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Modulation of *Escherichia coli* biofilm growth by cell-free spent cultures from lactobacilli

Research Article

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Abstract: *E. coli* biofilms cause serious problems in medical practice by contaminating surfaces and indwelling catheters. Due to the rapid development of antibiotic resistance, alternative approaches to biofilm suppression are needed. This study addresses whether products released by antagonistic bacteria – *Lactobacillus* isolates from vaginal and dairy-product samples could be useful for controlling *E. coli* biofilms. The effects of diluted cell-free supernatants (CFS) from late-exponential *Lactobacillus* cultures on the growth and biofilm production of *Escherichia coli* were tested. Most of the CFS applied as 10^{-2} had no impact on bacterial growth, biofilm development however was influenced even by 10^{-4} of CFS. Initial screening by crystal violet assay showed that biofilm modulation varied between different CFS and *E. coli* combinations from inhibition to activation; however three of the tested CFS showed consistency in biofilm suppression. This was not due to antibacterial activity since Live/Dead fluorescence labeling showed insignificant differences in the amount of dead cells in control and treated samples. Some *E. coli* strain-specific mechanisms of response to the three CFS included reduction in hydrophobicity and motility. Released exoploysaccharides isolated from the three CFS stimulated sessile growth, but proteinase K reduced their inhibitory activities implying participation of protein or peptide biofilm suppression factor(s).

Keywords: *Biofilm modulation • E. coli • Lactobacillus spent cultures*

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1. Introduction

The sessile growth of bacteria as biofilms causes serious problems in medical practice. Biofilms developed in clinical environments can be a source of nosocomial infections. They can also contaminate indwelling medical devices and cause both device malfunctions and infection complications of the patient.

In urinary-tract infections (UTI), *E. coli* are among the most frequent causative agents. Together with other virulence factors, their biofilm-forming capacity is of special importance for longer-term persistence and recurrence [1], as well as for catheter-associated infections [2-5]. Biofilm microorganisms are much more resistant to antibiotic treatments than plankton [3,6,7]. The problems caused by sessile bacteria require alternative approaches to biofilm control.

Natural biofilms are complicated consortia of species and strains. Interactions between them vary from synergism to antagonism [5,8-10]. Antagonistic relations have been studied by many authors. The potential of commensal probiotic bacteria to interfere with the ability of pathogens in colonizing the host has been explored with the aim to develop alternative

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approaches for supportive medical applications. One very important group in focus is lactobacilli. A variety of *Lactobacillus* strains were reported as producers of inhibitory substances against other bacterial species, including pathogens [2,11-13]. Released antibacterial substances in cell-free culture supernatants (CFS) can inhibit both the growth of different pathotypes of *E. coli* [14-16] and the expression of virulence factors [17]. In spite of the observation that in mixed biofilms lactobacilli can influence biofilm growth of other microorganisms or displace them from already formed biofilms [13,18-20], the effects of secreted *Lactobacillus* metabolites on biofilm formation have only rarely been addressed [20,21].

This study is focused on the possibility that released lactobacillus products could interfere with biofilm formation of *E. coli* laboratory and UTI strains on abiotic surfaces. As a source of CFS, 11 *Lactobacillus* vaginal and dairy-product isolates from the laboratory collection of the Institute of Microbiology were selected on the basis of their recently reported broad-spectrum antibacterial activities [22-24]. We collected evidence suggesting that highly diluted non-inhibitory amounts of CFS (10^{-2} to 10^{-4}) can still have a biofilm-modulation capacity and that this is probably due to factors different from those involved in their antibacterial activity.

2. Experimental Procedures

2.1 Microorganisms, growth media and preparation of CFS

The microbial cultures used in this study are listed in Table 1. The *E. coli* strains were selected based on previous tests on biofilm formation [25]. They were kept frozen at -20°C in TSB (Difco) containing 20% glycerol. Before the experiments, the strains were streaked on TS agar. Three single colonies were selected, inoculated separately in TSB and subcultivated twice in TSB at 37°C. From each of these, samples were applied on slant agar and used as a source of inocula.

The *Lactobacillus* strains were isolated from vaginal samples of healthy volunteer women or from dairy products (Table 1). They are a part of laboratory collection and were identified to the species level in our previous work [22,26]. The strain *L. plantarum* Lb26 (LB7) is deposited in NBPMCC, Bulgaria with N°8664. Before CFS collection, they were subcultivated twice in DE MAN Rogosa and Sharpe (MRS) broth (Merck)

