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Impairment of Swimming Motility by Antidiarrheic *Lactobacillus acidophilus* Strain LB Retards Internalization of *Salmonella enterica* Serovar Typhimurium within Human Enterocyte-Like Cells▽

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We report that both culture and the cell-free culture supernatant (CFCS) of *Lactobacillus acidophilus* strain LB (Lactéol Boucard) have the ability (i) to delay the appearance of *Salmonella enterica* serovar Typhimurium strain SL1344-induced mobilization of F-actin and, subsequently, (ii) to retard cell entry by *S.* Typhimurium SL1344. Time-lapse imaging and Western immunoblotting showed that *S.* Typhimurium SL1344 swimming motility, as represented by cell tracks of various types, was rapidly but temporarily blocked without affecting the expression of FliC flagellar propeller protein. We show that the product(s) secreted by *L. acidophilus* LB that supports the inhibitory activity is heat stable and of low molecular weight. The product(s) caused rapid depolarization of the *S.* Typhimurium SL1344 cytoplasmic membrane without affecting bacterial viability. We identified inhibition of swimming motility as a newly discovered mechanism by which the secreted product(s) of *L. acidophilus* strain LB retards the internalization of the diarrhea-associated pathogen *S. enterica* serovar Typhimurium within cultured human enterocyte-like cells.

Probiotic strains are defined as live microorganisms which, when consumed in appropriate amounts in food, confer a health benefit on the host (14). A small number of heat-inactivated probiotic cultures have been previously shown to conserve the probiotic activities of the parental live strains (49). The antidiarrheal drug Lactéol Fort is based on a lyophilized, heat-inactivated culture of *Lactobacillus acidophilus* strain LB (Lactéol Boucard) isolated from a human intestinal microbiota. The results of two recent randomized, double-blind, placebo-controlled clinical studies have demonstrated that, in children between the ages of 3 months and 4 years, established bacterial diarrhea was resolved more rapidly in members of groups receiving Lactéol Fort treatment plus oral rehydration salts (ORS) than in those receiving ORS alone (32, 47). Our team had previously reported that both live and heat-inactivated cultures and a 2-fold-concentrated cell-free culture supernatant of *L. acidophilus* strain LB culture displayed antibiotic-like activity against enterovirulent bacteria, including *Salmonella enterica* serotype Typhimurium (6, 8, 9, 10, 31).

Salmonella is the major food-borne pathogen contributing to food-borne gastroenteritis in humans (3). *S. enterica* serotypes Typhimurium and Enteritidis have been found to be the most common serotypes associated with illness, and 34.2% and 21.9% of isolates are resistant to two and five antibiotic subclasses, respectively (2). The ability to interact with, invade, and then live intracellularly in host cells is a prerequisite for the virulence of *Salmonella* (20). For cell entry into brush border-expressing enterocytes, motility and a molecular syringe apparatus known as the type-III secretion system (T3SS) act in tandem to trigger rapid and efficient bacterial internalization. For rapid cell entry into brush border-expressing enterocytes, flagella motility triggers rapid and efficient contact between the pathogen and host cells. Each flagellum is driven by a motor at its base that uses proton motive force as its energy source (5, 21, 36). Once *Salmonella* has attached itself to the host intestinal cells, dramatic membrane remodeling is triggered in the immediate vicinity of the adhering bacteria, resulting in the localized reorganization of the actin cytoskeleton (16) triggered by a complex set of *Salmonella* effector molecules that hijack host cell signaling pathways (19, 43). *Salmonella* delivers pathogenicity island-1 (SPI-1) and SPI-2 effectors into the cytosol of the host cell via its T3SS molecular syringe apparatus, which is also composed of three distinct substructures: a multiring base, an inner rod, and a needle (18).

The molecular exploration of the mechanisms of action of the strain-specific effects of probiotic *Lactobacillus* carried out during the last 10 years has shown that particular whole-cell surface molecules or secreted proteins and metabolites are involved (30). Our group has previously reported that secreted molecules present in both live and heat-inactivated cultures of *L. acidophilus* strain LB inhibited entry of invasive human pathogens into cultured human cultured enterocyte-like Caco-2 cells *in vitro* (6–8). The mechanism underlying the antagonism of cell internalization of *Salmonella* species by probiotic *Lactobacillus* strains is unknown. In order to try to explain why products secreted by the LB strain of *L. acidophilus* antagonize the entry of *S. enterica* serotype Typhimurium strain SL1344 (*S.* Typhimurium SL1344) into cultured human enterocyte-like Caco-2 cells, we investigated whether inhibition of flagellar motility and/or T3SS-dependent cell entry played any role in the process. We also examined the characteristics of the substance(s)

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secreted by the *L. acidophilus* strain that inhibited the swimming motility and retarded cell entry of *S.* Typhimurium SL1344.

MATERIALS AND METHODS

Bacterial strains. *S.* Typhimurium SL1344 (24) was a gift from B.A.D. Stocker (Stanford University, Stanford, CA). Flagellated nonmotile TH4482 (*fliD*), TH4475 (*flgK*), and χ3421 (*mot*$^-$, *fla*$^+$) mutants were kindly provided by K. T. Hughes (University of Utah, Salt Lake City, UT) and R. Curtiss III (Arizona State University, Tempe, AZ) and mutant EE633 (SL1344 *sipA*::Tn*5lacZY*) by S. I. Miller (University of Washington, Seattle, WA) (26). An *S.* Typhimurium SL1344 *prgH*::*gfp*$^+$-derivative strain was constructed as described by Hautefort et al. (22) with the P22 lysate containing the *prgH-gfp*$^+$ reporter construct from strain JH3010 kindly provided by M.A. Jepson (University of Bristol, United Kingdom). Strains were grown overnight in Luria-Bertani broth (LB) (Difco Laboratories, Detroit, MI) at 37°C as a static culture. Before use, cultures were diluted in LB and grown for 3 h in an orbital shaker. Prior to the experiments, the exponentially growing bacteria were washed twice with LB.

Clinical *S.* Typhimurium isolates (S1, S2, S3, S6, S9, S10, S1*, S2*, S3*, S5*, S9*, and S10*) were collected from hospitalized children with community-acquired *Salmonella* infection (Pediatric Unit, Cochin-Saint-Vincent-de-Paul hospital, Paris, France) during 1995 to 1999. Community-acquired salmonellosis requiring hospitalization was defined as an illness that started <10 days before enrollment and resulted in one of the following symptoms: (i) acute diarrhea (>3 soft or liquid stools produced within the previous 24 h) with fever, vomiting, or dehydration and no possibility of ambulatory treatment; (ii) bloody-mucoid stools (dysentery) with fever; or (ii) a persistent (one or more days in duration) temperature of >38.5°C together with soft or normal stools and no identifiable cause of fever other than salmonellosis. *Salmonella* strains in stool samples were isolated and identified (API 20E; Mérieux Diagnostics, Marcy l'Etoile, France) (M. Lorrot, J. Raymond, and D. Gendrel, personal communication). A total of 71% of the clinical isolates were resistant to ampicillin, chloramphenicol, and tetracycline, and all strains were sensitive to ceftriaxone. All strains were motile, positive for *S.* Typhimurium virulence genes (*invA*, *spiA*, *mgtC*, *orfL*, *sopB*, *spvC*, *sopE*, *sodC*, *sifA*, *sifB*, *ssel*, *sspH2*, *mart*, *sugR*, *pefC*, and *IpfA*) and had the ability to invade cultured human intestinal Caco-2/TC7 cells (A.-B. Blanc-Potard, personal communication).

L. acidophilus strain LB (Lactéol Boucard [Lactéol Fort]) was provided by Axcan Pharma (Houdan, France). *L. acidophilus* strain LB was grown in De Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories) for 18 h at 37°C (*L. acidophilus* LB culture). Isolated *L. acidophilus* LB bacteria and cell-free culture supernatant (LB-CFCS) were obtained by centrifuging an 18-h culture of *L. acidophilus* LB at 10,000 × *g* for 30 min at 4°C. A heat-inactivated *L. acidophilus* strain LB culture and CFCS were obtained by heating at 100°C for 1 h. Batches of heated and lyophilized *L. acidophilus* LB culture plus their spent culture medium (Lactéol Fort pharmaceutical preparation) were provided by Axcan Pharma. To obtain an equivalent to the CFCS of *L. acidophilus* LB culture, the batches were reconstituted by dissolving 200 mg in 2.6 ml of sterile phosphate-buffered saline (PBS). After being centrifuged, all CFCSs were passed through a sterile 0.22-μm-pore-size Millex GS filter unit (Millipore, Molsheim, France) before use.

