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Antibacterial Effect of the Adhering Human *Lactobacillus acidophilus* Strain LB

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The spent culture supernatant of the human *Lactobacillus acidophilus* strain LB produces an antibacterial activity against a wide range of gram-negative and gram-positive pathogens. It decreased the in vitro viability of *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Shigella flexneri*, *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. In contrast, it did not inhibit lactobacilli and bifidobacteria. The activity was heat stable and relatively sensitive to enzymatic treatments and developed under acidic conditions. The antimicrobial activity was independent of lactic acid production. Activity against *S. typhimurium* SL1344 infecting human cultured intestinal Caco-2 cells was observed as it was in the conventional C3H/He/oujco mouse model with *S. typhimurium* C5 infection and oral treatment with the LB spent culture supernatant.

Laboratory and clinical studies lend supporting evidence that lactobacilli play a protective role against urogenital infections (46, 47). Lactobacilli have been recognized as an important component of the normal urovaginal flora (45). Urovaginal lactobacillus strains adhere to uroepithelial (7) and vaginal (1) epithelial cells. Previous observations of Reid and coworkers (7, 8) concerning the interference activity of urovaginal lactobacilli support the hypothesis that the blockage of the attachment of enteropathogens could result from steric hindrance caused by the adhesion of lactobacilli. Laboratory studies suggest a protective role for lactobacilli against urinary tract infections and bacterial vaginosis, since antimicrobial substances (35) and H_2O_2 (15, 29, 30, 36) produced by lactobacilli inhibit the growth of pathogens. Moreover, intravaginal instillation of lactobacilli in animal models and human studies may be useful therapeutically (6, 45).

Lactobacilli are also components of the normal intestinal flora of healthy humans (33). Selected *Lactobacillus* strains isolated from human stools adhere to cultured human enterocyte-like Caco-2 cells (3, 10, 12, 14), although the mechanism of adhesion remains controversial (19). Recent work in our laboratory has demonstrated that some of them have the capability of inhibiting the adhesion of enterovirulent bacteria to cultured human intestinal cells (3, 9, 11, 22) and consequently blocking the invasion of the cells by pathogens which have the capacity to enter the cells (3, 11, 22). *Lactobacillus casei* GG, which adheres to the cultured human intestinal cells (14), is effective in shortening the course of acute diarrhea in children (24, 25, 27). *L. casei* GG produces an antimicrobial substance (50). A recent report shows the antimicrobial effect of *Lactobacillus acidophilus* LA1 supernatant against the peptic ulcer-associated pathogen *Helicobacter pylori*, both in vitro and in human volunteers (37). Altogether, these results suggest that secretory components could be involved in the antimicrobial activity of lactobacilli. The aim of this report was to examine whether the inhibitory activity of the adhering human *L. acidophilus* strain LB (10) against the process of pathogenicity of enterovirulent bacteria (9, 11) is the consequence of the production of secreted antimicrobial substances.

MATERIALS AND METHODS

Bacteria. *Salmonella typhimurium* SL1344 (16) was a gift of B. A. D. Stocker (Stanford, Calif.), *S. typhimurium* C5 (40) was provided by M. Y. Popoff (Institut Pasteur, Paris, France), *Listeria monocytogenes* EGD (Hly^+) and *Enterobacter* and *Klebsiella* spp. were provided by J. L. Gaillard (Faculté Necker-Enfants Malades, Paris, France), *Escherichia coli* C1845 was a gift of S. Bilge (University of Washington, Seattle), and *Shigella flexneri* was provided by P. Sansonetti (Institut Pasteur). *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus* strain D, and *Pseudomonas aeruginosa* were stock clinical isolates from the microbiological laboratory of the Faculté de Pharmacie Paris XI, Châtenay-Malabry, France. *L. acidophilus* LA1 and bifidobacterium strains *Bifidobacterium infantis* 1, *B. breve* 4, and B28 were from the Nestec culture collection (Lausanne, Switzerland).

L. acidophilus LB was an isolate from a human stool collected by the Lacteol Laboratory, Houdan, France. LB bacteria were grown in MRS broth (Biokar, Pantin, France) for 18 h at 37°C (10). A pH ranging from 4 to 4.5 was observed for different LB spent culture supernatant (LB-SCS) productions. The pH of tested LB-SCS was adjusted to 4.5 with HCl for all experiments. Concentrated suspensions (2.5- and 5-fold concentrate, pH 4.5) of the LB-SCS were obtained by freeze-drying.

Antimicrobial testing. The radial-diffusion assay (32) was used to determine the antimicrobial activity in the LB-SCS. *S. typhimurium* SL1344 was grown for 18 h at 37°C in Luria broth. To obtain mid-logarithmic-phase organisms, 10 ml of fresh Trypticase soy broth (TSB) was inoculated with 200 μl of cultured Luria broth and incubated for an additional 3 h at 37°C. The bacteria were pelleted by centrifugation at 5,500 × *g* for 5 min at 4°C, washed once with phosphate-buffered saline (PBS), and resuspended in PBS. *S. typhimurium* organisms were counted, and a volume containing 1×10^6 or 5×10^6 CFU/ml was added to 10 ml of autoclaved, warm (42°C) 10 mM sodium phosphate buffer (NAPB) containing 3 mg of powdered TSB medium, 1% (wt/vol) agarose, and Tween 20 at a final concentration of 0.02% (vol/vol). After rapid dispersion with a laboratory vortex mixer, the agar was poured into a square petri dish to form a uniform layer approximately 1 mm deep. A 3-mm-diameter gel punch was used to make nine evenly spaced wells per dish. After the addition of 5 μl of test material per well, the plates were incubated for 3 h at 37°C and then overlayed with 10 ml of sterile agar (6% [wt/vol] TSB and 1% [wt/vol] agarose). After incubation for 18 to 24 h at 37°C, the diameter of the clear zone surrounding each well was measured. The diameter of the clearing was expressed in units (0.1 mm = 1 U) and was calculated after subtracting the diameter of the central well (3 mm = 30 U).

