The Human *Lactobacillus acidophilus* Strain LA1 Secretes a Nonbacteriocin Antibacterial Substance(s) Active In Vitro and In Vivo

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The adhering human *Lactobacillus acidophilus* strain LA1 inhibits the cell association and cell invasion of enteropathogens in cultured human intestinal Caco-2 cells (M. F. Bernet, D. Brassard, J. R. Neeser, and A. L. Servin, Gut 35:483–489, 1994). Here, we demonstrate that strain LA1 developed its antibacterial activity in conventional or germ-free mouse models orally infected by *Salmonella typhimurium*. We present evidence that the spent culture supernatant of strain LA1 (LA1-SCS) contained antibacterial components active against *S. typhimurium* infecting the cultured human intestinal Caco-2 cells. The LA1-SCS antibacterial activity was observed in vitro against a wide range of gram-negative and gram-positive pathogens, such as *Staphylococcus aureus*, *Listeria monocytogenes*, *S. typhimurium*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*. By contrast, no activity was observed against species of the normal gut flora, such as lactobacilli and bifidobacteria. The LA1-SCS antibacterial activity was insensitive to proteases and independent of lactic acid production.

Recent reports have documented the role of exogenous lactobacilli in the prevention and treatment of gastrointestinal disorders. To explain this effect, several authors have suggested that exogenous lactobacilli could exert a "barrier effect" against pathogens. This hypothesis is based on the work of Reid and coworkers (6, 47), who have reported that several living lactobacilli, which adhere to freshly isolated uroepithelial cells or cell wall fragments, possess inhibiting properties against the adhesion of uropathogenic *Escherichia coli*. Consistent findings with well-characterized human intestinal cell models have brought insights into the action of lactobacilli. Several human *Lactobacillus acidophilus* strains exhibit adherence properties (3, 8), although the mechanism of adhesion remains controversial (17). Recent studies have produced evidence concerning the effects of enteroadherent lactobacilli against enteropathogens (3, 7, 9). For example, incubation of the LA1 strain together with the enteropathogens results in important inhibition of pathogen attachment to the enterocyte-like Caco-2 cells (3). This result is in agreement with the hypothesis of Chan et al. (6), i.e., the presence of nonspecific steric hindrance of the apical enterocytic receptors for pathogens by the *Lactobacillus* whole cells. Moreover, we have observed that in monolayers of Caco-2 cells already infected with enteropathogens, exposure of the infected cells to the LA1 bacteria with their spent culture supernatant (LA1-SCS) promoted a highly significant decrease in the attachment of pathogens (3). Two hypotheses could explain this result: (i) the *Lactobacillus* whole cells are able to displace the attached pathogens; or (ii) antimicrobial components are present in LA1-SCS. The latter hypothesis is supported by several reports suggesting a protective role of lactobacilli against pathogens through the production of several antimicrobial substances that inhibit the growth of pathogens. Certain lactobacillus antimicrobial compounds are related to the bacteriocin family

(25, 28), whereas others appear unrelated to bacteriocins; however, they remain unidentified (1, 2, 18, 35, 51, 53, 57, 58).

The aim of this study was to examine whether the inhibitory activity of the adhering human *L. acidophilus* strain LA1 against the process of pathogenicity of enterovirulent bacteria (3) is caused by the production of secreted antimicrobial substances.

MATERIALS AND METHODS

Bacteria. *L. acidophilus* LA1 and LA10 were from the Nestec (Lausanne, Switzerland) collection. The bacteria were grown under anaerobic conditions (Gaspak H_2+CO_2) in De Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostic, Beauvais, France) for 24 h at 37°C. Spent culture supernatant (LA1-SCS) was obtained by centrifugation at $10.000 \times g$ for 30 min at 4°C. Centrifuged LA1-SCS was passed through a sterile 0.22-µm-pore-size Millex GS filter unit (Millipore, Molsheim, France). Filtered LA1-SCS was checked for the absence of LA1 bacteria by being plated on tryptic soy agar (TSA) to confirm the absence of bacterial colonies. Concentrated LA1-SCS were obtained by freeze-drying.

Salmonella typhimurium SL1344 was a gift of B. A. D. Stocker (Stanford University, Stanford, Calif.) (12); *S. typhimurium* C5 was provided by M. Y. Popoff (Institut Pasteur, Paris, France) (40). *Listeria monocytogenes* EGD [HLY+] was provided by J. L. Gaillard (INSERM U411, Hôpital Necker, Paris, France). *Enterobacter cloacae* and *Klebsiella pneumoniae* were clinical isolates provided by A. Darfeuille-Michaud (Université Clermont I) (32). *Shigella flexneri* FL5M90T was provided by P. Sansonetti (Institut Pasteur, Paris, France). *Staphylococcus aureus*, *Streptococcus* group D, and *Pseudomonas aeruginosa* were stock clinical isolates from the microbiological laboratory of the Faculté de Pharmacie, Université Paris XI, Châtenay-Malabry, France. Human *L. acidophilus* LB was from Lacteol Laboratory, Houdan, France. Human *L. acidophilus* BG2FO4 and human *L. casei* GG were from T. R. Klaenhammer (North Carolina University, Raleigh, N.C.) and S. I. Gorbach (Tufts University, Boston, Mass.), respectively. The human bifidobacteria *Bifidobacterium breve* 4, *B. infantis* 1, and the adult human isolate B28 were from the Nestec collection.

