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

Francesca Turroni[a], Fausta Serafini[b,c], Elena Foroni[b,c], Sabrina Duranti[b,c], Mary O'Connell Motherway[a], Valentina Taverniti[d], Marta Mangifesta[b,c], Christian Milani[b,c], Alice Viappiani[b,c], Tommaso Roversi[d], Borja Sánchez[e], Andrea Santoni[c], Laura Gioiosa[f], Alberto Ferrarini[g], Massimo Delledonne[g], Abelardo Margolles[e], Laura Piazza[d], Paola Palanza[f], Angelo Bolchi[c], Simone Guglielmetti[d], Douwe van Sinderen[a], and Marco Ventura[b,c,1]

[a]Department of Microbiology, Alimentary Pharmabiotic Centre, Bioscience Institute, National University of Ireland, Cork, Ireland; [b]Laboratory of Probiogenomics, [c]Department of Life Sciences, and [f]Department of Neurosciences, University of Parma, 43124 Parma, Italy; [d]Department of Food, Environmental, and Nutritional Sciences, University of Milan, 20133 Milan, Italy; [e]Departamento de Microbiologia y Bioquimica de Productos Lacteos, Instituto de Productos Lácteos de Asturias, Consejo Superior de Investigaciones Cientificas, Villaviciosa, 33300 Asturias, Spain; and [g]Department of Biotechnology, Functional Genomic Center, University of Verona, 37134 Verona, Italy

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*).

MICROBIOLOGY

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. S3B). 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. S3E). 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. S3E) 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
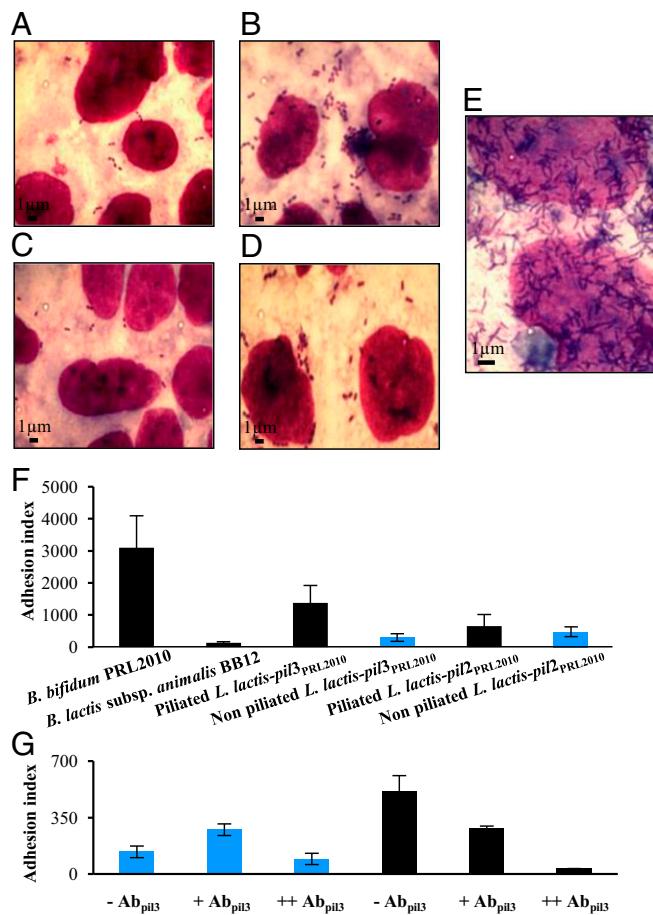
Fig. 2. Adhesion of *B. bifidum* PRL2010 sortase-assembled pili to human intestinal cells. (*A* and *B*) Display of the adhesion phenotype of nonpiliated (*A*) and piliated (*B*) *L. lactis-pil*3$_{PRL2010}$ cells to the Caco-2 cell monolayer. (*C* and *D*) The adhesion phenotype of nonpiliated (*C*) and piliated (*D*) *L. lactis-pil*2$_{PRL2010}$ 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-pil*2PRL2010 cells, as well as *L. lactis-pil*3PRL2010 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-pil*3$_{PRL2010}$ cells, which had first been treated with anti-pil3 antibodies (Ab$_{pil3}$). Piliated *L. lactis-pil*3$_{PRL2010}$ cells (black pillar) and nonpiliated (blue pillar) *L. lactis-pil*2$_{PRL2010}$ 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-pil*2$_{PRL2010}$ was significantly reduced when mannose or fucose was present during the binding assay. Binding ability of piliated *L. lactis-pil*3$_{PRL2010}$ was also affected by glucose and galactose, but not by the polysaccharide xylan (Fig. 3D). 