Time-kill studies. Bacteria were grown overnight for 18 h at 37°C in Luria broth (*S. typhimurium*, *Enterobacter*, *Shigella*, and *Klebsiella*), colonization factor antigen (CFA) broth (*E. coli*), or TSB (*Listeria*, *S. aureus*, *B. subtilis*, *Streptococcus* strain D, and *P. aeruginosa*). Bacterial culture was centrifuged at 5,500 × *g* for 5 min at 4°C. The culture medium was discarded, and the bacteria were washed once with PBS and resuspended in PBS. Colony count assays were performed by incubating approximately 10^8 CFU of bacteria per ml with or without the concentrated LB-SCS at 37°C in appropriate culture medium with test material at 37°C. Initially and at predetermined intervals, aliquots were removed, serially

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diluted, and plated on tryptic soy agar (TSA) to determine bacterial colony counts.

Sensitivity to heat and proteolytic and LDH enzymes. *L. acidophilus* LB was grown in MRS broth for 24 h. The culture supernatant was obtained by centrifugation at 5,500 × *g* for 5 min at 4°C. The LB-SCS obtained was heated at 110°C for 1 h. To test the sensitivity to protease, the LB-SCS was incubated at 37°C for 1 h with and without pronase (200 μg/ml), trypsin (200 μg/ml), proteinase K (100 μg/ml), or pepsin (200 μg/ml). To determine if the lactic acid in the LB-SCS participates in the LB-SCS antimicrobial activity, the LB-SCS was subjected to lactate dehydrogenase (LDH) treatment (250 μg/ml, 2 h at 37°C). The remaining activity against *S. typhimurium* SL1344 in both treated samples was determined by the radial-diffusion assay (32).

Gel filtration. For gel filtration, LB-SCS powder obtained by freeze-drying was used. Extraction of the antimicrobial component(s) was conducted as reported by Pulusani et al. (42). The powdery residue was dispersed in cold reagent-grade methanol at a ratio of 1:3 (wt/vol) and stirred for 2 h at 4 to 10°C. After centrifugation (10 min at 5,500 × *g*), the supernatant was collected. The residue was extracted two more times with cold methanol. The three methanol extracts were pooled and concentrated in vacuo, which yielded a few milliliters of yellow residue. The concentrated methanol extract was further extracted with cold acetone (4 to 10°C) (1:1, vol/vol). The extraction protocol with acetone was the same as that with methanol. The pooled acetone extracts were concentrated under reduced pressure. The product was termed methanol-acetone extract. This methanol-acetone extract (700 μl) was applied to a BioGel P2 column (exclusion, 100 to 1,800 Da; 1.5 by 30 cm; Bio-Rad Laboratories, Richmond, Calif.) pre-equilibrated with HEPES-citrate-Tris (20, 40, and 60 mM, respectively) buffer (pH 4.5). The column was eluted with this buffer at a flow rate of 0.28 ml/min, and 1-ml fractions were collected. The absorbance at 280 nm in each sample was determined, and the activity against *S. typhimurium* SL1344 was determined by the radial-diffusion assay (32). Moreover, the concentration of lactic acid was enzymatically determined in all fractions.

Determination of lactic acid. For determining the lactic acid concentration in the LB-SCS and in the BioGel column fractions, a commercial kit for the determination of D- and L-lactic acid was used (Test-Combination D-lactic acid/L-lactic acid UV-method; Boehringer Mannheim GmbH, Mannheim, Germany).

Caco-2 cell culture. Human colon adenocarcinoma Caco-2 cells (Jorgen Fogh, Sloan Kettering Memorial Cancer Center, Rye, N.Y.) (17), which model the mature enterocytes of the small intestine (41), from passages 60 to 90 were used. Cells were routinely grown in Dulbecco modified Eagle's minimal essential medium (25 mM glucose) (ATGC, Paris, France), supplemented with 20% heat-inactivated (30 min, 56°C) fetal calf serum (Life Technology, Paris, France) and 1% nonessential amino acids (ATCG) as previously described (10). Caco-2 culture cells were used at postconfluence after 15 days of culture (differentiated cells) for cell association and cell invasion assays using *S. typhimurium*.

Infection of Caco-2 cells by *S. typhimurium*. *S. typhimurium* SL1344 was cultured at 37°C for 18 h in Luria broth. The cell infection assay was conducted as previously reported (11). Briefly, prior to infection, the Caco-2 monolayers were washed twice with PBS. *S. typhimurium* SL1344 organisms were suspended in the culture medium, and a total of 1 ml (10^8 CFU/ml) of this suspension was added to each well of the tissue culture plate. The plates were incubated for 60 min at 37°C in 10% CO_2–90% air and then were washed three times with sterile PBS.

