

## ***In vitro* anti-bacterial and anti-adherence effects of *Lactobacillus delbrueckii* subsp *bulgaricus* on *Escherichia coli***

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### **Abstract**

Considering the emergence of antibiotic resistance, scientists are interested in using new antimicrobial agents in the treatment of infectious diseases including infections of the enteric systems. Lactic acid bacteria have the great potential to produce antimicrobial compounds that inhibit and control pathogenic bacteria. The aim of this study was to determine the anti-bacterial and anti-adherence properties of *Lactobacillus delbrueckii* subsp *bulgaricus* against *Escherichia coli*. The antibacterial activity of *L. delbrueckii* was investigated using disc diffusion and spot on lawn methods. *In vitro* anti-adhesion effect of *L. delbrueckii* against *E. coli* was examined using Caco-2 cells. In anti-adhesion assay, three competition conditions including competitive inhibition, adhesion inhibition, and displacement were examined. In spot on lawn method the zone of growth inhibition of *E. coli* by *L. delbrueckii* was 21.1 mm. The cell free supernatant of *L. delbrueckii* showed a good antibacterial activity against *E. coli* which was mainly related to lactic acid produced by *L. delbrueckii*. When two bacteria added simultaneously (competitive inhibition) degree of inhibition of *E. coli* binding by *L. delbrueckii* was 77%. In adhesion inhibition assay, *L. delbrueckii* was able to exclude *E. coli* adherence by around 43.5%. Displacement assay showed that *L. delbrueckii* had strong displacement ability toward *E. coli* and reduction of *E. coli* attachment by bound *L. delbrueckii* was 81.3%. The results suggest that *L. delbrueckii* may be able to inhibit *E. coli* infection in the gut; however more studies including *in vivo* studies need to be performed.

**Keywords:** Anti-bacterial; Anti-adherence; *Escherichia coli*, *Lactobacillus delbrueckii*

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### **INTRODUCTION**

*Lactobacillus delbrueckii* subsp *bulgaricus* is a gram-positive bacteria belonging to lactic acid bacteria. This bacterium is a yoghurt starter culture which produces lactic acid and gives yogurt its flavor and textural properties (1). Previous studies have reported that consumption of yoghurt containing viable bacteria (*Streptococcus thermophilus* and *L. delbrueckii*) improved lactose digestion and decreased lactose intolerance (2). Recent studies have shown that *L. delbrueckii* has a potential probiotic function. Guglielmotti and coworkers reported that some commercial strains of *L. delbrueckii* subsp *bulgaricus* have shown high hydrophobicity values,  $\beta$ -galactosidase activity, good lysozyme tolerance and poor bile resistance. They indicated that these bacteria also possess antibacterial activity

toward tested pathogens and can block the invasion of *Salmonella enterica* serovar Enteritidis into Caco-2/TC-7 cells (3).

Recently, scientific communities have focused on probiotics as health promoters. Probiotics are live microorganisms which beneficially affect their host by improving the intestinal microbial balance (4). Previous studies reported that some probiotic strains have antagonistic activities against gastrointestinal pathogens (5-6). Many mechanisms for these observations have been proposed which include production of antimicrobial compounds, change in the environmental conditions such as gut pH, competition for same nutrients and adhesion sites of like pathogens, and stimulation of the immune and non-immune defense mechanisms of the host (7). One of the common virulence strategies of pathogenic strains is adhesion to the host cells

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which provides a new target for treatment strategies (8). There are many approaches which inhibit bacterial attachment to the host cells. Some studies reported that sub-lethal concentrations of current antibiotics may inhibit bacterial adhesion (9). Today, the widespread use of antibiotics, repeatedly and incorrectly, increases antibiotic resistances causing inefficacy of antibiotics against bacteria which form biofilm and hospital acquired infections. Considering the emergence of antibiotic resistance, scientists are interested in using new antimicrobial agents in the treatment of infectious diseases including infections of the enteric system (10-11).

There is evidences that some probiotics can inhibit gastrointestinal infections by blocking adherence of the pathogens to the intestinal epithelium cells (6, 12). However, this effect of probiotics depends on both the specific probiotic strain and the pathogen (13).

