



Evaluation of adhesion properties of lactobacilli probiotic candidates

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Abstract

Bacterial adhesion is a complex phenomenon implicated in the host-bacterial interaction that is pivotal for probiotic activity. Eight probiotic lactobacilli candidates (*Lactobacillus reuteri*, *L. plantarum*, *L. mucosae*, *L. murinus*) were screened for their ability to adhere to abiotic and biotic surfaces in vitro. Adhesion to hydrocarbons was used for hydrophobicity assessment. Three strains of *L. reuteri* and *L. murinus* C were evaluated as hydrophobic, others as intermediate. All tested strains were able to form the biofilm on polystyrene. *L. mucosae* D and *L. reuteri* E were tested for adhesion to epithelial cell lines (HeLa and Caco-2). Both were more adherent to HeLa than to Caco-2. The adhesivity degree in HeLa reached the highest value after 8 h of co-cultivation in both lactobacilli tested, then decreased. In Caco-2, adhesion was increased within 24 h from the beginning of the co-cultivation. Mucus-binding protein gene, implicated in adhesion, was detected in *L. mucosae* D. Therefore, the involvement of proteinaceous substances in binding process was investigated. Cells of *L. mucosae* D were digested by three proteolytic enzymes (proteinase K, pronase E, trypsin) and evaluated for time-dependent adhesivity changes to HeLa, Caco-2, and L929 cell lines. Results confirmed that proteins are most likely to play an important role in binding of lactobacilli to eukaryotic cells. One hour after treatment, *L. mucosae* D was able to overcome the effect of proteolytic cleavage. We assume that it was due to the restoration of its cell-surface binding structures. Co-cultivation of HeLa and *L. mucosae* D led to protuberance and communication channels formation in eukaryotic cells.

Graphical abstract



Keywords *Lactobacillus* · Adherence · Mucus-binding protein · Electron microscopy · Hydrocarbons · Monolayers

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Introduction

Bacterial adherence to the host mucosa and epithelial cells (ECs) is one of the most important prerequisites of probiotic strains for host colonization [1]. It leads to direct interactions that may result in the competitive exclusion of pathogens and host immune response modulation. The adherence is a complex process of binding between the

bacterial cell wall and interacting surface (biotic or abiotic) that includes non-specific (e.g., surface hydrophobicity, determined as bacterial adherence to hydrocarbons, BATH) and specific mechanisms [2]. Among specific mechanisms on microbial site, several structures are responsible for binding: e.g., sortase-dependent proteins, S-layer proteins, proteins mediated adhesion to extracellular matrix components of ECs, non-protein adhesins (lipoteichoic acid, exopolysaccharides) [3]. In several strains of *L. reuteri*, *L. acidophilus*, *L. rhamnosus*, and *L. mucosae* mucus-binding proteins (MUBs) were found that belong to sortase-dependent ones, and their implication in adhesion process was confirmed [4].

There exists a positive correlation between adherence and biofilm formation ability. Biofilm formation of lactobacilli strains present in gastrointestinal and female urogenital tracts may have a dual protective role: protection of host mucosa against microbial pathogens colonization, and protection of lactobacilli cells against surrounding environmental conditions [5, 6]. Both properties, adherence and biofilm formation capacity, are possibly related to quorum sensing (QS) signaling molecules, e.g., autoinducer-2 [7]. Employment of QS was proved before in bioluminescence and virulence [8].

As adhesivity is a multifactorial process, this study deals with several methods involved in bacterial cell binding assessment. Eight potentially probiotic lactobacilli were tested for their biofilm formation potential, surface hydrophobicity, and adherence to different mammalian cell lines (HeLa, Caco-2, and L929) at miscellaneous in vitro conditions. Finally, the presence of *mub* genes coding MUBs in the genomes of tested strains was studied.