Treatment of *S.* Typhimurium SL1344. An exponentially growing culture (3 h) of *S.* Typhimurium SL1344 was used. For inhibitory experiments with heat-treated or live *L. acidophilus* LB culture, 500 μl of *S.* Typhimurium SL1344 culture was incubated for 1 h at 25°C in the presence of a live or heat-treated *L. acidophilus* LB 18-h culture and 500 μl of Dulbecco's modified Eagle's minimal essential medium (DMEM). For inhibitory experiments with *L. acidophilus* LB-CFCSs, 500 μl of the CFCS of a heat-treated or untreated *L. acidophilus* LB 18-h culture or the CFCS of reconstituted Lactéol Fort was incubated with 500 μl of *S.* Typhimurium SL1344 culture and 500 μl of DMEM for 1 h at 25°C. After centrifugation and washing with LB, untreated and treated *S.* Typhimurium SL1344 bacteria were resuspended in 500 μl of DMEM for experiments.

Swimming motility assays and quantification. The swimming motility of untreated, mutant, or treated *S.* Typhimurium SL1344 bacteria was observed under a light microscope (Leitz Aristoplan). In addition, we went on to use videomicroscopy to examine the swimming motility of untreated and treated *S.* Typhimurium SL1344. An exponentially growing culture of *Salmonella* SL1344, with or without treatment, was diluted 50-fold in buffer containing 0.0001% Brij 35 and 0.1 M glucose (Sigma-Aldrich Chimie SARL, L'Isle d'Abeau Chesnes, France) as previously described (54). About 50 μl was pressed between coverslips to form a layer about 50 μm thick, and the pressed mixture was immediately examined visually using a phase-contrast microscope. In addition, cell movements and trajectories were observed by time-lapse imaging using an inverted AxioObserver Z1_Colibri microscope (Zeiss, Germany) equipped with an AxioCam MRm charge-coupled-device (CCD) camera (6.45-μm-pixel size) and an Achroplan 10×/0.25 NO dry objective lens at 23°C (maintained by air conditioning). Images of cells were recorded at video rates (14 frames per s). Time-lapsed images were recorded with a 10-s exposure time at 10-min intervals, with the halogen light source shuttered between image acquisitions, for a total time of 3 h for all experimental conditions. The video images were then analyzed for motility data by extracting the frames by the use of the operating software for the Z1 microscope. Images were exported to ImageJ (http://rsb.info.nih.gov/ij/) for analysis.

Membrane depolarization. The cytoplasmic membrane depolarization was determined with the membrane potential-sensitive dye di$SC_3$5 (3,3-dipropylthiacarbocyanine) (Molecular Probes) (59) and *S.* Typhimurium SL1344. Bacterial cells in the mid-logarithmic phase were centrifuged, washed in LB, and resuspended in sodium phosphate (pH 7.4) at an optical density at 600 nm of 0.40. To permeabilize the outer membrane in order to allow the dye to be taken up, the bacteria were first treated with 0.2 mM EDTA (pH 8.0); di$SC_3$5 was then added to achieve a final concentration of 1.0 μM. The mix was then incubated at room temperature for 30 min in the dark. A 2-ml cell suspension was placed in a 1-cm cuvette, and aliquots (100 μl) of heat-treated *L. acidophilus* LB-CFCS or pooled fractions 25 to 27 were added for testing using Sephadex G-10 gel filtration chromatography. Changes in fluorescence due to the disruption of the membrane potential gradient ($\Delta\psi$) across the cytoplasmic membrane were recorded using a Genios spectrofluorimeter (Tecan, Trappes, France) at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

Determination of bacterial membrane injury. Membrane permeabilization was determined with a Hoechst 33258 DNA-binding probe (Invitrogen, Cergy-Pontoise, France) as previously described (5). Passage of the probe across the cell membrane, which increases its fluorescence after binding to DNA, was measured using a Genios spectrofluorimeter (Tecan, Trappes, France) at an excitation wavelength of 360 nm and an emission wavelength of 465 nm.

Isolation of flagella and Western blotting. The method described by Sojka et al. was used (50). A 20-ml aliquot of an exponential-phase culture (3 h) of *S.* Typhimurium SL1344 in DMEM (adjusted to an optical density of 0.4 at 600 nm) was exposed for 1 h at 37°C to 20 ml of *Lactobacillus* LB-CFCS or MRS broth (pH 4.5) or MRS broth containing increasing concentrations of lactic acid. Bacteria were pelleted by centrifugation at 5,000 × *g* for 5 min, resuspended in 10 ml of 500 mM Tris (pH 8.0), and then blended at high speed for 60 s in a Sorvall Omni mixer (Sorvall Products, Newton, CO). Bacteria and debris were pelleted by centrifugation at 8,000 × *g* for 15 min, and the supernatant was clarified by filtration (0.8 μm pore size). Flagella were pelleted by centrifugation at 100,000 × *g* for 1 h and then resuspended in loading buffer and boiled at 95°C for 5 min. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%). The gels were transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech). To identify FliC, the flagellar propeller protein of *S.* Typhimurium SL1344, the membranes were washed with PBS containing 0.1% Tween, blocked in Tris-buffered saline (TBS) (0.1% Tween, 3% bovine serum albumin [BSA], 0.5% gelatin), and probed with monoclonal antibody (MAb) 114/2 (which recognizes the phase-1 antigen) and with MAb 109/5/2 (which recognizes the common epitopes of *Salmonella* flagellin). Both antibodies were kindly provided by M. J. Woodward (Department of Bacterial Diseases, Veterinary Laboratories Agency, Addlestone, United Kingdom) (50). Blots were then incubated with horseradish peroxidase-linked secondary antibody (Ozyme), followed by ECL chemiluminescence detection performed according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Cell culture and infection. The TC7 clone (Caco-2/TC7) (4) established from the parental, human, enterocyte-like Caco-2 cell line was used. Cells were routinely grown in DMEM (Life Technologies, Cergy, France) (25 mM glucose), supplemented with 15% heat-inactivated (30 min, 56°C) fetal calf serum (FCS) (Life Technologies) and 1% nonessential amino acids. For maintenance purposes, cells were subjected to passage weekly using 0.02% trypsin in $Ca^{2+}Mg^{2+}$-free PBS containing 3 mM EDTA. The experiments and cell maintenance were carried out at 37°C in an atmosphere of 10% CO_2 and 90% air. The culture medium was changed daily. Fully differentiated cells were used at postconfluence after 15 days in culture (9).

Prior to infection, the Caco-2/TC7 monolayers prepared in 24-well tissue culture plates (ATGC, Marne la Vallée, France) were washed twice with PBS (9). The cells were infected with an exponentially growing culture of untreated or treated *S.* Typhimurium SL1344 in DMEM (500 μl; 5 × 10^7 CFU/well) at 37°C in an atmosphere of 10% CO_2 and 90% air for the times indicated.

Quantification of *S.* Typhimurium SL344 internalization. The internalization of *S.* Typhimurium SL1344 was measured by determining the number of bacteria located within the infected monolayers by the use of the aminoglycoside antibiotic assay (9). Infected monolayers were washed twice with sterile PBS and then incubated for 1 h in the presence of DMEM containing gentamicin (100 μg/ml) at 37°C in an atmosphere of 10% CO_2 and 90% air. Bacteria that adhered to the Caco-2/TC7 brush border were soon killed, whereas those located within the cells survived. The monolayer was washed twice with sterile PBS and then lysed with sterilized water. A bacterial colony count was performed as described above.

Imaging and quantization of *S.* Typhimurium-induced F-actin accumulation. Monolayers of Caco-2/TC7 cells were prepared on glass coverslips, which were placed in 24-well tissue culture plates. After infection, the cell preparations were fixed for 15 min at room temperature in PBS–3% paraformaldehyde. They were washed three times with PBS and then treated with 50 mM NH_4Cl for 10 min. The cells were permeabilized with 0.1% Triton X-100. Examination of F-actin was carried out by direct immunofluorescence labeling using Alexa-488-labeled phalloidin (Molecular Probes, Junction City, OR) (9). Samples were examined using a confocal laser scanning microscope (CLSM) (model LSM 510 META; Zeiss, Germany) equipped with a 488-nm, air-cooled argon ion laser and a 543-nm helium neon laser and configured with an Axiovert 100 M microscope using a Plan Apochromat 40×/1.2 numerical aperture (NA) Corr. water objective lens. To quantify the *S.* Typhimurium-induced F-actin accumulations, the projections obtained were analyzed and F-actin spots were counted using Image J software (version 1.42) (NIH). To determine the area of F-actin accumulations, the confocal microscope images were analyzed using Imaris software (version 6.21; Bitplane, Zurich, Switzerland). The identity of the images was hidden from the person quantifying the F-actin spots and assessing their morphology in order to eliminate any possible bias. For each sample, images of at least 10 randomly selected fields, each representing ~100 cells, were recorded. Photographic images were resized, organized, and labeled using Adobe Photoshop software (San Jose, CA).

