Applied and Environmental Microbiology, Nov. 2006, p. 6894–6901 Vol. 72, No. 11
0099-2240/06/$08.00+0 doi:10.1128/AEM.00928-06

Inactivation of Adhesion and Invasion of Food-Borne *Listeria monocytogenes* by Bacteriocin-Producing *Bifidobacterium* Strains of Human Origin▽

Olivier Moroni,[1] Ehab Kheadr,[1,2] Yvan Boutin,[3] Christophe Lacroix,[4] and Ismaïl Fliss[1]*

STELA Dairy Research Center, Nutraceuticals and Functional Foods Institute (INAF), Pavillon Paul Comtois, Université Laval, Québec, Québec, Canada G1K 7P4[1]; Department of Dairy Science and Technology, Faculty of Agriculture, University of Alexandria, Alexandria, Egypt[2]; TransBiotech, CEGEP Lévis-Lauzon, Lévis, Québec, Canada G6V 9V6[3]; and Institute of Food Science and Nutrition, Swiss Federal Institute of Technology, ETH Zentrum, LFO F18, CH-8092 Zurich, Switzerland[4]

Received 19 April 2006/Accepted 14 August 2006

Three bacteriocin-producing bifidobacterial isolates from newborns were identified as *Bifidobacterium thermacidophilum* (two strains) and *B. thermophilum* (one strain). This study was undertaken to evaluate the ability of these strains to compete with food-borne *Listeria monocytogenes* for adhesion and invasion sites on Caco-2 and HT-29 cells. The bifidobacteria adhered at levels ranging from 4% to 10% of the CFU added, but none of the bifidobacteria were able to invade cells. The abilities of *Listeria* to adhere to and to invade cells varied widely depending on the strain tested. Three groups of *Listeria* were identified based on invasiveness: weakly invasive, moderately invasive, and highly invasive strains. One strain from each group was tested in competition with bifidobacteria. *B. thermacidophilum* RBL70 was the most effective in blocking invasion of *Listeria*, and the decreases in invasion ranged from 38% to 90%. For all three bifidobacterial strains, contact between the cell monolayer and the bifidobacteria for 1 h before exposure to *Listeria* increased the degree of inhibition. Finally, visualization of competition for adhesion sites on cells by fluorescent in situ hybridization suggested that the two bacteria tended to adhere in close proximity.

Listeria monocytogenes is a facultatively intracellular, gram-positive bacterium responsible for severe food-borne infections leading to meningitis, encephalitis, and gastroenteritis (10, 41). Despite its low incidence in Western countries, *L. monocytogenes* is ranked first among the food-borne pathogenic bacteria in terms of death rate (40%), far ahead of *Campylobacter*, *Salmonella*, and *Escherichia coli* O157:H7. This high mortality rate has made *L. monocytogenes* and its spread the subject of intensive research. *L. monocytogenes* has to cross the intestinal barrier in order to produce central nervous system or feto-placental unit infections (41). The passage through the intestinal barrier is probably the most critical step. Recently, Lecuit et al. (24) showed that adhesion of *L. monocytogenes* and entry into epithelial cells of the small intestine involve several bacterial cell surface proteins. One of these proteins, internalin A, promotes entry into human cells by interacting with the host receptor E-cadherin (8, 19, 24). Internalin A is covalently anchored to the cell wall and belongs to a large family of leucine-rich repeat proteins (6). Recently, it was shown that an *L. monocytogenes* mutant with a mutation in the internalin A gene was completely deficient in attachment to the epithelial surface and was unable to invade (30). Animal studies have shown that the absence of either the bacterial surface protein or the intestinal cell receptor leads to a significant decrease in *Listeria* infectivity.

As it is for most enteric bacterial pathogens, the small intestine, more specifically the ileum, is the preferred site for adhesion and invasion of infectious *L. monocytogenes* (23). In this environment, the resident flora is less abundant than it is in the colon (10^7 CFU/g in the ileum compared to 10^{10} CFU/g in the colon), and this probably increases the capacity for adhesion to epithelial cells. However, the concentration of bifidobacteria, a normal component of the "autochthonous microbiota," is relatively high in the human ileum, up to 10^7 CFU/g (23, 27), suggesting that the genus *Bifidobacterium* may contain bacteria that are suitable probiotics for limiting *Listeria* infection.

The genus *Bifidobacterium* has been extensively studied because of its beneficial effects on health, especially protection of the intestinal tract from microbial infection (1, 16, 21, 26, 28). Several mechanisms have been proposed to explain the efficacy of bifidobacteria in preventing infection. These mechanisms include production of antimicrobial agents, such as organic acids, blocking the adhesion of pathogens and toxins to epithelial cells, and modulating the immune response of the host (32). Inhibition of enteric pathogens by bifidobacteria is well documented, especially for gram-negative bacteria. Gagnon et al. (12) showed that bifidobacteria isolated from feces of newborns were able to inhibit in vitro adhesion of *E. coli* O157:H7 to the Caco-2 and HT-29 cell lines. The level of inhibition varied from 0.1 to 12% depending on the bifidobacterial strain tested and its initial concentration. Similar results have been obtained for inhibition of *Salmonella enterica* serovar Typhimurium in both in vitro and in vivo studies (2, 25).

Recently, our group has isolated several new strains of *Bifidobacterium* from infant feces. Three of these strains, strains RBL67, RBL68, and RBL70, have been shown to produce bacteriocin-like peptides with strong inhibitory activity against *L. monocytogenes* (38). In the present study, we iden-

* Corresponding author. Mailing address: Department of Food and Nutrition, Pavillon Paul-Comtois, Laval University, Quebec, PQ, Canada G1K 7P4. Phone: (418) 656-2131, ext. 6825. Fax: (418) 656-3353. E-mail: ismail.fliss@aln.ulaval.ca.

▽ Published ahead of print on 25 August 2006.

TABLE 1. Bacterial strains used in this study

Strain	Reference[a]	Source	Serotype
B. thermophilum subsp. *infantis* RBL67	STELA	Infant feces	Unknown
B. thermacidophilum subsp. *suis* RBL68	STELA	Infant feces	Unknown
B. thermacidophilum subsp. *suis* RBL70	STELA	Infant feces	Unknown
L. ivanovii LSD5	CFIA	Unknown	Unknown
L. seeligeri LSD11	CFIA	Unknown	Unknown
L. welshimeri LSD12	CFIA	Unknown	Unknown
L. murrayi LSD14	CFIA	Unknown	Unknown
L. monocytogenes LSD15	CFIA	Frozen whole egg	Unknown
L. monocytogenes LSD338	CFIA	Cheese	Unknown
L. monocytogenes LSD339	CFIA	Egg	Unknown
L. monocytogenes LSD340	CFIA	Ice cream	Unknown
L. monocytogenes LSD341	CFIA	Cheese	Unknown
L. monocytogenes LSD346	CFIA	Milk	Unknown
L. monocytogenes LSD348	CFIA	Frozen yolk	Unknown
L. monocytogenes LSD523	CFIA	Unknown	1c
L. monocytogenes LSD524	CFIA	Unknown	1/2c
L. monocytogenes LSD526	CFIA	Unknown	4e
L. monocytogenes LSD529	CFIA	Unknown	3a
L. monocytogenes LSD530	CFIA	Unknown	3b
L. monocytogenes LSD531	CFIA	Unknown	3c
L. monocytogenes LSD535	CFIA	Unknown	1/2a

[a] STELA, STELA Dairy Research Group, Pavillon Comtois, Université Laval, Quebec, Canada; CFIA, Laboratory Services Division, Canadian Food Inspection Agency, Ottawa, Ontario, Canada.

tified the species to which the three bifidobacterial isolates belong and evaluated the potential of these three strains to inhibit in vitro adhesion and invasion of *L. monocytogenes* in human epithelial cell lines. To our knowledge, this is the first study that demonstrated that there is competition between *L. monocytogenes* and bifidobacteria for adhesion sites on enteric cells and that there is inhibition of pathogen invasion.

