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 × *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 *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*-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.

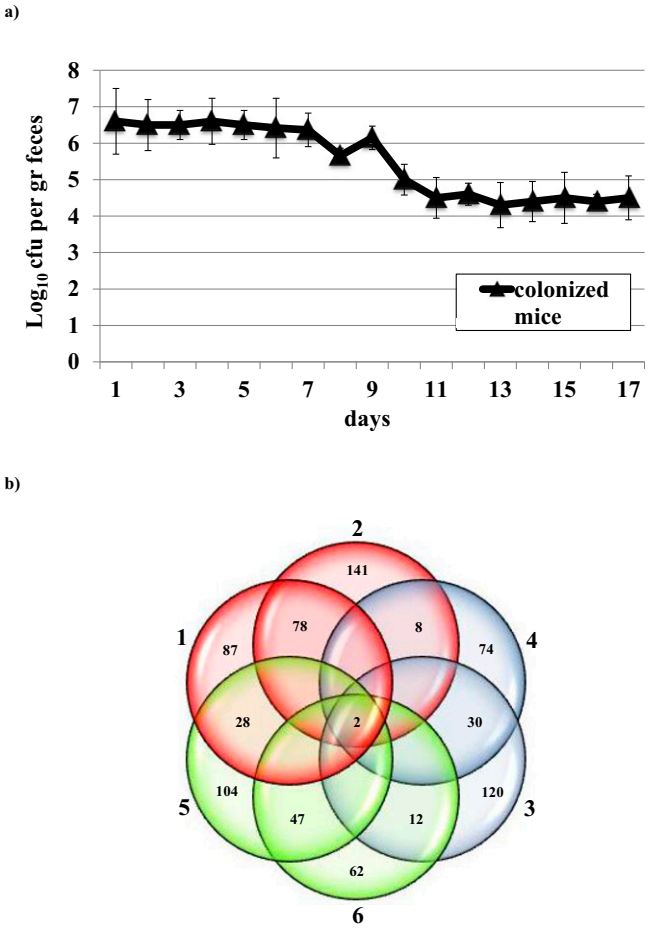

Fig. S1. Population sizes of *B. bifidum* PRL2010 colonizing the intestine of BALB/c mice (*A*) and Venn diagram representing the genes expressed under in vivo conditions, upon contact with Caco-2 cells and in synthetic media using different reference conditions (*B*). In *A*, each point represents the 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.