RT-PCR. Total RNA of untreated *S.* Typhimurium SL1344 or *S.* Typhimurium SL1344 subjected for 1 h to MRS broth (pH 4.5) or *L. acidophilus* LB CFCS was isolated using a Qiagen RNeasy Midi kit (France Qiagen SA, Courtaboeuf, France). First-strand cDNA was generated using reverse transcription (RT) of 5 μg of total RNA and a first-strand cDNA synthesis kit (Takara Bio Europ/Clontech, Saint Germain-en-Lay, France). The primers for amplification of the cDNA fragment were as follows: for *invH*, the forward primer was 5′-ATGGC GCCTCATTCTTCT-3′ and the reverse primer was 5′-AGCCAACGGTGATA TGGC-3′ (486-bp PCR product); for *sipA*, the forward primer was 5′-ACGAA TCTTGCGGCGAAT-3′ and the reverse primer was 5′-GTAGCGTCTTCGCC TCAG-3′ (274-bp PCR product); for *sipB*, the forward primer was 5′-ACTGG CGGCAGTATGCCA-3′ and the reverse primer was 5′-TCCGTGGCCTTATC TAAG-3′ (893-bp PCR product); for *sipD*, the forward primer was 5′-ATCGT TGCCGAACGGCCG-3′ and the reverse primer was 5′-TTCCGCTTCAGTG GCTAC-3′ (648-bp PCR product); and for *sopD*, the forward primer was 5′-A CCATGCGCTGGAAGTGT-3′ and the reverse primer was 5′ CTCCTGAAG TAATTCCGG-3′ (353-bp PCR product).

Gel filtration chromatography. CFCS was applied to a Sephadex G-10 column (Pharmacia, Uppsala, Sweden) (1.5 by 30 cm) preequilibrated with Milli-Q water, which was also used as the eluent. The column was eluted at a flow rate of 0.50 ml/min using a peristaltic pump, and fractions of 2 ml were collected at 4°C. Absorbance at 280 nm in each fraction was measured, and the inhibitory activity against *S.* Typhimurium SL1344 motility was determined as described above. The concentrations of lactic acid in all fractions were evaluated enzymatically.

Statistics. Data are expressed as means ± standard deviations (SD). The statistical significance was assessed using Student's *t* test. Differences were considered significant at a P value of <0.01.

RESULTS

***L. acidophilus* strain LB culture has the ability to delay *S.* Typhimurium SL1344-induced membrane remodeling and cell entry in Caco-2/TC7 cells.** We examined the kinetics of F-actin mobilization at the brush border and cell entry of *L. acidophilus* LB culture-treated *S.* Typhimurium SL1344. It is known that, in order to enter epithelial cells, *Salmonella* surrounds itself with a large extension of the host cell membrane corresponding to the size and extent of an F-actin dense region (16). Infected cells were fixed and stained with Alexa 488-conjugated phalloidin to visualize the F-actin, and confocal laser microscopy (CLM) was used to observe and quantify the mobilization of F-actin that accompanies bacterial internalization. Since the rearrangements that occur at later times postinfection (p.i.) reflect the consequences rather than the cause of *Salmonella* internalization, F-actin accumulation and internalization levels were observed and measured over a 3-h time course p.i. In untreated, wild-type *S.* Typhimurium SL1344-infected cells, numerous dense and localized F-actin spots were observed at the cell surface (Fig. 1A). When the cells were infected with *S.* Typhimurium SL1344 in the presence of *L. acidophilus* LB culture (Fig. 1A and B), we found that the number of F-actin spots was dramatically lower than in cells infected with the untreated wild-type bacterium alone at both 5 min and 1 h p.i. In control experiments, there was no F-actin mobilization in cells incubated for 3 h in the presence of *L. acidophilus* LB culture (not shown). Since concentrated *L. acidophilus* LB-CFCS is known to kill *S.* Typhimurium SL1344 (6, 8, 9), we checked the viability of *S.* Typhimurium SL1344 under our experimental conditions. In the presence of nonconcentrated *L. acidophilus* LB culture, we found no change in *S.* Typhimurium SL1344 viability (for *S.* Typhimurium SL1344, 7.65 ± 0.32 log CFU/ml; for *S.* Typhimurium SL1344 in the presence of *L. acidophilus* LB culture, 7.24 ± 0.40 log CFU/ml). At 3 h p.i., the same numbers of F-actin spots were detected in cells infected with *S.* Typhimurium SL1344 in the presence of *L. acidophilus* LB culture as in cells infected with untreated *S.* Typhimurium SL1344 (Fig. 1A and B). However, the F-actin spots appeared to be bigger than those in cells exposed to untreated *S.* Typhimurium SL1344, and this was confirmed by measuring their sizes (Table 1). Enlarged F-actin spots that correlated with a delay in cell entry have been observed previously as a result of a mutation in the SPI-I-associated *sipA* gene (23, 27, 44, 46, 60). Here, we found that the *S.* Typhimurium SL1344 EE633 (*sipA*) mutant induced diffuse spots 4 times larger than those observed in cells infected with wild-type strain and twice the size of those observed in cells infected with *L. acidophilus* LB culture- or CFCS-treated *S.* Typhimurium SL1344 (Table 1). We next determined the numbers of cell-associated or internalized bacteria seen during a given time period in cells infected with untreated *S.* Typhimurium SL1344 or *S.* Typhimurium SL1344 plus *L. acidophilus* LB culture (Fig. 1C). We found that the decrease in *S.* Typhimurium SL1344 cell association and cell entry observed in the presence of *L. acidophilus* LB culture paralleled the failure to promote membrane remodeling. At 5 min and at 1 h p.i., no *S.* Typhimurium SL1344 had entered the cells in the presence of *L. acidophilus* LB culture; by 3 h p.i., some internalization had occurred. Overall, our results show that, in the presence of *L. acidophilus* LB culture, *S.* Typhimurium SL1344 infecting Caco-2/TC7 cells displays a markedly delayed capacity to promote F-actin mobilization at the cell membrane and to enter enterocyte-like Caco-2/TC7 cells.

We next examined whether isolated *L. acidophilus* LB bacteria suspended in DMEM and/or *L. acidophilus* LB-CFCS inhibited the development of *S.* Typhimurium SL1344-induced F-actin spots and entry into infected Caco-2/TC7 cells. As shown in Fig. 2A, the presence of *L. acidophilus* LB-CFCS as the *L. acidophilus* LB culture inhibited growth of the *S.* Typhi-