MATERIALS AND METHODS

Bacterial strains and growth media. The sources of food-borne *Listeria* strains and *Bifidobacterium* sp. strains RBL67, RBL68, and RBL70 are listed in Table 1. All strains were maintained as 20% glycerol stock preparations at −80°C. The bifidobacteria used in this study were previously isolated from fecal samples from 1-week-old breast-fed infants by Touré et al. (38). Isolates RBL57, RBL68, and RBL70 have been shown to produce antibacterial substances similar to a bacteriocin that have high antilisterial activity (38).

All *Listeria* strains were grown at 37°C for 16 h in tryptic soy broth (Difco Laboratories, Detroit, MI) supplemented with 0.6% (wt/vol) yeast extract (Difco). Bifidobacteria were cultured in De Man-Rogosa-Sharpe (MRS) broth (9) obtained from Rosell Institute Inc. (Montreal, Québec, Canada) supplemented with 0.05% (wt/vol) L-cysteine hydrochloride (Sigma Chemical Co., St. Louis, MO) and incubated anaerobically at 37°C for 16 h in Oxoid jars using an atmosphere generation system (Oxoid AnaeroGen; Oxoid Ltd., Basingstoke, Hampshire, England).

Identification of bifidobacteria. The identities of *Bifidobacterium* sp. isolates RBL67, RBL68, and RBL70 were confirmed by 16S rRNA gene sequence analysis, using the PCR amplification protocol proposed previously (35). The genomic 16S rRNA gene was amplified using *Bifidobacterium* genus-specific 16S rRNA gene primers lm26 (5′-GATTCTGGCTCAGGATGAACG-3′) and lm3 (5′-CGGGTGCTI*CCCACTTTCATG-3′) (20). Bifidobacterial purity was verified by using universal bacterial primers Bak4 (5′-AGGAGGTGATCCARCC GCA-3′) and BakLLW (5′-AGTTTGATCMTGGCTCAG-3′) developed by Schürch (35). PCR amplification was carried out with a GeneAmp 2400 PCR system (Perkin-Elmer, Norwalk, CT). Amplified products were separated by electrophoresis (pH 8.0, 100 V for 45 min) using an i-Mupid mini-electrophoresis unit (Cosmo Bio Co. Ltd., Tokyo, Japan) in a 1% agarose gel (Invitrogen, Burlington, Ontario, Canada) and were stained with ethidium bromide. PCR-amplified products that were approximately 1.4 kb long were excised from gels and purified using QIAGEN columns (QIAGEN, Mississauga, Ontario, Canada). Automated DNA sequence analysis was carried out for both strands with a DNA sequencer (ABI Prism 3100; Applied Biosystems, Foster City, CA). Sequence data were assembled and analyzed using GCG Wisconsin package 10 (Accelrys, San Diego, CA). Database homology searches were performed using the FASTA network service at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The bootstrap method was employed to determine the statistical confidence of phylogenetic relationships (11).

Epithelial cell lines. Enterocyte-like Caco-2 (ATCC HTB-37) and HT-29 (ATCC HTB-38) cells were obtained from the American Type Culture Collection (Rockville, MD). Caco-2 cells were cultured as monolayers in 75-cm^2 flasks (Falcon, Becton and Dickinson Company, Franklin Lakes, NJ) with Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, Burlington, Ontario, Canada) containing 20% (vol/vol) fetal bovine serum (Wisent Inc., St. Bruno, Québec, Canada), 1% (vol/vol) nonessential amino acids (Gibco), and 1% (vol/vol) antibiotic solution (100 U/ml penicillin and 100 μg/ml streptomycin; Gibco). HT-29 cells were cultured in RPMI 1640 medium (Gibco) containing 10% (vol/vol) fetal bovine serum, 2 mM L-glutamine (Gibco), and 1% (vol/vol) antibiotic solution. For adhesion and invasion assays, Caco-2 or HT-29 cells were seeded in 24-well plates (2 cm^2/well; Falcon) to obtain semiconfluent monolayers (10^6 cells/well) in 3 days. Cell cultures were replenished with DMEM and RPMI 1640 without antibiotics 24 h before the assay was performed. All cell cultures were incubated at 37°C in a 5% (vol/vol) CO_2 atmosphere.

Adhesion assays. The adhesion of *L. monocytogenes* and the adhesion of bifidobacteria to epithelial cells were evaluated separately and in a competition assay. Briefly, fresh overnight cultures of *L. monocytogenes* and bifidobacteria were harvested by centrifugation at 10,000 × *g* for 10 min and washed twice with sterile phosphate-buffered saline (PBS). The washed pellets were suspended in DMEM for assays with Caco-2 cells and in RPMI 1640 medium for assays with HT-29 cells at final concentrations of 5×10^8 CFU/ml for *L. monocytogenes* and 5×10^6 to 5×10^8 CFU/ml for bifidobacteria. Cell monolayers in 24-well plates were then inoculated with 250 μl of a single strain for individual adhesion assays or with a combination of *Listeria* and *Bifidobacterium* for competition assays. For the latter assays, bifidobacteria were added either at the same time that *Listeria* was added or 1 h before or 1 h after *Listeria* was added. Plates were then incubated under anaerobic conditions at 37°C for 30 min, after which free bacteria were eliminated by washing the cell layers twice with PBS. Cells with adherent bacteria were harvested with trypsin, centrifuged at 10,000 × *g* for 5 min, and suspended in PBS. Adherent *L. monocytogenes* cells were enumerated on *Listeria* selective media (Oxoid) and incubated for 24 to 48 h at 37°C in aerobic conditions. Adherent bifidobacteria were enumerated on MRS agar media and incubated for 24 to 48 h at 37°C in anaerobic conditions. The adhesion capacity was expressed as the number of adherent bacteria divided by total number of bacteria added, multiplied by 100. The inhibition of adhesion of *L. monocytogenes* in a competition assay was expressed as a percentage, using the following formula: inhibition of adhesion = $100(1 - T_1/T_2)$, where T_1 and T_2 are the numbers of adherent *Listeria* cells (CFU/well) in the presence and absence of bifidobacteria, respectively.