Mice. Conventional and germfree animals (Iffa Credo, L'Arbresle 69, France) were adult female C3H/He/Oujco mice aged 7 to 8 weeks. They were housed and fed in accordance with the relevant national legislation. Germfree mice (Cesal, Orléans, France) were reared in Trexler-type isolators fitted with a rapid transfer system (La Calhène, Vélizy Villacoublay, France). Germfree and conventional mice were given ad libitum a commercial diet (RO3; UAR, Villemoisson/Orge, France) irradiated at 40 kGy and autoclaved demineralized water or a nonirradiated diet, respectively.

Caco-2 cell culture. Human Caco-2 colon adenocarcinoma cells (provided by Jorgen Fogh, Sloan Kettering Memorial Cancer Center, Rye, N.Y.) (13), a model of mature enterocytes of the small intestine (44), were used between the

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60th and 90th passages. The cells were routinely grown in Dulbecco's modified Eagle's minimal essential medium (25 mM glucose) (ATGC, Paris, France) supplemented with 20% heat-inactivated (30 min at 56°C) fetal calf serum (Life Technology, Paris, France) and 1% nonessential amino acids (ATCG) as previously described (3). Caco-2 cultured cells were used at postconfluence after 15 days of culture (differentiated cells) for cell association and cell invasion assays with *S. typhimurium*.

Infection of conventional and germ free mice by *S. typhimurium* C5. *S. typhimurium* C5 (40) was grown in Luria broth for 18 h at 37°C. The culture was harvested in phosphate-buffered saline (PBS). Viable bacteria were numbered after plating a suitable dilution on TSA and incubating it at 37°C for 18 h.

Adult 7- to 8-week-old female conventional C3H/He/Oujco mice (two groups of six mice) 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 fresh MRS broth per os daily. The LA1-treated group received 0.2 ml of LA1 culture in MRS broth (10^9 CFU/ml) per os daily.

L. acidophilus LA1 or LA10 (six mice per group) were inoculated into germfree C3H/He/Oujco mice as a single dose of a 100-fold-diluted fresh culture in bottled water 1 week before challenge with *S. typhimurium* C5. Inoculation of *S. typhimurium* C5 in germfree mice (six mice) or monoassociated mice was as follows: a single dose of 2×10^6 CFU/mouse was given in bottled water to the animals, which had been deprived of water since the day before.

The feces of control infected and LA1-treated infected conventional mice were collected individually 1, 4, 7, and 10 days postinfection, weighed, and dispersed in PBS. Viable *S. typhimurium* was determined after serial dilutions were plated on salmonella-shigella agar (SS agar medium, Difco) to determine bacterial colony counts. Black colonies of *S. typhimurium* were easily distinguishable from other normal resident enterobacteria. The results were expressed as the mean ± standard error of log CFU of viable bacteria per gram of feces. Feces of *L. acidophilus* LA1- or LA10-monoassociated germfree mice infected with *S. typhimurium* C5 were individually collected. The feces were weighed and diluted 10-fold in PBS. Fecal counts of lactobacilli were obtained by plating 0.1 ml of each 10-fold serial dilution on MRS agar (pH 4.5). The plates were incubated at 37°C for 48 h.

Infection of Caco-2 cells by *S. typhimurium* SL1344. The cell infection assay was conducted as previously reported (3, 7, 10). Briefly, prior to infection, the Caco-2 monolayers were washed twice with PBS. *S. typhimurium* SL1344 (12) was suspended in the culture medium, and 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 subsequently washed three times with sterile PBS.

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

Quantitative determination of *S. typhimurium* internalization was conducted by the method established by Isberg and Leong (22) with the aminoglycoside antibiotic gentamicin. After incubation, monolayers were washed twice with sterile PBS and then incubated for 60 min in a medium containing gentamicin (50 μg/ml). Bacteria adherent to the Caco-2 brush border membranes were rapidly killed, whereas those located within Caco-2 cells were not. The monolayers were washed with PBS and osmotically lysed with sterile H_2O. Appropriate dilutions were plated on TSA to determine the number of viable cell-associated bacteria by bacterial colony counts. Extracellular *S. typhimurium* was 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 LA1-SCS was determined by preincubating the pathogen (10^8 CFU/ml) with 2.5-fold-concentrated LA1-SCS for 1 and 2 h at 37°C. After centrifugation ($5,500 \times g$ for 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* organisms and determination of the viable extracellular and intracellular *S. typhimurium* counts were carried out as described above.

Transmission electron microscopy. An inoculum (10^8 CFU/ml) of *S. typhimurium* SL1344 was subjected to LA1-SCS for 2 h at 37°C. After centrifugation ($5,500 \times g$ for 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 E-320 transmission electron microscope at 60 kV.