*Escherichia coli* is a gram-negative bacteria which is a member of Enterobacteriaceae family. *E. coli* can colonize in the body especially in the lower intestine and be transmitted through the oral-fecal route. Pathogenic strains of the bacterium can cause diseases from gastroenteritis to extra-intestinal infections of the urinary tract, pulmonary and nervous system (14).

The aim of the current study was to determine the antibacterial properties of *L. delbrueckii* against *E. coli* and also to assess whether intestinal epithelium adhesion and viability of potentially adherent *E. coli* can be reduced by *L. delbrueckii*.

## MATERIALS AND METHODS

### Chemicals

Chemicals used in tissue culture assays were purchased from Gibco (Scotland) via local vendors. Other chemicals and reagents used in this study were of analytical grade and obtained from Merck (Darmstadt, Germany).

### Bacterial strains and growth conditions

*L. delbrueckii* subsp *bulgaricus* (DSM 20081) was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *L. delbrueckii* was grown in Man-Rogosa-Sharpe (MRS) broth at

37°C for 48 h. *E.coli* (PTCC 1330) was obtained from Persian Type Culture Collection and subcultured on Brain-heart infusion (BHI) broth and incubated at 37°C.

### Caco-2 cell culture

The human colon adenocarcinoma cell line, Caco-2, was supplied by Pasteur Institute of Iran, Tehran. It was grown in Dulbecco's modified Eagle's Minimal Essential Medium (DMEM) supplemented with 20% (v/v) Fetal Bovine Serum (FBS), penicillin-streptomycin (100 IU/ml and 100 µg/ml, respectively) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. For adhesion assay, the Caco-2 cells were seeded at a density of 2×10<sup>5</sup> cells/well in 6-well tissue culture plates. The culture was refed every 2 days to obtain monolayer, and then further cultivated for 7-10 days to obtain differentiated cells. Then, Caco-2 monolayers were incubated with antibiotic-free medium for 24 h to perform the adhesion assay.

### Agar spot on lawn method

Three µL of *L. delbrueckii* overnight culture (1×10<sup>7</sup> CFU/ml) was spotted on the surface of MRS agar plates and incubated overnight at 37 °C. Next day 200 µL of *E. coli* overnight culture (1×10<sup>7</sup> CFU/ml) was added into 7 mL of soft agar (0.7%). This soft agar contained a 1:1 mixture of BHI and MRS. The mixture was overlaid on the MRS agar plate containing the spots of *L. delbrueckii*. The zone free of bacterial growth observed around the spots was measured in millimeters.

### Disk diffusion method

Overnight culture of *L. delbrueckii* was centrifuged at 4000g for 10 min. The cell-free supernatant (CFS) was separated and passed through a 0.22 µ filter. The antibacterial activity of filtrate of CFS was investigated using disc diffusion method. To evaluate the effects of lactic acid and pH on the antibacterial activity of the CFS supernatant, following conditions were examined: 1) pH of CFS was adjusted to 6.5 using 0.1 M NaOH, 2) pH was adjusted to the pH values normally achieved by with *L. delbrueckii* by addition of enough lactic acid to MRS broth (without bacteria), 3) pH was adjusted to the pH values normally reached by *L. delbrueckii* by addition

of enough HCl to MRS broth (without bacteria), 4) CFS without any treatment and 5) MRS broth. Sterile paper discs were located on BHI agar plates inoculated with *E. coli*. Samples of 50 µl were added to paper discs and incubated overnight at 37°C. The following day, the zone of inhibition in millimeters was measured.

#### Adhesion assays

The overnight culture of *E. coli* and *L. delbrueckii* were centrifuged and after washing with ringer solution, the bacterial pellets were resuspended with DMEM medium (pH 4.5). Bacterial adhesion on Caco-2 cells was evaluated using 6-well plates. One ml of bacterial suspension ( $0.5 \times 10^5$  colony forming unit (CFU/ml)) was added to each well containing Caco-2 monolayer and incubated at 37°C for 1 h. Unbound bacteria were eliminated by three times washing with phosphate buffered saline (PBS).

