of their *ileS* genes using primers *ileS*1 (5′-GAGTTCGTGTTCT TCGAC-3′) and *ileS*2 (5′-GACACGGTGGTGTCCTG-3′) and primers *ileS*7 (5′-CAGTTCGGTAAGTGGCT-3′) and *ileS*4 (5′-GTAGTAGGAGCTCCACAC-3′), followed by DNA sequencing. We analyzed two *ileS* gene sequence regions consisting of 54 and 234 bp, respectively, corresponding to codons 215 to 264 and 1737 to 1971 of *ileS* of *B. bifidum* PRL2010. These regions, named RG1 and RG2, are directly involved in forming the ligand binding site, and for this reason the amino acid residues encompassing these regions are considered to be crucial for resistance to the ligand mupirocin (Fig. 3). Notably, the alignments of RG1 and RG2 display high conservation at the amino acid level among all of the species tested (RG1 consensus sequence, HYGH; RG2 consensus sequence, KMSK). All of the IleS sequences analyzed display a tyrosine residue in RG1 that, based on structural analyses of the IleS-mupirocin structures, is believed to be responsible for providing resistance to mupirocin (see above).

Conclusion. This study investigated the genetic basis of the intrinsic mupirocin resistance displayed by bifidobacteria. For this reason, mupirocin is widely used as a selective agent for the isolation of bifidobacteria from environmental samples (e.g., fecal samples, intestinal biopsy specimens, and food products) by the addition of mupirocin to different synthetic media used for cultivation of bifidobacteria. However, so far, little is known about the mupirocin susceptibility exhibited by the currently recognized bifidobacterial species. In this report, we show that bifidobacteria display variable levels of susceptibility to mupirocin, and this knowledge will be important for the development of novel and effective selection protocols for bifidobacteria based on antibiotic inclusion in selective media. Furthermore, we investigated the genetic basis of resistance to mupirocin in bifidobacteria and provided molecular evidence as to how the intrinsic mupirocin resistance of bifidobacteria is supported by the product encoded by the *ileS* gene and how a particular amino acid, tyrosine, in the targeted enzyme (IleS) may be responsible for the high level of mupirocin resistance observed in bifidobacteria. Bifidobacteria are generally not or only poorly genetically accessible, which at this time prevents us from performing particular confirmatory experiments. Future functional genomic investigations directed to the silencing of the *ileS* gene, as well as whole-genome transcription profil-

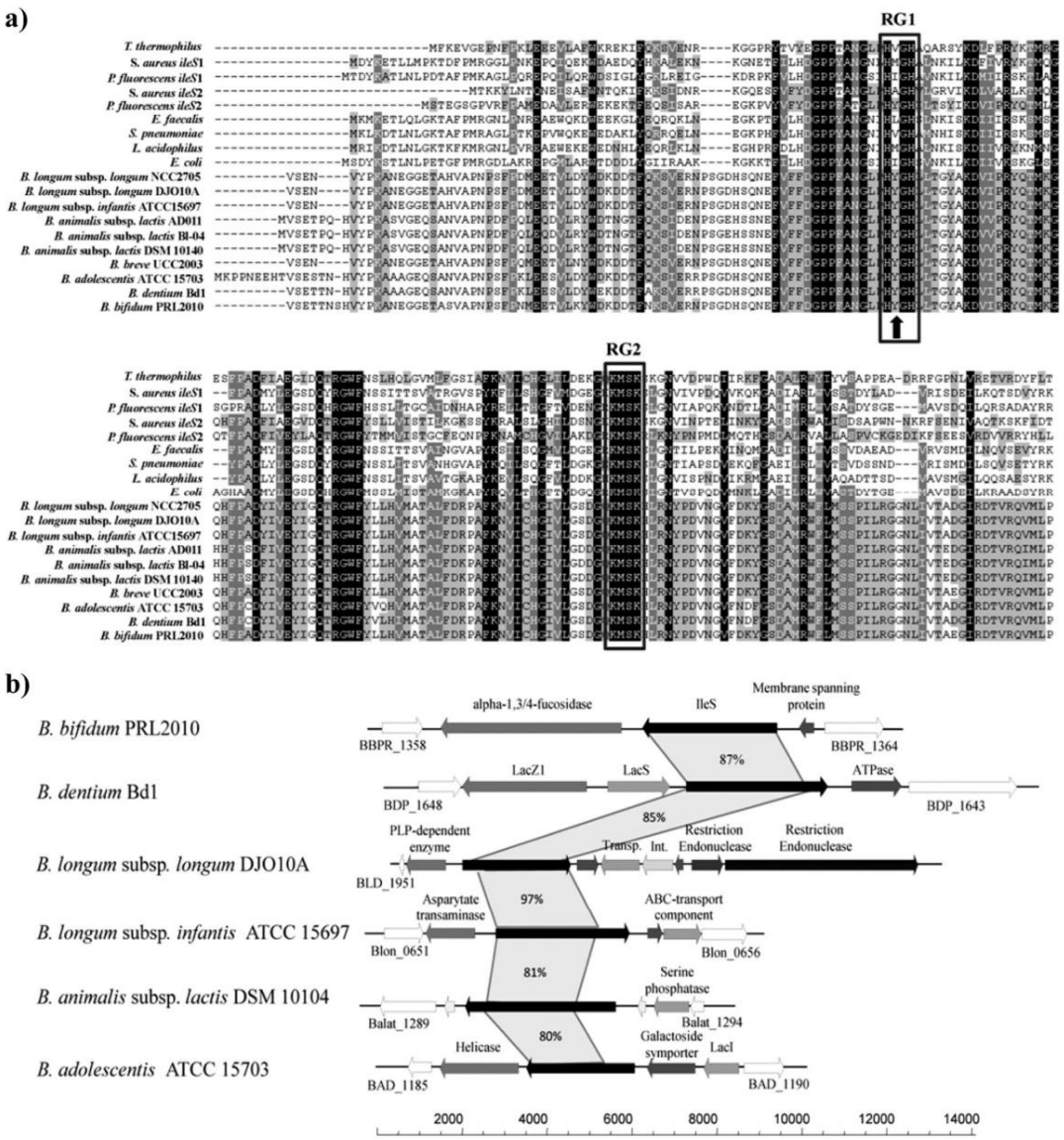

FIG. 2. Comparative analysis of isoleucyl-tRNA synthetase sequences. Multiple-sequence alignment of bacterial isoleucyl-tRNA synthetases displaying various levels of susceptibility to mupirocin (resistant versus susceptible) (a). When there are multiple *ileS* gene copies in the same organism (e.g., *ileS2* of *S. aureus* and *ileS2* of *P. fluorescens*), both of the protein sequences encoded are displayed. The amino acid residues constituting the RG1 and RG2 regions are highlighted. (b) Comparative schematic representation of the *ileS* locus of *B. bifidum* PRL2010 and those of various other bifidobacterial strains. Each arrow indicates an open reading frame, the size of which is proportional to the length of the arrow. The predicted function of the protein is indicated above each arrow.

ing experiments involving bifidobacteria grown in the presence of mupirocin, will allow us to identify the precise genetic requirements responsible for the high resistance to this antibiotic displayed by bifidobacteria. Furthermore, sequence analyses of the DNA regions surrounding the *ileS* gene in the genomes of bifidobacteria have not revealed any genetic features (e.g., atypical codon usage or atypical GC content or dinucleotide frequencies) or, except for the genome of *B. longum* subsp.

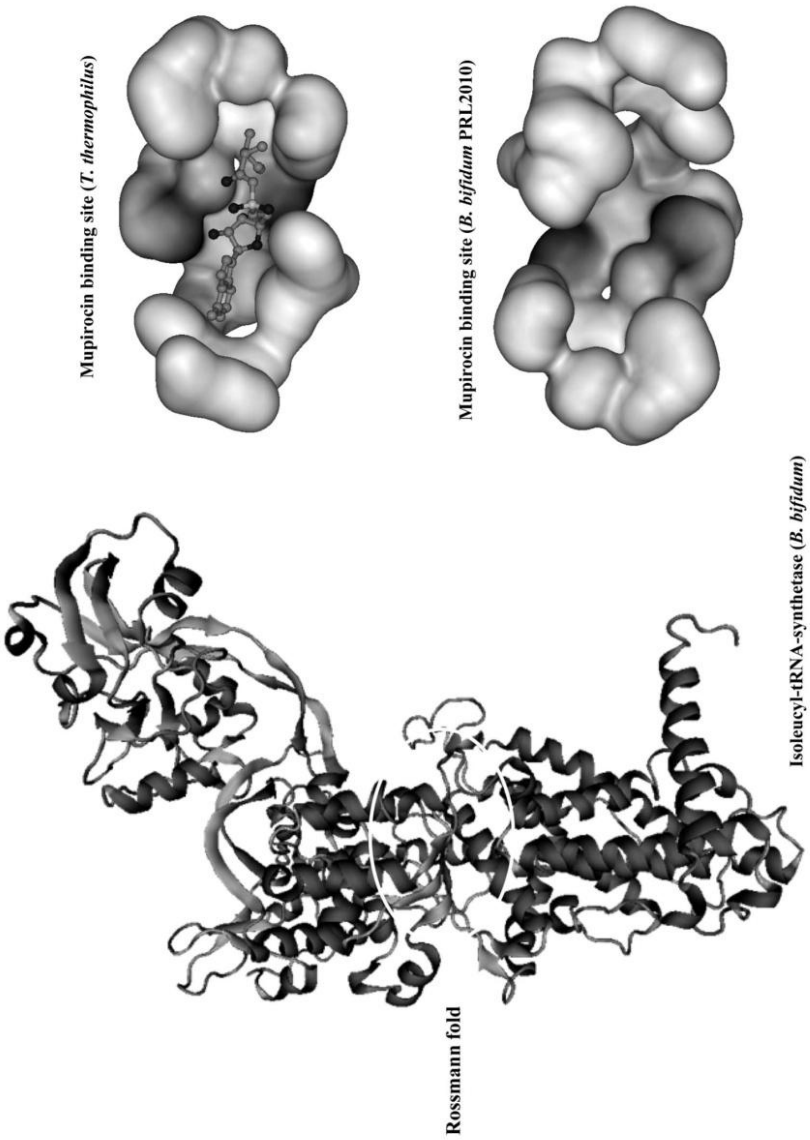

FIG. 3. Predicted 3D structure of the isoleucyl-tRNA synthetase from *B. bifidum* PRL2010 obtained by homology modeling using *T. thermophilus* as the template. This image is based on a docking model. The magnified sections represent the active site characterized by a Rossmann-type folding of the isoleucyl-tRNA synthetase from *T. thermophilus* as retrieved from the PDB and interacting with the mupirocin molecule, as well as the homologous region of the isoleucyl-tRNA synthetase from *B. bifidum* PRL2010. These structures are depicted as ball-and-stick structures shaded according to atom type. The different levels of interaction with the IleS binding cavity are indicated by various levels of gray shading.

longum NCC2705, the presence of nearby mobile elements (e.g., in the genome of *B. bifidum* PRL2010, the closest mobile element is represented by a transposases placed 203 kb downstream of the *ileS* gene), which indicates that mupirocin resistance in bifidobacteria is not mediated by a transferable genetic factor, minimizing the risk for horizontal transmission of resistance to other microorganisms in the digestive tract.

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REFERENCES

1. **Barrangou, R., et al.** 2009. Comparison of the complete genome sequences of *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and Bl-04. J. Bacteriol. **191:**4144–4151.
2. **Charteris, W. P., P. M. Kelly, L. Morelli, and J. K. Collins.** 1998. Antibiotic susceptibility of potentially probiotic *Bifidobacterium* isolates from the human gastrointestinal tract. Lett. Appl. Microbiol. **26:**333–337.
3. **Eltringham, I.** 1997. Mupirocin resistance and methicillin-resistant Staphylococcus aureus (MRSA). J. Hosp. Infect. **35:**1–8.
4. **Hill, R. L., G. J. Duckworth, and M. W. Casewell.** 1988. Elimination of nasal carriage of methicillin-resistant Staphylococcus aureus with mupirocin during a hospital outbreak. J. Antimicrob. Chemother. **22:**377–384.
5. **Hodgson, J. E., et al.** 1994. Molecular characterization of the gene encoding high-level mupirocin resistance in *Staphylococcus aureus* J2870. Antimicrob. Agents Chemother. **38:**1205–1208.
6. **Hughes, J., and G. Mellows.** 1980. Interaction of pseudomonic acid A with Escherichia coli B isoleucyl-tRNA synthetase. Biochem. J. **191:**209–219.
7. **Hurdle, J. G., A. J. O'Neill, E. Ingham, C. Fishwick, and I. Chopra.** 2004. Analysis of mupirocin resistance and fitness in *Staphylococcus aureus* by molecular genetic and structural modeling techniques. Antimicrob. Agents Chemother. **48:**4366–4376.
8. **Lee, J. H., et al.** 2008. Comparative genomic analysis of the gut bacterium Bifidobacterium longum reveals loci susceptible to deletion during pure culture growth. BMC Genomics **9:**247.
9. **Margolles, A., A. B. Florez, J. A. Moreno, D. van Sinderen, and C. G. de los Reyes-Gavilan.** 2006. Two membrane proteins from Bifidobacterium breve UCC2003 constitute an ABC-type multidrug transporter. Microbiology **152:**3497–3505.
10. **Margolles, A., J. A. Moreno, D. van Sinderen, and C. G. de Los Reyes-Gavilan.** 2005. Macrolide resistance mediated by a *Bifidobacterium breve* membrane protein. Antimicrob. Agents Chemother. **49:**4379–4381.
11. **Masco, L., K. Van Hoorde, E. De Brandt, J. Swings, and G. Huys.** 2006. Antimicrobial susceptibility of Bifidobacterium strains from humans, animals and probiotic products. J. Antimicrob. Chemother. **58:**85–94.
12. **Nakama, T., O. Nureki, and S. Yokoyama.** 2001. Structural basis for the recognition of isoleucyl-adenylate and an antibiotic, mupirocin, by isoleucyl-tRNA synthetase. J. Biol. Chem. **276:**47387–47393.
13. **Ouwehand, A. C., S. Salminen, and E. Isolauri.** 2002. Probiotics: an overview of beneficial effects. Antonie Van Leeuwenhoek **82:**279–289.
14. **Patel, J. B., R. J. Gorwitz, and J. A. Jernigan.** 2009. Mupirocin resistance. Clin. Infect. Dis. **49:**935–941.
15. **Schell, M. A., et al.** 2002. The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract. Proc. Natl. Acad. Sci. U. S. A. **99:**14422–14427.
16. **Simpson, P. J., G. F. Fitzgerald, C. Stanton, and R. P. Ross.** 2004. The evaluation of a mupirocin-based selective medium for the enumeration of bifidobacteria from probiotic animal feed. J. Microbiol. Methods **57:**9–16.
17. **Sutherland, R., et al.** 1985. Antibacterial activity of mupirocin (pseudomonic acid), a new antibiotic for topical use. Antimicrob. Agents Chemother. **27:**495–498.
18. **Thomas, C. M., J. Hothersall, C. L. Willis, and T. J. Simpson.** 2010. Resistance to and synthesis of the antibiotic mupirocin. Nat. Rev. Microbiol. **8:**281–289.
19. **Turroni, F., et al.** 2009. Exploring the diversity of the bifidobacterial population in the human intestinal tract. Appl. Environ. Microbiol. **75:**1534–1545.
20. **Ventura, M., et al.** 2007. Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum. Microbiol. Mol. Biol. Rev. **71:**495–548.
21. **Ventura, M., et al.** 2009. The Bifidobacterium dentium Bd1 genome sequence reflects its genetic adaptation to the human oral cavity. PLoS Genet. **5:**e1000785.
22. **Yanagisawa, T., and M. Kawakami.** 2003. How does Pseudomonas fluorescens avoid suicide from its antibiotic pseudomonic acid? Evidence for two evolutionarily distinct isoleucyl-tRNA synthetases conferring self-defense. J. Biol. Chem. **278:**25887–25894.
23. **Yanagisawa, T., J. T. Lee, H. C. Wu, and M. Kawakami.** 1994. Relationship of protein structure of isoleucyl-transfer RNA synthetase with pseudomonic acid resistance of *Escherichia coli*. A proposed mode of action of pseudomonic acid as inhibition of isoleucyl-tRNA synthetase. J. Biol. Chem. **269:**24304–24309.

4. Evaluation of adhesion properties and antibacterial activities of the infant gut commensal *Bifidobacterium bifidum* PRL2010

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Evaluation of adhesion properties and antibacterial activities of the infant gut commensal *Bifidobacterium bifidum* PRL2010

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ABSTRACT

Bifidobacteria are extensively exploited by the food industry as health-promoting microorganisms. However, very little is known about the molecular mechanisms responsible for these beneficial activities, or the molecular players that sustain their ability to colonize and persist within the human gut. Here, we have investigated the enteric adaptation features of the gut commensal *Bifidobacterium bifidum* PRL2010, originally isolated from infant feces. This strain was able to survive under gastrointestinal challenges, while it was shown to adhere to human epithelial intestinal cell monolayers (Caco 2 and HT-29), thereby inhibiting adhesion of pathogenic bacteria such as *Escherichia coli* and *Cronobacter sakazakii*.

1. Introduction

Certain members of the genus *Bifidobacterium* are considered to possess health-promoting activities [1]. In this respect, particular strains of the *Bifidobacterium bifidum* species are recognized to play important roles in providing health benefits to their human host, including antibacterial activities against pathogens such as *Helicobacter pylori* [2,3], reduction of apoptosis in the intestinal epithelium of infants suffering from necrotizing enterocolitis [4], modulation of the host-immune system [5,6], and eliciting anti-inflammatory activities to alleviate certain chronic large bowel diseases [7–9]. Furthermore, *B. bifidum* as well as other bifidobacterial species such as *Bifidobacterium breve* and *Bifidobacterium longum* subsp. *infantis* are believed to be important for the establishment of a well-balanced, autochthonous intestinal microbiota in newborns [10]. In this context, *B. bifidum* was recently shown to be one of the most dominant components of the gut microbiota of infants, especially from those that are breast-fed [11,12].

Although there is experimental evidence supporting functional claims for specific bifidobacterial strains, the molecular mechanisms behind such health-promoting activities remain largely unknown. The decoding of microbial genome sequences, i.e. microbial genomics, offers the possibility of accelerating research into the mechanisms of action of health-promoting or probiotic bacteria. Genome sequencing has also directed its interest towards food-related bacteria, intestinal commensals and health-promoting bacteria. Very recently, a novel discipline, designated as probiogenomics, was coined, which aims to provide insights into the diversity and evolution of probiotic bacteria, while also trying to reveal the molecular basis for their health-promoting activities [13]. Probiogenomic efforts have led to the decoding of bifidobacterial genome sequences including *B. bifidum* PRL2010 [14], *B. longum* subsp. *longum* NCC2705 [15], *B. longum* subsp. *longum* DJ010A [16], *B. longum* subsp. *infantis* ATCC15697 [17], *B. breve* UCC2003 [18]. Notably, these investigations have provided

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clear evidence of genetic adaptation of these microorganisms to the human gut ecological niche, which is reflected by their fermentation abilities of diet-derived, undigested complex carbohydrates (for reviews see Refs. [12,19] as well as from host-derived glycans such as mucin Refs. [14,20] and human milk oligosaccharides [17]. Furthermore, genomics coupled with transcriptome analyses have revealed the production of specific extracellular structures by bifidobacteria that mediate host-microbe interaction and support their colonization and persistence in the gut [18,21,22].

The above mentioned data indicate that even though interesting genomic results have been achieved for members of the genus *Bifidobacterium*, just a small number of studies have addressed physiological and metabolic features of *B. bifidum*. Hence, the aim of this study was to provide information on the capabilities of *B. bifidum* PRL2010 to interact with intestinal epithelial cells and to counteract adhesion of pathogenic bacteria to human cell monolayers.

2. Materials and methods

2.1. Isolation of bifidobacteria from human origin

2.1.1. Recovery of bifidobacteria from feces on selective medium

Serial dilutions of the supernatants derived from 29 fecal samples from infants were plated onto *Bifidobacterium* agar (BSM) for selective outgrowth of bifidobacteria. The BSM selective medium was prepared by the addition of 0.05% (wt/vol) L-cysteine-HCl and 50 μg mupirocin (Delchimica, Italy) per liter of MRS as described previously [23]. Agar plates were incubated in an anaerobic cabinet (Ruskin) in which the atmosphere consisted of 10% CO_2, 80% N_2, and 10% H_2 at 37 °C for 72 h. To analyze the dominant *Bifidobacterium* population of each subject, about 100–200 colonies (20–30 colonies from each plate), were randomly selected from each fecal sample, and subcultured in MRSc broth (Man–Rogosa–Sharpe (MRS) medium (Sharlau) supplemented with 0.05% L-cysteine-HCl) for 24–48 h. DNA was extracted from each isolate through rapid mechanical cell lysis as described previously [24]. PCR was used to amplify most of the 16S rRNA-encoding gene as well as the Internal Transcribed Spacer (ITS) sequences of all suspected *Bifidobacterium* isolates using previously described primers BIF-specific and 23S_bif [11].

2.1.2. Evaluation of growth capabilities of fecal Bifidobacterium isolates on mucin

All identified bifidobacterial strains from the fecal samples were assayed for their growth abilities on mucin as the sole source of carbohydrate. Cell growth curves on this substrate were monitored for 48 h by a plate reader (Biotek, Winooski, VT), which was set as described previously [14]. About 10^9 CFU/ml for each bifidobacterial strain were inoculated in basic medium [25], supplemented with 0.5% (w/v) mucin.

2.2. Probiotic potential of Bifidobacterium strains

2.2.1. Bacterial strains and growth conditions

The bifidobacterial strains used in this study are listed in Table 1, which were grown anaerobically in MRSc medium at 37 °C for 16 h. Pathogens used were *Cronobacter sakazakii* LMG570, *Salmonella enterica* serovar Tiphymurium LMG15860, *Escherichia coli* LMG2092, *Shigella sonnei* LMG10473 and *Listeria monocytogenes* LMG13305. All these strains were grown in Gifu Anaerobic Medium (GAM, Nissui Pharmaceutical, Tokyo, Japan) with 0.25% (w/v) L-cysteine-HCl under identical anaerobic conditions.

Table 1
Bacterial strains used in this study.

Strains	Ecological origin	Reference
B. bifidum PRL2010	Infant stool sample	[14]
B. animalis subsp. *lactis* BB12	Fermented milk product	[55]
B. breve 12L	Human milk	[56]
B. adolescentis 22L	Human milk	[56]
B. longum subsp. *longum* 296B	Infant stool sample	In this study
B. longum subsp. *infantis* 2256	Infant intestine	In this study

2.3. Adhesion and antibacterial properties of Bifidobacterium bifidum PRL2010

2.3.1. Adhesion to Caco-2 cells

Human colon adenocarcinoma Caco-2 cells (ATCC HTB-37) were used 15 days after confluence (to obtain fully differentiated cells). Caco-2 cells were routinely grown in 3-cm petri plates on microscopy cover glasses in Dulbecco's modified Eagle's medium (Lonza) supplemented with 10% (vol/vol) heat-inactivated (30 min at 56 °C) fetal calf serum, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, 0.1 mM non-essential amino acids and 2 mM L-glutamine, and incubated at 37 °C in a water-jacketed incubator in an atmosphere of 95% air and 5% CO_2. The culture medium was changed twice weekly. For adhesion assays, cells were used 15 days after seeding (in confluent monolayer and fully differentiated state). Cell monolayers were carefully washed twice with phosphate-buffered saline (PBS) (pH 7.3) before bacterial cells were added. For each of the six strains under study, the bacterial number of a culture grown overnight was determined microscopically. Approximately 2×10^8 cells of each strain resuspended in PBS (pH 7.3) were incubated with a monolayer of Caco-2 cells. After 1 h at 37 °C, all monolayers were washed three times with PBS to remove unbound bacteria. Cells were then fixed with 1 ml of methanol and incubated for 8 min at room temperature. After methanol was removed, cells were stained with 1.5 ml of Giemsa stain solution (1:20) (Carlo Erba, Milan, Italy) and left for 30 min at room temperature. Wells were then washed until no color was observed in the washing solution and dried in an incubator for 10 min. Microscopy cover glasses were then removed from the petri plate and examined microscopically (magnification, ×100), employing oil immersion. Adherent bacteria in 20 randomly selected microscopic fields were counted and averaged. An unpaired Student *t* test was run for statistically significant differences. To investigate the role of sugar residues in host–bacteria interaction, *B. bifidum* PRL2010 cultures were re-suspended in PBS containing 25 mM fucose or mannose (Sigma) before initiation of the adhesion experiments in the conditions previously described. Adherence capability was also tested after incubating bacterial cells at 37 °C with 3 g/l oxgall bile salts (Difco) for 1 h (bile shock, followed by a wash with PBS). All assays were run at least in triplicate.

2.4. Resistance to food and gastrointestinal challenges

2.4.1. Microplate reader experiments

Resistance to varying concentrations of bile salts (oxgall), NaCl, and pH was evaluated by monitoring Optical density (OD values) of selected bifidobacterial isolates on 96-well plates. In different wells, MRSc medium was supplemented with 0.5%, 1% or 2% (wt/vol) oxgall, or with 2%, 6% or 10% NaCl (wt/vol); strains were also cultivated in MRSc medium set by addition of HCl at pH 2.0, pH 3.0, or pH 4. Growth was monitored at OD600 using a microplate reader (Biotek) for 42 h of incubation at 37 °C under anaerobic conditions. The resistance level to the imposed bile, salt or pH stress was calculated in each case by comparing the maximum OD600 reached

in a particular medium with that of the control medium (MRSc). Assays were performed in triplicate as independent experiments.

2.4.2. Simulation of the microbial passage through the gastrointestinal tract

After 24 h of growth at 37 °C under anaerobic conditions, viable bifidobacteria were washed twice in saline solution (0.09%, wt/vol) and brought to an inoculum of 10^8–10^9 CFU/ml. In order to simulate passage through the gastrointestinal tract, an inoculum of 1% (vol/vol) bacteria was added to 10 ml of 0.5% NaCl, pH 3 solution, after which the resulting suspension was incubated at 37 °C under anaerobic conditions for 2 h. The cell suspension was then neutralized by washing with phosphate buffer at pH 7.0, and cells were then resuspended in pancreatin solution and incubated at 37 °C under anaerobic conditions for 4 h. Pancreatic solution was prepared immediately before use as follows: pancreatin from porcine pancreas (1 g/liter; Sigma–Aldrich) was resuspended in sterile saline solution (0.5%, wt/vol), and the pH was adjusted to 8.0 as described previously [2]. Samples taken at 0, 90, and 120 min in the first stage, which consists in the treatment of cells with a solution of 0.5% NaCl, pH 3, followed by a second stage where the cells were treated with pancreatin and collected at 0, 60, and 240 min. Subsequently cell viability was analyzed by plate counts. To monitor viability, the strain under investigation was inoculated in saline solution (0.09%, wt/vol) and incubated under identical conditions as the test samples; aliquots were taken at the beginning, after 120 min, and at the end of the assay. Assays were performed in triplicate and data were obtained from independent experiments.

2.4.3. Sensitivity to antibiotics

Bifidobacterial susceptibility to antibiotics was analyzed by MIC assays, which consisted of cultivating different bifidobacterial strains in the presence of various concentrations of antibiotic ranging from 0.125 to 1024 μg/ml. The MIC values of PRL2010 for seven antimicrobial agents were determined using the standardized MRS broth formulation as described above. MIC was determined according to European Food Safety Authority (EFSA) recommendations [26]. Assays were performed in triplicate, in each case employing an independent experimental set up.

2.5. In vitro antagonism against pathogen adhesion to HT-29 cells

The epithelial intestinal cell line HT-29 was maintained in McCoy's Medium (Sigma–Aldrich, USA) with antibiotics (final concentration 50 μg ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 50 μg ml^{-1} gentamicin and 1.25 μg ml^{-1} amphotericin B, Sigma). HT-29 cells (1×10^5 cells/ml) were seeded in 24-well plates and incubated to reach confluence (about 1×10^7 cells/ml, 14 ± 1 days) at 37 °C, 5% CO_2 in an SL Waterjacked CO_2 incubator (Sheldon Mfg. Inc., Cornelius, OR, USA). *B. bifidum* PRL2010 and pathogens grown in conditions as described above, were harvested by centrifugation ($950 \times g$ for 8 min), washed twice with Dulbelcco's PBS buffer (Sigma–Aldrich, USA) and resuspended in McCoy's medium (without antibiotic) to a final concentration of about 1×10^8 cells/ml. The HT-29 monolayers were washed twice with Dulbelcco's PBS buffer and then the bacterial suspension was added in a bacterial to epithelial intestinal cell ratio of 10:1. Plates were incubated at 37 °C, 5% CO_2 in a Heracell 240 incubator (Thermo Electron LDD GmbH, Langenselbold, Germany) for 2 h. After the incubation period, supernatants were removed and wells were gently washed three times with Dulbecco's PBS buffer to remove non-attached bacteria. Finally, HT-29 monolayers were treated with a 0.25% trypsin-EDTA solution (Sigma–Aldrich) and bacterial counts were carried out by deep plating in MRSc agar after incubation under anaerobic conditions for 48 h at 37 °C for *B. bifidum* PRL2010, and by surface plating on GAM agar after incubation under aerobic conditions for 48 h at 37 °C for pathogens. Results were expressed as the percentage of bacteria adhered relative to the amount of bacteria added (cfu bacteria adhered/cfu bacteria added × 100%). Experiments were independently carried out three times (each performed in duplicate to determine inter-assay variation). For the experimental designs, procedures previously described were used [27,28], to determine the inhibition of pathogen adhesion by *B. bifidum* PRL2010: strain PRL2010 was first added and after 1 h of incubation, the HT-29 monolayer was carefully washed, followed by the addition of 10^9 cfu of a particular pathogen, after which the mixture was incubated for 1 h. When the displacement of a particular pathogen by *B. bifidum* PRL2010 was investigated, the same procedure as indicated above for pathogen inhibition was used except that the pathogen was added first, followed by the addition of *B. bifidum* PRL2010 cells. When we wanted to investigate competition between a pathogen and *B. bifidum* PRL2010 for adherence to human intestinal cells, we added bifidobacteria and a pathogen simultaneously to the HT-29 cells, followed by incubation for 2 h.

3. Results and discussion

3.1. Isolation, identification and characterization of bifidobacteria from infant feces

A bifidobacterial survey of 29 fecal samples from healthy infants was performed using a polyphasic approach consisting of culture dependent and molecular-approaches. The bifidobacterial population of the collected fecal samples was assayed using mupirocin-based medium (BSM), previously shown to be highly selective for bifidobacteria [11,20]. Each bifidobacterial isolate, from a total collection of 202 isolates, was taxonomically characterized by sequencing PCR products corresponding to the 16S rRNA and Internal Transcribed Spacer (ITS) sequences as previously described [11]. A phylogenetic analysis based on the 16S rRNA gene sequences of the type strains plus other strains of each recognized species of the genus *Bifidobacterium* was performed. The results of this analysis were shown to be consistent with a previously described bifidobacterial taxonomic analysis, *Bifidobacterium longum* being the most abundant species [11]. Each bifidobacterial isolate was assayed for its growth abilities on a medium containing mucin, therefore simulating the human gut environment (Fig. 1). Notably, a large variability of growth on mucin-based medium was evident among bifidobacterial species, with best growth performance among isolates belonging to the *B. bifidum* species, confirming previously published data [14,29,30]. Notably, among the 16 *B. bifidum* isolates investigated, the *B. bifidum* PRL2010 strain, which was isolated from a three-month old healthy infant, showed the most vigorous ability to grow on mucin-based medium (Fig. 1), thus prompting us to further investigate the behavior of this strain in order to obtain a better understanding into its molecular adaptation to utilize this host-glycan product [14].

3.2. Evaluation of B. bifidum PRL2010 to human gut challenges

B. bifidum PRL2010 displays *in vitro* adhesive properties that may reflect its natural adaptation to the human gut. In order to further validate these findings, other features that would support adaptation of this microorganism to the human gut, such as resistance to acid and osmotic stresses, were investigated. Comparative analyses aimed at highlighting the ability of PRL2010 cells to cope with the above mentioned stressful conditions were carried out by including other bacteria such as the well characterized probiotic microorganism *Bifidobacterium animalis* subsp. *lactis* BB12 [31,32], as well

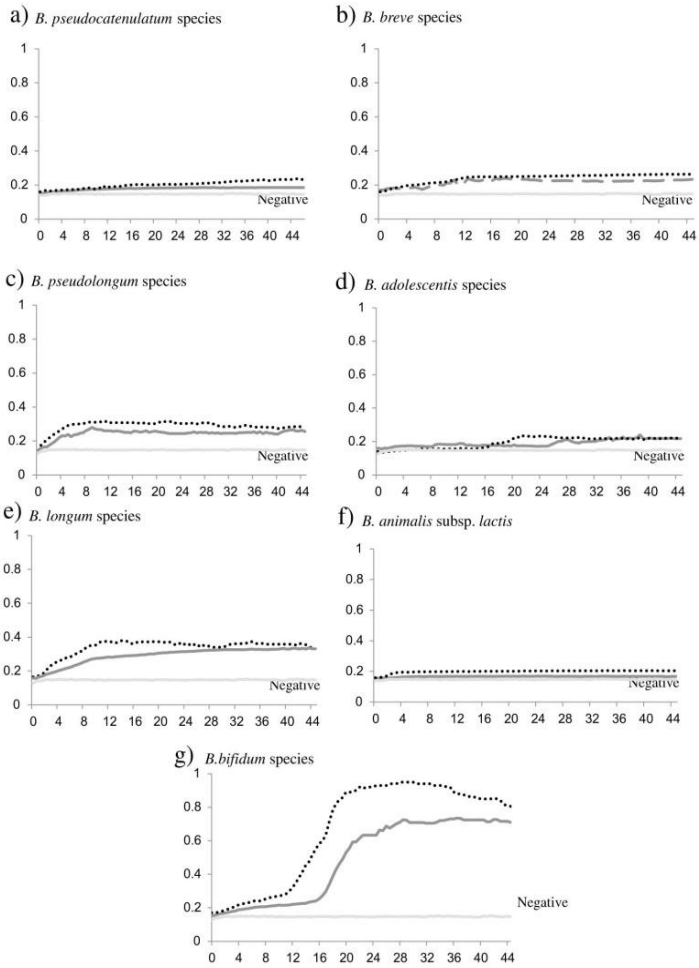

Fig. 1. Mucin-metabolizing capabilities of bifidobacterial isolates belonging to *B. bifidum*, *B. breve*, *B. pseudocatenulatum*, *B. longum*, *B. pseudolongum*, *B. animalis* subsp. *lactis* and *B. adolescentis* species. Each panel shows obtained growth curves (optical density [OD] versus time [in h]) of fecal bifidobacterial isolates on mucin. The curves representing the minimal (continuous line) and the maximal (dotted line) growth detected for each species are indicated. In the case of *B. bifidum* species (panel g) the maximal growth matches with that observed for *B. bifidum* PRL2010. "Negative" represents the OD values detected in the basal medium without inoculum.

as other enteric bifidobacterial strains previously isolated in our laboratory (Table 1).

The assay for measuring resistance to gastrointestinal juices simulates the conditions to which bacteria are exposed during their passage through the gastrointestinal tract (GIT). *In vitro* estimation of resistance of PRL2010 strain to the harsh environmental conditions that resemble the gastrointestinal passage was determined as the number of viable cells at each stage of the assay and the percentage of viable cells with respect to the viability level at the beginning of the experiment. Viability of PRL2010 upon treatment with pancreatin was 71.7% relative to untreated PRL2010 cells, which is slightly less than that noticed for BB12 strain, but considerably higher than those revealed for the other strains tested (Fig. 2).

Exposure to acid conditions revealed that strain PRL2010 exhibited the best survival rate compared to other bifidobacteria investigated (Table 2). Survival rate of PRL2010 was furthermore evaluated following exposure to biliary salts, i.e. presence of oxgall, or to osmotic stress. As displayed in Table 2, strain PRL2010 was shown to exhibit considerable resistance to oxgall, even though strain BB12 appeared to be slightly more resistant. When resistance to varying amounts of NaCl was assayed, both strains exhibited an equally high tolerance, i.e. above 2% (wt/vol) in the medium (Table 2).

As recently indicated by the European Food Safety Authority (EFSA), a key feature of a potential probiotic microorganism is represented by its susceptibility to antibiotics [26]. Thus, we analyzed the Minimum Inhibitory Concentrations (MIC) exhibited

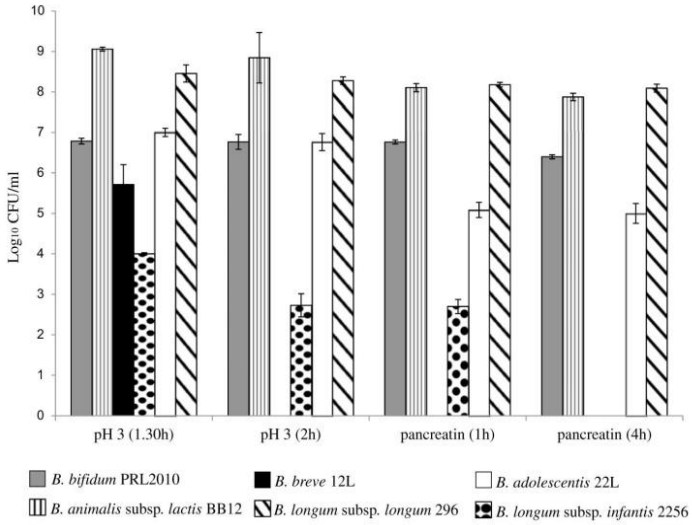

Fig. 2. Effect of the conditions that simulate the gastrointestinal tract on the survival rate of different strains tested. Each column represents the survival rate of bacterial cells after acidic treatment at pH 3 for 1 h 30 min or 2 h; and upon pancreatin treatment for 2 or 4 h. The data represents the means of at least three independent experiments conducted in duplicate. The vertical bars indicate standard deviations.

by PRL2010 to seven antibiotics, which belong to the list of antimicrobial compounds indicated by EFSA [26]. As shown in Table 3, PRL2010 strain does not show MIC values higher than those indicated by EFSA as critical values except for an atypically high level of resistance to streptomycin. However, a high level of resistance to this antibiotic has been noticed for several other bifidobacterial strains, including the probiotic BB12 strain [33]. Notably, the genetic basis of resistance to streptomycin was found to be a chromosomal mutation and thus unlikely to be transferred to other bacteria [33]. Furthermore, when MIC assays were performed for the other bifidobacterial strains, we obtained a varying range of susceptibility to antibiotics tested (Table 3).

3.3. Adhesive features of B. bifidum PRL2010 to epithelial intestinal cell lines

Adhesion to intestinal cells is considered to be an important trait for adaptation to the human gut environment as well as for potential health-promoting bacteria that are isolated from the autochthonous members of the human gut microbiota [34,35]. The ability of human gut commensals and probiotic bacteria to adhere to intestinal mucosa is recognized to be a crucial property in order to sustain host-microbe interactions, and this may thus play a pivotal role in intestinal homeostasis [36,37]. In order to investigate the adhesive abilities of *B. bifidum* PRL2010 to human intestinal mucosa, we exposed Caco-2 layers to cells of strain PRL2010 according to the protocol previously set up by Guglielmetti et al. [37]. After extensive washing with PBS, the proportion of bacterial cells that remained attached to the Caco-2 monolayer was determined in order to analyze the extent of specific host-microbe interaction. The human intestinal cell lines used here display typical epithelial enterocyte differentiation and are frequently applied as validated human intestinal cell models [38,39]. Adhesion capabilities of PRL2010 cells were measured and shown to be higher compared to those observed for other bifidobacteria including the *B. animalis* subsp. *lactis* BB12, which is widely used as a probiotic strain (Fig. 3A). The adhesion index represents a measure of the number of bacterial cells adhering to 100 Caco-2 cells [37], which in the case of *B. bifidum* PRL2010 cells was determined to be 15,871 ($P < 0.01$), which, according to previously published data, represents a high level of adhesion [37]. In contrast, a much lower adhesion index of 583 ($P < 0.01$) was determined for BB12 cells. These results were confirmed with the cell line HT-29, which in a monolayer state is known to differentiate to absorptive epithelial cells and Goblet

Table 2
Resistance of different bifidobacterial strains to exposure to bile salts, NaCl and to acidic conditions.

Strains	% Survival[a]								
	pH2	pH3	pH4	0.5% ox-gall	1% ox-gall	2% ox-gall	2% NaCl	6% NaCl	10% NaCl
B. bifidum PRL2010	15.8 ± 1.31	16.18 ± 1.82	16.3 ± 1.13	36.16 ± 12.48	32.37 ± 3.69	29.94 ± 6.76	62.67 ± 8.24	15.85 ± 1.11	14.53 ± 1.49
B. animalis subsp. *lactis* BB12	9.99 ± 0.30	10.85 ± 0.30	20.59 ± 1.61	47.08 ± 2.02	54.21 ± 3.55	79.73 ± 2.30	66.88 ± 3.82	26.93 ± 1.40	23.90 ± 8.03
B. breve 12L	14.87 ± 0.50	15.27 ± 0.21	16.26 ± 0.15	42.58 ± 5.56	58.57 ± 2.51	52.02 ± 4.03	59.85 ± 3.06	15.75 ± 0.44	13.21 ± 1.45
B. adolescentis 22L	10.17 ± 0.78	12.26 ± 1.64	13.00 ± 0.16	30.94 ± 5.77	26.25 ± 4.63	17.84 ± 0.73	87.07 ± 5.26	25.07 ± 0.15	11.71 ± 0.8
B. longum subsp. *infantis* 2256	10.85 ± 0.61	14.37 ± 0.38	21.87 ± 1.37	45.19 ± 6.35	44.00 ± 3.06	29.47 ± 1.12	26.64 ± 0.86	15.86 ± 1.01	14.80 ± 2.85
B. longum subsp. *longum* 296	15.53 ± 1.45	17.43 ± 1.70	26.13 ± 3.25	70.43 ± 7.71	51.29 ± 19.05	34.87 ± 2.37	50.97 ± 9.72	10.07 ± 0.28	9.80 ± 1.27

[a] %Survival was calculated in each case by comparing the maximum OD600 in the supplemented medium with that of the control medium (MRSc).

Table 3
Sensitivities to antibiotics of different bifidobacterial strains.

Antibiotic	MIC (μg/ml)					
	B.bifidum PRL2010	*B. animalis* subsp. *lactis* BB12	*B. breve* 12L	*B. adolescentis* 22L	*B. longum* subsp. *longum* 296	*B. longum* subsp. *infantis* 2256
Ampicillin	0.25	0.5	2	0.25	0.5	0.25
Chloramphenicol	1	1	2	2	4	2
Erytromycin	0.125	0.125	0.125	0.125	256	0.125
Kanamicyn	>1024	>1024	>1024	1024	>1024	128
Rifampicin	0.25	2	256	2	8	8
Streptomycin	>1024	1024	256	256	512	>1024
Tetracycline	4	32	128	8	64	1

The antibiotics were tested over a concentration range of 0.125–1024 μg/ml.

mucus-producing cells [40]. The adhesion level of *B. bifidum* PRL2010 to HT-29 was 11.5 ± 4.4%, significantly (one-way ANOVA test, $p < 0.001$) higher than that of BB12 (0.7 ± 0.4%) [41]. Therefore, the results obtained with both cell lines suggest a good transient persistence of strain PRL2010 in the gut environment. We performed adhesion assays to Caco-2 under different conditions and the obtained results were expressed as a percentage bacterial adhesion relative to PRL2010 grown in cMRS containing glucose (standard conditions) (Fig. 3B). Bifidobacterial cells, grown under standard conditions, were re-suspended in PBS containing 25 mM fucose or mannose prior to the adhesion test. Testing these two monosaccharides, which are reported to be the most abundant sugars present in Caco-2 glycoproteins [42], revealed that the adhesion efficiency increased by about 25% when PRL2010 cells were incubated with fucose as previously demonstrated for other *B. bifidum* strains [37]. In contrast, a marked decrease (~50%) in adhesion of PRL2010 cells was noticed when mannose was used, which suggests that mannose represents (part of) the specific ligand for PRL2010 cells. This carbohydrate is typically found in the human gut and as previously noticed for other enteric bacteria constitutes the microbial receptor for adhesion to the intestinal mucosa [43]. Further evaluation of the possible effects of other typically human gut compounds such as bile salts on the strength of PRL2010 adhesion to Caco-2 epithelial monolayer was carried out. Notably, the pre-treatment of PRL2010 cells with Oxgall caused a considerably reduction of adhesion efficacy by about 75%. Since

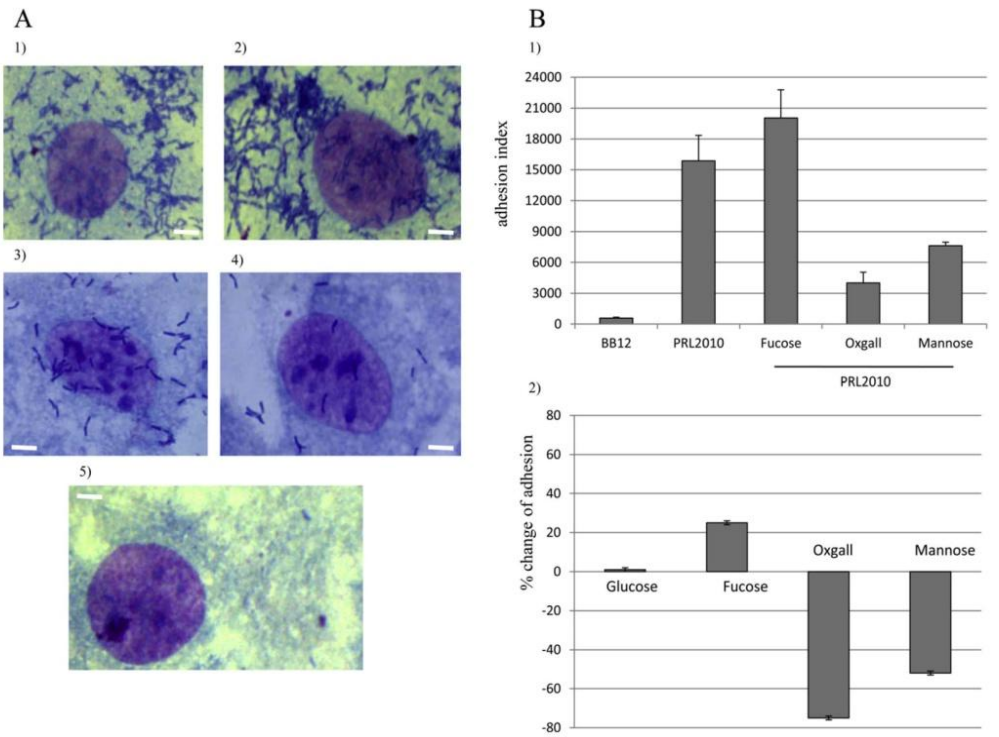

Fig. 3. Adherence of bacterial strains to Caco-2 cell monolayers. Panel A, displays the light microscopic images of Caco-2 cell monolayers as observed with Giemsa staining of *B. bifidum* PRL2010 cells grown under standard conditions (1); *B. bifidum* PRL2010 cells treated with fucose (2); *B. bifidum* PRL2010 cells treated with mannose (3); *B. bifidum* PRL2010 cells treated with oxgall (4); *B. animalis* subsp. *lactis* BB12 cells grown under standard conditions (5). Scale bar 5 μm. Panel B depicts the quantification of adhesion ability of *B. bifidum* PRL2010 cells and *B. animalis* subsp. *lactis* BB12 cells under the various conditions tested, expressed as the adhesion index (1), as well as the change in adhesion upon treatment with fucose, mannose, oxgall with respect to the standard (glucose) condition (2). The data represent the means of at least three independent experiments conducted in duplicate. The vertical bars indicate standard deviations.

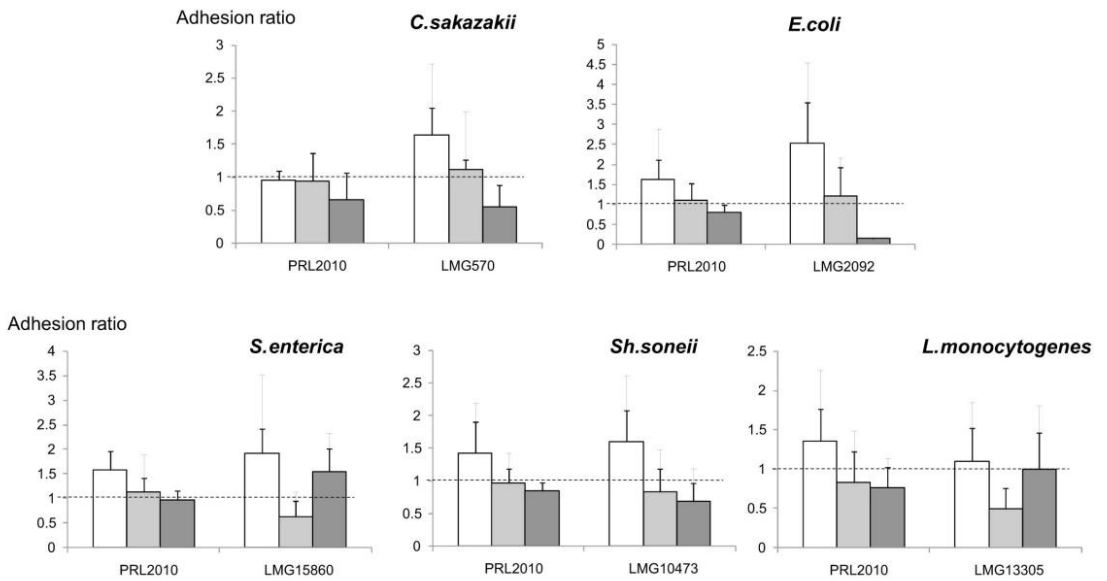

Fig. 4. Capability of *Bifidobacterium bifidum* PRL2010 to compete (white bars), to displace (light-grey bars) or to inhibit (dark-grey bars) the adhesion of different pathogens to the epithelial intestinal cell line HT-29. The adhesion ratio was calculated as the percentage of adhesion of the probiotic or the pathogen added in combination divided by the percentage of adhesion of the probiotic or the pathogen added alone. The dotted line (ratio = 1) represents the adhesion value to HT-29 of each strain added alone. The data represents the means of at least three independent experiments conducted in duplicate. The vertical bars indicate standard deviations.

strain PRL2010 displays a considerable resistance to oxgall after 42 h of incubation (Table 2), this reduced adhesion to Caco-2 cells after exposure to bile for 1 h is unlikely to be a consequence of bacterial lysis following bile treatment.

These data indicate that the PRL2010 molecule promoting the adhesion to Caco-2 cells is an extracellular protein, whose integrity and functionality is dramatically reduced upon bile salt treatment, in a fashion similar to what has been described previously [44].

3.4. Antagonist capability of B. bifidum PRL2010 against pathogen adhesion to HT-29 cell line

Another desirable property exhibited by beneficial human gut commensals is an ability to prevent/reduce adhesion of pathogenic bacteria to intestinal epithelial cells [45]. In order to explore the potential antagonistic properties of *B. bifidum* PRL2010 against pathogenic bacteria we used an *in vitro* model based on human epithelial intestinal HT-29 cells. A set of pathogens wasere employed in this study, including *Cronobacter sakazakii* LMG570, *S. enterica* serovar Typhimurium LMG15860, *E. coli* LMG2092, *S. sonnei* LMG10473 and *L. monocytogenes* LMG13305, which are responsible of severe gastrointestinal diseases such as dysentery, bacteraemia and necrotizing enterocolitis [46–48], and acute infectious diarrhea [49].

Notably, of the five pathogens tested *C. sakazakii* and *E. coli* cells were shown to be inhibited by *B. bifidum* PRL2010 in their ability adhere to an HT-29 monolayer (Fig. 4). We used this human cell monolayer since it was previously employed for similar trials [50]. Probably, the initial adhesion of the bifidobacteria avoided the attachment of these pathogen strains to similar eukaryotic cell receptors. Under the conditions tested no evidence for displacement and/or competition was noticed for most pathogen strains tested, although *L. monocytogenes* appeared to be displaced by PRL2010 (Fig. 4). In this case, the release of HT-29-bound *Listeria* induced by the *B. bifidum* strain may be due to the production of some antimicrobial substance by the potential probiotic. *C. sakazakii* is the etiological agent of necrotizing enterocolitis, which is one of the main causes of death in preterm infants, as well as the cause of bloodstream and central nervous system infections in infants [46]. *L. monocytogenes* is the etiological agent of listeriosis acquired through the ingestion of contaminated foods, such as unpasteurized fresh cheeses; this bacterial infection is a potential cause of mortality in newborns, elderly and immuno-compromised patients, while it also represents a major risk factor for pregnant woman in transmitting the infection to the fetus [10].

Altogether these results suggest that PRL2010 is effective as an antagonistic microorganism under *in vitro* conditions, but only when an HT-29 monolayer is first colonized with PRL2010 cells and then exposed to pathogens. This indicates that PRL2010 cells stably occupy a sufficient number of adhesion sites on the surface of enterocytes to cause adhesion reduction for certain pathogens. In contrast, when PRL2010 and pathogens were co-cultivated simultaneously on an HT-29 monolayer, no appreciable antagonistic effects of PRL2010 cells were noticed, which can be explained by the reduced ecological fitness of bifidobacterial cells compared to pathogens, at least under the conditions tested (in terms of cell growth rate or/and resistance to stressful conditions). A similar scenario has previously been described for other probiotic bacteria such as *Lactobacillus casei* Shirota and *Lactobacillus rhamnosus* GG against enteropathogens [51,52].

4. Conclusions

The data presented here show that *B. bifidum* PRL2010 possesses several key features sustaining its adaptation to the ecological niche of the human gut, while also showing potential as a probiotic

microorganism. The capabilities of enteric commensals to adhere to enterocytes are considered a critical property for such bacteria [53]. The strong adhesive behavior displayed by PRL2010 cells may thus reflect its ecological potential within the highly competitive environment of the human gut. In fact, the capabilities of an enteric bacterium to adhere to the intestinal mucosa is consequently linked to its abilities to stably colonize and persist in the intestine, and therefore contributing to its abilities to prevent the establishment of enteric pathogens. Previous studies have described how this strain can be used as a prototypical bacterium being representative of the *Bifidobacterium* genus for the utilization of host-derived glycans like those found in human mucus [14,20]. Genome analysis of *B. bifidum* PRL2010 revealed interesting metabolic traits involved in mucin utilization, underpinning a key example of host-microbe co-evolution [14,20]. The data presented here reinforce the notion of PRL2010 being an autochthonous member of the human intestinal microbiota. We found that adhesion decreased considerably when PRL2010 cells were placed in a medium containing mannose. This sugar represents the most abundant carbohydrate fraction present on the surface of enterocytes [42], and we speculate that this sugar acts as (part of) a receptor for PRL2010 adhesion. Previous studies have shown how bifidobacteria are decorated at their cell surfaces by the presence of extracellular structures resembling sortase-dependent and type-IV pili [18,22], which are believed to be critical players for adhesion and thus colonization.

The presence of different pili suggests that PRL2010 may recognize different receptors in the intestinal tract, including molecules expressed by epithelial cells. Molecular players which may mediate such a specific interaction between bacteria and enterocytes are represented by surface lipoproteins such as BopA, which in *B. bifidum* MIMBb75 has been shown to promote adhesion to Caco-2 cells [37]. In a similar fashion to what was found in other bifidobacterial strains [54], molecules such as lipoteichoic acids can also support the adhesion of *B. bifidum* PRL2010 to enterocytes, although no evident displacement and competition by *B. bifidum* PRL2010 was demonstrated against any of the pathogens tested. Although the mechanism of pathogen displacement is not known, a higher affinity of a *Bifidobacterium* strain for the receptors present in the mucus may explain such a phenomenon. In agreement with previous reports [28,54] no direct correlation was found between the overall adhesion level of a given *Bifidobacterium* strain and its ability to inhibit binding to or displace pathogens. Further studies will be needed in order to elucidate the precise molecular mechanisms responsible for the observed strong adhesion phenotype of *B. bifidum* PRL2010.

