Dual effects of medicinal plant extracts on mature biofilms of Escherichia coli dependent on time of exposure

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Abstract. Using a relatively simple experimental model with mature biofilms of Escherichia coli we showed that 1 h incubation with Vaccinium vitis-ideae, Betula pendula and Laminaria japonica extracts promoted biofilm dispersion inducing oxidative stress in the biofilms. However, a prolonged 3 h treatment completely changed mode of action of these extracts to strong prebiotic effects which were related to intracellular iron chelation.

1. Introduction

Sessile bacterial communities play a significant role in gut ecosystems contributing to state of health of host macroorganism [1]. On the other hand, communities of pathogenic biofilm-producing bacteria represent a real threat because of an increased resistance to antimicrobials [2]. Therefore, investigations of factors both promoting biofilm formation in commensal microbiota and inhibiting growth of biofilms of pathogens are in a great demand.

Special attention is given to compounds of plant origin known as polyphenols. These substances may demonstrate dual behavior in vivo and in vitro as antioxidants (scavenge free radicals and prevent their formation by chelating Fe^{2+} and thus inhibiting free radical production) and prooxidants (production of free radicals). Depending on a number of conditions, certain polyphenols and polyphenol-containing plant extracts may protect bacterial cells against oxidative stress and induce biofilm formation or, alternatively, produce bactericidal and anti-biofilm effects [3-6].

The aim of the present work was to study dynamic effects of medicinal plant extracts on colony-forming ability and antioxidant gene expression in mature biofilms of Escherichia coli. Extracts from Arctostaphylos uva-ursi, Vaccinium vitis-ideae, black and green teas contained high levels of polyphenols and showed a high ability to chelate iron and produce H_{2}O_{2} during autoxidation. All of them could stimulate biofilm formation. For comparison, extracts from Betula pendula and Laminaria japonica were used, which only slightly influenced the formation of biofilms, contained low levels of polyphenols and had a low ability to chelate iron and generate hydrogen peroxide [4]. While green and black teas are commonly consumed worldwide with everyday meals, other studied extracts are prescribed as anti-inflammatory agents during treatment or prophylaxis of bacterial infections.
2. Materials and methods

2.1. Preparation of the plant extracts

Water extracts of medicinal herbs Arctostaphylos uva-ursi, Vaccinium vitis-idaea, Betula pendula, Laminaria japonica were prepared from commercial pharmaceutical preparations (OAO Krasnogorskleskredstva and ZAO Ivan-Chai, Russia) and commercial samples of green and black tea (Greenfield “Golden Ceylon”, Greenfield tea Ltd., London, W1U 2HQ, UK) were prepared as previously described [6]. Dry herbs (1 g) were boiled in 30 ml of distilled water in a water-bath during 30 min, cooled and sequentially filtrated through paper and membrane (0.45 µm pore size) filters. This initial extract was concentrated by 8 times using a rotary evaporator IKA RV 10 basic (Germany). The final dose applied in the cultural medium 6.6 mg dry herb/ml. This dose was chosen as the maximal which did not result in precipitation during the incubation period. Fresh extracts were used in all experiments. Reagents including thiamine, casamino acids, agar, Luria-Bertani broth were from Sigma-Aldrich Chemical Co (St Louis, MO, USA). Other reagents were of analytical grade (Reachim, Russia).