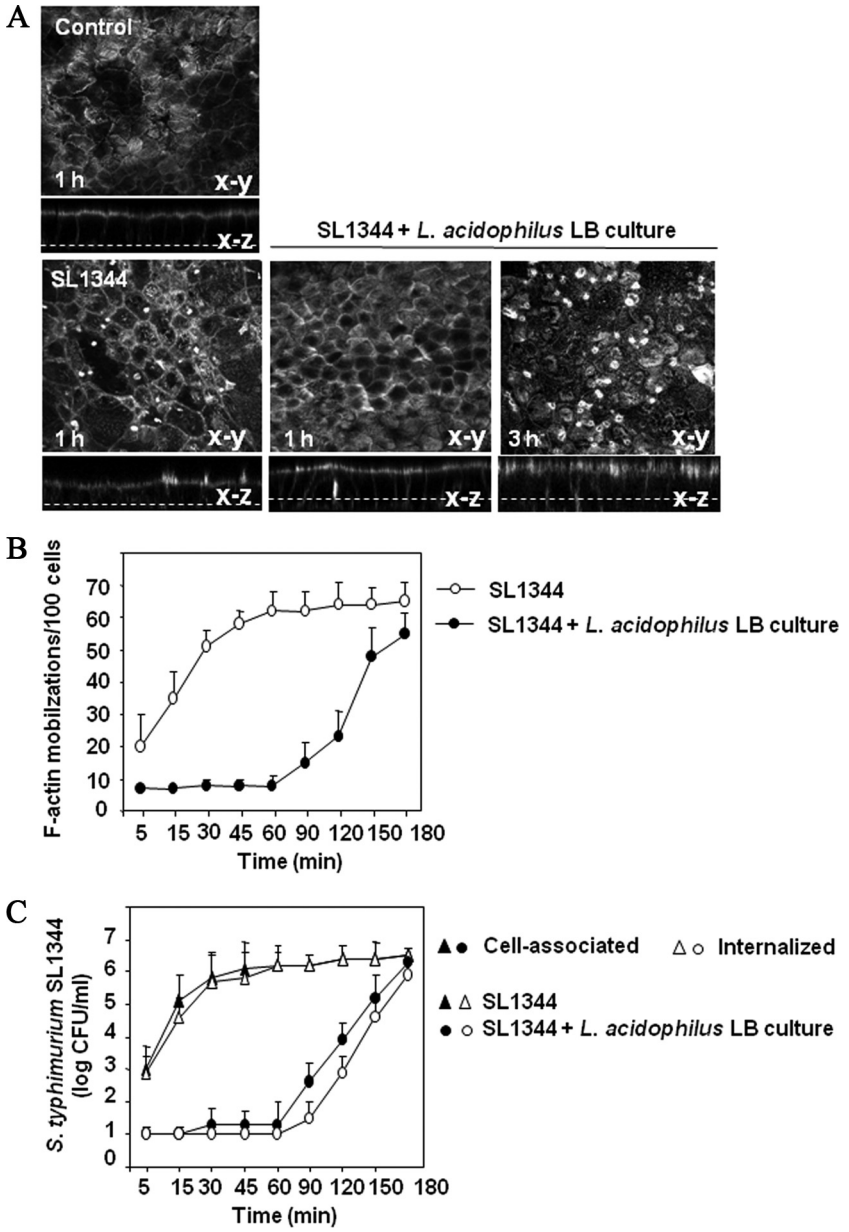

FIG. 1. *L. acidophilus* LB culture delayed *S.* Typhimurium SL1344-induced F-actin mobilization and entry into cultured human enterocyte-like Caco-2/TC7 cells. Untreated or treated wild-type *S.* Typhimurium SL1344 bacteria (5×10^7 CFU/well) were incubated in DMEM with fully differentiated Caco-2/TC7 cells without centrifugation for the indicated incubation times at 37°C in an atmosphere of 10% CO_2 and 90% air. (A) F-actin spots at the apical cell domain were monitored by direct labeling with fluorescein-labeled phalloidin and confocal laser microscopy for examination of Caco-2/TC7 cells infected with untreated or *L. acidophilus* LB culture-treated *S.* Typhimurium SL1344. (B) Quantification of F-actin during a time course of infection. (C) Cell-associated and internalized bacteria were quantified by colony counts using the aminoglycoside antibiotic assay. Images represent projections of confocal optical *x-y* section slices that show the presence or absence of apical F-actin mobilization (*x-z* reconstructions show the cell monolayer). Hatched bars represent the basolateral domain of cell monolayers. The images in panel A are representative of the results of three experiments, each performed in duplicate. Each value shown represents the mean ± SD of the results from three experiments (three successive passages of Caco-2/TC7 cells).

murium SL1344-induced F-actin spots at 5 min and 1 h p.i. but not at 3 h p.i. Measurement of the areas of the dense, localized F-actin spots revealed no differences in the sizes of the F-actin spots induced by *S.* Typhimurium SL1344 treated with *L. acidophilus* LB culture or CFCS (Table 1). In the *L. acidophilus* LB culture experiment, *L. acidophilus* LB-CFCS inhibited the cell entry of *S.* Typhimurium SL1344 at 5 min and 1 h p.i. but not at 3 h p.i. (Fig. 2B). In contrast, isolated *L. acidophilus* LB bacteria showed no inhibitory activity against the *S.* Typhimurium SL1344-induced F-actin spots and cell entry (Fig. 2A and B). In the control experiments, there were no F-actin spots seen in cells incubated for 3 h in the presence of *L. acidophilus* LB-CFCS or isolated *L. acidophilus* LB bacteria (not shown).

These findings indicate that the T3SS needle was probably

TABLE 1. Comparison of the sizes of F-actin spots induced at the brush border membrane of Caco-2/TC7 cells by wild-type, EE633 mutant, and *L. acidophilus* LB culture- or LB-CFCS-treated *S.* Typhimurium SL1344

Strain and treatment	Diam of membrane F-actin rearrangements[a] (μm)	Morphology of membrane F-actin rearrangements
SL1344	22.5 ± 2.0	Dense and localized
SL1344 + *L. acidophilus* LB culture	39.8 ± 2.3[b]	Dense and enlarged
SL1344 + *L. acidophilus* LB-CFCS	40.7 ± 1.9[b]	Dense and enlarged
EE633 (*sipA*)	92.3 + 3.9[b]	Diffuse and enlarged

[a] Each value shown represents the mean ± SD of data from three experiments (three successive passages of Caco-2/TC7 cells).
[b] $P < 0.01$ versus SL1344.

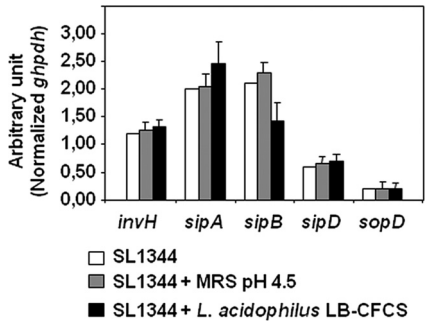

FIG. 3. *L. acidophilus* LB-CFCS-treated *S.* Typhimurium SL1344 normally expresses the T3SS and SPI-1 genes. Expression levels measured by RT-PCR of T3SS *invH* and SPI-1 *sipA*, *sipB*, *sipD*, and *sopD* genes in untreated or MRS broth (pH 4.5)-treated or *L. acidophilus* LB-CFCS-treated (1 h) *S.* Typhimurium SL1344. Each value shown represents the mean ± SD of the results from three experiments.

present and able to deliver SPI-1 effectors (19, 43) in *L. acidophilus* strain LB culture- and LB-CFCS-treated *S.* Typhimurium SL1344. We therefore went on to examine by RT-PCR whether expression of genes coding for several known structural components of the T3SS needle or SPI-1 effectors was modified in MRS broth (pH 4.5)- or *L. acidophilus* LB-CFCS-treated (1 h) *S.* Typhimurium SL1344. As shown in Fig. 3, the expression level of the *invH* gene, which codes for a component of the functional outer membrane translocation T3SS complex (11), was the same in *L. acidophilus* LB-CFCS-treated *S.* Typhimurium SL1344 as in untreated or MRS broth (pH 4.5)-treated *S.* Typhimurium SL1344. In order to confirm that the T3SS needle remained present in *L. acidophilus* LB-CFCS-treated *S.* Typhimurium SL1344, we investigated *prgH* expression by the use of the *S.* Typhimurium SL1344 *prgH*::*gfp*$^+$ strain as the test strain and found that *prgH*::*gfp*$^+$ expression was not modified in *L. acidophilus* LB-CFCS-treated *S.* Typhimurium SL1344 (not shown). When we examined the SPI-1 effector genes, we found that the expression levels of the *sipA*, *sipB*, *sipD*, and *sopD* genes were the same in *L. acidophilus* LB-CFCS-treated *S.* Typhimurium SL1344 as in untreated or MRS broth (pH 4.5)-treated *S.* Typhimurium SL1344 (Fig. 3).

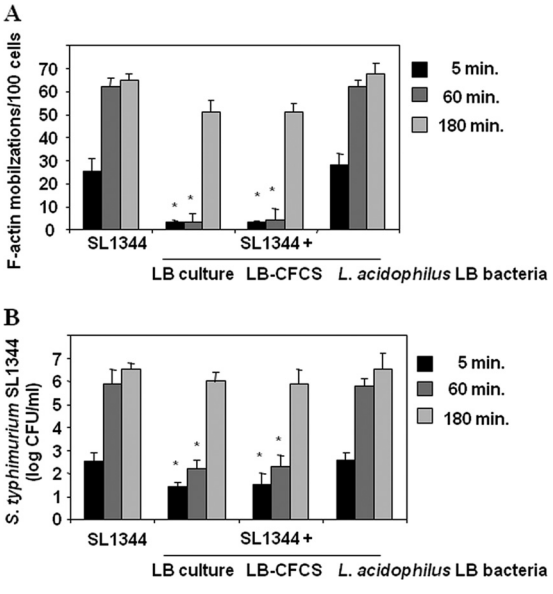

FIG. 2. Time course of *S.* Typhimurium SL1344-induced F-actin spot development and *S.* Typhimurium SL1344 cell entry in polarized Caco-2/TC7 cells infected with untreated or *L. acidophilus* LB-CFCS- or isolated *L. acidophilus* LB bacterium-treated *S.* Typhimurium SL1344. (A) Inhibition and time-dependent restoration of the development of apical F-actin spots. (B) Inhibition and time-dependent restoration of cell entry. Each value shown represents the mean ± SD of the results from three experiments (three successive passages of Caco-2/TC7 cells). *, $P < 0.01$ versus nontreated *S.* Typhimurium SL1344.