Invasion assays. The invasion of epithelial cells by *Listeria* or bifidobacteria was studied using the gentamicin-based assay described by Bunduki et al. (3) and Gaillard et al. (13). A bacterial suspension (250 μl, like the suspension used for the adhesion assay) was spread on cell monolayers and incubated at 37°C for 1.5 h before it was washed two times with 250 μl of PBS. For competition assays, *L. monocytogenes* and bifidobacteria were added either together or 1 h apart as described above for the adhesion assays. The monolayers were washed twice with PBS before addition of 250 μl of 1 mg/ml gentamicin (Sigma) and incubation for an additional 1.5 h under the same conditions to kill noninvading bacteria. The monolayers were then washed with PBS and lysed with 250 μl of ice-cold 0.1% (vol/vol) Triton X-100 (Sigma). Appropriate dilutions were prepared in PBS and plated on *Listeria* selective agar (aerobic conditions, 37°C, 48 h) and MRS agar (anaerobic conditions, 37°C, 48 h) for enumeration of *L. monocytogenes* and bifidobacteria, respectively. The invasion capacity was determined by multiplying the number of invading bacterial cells/total number of adherent bacterial cells by 100. The inhibition of invasion was expressed as a percentage, using the relative decrease in invasion by *Listeria* in the presence of bifidobacteria, as determined by the following formula: inhibition of invasion = $100(1 - T_B/T_L)$, where T_B and T_L are the numbers of invading *Listeria* cells (CFU/well) in the presence and absence of bifidobacteria, respectively.

FISH. All fluorescent in situ hybridization (FISH) experiments were performed by using cell monolayers with adherent and invading *Listeria* and bifidobacteria (from competition and noncompetition assays) and an adaptation of a protocol described by Wagner et al. (43). Monolayers were harvested by addition of 100 μl of trypsin and washed two times with PBS. Cells were then fixed for 3 h at 4°C in PBS containing 4% (wt/vol) paraformaldehyde. These cells were kept frozen in a 50% ethanol–PBS solution before hybridization. Fifteen

TABLE 2. Adhesion and invasion of bifidobacterial and listerial strains with Caco-2 and HT-29 epithelial cells[a]

Bacterial strain	Adhesion (%)		Invasion (%)	
	Caco-2 cells	HT-29 cells	Caco-2 cells	HT-29 cells
B. thermophilum subsp. *infantis* RBL67	5.43 ± 0.58 DEF	6.23 ± 0.55 CDE		
B. thermacidophilum subsp. *suis* RBL68	3.24 ± 1.02 FGHI	3.45 ± 0.91 FGH		
B. thermacidophilum subsp. *suis* RBL70	7.68 ± 1.09 BCD	8.37 ± 1.54 C		
L. ivanovii LSD5	4.53 ± 0.65 EFG	7.92 ± 0.10 BCD	1.84 ± 0.45 M	2.66 ± 0.70 KL
L. seeligeri LSD11	1.18 ± 0.07 HIJ	1.43 ± 0.15 EFG	4.69 ± 0.33 L	5.02 ± 1.23 JK
L. welshimeri LSD12	1.03 ± 0.5 HIJ	0.55 ± 0.09 G	18.33 ± 1.25 F	27.94 ± 0.91 C
L. murrayi LSD14	34.6 ± 1.2 A	65.7 ± 9.7 A	7.46 ± 1.02 K	8.96 ± 0.28 HI
L. monocytogenes LSD15	1.67 ± 0.66 HIJ	1.41 ± 0.33 EFG	13.48 ± 1.17 HI	13.18 ± 1.41 FG
L. monocytogenes LSD338	1.02 ± 0.38 HIJ	0.81 ± 0.17 EFG	34.89 ± 0.32 C	42.64 ± 2.13 B
L. monocytogenes LSD339	0.32 ± 0.24 J	0.46 ± 0.10 G	11.24 ± 1.12 IJ	10.57 ± 0.31 GH
L. monocytogenes LSD340	1.14 ± 0.51 HIJ	1.38 ± 0.31 EFG	22.57 ± 2.06 E	26.62 ± 0.47 CD
L. monocytogenes LSD341	0.66 ± 0.21 IJ	0.44 ± 0.11 G	38.72 ± 3.26 B	42.97 ± 7.59 AB
L. monocytogenes LSD346	0.012 ± 0.017 J	0.0037 ± 0.0014 G	15.26 ± 1.47 GH	16.47 ± 1.09 E
L. monocytogenes LSD348	1.04 ± 0.03 HIJ	1.27 ± 0.19 EFG	44.65 ± 0.85 A	45.49 ± 0.82 A
L. monocytogenes LSD523	1.89 ± 0.19 HIJ	2.29 ± 0.48 DEFG	0.64 ± 0.14 M	0.74 ± 0.33 L
L. monocytogenes LSD524	0.12 ± 0.01 J	0.22 ± 0.06 G	0.9 ± 0.29 M	0.83 ± 0.06 L
L. monocytogenes LSD526	0.42 ± 0.03 J	0.4 ± 0.13 G	0.78 ± 0.17 M	0.99 ± 0.34 L
L. monocytogenes LSD529	6.88 ± 1.15 BCDE	7.44 ± 0.36 BCD	7.41 ± 1.09 K	9.57 ± 0.61 HI
L. monocytogenes LSD530	6.42 ± 0.36 CDE	9.45 ± 1.13 B	0.0046 ± 0.0011 M	0.0056 ± 0.007 L
L. monocytogenes LSD531	0.31 ± 0.03 J	0.33 ± 0.11 G	9.14 ± 1.15 JK	10.04 ± 1.21 HI
L. monocytogenes LSD535	0.56 ± 0.30 IJ	0.65 ± 0.16 FG	25.18 ± 1.53 DE	26.31 ± 0.82 CD

[a] The data are means ± standard variations values followed by different letters in the same column are significantly different as determined by a least-significant-difference test at a P value of <0.05.

microliters of diluted paraformaldehyde-fixed cells was transferred to eight-well microscope slides and dried for 10 min at 60°C. The slides had previously been washed with a 70% (vol/vol) ethanol solution containing 0.1% (vol/vol) hydrochloride acid (HCl) and treated with 0.01% poly-L-lysine. The fixed cells were dehydrated successively in 50%, 80%, and 100% ethanol baths (3 min each) at room temperature and then treated with 20 μl of a 10-mg/ml lysozyme (Sigma) solution for 20 min at room temperature, followed by washing with distilled water and repetition of the ethanol dehydration procedure. Fixed samples were then treated at room temperature with a proteinase K solution (0.05 mg/ml; Sigma) for 5 min. After one wash with distilled water and a final dehydration procedure, the slides were dried at room temperature for 20 min and hybridized for 16 h at 46°C in a hybridization solution (0.09 M NaCl, 20 mM Tris-HCl, 0.1% sodium dodecyl sulfate, 35% formamide; pH 7.2) containing 50 ng/ml of *Listeria* genus-specific probe Lis1255 (5′-fluoro-ACCTCGCGGCTTCGCGAC-3′) (34, 39) and 50 ng/ml of *Bifidobacterium* genus-specific probe Bif164 (5′-Cy3-CATCCGGCA TTACCACCC-3′) (22, 33). Each slide was then washed with hybridization buffer and incubated in washing buffer (0.07 M NaCl, 20 mM Tris-HCl, 0.1% sodium dodecyl sulfate; pH 7.2) for 20 min at 48°C. After a final wash with distilled water, the slides were allowed to dry completely at room temperature before they were treated with a Slowfade antifade kit (Molecular Probes, Invitrogen) used according to the manufacturer's instructions and were visualized with an Olympus fluorescent microscope (Olympus, Melville, NY).

Statistical analysis. Adhesion and invasion assays were repeated three times, and strain differences were tested by analysis of variance. The significance of differences between strains was determined by Fisher's least-significant-difference test at a level of certainty of 5% with the StatGraphics Centurion 15.0 software (Statpoint Inc., Herndon, VA).