2.2. Bacterial strains and growth conditions

The strain of E. coli BW25113 was obtained from Keio collection [7]. The strains carrying transcriptional gene fusions katG::lacZ were constructed by transformation of the parental strain with pKT1033 plasmid [8]. Strain E. coli BN407 carrying iucC::lacZ fusion was from Dr. J. Imlay [9]. Bacteria were grown overnight at 37 °C without shaking in minimal M9 medium supplemented with glucose (4 g/l), 0.2 % casamino acids and 10 µg/ml thiamine [10]. After centrifugation at 6000 g for 4 min, the cells were resuspended in fresh M9 to initial optical density at 600 nm (OD<sub>600</sub>) of 0.1. This culture was transferred to 96-well polystyrene microtiter plates (200 µl per well) and incubated statically at 37 °C for 22 h to obtain biofilms. Mature biofilms were washed twice with 0.9% NaCl. Then 200 µl of fresh M9 (4 g/l glucose) medium supplemented with 0.2% casamino acids, 10 µg/ml thiamine and 5 µl of extract were added in each well. The plates were incubated at 37 °C with shaking (340 rpm) in Shaker Thermostat Sky Line (ELMI, USA) for 3 h.

2.3. Estimation of effects of the plant extracts on colony-forming ability

In order to determine colony-forming ability (CFU/ml) in biofilms, medium was removed, the biofilms were washed with sterile saline and sonicated by two pulses (37 kHz, 30W) for 1 min each with pause time of 1 min in a water bath sonicator (Ultrasonic cleaning unit Elmasonic S10 H, Elma, Germany). Then, OD<sub>600</sub> was measured using xMark™ spectrophotometer (Bio-Rad, USA) and 10-µl drops of serial dilutions were plated on LB-agar. Colonies were counted in 24 h after incubation at 37 °C.

The rate of plant extract-induced specific rate of CFU decline (ψ) was calculated based on the decline of the density of viable bacteria over a defined period, using the equation ψ = [ln(N<sub>t</sub>/N<sub>0</sub>)]/t, where N<sub>t</sub> is the cell density (CFU/ml) at time t; N<sub>0</sub> is the initial cell density before extract addition and t is the time in hours (1 and 3 hours in the current study) [11].

2.4. Measurement of β-galactosidase activity

Gene expression was estimated when measuring β-galactosidase activity in reporter strains carrying fusions of the genes katG and iucC with the gene lacZ [10] with the help of SmartSpec Plus Spectrophotometer (Bio-Rad, USA). In biofilms, broth was removed and the plate wells were rinsed to remove nonadherent bacteria. Fresh M9 was further added to each well and biofilms were sonicated by two 1 min pulses (37 kHz, 30W) with 1 min pause time between them in a water bath sonicator (Ultrasonic cleaning unit Elmasonic S10 H, Elma, Germany). Aliquots of 340 µl of similar planktonic culture samples were collected from two similar wells and OD<sub>600</sub> was measured. Then 170 µl of these aliquots were transferred to two tubes each containing 690 µl of reducing buffer [10]. Then, 5 µl of sodium deoxycholate and 5 µl of toluene were added to each tube and incubated for 30 min at 37°C.
Then 170 µl of 2-nitrophenyl-β-D-galactopyranoside (ONPG) were added to each test tube and incubated for 5 min. “Blank” tube was supplied with 170 µl of reducing buffer instead. The reaction was stopped by addition of 170 µl of K₂CO₃ and OD₄₂₀ and OD₅₅₀ were measured. β-galactosidase activity was expressed in Miller units, calculated using the formula:

\[(\text{OD}_{420} - 1.75 \times \text{OD}_{550} / \Delta \text{OD}_{600} \times t) \times 6000\]

where OD420 and OD550 – optical density of the samples, ΔOD600 is the difference between OD600 of bacterial culture and OD600 of bacteria-free medium, t – duration of exposition with ONPG, 6000 – coefficient taking into account dilution of the culture.

Each result is indicated as the mean value of at least five independent experiments ± the standard error of the mean (SEM). Significant difference was analyzed by Student’s t-test. A P-value of 0.05 was used as the cut-off for statistical significance. Results were analyzed by means of Statistica 6 (ver. 6, 2001; StatSoft Inc.).