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References

[1] Marco ML, Pavan S, Kleerebezem M. Towards understanding molecular modes of probiotic action. Curr Opin Biotechnol 2006;17:204–10.
[2] Chenoll E, Casinos B, Bataller E, Astals P, Echevarria J, Iglesias JR, et al. Novel probiotic Bifidobacterium bifidum CECT 7366 strain active against the pathogenic bacterium Helicobacter pylori. Appl Environ Microbiol 2011;77:1335–43.
[3] Shirasawa Y, Shibahara-Sone H, Iino T, Ishikawa F. Bifidobacterium bifidum BF-1 suppresses Helicobacter pylori-induced genes in human epithelial cells. J Dairy Sci 2010;93:4526–34.
[4] Khailova L, Mount Patrick SK, Arganbright KM, Halpern MD, Kinouchi T, Dvorak B. Bifidobacterium bifidum reduces apoptosis in the intestinal epithelium in necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 2010;299:G1118–27.
[5] Philippe D, Heupel E, Blum-Sperisen S, Riedel CU. Treatment with Bifidobacterium bifidum 17 partially protects mice from Th1-driven inflammation in a chemically induced model of colitis. Int J Food Microbiol 2011;149:45–9.
[6] Fu YR, Yi ZJ, Pei JL, Guan S. Effects of Bifidobacterium bifidum on adaptive immune senescence in aging mice. Microbiol Immunol 2010;54:578–83.
[7] Guglielmetti S, Mora D, Gschwender M, Popp K. Randomised clinical trial: Bifidobacterium bifidum MIMBb75 significantly alleviates irritable bowel syndrome and improves quality of life–a double-blind, placebo-controlled study. Aliment Pharmacol Ther 2011;33:1123–32.
[8] Malago JJ, Tooten PC, Koninkx JF. Anti-inflammatory properties of probiotic bacteria on Salmonella-induced IL-8 synthesis in enterocyte-like Caco-2 cells. Benef Microbe 2010;1:121–30.
[9] Mouni F, Aissi E, Hernandez J, Gorocica P, Bouquelet S, Zenteno E, et al. Effect of Bifidobacterium bifidum DSM 20082 cytoplasmic fraction on human immune cells. Immunol Invest 2009;38:104–15.
[10] Tabbers MM, de Milliano I, Roseboom MG, Benninga MA. Is Bifidobacterium breve effective in the treatment of childhood constipation? Results from a pilot study. Nutr J 2011;10:19.
[11] Turroni F, Foroni E, Pizzetti P, Giubellini V, Ribbera A, Merusi P, et al. Exploring the diversity of the bifidobacterial population in the human intestinal tract. Appl Environ Microbiol 2009;75:1534–45.
[12] Turroni F, Strati F, Foroni E, Serafini F, Duranti S, van Sinderen D, et al. Analysis of predicted carbohydrate transport systems encoded by Bifidobacterium bifidum PRL2010. Appl Environ Microbiol 2012.
[13] Ventura M, O'Flaherty S, Claesson MJ, Turroni F, Klaenhammer TR, van Sinderen D, et al. Genome-scale analyses of health-promoting bacteria: probiogenomics. Nat Rev Microbiol 2009;7:61–71.
[14] Turroni F, Bottacini F, Foroni E, Mulder I, Kim JH, Zomer A, et al. Genome analysis of Bifidobacterium bifidum PRL2010 reveals metabolic pathways for host-derived glycan foraging. Proc Natl Acad Sci U S A 2010;107:19514–9.
[15] Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, et al. The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci U S A 2002;99:14422–7.
[16] Lee JH, Karamychev VN, Kozyavkin SA, Mills D, Pavlov AR, Pavlova NV, et al. Comparative genomic analysis of the gut bacterium Bifidobacterium longum reveals loci susceptible to deletion during pure culture growth. BMC Genomics 2008;9:247.
[17] Sela DA, Chapman J, Adeuya A, Kim JH, Chen F, Whitehead TR, et al. The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome. Proc Natl Acad Sci U S A 2008;105:18964–9.
[18] O'Connell Motherway M, Zomer A, Leahy SC, Reunanen J, Bottacini F, Claesson MJ, et al. Functional genome analysis of Bifidobacterium breve UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. Proc Natl Acad Sci U S A 2011;108:11217–22.
[19] Pokusaeva K, Fitzgerald GF, van Sinderen D. Carbohydrate metabolism in Bifidobacteria. Genes Nutr 2011;6:285–306.
[20] Turroni F, Foroni E, Montanini B, Viappiani A, Strati F, Duranti S, et al. Global genome transcription profiling of Bifidobacterium bifidum PRL2010 under in vitro conditions and identification of reference genes for quantitative Real-Time PCR. Appl Environ Microbiol 2011.
[21] Fanning S, Hall LJ, Cronin M, Zomer A, MacSharry J, Goulding D, et al. Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. Proc Natl Acad Sci U S A 2012;109:2108–13.
[22] Foroni E, Serafini F, Amidani D, Turroni F, He F, Bottacini F, et al. Genetic analysis and morphological identification of pilus-like structures in members of the genus Bifidobacterium. Microb Cell Fact 2011;10(Suppl. 1):S16.
[23] Simpson PJ, Fitzgerald GF, Stanton C, Ross RP. The evaluation of a mupirocin-based selective medium for the enumeration of bifidobacteria from probiotic animal feed. J Microbiol Methods 2004;57:9–16.
[24] Ventura M, Elli M, Reniero R, Zink R. Molecular microbial analysis of Bifidobacterium isolates from different environments by the species-specific amplified ribosomal DNA restriction analysis (ARDRA). FEMS Microbiol Ecol 2001;36:113–21.
[25] Ryan SM, Fitzgerald GF, van Sinderen D. Screening for and identification of starch-, amylopectin-, and pullulan-degrading activities in bifidobacterial strains. Appl Environ Microbiol 2006;72:5289–96.
[26] Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. EFSA J 2008;732:1–15.
[27] Rinkinen M, Westermarck E, Salminen S, Ouwehand AC. Absence of host specificity for in vitro adhesion of probiotic lactic acid bacteria to intestinal mucus. Vet Microbiol 2003;97:55–61.
[28] Collado MC, Gueimonde M, Hernandez M, Sanz Y, Salminen S. Adhesion of selected Bifidobacterium strains to human intestinal mucus and the role of adhesion in enteropathogen exclusion. J Food Prot 2005;68:2672–8.

[29] Ruas-Madiedo P, Gueimonde M, Fernandez-Garcia M, de los Reyes-Gavilan CG, Margolles A. Mucin degradation by Bifidobacterium strains isolated from the human intestinal microbiota. Appl Environ Microbiol 2008;74:1936–40.
[30] Ruiz L, Gueimonde M, Coute Y, Salminen S, Sanchez JC, de los Reyes-Gavilan CG, et al. Evaluation of the ability of Bifidobacterium longum to metabolize human intestinal mucus. FEMS Microbiol Lett 2011;314:125–30.
[31] Moussavi M, Adams MC. An in vitro study on bacterial growth interactions and intestinal epithelial cell adhesion characteristics of probiotic combinations. Curr Microbiol 2010;60:327–35.
[32] Saxelin M, Lassig A, Karjalainen H, Tynkkynen S, Surakka A, Vapaatalo H, et al. Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. Int J Food Microbiol 2010;144:293–300.
[33] Kiwaki M, Sato T. Antimicrobial susceptibility of Bifidobacterium breve strains and genetic analysis of streptomycin resistance of probiotic B. breve strain Yakult. Int J Food Microbiol 2009;134:211–5.
[34] Sonnenburg JL, Angenent LT, Gordon JI. Getting a grip on things: how do communities of bacterial symbionts become established in our intestine? Nat Immunol 2004;5:569–73.
[35] Ouwehand AC, Tuomola EM, Tolkko S, Salminen S. Assessment of adhesion properties of novel probiotic strains to human intestinal mucus. Int J Food Microbiol 2001;64:119–26.
[36] Rautava S, Walker WA. Commensal bacteria and epithelial cross talk in the developing intestine. Curr Gastroenterol Rep 2007;9:385–92.
[37] Guglielmetti S, Tamagnini I, Mora D, Minuzzo M, Scarafoni A, Arioli S, et al. Implication of an outer surface lipoprotein in adhesion of Bifidobacterium bifidum to Caco-2 cells. Appl Environ Microbiol 2008;74:4695–702.
[38] Greene JD, Klaenhammer TR. Factors involved in adherence of lactobacilli to human Caco-2 cells. Appl Environ Microbiol 1994;60:4487–94.
[39] Vizoso Pinto MG, Schuster T, Briviba K, Watzl B, Holzapfel WH, Franz CM. Adhesive and chemokine stimulatory properties of potentially probiotic Lactobacillus strains. J Food Prot 2007;70:125–34.
[40] Hilgendorf C, Spahn-Langguth H, Regardh CG, Lipka E, Amidon GL, Langguth P. Caco-2 versus Caco-2/HT29-MTX co-cultured cell lines: permeabilities via diffusion, inside- and outside-directed carrier-mediated transport. J Pharm Sci 2000;89:63–75.
[41] López PMD, Gueimonde M, de los Reyes-Gavilán CG, Margolles A, Suárez A, Ruas-Madiedo P. Exopolysaccharide-producing Bifidobacterium strains elicit different in vitro responses upon interaction with human cells. Food Res Int 2012;46:99–107.
[42] Youakim A, Herscovics A. Cell surface glycopeptides from human intestinal epithelial cell lines derived from normal colon and colon adenocarcinomas. Cancer Res 1985;45:5505–11.
[43] Farfan MJ, Cantero L, Vidal R, Botkin DJ, Torres AG. Long polar fimbriae of enterohemorrhagic *Escherichia coli* O157:H7 bind to extracellular matrix proteins. Infect Immun 2011;79:3744–50.
[44] Guglielmetti S, Tamagnini I, Minuzzo M, Arioli S, Parini C, Comelli E, et al. Study of the adhesion of Bifidobacterium bifidum MIMBb75 to human intestinal cell lines. Curr Microbiol 2009;59:167–72.
[45] Freter M. Factors affecting the microecology of the gut 1992. Glasgow, UK.
[46] Healy B, Cooney S, O'Brien S, Iversen C, Whyte P, Nally J, et al. Cronobacter (Enterobacter sakazakii): an opportunistic foodborne pathogen. Foodborne Pathog Dis 2010;7:339–50.
[47] Haraga A, Ohlson MB, Miller SI. Salmonellae interplay with host cells. Nat Rev Microbiol 2008;6:53–66.
[48] Thielman NM, Guerrant RL. Clinical practice. Acute infectious diarrhea. N Engl J Med 2004;350:38–47.
[49] Ina K, Kusugami K, Ohta M. Bacterial hemorrhagic enterocolitis. J Gastroenterol 2003;38:111–20.
[50] Dhanani AS, Bagchi T. Lactobacillus plantarum CS24.2 prevents *E. coli* adhesion to HT-29 cells and also down-regulates enteropathogen induced TNF-alpha and IL-8 expression. Microbiol Immunol 2013.
[51] Lee YK, Puong KY, Ouwehand AC, Salminen S. Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. J Med Microbiol 2003;52:925–30.
[52] Bujnakova D, Kmet V. Functional properties of Lactobacillus strains isolated from dairy products. Folia Microbiol (Praha) 2012.
[53] Izquierdo E, Medina M, Ennahar S, Marchioni E, Sanz Y. Resistance to simulated gastrointestinal conditions and adhesion to mucus as probiotic criteria for *Bifidobacterium longum* strains. Curr Microbiol 2008;56:613–8.
[54] Bibiloni R, Fernando P, De Antoni GL. Will a high adhering capacity in a probiotic strain guarantee exclusion of pathogens from intestinal epithelia. Anaerobe 1999;5:519–24.
[55] Meile L, Ludwig W, Rueger U, Gut C, Kaufmann P, Dasen G, et al. *Bifidobacterium lactis* sp. nov, a moderately oxygen tolerant species isolated from fermented milk. Syst Appl Microbiol 1997;20:57–64.
[56] Turroni F, Foroni E, Serafini F, Viappiani A, Montanini B, Bottacini F, et al. Ability of *Bifidobacterium breve* to grow on different types of milk: exploring the metabolism of milk through genome analysis. Appl Environ Microbiol 2011;77:7408–17.

5. An efficient and reproducible method for transformation of genetically recalcitrant bifidobacteria

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RESEARCH LETTER

An efficient and reproducible method for transformation of genetically recalcitrant bifidobacteria

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bifidobacteria; genomics; transformation; genetic manipulation.

Abstract

This study describes an efficient transformation system for the introduction of plasmid DNA into *Bifidobacterium bifidum* PRL2010 and *Bifidobacterium asteroides* PRL2011, for which to the best of our knowledge no transformation data have been reported previously. The method is based on electroporation of bifidobacterial cells, which were made competent by an optimized methodology based on varying media and growth conditions. Furthermore, the transformation protocol was applied in order to design a PRL2010-derivative, which carries antibiotic resistance against chloramphenicol and which was used to monitor PRL2010 colonization in a murine model.

Introduction

Bifidobacteria are Gram-positive G+C%-rich, anaerobic/microaerophilic, fermentative bacteria, which are often Y- or V-shaped (Ventura *et al.*, 2007). *Bifidobacterium* represents one of the most numerically abundant bacterial genera of the human gut microbiota in infants and is presumed to play a fundamental role in host health, which drives their wide-spread use as probiotic bacteria in many functional foods. This commercial exploitation of probiotic bifidobacterial strains has fuelled scientific interest in these bacteria to identify the genomic traits that are responsible for the claimed beneficial activities.

To exploit the full potential of these microorganisms for applications as probiotic ingredients, further knowledge is required on their molecular biology and genetics. However, molecular studies of *Bifidobacterium* are severely hampered by the absence of effective genetic tools, including efficient transformation protocols. So far, several *Bifidobacterium* strains, including members of *Bifidobacterium bifidum* and *Bifidobacterium asteroides*, have been shown to be non-transformable or very poorly transformable (Argnani *et al.*, 1996). Many factors may contribute to bifidobacterial recalcitrance for acquiring exogenous DNA, such as the presence of a thick (multilayered) and complex cell wall (Fischer *et al.*, 1987), intracellular restriction/modification barriers (Hartke *et al.*, 1996; Schell *et al.*, 2002; O'Connell Motherway *et al.*, 2009), and sensitivity to environmental stresses, in particular oxygen, to which these strictly anaerobic bacteria are exposed to during the preparation of competent cells and transformation procedure.

With the advent of the genomics era, many bifidobacterial genomes have been fully decoded (for reviews, see Turroni *et al.*, 2011; Ventura *et al.*, 2009), which has thus

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provided a huge amount of genetic data that can be exploited to study genome functionality. Such studies are needed to understand the molecular mechanisms sustaining the interaction of bifidobacteria with its host as well as with other members of the gut microbiota (Hartke *et al.*, 1996; Schell *et al.*, 2002; Sela *et al.*, 2008; Ventura *et al.*, 2009; Turroni *et al.*, 2011).

However, to perform such functional genomic investigations, it will be necessary to develop transformation protocols as well as to implement gene knock-out methodologies effective for bifidobacteria. In this report, we describe the development of a protocol for efficient and reproducible genetic transformation of *B. bifidum* PRL2010 by electroporation using the shuttle vector pNZ8048 (de Ruyter *et al.*, 1996). The protocol of transformation is based on the preparation of electro-competent cells and subsequent electroporation and on the optimization of several parameters such as growth conditions, washing solutions, and electroporation voltage.

Materials and methods

Bacterial strains and plasmids

The *Bifidobacterium* strains used are described in Table 1. Plasmid pNZ8048 is a broad-host shuttle vector, which possesses the nisin-inducible *nisA* promoter and a chloramphenicol resistance gene as the selection marker (de Ruyter *et al.*, 1996).

Media and growth conditions

Escherichia coli strain DH10B, used as host strain for propagating the shuttle vector, was cultivated in LB medium (Savino *et al.*, 2011) supplemented with chloramphenicol (Sigma) at a final concentration of 10 μg mL^{-1}. The susceptibility to chloramphenicol of the bifidobacterial strains PRL2010 and PRL2011 was tested by means of a Minimal Inhibitor Concentration (MIC) assay, according to a previously described procedure (Serafini *et al.*, 2011).

Bifidobacteria were cultivated in de Man–Rogosa–Sharpe (MRS) medium supplemented with 0.05% cysteine-HCl (cMRS) in an anaerobic chamber (Concept 400, Ruskin; 2.99% H_2, 17.01% CO_2 and 80% N_2) at 37 °C for 24–72 h. In case of cultivation of bifidobacterial transformants, chloramphenicol was added to the growth medium cMRS agar at a final concentration of 3 μg mL^{-1}.

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains		Reference
B. bifidum PRL2010		Turroni *et al.* (2010)
B. asteroides PRL2011		Unpublished data
Plasmids		
Code	Size (bp)	
pNZ8048	3349 bp	de Ruyter *et al.* (1996)

DNA isolation procedures

Plasmid DNA was isolated from *E. coli* as well as from bifidobacterial transformants using a Qiagen Plasmid Mini Kit. For Bifidobacteria, an additional incubation step in 20 mg mL^{-1} lysozyme at 37 °C for 40 min was performed before beginning the Qiagen kit protocol (Guglielmetti *et al.*, 2008).

Preparation of bacteria for electroporation

An overnight culture of *Bifidobacterium* (10%) was used to inoculate fresh MRS broth supplemented with 0.05% (final concentration) cysteine-HCl and 16% (v/w) fructo-oligosaccharides (FOS) (Actilight®; Beneo-Orafti), a commercial product comprising a mix of short-chain FOS (1-kestose, nystose, and fructosylnystose; FOS) or 10% galacto-oligosaccharides (GOS) (Sigma), and cultivated overnight at 37 °C under anaerobic conditions. This overnight culture was diluted 1 : 10 in fresh MRS broth supplemented with 16% FOS or 10% GOS and cultivated at 37 °C until an $OD_{600\,nm}$ of 0.6–0.7 was reached. Then, bacteria were chilled on ice, harvested by centrifugation (4500 r.p.m. for 15 min), and washed twice with washing buffer composed of 1 mM citrate buffer supplemented with 16% FOS or 10% GOS (pH 6.0). Finally, cells were resuspended in about 1/250 of the original culture volume of ice-cold washing buffer, dispensed in Eppendorf tubes and incubated at 4 °C for 30 min to 3 h.

Electroporation

Plasmid DNA (200 ng) was mixed with 80 μL bacterial suspension in a precooled Gene Pulser disposable cuvette with an interelectrode distance of 0.2 cm (Eppendorf). A high-voltage electric pulse was delivered employing a Gene Pulser apparatus (BioRad, UK) using 25 μF capacity and a parallel resistance of 200 Ω. Following electroporation, bacteria were diluted with 920 μL cMRS broth. Bacteria were incubated for 3 h at 37 °C in an anaerobic cabinet to facilitate cell recovery and expression of the antibiotic resistance marker, after which cells were plated on cMRS agar supplemented with 3 μg mL^{-1} chloramphenicol. Plates were then incubated anaerobically at 37 °C for 48–72 h.

Selection of the transformants

Transformants were cultivated on cMRS supplemented with chloramphenicol at a final concentration of 3 μg mL^{-1}.

DNA was extracted from colonies using GeneReleaser (BioVentures), and the presence of pNZ8048 in transformants was confirmed by PCR using the primers pNZFw (5′-TTTGCAGCGAAGATGTTGTC-3′) and pNZRv (5′-CTATAGCTAACGCCGCAACC-3′) targeting DNA regions on this plasmid. The transformation efficiency was calculated according to the following formula:

$$E = \frac{[\sum c \times (1 \times 10^{x})]}{(\text{DNA } \mu\text{g})}$$

where Σ c is the total number of transformants and x is the dilution factor applied.

Transformation experiments were performed in triplicate.

Plasmid stability studies

Transformants were inoculated into fresh broth in the presence of chloramphenicol and grown for 24 h. These cultures were then screened for plasmid content prior to the start of the experiment to ensure that plasmid pNZ8048 was present. Cultures were then diluted (1%) in fresh broth without chloramphenicol, followed by continuous subcultivation for 15 days by dilution into fresh broth every 24 h in the absence of antibiotic selection. To determine plasmid stability, at least 50 colonies from each tested transformant were transferred to cMRS agar plates with or without chloramphenicol (3 μg mL^{-1}). Growth of these colonies was monitored following 24 h of incubation, and plasmid extractions were performed where relevant.

Mouse colonization

All animals used in this study were cared for in compliance with guidelines established by the Italian Ministry of Health. All procedures were approved by the University of Parma, as executed by the Institutional Animal Care and Use Committee (Dipartimento per la Sanità Pubblica Veterinaria, la Nutrizione e la Sicurezza degli Alimenti Direzione Generale della Sanità Animale e del Farmaco Veterinario). Two groups, each containing six animals of 3-month-old female BALB/c mice, were orally inoculated with bacteria or with water. Bacterial colonization was established by five consecutive daily administrations whereby each animal received 20 μL of 10^9 mL^{-1} of cells using a micropipette tip placed immediately behind the incisors (Sleator *et al.*, 2001). Bifidobacterial inocula were prepared by growing *B. bifidum* PRL2010 containing pNZ8048 anaerobically overnight at 37 °C in cMRS broth containing 3 μg mL^{-1} chloramphenicol. Cultures were harvested by centrifugation (950 ***g*** for 8 min), washed, and resuspended in 100 μL of water. The viable count of each inoculum was determined by retrospective plating on cMRS containing the antibiotic. To estimate the number of *B. bifidum* PRL2010 cells per gram of feces, individual fecal samples were weighed and followed by serial dilution and culturing on selective cMRS agar with chloramphenicol. Following enumeration of *B. bifidum* PRL2010 in fecal samples, 100 random colonies were further tested to verify their identity by the use of PCR primers targeting the *pil*2 and *pil*3 loci (Foroni *et al.*, 2011).

Results and discussion

It has previously been reported that *B. bifidum* cells are practically nontransformable (Argnani *et al.*, 1996). To corroborate such findings, we employed a previously described transformation protocol for *B. bifidum* PRL 2010 (Turroni *et al.*, 2010) and *B. asteroides* PRL2011 (F. Bottacini, F. Turroni, and M. Ventura, unpublished data), which is highly effective for other bifidobacterial strains, such as *Bifidobacterium breve* UCC2003 (O'Connell Motherway *et al.*, 2009). However, as displayed in Table 2, no PRL2010 transformants were obtained using this procedure. Thus, to genetically access *B. bifidum* PRL2010 and *B. asteroides* PRL2011, for which the genome sequences are currently available (F. Bottacini, F. Turroni, and M. Ventura, unpublished data), an efficient transformation protocol is required. Accordingly, we assessed and varied various critical parameters of the bacterial transformation protocol, such as preparation of

Table 2. Effect of various parameters on *Bifidobacterium bifidum* PRL2010 electroporation rates.

Parameters			Rate of transformation (CFU per μg DNA)
Carbohydrate		Growth phase	
Modified Rogosa Medium (O'Connell Motherway *et al.*, 2009)		OD value of 0.6–0.7	0
FOS		OD value of 0.4	0
		OD value of 0.7	1.3×10^3
GOS		OD value of 0.4	0
		OD value of 0.7	3.7×10^3
Resistances		Voltages (kV cm^{-1})	
FOS	100 Ω	7.5	0
		12.5	0
GOS		7.5	0
		12.5	0
FOS	200 Ω	7.5	2.0×10^2
		12.5	1.3×10^3
GOS		7.5	4.1×10^2
		12.5	3.7×10^3

electro-competent cells, electroporation buffers, and electroporation conditions, which are discussed below. Furthermore, susceptibility to the antibiotic used to select transformants (chloramphenicol) was tested for both *B. bifidum* PRL2010 and *B. asteroides* PRL2011 using the MIC assays, which showed a resistance level below 0.5 μg mL^{-1}.

Preparation of electro-competent cells

The presence of a thick and multilayered cell wall in bacteria generally represents a barrier for the uptake of exogenous DNA molecules (Kullen & Klaenhammer, 2000). Bifidobacteria possess a very thick and complex cell wall (Fischer *et al.*, 1987). In particular, for the *B. bifidum* taxon, the peptidoglycan structure differs from that of other bifidobacteria by the existence of specific cross-linking dipeptide bond between the 5-amino group of ornithine and the carboxyl group of C-terminal D-alanine (Veerkamp & van Schaik, 1974). Thus, we attempted to adapt our methodology so as to overcome this physical barrier by varying several parameters such as (1) cultivation of bifidobacteria/transformants in the presence of high concentration of complex carbohydrates; (2) the use of bacterial cells collected at the exponentially growth phase; (3) osmotic stabilizers in washing and electroporation buffers; and (4) maintenance of cells at low temperatures during all steps of the transformation procedure.

Growth media

The addition of carbohydrates at high concentration to the growth medium is a strategy previously described to be effective for transformation of other bifidobacterial species such as *Bifidobacterium animalis, Bifidobacterium longum* subsp. *infantis,* and *Bifidobacterium longum* subsp. *longum* (Argnani *et al.*, 1996; Rossi *et al.*, 1996; Guglielmetti *et al.*, 2007, 2008). In fact, the presence of a high concentration of carbohydrates in the growth medium and in the electroporation buffer has proven to be essential, as no transformants were observed when bacteria were cultivated in the absence of an osmotic stabilizer (Argnani *et al.*, 1996). A similar strategy was followed also for the preparation of the electro-competent *B. bifidum* PRL2010 cells, which were cultivated in the presence of different complex carbohydrates such as FOS or GOS.

Interestingly, PRL2010 transformants were isolated when cells were grown in MRS supplemented with FOS at a final content of 16% as well as with MRS enriched by 10% GOS with a transformation efficiency of 10^3 CFU μg^{-1} DNA (Table 2). Such findings may be explained by the effects that these oligosaccharides have on the composition of the cell wall as well as on other cell envelope constituents (e.g. decreased thickness of capsular polysaccharide layers and/or reduction of the cell wall/capsular complexity). Furthermore, the presence of a high amount of complex carbohydrates in the growth medium may exert a protective action against the stressful conditions encountered by bifidobacterial cells during transformation (Guglielmetti *et al.*, 2008).

Growth phase

Previous studies have reported that the composition of the bacterial cell wall, and consequently the efficiency of DNA uptake, seems to be significantly influenced by the growth phase of the bacterial cells (Rossi *et al.*, 1996). Thus, based on the growth curve of *B. bifidum* PRL2010 cells cultivated on MRS, we harvested PRL2010 cells at different time points corresponding to early (OD value of 0.4) and late exponential phase (OD value of 0.7) (Fig. 1). Subsequently, such cells were submitted to the electroporation procedure, and corresponding transformation efficiency was evaluated (Table 2). Notably, the maximal transformation efficiency was observed when PRL2010 cells were collected at late log phase (Table 2).

Electroporation buffers

Incubation of the cells in an electroporation buffer was found to be crucial for *Bifidobacterium* transformation (Argnani *et al.*, 1996). We observed that storage of bacterial cells for two hours before electroporation at 4 °C in an electroporation buffer composed of 16% FOS or 10% GOS and 1 mM citrate buffer (pH 6.0) significantly improved their transformation efficiency, increasing from $< 10^2$ to 10^4 CFU per μg DNA. Under these conditions, we assume that the low molarity of ammonium citrate acts as an osmotic stabilizer that supports controlled cell envelope removal/degradation without affecting cell viability, which may then result in improved cell wall permeability for exogenous DNA.

Electroporation condition and identification of PRL2010 transformants

Resistances of 100 or 200 Ω and voltages between 7.5 and 12.5 kV cm^{-1} were tested. Optimal results were obtained when the voltage applied to the cuvette was 12.5 kV cm^{-1} and the resistance was set at 200 Ω. When the resistance was set at 100 Ω, no transformants was observed. The transformation efficiency achieved with a voltage of 7.5 kV cm^{-1} and a resistance of 200 Ω was low (Table 2). After incubation, the transformants were selected on MRS supplemented with chloramphenicol and incubated at 37 °C. The presumptive transformants were

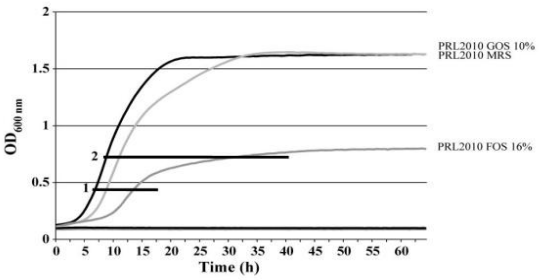

Fig. 1. Growth curves of *Bifidobacterium bifidum* PRL2010 (10%) in a growth medium containing different carbohydrates as carbon sources. Times of cell collection corresponded to early (OD value of 0.4) and late exponential phase (OD value of 0.7).

verified by colony PCR using primers based on the DNA sequence of pNZ8048. The transformation efficiency was calculated to be 1.35×10^3 CFU per μg DNA when the strain was grown in FOS, and 3.7×10^3 CFU per μg DNA when grown in GOS (Table 2).

Plasmid stability was evaluated by continuous cultivation for 15 days of five PRL2010 transformants in the absence of chloramphenicol selection by PCR assays. Notably, all PRL2010 transformants tested did not exhibit any plasmid loss during this period, despite the absence of antibiotic selection.

Transformation of *B. asteroides*

To evaluate the general usefulness of the transformation protocol developed here, we decided to apply it to another *Bifidobacterium* species, *B. asteroides* PRL2011, whose genome was recently decoded (F. Bottacini, F. Turroni and M. Ventura, unpublished data). Interestingly, the *B. asteroides* species represents a distantly related taxon with respect to *B. bifidum*, while it also occupies a different ecological niche, that is, the hindgut of honeybee (Veerkamp & van Schaik, 1974; Fischer *et al.*, 1987; Argnani *et al.*, 1996; de Ruyter *et al.*, 1996; Hartke *et al.*, 1996; Rossi *et al.*, 1996; Kullen & Klaenhammer, 2000; Sleator *et al.*, 2001; Schell *et al.*, 2002; Ventura *et al.*, 2006, 2007, 2009; Guglielmetti *et al.*, 2007, 2008; Sela *et al.*, 2008; O'Connell Motherway *et al.*, 2009; Turroni *et al.*, 2010, 2011; Foroni *et al.*, 2011; Serafini *et al.*, 2011). Thus, one may argue that the *B. asteroides* species possesses a different cell envelope composition (e.g. exopolysaccharides, extracellular proteins) compared to that of *B. bifidum*. When the transformation protocol optimized on *B. bifidum* PRL2010 cells was employed for transforming *B. asteroides* PRL2011 using pNZ8048, a higher transformation efficiency (1.6×10^4 CFU per μg DNA) was obtained as compared to *B. bifidum* PRL2010.

Bifidobacterium bifidum PRL2010 in colonization *in vivo* experiments

A direct application from the results of the successful transformation protocol described in this study was to monitor the colonization efficiency of *B. bifidum* PRL2010 in a murine model. In fact, so far, it has been proven impossible to generate stable antibiotic-resistant *B. bifidum* PRL2010 derivatives by spontaneous mutation such as those in other bacterial species might be obtained upon repeated cultivation in the presence of antibiotics. Thus, to discriminate the presence of PRL2010 cells from other members of the gut microbiota of mice, we employed a derivative PRL2010 strain that contained a plasmid carrying an antibiotic resistance gene to act as a selective marker.

The normal microbiota of mice encompasses microorganisms that are sensitive to chloramphenicol (Savino *et al.*, 2011), thus indicating that this antibiotic can be used in selective media. Colonization and clearance of PRL2010 were monitored over a 15-day period by determining viable counts recovered from fecal samples. Two groups of six mice were fed orally on a daily basis with either PRL2010 containing pNZ8048 (designated here as $PRL2010_{pNZ8048}$) or water for 1 week. In addition, 5% (w/v) FOS was orally administered to the mice, in water suspension, throughout the experiment to further facilitate *Bifidobacterium* colonization. After 1 week, the $PRL2010_{pNZ8048}$ supplementation was discontinued, and after one additional week, the animals were killed.

To follow $PRL2010_{pNZ8048}$ colonization, fecal samples were collected periodically (on days 0, 2, 5, 9, 12, and 15), and $PRL2010_{pNZ8048}$ cell enumeration was performed by plating fecal material on MRS–Cys–Agar supplemented with chloramphenicol. After incubation at 37 °C, the identity of colonies grown on MRS supplemented with chloramphenicol was further evaluated using PCR and employing PRL2010-specific primers that target pili-encoding loci, which have been described previously (Turroni *et al.*, 2010; Foroni *et al.*, 2011). The inoculated bacterial population increased in number (Fig. 2), reaching a maximum of 10^7 CFU g^{-1} feces at day 5.

Interestingly, following this rapid increase of PRL2010 cell numbers during the period of bacterial supplementation, the level of PRL2010 cells decreased to reach a plateau of approximately 10^5 CFU that appeared to remain stable during the full length of the post-treatment period (Fig. 2). Notably, the presence of high numbers of $PRL2010_{pNZ8048}$ cells upon a period of 7 days without any supplementation with bifidobacterial cells reinforces the notion that the plasmid is stable. Altogether these data indicate that PRL2010 is capable of colonizing the intestine of mouse, which will open new avenues in the

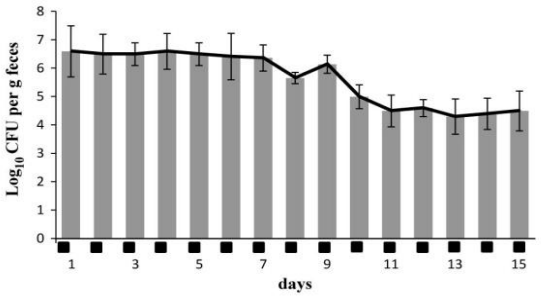

Fig. 2. Population sizes of *Bifidobacterium bifidum* PRL2010 colonizing the intestine of BALB/c mice. Each point represents the average of the log-transformed population size ± standard deviation for six mice. Squares represent the population size of PRL2010 in control mice.

exploration of host–microbe interactions of this microorganism using an *in vivo* murine model (O'Connell Motherway *et al.*, 2011).

Conclusion

This study describes an optimized protocol for the transformation of bifidobacteria that enables the establishment of plasmid DNA into two very distantly related species, that is, *B. bifidum* and *B. asteroides* taxa, where in the latter case it represents the first report on plasmid-mediated transformability. The transformation rates achieved were sufficiently high for cloning purposes; nonetheless, the experiments so far performed highlighted transformation efficiency of 10^4 CFU μg^{-1} which is not yet high enough for site-directed mutagenesis and for an effective selection of transformants in gene knock-out experiments (O'Connell Motherway *et al.*, 2009). The next step will be to improve the transformation efficiency, which could be achieved by overcoming the restriction modification systems of this microorganism (O'Connell Motherway *et al.*, 2009). Genetic tools to manipulate bifidobacteria are still largely undeveloped and represent a bottleneck in the advancing of knowledge on this important group of microorganisms. Thus, the transformation protocol and subsequent colonization model described in this study offer two important adjuncts in exploring genomic functionalities of bifidobacteria under *in vitro* as well as *in vivo* conditions.

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References

Argnani A, Leer RJ, van Luijk N & Pouwels PH (1996) A convenient and reproducible method to genetically transform bacteria of the genus *Bifidobacterium*. *Microbiology* **142**(Pt 1): 109–114.

de Ruyter PG, Kuipers OP & de Vos WM (1996) Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl Environ Microbiol* **62**: 3662–3667.

Fischer W, Bauer W & Feigel M (1987) Analysis of the lipoteichoic-acid-like macroamphiphile from *Bifidobacterium bifidum* subspecies pennsylvanicum by one- and two-dimensional 1H- and 13C-NMR spectroscopy. *Eur J Biochem* **165**: 647–652.

Foroni E, Serafini F, Amidani D *et al.* (2011) Genetic analysis and morphological identification of pilus-like structures in members of the genus *Bifidobacterium*. *Microb Cell Fact* **10** (Suppl 1): S16.

Guglielmetti S, Karp M, Mora D, Tamagnini I & Parini C (2007) Molecular characterization of *Bifidobacterium longum* biovar *longum* NAL8 plasmids and construction of a novel replicon screening system. *Appl Microbiol Biotechnol* **74**: 1053–1061.

Guglielmetti S, Ciranna A, Mora D, Parini C & Karp M (2008) Construction, characterization and exemplificative application of bioluminescent *Bifidobacterium longum* biovar *longum*. *Int J Food Microbiol* **124**: 285–290.

Hartke A, Benachour A, Boutibonnes P & Auffray Y (1996) Characterization of a complex restriction/modification system detected in a *Bifidobacterium longum* strain (vol 45, pg 132, 1995). *Appl Microbiol Biotechnol* **46**: 202.

Kullen MJ & Klaenhammer TR (2000) Genetic modification of intestinal lactobacilli and bifidobacteria. *Curr Issues Mol Biol* **2**: 41–50.

O'Connell Motherway M, O'Driscoll J, Fitzgerald GF & Van Sinderen D (2009) Overcoming the restriction barrier to plasmid transformation and targeted mutagenesis in *Bifidobacterium breve* UCC2003. *Microb Biotechnol* **2**: 321–332.

O'Connell Motherway M, Zomer A, Leahy SC *et al.* (2011) Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. *P Natl Acad Sci USA* **108**: 11217–11222.

Rossi M, Brigidi P, Gonzalez Vara y Rodriguez A & Matteuzzi D (1996) Characterization of the plasmid pMB1 from *Bifidobacterium longum* and its use for shuttle vector construction. *Res Microbiol* **147**: 133–143.

Savino F, Roana J, Mandras N, Tarasco V, Locatelli E & Tullio V (2011) Faecal microbiota in breast-fed infants after antibiotic therapy. *Acta Paediatr* **100**: 75–78.

Schell MA, Karmirantzou M, Snel B *et al.* (2002) The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *P Natl Acad Sci USA* **99**: 14422–14427.

Sela DA, Chapman J, Adeuya A *et al.* (2008) The genome sequence of *Bifidobacterium longum* subsp. *infantis* reveals adaptations for milk utilization within the infant microbiome. *P Natl Acad Sci USA* **105**: 18964–18969.

Serafini F, Bottacini F, Viappiani A, Baruffini E, Turroni F, Foroni E, Lodi T, van Sinderen D & Ventura M (2011) Insights into physiological and genetic mupirocin susceptibility in bifidobacteria. *Appl Environ Microbiol* **77**: 3141–3146.

Sleator RD, Gahan CG & Hill C (2001) Mutations in the listerial *pro*B gene leading to proline overproduction: effects on salt tolerance and murine infection. *Appl Environ Microbiol* **67**: 4560–4565.

Turroni F, Bottacini F, Foroni E *et al.* (2010) Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. *P Natl Acad Sci USA* **107**: 19514–19519.

Turroni F, van Sinderen D & Ventura M (2011) Genomics and ecological overview of the genus *Bifidobacterium*. *Int J Food Microbiol* **149**: 37–44.

Veerkamp JH & van Schaik FW (1974) Biochemical changes in *Bifidobacterium bifidum* var. Pennsylvanicus after cell wall inhibition. VII. Structure of the phosphogalactolipids. *Biochim Biophys Acta* **348**: 370–387.

Ventura M, Canchaya C, Del Casale A, Dellaglio F, Neviani E, Fitzgerald GF & van Sinderen D (2006) Analysis of bifidobacterial evolution using a multilocus approach. *Int J Syst Evol Microbiol* **56**: 2783–2792.

Ventura M, Canchaya C, Tauch A, Chandra G, Fitzgerald GF, Chater KF & van Sinderen D (2007) Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum. *Microbiol Mol Biol Rev* **71**: 495–548.

Ventura M, O'Flaherty S, Claesson MJ, Turroni F, Klaenhammer TR, van Sinderen D & O'Toole PW (2009) Genome-scale analyses of health-promoting bacteria: probiogenomics. *Nat Rev Microbiol* **7**: 61–71.

6. Genetic analysis and morphological identification of pilus-like structures in members of the genus *Bifidobacterium*

Foroni E., Serafini F., Amidani D., Turroni F., He F., Bottacini F.,O'Connell Motherway M., Viappiani A., Zhang Z., Rivetti C., van Sinderen D., Ventura M.

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Genetic analysis and morphological identification of pilus-like structures in members of the genus *Bifidobacterium*

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Abstract

Background: Cell surface pili in Gram positive bacteria have been reported to orchestrate the colonization of host tissues, evasion of immunity and the development of biofilms. So far, little if any information is available on the presence of pilus-like structures in human gut commensals like bifidobacteria.

Results and discussion: In this report, Atomic Force Microscopy (AFM) of various bifidobacterial strains belonging to *Bifidobacterium bifidum*, *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium dentium*, *Bifidobacterium adolescentis* and *Bifidobacterium animalis* subsp. *lactis* revealed the existence of appendages resembling pilus-like structures. Interestingly, these microorganisms harbour two to six predicted pilus gene clusters in their genome, with each organized in an operon encompassing the major pilin subunit-encoding gene (designated *fim*A or *fim*P) together with one or two minor pilin subunit-encoding genes (designated as *fim*B and/or *fim*Q), and a gene encoding a sortase enzyme (*str*A). Quantitative Real Time (qRT)-PCR analysis and RT-PCR experiments revealed a polycistronic mRNA, encompassing the *fim*A/P and *fim*B/Q genes, which are differentially expressed upon cultivation of bifidobacteria on various glycans.

Introduction

Pili or fimbriae are hair-like appendages commonly found in various Gram negative and Gram positive bacteria (for a review see [1]). Unlike Gram negative pili, whose subunits associate via non-covalent interactions, most pili detected in Gram positive bacteria are formed by covalent polymerization of pilin subunits, orchestrated by transpeptidase enzymes called sortases [2,3]. The general principles of pilus assembly/structure in Gram positive bacteria were first established for the pathogens *Corynebacterium diphtheriae* and *Actinomyces naeslundii*[2-5]. In these microorganisms, the genes for pilus formation and assembly are arranged in pathogenic islets that each encode one major pilin (represented by either SpaA, SpaD, or SpaH in *C. diphtheriae*, or FimA or FimP in *A. naeslundii*; Spa stands for sortase-mediated pilus assembly and Fim for fimbria-associated adhesion, respectively), one or two minor pilins (represented by the SpaB and SpaC, SpaE and SpaF, or SpaG and SpaI subunit couples in *C. diphtheriae*, or the FimB or FimQ subunits in *A. naeslundii*), and a pilus-specific sortase) ([1,2,4,6,7]. In *Actinomyces* the *fim*QP and the *fim*AB-loci encode fimbrial structures composed of a shaft protein, being either FimA or FimP, and a tip protein, represented by either FimB or FimQ, respectively [3,8]. Pilus gene clusters that encode a sortase have been found in many important Gram positive pathogens including *Clostridium perfringens*, enterococci and various streptococcal species,

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as well as in the *Actinomyces* taxon [1,2,7,9]. Recently, two pilus gene clusters have been identified in the genome of a human gut commensal *Lactobacillus rhamnosus* GG [10,11].

It has been shown that pili are involved in the attachment/colonization of members of the human microbiota to host tissues, as pilus-mediated adherence is a critical step in the establishment of infection by several Gram negative pathogens [12][13] as well as in Gram positive bacteria like corynebacteria [14] and *Actinomyces*[8]. In *Enterococcus faecalis*, pili are needed for biofilm production and non-piliated mutants are dramatically attenuated in an endocarditis model [9]. Furthermore, the presence of pili in streptococci was demonstrated to stimulate the host inflammatory response [13]. Recently, the mucosal adhesion features of the human intestinal bacterium, *Lactobacillus rhamnosus* GG were demonstrated to be due to the SpaCBA and SpaFED pilin subunits encoded by this organism [11].

Bifidobacteria are the most numerous bacterial members of the gut microbiota of infants [15,16]. They have been claimed to elicit several health-promoting or probiotic effects, such as strengthening of the intestinal barrier, modulation of the immune response and exclusion of pathogens [17,18]. Although there is some evidence to support each of these functional claims for particular bifidobacterial strains, the molecular mechanisms by which these activities are achieved remain largely unknown. Genome sequencing efforts have started to highlight the genetic strategies followed by bifidobacteria in order to colonize the human gut [19-23]. In a recent study, we discovered that the genome of *Bifidobacterium bifidum* PRL2010 harbors a large gene set involved in the utilization of host-derived glycans, such as those found in the outermost layer of the intestinal mucosa [24]. These findings therefore represent a clear example of host-microbe co-evolution, and presented *B. bifidum* PRL2010 as a bifidobacterial prototype for the analysis of the interaction between microbes and the intestinal mucosa.

The current study provides the first morphological evidence of pili-like structures decorating the cell surface of various bifidobacterial species. It further investigates the genetic organization and transcriptional profiling of the presumed bifidobacterial pilus encoding-gene clusters in response to different growth substrates.

Material and methods

Bacterial strains and culture conditions

Bifidobacterial cultures were incubated in an anaerobic atmosphere (2.99 % H_2, 17.01 % CO_2 and 80 % N_2) in a chamber (Concept 400, Ruskin) in the Man-Rogosa-Sharp (MRS) (Scharlau Chemie, Barcelona, Spain) supplemented with 0.05 % (w/v) L-cysteine hydrochloride and incubated at 37°C for 16 h. Bifidobacterial cells were also cultivated on MRS where glucose was replaced with 2% (w/v) of an alternative carbon source (lactose, Fructo Oligosaccharides [FOS], mucin or N-acetyl glucosamine).

Sample preparation and AFM imaging

Bacteria from four ml of a bacterial culture were harvested by centrifugation at 4000 rpm and resuspended in 200 μl of PBS (or 20 mM Hepes 7.5, 1 mM EDTA). 200 μl of 5% glutaraldehyde was added, followed by gentle mixing and incubation for 1 minute at room temperature. Thereafter, bacteria were washed four times with PBS by repeated resuspension and collection by centrifugation (4000 rpm). The washed pellet was then resuspended in 200 μl of PBS and kept on ice until AFM imaging.

To facilitate adhesion of bacteria to the mica support used for AFM imaging, mica was coated with polylysine (PL) as follows: 10 μl of a polylysine solution (10 ng/ml) was deposited onto freshly-cleaved mica for one minute. Mica was then rinsed with milliQ water (Millipore) and dried with nitrogen. After this, 20 μl of bacterial suspension was deposited onto PL-coated mica for 2-5 minutes depending on the particular strain or specific cultivation conditions. The mica disk was then rinsed with milliQ water and dried under a weak gas flow of nitrogen. Quality of the sample and density of surface-bound bacteria were verified with an optical microscope.

AFM imaging was performed on dried samples with a Nanoscope III microscope (Digital Instruments) equipped with scanner J and operating in tapping mode. Commercial diving board silicon cantilevers (Mikro-Masch) were used. Best image quality was obtained with high driving amplitude (1-3V) and low scan rate (0.5 Hz). Filamentous structures at the periphery of bacteria were visible in images of 512 x 512 pixels, representing a scansize of 10 μm or less. While imaging both height and amplitude signals were collected. Height images were flattened using Gwyddion software.

Phylogenetic analyses

Genomic survey of the pili-encoding genes were performed by BLAST analysis against the NCBI database of the bifidobacterial FimA/P (cutoff: E-value 1 x 10-4 and 30% identity over at least 80% of both protein sequences).

Phylogeny calculations, including distance calculations and the generation of phylogenetic trees, were performed using PHYLIP (Phylogeny Inference Package) [25] version 3.5c. Trees were calculated using the neighbour-joining method under Kimura's two-parameter substitution model [26]. Bootstrap values were computed by performing 1000 re-samplings. Dendograms

from gene sequences were drawn using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and were visualized with the TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

3D structure prediction of pilin major subunit

The 3D structures of pilin major subunits encoded by the so-called *pil* loci of *B. bifidum* PRL2010 (BBPR_1707, BBPR_1801 and BBPR_0283) were predicted using the fold recognition method. Taking the structure prediction of BBPR_1707 as an example, the structure prediction procedures are briefly described as follows. To obtain the structure template, we first submitted the sequence of BBPR_1707 to the protein structure prediction metaserver (http://meta.bioinfo.pl/submit_wizard.pl), which integrated a series of well-established fold recognition methods. Using the top hit ranked by the metaserver, the *Corynebacterium diphtheriae* major pilin subunit (SpaA; PDB code: 3HR6; X-ray resolution: 1.6 Å) was chosen as the structure template, which was based on the fold recognition result of the FFAS algorithm [27]. Then, the corresponding sequence alignment between BBPR_1707 and 3HR6 was also obtained from the FFAS algorithm. Finally, the 3D model of BBPR_1707 was generated and refined by the SCWRL program [28]. Similarly, the structures of BBPR_1801 and BBPR_0283 were predicted based on the same template used for BBPR_1707.

RNA isolation

Total RNA was isolated using the methods described previously [29]. Briefly, cell pellets were resuspended in 1 ml of QUIAZOL (Quiagen, UK) and placed in a tube containing 0.8 g of glass beads (diameter, 106 μm; Sigma). The cells were lysed by shaking the mix on a BioSpec homogenizer at 4°C for 2 min (maximum setting). The mixture was then centrifuged at 12,000 rpm for 15 min, and the upper phase containing the RNA-containing sample was recovered. The RNA sample was further purified by phenol extraction and ethanol precipitation according to an established method [30].

Reverse transcription -PCR analysis

Five micrograms of mRNA was treated with DNase (Roche, United Kingdom) and used as template in a 100 μl reaction mixture containing 20 ng of random primers, each deoxyribonucleoside triphosphate at a concentration of 0.125 mM, and Superscript enzyme (Invitrogen, Paisley, United Kingdom) used according the manufacturer's instructions to produce cDNA. The cDNA generated was then used as a template for reverse transcription (RT)-PCRs to determine the arrangement of the transcript encompassing the *pil* loci of *B. bifidum* PRL2010 using the primers listed in Additional file 1.

Quantitative real-time reverse transcription PCR (qRT-PCR)

qRT-PCR primers (Additional file 2) were used to amplify the genes encompassing the *pil* loci as indicated in Figure 1, and the reference genes *atp*D, *tuf*A, *rpo*B and *ldh*. Criteria for primer design were based on a desired melting temperature ™ values between 58 and 60°C and amplicon size of approximately 100 base pairs. qRT-PCR was performed using the CFX96 system (BioRad, CA, USA), fold change was evaluated through the estimation of the CT values with the aid of the CFX96 software (BioRad, CA, USA). PCR products were detected with SYBR Green fluorescent dye and amplified according to the following protocol: one cycle of 95°C for 3 minutes, followed by 39 cycles of 95°C for 5 s and 60°C for 20 s. Melting curve: 65°C to 95°C with increments of 0.5°C/s.

Each PCR reaction mix contained the following: 12.5 μl 2x SYBR SuperMix Green (BioRad, CA, USA), 1 μl of cDNA dilution, each of the forward and reverse primers at 0.5 μM and nuclease-free water was added to obtain a final volume of 20 μl. In each run, negative controls (no cDNA) for each primer set were included.

Fold change was calculated using the CFX96 software (BioRad

Results and discussion

Cell morphology and pili structures

The cell surface of bifidobacterial cells belonging to different species spanning a variety of ecological origins (Table 1) was analyzed by Atomic Force Microscopy (AFM). Bacterial cells were cultivated on various carbon sources, including simple carbohydrates, i.e. glucose and lactose, as well as more complex sugars such as fructo-oligosaccharides (FOS), representing those that are expected to be present in the human gut [31]. Such analyses revealed the presence of pilus-like structures on the cell surfaces of various bifidobacteria (Fig. 1). Interestingly, in a couple of cases, such as *B. bifidum* PRL2010 the cell surface was shown to be densely piliated with the majority of pili located at cell-polar position. In contrast, under the conditions examined pilus-like structures were shown to be rare if at all present in *B. longum* subsp. *infantis* ATCC15696 (Fig. 1). When we investigated the production of pilus-like structures in several bifidobacteria propagated under various culture conditions, i.e., growth on different carbon sources, by AFM, we observed differential behavior among the strain/species tested (Fig. 1). Notably, in the case of *B. bifidum* PRL2010 cultivation in MRS medium supplemented with FOS caused a high production of pilus-like structures (Fig. 1), which were often seen as polar tufts. In contrast, a different response, i.e., a less abundant production of pili, to FOS cultivation was noticed on the cell surface of *B. dentium* Bd1. The

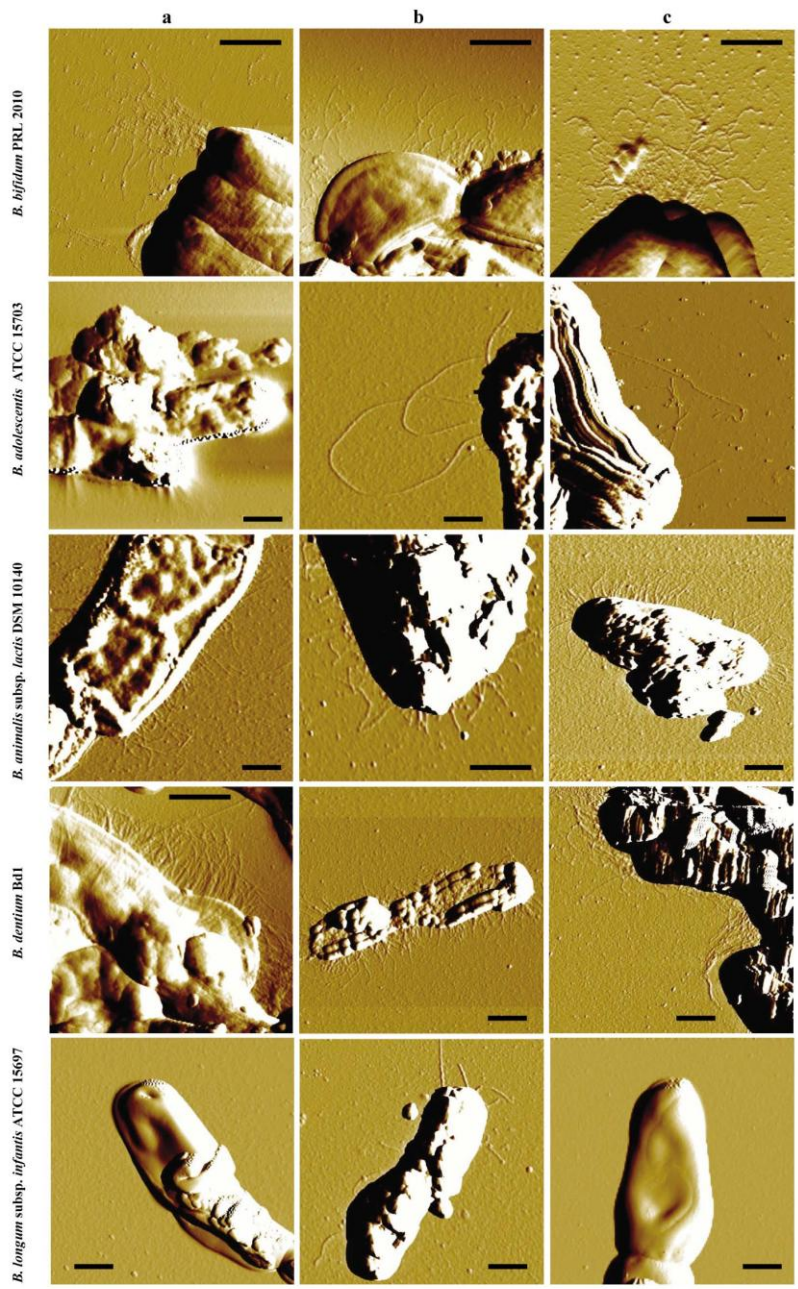

Figure 1 Presence and morphology of pilus-like structures in various bifidobacterial species. Samples were viewed by Atomic Force Microscope**.** Panels a, b and c show pilus-like structures of bifidobacteria cultivated on glucose, lactose or FOS, respectively, as the sole carbon source. Scale bar 0.5 μm.

Table 1 Strains used in this study, their origin and effects of the different growth conditions on the on pilus gene/ protein expression.