In order to determine the cell-associated *S. typhimurium* (extracellular plus intracellular bacteria), the infected cell monolayers were lysed by adding H_2O. Appropriate dilutions were plated on TSA to determine the number of viable cell-associated bacteria by bacterial colony counts.

S. typhimurium internalization was determined by quantitative determination of bacteria located within the infected monolayers by the aminoglycoside antibiotic assay. After incubation, monolayers were washed twice with sterile PBS and afterwards incubated 60 min in a medium containing gentamicin (50 μg/ml). Bacteria that adhere to the Caco-2 brush border were rapidly killed, whereas those located within Caco-2 cells were not. The monolayer was washed with PBS and lysed with sterilized H_2O. Appropriate dilutions were plated on TSA to determine the number of viable cell-associated bacteria by bacterial colony counts. The numbers of extracellular *S. typhimurium* were evaluated by subtracting the number of intracellular bacteria from the number of cell-associated bacteria. Each assay was conducted in triplicate with three successive passages of Caco-2 cells.

Inhibition assays of *S. typhimurium* cell association and cell invasion. The inhibition of *S. typhimurium* SL1344 cell association or invasion by antibiotics or LB-SCS was determined by preincubating the pathogen (10^8 CFU/ml) with antibiotics in PBS or concentrated LB-SCS for 1 h at 37°C. After centrifugation (5,500 × *g*, 10 min at 4°C), the bacteria were washed with PBS and resuspended in the Caco-2 cell culture medium. Contact between the Caco-2 cells and the treated *S. typhimurium* and determination of the numbers of viable extracellular and intracellular *S. typhimurium* were conducted as described under Infection of Caco-2 cells by *S. typhimurium* above.

The activity of antibiotics or LB-SCS against intracellular *S. typhimurium* was determined by using preinfected Caco-2 cells. Differentiated Caco-2 cells were infected by *S. typhimurium* SL1344 (10^8 CFU/ml, 1 h). After two washings of the cells with PBS, the extracellular bacteria were killed by gentamicin (50 μg/ml, 1 h at 37°C) and the infected cells were washed with PBS to remove the killed bacteria. PBS, MRS, antibiotics, or LB-SCS was added apically, and the cells were incubated for 1 h at 37°C. Determination of the viable intracellular *S. typhimurium* was conducted as described under Infection of Caco-2 cells by *S. typhimurium* above.

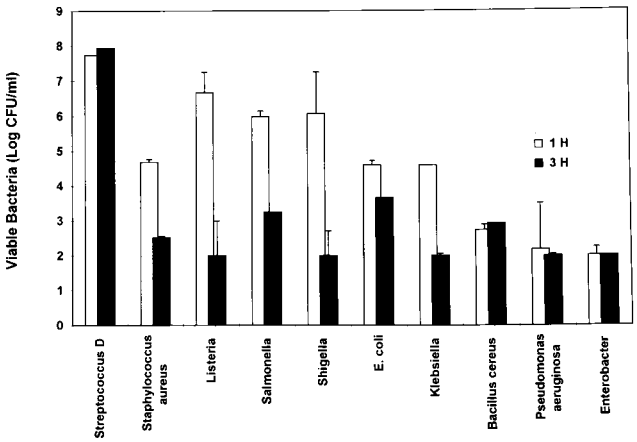

FIG. 1. Effects of LB-SCS on the viabilities of gram-positive and gram-negative bacteria, measured after 1 and 3 h of contact. For each pathogen, the inoculum size was 10^8 CFU/ml. The inoculum remained stable after 1 and 3 h of culture. LB-SCS was used at a 2.5-fold concentration. Experimental conditions are described in the text. Each value shown is the mean ± standard error of the mean (error bar) from three experiments. Statistical analysis was performed with a Student test.

Infection of mice. *S. typhimurium* C5 was grown in Luria broth for 18 h at 37°C. The culture was harvested in PBS. Viable bacteria were counted after suitable dilutions were plated on TSA and incubated at 37°C for 18 h. Adult female mice (7 to 8 weeks old; conventional C3H/He/oujco; Iffa Credo) were housed and fed in accordance with the relevant national legislation. Two groups of mice (six mice per group) were infected orally with a fixed concentration of *S. typhimurium* C5 (0.2 ml, 10^8 CFU/mouse). The control group received 0.2 ml of (fivefold) concentrated MRS per os daily. The LB-SCS-treated group received 0.2 ml of (fivefold) concentrated LB-SCS per os daily. Feces were collected individually 1, 4, and 7 days postinfection, weighed, and dispersed in PBS. The numbers of viable *S. typhimurium* were determined after serial dilution and plating on TSA to determine bacterial colony counts. Black colonies of *S. typhimurium* were easily distinguishable from other normal resident enterobacteria. Results were expressed as the mean ± standard error of the mean of viable bacteria (log CFU/gram of feces).

Transmission Electron Microscopy (TEM). *S. typhimurium* SL1344 inoculum (10^8 CFU/ml) was subjected to LB-SCS treatment for 2 h at 37°C. After centrifugation (5,500 × *g*, 10 min at 4°C), the bacteria were washed with PBS. After negative staining with phosphotungstic acid (2% [wt/vol] in H_2O), the specimens were examined with a Philips model E-320 transmission electron microscope at 60 kV.

