# Surface Adhesins of Lactobacilli Loosely Connected to the Cell Wall and Eliminated into the Environment during Culturing in Liquid Nutrient Media

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Six lactobacillus species and 4 clones of one of them were studied in order to clear out the ratio between the adhesion capacities of concanavalin A-reactive glycoprotein adhesins on the surface of the bacterial cell and glycoprotein adhesins released into the broth during culturing in liquid nutrient media. The adhesive activity of cultures is largely determined by the strain rather than species appurtenance. Elimination of glycoprotein adhesins from the bacterial cell and their antagonistic activity towards *Candida albicans* were demonstrated in specific interactions of glycoprotein adhesins with immune serum and concanavalin A.

**Key Words:** *probiotics; adhesion; vaginal epithelium*

Lactobacilli are components of the protective lining of epitheliocytes (EC) of the terminal microecological niches of human body; this prompted their use for the creation of probiotic drugs [6].

The formation of a biological film on EC is not an obligatory manifestation of defense mechanism for other lactobacillus-based probiotics. These lactobacillus strains express surface adhesin of glycoprotein nature (GPA) loosely connected to the cell surface and capable of eliminating from the microorganism walls into the environment. These GPA in complex with lactobacilli and metalloproteinases block the opportunistic microflora vegetating in the terminal microecological niches, thus preventing its translocation into the internal medium of the macroorganism.

Some variants of lactobacillus GPA bind concanavalin A (Con A) [4], *L. fermentum* carry on their surface GPA loosely bound to the cell wall, released into culture medium, and blocking *S. dublin* fixation on enteric EC in preincubation experiments [8,9]. A similar GPA can be found on the surface of *L. fermentum* strain 90 TS-4 and in its culture medium [3]. Moreover, Con A is bound by GPA on the surface of strain 90 TS-4 cells and by its form eliminated from bacterial cell surface into the environment. It remains unclear whether the adhesive activities of Con A-reacting GPA retaining on the bacterial cell surface and GPA forms released into the environment are comparable. This comparative analysis became the aim of our study.

## MATERIALS AND METHODS

The study was carried out on a collection of lactobacillus strains: *L. buchneri* (1 isolate), *L. fermentum* (3 isolates), *L. plantarum* (3 isolates), *L. casei* (2 isolates), *L. paracasei* (1 isolate), and *L. acidophilus* (1 isolate). Four clones of *L. fermentum* strain 90 TS-4 (21) were studied, which were selected by high agglutination by Con A in the culture. The antagonistic activity of GPA was studied using a reference *E. coli* M17 strain and clinical strain of

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*C. albicans* yeast-like fungi isolated from a woman with symptoms of candidal vaginitis. The studied lactobacilli were verified by biochemical characteristics using API 50 CHL system (bioMerieux); *C. albicans* were identified using Nickerson medium (HiMedia). Lactobacillus strains were cultured in MPC-1 at 37°C and 5% $CO_2$, *C. albicans* in Saburo broth at 37°C. Vaginal EC from two donors served as the target cells for bacterial adhesion (the donors gave informed consent for participation in the study). Vaginal EC were collected on days 15-18 of the menstrual cycle, washed 3 times in buffered saline (pH 7.2) by 3-min centrifugation at 1000 rpm.

Adhesion of lactobacilli of different species and of *C. albicans* yeast-like fungi on vaginal EC was evaluated in direct experiments.

After completion of culturing cycle, lactobacilli and yeast-like fungi were washed from the culture medium by centrifugation in buffered saline at 7000 rpm. The concentrations of lactobacillus and fungal suspensions were formed by the opacity standard (5 Units). Bacterial adhesion on vaginal EC was carried out at 20°C by uniting suspensions of target cells and bacteria (100 bacterial bodies per vaginal EC). After 1 h the mixture was washed 3 times in buffered saline, centrifuged at 1500-2000 rpm for 2 min, and the preparation was stained after Gram or with Evans blue (Sigma). After washout the bacteria were counted in 31 visual fields. Microscopy of the smears was carried out under an MBI-15-2 light microscope (LOMO) [2].