Organism	Origin	Pilus gene cluster	Carbon sources											
			Glucose*		lactose		FOS		mucin		Bovine milk		N-acetylglucosamine	
			AFM	qRT-PCR	AFM	qRT-PCR	AFM	qRT-PCR	AFM	qRT-PCR	AFM	qRT-PCR	AFM	qRT-PCR
B. bifidum PRL2010	Feces of breast-fed infant	282-284		nd		nd		nd		nd		yes		nd
		1707-1709	+	nd	+	nd	+	yes	-	nd	-	nd	-	nd
		1820-1822		nd		nd		nd		nd		yes		nd
B. longum subsp. *infantis* ATCC15697	Intestine of infant	-	nd	-	+		nd		-		-		-	
B. dentium Bd1	Dental caries	142-144		nd		nd		nd		nd		nd		nd
		197-200		nd		nd		nd		nd		nd		nd
		276-278		-		-		-		-		-		-
		534-536	+	-	+	-	nd	-	-	-	-	-	-	-
		1874-1876		-		-		-		-		-		-
		2000-2002		nd		yes		nd		nd		nd		nd
		2188-2191		-		-		-		-		-		-
B. animalis subsp. *lactis* DSM10140	Yogurt	1488-1486	+	nd	+	+	+	+	-	nd	-	nd	-	nd
B. adolescentis ATCC15703	Intestine of adult	1467-1470	nd	nd	+	nd	+	+	-	nd	-	+	-	+

*Glucose was used as reference condition for normalizing the qRT-PCR experiments; nd, not identified; +, identified ; -, not performed or present in the genome; ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen

observed differential phenotypes of *B. bifidum* PRL2010 versus *B. dentium* Bd1 may be linked to the different ecological niches occupied by these micro organisms, represented by the human intestine and oral cavity, respectively.

The observed sizes of these identified pilus-like structures appeared to be highly variable among the different bifidobacterial species analysed, as well as within the same cell. In particular the length of the filaments was ranging from 100 nm to several micrometers, the width from 10 to 30 nm and the height from 0.5 to 2 nm (Additional file 3). Although the width and the height of these biological structures are affected by the AFM tip size and compression, these measurements indicate the existence of a variable number of pilus-types within the genus *Bifidobacterium*.

Sequence analysis of bifidobacterial pili-loci

As previously described [1,32,33] pilus-encoding gene clusters in genomes of Gram positive bacteria consist of one to three genes specifying pilus subunits and an associated sortase-encoding gene and can thus be identified based on sequence similarities. Recently, the genome sequences of *B. dentium* Bd1, *B. longum* subsp. *infantis* ATCC 15697, *Bifidobacterium longum* subsp. *longum* NCC2705,*Bifidobacterium longum* subsp. *longum* DJO10A, *Bifidobacterium bifidum* PRL2010, *Bifidobacterium adolescentis* ATCC15703, and *Bifidobacterium animalis* subsp. *lactis* DSM10140 genomes [19-24] have become available for such purposes. Screening for pilin subunit- and sortase-encoding genes in these genomes revealed the presence of putative pilus gene clusters in all of these genomes with the exception of *B. longum* subsp. *infantis* ATCC15697 (Fig. 2). These putative pilus genetic loci occupy various genomic positions (name designations and genome positions are indicated in Figure 2). Notably, the largest number of pilus gene clusters was identified in the genome of *B. dentium* Bd1, which suggests that this microorganism possesses expanded capabilities to adapt to different ecological environments (e.g., oral cavity as well as intestine and fecal material).

The typical predicted pilus gene cluster identified in these bifidobacterial genomes encompasses genes encoding a major pilin subunit (similar to FimA or FimP) and one or two ancillary minor pilin subunits (similar to FimB and FimQ), which were identified on the basis of amino acid identity with pilin sununits identified in other Gram positive bacteria such as *Actinomyces* spp. and streptoccocae. Furthermore, the sequences of the predicted *fim*AB, *fim*PQ gene products contained the anticipated consensus motifs and domains characteristic of a pilin primary structure, including a Sec-dependent secretion signal, the sortase recognition site (CWSS

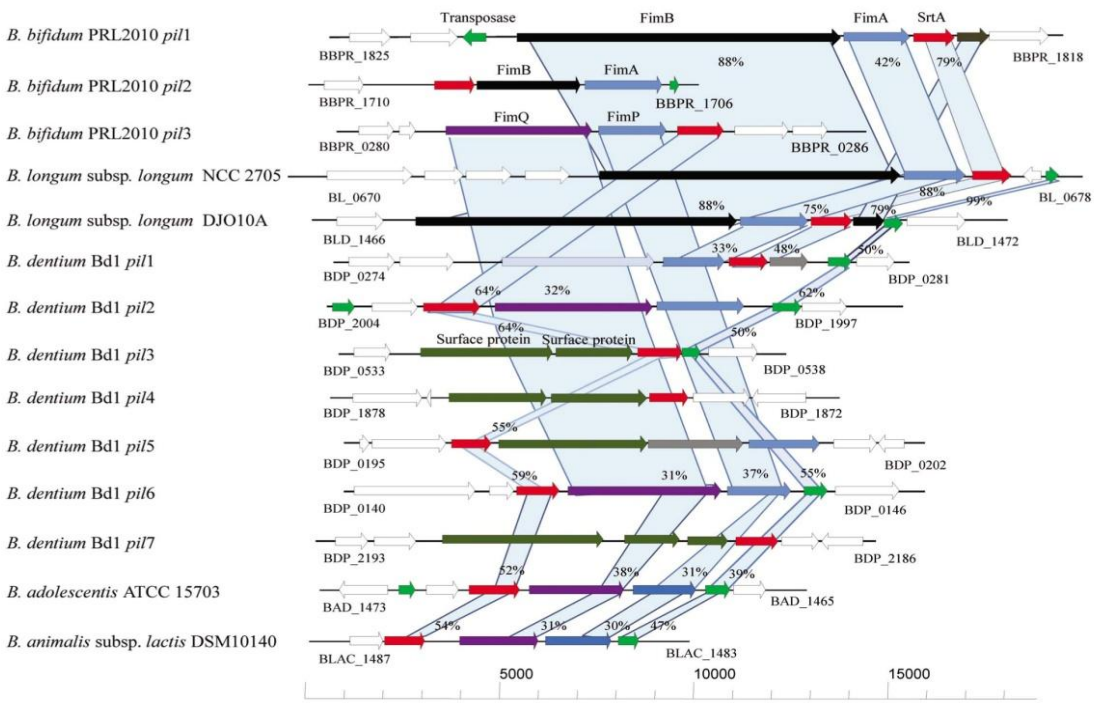

Figure 2 Schematic comparative representation of pilus-encoding loci of various bifidobacterial strains. Each arrow indicates an ORF, the size of which is proportional to the length of the arrow. Colouring of the arrows represents the different function of the gene as indicated indicated above each arrow. The amino acid identity of the relevant encoded proteins is indicated in percentages.

Table 2 Genetic features of the major pilin protein in bifidobacteria.

Strain	Major pilin protein			
	ORF	CWSS	Pilin motif	E box
B. bifidum PRL2010	283	**LP**KT**G**A	GDSTAE**V**DM**K**	YTVT**ET**AVAD**G**Y
B. bifidum PRL2010	1707	**LP**LT**G**G	VYSSGSIDM**K**	YTIE**EI**AAPN**G**Y
B. bifidum PRL2010	1821	**LP**G**TG**G	HSTTVG**V**DI**K**	YTLT**ET**EAPA**G**Y
B.dentium Bd1	144	**LP**LT**G**A	NHGDNT**V**NM**K**	YTVS**ET**KVAT**G**Q
B.dentium Bd1	200	**LP**LT**G**G	QTLSYN**V**TA**K**	YTVK**ET**KAPA**G**Y
B.dentium Bd1	277	**LP**ET**G**G	VAGN**V**TITP**K**	YVLT**ET**KTPA**G**Y
B.dentium Bd1	535	**LP**LT**G**A	GQTLGV**V**NV**K**	YDVV**ET**DAPA**G**Y
B.dentium Bd1	1875	**LP**ITGA	HPAQTIDVK**K**	YTVT**ET**VVPA**G**F
B.dentium Bd1	2190	**LP**ST**G**G	FEKINS**V**KV**K**	YVLS**ET**KTPE**G**Y
B.dentium Bd1	1999	**LP**LT**G**A	PLTLGT**V**VA**K**	YTVK**ET**ATREDL
B. longum subsp. *longum* NCC2705	675	**LP**DT**G**G	KSEYPT**V**DKT	YYLK**ET**FAPK**G**Y
B. longum subsp. *longum* DJ010A	1468	**LP**G**TG**G	KGSLPT**V**DK**K**	YTLT**ET**KAPA**G**Y
B. adolescentis ATCC 15703	1463	**LP**LT**G**A	INAVGMFVA**K**	YTLK**ET**GFAS**G**Y
B. animalis subsp. *lactis* DSM10140	1484	**LP**LT**G**A	KPSGTITLG**K**	YKVT**ET**DVLSRY
A. naeslundii T14V	FimP	**LP**LT**G**A	WNYNVH**V**YP**K**	YCLV**ET**KAPE**G**Y
A. naeslundii T14V	FimA	**LP**LT**G**A	WIYDVH**V**YP**K**	YVLV**ET**KAPA**G**Y
Consensus sequences		**LP**x**TG**	xxxxxTVxx**K**	Yxxx**ET**xAPx**G**Y

motif), the pilin-like motif (TVXXK) and the E box (Table 2) [1,3]. Interestingly, all the identified bifidobacterial pilus gene clusters are flanked by transposon elements, indicative of their acquisition by horizontal gene transfer (HGT) (Fig. 2 and see below).

Notably, in the genome of PRL2010 the ORF1822, encoding a putative FimB, appears to be a pseudogene due to a frame-shift within a stretch of nine guanine residues. However, it is possible that transcriptional slippage along the guanine residues allows expression of this fimbrial subunit under certain environmental conditions [34].

Structural investigation of pili-like proteins encoded by bifidobacteria

In order to corroborate these findings a structural investigation of *B. bifidum* PRL2010 major subunits encoded by the three *pil* loci present in its genome, was performed. The 3D structures of major pilin subunits (BBPR_1707, BBPR_1821 and BBPR_0283) were predicted based on the *Corynebacterium diphtheriae* major pilin subunit (SpaA, PDB code: 3HR6; X-ray resolution: 1.6 Å) through the FFAS03 program [27]. Similar to the 3D structure of 3HR6 [35], the predicted 3D model also comprises three tandem Ig-like domains. The 3D models of BBPR_1707, BBPR_1821 and BBPR_0283 cover residues of their original sequences from 36-487, 89-470 and 143-467, respectively (Fig. 3). Although the overall protein sequence identity between the three major pili subunits and 3HR6 is only in a range of 22–25 %, the results from fold recognition revealed that the predicted structures of the three major pilin subunits are similar to the 3D structure of 3HR6, which contains three tandem Ig-like domains [35].

In addition, in order to model the general structure of the three major bifidobacterial pilin subunits, the predicted 3D models allow the spatial location of some important functional motifs. As reported previously, the threonine-glycine bond within the LPXTG motif (CWSS motif), which is located at the C-terminal of pilin subunit, is recognized and cleaved by a dedicated sortase. Following this cleavage, the inter-molecular covalent linkage between the threonine of the cleaved LPXTG motif in one pilin subunit and a conserved lysine present within the pilin motif of its neighboring pilin subunit leads to fiber assembly by covalent linkages [36]. Additionally, the glutamic acid within the E-box of the major subunit has been reported to be involved in the incorporation of the minor subunit into the pilus fiber. Based on the predicted 3D models, the corresponding pilin motifs as well as the conserved lysines in the three major pilin subunits can be annotated (Fig. 3). Moreover, the E-box motifs in these three major pilin subunits were also identified (Fig. 3).

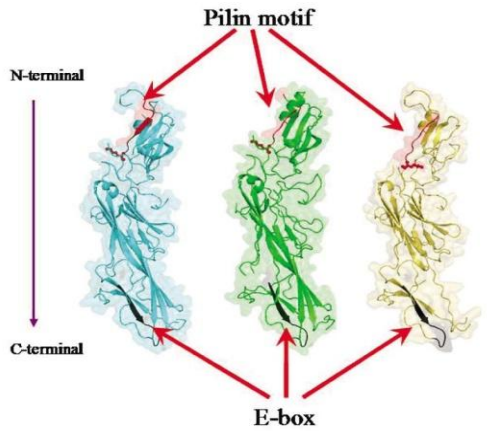

Figure 3 Structural representation of the major pilin subunits of *B. bifidum* PRL2010. BBPR_1707, BBPR_1821 and BBPR_0283 are colored in cyan, green and yellow, respectively. The pilin motifs (residues 188-197 in BBPR_1707, 184-191 in BBPR_1821 and 224-231 in BBPR_0283) are highlighted in red. The side chains of the conserved lysines in these three pilin motifs (K197 in BBPR_1707, K191 in BBPR_1821 and K231 in BBPR_0283) are shown as sticks and balls. The E-box motif in each model is colored in black (residues 445-456 in BBPR_1707, 428-439 in BBPR_1821 and 438-449 in BBPR_0283).

Polymerization of the pilin subunits is catalysed by an enzyme with homology to sortases [37]. Notably, all the detected pili genetic loci encompass a sortase encoding gene (*str*A) belonging to the class C family (Fig. 2). Furthermore, genome analyses revealed the presence of a variable number of additional sortases belonging to the housekeeping sortases, also referred as the class A (data not shown). The class A sortases are usually encoded by genes whose chromosomal locations are unlinked to the genes that encode their specific surface protein substrates [38]. These housekeeping sortases might exert an accessory role in the assembly of pili in bifidobacteria, similar to their role in *C. diphtheriae*, where housekeeping sortases are required for the correct covalent attachment of the major pilin subunits to the cell wall [38].

Phylogenetic analyses of bifidobacterial pilus-associated genes

It has been previously shown that pilus-encoding genes in other microorganisms such as corynebacteria have been acquired through Horizontal Gene Transfer (HGT) events [7,39]. We therefore investigated if this may also have been the case for the pilus-encoding genes of bifidobacteria.. In order to assess the distribution of *fim*A/P

homologs across bacteria we surveyed currently available genomic data, representing members of *Actinobacteria*, *Firmicutes* and various Gram negative bacteria (Additional file 4) for the presence of genes specifying predicted major pilus subunits. This analysis revealed that predicted FimA/P-encoding genes are not uniformly present in all bacteria studied to date (data not shown). The distribution of *fim*A/P homologs might be a consequence of the organism's development following a vertical- or horizontal- evolution mechanism. Alignment of the presumed FimA/P-encoding genes was performed using ClustalW and resulted in a rooted neighbour joining phylogenetic tree (Fig. 4). Notably, bifidobacterial FimA/P does form a monophyletic group with other high G+C Gram positive bacteria such as *Actinomyces* spp., as well as with the FimA/P sequences of *Firmicutes* (Fig. 4). Furthermore, bifidobacterial FimA/P sequences cluster together with homologs sequences from Gram negative bacteria such as *Bacteroidetes* spp. (Fig. 4). These phylogenetic inconsistencies based on FimA/P sequences, can be explained by assuming that the bifidobacterial FimA/P-encoding genes, similar to *fimA/P* genes from other high G+C Gram positive, were acquired through HGT events. Other findings that support this hypothesis are the deviating G+C% content of the bifidobacterial *fim*A/P genes compared to the average G+C% content of their genome [e.g. between 5 % lower (in case of *B. longum* subsp. *longum* DJO10A) to 9 % higher (in the case of *B. bifidum* PRL2010 *pil*1) than the G+C average value], as well as by the different codon usage bias for the predicted FimA/P proteins (results not shown).

Transcriptional analysis of bifidobacterial pili loci

In order to determine if the genes encompassing the pili loci detected in bifidobacteria are differentially transcribed upon exposure to different growth substrates simulating those which may be encountered by bifidobacteria in their natural ecological niche, the amount of predicted pilin gene-specific mRNAs was determined by quantitative real-time PCR (qRT-PCR) assays. Such experiments were performed using mRNA samples extracted from exponentially grown cultures of *B. longum* subsp. *longum* DJ010A, *B. bifidum* PRL2010, *B. animalis* subsp. *lactis* DSM10140, *B. adolescentis* ATCC15703 and *B. dentium* Bd1, which had been resuspended in pre-warmed MRS medium containing one of a varied set of growth substrates [lactose, fructooligosaccharides (FOS), bovine milk, N-acetyl glucosamine and mucin]. The observed induction level of putative pilus subunit/pilin- or sortase-encoding genes in response to these growth substrates was shown to be variable between the different bifidobacterial species and highly variable between different pilus gene clusters harboured by the same organism (Fig. 5). In *B. adolescentis* ATCC15703 growth on any of these carbohydrate substrates produced an increase of pilin/sortase gene transcription relative to glucose, with the highest level of transcription observed when this strain was cultivated on bovine milk, FOS, or N-acetyl glucosamine (Fig. 5). In contrast, in another intestinal bifidobacterial strain, *B. bifidum* PRL2010, transcription of the putative pilin/sortase-encoding genes was significantly increased relative to glucose-based growth upon cultivation in bovine milk or FOS (Fig. 5). Conversely, the level of pilin-encompassing mRNA of *B. dentium* Bd1, as well as *B. animalis* subsp. *lactis* DSM10140 did not change significantly when this strain was cultivated on any of these substrates (Fig. 5). However, the expression level of pilus-encoding genes in *B. dentium* Bd1 and *B. animalis* subsp. *lactis* DSM10140 changes significantly when such microorganisms were cultivated in the presence of lactose.

The observed differential transcription patterns of the predicted pilus-encoding genes in these bifidobacterial species, i.e., *B. bifidum*, *B. animalis* subsp. *lactis*, *B. adolescentis* and *B. dentium*, may be linked to the specific ecological niche that each of these bacteria occupy (intestine vs. oral cavity as in the case of *B. adolescentis*, *B. bifidum* vs. *B. dentium*, or different niches within the colon, i.e. human intestine vs. animal intestine, such as in the case of *B. bifidum*, *B. adolescentis* vs. *B. animalis* subsp. *lactis*) [15] and thus may be pivotal for their specific colonization strategy. The different pilus gene clusters carried by the genome of a single microorganism (e.g., the *pil*1, *pil*2 and *pil*3 of *B. bifidum* PRL2010) appear to undergo varying levels of transcriptional induction in response to a particular carbohydrate (Fig. 5), which may be explained by the existence of alternative genetic strategies for bacterial colonization and/or microbe-microbe interactions evolved by a specific bifidobacterial strain, similar to what was previously described for *Actinomyces*[8,40]. Furthermore, such findings suggest that these pili promote adhesion to different molecules (e.g., mucin, epithelial cells, enamel).

Operon organization of the pilus-gene clusters in bifidobacteria

The genetic organization of the pili gene clusters identified in the individual genomes of investigated bifidobacterial species suggests that the pilus subunit-encoding genes and the adjacent sortase-specifying gene in each cluster are co-transcribed. To examine this further, we selected the pilus-encoding gene clusters identified in the genome of *B. bifidum* PRL2010 for transcriptional analysis using RT-PCR experiments. cDNA templates were obtained by RT of total *B. bifidum* PRL2010 mRNAs, isolated from stationary growth stage cells that

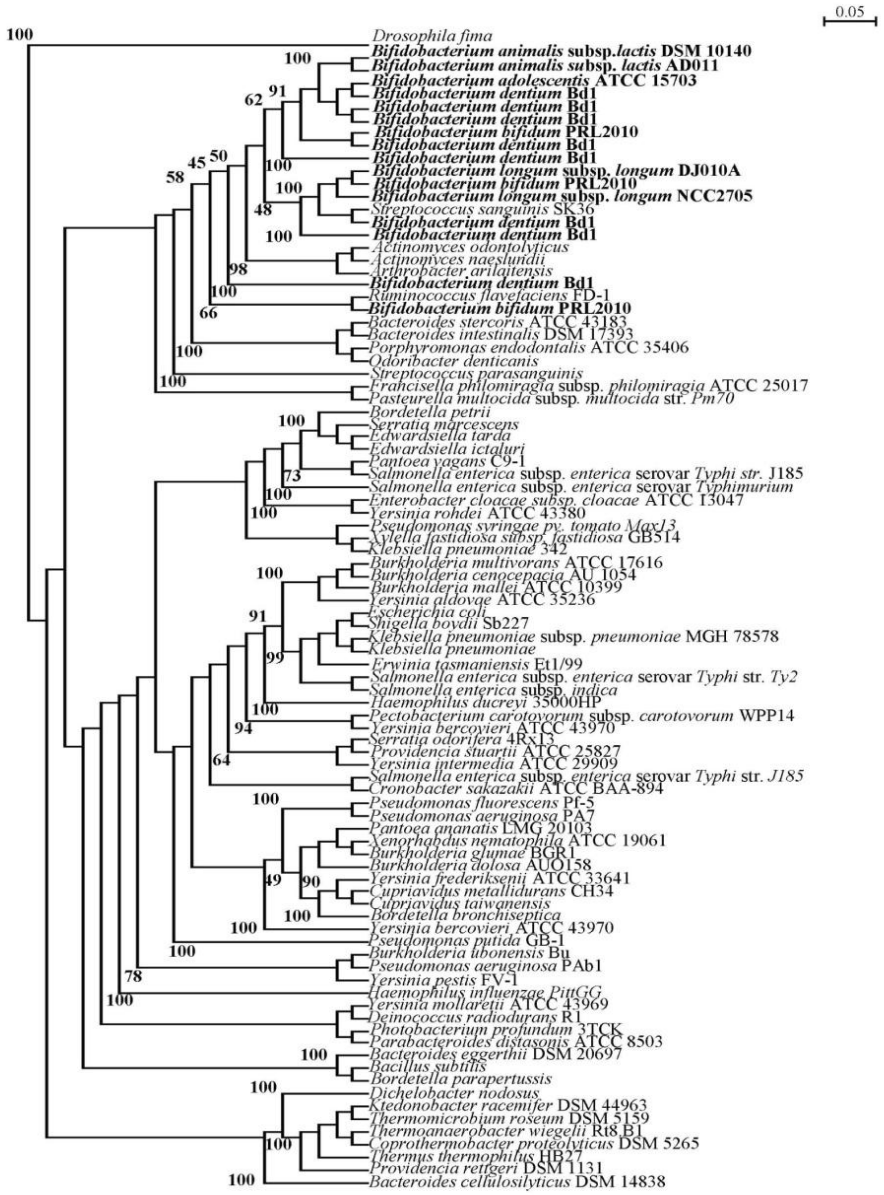

Figure 4 Phylogenetic tree obtained using presumed FimA/P homologs from various bacteria. Bar scale indicates phylogenetic distances. The FimA/P protein sequences from bifidobacteria are typed in bold.

had been cultivated on bovine milk and/or FOS. Each cDNA sample was used as template in various PCR reactions that targeted various parts of the pilus-encoding gene cluster and flanking genes through the use of different combinations of primer pairs (Fig. 6). Notably, amplicons of the expected size were achieved using the internal PCR primer pairs spanning the pilin subunits genes (*fim*A/P and *fim*B/Q). In contrast, no PCR

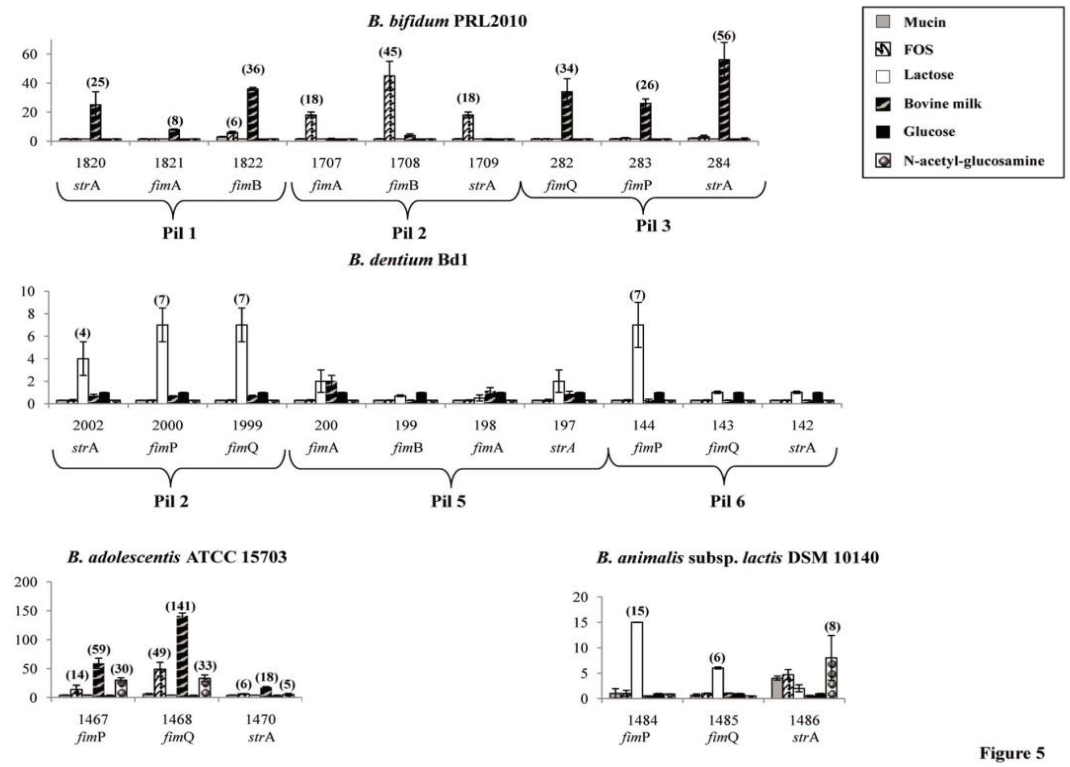

Figure 5

Figure 5 Relative transcription levels of pilus-encoding genes from *B. bifidum* PRL2010, *B. adolescentis* ATCC15703, *B. dentium* Bd1 and *B. animalis* subsp. *lactis* DSM10140 upon cultivation in MRS supplemented with various carbon sources (lactose, FOS, N-acetylglucosamine, mucin), or bovine milk, versus growth in MRS supplemented with glucose as unique carbon source as analysed by quantitative real-time PCR assays. The histograms indicate the relative amounts of the pilin/sortase mRNAs for the specific samples. In each panel, the ORF numbering indicates the gene code according to Figure 1. The values on the y-axes as well as the value (between brackets) represent the fold fold change relative to reference genes as indicated in the materials and method section. For *B. dentium* Bd1 qRT-PCR experiments were performed only on those pili-encoding loci, i.e., *pil*2, *pil*5 and *pil*6, which display a typical gene constellation of the pilus locus (see Figure 2).

products were obtained, when PCR primers were used that targeted the amplification of the intergenic region between the *str*A and the pilin-encoding genes (Fig. 6). When the same strategy was employed to investigate the co-transcription of the pilus gene locus with the other flanking genes, no RT-PCR products were obtained. Therefore, these data indicate that the genes encompassing the *fim*A/P, *fim*B/Q loci produce a single polycistronic mRNA transcript and are thus organized in an operon, a characteristic of known pilus gene clusters [9,41]. Such findings are also corroborated by the evaluation of the change of mRNA levels of the different genes encompassing the pili-loci obtained by qRT-PCR analysis (Fig.5). Notably, the sortase-encoding gene displayed a different level of induction compared to its neighbouring *fim*A/B/P/Q gene, while in the case where no induction was noticed for these latter genes, the sortase gene sometimes displayed the opposite behaviour (e.g., in the case of the pilus-operon of *B. animalis* subsp. *lactis* DSM10140).

Analysis of the nucleotide sequence of the pili loci revealed that the gene clusters were flanked at their 3' end by an inverted repeat (ΔG values ranging from -22.4 to 34.1 Kcal) that is expected to function as a rho-independent transcriptional terminator structure (Fig. 6), whereas no such sequences were found between the genes of the presumed pilus subunit-encoding genes.

Conclusions

Although pili are described in both Gram negative and Gram positive bacteria, very little is so far known about the presence of these extracellular structures in

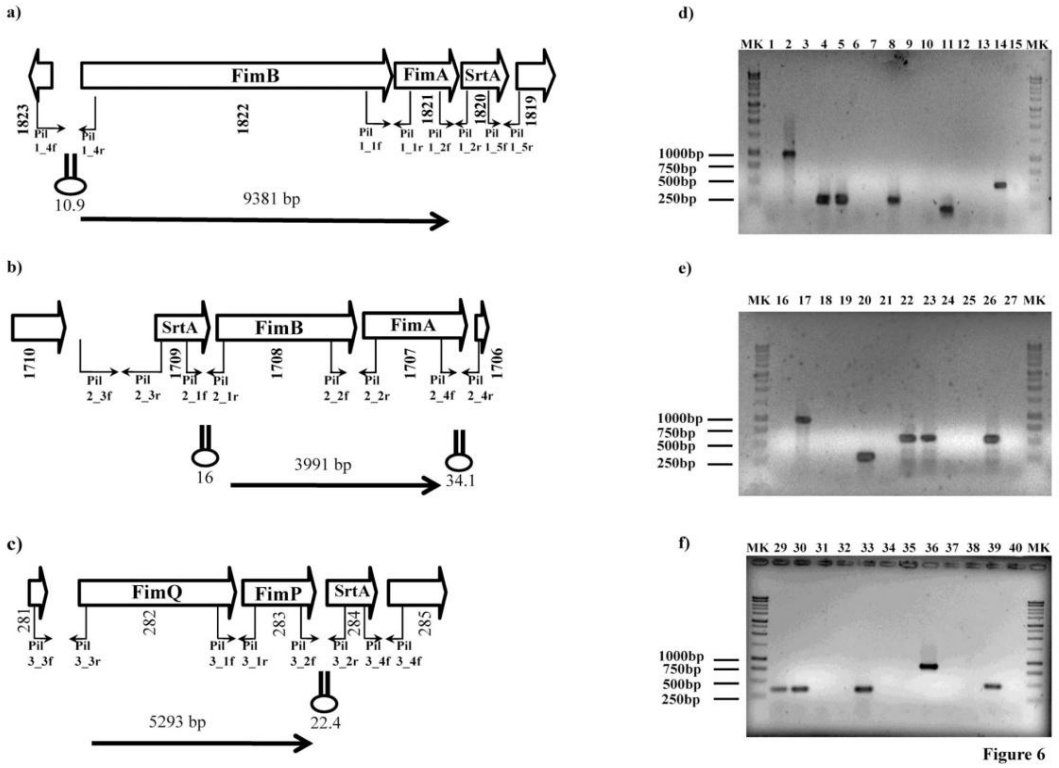

Figure 6

Figure 6 Transcriptional organization of the bifidobacterial pili loci in *B. bifidum* PRL2010. (panel **a-c**) Schematic diagram of bifidobacterial pilus locus. Hairpin symbols indicate predicted secondary structures. Panels **d-f**, products generated by RT-PCR. PCR products were obtained with primers spanning the intergenic regions between the pilin encoding subunits (primers Pil1_1f/ Pil1_1r, Pil2_2f/Pil2_2r and Pil3_1f/Pil3_1r) (lanes 4-5, 22-24, 29-31), between pilin encoding subunits and the *srt*A gene (primers Pil1_2f/Pil1_2r, Pil2_1f/Pil2_1r and Pil3_2f/Pil3_2r) (lanes 7-8, 19-21, 32-34), between pilin encoding subunits and ORF1823, ORF1706, ORF281 (primers Pil1_4f/Pil1_4r; Pil2_4f/Pil2_4r and Pil3_3f/Pil3_3r) (lanes 1-3, 16-18, 35-37), and between the *srt*A gene and ORF 1819, 1710 and 285 (primers Pil1_5f/Pil1_5r; Pil2_3f/Pil2_3r and Pil3_4f/Pil3_4f) (lanes 10-12, 25-27, 38-40). The positions of the primer pairs used in RT-PCR experiments are shown in panels a-c. Products of the PCR were derived under the following conditions: cDNA prepared from *B. bifidum* PRL2010 RNA cultivated on MRS supplemented with bovine milk and/or FOS (lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 29, 32, 35, 38). A positive control control *B. bifidum* PRL2010 DNA (lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 30, 33, 36, 39). A negative control in which *B. bifidum* PRL2010 RNA was used but reverse transcriptase was omitted (lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 31, 34, 37, 40). Lanes MK contained DNA molecular marker 1kb

bifidobacteria [15,42]. In this study we provide for the first time visual evidence of the existence of pilus-like structures on the surface of bifidobacterial cells belonging to different species (*B. bifidum*, *B. dentium*, *B. longum* subsp. *longum*, *B. adolescentis* and *B. animalis* subsp. *lactis*). However, the function of pili in bifidobacteria remains still to be elucidated. In close related organisms, such as *C. diphtheriae* and *Actinomyces* spp. pilus fibres are important for bacterial adherence to specific host cells, including epithelial cells, erythrocytes and polymorphonuclear leukocytes [5,14] or for the binding of *Actinomyces* to oral streptococci [43,44]; a scenario which is also found for human gut commensals such as lactobacilli [10,11], therefore suggesting that the adhesive function played by pili in bacteria represents a conserved functional role of these structures.

The past few years have seen dramatic advances in our knowledge on the genetics of probiotic bacteria such as bifidobacteria through genome sequencing of a large number of strains [19-24]. However, the mechanisms based on which such commensals recognize and interact with the human host are still unclear. The identification of possible receptors recognized by pili and the function of these receptors in signaling and

host defense mechanisms might provide new insights into the molecular mechanisms of microbe-host interactions. In this study we have shown how the expression of the genes encompassing the putative pilus-encoding loci is largely affected by the different composition of the substrate of cultivation, in particular with respect the presence of host-derived products (e.g., mucin or milk). It has been demonstrated that the corynebacterial minor pilin subunits (SpaC and SpaF) plays a pivotal role as receptor in adhesion of the pili to epithelial cells [14]. Considering that many known adhesive receptors in human cells are represented by carbohydrates [45], it is possible that the presence of specific glycans might trigger the expression of pilin-encoding genes. Bifidobacterial strains that were derived from a different ecological origin (e.g., oral vs. intestinal or human vs. animal) display very different transcription induction patterns of the presumptive pilus-gene clusters upon exposure to glycans, which might be a consequence of the fact that these bifidobacteria are adapted to different ecological niches, where the glycan composition is diverse. Furthermore, the identification of pilus-resembling structures decorating the cell surfaces of bifidobacteria whose genomes do not appear to specify sortase-dependent pili, such as *B. longum* subsp. *infantis* ATCC15697, might suggest the existence of additional types of pilus-like structures in bifidobacteria.

At this point we cannot conclusively state that the observed pilus-like surface appendages are encoded by the clusters of genes described in this study. However, recent investigations about the extracellular proteome of *B. animalis* subsp. *lactis* lead to identification of pilin subunits, which correspond to the *B. animalis* subsp. *lactis* pilus-encoding gene cluster (BLAC_1486-BLAC_1484) described in this study [46].

Most bifidobacteria are poorly, if at all, genetically accessible, and therefore it is currently not feasible to perform those experiments needed to confirm the link between the observed pilus structures and their suspected genetic determinants. Future functional genomics investigations directed to the silencing or mutagenesis of the pilus-encoding genes as well as immuno-EM experiments involving antibodies targeting the different pilin subunit will allow an in depth characterization of the genetics of the pilus-like surface appendages displayed by bifidobacteria.

In addition, further studies will be necessary to evaluate the binding activities of these identified pili and to explore how the various pilus-encoding gene clusters from a single microorganism might be expressed in response to colonization of diverse ecological niches and/or exposure to a different microbiota.

Additional material

Additional file 1: Primers used in RT-PCR experiments.

Additional file 2: Primers used in qRT-PCR experiments.

Additional file 3: Profile plot of pili-like structure obtained from the AFM-height images. The noise in the background is mainly due to polylysine used for coating mica.

Additional file 4: List of the pili-encoding genes used for the pyhlogenetic anlyses.

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Competing interests

The authors declare that they have no competing interests.

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References

1. Scott JR, Zahner D: **Pili with strong attachments: Gram-positive bacteria do it differently.** *Mol Microbiol* 2006, **62**:320-330.
2. Ton-That H, Schneewind O: **Assembly of pili on the surface of Corynebacterium diphtheriae.** *Mol Microbiol* 2003, **50**:1429-1438.
3. Ton-That H, Marraffini LA, Schneewind O: **Sortases and pilin elements involved in pilus assembly of Corynebacterium diphtheriae.** *Mol Microbiol* 2004, **53**:251-261.
4. Mishra A, Das A, Cisar JO, Ton-That H: **Sortase-catalyzed assembly of distinct heteromeric fimbriae in Actinomyces naeslundii.** *J Bacteriol* 2007, **189**:3156-3165.
5. Mishra A, Wu C, Yang J, Cisar JO, Das A, Ton-That H: **The Actinomyces oris type 2 fimbrial shaft FimA mediates co-aggregation with oral streptococci, adherence to red blood cells and biofilm development.** *Mol Microbiol* 2010.
6. Mora M, Bensi G, Capo S, Falugi F, Zingaretti C, Manetti AG, Maggi T, Taddei AR, Grandi G, Telford JL: **Group A Streptococcus produce pilus-like structures containing protective antigens and Lancefield T antigens.** *Proc Natl Acad Sci U S A* 2005, **102**:15641-15646.
7. Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G: **Pili in gram-positive pathogens.** *Nat Rev Microbiol* 2006, **4**:509-519.
8. Yeung MK, Ragsdale PA: **Synthesis and function of Actinomyces naeslundii T14V type 1 fimbriae require the expression of additional fimbria-associated genes.** *Infect Immun* 1997, **65**:2629-2639.
9. Nallapareddy SR, Singh KV, Sillanpaa J, Garsin DA, Hook M, Erlandsen SL, Murray BE: **Endocarditis and biofilm-associated pili of Enterococcus faecalis.** *J Clin Invest* 2006, **116**:2799-2807.

10. Kankainen M, Paulin L, Tynkkynen S, von Ossowski I, Reunanen J, Partanen P, Satokari R, Vesterlund S, Hendrickx AP, Lebeer S, *et al*: **Comparative genomic analysis of Lactobacillus rhamnosus GG reveals pili containing a human- mucus binding protein.** *Proc Natl Acad Sci U S A* 2009, **106**:17193-17198.
11. von Ossowski I, Reunanen J, Satokari R, Vesterlund S, Kankainen M, Huhtinen H, Tynkkynen S, Salminen S, de Vos WM, Palva A: **Mucosal adhesion properties of the probiotic Lactobacillus rhamnosus GG SpaCBA and SpaFED pilin subunits.** *Appl Environ Microbiol* 2010, **76**:2049-2057.
12. Pizarro-Cerda J, Cossart P: **Bacterial adhesion and entry into host cells.** *Cell* 2006, **124**:715-727.
13. Barocchi MA, Ries J, Zogaj X, Hemsley C, Albiger B, Kanth A, Dahlberg S, Fernebro J, Moschioni M, Masignani V, *et al*: **A pneumococcal pilus influences virulence and host inflammatory responses.** *Proc Natl Acad Sci U S A* 2006, **103**:2857-2862.
14. Mandlik A, Swierczynski A, Das A, Ton-That H: **Corynebacterium diphtheriae employs specific minor pilins to target human pharyngeal epithelial cells.** *Mol Microbiol* 2007, **64**:111-124.
15. Ventura M, Canchaya C, Tauch A, Chandra G, Fitzgerald GF, Chater KF, van Sinderen D: **Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum.** *Microbiol Mol Biol Rev* 2007, **71**:495-548.
16. Turroni F, Ribbera A, Foroni E, van Sinderen D, Ventura M: **Human gut microbiota and bifidobacteria: from composition to functionality.** *Antonie Van Leeuwenhoek* 2008, **94**:35-50.
17. Marco ML, Pavan S, Kleerebezem M: **Towards understanding molecular modes of probiotic action.** *Curr Opin Biotechnol* 2006, **17**:204-210.
18. O'Hara AM, Shanahan F: **Mechanisms of action of probiotics in intestinal diseases.** *ScientificWorldJournal* 2007, **7**:31-46.
19. Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zwahlen MC, Desiere F, Bork P, Delley M, *et al*: **The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract.** *Proc Natl Acad Sci U S A* 2002, **99**:14422-14427.
20. Sela DA, Chapman J, Adeuya A, Kim JH, Chen F, Whitehead TR, Lapidus A, Rokhsar DS, Lebrilla CB, German JB, *et al*: **The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome.** *Proc Natl Acad Sci U S A* 2008, **105**:18964-18969.
21. Barrangou R, Briczinski EP, Traeger LL, Loquasto JR, Richards M, Horvath P, Coute-Monvoisin AC, Leyer G, Rendulic S, Steele JL, *et al*: **Comparison of the complete genome sequences of Bifidobacterium animalis subsp. lactis DSM 10140 and Bl-04.** *J Bacteriol* 2009, **191**:4144-4151.
22. Lee JH, Karamychev VN, Kozyavkin SA, Mills D, Pavlov AR, Pavlova NV, Polouchine NN, Richardson PM, Shakhova VV, Slesarev AI, *et al*: **Comparative genomic analysis of the gut bacterium Bifidobacterium longum reveals loci susceptible to deletion during pure culture growth.** *BMC Genomics* 2008, **9**:247.
23. Ventura M, Turroni F, Zomer A, Foroni E, Giubellini V, Bottacini F, Canchaya C, Claesson MJ, He F, Mantzourani M, *et al*: **The Bifidobacterium dentium Bd1 genome sequence reflects its genetic adaptation to the human oral cavity.** *PLoS Genet* 2009, **5**:e1000785.
24. Turroni F, Bottacini F, Foroni E, Mulder I, Kim JH, Zomer A, Sanchez B, Bidossi A, Ferrarini A, Giubellini V, *et al*: **Genome analysis of Bifidobacterium bifidum PRL2010 reveals metabolic pathways for host-derived glycan foraging.** *Proc Natl Acad Sci U S A* 2010, **107**:19514-19519.
25. Felsenstein J: **An alternating least squares approach to inferring phylogenies from pairwise distances.** *Syst Biol* 1997, **46**:101-111.
26. Kimura M: **A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences.** *J Mol Evol* 1980, **16**:111-120.
27. Jaroszewski L, Rychlewski L, Li Z, Li W, Godzik A: **FFAS03: a server for profile–profile sequence alignments.** *Nucleic Acids Res* 2005, **33**:W284-288.
28. Canutescu AA, Shelenkov AA, Dunbrack RL Jr.: **A graph-theory algorithm for rapid protein side-chain prediction.** *Protein Sci* 2003, **12**:2001-2014.
29. Turroni F, Foroni E, O'Connell Motherway M, Bottacini F, Giubellini V, Zomer A, Ferrarini A, Delledonne M, Zhang Z, van Sinderen D, Ventura M: **Characterization of the serpin-encoding gene of Bifidobacterium breve 210B.** *Appl Environ Microbiol* 2010, **76**:3206-3219.
30. **Molecular Cloning. A manual laboratory.** Sambrook 1989.
31. Almagro-Moreno S, Boyd EF: **Insights into the evolution of sialic acid catabolism among bacteria.** *BMC Evol Biol* 2009, **9**:118.
32. Ton-That H, Schneewind O: **Assembly of pili in Gram-positive bacteria.** *Trends Microbiol* 2004, **12**:228-234.
33. Mandlik A, Swierczynski A, Das A, Ton-That H: **Pili in Gram-positive bacteria: assembly, involvement in colonization and biofilm development.** *Trends Microbiol* 2008, **16**:33-40.
34. Gesteland RF, C TR, Atkins JF: **RNA World.**, 4 2009.
35. Kang HJ, Paterson NG, Gaspar AH, Ton-That H, Baker EN: **The Corynebacterium diphtheriae shaft pilin SpaA is built of tandem Ig-like modules with stabilizing isopeptide and disulfide bonds.** *Proc Natl Acad Sci U S A* 2009, **106**:16967-16971.
36. Kline KA, Dodson KW, Caparon MG, Hultgren SJ: **A tale of two pili: assembly and function of pili in bacteria.** *Trends Microbiol* **18**:224-232.
37. Marraffini LA, Dedent AC, Schneewind O: **Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria.** *Microbiol Mol Biol Rev* 2006, **70**:192-221.
38. Swaminathan A, Mandlik A, Swierczynski A, Gaspar A, Das A, Ton-That H: **Housekeeping sortase facilitates the cell wall anchoring of pilus polymers in Corynebacterium diphtheriae.** *Mol Microbiol* 2007, **66**:961-974.
39. Filloux A: **A variety of bacterial pili involved in horizontal gene transfer.** *J Bacteriol* 2010, **192**:3243-3245.
40. Yeung MK, Cisar JO: **Cloning and nucleotide sequence of a gene for Actinomyces naeslundii WVU45 type 2 fimbriae.** *J Bacteriol* 1988, **170**:3803-3809.
41. Sillanpaa J, Nallapareddy SR, Prakash VP, Qin X, Hook M, Weinstock GM, Murray BE: **Identification and phenotypic characterization of a second collagen adhesin, Scm, and genome-based identification and analysis of 13 other predicted MSCRAMMs, including four distinct pilus loci, in Enterococcus faecium.** *Microbiology* 2008, **154**:3199-3211.
42. Ventura M, O'Flaherty S, Claesson MJ, Turroni F, Klaenhammer TR, van Sinderen D, O'Toole PW: **Genome-scale analyses of health-promoting bacteria: probiogenomics.** *Nat Rev Microbiol* 2009, **7**:61-71.
43. McIntire FC, Vatter AE, Baros J, Arnold J: **Mechanism of coaggregation between Actinomyces viscosus T14V and Streptococcus sanguis 34.** *Infect Immun* 1978, **21**:978-988.
44. Yeung MK: **Molecular and genetic analyses of Actinomyces spp.** *Crit Rev Oral Biol Med* 1999, **10**:120-138.
45. Kobayashi N, Seto K, Orikawa Y, Hamano H, Yoshinaga K, Takei M: **Z-360, a novel cholecystokinin-2/gastrin receptor antagonist, inhibits gemcitabine-induced expression of the vascular endothelial growth factor gene in human pancreatic cancer cells.** *Biol Pharm Bull* 2010, **33**:216-222.
46. Gilad O, Svensson B, Viborg AH, Stuer-Lauridsen B, Jacobsen S: **The extracellular proteome of Bifidobacterium animalis subsp. lactis BB-12 reveals proteins with putative roles in probiotic effects.** *Proteomics* 2011.

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7. Role of sortase-dependent pili of *Bifidobacterium bifidum* PRL2010 in modulating bacterium-host interactions

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Role of sortase-dependent pili of *Bifidobacterium bifidum* PRL2010 in modulating bacterium–host interactions

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Bifidobacteria represent one of the dominant groups of microorganisms colonizing the human infant intestine. Commensal bacteria that interact with a eukaryotic host are believed to express adhesive molecules on their cell surface that bind to specific host cell receptors or soluble macromolecules. Whole-genome transcription profiling of *Bifidobacterium bifidum* PRL2010, a strain isolated from infant stool, revealed a small number of commonly expressed extracellular proteins, among which were genes that specify sortase-dependent pili. Expression of the coding sequences of these *B. bifidum* PRL2010 appendages in nonpiliated *Lactococcus lactis* enhanced adherence to human enterocytes through extracellular matrix protein and bacterial aggregation. Furthermore, such piliated *L. lactis* cells evoked a higher TNF-α response during murine colonization compared with their nonpiliated parent, suggesting that bifidobacterial sortase-dependent pili not only contribute to adherence but also display immunomodulatory activity.

genomics | host–microbe interaction | probiotics | transcriptomics

The interaction between bacteria and their human host affects the latter in several ways. In some cases, this interaction may impact negatively on the health status of the host (pathogenesis), whereas in other cases may not influence host health at all (commensalism). In addition, certain bacterium–host interactions that represent symbiotic and probiotic relationships promote the health status of the host (1). Currently, the molecular mechanisms underlying the presumed health-promoting activities are largely unknown, despite the widely held view that microbial populations residing in the human gastrointestinal tract exert activities that positively affect host health (2). Bifidobacteria represent prominent commensals of the human infant gut (3) where they modulate metabolic and immune activities of their host (4–6). Through functional genomic approaches, significant progress has been made in unraveling bifidobacterial gut colonization strategies (1). In a recent study, we found that the genome of *Bifidobacterium bifidum* PRL2010 harbors an extensive gene set involved in the utilization of host-derived glycans, such as those found in the outermost layer of the intestinal mucosa (7). These findings are very suggestive of host-microbe coevolution, and signify *B. bifidum* PRL2010 as a bifidobacterial prototype for analysis of interactions between microbes and the intestinal mucosa. Many commensal and pathogenic microorganisms that interact with eukaryotic hosts express adhesive structures on their cell surface that mediate physical contacts between such bacteria and specific host cell receptors or soluble macromolecules (8). Bacterial surface appendages, such as pili or fimbrial adhesins in Gram-negative bacteria, have historically been considered to be the predominant bacterial structures involved in host–microbe interaction (8). Gene clusters responsible for the biosynthesis of pili have been identified in the genomes of many Gram-negative and Gram-positive bacteria, not only in pathogens (9–12) but also in gut inhabitants, such as *Lactobacillus rhamnosus* GG (13). Notably, sortase-dependent pili encoded by *L. rhamnosus* GG were demonstrated to be pivotal for efficient adherence and immunomodulatory interactions with human gut cells (14). In bifidobacteria, experimental evidence of the existence of sortase-dependent and type IV pili was reported only very recently (15, 16). Here, we describe in vivo analyses of the gut commensal *B. bifidum* PRL2010, with a focus on the role of sortase-dependent pili in host–microbe interaction.

Results and Discussion

Introduction of *B. bifidum* PRL2010 to the Murine Gut. Conventional female BALB/c mice were administered a single daily dose of 10^9 CFU *B. bifidum* PRL2010 (*SI Materials and Methods*). Mice were a priori checked for the presence of bifidobacteria in fecal samples by PCR using *Bifidobacterium*-specific primers (17), which revealed that bifidobacteria were either absent or below the limit of detection. Animals were killed 12 d later, allowing sufficient time for several cycles of turnover of the intestinal epithelium and its overlying mucus layer (18). Microbial evaluation of the murine gut showed the presence of strain PRL2010 at stable numbers, reminiscent of at least transient colonization over time with the highest numbers of this strain recorded in the cecum and colon (Fig. S1). Given the robustness of its colonization of the distal gut, we focused on determining adaptations of PRL2010 to the caecal habitat.

***B. bifidum* PRL2010 Transcriptome Under in Vitro and in Vivo Conditions.** To investigate possible interactions of PRL2010 with its natural ecological niche, the gut, we performed global genome transcription profiling of this strain in an in vitro human gut model using HT29 cells, as well as upon colonization of PRL2010 of the murine gut using a custom-made *B. bifidum* PRL2010 array representing 90% of the identified genes of this organism (7). The global gene expression profile of *B. bifidum* PRL2010 was conserved in the caeca between different mice (Fig. 1*A*).

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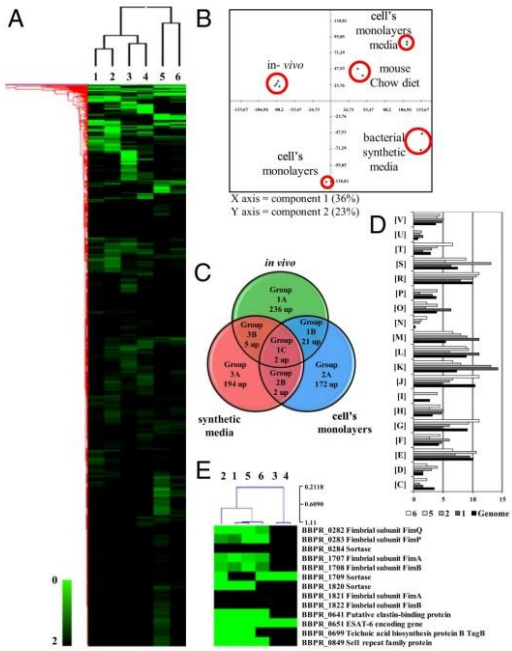

Fig. 1. Identification of *B. bifidum* PRL2010 differentially expressed genes by transcriptome analysis in response to contact with the host. (*A*) Heat-map displaying the change in PRL2010 gene expression upon colonization of murine caeca (lanes 1 and 2), when grown in DMEM synthetic medium (lanes 3) or in fresh chow diet (lane 4), and following incubation with human intestinal HT29 cells (lanes 5 and 6). Each row represents a separate transcript and each column represents a separate sample. Color legend is on the bottom of the microarray plot; green indicates increased transcription levels compared with the reference samples. The reference conditions used were as follows: lane 1, fresh chow diet; lanes 2–4 and 6, MRS medium; lane 5, DMEM. Dendrogram on the left margin of the heat-map represents the hierarchical clustering algorithm result based on average linkage and Euclidean distance of the gene dataset. (*B*) The clustering of PRL2010 transcriptomes under in vitro and in vivo conditions by principal component analysis. (*C*) Venn diagram showing the number of genes expressed during the different conditions: in vitro (exposure to a human cell line), in vivo, and DMEM synthetic medium. (*D*) Depiction of a functional annotation of the in vitro- and in vivo-expressed genes of *B. bifidum* PRL2010 according to their COG categories. Each COG family is identified by a one-letter abbreviation (National Center for Biotechnology Information database). For each category, the black bar represents the percentage of genes in that category as detected in the sequenced genome of PRL2010 (7). The other bar shows the percentages of genes transcribed during murine colonization by PRL2010 (conditions 1 and 2), and following exposure of PRL2010 cells to human intestinal cells (conditions 5 and 6). The percentage was calculated as the percentage of transcribed genes belonging to the indicated COG category with respect to all transcribed genes. (*E*) Selected genes that were up-regulated when PRL2010 were cultivated in the conditions indicated in *A*.

According to a principal component analysis, these profiles were clearly different from the transcriptomes of PRL2010 obtained under in vitro conditions (HT29 monolayer or laboratory cultures) (Fig. 1*B*). This finding suggests, not unexpectedly, distinctly different transcriptional responses of PRL2010 to each of these environments. A total of 104 or 62 genes exhibited a ≥twofold change ($P < 0.0005$) in transcription upon bringing PRL2010 cells in contact with HT29 cells, using the transcriptome of PRL2010 grown in DMEM or Man-Rogosa-Sharp (MRS) synthetic medium as a reference, respectively (Fig. S1*B*). Analysis of the transcriptome of PRL2010 obtained when this microorganism was present in the cecum of conventional BALB/c mice showed that transcription of 87 or 141 genes was increased more than twofold compared with their transcription level in PRL2010 cells when obtained from the caecal contents of mice fed on fresh Chow diet or when grown on MRS, respectively. The comparative analysis shown in Fig. 1*A* and Fig. S1*B* yielded three groups of regulated genes, including those specific to in vivo conditions (group 1), to in vitro experiments (i.e., exposure to a human cell line, group 2), and to growth in synthetic medium under laboratory conditions (group 3). We also categorized genes from these three groups into three subgroups based on whether the genes were contributed from a single environmental condition (i.e., human cell line model, in vivo, or synthetic media datasets) (subgroups A), or two conditions (subgroups B), or all three conditions (subgroup C) (Fig. 1*C*). Assignment of genes into these groups may reflect bacterial responses to differences in host structures. These differences could be species-specific (human vs. murine), tissue-specific (colon vs. cecal mucosa), or they could also be linked to differential carbohydrate availability or to the effect of the residential intestinal microorganisms. We used cluster of orthologous groups (COG) analysis to identify differentially transcribed genes that contribute to specific biological functions. As illustrated in Fig. 1*D*, carbohydrate metabolism, corresponding to COG category [G], is one of the COG functions of PRL2010 most significantly affected by the interaction with the murine host, which is probably because of a response to the presence of specific host glycans, in particular mucin (7). Various members of this COG function were significantly up-regulated (≥twofold; $P < 0.0005$) under in vivo conditions, encompassing genes involved in breakdown of glycoproteins (Fig. 1*A*).