RESULTS

LB-SCS-sensitive microorganisms. The viability of a variety of gram-positive and gram-negative microorganisms subjected to the LB-SCS (2.5-fold concentrate, pH 4.5) was examined after 1 and 3 h of contact (Fig. 1). *Streptococcus* strain D was not affected by LB-SCS. In contrast, *S. aureus* and *L. monocytogenes* were affected after 3 h of contact. All the gram-negative bacteria examined were sensitive to LB-SCS. *Salmonella*, *Shigella*, *E. coli*, and *Klebsiella* were affected after 3 h of contact, whereas the viabilities of *Bacillus cereus*, *P. aeruginosa*, and *Enterobacter* were rapidly altered after 1 h of contact. No LB-SCS antibacterial activity against bacteria of the normal human flora such as *L. acidophilus* LA1 and the bifidobacterium strains *B. breve* 4, *B. infantis* 1, and B28 was observed (not shown).

A comparison of the LB-SCS antibacterial activity with that of antibiotics was carried out (Fig. 2). For this purpose, *S. typhimurium* SL1344 was used as an indicator. A preliminary selection of antibiotics to which *S. typhimurium* SL1344 was sensitive was conducted by the radial-diffusion method using

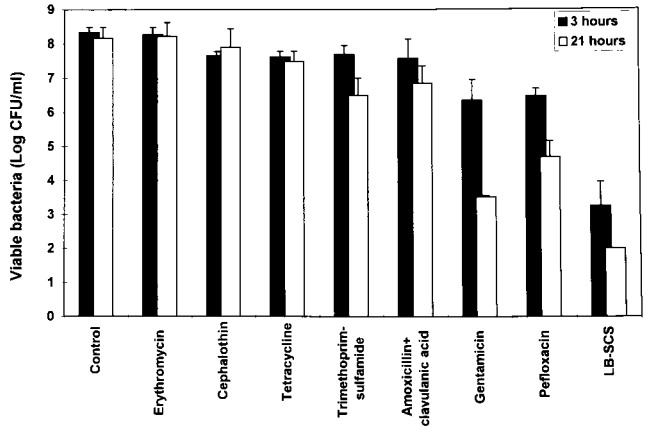

FIG. 2. Effects of antibiotics on the viability of *S. typhimurium* SL1344, measured after 3 and 21 h of contact. The values for the antibiotics are shown in comparison with those for the LB-SCS. The following compounds were used: erythromycin (15 μg/ml); gentamicin (15 μg/ml); cephalothin (30 μg/ml); tetracycline (30 μg/ml); pefloxacin (5 μg/ml); trimethoprim-sulfamide (1.25 and 23.75 μg/ml, respectively); amoxicillin-clavulanic acid (20 and 10 μg/ml, respectively), and LB-SCS (2.5-fold concentrate). The inoculum size of each pathogen was 10^8 CFU/ml. Experimental conditions are described in the text. Each value shown is the mean ± standard error of the mean (error bar) from three experiments. Statistical analysis was performed with a Student test.

preloaded antibiotic disks (not shown). The viability of *S. typhimurium* SL1344, subjected to one of these antibiotics or to LB-SCS for 3 or 21 h, was examined. Erythromycin (15 μg/ml), to which *S. typhimurium* SL1344 was insensitive, was used as a negative control. The viability of *S. typhimurium* SL1344 was insignificantly altered when subjected to treatment with cephalothin (30 μg/ml) or tetracycline (30 μg/ml). A significant decrease in viability was observed after treatment with trimethoprim-sulfonamide (1.25 and 23.75 μg/ml, respectively) or amoxicillin-clavulanic acid (20 and 10 μg/ml, respectively). A highly significant decrease in viability occurred when *S. typhimurium* SL1344 was subjected to treatment with LB-SCS, gentamicin (15 μg/ml) or pefloxacin (5 μg/ml).

Characteristics of LB-SCS antibacterial activity. The production of antibacterial activity during the *L. acidophilus* LB time course in culture was examined. As shown in Fig. 3, activity appeared after 12 h in culture and remained stable

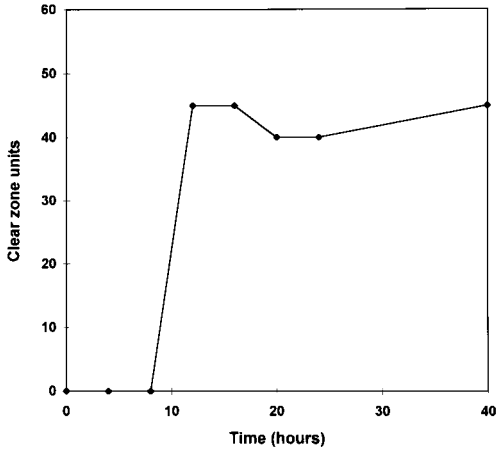

FIG. 3. Production of antibacterial activity against *S. typhimurium* SL1344 during the time course in the LB strain culture. Experimental conditions are described in the text. Each value shown is the mean from two experiments.