RESULTS

Identification of bifidobacterial species. The sequence data for the 16S rRNA gene (approximately 1.4 kb) of bifidobacterial isolates RBL67, RBL68, and RBL70 were compared with the available sequences in the GenBank database. The 16S rRNA gene sequences of isolates RBL68 and RBL70 exhibited the highest levels of similarity to the sequences of *B. thermacidophilum* subsp. *suis* (GenBank accession no. AY148470.1) (99%) and bacterial isolate mpn (GenBank accession no. AF357558) (98%). The sequence of the 16S rRNA gene of strain RBL67 was also related to the *B. thermacidophilum* subsp. *suis* sequence (99%). Using DNA-DNA hybridization, von Ah et al. (42) reported levels of genome homology of 77.2, 77.9, and 86.25% between strain RBL67 and *B. thermacidophilum* subsp. *suis* LMG21689^T, *B. thermacidophilum* LMG21395^T, and *B. thermophilum* DSM20210^T, respectively. Strain RBL67 was found to differ from *B. thermophilum* DSM20210^T in its tolerance to low pH and carbohydrate fermentation characteristics. Based on these results, a novel subspecies of *B. thermophilum*, *Bifidobacterium thermophilum* subsp. *infantis*, was created for strain RBL67 (GenBank accession no. DQ340558).

Bacterial adhesion on epithelial cells. The efficiencies of adhesion of the bifidobacterial and *Listeria* strains to Caco-2 and HT-29 cells are shown in Table 2. The levels of adhesion of bifidobacteria ranged from 3.24% to 7.68% for Caco-2 cells and from 3.45% to 8.37% for HT-29 cells. In general, there was no significant difference between adhesion of bifidobacteria to Caco-2 cells and adhesion of bifidobacteria to HT-29 cells. *B. thermacidophilum* subsp. *suis* RBL70 was the most efficient strain in terms of adhesion to epithelial cells, and the values were 7.68% and 8.37% with Caco-2 and HT-29 cells, respectively. The least efficient of the bifidobacteria was strain RBL68, while the values for strain RBL67 were intermediate.

The adhesion of *Listeria* strains was more variable, and the levels ranged from 0.012% and 0.0037% with Caco-2 and HT-29 cells, respectively, for *L. monocytogenes* LSD346 to 34.6% and 65.71% with Caco-2 and HT-29 cells, respectively, for *L. murrayi* LSD14. Three strains exhibited levels of adhesion ranging from 4.53% to 6.88% with Caco-2 cells and from 7.44% to 9.45% with HT-29 cells. For the remaining strains, the levels of adhesion ranged from 0.1% to 2.0% with both Caco-2 and HT-29 cells. As observed with bifidobacteria, no significant difference between adhesion on Caco-2 cells and adhesion on HT-29 cells was noted.

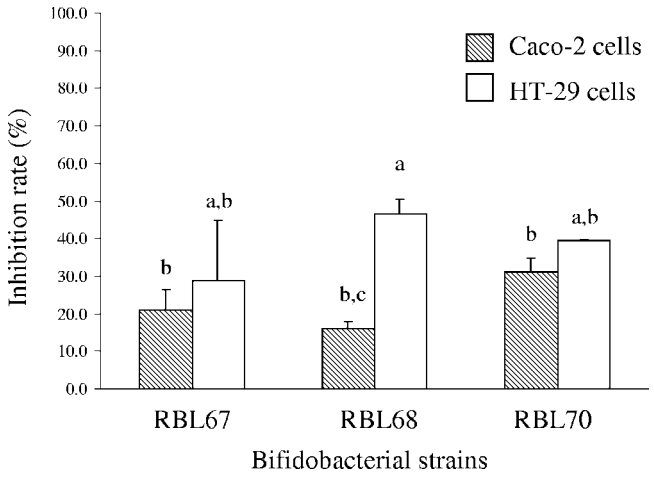

FIG. 1. Effect of 1 h of preincubation with bifidobacteria on adhesion of *L. monocytogenes* strain LSD348 to epithelial cells. Different letters above columns indicate that the values are significantly different ($P < 0.05$).

With the exception of *L. murrayi* LSD14, which adhered more than any other strain, the adhesion of non-*L. monocytogenes Listeria* strains, including *L. ivanovii* LSD5, *L. seeligeri* LSD11, and *L. welshimeri* LSD12, did not differ from the adhesion of *L. monocytogenes* strains.

Bacterial invasion of epithelial cells lines. The abilities of bifidobacteria and *Listeria* to invade the Caco-2 and HT-29 epithelial cells lines are also summarized in Table 2. First, no invasion of either cell line was observed with the three bifidobacterial strains.

As observed for adhesion, invasion of both epithelial cell lines by *Listeria* strains was highly variable. The highest levels of invasion were obtained with *L. monocytogenes* LSD348 (44.65 and 45.49% for Caco-2 and HT-29 cells, respectively). The lowest levels, 0.0046 and 0.0056%, were observed with *L. monocytogenes* LSD530. Again, there was no significant difference between invasion of Caco-2 cells and invasion of HT-29 cells, except for strains LSD12, LSD338, LSD340, and LSD341, for which the level of invasion was always higher with HT-29 cells. In addition, no correlation between adhesion and invasion was observed for any *Listeria* strain. For example, the highly adherent strain LSD530 showed a very low level of invasion, while the weakly adherent strain LSD346 was highly invasive (15.26 and 16.47% for Caco-2 and HT-29 cells, respectively).

Competition for adhesion. Three *L. monocytogenes* strains (LSD348, LSD339, and LSD530) were selected and tested in adhesion and invasion competition assays. The invasion percentages for these three strains were very different.

Three concentrations (10^6, 10^7, and 10^8 CFU/ml) were tested for each bifidobacterial strain. When *Listeria* and bifidobacteria were added simultaneously, no inhibition of the