PRL2010 cell surface properties also appear to be modified in response to tissue contact, as indicated by the increased transcription of genes encoding several extracellular and membrane-spanning proteins, many of which are predicted to mediate interaction with eukaryotic cells (Fig. 1*E*). Adhesion of bacteria to human intestinal mucosa or extracellular matrix (ECM) proteins represents a key strategy for intestinal colonization, and bifidobacteria can indeed adhere to intestinal cells (19, 20). Genes that specify putative adhesion functions for PRL2010 include BBPR_0641, which specifies a putative elastin-binding protein and whose transcription was significantly induced under in vivo conditions as well as upon exposure to HT29 cells (18- to 24-fold). It is known that elastin-binding proteins promote recognition of mammalian ECM, thus allowing colonization of the host by gut bacteria (21). Furthermore, transcription of BBPR_0651, whose protein product displays similarity to early secretory antigen target 6 (ESAT-6), was shown to be highly induced following HT29 exposure and when PRL2010 was present in the murine gut. The small ESAT-6 protein appears to be of fundamental importance in virulence and protective immunity in *Mycobacterium tuberculosis* and *Staphylococcus aureus* (22), suggesting that the homologous protein of PRL2010 acts as a protective immunity determinant. PRL2010 contact with HT29 cells and its presence in mice also triggered the transcription of another gene (BBPR_0699), predicted to encode a protein involved in the biosynthesis of teichoic acids, which for *Lactobacillus acidophilus* NCFM have been shown to modulate host–microbe interaction (23). Two of the three pilus clusters identified on the PRL2010 genome (7, 15) [i.e., $pil2_{PRL2010}$ (BBPR_1707-BBPR_1709), and $pil3_{PRL2010}$ (BBPR_282-BBPR_284)] were shown to be expressed under both in vitro and in vivo conditions. Notably, and in contrast to the adjacent pilin subunit-encoding genes (BBPR_1707 and BBPR_1708), BBPR_1709, which specifies a predicted sortase, was shown to be expressed when PRL2010 was grown in MRS medium, suggesting that this sortase also processes other cell wall-anchored proteins. This finding is supported by

a previously reported finding that the BBPR_1709 gene is transcribed separately from the other components of this pilus gene cluster (15). The predicted pilus proteins are similar to subunits of so-called sortase-dependent pili, which are typically composed of a major pilin subunit (represented by $FimA_{PRL2010}$ or $FimP_{PRL2010}$ for the $pil2_{PRL2010}$ and $pil3_{PRL2010}$ clusters, respectively), and one or two ancillary minor pilin subunits (represented by $FimB_{PRL2010}$ and $FimQ_{PRL2010}$ for the $pil2_{PRL2010}$ and $pil3_{PRL2010}$ clusters, respectively) (Fig. S2) (15). When we compared $FimA_{PRL2010}$ to FimA homologs encoded by other *B. bifidum* strains, their amino acid sequences were shown to display much higher variability compared with the FimP homologs (Fig. S2). In addition, $FimA_{PRL2010}$ contains a CnaB-type domain, which is described to act as a stalk in binding to components of the ECM of the host, such as fibronectin, collagen types I to XV, and laminin (24). Atomic force microscopy (AFM) assays of PRL2010 cells that had been exposed to human cell lines (Caco-2 or HT29) revealed a highly piliated cell morphology (Fig. S3*B*). Interestingly, we found by AFM that pili were also present in PRL2010 cultivated in liquid media or on agar plates, although pili appeared to be less abundant compared with the numbers seen upon contact with a human cell line (Fig. S3 *A*–*D*). Furthermore, Western blot analysis using antibodies that had been raised against the major subunit protein of the *pil*2 or *pil*3 loci, $FimA_{PRL2010}$ and $FimP_{PRL2010}$, respectively (Ab_{pil2} or Ab_{pil3}) (*SI Materials and Methods*) was performed on PRL2010 cells that had been in contact with Caco-2 monolayers or cultivated on agar plates. Clear signals representing a protein of 55.87 kDa and 55.43 kDa were noticed when a crude extract of PRL2010 cells, previously exposed to Caco-2 monolayers, was probed in a Western blot using Ab_{pil2} or Ab_{pil3}, respectively (Fig. S3*E*). In contrast, Western blot signals of lower intensity were observed using protein extracts from PRL2010 cultivated on agar plates or MRS plus lysine. The higher molecular weight signals above 100 kDa detected in each immunoblot image (Fig. S3*E*) likely represent the covalently linked polymers of $FimA_{2010}$ and $FimP_{2010}$, a typical feature of sortase-dependent pili (12, 25).

Differential Binding to Human Epithelial Cells Mediated by PRL2010 Pili. To obtain further insight into the functional roles exerted by pili encoded by PRL2010, we expressed the $pil2_{PRL2010}$ and $pil3_{PRL2010}$ gene clusters in the Gram-positive host *Lactococcus lactis* NZ9000 (*SI Materials and Methods*), because genetic manipulation of PRL2010, such as creating knockout mutants, is currently not possible. *L. lactis* has previously been used successfully as a heterologous host for expression of bifidobacterial proteins (26, 27), as well as a host to display full-length forms of microbial surface structures (28). AFM analysis revealed that both *L. lactis-pil2*$_{PRL2010}$ and *L. lactis-pil3*$_{PRL2010}$ clones displayed evident piliated morphology when pilus expression had been induced with nisin, but no pili were observed in noninduced *L. lactis* controls (Fig. S4 *A*–*C*). Furthermore, the cell surface of (nisin-induced) *L. lactis-pil2*$_{PRL2010}$ and *L. lactis-pil3*$_{PRL2010}$ clones was less densely piliated than that of the wild strain *B. bifidum* PRL2010 (Figs. S3 *A*–*D* and S4 *B* and *D*).

Based on previous findings for analogous structures in *Actinomyces oris* (29), we decided to evaluate possible interactions between *B. bifidum* PRL2010 pili structures and a Caco-2 human intestinal epithelial cell line, to establish if they are involved in bacterial adhesion to human enterocytes. To this aim, we used *L. lactis-pil2*$_{PRL2010}$ and *L. lactis-pil3*$_{PRL2010}$ cells in adhesion experiments using a Caco-2 differentiated cell layer. Remarkably, following nisin induction *L. lactis-pil3*$_{PRL2010}$ displayed a significant enhancement in adhesion to eukaryotic cells compared with noninduced *L. lactis-pil3*$_{PRL2010}$ (Fig. 2 *A* and *B*). In contrast, *L. lactis-pil2*$_{PRL2010}$ cells did not display any significant change in adhesion properties under the conditions tested (Fig. 2 *C* and *D*). A clear adhesion phenotype was also noticed in the wild-type strain *B. bifidum* PRL2010 (Fig. 2 *E* and *F*); the observed Caco-2-adhesive behavior was displayed by PRL2010, which is clearly more pronounced than that observed for nisin-induced *L. lactis-pil2*$_{PRL2010}$ cells, and which may be because of the elevated abundance of pili structures in PRL2010 cells compared with those produced in the heterologous host, or the result of the presence of additional adhesion promoting cell-surface molecules on PRL2010, such as BopA (19). These results therefore demonstrate direct involvement of bifidobacterial sortase-dependent pili structures in mediating adhesion to human intestinal cells, thus implicating these extracellular structures in host colonization by the infant intestinal commensal *B. bifidum* PRL2010. To substantiate these findings, competitive adhesion assays involving piliated *L. lactis*-pil3$_{PRL2010}$ cells that had first been treated with Ab_{pil3}, were performed. This experiment showed that treatment with Ab_{pil3} decreased adhesion of piliated *L. lactis*-pil3$_{PRL2010}$ cells to Caco-2 cells by maximum 17-fold ($P < 0.05$) (Fig. 2*G*).

Bacterial Aggregation and PRL2010 Pili. Sortase-assembled pili are also known to promote bacterial coaggregation (30). We therefore investigated if a similar scenario would apply to the *B. bifidum* PRL2010 pili. We performed aggregation experiments involving piliated *L. lactis-pil3*$_{PRL2010}$, *L. lactis-pil2*$_{PRL2010}$, and their nonpiliated *L. lactis* equivalent strains. Notably, in the case of nisin-induced *L. lactis-pil2*$_{PRL2010}$ and *L. lactis-pil3*$_{PRL2010}$ cells (from here on referred as piliated *L. lactis-pil2*$_{PRL2010}$ and *L. lactis-pil3*$_{PRL2010}$ cells), aggregation levels enhanced 13- and 21-fold with respect to their uninduced equivalents ($P < 0.05$) (from here on referred as nonpilated *lactis-pil2*$_{PRL2010}$ and *L. lactis-pil3*$_{PRL2010}$ cells), respectively (Fig. S5). Taken together, these data implicate $Pil2_{PRL2010}$ and $Pil3_{PRL2010}$ as important factors that promote bacterial autoaggregation, possibly with a contributing role in gut colonization. These findings corroborate the view that bacterial aggregation represents a mechanism by which gastrointestinal commensals adhere to each other and, as a result, may colonize persistently in biofilms on the host's mucosa (31).

Human Receptors for PRL2010 Pili. Although we identified sortase-dependent pili as a PRL2010 adhesion factor that mediates binding to epithelial cells, the receptors involved in their recognition are unknown. As enteropathogens are known to adhere to intestinal tissue by pili-mediated binding to ECM proteins (32–34), we examined the ability of PRL2010 to adhere to the ECM proteins fibrinogen, plasminogen, fibronectin, laminin, and collagen type IV. Notably, *B. bifidum* PRL2010 cells showed higher adhesion to fibronectin, plasminogen, and laminin, compared with the other ECM proteins (Fig. 3*A*). A similar scenario was noticed when piliated *L. lactis-pil3*$_{PRL2010}$ as well as *L. lactis-pil2*$_{PRL2010}$ clones were used. In contrast, nonpiliated *L. lactis* cells displayed very limited adhesion to these ECM substrates, as confirmed by microscopic examination of the samples (Fig. 3*A*). Binding of piliated *L. lactis-pil3*$_{PRL2010}$ and *L. lactis-pil2*$_{PRL2010}$ cells to serial dilutions of ECM substrates was evaluated, showing that saturation of binding already occurred at low concentrations of the ECM protein tested (Fig. S6). To evaluate whether the binding of *B. bifidum* PRL2010 to fibronectin, which appeared to be the most effective ECM substrate, occurs for strains besides our reference strain, other bifidobacterial strains were tested. Notably, within the *B. bifidum* species, strain PRL2010 displayed the highest adhesion level, whereas other bifidobacterial strains belonging to *Bifidobacterium breve*, *Bifidobacterium adolescentis*, and *Bifidobacterium longum* subsp. *infantis*, displayed much lower levels of binding to fibronectin, plasminogen, and laminin (Fig. 3*B*), which may reflect different strategies used by these bacteria to colonize the human gut. All these strains except *B. longum* subsp. *infantis* ATCC15697 encode putative sortase-dependent pili, and they were treated and cultivated under the same conditions as PRL2010. Considering that fibronectin is a glycoprotein and that carbohydrate residues have been shown to be involved in fimbrial binding to fibronectin (35), we addressed the possible involvement of fibronectin-associated glycans in PRL2010 pili binding. Fibronectin deglycosylation of *O*-linked and *N*-linked oligosaccharides

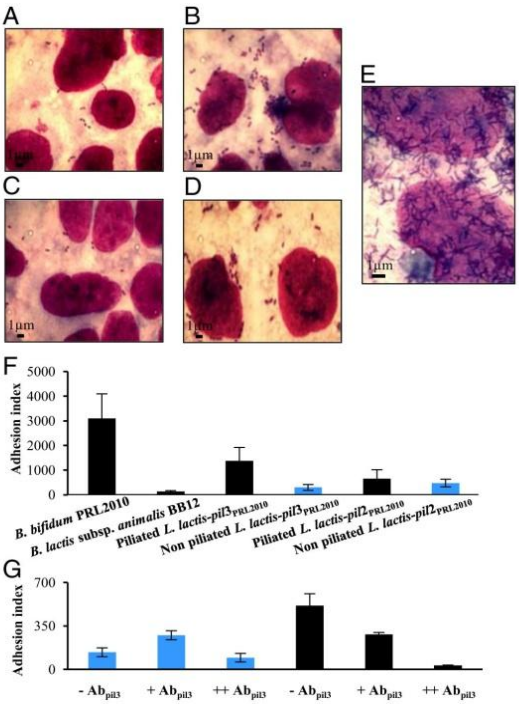

Fig. 2. Adhesion of *B. bifidum* PRL2010 sortase-assembled pili to human intestinal cells. (*A* and *B*) Display of the adhesion phenotype of nonpiliated (*A*) and piliated (*B*) *L. lactis-pil3*$_{PRL2010}$ cells to the Caco-2 cell monolayer. (*C* and *D*) The adhesion phenotype of nonpiliated (*C*) and piliated (*D*) *L. lactis-pil2*$_{PRL2010}$ cells to the Caco-2 cell monolayer. (*E*) The adhesion of *B. bifidum* PRL2010 cells to Caco-2 cell monolayer. (*F*) The adhesion efficiency (adhesion index) of *B. bifidum* PRL2010, piliated (black pillar) and nonpiliated (blue pillar) *L. lactis-pil2*PRL2010 cells, as well as *L. lactis-pil3*PRL2010 cells determined in terms number of adhered bacterial cells per 100 Caco-2 cells. The negative control is represented by *B. animalis* subsp. *lactis* BB12 cells. (*G*) Depiction of inhibition pili-mediated adhesion to the Caco-2 cell monolayer by *L. lactis*-pil3$_{PRL2010}$ cells, which had first been treated with anti-pil3 antibodies (Ab$_{pil3}$). Piliated *L. lactis-pil3*$_{PRL2010}$ cells (black pillar) and nonpiliated (blue pillar) *L. lactis-pil2*$_{PRL2010}$ cells were used. Two different concentrations of antibodies were used. +, Represents the use of diluted (1:50) Ab$_{pil3}$; ++, undiluted Ab$_{pil3}$. Bars represent mean values of three independent experiments, and the error bars indicate the SD ($P < 0.05$).

caused a significant reduction in PRL2010 pili-mediated binding ability compared with untreated fibronectin (Fig. 3c), suggesting that *N*- and *O*-linked glycoproteins are involved in adhesion of PRL2010 pili to fibronectin. Because it was previously shown that certain carbohydrates bind to sortase-dependent pili, thereby competing with the actual pilus receptor (32, 33, 35), we evaluated the effect of various carbohydrates on binding of PRL2010 Pil2 and Pil3 to fibronectin. Interestingly, we found that the binding of this ECM protein to piliated *L. lactis-pil2*$_{PRL2010}$ was significantly reduced when mannose or fucose was present during the binding assay. Binding ability of piliated *L. lactis-pil3*$_{PRL2010}$ was also affected by glucose and galactose, but not by the polysaccharide xylan (Fig. 3*D*). This finding suggests that mannose and fucose act as potential receptors for Pil2 of *B. bifidum* PRL2010 reminiscent of the pili behavior of enteric bacteria (32, 33, 35), whereas the putative receptors of Pil3 seem to include a wider spectrum of carbohydrates.

Immunomodulatory Activity Exerted by PRL2010 Pili. Similar to other extracellular structures encoded by several enteropathogens colonizing the human gut (36), we wanted to explore the possible roles played by *B. bifidum* PRL2010 pili in triggering (aspects of) the immune system of its human host. When we assayed the impact of the *L. lactis* clones producing Pil2$_{PRL2010}$ and Pil3$_{PRL2010}$ on cytokine expression by human macrophage-like cell line U937, we noticed a different cytokine modulation exerted by Pil2 and Pil3. Notably, piliated *L. lactis-pil3*$_{PRL2010}$ clones displayed a significant induction (10-fold; $P < 0.05$) of the TNF-α mRNA levels compared with nonpiliated *L. lactis-pil3*$_{PRL2010}$ (Fig. S7*A*). In contrast, piliated *L. lactis-pil2*$_{PRL2010}$ clones did not appear to have any effect on the expression of the four cytokines that we assessed (Fig. S7*A*). Presence or absence of PRL2010 pili might therefore explain the difference in TNF-α response. To test this possibility directly, the TNF-α response was measured in challenging mice treated with piliated *L. lactis-pil3*$_{PRL2010}$ cells.

Pili of PRL2010 Affect the Cytokine Profiles in a Mouse Model. To investigate the role of sortase-dependent pili in PRL2010 colonization in mammals, piliated *L. lactis-pil3*$_{PRL2010}$ and non piliated *L. lactis-pil3*$_{PRL2010}$ were used in murine models. To mimic the natural route of gut microbial colonization, a 10^9 CFU dose of microencapsulated lactococci (to prevent pili removal from *L. lactis* during gastric transit) was orally administered daily to 12-wk-old BALB/c mice. Production of pili was induced before microencapsulation of lactococci by the addition of nisin (*SI Materials and Methods*). Furthermore, to ensure proper delivery of piliated/nonpiliated lactococci, we used alginate microencapsulation, which is known to release encapsulated bacteria following gastric transit (37). Mice were killed 4 h following the last lactococcal administration, and cytokine expression profiles were determined (Fig. S7*B*). Notably, under these in vivo conditions the piliated *L. lactis-pil3*$_{PRL2010}$ evoked TNF-α expression and a significantly lower IL-10 response compared with the nonpiliated *L. lactis-pil3*$_{PRL2010}$ (Fig. S7*B*) in murine cecum mucosa samples. These results reinforce the notion that pili of PRL2010 can influence the host innate immunity in a similar manner as previously outlined for other human gut commensals, such as *Bacteroides* (38).

Reportedly, and in accordance with our results, bifidobacteria can be strong inducers of TNF-α but weak inducers of other proinflammatory cytokines (39, 40), which are more specifically involved in mounting responses at systemic level, such as IL-12 (41, 42). Therefore, the immunomodulatory effects elicited by PRL2010 Pil3 may be delimited at local level, as previously suggested for other bifidobacterial strains, potentially because of insufficient induction of antigen-presenting cell maturation (41, 42). More specifically, a local induction of TNF-α could be important for the initiation of cross-talk among immune cells without causing any inflammation or detrimental effects (43).

Conclusions

Various ecological studies have demonstrated that bifidobacteria are a dominant bacterial group of the (human) infant gut microbiota, as well as part of the intestinal microbiota of an adult human being (3, 17). However, relatively little is known about the molecular basis sustaining their ability to colonize the human gut and to interact with the intestinal mucosa. Bifidobacteria arguably use a variety of mechanisms that may facilitate interactions with the intestinal mucosa at different life stages of the host, but perhaps also pertaining to different compartments of the gastrointestinal tract of the host (5, 16). The intimate attachment to the intestinal mucosa is presumed to be pivotal to allow colonization by gut commensals. Here, we describe the presence of a number of extracellular protein-encoding genes whose transcription is specifically up-regulated when our model bacterium *B. bifidum* PRL2010 was placed in contact with

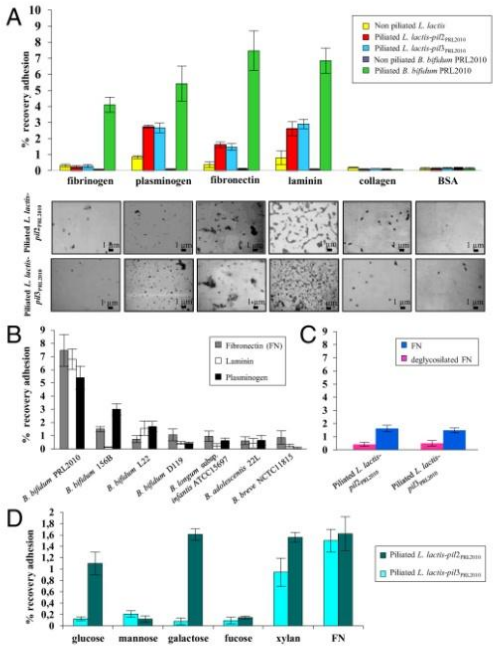

Fig. 3. Involvement of pili produced by *B. bifidum* PRL2010 in binding to ECM proteins. (*A*) Adhesion of *B. bifidum* PRL2010 cells, *L. lactis-pil2*$_{PRL2010}$ cells, and *L. lactis-pil3*$_{PRL2010}$ cells to various ECM proteins. Below each pillar a picture is placed to indicate how piliated and nonpiliated *L. lactis-pil2*$_{PRL2010}$ cells and *L. lactis-pil3*$_{PRL2010}$ cells appeared under the microscope following exposure to ECM substrates. For each of these experiments, adhesion of microbial cells to BSA was used as negative control. Piliated PRL2010 represent PRL2010 cells grown under conditions that promote the production of sortase-dependent pili (cells grown on MRS agar or grown to stationary phase in MRS broth plus lysine), and nonpiliated PRL2010 represent PRL2010 cells grown under conditions that reduce production of sortase-dependent pili (cells grown in MRS broth to exponential phase). (*B*) Adhesion of different bifidobacterial strains to fibronectin, plasminogen and laminin. (*C*) Adhesion of piliated *L. lactis-pil2*$_{PRL2010}$ and *L. lactis-pil3*$_{PRL2010}$ cells to deglycosylated fibronectin. (*D*) Adhesion ability of piliated *L. lactis-pil2*$_{PRL2010}$ and *L. lactis-pil3*$_{PRL2010}$ to fibronectin in the presence of different carbohydrates. Bars represent mean values for three independent experiments, and the error bars indicate the SD ($P < 0.05$).

human cell lines or when present in the murine gut. Remarkably, among these specific host-induced genes we identified two loci that encode sortase-dependent pili. These appendages that decorate the bacterial surface are considered key molecules in mediating bacterial adherence to the host epithelium and may thus influence mucosal immune responses (12, 44). It was recently shown that type IV or Tad pili encoded by *B. breve* UCC2003 are required for colonization and persistence of this bacterium in the murine gut (16). However, in the case of *B. bifidum* PRL2010, the role of Tad pili in gut colonization and gut adhesion is currently not known. The genes that are responsible for the biosynthesis of the Tad pili are strictly conserved within bifidobacterial genomes, but the gene clusters that specify sortase-dependent pili production vary in number and sequence, and may thus represent strain-specific pili combinations. Thus, in contrast to Tad pili, sortase-dependent pili may impart unique features to a bifidobacterial strain among the complex microbiota present in the human gut, such as modulating specific host-microbe responses. In this context we noticed that piliated *L. lactis-pil3*$_{PRL2010}$ clones evoked a higher TNF-α response during mouse colonization, compared with nonpiliated *L. lactis-pil3*$_{PRL2010}$ clones, suggesting that PRL2010 pili not only contribute to adherence but also act as immunogenic effectors. Triggering of increased TNF-α production by pili encoded by *B. bifidum* PRL2010 may be an interesting feature of this species as one of the first colonizers of the human gut (3). In fact, cytokines belonging to the TNF-α superfamily are not only linked to the occurrence of inflammatory diseases (45), but also play a role in the rejection of tumors and the response to infections (46, 47). In addition, the induction of TNF-α may be important for the initiation of cross-talk among immune cells without causing any inflammation or detrimental effects (43). In fact, the infant's immune system is immature and the presence of proinflammatory stimuli, such as those exerted by pili encoded by *B. bifidum*, may be crucial in developmental immunological programming. In this context, it is well known that decreased antigenic exposure has adverse effects on the budding immune system and increases the likelihood of developing atopic disorders (48). In addition, a transient inflammatory state could aid host defense. Thus, as recently suggested, the difference between pathogenic and gut commensal bacteria is in the magnitude of the immune response evoked, which can be defined as strong, intermediate or homeostatic (49).

The genome of *B. bifidum* PRL2010 encompasses three different loci encoding predicted sortase-dependent pili, of which only *pil*2 and *pil*3 appear to be functional as one of the pilus subunit-encoding genes of *pil*1 contains a frameshift (15). Here, we have shown that expression of *pil*2 and *pil*3 enhances adherence to enterocytes and modulates the host inflammatory response (for *pil*3), but it also promotes bacterial aggregation (for *pil*2). A possible model explaining the role of pili in host-PRL2010 interactions envisages an initial attachment of planctonic PRL2010 cells to the enterocytes by extending their pili, either sortase-dependent or Tad pili, toward the apical surface of the host cells. This initial adhesion to enterocytes is followed by a more intimate attachment driven by the establishment of the linkage between pili and specific host receptors, such as host-glycoproteins. In addition, pilus-mediated PRL2010 aggregation further assists the formation of a microbial community in the proximity of the colonized enterocytes. Pil2 and Pil3 were shown to exhibit different binding abilities with respect to carbohydrates, where Pil2 is able to adhere to typical mammalian gut carbohydrates, which appear to include fucose and mannose, and Pil3 showed an apparent ability to adhere to a wider set of carbohydrates, many of which would be expected to be present in the diet, thus suggesting that such diet-derived carbohydrates modulate PRL2010 gut colonization. Colonization as a result of a wide variety of host and bacterial factors, together with increased bacterial cell density, can lead to an enhanced innate immune response. This finding is in line with the notion that mammals depend on critical gene products from their gut microbiota to fully develop their immune system (50). Hence, the presence or absence of distinct *B. bifidum* sortase-dependent pili may represent pivotal molecules in colonization and persistence within the human gut, and may have a profound effect in terms of the developmental programming of the host immune system, which is in line with observations previously noted for other gut symbionts (51).

Materials and Methods

B. bifidum PRL2010 and *L. lactis* were manipulated and used as described in *SI Materials and Methods*. Detailed descriptions of bacterial strains, plasmids, and oligonucleotides (Table S1) used in this study, as well as methods for gene expression analyses, AFM investigations, bacterial adhesion assays, activation of human macrophage cell line, murine trials, and Western blot experiments are provided in *SI Materials and Methods*. The transcriptional array data have been deposited in the GEO database under accession no. GSE36442.

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1. Ventura M, et al. (2009) Genome-scale analyses of health-promoting bacteria: Probiogenomics. *Nat Rev Microbiol* 7(1):61–71.
2. Guarner F, Malagelada JR (2003) Gut flora in health and disease. *Lancet* 361(9356): 512–519.
3. Turroni F, et al. (2012) Diversity of bifidobacteria within the infant gut microbiota. *PLoS ONE* 7(5):e36957.
4. Ventura M, Turroni F, Motherway MO, MacSharry J, van Sinderen D (2012) Host-microbe interactions that facilitate gut colonization by commensal bifidobacteria. *Trends Microbiol* 20(10):467–476.
5. Fanning S, et al. (2012) Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. *Proc Natl Acad Sci USA* 109(6):2108–2113.
6. Macfarlane S, Bahrami B, Macfarlane GT (2011) Mucosal biofilm communities in the human intestinal tract. *Adv Appl Microbiol* 75:111–143.
7. Turroni F, et al. (2010) Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. *Proc Natl Acad Sci USA* 107(45): 19514–19519.
8. Kline KA, Fälker S, Dahlberg S, Normark S, Henriques-Normark B (2009) Bacterial adhesins in host-microbe interactions. *Cell Host Microbe* 5(6):580–592.
9. Ton-That H, Schneewind O (2004) Assembly of pili in Gram-positive bacteria. *Trends Microbiol* 12(5):228–234.
10. Nallapareddy SR, et al. (2006) Endocarditis and biofilm-associated pili of *Enterococcus faecalis*. *J Clin Invest* 116(10):2799–2807.
11. Scott JR, Zähner D (2006) Pili with strong attachments: Gram-positive bacteria do it differently. *Mol Microbiol* 62(2):320–330.
12. Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G (2006) Pili in gram-positive pathogens. *Nat Rev Microbiol* 4(7):509–519.
13. Kankainen M, et al. (2009) Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human-mucus binding protein. *Proc Natl Acad Sci USA* 106(40):17193–17198.
14. Lebeer S, et al. (2012) Functional analysis of *Lactobacillus rhamnosus* GG pili in relation to adhesion and immunomodulatory interactions with intestinal epithelial cells. *Appl Environ Microbiol* 78(1):185–193.
15. Foroni E, et al. (2011) Genetic analysis and morphological identification of pilus-like structures in members of the genus *Bifidobacterium*. *Microb Cell Fact* 10(Suppl 1):S16.
16. O'Connell Motherway M, et al. (2011) Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. *Proc Natl Acad Sci USA* 108(27):11217–11222.
17. Turroni F, et al. (2009) Exploring the diversity of the bifidobacterial population in the human intestinal tract. *Appl Environ Microbiol* 75(6):1534–1545.
18. Wolfensohn S, Lloyd M (2003) *Handbook of Laboratory Animal Managment and Welfare* (Blackwell, Oxford).
19. Guglielmetti S, et al. (2008) Implication of an outer surface lipoprotein in adhesion of *Bifidobacterium bifidum* to Caco-2 cells. *Appl Environ Microbiol* 74(15):4695–4702.
20. Guglielmetti S, et al. (2009) Study of the adhesion of *Bifidobacterium bifidum* MIMBb75 to human intestinal cell lines. *Curr Microbiol* 59(2):167–172.
21. Walsh EJ, O'Brien LM, Liang X, Hook M, Foster TJ (2004) Clumping factor B, a fibrinogen-binding MSCRAMM (microbial surface components recognizing adhesive matrix molecules) adhesin of *Staphylococcus aureus*, also binds to the tail region of type I cytokeratin 10. *J Biol Chem* 279(49):50691–50699.
22. Pallen MJ (2002) The ESAT-6/WXG100 superfamily—And a new Gram-positive secretion system? *Trends Microbiol* 10(5):209–212.
23. Mohamadzadeh M, et al. (2011) Regulation of induced colonic inflammation by *Lactobacillus acidophilus* deficient in lipoteichoic acid. *Proc Natl Acad Sci USA* 108:(Suppl 1):4623–4630.
24. Deivanayagam CC, et al. (2000) Novel fold and assembly of the repetitive B region of the *Staphylococcus aureus* collagen-binding surface protein. *Structure* 8(1):67–78.
25. Ton-That H, Marraffini LA, Schneewind O (2004) Sortases and pilin elements involved in pilus assembly of *Corynebacterium diphtheriae*. *Mol Microbiol* 53(1):251–261.
26. Alvarez-Martin P, et al. (2012) A two-component regulatory system controls autoregulated serpin expression in *Bifidobacterium breve* UCC2003. *Appl Environ Microbiol* 78(19):7032–7041.
27. Ruiz L, Zomer A, O'Connell-Motherway M, van Sinderen D, Margolles A (2012) Discovering novel bile protection systems in *Bifidobacterium breve* UCC2003 through functional genomics. *Appl Environ Microbiol* 78(4):1123–1131.
28. Corrigan RM, Miajlovic H, Foster TJ (2009) Surface proteins that promote adherence of *Staphylococcus aureus* to human desquamated nasal epithelial cells. *BMC Microbiol* 9:22.
29. Mishra A, et al. (2010) The *Actinomyces oris* type 2 fimbrial shaft FimA mediates coaggregation with oral streptococci, adherence to red blood cells and biofilm development. *Mol Microbiol* 7:841–854.
30. Yeung MK (2000) Actinomyces: Surface macromolecules and bacteria-host interactions. *Gram-Positive Pathogens*, eds Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JI (American Society for Microbiology, Washington, DC), pp 583–593.
31. Kolenbrander PE (1995) Coaggregations among oral bacteria. *Methods Enzymol* 253: 385–397.
32. Farfan MJ, Cantero L, Vidal R, Botkin DJ, Torres AG (2011) Long polar fimbriae of enterohemorrhagic *Escherichia coli* O157:H7 bind to extracellular matrix proteins. *Infect Immun* 79(9):3744–3750.
33. Torres AG, Slater TM, Patel SD, Popov VL, Arenas-Hernández MM (2008) Contribution of the Ler- and H-NS-regulated long polar fimbriae of *Escherichia coli* O157:H7 during binding to tissue-cultured cells. *Infect Immun* 76(11):5062–5071.
34. Farfan MJ, Inman KG, Nataro JP (2008) The major pilin subunit of the AAF/II fimbriae from enteroaggregative *Escherichia coli* mediates binding to extracellular matrix proteins. *Infect Immun* 76(10):4378–4384.
35. Saarela S, Westerlund-Wikström B, Rhen M, Korhonen TK (1996) The GafD protein of the G (F17) fimbrial complex confers adhesiveness of *Escherichia coli* to laminin. *Infect Immun* 64(7):2857–2860.
36. Sampaio SC, et al. (2009) The flagella of an atypical enteropathogenic *Escherichia coli* strain are required for efficient interaction with and stimulation of interleukin-8 production by enterocytes in vitro. *Infect Immun* 77(10):4406–4413.
37. Islam MA, Yun CH, Choi YJ, Cho CS (2010) Microencapsulation of live probiotic bacteria. *J Microbiol Biotechnol* 20(10):1367–1377.
38. Sonnenburg JL, Chen CT, Gordon JI (2006) Genomic and metabolic studies of the impact of probiotics on a model gut symbiont and host. *PLoS Biol* 4(12):e413.
39. Okada Y, et al. (2009) Anti-inflammatory effects of the genus *Bifidobacterium* on macrophages by modification of phospho-I kappaB and SOCS gene expression. *Int J Exp Pathol* 90(2):131–140.
40. Weiss G, et al. (2010) *Bifidobacterium bifidum* actively changes the gene expression profile induced by *Lactobacillus acidophilus* in murine dendritic cells. *PLoS ONE* 5(6): e11065.
41. Fink LN, et al. (2007) Distinct gut-derived lactic acid bacteria elicit divergent dendritic cell-mediated NK cell responses. *Int Immunol* 19(12):1319–1327.
42. Fink LN, Zeuthen LH, Ferlazzo G, Frøkiaer H (2007) Human antigen-presenting cells respond differently to gut-derived probiotic bacteria but mediate similar strain-dependent NK and T cell activation. *FEMS Immunol Med Microbiol* 51(3):535–546.
43. Galdeano CM, de Moreno de LeBlanc A, Vinderola G, Bonet ME, Perdigón G (2007) Proposed model: Mechanisms of immunomodulation induced by probiotic bacteria. *Clin Vaccine Immunol* 14(5):485–492.
44. Mandlik A, Swierczynski A, Das A, Ton-That H (2008) Pili in Gram-positive bacteria: Assembly, involvement in colonization and biofilm development. *Trends Microbiol* 16(1):33–40.
45. Yasutake N, et al. (1999) The role of tumor necrosis factor (TNF)-alpha in the antitumor effect of intrapleural injection of *Lactobacillus casei* strain Shirota in mice. *Med Microbiol Immunol (Berl)* 188(1):9–14.
46. Wajant H, Pfizenmaier K, Scheurich P (2003) Tumor necrosis factor signaling. *Cell Death Differ* 10(1):45–65.
47. Lebeer S, Vanderleyden J, De Keersmaecker SC (2010) Host interactions of probiotic bacterial surface molecules: Comparison with commensals and pathogens. *Nat Rev Microbiol* 8(3):171–184.
48. Bach JF (2002) The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 347(12):911–920.
49. Otte JM, et al. (2009) Probiotics regulate the expression of COX-2 in intestinal epithelial cells. *Nutr Cancer* 61(1):103–113.
50. Mazmanian SK, Round JL, Kasper DL (2008) A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* 453(7195):620–625.
51. Kaplan JL, Shi HN, Walker WA (2011) The role of microbes in developmental immunologic programming. *Pediatr Res* 69(6):465–472.

Supporting Information

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SI Text

Bacterial Strains and Culture Conditions. Bifidobacterial cultures were incubated in an anaerobic atmosphere [2.99% (vol/vol) H_2, 17.01% (vol/vol) CO2, and 80% (vol/vol) N_2] in a chamber (Concept 400, Ruskin) in the Man-Rogosa-Sharp (MRS) (Scharlau Chemie) supplemented with 0.05% (wt/vol) L-cysteine hydrochloride and incubated at 37 °C for 16 h. *Lactococcus lactis* was routinely cultured at 30 °C in GM17 (M17 supplemented with 0.5% glucose) for 16 h. Microencapsulation of lactococci was performed as previously described (1). To facilitate the inclusion of lactococci in a polymer shell, microencapsulation was performed using a co-extrusion method, which had been previously optimized for the production of water-in-water hydrogel microbeads (1, 2).

Sample Preparation and Atomic Force Microscopy Imaging. Bacteria from four ml bacterial culture or obtained following contact with Caco-2 or HT29 were harvested by centrifugation and resuspended in 200 μL of PBS (or 20 mM Hepes 7.5, 1 mM EDTA). Next, 200 μL of 5% glutaraldehyde was added, followed by gentle mixing and incubation for 1 min at room temperature. Thereafter, bacteria were washed four times with PBS by repeated resuspension and collection by centrifugation (1,700 × *g*). The washed pellet was then resuspended in 200 μL of PBS and kept on ice until atomic force microscopy (AFM) imaging. To facilitate adhesion of bacteria to the mica support used for AFM imaging, mica was coated with polylysine as follows: 10 μL of a polylysine solution (10 ng/mL) was deposited onto freshly cleaved mica for 1 min. Mica was then rinsed with milliQ water (Millipore) and dried with nitrogen. After this process, 20 μL of bacterial suspension was deposited onto polylysine-coated mica for 2–5 min, depending on the particular strain or specific cultivation conditions. The mica disk was then rinsed with milliQ water and dried under a weak gas flow of nitrogen. Quality of the sample and density of surface-bound bacteria were verified with an optical microscope.

AFM imaging was performed on dried samples with a Nanoscope III microscope (Digital Instruments) equipped with scanner J and operating in tapping mode. Commercial diving board silicon cantilevers (MikroMasch) were used. Best image quality was obtained with high driving amplitude (1–3 V) and low scan rate (0.5 Hz). Filamentous structures at the periphery of bacteria were visible in images of 512 × 512 pixels, representing a scan size of 10 μm or less. While imaging both height and amplitude signals were collected, height images were flattened using Gwyddion software.

Cloning of Pili-Encoding Genes in *L. lactis*. Chromosomal DNA was isolated from *Bifidobacterium bifidum* PRL2010 as previously described (3). Minipreparation of plasmid DNA from *L. lactis* was achieved using the Qiaprep spin plasmid miniprep kit (Qiagen) as described previously (4). Single-stranded oligonucleotide primers used in this study were synthesized by MWG Biotech. Standard PCRs were performed using TaqPCR mastermix (Qiagen), and high-fidelity PCR was achieved using KOD polymerase (Novagen). PCR fragments were purified using the Qiagen PCR purification kit (Qiagen). Electroporation of plasmid DNA into *L. lactis* was performed as described by Wells et al. (5).

For the construction of plasmids pNZ8150-*pil*2, pNZ8048-*pil*3 and pNZEM-SRT$_{BBPR_0285}$ DNA fragments encompassing the *pil*2 (BBPR_1709-BBPR_1707), *pil*3 (BBPR_0282-BBPR_0283) or *srt*$_{BBPR\_0285}$ (BBPR_0284) genes were generated by PCR amplification from chromosomal DNA of *B. bifidum* PRL2010 using KOD polymerase and primer combinations pil2F and pil2R, pil3F and pil3R, or pil3srtF and pil3srtR, respectively (Table S1). NcoI or EcoRV, and XbaI restriction sites were incorporated at the 5′ ends of each forward and reverse primer combination, respectively (Table S1). The four generated amplicons were digested with NcoI/EcoRV and XbaI, and ligated into NcoI/ScaI- and XbaI-digested nisin-inducible translational fusion plasmids pNZ8048, pNZ8150, or pNZ8048-Em as appropriate (6). The ligation mixtures were introduced into *L. lactis* NZ9000 by electrotransformation, and transformants were selected based on chloramphenicol resistance for pNZ8048 and pNZ8150, or erythromycin resistance for pNZ8048-EM transformants. The plasmid content of a number of transformants was screened by restriction analysis and the integrity of positively identified clones was verified by sequencing. For the construction of pNZ-pil2$_{BBPR1707}$, pNZ-pil3$_{BBPR0282}$, or pNZ-pil3$_{BBPR0283}$, DNA fragments of the pilin-encoding genes, BBPR_1707, BBPR_0282, and BBPR_0283, without the signal sequence-encoding DNA were amplified with 1707F and 1707R, 282F and 282R, or 283F and 283R (Table S1), respectively. These DNA fragments represent codons 30–517 of BBPR_1707, codons 40–1137 of BBPR_0282, and codons 30–495 of BBPR_0283. NcoI and XbaI restriction sites were incorporated at the 5′ end of each forward and reverse primer combination, respectively (Table S1). In addition, an in-frame His_{10}-encoding sequence was incorporated into each of the forward primers to facilitate downstream protein purification using the Ni-NTA affinity system (Qiagen). The three generated amplicons were digested with NcoI and XbaI, and ligated into similarly-digested, nisin-inducible translational fusion plasmid pNZ8048 (6). The ligation mixtures were introduced into *L. lactis* NZ9000 by electrotransformation, and transformants were selected based on chloramphenicol resistance. The plasmid content of a number of Cm^r transformants was screened by restriction analysis and the integrity of positively identified clones was verified by sequencing.

Protein Overproduction and Purification. M17 broth (400 mL) supplemented with 0.5% glucose was inoculated with a 2% inoculum of a particular *L. lactis* strain, followed by incubation at 30 °C until an optical density (OD at wavelength 600 nm) of 0.5 was reached, at which point protein expression was induced by the addition of purified nisin (5 ng·mL^{-1}) followed by continued incubation at 30 °C for 90 min. Cells were harvested by centrifugation, washed and concentrated 40-fold in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole; pH 8.0). Cell extracts were prepared using 106-μm glass beads and the minibeadbeater-8 cell disrupter (Biospec Products). After homogenization, the glass beads and cell debris were removed by centrifugation, and the supernatant containing the cytoplasmic fractions was retained. Protein purification from the cytoplasmic fraction was performed using Ni-NTA matrices in accordance with the manufacturer's instructions (Qiagen). Elution fractions were analyzed by SDS/PAGE, as described by Laemmli (7), on a 12.5% polyacrylamide gel. After electrophoresis the gels were fixed and stained with Commassie Brilliant blue to identify fractions containing the purified protein. Rainbow prestained low molecular weight protein markers (New England Biolabs) were used to estimate the molecular weight of the purified proteins.

Preparation of Antisera. Antiserum specific for FimA$_{PRL2010}$ (Ab$_{FimA}$), and FimP$_{PRL2010}$ (Ab$_{FimP}$) recombinant His-tagged proteins were produced by Eurogentec according to their standard

procedures. In brief, for each protein groups of two rabbits were injected subcutaneously with a 1-mL volume of 400 μg purified recombinant BBPR_1707 or BBPR_0283 proteins and Freund's complete adjuvant (1:1 mixture). One milliliter of subcutaneous booster injections of 200 μg of BBPR_1707 or BBPR_0283 protein in Freund's incomplete adjuvant (1:1 mixture) were administered at 3-wk intervals over a 9-wk period. Blood was collected 14 d after the last booster injection and the antiserum was prepared.

Western Blotting. Overnight cultures were harvested by centrifugation and resuspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea pH 8). This bacterial cell suspension was then subjected to sonication using six 10-s bursts at 200–300 W with a 10-s cooling period between each burst. Lysed bacteria were then centrifuged at 3,500 × *g* for 2 min, the pellets were resuspended in 60 μL Sample Buffer (Laemmli), and boiled at 100 °C for 15 min. Proteins were separated on a 10% SDS-polyacrylamide gel and then transferred onto a 0.2 μm pore size nitrocellulose membrane (Bio-Rad), using a wet/tank blotting apparatus (Bio-Rad Criterion Blotter). The membrane was successively blocked overnight at 4 °C in TBS supplemented with 0.05% Tween 20 (TBST) containing 5% skim milk powder (blocking solution). Membranes were washed three times with TBST for 15 min and then incubated with the polyclonal antibody Ab_{FimA} or FimP Ab_{FimP} (diluted 1:5,000 in TBST) for 2 h at room temperature. Immunoblots were then washed three times for 15 min with TBST followed by incubation with LiCor IRDye 680 Goat anti-rabbit for 1 h. Immunodetection was performed with the Odyssey Infrared Imager (LiCor).

Bioinformatics Analyses. The estimation of the sequence diversity was established from the calculation of the average π of differences between pairs of sequences, or from the number of segregation sites (Watterson's estimator θ). The nucleotide diversity π (8) the Watterson's estimator θ (9) were computed using DnaSP 4.10 (10), using a sliding window length of 100 bp shifted by 25 bp at each sliding step.

RNA Isolation. Total RNA was isolated using a previously described method (11). Briefly, cell pellets/tissue materials were resuspended in 1 mL of QUIAZOL (Qiagen) and placed in a tube containing 0.8 g of glass beads (diameter, 106 μm; Sigma). The cells were lysed by shaking the mix on a BioSpec homogenizer at 4 °C for 2 min (maximum setting). The mixture was then centrifuged at 15,000 × *g* for 15 min, and the upper phase containing the RNA-containing sample was recovered. The RNA sample was further purified by phenol extraction and ethanol precipitation according to an established method (12). The quality of the RNA was checked by analyzing the integrity of rRNA molecules by Experion (Bio-Rad).

Microarray, Description, Labeling, and Hybridizations. Microarray analysis was performed with an oligonucleotide array based on the *B. bifidum* PRL2010 genome: a total of 39,249 oligonucleotide probes of 35 bp in length were designed on 1,644 ORFs using OligoArray 2.1 software (13). The Oligos were synthesized in triplicate on a 2 × 40k CombiMatrix array (CombiMatrix). Replicates were distributed on the chip at random, nonadjacent positions. A set of 74 negative control probes designed on phage and plant sequences were also included on the chip.

Reverse transcription and amplification of 500 ng of total RNA was performed with MessageAmp II-Bacteria kit (Ambion) according to the manufacturer's instructions. Five micrograms of RNA was then labeled with ULS Labeling kit for Combimatrix arrays with Cy5 (Kreatech). Hybridization of labeled DNA to *B. bifidum* PRL2010 arrays was performed according to CombiMatrix protocols (www.combimatrix.com).

Microarray Data Acquisition and Treatment. Fluorescence scanning was performed on an InnoScan 710 microarray scanner (Innopsys). Signal intensities for each spot were determined using GenePix Pro-7 software (Molecular Devices). Signal background was calculated as the mean of negative controls plus two times the SD (14). A global quantile normalization was performed (15) and $\log_2$ ratios between the reference sample and the test samples were calculated. The distribution of the $\log_2$-transformed ratios was separately calculated for each hybridization reaction.

Study of the Activation of U937 Human Macrophage Cell Line. Cell line U937 (ATCC CRL-1593.2) was derived from a human histiocytic lymphoma (16). For immunological experiments on human U937 macrophages, bacterial cells from an overnight culture were collected, washed twice with sterile PBS, and then resuspended in the same medium used to culture human cells. Bacteria were tested at a multiplicity of infection of 10 and 100. To prevent underestimation of the number of cells used because of the coaggregation exerted by PRL2010 pili, *L. lactis*-$pil2_{PRL2010}$ and *L. lactis* $pil3_{PRL2010}$ cells were enumerated through the use of a Petroff–Hausser counting chamber and normalized prior the induction of pili by the addition of nisin as mentioned above. U937 cells are maintained as replicative, nonadherent cells and have many of the biochemical and morphological characteristics of blood monocytes (17). When treated with phorbol myristate acetate, U937 cells differentiate to become adherent, nonreplicative cells with characteristics similar to tissue macrophages, including similar isoenzyme patterns (18) and other phenotypic markers (17). The normal growth medium for the U937 cells consisted of RPMI medium 1640 (Lonza) supplemented with 10% (vol/vol) FBS (FBS) (Gibco-BRL, Life Technologies), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich). U937 cells were seeded at a density of 5×10^5 cells/well in 12-well plates and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Differentiation was induced by the addition of phorbol myristate acetate (Sigma-Aldrich) into the cellular medium at a final concentration of 100 nM and was allowed to proceed for 48 h. Following this, cells were washed once with sterile PBS buffer to remove all nonadherent cells. One hour before the bacteria were added to the cells, the culture medium was replaced with RPMI 1640 supplemented with 1% (vol/vol) FBS to allow the cells to adapt. Bacteria were used at a multiplicity of infection of 10 and 100. An untreated sample [i.e., only RPMI medium 1640 with 1% (vol/vol) FBS] was used as control.

Preparation of RNA and Reverse Transcription. Following incubation of macrophages at 37 °C for 4 h, the supernatant was carefully removed from each well and total cellular RNA was isolated from the adhered cells with a Bio-Rad Aurum Total RNA Fatty and Fibrous Tissue Pack kit. RNA concentration and purity was then determined with a Bio-Rad Smart Spec Spectrophotometer and the quality and integrity of the RNA was checked by Experion (Bio-Rad) analysis. Reverse transcription to cDNA was performed with the iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories) using the following thermal cycle: 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C. The mRNA expression levels of cytokines were analyzed with SYBR Green technology in RT-quantitative PCR using SoFast EvaGreen Supermix (Bio-Rad) on a Bio-Rad CFX96 system according to the manufacturer's instructions (Bio-Rad). The primers used are indicated in Table S1. Quantitative PCR was carried out according to the following cycle: initial hold at 96 °C for 30 s and then 40 cycles at 96 °C for 2 s and 60 °C for 5 s. Gene expression was normalized to the housekeeping gene coding for the18S rRNA. The amount of template cDNA used for each sample was 12.5 ng. All results regarding cytokine mRNA expression levels are reported as the fold-of-induction in comparison with the control (namely

unstimulated macrophages), to which we attributed an fold-of-induction of 1.

Tissue-Culture Experiments. All cell-culture reagents unless specified otherwise were from Sigma-Aldrich. For cell culture experiments, 2×10^5 Caco-2 or HT29 cells in 1.5 mL of DMEM (high glucose, Hepes) medium supplemented with 10% heat-inactivated FBS (Gibco), penicillin (100 U/mL), streptomycin (0.1 mg/mL) and amphotericin B (0.25 μg/mL), and 4 mM L-glutamine were seeded into the upper compartments of a six-well transwell plate (Corning). The lower compartments contained 3.0 mL of the same medium. The cells were incubated at 37 °C in a 5% CO_2 atmosphere until they reached 3 d postconfluence. The cells were then washed with Hanks' solution and stepped-down in DMEM supplemented with L-glutamine (4 mM), sodium selenite (0.2 μg/mL), and transferrin (5 μg/mL) for 24 h. These transwell inserts were transferred to an anaerobic culture box within an MACS-MG-1000 anaerobic workstation at 37 °C and each insert filled with anaerobic DMEM cell medium. A culture of *B. bifidum* PRL2010 at exponential phase was harvested by centrifugation at 3,500 × *g* for 5 min and washed with 10 mL of anaerobic DMEM. The pellet was resuspended in 0.8 mL of the same medium. Next, 100 μL of bacterial suspension (10^8 cfu/mL) was added to experimental wells; the control wells received the same amount of medium without bacterial cells. As an additional control bacterial cells incubated without Caco-2 and HT29 cells was used.

Bacterial cells were harvested for analyses after 4 h of incubation. Microbial cells were collected into 1.5-mL tubes, centrifuged at 3,500 × *g* for 5 min, and the resulting pellet resuspended in 400 μL of RNAlater and submitted to RNA extraction following the protocol described above. Caco-2 cells or HT29 cells were harvested from the wells, pooled, and stored in RNA later at 4 °C.

Bacterial Adhesion to Caco-2 Cells. Caco-2 cells were routinely grown in 3-cm Petri plates on microscopy cover glasses in DMEM supplemented with 10% (vol/vol) heat-inactivated (30 min at 56 °C) FCS, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.1 mM nonessential amino acids, 2 mM L-glutamine (complete medium), and incubated at 37 °C in a water-jacketed incubator in an atmosphere of 95% air and 5% carbon dioxide. The culture medium was changed twice weekly. For adhesion assay experiments, cells were used 15 d after confluence (fully differentiated cells). Cell monolayers were carefully washed twice with PBS pH 7.3 (PBS) before addition of bacterial cells. The bacterial cell concentration of an overnight culture was determined microscopically with Neubauer Improved counting chamber (Marienfeld). Approximately 2×10^8 cells for each strain were incubated with a monolayer of fully differentiated Caco-2 cells. After 1 h at 37 °C in anaerobic conditions, all monolayers were washed three times with PBS to release unbound bacteria. Cells were then fixed with 3 mL of methanol and incubated for 8 min at room temperature. After removal of methanol, cells were stained with 3 mL of Giemsa stain solution (1:20) (Carlo Erba) and left 30 min at room temperature. Wells were then washed until no color was observed in the washing solution and dried in an incubator for 1 h. Microscopy cover glasses were then removed from the Petri plate and examined microscopically (magnification, 100×) under oil immersion. The adherent bacteria in 20 randomly selected microscopic fields were counted and averaged.

Inhibition of the Pili-Mediated Adhesion Using Anti-Pili Antibodies. Before the adhesion assay on polarized Caco-2 cells, *L. lactis*-$pil3_{PRL2010}$ (nisin-induced or uninduced) cells were incubated at room temperature for 1 h with or without Ab_{pil3}. Two different concentrations of Ab_{pil3} were considered: 50 μl of undiluted Ab_{pil3} or 50 μl of 1:50 diluted Ab_{pil3} were added to 1 mL of bacterial suspension containing 10^8 cells in PBS (pH 7.3). Following incubation, bacterial cells were washed once with PBS and tested in adhesion experiments as described above.

Bacterial Aggregation. *L. lactis* clones and bifidobacterial species were incubated at 30 °C to 37 °C respectively in M17 and MRS broth, respectively. Pili synthesis in *L. lactis* clones were induced by the addition of nisin as described above. After incubation for 3 and 24 h, 1 mL of the upper suspension was transferred to another tube and the OD was measured at 600 nm. The aggregation was expressed as follows: 1 − (OD upper suspension/OD total bacterial suspension) × 100 (19).

Quantification of Bacterial Binding to Extracellular Matrix Proteins. Ninty-six MicroWell plates (Maxisorp Nunc) were coated with a solution of 500 μg/mL of extracellular matrix (ECM) protein in 100 μL PBS (PBS). The ECM proteins used included fibrinogen, plasminogen, fibronectin, laminin, and collagen type IV, which were purchased from Sigma. Unbound protein was removed by washing the plates two times with PBS containing and was subsequently blocked with 1% BSA (BSA) in PBS for 30 min at 37 °C. The blocking buffer was removed, and the wells were washed twice before the addition of bacterial cells in a 100-μL final volume. Incubation with the bacteria was performed for 1 h at growth bacterial temperature. After the wells were washed with PBS, the bacterial cells that adhered to the wells were collected by scraping them into PBS with 0.5% (vol/vol) Triton X-100; serial dilutions were plated onto MRS or GM17 agar plates. The number of adherent bacteria was determined by counting the resulting colonies in duplicate. Deglycosylation of nondenatured fibronectin was carried out by digestion at 37 °C for 4 d with *N*-glycanase, Sialidase A, O-glycanase, β1,4-galactosidase, and β-*N*-acetylglucosaminidase according to the manufacturer's protocols (Prozyme). To evaluate the role of mannose, fucose, galactose, glucose, and xylan on adhesion of the pili encoded by PRL2010, bacteria were incubated in PBS with 1% of each of the above mentioned carbohydrates.

Light Microscopy. Glass coverslips were coated with each ECM as described above. After washing with PBS, 100 μL of PBS containing ~1×10^8 bacteria was added, and the plate was incubated at 30 °C for 1 h. After fixation samples were rinsed with PBS to remove the unbound bacterial cells and then analyzed by light microscopy.

Murine Colonization. All animals used in this study were cared for in compliance with guidelines established by the Italian Ministry of Health. All procedures were approved by the University of Parma, as executed by the Institutional Animal Care and Use Committee (Dipartimento per la Sanità Pubblica Veterinaria, la Nutrizione e la Sicurezza degli Alimenti Direzione Generale della Sanità Animale e del Farmaco Veterinario). Two groups, each containing five animals of 3-mo-old female BALB/c mice, were orally inoculated with bacteria. Bacterial colonization was established by five consecutive daily administrations whereby each animal received a dose of 10^9 cells using a micropipette tip placed immediately behind the incisors (20). Bacterial inocula were prepared by feeding mice with 10^9 CFU doses of *B. bifidum* PRL2010 or by microencapsulated lactococci.

To estimate the number of *B. bifidum* PRL2010 cells per gram of feces, individual fecal samples were serially diluted and cultured on selective agar (MRS) containing 3 μg/mL chloramphenicol. Following enumeration of *B. bifidum* PRL2010 in fecal samples, 100 random colonies were further tested to verify their identity using PCR primers targeting the *pil2* and *pil3* loci (21).

Animals were killed by cervical dislocation and their individual gastrointestinal tracts were removed, immediately treated with RNA-later and subsequently used for RNA extraction.

In vivo evaluation of the immune-modulatory activities exerted by sortase-dependent pili encoded by PRL2010 was performed by daily supplementation of two groups of five mice each with microencapsulated piliated *L. lactis-pil3*$_{PRL2010}$ and microencapsulated nonpiliated *L. lactis-pil3*$_{PRL2010}$ for 3 d. Production of pili was induced before microencapsulation of lactococci through the addition of nisin to the culture as mentioned above. To prevent underestimation of cell numbers because of coaggregation, *L. lactis*-pil2$_{PRL2010}$ and *L. lactis* pil3$_{PRL2010}$ cells were enumerated by means of a Petroff–Hausser counting chamber and normalized prior the induction of pili by the addition of nisin as mentioned above. Four hours from the last lacococcal administration, mice were killed and cecum sections were removed and stored in RNAlater, were diluted 1:1 in an equal volume of sterile PBS, followed by centrifugation at 5,000 × *g* for 10 min at 4 °C.