Results and discussion

Lactobacilli biofilm formation in vivo is probably a long-term evolutionary process that is highly specific for host ECs selection on one side and a certain *Lactobacillus* strain on the other [9, 10]. Biofilm formation by probiotics can contribute to maintaining host health/disease. It affects antibiotic susceptibility and stress resistance of probiotic cells and indirectly indicates their ability to colonize gut and female urogenital tract [5, 11]. Another benefit of biofilm formation is a production of antimicrobial substances on the basis of QS management. It was observed that *L. reuteri* strains bound in biofilms produced higher amounts of potent anti-pathogenic compound reuterin than planktonic cells [5]. Furthermore, lactobacilli organized in biofilm were able to affect immunomodulation properties of human monocytes/macrophages, e.g., TNF production [5]. All lactobacilli strains tested in this study adhered to polystyrene, although their capabilities varied. The

strongest biofilm formation ability demonstrated *L. mucosae* D (Table 1). Very strong biofilm forming capacity also demonstrated *L. reuteri* E.

Hydrophobic nature of the bacterial surface is required for their attachment to the host tissues and hydrophobicity is crucial in the first contact of bacterium and ECs or mucus [12]. All animal lactobacilli strains tested exhibited intermediate hydrophobic or hydrophobic properties (Table 1). In BATH test, the lowest hydrophobicity was determined in collection strain *L. reuteri* CCM 3625 ($32.8 \pm 0.4\%$) originated from paste rennet. This confirms the previous findings of Vinderola et al. [13] that starter and dairy-associated lactobacilli exhibit lower degree of hydrophobicity in comparison with gastrointestinal tract originated strains. BATH test is a valid quantitative phenomenological approach for estimation of strain epithelial adherence [14], however, some authors stated that there is no correlation between lactobacilli cells hydrophobicity and their adherence capacity to Caco-2 cells. These discrepancies could be explained by different methodological approaches [15].

Bacterial adherence is a process that involved interaction between ligand expressed on bacterial cell surface and receptors located on ECs. On the base of previous results (bile salt resistance, antibacterial properties, immunomodulation potential, biological activities [16–19]) and BATH test and biofilm formation experiments presented in this study, we chose among eight lactobacilli the two most promising probiotic candidates, *L. mucosae* D and *L. reuteri* E, for testing of time-dependent manner of their adherence to mammalian cell lines HeLa and Caco-2 (Fig. 1). HeLa cells are considered as common mammalian cells model. Caco-2 represents the intestinal cells [20]. Both HeLa and Caco-2 are cancer cells, however, they were used in our experiments because of their less problematic cultivation in comparison with primary isolates derived from volunteers' colon.

For both tested lactobacilli strains, the percentage of HeLa cells adhered with bacteria grew until the 8th hour. The higher binding capacity demonstrated *L. reuteri* E that adhered significantly more effectively than *L. mucosae* D. After 8 h of co-incubation, the adhesion of both strains significantly decreased (Fig. 1).

In comparison with HeLa cells, in Caco-2 tests, we observed different course. *L. mucosae* D adhesivity significantly increased until the 24th hour of co-cultivation, while in *L. reuteri* E, a not significant change was detected. Overall, HeLa cells bound lactobacilli more effectively than Caco-2 (Fig. 1).

In certain strains of *L. reuteri* and *L. mucosae*, MUB protein that is involved in adhesion process was identified. All isolated MUB proteins carried the N-terminal signal peptide and C-terminal LPXTG anchoring motif [21].

Table 1 Lactobacilli BATH test and biofilm formation on polystyrene

Strain	Hydrophobicity \pm SD/%	Degree of hydrophobicity	Biofilm formation \pm SD (OD ₅₇₀)	Biofilm forming capacity
<i>L. murinus</i> C	79.4 \pm 0.8	**	0.577 \pm 0.091	***
<i>L. mucosae</i> D	35.6 \pm 0.3	*	0.678 \pm 0.135	***
<i>L. reuteri</i> E	57.5 \pm 1.2	*	0.319 \pm 0.091	***
<i>L. reuteri</i> KO4b	94.1 \pm 0.5	**	0.193 \pm 0.008	**
<i>L. reuteri</i> KO4m	75.4 \pm 0.8	**	0.258 \pm 0.057	**
<i>L. reuteri</i> KO5	96.3 \pm 1.2	**	0.269 \pm 0.055	**
<i>L. plantarum</i> KG1z	56.2 \pm 0.6	*	0.215 \pm 0.085	**
<i>L. plantarum</i> KG4	60.9 \pm 1.1	*	0.330 \pm 0.041	***
<i>L. reuteri</i> CCM 3625	32.8 \pm 0.4	*	0.156 \pm 0.051	**