A product(s) secreted by *L. acidophilus* strain LB impaired *S.* Typhimurium SL1344 swimming motility without affecting flagellum expression. We investigated the swimming motility of *S.* Typhimurium SL1344 treated with *L. acidophilus* strain LB culture or LB-CFCS or isolated *L. acidophilus* LB bacteria. As shown in Fig. 4A, ~98% of untreated *S.* Typhimurium SL1344 cells remained highly motile throughout the experiment. In contrast, *S.* Typhimurium SL1344 treated with *L. acidophilus* LB culture or LB-CFCS, and then replaced in DMEM cell culture medium, showed a transient inhibition of motility compared with untreated *S.* Typhimurium SL1344. Interestingly, isolated *L. acidophilus* LB bacteria did not inhibit *S.* Typhimurium SL1344 motility (Fig. 4A). Importantly, it was noted that the temporary decrease in motility paralleled the delayed cell entry of *L. acidophilus* strain LB-treated *S.* Typhimurium SL1344 reported above (Fig. 1B and C).

Flagella were isolated, and Western blot analysis showed that flagellar propeller FliC protein expression in *S.* Typhimurium SL1344 treated for 1 h with *L. acidophilus* LB culture or LB-CFCS was the same as that seen with untreated *S.* Typhimurium SL1344 (Fig. 4B). These findings collectively showed that, when *S.* Typhimurium SL1344 was exposed to *L. acidophilus* LB culture or LB-CFCS, the swimming motility of the pathogen was transiently inhibited without any change in flagellar propeller expression.

The distribution of the different types of swim cell tracks of untreated *S.* Typhimurium SL1344 and *S.* Typhimurium SL1344 subjected to *L. acidophilus* LB-CFCS was examined by time-lapse videomicroscopy. As described by Turner et al. (54, 55), untreated *S.* Typhimurium SL1344 cells moved

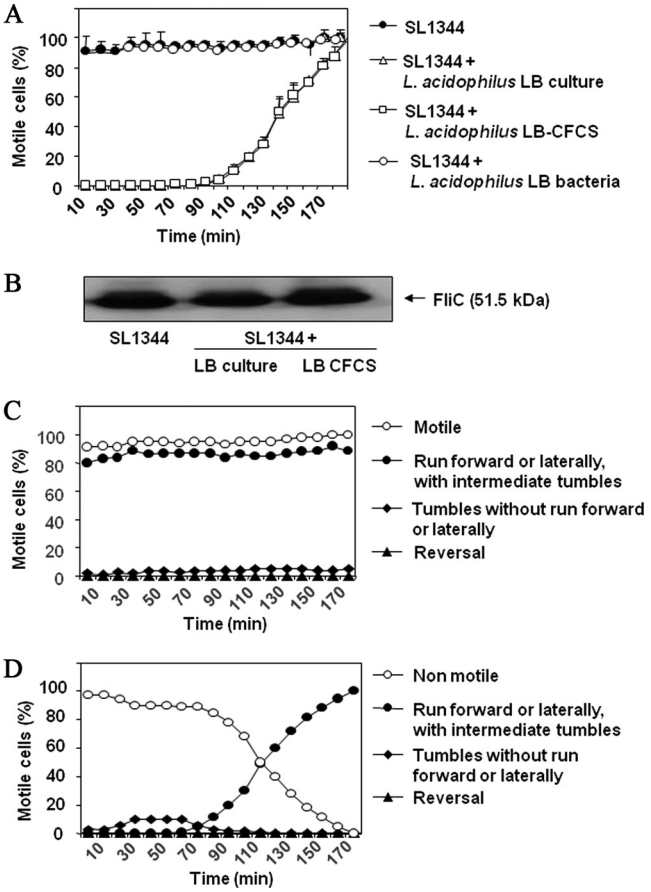

FIG. 4. Treatment with *L. acidophilus* LB culture or LB-CFCS transiently impaired swimming motility of *S.* Typhimurium SL1344 without affecting expression of FliC, the flagellar propeller protein. FliC protein was revealed by Western blotting using MAbs 114/2 and 109/5/2. (A) Inhibition and time-dependent restoration of swimming motility of *S.* Typhimurium SL1344 left untreated or treated for 1 h with *L. acidophilus* LB culture or LB-CFCS or isolated LB bacteria. Swimming motility was observed and quantified by light microscopy as described in Materials and Methods. (B) Western blot analysis revealing the presence of FliC, the flagellar protein, in untreated and *L. acidophilus* LB culture- or LB-CFCS-treated *S.* Typhimurium SL1344. (C) Cell tracks of untreated *S.* Typhimurium SL1344 over a given time course. (D) Inhibition and time-dependent restoration of cell tracks of *S.* Typhimurium SL1344 exposed to *L. acidophilus* LB-CFCS. Cell tracks were analyzed and quantified by time-lapse imaging using an AxioObserver Z1_Colibri inverted microscope as described in Materials and Methods. In panels A, C, and D, each value shown represents the mean of the results of three experiments (SD not shown). In panel B, the immunoblot shown is representative of the results of two experiments.

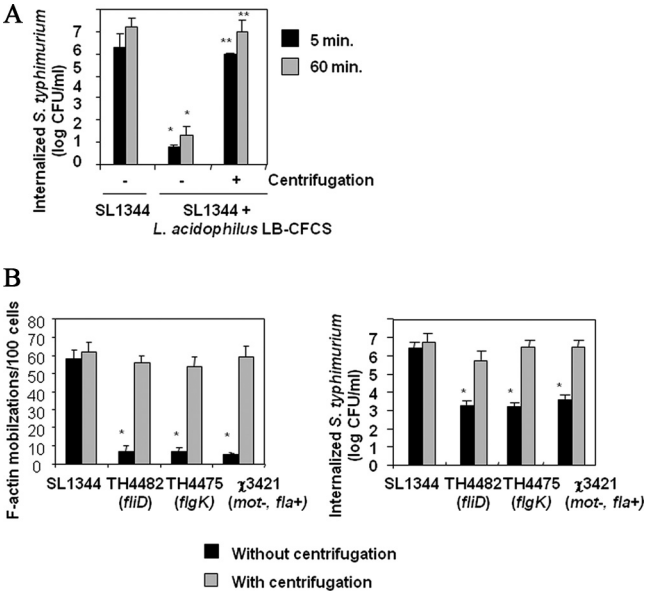

FIG. 5. Mild centrifugation restored the capacity of *L. acidophilus* LB-CFCS-treated *S.* Typhimurium SL1344 to induce F-actin mobilization and enter into Caco-2/TC7 cells. (A) Cell entry of untreated *S.* Typhimurium SL1344 and *L. acidophilus* LB-CFCS-treated *S.* Typhimurium SL1344 at 5 min and 60 min after infection, with or without the application of mild centrifugal force (500 × *g* for 10 min). *, $P < 0.01$ versus *S.* Typhimurium SL1344 without centrifugation; **, $P < 0.01$ versus treated *S.* Typhimurium SL1344 without centrifugation. (B) Quantification of F-actin spots and bacterial internalization in cells infected for 60 min with flagellated nonmotile *S.* Typhimurium mutants with or without mild centrifugation. *, $P < 0.01$ versus *S.* Typhimurium SL1344 without centrifugation. Each value shown represents the mean ± SD of the results from three experiments (three successive passages of Caco-2/TC7 cells).

in spurts by arresting their runs for relatively short periods during which the cells moved erratically on the spot with little net displacement (tumbling) and afterward ran forward (run) or sideways (laterally) (Fig. 4C). After treatment with *L. acidophilus* LB-CFCS, the videomicroscopy results showed that *S.* Typhimurium SL1344 cells were immobile for the first hour and that their swimming motility was subsequently restored (Fig. 4D). The first movement observed was tumbling, without any forward or lateral runs. This was followed by normal runs in either a forward or a lateral direction, with intermediate tumbling also observed in untreated *S.* Typhimurium SL1344.

