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Original article

Heat-killed *Lactobacillus acidophilus* inhibits adhesion of *Escherichia coli* B41 to HeLa cells

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Summary — Escherichia coli B41 (O101: K99: F41: ST⁺) adheres to HeLa 229 cells in a diffuse pattern. Heat-killed (100–105 °C) Lactobacillus acidophilus (Lactéol strain) was found to inhibit this adhesion in a dose-dependent manner. This inhibitory action was lost after lysis of the *L* acidophilus, suggesting steric hindrance of *E coli* adhesion sites rather than competition for a common binding site. A thermostable factor (100–105 °C) excreted by *L* acidophilus into the medium may be required for the adhesion of *L* acidophilus to HeLa cells, and for the inhibition of adhesion of *E coli* to these cells.

Escherichia coli / Lactobacillus acidophilus / adhesion / inhibition

Résumé — Inhibition de l'adhésion de Escherichia coli B41 aux cellules HeLa par Lactobacillus acidophilus tué. Escherichia coli B41 (O101: K99: F41: ST+) adhère aux cellules HeLa 229 en donnant une adhésion de type diffus. L'addition simultanée sur le tapis cellulaire de Lactobacillus acidophilus (souche Lactéol) tué par chauffage à 100–105 °C en suspension dans son milieu de culture inhibe l'adhésion de E coli de façon proportionnelle à la concentration en L acidophilus mise en œuvre. Lorsque L acidophilus a été soumis à un broyage (billes de verre, puis ultrasons), l'effet inhibiteur n'existe plus. Le mécanisme d'inhibition ne ferait donc pas appel à une compétition pour un site commun d'adhésion, mais serait plutôt dû à un encombrement stérique des sites d'adhésion de E coli par les lactobacilles adhérents. L'adhésion de L acidophilus aux cellules HeLa et, de ce fait, l'expression de son pouvoir inhibiteur, semblent nécessiter la présence d'un facteur excrété par L acidophilus dans son milieu de culture et thermostable à 100–105 °C.

Escherichia coli / Lactobacillus acidophilus / adhésion / inhibition

INTRODUCTION

An essential stage in the pathogenesis of intestinal infections, especially diarrhoea of bacterial origin, involves the adhesion of the micro-organisms concerned to the intestinal epithelial cells. In principle, bacterial diarrhoea could be treated by preventing adhesion of enteropathogenic bacteria. Three main methods have been described: i) vaccination against bacterial adhesion factors (Levine *et al*, 1983; Evans *et al*, 1984; Runnels *et al*, 1987; Duchet-Suchaux, 1988; Sack *et al*, 1988); ii) administration of antibiotics which inhibit expression of adhesion factors (De-

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necke *et al*, 1985; Chopra and Hacker, 1986); and iii) oral administration of substances containing structures similar to those of the adhesion factors of pathogens (*eg* non enterotoxinogenic strains possessing the same adhesion factors, carbohydrates or glycoproteins) (Duval-Iflah *et al*, 1982; Borriello and Barclay, 1985; Neeser *et al*, 1988) or structures that mimic receptors of the intestinal mucosa (Mouricout *et al*, 1986).

Oral administration of certain strains of Lactobacillus has also been found to inhibit implantation of various pathogens in the digestive tract (Watkins et al, 1982; Lidbeck et al. 1987; Itoh and Freter, 1989), and has been shown to have a beneficial effect on diarrhea in both man and animals (Beck and Necheles, 1961; Bodilis, 1983; Clements et al, 1983; Gorbach et al, 1987; Fuller, 1989; Perdigon et al, 1990). This action may be due to non-specific competitive inhibition of the adhesion of pathogenic strains to epithelial cells of the digestive tract. Inhibition of this type has been demonstrated in vivo in the pig (Muralidhara et al, 1973; Barrow et al, 1980) and mouse (Moyen et al, 1986), and in vitro with epithelial cells and isolated enterocytes (Makrides and MacFarlane, 1982; Conway, 1988).