Strain	Collection, [References]
Escherichia coli strains:	
Escherichia coli W1655 F lac$^+$ Str-s, Met-	J. Gumpert, Inst Mol Biotechnol, Jena, DE [40,41]
Escherichia coli 406, Strain K-12 AB sfa, lac$^+$	NBIMCC*
Escherichia coli 420, Strain K-12 C600, F$^-$, lac$^+$	NBIMCC
Escherichia coli PU-1, Amp-r	Urine, woman with pyelonephritis, BM-PU** [42]
Escherichia coli PU-3	Urine, woman with pyelonephritis, BM-PU [42]
Escherichia coli PU-13 Am-r, Sxt-r	Urine, man with cystitis, BM-PU [42]
Lactobacillus strains:	
Lactobacillus gasseri Lb821 (LB1)****	Vagina, healthy woman/ IMS*** Lab. Collection
Lactobacillus salivarius Lb832 (LB2)****	Vagina, healthy woman/ IMS Lab. Collection
Lactobacillus fermentum Lb304 (LB3)****	Vagina, healthy woman/ IMS Lab. Collection [25]
Lactobacillus fermentum Lb364 (LB4)****	Vagina, healthy woman/ IMS Lab. Collection [25]
Lactobacillus fermentum Lb362 (LB5)****	Vagina, healthy woman/ IMS Lab. Collection
Lactobacillus plantarum Lb34 (LB6)****	White brined cheese/ IMS Lab. Collection [22]
Lactobacillus plantarum Lb26 (LB7)****	White brined cheese / IMS Lab. Collection [22,25]
Lactobacillus plantarum LbKCB1 (LB8)****	White brined cheese / IMS Lab. collection
Lactobacillus plantarum LbKCC1 (LB9)****	White brined cheese / IMS Lab. collection
Lactobacillus plantarum LbS11 (LB10)****	White brined cheese / IMS Lab. collection
Lactobacillus sp. Lb K4 (LB11)****	Kumis/ IMS Lab. collection

Table 1. Bacterial strains.*NBIMCC, National Bank of Industrial Microorganisms and Cell Cultures, Sofia, Bulgria; **BM-PU, Collection of the Department of Biochemistry and Microbiology, Plovdiv University, Plovdiv, Bulgaria; ***IMS- Institute of Microbiology, Sofia, Bulgaria. ****Abbreviation used in the text for designation of the spent culture media of the strains.

with a pH of 6.5 for 24 h at 37°C by anaerobiosis (BBL® GasPak Anaerobic system). 10% (v/v) of these were used as inoculum for fresh MRS. Following overnight cultivation until OD_{600} of each of the cultures reached the pre-established specific values corresponding to late exponential growth phases of each of the *Lactobacillus* strains [27,28]. The 11 *Lactobacillus* cultures are thus in in optimal growth status for metabolite secretions and antibacterial compound production [22-24]. The cells were then removed by centrifugation (18000x*g*, 10 min, 4°C) and the supernatant was filter-sterilized (0.22 µm). The CFS were aliquot-frozen and kept at -20°C for up to 6 months.

Biofilm growth experiments were performed in minimal salt M63 medium (0.02 M KH_2PO_4, 0.04 M K_2HPO_4, 0.02 M $(NH_4)_2SO_4$, 0.1 mM $MgSO_4$ and 0.04 M glucose, pH 7.5). The medium was supplemented with 10^{-2} MRS (control) or with the decimal dilutions of the CFS specified below.

2.2 Effects of 10^{-2} diluted CFS on *E. coli* planktonic growth

To avoid interference of antibacterial and antibiofilm activities of the CFS, non-inhibitory dilutions of the CFS were applied throughout this study. These were determined by an initial disk-diffusion assay. *E. coli* were plated on Müller-Hinton agar. Disks (5 mm) were applied onto the plates and loaded with 10 µl of the LB filtrates – non-diluted, or decimally diluted in M63 medium. Inhibition was estimated after 24 h of growth at 37°C. Antibacterial activities of the 11 *Lactobacillus* spent cultures were confirmed for non-diluted and diluted 10^{-1} CFS. These varied from strongly bactericidal to low bacteriostatic activity. No inhibition was registered with higher-order dilutions, 10^{-2}, 10^{-3}, and 10^{-4} which were further used in biofilm tests.

The influence of the 10^{-2} diluted CFS on planktonic growth was checked in M63 medium. The *E. coli* strains were cultivated overnight at 37°C in TSB and diluted 1:100 in M63 medium supplemented with 10^{-2} of either MRS (control) or CFS. Samples of 150 µl were placed in 96-well U-shaped microtitre plates (Nunc). Each variant was repeated in 6 wells. The samples were incubated at 37°C and turbidity was measured hourly by a LP 400 ELISA reader at 620 nm.

2.3 Hydrophobicity

The *E. coli* strains were cultivated for 18 hours in M63 medium supplemented with 10^{-2} of MRS (control) or LB1, LB5 and LB10. The bacteria were pelleted and resuspended in PBS to OD_{620} 1.0. The hydrophobicity test using hexadecane was applied as described by Li and McLandsborough [29]. Each bacterial suspension was divided into 1-ml samples that were placed into 6 separate tubes. Two of them were used to estimate OD of the whole culture, and 250 µl of hexadecane was added to the other 4 tubes. The samples were vortexed and left for 30 min at 37°C for the phases to separate. Two 250 µl aliquots of each of the hexadecane-untreated tubes, or from the water phases of the tubes with hexadecane, were placed in the wells of a 96-well plate and OD_{620} was measured. The percentage of hydrophobic cells in each sample was calculated according to the formula $A_o–A_{hex}/A_o x100$ where A_o is the absorbance of the sample without hexadecane, and A_{hex} is the absorbance of the sample with hexadecane.

2.4 Motility

Motility of the strains was estimated in 0.3% agar prepared in M63 medium supplemented with 10^{-2} of either MRS (control) or LB1, LB5 and LB10. Overnight *E. coli* cultures were needle-inoculated into the agar. Motility halos were measured after 7 hours incubation at 22°C. Each variant was repeated in triplicate.