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-pil*3$_{PRL2010}$ clones displayed a significant induction (10-fold; $P < 0.05$) of the TNF-α mRNA levels compared with nonpiliated *L. lactis-pil*3$_{PRL2010}$ (Fig. S7*A*). In contrast, piliated *L. lactis-pil*2$_{PRL2010}$ 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-pil*3$_{PRL2010}$ 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-pil*3$_{PRL2010}$ and non piliated *L. lactis-pil*3$_{PRL2010}$ 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-pil*3$_{PRL2010}$ evoked TNF-α expression and a significantly lower IL-10 response compared with the nonpiliated *L. lactis-pil*3$_{PRL2010}$ (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
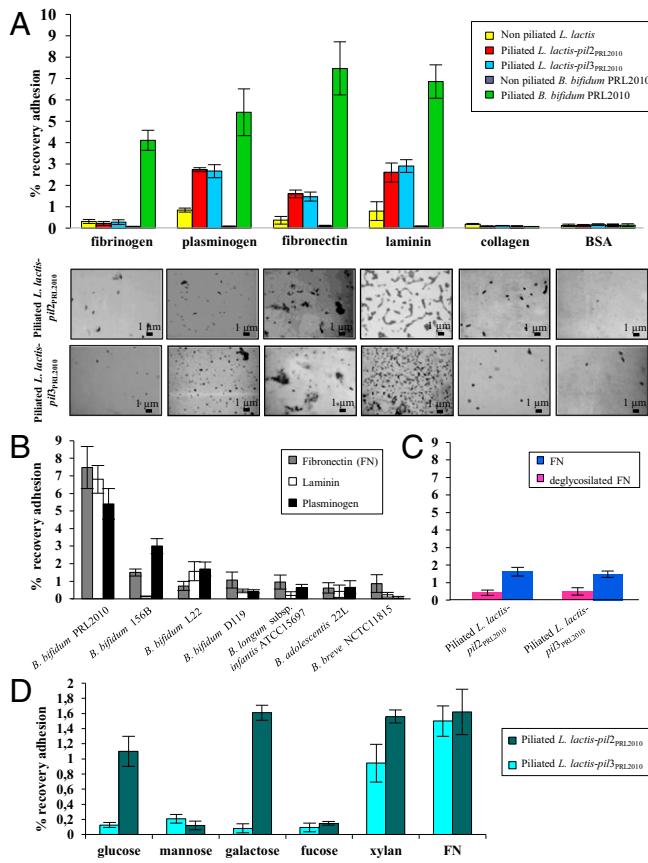
Fig. 3. Involvement of pili produced by *B. bifidum* PRL2010 in binding to ECM proteins. (*A*) Adhesion of *B. bifidum* PRL2010 cells, *L. lactis-pil2*$_{PRL2010}$ cells, and *L. lactis-pil3*$_{PRL2010}$ cells to various ECM proteins. Below each pillar a picture is placed to indicate how piliated and 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 *pil2* and *pil3* appear to be functional as one of the pilus subunit-encoding genes of *pil1* contains a frameshift (15). Here, we have shown that expression of *pil2* and *pil3* enhances adherence to enterocytes and modulates the host inflammatory response (for *pil3*), but it also promotes bacterial aggregation (for *pil2*). 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.

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

Turroni et al. 10.1073/pnas.1303897110

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 ($1,700 \times g$). 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-*pil2*, pNZ8048-*pil3* 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 minibead-beater-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 $3,500 \times g$ 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 $15,000 \times g$ 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 to 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 $3,500 \times g$ 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 $3,500 \times g$ 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 $\sim 1 \times 10^8$ 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 *pil*2 and *pil*3 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*-pil2PRL2010 and *L. lactis* pil3PRL2010 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.