The mean number of adherent bacteria per target cell and the error of the mean were estimated. The significance of differences was evaluated by Student's *t* test, *r* coefficient of correlations using the BIOSTAT software.

Routine microscopy, immunofluorescent analysis of lactobacteria fixed on vaginal EC (ML-2 fluorescent microscope, PROTVA device; Nikon Coolpix camera (dpi 3.4)) were used in the study. Rabbit immune serum specific to *L. fermentum* 90 TS-4 and FITC-conjugated guinea pig anti-rabbit immunoglobulin (N. F. Gamaleya Institute of Epidemiology and Microbiology) were used in indirect test.

Culture medium after lactobacillus culturing was purified and concentrated on AMICON cells with Diaflo PM-30 membranes and Millipore sterilizing membranes with 0.02-μ pores. For this purpose 24-h culture medium was centrifuged for 15 min at 3000 rpm (R=9 cm) and filtered through the first membrane (0.02 μ), due to which microorganisms and small particles present in the culture medium were eliminated. The material was then washed 3 times on a PM-30 membrane and the resultant concentrate (10 ml) was collected and stored at -20°C. Protein content in culture medium was measured by the method of Lowry [2] before and after concentration. Calibration curve was plotted using BSA.

Agglutination characteristics of lactobacilli with Con A were studied by the micromethod. The results were recorded after 24 h. The capacity of culture medium to react with Con A was evaluated in the annular precipitation test [2].

## RESULTS

First, we evaluated adhesive activity of all lactobacillus strains in the collection. The data indicate that adhesive activity of the test cultures is largely determined by the strain, rather than species appurtenance (Fig. 1). *L. fermentum* strains with high (53.9±4.3 bac.cells/vaginal EC; strain 90 TS-4 (21) clone 4) and low adhesion (5.97±1.30 bac.cells/vaginal EC; A-4 strain) were detected. For *L. plantarum* 8 RA-3 strain the mean adhesion level was 4.12±0.80 bac.cells/vaginal EC, while for *L. plantarum* 39 strain it was 22.23±2.50 bac.cells/vaginal EC.

Then, we studied the relationship between Con A agglutination by lactobacilli and their adhesion to vaginal EC. Estimation of coefficient of paired correlation between agglutination titer (Table 1) and mean level of adhesion to target cells showed $r$=0.15, in other words, no correlation between these parameters, which confirms our previous conclusion on the presence of other adhesin types (in addition to Con A-reactive lectin-dependent adhesin) providing tropism in fixation on various target cells [5,7].

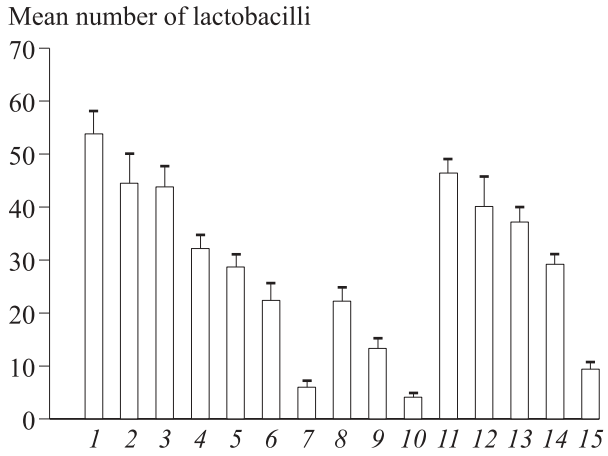


**Fig. 1.** Lactobacillus adhesion to the surface of vaginal EC. *1*) *L. fermentum* 90 TS-4 (clone 4); *2*) *L. fermentum* (production strain); *3*) *L. fermentum* (B-2); *4*) *L. fermentum* 90 TS-4 (clone 2); *5*) *L. fermentum* 90 TS-4 (clone 1); *6*) *L. fermentum* 90 TS-4 (clone 3); *7*) *L. fermentum* (A-4); *8*) *L. plantarum* 39; *9*) *L. plantarum* 38; *10*) *L. plantarum* 8 RA-3; *11*) *L. casei* 37; *12*) *L. casei*; *13*) *L. paracasei*; *14*) *L. buchneri* P0; *15*) *L. acidophilus* $K_3Sh_{24}$.