3. Results and discussion

Mature biofilms of E. coli BW25113 before the exposure to fresh medium demonstrated an initial value of CFU*10⁵ equal to 1190 ± 75. Without extracts, after 1 h incubation in the fresh medium a dispersion of biofilm mass (data not shown) was accompanied with a CFU decrease down to 60 ± 5 which was further followed by a low growth of biofilm mass and a slight increase of CFU. Interestingly, during the first hour of incubation in the presence of A. uva-ursi extract decrease of CFU was 2-times smaller, while treatment with V. vitis-ideae resulted in 7.5-time more efficient biofilm dispersion compared to the cells not treated with the extracts. A 2-time enhancement of biofilm dispersion was found with B. pendula and L. japonica extracts as well (figure 1). This coincided with the values of specific rates of CFU decline after 1 h incubation in the presence of the studied extracts (figure 2). The observed CFU decline after 1 h incubation in the presence of V. vitis-ideae, B. pendula and L. japonica was accompanied with an almost 4- and 2-time rise in expression of katG gene, respectively (figure 3). This gene is specifically induced by hydrogen peroxide. Therefore, pro-oxidant action of these extracts could contribute to CFU decline in the mature biofilms of E. coli after 1 h incubation. As the incubation continued further, an alternative situation could be seen. CFU decline in the presence of V. vitis-ideae disappeared and, instead, a gradual growth of CFU was observed. CFU decline in biofilms was the highest (figure 1). CFU decline in the presence of V. vitis-ideae disappeared and, instead, a gradual growth of CFU was observed. In case of green tea CFU stimulation in biofilms was the highest (figure 1). CFU decline was absent after 3 h incubation with all the extracts but even a slight stimulation of growth was found with the exception for the black tea (figure 2).

![Figure 1](image1.png)

**Figure 1.** Colony-forming ability in mature biofilms in the presence of the extracts at different times of incubation.

![Figure 2](image2.png)

**Figure 2.** Specific rates of CFU decline in mature biofilms at 1 and 3 h of incubation with the extracts.
In our previous studies we have shown that under similar conditions mass biofilm formation in E. coli QC771 was stimulated in the presence of A. uva-ursi, V. vitis-ideae, green and black tea after 3 h incubation [6].

Elevation in expression of iucC gene involved in expression of Fe$^{2+}$ ions transporter [9] may also be used as an indicator of intracellular iron chelation by the extracts which pointed out antioxidant action of green tea already after 1 h of incubation (figure 4). Other extracts exhibited this type of antioxidant action (intracellular iron-chelation) in mature biofilms only after 3 h incubation which could contribute to the loss of CFU decline effect.

Recently published results stated strong anti-biofilm and bactericidal activity of V. vitis-ideae and B. pendula extracts [12] after 24 h incubation. We used a model experiment with mature biofilms that seemed more tolerant to external stress, however our results revealed that 1 h incubation of mature biofilms with V. vitis-ideae, B. pendula and L. japonica extracts promotes biofilm dispersion and therefore might be helpful in treatment of solid surfaces in order to prevent growth of biofilms. At the same time, prolonged treatment with green tea and other studied medicinal extracts could promote strong prebiotic effects.

4. Conclusions
Considering dual effects observed, our findings highlight the need to develop better biofilm model systems to see the whole range of polyphenol-containing medicinal plant extracts modulating effects.

E. coli is a convenient model microorganism which is a representative of normal human microbiota, however, at the same time, certain E. coli strains may become pathogenic and cause severe infections.

Thus, our results might have a practical interest because we have clearly shown that varying exposure time with the plant extracts might establish dual two-phase mode of influence on mature bacterial biofilms that includes promotion of biofilm dispersion followed by biofilm formation stimulation. This approach might be helpful in creating novel medications combining antimicrobial and prebiotic effects to treat antibiotic resistant infections caused by biofilm-associated strains.

Acknowledgements
This work was supported by state assignment 01201353246, also by the grant of Russian Foundation of Basic Research RFBR-Ural 19-44-590009 and by the grant from President of Russian Federation
for young scientists MK-3376.2018.4. The authors are grateful to Dr. J. Imlay for kindly providing bacterial strain.

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