Antimicrobial testing. The ultrasensitive assay of Lehrer et al. (30) for endogenous antimicrobial polypeptides was used to determine the antimicrobial activity in LA1-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 tryptic 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 $5,500 \times g$ for 5 min at 4°C, washed once with 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 containing 3 mg of powdered TSB medium, 1% (wt/vol) agarose, and a final concentration of 0.02% (vol/vol) Tween 20. After rapid dispersal with a laboratory vortex mixer, the agar was poured into a square Petri dish to form
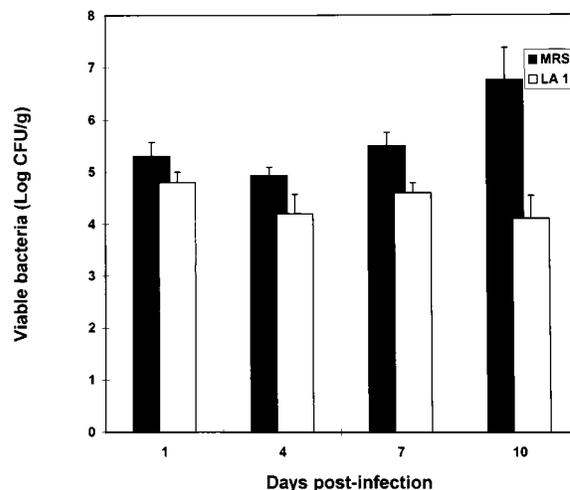

FIG. 1. Effect of the LA1 culture on infection of the conventional C3H/He/ Oujco mice by *S. typhimurium* C5. A significant decrease in the fecal contents of *S. typhimurium* occurs on day 7 postinfection in the LA1-treated group of mice (0.2 ml [10^9 CFU/ml]/day per os). The decrease in the level of the fecal contents of *S. typhimurium* is still present on day 10 postinfection, although the LA1 treatment was stopped on day 7 postinfection. Experimental conditions are described in the text. Each value shown is the mean ± standard error for six mice per group. Statistical analysis was performed by a Student *t* test.

a uniform layer approximately 1 mm deep. A 3-mm-diameter gel punch was used to make nine evenly spaced wells per dish. Samples (5 μl) were added to each well. The plates were incubated for 3 h at 37°C and then overlaid with 10 ml of sterile agar (TSB [6%, wt/vol], agarose [1%, wt/vol]). After incubation for 18 to 24 h at 37°C, the diameter of the inhibition area surrounding the wells was measured. The diameter of 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).

For time-kill studies, bacteria were grown overnight for 18 h at 37°C in Luria broth (*S. typhimurium*, *E. cloacae*, *Shigella flexneri*, and *K. pneumoniae*), or TSB (*L. monocytogenes*, *Staphylococcus aureus*, *Streptococcus* group D, and *P. aeruginosa*). Spent culture medium was centrifuged at $5,500 \times g$ for 5 min at 4°C, and the bacteria were washed once with PBS and resuspended in PBS. Colony count assays were performed by incubating approximately 10^8 CFU/ml of mid-logarithmic-phase bacteria with or without concentrated LA1-SCS at 37°C in the appropriate culture medium with test material. At predetermined intervals, aliquots were removed, serially diluted, and plated on TSA to determine bacterial colony counts.

Sensitivity of LA1-SCS to proteolytic and LDH enzymes. To test the sensitivity to protease, LA1-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 participates in the LA1-SCS antimicrobial activity, the LA1-SCS was subjected to lactate dehydrogenase (LDH) treatment (250 μg/ml for 2 h at 37°C). The remaining antimicrobial activity in the treated LA1-SCS was tested against *S. typhimurium* SL1344 by the radial diffusion assay.