In a previous study (Fourniat et al, 1986), we showed that oral administration of a suspension of a heat-killed Lactobacillus acidophilus (Lactéol strain) increased survival of suckling OF1 mice infected with Escherichia coli B41 (O101: K99: F41: ST⁺). This strain of *E coli* of bovine origin, like all enterotoxinogenic (ETEC) strains of E coli, possesses 2 types of virulence factors (Bertin, 1985): adhesion factors which are responsible for binding to epithelial cells in the intestine (antigens K99 and F41), and an enterotoxin (thermostable enterotoxin = ST). Two possible mechanisms for this beneficial effect of L acidophilus can be evoked: an action on the adhesion of *E coli* B41, or an inhibition of production of the ST toxin. The first possibility is supported by the results of Moyen *et al* (1986), who found that oral administration of a preparation of heat-killed *L acidophilus* to mice monoxenic to *Campylobacter jejuni* reduced the number of *C jejuni* in the colonic mucosa (< 10^2 C *jejuni*/g intestine *versus* $10^{6.2}$ C *jejuni/g* intestine in mice treated with sterile water) on the first day after treatment. Similar results were observed in the ileum 5 days after treatment with heat-killed *L acidophilus*.

We present here the results of a preliminary study on the influence of killed *L acidophilus* (Lactéol strain) on the adhesion of *E coli* B41 to HeLa cells in culture. HeLa cells were chosen because they are commonly used to study the adhesion of intestinal bacteria (Scalestsky *et al*, 1984; Milon *et al*, 1990) and their culture is relatively easy.

MATERIALS AND METHODS

Bacterial strains

The strain of Escherichia coli B41 (O101: K99: F41: ST+) of bovine origin (P Gouet, INRA, Theix, Ceyrat) was kept under paraffin oil at 4 °C after being cultured on a Mueller-Hinton agar slope (Bio-Mérieux, Marcy-l'Etoile). On the day before each experiment, the strain was reseeded on minimum broth according to the method of De Graaf and Roodra (1982). This medium is the most suitable medium (with Minca medium) for obtaining high production of K99 and F41 antigens (De Graaf and Roodra, 1982). The K99 antigen production in the used broth was verified using an ANI E coli K99 kit (Sirebio Teck, Le Blanc-Mesnil). After incubation for 18 h at 37 °C, the culture was centrifuged. The bacterial pellet was washed once in Hanks medium (Flow, Puteaux) and then resuspended in the same medium buffered to pH 7.0-7.4 with Hepes (Flow) to obtain a bacterial concentration of around 2 x 10⁹ cells/ml.

The Lactobacillus acidophilus Lactéol strain was first isolated from a human stool specimen and heat-killed in its culture medium. It has been used as an antidiarrhoeal drug in man and animals since 1907 (Gautrelet, 1968). This strain (supplied by the Laboratoire du Lactéol, Houdan) was kept by seeding once a week in a whey-broth (atomized cheese-making quality whey 100 g/l, Solaipat, Vimoutiers) used for the industrial production.

Preparations of L acidophilus tested

Suspension of *L acidophilus* killed by heating in spent culture medium

Most of the experiments were conducted with the suspension of heat-killed *L* acidophilus prepared and supplied by Laboratoire du Lactéol. After culture for 16–18 h in the industrial wheybroth, the culture was centrifuged and the bacterial pellet resuspended at high concentration $(1-2 \times 10^{10} L acidophilus/ml)$ in the spent broth culture supernatant. This suspension was adjusted to pH 5.0 ± 0.3 with CaCO₃ powder, and then autoclaved for 10 min at 100–105 °C. The suspension used in the experiments contained 1.2 x $10^{10} L$ acidophilus/ml counted in a Petit-Salumbeni cell (Préciss, Arcueil) on microscopic observation.

A portion of this suspension was treated to lyse the bacteria as follows: shaking with glass beads (0.22-mm diameter) in a Virtiss homogenizer (500 rpm) for 1 h, and then sonication (Branson B30) for 30 min. After these treatments, the suspension was devoid of whole bacteria (checked microscopically).

Another portion was centrifuged. The supernatant (designated as supernatant S) was kept at 4 °C to study its influence on the *E coli* adhesion and on the anti-adhesive effect of *L acidophilus* heat-killed in water.