Statistical Analysis. Statistical significance between means was analyzed using the unpaired Student *t* test with a threshold $P < 0.05$. Values are expressed as the means ± the SEMs of three experiments. Multiple comparisons are analyzed using one-way ANOVA and Bonferroni tests. Statistical calculations were performed using the software program GraphPad Prism 5.

1. Piazza L, Roversi T (2011) Preliminary study on microbeads production by co-extrusion technology. *Procedia Food Science* 1(1):1374–1380.
2. Piazza L, Guglielmetti S, Roversi T, Valsecchi L (2012) Functional structure processing by co-extrusion in the production of probiotics-enriched microbeads. *Proceedings CeFOOD* (May 23–26, 2012, Novi Sad, Serbia), pp 28.
3. Turroni F, et al. (2010) Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. *Proc Natl Acad Sci USA* 107(45): 19514–19519.
4. O'Connell Motherway M, et al. (2008) Characterization of ApuB, an extracellular type II amylopullulanase from *Bifidobacterium breve* UCC2003. *Appl Environ Microbiol* 74(20):6271–6279.
5. Wells JM, Wilson PW, Le Page RW (1993) Improved cloning vectors and transformation procedure for *Lactococcus lactis*. *J Appl Bacteriol* 74(6):629–636.
6. Mierau I, Kleerebezem M (2005) 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. *Appl Microbiol Biotechnol* 68(6):705–717.
7. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259):680–685.
8. Lynch M, Crease TJ (1990) The analysis of population survey data on DNA sequence variation. *Mol Biol Evol* 7(4):377–394.
9. Watterson GA (1975) On the number of segregating sites in genetical models without recombination. *Theor Popul Biol* 7(2):256–276.
10. Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19(18):2496–2497.
11. Turroni F, et al. (2011) Global genome transcription profiling of *Bifidobacterium bifidum* PRL2010 under in vitro conditions and identification of reference genes for quantitative real-time PCR. *Appl Environ Microbiol* 77(24):8578–8587.
12. Sambrook J, Fritsch EF, Maniatis T eds (1989) *Molecular Cloning. A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
13. Rouillard JM, Zuker M, Gulari E (2003) OligoArray 2.0: Design of oligonucleotide probes for DNA microarrays using a thermodynamic approach. *Nucleic Acids Res* 31(12):3057–3062.
14. Bilban M, Buehler LK, Head S, Desoye G, Quaranta V (2002) Defining signal thresholds in DNA microarrays: Exemplary application for invasive cancer. *BMC Genomics* 3(1):19.
15. Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 19(2):185–193.
16. Sundström C, Nilsson K (1976) Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int J Cancer* 17(5):565–577.
17. Harris P, Ralph P (1985) Human leukemic models of myelomonocytic development: A review of the HL-60 and U937 cell lines. *J Leukoc Biol* 37(4):407–422.
18. Radzun HJ, Parwaresch MR, Sundström C, Nilsson K, Eissner M (1983) Monocytic origin of the human hematopoietic cell line U-937 and its convertibility to macrophages evidenced by isoenzyme mapping. *Int J Cancer* 31(2):181–186.
19. Del Re B, Sgorbati B, Miglioli M, Palenzona D (2000) Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Lett Appl Microbiol* 31(6): 438–442.
20. Sleator RD, Gahan CG, Hill C (2001) Mutations in the listerial proB gene leading to proline overproduction: Effects on salt tolerance and murine infection. *Appl Environ Microbiol* 67(10):4560–4565.
21. Foroni E, et al. (2011) Genetic analysis and morphological identification of pilus-like structures in members of the genus *Bifidobacterium*. *Microb Cell Fact* 10(Suppl 1):S16.

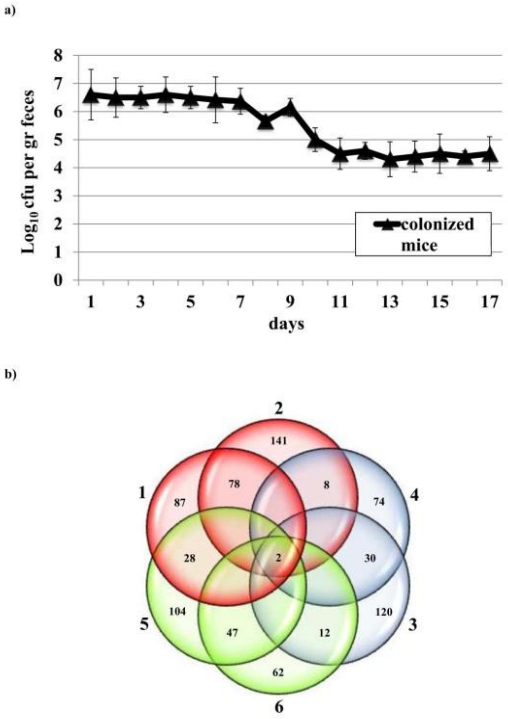

Fig. S1. Population sizes of *B. bifidum* PRL2010 colonizing the intestine of BALB/c mice (*A*) and Venn diagram representing the genes expressed under in vivo conditions, upon contact with Caco-2 cells and in synthetic media using different reference conditions (*B*). In *A*, each point represents the average of the log-transformed population size ± SD for five mice. In *B* the numbering of the cluster refers to the conditions described in Fig. 1.

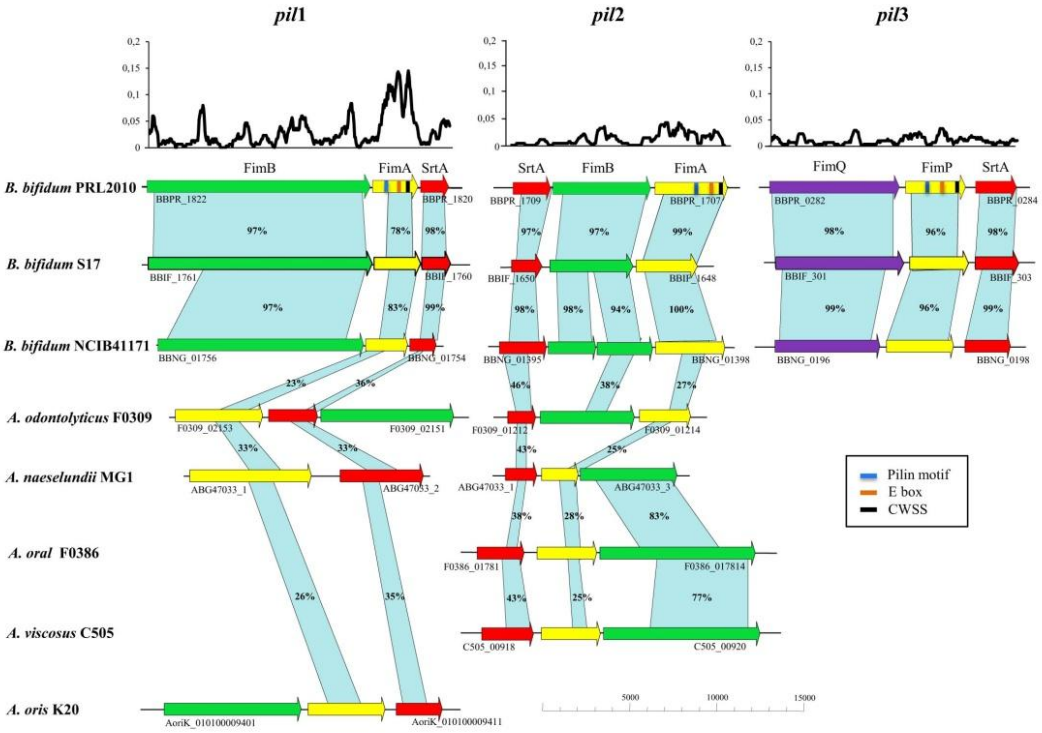

Fig. S2. Schematic comparative representation of pilus-encoding loci of *B. bifidum* PRL2010. Each arrow indicates an ORF, the size of which is proportional to the length of the arrow. Coloring of the arrows represents the different function of the gene as indicated above each arrow. The amino acid identity of the relevant encoded proteins is indicated in percentages. A plot of genetic diversity is indicated at the top of each pilus genetic maps. Nucleotide diversity π and Watterson estimator θ of the population mutation rate per site measured (represented on the *y* axis) along the sequence (represented on the *x* axis).

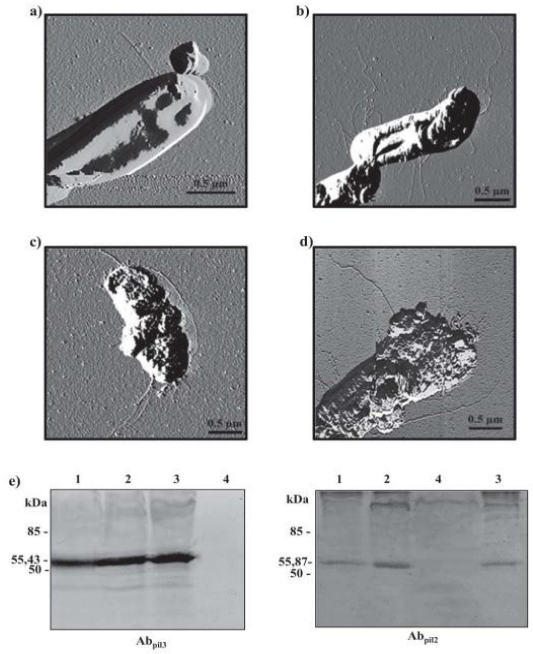

Fig. S3. Pili production of PRL2010 cultivated under different growth conditions. (*A* and *B*) PRL2010 cell's morphology upon cultivation on solid substrate (*A*, MRS agar) and exposure to Caco-2 cells (*B*). (*C* and *D*) PRL2010 cell's morphology upon cultivation in liquid medium supplemented with lysine (*C*, MRS broth plus lysine) and solid substrate enriched with lysine (*D*, MRS agar plus lysine). (*E*) Western blot analyses using crude extracts of PRL2010 cells upon cultivation on MRS broth (lane 4), or treatment with Caco-2 cells (lane 3), or growth on MRS agar (lane 2), or cultivation on MRS broth plus lysine (lane 1). The antibodies used are indicated below each immunoblot image.

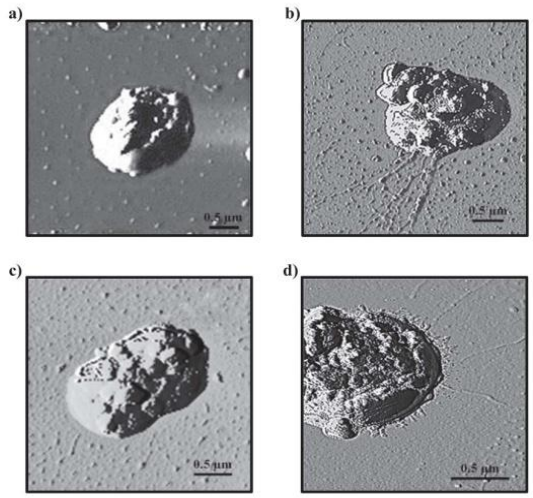

Fig. S4. Cell morphology of *L. lactis* clones expressing PRL2010 pili assayed by AFM. (*A* and *B*) *L. lactis-pil2*$_{PRL2010}$ cell morphology before (*A*) and after (*B*) treatment with nisin. (*B* and *C*) *L. lactis-pil3*$_{PRL2010}$ cell morphology by AFM before (*C*) and after (*D*) treatment with nisin.

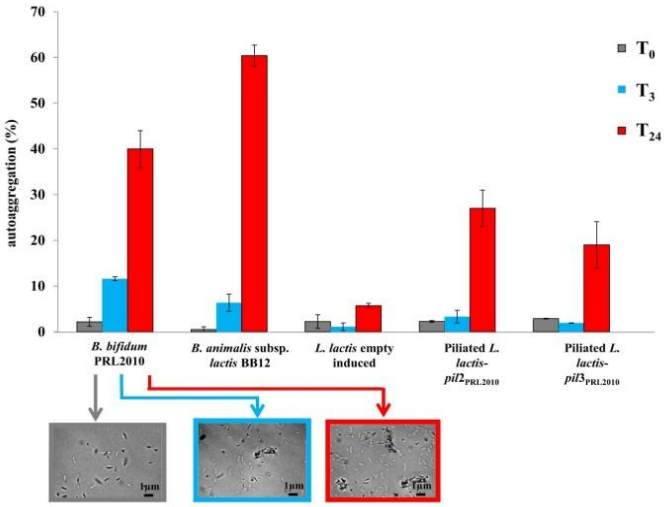

Fig. S5. Aggregation phenotype provided by pili encoded by *B. bifidum* PRL2010. Each pillar represents the coaggregation strength of cells from different microorganisms (*B. bifidum* PRL2010, *Bifidobacterium breve* 12L, *Bifidobacterium animalis* subsp. *lactis* BB12, *L. lactis* MG1363, *L. lactis-pil2*$_{PRL2010}$, and *L. lactis-pil3*$_{PRL2010}$) following different incubation time. The bars represent the mean values for three experiments and the error bars indicate the SDs ($P < 0.05$).

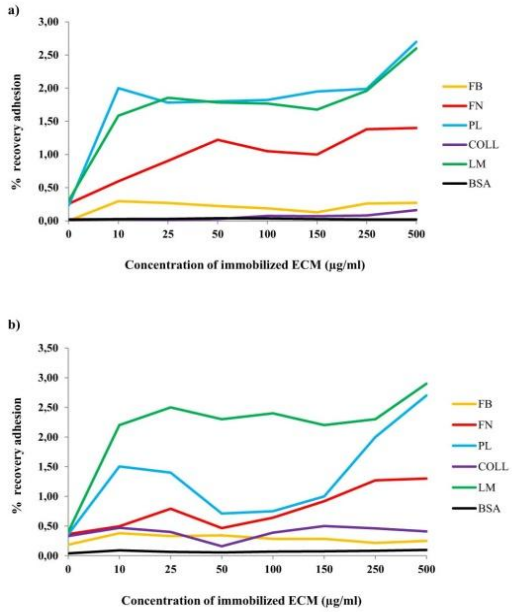

Fig. S6. Evaluation of the adhesion capabilities of piliated *L. lactis-pil2*$_{PRL2010}$- and *L. lactis-pil3*$_{PRL2010}$ cells to different amount of ECM substrates. FB, fibrinogen; FN, fibronectin; PL, plasminogen; COLL, collagen type IV; LM, laminin; BSA, BSA.

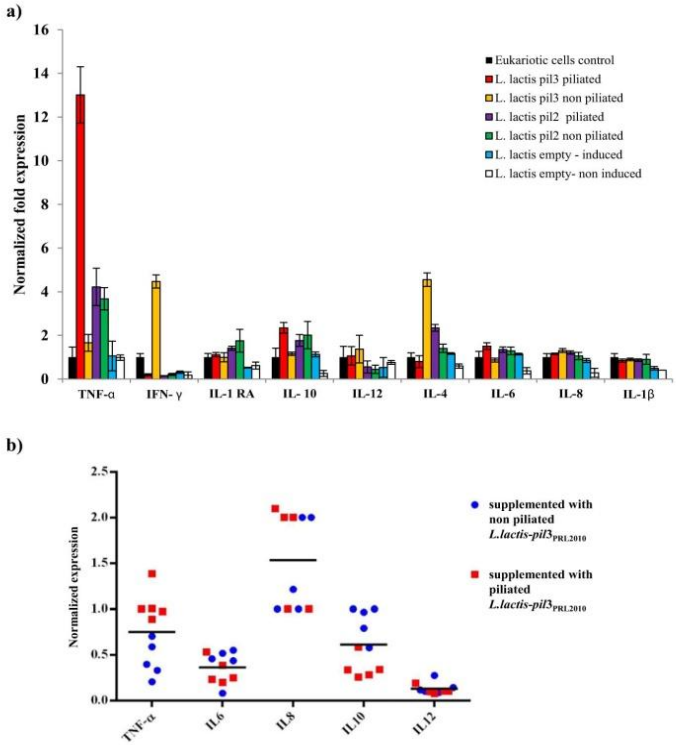

Fig. S7. Evaluation of the host cytokine profiles driven by pili-sortase dependent of *B. bifidum* PRL2010. (*A*) The relative transcription levels of different cytokine upon contacts of *L. lactis-pil2*$_{PRL2010}$ and *L. lactis-pil3*$_{PRL2010}$ with eukaryotic macrophages. The histograms indicate the relative amounts of the cytokine mRNAs for the specific samples. The values on the *y* axis represent the fold-fold change relative to reference genes as indicated in *SI Materials and Methods*. The bars represent the means for three experiments and the error bars indicate the SDs ($P < 0.05$). (*B*) The relative transcription levels of different cytokines upon *L. lactis-pil3*$_{PRL2010}$ colonization of mice. Results from individual mice are shown. Horizontal lines represent the medians. The *y* axis represents the level of expression as normalized expression (ΔΔCt) according to CFX96 Bio-Rad software.

Table S1. Primers used in this study

Primer	Sequence (5′–3′)
pil2F	CTGGAC**GATATC**ATGCCGGCATCGTCGAAGACCATC
pil2R	TGCGCA**TCTAGA**CTCATCGCCGTTCCTTTCCGTATG
pil3F	CTGGAC**CCATGG**TGGAAGGGATGCTTATCGTG
pil3R	AGCTC**TCTAGA**TCAGACGAGCCGACGCTTGCG
pil3srtF	CTGGAC**CCATGG**GCATGGCATCGAGGAG
pil3srtF	TGCGCA**TCTAGA**TCACCCACGCATGCGCCAC
1707F	GACAAG**CCATGG**AG*CATCACCATCACCATCACCATCACCATCAC*GTCGTCACGGACGGTGAGAAG
1707R	AGCTC**TCTAGA**GCATCCGTCAGACCGTTC
282F	GACAAG**CCATGG**AG*CATCACCATCACCATCACCATCACCATCAC*GACAGCAGTGATCCGAAGGGC
282F	AGCTC**TCTAGA**TCAGACGAGCCGACGCTTGCG
283F	GACAAG**CCATGG**AG*CATCACCATCACCATCACCATCACCATCAC*GCATACAAGATCGCGGACTAC
283F	AGCTC**TCTAGA**GTTATCGTGTCAGTCAGCGCAG
18S rRNA -F	ATCCCTGAAAAGTTCCAGCA
18S rRNA-R	CCCTCTTGGTGAGGTCAATG
IL-10- F	AGCAGAGTGAAGACTTTCTTTC
IL-10-R	CATCTCAGACAAGGCTTGG
TNF-α-F	AACTAATGGGAGTTGCCTGG
TNF-α-R	CCACCTGGTACATCTTCAAGTC
IL-1β-F	AGCTTGGTGATGTCTGGTCCA
IL-1β-R	ACACGCAGGACAGGTACAGATT
IL-RA-F	GTACCCATTGAGCCTCATGCT
IL-1RA-R	GTTCTCGCTCAGGTCAGTGATG
IL-12p35-F	AACTAATGGGAGTTGCCTGG
IL-12p35-R	CCACCTGGTACATCTTCAAGTC
IL-4-F	AGCTTCTCCTGATAAACTAATTGCC
IL-4-R	CAGCAAAGATGTCTGTTACGG
IL-6-F	GAACCTTCCAAAGATGGC
IL-6-R	CAAATCTGTTCTGGAGGT
IL-8-F	GAGTGCTAAAGAACTTAGATGTCAG
IL-8-R	AAACTTCTCCACAACCCTC
IFN-γ-F	CAGGTCATTCAGATGTAGC
IFN-γ-R	CACCGAATAATTAGTCAGC

Restriction enzyme sequences incorporated into forward and reverse primers are indicated in bold. His-tag encoding sequences incorporated into forward primers are indicated in italics.

8. Expression of sortase-dependent pili of *Bifidobacterium bifidum* PRL2010 in response to environmental gut conditions

Manuscript in preparation.

Abstract

Cell surface pili have recently been found in many different bifidobacterial species, including the infant gut commensal *Bifidobacterium bifidum* PRL2010. Pili produced by PRL2010 have been shown to be important molecular mediators for bacterial interaction with its human host. However, nothing is known about the modulation of their expression in response to cues that reflect the gastro intestinal environment, such as thermal, acidic and osmotic challenges, or the presence of other gut micro-organisms. Here, we have investigated how different stress conditions that simulate the gastro-intestinal niche influence the expression of PRL2010 sortase-dependent pili, and how this may impact on the coexistence and interaction with other human gut commensals.

INTRODUCTION

Bifidobacteria are common gut inhabitants of mammals, in particular during the first phase of life when they represent one of the most dominant members of the intestinal microbiota (Turroni, Peano et al. 2012, Ventura, Turroni et al. 2012). Bifidobacteria are able to establish themselves in such ecosystem thanks to different genetic strategies involving metabolic adaptation to natural substrates present in the gut such as undigested diet-derived poly/oligo-saccharides, and/or host-derived glycans [for a review see (Pokusaeva, Fitzgerald et al. 2011)]. Pilus-like structures have recently been described to be encoded by various bifidobacterial species (Foroni, Serafini et al. 2011, Motherway, Zomer et al. 2011, Turroni, Serafini et al. 2013), and reported to be pivotal for bifidobacterial gut colonization as well as for human host interactions. In this context, *Bifidobacterium bifidum* PRL2010, a strain isolated from infant stool, revealed the abundant presence of appendages known as sortase-dependent pili, which decorate the bacterial cell surface (Turroni, Bottacini et al. 2010, Turroni, Serafini et al. 2013). These sortase-dependent pili are highly expressed during *in vivo* colonization of mice as well as upon contact with a monolayer of human cells (Turroni, Serafini et al. 2013). Notably, heterologous expression of the coding sequences of these *B. bifidum* PRL2010 appendages in non-piliated *Lactococcus lactis* enhanced adherence to human enterocytes, promoted interaction with extracellular matrix proteins and stimulated bacterial aggregation. Furthermore, *in vivo* murine challenges with such piliated *L. lactis* cells evoked a higher TNFα response, supporting the notion that PRL2010 sortase-dependent pili not only contribute to adherence but also display immunomodulatory activity (Turroni, Serafini et al. 2013).

In *B. bifidum* PRL2010 as well as in other bifidobacteria, the genes for pilus formation and assembly are arranged in one or more loci, each of which encode one major pilin (represented by a FimA or FimP homolog), one minor pilins (represented by a FimB or FimQ homolog), and a pilus-specific sortase (Foroni, Serafini et al. 2011, Turroni, Serafini et al. 2013), in a similar fashion as described for *Actinomyces naeslundii* (Ton-That and Schneewind 2003, Mishra, Wu et al. 2010). In *Actinomyces* the *fim*QP and the *fim*AB-loci encode fimbrial structures composed of

a shaft protein, being either multimers of FimA or FimP, and a tip protein, represented by either FimB or FimQ, respectively (Yeung and Ragsdale 1997). Transcriptomics experiments have highlighted how the subunits that form the sortase-dependent pili of *B. bifidum* PRL2010 are differently expressed upon cultivation on different carbon sources, including diet-based carbohydrates or host-produced glycans (Foroni, Serafini et al. 2011). However, nothing is known about how pilus production may be influenced by environmental factors, such as those encountered by enteric bacteria during the approaching steps ahead of their establishment in their natural ecological niche or upon colonization of the human gut. Furthermore, biofilm formation involving microbe-microbe interaction has been shown to be largely influenced by pili synthesis in oral pathogens like *Actinomyces* and oral streptococci, i.e., *Streptococcus gordonii*, *Streptococcus sanguinis*, *Streptococcus mitis* and *Streptococcus oralis* (Okahashi, Nakata et al. 2011). In the human gut a large plethora of bacteria are believed to co-exist and may interact with each other (Sonnenburg, Chen et al. 2006), which may ultimately affect the expression of host-responsive molecules like pili. Here, we evaluate the expression of sortase-dependent pili of *B. bifidum* PRL2010 in response to environmental cues that are reflective of the gastro intestinal environment, and to the co-cultivation with various gut micro-organisms.

Materials and Methods

Bacterial strains and growth conditions. Bifidobacterial strains used in this study are listed in Table 1, and were cultivated in an anaerobic atmosphere (2.99 % H_2, 17.01 % CO_2 and 80 % N_2) in a chamber (Concept 400, Ruskin) on De Man-Rogosa-Sharp (MRS) (Scharlau Chemie, Barcelona, Spain) supplemented with 0.05 % (w/v) L-cysteine hydrochloride (MRSc) and incubated at 37°C for 16 h. *Lactobacillus paracasei* LMG9192 was cultivated in MRSc at 37°C under aerobic conditions. *Saccharomyces cerevisiae* BY4741 was cultivated in Yeast Extract Peptone Dextrose (YPD) (Oxoid) medium at 30°C under aerobic conditions.

Response to different physiological conditions. Following 24 h of growth at 37°C under anaerobic conditions, bifidobacteria were harvested by centrifugation, washed twice in saline solution (0.09 % NaCl, wt/vol), and brought to an inoculum of 10^8 to 10^9 CFU/ml. In order to simulate passage through the gastrointestinal tract, an inoculum of 10^7 CFU of bacteria was added to 10 ml of 2, 6 or 10 % of a saline solution (9 g/L NaCl), pH 3 solution, after which the resulting suspension was incubated at 37°C under anaerobic conditions for 16 hours as previously described (Sincock and Robinson 2001). The same procedure was used to inoculate *B. bifidum* PRL2010 in oxgal (Sigma-Aldrich) 0.5 % or at pH 4. In the case of pancreatin exposure, the cell suspension was neutralized by washing with phosphate buffer at pH 7.0, and cells were then resuspended in pancreatin solution and incubated at 37°C under anaerobic conditions for 4 h. Pancreatic solution was prepared immediately before use as follows: pancreatin from porcine pancreas (1% wt/vol; Sigma-Aldrich) was resuspended in sterile saline solution (0.5 %, wt/vol), and the pH was adjusted to 8.0 as described previously (Chenoll, Casinos et al. 2011). Moreover, the response of bifidobacteria to thermal fluctuations (25 and 30°C) was tested according to the protocol previously described (Ventura, Canchaya et al. 2004), while acidic stress was examined as previously described (Ventura, Canchaya et al. 2004). Samples were taken after four or sixteen hours, after which RNA extractions were performed. Assays were performed in triplicate and data were obtained from independent experiments.

Co-cultivation assays. Approximately 10^8 cells/ml of each of the following strains: *B. bifidum* PRL2010, *B. breve* 12L, *B. adolescentis* 22L, *B. animalis* subsp. *lactis* Bl12, *L. paracasei* LMG9192, *S. cerevisiae* BY4741 or these strains in co-cultivation with *B. bifidum* PRL2010 was inoculated in 6 ml of MRSc. Cell suspensions were mixed and incubated overnight at 37°C in anaerobic conditions in order to allow growth of PRL2010 cells. The suspensions were harvested and RNA extraction was performed to analyze the expression of pili through real time analyses (see below).

Evaluation of the cell density of PRL2010 in co-cultivation trials. Possible enhancement and/or reduction of PRL2010 growth as a consequence of the co-cultivation with other bacteria was monitored by quantitative real-time reverse transcription PCR (qRT-PCR). The copy-number of a gene for a given strain used in the co-cultivation experiments was evaluated and normalized according to the genome size and compared to the growth rate of each individually cultivated micro-organism. In the case of PRL2010 we used the following primer couple: BBPR_0282-UNI (5'-GCGAACAATGATGGCACCTA-3') and BBPR_282-REV (5'-GTCGAACACCACGACGATGT-3'). In the case of *B. breve* 12L, we used 12L-UNI (5'-CGAAGTTCCAGTTAACCAT-3') and 12L-REV (5'-GTTCTTCGCGTTCCAGATGT-3'); for *B. adolescentis* 22L, we employed the PCR primers 22L-UNI (5'-GACCAAGCCAACCAGTTCAT-3') and 22L-REV (5'-TTGGTGGCCTTGTAGTAGCC-3'); for *B. animalis* subsp. *lactis* Bl12, the following PCR primers l12-UNI (5'CGGTCATCCAGGTCGATA-3') and l12-REV (5'-CAGGGTATATGTGCGGTCAG-3'); for *S. cerevisiae* BY4741, the following PCR primer pair BY4741-UNI (5'-GGACTCTGGACATGCAA-3') and BY4741-REV (5'-ATACCCTTCTTAACACCT-3'); for *L. paracasei* LMG9192, primers 9192-UNI (5'-AGCAGTAGGGAATCTTC-3') and 9192-REV (5'-CATGGAGTTCCACTGTC-3').

Auto-aggregation and co-aggregation assays. These experiments were performed using PRL2010 cells placed in contact with cells from another micro-organism (Table 1). Auto-aggregation assays were carried out according to Del Re *et al*. (Del Re, Sgorbati et al. 2000) with

subsequently described modifications (Kos, Suskovic et al. 2003). Briefly, bacteria were grown for 16 h at 37°C in MRSc under anaerobic conditions. Cells were harvested by centrifugation at 5000 g for 15 min, washed twice and resuspended in phosphate buffered saline (PBS). Approximately 10^8 cells/ml of each strain or both strains was inoculated in 10 ml PBS, then cell suspensions were mixed and the auto-aggregation and co-aggregation was determined during five hours of incubation at 37°C under anaerobic conditions. Every hour the absorbance (A) of 0.5 ml of the upper suspension was measured at 600nm. The auto-aggregation percentage is expressed as:

1- (A_t/A_0) x 100, where A_t is the absorbance at time t=1, 2, 3, 4 or 5 h and A_0 the absorbance at t=0.

The percentage of co-aggregation was calculated using the following equation (Handley, Harty et al. 1987):

$$\frac{(Ax + Ay) \div 2 - A(x + y)}{(Ax + Ay) \div 2} \times 100$$

where x and y represent each of the two strains in the control tubes, and (x + y) the mixture. Results are expressed as the percentage reduction in the absorbance of a mixed suspension compared with the single bacterial suspension.

Flow Cytometry assay. In flow cytometry (FCM) particles/cells passing through the beam will scatter light, which is detected as forward scatter (FSC) and side scatter (SSC). FSC correlates with the cell size, shape and cell aggregates, whereas SSC depends on the density of the particle/cell (i.e. number of cytoplasmic granules, membrane size), and in this manner cell populations can often be distinguished based on differences in their size and density (Gunasekera, Veal et al. 2003, Cronin and Wilkinson 2010). The combination of scattered and fluorescent light is measured by a number of detectors as they pass an interrogation point in a fluid stream (Sincock and Robinson 2001). The effect of culture association between piliated PRL2010 cells and other microorganisms (*Bifidobacterium* sp., *L. paracasei*, and *S. cerevisiae*) on the dimension of cell aggregates of *B. bifidum* PRL2010 have been investigated by FCM

evaluating the FSC parameter. Cell suspensions prepared, as described before, were analyzed using the flow cytometer Accuri C6 (BD Biosciences, Milan, Italy) using the following threshold settings: FSC 5000 and SSC 4000, 20,000 total events collected. All parameters were collected as logarithmic signals. The 488 nm laser was used to measure the FSC values. The rate of events in the flow was generally lower than 2500 events s^{-1}. The data obtained were analyzed using the BD Accuri™ C6 software version 1.0 (BD Biosciences, Milan, Italy). Previous experiments were carried out using the same instrument settings but labeling the cell suspension with SYBR green I (SIGMA Aldrich, Milan, Italy) and evaluating the relative green fluorescence intensity (excitation 488 nm, emission filter 530/30) in order to estimate the amount of particles background in the cell suspension. Simultaneous with the FCM analysis the cell suspension was observed using a phase contrast microscope Axio Scope A.1 (Zeiss, Milan, Italy), and images were acquired using a TiEsseLab DV5000 camera (TiEsseLab srl, Milan, Italy) a Bel Eurisko v. 2.9 (BEL Engineering srl – Monza, italy).

RNA isolation. Total RNA was isolated using a previously described method (Turroni, Foroni et al. 2011). Briefly, cell pellets/tissue materials were resuspended in 1 ml of QUIAZOL (Qiagen, UK) and placed in a tube containing 0.8 g of glass beads (diameter, 106 µm; Sigma). Cells were lysed by shaking the mix on a BioSpec homogenizer at 4°C for 2 min (maximum setting). The mixture was then centrifuged at 12,000 rpm for 15 min, and the upper phase containing the RNA-containing sample was recovered. The RNA sample was further purified by phenol extraction and ethanol precipitation according to an established method. The quality of the RNA was checked by analysing the integrity of rRNA molecules by Experion (BioRad).

qRT-PCR. cDNA was synthesized and purified using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), according to the supplier's instructions. qRT-PCR was performed using the CFX96 system (BioRad, CA, USA), fold change was evaluated through the estimation of the CT values with the aid of the CFX96 software (BioRad, CA, USA). Each PCR reaction mix contained the following: 12.5µl 2x SYBR SuperMix Green (BioRad, CA, USA), 1 µl of cDNA dilution, each of the forward and reverse primers at 0.5 µM and nuclease-free water was added to

obtain a final volume of 20 µl. PCR products were detected with SYBR Green fluorescent dye and amplified according to the following protocol: one cycle of 95°C for 3 minutes, followed by 39 cycles of 95°C for 5 s and 60°C for 20 s. Melting curve: 65°C to 95°C with increments of 0.5°C/s. In each run, negative controls (no cDNA) for each primer set were included. The primers involved in qRT-PCR experiments were previously described (Foroni, Serafini et al. 2011, Turroni, Serafini et al. 2013). Fold change was calculated using the CFX96 software (BioRad). The expression was considered significant when the fold-induction was more than two.

Western Blotting. Overnight cultures were harvested by centrifugation and resuspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea pH 8). This bacterial cell suspension was then subjected to sonication using six 10 s bursts at 200–300 W with a 10 s cooling period between each burst. Bacterial cell envelopes were collected by centrifugation at 6,000 rpm for 2 minutes, the pellets were resuspended in 60 µl of PBS-lysis buffer containing SDS and β-mercaptoethanol, and boiled at 100°C for 15 min. Proteins were separated on a 10 % SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane, which was blocked overnight at 4 C in Tris Buffered Saline (TBS) supplemented with 0.05 % Tween 20 (TBST) containing 5 % skim milk powder (blocking solution). Membranes were washed three times with TBST for 5 min and then incubated with the polyclonal antibody Ab_{pil3} (diluted 1:5000 in TBST) for two hours at RT. Immunoblots were then washed three times for 10 min with TBST followed by immunodetection with IR Dye 680 Goat anti-rabbit for one hour. The nitrocellulose membrane (BioRad) was scanned using the Odyssey Infrared Imager at an intensity of 3.

RESULTS AND DISCUSSION

Response to different physiological conditions. Expression of pilus-encoding genes from various pathogenic bacteria, such as *Streptococcus pyogenes* and *Enterococcus faecium*, have previously been shown to be affected by various environmental factors, e.g. temperature and acidity, encountered by such microorganisms during their transit through or residence in various compartments of the human body (Hendrickx, Bonten et al. 2008, Nakata, Koeller et al. 2009, Jiang, Park et al. 2012). Thus, we decided to investigate if environmental stresses including exposure to thermal, osmotic and acidity fluctuations, which resemble those encountered by a microbial cell when it approaches and passes through the gastrointestinal (GIT) tract, might influence the production of sortase-dependent pili using our model gut microorganism of *B. bifidum* PRL2010. Furthermore, it has previously been suggested that environmental stresses may stimulate pre-adaption of enteric bacteria to the gut environment through for example the production of capsular structures (Hidalgo-Cantabrana, Sanchez et al. 2013), and it may therefore be that the synthesis of other microbe-host interaction effector molecules such as pili are also influenced [for review see (Ventura, Turroni et al. 2012)].

Common stresses to which enteric bacteria may be exposed are represented by osmotic insults derived from osmotic fluctuations of the diet as well as from temperature challenges [for a review see (Ventura, Canchaya et al. 2006)]. In order to determine if transcription of the major pilin subunit-encoding genes (BBPR_1707 *fim*A and BBPR_0283 *fim*P genes), the minor pilin subunit-encoding genes (BBPR_1708 *fim*B and BBPR_0282 *fim*Q genes) or the sortase-encoding genes (BBPR_1709 and BBPR_284) of the *B. bifidum* PRL2010 *pil*2 and *pil*3 loci, respectively, is altered following temperature fluctuations or osmotic shock, a qRT-PCR procedure was employed. The reference condition used were mRNA samples isolated from PRL2010 cultures cultivated in MRS broth at 37°C without lysine, which is a condition that do not allow the production of pili sortase-dependent by PRL2010 cells (Turroni, Serafini et al. 2013). The mRNA samples used in these experiments were isolated from *B. bifidum* PRL2010 cultures grown at temperatures ranging from 25°C to 37°C (Fig. 1), which might simulate thermal

conditions encountered by bacterial cells prior the approaching to the intestine (e.g., diet as well as in the upper GIT compartments), or in medium with a NaCl concentrations ranging of 2 % to 10 % (Fig. 1). Based on the qRT-PCR results, the highest transcription level of the *pil*3 locus was shown to occur following cultivation of PRL2010 cells at 30°C or incubation in a medium containing 2 % NaCl (Fig. 1). The transcriptional levels obtained under NaCl concentrations of 6 % and 10 % are not included here as we obtained a low survival of the cells. Such environmental conditions (cultivation at 30°C or incubation in a medium containing 2 % NaCl) increased the mRNA levels of *fim*P, specifying the major subunit of *pil*3, by approximately 30 and 23-fold, respectively, with respect to the highest expression (37-fold) at 37°C which represents the positive control where *B. bifidum* PRL2010 is piliated (Fig. 1). Temperatures closer to 30°C are commonly found in the upper regions of the human GIT (Kim, Kim et al. 2013), thus suggesting that an exposure of PRL2010 cells to such a temperature pre-adapts cells for their subsequent colonization of the human gut. Furthermore, Western blot analyses using antibodies (Ab_{pil3}) that had been raised against the major subunit protein of *pil*3 loci, $FimP_{PRL2010}$, were performed on PRL2010 cells grown using the identical range of incubation temperatures and NaCl concentrations described above. Clear signals representing a protein of 55.4 kDa were observed when crude extracts of PRL2010 cells, which had undergone cultivation at 30°C or in the presence of 2 % NaCl, were probed in Western blots using Ab_{pil3} (Fig. 1). Interestingly, only the monomeric form of *fim*P was detected at 25°C while no apparent polymerization into polymeric pili structures was observed (Fig. 1). The fact that the major pilin protein was expressed indicates that the polymerization of monomers into a complete pilus structure may readily occur upon a temperature switch as has previously been noticed for pili of *Enterococcus faecium* (Hendrickx, Bonten et al. 2008). However, we cannot exclude the possibility that the polymerized form of *fim*P is not easily accessible and recognizable by the antibody compared to the monomeric form. qRT-PCR analyses demonstrated a transcription of the monomeric subunits approximately of 10-fold respect the positive control (37-fold) where *B. bifidum* PRL2010 is piliated. Higher molecular weight signals of above 100 kDa detected in each immunoblot image (Fig. 1) likely

represent the covalently linked polymers of FimP, a typical feature of sortase-dependent pili (Ton-That and Schneewind 2004, Telford, Barocchi et al. 2006, Turroni, Serafini et al. 2013).

Other environmentally relevant cues, such as fluctuations in pH and exposure to bile salts, may also influence pilus expression as described previously for other enteric organisms (Hendrickx, Bonten et al. 2008). Thus, transcription of the genes cluster encoding *pil*3 in *B. bifidum* PRL2010 cells cultivated in MRS broth displaying different level of acidity (pH) of 2 or 4, or concentration of bile salts of 0.5 %, was investigated at both mRNA and protein level by using qRT-PCR and Western blot approaches, respectively. Transcripts from *fim*P were detected for all pH values tested, with mRNA levels corresponding to *fim*P showing a considerable increase following cultivation of PRL2010 cells in MRS at pH 4 (41-fold) respect the positive control (37-fold) (Fig. 1). The reference condition used involves PRL2010 cultures cultivated in MRS broth at 37°C without lysine, which is a condition that do not allow the production of pili sortase-dependent by PRL2010 cells (Turroni, Serafini et al. 2013). Similarly, *fim*P mRNAs reaches a high amount at 0.5 % of bile salts added to the substrate (27-fold) (Fig. 1). In order to simulate the gastric passage, we grew *B. bifidum* PRL2010 cells in presence of pancreatin as previously published (Serafini, Strati et al. 2013). In this growth condition the level of *fim*P transcripts increased 14-fold. Furthermore, pilin proteins extracted from the same PRL2010 cultures used for qRT-PCR trials were assayed using specific Ab_{pil3} antibody. Such Western blot analyses revealed a higher amount of major pilin subunits when PRL2010 cells were cultivated in MRS broth at pH 4 or in the presence of 0.5 % bile salts as well as upon cell treatment with pancreatin (Fig. 1). No relevant expression of *pil*2 locus has been detected under the tested conditions.

Pili production of *B. bifidum* PRL2010 in response to co-cultivation with other gut organisms. In a previous study it has been shown that sortase-dependent pili of PRL2010 mediate microbial co-aggregation (Turroni, Serafini et al. 2013). Furthermore, sortase-dependent pili play a crucial role in biofilm initiation and maturation in complex bacterial communities such as those encountered in the human oral cavity [for a review see (Danne and Dramsi 2012)]. Thus, we were interested to explore if the expression of sortase-dependent pili of *B. bifidum* PRL2010

is influenced by the presence of other bacterial gut inhabitants such as *B. breve* 12L, *B. adolescentis* 22L, *B. animalis* subsp. *lactis* Bl12 (Milani, Duranti et al. 2013), which represent bifidobacterial species sharing a common ecological niche, and *L. paracasei* LMG9192, or by the eukaryotic organism *S. cerevisiae* BY4741. Evaluation of the transcription levels of individual genes of the *pil*2 and *pil*3 loci of *B. bifidum* PRL2010 upon co-cultivation of PRL2010 cells with these organisms, followed by extraction of RNA samples was assayed through qRT-PCR. Furthermore, possible enhancement or reduction of the cells density of PRL2010 as consequence of the co-cultivation with other gut organisms was monitored. As displayed in Fig. 2, no major effect in terms of variation of the cell density was noticed when PRL2010 was co-cultivated with the above mentioned organisms except for *S. cerevisiae* BY4741, which displays a slight enhancement of cell density upon co-cultivation with PRL2010 cells. Whereas, an increase of the mRNA levels of the major subunit-encoding gene of *pil*3 and *pil*2 by approximately eight and three-fold, respectively, was noticed when PRL2010 was co-cultivated with *L. paracasei* LMG9192. In the co-cultivation experiments of PRL2010 with *B. breve* 12L the enhancement of the major subunit-encoding gene of *pil*3 expression was about three-fold while the major subunit of *pil*2 does not appeared to be expressed (Fig. 3). No increase in expression was noticed, for the major subunit encoding gene of *pil*2 and *pil*3, when *B. bifidum* PRL2010 was co-cultivated with the other organisms here assayed (Fig. 3). Crude protein extracts from the set of microbial co-cultivations displaying a remarkable expression of *fim*P gene, were also subjected to Western blot analysis using Ab_{pil3}. Clear signals representing a protein of 55.4 kDa were noticed in the case of association of PRL2010 with *B. breve* 12L or *L. paracasei* LMG9192 as compared with the control piliated PRL2010 cells (PRL2010 cultivated in MRSc plus lysine) (Fig. 3). Nevertheless, a faint signal was also noticed on protein extract from *B. breve* 12L, which may indicate a possible cross-reaction with the sortase-dependent pilus protein produced by this bacterium. In fact, *in silico* analyses of the genome sequences of this microorganism highlighted the existence of sortase-dependent pilus loci in its chromosome (Bottacini et al., submitted ms), which suggests a cross-hybridization of Ab_{pil3} with such extracellular structures.

Evaluation of the aggregation properties of *B. bifidum* PRL2010. As previously described, pili of PRL2010 are able to promote the co-aggregation of *Lactococcus lactis* expressing both $Pil2_{PRL2010}$ and $Pil3_{PRL2010}$ (Turroni, Serafini et al. 2013). However, nothing is known about the putative role of pili of PRL2010 in driving aggregation with other bifidobacteria as well as other microorganisms that share their ecological niche with PRL2010. Aggregation following co-cultivation of *B. bifidum* PRL2010 together with a set of commensal bacteria of the human gut (*B. breve* 12L, *B. adolescentis* 22L, *B. animalis* subsp. *lactis* Bl12, *B. angulatum* LMG11039, *B. catenulatum* LMG16992, *B. gallicum* DSM20093, *B. longum* subsp. *infantis* ACC15687, *B. kashiwanohense* DSM21854, *B. longum* subsp. *longum* NCC2705, *B. pseudolongum* subsp. *pseudolongum* LMG11571, *L. paracasei* LMG9192), or the eukaryote *S. cerevisiae* BY4741, was evaluated as described in the Materials and Methods section and compared to non-pilated PRL2010 cells (reference condition). After five hours of incubation, *B. bifidum* PRL2010 cells showed an increase in aggregation ($p<0.05$) when co-incubated with *B. angulatum* LMG11039 (5.4%) *B. gallicum* DSM20093 (27.1%), *B. kashiwanohense* DSM21854 (5.5%) and *L. paracasei* LMG9192 (5%) cells, with respect to the reference condition (Fig. 4). In all the other bacterial mixes the strength of co-aggregation estimated was lower than or equal to the auto-aggregation levels of PRL2010 (Fig. 4). These results were confirmed by the presence/absence of FimP in the crude microbial extracts of the above mentioned bacterial mixes through Western blot analyses (Fig. 4). When *B. bifidum* PRL2010 cells were co-cultivated with the organisms for which we noticed a high percentage of co-aggregation, a signal of 55.4 kDa was evident corresponding to the major pilin subunit as well as higher bands suggesting the occurrence of a pilus unit polymerization under the conditions tested (Fig. 4).

Evaluation of the change in aggregation by flow cytometry. Flow Cytometry **(**FCM) analysis revealed that the frequency of cell aggregates present in piliated *B. bifidum* PRL2010 cell suspension, and characterized by a wide range of FSC values measured (Fig. 5), was modified when piliated *B. bifidum* PRL2010 cells were in association with other microorganisms. While the FSC profile of the association between piliated PRL2010 cells and *S. cerevisiae* BY4741

cells (Fig. 5f) results to be the superimposition of the FSC profiles of each independent cell suspension, thus it was possible to distinguish the two populations. This was not the case of the FSC profiles for the other associations. In fact, when piliated PRL2010 cells were grown in association with *L. paracasei* LMG9192 or *B. gallicum* DSM20093 cells, the FSC profile of the association indicated an increase or decrease of the frequency and/or dimension of cell aggregates if compared to those detected in piliated PRL2010 cells or *B. gallicum* DSM20093 cells or *L. paracasei* LMG9192 cells alone (Fig. 5). Conversely, the FSC profile of piliated PRL2010 in association with *B. kashiwanohense* DSM21854 cells or with *B. longum* subsp. *infantis* ATCC15697 cells resembled those of strain DSM21854 and ATCC15697 alone, thus indicating a decrease of the frequency and/or dimension of cell aggregates as confirmed by phase contrast microscope observation (Fig. 5). FCM analyses clearly demonstrated that the co-aggregates of piliated PRL2010 cells when placed together with cells of another gut organisms are disaggregated and are prone to a direct interaction between PRL2010 cells and cells of other gut associated micro-organisms. Such behaviour is essential to sustain microbe-microbe interaction in complex bacterial communities such as those encountered in the human gut.

Conclusions

Although pili have only recently been described in bifidobacteria (Foroni, Serafini et al. 2011, Motherway, Zomer et al. 2011, Turroni, Serafini et al. 2013), very little is so far known about the environmental factors that trigger their expression *in vivo*, i.e. when bifidobacteria reside in their ecological niches. In this study, we demonstrated for the first time how different growth conditions or environmental stresses simulating those encountered by bifidobacteria during their passage through the gastrointestinal tract (e.g., heat and acidic fluctuations) as well as during the establishment of bifidobacterial cells in the gut (e.g., osmotic stress and/or bile salt challenges) influence the expression bifidobacterial pili sortase-dependent genes and consequently the synthesis of pili subunits. Considering that bifidobacteria are largely exploited by food industries as health promoting bacteria it is crucial importance to develop reliable formulations that predispose the microbial cells to interact with the human host. Thus, pre-exposure of bacterial

cells to environmental conditions that may cause differential expression of key host-microbe effectors like pili is important for the development of effective probiotic formulations.

Here, we have also explored the putative role of sortase-dependent pili as key mediators for the physical interaction with other member of the human gut microbiota. We noticed that the level of expression of *pil*3 locus of *B. bifidum* PRL2010 is considerably enhanced upon co-cultivation of PRL2010 cells with *L. paracasei* and at lesser extent with *B. breve* 12L. We do not know if this enhancement in the synthesis of pili of PRL2010 is triggered by a quorum sensing stimulus or is dependent on the presence of capsular carbohydrates released in the environment by such gut microorganisms. In fact, as previously noticed the expression of sortase-dependent pili by *B. bifidum* PRL2010 is influenced by the occurrence of complex carbohydrates in the media (Foroni, Serafini et al. 2011). Also, sortase-dependent pili produced by PRL2010 cells may represent key appendages involved in the interaction with other bacteria through heterogeneous aggregation. Such observations reinforce the notion that sortase-dependent pili of bifidobacteria do not only support the interaction with the intestinal mucosa of the human intestine but are also pivotal for providing a physical bridge between a heterogeneous population of bacterial cells. In addition, we postulated that sortase-dependent pili are playing a key role in modulating the microbe-microbe interaction through the development of hetero-aggregates between the members of complex microbial communities such as those found in the human intestine.

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References

1. **Turroni F, Peano C, Pass DA, Foroni E, Severgnini M, Claesson MJ, Kerr C, Hourihane J, Murray D, Fuligni F, Gueimonde M, Margolles A, De Bellis G, O'Toole PW, van Sinderen D, Marchesi JR, Ventura M.** 2012. Diversity of bifidobacteria within the infant gut microbiota. PLoS One **7:**e36957.
2. **Ventura M, Turroni F, Motherway MO, MacSharry J, van Sinderen D.** 2012. Host-microbe interactions that facilitate gut colonization by commensal bifidobacteria. Trends Microbiol **20:**467-476.
3. **Pokusaeva K, Fitzgerald GF, van Sinderen D.** 2011. Carbohydrate metabolism in Bifidobacteria. Genes Nutr **6:**285-306.
4. **Foroni E, Serafini F, Amidani D, Turroni F, He F, Bottacini F, Motherway MO, Viappiani A, Zhang ZD, Rivetti C, van Sinderen D, Ventura M.** 2011. Genetic analysis and morphological identification of pilus-like structures in members of the genus Bifidobacterium. Microbial cell factories **10**.
5. **Motherway MO, Zomer A, Leahy SC, Reunanen J, Bottacini F, Claesson MJ, O'Brien F, Flynn K, Casey PG, Munoz JAM, Kearney B, Houston AM, O'Mahony C, Higgins DG, Shanahan F, Palva A, de Vos WM, Fitzgerald GF, Ventura M, O'Toole PW, van Sinderen D.** 2011. Functional genome analysis of Bifidobacterium breve UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. P Natl Acad Sci USA **108:**11217-11222.
6. **Turroni F, Serafini F, Foroni E, Duranti S, Motherway MO, Taverniti V, Mangifesta M, Milani C, Viappiani A, Roversi T, Sanchez B, Santoni A, Gioiosa L, Ferrarini A, Delledonne M, Margolles A, Piazza L, Palanza P, Bolchi A, Guglielmetti S, van Sinderen D, Ventura M.** 2013. Role of sortase-dependent pili of Bifidobacterium bifidum PRL2010 in modulating bacterium-host interactions. P Natl Acad Sci USA **110:**11151-11156.
7. **Turroni F, Bottacini F, Foroni E, Mulder I, Kim JH, Zomer A, Sanchez B, Bidossi A, Ferrarini A, Giubellini V, Delledonne M, Henrissat B, Coutinho P, Oggioni M, Fitzgerald GF, Mills D, Margolles A, Kelly D, van Sinderen D, Ventura M.** 2010. Genome analysis of Bifidobacterium bifidum PRL2010 reveals metabolic pathways for host-derived glycan foraging. P Natl Acad Sci USA **107:**19514-19519.
8. **Ton-That H, Schneewind O.** 2003. Assembly of pili on the surface of Corynebacterium diphtheriae. Molecular microbiology **50:**1429-1438.
9. **Mishra A, Wu CG, Yang JH, Cisar JO, Das A, Ton-That H.** 2010. The Actinomyces oris type 2 fimbrial shaft FimA mediates co-aggregation with oral streptococci, adherence to red blood cells and biofilm development. Molecular microbiology **77:**841-854.
10. **Yeung MK, Ragsdale PA.** 1997. Synthesis and function of Actinomyces naeslundii T14V type 1 fimbriae require the expression of additional fimbria-associated genes. Infect Immun **65:**2629-2639.
11. **Okahashi N, Nakata M, Terao Y, Isoda R, Sakurai A, Sumitomo T, Yamaguchi M, Kimura RK, Oiki E, Kawabata S, Ooshima T.** 2011. Pili of oral Streptococcus sanguinis bind to salivary amylase and promote the biofilm formation. Microbial pathogenesis **50:**148-154.
12. **Sonnenburg JL, Chen CTL, Gordon JI.** 2006. Genomic and metabolic studies of the impact of probiotics on a model gut symbiont and host. PLoS biology **4:**2213-2226.
13. **Sincock SA, Robinson JP.** 2001. Flow cytometric analysis of microorganisms. Methods in cell biology **64:**511-537.
14. **Chenoll E, Casinos B, Bataller E, Astals P, Echevarria J, Iglesias JR, Balbarie P, Ramon D, Genoves S.** 2011. Novel probiotic Bifidobacterium bifidum CECT 7366

strain active against the pathogenic bacterium Helicobacter pylori. Appl Environ Microbiol **77:**1335-1343.

15. **Ventura M, Canchaya C, Zink R, Fitzgerald GF, van Sinderen D.** 2004. Characterization of the groEL and groES loci in Bifidobacterium breve UCC 2003: genetic, transcriptional, and phylogenetic analyses. Appl Environ Microbiol **70:**6197-6209.
16. **Ventura M, Canchaya C, van Sinderen D, Fitzgerald GF, Zink R.** 2004. Bifidobacterium lactis DSM 10140: identification of the atp (atpBEFHAGDC) operon and analysis of its genetic structure, characteristics, and phylogeny. Appl Environ Microbiol **70:**3110-3121.
17. **Del Re B, Sgorbati B, Miglioli M, Palenzona D.** 2000. Adhesion, autoaggregation and hydrophobicity of 13 strains of Bifidobacterium longum. Lett Appl Microbiol **31:**438-442.
18. **Kos B, Suskovic J, Vukovic S, Simpraga M, Frece J, Matosic S.** 2003. Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92. J Appl Microbiol **94:**981-987.
19. **Handley PS, Harty DW, Wyatt JE, Brown CR, Doran JP, Gibbs AC.** 1987. A comparison of the adhesion, coaggregation and cell-surface hydrophobicity properties of fibrillar and fimbriate strains of Streptococcus salivarius. Journal of general microbiology **133:**3207-3217.
20. **Gunasekera TS, Veal DA, Attfield PV.** 2003. Potential for broad applications of flow cytometry and fluorescence techniques in microbiological and somatic cell analyses of milk. Int J Food Microbiol **85:**269-279.
21. **Cronin UP, Wilkinson MG.** 2010. The potential of flow cytometry in the study of Bacillus cereus. J Appl Microbiol **108:**1-16.
22. **Turroni F, Foroni E, Montanini B, Viappiani A, Strati F, Duranti S, Ferrarini A, Delledonne M, van Sinderen D, Ventura M.** 2011. Global Genome Transcription Profiling of Bifidobacterium bifidum PRL2010 under In Vitro Conditions and Identification of Reference Genes for Quantitative Real-Time PCR. Applied and environmental microbiology **77:**8578-8587.
23. **Turroni F, Serafini F, Foroni E, Duranti S, O'Connell Motherway M, Taverniti V, Mangifesta M, Milani C, Viappiani A, Roversi T, Sanchez B, Santoni A, Gioiosa L, Ferrarini A, Delledonne M, Margolles A, Piazza L, Palanza P, Bolchi A, Guglielmetti S, van Sinderen D, Ventura M.** 2013. Role of sortase-dependent pili of Bifidobacterium bifidum PRL2010 in modulating bacterium-host interactions. Proc Natl Acad Sci U S A.
24. **Foroni E, Serafini F, Amidani D, Turroni F, He F, Bottacini F, O'Connell Motherway M, Viappiani A, Zhang Z, Rivetti C, van Sinderen D, Ventura M.** 2011. Genetic analysis and morphological identification of pilus-like structures in members of the genus Bifidobacterium. Microbial cell factories **10 Suppl 1:**S16.
25. **Nakata M, Koeller T, Moritz K, Ribardo D, Jonas L, McIver KS, Sumitomo T, Terao Y, Kawabata S, Podbielski A, Kreikemeyer B.** 2009. Mode of Expression and Functional Characterization of FCT-3 Pilus Region-Encoded Proteins in Streptococcus pyogenes Serotype M49. Infect Immun **77:**32-44.
26. **Hendrickx APA, Bonten MJM, van Luit-Asbroek M, Schapendonk CME, Kragten AHM, Willems RJL.** 2008. Expression of two distinct types of pili by a hospital-acquired Enterococcus faecium isolate. Microbiol-Sgm **154:**3212-3223.
27. **Jiang SM, Park SE, Yadav P, Paoletti LC, Wessels MR.** 2012. Regulation and Function of Pilus Island 1 in Group B Streptococcus. Journal of bacteriology **194:**2479-2490.
28. **Hidalgo-Cantabrana C, Sanchez B, Milani C, Ventura M, Margolles A, Ruas-Madiedo P.** 2013. Exopolysaccharide biosynthesis in Bifidobacterium spp.: biological functions and a genomic overview. Applied and environmental microbiology.