RESULTS

Activity of LA1 against *S. typhimurium* infection of conventional mice. We examined if the human *L. acidophilus* LA1 develops antimicrobial activity in mice infected by *S. typhimurium*. The conventional C3H/He/Oujco mouse model was infected orally by *S. typhimurium* C5 (40). As shown in Fig. 1, the kinetics of the appearance of *S. typhimurium* in the feces of the infected mice receiving fresh MRS (0.2 ml/day per os) as a control showed that a stable excretion level of 5 log CFU of bacteria per g of feces was present from days 1 to 7 postinfection. Then the level of *S. typhimurium* increased significantly on day 10 postinfection to reach 7 log CFU of bacteria/g. When a group of infected mice was treated with the LA1 culture (0.2 ml of LA1 culture in MRS [10^9 CFU/ml] per os) for 4 days postinfection, no significant change in the *S. typhimurium* excretion level was observed compared to that in the group of mice receiving the fivefold-concentrated MRS. In contrast, a
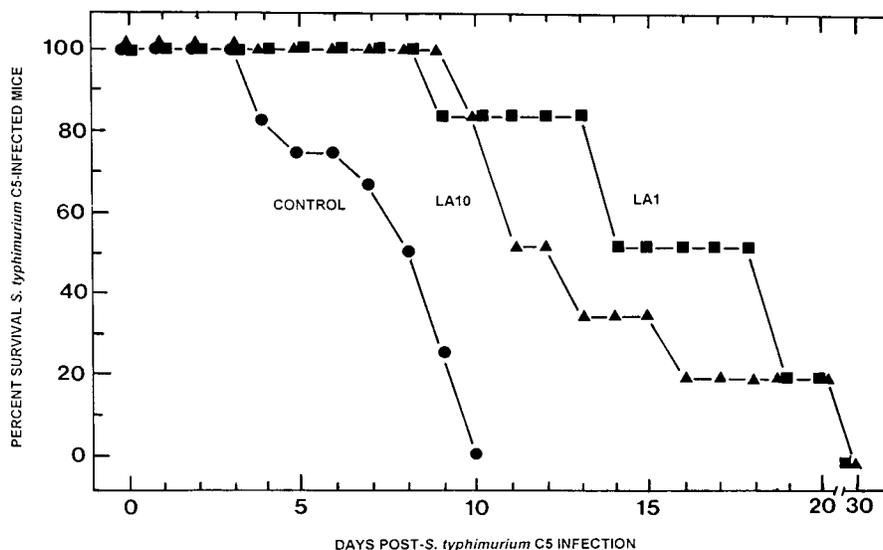
FIG. 2. Mortality due to *S. typhimurium* C5 infection in germfree C3/He/Oujco mice associated or not associated with *L. acidophilus* LA1 or LA10. C5 was given in drinking water at 2.5×10^6 CFU per mouse; *L. acidophilus* LA1- or LA10-monoassociated mice received a single administration of lactobacilli 8 days before the challenge with C5. A highly significant displacement of the curves was observed in the LA10- and LA1-monoassociated mice compared with the germfree C5-infected mice.

significant decrease ($P < 0.01$) in the amount of *S. typhimurium* in feces was observed on day 7 postinfection in the LA1-treated group of mice. This result suggests a protective effect of *L. acidophilus* LA1 against *Salmonella* infection in vivo.

We have examined how the *Salmonella* fecal content evolved in the LA1-treated group of mice after day 7 postinfection, when the LA1 treatment was stopped. The level of *S. typhimurium*, measured at 10 days postinfection, remained stable compared with the level of *S. typhimurium* in the group of LA1-treated mice on day 7 postinfection. In contrast, comparison with the group of MRS-treated mice showed that a highly significant decrease in the fecal contents of *S. typhimurium* occurred in the group of LA1-treated mice although the treatment was stopped (MRS-treated versus LA1-treated, $P < 0.001$). This result suggests that (i) the LA1 bacteria are able to survive in the intestine of mice and (ii) the LA1 bacteria remaining after the LA1 administration was stopped are able to develop antimicrobial effect in vivo.

Activity of LA1 against *S. typhimurium* infection of germfree mice. To examine the role of the human *L. acidophilus* LA1 colonizing the intestine of the mice, we used germfree C3H/He/Oujco mice receiving *L. acidophilus* LA1 as a single dose. At 7 days postadministration, *L. acidophilus* LA1 was established in the intestine, since 8.6 log CFU/g of LA1 lactobacilli was observed in the feces of the LA1-monoassociated germfree mice. To investigate if the LA1-monoassociated bacteria are able to protect the germfree mice against *Salmonella* infection, mice were infected with *S. typhimurium* C5. As shown in Fig. 2, a highly significant delayed mortality was observed in the LA1-monoassociated C5-infected mice compared with the germfree C5-infected mice ($P < 0.01$). Indeed, the first death was observed on day 4 postinfection in the germfree C5-infected mice but not until day 8 postinfection in the LA1-monoassociated C5-infected mice. A 100% mortality rate was observed by day 9 postinfection in the germfree C5-infected mice but not until day 30 postinfection in the LA1-monoassociated C5-infected mice. This result indicates that (i) the LA1 strain is able to colonize the digestive tract of the mice and (ii) the established LA1 bacteria exert antimicrobial activity in vivo.

Considering our previous report showing that the LA1 strain was able to bind to enterocyte-like cells in vitro whereas the LA10 strain was not (3), an additional experiment was conducted with *L. acidophilus* LA10, which exerts antimicrobial activity in vitro (data not shown). The LA10-monoassociated mice infected with *S. typhimurium* C5 showed an intermediate curve of mortality but not significantly different from that for the LA1-monoassociated mice (Fig. 2). A strong difference in the maintenance of the nonadhering lactobacilli compared with the adhering lactobacilli in the intestine of C5 infected mice was observed. Indeed, when examining how the level of lactobacilli in the feces in these two groups of mice evolved, we found that both LA1 and LA10 were equally established at 7 days after a single administration (8.6 ± 0.1 and 8.9 ± 0.1 log CFU/g, respectively). However, at 1 day after infection with *S. typhimurium* C5, the level of LA10 lactobacilli in the feces was dramatically decreased (4.9 ± 0.4 log CFU/g), whereas, in contrast, there was a slight decrease in the level of LA1 lactobacilli (9.4 ± 0.1, 7.8 ± 0.5, and 7.0 ± 0.2 log CFU/g on days 1, 2, and 5 postinfection, respectively).

