Inhibition of in vitro growth of enteropathogens by new *Lactobacillus* isolates of human intestinal origin

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Abstract

Three human *Lactobacillus* strains, coded B21060, B21070 and B21190, have recently been isolated. The strains show a series of features (acid and bile resistance, adhesion to various types of mucosal cell) which make them particularly promising for the preparation of probiotic products. In the present study, the ability of the strains to inhibit the growth of pathogens in coculture was investigated. Lactobacilli were incubated simultaneously or after one overnight growth with enterotoxigenic *Escherichia coli*, *Salmonella enteritidis* or *Vibrio cholerae*. After 24 and 48 h, bacterial counts of the pathogens and of the lactobacilli were performed. The results showed that these *Lactobacillus* strains inhibited the in vitro growth of *E. coli* and *S. enteritidis* under both conditions. Moreover, a cumulative effect was observed for mixtures of lactobacilli. In contrast, no significant inhibition of *Vibrio cholerae* growth was observed, provided that the pH of the medium was kept constant. The presence of the pathogens did not affect the growth of the *Lactobacillus* strains. Moreover, each of the *Lactobacillus* strains showed coaggregation ability with two pathogenic *E. coli* strains, namely ATCC 25922 and ATCC 35401.

1. Introduction

Gastrointestinal disorders of infectious etiology are endemic and constitute a significant health problem in certain countries. The major manifestation of enteric infection is diarrhea which under certain circumstances can reach life-threatening levels. The two main classes of agents that are responsible for diarrhea of infectious origin are enteropathogenic viruses and bacteria. Among the bacteria, *Escherichia* and *Salmonella* are the most common culprits. The same bacterial genera, together with *Shigella*, *Campylobacter* and *Vibrio* sp., are often recognized as the causative agents of diarrhea in children in developing countries [1], which still constitutes one of the commonest illnesses and one of the major causes of infant and childhood mortality in these countries. In addition, bacterial enteropathogens cause at least 80% of traveller's diarrhea.

The management of bacterial associated diarrhea usually ranges from no treatment to oral rehydration therapy and to antimicrobial drugs, although there may be problems associated with the routine use of antibiotics, which have potentially serious side ef-

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Table 1
Bacterial growth (log CFU/ml) 24 h after simultaneous inoculation of *Lactobacillus* strains and either *E. coli* ATCC 35401, *S. enteritidis* IMM 2 or *V. cholerae* El Tor[a]

Strain	*Lactobacillus*	*E. coli* ATCC 35401
B21060	9.8 (9.9)	5.5 (9.5)
B21070	9.7 (9.5)	5.3 (9.5)
B21190	9.6 (9.6)	5.6 (9.5)
Strain	*Lactobacillus*	*S. enteritidis*
B21060	8.4 (9.8)	<3 (9.5)
B21070	8.3 (9.9)	<3 (9.5)
B21190	8.0 (9.5)	<3 (9.5)
Strain	*Lactobacillus*	*V. cholerae*
B21060	8.2 (8.6)	9.0 (9.2)
B21070	8.0 (8.7)	9.0 (9.2)
B21190	7.9 (8.6)	9.1 (9.2)

[a]Values in parentheses show the growth of controls, i.e. of pure cultures, under the same conditions.

fects. The use of probiotics has been suggested as a safer alternative to chemotherapy in the management of gastrointestinal disorders caused by infectious agents, and one with the potential for preventing such disorders. In particular, positive results have been reported for *Enterococcus faecium* [2], *Lactobacillus acidophilus* [3,4], *Lactobacillus* GG [5–8], and *Bifidobacterium bifidum* in combination with *Streptococcus thermophilus* [9].

Following up on some earlier studies [10], we have recently isolated three *Lactobacillus* strains, coded B21060, B21070, and B21190, from feces of newborns or from weaned babies. Isolation was on the basis of fecal load (prevalent strains) and presence for several consecutive days (permanent strains) [11]. The B21060 and B21070 strains were both classified as *Lactobacillus paracasei*, while B21190 was classified as *Lactobacillus acidophilus*. The strains are all acid and bile resistant. In addition, they share with other strains that are currently the subject of intensive study strong adhesive properties in vitro to both buccal and intestinal mucosal cells [11]. These features, taken together, render these strains exploitable for the preparation of probiotic products. It is, however, well known that probiotics may hamper the virulence of pathogens through mechanisms other than competitive adhesion to target cells, for example through production of antibacterial substances

and bacterial aggregation. In order to test whether such mechanisms are relevant in the case of our *Lactobacillus* strains, we have undertaken a series of experiments aimed at evaluating inhibition of pathogen growth in coculture.