Hydrophobicity was calculated from three independent measurements \pm SD. The degree of hydrophobicity was evaluated according to Thapa et al. [30], $\geq 75\%$ as hydrophobic (**), 26–74% as intermediate (*). Biofilm formations are average numbers of four measurements \pm SD. *** Very strong biofilm forming capacity; ** strong biofilm forming capacity [11]

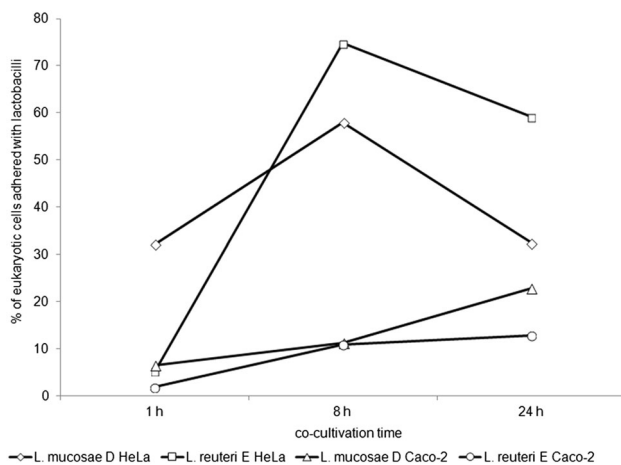


Fig. 1 Time-dependent adherence of *L. mucosae* D and *L. reuteri* E to HeLa and Caco-2 cells. A percentage of eukaryotic cells adhered at least with one bacterial cell. In each sample, 1000 eukaryotic cells were evaluated

MUB in *L. reuteri* ATCC 53608 possesses several copies of two related repeats, six copies of Mub1 repeat (31–87% amino acids identity) and eight copies of Mub2 repeat (83–100% amino acid identity) [4]. Gene-specific PCR primers targeting *mub1* were used for detection of *mub* gene in the genome of all eight lactobacilli tested. PCR product of expected length, ca. 600 bp, was obtained only in the case of *L. mucosae* D, in samples of other isolates variety of unspecific amplicons appeared. Sequencing of 546 bp amplicon confirmed the presence of *mub1* sequence in the genome of *L. mucosae* D (KX517913). Its nucleotide sequence was homologous for 97% with *L. mucosae* LM1 (gblCP011013.1) *mub* gene fragment with no gaps. Although the presence of a *mub1* fragment is not a guarantee of MUB expression, we suppose that this potential attribute may be jointly responsible for the good adherent properties of *L. mucosae* D.

To identify the nature of lactobacilli cells' surface structures responsible for host-bacterium interaction, we treated *L. mucosae* D with three proteolytic enzymes (proteinase K, trypsin, pronase E). Afterwards, their adhesivity potential to mammalian cells was tested. For this experiment, three different cell lines: HeLa, Caco-2 and L929 were used. The cell line L929 was covered in the present study, because of a primary source of most extracellular matrix (ECM) components, including collagen [22]. It was estimated, that some species of lactic acid bacteria produce proteins binding ECM components; e.g., collagen-binding protein [23–26]. According to our results (Figs. 2, 3, 4), it seems that proteins play an important role in lactobacilli adhesion process. The binding capacity of enzymatically treated lactobacilli to eukaryotic cells after the 1st hour of co-cultivation was decreased or not significantly changed. The exceptions were pronase E treated