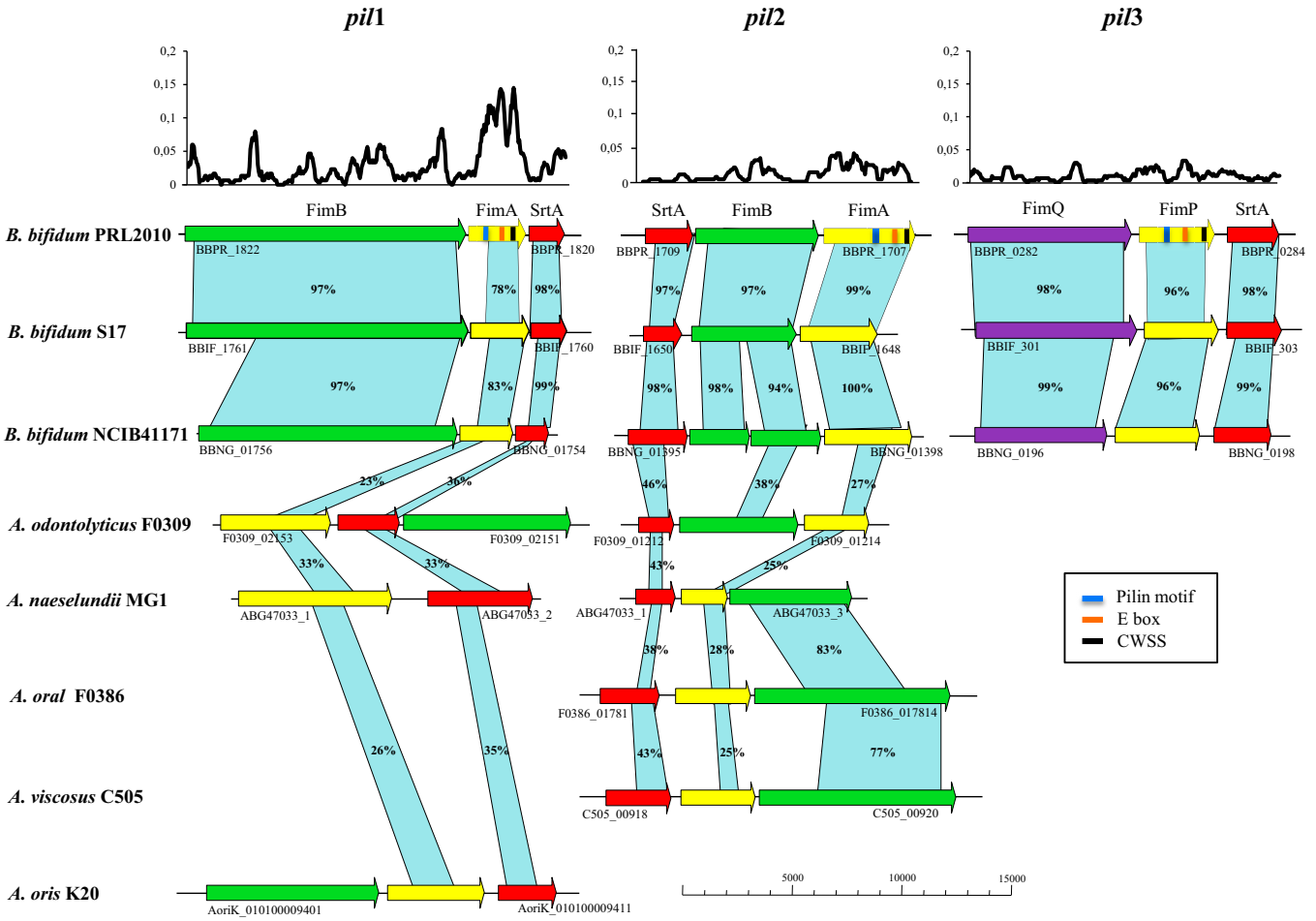

Fig. S2. Schematic comparative representation of pilus-encoding loci of *B. bifidum* PRL2010. Each arrow indicates an ORF, the size of which is proportional to the length of the arrow. Coloring of the arrows represents the different function of the gene as indicated above each arrow. The amino acid identity 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).

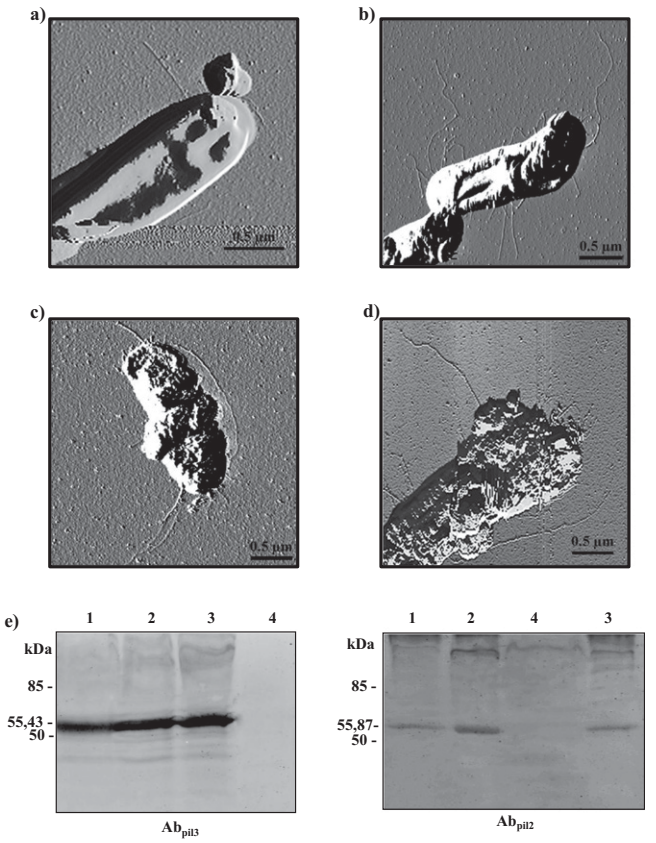

Fig. S3. Pili production of PRL2010 cultivated under different growth conditions. (*A* and *B*) PRL2010 cell's morphology upon cultivation on solid substrate (*A*, MRS agar) and exposure to Caco-2 cells (*B*). (*C* and *D*) PRL2010 cell's morphology upon cultivation in liquid medium supplemented with 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.