Mild centrifugal force reverses the impairment of cell entry of *L. acidophilus* LB culture-treated *S.* Typhimurium SL1344. It had previously been reported that mild centrifugal force, which accelerates the contact between bacteria and cultured cells, reverses the impairment of the invasion of flagellated, nonmotile *Salmonella* strains but not that of T3SS-deficient strains (13, 28). When mild centrifugal force was applied, the *L. acidophilus* LB-CFCS-treated *S.* Typhimurium SL1344 showed the same cell entry capacity as untreated *S.* Typhimurium SL1344 without centrifugation (Fig. 5A). We conducted a control experiment using flagellated nonmotile TH4482 (*fliD*), TH4475 (*flgK*), and χ3421 (*mot*$^-$, *fla*$^+$) *S.* Typhimurium mutants. As expected, we observed that (i) flagellated nonmotile mutants displayed a delayed capacity to promote F-actin mobilization and enter Caco-2/TC7 cells and (ii) mild centrifugation promoted the manifestation of F-actin spots and internalization of the mutants (Fig. 5B).

Characteristics of the *L. acidophilus* LB-induced inhibition of *S.* Typhimurium SL1344 swimming motility. We conducted a set of experiments intended to analyze the characteristics of the inhibitory activity of *L. acidophilus* strain LB against *S.* Typhimurium SL1344 swimming motility. We investigated whether nonfermented MRS broth at pH 4.5 would affect the swimming motility of *S.* Typhimurium SL1344 and found that

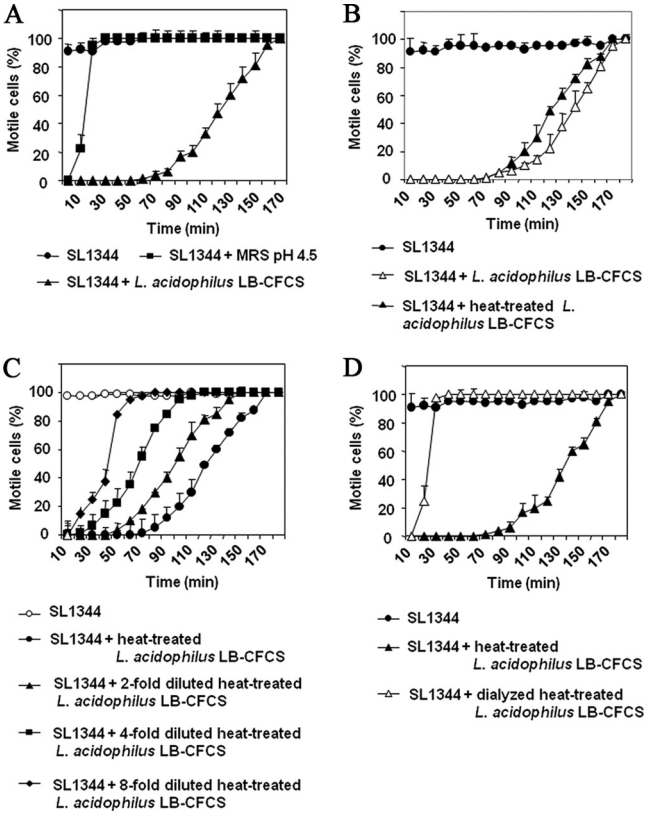

FIG. 6. Characteristics of the *L. acidophilus* LB-CFCS inhibitory activity against *S.* Typhimurium SL1344 swimming motility. (A) Inhibition and time-dependent restoration of *S.* Typhimurium SL1344 swimming motility treated with MRS broth at pH 4.5 compared with *L. acidophilus* LB-CFCS treatment. (B) Inhibition and time-dependent restoration of swimming motility of *S.* Typhimurium SL1344 treated with nonheated or heated *L. acidophilus* LB-CFCSs. (C) Inhibition and time-dependent restoration of *S.* Typhimurium SL1344 swimming motility as a function of concentrations of heated *L. acidophilus* LB-CFCS. (D) Disappearance of the inhibition of *S.* Typhimurium SL1344 swimming motility treated with dialyzed (cutoff, 1,000 Da) heat-treated *L. acidophilus* LB-CFCS. Each value shown represents the mean ± SD of the results of three experiments.

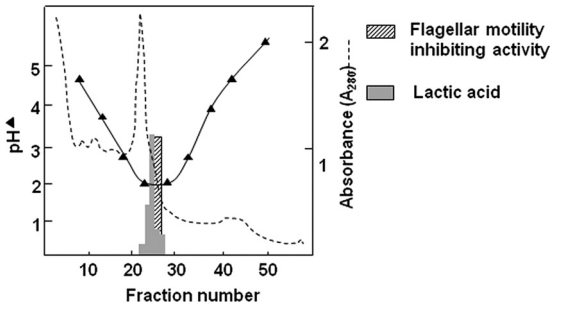

FIG. 7. Separation of molecules present in *L. acidophilus* LB-CFCS and inhibitory activity of the fractions against *S.* Typhimurium SL1344 swimming motility. Results of fractionation of *L. acidophilus* LB-CFCS by the use of gel chromatography and a Sephadex G10 column are shown. The eluate was deionized water. Absorbance at 280 nm, pH, lactic acid levels, and inhibitory activity against *S.* Typhimurium SL1344 swimming motility were determined. Each value shown represents the mean ± SD of the results from three experiments. *, $P < 0.01$ versus *S.* Typhimurium SL1344. An elution profile representative of the results of three experiments is shown.

swimming motility was transiently impaired for a short time posttreatment but with a kinetic pattern differing from that of *L. acidophilus* LB-CFCS (Fig. 6A). Heat-treated *L. acidophilus* LB-CFCS displayed inhibitory activity against *S.* Typhimurium SL1344 swimming motility that was not significantly different from that of the inhibitory activity of nonheated *L. acidophilus* LB-CFCS (Fig. 6B). Inhibition of *S.* Typhimurium SL1344 swimming motility by *L. acidophilus* LB-CFCS developed in a concentration-dependent manner (Fig. 6C). The inhibitory activity of *L. acidophilus* LB-CFCS disappeared after dialysis (cutoff, 1,000 Da) (Fig. 6D). The chromatographic profile of the antagonist activity of *L. acidophilus* LB-CFCS was investigated using a Sephadex G-10 column, which fractionated molecules with a molecular mass of less than 700 Da. A typical elution pattern is presented in Fig. 7. Antagonist activity against *S.* Typhimurium SL1344 swimming motility corresponded to fractions 25 to 27. The lactic acid elution profile corresponded to fractions 22 to 27 and peaked in fraction 25. Collectively, these results showed that the transient inhibitory activity of *L. acidophilus* strain LB against *S.* Typhimurium SL1344 swimming motility was implemented by heat-stable, low-molecular-mass, secreted molecule(s).

The antidiarrheal drug Lactéol Fort has been used in clinical studies (32, 47) and contains a lyophilized heat-inactivated culture of *L. acidophilus* strain LB. We checked whether the CFCS of a Lactéol Fort pharmaceutical preparation displayed the properties reported above for *L. acidophilus* LB culture and nonheated or heated *L. acidophilus* LB-CFCSs. Results presented in Fig. 8 show that *S.* Typhimurium SL1344 treated with heat-treated *L. acidophilus* LB-CFCS displayed transient inhibition of swimming motility (Fig. 8A), F-actin mobilization (Fig. 8B), and cell entry (Fig. 8C). We found no detectable difference between the inhibitory effects of CFCS of reconstituted Lactéol Fort and those of heat-treated *L. acidophilus* LB-CFCS.

We examined whether clinical *S.* Typhimurium isolates were sensitive to heat-treated LB-CFCS and CFCS of reconstituted Lactéol Fort. To do this, 12 clinical *S.* Typhimurium strains isolated from hospitalized children with community-acquired *Salmonella* infection were left untreated or treated for 1 h with heat-treated LB-CFCS or CFCS of reconstituted Lactéol Fort. As shown in Table 2, treated clinical *S.* Typhimurium isolates showed transient inhibition of swimming motility as observed above for *S.* Typhimurium SL1344.