2.5 Biofilm growth estimation by crystal violet (CV) assay

The CV test was applied following the test medium and protocol described for UPEC biofilms [1]. In this series of studies, M63 medium supplemented with 10^{-2} MRS (control) or 10^{-2}, 10^{-3}, and 10^{-4} of the CFS was used. The *E. coli* were pre-cultivated similarly as in the test for planktonic growth and diluted 1:100 in the media. Samples of 100 µl were placed in 96-well U-shaped microtitre plates (Nunc). Six wells per variant were included in the plates and peripheral wells were avoided. Each plate contained one control row of 6 wells. Biofilm developed for 24 h at 22°C. Plankton was removed, the wells were washed with 0.85% NaCl and the biofilm was stained for 15 min with 0.1% crystal violet (CV). The dye was solubilised with 75% ethanol and the absorbance was measured at 550 nm. For each of the *E. coli* strains, samples from three single colonies were processed in parallel on three separate plates, each applied in 6 wells per variant.

2.6 Bacterial vitality in biofilms estimation by Live/Dead fluorescent test

Biofilms were statically cultivated for 24 h on microscope cover glasses spin-coated with methyl methacrylate. The glasses were placed in 6 cm glass Petri dishes and sterilized for 4 h at 180°C. Overnight *E. coli* cultures were dissolved 1:100 in M63 supplemented with 10^{-2} of either MRS (control) or CFS from LB1, LB5 or LB10, and 3 ml of the bacteria suspension was carefully applied over the cover glasses. Following 24 h of cultivation

at 22°C, plankton was removed and the glasses were delicately rinsed with 0.85% NaCl. The biofilms were colored by L13152 Live/Dead BacLight bacterial viability kit (Molecular Probes) using the protocol recommended by the provider. Observations were made on laser scanning microscope Nikon Eclipse Ti-U.

2.7 Proteinase K treatment of CFS

CFS samples from LB1, LB5 and LB10 were incubated for 1 hour at 37°C in the presence of 10 µg/ml of proteinase K (Boehringer Ingelheim) and the enzyme was inactivated for 10 min at 70°C. CV test was performed on biofilms grown in M63 alone (control) or supplemented with 10^{-2} of untreated or proteinase K-treated CFS.

2.8 Preparation and application of samples from released exopolysaccharides (rPS)

Two ml quota of the CFS were extensively dialyzed overnight against dH_2O and checked for rPS concentrations by the phenol-sulfuric acid method using a glucose standard. rPS was isolated from 10 ml of CFS of LB1, LB5 and LB10 by cold ethanol precipitation. The samples were dialysed and the amount of rPS was estimated as abovE. M63 medium was supplemented with 10 µg/ml of each rPS and the effects on biofilm growth were checked.

2.9 Statistical analysis

Calculation of mean values and standard deviations were done using Microsoft Excell. Comparisons of the mean values and estimation of differences between test and control samples were conducted *via* Student testing by the 'Statistika' software package (http://sites.google.com/site/borjanaboeva). In comparing different samples, probability values for $P<0.05$ were accepted as indicative for statistically significant differences.

3. Results

3.1 Effects of *Lactobacillus* cell-free supernatants on *E. coli* growth

To check for the possible interference of CFS with *E. coli* growth dynamics under the nutrient conditions applied further to biofilm growth, turbidity of samples inoculated in M63 medium with or without supplements was measured hourly. Most of the CFS had no notable effect on bacterial growth curves. As an exception, LB6 and LB7 suppressed growth during early stages of culture - hours 2 to 6, resulting in prolonged lag phase of *E. coli* cultures (Figure 1).

3.2 Modulation of *E. coli* biofilm growth by non-inhibitory amounts of the *Lactobacillus* cell-free supernatants

The six *E. coli* strains differed in their biofilm-forming capacity. Strain *E. coli* 420 produced much higher amounts of biofilm (A_{550} 0.820±0.092) than the other strains. It was followed by *E. coli* W1655 (A_{550} 0.275±0.087), and then the other strains for which A_{550} was within the range between 0.150 and 0.100. The biofilm-modulating effects of the CFS were registered at three decimal dilutions (10^{-2}, 10^{-3} and 10^{-4}). For easier comparison of CFS effects between the *E. coli* strains, the results were graphically represented as percent values from controls (the mean absorbance value in each plate of biofilm growth in the absence of CFS). Biofilm production was influenced by all *Lactobacillus* filtrates (Figure 2). Biofilm modulation varied from significant inhibition, though no statistically significant effect, to stimulation dependent on the combinations of *E. coli* strains and LB filtrates. In spite of the *E. coli* strain-to-strain differences, CFS from vaginal lactobacilli were more successful in suppressing *E. coli* sessile growth. The effects were more pronounced for *E. coli* K-12 strains than for clinical isolates. A similar trend characterized the spent media of two of the dairy-product lactobacilli, LB10 and LB11, and statistically significant biofilm reduction occurred in most strains of *E. coli*.

Biofilm-modulation activities of LB6 and LB7 are of particular interest. Even in non-inhibitory amounts, these two supernatants slowed down *E. coli* growth (see Figure 1). In the CV assay, LB6 unexpectedly increased biofilm production in three of the examined *E. coli* strains and had no effect on the other ones, and LB7 stimulated sessile growth in one and had no effect on three *E. coli* strains.