**TABLE 1.** Agglutination of Lactobacilli by Con A

| Lactobacillus spp. | Con A agglutination | |
|---|---|---|
| | order of dilution | Con A concentration ($1\times10^{-4}$ mg/ml) |
| *L. fermentum* 90 TS-4 (producer strain) | 8 | $3.5\times10^{-4}$ |
| *L. fermentum* 90 TS-4 (21) clone 1 | 9 | $1.75\times10^{-4}$ |
| *L. fermentum* 90 TS-4 (21) clone 2 | 10 | $0.875\times10^{-4}$ |
| *L. fermentum* 90 TS-4 (21) clone 3 | 10 | $0.875\times10^{-4}$ |
| *L. fermentum* 90 TS-4 (21) clone 4 | 9 | $1.75\times10^{-4}$ |
| *L. fermentum* A-4 | 5 | $30\times10^{-4}$ |
| *L. fermentum* B-2 | 8 | $3.5\times10^{-4}$ |
| *L. plantarum* 8RA-3 | 6 | $15\times10^{-4}$ |
| *L. plantarum* 39 | No reaction | No reaction |
| *L. plantarum* 38 | No reaction | No reaction |
| *L. buchneri* P0 | 5 | $30\times10^{-4}$ |
| *L. acidophilus* $K_3Sh_{24}$ | No reaction | No reaction |
| *L. paracasei* | No reaction | No reaction |
| *L. casei* | No reaction | No reaction |
| *L. casei* 37 | No reaction | No reaction |

Our next step was to clear out whether GPA selectively reacting with Con A can be detected not only on lactobacillus surface, but in culture medium as well. Precipitation test with Con A showed the presence of GPA eliminated into culture medium in all the studied samples of culture medium (Table 2).

If lectin-binding GPA released into culture medium retains its adhesive activity, it can be expected to inhibit fixation of other bacteria on the target cells. This hypothesis was verified in a direct experiment. Culture medium specimens obtained after culturing of strains with high expression of GPA (*L. fermentum* 90 TS-4, clones 2, 3; *L. plantarum* 8 RA-3) and after culturing of strains producing virtually no GPA (*L. plantarum* 38, *L. plantarum* 39) were preincubated with vaginal EC. After 20-min incubation at 37°C, the target cells were washed and suspension of *C. albicans* yeast-like fungi was added, after which the mixture was incubated for 20 min at 37°C and washed. In the control, adhesion of yeast-like fungi to vaginal EC not pretreated with culture medium was evaluated (Fig. 2). A significant reduction (in comparison with the control) in the number of yeast-like fungi adhered to one target cell was noted in three cases of vaginal EC preincubation with culture medium containing GPA. No inhibition of *C. albicans* adhesion was detected after target cell treatment with culture medium in which strains producing no GPA were cultured. These data indicate that lectin-dependent substrate can be desquamated from the lactobacillus surface and, when free, block adhesion sites, thus preventing fixation of yeast-like fungi on target cells (the surface of these fungi carrying adhesins complementary to the blocked site).

**TABLE 2.** Annular Precipitation Test with Culture Medium and Con A and Protein Concentration in the Studied Lactobacterial Culture Media

| Parameter | *L. fermentum* 90 TS-4 (production strain) | *L. fermentum* 90 TS-4 (21) clone 2 | *L. fermentum* 90 TS-4 (21) clone 3 | *L. plantarum* 8 RA-3 | *L. plantarum* 39 |
|---|---|---|---|---|---|
| Annular precipitation test with 0.1% Con A | +++ | +++ | ++++ | +++ | + |
| Protein concentration in culture medium, μg/ml | 1400 | 2100 | 8450 | 650 | 900 |