Suspensions of *L acidophilus* killed by heating in water

A suspension of *L* acidophilus was prepared as described above except that the spent broth was replaced with distilled water. This suspension was then freeze-dried (2.7×10^{11} heat-

killed *L* acidophilus/g solid). For the adherence inhibition assays stock suspensions of the freeze-dried bacteria $(1-1.5 \times 10^{10} L acidophilus/ml)$ were prepared either in sterile distilled water or in the supernatant S.

Suspension of live L acidophilus

On the day before each experiment, the strain of *L* acidophilus was seeded on whey-broth or Man–Rogosa–Sharpe broth (MRS broth, Merck, Nogent). After incubation for 18–24 h at 37 °C, the culture was centrifuged and the bacterial pellet was resuspended in the corresponding supernatant liquid to produce a concentration of $1-1.5 \times 10^{10}$ bacteria/ml.

Cells

The HeLa 229 cells (Flow) used in the experiments were cultured in 25-cm² Falcon flasks or in Leighton tubes containing a cover slip (11 x 24 mm) in Eagle minimum essential medium containing Earles salts (Flow) supplemented with 10% fetal calf serum (Flow), 5% 5 mM glutamine (Flow), 1% non-essential amino acids (Flow) and antibiotics (200 IU/ml penicillin, 40 μ g/ml streptomycin). The cells were used after culture for 48 h at 37 °C (confluent mono-layer).

The cultures in Falcon flasks were used for a quantitative study of *L* acidophilus influence on *E* coli adhesion, and the cultures in Leighton tubes were used for microscopic examination of bacterial adhesions.

Determination of the index of adhesion of E coli to HeLa cells by subculture method

The method described by Karam *et al* (1986) was used to count the viable bacteria adhering to cells. After removing the growth medium, the cell layer present in Falcon flasks was washed 3 times with phosphate buffered saline (PBS) pH 7–7.2. It was then covered with 4 ml of the suspension of *E coli* diluted in Hanks medium (1 x 10^9 *E coli*/ml, *ie* 5–10 times the

hesion index during preliminary assays by microscopic examination). After incubation for 1 h at 37 °C, the non-adherent bacteria were removed by 8 successive washings with PBS. The cell layer was then treated with trypsin to separate cells and remove adhering bacteria. The suspension was well shaken and then diluted in sterile glucose/saline (8.5 g NaCl, 1 g pancreatic casein peptone, and 1 g glucose/1 distilled water). The HeLa cells were counted in a Malassez hemocytometer (Préciss). The viable adhering E coli were counted in serial dilutions in trypticase soy agar medium (Merck). The adhesion index (AI) was expressed as the ratio of the number of viable adhering bacteria to the total number of cells (AI in CFU/cell).

Influence of L acidophilus suspensions on adhesion of E coli

Suspension of *L acidophilus* killed by heating in spent whey-broth

Adhesion of E coli to HeLa cells was determined in the presence of various concentrations of heat-killed L acidophilus: 1.2 x 109, 6×10^8 , 2.4 x 10⁸, 1.2 x 10⁸ and 6 x 10⁷ L acidophilus/ml in contact with the cells (the concentration of 1.2 x 109 L acidophilus/ml provided the maximal adhesion index during preliminary assays by microscopic examination with an Al of 22 bacteria/cell). Two ml of diluted L acidophilus suspension (prepared at 2 x final concentration in Hanks medium buffered with HEPES (pH 7-7.4)) were added to the cell layer grown in Falcon flasks immediately after addition of 2 ml of the suspension of E coli (2 x 10⁹ cells/ml). The adhesion test was carried out as described above.

Other suspensions of *L acidophilus* (lysed, heat-killed in water or live)

A single concentration was employed of $5-6 \times 10^8 L$ acidophilus/ml of medium in contact with the HeLa cells for all bacterial preparations. The experiment was conducted as described above.

Statistical analysis

For each test, the adhesion index of *E coli* was determined simultaneously in the absence (= control) and in the presence of *L acidophilus* (= assay) on similar cell layers. Each test was performed 5 times on different days. The results were analyzed using Student's *t*-test for paired series (assays *vs* controls) (Schwartz, 1963).