In our study following type and order of bacterial additions were evaluated: 1) *L. delbrueckii* alone (L), 2) *E. coli* alone (E), 3) addition of simultaneous *L. delbrueckii* and *E. coli* (L+E) 4) addition of *E. coli* after *L. delbrueckii* (L/E) and 5) addition of *L. delbrueckii* after *E. coli* (E/L). To evaluate the possibility of substitution of *E. coli* by *L. delbrueckii* or vice versa, after three times washing with PBS, second bacteria was added and incubated. Then, Caco-2 cells were disrupted with 0.05% Triton X-100 for 5 min, and bound *L. delbrueckii* and *E. coli* were evaluated using plate counting on MRS and violet red bile (VRB) agar (CFU/ml), respectively. The MRS and VRB plates were

incubated at 37°C for 48 and 24 h, respectively.

#### Statistical analysis

Each assay was repeated three times to ensure reproducibility of the results. All data are presented as mean  $\pm$  standard deviation. Significant differences were calculated by analysis of variance (ANOVA) using SPSS version 16 and Tukey test was used to evaluate the difference between groups.  $P < 0.05$  was considered significant.

## RESULTS

#### Agar spot on lawn

Results of spot on lawn method showed that *L. delbrueckii* inhibited the growth of *E. coli* and the zone of growth inhibition of *E. coli* by *L. delbrueckii* was  $21.1 \pm 3$  mm (n=12).

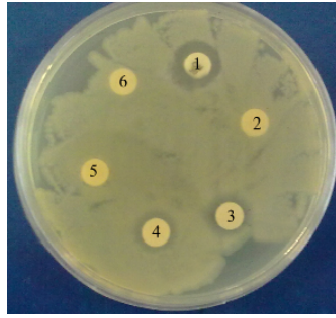
#### Disk diffusion

The antibacterial activity of CFS of *L. delbrueckii* was investigated using disc diffusion method (Table 1). The zone of inhibition for pH adjusted MRS broth with lactic acid and CFS (unadjusted pH) were significantly more than that of the negative control (PBS). The zone of inhibition for pH adjusted MRS with HCl was not significantly larger than that of the negative control (PBS). The results also showed that antibacterial effect of CFS did not significantly differ from that of pH adjusted MRS broth with lactic acid. After neutralizing pH to 6.5, antibacterial effect of CFS diminished and zone of inhibition for pH adjusted CFS was equal to that of the negative control (Fig. 1).

Table 1. Antibacterial activity of *L. delbrueckii* against *E. coli* using disc diffusion method. (n=9)

CFS	Zone of inhibition (mm) after 24 h					Cephalexin
	PH adjusted CFS	MRS	pH adjusted MRS with lactic acid	pH adjusted MRS with HCl	PBS	
9.1 $\pm$ 0.8	6.0 $\pm$ 0	6.0 $\pm$ 0	8.8 $\pm$ 1.0	7.5 $\pm$ 0.5	6.0 $\pm$ 0	14.4 $\pm$ 1.4

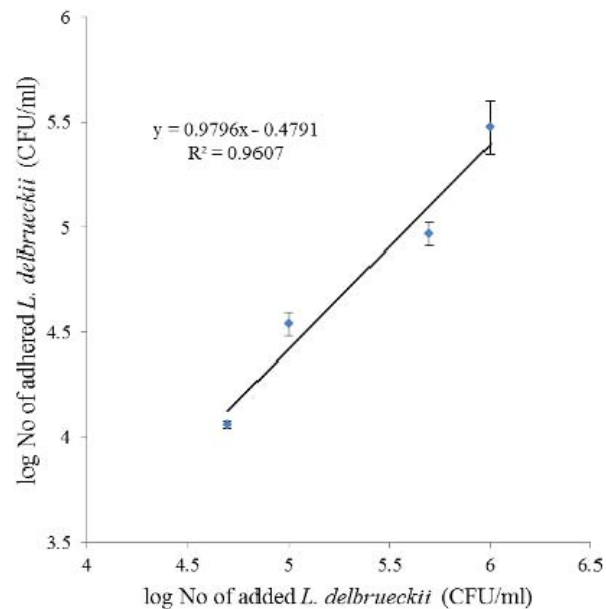
CFS: Cell free supernatant; pH-adjusted CFS: pH of CFS was adjusted to 6.5 using 0.1 M NaOH; MRS: Man-Rogosa-Sharpe broth; pH-adjusted MRS with lactic acid: pH was adjusted to the pH values reached normally by *L. delbrueckii* by adding enough lactic acid to MRS broth; pH adjusted MRS with HCl: pH was adjusted to the pH values normally reached by *L. delbrueckii* by adding enough HCl to MRS broth; PBS: phosphate buffered saline as negative control; Cephalexin: 30 µg cephalexin as positive control. Zone of inhibition of 6 mm means no inhibition of bacterial growth, as it is equal to the diameter of the disks used in this experiment. That is why the SD of some cases is 0.