a)

b)
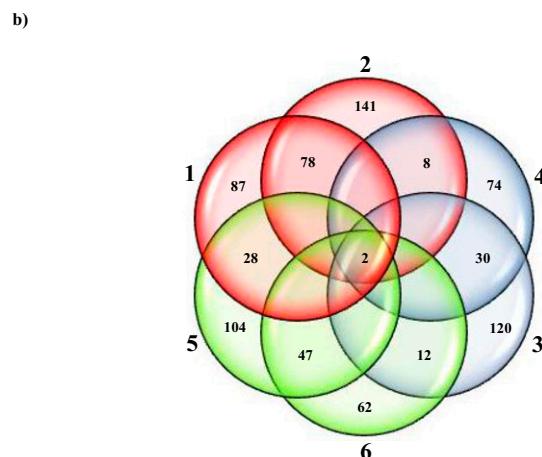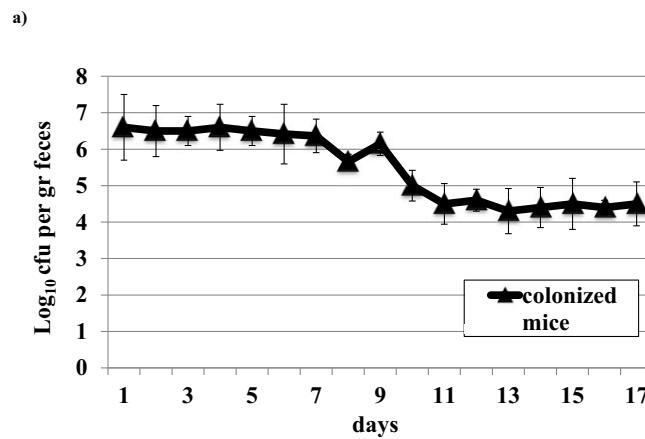
Fig. S1. Population sizes of *B. bifidum* PRL2010 colonizing the intestine of BALB/c mice (*A*) and Venn diagram representing the genes expressed under in vivo conditions, upon contact with Caco-2 cells and in synthetic media using different reference conditions (*B*). In *A*, each point represents the 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.
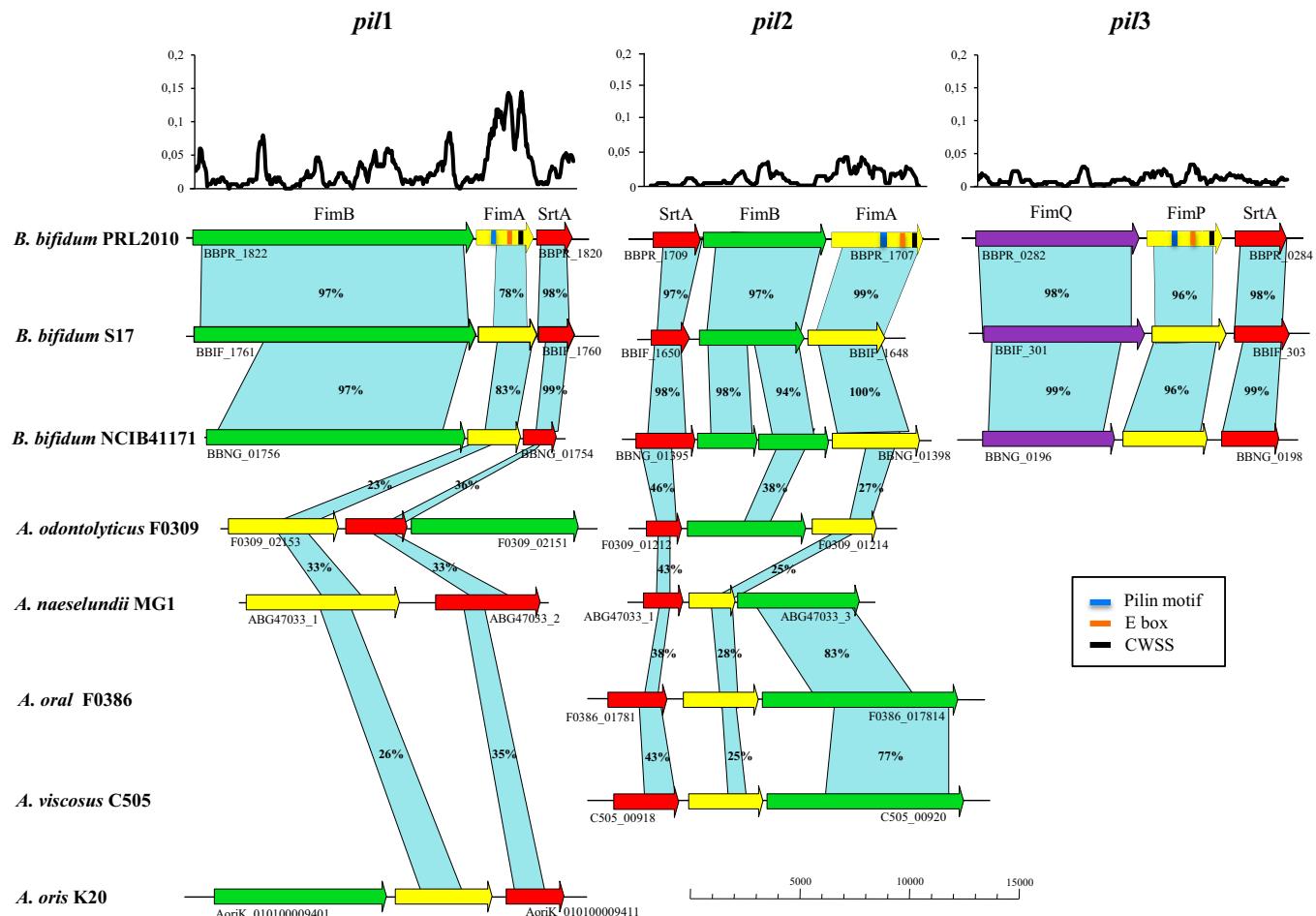
Fig. S2. Schematic comparative representation of pilus-encoding loci of *B. bifidum* PRL2010. Each arrow indicates an ORF, the size of