Probiotic activities of *Lactobacillus casei rhamnosus*: in vitro adherence to intestinal cells and antimicrobial properties

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**Abstract** – The interest of probiotics as remedies for a broad number of gastrointestinal and other infectious diseases has gained wide interest over the last few years, but little is known about their underlying mechanism of action. In this study, the probiotic activities of a human isolate of *Lactobacillus casei* subsp. *rhamnosus* strain (Lcr35) were investigated. Using intestinal Caco-2 cell line in an in vitro model, we demonstrated that this strain exhibited adhesive properties. The inhibitory effects of Lcr35 organisms on the adherence of three pathogens, enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and *Klebsiella pneumoniae*, were determined. A decrease in the number of adhering pathogens was observed, using either preincubation, postincubation or coincubation of the pathogens with Lcr35. Moreover, the antibacterial activities of cell-free Lcr35 supernatant was examined against nine human pathogenic bacteria, *ETEC*, *EPEC*, *K. pneumoniae*, *Shigella flexneri*, *Salmonella typhimurium*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Clostridium difficile*. The growth of all strains was inhibited, as measured by determining the number of viable bacteria over time, but no bactericidal activity was detected in this in vitro assay. Together, these findings suggest that this probiotic strain could be used to prevent colonization of the gastrointestinal tract by a large variety of pathogens. © 2001 Éditions scientifiques et médicales Elsevier SAS

**Lactobacillus casei subsp. rhamnosus / probiotics / adherence / gut**

1. **Introduction**

   Maintenance of the intestinal ecological flora is important in preventing disease by controlling overgrowth of potentially pathogenic bacteria. Widespread prescription of antibiotics not only has led to an increase in antibiotic-resistant bacterial pathogen strains, but is often associated with the disruption of the protective flora, leading to predisposition to infections. For these reasons, the control of infections through a nonantibiotic approach is urgently needed and bacterial replacement therapy using nonpathogenic bacteria from the natural flora represents an promising alternative. Probiotic bacteria are live microorganisms belonging to the natural flora with no pathogenicity, but with functions of importance to the health and well being of the host. It is increasingly accepted that these bacteria might represent effective tools for controlling overgrowth of pathogens and thereby control or prevent infections. Indeed, numerous in vitro and in vivo studies performed with different genera of probiotics bacteria have shown the capacities of these bacteria to interfere with both growth and virulence properties of various pathogens [1, 8, 22, 33, 34]. The underlying mechanism remains unclear; it could be due to pH reduction linked to organic acid production, to intrinsic activities of metabolites [23] or to synthesis of antimicrobial substances [5, 6, 31]. In addition, adherence of probiotics to intestinal epithelial cells and the ensuing temporary colonization of the gut is probably of crucial importance for their beneficial health effect [4, 17, 20].

   The aim of this study was to further characterize a *Lactobacillus casei rhamnosus* strain successfully exploited commercially as a pharmaceutical product for its antidiarrheal properties for more than 20 years. No pathogenic behavior has ever been associated with this strain and it shows high resistance to technological processes, two properties required for probiotic la-
beling. We investigated the in vitro adherence capacities of this strain using human intestinal cells as well as its ability to impair adherence of several pathogens. Furthermore, the antimicrobial activity of metabolic products from this probiotic was determined against a wide variety of Gram-positive and Gram-negative human pathogens.

2. Materials and methods

2.1. Bacterial strains

*L. casei rhamnosus* (Lcr35) was obtained from Laboratoires Lyocentre, France and was grown in De Man, Rogosa, Sharpe (MRS) medium (Oxoid Ltd, Basingstoke Hampshire, England) at 37°C in the presence of CO₂ (Biomérieux, Marcy l’Etoile, France). *Escherichia coli* H10407 and 2348/69 were used as reference strains for respectively enterotoxigenic (ETEC) and enteropathogenic (EPEC) *E. coli* strains [2, 12]. *Klebsiella pneumoniae* LM21 was previously described [14]. *Enterococcus faecalis*, *Shigella flexneri*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Enterobacter cloacae* and *Clostridium difficile* were clinical isolates kindly provided by J. Sirot. The nonlactic bacteria were grown in either Luria-Bertani (LB) medium, Schaedler broth or Columbia agar supplemented with 5% sheep blood (Biomérieux, Marcy l’Etoile, France) at 37°C under aerobic or anaerobic conditions.