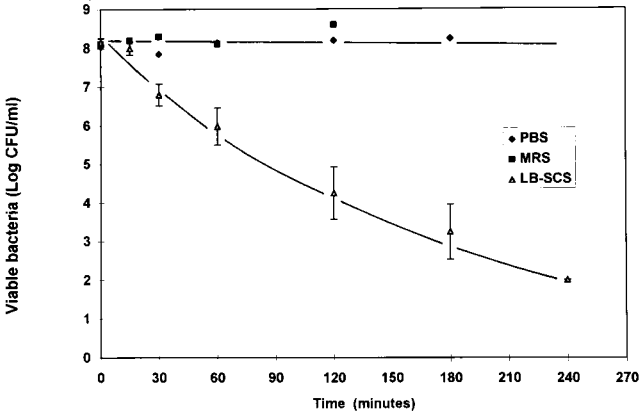

FIG. 4. Evaluation of the bactericidal activity of LB-SCS on the viability of *S. typhimurium* SL1344 as a function of time. Experimental conditions are described in the text. Each value shown is the mean ± standard error of the mean (error bar) from three experiments.

afterwards. Lactic acid concentrations evolved during the time course in culture and were 53 mM at 8 h, 113 mM at 12 and 16 h, and 150 mM at 20, 24, and 40 h. Moreover, the pH determined during the time course ranged between 4 and 4.5.

The characteristics of the antibacterial activity of the LB-SCS were examined, and *S. typhimurium* SL1344 was chosen as an indicator. The antibacterial activity of the LB-SCS (2.5-fold concentrate, pH 4.5) was examined as a function of time (Fig. 4). A rapid decrease in viability, i.e., 2 logs of decrease after 1 h of contact, was observed. After 4 h of contact, a dramatic decrease (6 logs) in viability was obtained. The controls PBS and MRS (2.5-fold concentrate) adjusted to pH 4.5 showed no activity.

Physical and chemical characteristics of the antibacterial activity of the LB-SCS (fivefold concentrate, pH 4.5) (Fig. 5) were examined by the ultrasensitive assay for endogenous antimicrobial polypeptides developed by Lehrer et al. (25). Fresh MRS at pH 4.5 showed no activity. After heating for 1 h at 100°C, the LB-SCS activity was unchanged, indicating that the

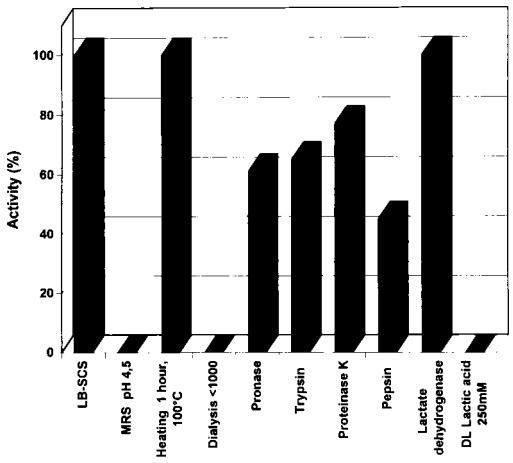

FIG. 5. Effects of physical or chemical treatments on the antimicrobial activity of LB-SCS against *S. typhimurium* SL1344. The activities are shown in comparison with that of an untreated LB-SCS (100%). Experimental conditions are described in the text. Each value shown is the mean from three experiments. The variation in reproducibility was less than 5%. Dialysis <1000, dialysis with a molecular mass cutoff of 1,000 Da.

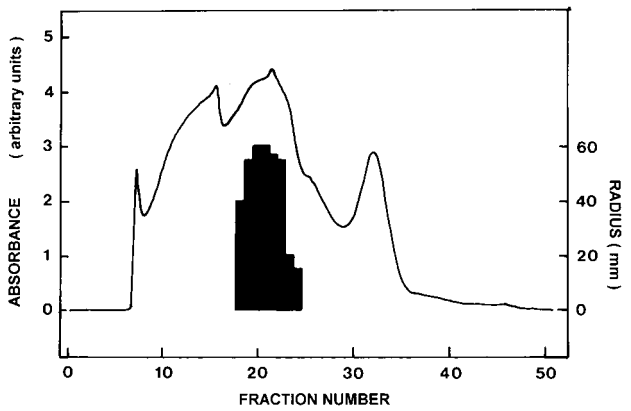

FIG. 6. BioGel P2 chromatography of the methanol-acetone extract of freeze-dried LB-SCS. Experimental conditions are given in Materials and Methods. The line shows the absorbance at 280 nm. The antimicrobial activity of the eluate against *S. typhimurium* SL1344 was determined by the radial-diffusion assay (23), and the radius of the clear zone was measured. The eluate showed a single peak of antimicrobial activity in fractions 18 to 25.

antibacterial activity produced by *L. acidophilus* LB was supported by a heat-stable component(s). After dialysis (molecular mass cutoff, 1,000 Da), the activity disappeared. The activity decreased slightly after enzymatic treatments with pronase, trypsin, and proteinase K (20 to 40% decrease of activity), whereas pepsin treatment decreased the activity twofold. A decrease in activity was observed when the LB-SCS was exposed to an alkaline environment, indicating that the component(s) supporting the antimicrobial activity was acidic or required an acidic environment to optimally develop its activity.