which is proportional to the length of the arrow. Coloring of the arrows represents the different function of the gene as indicated above each arrow. The amino acid identity 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).
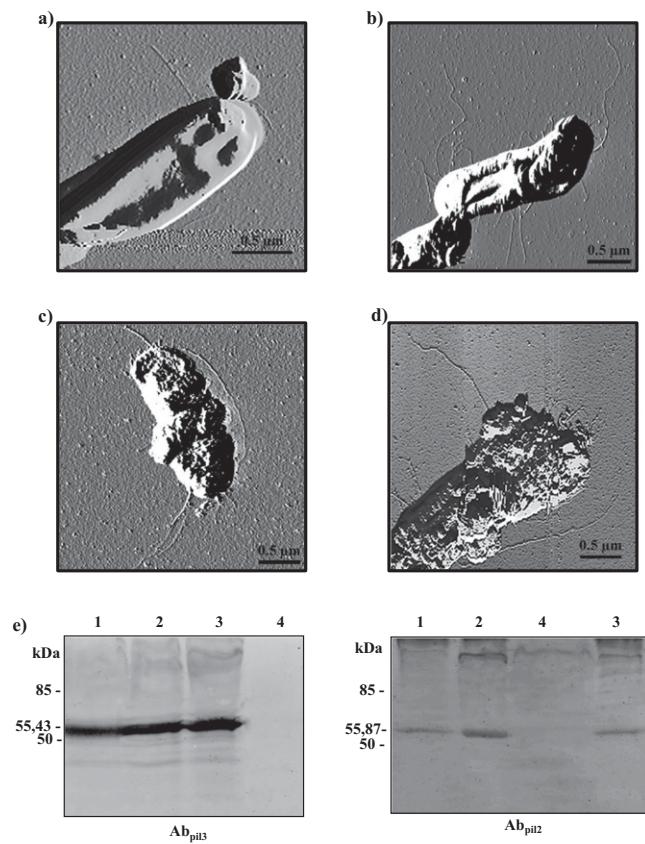
Fig. S3. Pili production of PRL2010 cultivated under different growth conditions. (*A* and *B*) PRL2010 cell's morphology upon cultivation on solid substrate (*A*, MRS agar) and exposure to Caco-2 cells (*B*). (*C* and *D*) PRL2010 cell's morphology upon cultivation in liquid medium supplemented with 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.