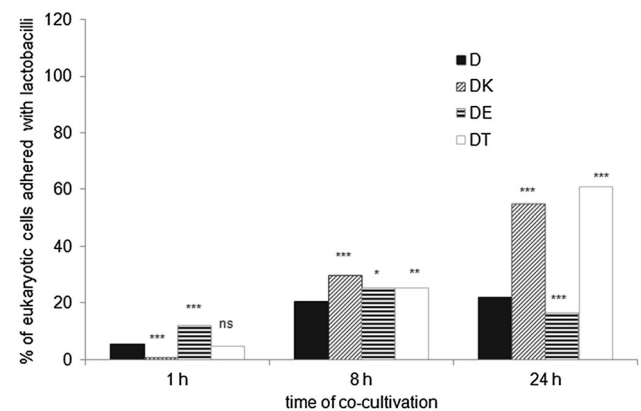


Fig. 2 Time-dependent adherence of *L. mucosae* D to Caco-2 cells under different conditions. A percentage of eukaryotic cells adhered at least with one bacterial cell. Statistical difference between control and sample, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns no statistical significant difference. D—*L. mucosae* D, control sample, DK—*L. mucosae* D cells treated with proteinase K, DE—*L. mucosae* D cells treated with pronase E, DT—*L. mucosae* D cells treated with trypsin

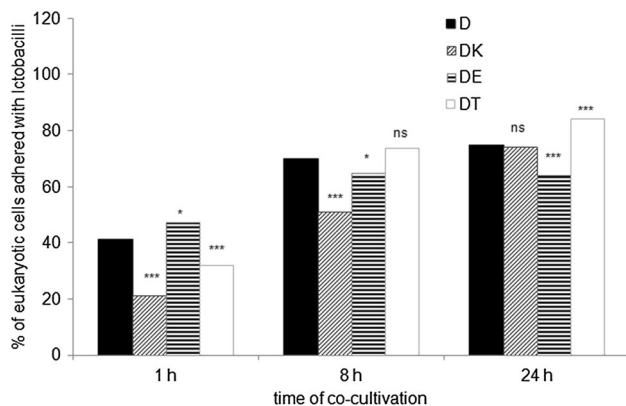


Fig. 3 Time-dependent adherence of *L. mucosae* D to HeLa cells under different conditions. A percentage of eukaryotic cells adhered at least with one bacterial cell. Statistical difference between control and sample, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns no statistical significant difference. D—*L. mucosae* D, control sample, DK—*L. mucosae* D cells treated with proteinase K, DE—*L. mucosae* D cells treated with pronase E, DT—*L. mucosae* D cells treated with trypsin

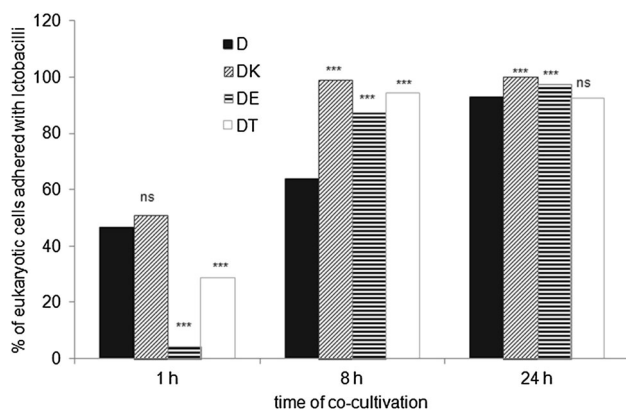


Fig. 4 Time-dependent adherence of *L. mucosae* D to L929 cells under different conditions. A percentage of eukaryotic cells adhered at least with one bacterial cell. Statistical difference between control and sample, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns no statistical significant difference. D—*L. mucosae* D, control sample, DK—*L. mucosae* D cells treated with proteinase K, DE—*L. mucosae* D cells treated with pronase E, DT—*L. mucosae* D cells treated with trypsin

lactobacilli adhering to Caco-2 (Fig. 2) and L929 (Fig. 4) cells. In this case, both eukaryotic cell lines bound *L. mucosae* D more effectively than control. Comparing results obtained after 1 h co-incubation of eukaryotic cells with *L. mucosae* D treated with different proteolytic enzymes, we deduce that diverse proteinaceous structures on lactobacilli surface are incorporated in their binding to eukaryotic cells. According to obtained results, we suppose that after the 8th hour, lactobacilli were able to overcome the effect of proteolytic digestion and restored the surface binding structures on the degree comparable to the control sample or even higher. We assumed that *L. mucosae* D may dispose also of structures binding collagen, because the overall rate of adhesion ability to fibroblasts L929 (Fig. 4) was higher than to intestinal line Caco-2 (Fig. 2).