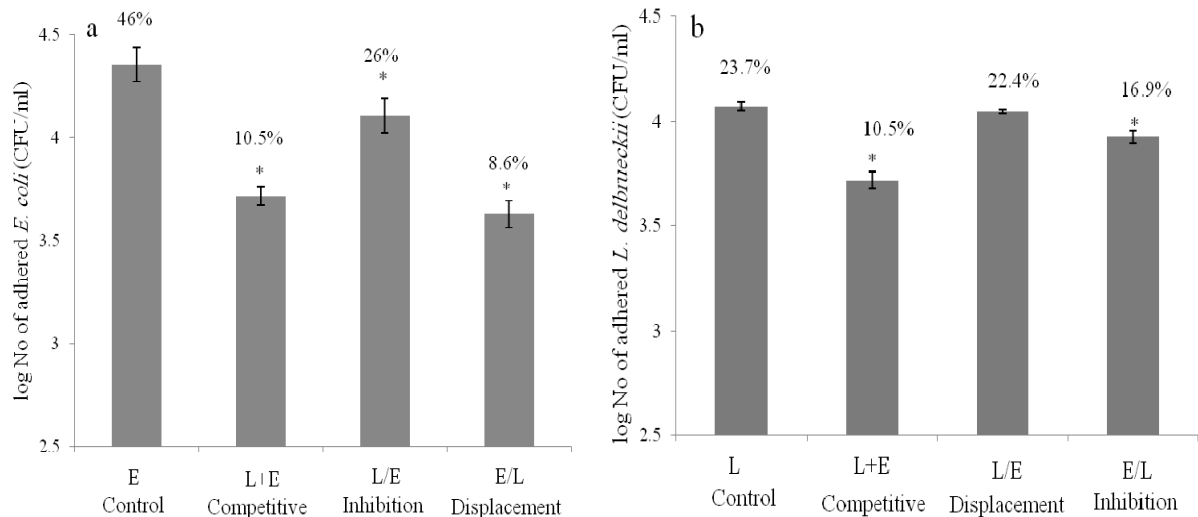
**Fig. 1.** Evaluation of antibacterial activity using disk diffusion method. 1: positive control (cephalexin); 2: negative control (PBS); 3: cell free supernatant (CFS) without any treatment; 4: MRS broth whose pH was adjusted to the pH values normally reached by each *L. delbrueckii* (pH of CFS) by adding enough lactic acid; 5: CFS whose pH was adjusted to 6.5 using 0.1 M NaOH; and 6 : MRS broth.