29. **Ventura M, Canchaya C, Zhang ZD, Bernini V, Fitzgerald GF, van Sinderen D.** 2006. How high G+C Gram-positive bacteria and in particular bifidobacteria cope with heat stress: protein players and regulators. Fems Microbiol Rev **30:**734-759.
30. **Kim HK, Kim KS, Kim ME.** 2013. Influence of Test Site and Baseline Temperature on Orofacial Thermal Thresholds. J Orofac Pain **27:**263-270.
31. **Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G.** 2006. Pili in Gram-positive pathogens. Nat Rev Microbiol **4:**509-519.
32. **Ton-That H, Schneewind O.** 2004. Assembly of pill in Gram-positive bacteria. Trends in microbiology **12:**228-234.
33. **Serafini F, Strati F, Ruas-Madiedo P, Turroni F, Foroni E, Duranti S, Milano F, Perotti A, Viappiani A, Guglielmetti S, Buschini A, Margolles A, van Sinderen D, Ventura M.** 2013. Evaluation of adhesion properties and antibacterial activities of the infant gut commensal Bifidobacterium bifidum PRL2010. Anaerobe **21:**9-17.
34. **Danne C, Dramsi S.** 2012. Pili of Gram-positive bacteria: roles in host colonization. Res Microbiol **163:**645-658.
35. **Milani C, Duranti S, Lugli GA, Bottacini F, Strati F, Arioli S, Foroni E, Turroni F, van Sinderen D, Ventura M.** 2013. Comparative Genomics of Bifidobacterium animalis subsp lactis Reveals a Strict Monophyletic Bifidobacterial Taxon. Applied and environmental microbiology **79:**4304-4315.
36. **Turroni F, Bottacini F, Foroni E, Mulder I, Kim JH, Zomer A, Sanchez B, Bidossi A, Ferrarini A, Giubellini V, Delledonne M, Henrissat B, Coutinho P, Oggioni M, Fitzgerald GF, Mills D, Margolles A, Kelly D, van Sinderen D, Ventura M.** 2010. Genome analysis of Bifidobacterium bifidum PRL2010 reveals metabolic pathways for host-derived glycan foraging. Proc Natl Acad Sci U S A **107:**19514-19519.
37. **Turroni F, Foroni E, Serafini F, Viappiani A, Montanini B, Bottacini F, Ferrarini A, Bacchini PL, Rota C, Delledonne M, Ottonello S, van Sinderen D, Ventura M.** 2011. Ability of Bifidobacterium breve to grow on different types of milk: exploring the metabolism of milk through genome analysis. Appl Environ Microbiol **77:**7408-7417.
38. **Miyake T, Watanabe K, Watanabe T, Oyaizu H.** 1998. Phylogenetic analysis of the genus Bifidobacterium and related genera based on 16S rDNA sequences. Microbiol Immunol **42:**661-667.
39. **Scardovi V, Crociani F.** 1974. Bifidobacterium-Catenulatum, Bifidobacterium-Dentium, and Bifidobacterium-Angulatum - 3 New Species and Their Deoxyribonucleic-Acid Homology Relationships. International journal of systematic bacteriology **24:**6-20.
40. **Sela DA, Chapman J, Adeuya A, Kim JH, Chen F, Whitehead TR, Lapidus A, Rokhsar DS, Lebrilla CB, German JB, Price NP, Richardson PM, Mills DA.** 2008. The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome. Proc Natl Acad Sci U S A **105:**18964-18969.
41. **Morita H, Nakano A, Onoda H, Toh H, Oshima K, Takami H, Murakami M, Fukuda S, Takizawa T, Kuwahara T, Ohno H, Tanabe S, Hattori M.** 2011. Bifidobacterium kashiwanohense sp. nov., isolated from healthy infant faeces. Int J Syst Evol Microbiol **61:**2610-2615.
42. **Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zwahlen MC, Desiere F, Bork P, Delley M, Pridmore RD, Arigoni F.** 2002. The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci U S A **99:**14422-14427.
43. **Collins MD, Phillips BA, Zanoni P.** 1989. Deoxyribonucleic-Acid Homology Studies of Lactobacillus-Casei, Lactobacillus-Paracasei Sp-Nov, Subsp Paracasei and Subsp Tolerans, and Lactobacillus-Rhamnosus Sp-Nov, Comb-Nov. International journal of systematic bacteriology **39:**105-108.

Table 1. Strains used in this study.

Strains	References
B. bifidum PRL2010	(Turroni, Bottacini et al. 2010)
B. animalis subsp. *lactis* Bl12	(Milani, Duranti et al. 2013)
B. breve 12L	(Turroni, Foroni et al. 2011)
B. adolescentis 22L	(Turroni, Foroni et al. 2011)
B. angulatum LMG11039	(Miyake, Watanabe et al. 1998)
B. catenulatum LMG16992	(Scardovi and Crociani 1974)
B. gallicum DSM20093	(Miyake, Watanabe et al. 1998)
B. longum subsp. *infantis* ATCC15697	(Sela, Chapman et al. 2008)
B. kashiwanohense DSM21854	(Morita, Nakano et al. 2011)
B. longum subsp. *longum* NCC2705	(Schell, Karmirantzou et al. 2002)
B. pseudolongum subsp. *pseudolongum* LMG11571	(Miyake, Watanabe et al. 1998)
L. paracasei LMG9192	(Collins, Phillips et al. 1989)
S. cerevisiae BY4742	Yeast genetic Stock Center (Berkeley, CA)

Legends

Figure 1. Pili production of PRL2010 cultivated under different growth conditions. Panel a shows relative transcription levels of pilus-encoding genes *pil*2 and *pil*3 of *B. bifidum* PRL2010 cultures subjected to different stress conditions. BBPR_0282 encodes the minor subunit (*fim*Q), BBPR_0283 encodes the major subunit (*fim*P), and BBPR_0284 specifies the sortase of the *pil*3 locus. BBPR_1707 encodes the major subunit (*fim*A), BBPR_1708 encodes the minor subunit (*fimB*), and BBPR_1709 specifies the sortase of the *pil*2 locus. The reference condition used involves PRL2010 cultures cultivated in MRS broth at 37°C without lysine, which is a condition that do not allow the production of pili sortase-dependent by PRL2010 cells (Turroni, Serafini et al. 2013). Panel b displays Western blot analyses of crude protein extracts of *B. bifidum* PRL2010 cultures previously exposed to different stressful conditions. Lane 1, negative control (MRS without lysin); lane 2, positive control (37°C in MRS plus lysin); lane 3, 30°C; lane 4, 25°C; lane 5, oxgal 0.5 %; lane 6, pH 4; lane 7, NaCl at 2 %; lane 8, pancreatin.

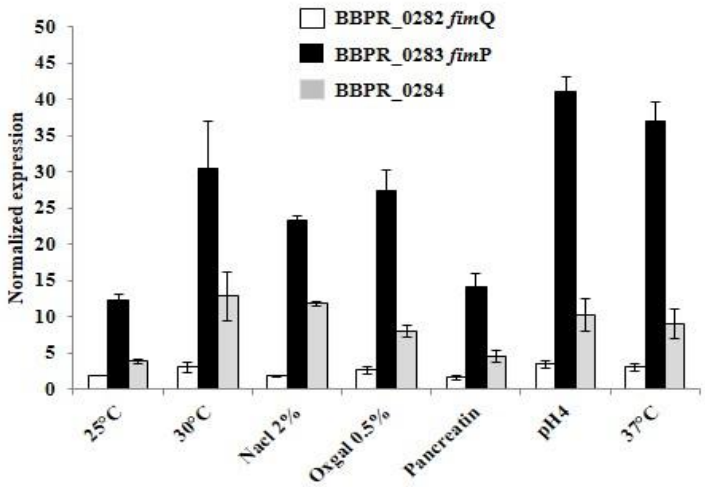
Figure 2. Growth of *B. bifidum* PRL2010 co-cultivated with other micro organisms. The histogram represents, through RT-PCR analyses, the genome copy number/ml broth of *B. breve* 12L, *B. adolescentis* 22L, *B. animalis* subsp. *lactis* Bl12, *S. cerevisiae* BY4741, *L. paracasei* LMG9192 alone and co-cultivated with *B. bifidum* PRL2010.

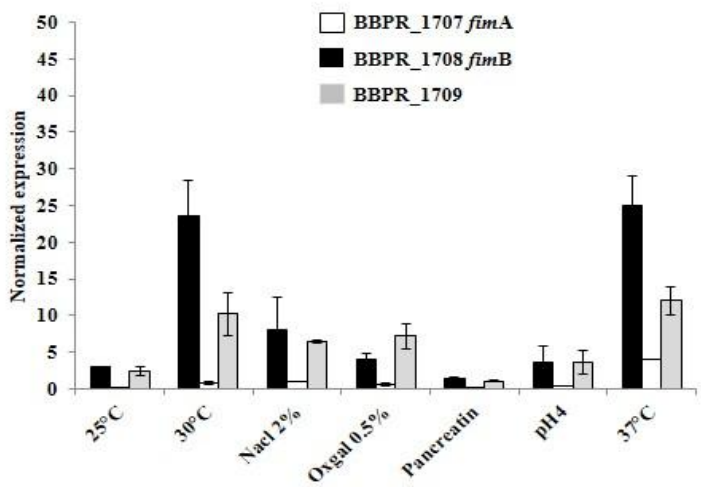
Figure 3. Modulation of the expression of sortase-dependent pili of *B. bifidum* PRL2010 upon co-cultivation with other bacteria. Panel a displays the relative transcription levels of pilus-encoding genes *pil*2 and *pil*3 from *B. bifidum* PRL2010 upon co-cultivation with different organisms. Panel b shows Western blot analyses using crude extracts of PRL2010 cells upon co-cultivation with various organisms. Lane 1, negative control consisting of the crude extracts of non-piliated *B. bifidum* PRL2010 cells; lane 2, crude extracts of *B. breve* 12L; lane 3, crude extracts of cultures consisting of *B. bifidum* PRL2010 co-cultivated with *B. breve* 12L; lane 4,

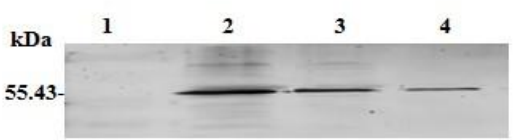
crude extracts of *B. bifidum* PRL2010 co-cultivated with *L. paracasei* LMG9192; lane 5, crude extracts of *L. paracasei* LMG9192.

Figure 4. Co-aggregation of *B. bifidum* PRL2010 cells with different gut organisms. The graph shows the comparison of co-aggregation ability of piliated *B. bifidum* cells with other strains compared to the percentage of auto-aggregation of non-piliated PRL2010 cells grown alone. The level of auto-aggregation for the other organisms tested alone is also displayed. Above each pillar showing the co-aggregation level measured for the microbial mixture, the corresponding Western blot profile of the crude extracts probed with Ab_{pil3} is displayed.

Figure 5. FCM analysis and phase contrast microscopic pictures of *B. bifidum* PRL2010 alone and in association with *B. angulatum* LMG11039, *B. kashiwanohense* DSM21854, *B. longum* subsp. *infantis* ATCC15697, *L. paracasei* LMG9192 and *S. cerevisiae* BY4741. FCM plots report the FSC parameter as area logarithmic signals (FSC-A). Dark lines refer to the FSC profile of PRL2010 cell suspension. Red lines referred to the FSC profile of the other microorganisms alone. Blue lines refer to the FSC profile of culture associations between PRL2010 and one of the other microorganisms as indicated in the figure. Panel a shows PRL2010 alone. Panel b, display the association between PRL2010 and *B. gallicum* DSM20093. Panel c depicts the association between PRL2010 and *B. longum* subsp. *infantis* ATCC15697. Panel d exhibits the association between PRL2010 and *B. kashiwanohense* DSM21854. Panel e evidences the association between PRL2010 and *L. paracasei* LMG9192. Panel f shows the association between PRL2010 and *S. cerevisiae* BY4741. All pictures, except that represented in panel a referred to culture association.


BBPR_0282 fimQ
BBPR_0283 fimP
BBPR_0284
Normalized expression
50
45
40
35
30
25
20
15
10
5
0
25°C
30°C
NaCl 2%
Oxgal 0.5%
Pancreatin
pH4
37°C


BBPR_1707 fimA
BBPR_1708 fimB
BBPR_1709
Normalized expression
50
45
40
35
30
25
20
15
10
5
0
25°C
30°C
NaCl 2%
Oxgal 0.5%
Pancreatin
pH4
37°C


kDa
1
2
3
4
55.43-

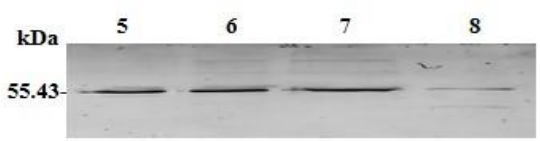

kDa
5
6
7
8
55.43-

Figure 1

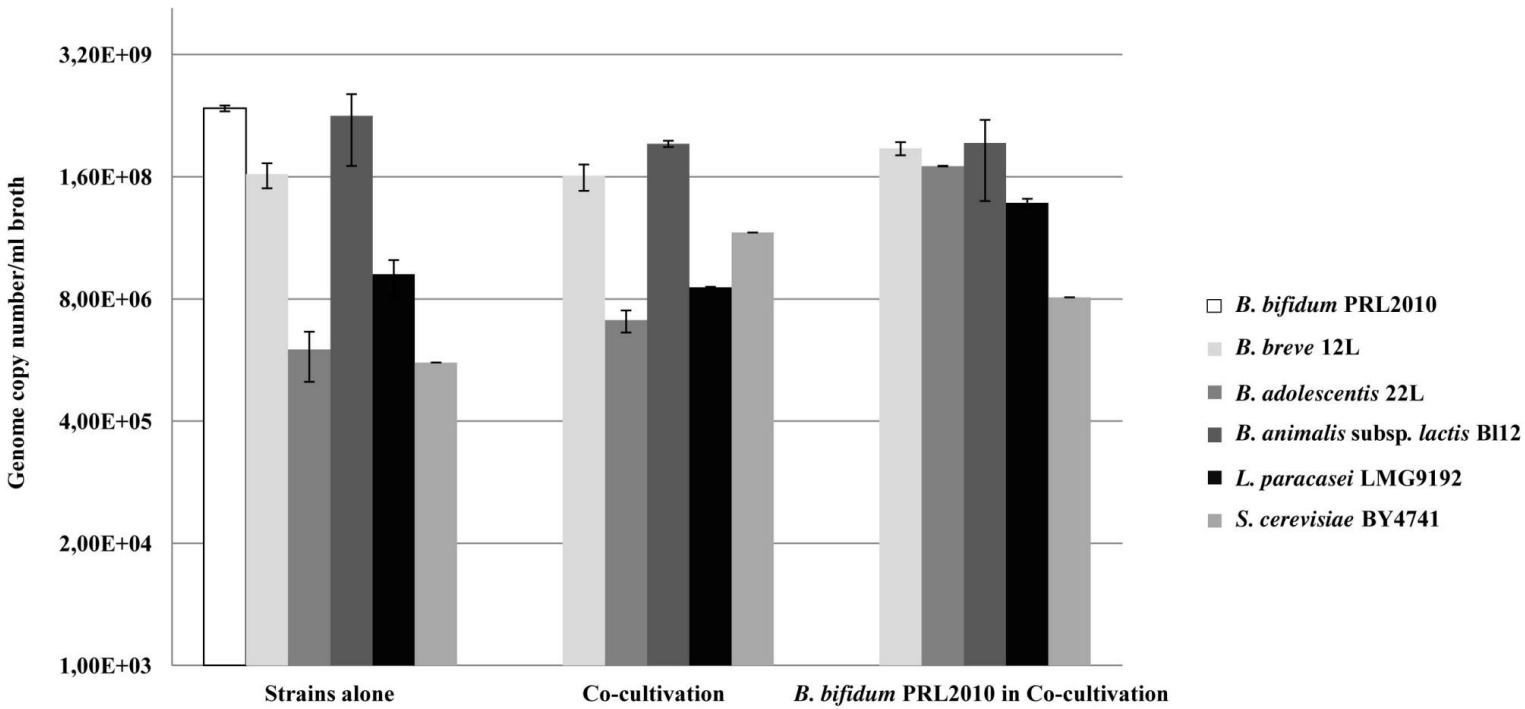
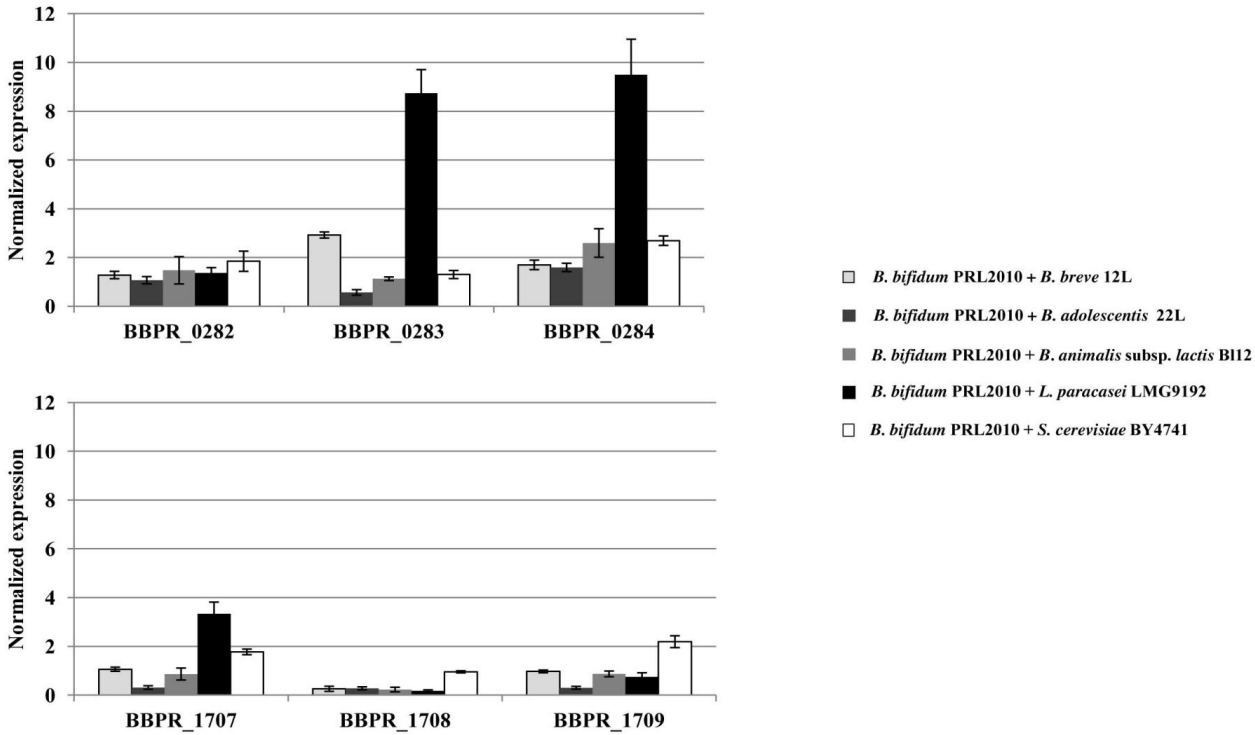

3,20E+09
1,60E+08
8,00E+06
4,00E+05
2,00E+04
1,00E+03
Genome copy number/ml broth
Strains alone
Co-cultivation
B. bifidum PRL2010 in Co-cultivation
B. bifidum PRL2010
B. breve 12L
B. adolescentis 22L
B. animalis subsp. lactis Bl12
L. paracasei LMG9192
S. cerevisiae BY4741

Figure 2


Normalized expression
12
10
8
6
4
2
0
BBPR_0282
BBPR_0283
BBPR_0284
Normalized expression
12
10
8
6
4
2
0
BBPR_1707
BBPR_1708
BBPR_1709
B. bifidum PRL2010 + B. breve 12L
B. bifidum PRL2010 + B. adolescentis 22L
B. bifidum PRL2010 + B. animalis subsp. lactis Bl12
B. bifidum PRL2010 + L. paracasei LMG9192
B. bifidum PRL2010 + S. cerevisiae BY4741

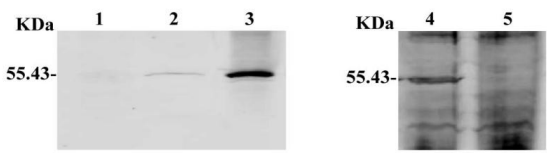

KDa
1
2
3
55.43-
KDa
4
5
55.43-

Figure 3

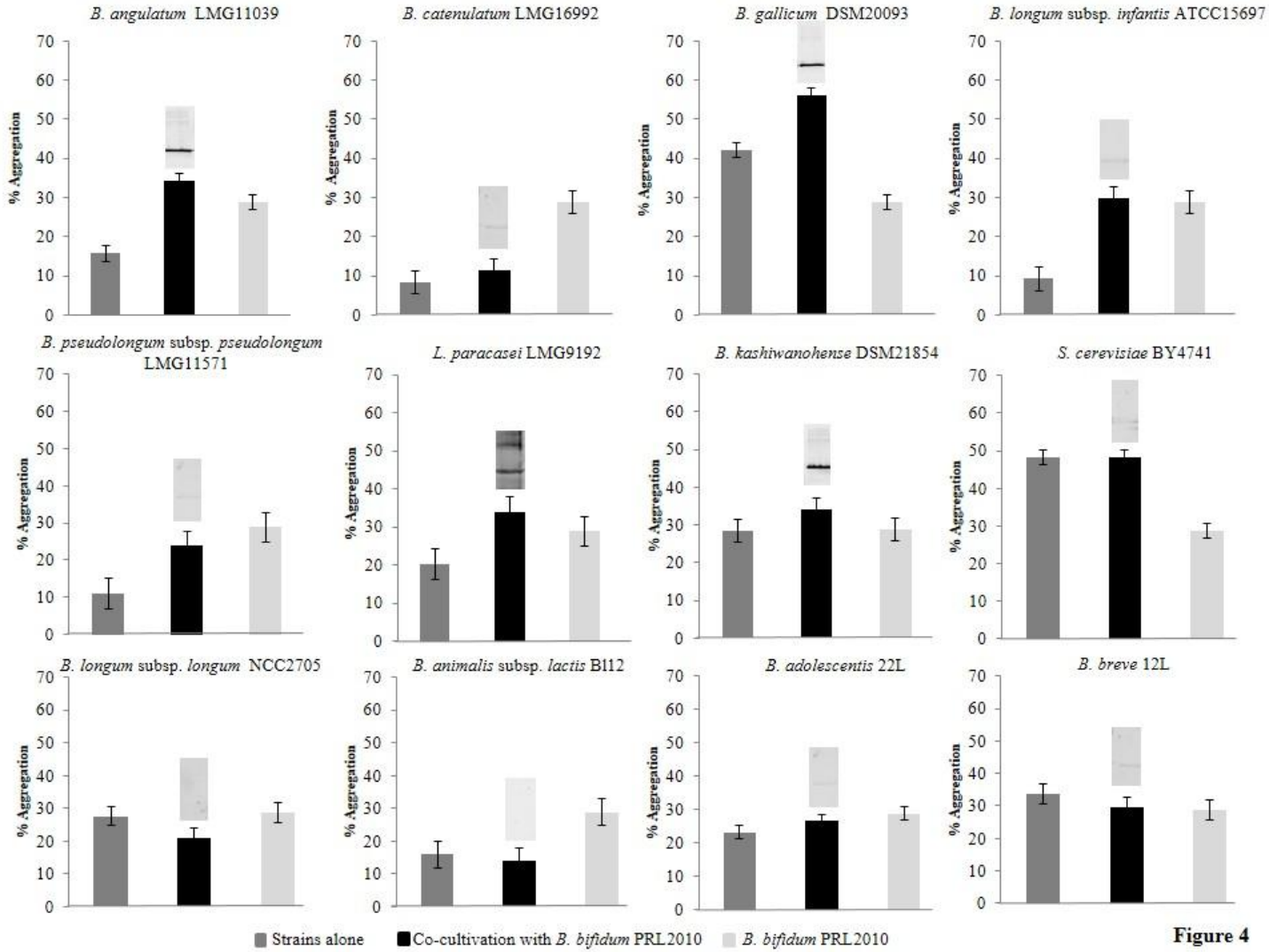

B. angulatum LMG11039
B. catenulatum LMG16992
B. gallicum DSM20093
B. longum subsp. infantis ATCC15697
B. pseudolongum subsp. pseudolongum LMG11571
L. paracasei LMG9192
B. kashiwanohense DSM21854
S. cerevisiae BY4741
B. longum subsp. longum NCC2705
B. animalis subsp. lactis BI12
B. adolescentis 22L
B. breve 12L
% Aggregation
Strains alone
Co-cultivation with B. bifidum PRL2010
B. bifidum PRL2010

Figure 4

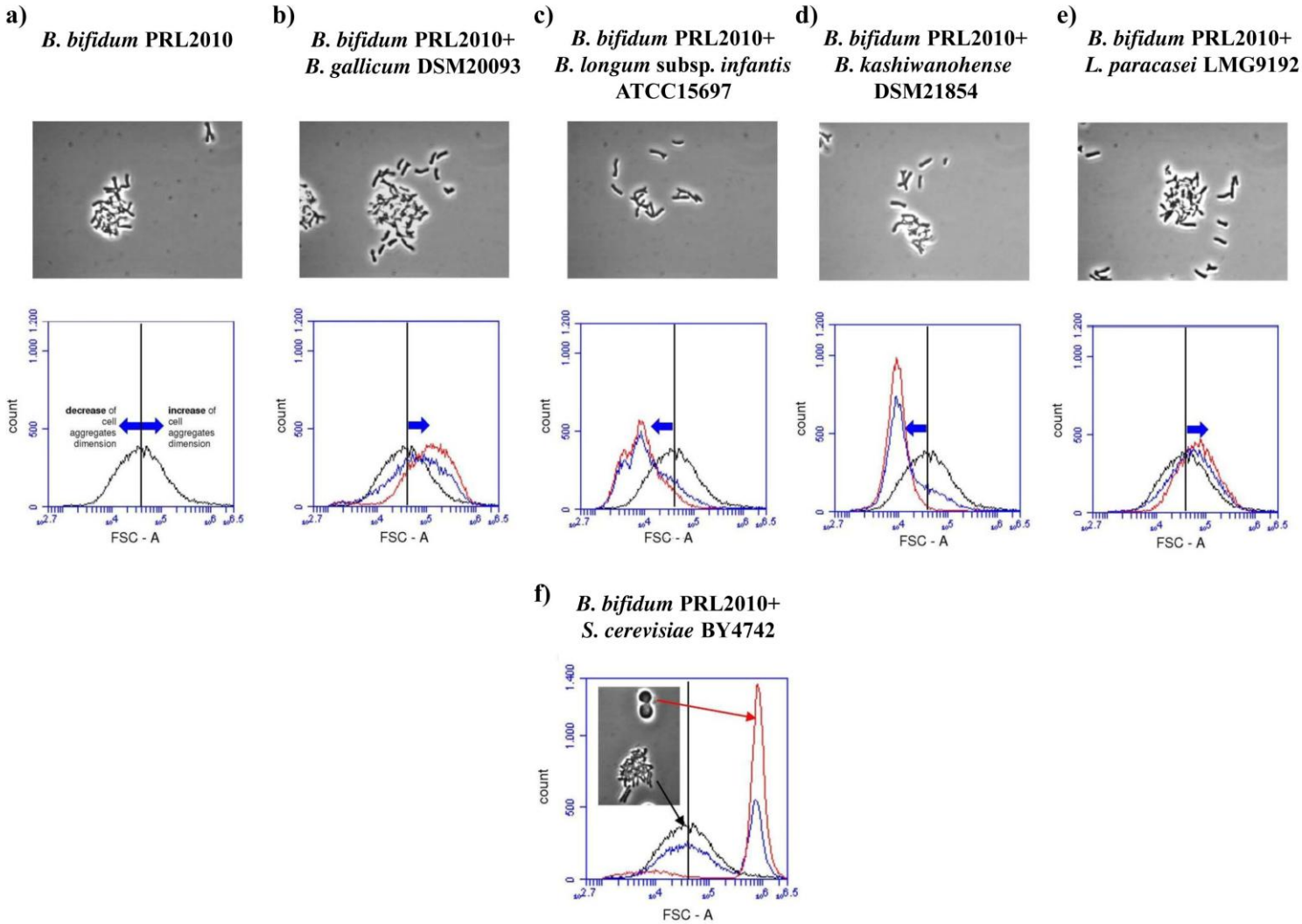

a)
B. bifidum PRL2010
b)
B. bifidum PRL2010+
B. gallicum DSM20093
c)
B. bifidum PRL2010+
B. longum subsp. infantis
ATCC15697
d)
B. bifidum PRL2010+
B. kashiwanohense
DSM21854
e)
B. bifidum PRL2010+
L. paracasei LMG9192
f)
B. bifidum PRL2010+
S. cerevisiae BY4742
count
FSC - A
decrease of cell aggregates dimension
increase of cell aggregates dimension

Figure 5

9. Interplay between *Bifidobacterium bifidum* PRL2010 and the microbiota of fermented milk kefir: insights from metagenomics and transcriptomics

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Abstract

Bifidobacteria constitute one of the dominant groups of microorganisms colonizing the human gut of infants. Their ability to utilize various host-derived glycans as well as dietary carbohydrates has received considerable scientific attention. However, very little is known about the role of fermented foods, such as kefir, or their constituent glycans, such as kefiran, as substrates for the growth of bifidobacteria and for the modulation of the expression of bifidobacterial host-effector molecules. Here, we show that *Bifidobacterium bifidum* PRL2010 exhibits high growth performance among the bifidobacterial strains tested when cultivated on kefir and/or kefiran polymer. Furthermore, a 16S rRNA metagenomic approach revealed that the microbiota of kefir is modified upon the addition of PRL2010 cells to the kefir matrix. Finally, our results show that kefir and kefiran are able to influence the transcriptome of *B. bifidum* PRL2010 causing increased transcription of genes involved in the metabolism of dietary glycans as well as genes that act as host-microbe effector molecules such as pili.

1. Introduction

Kefir is a fermented milk beverage that originated in Eastern Europe (Wouters, Ayad et al. 2002). It is a self-carbonated dairy product with a slightly acidic taste, yeasty flavor, creamy consistency, and low alcohol content (Marshall and Cole 1985, Garrote GL 2010, E (2005)). Many health benefits have been attributed to kefir, including the enhancement of the immune system and improvement of digestive health, as well as antimicrobial, antitumor, antiviral, antimutagenic, and antioxidant activity (Otles, Cagindi et al. 2003, Santos, San Mauro et al. 2003, Liu, Chen et al. 2005, Rodrigues, Carvalho et al. 2005, de LeBlanc, Matar et al. 2007, Vardjan, Lorbeg et al. 2013). Kefir is produced by the fermentation of milk with kefir grains. The grains contain a cocktail of microorganisms that co-exist in a complex symbiotic relationship, and include species of lactic acid bacteria (e.g., lactobacilli, lactococci, leuconostoc, streptococci), yeasts, acetic acid bacteria and mycelia-forming fungi (Garbers I-M 2004, Vardjan, Lorbeg et al. 2013). Speciation of individual microbes present in such a complex microbial consortium is complicated by the fact that some of the constituent microorganisms cannot be grown on currently available synthetic growth media (Muyzer, de Waal et al. 1993, Wyder, Meile et al. 1999).

Following the fermentation process the kefir grains can be recovered, reused, and cultivated, often over long periods. In addition to the value of the kefir-associated microbial community as a whole, specific strains isolated from kefir may have value as probiotics (Golowczyc, Gugliada et al. 2008), as kefir is considered an example of a probiotic mixture of bacteria and yeast (Simova, Beshkova et al. 2002).

The exopolysaccharide (EPS) produced by the kefir microflora, named kefiran, is a heteropolymer (with a hexasaccharide repeated unit) containing glucose and galactose (Micheli, Uccelletti et al. 1999). Several health-promoting properties of kefiran, such as immunomodulation or epithelium protection, have been reported (Vinderola, Perdigon et al. 2006, Medrano, Perez et al. 2008). In addition, EPS synthesized by kefir bacteria could act as a

fermentable substrate for other microorganisms in the kefir milk, modifying interactions among kefir populations. Within this context, there is a growing commercial interest to use kefir as a suitable food matrix for the supplementation with health-promoting bacteria. Here, we wanted to explore the interactions that may occur between kefir and kefiran, and common human gut residents such as bifidobacteria, and to investigate if these substrates modulate expression of specific host-responsive molecule effectors, e.g. pili (Ventura, Turroni et al. 2012, Turroni, Serafini et al. 2013).

Bifidobacteria enjoy intensive commercial exploitation by food industry due to the presumed health beneficial effects they exert on the human host (Masco, Huys et al. 2005). Various molecules, such as capsular polysaccharides and fimbriae/pili produced by bifidobacteria, are considered crucial in mediating host interactions and are believed to cover or protrude from the bacterial cell, thus being exposed to the external environment of the bacterial cell (Motherway, Zomer et al. 2011, Fanning, Hall et al. 2012, Ventura, Turroni et al. 2012, Turroni, Serafini et al. 2013). However, the molecular mechanisms underlying the presumed health-promoting activities of bifidobacteria, or the dietary compounds that may be responsible for driving the expression of the above-mentioned host-bifidobacterial responsive molecules are still largely unknown.

Here, we assessed the possibility to utilize kefir as a substrate for the growth of bifidobacteria, with specific emphasis on the infant gut commensal *Bifidobacterium bifidum* PRL2010, serving as our bifidobacterial model (Turroni, Bottacini et al. 2010, Turroni, Strati et al. 2012, Serafini, Strati et al. 2013, Turroni, Serafini et al. 2013). Furthermore, we have evaluated if the presence of PRL2010 cells influences the kefir bacterial microbiota using 16S rRNA gene metagenomic approach and explored the impact of kefir compounds on the transcriptome of *B. bifidum* PRL2010.

2. Materials and methods

2.1. Bacterial strains and growth conditions.

Bifidobacterial strains used in this study are listed in Table 1, and were cultivated in an anaerobic atmosphere (2.99 % H_2, 17.01 % CO_2 and 80 % N_2) in a chamber (Concept 400, Ruskin) on De Man-Rogosa-Sharp (MRS) (Scharlau Chemie, Barcelona, Spain) supplemented with 0.05 % (w/v) L-cysteine hydrochloride (MRSc) and incubated at 37°C for 16 h.

2.2. Kefir Grains.

Kefir grains were sampled four times, once per month. Between two consecutive samplings, kefir grains were transferred daily and propagated at 23°C in pasteurized milk according to a dairy routine protocol (Generoso, Wolf et al. 2005, Kok-Tas, Seydim et al. 2013).

2.3. Growth of bifidobacteria in Kefir by Culture-Dependent Methods.

Kefir grains were incubated in sterile 10 % reconstituted skim milk at 23°C for 24 h. The grains were removed by filtration and the obtained fermented kefir milk was inoculated with 2 % of each strain to be tested (Table 1). In order to monitor the specific strain survival over the course of the kefir fermentation, kefir milk samples were first homogenized, 10-fold serial dilutions were then prepared and appropriate dilutions were spread plated onto MRSc supplied with 50 □g/mL mupirocin (Oxoid Ltd, Hampshire, UK). Plates were incubated overnight anaerobically at 37°C. In order to evaluate if bifidobacterial cells were still present in the kefir at the end of fermentation, ten colonies from each condition were randomly picked in MRS broth and incubated overnight anaerobically at 37°C. The strain identity was assayed by PCR using strain-specific primers (Table 2).

2.4. Purification and chemical analysis of Kefiran.

Three different batches of fermented milk kefir, made by the same manufacturer, were purchased from the supermarket (stated carbohydrate, protein, and fat contents were 4 %, 3.8 % and 4.5 %, respectively). These fermented milks were used to isolate the EPS kefiran using a previously described procedure by Burns and co-workers (Burns, Vinderola et al. 2011). In short, series of glass flasks containing 40 g kefir were mixed with 8 ml of 60 % TCA solution (final TCA 12 %)

and vigorously stirred for 45 min at room temperature. Then, samples were centrifuged (10,000 x g, 4ºC, 30 min) to remove the surface layer of fat as well as the precipitated proteins and bacteria. The pH of the supernatants was increased with 10 M NaOH up to 4.5 and introduced in cellulose dialysis membranes (12-14 kDa MWCO, Sigma Chemical Co. St. Louis, MO, USA). Intensive dialysis was carried out against ultra-pure water at 4ºC during three days with two changes of water per day. Finally, dialysed samples were frozen at -80ºC and free-dried in the Freezemobile 12L lyophilizer (VirTis, Thermo-Fisher Scientific, Madrid, Spain) to obtain the lyophilized power. The protein content of this powder, as determined by the BCA protein assay kit (Pierce, IL, USA), was 22.4±5.2%. The kefiran yield, as well as the distribution of molecular weight and radius of gyration of the polymer, in the lyophilized powder was determined by means of size exclusion chromatography (SEC) as previously described (Salazar, Prieto et al. 2009). An HPLC system from Waters (Milford, MA, USA) was used, which was composed of the Alliance 2690 module injector, the Photodiode Array PDA966 detector, and the Refractive Index RI410 detector. These detectors were coupled in series with the multiangle laser light scattering (MALLS) detector Dawn Heleos II (Wyatt Europe GmbH, Dembach, Germany). The analysis of data was achieved by the Astra V 5.3.4.19 software (Wyatt Europe). Then, 10 mg lyophilized powder was dissolved in 0.1 M $NaNO_3$ and separated by chromatography using TSK-Gel G3000 PWXL and TSK-Gel G5000 PWXL (Supelco-Sigma) columns placed in series and protected with a TSK-guard column. The same solution was used as the mobile phase and the separation took place at 40ºC at 0.45 ml/min flow rate for up to 60 min. The kefiran yield was calculated from the RI data using the corresponding regression equations ($R2 \geq 0.9999$) obtained from dextran standards (Fluka-Sigma) of similar size ($4.9x10^6$ Da and $2.7x10^5$ Da). The PDA detector set at 280 nm was used to check the absence of protein in the peaks corresponding to kefiran. The weight average molar mass (Mw) and the radius of gyration (Rw) were directly measured with the MALLS detector and used to calculate the coefficient ν [$\nu = (\log Rw / \log Mw)$].

2.5. Growth of *B. bifidum* PRL2010 in kefiran-containing medium by Culture-Dependent Methods.

A pre-inoculum of PRL2010 was washed with PBS and resuspended in the same volume of carbohydrate-free MRSc (CF-MRSc) formulated according to the composition of the Difco medium (BD, Biosciences, San Diego, CA) but without glucose. Then, *B. bifidum* PRL2010 was inoculated (2 %) in 10 ml of CF-MRSc containing 0.3 % (wt/vol) kefiran and incubated at 37ºC under anaerobic conditions. CF-MRSc and this medium supplemented with 0.3 % glucose were used as controls. To monitor the growth performance of PRL2010, bacterial counts were carried out by deep plating in MRSc agar following incubation under anaerobic conditions for 8 h, 24 h, 48 h and 72 h at 37°C. Strain identity was assayed by PCR using specific primers based on the *pil*3 locus (Table 2) (Turroni, Serafini et al. 2013). Furthermore for each time point the pH value was measured.

2.6. Kefir microbiota identification by 16S rRNA gene amplification.

DNA was extracted from kefir samples by acid guanidinium thiocyanate-phenol-chloroform extraction (Chirgwin, Przybyla et al. 1979, Chomczynski and Sacchi 1987, Boom, Sol et al. 1990). Partial 16S rRNA gene sequences were amplified from extracted DNA using primer pair Probio_Uni and /Probio_Rev, which target the V3 region of the 16S rRNA gene sequence (Milani, Hevia et al. 2013). These primers were designed to include at their 5' end one of the two adaptor sequences used in the Ion Torrent-sequencing library preparation protocol linking a unique Tag barcode of 10 bases to identify different samples. The PCR conditions used were 5 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 55°C and 90 s at 72°C, followed by 10 min at 72°C. Amplification was carried out using a Verity Thermocycler (Applied Biosystems). The integrity of the PCR amplicons was analyzed by electrophoresis on an Experion workstation (BioRad, UK).

2.7. Ion Torrent PGM sequencing of 16S rRNA gene-based amplicons.

PCR products derived from amplification of specific 16S rRNA gene hypervariable regions were purified by electrophoretic separation on an 1.5 % agarose gel and the use of a Wizard SV Gen

PCR Clean-Up System (Promega), followed by an additional purification step involving the Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) in order to remove primer dimers. DNA concentration of the amplified sequence library was estimated through the Experion system (BioRad). From the concentration and the average size of each amplicon library, the amount of DNA fragments per microliter was calculated and libraries for each run were diluted to $3E^9$ DNA molecules prior to clonal amplification. Emulsion PCR was carried out using the Ion OneTouch™ 200 Template Kit v2 DL (Life Technologies) according to the manufacturer's instructions. Sequencing of the amplicon libraries was carried out on a 316 chip using the Ion Torrent PGM system and employing the Ion Sequencing 200 kit (Life Technologies) according to the supplier's instructions. Sequencing was performed at the DNA sequencing facility of GenProbio srl (www.genprobio.com). Following sequencing, the obtained individual sequence reads were filtered by the PGM software to remove low quality and polyclonal sequences. Sequences matching the PGM 3' adaptor were also automatically trimmed. All PGM quality-approved, trimmed and filtered data were exported as sff files.

2.8. Sequence-based microbiota analysis.

The sff files were processed using QIIME (Caporaso, Kuczynski et al. 2010). Quality control retained sequences with a length between 150 and 200 bp, mean sequence quality score >25, with truncation of a sequence at the first base if a low quality rolling 10 bp window was found Presence of homopolymers >7 bp, and sequences with mismatched primers were omitted. In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA Operational Taxonomic Units (OTUs) were defined at ≥ 97 % sequence homology. All reads were classified to the lowest possible taxonomic rank using QIIME and a reference dataset from the Ribosomal Database Project (Cole, Wang et al. 2009). OTUs were assigned using uclust (Edgar 2010). Further MEGAN analyses allowed the classification of reads down to species level (Huson, Mitra et al. 2011). The distance values based on population

profiles were calculated by Weighted uniFrac diversity values (Lozupone and Knight 2005). The range of distance is calculated between the values 0 and 1.

2.9. RNA isolation.

Total RNA was isolated using a previously described method (Turroni, Foroni et al. 2011). Briefly, cell pellets/tissue materials were resuspended in 1 ml of QUIAZOL (Qiagen, UK) and placed in a tube containing 0.8 g of glass beads (diameter, 106 µm; Sigma). The cells were lysed by shaking the mix on a BioSpec homogenizer at 4°C for 2 min (maximum setting). The mixture was then centrifuged at 12,000 rpm for 15 min, and the upper phase containing the RNA-containing sample was recovered. The RNA sample was further purified by phenol extraction and ethanol precipitation according to an established method (Sambrook 1989). The quality of the RNA was checked by analysing the integrity of rRNA molecules by Experion (Bio Rad).

2.10. Microarray, description, labelling and hybridizations.

Microarray analysis was performed with an oligonucleotide array based on the *B. bifidum* PRL2010 genome: a total of 45,220 oligonucleotide probes of 60 bp in length were designed on 1707 ORFs using eArray5.0 (Agilent Technologies). 5 Oligos were designed for each gene on a 4x44k Agilent Microarrays (Agilent Technologies, Santa Clara, CA, USA). Replicates were distributed on the chip at random, non-adjacent positions. A set of 152 negative control probes designed on phage and plant sequences were also included on the chip. Reverse transcription and amplification of 500 ng of total RNA was performed with ImProm-IITM Reverse Transcriptase (Promega, Madison, USA) according to the manufacturer's instructions. Five µg of cDNA was then labeled with ULS Labeling kit with Cy5 or Cy3 (Kreatech, The Netherlands). For the purpose of the tiling array, samples containing kefir/kefiran cDNA was labeled with Cy5, while control samples were labeled with Cy3.

Labeled cDNA was hybridized using the Agilent Gene Transcription hybridization kit (part number 5188-5242) as described in the Agilent Two-Color Microarray-Based Gene Transcription Analysis v4.0 manual (G4140-90050). Following hybridization, microarrays were washed in accordance with Agilent's standard procedures.

2.11. Microarray data acquisition and treatment.

Fluorescence scanning was performed on an InnoScan 710 microarray scanner (Innopsys, France). Signal intensities for each spot were determined using Mapix5.5 software (Innopsys, Carbonne, France). Differential transcription tests were performed with the Cyber-T implementation of a variant of the t-test, as described previously (Baldi and Long 2001). A gene was considered differentially expressed between a test condition and a control when an expression ratio of 2 or 0.5 relative to the result for the control was obtained with a corresponding P-value ≤ 0.001.

2.12. Quantitative real-time reverse transcription PCR (qRT-PCR).

The quality and integrity of the RNA were checked by Experion (Bio-Rad) analysis. cDNA was synthesized and purified using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), according to the supplier's instructions. qRT-PCR primers were used to amplify the genes encompassing the pilus loci, and the reference genes (*atp*D, *rpo*B and *ldh*). Criteria for primer design were based on a desired melting temperature ™ values between 58 and 60°C and amplicon size of approximately 100 base pairs. qRT-PCR was performed using the CFX96 system (BioRad, CA, USA), fold change was evaluated through the estimation of the CT values with the aid of the CFX96 software (BioRad, CA, USA). Each PCR reaction mix contained the following: 12.5µl 2x SYBR SuperMix Green (BioRad, CA, USA), 1 µl of cDNA dilution, each of the forward and reverse primers at 0.5 µM and nuclease-free water was added to obtain a final volume of 20 µl. PCR products were detected with SYBR Green fluorescent dye and amplified according to the following protocol: one cycle of 95°C for 3 minutes, followed by 39 cycles of 95°C for 5 s and 60°C for 20 s. Melting curve: 65°C to 95°C with increments of 0.5°C/s. In each run, negative controls (no cDNA) for each primer set were included. Fold change was calculated using the CFX96 software (BioRad).

2.13. Western Blotting.

Overnight cultures were harvested by centrifugation and resuspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea pH 8). This bacterial cell suspension was then subjected to sonication using six 10 s bursts at 200–300 W with a 10 s cooling period between each burst. Bacterial cell envelopes were collected by centrifugation at 6,000 rpm for 2 minutes, the pellets were resuspended in 60 µl of PBS-lysis buffer containing SDS and β-mercaptoethanol, and boiled at 100°C for 15 min. Proteins were separated on a 10 % SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane, which was blocked overnight at 4° C in Tris Buffered Saline (TBS) supplemented with 0.05 % Tween 20 (TBST) containing 5 % skim milk powder (blocking solution). Membranes were washed three times with TBST for 5 min and then incubated with the polyclonal antibody Ab_{pil2} or Ab_{pil3} (diluted 1:5000 in TBST) for two hours at RT. Immunoblots were then washed three times for 10 min with TBST followed by immunodetection with IR Dye 680 Goat anti-rabbit for one hour. The nitrocellulose membrane (BioRad) was scanned using the Odyssey Infrared Imager at an intensity of 3.

2.14. Microarray and nucleotide accession number.

The transcriptional array data have been deposited in the GEO database under accession number GSE50506. The raw sequences reported in this article have been deposited in the NCBI Short Read Archive (SRA) (SRP029313).

3. Results and discussion

3.1. Evaluation of growth capabilities of *Bifidobacterium bifidum* PRL2010 in kefir and in kefiran.

We were interested in evaluating the growth capabilities of different bifidobacterial strains (Table 1) on kefir. Growth performances on fermented milk kefir were monitored by cell plating of the strains to be tested on MRSc containing mupirocin, which is a selective antibiotic for the isolation of bifidobacteria (Serafini, Bottacini et al. 2011). In order to verify the identity of those bifidobacterial cells that had retained viability in the kefir upon cultivation, ten colonies were randomly picked and strain identity was assayed by PCR using strain-specific primers, which were based on strain-specific genes (Table 2). In the case of PRL2010 the strain identity was verified by PCR using PRL2010-specific primers, which were designed based on the *pil*2 locus (Turroni, Serafini et al. 2013). As displayed in Figure 1, among the tested bifidobacteria, *B. bifidum* PRL2010 displayed one of the highest viability level, 2 x 10^6 CFU/ml, following 24 hour of cultivation.

Furthermore, we were interested in assessing the prebiotic features of kefiran, which is the heteropolysaccharide isolated from kefir samples on *B. bifidum* PRL2010. Growth performance of PRL2010 on CF-MRSc broth containing 0.3 % of kefiran as the sole carbon source, was monitored by cell plating at different time points, i.e., 8 h, 24 h, 48 h and 72 h. As displayed in Figure 1, upon 24 hours of incubation in kefiran, PRL2010 cells reached a cell density of 5.5 x 10^8 CFU/ml, suggesting that *B. bifidum* PRL2010 cells are capable of utilizing this kefir-derived glycan. It is worth mentioning that kefiran was obtained from fermented milk kefir by means of an extensive purification process involving several and recurring chromatographic and dialysis steps. A table showing a representative SEC-MALLS chromatogram and the values of these parameters obtained for the purified kefiran are presented as supplementary material (Table S1 and Figure S1).

Moreover, the pH at all sampling points was measured, revealing an increase of acidity of the medium which was directly correlated to growth of *B. bifidum* PRL2010 (Fig. 1).

3.2. Evaluation of the kefir microbiota upon addition with PRL2010 cells.

We were interested in assessing if the addition of *B. bifidum* PRL2010 cells to kefir would affect the overall composition of the microbiota of this substrate. Thus, we analyzed the microbiota composition of fermented milk kefir by means of 16S rRNA gene-based profiling using the Ion Torrent PGM technology. DNA was isolated from samples of kefir to which *B. bifidum* PRL2010 had been added, and then incubated for 0 (pre-inoculum, reference condition), 12 and 24 hours. Partial 16S rRNA gene sequences were amplified from extracted DNA using primer pair Probio_Uni and /Probio_Rev, which had previously been used for similar studies (Milani, Hevia et al. 2013), and which targets the V3 region of the 16S rRNA gene sequence.

In total, 246,427 sequence reads, representing 79,374, 111,206 and 55,847 reads for the reference condition (kefir), and test samples (kefir plus PRL2010) after 12 and 24 hours, respectively, were generated on the Ion Torrent PGM machine (Table 3). A large part of the diversity in these libraries had been identified as demonstrated by the decrease in the rate of phylotype detection and the plateauing of the diversity index for the different samples analysed (Fig. 2). Clustering of de-noised high quality reads generated 2122 Operation Taxonomic Units (OTUs) that were taxonomically assigned down to genera level. Taxonomic allocation of these reads at phyla level revealed that *Firmicutes* were the dominant phyla comprising 90.35 % of the total sequences in kefir samples, a total of 15,453 kefir-associated reads were assigned to the *Lactobacillaceae* family (corresponding to 86.10 % of total assigned sequences) (Fig. 2). Furthermore, MEGAN analysis allowed the classification of the obtained reads at a species level and showed a dominance of 16S rRNA reads harboring to *Lactobacillus kefiranofaciens* subsp. *kefirgranum* (86.10 %), *Lactobacillus parakefiri* (2.29 %), and *L. delbrueckii* subsp. *sunki* (10.43 %). Additionally, *Clostridiaceae* families accounted for just 0.2 %. The *Proteobacteria* phylum was a minor component of the kefir sample (0.03 %), while *Bacteroidetes* and *Actinobacteria* accounted for 0.1 % and 0.2 %, respectively. Among the *Actinobacteria*, members of the genus *Bifidobacterium* was detected at a low level (0.2 %) in the kefir sample, suggesting that bifidobacteria are not a major natural inhabitant of this environment. When *B. bifidum* PRL2010

was added to kefir, the levels of 16S rRNA reads corresponding to *Actinobacteria* and specifically of *Bifidobacterium* increased to 52.41 % after 12 hours, and to 22.47 % at 24 hours, with a decrease of the rate of reads belonging to *Lactobacillus* genus to 46.79 % and 76.95 %, respectively (Fig. 3). Weighted uniFrac diversity values based on population profiles (Lozupone and Knight 2005) at T0 and T24 are very similar, whereas it reaches the highest value (0.23) upon 12 hours. These data suggested that 12 hours after the inoculum *B. bifidum* becomes the predominant species but then decreases, and at T24 the initial microbiota of the kefir is partially restored.

3.3. Analysis of the global transcription profiling of PRL2010 cultivated in kefir and kefiran.

We were further interested in obtaining insights into the *B. bifidum* PRL2010 genes whose transcription was affected when such bacterial cells were cultivated on kefir and kefiran. In order to explore the transcriptome of PRL2010 grown on kefir and kefiran-containing medium, we performed global transcription profiling using PRL2010-based microarrays hybridized with cDNA derived from the RNA samples of *B. bifidum* PRL2010 cultivated on these substrates.

We isolated mRNA from *B. bifidum* PRL2010 cells collected from a culture of kefir and from PRL2010 cultivated on CF-MRSc plus kefiran at 12 hours following inoculation.

Genes were scored as being transcribed if their signal was identified in all hybridization experiments, while also fulfilling additional signal strength criteria as outlined in the Materials and Methods section. A total of 40 or 19 genes were shown to be meeting these criteria upon grown in kefir or kefiran, respectively, and were thus considered to be transcribed under these *in vitro* conditions (Table 4 shows a selection of these genes). We used Cluster Orthologues Gene (COG) analysis in order to identify differentially transcribed genes that contribute to specific biological functions. As illustrated in Figure 4, carbohydrate metabolism is one of the COG functions of PRL2010 most significantly affected by the cultivation of this microorganism on kefir or on kefiran. Compared with their abundance as a functional category in the genome, the transcriptomes of PRL2010 on kefir or on kefiran were somewhat enriched in functions related to

transport and metabolism of carbohydrates, which in the case of kefir is probably due to a response to the presence of this specific dietary glycan, whereas in the case of kefiran is due to its structure. In fact, this exopolysaccharide consists of approximately equal proportions of galactose and glucose residues (Generoso, Wolf et al. 2005). This can explain the observed high level of transcription of glycoside hydrolase-encoding gene, such as the predicted β-galactosidase-specifying gene BBPR_0482 (Table 4).

3.4. qRT-PCR analyses.

DNA loci such as the sortase-dependent pili-encoding *pil*1 (ORF1707-1709) and *pil*3 (ORF0282-0284), which have previously been described to be pivotal in the interaction with the host (Turroni, Serafini et al. 2013), were not represented on the DNA microarray that we used. Thus, we decided to investigate if these genes were (differentially) transcribed during growth of *B. bifidum* PRL2010 cells on kefir or kefiran using a qRT-PCR approach. Interestingly, BBPR_0283, encoding the major subunit of *pil*3, FimP, was shown to be transcribed 250-fold higher in kefir as compared with the reference condition (PRL2010 on MRSc) (Fig. 5). A similar transcription pattern was noticed when *B. bifidum* PRL2010 cells were cultivated on CF-MRSc plus kefiran, BBPR_0282, which encodes the minor subunit of *pil*3, and which was shown to be transcribed 30-fold higher with respect to the reference condition. (Fig. 5). In contrast, the genes spanning the *pil*1 locus did not appear to be transcribed under these conditions (i.e. growth in kefir or kefiran-containing medium).