Microscopic examination of E coli *and* L acidophilus *adhesion*

Adhesion was also assessed in Leighton tubes using the conventional method by microscopic examination (Karam *et al*, 1986) in order to control the adhesiveness of studied bacteria.

After culture the cell layer was washed with PBS, then covered with 1 ml of bacterial suspension. After a 1-h incubation at 37 °C the nonadherent bacteria were eliminated by 8 washings with PBS. After fixation of cells with methanol. Gram staining and mounting on glass slides, the coverslips were examined under a light microscope (x1 000). For E coli the used bacterial suspensions contained 1 x 109 bacteria/ml. For L acidophilus the different suspensions studied (live, heat-killed) were used at a concentration of 5-6 x 108/ml. When E coli and L acidophilus preparations were added simultaneously on the cell layer, a mixture containing the same concentrations of bacteria/ml was used.

RESULTS

Adhesion of E coli B41 and of L acidophilus to HeLa cells

The B41 strain of *E coli* consistently adhered to HeLa cells with a mean adhesion index determined by sub-culture method of 19.5 ± 4.1 CFU/cell over a total of 35 experiments (1 x 10^9 *E coli/*ml in contact with the cells). Using the conventional method (microscopic examination after Gram staining), 40–50% of cells did not

appear to bind *E coli* B41 (< 5 bacteria/ cell). On the other hand, some cells bound a large number of *E coli* (up to 120 bacteria/cell) in a diffuse pattern over the whole surface of the cell. The mean number of *E coli* adhering per cell was 19.3 (mean of 3 experiments, 100 cells examined per slide).

As for *E coli*, only a fraction (50–60%) of the cells were observed to bind *L acidophilus* (live or heat-killed in spent medium, 5–6 x 10⁸ bacteria/ml in contact with the cells) on microscopic examination. The adhesion was also observed in a diffuse pattern with an Al of 17.2 bacteria/cell for heat-killed *Lactobacillus*, and 21.3 bacteria/cell for living *Lactobacillus* (means of 3 experiments, 100 cells examined per slide).

When the 2 species of bacteria were added on the HeLa cells, the cells which did not bind *L* acidophilus did not bind *E* coli either. For the other cells, the lacto-bacilli completely masked any *E* coli that might have bound. Furthermore, a fraction of heat-killed lactobacilli was Gram-negative. Consequently we chose to quantify *E* coli adhering to the HeLa cells by counting viable microorganisms on subculture.

Influence of L acidophilus suspensions on the adhesion of E coli B41 to HeLa cells

L acidophilus killed by heating in spent culture medium

The adhesion index of *E coli* B41 to HeLa cells was determined at 5 different concentrations of heat-killed *L acidophilus*. As shown in table I, the heat-killed *L acidophilus* inhibited adhesion of *E coli* in a dose-dependent manner, with 50% inhibition at 2.4 x 10^8 *L acidophilus*/ml in contact with the cells.

The lactobacilli killed by heating in their culture medium and then lysed did not inhibit adhesion of *E coli* B41 (table II). The supernatant of the suspension of *L acidophilus* killed in culture medium was also without effect on the adhesion of *E coli* B41 (table II).

L acidophilus killed by heating in water

The water suspension of *L* acidophilus killed by heating in water did not affect the adhesion of *E* coli B41 to HeLa cells. How-

Concentration of L acidophilus	Adhesion index ª (CFU/cell)	Inhibition ^a (in %)	٩٥	
0	16.7 ± 0.5	0	_	
1.2 x 10 ⁹	1.6 ± 0.2	90.8 ± 1.3	P < 0.001	
6.0 x 10 ⁸	3.7 ± 1.0	77.8 ± 6.5	<i>P</i> < 0.001	
2.4 x 10 ⁸	8.4 ± 1.0	49.0 ± 6.9	0.001 < <i>P</i> < 0.01	
1.2 x 10 ⁸	13.0 ± 0.9	21.8 ± 5.5	0.02 < <i>P</i> < 0.05	
6.0 x 10 ⁷	14.0 ± 1.1	16.0 ± 6.3	0.05 <i>< P <</i> 0.10	

Table I. Influence of *L* acidophilus (Lactéol strain) killed by heating in spent whey-broth on the adhesion of *E* coli B41 (1 x 10^9 /ml in contact with the cells) to HeLa cells.