Biofilm suppression characteristics were most consistent in the presence of LB1, LB5 and LB10 CFS. When applied diluted 10^{-2}, LB1 and LB5 inhibited more than 50% the biofilm growth of all the *E. coli* K-12 strains and the clinical isolate *E. coli* PU-3, and were also effective with the other two uropathogenic strains. LB10 suppressed more than 50% the biofilms of *E. coli* 420 and PU-3, and significantly, though less effectively, the other tested *E. coli*. These filtrates seem promising as sources of biofilm inhibitors. For this reason, their impact on biofilm structure was examined, and an attempt was made to identify the nature of the active biofilm-suppressive substance(s).

3.3 Effects of LB1, LB5 and LB10 on *E. coli* biofilm morphology

In scanning electron microscopy experiments, RR was applied in the fixatives to preserve the extracellular

matrix during dehydration. Biofilm morphology in controls differed between strains. *E. coli* 420 produced a thick film with a rough surface and large cell patches protruding upwards. Sessile growth in the presence of LB1 and LB5 (10^{-2}) resulted in a sticky morphology with flattened cell patches and fracture-like spaces between them. LB10 had an apparently stronger deteriorating effect on this strain. All other strains formed flat sessile communities, and *E. coli* PU-1 is shown as a representative example of this biofilm morphotype. CFS application resulted in uneven substratum coverage, with alternation between cell monolayers and more or less thicker films.

To check for the vitality of cells in the biofilms, the Live/Dead fluorescence stain test was applied. This is a combination of Syto 9® which colors all cells green, and propidium iodide which penetrates dead cells only. The test showed no substantial differences between control and LB-treated samples regarding the amounts of dead cells (Figure 3).

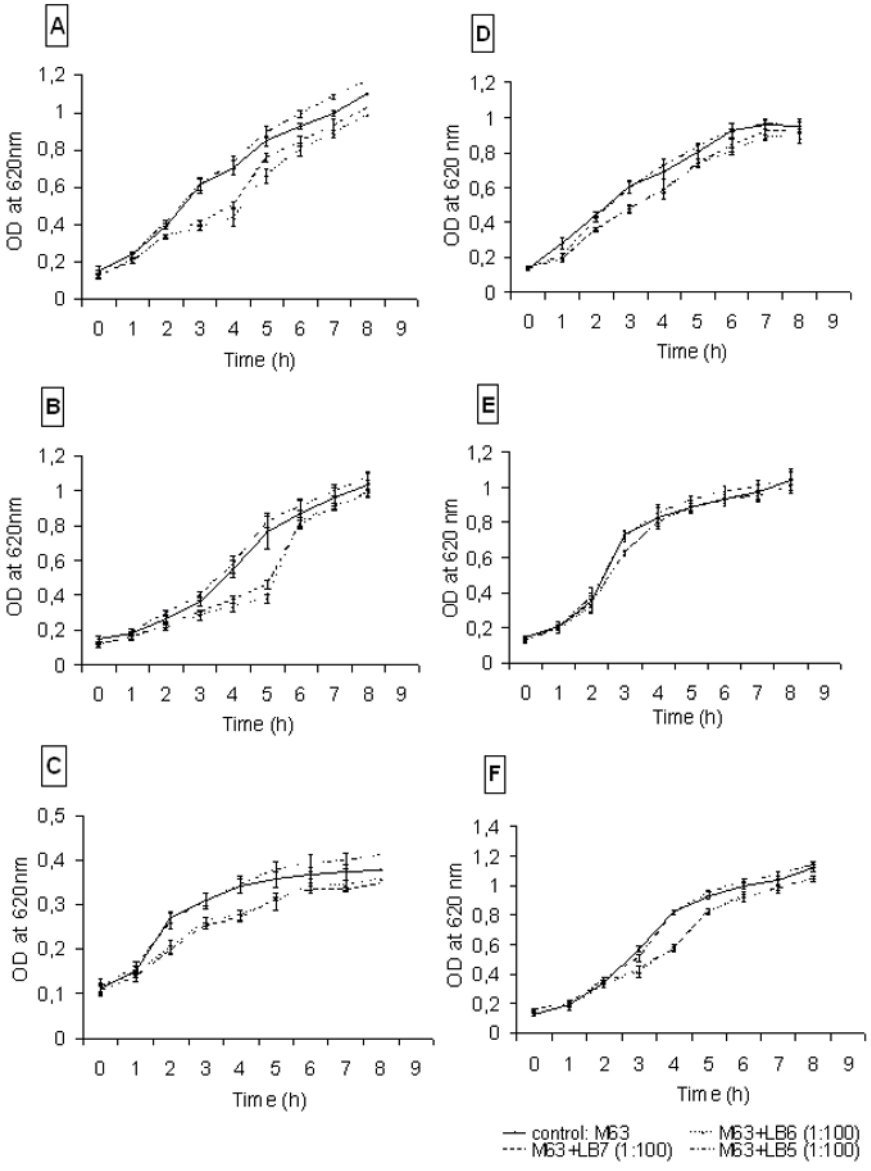

Figure 1. Growth of *E. coli* W1655 (A), *E. coli* 420 (B), *E. coli* 406 (C), *E. coli* PU-1 (D), *E. coli* PU-3 (E) and *E. coli* PU-13 (F) in minimal M63 medium supplemented with 10^{-2} MRS (control) or 10^{-2} CFS from LB5, LB6 and LB7 slowed down growth during early intervals. The other LB filtrates caused no notable differences from controls, and LB5 is included as a representative sample for them. Each time point represents the mean absorbance of 6 wells, error bars stand for standard deviations.