2. Materials and methods

2.1. Microorganisms

Bacterial strains B21060 and B21070, which are *L. paracasei*, and strain B21190, which is a *L. acidophilus*, were isolated and characterized as previously described [11]. Enterotoxigenic and uropathogenic *Escherichia coli* (ATCC 35401 and ATCC 25922, respectively, American Type Culture Collection, Rockville, MD, USA), *Salmonella enteritidis* (IMM 2, isolated by us from an infant affected by salmonellosis) and *V. cholerae* El Tor (obtained from Istituto Sieroterapico Milanese, Milan, Italy) were utilized as pathogenic target strains.

2.2. Bacterial growth

Lactobacillus strains were grown in de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) in 5% CO_2 atmosphere at 37°C for 24 h. *E. coli* (ATCC 35401 and ATCC 25922 strains) and *S. enteritidis* were cultured in Mueller-Hinton (MH) broth (Oxoid, Basingstoke, UK) in 5% CO_2 at 37°C for 24 h, while *V. cholerae* was grown in a modified TSB medium consisting of tryptic soy broth (TSB, Difco) in 5% CO_2 at 37°C for 24 h to which 8 g l^{-1} of bacto beef extract (Difco) and 4 g l^{-1} of bacto yeast extract (Difco) had been added.

2.3. Coculture growth curves

The interference of lactobacilli with the growth of pathogenic strains was evaluated by coincubating *E. coli* ATCC 35401, *S. enteritidis*, and *V. cholerae* individually with each *Lactobacillus* strain. For each experiment, a tube containing 5 ml of MRS broth and 5 ml of MH broth was inoculated with 10^5 CFU/ml of both the *Lactobacillus* and the enteropathogen strain. For experiments including *V. cholerae*, modified TSB was used instead of MH broth.
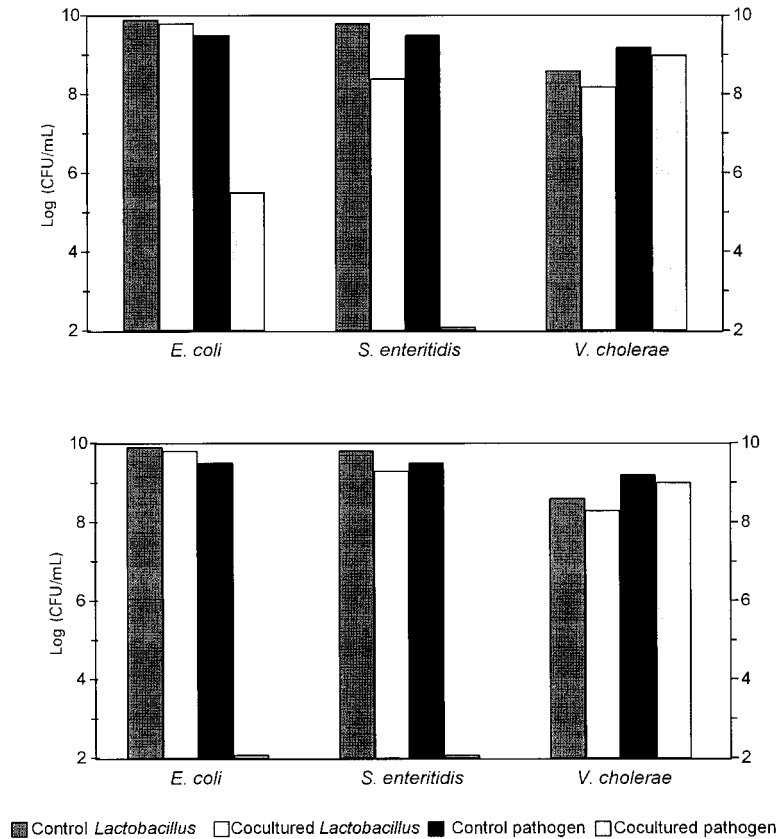
Fig. 1. Inhibition of in vitro growth of *E. coli* ATCC 35401, *S. enteritidis* IMM 2 and *V. cholerae* El Tor by *Lactobacillus* strain B21060 after 24 h coincubation. Only the data obtained for *Lactobacillus* strain B21060 were represented, since those for strains B21070 and B21190 were very similar (see Tables 1 and 2). Top: *Lactobacillus* strain B21060 and a pathogenic strain were inoculated simultaneously. Bottom: The pathogenic strains were inoculated after overnight growth of *Lactobacillus* strain B21060. Values of log (CFU/ml) < 3 are only indicative.