3.5. Western blotting analyses.

Furthermore, Western blot analysis using antibodies Ab_{pil3} that had been raised against the major subunit protein of the *pil*3 locus, FimP (specified by BBPR_0283), was performed on PRL2010 cells that had been collected upon cultivation on kefir or on kefiran for 12 h. We decided to use this particular time point as this coincided with the highest level of transcription of BBPR_0283. Clear signals representing a protein of 55.43 kDa were noticed in both conditions, even if at lower intensity than that identified for PRL2010 cells cultivated in MRSc plus lysine (positive

control) (Turroni, Serafini et al. 2013) (Fig. 5). In fact, as previously described the presence of lysine in the medium stimulated the production of sortase dependent pili by PRL2010 cells (Turroni, Serafini et al. 2013). Whereas, as previously noticed PRL2010 does not produce any pili-like structures when cultivated on simple MRS substrate (without lysine) (Turroni, Serafini et al. 2013). Thus, we can argue that kefir and kefiran are important growth substrates that can be employed in order to modulate the expression of structures such as sortase-dependent pili that are known to be involved in host-microbe interaction (Turroni, Serafini et al. 2013).

4. Conclusions

In this study, growth performance of bifidobacterial strains from human gut origin was tested, revealing that *B. bifidum* PRL2010, which was previously shown to utilize different host glycans such as mucin and human milk oligosaccharides (Turroni, Bottacini et al. 2010, Turroni, Milani et al. 2011), is also able to utilize dietary glycans such as kefiran. Also, our 16S rRNA-based metagenomics analyses highlight that PRL2010 is able to (temporarily) colonize kefir milk, thereby reducing the levels of other kefir microbiota members such as lactobacilli. This clearly suggests the existence of nutrient competition that may be linked to a different efficiency in the uptake of dietary glycans as well as by a diverse repertoire of extracellular enzymes involved in the breakdown of these carbohydrates. Furthermore, the kefir matrix was shown to modulate the expression of particular PRL2010 genes such as sortase-dependent pili, which have previously been demonstrated to play a pivotal role in host-interaction. Thus, particular food substrates like kefir may be used for the administration of bifidobacteria to humans and may represent a valuable food matrix to pre-adapt bifidobacterial cells to the host in order to enhance the effects of the probiotic administration.

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References

Baldi, P., Long, A.D., 2001. A Bayesian framework for the analysis of microarray expression data: regularized t -test and statistical inferences of gene changes. Bioinformatics 17, 509-519.

Boom, R., Sol, C.J., Salimans, M.M., Jansen, C.L., Wertheim-van Dillen, P.M., van der Noordaa, J., 1990. Rapid and simple method for purification of nucleic acids. J Clin Microbiol 28, 495-503.

Burns, P., Vinderola, G., Reinheimer, J., Cuesta, I., de los Reyes-Gavilan, C.G., Ruas-Madiedo, P., 2011. Technological characterization and survival of the exopolysaccharide-producing strain Lactobacillus delbrueckii subsp lactis 193 and its bile-resistant derivative 193+in simulated gastric and intestinal juices. Journal of Dairy Research 78, 357-364.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Tumbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7, 335-336.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., Rutter, W.J., 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294-5299.

Chomczynski, P., Sacchi, N., 1987. Single-Step Method of Rna Isolation by Acid Guanidinium Thiocyanate Phenol Chloroform Extraction. Analytical Biochemistry 162, 156-159.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M., 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Research 37, D141-D145.

de LeBlanc, A.D.M., Matar, C., Farnworth, E., Perdigon, G., 2007. Study of immune cells involved in the antitumor effect of kefir in a murine breast cancer model. Journal of Dairy Science 90, 1920-1928.

E, F., (2005). Kefir – a complex probiotic. Food Sci Technol Bull: Funct Foods 2, 1–17.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460-2461.

Fanning, S., Hall, L.J., Cronin, M., Zomer, A., Macsharry, J., Goulding, D., O'Connell Motherway, M., Shanahan, F., Nally, K., Dougan, G., van Sinderen, D., 2012. Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. Proc Natl Acad Sci U S A 109, 2108-2113.

Garbers I-M, B.T.W.R., 2004. PCR-based denaturing gradient gel electrophoretic typification and identification of the microbial consortium present in kefir grains. World Journal of Microbiology & Biotechnology 20, 687–693.

Garrote GL, A.A.D.A.G., 2010. Microbial interactions in Kefir: a natural probiotic drink. Biotechnology of Lactic Acid Bacteria: Novel Applications, pp. 327–340.

Generoso, M., Wolf, M., Dondi, C.A., Vecchio, C., De Rosa, M., 2005. Kefir: a border line probiotic between innovation and tradition, Probiotics & Prebiotics, Rome.

Golowczyc, M.A., Gugliada, M.J., Hollmann, A., Delfederico, L., Garrote, G.L., Abraham, A.G., Semorile, L., De Antoni, G., 2008. Characterization of homofermentative lactobacilli isolated from kefir grains: potential use as probiotic. J Dairy Res 75, 211-217.

Huson, D.H., Mitra, S., Ruscheweyh, H.J., Weber, N., Schuster, S.C., 2011. Integrative analysis of environmental sequences using MEGAN4. Genome Research 21, 1552-1560.

Kok-Tas, T., Seydim, A.C., Ozer, B., Guzel-Seydim, Z.B., 2013. Effects of different fermentation parameters on quality characteristics of kefir. J Dairy Sci 96, 780-789.

Liu, J.R., Chen, M.J., Lin, C.W., 2005. Antimutagenic and antioxidant properties of milk-kefir and soymilk-kefir. J Agric Food Chem 53, 2467-2474.

Lozupone, C., Knight, R., 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Applied and Environmental Microbiology 71, 8228-8235.

Marshall, V.M., Cole, W.M., 1985. Methods for Making Kefir and Fermented Milks Based on Kefir. Journal of Dairy Research 52, 451-456.
Masco, L., Huys, G., De Brandt, E., Temmerman, R., Swings, J., 2005. Culture-dependent and culture-independent qualitative analysis of probiotic products claimed to contain bifidobacteria. Int J Food Microbiol 102, 221-230.
Mattarelli, P., Bonaparte, C., Pot, B., Biavati, B., 2008. Proposal to reclassify the three biotypes of Bifidobacterium longum as three subspecies: Bifidobacterium longum subsp. longum subsp. nov., Bifidobacterium longum subsp. infantis comb. nov. and Bifidobacterium longum subsp. suis comb. nov. Int J Syst Evol Microbiol 58, 767-772.
Medrano, M., Perez, P.F., Abraham, A.G., 2008. Kefiran antagonizes cytopathic effects of Bacillus cereus extracellular factors. Int J Food Microbiol 122, 1-7.
Micheli, L., Uccelletti, D., Palleschi, C., Crescenzi, V., 1999. Isolation and characterisation of a ropy Lactobacillus strain producing the exopolysaccharide kefiran. Appl Microbiol Biotechnol 53, 69-74.
Milani, C., Duranti, S., Lugli, G.A., Bottacini, F., Strati, F., Arioli, S., Foroni, E., Turroni, F., van Sinderen, D., Ventura, M., 2013. Comparative Genomics of Bifidobacterium animalis subsp. lactis Reveals a Strict Monophyletic Bifidobacterial Taxon. Appl Environ Microbiol 79, 4304-4315.
Milani, C., Hevia, A., Foroni, E., Duranti, S., Turroni, F., Lugli, G.A., Sanchez, B., Martin, R., Gueimonde, M., van Sinderen, D., Margolles, A., Ventura, M., 2013. Assessing the Fecal Microbiota: An Optimized Ion Torrent 16S rRNA Gene-Based Analysis Protocol. PLoS One 8, e68739.
Motherway, M.O., Zomer, A., Leahy, S.C., Reunanen, J., Bottacini, F., Claesson, M.J., O'Brien, F., Flynn, K., Casey, P.G., Munoz, J.A.M., Kearney, B., Houston, A.M., O'Mahony, C., Higgins, D.G., Shanahan, F., Palva, A., de Vos, W.M., Fitzgerald, G.F., Ventura, M., O'Toole, P.W., van Sinderen, D., 2011. Functional genome analysis of Bifidobacterium breve UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. Proceedings of the National Academy of Sciences of the United States of America 108, 11217-11222.
Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59, 695-700.
Otles, S., Cagindi, O., Akcicek, E., 2003. Probiotics and health. Asian Pac J Cancer Prev 4, 369-372.
Rodrigues, K.L., Carvalho, J.C., Schneedorf, J.M., 2005. Anti-inflammatory properties of kefir and its polysaccharide extract. Inflammopharmacology 13, 485-492.
Salazar, N., Prieto, A., Leal, J.A., Mayo, B., Bada-Gancedo, J.C., de los Reyes-Gavilan, C.G., Ruas-Madiedo, P., 2009. Production of exopolysaccharides by Lactobacillus and Bifidobacterium strains of human origin, and metabolic activity of the producing bacteria in milk. Journal of Dairy Science 92, 4158-4168.
Sambrook, 1989. Molecular Cloning. A manual laboratory., in: Press, C. (Ed.).
Santos, A., San Mauro, M., Sanchez, A., Torres, J.M., Marquina, D., 2003. The antimicrobial properties of different strains of Lactobacillus spp. isolated from kefir. Syst Appl Microbiol 26, 434-437.
Serafini, F., Bottacini, F., Viappiani, A., Baruffini, E., Turroni, F., Foroni, E., Lodi, T., van Sinderen, D., Ventura, M., 2011. Insights into physiological and genetic mupirocin susceptibility in bifidobacteria. Appl Environ Microbiol 77, 3141-3146.
Serafini, F., Strati, F., Ruas-Madiedo, P., Turroni, F., Foroni, E., Duranti, S., Milano, F., Perotti, A., Viappiani, A., Guglielmetti, S., Buschini, A., Margolles, A., van Sinderen, D., Ventura, M., 2013. Evaluation of adhesion properties and antibacterial activities of the infant gut commensal Bifidobacterium bifidum PRL2010. Anaerobe 21, 9-17.
Simova, E., Beshkova, D., Angelov, A., Hristozova, T., Frengova, G., Spasov, Z., 2002. Lactic acid bacteria and yeasts in kefir grains and kefir made from them. Journal of Industrial Microbiology & Biotechnology 28, 1-6.

Turroni, F., Bottacini, F., Foroni, E., Mulder, I., Kim, J.H., Zomer, A., Sanchez, B., Bidossi, A., Ferrarini, A., Giubellini, V., Delledonne, M., Henrissat, B., Coutinho, P., Oggioni, M., Fitzgerald, G.F., Mills, D., Margolles, A., Kelly, D., van Sinderen, D., Ventura, M., 2010. Genome analysis of Bifidobacterium bifidum PRL2010 reveals metabolic pathways for host-derived glycan foraging. Proc Natl Acad Sci U S A 107, 19514-19519.

Turroni, F., Foroni, E., Montanini, B., Viappiani, A., Strati, F., Duranti, S., Ferrarini, A., Delledonne, M., van Sinderen, D., Ventura, M., 2011. Global genome transcription profiling of Bifidobacterium bifidum PRL2010 under in vitro conditions and identification of reference genes for quantitative real-time PCR. Appl Environ Microbiol 77, 8578-8587.

Turroni, F., Foroni, E., Serafini, F., Viappiani, A., Montanini, B., Bottacini, F., Ferrarini, A., Bacchini, P.L., Rota, C., Delledonne, M., Ottonello, S., van Sinderen, D., Ventura, M., 2011. Ability of Bifidobacterium breve to grow on different types of milk: exploring the metabolism of milk through genome analysis. Appl Environ Microbiol 77, 7408-7417.

Turroni, F., Milani, C., van Sinderen, D., Ventura, M., 2011. Genetic strategies for mucin metabolism in Bifidobacterium bifidum PRL2010: an example of possible human-microbe co-evolution. Gut Microbes 2, 183-189.

Turroni, F., Serafini, F., Foroni, E., Duranti, S., O'Connell Motherway, M., Taverniti, V., Mangifesta, M., Milani, C., Viappiani, A., Roversi, T., Sanchez, B., Santoni, A., Gioiosa, L., Ferrarini, A., Delledonne, M., Margolles, A., Piazza, L., Palanza, P., Bolchi, A., Guglielmetti, S., van Sinderen, D., Ventura, M., 2013. Role of sortase-dependent pili of Bifidobacterium bifidum PRL2010 in modulating bacterium-host interactions. Proc Natl Acad Sci U S A 110, 11151-11156.

Turroni, F., Strati, F., Foroni, E., Serafini, F., Duranti, S., van Sinderen, D., Ventura, M., 2012. Analysis of predicted carbohydrate transport systems encoded by Bifidobacterium bifidum PRL2010. Appl Environ Microbiol.

Vardjan, T., Lorbeg, P.M., Rogelj, I., Majhenic, A.C., 2013. Characterization and stability of lactobacilli and yeast microbiota in kefir grains. Journal of Dairy Science 96, 2729-2736.

Ventura, M., Turroni, F., van Sinderen, D., 2012. Probiogenomics as a tool to obtain genetic insights into adaptation of probiotic bacteria to the human gut. Bioeng Bugs 3.

Vinderola, G., Perdigon, G., Duarte, J., Farnworth, E., Matar, C., 2006. Effects of the oral administration of the exopolysaccharide produced by Lactobacillus kefiranofaciens on the gut mucosal immunity. Cytokine 36, 254-260.

Wouters, J.T.M., Ayad, E.H.E., Hugenholtz, J., Smit, G., 2002. Microbes from raw milk for fermented dairy products. International Dairy Journal 12, 91-109.

Wyder, M.T., Meile, L., Teuber, M., 1999. Description of Saccharomyces turicensis sp. nov., a new species from kefyr. Syst Appl Microbiol 22, 420-425.

Table 1. Bacterial strains used in this study.

Strains	Ecological	References
B. bifidum PRL2010	Infant stool	(Turroni, Bottacini et al. 2010)
B. animalis subsp. *lactis* Bl12	Human intestine	(Milani, Duranti et al. 2013)
B. breve 12L	Human milk	(Turroni, Foroni et al. 2011)
B. adolescentis 22L	Human milk	(Turroni, Foroni et al. 2011)
B. longum subsp. *longum* 296B	Infant stool	(Serafini, Strati et al. 2013)
B. longum subsp. *infantis*	Infant intestine	(Mattarelli, Bonaparte et al.

Table 2. Strain specific primers

Primer	Sequence (5'-3')	Targeted ORF/gene	Organism
BLAC1485Fw	CGGTCATCCAGGTCGATACT	BLAC1485	*B. animalis* subsp. *lactis* Bl12
BLAC1485Rv	CACGGTATATGTGCGGTCAG	BLAC1486	*B. animalis* subsp. *lactis* Bl12
BAD_PilV_Fw	GAGGTATTGGTGGCGATTGT	22L_0586	*B. adolescentis* 22L
BAD_PilV_Rv	GGATGGCGCAGAAGTATGAT	22L_0586	*B. adolescentis* 22L
B.inf_101Fw	GATGTGCTTCATGACCTTGG	B.lon 0101	*B.longum* subsp. *infantis* ATCC15697
B.inf_101Rv	AACCGACGTAGTCGGAGATG	B.lon 0101	*B.longum* subsp. *infantis* ATCC15697
BR12L_105Fw	CCGAAGTTCCAGTTGACCAT	Br_Milk_0105	*B. breve* 12L
BR12L_105Rv	GTTCTTCGCGTTCCAGATGT	Br_Milk_0105	*B. breve* 12L
BBP0283Fw	CACGGTGGAAAACAACCTGA	BBP0283	*B. bifidum* PRL2010
BBP0283 Rv	GGCGTTGTAGGTGATGGTGA	BBP0283	*B. bifidum* PRL2010
BiLon-1	TTCCAGTTGATCGCATGGTC	16S rRNA	*B. longum* subsp. *longum* 296B
BiLon-2	GGGAAGCCGTATCTCTACGA	16S rRNA	*B. longum* subsp. *longum* 296B

Table 3. Quantitative data of the 16S rRNA gene sequence datasets used in this study.

Sample	Number of reads	Number of reads removed because of:						Final read number	Reduced by (%)
		Outside bounds (140-400)	Ambiguous bases	Mean quality <15	Homopolymer runs >7bp	Primer mismatch >1	Low quality window truncation results in <140bp		
T0	121162	19900	0	0	48	11213	10606	79374	34.5
T12	155120	24212	0	0	241	9544	9909	111206	28.30
T24	82142	11251	0	0	49	9498	5491	55847	32.01
TOTAL	**358424**							**246427**	**31.24**

Table 4. Selected transcripts up-regulated in cell cultivated on fermented milk kefir or kefiran-containing CF-MRSc compared to *B. bifidum* PRL2010 cells grown in MRSc.

	Gene	Product	Fold change	p-value
Kefir	BBPR_0379	Endo-1,4-beta-xylanase	2.270	2.48E-04
	BBPR_0666	Two-component response regulator	2.310	1.93E-05
	BBPR_0854	ABC-type uncharacterized transport system. ATPase component	2.110	3.96E-03
	BBPR_1202	Solute-binding protein of ABC transporter system	2.326	2.01E-04
	BBPR_1283	Two component system histidine kinase	2.277	3.97E-04
	BBPR_1399	Glucose uptake protein	2.221	4.85E-04
	BBPR_1436	Response regulator of two-component system	2.642	5.67E-04
	BBPR_1714	Bgl1 Beta-glucosidase	2.566	3.36E-04
	BBPR_1717	PTS system, cellobiose-specific component IIC	2.030	2.50E-04
Kefiran	BBPR_0031	PTS system, lactose/cellobiose-specific IIB component	2.587	3.01E-05
	BBPR_0239	PTS system, glucose-specific IIA component	2.061	2.65E-05
	BBPR_0362	Permease protein of ABC transporter system	2.255	1.46E-05
	BBPR_0363	Permease protein of ABC transporter system	3.015	4.15E-05
	BBPR_0482	LacZ Beta-galactosidase	2.471	1.99E-05
	BBPR_0517	Linoleic acid isomerase	2.924	4.43E-05
	BBPR_0562	Fructokinase	2.117	3.27E-05
	BBPR_0563	N-acetylglucosamine repressor	2.073	8.20E-05
	BBPR_1529	NagZ Beta-N-acetylhexosaminidase	3.702	3.72E-05

LEGENDS

Fig. 1. Panel a: Growth performance of different bifidobacterial strains on kefir after 24 hours. Panel b displays the decrease of the pH of the media of the *B. bifidum* PRL2010 on kefiran, MRSc with glucose, MRSc w/o carbon source at 8, 24, 48, 72 hours. Panel c showes the growth (CFU/ml) of the *B. bifidum* PRL2010 on kefiran, MRSc with glucose, MRSc w/o carbon source at 8, 24, 48, 72 hours. The data represent the means of at least two independent experiments conducted in duplicate. The vertical bars indicate standard deviations.

Fig. 2. Microbial composition of kefir grains. Panel a shows the diversity at phylum level of kefir milk microbiota, expressed as percentage of the total reads sequenced, at 0, 12 and 24 hours after inoculation of *B. bifidum* PRL2010. Bar shading represents the relative proportion of assignable tags present in each sample. Panel b displays rarefaction curves generated for 16S rRNA gene sequences using the Chao1 index.

Fig. 3. Microbial diversity of kefir milk (at species level) before and after (two time points) addition of *B. bifidum* PRL2010. Weighted uniFrac diversity values based on population profiles are shown on the arrows of the pie chart.

Fig. 4. Functional analysis according to COG categories of *B. bifidum* PRL2010 genes transcribed upon cultivation on kefir or kefiran as determined by global transcription profiling. For each category, the black bar represents the percentage of genes in that category as detected in the sequenced genome of PRL2010 (Turroni, Bottacini et al. 2010). The other bar shows the percentages of genes transcribed upon growth on kefir (grey bar) or kefiran-containing medium (white bar) that belong to a particular category. The percentage was calculated as the percentage of transcribed genes belonging to the indicated COG category with respect to all transcribed genes.

Fig. 5. Relative transcription levels of pilus-encoding genes from *B. bifidum* PRL2010 upon cultivation in kefir and on CF-MRSc supplemented with kefiran assayed by qRT-PCR analysis (panels a and b, respectively) and Western blotting analysis (panel c). The histograms indicate the relative amounts of the pili mRNAs for the specific samples versus growth in MRSc supplemented with glucose as unique carbon source. In each panel, the ORF numbering indicates the gene code. The value on the y-axes represents the fold change relative to reference genes as indicated in the materials and method section. Results are shown as mean-standard deviation (SD) of triplicate analysis. Panel c shows Western blot analyses of PRL2010 cell extracts cultivated under different substrates probed with Ab_{pil3}. *B. bifidum* PRL2010 grown on: MRSc without lysine (lane 1); kefir (lane 2); CF-MRSc-kefiran (lane 3) and MRSc with lysine (lane 4).

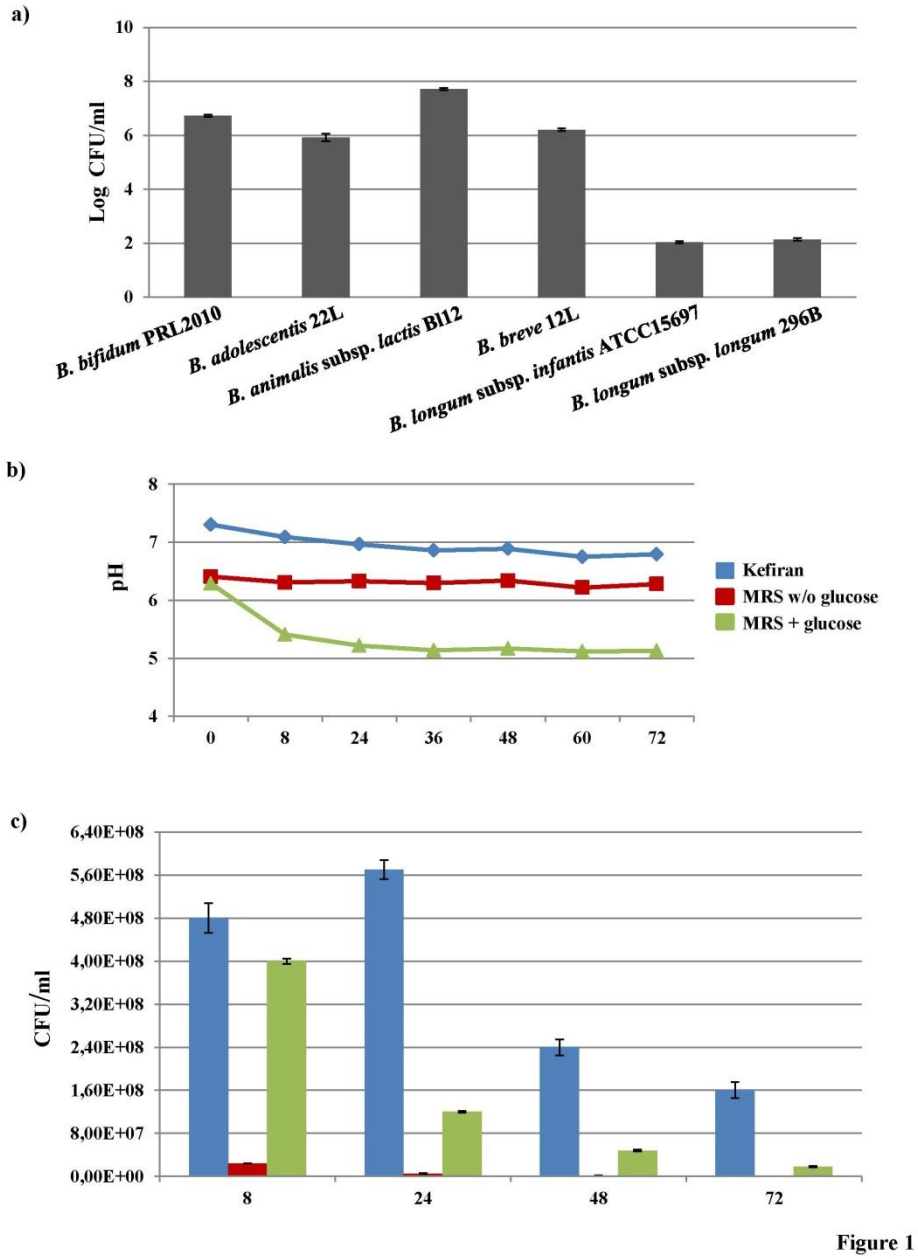
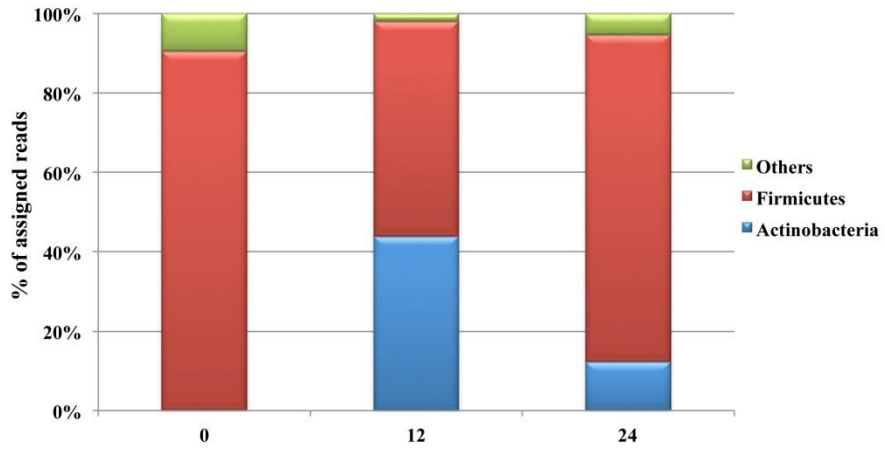

a)
Log CFU/ml
10
8
6
4
2
0
B. bifidum PRL2010
B. adolescentis 22L
B. animalis subsp. lactis Bl12
B. breve 12L
B. longum subsp. infantis ATCC15697
B. longum subsp. longum 296B
b)
pH
8
7
6
5
4
0
8
24
36
48
60
72
Kefiran
MRS w/o glucose
MRS + glucose
c)
CFU/ml
6,40E+08
5,60E+08
4,80E+08
4,00E+08
3,20E+08
2,40E+08
1,60E+08
8,00E+07
0,00E+00
8
24
48
72

Figure 1

a)


100%
80%
60%
40%
20%
0%
% of assigned reads
0
12
24
Others
Firmicutes
Actinobacteria

b)

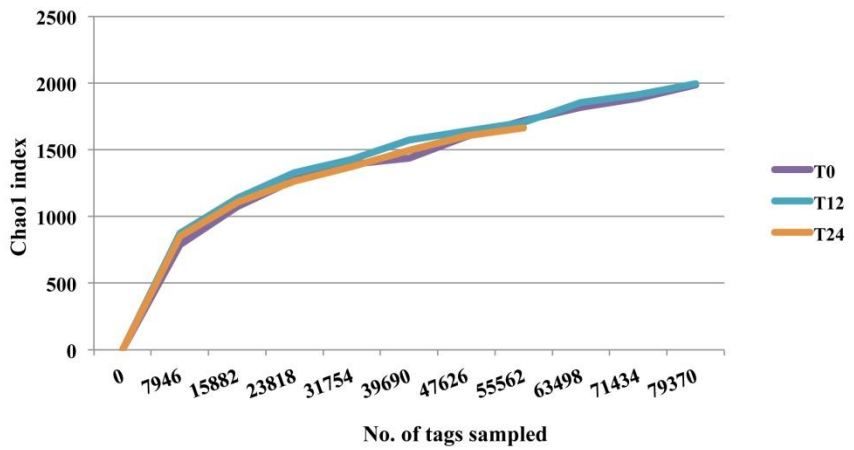

2500
2000
1500
1000
500
0
Chao1 index
0
7946
15882
23818
31754
39690
47626
55562
63498
71434
79370
No. of tags sampled
T0
T12
T24

Figure 2

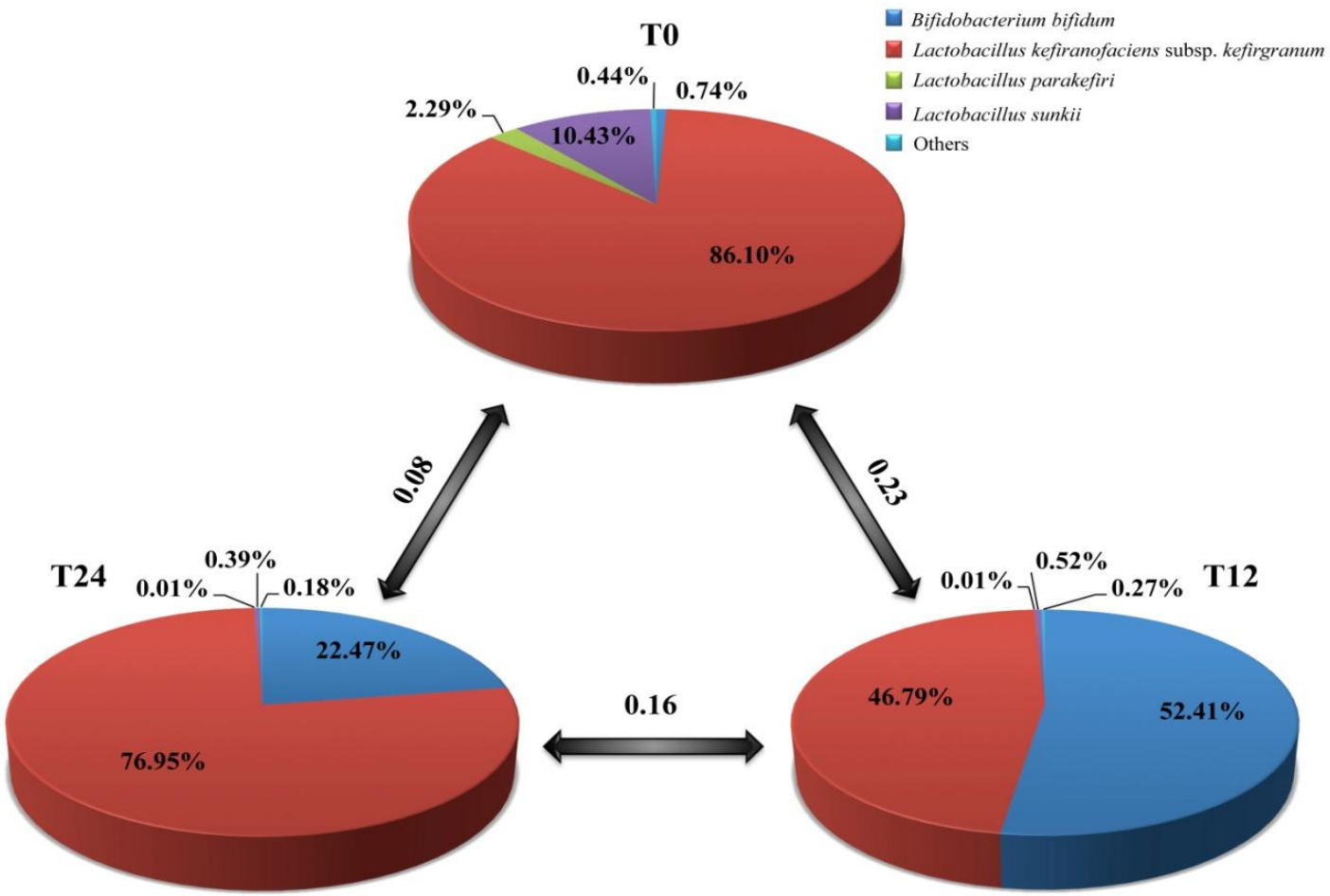

T0
0.44%
0.74%
2.29%
10.43%
86.10%
Bifidobacterium bifidum
Lactobacillus kefiranofaciens subsp. kefirgranum
Lactobacillus parakefiri
Lactobacillus sunkii
Others
0.08
0.23
T24
0.39%
0.01%
0.18%
22.47%
76.95%
0.16
T12
0.01%
0.52%
0.27%
46.79%
52.41%

Figure 3

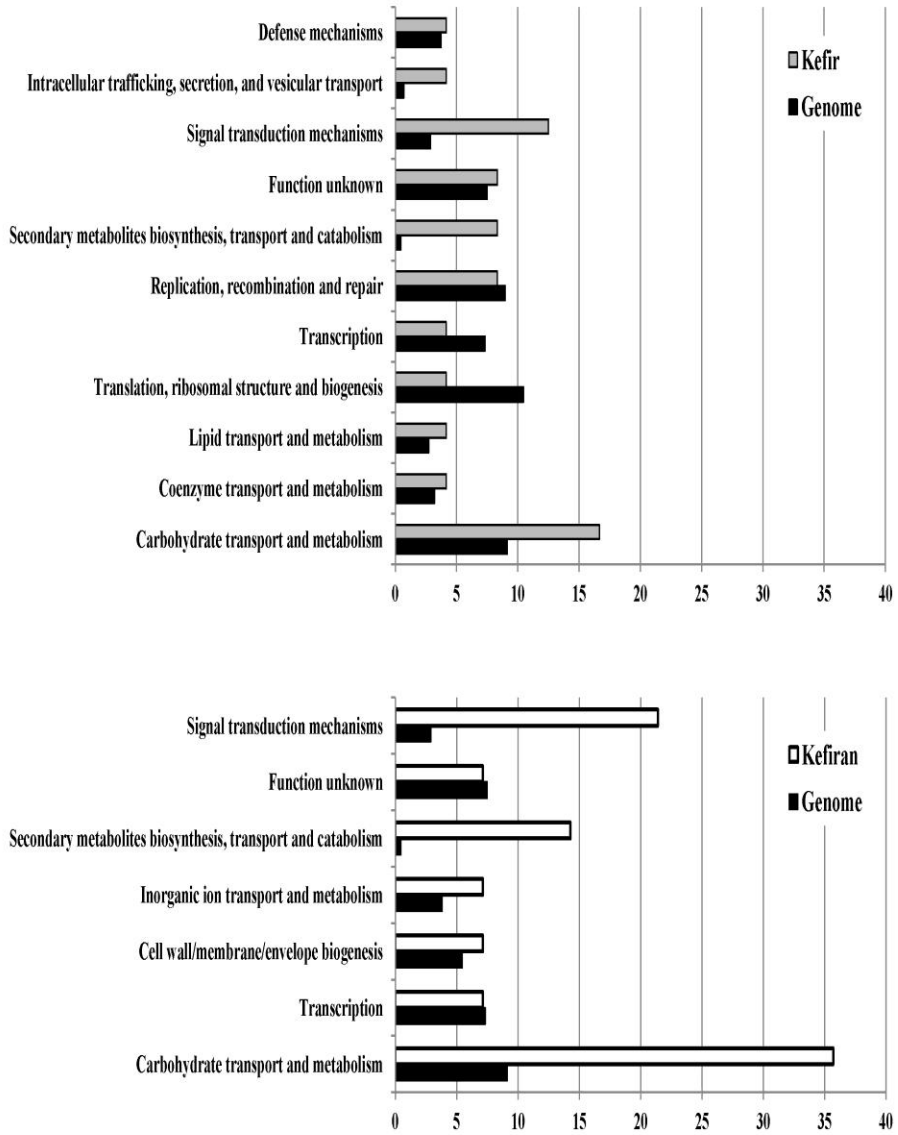

Defense mechanisms
Intracellular trafficking, secretion, and vesicular transport
Signal transduction mechanisms
Function unknown
Secondary metabolites biosynthesis, transport and catabolism
Replication, recombination and repair
Transcription
Translation, ribosomal structure and biogenesis
Lipid transport and metabolism
Coenzyme transport and metabolism
Carbohydrate transport and metabolism
Kefir
Genome
0
5
10
15
20
25
30
35
40
Signal transduction mechanisms
Function unknown
Secondary metabolites biosynthesis, transport and catabolism
Inorganic ion transport and metabolism
Cell wall/membrane/envelope biogenesis
Transcription
Carbohydrate transport and metabolism
Kefiran
Genome
0
5
10
15
20
25
30
35
40

Figure 4

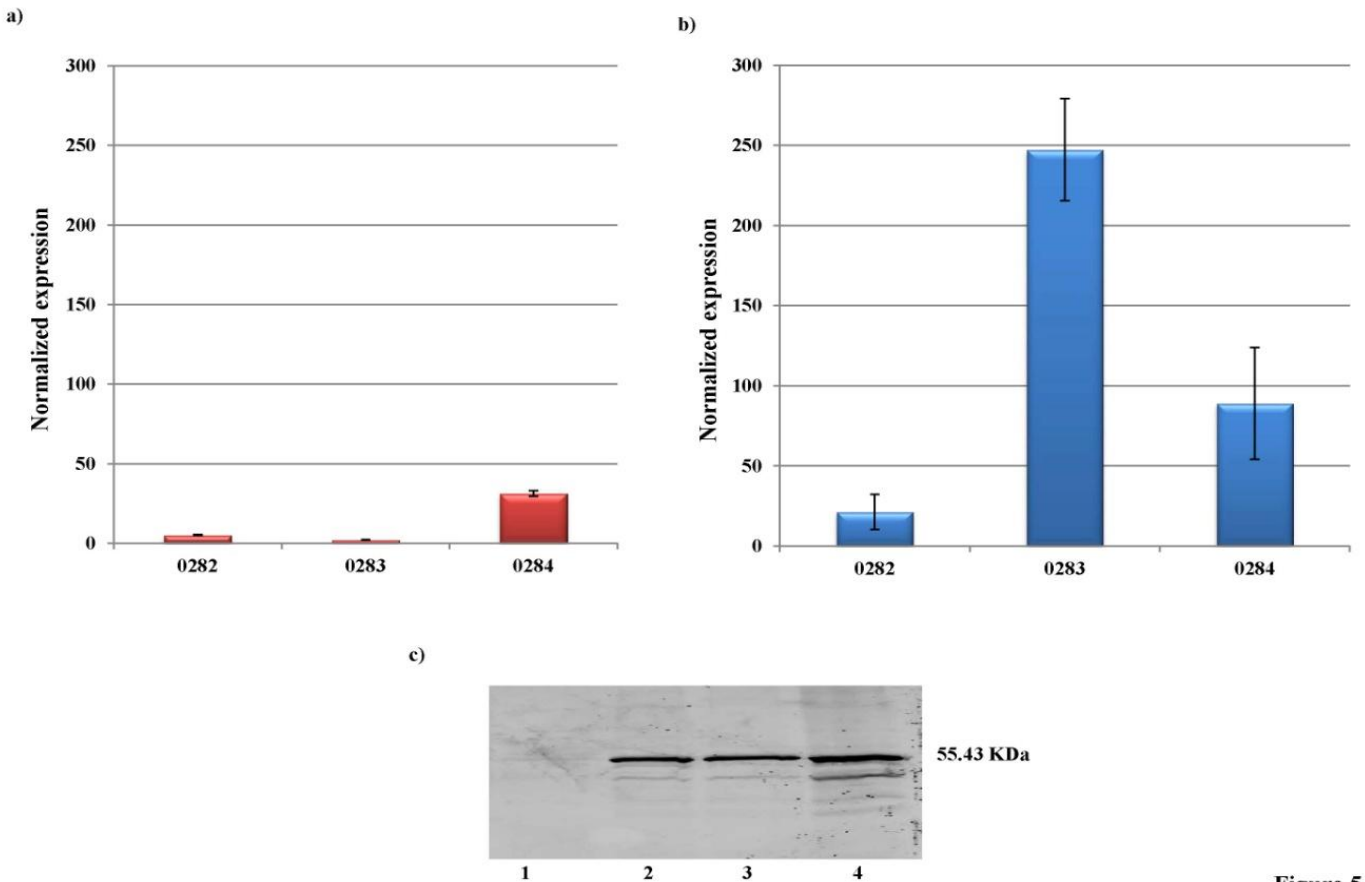

a)
Normalized expression
300
250
200
150
100
50
0
0282
0283
0284
b)
Normalized expression
300
250
200
150
100
50
0
0282
0283
0284
c)
55.43 KDa
1
2
3
4

Figure 5

10. Conclusions

10.1 Physiological characterization of *B. bifidum* PRL2010

The first part of this thesis elucidates the features of *B. bifidum* PRL2010, as a key member of the human gut microbiota.

In the chapter 3 it was showed variable levels of susceptibility to mupirocin in bifidobacteria. These data are important for effective selection protocols for isolation as well as enumeration of bifidobacteria based on antibiotic inclusion in selective media. Furthermore, it was investigated the genetic basis of resistance to mupirocin in bifidobacteria and showed how this is conferred by the product encoded by the *ileS* gene. This gene cloned in a mupirocin-sensitive *Escherichia coli* strain, has showed how a particular amino acid residue, tyrosine, in the targeted enzyme (IleS) may be responsible for the high level of mupirocin resistance observed in bifidobacteria.

Data presented in the chapter 4, show that *B. bifidum* PRL2010 possesses several features sustaining its adaptation to the human gut. The strong adhesive behavior displayed by PRL2010 cells may thus reflect its ecological potential within the highly competitive environment of the human gut. In fact, the capabilities of an enteric bacterium to adhere to the intestinal mucosa is consequently linked to its abilities to stably colonize and persist in the intestine, and therefore preventing the establishment of enteric pathogens.

10.2 Novel methods for transformation and genetic manipulation

Molecular studies of *Bifidobacterium* are severely hampered by the absence of effective genetic tools, including efficient transformation protocols. In chapter 5 of this thesis it was described the development of a protocol for efficient and reproducible genetic transformation of *B. bifidum* PRL2010 by electroporation. The efficiency of transformation achieved in this study (10^4 UCF/µg) is sufficiently high for cloning purposes but it is not yet high enough for site-directed mutagenesis (O'Connell Motherway, O'Driscoll et al. 2009).

10.3 Molecular validation of host-microbe effector molecules such as pili

The fulcrum of the whole thesis is to investigate and to characterize candidate genes involved in the interaction between the bifidobacteria and the human host. Pili are involved in the attachment/colonization of members of the human microbiota to host tissues. However, very little is known about their occurrence in bifidobacteria.

Chapter 6 provides the first morphological evidence of pili-like structures decorating the cell surface of various bifidobacterial species. It further investigates the genetic organization and transcriptional profiling of the bifidobacterial pilus in response to different growth substrates. In the chapter 7 it was elucidated the function of sortase-dependent pili in bifidobacteria. Like in other Gram-positive organisms, such as *C. diphtheriae* and *Actinomyces* spp. (Ton-That and Schneewind 2003, Mishra, Wu et al. 2010), in *B. bifidum* PRL2010, sortase-dependent pili are crucial for bacterial adherence to host cells. They may influence mucosal immune responses, in a similar scenario previously described for other human gut commensals such as lactobacilli (Kankainen, Paulin et al. 2009, von Ossowski, Reunanen et al. 2010).

Moreover in chapter 8 it was investigated the expression and the modulation of pili sortase-dependent genes of *B. bifidum* PRL2010 in response to the stress conditions simulating those encountered by bifidobacteria during their passage through the gastrointestinal tract (e.g., heat and acidic stresses) as well as during the establishment of bifidobacterial cells in the gut (e.g.,

osmotic stress as well as bile salt challenges). It was investigated the putative role of sortase-dependent pili in the interaction with other member of the human gut microbiota.

The results here presented reinforce the notion that sortase-dependent pili of bifidobacteria might supporting the interaction with the other commensal of the GIT through the development of hetero-aggregates between the members of complex microbial communities such as those found in the human intestine.

In the last chapter of this thesis it was investigated the prebiotic role of fermented foods, such as kefir, and their constituent glycans, such as kefiran, as substrates for the growth of bifidobacteria and for the modulation of the expression of pili. It has been shown that kefir and kefiran are able to influence the transcriptome of *B. bifidum* PRL2010, by increasing the transcription of genes involved in the metabolism of dietary glycans as well as genes that act as host-microbe effector molecules such as pili. The complex food matrix of kefir may be used for the supplementation of bifidobacteria to humans and may represent a medium to pre-adapt bifidobacterial cells to the host in order to enhance the effects of the probiotic administration.

Considering that bifidobacteria are largely exploited by food industries as health promoting bacteria, it is crucial to develop appropriated formulation to predispose microbial cells to interact with the human host.

References

Alvarez-Martin, P., M. O. Motherway, F. Turroni, E. Foroni, M. Ventura and D. van Sinderen (2012). "A Two-Component Regulatory System Controls Autoregulated Serpin Expression in Bifidobacterium breve UCC2003." Applied and Environmental Microbiology **78**(19): 7032-7041.

Alvarez-Martin, P., M. O'Connell Motherway, F. Turroni, E. Foroni, M. Ventura and D. van Sinderen (2012). "A two-component regulatory system controls autoregulated serpin expression in Bifidobacterium breve UCC2003." Appl Environ Microbiol **78**(19): 7032-7041.

Argnani, A., R. J. Leer, N. van Luijk and P. H. Pouwels (1996). "A convenient and reproducible method to genetically transform bacteria of the genus Bifidobacterium." Microbiology **142 (Pt 1)**: 109-114.

Asakuma, S., T. Urashima, M. Akahori, H. Obayashi, T. Nakamura, K. Kimura, Y. Watanabe, I. Arai and Y. Sanai (2008). "Variation of major neutral oligosaccharides levels in human colostrum." Eur J Clin Nutr **62**(4): 488-494.

Ashida, H., M. Ogawa, M. Kim, H. Mimuro and C. Sasakawa (2012). "Bacteria and host interactions in the gut epithelial barrier." Nat Chem Biol **8**(1): 36-45.

Backhed, F., H. Ding, T. Wang, L. V. Hooper, G. Y. Koh, A. Nagy, C. F. Semenkovich and J. I. Gordon (2004). "The gut microbiota as an environmental factor that regulates fat storage." Proc Natl Acad Sci U S A **101**(44): 15718-15723.

Backhed, F., R. E. Ley, J. L. Sonnenburg, D. A. Peterson and J. I. Gordon (2005). "Host-bacterial mutualism in the human intestine." Science **307**(5717): 1915-1920.

Baldi, P. and A. D. Long (2001). "A Bayesian framework for the analysis of microarray expression data: regularized t -test and statistical inferences of gene changes." Bioinformatics **17**(6): 509-519.

Barrangou, R., E. P. Briczinski, L. L. Traeger, J. R. Loquasto, M. Richards, P. Horvath, A. C. Coute-Monvoisin, G. Leyer, S. Rendulic, J. L. Steele, J. R. Broadbent, T. Oberg, E. G. Dudley, S. Schuster, D. A. Romero and R. F. Roberts (2009). "Comparison of the complete genome sequences of Bifidobacterium animalis subsp. lactis DSM 10140 and Bl-04." J Bacteriol **191**(13): 4144-4151.

Biavati, B. and P. Mattarelli (1991). "Bifidobacterium-Ruminantium Sp-Nov and Bifidobacterium-Merycicum Sp-Nov from the Rumens of Cattle." International Journal of Systematic Bacteriology **41**(1): 163-168.

Biavati, B., P. Mattarelli and F. Crociani (1991). "Bifidobacterium-Saeculare - a New Species Isolated from Feces of Rabbit." Systematic and Applied Microbiology **14**(4): 389-392.

Bik, E. M., P. B. Eckburg, S. R. Gill, K. E. Nelson, E. A. Purdom, F. Francois, G. Perez-Perez, M. J. Blaser and D. A. Relman (2006). "Molecular analysis of the bacterial microbiota in the human stomach." Proc Natl Acad Sci U S A **103**(3): 732-737.

Bonnassie, S., J. F. Burini, J. Oreglia, A. Trautwetter, J. C. Patte and A. M. Sicard (1990). "Transfer of plasmid DNA to Brevibacterium lactofermentum by electrotransformation." J Gen Microbiol **136**(10): 2107-2112.

Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen and J. van der Noordaa (1990). "Rapid and simple method for purification of nucleic acids." J Clin Microbiol **28**(3): 495-503.

Bottacini, F., D. Medini, A. Pavesi, F. Turroni, E. Foroni, D. Riley, V. Giubellini, H. Tettelin, D. van Sinderen and M. Ventura (2010). "Comparative genomics of the genus Bifidobacterium." Microbiology **156**(Pt 11): 3243-3254.

Bottacini, F., D. Medini, A. Pavesi, F. Turroni, E. Foroni, D. Riley, V. Giubellini, H. Tettelin, D. van Sinderen and M. Ventura (2010). "Comparative genomics of the genus Bifidobacterium." Microbiology-Sgm **156**: 3243-3254.

Bottacini, F., C. Milani, F. Turroni, B. Sanchez, E. Foroni, S. Duranti, F. Serafini, A. Viappiani, F. Strati, A. Ferrarini, M. Delledonne, B. Henrissat, P. Coutinho, G. F. Fitzgerald, A. Margolles, D. van Sinderen and M. Ventura (2012). "Bifidobacterium asteroides PRL2011 Genome Analysis Reveals Clues for Colonization of the Insect Gut." Plos One **7**(9).

Burns, P., G. Vinderola, J. Reinheimer, I. Cuesta, C. G. de los Reyes-Gavilan and P. Ruas-Madiedo (2011). "Technological characterization and survival of the exopolysaccharide-producing strain Lactobacillus delbrueckii subsp lactis 193 and its bile-resistant derivative 193+in simulated gastric and intestinal juices." Journal of Dairy Research **78**(3): 357-364.
Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Pena, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld and R. Knight (2010). "QIIME allows analysis of high-throughput community sequencing data." Nature Methods **7**(5): 335-336.
Chenoll, E., B. Casinos, E. Bataller, P. Astals, J. Echevarria, J. R. Iglesias, P. Balbarie, D. Ramon and S. Genoves (2011). "Novel probiotic Bifidobacterium bifidum CECT 7366 strain active against the pathogenic bacterium Helicobacter pylori." Appl Environ Microbiol **77**(4): 1335-1343.
Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald and W. J. Rutter (1979). "Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease." Biochemistry **18**(24): 5294-5299.
Chomczynski, P. and N. Sacchi (1987). "Single-Step Method of Rna Isolation by Acid Guanidinium Thiocyanate Phenol Chloroform Extraction." Analytical Biochemistry **162**(1): 156-159.
Cole, J. R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R. J. Farris, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, T. Marsh, G. M. Garrity and J. M. Tiedje (2009). "The Ribosomal Database Project: improved alignments and new tools for rRNA analysis." Nucleic Acids Research **37**: D141-D145.
Collins, J. K., G. Thornton and G. O. Sullivan (1998). "Selection of probiotic strains for human applications." International Dairy Journal **8**(5-6): 487-490.
Collins, M. D., B. A. Phillips and P. Zanoni (1989). "Deoxyribonucleic-Acid Homology Studies of Lactobacillus-Casei, Lactobacillus-Paracasei Sp-Nov, Subsp Paracasei and Subsp Tolerans, and Lactobacillus-Rhamnosus Sp-Nov, Comb-Nov." International Journal of Systematic Bacteriology **39**(2): 105-108.
Cronin, U. P. and M. G. Wilkinson (2010). "The potential of flow cytometry in the study of Bacillus cereus." J Appl Microbiol **108**(1): 1-16.
Cruz-Rodz, A. L. and M. S. Gilmore (1990). "High efficiency introduction of plasmid DNA into glycine treated Enterococcus faecalis by electroporation." Mol Gen Genet **224**(1): 152-154.
D'Elia, M. A., J. A. Henderson, T. J. Beveridge, D. E. Heinrichs and E. D. Brown (2009). "The N-acetylmannosamine transferase catalyzes the first committed step of teichoic acid assembly in Bacillus subtilis and Staphylococcus aureus." J Bacteriol **191**(12): 4030-4034.
Danelishvili, L., Y. Yamazaki, J. Selker and L. E. Bermudez (2010). "Secreted Mycobacterium tuberculosis Rv3654c and Rv3655c proteins participate in the suppression of macrophage apoptosis." PLoS One **5**(5): e10474.
Danne, C. and S. Dramsi (2012). "Pili of Gram-positive bacteria: roles in host colonization." Research in Microbiology **163**(9-10): 645-658.
Danne, C. and S. Dramsi (2012). "Pili of gram-positive bacteria: roles in host colonization." Res Microbiol **163**(9-10): 645-658.
de LeBlanc, A. D. M., C. Matar, E. Farnworth and G. Perdigon (2007). "Study of immune cells involved in the antitumor effect of kefir in a murine breast cancer model." Journal of Dairy Science **90**(4): 1920-1928.
Del Re, B., B. Sgorbati, M. Miglioli and D. Palenzona (2000). "Adhesion, autoaggregation and hydrophobicity of 13 strains of Bifidobacterium longum." Letters in Applied Microbiology **31**(6): 438-442.
Delcenserie, V., B. Taminiau, F. Gavini, M. A. de Schaetzen, I. Cleenwerck, M. Theves, M. Mahieu and G. Daube (2013). "Detection and characterization of Bifidobacterium crudilactis and

B. mongoliense able to grow during the manufacturing process of French raw milk cheeses." BMC Microbiol **13**(1): 239.
Dubail, I., A. Bigot, V. Lazarevic, B. Soldo, D. Euphrasie, M. Dupuis and A. Charbit (2006). "Identification of an essential gene of Listeria monocytogenes involved in teichoic acid biogenesis." Journal of Bacteriology **188**(18): 6580-6591.
E, F. ((2005)). "Kefir – a complex probiotic." Food Sci Technol Bull: Funct Foods **2**(1): 1–17.
Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson and D. A. Relman (2005). "Diversity of the human intestinal microbial flora." Science **308**(5728): 1635-1638.
Edgar, R. C. (2010). "Search and clustering orders of magnitude faster than BLAST." Bioinformatics **26**(19): 2460-2461.
Endo, A., Y. Futagawa-Endo, P. Schumann, R. Pukall and L. M. Dicks (2012). "Bifidobacterium reuteri sp. nov., Bifidobacterium callitrichos sp. nov., Bifidobacterium saguini sp. nov., Bifidobacterium stellenboschense sp. nov. and Bifidobacterium biavatii sp. nov. isolated from faeces of common marmoset (Callithrix jacchus) and red-handed tamarin (Saguinus midas)." Syst Appl Microbiol **35**(2): 92-97.
Erney, R. M., W. T. Malone, M. B. Skelding, A. A. Marcon, K. M. Kleman-Leyer, M. L. O'Ryan, G. Ruiz-Palacios, M. D. Hilty, L. K. Pickering and P. A. Prieto (2000). "Variability of human milk neutral oligosaccharides in a diverse population." J Pediatr Gastroenterol Nutr **30**(2): 181-192.
Ewaschuk, J. B., H. Diaz, L. Meddings, B. Diederichs, A. Dmytrash, J. Backer, M. Looijer-van Langen and K. L. Madsen (2008). "Secreted bioactive factors from Bifidobacterium infantis enhance epithelial cell barrier function." Am J Physiol Gastrointest Liver Physiol **295**(5): G1025-1034.
F. Guarner, J. R. M. (2003). Gut flora in health and disease.
Fanning, S., L. J. Hall, M. Cronin, A. Zomer, J. Macsharry, D. Goulding, M. O'Connell Motherway, F. Shanahan, K. Nally, G. Dougan and D. van Sinderen (2012). "Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection." Proc Natl Acad Sci U S A **109**(6): 2108-2113.
Fischer, W. (1987). "'Lipoteichoic acid' of Bifidobacterium bifidum subspecies pennsylvanicum DSM 20239. A lipoglycan with monoglycerophosphate side chains." Eur J Biochem **165**(3): 639-646.
Fischer, W. (1994). "Lipoteichoic acid and lipids in the membrane of Staphylococcus aureus." Med Microbiol Immunol **183**(2): 61-76.
Foroni, E., F. Serafini, D. Amidani, F. Turroni, F. He, F. Bottacini, M. O. Motherway, A. Viappiani, Z. D. Zhang, C. Rivetti, D. van Sinderen and M. Ventura (2011). "Genetic analysis and morphological identification of pilus-like structures in members of the genus Bifidobacterium." Microbial Cell Factories **10**.
Foroni, E., F. Serafini, D. Amidani, F. Turroni, F. He, F. Bottacini, M. O'Connell Motherway, A. Viappiani, Z. Zhang, C. Rivetti, D. van Sinderen and M. Ventura (2011). "Genetic analysis and morphological identification of pilus-like structures in members of the genus Bifidobacterium." Microb Cell Fact **10 Suppl 1**: S16.
Forstner, G. (1995). "Signal transduction, packaging and secretion of mucins." Annu Rev Physiol **57**: 585-605.
Foster, P. L. (2005). "Stress responses and genetic variation in bacteria." Mutat Res **569**(1-2): 3-11.
Fronzes, R., H. Remaut and G. Waksman (2008). "Architectures and biogenesis of non-flagellar protein appendages in Gram-negative bacteria." EMBO J **27**(17): 2271-2280.
Garbers I-M, B. T. W. R. (2004). "PCR-based denaturing gradient gel electrophoretic typification and identification of the microbial consortium present in kefir grains." World J Microb Biot **20**: 687–693.
Garrote GL, A. A. D. A. G. (2010). "Microbial interactions in Kefir: a natural probiotic drink." Biotechnology of Lactic Acid Bacteria: Novel Applications: pp. 327–340.