3.4 Influence of LB1, LB5 and LB10 on *E. coli* hydrophobicity and motility

One possible way by which the selected CFS could reduce biofilms is *via* changes in cell surface hydrophobicity, important especially during initial adherence to the hydrophobic surface of the polystyrene plates. When the *E. coli* strains were cultivated in M63 medium supplemented with 10^{-2} of the three CFS, a general trend of hydrophobicity diminution was observed (Table 2). The motility test was performed at 22°C in 0.3% agar in either M63 medium alone, or M63 supplemented with 10^{-2} of LB1, LB5 or LB10. Motility halos developed 7 hours later around only two of the UPEC strains, with distinct reduction of the diameters measured in the cases of CFS supplementation (Table 2). Under the experimental conditions, all other strains were non-motile.

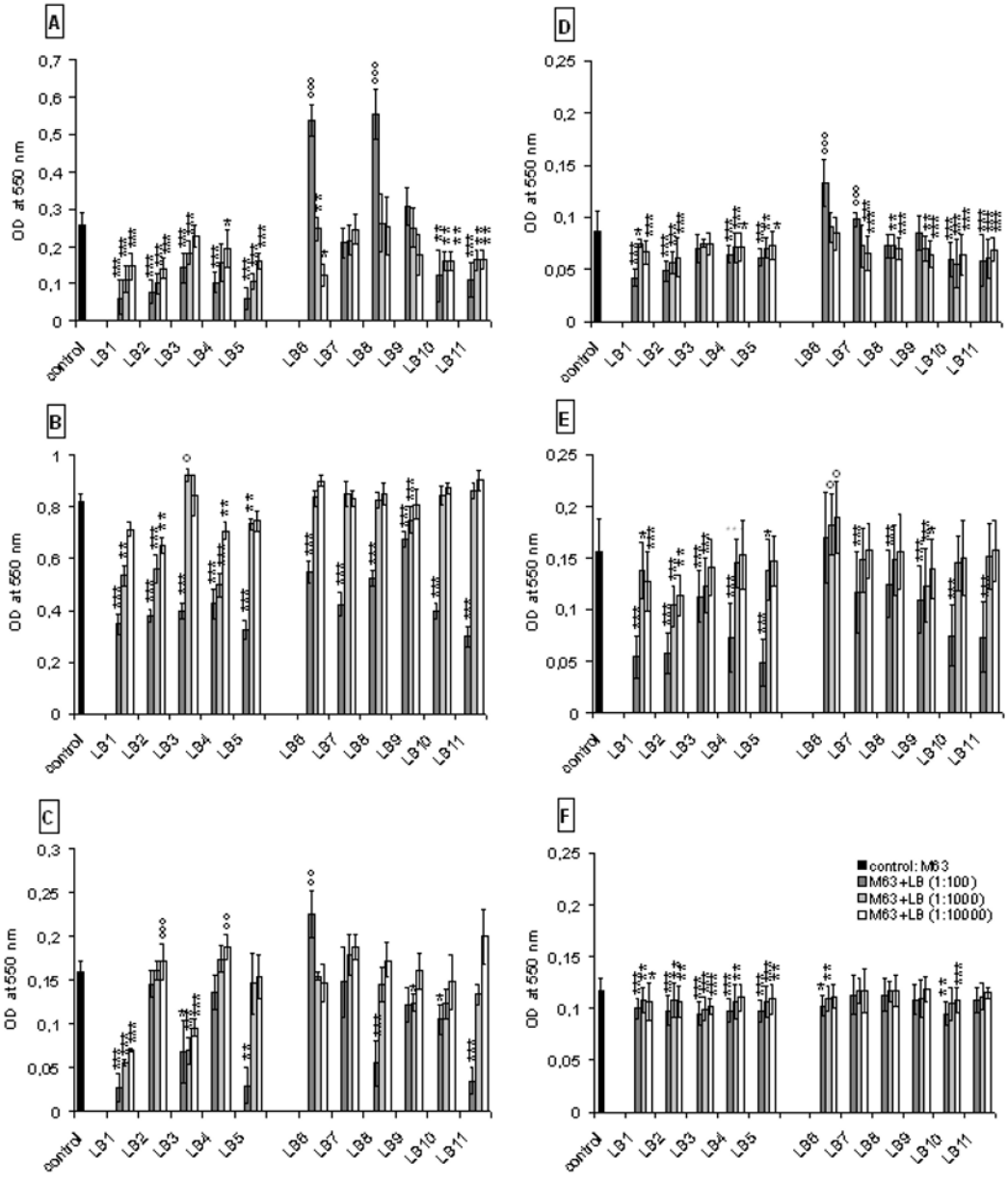

Figure 2. Biofilm growth, CV test, of *E. coli* W1655 (A), *E. coli* 420 (B), *E. coli* 406 (C), *E. coli*PU-1 (D), *E. coli* PU-3 (E), and *E. coli* PU-13 (F) in M63 medium supplemented 10^{-2} MRS (control, black bars) or diluted LB-CFS (10^{-2}, dark-grey bars; 10^{-3}, light-grey bars; 10^{-4}, white bars). The mean values of measured absorbances of 6 wells per variant for each of at least three independent experiments is included. Error bars represent the standard deviations between the experiments. Statistically significant differences from controls are labeled (decrease:***P<0.001, **P<0.01, *P<0.05; increase: °°°P<0.001, °°P<0.01, °P<0.05).