**Fig. 2.** Adhesion of *L. delbrueckii* to Caco-2 cells observed using light microscopy after Gram-staining



**Fig. 3.** The effect of added bacteria (CFU/ml) on the number of adhered *L. delbrueckii* to Caco-2 cells. (n=3)



**Fig. 4.** Adhesion of *L. delbrueckii* (a) and *E. coli* (b) to Caco-2 cells. The Caco-2 cell was incubated with DMEM medium containing  $0.5 \times 10^5$  CFU/ml of bacteria. L: *L. delbrueckii* alone; E: *E. coli* alone; L+E: *L. delbrueckii* and *E. coli* added simultaneously; L/E: *E. coli* added after *L. delbrueckii*; and E/L: *L. delbrueckii* added after *E. coli*. Asterisk (\*) indicates the means which were significantly different ( $P < 0.05$ ) from the control (Adhesion of *L. delbrueckii* alone (L) was considered as a control for adhesion assay *L. delbrueckii* and adhesion of *E. coli* alone (E) was considered as a control for adhesion assay *E. coli*. (n=9)

#### Adhesion assays

*In vitro* adhesion of *L. delbrueckii* was examined using Caco-2 cells. Adhesion of *L. delbrueckii* to Caco-2 cells observed using light microscopy after Gram-staining (Fig. 2). The number of adhered bacteria (CFU/ml) was dependent on the amount of added cells (Fig. 3) and their coloration was linear ( $r^2 = 0.96$ ).

Adhesion value was determined according to the following equation:

$$\% \text{ Adhesion} = (\text{Adhered bacteria} / \text{Added bacteria}) \times 100$$

The adherence values for *L. delbrueckii* and *E. coli* were 23.7% and 46%, respectively which proved that *E. coli* bind more effectively to Caco-2 cells than *L. delbrueckii*. In anti-adhesion assay, three competition conditions including competitive inhibition, adhesion inhibition, and displacements were examined. The numbers of adhered bacteria under different competition conditions are shown in Figs 4a and 4b for *L. delbrueckii* and *E. coli*, respectively. The results of competitive inhibition revealed that adhesion values of two bacteria reduced significantly ( $P < 0.05$ ) when *L. delbrueckii* and *E. coli* added simultaneously (Fig. 4). *E. coli* showed more reduction (77%) in its adhesion value compared to *L. delbrueckii* (53%). In displacement assay, bacteria were first allowed

to attach to Caco-2 cells before addition of second bacteria, and then the rate of reduction in the attachment of

first bacteria was measured. In E/L procedure, attachment of *E. coli* was decreased by *L. delbrueckii* from 4.35 to 3.63 log CFU/ml (Fig. 4a) and in L/E procedure adherence of *L. delbrueckii* was reduced by *E. coli* from 4.07 to 4.05 log CFU/ml (Fig. 4b). Displacement assay showed that *L. delbrueckii* substituted significantly ( $P < 0.05$ ) the attached *E. coli* (E/L) while *L. delbrueckii* displacement by *E. coli* (L/E) was not statistically significant. In adhesion inhibition, ability of pre-adhered bacteria to exclude attachment of second bacteria was evaluated. The degree of adhesion inhibition of *E. coli* by pre-adhered *L. delbrueckii* was 43.5 % and reduction of *L. delbrueckii* attachment by bound *E. coli* was 28.6%.

#### DISCUSSION

Lactic acid bacteria are the most widely used bacteria as starter cultures and have the great potential to produce antimicrobial compounds (15). The antimicrobial activity of lactic acid bacteria has been attributed to the production of different antimicrobials such as lactic acid, acetic acid, hydrogen peroxide,

carbon dioxide, bacteriocins and other low molecular mass compounds with antimicrobial activity (16).

Using spot on lawn method which shows direct interaction of live bacteria, the antagonistic effect of *L. delbrueckii* against *E. coli* was evaluated. The findings indicated that *L. delbrueckii* had a good inhibitory effect on the *E. coli* growth. Also, antibacterial activity of the supernatant of *L. delbrueckii* was determined using disc diffusion method which showed CFS of *L. delbrueckii* had a good antibacterial effect. This activity was diminished when pH of CFS was adjusted to 6.5 indicating that antimicrobial compounds produced by *L. delbrueckii* were acidic compound or need low pH for their optimum activities. To evaluate the effects of pH and lactic acid on antibacterial activity, CFS and pH adjusted MRS broth with lactic acid and HCl were compared. These findings indicated that lactic acid was the most potent inhibitor produced by *L. delbrueckii*. Our findings are in agreement with studies of Guglielmotti and coworkers. and De Keersmaecker and coworkers. According to their studies the antimicrobial activity of some commercial strains of *L. delbrueckii* was mainly related to the lactic acid, and not to the pH value (3). In other study, Vanderleyden and coworkers reported strong antimicrobial activity of *L. rhamnosus* GG against Salmonella that was mediated by lactic acid (17).