All media were used at twice the usual concentration. The tubes were incubated at 37°C under continuous agitation and microaerophilic conditions (5% CO_2). At 8–10 h intervals all media were refreshed to limit changes in growth due to pH variation or nutrient consumption; to achieve this, cultures were centrifuged for 15 min at $5000 \times g$ and pellets were resuspended in fresh medium. 24 and 48 h after inoculation, bacterial cells were collected by centrifugation (15 min at $5000 \times g$) and suspended in phosphate-buffered saline (PBS obtained from PBI International Milan, Italy) by vortex mixing for at least 1 min in order to disrupt all aggregates. Seven successive 1:10 dilutions were plated on MRS agar to evaluate the *Lactobacillus* growth and either on MacConkey

(MC) agar, on *Salmonella Shigella* (SS) agar or on tryptic soy agar (TSA) to evaluate the growth of *E. coli, S. enteritidis* or *V. cholerae*, respectively. The MRS agar plates were incubated for 48 h in 5% CO_2 at 37°C, while MC, SS and TSA agar plates were incubated for 24 h at 37°C. Pure cultures of each strain were subjected to the same treatments and used as controls.

Another series of experiments was performed in the same way, except that enteropathogens were inoculated (10^5 CFU/ml) in overnight cultures of lactobacilli (10^9 CFU/ml).

A final set of experiments was performed by incubating the pathogen strains together with various combinations of *Lactobacillus* strains. Mixed cultures

Table 2
Bacterial growth (log CFU/ml) at 24 h coculture of *Lactobacillus* strains and either *E. coli* ATCC 35401, *S. enteritidis* IMM 2 or *V. cholerae* El Tor[a]

Strain	*Lactobacillus*	*E. coli* ATCC 35401
B21060	9.8 (9.9)	< 3 (9.5)
B21070	9.8 (9.5)	< 3 (9.5)
B21190	9.6 (9.6)	< 3 (9.5)
Strain	*Lactobacillus*	*S. enteritidis*
B21060	9.3 (9.8)	< 3 (9.5)
B21070	9.9 (9.9)	< 3 (9.5)
B21190	9.3 (9.5)	< 3 (9.5)
Strain	*Lactobacillus*	*V. cholerae*
B21060	8.3 (8.6)	9.0 (9.2)
B21070	8.8 (8.7)	8.7 (9.2)
B21190	8.1 (8.6)	8.6 (9.2)

Pathogens were inoculated after overnight growth of *Lactobacillus* strains.
[a]Values in parentheses show the growth of controls, i.e. of pure cultures, under the same conditions.

of lactobacilli were used as controls. Other conditions were as above described.

2.4. Antimicrobial supernatant activity

Lactobacillus strains were grown in MRS broth for 48 h at 37°C in 5% CO_2 atmosphere, without regeneration of the culture medium, and removed by centrifugation ($5000 \times g$ for 10 min). The supernatants were adjusted to pH 7 with 1 M NaOH and sterilized by filtration (0.22 µm polycarbonate filter, Millipore type GTTP, Millipore Co., Bedford, MA, US). Inhibitory activity was determined against *E. coli* ATCC 35401, *S. enteritidis*, *V. cholerae* and *Lactobacillus* strains, by both a well-diffusion assay and a paper-disk assay [12].

The strains to be tested were added to molten MH agar at a final concentration of 10^5 CFU/m/L, distributed in plates and allowed to solidify. For the well-diffusion assay, some wells (diameter = 4 mm) were cut into agar and filled with the supernatants. After aerobic incubation for 24 h at 37°C, the diameters of the inhibition zones were evaluated. For the paper-disk assay, sterile disks (Oxoid) were imbibed with 20 µl of the supernatants and placed onto the agar plates. After incubation for 18 h at 37°C, the diameters of the inhibition zones were evaluated.