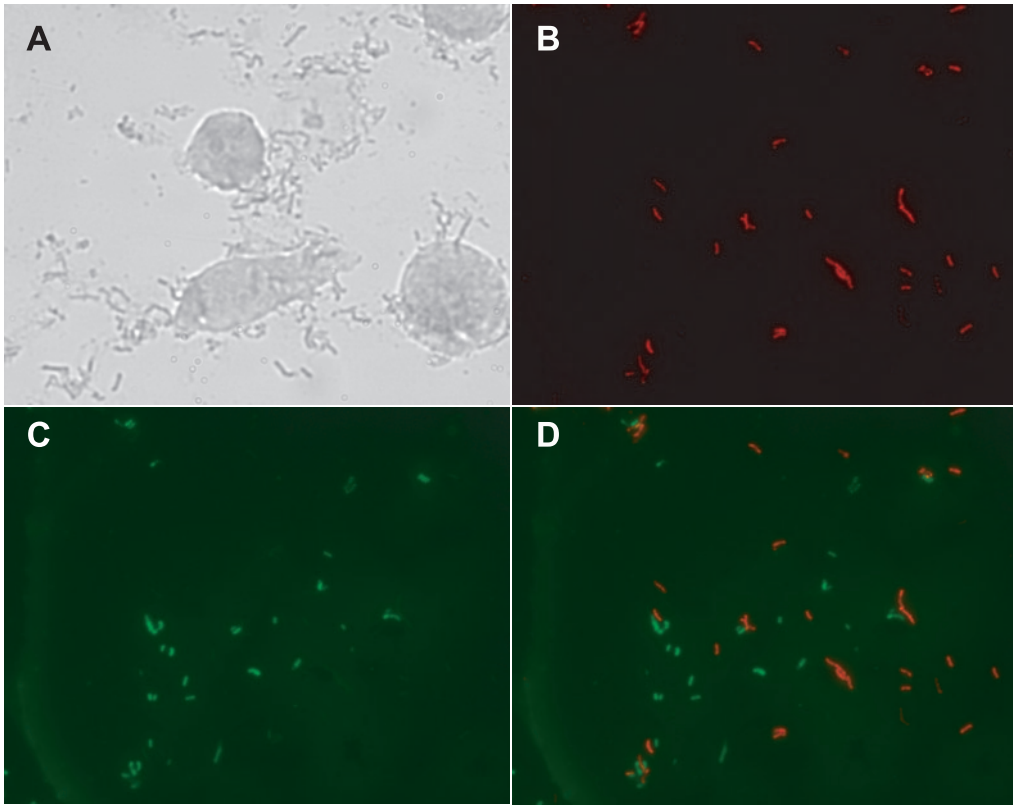

FIG. 2. Visualization of bacterial adhesion on HT-29 cells. (A) Phase-contrast image. Magnification, ×1,000. (B to D) FISH images of *B. thermacidophilum* subsp. *suis* RBL70 (red), *L. monocytogenes* LSD348 (green), and the two strains in competition, respectively.

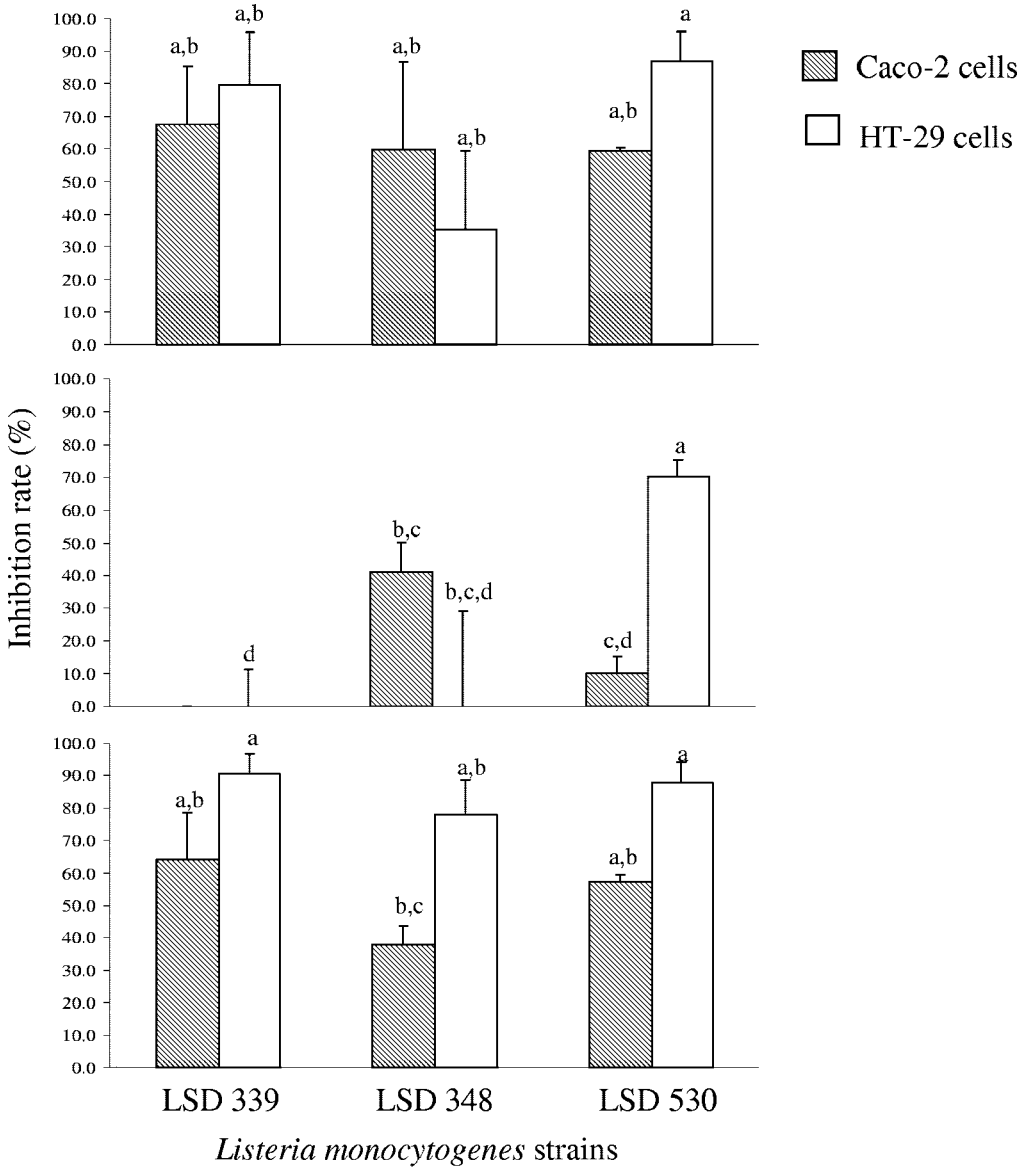

FIG. 3. Inhibition of *L. monocytogenes* LSD339, LSD348, and LSD530 invasion of epithelial cells by *B. thermophilum* subsp. *infantis* RBL67 (top) and *B. thermacidophilum* subsp. *suis* RBL68 (middle) and RBL70 (bottom). Different letters above columns indicate that the values are significantly different ($P < 0.05$).

adhesion of *Listeria* was observed (data not shown). Moreover, *Listeria* adhesion increased slightly when bifidobacteria were added at a concentration of 10^8 CFU/ml. Similar results were obtained when *L. monocytogenes* was added 1 h before bifidobacteria were added.

When bifidobacteria were added at a concentration of 10^8 CFU/ml 1 h before *Listeria* was added, strong inhibition of adhesion was observed, notably in the case of *L. monocytogenes* LSD348, the most invasive strain tested (Fig. 1). Decreases of 21, 16, and 31% in adhesion to Caco-2 cells were observed with *Bifidobacterium* strains RBL67, RBL68, and RBL70, respectively. Slightly greater decreases were observed for RBL67 and RBL70 with HT-29 cells (29 and 40%, respectively), while RBL68 decreased *L. monocytogenes* adhesion three times as much (47%) on HT-29 cells as on Caco-2 cells. Finally, no protective inhibition of pathogen adhesion was observed when bifidobacteria were added at concentrations of 10^6 and 10^7 CFU/ml (data not shown).

Figure 2 shows adhesion of bifidobacteria and *Listeria* to HT-29 cells, as visualized by phase-contrast microscopy (Fig. 2A) and FISH (Fig. 2B to D). Some of the bifidobacteria present on the cell monolayer are near *Listeria* cells.