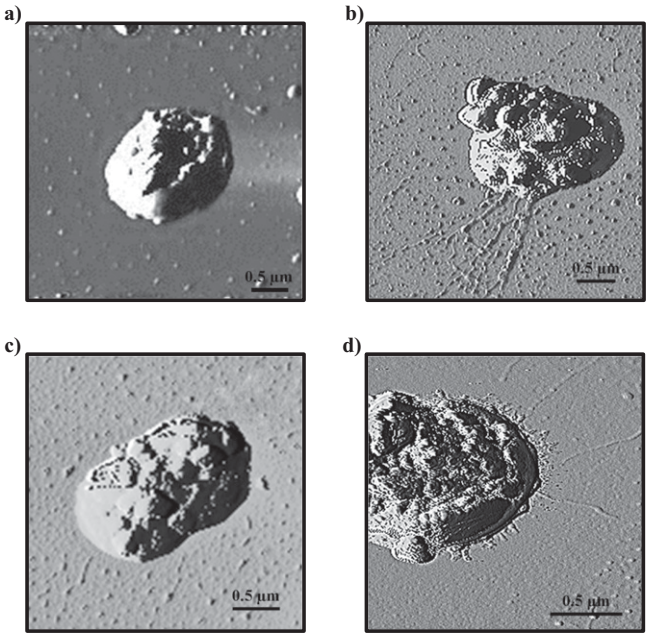

Fig. S4. Cell morphology of *L. lactis* clones expressing PRL2010 pili assayed by AFM. (*A* and *B*) *L. lactis-pil2*$_{PRL2010}$ cell morphology before (*A*) and after (*B*) treatment with nisin. (*B* and *C*) *L. lactis-pil3*$_{PRL2010}$ cell morphology by AFM before (*C*) and after (*D*) treatment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

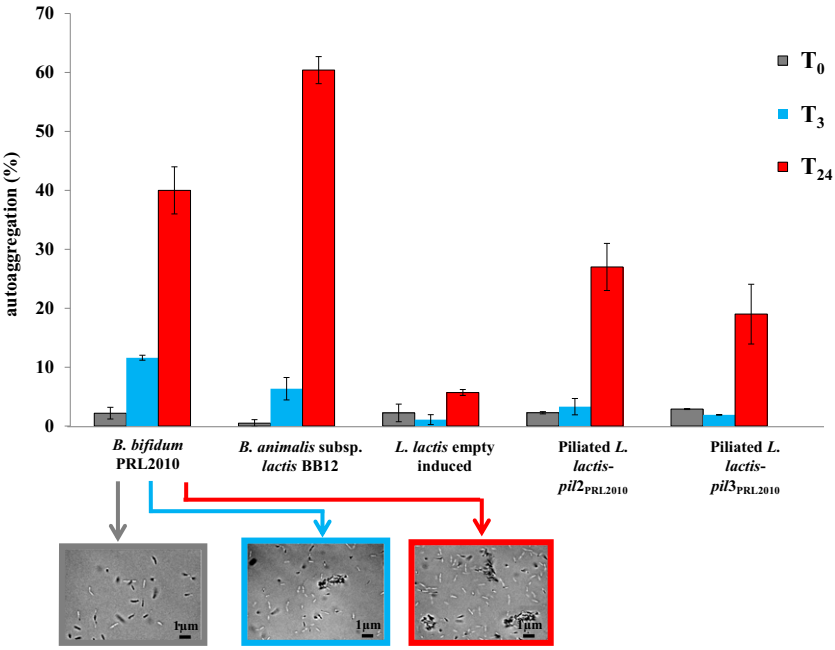

Fig. S5. Aggregation phenotype provided by pili encoded by *B. bifidum* PRL2010. Each pillar represents the coaggregation strength of cells from different microorganisms (*B. bifidum* PRL2010, *Bifidobacterium breve* 12L, *Bifidobacterium animalis* subsp. *lactis* BB12, *L. lactis* MG1363, *L. lactis-pil2*$_{PRL2010}$, 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 ($P < 0.05$).