2.5. Aggregation experiments

Lactobacillus strains B21060, B21070, B21190 and *E. coli* strains ATCC 25922 and ATCC 35401 were used for aggregation experiments. These were performed as described by Reid et al. [13]. Briefly, each strain was grown overnight at 37°C in MH broth (*E. coli*) or MRS broth (lactobacilli) in 5% CO_2 atmosphere. Bacteria were centrifuged for 10 min at $10\,000 \times g$ and 4°C, washed three times with PBS and suspended in PBS at a concentration of 10^9 CFU/ml.

For coaggregation experiments, 500 µl of each *Lactobacillus* suspension was mixed with 500 µl of *E. coli* suspension (either ATCC 25922 or ATCC 35401) for at least 10 s on a vortex mixer and then incubated in 24-well microtrays (Corning, Italy) at 37°C under agitation. Controls for autoaggregation consisted of a mixture of 500 µl of each strain suspended with 500 µl of PBS. After 4 h, the suspensions were observed by inversion light microscopy and scored for aggregation (from 0 for no aggregation to 4 for maximum aggregation) [14].

Scanning electron microscopy (SEM) was used to confirm the results. In detail, 10 µl of each suspension was placed onto a slide, air dried, fixed with 2.5% glutaraldehyde for 2 h at room temperature, washed three times in 0.1% (w/v) sodium cacodylate buffer pH 7 (Sigma Chemical Company, St. Louis, MO, USA) and postfixed with 1% (w/v) osmium tetroxide (Sigma) for 90 min at room temperature. Samples were dehydrated through a graded series of ethanol and acetone mixtures, critical point dried at 35°C and 1250 psi for 15 min, coated with gold and observed by SEM with a Leica S420 microscope (Leica Technology BV, Netherlands). Ten randomized fields were evaluated to independently estimate the degree of autoaggregation and coaggregation. The results were compared with those obtained by inversion light microscopy and a final score was assigned.

3. Results

3.1. Interference with the growth of intestinal pathogens

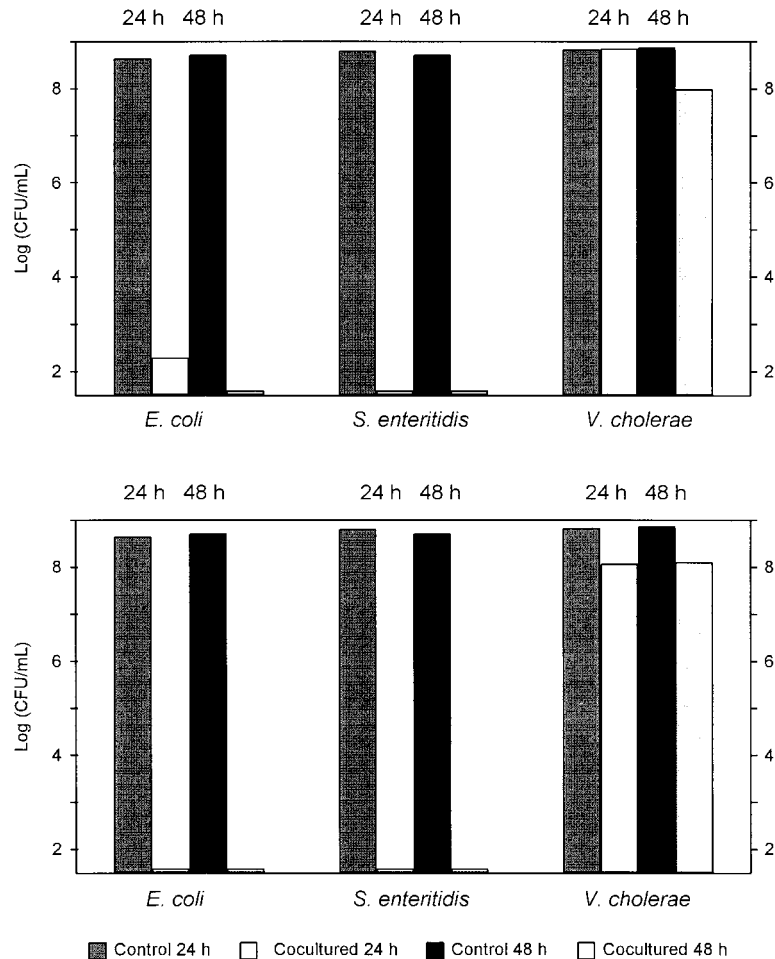The capability of the *Lactobacillus* strains B21060,

Fig. 2. Inhibition of in vitro growth of *E. coli* ATCC 35401, *S. enteritidis* IMM 2 and *V. cholerae* El Tor by a mixture of *Lactobacillus* strains B21060, B21070 and B21190 after 24 and 48 h coincubation. Top: The *Lactobacillus* and pathogenic strains were inoculated simultaneously. Bottom: The pathogenic strains were inoculated after overnight growth of the mixed *Lactobacillus* strains. Values of log (CFU/ml) < 2 are only indicative.