A product(s) present in *L. acidophilus* LB-CFCS depolarized the cytoplasmic membrane of *S.* Typhimurium S1344. The flagellar motor is a membrane-embedded nanomachine powered by the electrochemical potential difference across the cytoplasmic membrane (39). Paralyzed flagella of *Escherichia coli* and *Salmonella* have been observed as a result of a collapse of the transmembrane potential (40, 54, 57). A deenergizing effect of *L. acidophilus* LB products on the membrane potential can be postulated. To test this hypothesis, we conducted an assay to measure the disruption of an electrical potential gradient using untreated and *L. acidophilus* LB-CFCS-treated *S.* Typhimurium SL1344 and the membrane potential-sensitive dye DiSC3(5) (59). This probe is a caged cation that quenches its own fluorescence when it is inside the bacterium. When the

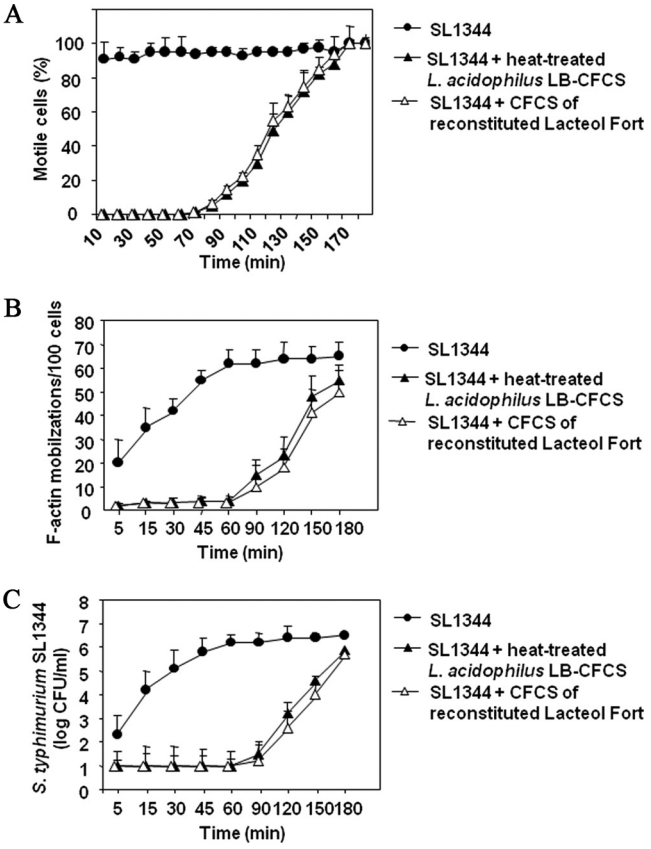

FIG. 8. Inhibitory activity of CFCS of reconstituted Lactéol Fort pharmaceutical preparation against swimming motility and cell entry of *S.* Typhimurium SL1344. (A) Inhibition and time-dependent restoration of *S.* Typhimurium SL1344 swimming motility. (B) Inhibition and time-dependent restoration of *S.* Typhimurium SL1344-induced F-actin mobilization. (C) Inhibition and time-dependent restoration of *S.* Typhimurium SL1344 cell entry. Each value shown represents the mean ± SD of the results of three experiments.

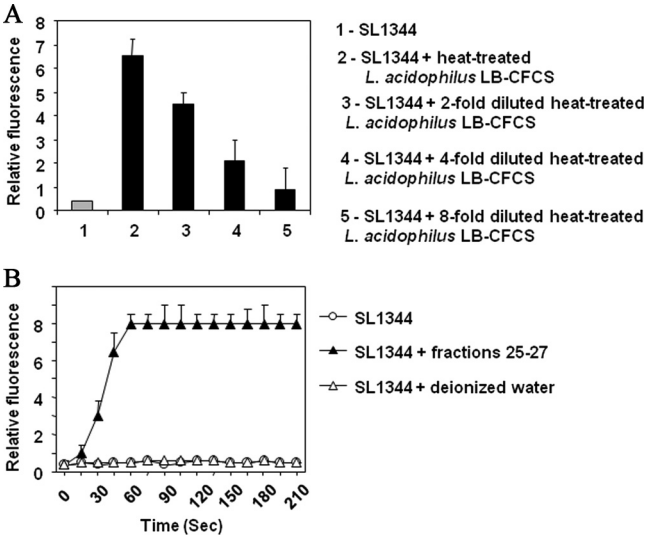

FIG. 9. Depolarization of the *S.* Typhimurium SL1344 membrane by *L. acidophilus* LB-CFCS. The release of DiSC3(5) dye from untreated and treated prelabeled DiSC3(5)-*S.* Typhimurium SL1344 was measured with a spectrophotometer. (A) Concentration-dependent release of DiSC3(5) from prelabeled DiSC3(5)-*S.* Typhimurium SL1344 in the presence of heat-treated *L. acidophilus* LB-CFCS. (B) Time-dependent release of DiSC3(5) from prelabeled DiSC3(5)-*S.* Typhimurium SL1344 in the presence of an aliquot of pooled fractions 25 to 27 obtained by gel chromatography (Sephadex G10) of *L. acidophilus* LB-CFCS. Each value shown represents the mean ± SD of the results from three experiments.

bacterial membrane is affected by antibacterial compounds (41, 59), the membrane potential is dissipated and the level of fluorescent probe released into the medium can be measured by fluorescent spectrophotometry. As shown in Fig. 9A, when decreasing concentrations of heat-treated *L. acidophilus* LB-CFCS were added to prelabeled DiSC3(5)-*S.* Typhimurium SL1344, a concentration-dependent release of fluorescence was observed. When an aliquot of pooled fractions 25 to 27 from the Sephadex G-10 column-separated compounds present in the *L. acidophilus* LB-CFCS was added to prelabeled DiSC3(5)-*S.* Typhimurium SL1344, a time-dependent increase in the release of fluorescence into the medium was observed (Fig. 9B). Alakomi et al. (1) have reported that lactic acid acts as an outer membrane-disintegrating agent. The observation that lactic acid was present in fractions 25 to 27, which displayed antagonistic activity, prompted us to investigate whether lactic acid underpins the observed inhibitory activity of *L. acidophilus* LB culture and LB-CFCS. To do this, *S.* Typhimurium SL1344 bacteria were treated with MRS broth containing increasing concentrations (5 to 200 mM) of lactic acid. Loss of the electrochemical gradient, in turn causing loss of swimming motility, can result from membrane destabilization. We checked whether the membrane of *S.* Typhimurium SL1344 was altered after exposure for 1 h to *L. acidophilus* LB-CFCS or MRS broth containing increasing concentrations of lactic acid. Membrane permeabilization measured using a Hoechst 33258 fluorescent DNA-binding probe, small amounts of which are able to pass through intact membranes, showed that passage was increased 2-fold in *L. acidophilus* LB-CFCS-treated *S.* Typhimurium SL1344 compared with untreated *S.* Typhimurium SL1344 results (Fig. 10A). As with the results reported by Alakomi et al. (1), we found that MRS broth containing lactic acid displays concentration-dependent membrane permeabilization in *S.* Typhimurium SL1344 (Fig. 10A). We investigated whether MRS broth containing lactic acid affects the swimming motility of *S.* Typhimurium SL1344 in a concentration-dependent manner. We observed a concentration-dependent decrease of *S.* Typhimurium SL1344 swimming motility in the

TABLE 2. Effect of LB-CFCS and CFCS of reconstituted Lactéol Fort on swimming motility of clinical *S.* Typhimurium isolates

Strain category	% motile cells at indicated time p.i.[a] (min)			
	30	60	120	180
Untreated strains	96 ± 3	98 ± 2	97 ± 3	98 ± 2
Heat-treated LB-CFCS-treated strains	2.5 ± 0.6[b]	2.8 ± 0.7[b]	25 ± 4[b]	95 ± 4
CFCS of reconstituted Lactéol Fort-treated strains	3.1 ± 0.4[b]	2.9 ± 0.5[b]	23 ± 5[b]	97 ± 4

[a] Each value shown represents the mean ± SD of data from three experiments.
[b] $P < 0.01$ versus untreated strains.