2.2. In vitro adherence assays

The adherence of the Lcr35 strain was examined using Caco-2 intestinal cells, a human colon carcinoma cell line that expresses several markers characteristic of normal small intestinal villous cells [27]. Cells were routinely grown in Dulbecco modified Eagle’s minimal essential medium (DMEM) supplemented with 20% inactivated fetal calf serum (PolyLabo, Strasbourg, France). Monolayers of Caco-2 cells were seeded at a concentration of 1.4 x 10⁴ cells/cm² in 24-well Nunc tissue plates (PolyLabo, Strasbourg, France), and incubated at 37°C in a 10% CO₂ air atmosphere. Cells were used at late post-confluence culture after 15 days, with a change of medium every other day. Just before use, the monolayer was washed twice with PBS and 0.5 mL of DMEM was added to each well. Cells from two wells were collected and their concentration was determined under optical microscopy using a Malassez chamber; the number of cells was within the range of 1 x 10⁶ to 2 x 10⁶/well.

Lcr35 was grown overnight in MRS broth, washed once with PBS and resuspended in 0.5 mL of either DMEM supplemented with 20% fetal calf serum or spent culture supernatant. The final concentrations of 2 x 10⁸, 10⁹, 2 x 10⁹ and 10¹⁰ CFUs/mL were used in order to test the multiplicities of infection (MOI) of respectively 100, 500, 1000 and 5000. These suspensions were added to each well of the tissue culture plate and incubated at 37°C in a 10% CO₂ air atmosphere. After 1 h of incubation, the monolayers were washed three times with PBS, the cells were lysed by addition of Triton-X100 0.05% solution and the number of viable adhering bacteria was determined by plating serial dilutions on MRS agar plates. For microscopic examination of the interactions between Lcr35 and Caco-2 cells, intestinal cells were seeded on glass coverslips placed in the same tissue culture plates. After the incubation period, cells were washed as previously described, fixed with methanol and stained with 20% Giemsa. The enterotoxigenic strain *E. coli* H10407 was used as a positive control at a MOI 100 and each adhesion assay was conducted in triplicate.

2.3. Adherence inhibition assay

Experiments examining the inhibition of adherence were performed with *E. coli* H10407, *E. coli* 2348/69 and *K. pneumoniae* LM21 which showed significant capacities to adhere to Caco-2 cells. Three different procedures were used in order to differentiate exclusion, competition or displacement of the pathogens by Lcr35. For exclusion tests, Caco-2 cell monolayers were cultured and washed as previously described and incubated with lactobacilli (10⁹/mL, MOI 500) for 30 min. Afterward, nonadhering lactobacilli were removed, *K. pneumoniae* or *E. coli* H10407 or *E. coli* 2348/69 (10⁹/mL, MOI 100) was added and incubation was continued for a further 30 min. For competition tests, lactobacilli and any of the pathogens and the intestinal cells were mixed and incubated for 1 h. For displacement tests, the pathogens and the Caco-2 cells were incubated together for 30 min; after removal of nonadhering pathogens lactobacilli were added and incubation was continued for a further 30 min. Lactobacilli were incubated with their
spent culture (1/2 volume) mixed with DMEM supplemented with 10% fetal calf serum. Adherence of the pathogens was performed in the presence of 0.5% methyl mannose to avoid type-1-mediated adherence, a nonspecific interaction observed with most Enterobacteriaceae. The number of bacteria adhering to the intestinal cells was determined as described above, by plating serial dilutions on LB or MRS agar plates. Each assay was conducted at least twice with two determinations per assay.

2.4. Detection of antimicrobial activity

Cells of Lcr35 from an overnight culture were pelleted at 10,000 g for 30 min at 4°C and the supernatant fluid was collected and filtered (Millex HV, 0.45 µm pore size) to remove any remaining bacteria. Strains to be tested were cultured overnight in the appropriate medium. The supernatant was discarded, bacteria were washed once with phosphate-buffered saline (PBS) and resuspended in LB (E. coli, K. pneumoniae, E. faecalis, S. flexneri, S. typhimurium, P. aeruginosa, E. cloacae) or Schaedler (C. difficile) broth to a density of 4 × 10^7 colony forming units (CFUs)/mL. The assay was performed by incubating 250 µL of this suspension (approximately 10^7 CFUs) with 250 µL of Lcr35 supernatant at 37°C, except for C. difficile and P. aeruginosa where only 125 and 100 µL of Lcr35 supernatant were used, respectively. Initially and then at predetermined intervals, aliquots were removed, serially diluted and plated on LB or Columbia blood agar to determine bacterial colony counts. Control experiments were performed by incubating the same amount of pathogens with MRS broth medium instead of Lcr35 supernatant and all assays were performed twice.