B21070, and B21190 to inhibit the in vitro growth of intestinal pathogens was evaluated in coculture experiments. In a first series of experiments, the *Lacto-* bacillus strains were inoculated simultaneously with the pathogens. The results are reported in Table 1 and for strain B21060 in Fig. 1, top. They indicate

Table 3
Aggregation score for lactobacilli incubated alone (autoaggregation) and with *E. coli* (coaggregation)[a]

	B21060	B21070	B21190
Autoaggregation	0	2	1
Coaggregation with *E. coli* ATCC 25922[b]	2	4	2
Coaggregation with *E. coli* ATCC 35401[b]	1	4	2

[a]The score is based upon the system described by Cisar et al. [14]. 0 = no aggregation; 1 = partial aggregation; 2 = good aggregation; 3 = marked aggregation; 4 = maximum aggregation. Results were confirmed by SEM so that autoaggregates were excluded from coaggregation scores.
[b]Autoaggregation for *E. coli* ATCC 25922 and ATCC 35401 was scored as 3 and 1, respectively.
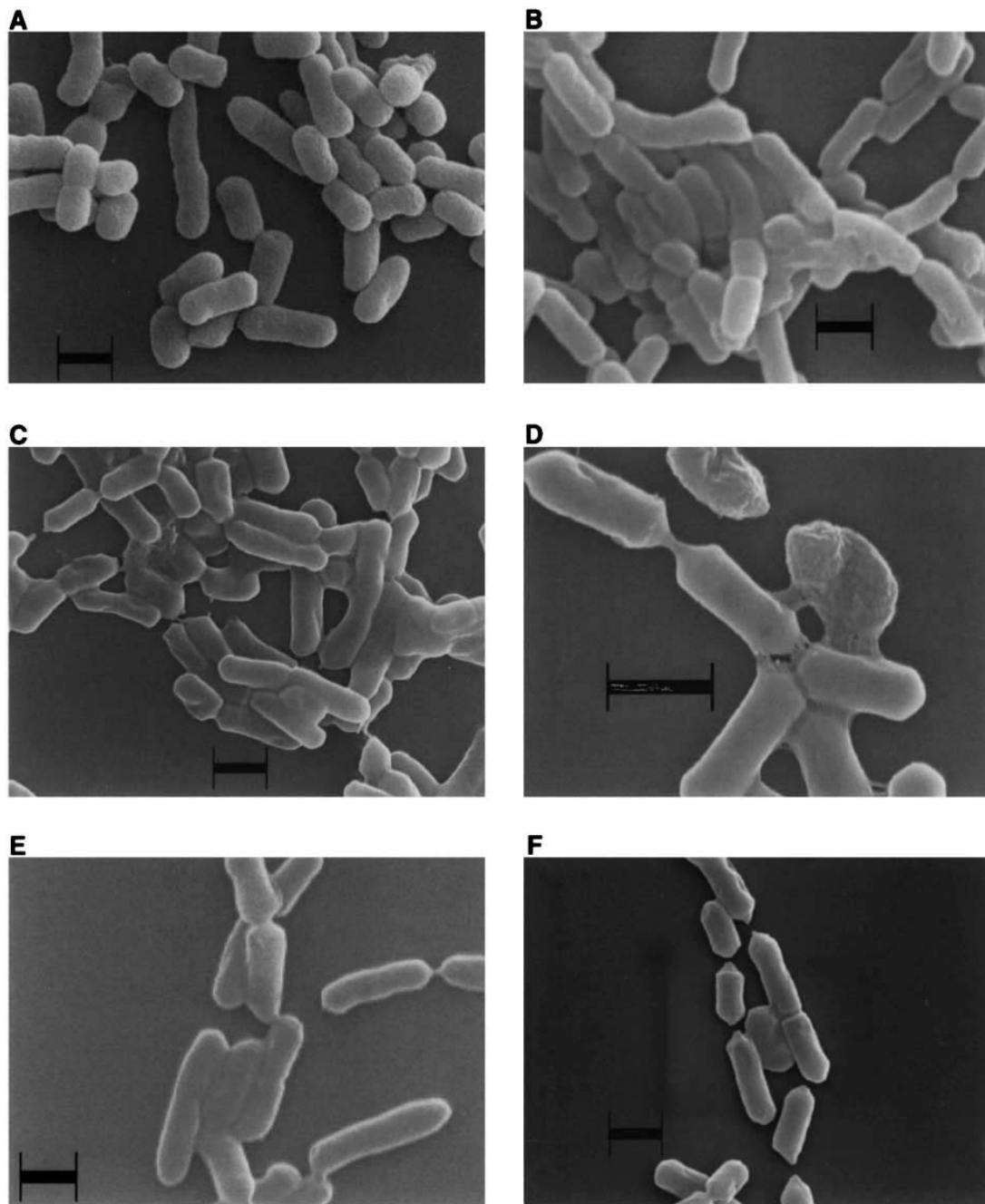
Fig. 3. Evaluation of aggregation by scanning electron microscopy (bar = 1 μm). A: *E. coli* ATCC 35401 pure culture. B: *L. paracasei* B21070 pure culture. C: *E. coli* ATCC 35401 plus *L. paracasei* B21070. D: Magnified portion of the same preparation as in C. E: *L. paracasei* B21060 pure culture. F: *E. coli* ATCC 35401 plus *L. paracasei* B21060.