HeLa cells co-cultivated with *L. mucosae* D 1 and 24 h were examined also by scanning electron microscopy (SEM). SEM showed time-dependent changes in HeLa cells morphology after 24 h co-cultivation. Co-cultivated HeLa expressed more cytoplasmic protuberances and communication channels (Fig. 5). It indicates that *L. mucosae* D represents an impulse for HeLa cells which induce their higher intercellular communication activity in comparison with lactobacilli-free HeLa cells. By production of various organic acids (acetic, propionic, lactic, butyric, formic acids), lactobacilli are responsible for acidification of their environment. Besides that, they are able to produce other extracellular substances (e.g., hydrogen peroxide, carbon dioxide, bacteriocins, and substances participating on adhesion). All mentioned lactobacilli products may serve as stress stimuli for eukaryotic cells and increase HeLa intercellular communication. Morphological and physiological changes in HeLa cells are not induced only by the direct cell to cell contact with lactobacilli, but are spread across the entire HeLa population.

Conclusion

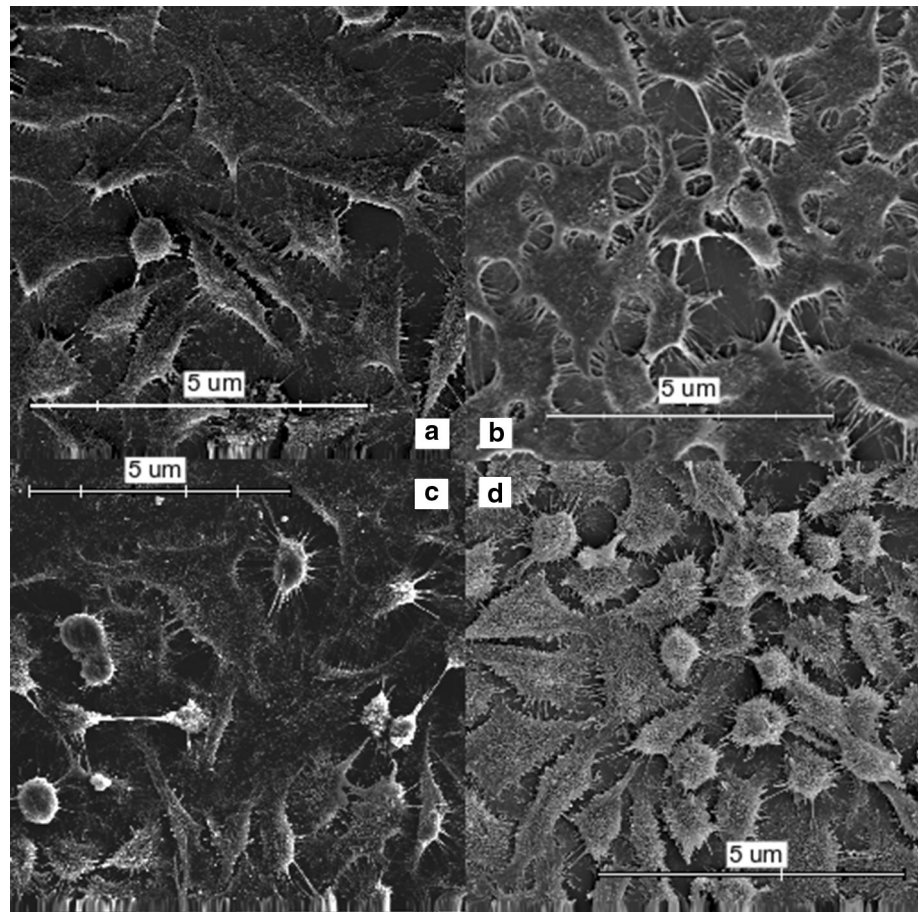
Our results indicate that there is not a distinct relationship between hydrophobicity, biofilm forming capacity and adherence of lactobacilli to eukaryotic cells. Instead of the similar hydrophobicity and biofilm forming capacity of *L. reuteri* E and *L. mucosae* D (for both strains intermediate and very strong, respectively), they displayed different degrees of adherence ability to cell lines HeLa and Caco-2. The presence of *mub* gene involved in adhesion was established in genome of *L. mucosae* D and its partial nucleotide sequence was gained. The adhesion of proteolytically treated *L. mucosae* D on three types of eukaryotic cell lines, HeLa, Caco-2, and L929 also confirmed that proteins play a crucial role in this process. However, *L. mucosae* D overcame the effect of proteolytic cleavage after 1 h, presumably by the restoration of its proteinaceous cell-surface binding structures. Noticeable communication in HeLa cells after co-cultivation with lactobacilli resulted in intensive formation of communication channels and protuberances between HeLa.