^a Mean ± SD of the mean from 5 different experiments. ^b Comparison of adhesion index means in paired series (assay *vs* control).

	Adhesion index ^a		Inhibition ^a	٩٥
	Control	Assay	(in %)	
L acidophilus c heat-killed in spent whey-t	proth			
Native suspension	16.7 ± 0.5	3.7 ± 1.0	77.7 ± 6.5	< 0.01
Supernatant (S) of this suspension	21.3 ± 1.9	21.0 ± 2.3	2.1 ± 2.6	NS
Suspension after bacterial lysis	19.7 ± 1.2	17.8 ± 1.9	10.4 ± 5.2	NS
L acidophilus ^c heat-killed in water				
Suspension in water	19 ± 1.7	17.9 ± 1.5	5.6 ± 3.0	NS
Suspension in supernatant S	20.6 ± 2.4	7.7 ± 0.5	59.8 ± 6.9	< 0.01
Live <i>L acidophilus</i> ^c				
Grown in whey-broth	21.9 ± 2.6	4.8 ± 0.4	78 ± 1.3	< 0.01
Grown in MRS broth	17.9 ± 1.9	6.3 ± 0.4	62.6 ± 5.2	< 0.01

Table II. Adhesion of *E coli* B41 (1 x 10^9 /ml in contact with the cells) to HeLa cells in the presence of different preparations of *L acidophilus* Lactéol strain (adhesion index expressed in CFU/ell).

^a Mean \pm SD of the mean from 5 different experiments. ^b Comparison of adhesion index means in paired series (assay *vs* control). NS: not significant (*P* > 0.10). ^c *L* acidophilus was used at a concentration of 5–6 x 10⁸/ml in contact with the cells.

ever, inhibition was observed when these bacteria were resuspended in the supernatant of lactobacilli killed in spent wheybroth (table II). On microscopic examination, adherence of the lactobacilli to HeLa cells was only observed with this bacterial preparation in the presence of supernatant from *L acidophilus* killed in culture medium or of culture medium of live cultures. In the presence of virgin culture broths, the lactobacilli killed in water did not adhere to the HeLa cells.

Live L acidophilus

At a concentration of 5–6 x 10^8 bacteria/ml in the medium in contact with the HeLa cells, live *L* acidophilus led to a comparable inhibition of adhesion to that observed with the same concentration of *L* acidophilus killed by heating in spent whey-broth (table II). No significant difference in effect from bacteria cultured in either of the 2 media (industrial whey or MRS broth) was observed. On microscopic examination, the live lactobacilli only adhered to the HeLa cells in the presence of medium in which they had been cultured.

DISCUSSION

The beneficial effect of oral administration of lactic bacteria in the prevention and treatment of diarrhea has been attributed to the production of antimicrobial substances, although no active substance has yet been identified within the intestine (Fuller, 1989). Another possibility, which is currently under investigation, is a competitive inhibitory effect on the adhesion of pathogens to epithelial cells of the digestive tract. It has been shown, for example, that adherent strains of *Lactobacillus* inhibit the adhesion of various microorganisms to epithelial cells *in vitro*. *L casei* NCTC 10302 inhibits the adhesion of *Candida albicans* MRL 3153 to HeLa cells (Makrides and MacFarlane, 1982); and *L acidophilus* inhibits adhesion of *Escherichia coli* K88 to porcine ileal cells (Conway, 1988). Some strains of *Lactobacillus* have been found to inhibit adhesion of uropathogenic bacteria (*Escherichia coli, Proteus mirabilis* and *Pseudomonas aeruginosa*) to human uro-epithelial cells (Chan *et al*, 1984, 1985; Reid *et al*, 1987).

In mouse monoassociated to *Campylobacter jejuni*, the administration *per os* of the heat-killed *L acidophilus* Lactéol strain reduces the number of *C jejuni* adhering to the intestinal mucosa (Moyen *et al*, 1986); however, these authors did not study the adhesion of lactobacilli.