that under these conditions the lactobacilli inhibited the growth of the pathogens *E. coli* and *S. enteritidis* after 24 h incubation: a decrease of 4–5 log and 6 log was observed for *E. coli* and *S. enteritidis*, respectively. In contrast, no significant difference compared to the control was observed for *V. cholerae*. Nevertheless, this apparently negative result gave us a useful indication that the experimental conditions were well chosen. In particular, the refreshment of the culture media proved effective in keeping constant the pH value, since it is known that *V. cholerae* does not grow at pH below 6 [15], a level which is rapidly obtained in any uncontrolled *Lactobacillus* culture, but that we wanted to avoid in order to find specific inhibition effects for our strains. In addition, it is worth noting that the growth of the *Lactobacillus* strains was not influenced by the simultaneous presence of any pathogen. Similar results were observed at 48 h.

In another series of experiments, lactobacilli from an initial overnight culture were coincubated with the pathogens. After 24 and 48 h of coincubation the bacterial counts of both pathogens and lactobacilli were evaluated. The results (Table 2 and Fig. 1, bottom) show that the *Lactobacillus* strains were effective in inhibiting the growth of the harmful microorganisms. Indeed, the growth of both *E. coli* and *S. enteritidis* was reduced by 6 log compared to the controls. The same degree of inhibition was observed both at 24 (Fig. 1, bottom) and 48 h (data not shown). As regards *V. cholerae*, a small reduction (1 log) in the pathogen count was observed after 48 h with lactobacilli grown overnight.

Fig. 2 reports how a mixture of the *Lactobacillus* strains B21060, B21070 and B21190 interfered with the growth of each pathogenic strain. The mixture of lactobacilli exerted a larger effect than individual strains alone. In fact, the mixture was able to almost completely inhibit the growth of *E. coli* and *S. enteritidis* as assessed at both 24 and 48 h. For both pathogens a 7 log decrease in growth compared to proper controls was observed. The same *Lactobacillus* mixture did not significantly affect the growth of *V. cholerae*. Similar results were observed with various combinations of two *Lactobacillus* strains (data not shown). The combinations of *Lactobacillus* strains did not mutually influence each other's growth.

3.2. Antimicrobial supernatant activity

Using both the well-diffusion assay and the paper-disk assay, no inhibition of enteropathogenic strains was observed for any of the tested supernatants.