Inhibition of invasion. Figure 3 shows the inhibition of invasion of enteric cells by *L. monocytogenes* strains LSD339, LSD348 and LSD530 in the presence of *Bifidobacterium* strains RBL67, RBL68, and RBL70 added at the same time. The degree of inhibition depended on both the bifidobacterial strain and the *Listeria* strain. With *B. thermophilum* subsp. *infantis* RBL67, the level of invasion inhibition varied from 35 to 87%; the latter value was obtained for *L. monocytogenes*

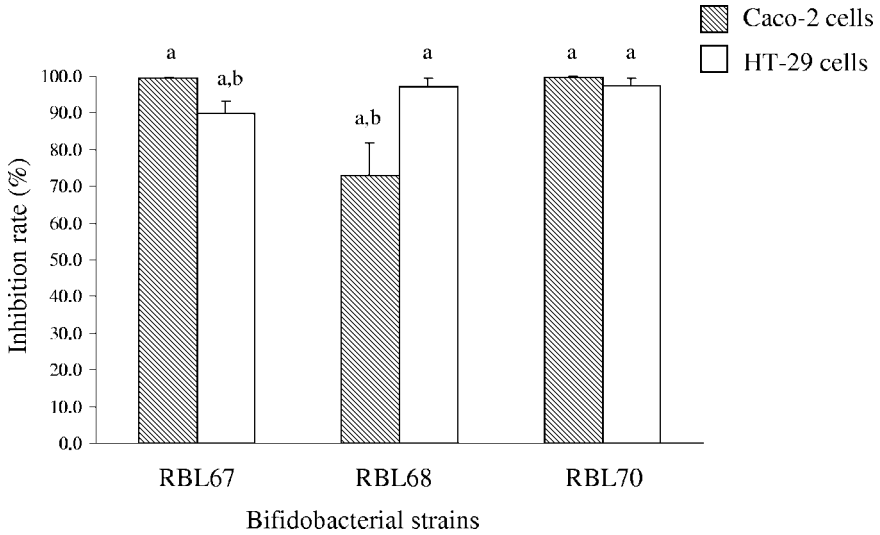

FIG. 4. Effect of 1 h of preincubation with bifidobacteria on invasion of epithelial cells by *L. monocytogenes* strain LSD348. Different letters above columns indicate that the values are significantly different ($P < 0.05$).

LSD530 on HT-29 cells. With *B. thermacidophilum* subsp. *suis* RBL68, 70% inhibition was observed for LSD530 on HT-29 cells, while LSD339 invasion was not inhibited at all for either cell line, nor was invasion of HT-29 cells by LSD348. With *B. thermacidophilum* subsp. *suis* RBL70, the level of inhibition of *L. monocytogenes* LSD339 on HT-29 cells was 91%, which was the highest level of inhibition observed, while the level of inhibition of LSD348 on Caco-2 cells was only 38%. The levels of invasion inhibition were higher on HT-29 cells with all strain combinations except RBL67 and RBL68 with LSD348. RBL70 inhibited invasion of HT-29 cells by LSD339, LSD348, and LSD530 by 91, 78, and 88%, respectively. *L. monocytogenes* LSD348, the most invasive strain tested, was more invasive than the other two strains except for Caco-2 cells in the presence of RBL68.

Figure 4 shows the effect of 1 h of prior contact between epithelial cells and bifidobacteria on invasion of *L. monocytogenes* LSD348. The efficacy of the three bifidobacteria for inhibiting invasion by *Listeria* is clear, with values ranging from 73 to 99.8%, compared to 0 to 78% when both bacteria were added simultaneously. Strain RBL68 was the least effective strain, while the two other bifidobacterial strains showed similar activities.

Finally, when the bifidobacteria were not added until 1 h after *L. monocytogenes* was added, strain RBL67 was still able to limit the invasion of the most invasive *Listeria* strain tested (Fig. 5) by as much as 67% on HT-29 cells. This was in stark contrast with the results obtained for the adhesion assay, which showed that there was no inhibition of *L. monocytogenes* adhesion when bifidobacteria were added 1 h after *Listeria* was added.

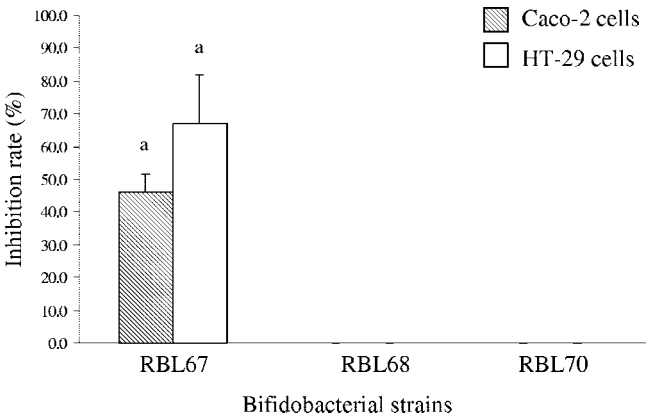

FIG. 5. Effect of contact with bifidobacteria 1 h after exposure to *L. monocytogenes* strain LSD348 on invasion of epithelial cells by the pathogen. The same letter above columns indicates that the values are not significantly different ($P > 0.05$).

DISCUSSION

In this study, we used two in vitro models to test the abilities of three bifidobacterial strains, isolated from infant feces, to adhere to intestinal cells using two epithelial cell models. The first model is based on the human colon adenocarcinoma cell line Caco-2, whose characteristics simulate structural and functional characteristics of mature enterocytes in vitro (31). The second model is based on the HT-29 cell line, which is also derived from a human colon adenocarcinoma. This cell line also has structural and functional features of mature human enterocytes, but when cultured in the presence of glucose, it maintains a constant proliferation rate with practically no further differentiation (44). Our results indicate that high percentages of *B. thermophilum* subsp. *infantis* RBL67 and *B. thermacidophilum* subsp. *suis* strain RBL68 and RBL70 cells adhered to both cell lines. The adhesion of strains RBL67 and RBL70 was found to be greater than that reported for *B. bifidum* RBL71, which we previously found to be highly adhesive (12), and *B. pseudolongum* ATCC 25526 (7), which is also considered highly adhesive. The adhesion by *B. thermacidophilum* subsp. *suis* strain RBL68 is comparable to the adhesion by *B. bifidum* RBL71. The three bifidobacterial strains tested in

the present study did not invade the intestinal epithelial cells, confirming that the genus *Bifidobacterium* is generally noninvasive. This criterion is an important safety consideration.

The ability of *L. monocytogenes* to invade intestinal cells is a good indicator of its virulence (18, 29, 32, 40). On this basis, we tested the abilities of 14 strains of *L. monocytogenes* and four other *Listeria* species isolated from food commodities in Canada to invade intestinal cell lines. Our results revealed widely different levels of adhesion and invasion among the strains tested. Such strain variability is very common and may explain the difference in virulence among *L. monocytogenes* strains. We also noted that adhesion and invasion were greater with HT-29 cells than with Caco-2 cells. This may have been due to facilitation of *L. monocytogenes* invasion by factors related to epithelial cell proliferation (44). Velge et al. (41) reported that entry of *L. monocytogenes* into Caco-2 and HT-29 cells was closely related to the proliferation process. These authors proposed that entry of *L. monocytogenes* into Caco-2 and HT-29 cells is mediated by interaction between E-cadherin on the surface of the proliferative cell and the bacterial cell surface protein "internalin." Since the proliferation rate in HT-29 cells is constant and is higher than that in Caco-2 cells (44), the interaction between *Listeria* and HT-29 cells is expected to be greater than the interaction between *Listeria* and Caco-2 cells.