We have shown that this *Lactobacillus* strain adheres to the HeLa 229 cells. *E coli* B41 also adhere to the same cells. In our model, a percentage of the cells (40–50%) did not bind a significant number of bacteria (more than 5). These can be assumed to be either immature cells which had not yet acquired adhesion sites or degenerating cells.

In the first set of experiments, we showed that heat-killed *L* acidophilus Lactéol strain inhibited adhesion of *E* coli B41 to HeLa cells in a dose-dependent manner. Live *L* acidophilus was found to be as effective as the bacteria killed by heating in spent culture medium. Thus heat treatment does not appear to alter the capacity of adhesion of lactobacilli to HeLa cells.

The fact that inhibition was not observed after lysis of the lactobacilli indicates that the inhibition is probably not a specific competition for common binding sites, but rather a steric hindrance of adhesion sites of *E coli*, as suggested by Chan *et al* (1985). In a study of the influence of a strain of *L casei* var *rhamnosus* isolated from a urethal sample, on adhesion of uropathogenic bacteria (*E coli*, *P*

aeruginosa and Klebsiella pneumoniae) onto ureoepithelial cells, these authors found that whole viable cells were more effective in blocking adhesion than fragments and extracts of bacterial walls.

Inhibition of the adhesion of enteropathogenic bacteria could thus account, at least in part, for the action of anti-diarrheal or probiotic preparations based on strains of *Lactobacillus*. Such a hypothesis suggests that it is essential to use adhering strains of lactic acid bacteria in this sort of product (Fuller, 1989).

The effect of L acidophilus Lactéol strain on the E coli B41 adhesion could account for the increased survival of suckling mice infected with E coli B41 that we observed previously (Fourniat et al, 1986). In the mucosa, the ability to adhere to cells confers to microorganisms an ecological advantage with respect to non-adherent microorganisms. Adherent microorganisms are protected from mechanical removal by secretions and benefit from a high level of nutrients which may sustain a higher growth rate (Zafriri et al, 1987). Furthermore, toxins produced by adherent bacteria are more likely to be active as they are secreted in contact with host cells, and can evade inhibitors such as antibodies (Ofek et al. 1990). By adhering to epithelial cells and inhibiting adhesion of enterotoxigenic E coli, L acidophilus mitigates these selective advantages and enhances the host's defenses.

Inhibition of the *E* coli adhesion by *L* acidophilus was found to require the presence of spent broth of the lactobacilli. The presence of spent medium was also found to be required for adhesion of live or heatkilled lactobacilli to HeLa cells. This could be accounted for by the excretion into the culture medium of a factor required for adhesion of *L* acidophilus to HeLa cells. It has been reported, for example, that adhesion of *L* fermentum strain 737 to gastric mucosa cells of the mouse is mediated by a protein factor excreted by *L fermentum* into its culture medium (Conway and Kjelleberg, 1989). Other Gram-negative bacilli adhere to epithelial cells after binding of an extracellular protein, *eg Listeria monocytogenes* (Kuhn and Goebel, 1989). However, the nature of the substance excreted by the studied strain of *L acidophilus* remains to be determined, although it is not destroyed by heating to 100–105 °C for 10 min in contrast to the factor described by Conway and Kjelleberg (1989).

These observations need to be confirmed in vivo in the suckling mouse as the in vitro system differs from the intestinal environment (absence of mucus, for example). In addition, the cells used (HeLa) are undifferentiated cells of non-intestinal origin and may have different surface receptors to those in the brush border of enterocytes. HeLa cells are, however, commonly employed to study adhesion of bacteria, especially human or animal strains of E coli of intestinal origin (Scalestsky et al, 1984; Milon et al, 1990; Cantey and Moseley, 1991), and our results are in agreement with those of Moyen et al (1986) in gnotobiotic mice monoassociated with Campvlobacter ieiuni. Moreover. the studied strain of L acidophilus adheres onto the brush border of cultured human enterocyte-like cell lines Caco-2 and HT-29 (Chauvrière et al, 1989). Future experiments may be carried out using on the one hand these cell lines to confirm the inhibitory effect of L acidophilus and the requirement of the adhesion-promoting factor present in spent broth, and on the other hand HeLa cells to detect this factor in spent broth extracts in order to identify it.

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