Experimental

Bacterial cultures and growth conditions

Lactobacillus murinus C, *L. mucosae* D, *L. reuteri* E were isolated previously from stomach mucosa of lamb (breeding station Očová, Slovakia) and identified [27]. *L. reuteri* KO4b, *L. reuteri* KO4m, *L. reuteri* KO5, *L. plantarum*

Fig. 5 Scanning electron microscopy of HeLa cells and HeLa cells co-cultivated with *L. mucosae* D. **a** HeLa cells in 1st hour; **b** HeLa cells co-cultivated with lactobacilli 1 h; **c** HeLa cells in 24th hour; **d** HeLa cells co-cultivated with lactobacilli 24 h



KG1z and *L. plantarum* KG4 were isolated from stomach mucosa of goatling (breeding station Teplý Vrch, Slovakia) and identified earlier [28]. Both animals were breast-fed and three weeks old. For comparison collection strain *L. reuteri* CCM 3625 (Czech Collection of Microorganisms, Brno, Czech Republic) was engaged in some experiments. Lactobacilli were cultivated in MRS broth (Oxoid, UK) in anaerobic conditions at 37 °C.

Hydrophobicity assay

BATH test was performed as described Vinderola and Reinheimer [29] with minor modifications. Overnight cultures of lactobacilli were harvested by centrifugation (10,000g/10 min), washed twice in 50 mM potassium phosphate buffer (pH 7.0), resuspended in the same buffer to an A_{560} of approximately 1.0. Thereafter, 1.5 cm³ of bacterial suspension was mixed with 1.5 cm³ of xylene (Lachema, Czech Republic) by vortexing for 2 min. After phase separation (30 min), A_{560} of an aqueous phase was determined. Hydrophobicity was expressed as a percentage of bacterial strain adhering to xylene ($H_{\%}$) according to the equation:

$$H_{\%} = ((A_0 - A)/A_0) \times 100,$$

where A_0 and A is absorbance before and after extraction with xylene, respectively. Results were reported as a percentage of adherence to xylene and appraised according to Thapa et al. [30]: $\geq 75\%$ as hydrophobic, 26–74% as intermediate.

Biofilm formation assay

A modified version of previously described methods [5, 11] was used. Lactobacilli after overnight cultivation were settled by centrifugation (10,000g/10 min) and resuspended in phosphate-buffered saline (PBS, pH 6.5) to reach the McFarland 2. Samples were diluted 1:1 in double concentrated MRS and 100 mm³ of each culture were inoculated in a well of a polystyrene microtiter plate (Greiner bio-one, Germany). Plates were incubated anaerobically at 37 °C for 20 h.

The biofilm formed in the plates' wells were washed six times with 200 mm³ of PBS and dried at 37 °C in an inverted position. Adhered cells were stained with 25 mm³ of crystal violet in ethanol (0.1% w/v) for 15 min at 37 °C. Crystal violet was discarded and the plates were washed

five times with 400 mm³ of distilled water. After drying at 37 °C, the crystal violet was redissolved with 200 mm³ of the ethanol-acetone (80:20, v/v) mixture and the OD₅₇₀ was determined (EpochTM Microplate Spectrophotometer, BioTek, USA). Values were assessed for OD₅₇₀ as following: ≤ 0.1—very low, 0.2–0.3—strong and ≥ 0.3 very strong biofilm forming capacity.