3. Results

Adherence of Lcr35 to Caco-2 cells was measured using different MOIs; 100, 500, 1000 and 5000 bacteria/Caco-2 cell. The number of adhering bacteria was dependent on the number of bacteria added and reached a maximum of 1.0 × 10^6 CFUs/mL with a MOI of 5000 (figure 1). In all cases, lower numbers of adhering bacteria were observed with Lcr35 than with the control E. coli strain, H10407 (1.4 × 10^6 CFUs/mL). In order to check the effects of molecules produced by Lcr35, adhesion assays were also performed in the presence of spent supernatant.

Whatever the MOI, only slightly higher numbers of adhering bacteria were obtained when the bacteria were incubated with their filtered spent supernatant (figure 1).

Live Lcr35 bacteria were also examined for their ability to impair the adherence of three pathogens to Caco-2 cells, an enterotoxinogenic E. coli strain (ETEC H10407), an enteropathogenic E. coli strain (EPEC 2348/69) and K. pneumoniaeLM21, a clinical isolate previously tested in an adherence assay [14]. Using a MOI of 100, the levels of adhesion of these pathogens were respectively 4.8 × 10^5, 6.0 × 10^5 and 7.1 × 10^5 CFUs/mL. In the presence of Lcr35, the adhesion of the pathogens was reduced (see figure 2), regardless of the protocol used: coincubation, post incubation or preincubation of Lcr35. The viability of the pathogens during the period of incubation was not affected (data not shown) and therefore did not account for the observed decrease in the adhesion capacity.

The antibacterial activity of Lcr35 was examined using nine bacterial pathogens, both Gram-negative and Gram-positive, aerobes and anaerobes: ETEC H10407, EPEC 2348/69, K. pneumoniae LM21, S. flexneri, S. typhimurium, E. cloacae, P. aeruginosa, E. faecalis and C. difficile. The viability of all these microorganisms was verified during a 7-h time course of incubation with L. casei rhamnosus supernatant. No or only slight inhibition effect was observed after 3 h of incubation, but after 5 h, the growth of all the bacterial species was slowed in the presence of Lcr35 supernatant (figure 3). The level of viable bacteria remained stable (inoculum 10^7 CFUs/mL), indicating that the substance present in Lcr35 supernatant...
did not lyse the bacteria but rather impaired their division.

4. Discussion

The *Lactobacillus* strain used in this study was initially isolated from the intestinal fluid of a child and was later characterized as *L. casei* subsp. *rhamnosus* using biochemical and metabolic parameters. Recent analysis of the nucleotide sequences of its 16S- and 16-23S intergenic regions confirmed this taxonomical identification (data not shown). This group of lactic acid bacteria contains the most thoroughly characterized probiotics strains, and therefore represents the most suitable group of bacteria for microbial interference treatment in preventing infectious diseases [3].

Since bacterial adhesion to intestinal cells is considered one of the most crucial selection criteria for probiotic strains [11], we determined the adherence capacities of the Lcr35 strain. The investigation was performed using Caco-2 cells, a cell line used as an in vitro model for intestinal epithelium [27]. Adherence of Lcr35 was dose- and concentration-dependent and reached a maximum of $10^6$ CFUs/mL with a MOI of 1/5000. Similar concentration dependent adherence levels had been observed previously with several *Lactobacillus* strains [16, 32], and probably reflect a nonsaturating interaction process with the epithelial cells. Several studies involving different *Lactobacillus* strains and Caco-2 cells have been previously published and slightly lower adhesion levels were obtained in our study compared to others [16, 19, 29, 32]. However, the adhesion levels obtained are not strictly comparable because of differences in the assay procedures. This could also be related to the fact that we determined only the number of viable adhering bacteria rather than the total number of adhering bacteria. A similar difference in the adhesion levels with the positive control, *E. coli* H10407, was observed between the different studies and therefore would support this hypothesis.