As recently reviewed by Servin (37) and Servin and Coconnier (36), one of the better-documented effects of probiotic bifidobacteria on human health is the prevention of infection. As observed for many invasive enteropathogens, *L. monocytogenes* pathogenicity involves adhesion of the bacteria to intestinal epithelial cells and invasion in two distinct steps (14, 18, 36). In this study, we examined the abilities of three bifidobacterial strains to block the adhesion and invasion of three strains of *L. monocytogenes* with different invasive potentials. All three bifidobacteria failed to inhibit the adhesion of *Listeria* when the two organisms were added simultaneously. We even observed a slight increase in *Listeria* adhesion at the highest concentration of bifidobacteria used. These results differ from those of Coconnier et al. (4), who showed that there was dose-dependent inhibition of adhesion by *L. monocytogenes* in competition with *Lactobacillus acidophilus* LB on Caco-2 cells. These authors tested live and heat-killed *Lactobacillus* and observed 75% and 80% inhibition, respectively, with 1×10^9 lactobacilli/ml. Considering that our incubation time was 30 min, compared to the 60 min used by Coconnier et al. (4), the levels of adhesion of our *Bifidobacterium* strains on epithelial cells should have been lower. Coconnier et al. did not detect any differences when heat-killed lactobacilli were added before the *Listeria* strain was added, while we observed significantly enhanced inhibition of the pathogen when live bifidobacteria were used.

Inhibition of adhesion of other enteric pathogen by bifidobacteria or *Lactobacillus* has also been reported. Gagnon et al. (12) showed that a *B. bifidum* strain isolated from infant feces reduced adhesion of *E. coli* O157:H7 to Caco-2 cells in a dose-dependent manner. Other studies have also reported inhibition of adhesion of enteropathogens, such as *E. coli*, *S. enterica* serovar Typhimurium, and *Staphylococcus aureus*, in a dose-dependent manner (2, 5, 15, 25). In a very limited number of studies workers have examined interactions between bifidobacteria and *Listeria*. Our study showed the potential probiotic activities of the *B. thermophilum* subsp. *infantis* RBL67 and *B. thermacidophilum* subsp. *suis* RBL68 and RBL70 strains from newborns to reduce the adherence of *Listeria* spp., particularly *L. monocytogenes*, to both epithelial cells lines and to reduce invasion of these cells lines.

While the bifidobacterial strains tested in this study were not very effective in inhibition of the adhesion of *L. monocytogenes* when the two bacteria were added simultaneously, they were quite effective in blocking the invasion of epithelial cells. In fact, two of the three strains tested inhibited invasion by 35 to 90% depending on the mode of addition and the cell line. Interestingly, when bifidobacteria were added 1 h before *Listeria* was added, the level of inhibition increased to nearly 100% for the majority of the strain combinations. One possible explanation for the differences between the adhesion and invasion inhibition results in this study is the different contact times (30 min for adhesion assays and 90 min for invasion assays). Since *Listeria* is able to invade epithelial cells as soon as it comes into contact with them (18), we limited the incubation time to 30 min in order to minimize invasion and obtain a better estimate of inhibition of adhesion. We also tested the inhibition of adhesion with a prolonged incubation time, but no significant differences were noted. Adhesion and invasion of *L. monocytogenes* occur by separate mechanisms involving different proteins located on the bacterial cell wall (14, 17, 18). The first step in the invasion process is adhesion of the bacteria on the epithelial cell, which is modulated by fibronectins. This step is believed to be nonspecific and may occur with a wide variety of cell types and other molecules (14). The second step involves a more specific interaction of other bacterial proteins called internalins with other receptors, which results in high-affinity host-cell-specific interactions and therefore in the internalization of the bacteria in the host cells (14, 18). Considering this mechanism, the greater ability of bifidobacteria to inhibit *L. monocytogenes* invasion may suggest that there is an interaction with specific receptors on epithelial cells that are essential for *Listeria* internalins to promote entry of the pathogen into the host cells.

Fluorescent in situ hybridization performed with a cell monolayer after a competition assay for invasion showed that some of the bifidobacteria present were physically near *Listeria* cells, suggesting that competition between the two bacteria for adhesion and invasion is possible. However, further experiments are needed to determine possible interactions between the two bacteria and the impact that such interactions could have on the inhibition of listerial invasion.

In conclusion, the bifidobacterial strains used in this study poorly blocked adhesion of *L. monocytogenes* strains isolated from food samples but were able to block invasion by *L. monocytogenes*. Our results also suggest that competition between probiotic and pathogenic bacteria should be analyzed by considering adhesion and invasion two distinct events, in order to characterize the mode of action by which the probiotic is able to decrease infection. Finally, the results obtained in this study reinforce previous data that suggest that probiotics are able to prevent infections by pathogens when sufficient numbers are present in the intestinal flora. The three bifidobacterial strains used in this study should be considered probiotic strains with interesting potential for preventing enteric infections in hu-

mans. In vivo studies using animal models for *Listeria* infection are currently under way to confirm the results obtained in vitro.

ACKNOWLEDGMENTS

This research and O.M. were financially supported by the Fonds Québecois de la Recherche sur la Nature et les Technologies (FQRNT).

We thank Cecile Cinquin for her help with FISH analysis.