LA1 antimicrobial activity against *S. typhimurium* infecting cultured human intestinal Caco-2 cells. *S. typhimurium* SL1344 infects the differentiated enterocyte-like Caco-2 cells (12). We have previously reported that LA1 plus LA1-SCS efficiently inhibited the association of *S. typhimurium* SL1344 with Caco-2 cells in a dose-dependent fashion (3). We observed that the LA1 bacteria separated from the SCS are unable to inhibit the association of *S. typhimurium* with Caco-2 cells (control *S. typhimurium*, 7.5 ± 0.3 log CFU/ml; *S. typhimurium* in the presence of LA1 bacteria, 7.2 ± 0.4 log CFU/ml). This results led us to suggest that LA1-SCS contains an antimicrobial substance(s). Indeed, the sterilized LA1-SCS efficiently inhibits the association of *S. typhimurium* with Caco-2 cells (Fig. 3). As a control, the *S. typhimurium* organisms (10^8 CFU/ml) were subjected to pretreatment with MRS (2.5-fold concentrate). No change in viability was observed. After 2 h of incubation, the majority of the MRS-pretreated *S. typhimurium* organisms were associated (extracellular plus intracellular bacteria) with the Caco-2 cells and efficiently invaded the cells.
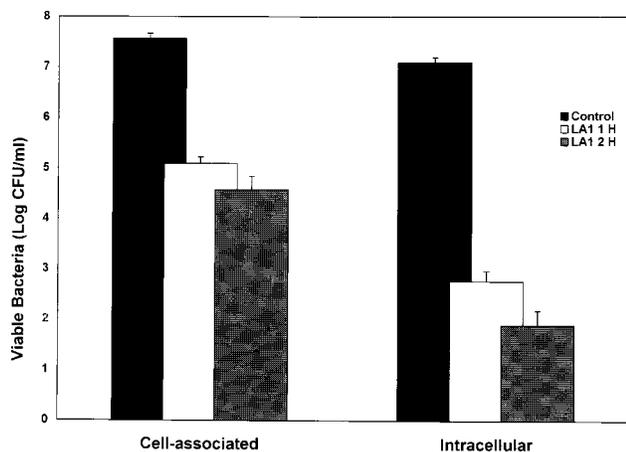
FIG. 3. Effect of the precontact of LA1-SCS on the capacity of *S. typhimurium* SL1344 to adhere to and invade the cultured human enterocyte-like Caco-2 cells, showing a dramatic decrease in *S. typhimurium* cell invasion. LA1 is the LA1-SCS 2.5-fold concentrate. Experimental conditions are described in the text. Each value shown is the mean ± standard error of the mean for three experiments (three successive passages of Caco-2 cells). Statistical analysis was performed by a Student *t* test.

When the *S. typhimurium* organisms (10^8 CFU/ml) were subjected to LA1-SCS (2.5-fold concentrate) for 1 h at 37°C, 5 × 10^5 CFU of bacteria per ml remained viable after the LA1-SCS exposure. These LA1-SCS-pretreated *S. typhimurium* organisms were then incubated with the Caco-2 cells for 1 and 2 h. After 1 and 2 h of incubation, the majority of the viable LA1-SCS-pretreated *S. typhimurium* organisms were efficiently associated with the Caco-2 cells. In contrast, a dramatic decrease in *S. typhimurium* cell invasion was observed (3 to 4 log decrease), indicating that the exposure of *S. typhimurium* to LA1-SCS selectively inhibits the invasion process.

Pathogen sensitivity to LA1-SCS. The viability of all the microorganisms tested (inoculum, 10^8 CFU/ml) was verified after 1 and 3 h of incubation with 2.5-fold-concentrated LA1-SCS (Fig. 4). As with gram-positive bacteria, several situations occurred. The viability of *Streptococcus* group D was not affected at any time points (1 and 3 h). By contrast, the viability of *Staphylococcus aureus* and *L. monocytogenes* was greatly decreased at both time points ($P < 0.01$). For gram-negative bacteria, the situation was more clear-cut, since all the genera and species tested showed altered viability when treated with LA1-SCS. The viability of *P. aeruginosa* was rapidly affected even after 1 h of contact ($P < 0.01$), whereas that of *Shigella flexneri*, *E. cloacae*, and *K. pneumoniae* was affected to a less important extent since the effect became evident only after 3 h of contact ($P < 0.01$). Interestingly, no antibacterial activity against other human lactobacilli or bifidobacteria, such as *L. acidophilus* LB and BG2FO4, *L. casei* GG, *B. breve* 4, *B. infantis* 1, and B28 strains, was evident (data not shown).