**Note.** Duration of reaction: "+" longer than 15 min, "+++" 5-10 sec, "++++" first seconds.

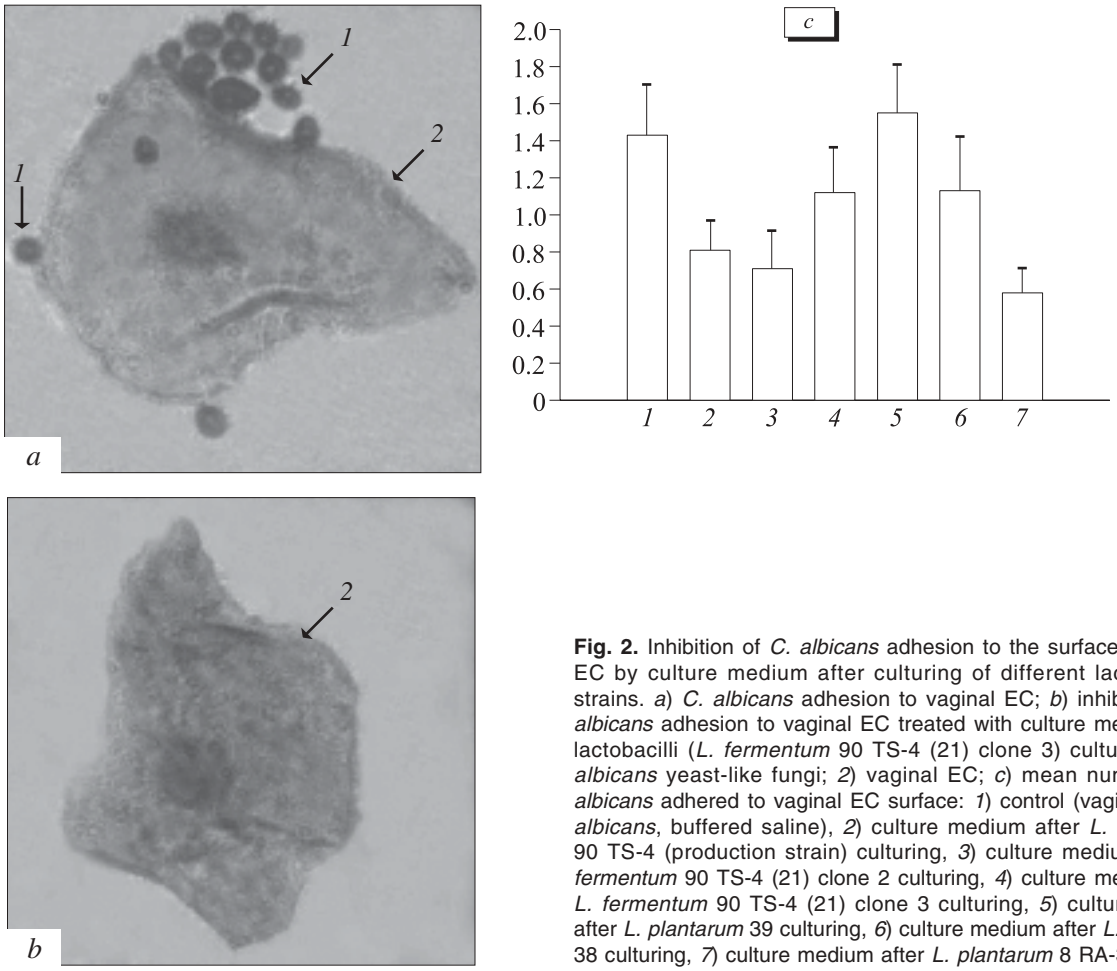


**Fig. 2.** Inhibition of *C. albicans* adhesion to the surface of vaginal EC by culture medium after culturing of different lactobacillus strains. *a*) *C. albicans* adhesion to vaginal EC; *b*) inhibition of *C. albicans* adhesion to vaginal EC treated with culture medium after lactobacilli (*L. fermentum* 90 TS-4 (21) clone 3) culturing: *1*) *C. albicans* yeast-like fungi; *2*) vaginal EC; *c*) mean number of *C. albicans* adhered to vaginal EC surface: *1*) control (vaginal EC, *C. albicans*, buffered saline), *2*) culture medium after *L. fermentum* 90 TS-4 (production strain) culturing, *3*) culture medium after *L. fermentum* 90 TS-4 (21) clone 2 culturing, *4*) culture medium after *L. fermentum* 90 TS-4 (21) clone 3 culturing, *5*) culture medium after *L. plantarum* 39 culturing, *6*) culture medium after *L. plantarum* 38 culturing, *7*) culture medium after *L. plantarum* 8 RA-3 culturing.