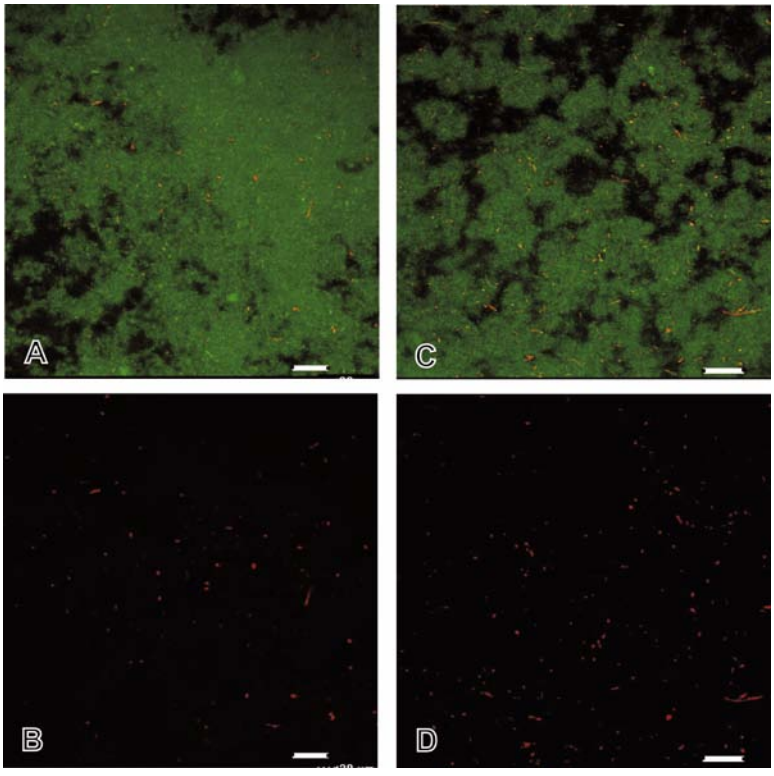

Figure 3. Fluorescence images of 24-h biofilms of *E. coli* 420, Live/Dead BacLight bacterial viability kit. Biofilm was developed for 24 h in M63 supplemented 10^{-2} MRS (controls, A and B) or LB10 (C and D). Images in A and C represent coloring of the live cells with Syto 9® - green, and of dead cells with propidium iodide – red. Images in B and D show only the red signal in, respectively, images A and C. Scale bars = 10 μm.

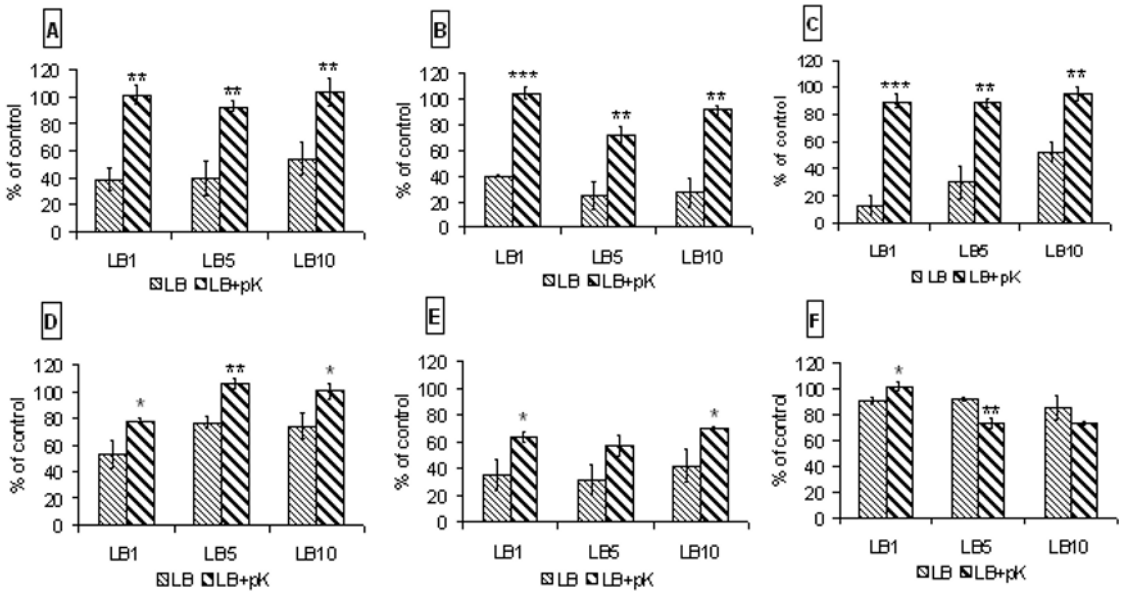

Figure 4. Effect of proteinase K treatment on the biofilm-modulating activities of the CFS, CV assay. Biofilms were grown in the presence of 10^{-2} of the total LB1, LB5 and LB10 or the CFS treated with proteinase K (LB1+pK, LB5+pK, and LB10+pK). The averages of control values (biofilms grown M63 medium supplemented 10^{-2} MRS not shown) were accepted as '100%', and the other values were normalized to this. Each bar represents the mean per cent from three independent experiments (including 6wells each), and error bars stand for standard deviations between the three experiments. Asterisks stand for statistically significant (***P<0.001, **P<0.01, *P<0.05) differences between the pK-treated samples and the corresponding untreated CFS.