Characteristics of LA1-SCS antibacterial activity. For this study, *S. typhimurium* SL1344 was chosen as an indicator. As shown in Fig. 5, the viability of *S. typhimurium* subjected to LA1-SCS (2.5-fold concentrate) decreased as a function of time. As a control, PBS and MRS (2.5-fold concentrate) adjusted to pH 4.5 were used; they showed no antibacterial activity. *S. typhimurium* SL1344 treated with LA1-SCS was examined by transmission electron microscopy. As demonstrated in Fig. 6B, bacterial cells exposed to LA1-SCS (2.5-fold concentrate) for 1 h at 37°C showed an altered cell surface. This suggests a general loss of large amounts of membrane-associated material from the *S. typhimurium* that had been exposed to LA1-SCS.

The characteristics of the antibacterial activity of LA1-SCS (fivefold concentrate) were examined by the radial diffusion assay developed by Lehrer et al. (30) to test antimicrobial polypeptides (Fig. 7). Fresh MRS (fivefold concentrate at pH 4.5) showed no activity. The activity of LA1-SCS remained
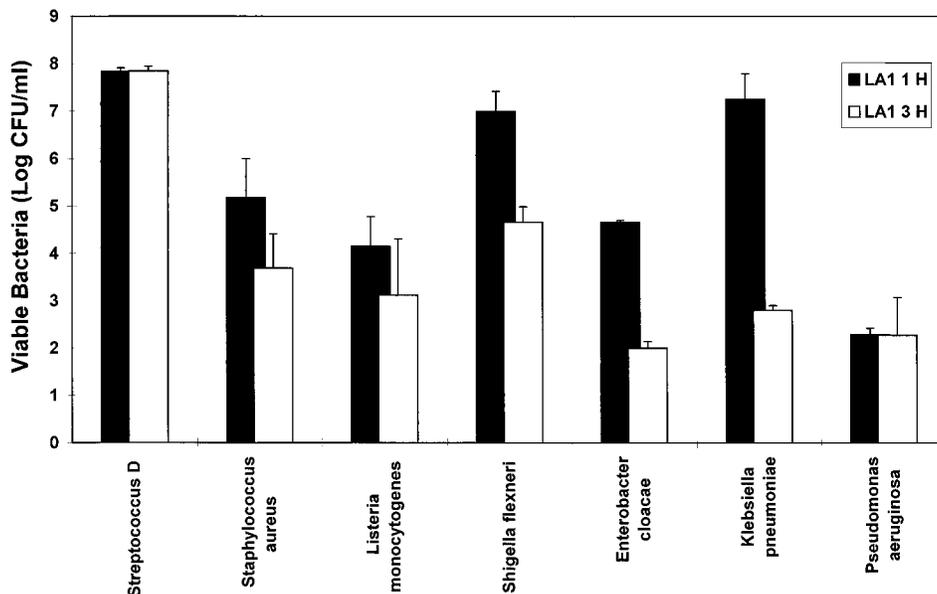

FIG. 4. Effect of LA1-SCS on the viability of gram-positive and gram-negative bacteria measured after 1 and 3 h of contact. For each pathogen, the inoculum was 10^8 CFU/ml. Each value shown is the mean ± standard error of the mean for three experiments. Statistical analysis was performed by a Student *t* test. Experimental conditions are described in the text. LA1 is the LA1-SCS 2.5-fold concentrate.
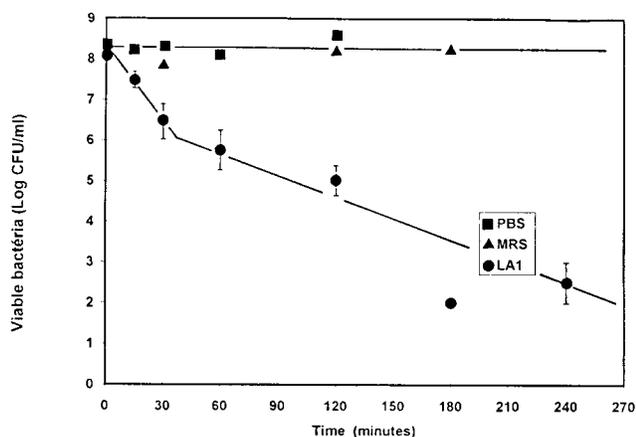
FIG. 5. Evaluation of the bactericidal activity of LA1-SCS against *S. typhimurium* SL1344, showing a decrease in viable counts as a function of time. Experimental conditions are described in the text. Each value shown is the mean ± standard error of the mean for three experiments. MRS and PBS are 2.5-fold concentrate; LA1 is the LA1-SCS 2.5-fold concentrate.
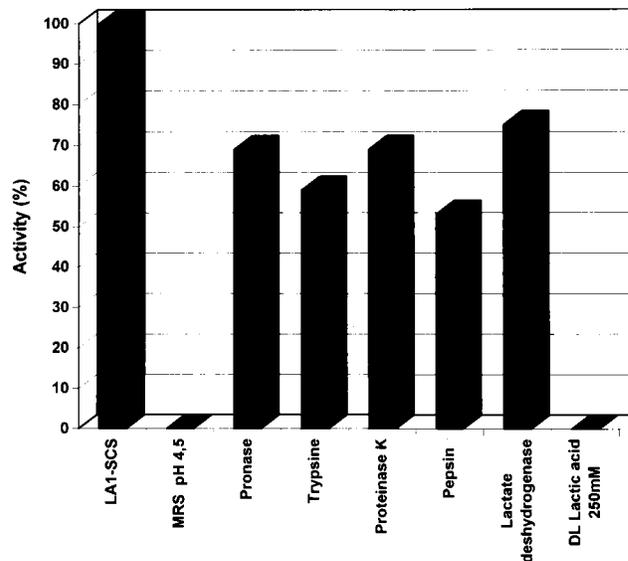