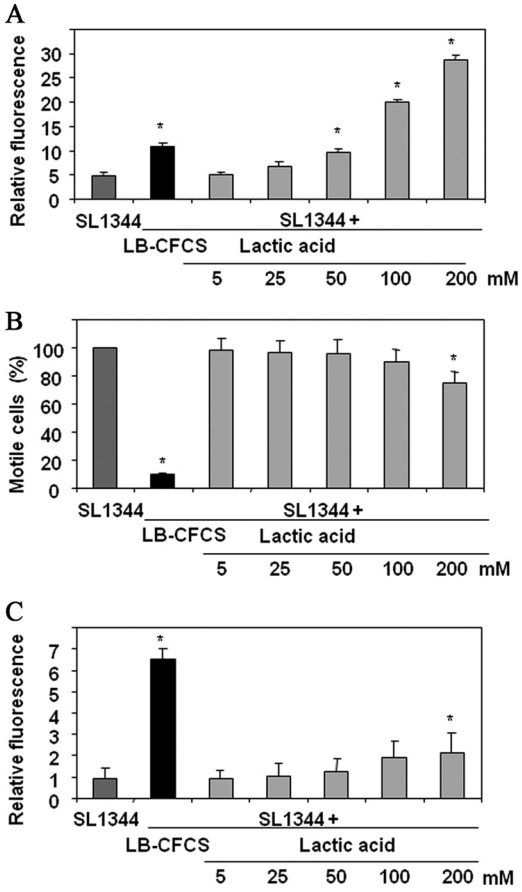

FIG. 10. Concentration-dependent membrane permeabilization, loss of swimming motility, and depolarization of membrane by lactic acid in *S.* Typhimurium SL1344. Exponential-phase *S.* Typhimurium SL1344 was treated for 1 h with MRS broth containing increasing concentrations of lactic acid (5 to 200 mM). (A) Membrane permeabilization measured using Hoechst 33258 fluorescent DNA-binding probe. (B) Swimming motility. (C) Release of DiSC3(5) from prelabeled DiSC3(5)-*S.* Typhimurium SL1344. Each value shown represents the mean ± SD of the results from three experiments. *, $P < 0.01$ versus *S.* Typhimurium SL1344.

presence of MRS broth containing lactic acid (Fig. 10B), although the effect was reduced compared to that seen in the presence of *L. acidophilus* LB-CFCS or CFCS of Lactéol Fort pharmaceutical preparation as reported above. Prelabeled DiSC3(5)-*S.* Typhimurium SL1344 showed a low level of membrane depolarization in the presence of MRS broth containing lactic acid compared with LB-CFCS treatment (Fig. 10C). The antidiarrheal drug Lactéol Fort includes a lyophilized heat-inactivated culture of *L. acidophilus* strain LB that contains lactic acid, and a heated and acidified, but not a nonheated, lactic acid solution destabilized membranes and promoted potassium leakage in stationary-phase *S.* Typhimurium (38). Experiments to determine whether or not the heated (110°C) MRS broth containing lactic acid modified the swimming of exponential-phase *S.* Typhimurium SL1344 showed that treatment with heated lactic acid at 50 or 100 mM did not modify *S.* Typhimurium SL1344 motility whereas treatment with a concentration of 200 mM resulted in a 2-fold increase in inhibitory activity compared with nonheated lactic acid results (not shown).

DISCUSSION

The data reported here show for the first time that *S.* Typhimurium SL1344 treated with probiotic *L. acidophilus* LB culture or LB-CFCS displays a transient loss of swimming motility. As with a previous observation of the presence of flagella in *L. acidophilus* LB culture- and LB-CFCS-treated *S.* Typhimurium SL1344 (8), we found that *L. acidophilus* LB culture- and LB-CFCS-treated *S.* Typhimurium SL1344 exhibited unchanged levels of expression of FliC flagellar propeller protein. Transient impairment of swimming motility meant that the treated *S.* Typhimurium SL1344 bacteria exhibited a delayed capacity to induce F-actin membrane remodeling and thus to enter cultured human enterocyte-like Caco-2/TC7 cells. This was confirmed by the observation that mild centrifugation applied at the beginning of the cell infection, which accelerated the contact between nonmotile *L. acidophilus* LB culture-treated *S.* Typhimurium SL1344 bacteria and Caco-2/TC7 cells, restored normal F-actin mobilization and cell entry. Observation of the presence of F-actin spots and the unchanged levels of expression of T3SS-related genes *prgH* and *invA* and of SPI-1 effector genes *sipA*, *sipB*, *sipD*, and *sopD* in *L. acidophilus* LB-CFCS-treated *S.* Typhimurium SL1344 indicated that the T3SS remained structurally and functionally present in treated *S.* Typhimurium SL1344. Our results agreed with the previously observed flagellum-dependent cell entry of *Salmonella* spp. Indeed, both aflagellate and flagellated, nonmotile *S.* Typhimurium mutant strains displayed a reduced capacity to enter both nonintestinal epithelial cells and undifferentiated or fully differentiated intestinal cells (12, 28, 29, 35, 56, 58). Moreover, similar flagellum motility-dependent cell entry has also been reported for Δ*fliC* mutants of *E. coli* K1 (42), an atypical enteropathogenic *E. coli* strain (48), and uropathogenic *E. coli* (45). In addition, previous reports have shown that *S.* Typhimurium with mutations affecting the flagellar motor has an impaired capacity to interact with and invade epithelial cells (34, 35, 56). Similarly, a Δ*motAB* mutant of a uropathogenic strain of *E. coli* failed to enter murine collecting-duct mpkCCD$_{cl4}$ cells (45).

Molecule(s) secreted by *L. acidophilus* strain LB may impair the swimming motility of the *Salmonella enterica* serovar Typhimurium flagellum by a variety of mechanisms. Consistent with a report indicating that the presence of lipopolysaccharide (LPS) and *Salmonella* motility are linked (53), it has previously been observed that the presence of anti-LPS Sal4 monoclonal immunoglobulin A antibody prevents *S.* Typhimurium from entering polarized Madin-Darby canine kidney (MDCK) cells (37) by paralyzing the flagellum (17). Our group has previously reported that 2-fold-concentrated *L. acidophilus* LB-CFCS induced membrane damage before killing *S.* Typhimurium SL1344. It does not appear that inhibition of the swimming motility of *S.* Typhimurium SL1344 induced by the secreted product(s) of *L. acidophilus* strain LB resulted from an alteration in the membrane of bacteria, since we found here that *S.* Typhimurium SL1344 exposed to nonconcentrated *L. acidophilus* LB-CFCS for 1 h showed no decrease in viability and no release of LPS (not shown). Altering the membrane potential

of pathogens prevents the flagella from rotating, thus rendering the bacteria nonmotile, as has been previously reported for *E. coli*, *Salmonella*, and *Helicobacter pylori* (40, 51, 54, 57). In agreement with those reports, we demonstrated that the product(s) present in *L. acidophilus* LB-CFCS has a deenergizing effect on *S.* Typhimurium SL1344. A few *Lactobacillus*-secreted antibacterial factors other than bacteriocins have previously been isolated and identified (30). The secreted product(s) of *L. acidophilus* strain LB, which underpins the inhibitory effect on the swimming motility-dependent cell entry of *S.* Typhimurium SL1344, is of low molecular weight and heat stable. Separation of compounds present in *L. acidophilus* LB-CFCS by Sephadex G-10 chromatography revealed that lactic acid was present in fractions showing inhibitory activity against *S.* Typhimurium SL1344 swimming motility. It has been previously reported that lactic acid acts as an outer membrane-disintegrating agent (1). Examining whether lactic acid influences the electrical potential gradient and impairs the swimming motility of *S.* Typhimurium SL1344, we observed membrane depolarization at high concentrations that paralleled the loss of swimming motility in treated *S.* Typhimurium SL1344. However, both effects were reduced compared to those observed with *L. acidophilus* LB-CFCS and developed at concentrations higher than the concentration of lactic acid present in the CFCS. These results suggested that a low-molecular-weight and heat-stable compound(s) other than lactic acid had dissipated the membrane potential gradient and swimming motility of *S.* Typhimurium SL1344. However, the possibility cannot be excluded that lactic acid produced by strain *L. acidophilus* strain LB permeabilizes the membrane of *S.* Typhimurium SL1344 and cooperates with the secreted low-molecular-weight and heat-stable compound(s) present in *L. acidophilus* LB-CFCS, contributing to the loss of flagella motility. Such cooperation between lactic acid and antibacterial compounds of lactic acid strains has been previously reported for probiotic *Lactobacillus* strains (49).

What is the impact of *L. acidophilus* strain LB-induced, transient inhibition of swimming motility and delayed cell entry on *Salmonella* virulence *in vivo*? The possession of active flagella appears to be an important factor in enabling *Salmonella* to penetrate the gastrointestinal mucus layer and the cells lining the epithelium (29). Furthermore, an aflagellate *S.* Typhimurium mutant (*fliGHI*) displayed a reduced capacity to approach the intestinal epithelium (52). *S.* Typhimurium treated with the Sal4 antibody, a potent inhibitor of flagellum-based motility, remained noninvasive in infected mice (17). Our group previously reported that (i) in conventional mice orally infected with *S.* Typhimurium and treated with CFCSs of live probiotic *Lactobacillus*, the levels of translocated *Salmonella* were found to be dramatically lower than in untreated mice (15) and that (ii) in pathogen-free mice orally infected with *S.* Typhimurium, the establishment of *L. rhamnosus* strain GG significantly decreased the translocation rate of *Salmonella* (25). It is possible that inhibition of *Salmonella* swimming motility had the effect of delaying the translocation of the pathogen across the intestinal barrier, thus exposing the pathogen for longer durations to intestinal host defenses in the lumen compartment, including host cells and microbiota-secreted antimicrobial substances (33).

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