The LB strain produced L- and D-lactic acid in its SCS. The activity of lactic acid towards *S. typhimurium* SL1344 was examined (Fig. 5). DL-Lactic acid at a concentration of 250 mM (pH 4.5), which was higher than the lactic acid concentration produced by the LB strain, showed no activity. Moreover, when subjected to LDH treatment, the LB-SCS activity remained unchanged. Since pyruvic acid formed from lactic acid by LDH treatment could have antimicrobial activity, we examined this possibility and found that pyruvic acid (250 mM, pH 4.5) had no activity. These results demonstrated that lactic acid did not participate in the antibacterial activity of the LB-SCS. Moreover, since hydrogen peroxide-producing lactobacilli, inhabitants of the normal vaginal flora, have been shown to develop antagonistic activity toward pathogens (15, 29, 30, 36), we examined the LB strain for hydrogen peroxide production and found it to be a nonproducing strain (not shown).

LB-SCS powder obtained by freeze-drying and subjected to methanol-acetone extraction showed that the antibacterial activity was extractable, as was the antibacterial activity of *L. casei* GG (50). The LB-SCS methanol-acetone extract was loaded on a BioGel P2 column (exclusion, 100 to 1,800 Da), and the column was eluted with a HEPES-citrate-Tris (20, 40, and 60 mM, respectively) buffer. The elution profile, shown in Fig. 6, indicated that the antimicrobial activity was eluted in fractions 18 to 23 (pH 5.3). Lactic acid is present in these fractions in a range of concentrations between 100 and 150 mM. It should be noted that at these concentrations, lactic acid was inactive against *S. typhimurium* SL1344, since a concentration of 250 mM showed no activity (Fig. 5).

TEM studies. To further characterize the bactericidal effect of the LB-SCS, we used TEM to examine *S. typhimurium* SL1344 treated with LB-SCS (Fig. 7). We found that bacterial cells exposed to LB-SCS (2.5-fold concentrate) for 1 h at 37°C showed an altered cell membrane morphology but no effect on the flagellae.

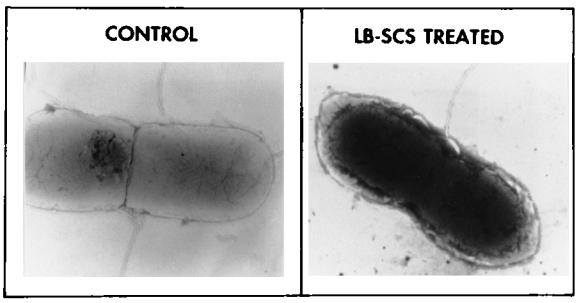

FIG. 7. Transmission electron micrographs of *S. typhimurium* SL1344 subjected to LB-SCS treatment. (A) Control; (B) *S. typhimurium* incubated for 1 h at 37°C with 2.5-fold concentrated LB-SCS. Experimental conditions are described in the text. The micrographs are representative of three separate experiments.

Inhibitory activity of LB-SCS against *S. typhimurium* SL1344 infection of cultured human intestinal Caco-2 cells. The activity of LB-SCS (2.5-fold concentrate) inhibiting *S. typhimurium* cell association and the entry of the pathogen within the Caco-2 cells was examined and compared with the activities of various antibiotics (Fig. 8). Pretreatment of the *S. typhimurium* SL1344 with the LB-SCS for 1 h at 37°C led to a significant decrease in *S. typhimurium* cell association, whereas the cell invasion was dramatically decreased. It was noticed that the proportion of extracellular *S. typhimurium* increased when the pathogen was pretreated with the LB-SCS, compared with the proportion of extracellular bacteria in the control, untreated pathogen. This suggests that the LB-SCS affects the cell entry of *S. typhimurium*, which is its major mechanism of pathogenicity. Pretreatment of *S. typhimurium* SL1344 with cephalothin (60 μg/ml), tetracycline (60 μg/ml), or amoxicillin-clavulanic acid (40 and 20 μg/ml, respectively) had no effect on *S. typhimurium* cell association. In contrast, a significant decrease

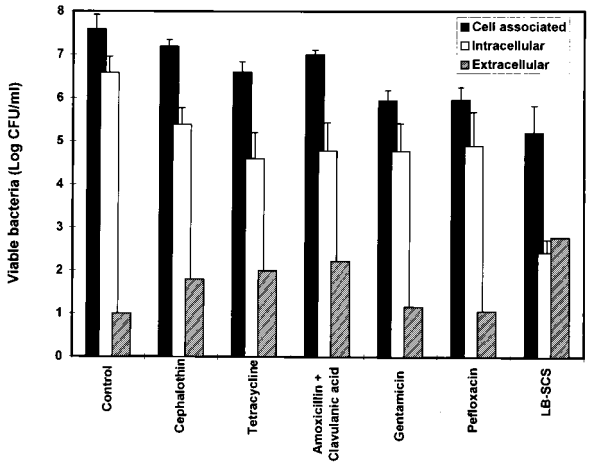

FIG. 8. Effects of the precontact of antibiotics or LB-SCS on the capacity of *S. typhimurium* SL1344 to adhere to and invade cultured human enterocyte-like Caco-2 cells. The following compounds were used: gentamicin (30 μg/ml), tetracycline (60 μg/ml), cephalothin (60 μg/ml), amoxicillin-clavulanic acid (40 and 20 μg/ml, respectively), pefloxacin (10 μg/ml), and LB-SCS (2.5-fold concentrate). Experimental conditions are described in the text. Each value shown is the mean ± standard error of the mean (error bar) from three experiments (three successive passages of Caco-2 cells). Statistical analysis was performed with a Student test.