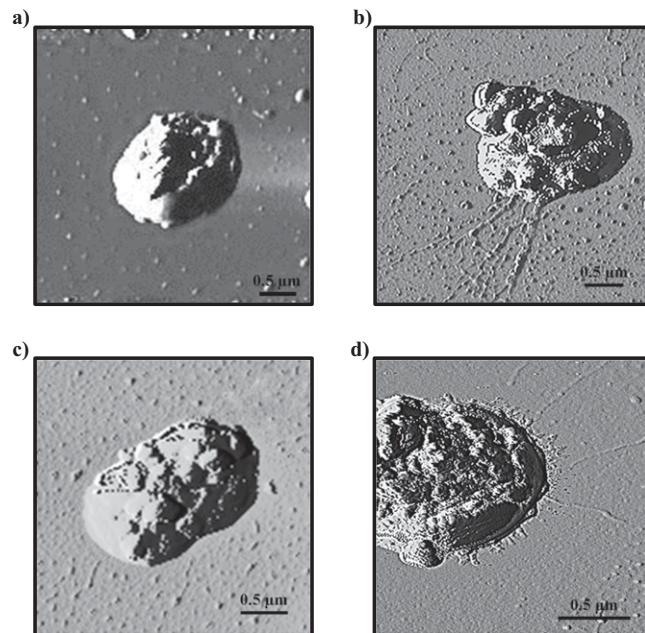
Fig. S4. Cell morphology of *L. lactis* clones expressing PRL2010 pili assayed by AFM. (*A* and *B*) *L. lactis-pil2*$_{PRL2010}$ cell morphology before (*A*) and after (*B*) treatment with nisin. (*B* and *C*) *L. lactis-pil3*$_{PRL2010}$ cell morphology by AFM before (*C*) and after (*D*) treatment with nisin.
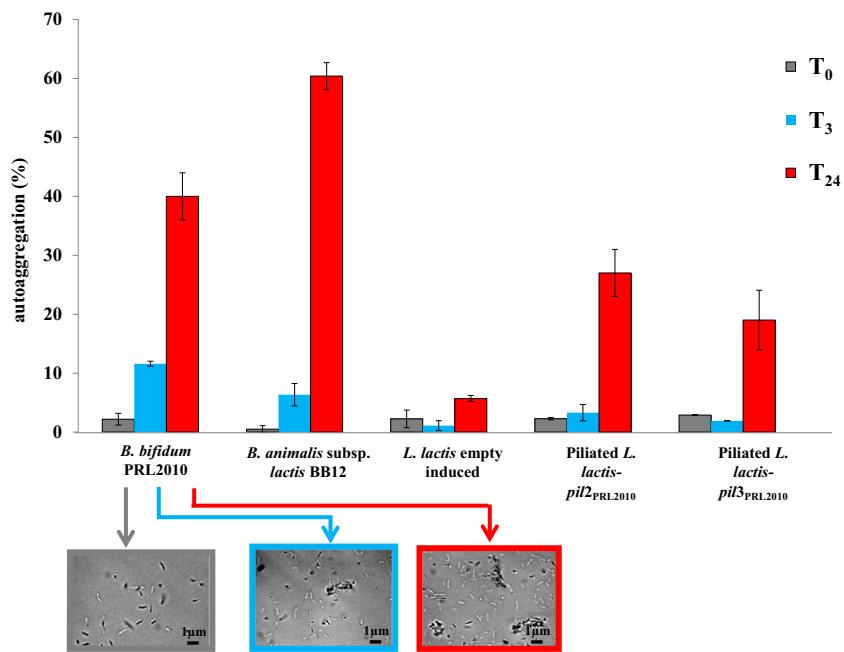
Fig. S5. Aggregation phenotype provided by pili encoded by *B. bifidum* PRL2010. Each pillar represents the coaggregation strength of cells from different microorganisms (*B. bifidum* PRL2010, *Bifidobacterium breve* 12L, *Bifidobacterium animalis* subsp. *lactis* BB12, *L. lactis* MG1363, *L. lactis-pil*2$_{PRL2010}$, and *L. lactis-pil*3$_{PRL2010}$) following different incubation time. The bars represent the mean values for three experiments and the error bars indicate the SDs ($P < 0.05$).