FIG. 7. Effect of chemical treatments on the antimicrobial activity of LA1-SCS against *S. typhimurium* SL1344. Experimental conditions are described in the text. Each value shown is the mean for three experiments. The variation in reproducibility was less than 5%.

relatively stable after proteolytic enzyme treatments (pronase, trypsin, proteinase K, or pepsin).

Lactic acid participates in the antimicrobial activity of lactobacilli (55). To determine if the lactic acid in LA1-SCS participates in the antimicrobial activity, LA1-SCS was subjected to LDH treatment. The antimicrobial activity of the LDH-treated LA1-SCS remained unchanged (Fig. 7). Moreover, no activity was found with DL-lactic acid at 250 mM, which was greater than the lactic acid concentration produced by LA1 during 18 h of culture. These results demonstrated that the lactic acid did not participate in the antibacterial activity of LA1-SCS.

DISCUSSION

Lactobacilli are the most frequently used species in products for human consumption and can be found in infant foods, cultured milks, and pharmaceutical preparations (27, 49). Fermented milks are claimed to contain a number of biologically active compounds which may contribute to human health (20,

38). In 1993, a critical review examining the bulk of the available publications particularly emphasized that the excessive extrapolation of results has been detrimental to the development of healthy or therapeutic products (34). Reviewing the recent publications, Lee and Salminen (29) concluded that recent advances in probiotic research have finally confirmed the health benefits of some lactic bacterial strains. *L. casei* GG survives in the human gastrointestinal tract (15) and causes clinically significant health benefits against human diarrhea (23, 24, 26). Adherent *L. gasseri* ADH is likely to survive passage through the gastrointestinal tract in human and thus has greater metabolic effects such as reduction in fecal bacterial enzyme activity (42). Other lactobacilli of nonintestinal origin express antimicrobial activities. *L. reuteri* MM53, isolated from human milk, survives in the human gastrointestinal
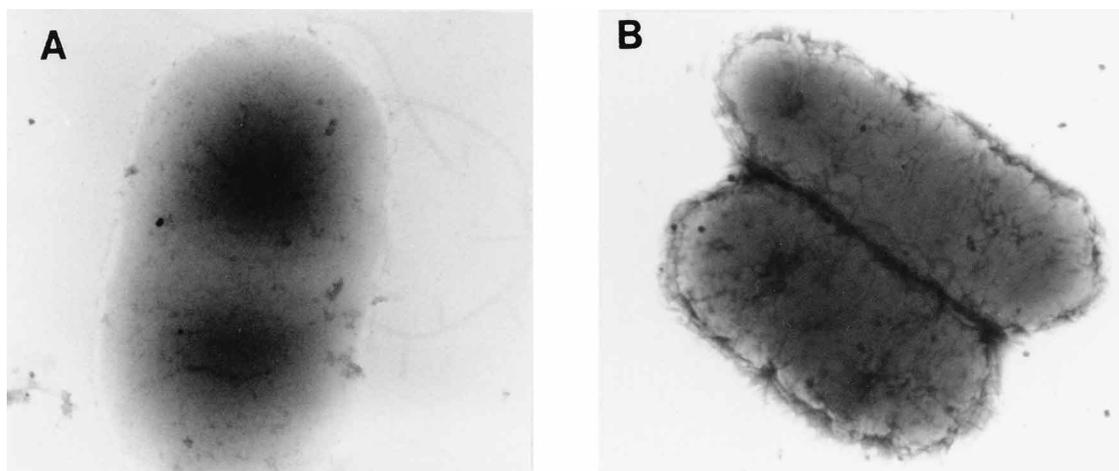

FIG. 6. Transmission electron micrographs of *S. typhimurium* SL1344 subjected to LA1-SCS. (A) *S. typhimurium* control incubated for 1 h at 37°C with 2.5-fold-concentrated MRS. (B) *S. typhimurium* incubated with 2.5-fold-concentrated LA1-SCS, showing alteration of the bacterial cell surface. Experimental conditions are described in the text. The micrographs are representative of three separate experiments.

tract (59). *L. acidophilus* AI induces metabolic modification of the human colonic flora (33).