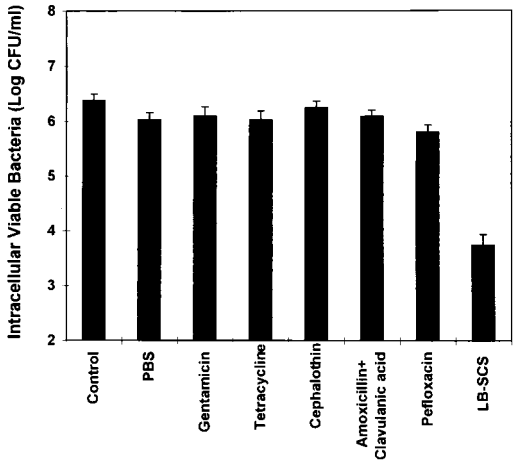

FIG. 9. Effect of antibiotics or LB-SCS on the viability of *S. typhimurium* SL1344 located intracellularly in infected cultured human enterocyte-like Caco-2 cells. The following compounds were used: gentamicin (30 μg/ml), tetracycline (60 μg/ml), cephalothin (60 μg/ml), amoxicillin-clavulanic acid (40 and 20 μg/ml, respectively), pefloxacin (10 μg/ml), and LB-SCS (2.5-fold concentrate). Experimental conditions are described in the text. Each value shown is the mean ± standard error of the mean (error bar) from three experiments (three successive passages of Caco-2 cells). Statistical analysis was performed with a Student test.

in *S. typhimurium* cell association was observed after pretreatment with gentamicin (30 μg/ml) or pefloxacin (10 μg/ml). Cephalothin, tetracycline, amoxicillin-clavulanic acid, gentamicin, or pefloxacin pretreatment of *S. typhimurium* SL1344 had less effect on *S. typhimurium* cell entry.

The activities of LB-SCS and antibiotics in Caco-2 cells preinfected with the *S. typhimurium* SL1344 were examined (Fig. 9). The LB-SCS (2.5-fold concentrate, pH 4.5) dramatically decreased the viable numbers of intracellular *S. typhimurium*, whereas PBS and fresh MRS (2.5-fold concentrate, pH 4.5) used as controls were inactive. In contrast, none of the antibiotics tested had an activity against intracellular *S. typhimurium*. This result suggests that the antimicrobial component(s) present in the LB-SCS was able to cross the epithelial membrane to act on the intracellular *S. typhimurium*.

Antibacterial activity of LB-SCS against *S. typhimurium* in the infected-mouse model. The LB-SCS antimicrobial activity in vitro against *S. typhimurium* SL1344 was in part destroyed after proteolytic enzyme treatment and decreased after the LB-SCS was subjected to alkaline conditions. Considering that in the intestine neutral conditions prevail, we examined if the LB-SCS antimicrobial activity develops in vivo. For this purpose, the conventional C3H/He/oujco mouse model with oral infection by *S. typhimurium* C5 was used. Results are presented in Fig. 10. In the infected-mouse control group, 4 to 5 log CFU of *S. typhimurium* C5 per g of feces was quantified daily postinfection. In the infected-mouse group receiving daily treatment with the LB-SCS (fivefold concentrate), a highly significant decrease in fecal *S. typhimurium* C5 contents was observed after 4 and 7 days of treatment. This result shows that when the LB-SCS is orally administered to *S. typhimurium*-infected mice, the LB-SCS antibacterial activity, previously observed in vitro, is maintained in vivo.

DISCUSSION

We have previously reported that heat-killed *L. acidophilus* LB in its SCS is able to inhibit adhesion of enteropathogens to cultured human intestinal cells (9), resulting in a decrease in cell entry for the enteroinvasive bacteria (11). Inhibition of pathogen adhesion by adhering lactobacilli (7, 8, 21) results from the steric hindrance of the pathogen host receptors by the whole-cell lactobacilli (7, 8) or from high-molecular-weight substances of lactobacilli exerting competitive exclusion or the adhesion of pathogens (5, 8, 39, 54). In this work, we found that the human *L. acidophilus* strain LB expressed antibacterial activity in its SCS. The antibacterial activity is manifest against a broad spectrum of gram-negative and gram-positive pathogenic bacteria in vitro. When the inhibitory activity was examined with the pathogenic strain *S. typhimurium* SL1344 as an indicator and cultured human intestinal cells as a model, we observed that the secreted antimicrobial component(s) is more effective against the invasive process of *S. typhimurium* than against the adhesive process. It is intriguing that compared with the activities of several antibiotics, the LB-SCS antibacterial activity against *S. typhimurium* SL1344 appears more rapid and efficient. Our results with the Caco-2 cell model showing low-level activity for some antibiotics when preincubating conditions were was used does not exclude the possibility that under other experimental conditions, such as a different time course and higher concentrations, these antibiotics would be able to kill cell-associated *S. typhimurium* SL1344. Moreover, we showed that the LB-SCS antimicrobial component(s) is able to cross the epithelial membrane of the preinfected cultured human intestinal Caco-2 cells to later kill invading *S. typhimurium*. By contrast, all the antibiotics tested were unable to kill intracellular *S. typhimurium* SL1344 in preinfected cells. This result suggests that the LB-SCS antimicrobial component(s) was able to cross the apical epithelial membrane, by an unknown mechanism, to kill the intracellular pathogen. For the antibiotics tested, inactivity could be explained by different transepithelial passages in the Caco-2 cell model (13, 23, 43, 44).