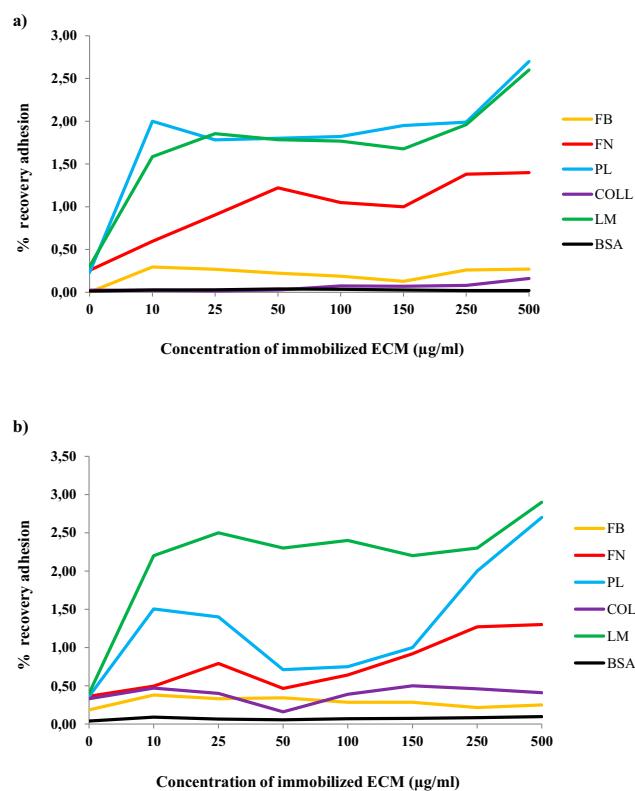
Fig. S6. Evaluation of the adhesion capabilities of piliated *L. lactis-pil*2$_{PRL2010}$- and *L. lactis-pil*3$_{PRL2010}$ cells to different amount of ECM substrates. FB, fibrinogen; FN, fibronectin; PL, plasminogen; COLL, collagen type IV; LM, laminin; BSA, BSA.
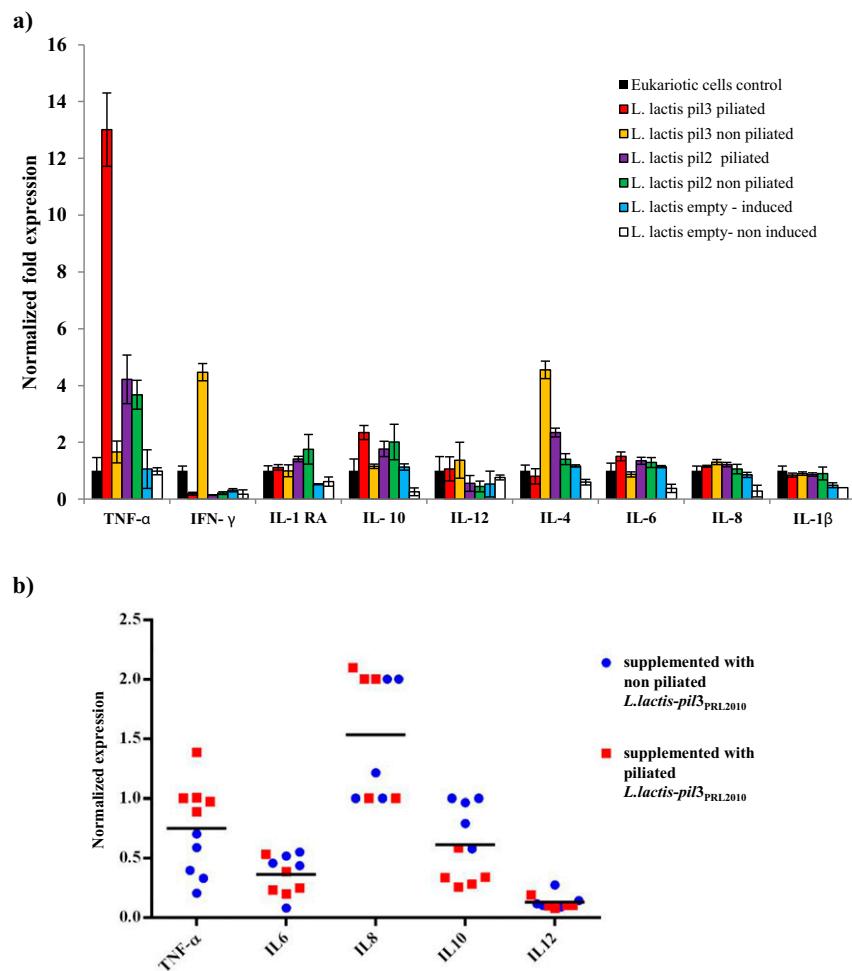
Fig. S7. Evaluation of the host cytokine profiles driven by pili-sortase dependent of *B. bifidum* PRL2010. (*A*) The relative transcription levels of different cytokine upon contacts of *L. lactis-pil2*$_{PRL2010}$ and *L. lactis-pil3*$_{PRL2010}$ with eukaryotic macrophages. The histograms indicate 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.