One of the interesting properties of lactic acid bacteria is the inhibition of microbial pathogens. In vitro studies have recently documented the antagonistic activity of lactobacilli against enteropathogens as a result of the competitive exclusion of adhesion of pathogenic bacteria to host cells (3, 4, 6–10, 19, 39, 47, 56). The antagonistic activity of lactobacilli against pathogens could exert itself in a different way from the model, implying competitive exclusion by whole cells and cell wall fragments. Metabolic products of lactobacilli inhibit the growth of pathogenic and nonpathogenic bacteria (55). When examined in vitro with cultured human intestinal cells as a model, the adherent human *L. acidophilus* LA1, which is capable of inhibiting the pathogenicity of enterovirulent bacteria in vitro (3), secretes in its SCS a substance(s) exhibiting antibacterial activity. Our results showing that the LA1 culture develops activity in the *S. typhimurium*-infected mice are of interest. We found that daily administration per os of the LA1 culture to conventional mice infected with *S. typhimurium* significantly decreased the level of *S. typhimurium* in the fecal contents. Moreover, we observed that strain LA1 exerted antimicrobial activity in vivo after the daily oral administration was arrested. The observation that the antagonistic effects were maintained after the end of intake suggests that the strain persists in the intestine. Recent clinical studies have confirmed this point. When administered to human volunteers, *L. acidophilus* LA1 was recovered from the feces and thus contributed to the increase in lactobacillus counts (32). An experiment conducted with germfree mice showed that the human strain LA1 should be able to render the mice resistant to an *S. typhimurium* infection.

Antimicrobial activities developed in vivo by lactobacilli have been previously reported. Heat-killed *L. acidophilus* LB administered with its SCS exerts antagonistic activity in *E. coli*- and *Campylobacter jejuni*-infected mice (14, 37). *L. casei* GG develops antagonistic activity in *S. typhimurium*-infected mice (21). *L. casei* LAB-2 increases antibody responses against cholera in mice (45) and enhances the resistance of mice against *S. typhimurium* infection (41). Feeding of mice with *L. acidophilus* and/or *L. casei* strains results in a systemic and local augmentation of the immune response against *S. typhimurium* infection (43). In contrast, the activity against *E. coli* RDEC-1 of *L. bulgaricus* and *Streptococcus thermophilus* strains observed in vitro was not confirmed in vivo in the just-weaned rabbit model infected by RDEC-1 (16). The mechanism of action of these different *Lactobacillus* strains has not been elucidated. However, in parallel with the antimicrobial activity, lactobacilli are known to stimulate immunological defenses against pathogens (43). It has also been demonstrated that when LA1 was orally administered to humans in fermented milk, it was able to increase the blood phagocytic activity (50), as well as both the total immunoglobulin A levels in serum and the specific immunoglobulin A titers to *S. typhi* Ty21a in serum (31). Furthermore, the antimicrobial effect of LA1-SCS against the peptic ulcer-associated pathogen *Helicobacter pylori*, both in vitro and in human volunteers, was recently demonstrated (36).

The nature of the antimicrobial component(s) secreted by LA1 strain in its SCS remains to be determined. Several features of the LA1 activity are similar to those of other lactobacilli (51, 53, 54, 57): its relative insensitivity to proteolytic enzymes and the unrelateness of the activity to lactic acid production. However, some specific properties of LA1-SCS can be underlined. In contrast to the adverse effect of *L. casei* GG against some gram-positive bacteria of the human normal flora (53), LA1-SCS is inactive against *Bifidobacterium* and *Lactobacillus* strains isolated from human stools. To date, the substance(s) which support(s) the antibacterial activity secreted by *L. acidophilus* LA1 remains unknown. However, examination of the spectrum of activity of LA1-SCS against enterovirulent bacteria revealed that the activity was evident in vitro against a broad spectrum of gram-positive and gram-negative bacteria, thus differentiating it from classical bacteriocins and microcins. By definition, bacteriocins are proteinaceous antimicrobial compounds which are produced by lactic acid bacteria and exhibit a bactericidal effect against taxonomically closely related bacteria with, generally, no effects against other microorganisms (25, 28). The genes encoding their expression have been characterized in some cases (28). Microcins are peptide antibiotics produced by members of the family *Enterobacteriaceae*, mostly *E. coli* strains (48); they have low molecular weights and are insensitive to proteases and active against gram-negative bacteria. Other antimicrobial substances produced by lactobacilli, active in vitro against both gram-negative and gram-positive bacteria and different from bacteriocins, have been reported (1, 2, 18, 35, 51, 53, 54, 57, 58). For example, *L. casei* GG, adhering to the cultured human enterocyte-like cells (11), produces a heat-stable antimicrobial substance with a low molecular weight ($>1,000$); this substance is soluble in acetone-water and distinct from lactic and acetic acid, developing inhibitory activity against *E. coli*, *Streptococcus* spp., *Pseudomonas* spp., *Salmonella* spp., and *Bacillus* spp. (53). Moreover, reports have described the production of antimicrobial substances in vitro by several dairy *Streptococcus* strains (46, 52).

In conclusion, the results previously reported (3) and those reported here demonstrated that the human *L. acidophilus* LA1 produces antibacterial activity that is effective in vitro against gram-positive and gram-negative pathogens. According to the data reported herein, it appears that the antipathogenic effects of LA1 observed in vitro and in vivo might be attributable to a secreted unknown antimicrobial substance different from lactic acid rather than to competitive exclusion of adhesion of pathogenic bacteria.

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