Generoso, M., M. Wolf, C. A. Dondi, C. Vecchio and M. De Rosa (2005). Kefir: a border line probiotic between innovation and tradition. Probiotics & Prebiotics, Rome.
Gerritsen, J., H. Smidt, G. T. Rijkers and W. M. de Vos (2011). "Intestinal microbiota in human health and disease: the impact of probiotics." Genes Nutr **6**(3): 209-240.
Gettins, P. G. (2002). "Serpin structure, mechanism, and function." Chem Rev **102**(12): 4751-4804.
Gill, S. R., M. Pop, R. T. Deboy, P. B. Eckburg, P. J. Turnbaugh, B. S. Samuel, J. I. Gordon, D. A. Relman, C. M. Fraser-Liggett and K. E. Nelson (2006). "Metagenomic analysis of the human distal gut microbiome." Science **312**(5778): 1355-1359.
Giraud, A., I. Matic, O. Tenaillon, A. Clara, M. Radman, M. Fons and F. Taddei (2001). "Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut." Science **291**(5513): 2606-2608.
Goldin, B. R. (1998). "Health benefits of probiotics." British Journal of Nutrition **80**(4): S203-S207.
Golowczyc, M. A., M. J. Gugliada, A. Hollmann, L. Delfederico, G. L. Garrote, A. G. Abraham, L. Semorile and G. De Antoni (2008). "Characterization of homofermentative lactobacilli isolated from kefir grains: potential use as probiotic." J Dairy Res **75**(2): 211-217.
Guarner, F. and J. R. Malagelada (2003). "Gut flora in health and disease." Lancet **361**(9356): 512-519.
Gunasekera, T. S., D. A. Veal and P. V. Attfield (2003). "Potential for broad applications of flow cytometry and fluorescence techniques in microbiological and somatic cell analyses of milk." Int J Food Microbiol **85**(3): 269-279.
Haeuw-Fievre, S., J. M. Wieruszeski, Y. Plancke, J. C. Michalski, J. Montreuil and G. Strecker (1993). "Primary structure of human milk octa-, dodeca- and tridecasaccharides determined by a combination of 1H-NMR spectroscopy and fast-atom-bombardment mass spectrometry. Evidence for a new core structure, the para-lacto-N-octaose." Eur J Biochem **215**(2): 361-371.
Handley, P. S., D. W. Harty, J. E. Wyatt, C. R. Brown, J. P. Doran and A. C. Gibbs (1987). "A comparison of the adhesion, coaggregation and cell-surface hydrophobicity properties of fibrillar and fimbriate strains of Streptococcus salivarius." J Gen Microbiol **133**(11): 3207-3217.
Hao, Y., D. Huang, H. Guo, M. Xiao, H. An, L. Zhao, F. Zuo, B. Zhang, S. Hu, S. Song, S. Chen and F. Ren (2011). "Complete genome sequence of Bifidobacterium longum subsp. longum BBMN68, a new strain from a healthy chinese centenarian." J Bacteriol **193**(3): 787-788.
Hayashi, H., M. Sakamoto and Y. Benno (2002). "Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods." Microbiol Immunol **46**(8): 535-548.
Hendrickx, A. P., J. M. Budzik, S. Y. Oh and O. Schneewind (2011). "Architects at the bacterial surface - sortases and the assembly of pili with isopeptide bonds." Nat Rev Microbiol **9**(3): 166-176.
Hendrickx, A. P. A., M. J. M. Bonten, M. van Luit-Asbroek, C. M. E. Schapendonk, A. H. M. Kragten and R. J. L. Willems (2008). "Expression of two distinct types of pili by a hospital-acquired Enterococcus faecium isolate." Microbiology-Sgm **154**: 3212-3223.
Hidalgo-Cantabrana, C., B. Sanchez, C. Milani, M. Ventura, A. Margolles and P. Ruas-Madiedo (2013). "Exopolysaccharide biosynthesis in Bifidobacterium spp.: biological functions and a genomic overview." Appl Environ Microbiol.
Hidalgo-Cantabrana, C., B. Sanchez, C. Milani, M. Ventura, A. Margolles and P. Ruas-Madiedo (2014). "Genomic Overview and Biological Functions of Exopolysaccharide Biosynthesis in Bifidobacterium spp." Appl Environ Microbiol **80**(1): 9-18.
Houwink, A. L. and I. W. van (1950). "Electron microscopical observations on bacterial cytology; a study on flagellation." Biochim Biophys Acta **5**(1): 10-44.
Hoyles, L., E. Inganas, E. Falsen, M. Drancourt, N. Weiss, A. L. McCartney and M. D. Collins (2002). "Bifidobacterium scardovii sp nov., from human sources." International Journal of Systematic and Evolutionary Microbiology **52**: 995-999.

Huson, D. H., S. Mitra, H. J. Ruscheweyh, N. Weber and S. C. Schuster (2011). "Integrative analysis of environmental sequences using MEGAN4." Genome Research **21**(9): 1552-1560.
Isolauri, E., S. Salminen and A. C. Ouwehand (2004). "Microbial-gut interactions in health and disease. Probiotics." Best Pract Res Clin Gastroenterol **18**(2): 299-313.
Jensen, P. H., D. Kolarich and N. H. Packer (2010). "Mucin-type O-glycosylation - putting the pieces together." Febs Journal **277**(1): 81-94.
Jiang, S. M., S. E. Park, P. Yadav, L. C. Paoletti and M. R. Wessels (2012). "Regulation and Function of Pilus Island 1 in Group B Streptococcus." Journal of Bacteriology **194**(10): 2479-2490.
Kachlany, S. C., S. B. Levery, J. S. Kim, B. L. Reuhs, L. W. Lion and W. C. Ghiorse (2001). "Structure and carbohydrate analysis of the exopolysaccharide capsule of Pseudomonas putida G7." Environ Microbiol **3**(12): 774-784.
Kainulainen, V., J. Reunanen, K. Hiippala, S. Guglielmetti, S. Vesterlund, A. Palva and R. Satokari (2013). "BopA does not have a major role in the adhesion of Bifidobacterium bifidum to intestinal epithelial cells, extracellular matrix proteins, and mucus." Appl Environ Microbiol **79**(22): 6989-6997.
Kanauchi, O., A. Andoh and K. Mitsuyama (2013). "Effects of the Modulation of Microbiota on the Gastrointestinal Immune System and Bowel Function." Journal of Agricultural and Food Chemistry **61**(42): 9977-9983.
Kang, H. J. and E. N. Baker (2012). "Structure and assembly of Gram-positive bacterial pili: unique covalent polymers." Curr Opin Struct Biol **22**(2): 200-207.
Kankainen, M., L. Paulin, S. Tynkkynen, I. von Ossowski, J. Reunanen, P. Partanen, R. Satokari, S. Vesterlund, A. P. Hendrickx, S. Lebeer, S. C. De Keersmaecker, J. Vanderleyden, T. Hamalainen, S. Laukkanen, N. Salovuori, J. Ritari, E. Alatalo, R. Korpela, T. Mattila-Sandholm, A. Lassig, K. Hatakka, K. T. Kinnunen, H. Karjalainen, M. Saxelin, K. Laakso, A. Surakka, A. Palva, T. Salusjarvi, P. Auvinen and W. M. de Vos (2009). "Comparative genomic analysis of Lactobacillus rhamnosus GG reveals pili containing a human- mucus binding protein." Proc Natl Acad Sci U S A **106**(40): 17193-17198.
Kaplan, H. and R. W. Hutkins (2000). "Fermentation of fructooligosaccharides by lactic acid bacteria and bifidobacteria." Appl Environ Microbiol **66**(6): 2682-2684.
Killer, J., J. Kopecny, J. Mrazek, I. Koppova, J. Havlik, O. Benada and T. Kott (2011). "Bifidobacterium actinocoloniiforme sp. nov. and Bifidobacterium bohemicum sp. nov., from the bumblebee digestive tract." Int J Syst Evol Microbiol **61**(Pt 6): 1315-1321.
Killer, J., J. Kopecny, J. Mrazek, V. Rada, O. Benada, I. Koppova, J. Havlik and J. Straka (2009). "Bifidobacterium bombi sp. nov., from the bumblebee digestive tract." Int J Syst Evol Microbiol **59**(Pt 8): 2020-2024.
Killer, J., J. Kopecny, J. Mrazek, V. Rada, S. Dubna and M. Marounek (2010). "Bifidobacteria in the digestive tract of bumblebees." Anaerobe **16**(2): 165-170.
Kim, H. K., K. S. Kim and M. E. Kim (2013). "Influence of Test Site and Baseline Temperature on Orofacial Thermal Thresholds." Journal of Orofacial Pain **27**(3): 263-270.
Kim, J. F., H. Jeong, D. S. Yu, S. H. Choi, C. G. Hur, M. S. Park, S. H. Yoon, D. W. Kim, G. E. Ji, H. S. Park and T. K. Oh (2009). "Genome Sequence of the Probiotic Bacterium Bifidobacterium animalis subsp. lactis AD011 (vol 191, pg 678, 2009)." Journal of Bacteriology **191**(6): 1995-1995.
Kim, J. H., H. J. An, D. Garrido, J. B. German, C. B. Lebrilla and D. A. Mills (2013). "Proteomic analysis of Bifidobacterium longum subsp. infantis reveals the metabolic insight on consumption of prebiotics and host glycans." PLoS One **8**(2): e57535.
Knol, J., P. Scholtens, C. Kafka, J. Steenbakkers, S. Gro, K. Helm, M. Klarczyk, H. Schopfer, H. M. Bockler and J. Wells (2005). "Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: more like breast-fed infants." J Pediatr Gastroenterol Nutr **40**(1): 36-42.
Kobata, A. and V. Ginsburg (1972). "Oligosaccharides of human milk. IV. Isolation and characterization of a new hexasaccharide, lacto-N-neohexaose." Arch Biochem Biophys **150**(1): 273-281.

Kohler, T., C. Weidenmaier and A. Peschel (2009). "Wall teichoic acid protects Staphylococcus aureus against antimicrobial fatty acids from human skin." J Bacteriol **191**(13): 4482-4484.
Kok-Tas, T., A. C. Seydim, B. Ozer and Z. B. Guzel-Seydim (2013). "Effects of different fermentation parameters on quality characteristics of kefir." J Dairy Sci **96**(2): 780-789.
Kos, B., J. Suskovic, S. Vukovic, M. Simpraga, J. Frece and S. Matosic (2003). "Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92." Journal of Applied Microbiology **94**(6): 981-987.
Kraus, D. and A. Peschel (2008). "Staphylococcus aureus evasion of innate antimicrobial defense." Future Microbiology **3**(4): 437-451.
Kreikemeyer, B., G. Gamez, I. Margarit, J. C. Giard, S. Hammerschmidt, A. Hartke and A. Podbielski (2011). "Genomic organization, structure, regulation and pathogenic role of pilus constituents in major pathogenic Streptococci and Enterococci." Int J Med Microbiol **301**(3): 240-251.
Kunin, V. and C. A. Ouzounis (2003). "The balance of driving forces during genome evolution in prokaryotes." Genome Res **13**(7): 1589-1594.
Kunz, C., S. Rudloff, W. Baier, N. Klein and S. Strobel (2000). "Oligosaccharides in human milk: structural, functional, and metabolic aspects." Annu Rev Nutr **20**: 699-722.
Kurokawa, K., T. Itoh, T. Kuwahara, K. Oshima, H. Toh, A. Toyoda, H. Takami, H. Morita, V. K. Sharma, T. P. Srivastava, T. D. Taylor, H. Noguchi, H. Mori, Y. Ogura, D. S. Ehrlich, K. Itoh, T. Takagi, Y. Sakaki, T. Hayashi and M. Hattori (2007). "Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes." DNA Res **14**(4): 169-181.
Lauer, E. (1990). "Bifidobacterium gallicum sp. nov. isolated from human feces." Int J Syst Bacteriol **40**(1): 100-102.
Lebeer, S., I. Claes, H. L. Tytgat, T. L. Verhoeven, E. Marien, I. von Ossowski, J. Reunanen, A. Palva, W. M. Vos, S. C. Keersmaecker and J. Vanderleyden (2012). "Functional analysis of Lactobacillus rhamnosus GG pili in relation to adhesion and immunomodulatory interactions with intestinal epithelial cells." Appl Environ Microbiol **78**(1): 185-193.
Lebeer, S., J. Vanderleyden and S. C. De Keersmaecker (2010). "Adaptation factors of the probiotic Lactobacillus rhamnosus GG." Benef Microbes **1**(4): 335-342.
Lee, J. H., V. N. Karamychev, S. A. Kozyavkin, D. Mills, A. R. Pavlov, N. V. Pavlova, N. N. Polouchine, P. M. Richardson, V. V. Shakhova, A. I. Slesarev, B. Weimer and D. J. O'Sullivan (2008). "Comparative genomic analysis of the gut bacterium Bifidobacterium longum reveals loci susceptible to deletion during pure culture growth." BMC Genomics **9**: 247.
Ley, R. E., D. A. Peterson and J. I. Gordon (2006). "Ecological and evolutionary forces shaping microbial diversity in the human intestine." Cell **124**(4): 837-848.
Linke-Winnebeck, C., N. G. Paterson, P. G. Young, M. J. Middleditch, D. R. Greenwood, G. Witte and E. N. Baker (2013). "Structural model for the covalent adhesion of the Streptococcus pyogenes pilus through a thioester bond." J Biol Chem.
Liu, J. R., M. J. Chen and C. W. Lin (2005). "Antimutagenic and antioxidant properties of milk-kefir and soymilk-kefir." J Agric Food Chem **53**(7): 2467-2474.
Lloyd, K. O., J. Burchell, V. Kudryashov, B. W. T. Yin and J. TaylorPapadimitriou (1996). "Comparison of O-linked carbohydrate chains in MUC-1 mucin from normal breast epithelial cell lines and breast carcinoma cell lines - Demonstration of simpler and fewer glycan chains in tumor cells." Journal of Biological Chemistry **271**(52): 33325-33334.
LoCascio, R. G., M. R. Ninonuevo, S. L. Freeman, D. A. Sela, R. Grimm, C. B. Lebrilla, D. A. Mills and J. B. German (2007). "Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation." J Agric Food Chem **55**(22): 8914-8919.
Lozupone, C. and R. Knight (2005). "UniFrac: a new phylogenetic method for comparing microbial communities." Applied and Environmental Microbiology **71**(12): 8228-8235.
Luchansky, J. B., P. M. Muriana and T. R. Klaenhammer (1988). "Application of electroporation for transfer of plasmid DNA to Lactobacillus, Lactococcus, Leuconostoc, Listeria, Pediococcus, Bacillus, Staphylococcus, Enterococcus and Propionibacterium." Mol Microbiol **2**(5): 637-646.

Mandlik, A., A. Swierczynski, A. Das and H. Ton-That (2008). "Pili in Gram-positive bacteria: assembly, involvement in colonization and biofilm development." Trends Microbiol **16**(1): 33-40.
Marshall, V. M. and W. M. Cole (1985). "Methods for Making Kefir and Fermented Milks Based on Kefir." Journal of Dairy Research **52**(3): 451-456.
Masco, L., G. Huys, E. De Brandt, R. Temmerman and J. Swings (2005). "Culture-dependent and culture-independent qualitative analysis of probiotic products claimed to contain bifidobacteria." Int J Food Microbiol **102**(2): 221-230.
Matsumura, H., A. Takeuchi and Y. Kano (1997). "Construction of Escherichia coli-Bifidobacterium longum shuttle vector transforming B. longum 105-A and 108-A." Biosci Biotechnol Biochem **61**(7): 1211-1212.
Mattarelli, P., C. Bonaparte, B. Pot and B. Biavati (2008). "Proposal to reclassify the three biotypes of Bifidobacterium longum as three subspecies: Bifidobacterium longum subsp. longum subsp. nov., Bifidobacterium longum subsp. infantis comb. nov. and Bifidobacterium longum subsp. suis comb. nov." Int J Syst Evol Microbiol **58**(Pt 4): 767-772.
Maynard, C. L., C. O. Elson, R. D. Hatton and C. T. Weaver (2012). "Reciprocal interactions of the intestinal microbiota and immune system." Nature **489**(7415): 231-241.
Medrano, M., P. F. Perez and A. G. Abraham (2008). "Kefiran antagonizes cytopathic effects of Bacillus cereus extracellular factors." Int J Food Microbiol **122**(1-2): 1-7.
Micheli, L., D. Uccelletti, C. Palleschi and V. Crescenzi (1999). "Isolation and characterisation of a ropy Lactobacillus strain producing the exopolysaccharide kefiran." Appl Microbiol Biotechnol **53**(1): 69-74.
Milani, C., S. Duranti, G. A. Lugli, F. Bottacini, F. Strati, S. Arioli, E. Foroni, F. Turroni, D. van Sinderen and M. Ventura (2013). "Comparative Genomics of Bifidobacterium animalis subsp lactis Reveals a Strict Monophyletic Bifidobacterial Taxon." Applied and Environmental Microbiology **79**(14): 4304-4315.
Milani, C., S. Duranti, G. A. Lugli, F. Bottacini, F. Strati, S. Arioli, E. Foroni, F. Turroni, D. van Sinderen and M. Ventura (2013). "Comparative Genomics of Bifidobacterium animalis subsp. lactis Reveals a Strict Monophyletic Bifidobacterial Taxon." Appl Environ Microbiol **79**(14): 4304-4315.
Milani, C., A. Hevia, E. Foroni, S. Duranti, F. Turroni, G. A. Lugli, B. Sanchez, R. Martin, M. Gueimonde, D. van Sinderen, A. Margolles and M. Ventura (2013). "Assessing the Fecal Microbiota: An Optimized Ion Torrent 16S rRNA Gene-Based Analysis Protocol." PLoS One **8**(7): e68739.
Mishra, A., C. Wu, J. Yang, J. O. Cisar, A. Das and H. Ton-That (2010). "The Actinomyces oris type 2 fimbrial shaft FimA mediates co-aggregation with oral streptococci, adherence to red blood cells and biofilm development." Mol Microbiol.
Mishra, A., C. G. Wu, J. H. Yang, J. O. Cisar, A. Das and H. Ton-That (2010). "The Actinomyces oris type 2 fimbrial shaft FimA mediates co-aggregation with oral streptococci, adherence to red blood cells and biofilm development." Molecular Microbiology **77**(4): 841-854.
Miyake, T., K. Watanabe, T. Watanabe and H. Oyaizu (1998). "Phylogenetic analysis of the genus Bifidobacterium and related genera based on 16S rDNA sequences." Microbiol Immunol **42**(10): 661-667.
Morita, H., A. Nakano, H. Onoda, H. Toh, K. Oshima, H. Takami, M. Murakami, S. Fukuda, T. Takizawa, T. Kuwahara, H. Ohno, S. Tanabe and M. Hattori (2011). "Bifidobacterium kashiwanohense sp. nov., isolated from healthy infant faeces." Int J Syst Evol Microbiol **61**(Pt 11): 2610-2615.
Motherway, M. O., A. Zomer, S. C. Leahy, J. Reunanen, F. Bottacini, M. J. Claesson, F. O'Brien, K. Flynn, P. G. Casey, J. A. M. Munoz, B. Kearney, A. M. Houston, C. O'Mahony, D. G. Higgins, F. Shanahan, A. Palva, W. M. de Vos, G. F. Fitzgerald, M. Ventura, P. W. O'Toole and D. van Sinderen (2011). "Functional genome analysis of Bifidobacterium breve UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization

factor." Proceedings of the National Academy of Sciences of the United States of America **108**(27): 11217-11222.
Mrazek, J., L. Strosova, K. Fliegerova, T. Kott and J. Kopecny (2008). "Diversity of insect intestinal microflora." Folia Microbiol (Praha) **53**(3): 229-233.
Muyzer, G., E. C. de Waal and A. G. Uitterlinden (1993). "Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA." Appl Environ Microbiol **59**(3): 695-700.
Na, S., T. Shen, W. Xiao and P. Jia (1995). "The factors affecting transformation efficiency of coryneform bacteria by electroporation." Chin J Biotechnol **11**(3): 193-198.
Nakata, M., T. Koeller, K. Moritz, D. Ribardo, L. Jonas, K. S. McIver, T. Sumitomo, Y. Terao, S. Kawabata, A. Podbielski and B. Kreikemeyer (2009). "Mode of Expression and Functional Characterization of FCT-3 Pilus Region-Encoded Proteins in Streptococcus pyogenes Serotype M49." Infection and Immunity **77**(1): 32-44.
Neish, A. S. (2009). "Microbes in Gastrointestinal Health and Disease." Gastroenterology **136**(1): 65-80.
O'Connell Motherway, M., J. O'Driscoll, G. F. Fitzgerald and D. Van Sinderen (2009). "Overcoming the restriction barrier to plasmid transformation and targeted mutagenesis in Bifidobacterium breve UCC2003." Microb Biotechnol **2**(3): 321-332.
Okahashi, N., M. Nakata, Y. Terao, R. Isoda, A. Sakurai, T. Sumitomo, M. Yamaguchi, R. K. Kimura, E. Oiki, S. Kawabata and T. Ooshima (2011). "Pili of oral Streptococcus sanguinis bind to salivary amylase and promote the biofilm formation." Microbial Pathogenesis **50**(3-4): 148-154.
Otles, S., O. Cagindi and E. Akcicek (2003). "Probiotics and health." Asian Pac J Cancer Prev **4**(4): 369-372.
Ouwehand, A. C., H. Lagstrom, T. Suomalainen and S. Salminen (2002). "Effect of probiotics on constipation, fecal azoreductase activity and fecal mucin content in the elderly." Annals of Nutrition and Metabolism **46**(3-4): 159-162.
Pallen, M. J. and B. W. Wren (2007). "Bacterial pathogenomics." Nature **449**(7164): 835-842.
Palmer, C., E. M. Bik, D. B. DiGiulio, D. A. Relman and P. O. Brown (2007). "Development of the human infant intestinal microbiota." PLoS Biol **5**(7): e177.
Pelicic, V. (2008). "Type IV pili: e pluribus unum?" Mol Microbiol **68**(4): 827-837.
Perez-Cobas, A. E., A. Artacho, H. Knecht, M. L. Ferrus, A. Friedrichs, S. J. Ott, A. Moya, A. Latorre and M. J. Gosalbes (2013). "Differential effects of antibiotic therapy on the structure and function of human gut microbiota." PLoS One **8**(11): e80201.
Perez, P. F., Y. Minnaard, E. A. Disalvo and G. L. De Antoni (1998). "Surface properties of bifidobacterial strains of human origin." Appl Environ Microbiol **64**(1): 21-26.
Podolsky, D. K. (1985). "Oligosaccharide Structures of Isolated Human Colonic Mucin Species." Journal of Biological Chemistry **260**(29): 5510-5515.
Pokusaeva, K., G. F. Fitzgerald and D. van Sinderen (2011). "Carbohydrate metabolism in Bifidobacteria." Genes Nutr **6**(3): 285-306.
Pokusaeva, K., G. F. Fitzgerald and D. van Sinderen (2011). "Carbohydrate metabolism in Bifidobacteria." Genes and Nutrition **6**(3): 285-306.
Pokusaeva, K., M. O'Connell-Motherway, A. Zomer, G. F. Fitzgerald and D. van Sinderen (2009). "Characterization of two novel alpha-glucosidases from Bifidobacterium breve UCC2003." Appl Environ Microbiol **75**(4): 1135-1143.
Qin, J., R. Li, J. Raes, M. Arumugam, K. S. Burgdorf, C. Manichanh, T. Nielsen, N. Pons, F. Levenez, T. Yamada, D. R. Mende, J. Li, J. Xu, S. Li, D. Li, J. Cao, B. Wang, H. Liang, H. Zheng, Y. Xie, J. Tap, P. Lepage, M. Bertalan, J. M. Batto, T. Hansen, D. Le Paslier, A. Linneberg, H. B. Nielsen, E. Pelletier, P. Renault, T. Sicheritz-Ponten, K. Turner, H. Zhu, C. Yu, S. Li, M. Jian, Y. Zhou, Y. Li, X. Zhang, S. Li, N. Qin, H. Yang, J. Wang, S. Brunak, J. Dore, F. Guarner, K. Kristiansen, O. Pedersen, J. Parkhill, J. Weissenbach, H. I. T. C. Meta, P. Bork, S. D. Ehrlich and J. Wang (2010). "A human gut microbial gene catalogue established by metagenomic sequencing." Nature **464**(7285): 59-65.

Rodrigues, K. L., J. C. Carvalho and J. M. Schneedorf (2005). "Anti-inflammatory properties of kefir and its polysaccharide extract." Inflammopharmacology **13**(5-6): 485-492.
Roediger, W. E. (1980). "Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man." Gut **21**(9): 793-798.
Rossi, M., P. Brigidi, A. G. V. Y. Rodriguez and D. Matteuzzi (1996). "Characterization of the plasmid pMB1 from Bifidobacterium longum and its use for shuttle vector construction." Research in Microbiology **147**(3): 133-143.
Ruas-Madiedo, P., M. Gueimonde, M. Fernandez-Garcia, C. G. de los Reyes-Gavilan and A. Margolles (2008). "Mucin degradation by Bifidobacterium strains isolated from the human intestinal microbiota." Appl Environ Microbiol **74**(6): 1936-1940.
Ryan, S. M., G. F. Fitzgerald and D. van Sinderen (2005). "Transcriptional regulation and characterization of a novel beta-fructofuranosidase-encoding gene from Bifidobacterium breve UCC2003." Appl Environ Microbiol **71**(7): 3475-3482.
Sakaguchi, K., J. He, S. Tani, Y. Kano and T. Suzuki (2012). "A targeted gene knockout method using a newly constructed temperature-sensitive plasmid mediated homologous recombination in Bifidobacterium longum." Appl Microbiol Biotechnol **95**(2): 499-509.
Salazar, N., A. Prieto, J. A. Leal, B. Mayo, J. C. Bada-Gancedo, C. G. de los Reyes-Gavilan and P. Ruas-Madiedo (2009). "Production of exopolysaccharides by Lactobacillus and Bifidobacterium strains of human origin, and metabolic activity of the producing bacteria in milk." Journal of Dairy Science **92**(9): 4158-4168.
Sambrook, Ed. (1989). Molecular Cloning. A manual laboratory.
Santos, A., M. San Mauro, A. Sanchez, J. M. Torres and D. Marquina (2003). "The antimicrobial properties of different strains of Lactobacillus spp. isolated from kefir." Syst Appl Microbiol **26**(3): 434-437.
Saxelin, M., S. Tynkkynen, T. Mattila-Sandholm and W. M. de Vos (2005). "Probiotic and other functional microbes: from markets to mechanisms." Current Opinion in Biotechnology **16**(2): 204-211.
Scardovi, V. and F. Crociani (1974). "Bifidobacterium-Catenulatum, Bifidobacterium-Dentium, and Bifidobacterium-Angulatum - 3 New Species and Their Deoxyribonucleic-Acid Homology Relationships." International Journal of Systematic Bacteriology **24**(1): 6-20.
Scardovi, V. and L. D. Trovatelli (1969). "New species of bifid bacteria from Apis mellifica L. and Apis indica F. A contribution to the taxonomy and biochemistry of the genus Bifidobacterium." Zentralbl Bakteriol Parasitenkd Infektionskr Hyg **123**(1): 64-88.
Scardovi, V., L. D. Trovatelli, B. Biavati and G. Zani (1979). "Bifidobacterium-Cuniculi, Bifidobacterium-Choerinum, Bifidobacterium-Boum, and Bifidobacterium-Pseudocatenulatum - 4 New Species and Their Deoxyribonucleic-Acid Homology Relationships." International Journal of Systematic Bacteriology **29**(4): 291-311.
Schell, M. A., M. Karmirantzou, B. Snel, D. Vilanova, B. Berger, G. Pessi, M. C. Zwahlen, F. Desiere, P. Bork, M. Delley, R. D. Pridmore and F. Arigoni (2002). "The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract." Proc Natl Acad Sci U S A **99**(22): 14422-14427.
Schreiner, H. C., K. Sinatra, J. B. Kaplan, D. Furgang, S. C. Kachlany, P. J. Planet, B. A. Perez, D. H. Figurski and D. H. Fine (2003). "Tight-adherence genes of Actinobacillus actinomycetemcomitans are required for virulence in a rat model." Proc Natl Acad Sci U S A **100**(12): 7295-7300.
Scott, J. R. and D. Zahner (2006). "Pili with strong attachments: Gram-positive bacteria do it differently." Mol Microbiol **62**(2): 320-330.
Sela, D. A., J. Chapman, A. Adeuya, J. H. Kim, F. Chen, T. R. Whitehead, A. Lapidus, D. S. Rokhsar, C. B. Lebrilla, J. B. German, N. P. Price, P. M. Richardson and D. A. Mills (2008). "The genome sequence of Bifidobacterium longum subsp infantis reveals adaptations for milk utilization within the infant microbiome." Proceedings of the National Academy of Sciences of the United States of America **105**(48): 18964-18969.

Sela, D. A., J. Chapman, A. Adeuya, J. H. Kim, F. Chen, T. R. Whitehead, A. Lapidus, D. S. Rokhsar, C. B. Lebrilla, J. B. German, N. P. Price, P. M. Richardson and D. A. Mills (2008). "The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome." Proc Natl Acad Sci U S A **105**(48): 18964-18969.
Serafini, F., F. Bottacini, A. Viappiani, E. Baruffini, F. Turroni, E. Foroni, T. Lodi, D. van Sinderen and M. Ventura (2011). "Insights into physiological and genetic mupirocin susceptibility in bifidobacteria." Appl Environ Microbiol **77**(9): 3141-3146.
Serafini, F., F. Strati, P. Ruas-Madiedo, F. Turroni, E. Foroni, S. Duranti, F. Milano, A. Perotti, A. Viappiani, S. Guglielmetti, A. Buschini, A. Margolles, D. van Sinderen and M. Ventura (2013). "Evaluation of adhesion properties and antibacterial activities of the infant gut commensal Bifidobacterium bifidum PRL2010." Anaerobe **21**: 9-17.
Shanahan, F. (2005). "Physiological basis for novel drug therapies used to treat the inflammatory bowel diseases - I. Pathophysiological basis and prospects for probiotic therapy in inflammatory bowel disease." American Journal of Physiology-Gastrointestinal and Liver Physiology **288**(3): G417-G421.
Silverman, G. A., P. I. Bird, R. W. Carrell, F. C. Church, P. B. Coughlin, P. G. Gettins, J. A. Irving, D. A. Lomas, C. J. Luke, R. W. Moyer, P. A. Pemberton, E. Remold-O'Donnell, G. S. Salvesen, J. Travis and J. C. Whisstock (2001). "The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature." J Biol Chem **276**(36): 33293-33296.
Simova, E., D. Beshkova, A. Angelov, T. Hristozova, G. Frengova and Z. Spasov (2002). "Lactic acid bacteria and yeasts in kefir grains and kefir made from them." Journal of Industrial Microbiology & Biotechnology **28**(1): 1-6.
Sincock, S. A. and J. P. Robinson (2001). "Flow cytometric analysis of microorganisms." Methods Cell Biol **64**: 511-537.
Snel, B., P. Bork and M. A. Huynen (2002). "Genomes in flux: the evolution of archaeal and proteobacterial gene content." Genome Res **12**(1): 17-25.
Sonnenburg, J. L., C. T. Chen and J. I. Gordon (2006). "Genomic and metabolic studies of the impact of probiotics on a model gut symbiont and host." PLoS Biol **4**(12): e413.
Sonnenburg, J. L., C. T. L. Chen and J. I. Gordon (2006). "Genomic and metabolic studies of the impact of probiotics on a model gut symbiont and host." Plos Biology **4**(12): 2213-2226.
Stackebrandt, E. and B. J. Tindall (2000). "Appreciating microbial diversity: rediscovering the importance of isolation and characterization of microorganisms." Environmental Microbiology **2**(1): 9-10.
Suzuki, T. and K. Yasui (2011). "Plasmid artificial modification: a novel method for efficient DNA transfer into bacteria." Methods Mol Biol **765**: 309-326.
Tap, J., S. Mondot, F. Levenez, E. Pelletier, C. Caron, J. P. Furet, E. Ugarte, R. Munoz-Tamayo, D. L. Paslier, R. Nalin, J. Dore and M. Leclerc (2009). "Towards the human intestinal microbiota phylogenetic core." Environ Microbiol **11**(10): 2574-2584.
Telford, J. L., M. A. Barocchi, I. Margarit, R. Rappuoli and G. Grandi (2006). "Pili in gram-positive pathogens." Nat Rev Microbiol **4**(7): 509-519.
Telford, J. L., M. A. Barocchi, I. Margarit, R. Rappuoli and G. Grandi (2006). "Pili in Gram-positive pathogens." Nature Reviews Microbiology **4**(7): 509-519.
Tissier (1900). Recherchers sur la flora intestinale normale et pathologique du nourisson. Thesis, university of Paris.
Tock, M. R. and D. T. Dryden (2005). "The biology of restriction and anti-restriction." Curr Opin Microbiol **8**(4): 466-472.
Tomich, M., P. J. Planet and D. H. Figurski (2007). "The tad locus: postcards from the widespread colonization island." Nat Rev Microbiol **5**(5): 363-375.
Ton-That, H., L. A. Marraffini and O. Schneewind (2004). "Sortases and pilin elements involved in pilus assembly of Corynebacterium diphtheriae." Mol Microbiol **53**(1): 251-261.
Ton-That, H. and O. Schneewind (2003). "Assembly of pili on the surface of Corynebacterium diphtheriae." Mol Microbiol **50**(4): 1429-1438.

Ton-That, H. and O. Schneewind (2003). "Assembly of pili on the surface of Corynebacterium diphtheriae." Molecular Microbiology **50**(4): 1429-1438.
Ton-That, H. and O. Schneewind (2004). "Assembly of pill in Gram-positive bacteria." Trends in Microbiology **12**(5): 228-234.
Trovatelli, L. D., F. Crociani, M. Pedinotti and V. Scardovi (1974). "Bifidobacterium pullorum sp. nov.: a new species isolated from chicken feces and a related group of bifidobacteria isolated from rabbit feces." Arch Microbiol **98**(3): 187-198.
Tsuchida, S., S. Takahashi, P. P. Mbehang Nguema, S. Fujita, M. Kitahara, J. Yamagiwa, A. Ngomanda, M. Ohkuma and K. Ushida (2013). "Bifidobacterium moukalabense sp. nov. isolated from the faeces of wild west lowland gorilla (Gorilla gorilla gorilla) in Gabon." Int J Syst Evol Microbiol.
Turroni, F., F. Bottacini, E. Foroni, I. Mulder, J. H. Kim, A. Zomer, B. Sanchez, A. Bidossi, A. Ferrarini, V. Giubellini, M. Delledonne, B. Henrissat, P. Coutinho, M. Oggioni, G. F. Fitzgerald, D. Mills, A. Margolles, D. Kelly, D. van Sinderen and M. Ventura (2010). "Genome analysis of Bifidobacterium bifidum PRL2010 reveals metabolic pathways for host-derived glycan foraging." Proc Natl Acad Sci U S A **107**(45): 19514-19519.
Turroni, F., F. Bottacini, E. Foroni, I. Mulder, J. H. Kim, A. Zomer, B. Sanchez, A. Bidossi, A. Ferrarini, V. Giubellini, M. Delledonne, B. Henrissat, P. Coutinho, M. Oggioni, G. F. Fitzgerald, D. Mills, A. Margolles, D. Kelly, D. van Sinderen and M. Ventura (2010). "Genome analysis of Bifidobacterium bifidum PRL2010 reveals metabolic pathways for host-derived glycan foraging." Proceedings of the National Academy of Sciences of the United States of America **107**(45): 19514-19519.
Turroni, F., E. Foroni, B. Montanini, A. Viappiani, F. Strati, S. Duranti, A. Ferrarini, M. Delledonne, D. van Sinderen and M. Ventura (2011). "Global genome transcription profiling of Bifidobacterium bifidum PRL2010 under in vitro conditions and identification of reference genes for quantitative real-time PCR." Appl Environ Microbiol **77**(24): 8578-8587.
Turroni, F., E. Foroni, B. Montanini, A. Viappiani, F. Strati, S. Duranti, A. Ferrarini, M. Delledonne, D. van Sinderen and M. Ventura (2011). "Global Genome Transcription Profiling of Bifidobacterium bifidum PRL2010 under In Vitro Conditions and Identification of Reference Genes for Quantitative Real-Time PCR." Applied and Environmental Microbiology **77**(24): 8578-8587.
Turroni, F., E. Foroni, M. O'Connell Motherway, F. Bottacini, V. Giubellini, A. Zomer, A. Ferrarini, M. Delledonne, Z. Zhang, D. van Sinderen and M. Ventura (2010). "Characterization of the serpin-encoding gene of Bifidobacterium breve 210B." Appl Environ Microbiol **76**(10): 3206-3219.
Turroni, F., E. Foroni, F. Serafini, A. Viappiani, B. Montanini, F. Bottacini, A. Ferrarini, P. L. Bacchini, C. Rota, M. Delledonne, S. Ottonello, D. van Sinderen and M. Ventura (2011). "Ability of Bifidobacterium breve to grow on different types of milk: exploring the metabolism of milk through genome analysis." Appl Environ Microbiol **77**(20): 7408-7417.
Turroni, F., J. R. Marchesi, E. Foroni, M. Gueimonde, F. Shanahan, A. Margolles, D. van Sinderen and M. Ventura (2009). "Microbiomic analysis of the bifidobacterial population in the human distal gut." ISME J **3**(6): 745-751.
Turroni, F., C. Milani, D. van Sinderen and M. Ventura (2011). "Genetic strategies for mucin metabolism in Bifidobacterium bifidum PRL2010: an example of possible human-microbe co-evolution." Gut Microbes **2**(3): 183-189.
Turroni, F., C. Peano, D. A. Pass, E. Foroni, M. Severgnini, M. J. Claesson, C. Kerr, J. Hourihane, D. Murray, F. Fuligni, M. Gueimonde, A. Margolles, G. De Bellis, P. W. O'Toole, D. van Sinderen, J. R. Marchesi and M. Ventura (2012). "Diversity of bifidobacteria within the infant gut microbiota." PLoS One **7**(5): e36957.
Turroni, F., A. Ribbera, E. Foroni, D. van Sinderen and M. Ventura (2008). "Human gut microbiota and bifidobacteria: from composition to functionality." Antonie Van Leeuwenhoek **94**(1): 35-50.

Turroni, F., F. Serafini, E. Foroni, S. Duranti, M. O. Motherway, V. Taverniti, M. Mangifesta, C. Milani, A. Viappiani, T. Roversi, B. Sanchez, A. Santoni, L. Gioiosa, A. Ferrarini, M. Delledonne, A. Margolles, L. Piazza, P. Palanza, A. Bolchi, S. Guglielmetti, D. van Sinderen and M. Ventura (2013). "Role of sortase-dependent pili of Bifidobacterium bifidum PRL2010 in modulating bacterium-host interactions." Proceedings of the National Academy of Sciences of the United States of America **110**(27): 11151-11156.

Turroni, F., F. Serafini, E. Foroni, S. Duranti, M. O'Connell Motherway, V. Taverniti, M. Mangifesta, C. Milani, A. Viappiani, T. Roversi, B. Sanchez, A. Santoni, L. Gioiosa, A. Ferrarini, M. Delledonne, A. Margolles, L. Piazza, P. Palanza, A. Bolchi, S. Guglielmetti, D. van Sinderen and M. Ventura (2013). "Role of sortase-dependent pili of Bifidobacterium bifidum PRL2010 in modulating bacterium-host interactions." Proc Natl Acad Sci U S A.

Turroni, F., F. Serafini, E. Foroni, S. Duranti, M. O'Connell Motherway, V. Taverniti, M. Mangifesta, C. Milani, A. Viappiani, T. Roversi, B. Sanchez, A. Santoni, L. Gioiosa, A. Ferrarini, M. Delledonne, A. Margolles, L. Piazza, P. Palanza, A. Bolchi, S. Guglielmetti, D. van Sinderen and M. Ventura (2013). "Role of sortase-dependent pili of Bifidobacterium bifidum PRL2010 in modulating bacterium-host interactions." Proc Natl Acad Sci U S A **110**(27): 11151-11156.

Turroni, F., F. Strati, E. Foroni, F. Serafini, S. Duranti, D. van Sinderen and M. Ventura (2012). "Analysis of predicted carbohydrate transport systems encoded by Bifidobacterium bifidum PRL2010." Appl Environ Microbiol.

Turroni, F., D. van Sinderen and M. Ventura (2011). "Genomics and ecological overview of the genus Bifidobacterium." International Journal of Food Microbiology **149**(1): 37-44.

Turroni, F., M. Ventura, L. F. Butto, S. Duranti, P. W. O'Toole, M. O. Motherway and D. van Sinderen (2014). "Molecular dialogue between the human gut microbiota and the host: a Lactobacillus and Bifidobacterium perspective." Cell Mol Life Sci **71**(2): 183-203.

Urashima, T., T. Saito, T. Nakamura and M. Messer (2001). "Oligosaccharides of milk and colostrum in non-human mammals." Glycoconj J **18**(5): 357-371.

Vardjan, T., P. M. Lorbeg, I. Rogelj and A. C. Majhenic (2013). "Characterization and stability of lactobacilli and yeast microbiota in kefir grains." Journal of Dairy Science **96**(5): 2729-2736.

Ventura, M., C. Canchaya, G. F. Fitzgerald, R. S. Gupta and D. van Sinderen (2007). "Genomics as a means to understand bacterial phylogeny and ecological adaptation: the case of bifidobacteria." Antonie Van Leeuwenhoek **91**(4): 351-372.

Ventura, M., C. Canchaya, A. Tauch, G. Chandra, G. F. Fitzgerald, K. F. Chater and D. van Sinderen (2007). "Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum." Microbiol Mol Biol Rev **71**(3): 495-548.

Ventura, M., C. Canchaya, D. van Sinderen, G. F. Fitzgerald and R. Zink (2004). "Bifidobacterium lactis DSM 10140: identification of the atp (atpBEFHAGDC) operon and analysis of its genetic structure, characteristics, and phylogeny." Appl Environ Microbiol **70**(5): 3110-3121.

Ventura, M., C. Canchaya, Z. D. Zhang, V. Bernini, G. F. Fitzgerald and D. van Sinderen (2006). "How high G+C Gram-positive bacteria and in particular bifidobacteria cope with heat stress: protein players and regulators." Fems Microbiology Reviews **30**(5): 734-759.

Ventura, M., C. Canchaya, R. Zink, G. F. Fitzgerald and D. van Sinderen (2004). "Characterization of the groEL and groES loci in Bifidobacterium breve UCC 2003: genetic, transcriptional, and phylogenetic analyses." Appl Environ Microbiol **70**(10): 6197-6209.

Ventura, M., S. O'Flaherty, M. J. Claesson, F. Turroni, T. R. Klaenhammer, D. van Sinderen and P. W. O'Toole (2009). "Genome-scale analyses of health-promoting bacteria: probiogenomics." Nat Rev Microbiol **7**(1): 61-71.

Ventura M, T. F., Lugli GA, van Sinderen D. (2014). "Bifidobacteria and humans: our special friends, from ecological to genomics perspectives." J Sci Food Agric. **94(2)**: 163-168.

Ventura, M., F. Turroni, C. Canchaya, E. E. Vaughan, P. W. O'Toole and D. van Sinderen (2009). "Microbial diversity in the human intestine and novel insights from metagenomics." Front Biosci (Landmark Ed) **14**: 3214-3221.

Ventura, M., F. Turroni, G. A. Lugli and D. van Sinderen (2014). "Bifidobacteria and humans: our special friends, from ecological to genomics perspectives." J Sci Food Agric **94**(2): 163-168.
Ventura, M., F. Turroni, M. O. Motherway, J. MacSharry and D. van Sinderen (2012). "Host-microbe interactions that facilitate gut colonization by commensal bifidobacteria." Trends in Microbiology **20**(10): 467-476.
Ventura, M., F. Turroni, M. O. Motherway, J. MacSharry and D. van Sinderen (2012). "Host-microbe interactions that facilitate gut colonization by commensal bifidobacteria." Trends Microbiol **20**(10): 467-476.
Ventura, M., F. Turroni and D. van Sinderen (2012). "Probiogenomics as a tool to obtain genetic insights into adaptation of probiotic bacteria to the human gut." Bioeng Bugs **3**(2).
Ventura, M., F. Turroni, A. Zomer, E. Foroni, V. Giubellini, F. Bottacini, C. Canchaya, M. J. Claesson, F. He, M. Mantzourani, L. Mulas, A. Ferrarini, B. Gao, M. Delledonne, B. Henrissat, P. Coutinho, M. Oggioni, R. S. Gupta, Z. Zhang, D. Beighton, G. F. Fitzgerald, P. W. O'Toole and D. van Sinderen (2009). "The Bifidobacterium dentium Bd1 genome sequence reflects its genetic adaptation to the human oral cavity." PLoS Genet **5**(12): e1000785.
Ventura, M., D. van Sinderen, G. F. Fitzgerald and R. Zink (2004). "Insights into the taxonomy, genetics and physiology of bifidobacteria." Antonie Van Leeuwenhoek **86**(3): 205-223.
Vinderola, G., G. Perdigon, J. Duarte, E. Farnworth and C. Matar (2006). "Effects of the oral administration of the exopolysaccharide produced by Lactobacillus kefiranofaciens on the gut mucosal immunity." Cytokine **36**(5-6): 254-260.
von Ossowski, I., J. Reunanen, R. Satokari, S. Vesterlund, M. Kankainen, H. Huhtinen, S. Tynkkynen, S. Salminen, W. M. de Vos and A. Palva (2010). "Mucosal adhesion properties of the probiotic Lactobacillus rhamnosus GG SpaCBA and SpaFED pilin subunits." Appl Environ Microbiol **76**(7): 2049-2057.
Wada, J., T. Ando, M. Kiyohara, H. Ashida, M. Kitaoka, M. Yamaguchi, H. Kumagai, T. Katayama and K. Yamamoto (2008). "Bifidobacterium bifidum lacto-N-biosidase, a critical enzyme for the degradation of human milk oligosaccharides with a type 1 structure." Appl Environ Microbiol **74**(13): 3996-4004.
Wada, J., T. Ando, M. Kiyohara, H. Ashida, M. Kitaoka, M. Yamaguchi, H. Kumagai, T. Katayama and K. Yamamoto (2009). "Bifidobacterium bifidum Lacto-N-Biosidase, a Critical Enzyme for the Degradation of Human Milk Oligosaccharides with a Type 1 Structure (vol 74, pg 3996, 2009)." Applied and Environmental Microbiology **75**(19): 6414-6414.
Walker, A., A. Cerdeno-Tarraga and S. Bentley (2006). "Faecal matters." Nat Rev Microbiol **4**(8): 572-573.
Ward, R. E., M. Ninonuevo, D. A. Mills, C. B. Lebrilla and J. B. German (2006). "In vitro fermentation of breast milk oligosaccharides by Bifidobacterium infantis and Lactobacillus gasseri." Appl Environ Microbiol **72**(6): 4497-4499.
Watabe, J., Y. Benno and T. Mitsuoka (1983). "Bifidobacterium-Gallinarum Sp-Nov - a New Species Isolated from the Ceca of Chickens." International Journal of Systematic Bacteriology **33**(2): 127-132.
Watanabe, K., H. Makino, M. Sasamoto, Y. Kudo, J. Fujimoto and S. Demberel (2009). "Bifidobacterium mongoliense sp nov., from airag, a traditional fermented mare's milk product from Mongolia." International Journal of Systematic and Evolutionary Microbiology **59**: 1535-1540.
Weidenmaier, C., J. F. Kokai-Kun, S. A. Kristian, T. Chanturiya, H. Kalbacher, M. Gross, G. Nicholson, B. Neumeister, J. J. Mond and A. Peschel (2004). "Role of teichoic acids in Staphylococcus aureus nasal colonization, a major risk factor in nosocomial infections." Nature Medicine **10**(3): 243-245.
Weidenmaier, C., J. F. Kokai-Kun, S. A. Kristian, J. J. Mond, T. Koprivnjak and A. Peschel (2004). "Role of teichoic acid polymers in staphylococcal infections: structure and function in host pathogen interaction." International Journal of Medical Microbiology **294**: 167-167.
Weidenmaier, C. and A. Peschel (2008). "Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions." Nat Rev Microbiol **6**(4): 276-287.

Wicken, A. J. and K. W. Knox (1975). "Characterization of group N streptococcus lipoteichoic acid." Infect Immun **11**(5): 973-981.
Wouters, J. T. M., E. H. E. Ayad, J. Hugenholtz and G. Smit (2002). "Microbes from raw milk for fermented dairy products." International Dairy Journal **12**(2-3): 91-109.
Wyder, M. T., L. Meile and M. Teuber (1999). "Description of Saccharomyces turicensis sp. nov., a new species from kefyr." Syst Appl Microbiol **22**(3): 420-425.
Xia, G., T. Kohler and A. Peschel (2010). "The wall teichoic acid and lipoteichoic acid polymers of Staphylococcus aureus." Int J Med Microbiol **300**(2-3): 148-154.
Xie, N., Y. Wang, Q. Wang, F. R. Li and B. Guo (2012). "Lipoteichoic acid of Bifidobacterium in combination with 5-fluorouracil inhibit tumor growth and relieve the immunosuppression." Bull Cancer **99**(5): E55-63.
Yanagawa, R. and K. Otsuki (1970). "Some properties of the pili of Corynebacterium renale." J Bacteriol **101**(3): 1063-1069.
Yanagawa, R., K. Otsuki and T. Tokui (1968). "Electron microscopy of fine structure of Corynebacterium renale with special reference to pili." Jpn J Vet Res **16**(1): 31-37.
Yeung, M. K. and P. A. Ragsdale (1997). "Synthesis and function of Actinomyces naeslundii T14V type 1 fimbriae require the expression of additional fimbria-associated genes." Infection and Immunity **65**(7): 2629-2639.
Yeung, M. K. and P. A. Ragsdale (1997). "Synthesis and function of Actinomyces naeslundii T14V type 1 fimbriae require the expression of additional fimbria-associated genes." Infect Immun **65**(7): 2629-2639.
Zhu, L., W. Li and X. Z. Dong (2003). "Species identification of genus Bifidobacterium based on partial HSP60 gene sequences and proposal of Bifidobacterium thermacidophilum subsp porcinum subsp nov." International Journal of Systematic and Evolutionary Microbiology **53**: 1619-1623.
Zhurina, D., A. Zomer, M. Gleinser, V. F. Brancaccio, M. Auchter, M. S. Waidmann, C. Westermann, D. van Sinderen and C. U. Riedel (2011). "Complete Genome Sequence of Bifidobacterium bifidum S17." Journal of Bacteriology **193**(1): 301-302.
Zilberstein, B., A. G. Quintanilha, M. A. Santos, D. Pajecki, E. G. Moura, P. R. Alves, F. Maluf Filho, J. A. de Souza and J. Gama-Rodrigues (2007). "Digestive tract microbiota in healthy volunteers." Clinics (Sao Paulo) **62**(1): 47-54.

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Studies

2011-2013 **Ph.D. in Biotechnology**. Department of Life Sciences, University of Parma, Italy

2007-2009 **Master degree in Industrial Biotechnology**. University of Parma, Italy

2004-2007 **Bachelor degree in Biotechnologies**. University of Parma, Italy

Practical experiences

2012 (1 month) **Ph.D project**, "Evaluation of the utilization of prebiotics matrix such as kefir and kefiran by bifidobacteria". Departamento de Microbiologia y Bioquimica de Productos Lacteos (IPLA e CSIC) Villaviciosa, Asturias, Spain.

2011 (3 months) **Ph.D. project**, "Optimization of a method to transform genetically recalcitrant bifidobacteria". University College Cork, Ireland (UCC).

2010 (9 months) **Junior Researcher,** "Isolation and molecular characterization of potential probiotic bacteria to adapt to colonize the human gut". Consorzio Spinner**.** University of Parma, Italy

Pubblications

1. Turroni F**, Serafini F**, Foroni E, Duranti S, O'Connell Motherway M, Taverniti V, Mangifesta M, Milani C, Viappiani A, Roversi T, Sánchez B, Santoni A, Gioiosa L,Ferrarini A, Delledonne M, Margolles A, Piazza L, Palanza P, Bolchi A, Guglielmetti S, van Sinderen D, Ventura M."Role of sortase-dependent pili of *Bifidobacterium bifidum* PRL2010 in modulating bacterium-host nteractions. Proc Natl Acad Sci U S A. 2013

2. **Serafini F**, Strati F, Ruas-Madiedo P, Turroni F, Foroni E, Duranti S, Milano F, Perotti A, Viappiani A, Guglielmetti S, Buschini A, Margolles A, van Sinderen D, Ventura M. Evaluation of adhesion properties and antibacterial activities of the infant gut commensal *Bifidobacterium bifidum* PRL2010. Anaerobe 2013

3. Bottacini F, Milani C, Turroni F, Sánchez B, Foroni E, Duranti S, **Serafini F**, Viappiani A, Strati F, Ferrarini A, Delledonne M, Henrissat B, Coutinho P, Fitzgerald GF, Margolles A, van Sinderen D, Ventura M. *Bifidobacterium asteroides* PRL2011 Genome Analysis Reveals Clues for Colonization of the Insect Gut. PLoS One. 2012 Sep 20.

4. **Serafini F**, Turroni F, Guglielmetti S, Gioiosa L, Foroni E, Sanghez V, Bartolomucci A, Motherway MO, Palanza P, van Sinderen D, Ventura M. An efficient and reproducible method for transformation of genetically recalcitrant bifidobacteria. FEMS Microbiol Lett. 2012 Aug

5. Turroni F, Strati F, Foroni E, **Serafini F**, Duranti S, van Sinderen D, Ventura M.Analysis of predicted carbohydrate transport systems encoded by *Bifidobacterium bifidum* PRL2010. Appl Environ Microbiol. 2012 Jul

6. Baruffini E, **Serafini F**, Ferrero I, Lodi T. Overexpression of DNA polymerase zeta reduces the mitochondrial mutability caused by pathological mutations in DNA polymerase gamma in yeast. PLoS One. 2012

7. Turroni F., Foroni E., Ser**afini F**., Viappiani A., Montanini B., Bottacini F., Ferrarini A., Bacchini PL., Rota C., Delledonne M., Ottonello S., van Sinderen D., Ventura M. The ability of *Bifidobacterium breve* to grow on different milk types: exploring milk metabolism through genome analysis. Appl Environ Microbiol. 2011 Aug 19.

8. Foroni E., **Serafini F**., Amidani D., Turroni F., He F., Bottacini F., O'Connell M. Motherway, Viappiani A., Zhang Z., Rivetti C., van Sinderen D. and Ventura M. Genetic analysis and morphological identification of pilus-like structures in members of the genus *Bifidobacterium*. Microbial Cell Factories. 2011

9. **Serafini F**, Bottacini F, Viappiani A, Baruffini E, Turroni F, Foroni E, Lodi T, van Sinderen D, Ventura M. Insights into physiological and genetic mupirocin susceptibility in bifidobacteria. Appl Environ Microbiol. 2011 May;77(9):3141-6.

10. Baruffini E., **Serafini F**. and Lodi T. (2009) Construction and characterization of centromeric, episomal and GFP-containing vectors for *Saccharomyces cerevisiae* prototrophic strains. J Biotechnol. 143:247-254

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