Since it is difficult to study bacterial adherence *in vivo*, especially in humans, adhesion has been evaluated using *in vitro* model. *In vitro* adhesion of *L. delbrueckii* was performed using Caco-2 cell line. This cell line is one of the most widely used cell lines for studies related to probiotic and pathogens adhesion to intestinal epithelium (18).

In the present study, the number of adhered bacteria (CFU/ml) was linearly related to the amount of added cells. Similar results were reported by other groups indicating concentration dependent kinetics of adhesion (19). In anti-adhesion assay, the ability of *L. delbrueckii* to inhibit attachment of *E. coli* or vice versa was examined under three competitive conditions. When *E. coli* and *L. delbrueckii* were added simultaneously,

degrees of inhibition were 77% and 53%, respectively.

Lee and coworkers reported 20-50% reduction in the attachment of strains of *E. coli* and *S. Typhimurium* to Caco-2 cells by *L. rhamnosus* (20). In another study, Lee and coworkers proposed that degree of adherence of two competitor bacteria depends on the affinity of adhesive molecules expressed on the surface of bacteria to adhesion receptor present on the surface of host cells that they are competing for (21). In adhesion inhibition assay, *L. delbrueckii* was able to reduce *E. coli* and *L. delbrueckii* adherence by around 43.5% and 28.6%. Similar findings were reported for *in vivo* study in gnotobiotic piglets which indicated competitive exclusion of *E. coli* by *L. gasseri* K7 (22). The possible mechanisms involved in inhibition of adherence of *E. coli* by *L. delbrueckii* are competition for common adhesion receptors, effects of substances present in the supernatant of *L. delbrueckii* and steric hindrance of adhered *L. delbrueckii* (23). However, the precise details of the proposed mechanisms are not understood. Displacement assay showed that *L. delbrueckii* has strong displacement ability toward *E. coli* and reduction of *E. coli* attachment by bound *L. delbrueckii* was 81.3%. In other displacement assay, attachment of *Staphylococcus aureus* in human intestinal mucus was inhibited 39-44% by *L. rhamnosus* GG, *Lactococcus lactis* subsp. *lactis* and *Propionibacterium freudenreichii* subsp. *shermanii*. (19) This displacement activity of probiotic bacteria may be explained by production of antimicrobial compounds or anti-adhesion factors and also competition for the same adhesion receptors (24). It is found that the anti-adhesion factors were able to degrade carbohydrate receptors of pathogens, to establish a biofilm and to induce production of biosurfactants and receptor analogues.

Our findings indicated that *L. delbrueckii* had a good anti-adhesion activity against *E. coli* in different competitive conditions. However, the ability of adhesion inhibition may depend on the specific probiotic strains and the pathogens. For example some commercial probiotic strains were not able to inhibit adherence of *E. coli*, *L. monocytogenes* and *Salmonella typhimurium* to human mucus

and even increased attachment of these pathogens to intestinal mucus (13).

However, the specific mechanism of action of *L. delbrueckii* *in vivo* remains to be elucidated. Some study showed that administration of some of lactobacillus strains can decrease pH of gut and feces(25-26). In human body, in addition to a change in gut pH, the mechanism of inhibitory effect of bacteria may consist of competition for the same nutrients and adhesion sites. For better understanding of mechanism of action of this bacterium it is necessary to design an *in vivo* study.

### CONCLUSION

It can be concluded from this study that *L. delbrueckii* is effective in inhibition of *E. coli* adhesion to Caco-2 cells under the conditions tested. The findings suggest that *L. delbrueckii* may be able to inhibit *E. coli* infection in the gut; however more studies including *in vivo* studies need to be performed. As this bacterium is used as a starter culture and considered safe, consumption of yoghurt containing viable *L. delbrueckii* may help to prevent and treat gastrointestinal infections caused by *E. coli*.

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