We tried to block adhesion of FimH-positive *E. coli* strain to vaginal EC in the same way, but addition of GPA to the system did not block the fixation of *E. coli* M17 strain on vaginal EC.

Culturing of some lactobacillus strains in liquid nutrient media leads to elimination of surface GPA, inhibiting adhesion of *C. albicans* (but not *E. coli* strain M17) to target cells. GPA capacity to react with Con A fragments can help disclose the nature of this phenomenon.

Another and more specific method for GPA testing can be the immunochemical method (immune serum to GPA-expressing strain can be specific to the target product, which is indirectly confirmed by stringent correlation between the agglutination activity of the immune serum and Con A ($r$=0.84) in lactobacterial strains studied [1]. Experiments demonstrating immunofluorescence arrest after Con A treatment of *L. fermentum* 90 TS-4 by the indirect Coons method [2] directly confirm this hypothesis (Fig. 3).

Con A blocks surface antigens of *L. fermentum* and these structures become inaccessible for antibodies (yellow-green fluorescence of objects in the control corresponds to staining with FITC-labeled antibodies; red fluorescence in the experiment corresponds to Evans blue staining; Fig. 3). In the other experiment immune serum was adsorbed by bacterial cells (*L. fermentum* strain 90 TS-4 (21) clone 3) pretreated and not (control) with Con A. The titer of initial (immune) serum was evaluated in the bacterial agglutination test (1:640). After adsorption of the serum with culture not treated with Con A the titer decreased to 1:20, while after adsorption with Con A-treated culture it decreased to 1:160.

Con A reduces the capacity of cultured *L. fermentum* strain 90 TS-4 (21) clone 3 to bind antibodies present in the immune serum, but some surface antigenic complex epitopes remain free. Due to this the titer of bacterial agglutination of Con A-treated culture does not reach the titer of the culture not treated with the lectin.

Hence, probiotics intended for vegetation in the terminal microecological niches and prepared from lactobacillus strains forming a biofilm on the surface of vaginal EC can be enriched with adhesins

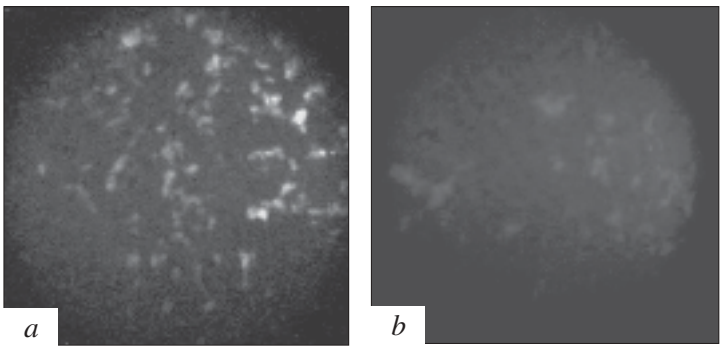

**Fig. 3.** Arrest of immunofluorescence after Con A treatment of *L. fermentum* 90 TS-4 (21) clone 3. *a*) lactobacillus fluorescence after treatment with FITC-labeled antispecies serum (no Con A treatment of lactobacilli); *b*) Evans blue staining of lactobacilli (after Con A treatment of lactobacilli).

eliminated from the surface of other lactobacterial strains and competing for adhesion sites for *C. albicans* yeast-like fungi. The validity of the drug in this situation can be controlled by testing their interactions with Con A and their activity with immune serum.

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