REFERENCES

1. **Asahara, T., K. Shimizu, K. Nomoto, T. Hamabata, A. Ozawa, and Y. Takeda.** 2004. Probiotic bifidobacteria protect mice from lethal infection with *Shiga* toxin-producing *Escherichia coli* O157:H7. Infect. Immun. **72:** 2240–2247.
2. **Bernet, M.-F., D. Brassart, J.-F. Neeser, and A. Servin.** 1993. Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions. Appl. Environ. Microbiol. **59:**4121–4128.
3. **Bunduki, M. C., C. M. Beliveau, and C. W. Donnelly.** 1993. Examination of attachment and phagocytic uptake of *Listeria* species by mammalian intestinal cells. Food Microbiol. **10:**507–516.
4. **Coconnier, M.-H., M.-F. Brenet, S. Kernéis, G. Chauvière, J. Fourniat, and A. L. Servin.** 1993. Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by *Lactobacillus acidophilus* strain LB decreases bacterial invasion. FEMS Microbiol. Lett. **110:**299–306.
5. **Coconnier, M.-H., V. Liévin, M.-F. Mernet-Camard, S. Hudault, and A. L. Servin.** 1997. Antibacterial effect of the adhering human *Lactobacillus acidophilus* strain LB. Antimicrob. Agents Chemother. **41:**1046–1052.
6. **Cossart, P., and P. J. Sansonetti.** 2004. Bacterial invasion: the paradigms of enteroinvasive pathogens. Science **304:**242–248.
7. **Crociani, J., J. P. Grill, M. Huppert, and J. Ballongue.** 1995. Adhesion of different bifidobacteria strains to human enterocyte-like Caco-2 cells and comparison with *in vivo* study. Lett. Appl. Microbiol. **21:**146–148.
8. **Daniels, J. J. D., I. B. Autrenrieth, and W. Goebel.** 2000. Interaction of *Listeria monocytogenes* with the intestinal epithelium. FEMS Microbiol. Lett. **190:**323–328.
9. **De Man, J. C., M. Rogosa, and M. E. Sharp.** 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. **23:**130–135.
10. **Doganay, M.** 2003. Listeriosis: clinical presentation. FEMS Immunol. Med. Microbiol. **35:**173–175.
11. **Felsenstein, J.** 1985. Confidence limit on phylogenies: an approach using bootstrap. Evolution **39:**783–791.
12. **Gagnon, M., E. E. Kheadr, G. Le Blay, and I. Fliss.** 2004. *In vitro* inhibition of *Escherichia coli* O157:H7 by bifidobacterial strains of human origin. Int. J. Food. Microbiol. **92:**69–78.
13. **Gaillard, J.-L., P. Berche, J. Mounier, S. Richard, and P. Sansonetti.** 1987. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infect. Immun. **55:**2822–2829.
14. **Gilot, P., P. André, and J. Content.** 1999. *Listeria monocytogenes* possesses adhesions for fibronectin. Infect. Immun. **67:**6698–6701.
15. **Gopal, P. K., J. Prasad, J. Smart, and H. S. Gill.** 2001. *In vitro* adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. Int. J. Food. Microbiol. **67:**207–216.
16. **Hamilton-Miller, J. M. T.** 2003. The role of probiotic in the treatment and prevention of *Helicobacter pylori* infection. Int. J. Antimicrob. Agents **22:** 360–366.
17. **Jacquet, C., E. Gouin, D. Jeannel, P. Cossart, and J. Rocourt.** 2002. Expression of ActA, Ami, InlB, and listeriolysin O in *Listeria monocytogenes* of human and food origin. Appl. Environ. Microbiol. **68:**616–622.
18. **Jaradat, Z. W., and A. K. Bhunia.** 2003. Adhesion, invasion, and translocation characteristics of *Listeria monocytogenes* serotype in Caco-2 cell and mouse models. Appl. Environ. Microbiol. **69:**3640–3645.
19. **Jaradat, Z. W., J. L. Wampler, and A. K. Bhunia.** 2003. A *Listeria* adhesion protein-deficient *Listeria monocytogenes* strain shows reduced adhesion primarily to intestinal cell lines. Med. Microbiol. Immunol. **192:**85–91.
20. **Kaufmann, P., A. Pfefferkorn, M. Teuber, and L. Meile.** 1997. Identification of *Bifidobacterium* species isolated from food with genus-specific 16S rRNA-targeted probes by colony hybridization and PCR. Appl. Environ. Microbiol. **63:**1268–1273.
21. **Kaur, I. P., K. Chopra, and A. Saini.** 2002. Probiotics: potential pharmaceutical applications. Eur. J. Pharm. Sci. **15:**1–9.
22. **Langendijk, P. S., F. Schut, G. J. Jansen, G. C. Raangs, G. R. Kamphuis, M. H. F. Wilkinson, and G. W. Welling.** 1995. Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl. Environ. Microbiol. **61:**3069–3075.
23. **Larpent, J.-P., N. Château, M. I. Castellanos, and J.-L. Larpent.** 1994. Les probiotiques en alimentation animale et humaine. Tec. & Doc., Lavoisier, Paris, France.
24. **Lecuit, M., S. Vandormael-Pournin, J. Lefort, M. Huerre, P. Gounon, C. Dupuy, C. Babinet, and P. Cossart.** 2001. A transgenic model of listeriosis: role of internalin in crossing the intestinal barrier. Science **292:**1722–1725.
25. **Liévin, V., I. Pieffer, S. Hubauld, F. Rochat, D. Brassart, J.-R. Neeser, and A. L. Servin.** 2000. *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity. Gut **47:**646–652.
26. **Mack, D. R., and S. Lebel.** 2003. Role of probiotic in the modulation of intestinal infection and inflammation. Curr. Opin. Gastroenterol. **20:**22–26.
27. **Mallory, A., D. Savage, F. Kern, Jr., and J. G. Smith.** 1973. Patterns of bile acids and microflora in the human small intestine. II. Microflora. Gastroenterology **64:**32–42.
28. **Mitsuoka, T.** 1990. Bifidobacteria and their role in human health. J. Ind. Microbiol. **6:**263–267.
29. **Olier, M., F. Pierre, J.-P. Lemaître, C. Divies, A. Rousset, and J. Guzzo.** 2002. Assessment of the pathogenic potential of two *Listeria monocytogenes* human faecal carriage isolates. Microbiology **148:**1855–1862.
30. **Pentecost, M., O. Glen, T. Julie, and M. Amieva.** 2006. *Listeria monocytogenes* invades the epithelial junction at sites of cell extrusion. PLOS Pathogen. **2:**29–40.
31. **Pinto, M., S. Robine-Leon, M. D. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assman, K. Haffen, J. Fogh, and A. Zweibaum.** 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol. Cell. **47:**323–330.
32. **Roche, S. M., P. Velge, E. Bottreau, C. Durier, N. Marquet-van der Mee, and P. Pardon.** 2001. Assessment of the virulence of *Listeria monocytogenes*: agreement between a plaque-forming assay with HT-29 cells and infection of immunocompetent mice. Int. J. Food Microbiol. **68:**33–44.
33. **Rochet, V., L. Rigottier-Gois, F. Béguet, and J. Doré.** 2001. Composition of human intestinal flora analysed by fluorescent *in situ* hybridization using group specific 16S rRNA-targeted oligonucleotide probes. Genet. Sel. Evol. **33:**S339–S352.
34. **Schmid, M., M. Walcher, A. Bubert, M. Wagner, M. Wagner, and K.-H. Scheifer.** 2003. Nucleic acid-based, cultivation-independent detection of *Listeria* spp. and genotypes of *L. monocytogenes*. FEMS Immunol. Med. Microbiol. **35:**215–225.
35. **Schürch, C.** 2002. Development of a novel DNA transformation system for bifidobacteria. Ph.D. thesis. Swiss Federal Institute of Technology, Zurich, Switzerland.
36. **Servin, A. L., and M.-H. Coconnier.** 2003. Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. Best Pract. Res. Clin. Gastroenterol. **17:**741–754.
37. **Servin, A. L.** 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol. Lett. **28:**405–440.
38. **Touré, R., E. Kheadr, C. Lacroix, O. Moroni, and I. Fliss.** 2003. Production of antimicrobial substances by bifidobacterial isolated from infant stool active against *Listeria monocytogenes*. J. Appl. Microbiol. **95:**1058–1069.
39. **van Langendonck, N., E. Brottreau, S. Bailly, M. Tabouret, J. Marly, P. Pardon, and P. Velge.** 1998. Tissue culture assays using Caco-2 cell line differentiate virulent from non-virulent *Listeria monocytogenes* strains. J. Appl. Microbiol. **85:**337–346.
40. **Vázquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Domínguez-Bernal, W. Goebel, B. González-Zorn, J. Wehland, and J. Kreft.** 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. **14:**584–640.
41. **Velge, P., E. Bottreau, N. Van-Langendonck, and B. Kaeffer.** 1997. Cell proliferation enhances entry of *Listeria monocytogenes* into intestinal epithelial cells by two proliferation-dependent entry pathways. J. Med. Microbiol. **46:**681–692.
42. **von Ah, U., C. Lacroix, V. Mozzetti, E. E. Kheadr, I. Fliss, and L. Meile.** Submitted for publication.
43. **Wagner, M., M. Schmid, S. Juretschko, K.-H. Trebesius, A. Bubert, W. Goebel, and K.-H. Scheifer.** 1998. *In situ* detection of a virulence factor mRNA and 16S rRNA in *Listeria monocytogenes*. FEMS Microbiol. Lett. **160:**159–168.
44. **Zweibaum, A., M. Pinto, G. Chevalier, E. Dussaulx, N. Triadou, B. Lacroix, K. Haffen, J. L. Brun, and M. Rousset.** 1985. Enterocytic differentiation of a subpopulation of the human colon tumor cell line HT-29 selected for growth in sugar-free medium and its inhibition by glucose. J. Cell Physiol. **122:**21–29.