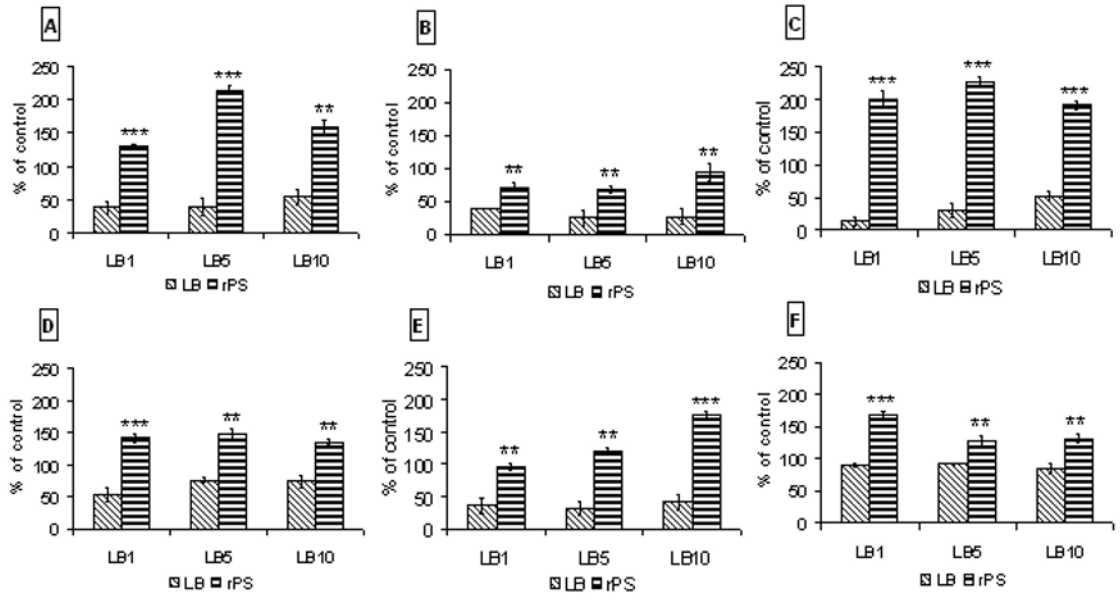

Figure 5. Comparison between the effects on biofilm growth (CV test) of 10^{-2} total LB1, LB5, and LB10 and of 10 μg/ml rPS isolated from them. The average of control values (biofilms grown in M63 supplemented with 10-2 MRS) were accepted as '100%' and the other values were normalized to it. Each bar represents the mean per cent from three independent experiments, and error bars stand for standard deviations between the experiments. Asterisks stand for statistically significant differences (***P<0.001, **P<0.01, *P<0.05) between the effects of total CFS and their respective rPS.

	Hydrophobicity (%)					Motility (mm)		
control	18.62±2.9	29.2±0.2	10.1±0.7	9.5±0.5	6.27±0.2	8.2±0.4	8.5±1	13.3±1.7
LB1	11.1±0.6**	27.6±1.4**	5.2±0.8**	7.5±0.4**	3.2±0.2***	6.4±2.2	4.3±0.7**	6.3±0.7**
LB5	5±0.5***	19.3±1.9**	9.7±1.4	5.6±1.1**	2.3±0.2***	1.3±0.4***	6±1*	7±0.5**
LB10	7.07±0.5***	16.5±0.7***	2.7±0.4***	8.4±0.5*	2.7±0.7**	2.4±0.3***	4.5±0.5****	5±1***
Strain	*E. coli* W1655	*E. coli* 420	*E. coli* 406	*E. coli* PU-13	*E. coli* PU-1	*E. coli* PU-3	*E. coli* PU-1	*E. coli* PU-3

Table 2. Changes in *E. coli* cell surface hydrophobicity and motility under the action of CFS All tests were repeated in triplicate. Statistically significant reduction of test sample mean values in comparisons with controls are presented as (*), P<0.05, (**), P<0.005 or (***), P<0.001.

3.5 Contribution of polypeptides and rPS to biofilm modulating activities of LB1, LB5, and LB10

To check for a possible contribution of secreted proteins or peptides to the activities of the three CFS, proteinase K treatment was applied. Removal of proteins and/or polypeptides from the CFS resulted in higher biofilm growth than in enzymatically non-treated CFS (Figure 4). However, with some strain-to-strain differences, this treatment did not completely restore the values of the control biofilm.

In addition, the possible role of extracellular rPs secreted during the *Lactobacillus* cultivation on *E. coli* sessile growth was estimated. With this aim, we first determined the rPS amounts in dyalised total CFS. The established values were 1200 μg/ml for LB1, 1700 μg/ml for LB5, and 800 μg/ml for LB10. We then checked the effect of 10 μg/ml (approximately - the rPS amount in 10^{-2} dilution of the three total CFS) to biofilm growth. Unexpectedly, the samples had a biofilm-stimulating effect rather than an inhibitory affect on all *E. coli* strains except *E. coli* 420 (Figure 5).

4. Discussion

The antibacterial activities of secreted metabolites from a variety of *Lactobacillus* strains against different

pathotypes of *E. coli* have been well-documented [14-16,21]. It has been shown that 80% CFS from *L. rhamnosus* GR-1 and *L. reuteri* RC-14 can influence surface membrane characteristics of an UPEC isolate by upregulation of OMPs and downregulation of type 1 and P fimbriae synthesis [17]. Furthermore, non-diluted CFS from *L. rhamnosus* GR-1 were able to influence pre-formed UPEC biofilms by increasing the number of dead cells and decreasing cell density [19]. Antibacterial activities of *Lactobacillus* non-diluted CFS may thus be a decisive factor for biofilm deterioration [19].