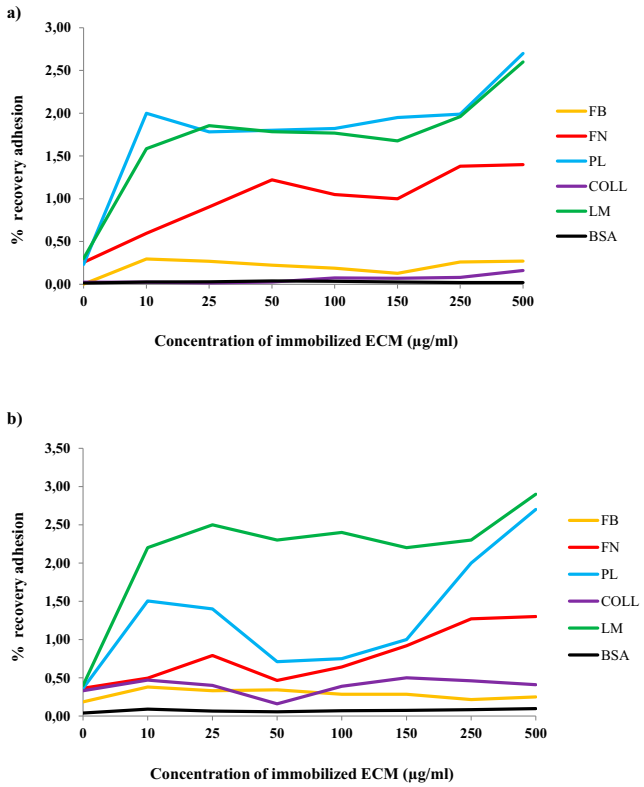

Fig. S6. Evaluation of the adhesion capabilities of piliated *L. lactis-pil2*$_{PRL2010}$- and *L. lactis-pil3*$_{PRL2010}$ cells to different amount of ECM substrates. FB, fibrinogen; FN, fibronectin; PL, plasminogen; COLL, collagen type IV; LM, laminin; BSA, BSA.

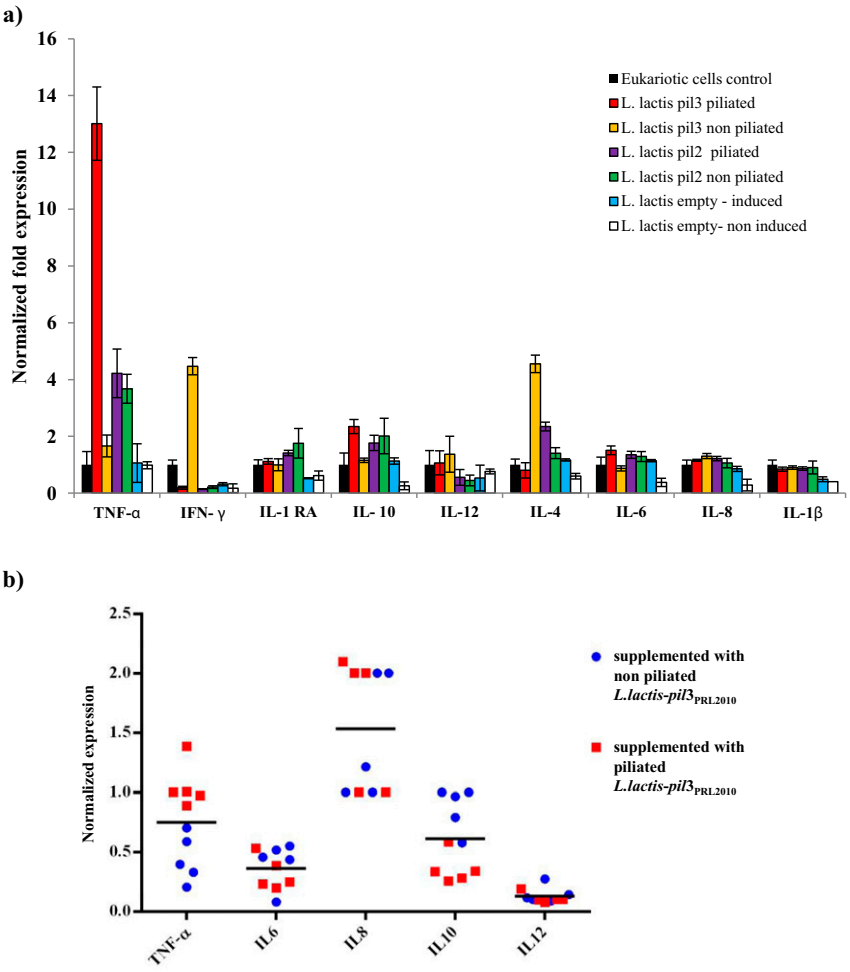

Fig. S7. Evaluation of the host cytokine profiles driven by pili-sortase dependent of *B. bifidum* PRL2010. (*A*) The relative transcription levels of different cytokine upon contacts of *L. lactis-pil2*$_{PRL2010}$ and *L. lactis-pil3*$_{PRL2010}$ with eukaryotic macrophages. The histograms indicate 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.