Adherence of the probiotic strain *Lactobacillus acidophilus* LA1 to Caco-2 cells was shown to be influenced by the presence of proteinaceous promoting factor present in the bacterial culture supernatant [4]. When adhesion assays were performed with Lcr35 in the presence of spent supernatant, only slight increases in the adhesion levels were observed (Figure 1). More recently, a nonproteinaceous component of the bacterial surface of this *Lactobacillus* LA1 strain was also shown to participate in the interactions with Caco-2 cells [15]. This would indicate that different structures are implicated in the interactions between probiotic strains and epithelial cells, reflecting the heterogeneity of the *Lactobacilli* group members.

Adherence of bacteria to the epithelial intestinal cells is an important prerequisite for colonization by microorganisms and virulence manifestations [13]. Inhibiting the adhesion of pathogenic bacteria to their receptor could decrease the intestinal colonization and in consequence modify the process of pathogenicity. In this study, we showed that Lcr35 interfered with the adhesion process to Caco-2 cells of three pathogens, enteropathogenic and enterotoxigenic *E. coli* and *Klebsiella pneumoniae*. The adherence of the three pathogens was decreased by addition of Lcr35, regardless of whether the Lcr35 was added before, during or after the incubation with the pathogen (Figure 2). The presence of Lcr35 may impede the access of pathogens to tissue receptors by steric hinderance and that may explain the decrease of adhesion of the pathogens in the presence of Lcr35. However, when tested individually, the level of adhesion of the pathogens were at least 10 times higher than those of Lcr35 at similar MOI. Therefore, this hypothesis could not account for the whole inhibition process. It has been recently demonstrated using the intestinal cell line HT29 that an increased ex-
Expression of mucins was elicited when the cells were incubated with a probiotic [24]. Since Caco-2 cells can also express significant levels of mucins [24], the presence of Lcr35 could interact with the level of mucins produced and thus impair the adhesion of pathogens. It is also possible that Lcr35-specific products inhibit the adhesion of Enterobacteriaceae; it has been previously shown that production of biosurfactants by some strains of Lactobacillus can prevent adhesion of pathogens to intestinal cells [28, 34]. In any case, the inhibition adhesion observed in our study results from a nonspecific mechanism, since it occurs at similar levels whatever the pathogen tested and the incubation parameters. This result differs from those obtained by Bernet et al. where a significantly lower inhibition was observed in postincubation experiments using E. coli H10407 and a L. acidophilus probiotic strain [4]. Since the protocols used in both studies are rather similar, the difference observed may be due to differences in the probiotic strain. Very little is known about the mechanism of inhibition of adhesion/invasion of pathogens by Lactobacilli, despite abundant literature including enteropathogens [4, 7, 9, 17]. However, using animal models, experiments showed an in vivo antagonist effect after oral administration of some probiotics strains [17, 20, 26], suggesting new therapeutic possibilities.

The antibacterial activity of probiotics may also partially explain their protective in vivo effect. In this study, we were able to demonstrate a growth-inhibitory effect of cell-free supernatant from Lcr35 against nine bacterial species including both Gram-negative and Gram-positive bacteria, aerobes and anaerobes. The inhibitory effect of Lactobacillus strains is variable even within a same species [19, 34]. Because of the wide range of activity of Lcr35, the antimicrobial mechanism involved is unlikely to be the production of classic bacteriocins, proteinaceous compounds produced by lactic acid bacteria that exhibit a bactericidal effect against taxonomically closely related bacteria [18, 21]. Other antibacterial substances referred to as ‘bacteriocin-like’, exhibiting broad activities and produced by different species of Lactobacilli, have been described [5, 10, 25, 30]. Moreover, treatment of Lcr35 culture supernatant with either protease or heat (110°C for 1 h) did not affect its activities and gel filtration experiments indicated the molecular mass of the active substance was below 3 kDa (data not shown). Thus the growth inhibition observed in this study could be due to the production by Lcr35 of a bacteriocin-like substance. Nevertheless, we cannot exclude that the production of organic acids by Lcr35 also plays a role in the antagonistic effect observed; further experiments are necessary to identify the chemical nature of the antibacterial compounds.

In conclusion, the lactobacilli used in this study may protect the intestinal epithelium through a series of barriers and interference mechanisms. Conse-
quently, they may be excellent candidates for eventual use as prophylactic and therapeutic agents. The protective role of probiotics against intestinal colonization by pathogenic microorganisms has gained more credibility and may represent a new effective tool to control pathogen overgrowth inside the gastrointestinal tract of patients. The extent to which these in vitro results correspond to in vivo conditions remains to be determined; clinical trials are needed to assess the benefits of probiotic organism consumption in the management of these diseases.

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References