Other organisms, e.g. *Pseudomonas aeruginosa*, *Hafnia alveyi,* and marine microorganisms such as *Pseudoalteromonas* sp and coral-associated actinomycetes, were shown to contain CFS component(s) which have biofilm-modulating activities *per se*, either stimulating or suppressing attached growth, and are not antimicrobial [30-34]. As a result of the increasing incidence of antibiotic-resistant strains, such results put into focus the search for substances that may be active in biofilm suppression without killing bacteria. Likely was the rationale of the present study, which was focused on the effects of highly diluted non-inhibitory amounts (10^{-2} to 10^{-4}) of CFS from a novel collection of *Lactobacillus* strains on *E. coli* biofilm formation.

The examined CFS from 11 *Lactobacillus* strains were previously shown to have a broad spectrum of antibacterial activity against Gram positive and Gram negative reference strains and antibiotic-resistant out-patient strains, two *Acinetobacter baumannii* and two *Pseudomonas aeruginosa*. Based on these results, strains LB6 and LB7 were considered as probiotic candidate strains with strong transit tolerance and biological activity [22-24]. The present results are in support of this. Even when diluted 10^{-2}, these two CFS, while not producing zones of inhibition on Mueller-Hinton agar, still slowed down the growth of the *E. coli* strains at early log phase (2-6 h) (Figure 1). This shows that antibacterial potential of the CFS does not necessarily predict an antibiofilm action.

The observed biofilm modulation by the CFS varied highly according to the combination of different CFS and *E. coli* strains. Similarly, a recent study on the effects of CFS from water-borne bacteria indicated variable outputs depending on strain-CFS combinations [35].

The results on *E. coli* biofilm modulation by LB6 andLB7 filtrates are of particular interest. Unlike their suppressing effects on *E. coli* growth, these CFS stimulated sessile growth in some *E. coli* strains, including one of the UPEC (Figure 2). Similarly, spent cultures of several probiotic bacterial species that had antibacterial activity against enteroaggregative *E. coli* stimulated instead of suppressing biofilm growth [21]. Such results indicate switching-on of protective mechanisms in *E. coli* and illustrate biofilm growth as an adaptation to unfavorable environmental clues.

Most consistent in their suppressive effects were LB1, LB5 and LB10. These were observed in most *E. coli* strains, not only with the 10^{-2}, but also with 10^{-3} and 10^{-4} dilutions. The Live/Dead fluorescence test did not show a significant increase of dead *E. coli* cells in biofilms grown in the presence of the three CFS. Hence, the deteriorating effects of these CFS on biofilm growth involve mechanisms different from bactericidal action. The results imply that the biofilm modulation activities of the CFS are not necessarily correlated with their antibacterial potential, and that biofilm suppression was due to factors and mechanisms other than antibacterial activity. Thus, the presently observed reduction of surface hydrophobicity and suppression of motility show that 10^{-2} dilutions of the three CFS can affect *E. coli* phenotypic characteristics important in the contacts with the substratum during the early stages of biofilm settlement.

To this moment, antibacterial activities of secreted metabolites from *Lactobacillus* strains have been attributed to several types of molecules: H_2O_2, lactic acid, biosurfactants, and antibacterial peptides [12]. Under our experimental conditions (use of frozen CFS samples at high dilutions), H_2O_2 was unlikely to have any contribution. Since supplementation of M63 with 10^{-2} to 10^{-4} CFS did not change the pH of the medium, the impact of organic acids was also not probable.

Other candidate molecules could be released polysaccharides [36] and secreted proteins [37,38]. Hence, we concentrated on the putative biofilm-modulation impact of released proteins and polysaccharides in LB1, LB5 and LB10. We demonstrated that proteolytic treatment reduced the biofilm-suppressive effects of LB1, LB5 and LB10 while 10 µg/ml of rPS from LB1, LB5 and LB10, an amount within the range in which rPS are present in these CFS under 10^{-2} dilution, had a biofilm-stimulating effect on most *E. coli* strains. Such an output of the rPS application differs from existing results, showing that polysaccharides released in bacterial culture media reduced biofilm production [36,39]. This could be due to differences in the rPS molecules, but is more likely due to the experimental protocol. Other authors included much higher quantities of rPS, while in this experimental protocol, it was applied in small amounts of the order in which it is present in the 10^{-2} diluted CFS. Taken together, our results imply that the presently observed biofilm suppression by 10^{-2} diluted LB1, LB5 and LB10 is, at least in part, due to peptide or protein factor(s). These probably antagonize the biofilm-promoting effects of the respective rPS.

In summary, the present results provided evidence that the examined set of *Lactobacillus* strains release into the culture medium substances with pronounced biofilm-modulating properties even at high-order dilutions (10^{-2} to 10^{-4}). The observed effects were most probably due to mechanisms different from bactericidal action. The *E. coli* strains showed individual responses to each of the 11 LB supernatants, but LB1, LB and LB10 were biofilm-suppressive throughout, and hence are a promising source of biofilm-suppression factor(s). The variety of strain-specific *E. coli* responses to the treatments indicated a multifactorial interaction. This may concern both the composition of the different CFS and strain-peculiar mechanisms of biofilm production in the tested set of *E. coli* strains.

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