It has been reported by several investigators that lactobacilli are able to produce antimicrobial substances. A group of substances named bacterocins have been isolated, and the genes controlling their production have been characterized (26, 28). Well-known metabolic end products of lactic acid fermentation, lactic and acetic acids and hydrogen peroxide, are capable on interfering with the growth of pathogens (53). Acidic and heat-stable, low-molecular-weight antimicrobial substances produced by lactobacilli are active against gram-negative and

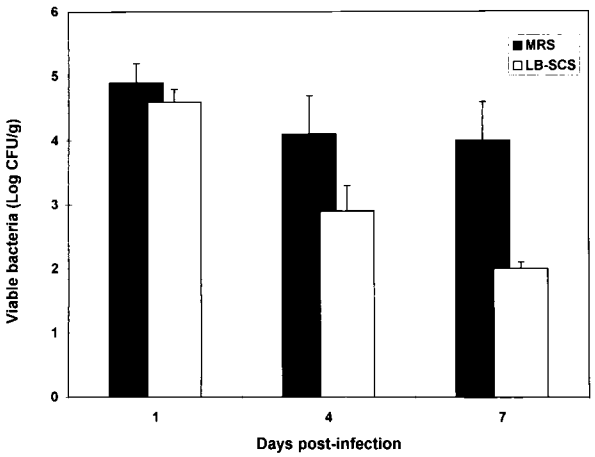

FIG. 10. Effect of LB-SCS treatment on infection of C3H/He/oujco mice by *S. typhimurium* C5. Experimental conditions are described in the text. Each value shown is the mean ± standard error of the mean (error bar) from six mice per group. Statistical analysis was performed with a Student test.

gram-positive bacteria (20, 35, 49, 50, 55, 56); however, they remain to be purified and chemically characterized. By contrast, the broad-spectrum antimicrobial substance named reuterin, produced by *Lactobacillus reuteri* during the fermentation of glycerol, is to our knowledge the only low-molecular-weight substance produced by lactobacilli which has been purified and characterized (52). We have not purified and characterized the LB-SCS antimicrobial component(s) described in the present study. However, we have examined several characteristics in vitro. The result indicated that the compound(s) produced by *L. acidophilus* LB which supports the antimicrobial activity had several characteristics in common with those of antimicrobial substances produced by other lactobacilli, such as insensitivity or low-level sensitivity to proteases, a low molecular mass in the range of 100 to 1,800 Da, and activity in vitro against both gram-positive and gram-negative bacteria which develops more under acidic than under alkaline conditions (20, 49, 50, 55, 56). Considering these characteristics, the LB-SCS component(s) supporting the antimicrobial activity appears different from bacteriocins and microcins. Indeed, the bacteriocins produced by lactobacilli are by definition proteinaceous antimicrobial compounds which exhibit a bactericidal effect against closely related bacteria and show little or no inhibition against other microorganisms (26, 28). The microcins, produced by a number of members of the family *Enterobacteriaceae*, mostly *E. coli* strains, are peptide antibiotics with low molecular weights, insensitive to proteases, and active against only gram-negative bacteria (2, 48).

The LB-SCS antimicrobial activity appeared in part sensitive to the proteolytic treatments, although the activity was not totally abolished. This raises the question of the activity in vivo. Upon examining this point, we found that the antibacterial activity develops in vivo, since we observed a significant decrease in fecal *S. typhimurium* contents in the *S. typhimurium* C5-infected mice treated orally with the LB-SCS. This last result was in agreement with previous observations showing in vivo activity of the heat-killed strain LB with its SCS against *E. coli* (18) and *Campylobacter jejuni* (38) infecting the mouse. We have recently reported that the in vitro antimicrobial activity of *L. casei* GG with its SCS against *S. typhimurium* infecting Caco-2 cells develops under acidic conditions and disappears under alkaline conditions (22). Despite this, we have found that the antagonistic activity of strain GG was able to develop in the conventional model or the germfree C3H/He/oujco mouse model with oral infection with *S. typhimurium* C5 (22). This activity resembles the antimicrobial activities of the small cationic peptides named defensins (31). Indeed, defensins are known to develop activity in vivo against microbes ingested by neutrophils in the phagosomal vacuoles which present an acidic environment (34, 51). Several defensins are also produced by the Paneth cells in the intestine, in which a neutral pH occurs. We have recently observed that antibacterial peptides are apically produced in cultured human enterocytic cells and develop in vitro activity against adherent enterovirulent *E. coli* under neutral conditions (4).

In conclusion, our results demonstrate that the human *L. acidophilus* strain LB produces an antibacterial activity effective (i) in vitro against gram-negative and gram-positive pathogens, (ii) in vitro against an enteroinvasive pathogen which adheres to and enters cultured human enterocytic cells, and (iii) in vivo in the *S. typhimurium*-infected-mouse model. Considering the results obtained with the pathogens and the characteristics of the activity, we believe that the component(s) secreted by *L. acidophilus* LB that supports the antimicrobial activity could contain an unusual acidic amino acid present in a novel peptidic agent. Our laboratory is attempting to further characterize and purify this secreted substance(s).

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