Mammalian cell lines and cultivation conditions

HeLa (human epithelioid cervix adenocarcinoma cells, ECACC 93021013, European Collection of Cell Cultures, Great Britain), Caco-2 cells (human colon adenocarcinoma cells, ECACC 86010202), and L929 (mice fibroblasts, ECACC 85011425) were kept in supplemented MEM medium (10% fetal bovine serum; antibiotics: amphotericin B 0.25 µg/cm³, penicillin 100 IU/cm³, streptomycin 0.1 mg/cm³; non-essential amino acids) for 48 h (HeLa and L929 cells) and for 2 weeks (Caco-2 cells). Reagents and media were purchased from PAA, Austria. All cell lines were cultivated in 5% CO₂ atmosphere at 37 °C (Steri-Cycle.CO2 Incubator, model 381, Thermo Electron Corporation, USA) in 24-well tissue culture plates (Schoeller Instruments, Czech Republic) on coverslips and cultivation medium was replaced every other day.

Adherence assay

Mammalian cells were washed twice with sterile PBS (pH 7.3) and well-mixed bacterial suspensions (*L. mucosae* D and *L. reuteri* E) at McFarland 0.5 in MEM medium without antibiotics and antimycotics were added. Cells were co-incubated for 1, 8, and 24 h (5% CO₂ atmosphere at 37 °C), then washed twice with PBS, fixed with methanol/acetic acid mixture (3:1) for 5 min, stained by May-Grünwald and Giemsa-Romanowski dyes and drained in acetone (all Penta, Czech Republic) and xylene mixtures (Lach-Ner s.r.o., Czech Republic). Samples were evaluated microscopically (magnification 1000) by counting 1000 eukaryotic cells and the percentage of cells adhered with at least one lactobacillus was expressed.

Detection of *mub* gene fragment

Chromosomal DNA of lactobacilli was isolated from 3 cm³ of an overnight culture using DNeasy Tissue Kit (Qiagen, Germany), according to the manufacturer's protocol. For *mub* gene amplification, PCR primers MucB1-RVIf and MucB2-RVIf according to MacKenzie et al. [31] were used. Reaction mixtures contained 1 × Taq polymerase buffer, MgCl₂ 2.5 mM, dNTPs 200 µM, a Taq polymerase 1.5 U, 20 pM of each primer, 2 mm³ of template DNA (final volume 25 mm³; all reagents were purchased from

Promega, Germany). Conditions of PCR were: predenaturation 94 °C/5 min, 35 cycles of denaturation 94 °C/30 s, annealing 55 °C/40 s, extension 72 °C/40 s; final extension 72 °C/7 min. PCR was carried out in Mastercycler personal (Eppendorf, Germany).

Obtained amplicons were separated on 1.5% agarose gels (Promega, Germany), stained with GoldView (SBS Genetech Co. Ltd., China) and visualized at λ = 254 nm. Sequence analysis of PCR products was performed by Genetion (Slovakia). The nucleotide sequences were processed in computer program Vector NTI 9.1 and compared with GenBank sequences (<http://www.ncbi.nlm.nih.gov/BLAST>). The nucleotide sequence of *L. mucosae* D was deposited in GenBank.

Enzymatic treatment of *L. mucosae* D

Bacterial cells of *L. mucosae* D (McFarland 0.5) were treated with proteolytic enzymes (trypsin (PAA, Austria), pronase E and proteinase K (both Merck KGaA, Germany) in final concentration 1 mg/cm³ for 1 h at 37 °C. After treatment, bacterial cells were washed twice by sterile PBS and the adherence assay (HeLa, Caco-2, L929 cell lines) was performed as described before. Values in each sample were compared with control (untreated *L. mucosae* D) by Student's pair *t* test of the difference of two relative values and *p* values were expressed.

Scanning electron microscopy

An overnight culture of *L. mucosae* D in 2.25 × 10⁶ CFU/cm³ in supplemented MEM medium was co-cultivated with HeLa cells 1 or 24 h. HeLa cells without lactobacilli in the 1st and 24th hour were used as a control. Cultivation conditions were the same as mentioned above. After that, cells were washed with sterile PBS twice to remove non-adherent cells and fixed with glutaraldehyde (3% v/w in phosphate buffer) for 1 and 3 h. Samples were dehydrated with 50 and 70% ethanol three times for 10 min with each. Coverslips were then air-dried, mounted on the stubs and coated with golden particles. Specimens were screened by JSM-6300 Scanning Microscope (Japan).

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