3.3. Aggregation assay

Coaggregation of lactobacilli with *E. coli* was evaluated following the procedures described in the literature [13] and by direct SEM observations. At first, *E. coli* strain ATCC 25922 was used to standardize the methodology. Subsequently, *E. coli* strain ATCC 25922 was replaced by strain ATCC 35401, the strain used in the coculture experiments presented above. The results are reported in Table 3 together with those of the autoaggregation experiments. In cases when autoaggregation was not negligible, SEM observations allowed us to distinguish autoaggregates from coaggregates, so that the reported coaggregation scores are the net results. In most coaggregates SEM showed the presence of large contact areas between lactobacilli and pathogens (Fig. 3).

The coaggregation scores ranged from maximal (in the case of B21070 for both pathogens) to partial (B21060 for *E. coli* ATCC 35401). Even strain B21060, which exhibited no autoaggregation, showed partial/good aggregation with the pathogens. This strain clearly showed a slightly different degree of coaggregation towards the two pathogens. B21070 turned out to be the most coaggregating strain with both pathogens; coaggregation was so pronounced that further experiments are being designed to study this phenomenon more fully.

4. Discussion

In the present study we have shown that the *Lactobacillus* strains B21060, B21070 and B21190, which have recently been isolated from the feces of newborns, effectively inhibit the growth of both *E. coli* and *S. enteritidis*, either when inoculated simultaneously or when cultured overnight and then incubated with the pathogens. In addition, mixtures of these *Lactobacillus* strains showed a cumulative inhibitory effect. In contrast, in all cases, the growth of the

lactobacilli was not influenced by the presence of the pathogens.

In the case of *V. cholerae*, no significant difference compared to the control was noticed when inoculated simultaneously with the lactobacilli. A small inhibitory effect of *V. cholerae* was observed after 48 h incubation with lactobacilli grown overnight. This lack of inhibition on *V. cholerae* was also due to our unlimiting experimental design, which provided for medium refreshment every 8–10 h, and therefore avoided the drop in pH that is characteristic for lactic acid producing bacteria and is badly tolerated by *V. cholerae* [15]. Previous findings [16] have shown that some bacteriocin producing *Lactobacillus* strains, although able to inhibit a variety of pathogenic bacteria, did not influence the growth of both *Salmonella* sp. and *V. cholerae*, when the effect of acids was excluded.

Lactobacilli may exert their antibacterial activity through the production of lactic acid and other metabolites such as hydrogen peroxide and short chain fatty acids. Also specific antibacterial compounds such as antibiotics or bacteriocins have been identified in the culture medium of several lactic acid producing bacteria. The mechanisms by which our strains inhibit pathogen growth are not fully understood at present. However, it is worth noting that in our tests the culture medium was periodically replaced by freshly prepared medium in order to keep the pH value constant over time. Consequently, the inhibition of the pathogens could not be ascribed simply to acidification of the culture medium due to the production of lactic acid. In addition, no inhibition of either pathogens or lactobacilli was observed by both the well-diffusion and paper-disk assays that were performed using the supernatants from cultures of lactobacilli incubated for 48 h in MRS broth. These experiments do not enable us to rule out the production of bacteriocins or antibiotics by our strains but suggest that if such compounds are produced they are too dilute in the culture medium to explain the observed inhibition.

Bacterial coaggregation was therefore considered among the possible mechanisms. Reid et al. [13] showed that certain *Lactobacillus* strains undergo coaggregation with uropathogens and suggested that this phenomenon is an important factor in the establishment and maintenance of a healthy urogenital flora. We found that our *Lactobacillus* strains coaggregate with both uropathogenic strain *E. coli* ATCC 25922 and enterotoxigenic strain *E. coli* ATCC 35401, although to different degrees. As a consequence of this phenomenon lactobacilli and the pathogen develop large contact areas. Possibly the inhibitory activity of certain metabolites is exacerbated in these areas ('inhibitory microenvironment'). In addition, contacts between bacterial membrane surfaces may create continuity zones between the two bacterial cytoplasms leading possibly to the transfer of intracellular metabolites.

Other studies, which are currently in progress, have shown that these *Lactobacillus* strains isolated from the feces of healthy newborns and weaned infants are endowed with promising probiotic characteristics, such as acid and bile resistance. In in vitro experiments they show a surprisingly high adhesion to both buccal and intestinal epithelial cells [11]. In this paper, it has been demonstrated that the same strains are able to inhibit the in vitro growth of the pathogenic strains *E. coli* and *S. enteritidis*. In addition, it is possible that coaggregation is involved in this process. In conclusion all these data suggest that *Lactobacillus* bacteria may rely on multiple factors in defeating pathogens.

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