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Influence of osmolites on dual-species biofilm formation

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Abstract

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In a large part of ecological niches, bacterial species are united in communities and develop together in the form of heterogeneous structures called biofilms, which are formed on surfaces of different nature and nature with the participation and as a result of the interaction of two or more bacterial species. In this study, we evaluated effects of the osmolality of the cultivating on the biofilm development and architecture of biofilms of the bacterium (*B. subtilis*) during their interactions with *Escherichia coli* K-12 1655 strain. Effects of the osmolality of the cultivating on bacterial biofilm formation and the mechanisms were analyzed by the crystal violet staining method combined with cultivated microbial analysis, and confocal laser scanning microscopy. A 24 h mature *Bacillus subtilis* and *Escherichia coli* dual-species biofilms were exposed to different concentration of NaCl of the cultivating. To investigate the effect of NaCl concentration, a series of experiments on the cultivation of biofilms in 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) were carried out according to the indicated scheme at the concentration of NaCl of 100 mM, 150 mM, and 200 mM. At cultivation in the cultural medium without NaCl, the bacterial growth rates of single-species biofilms of *Bacillus subtilis* strains and biofilms as a result of their interaction with *Escherichia coli* strains were similar and determines the mutualism between two strains in the structure of biofilms, but the temperature of the cultivating of the concentration of NaCl in the cultural medium over of 100 mM lead to the impeded growth and affect adversely the process of biofilm formation by the participation of *B. subtilis* 170 and *E. coli* K-12 1655, *B. subtilis* 168 and *E. coli* K-12 1655 strains, as a result of which a decrease in the value of the optical density, average thickness and relative spreading area and an inversely proportional increase in the ratio of the spreading area of the structures to their volume, but inhibits spore formation.

Keywords: Osmolality Regulation; Confocal laser scanning microscopy; *Bacillus subtilis* 170; Multispecies biofilms

Introduction

In a large part of ecological niches, bacterial species are united in communities and develop together in the form of heterogeneous structures called biofilms, which are formed on surfaces of different nature and nature with the participation and as a result of the interaction of two or more bacterial species (Rendueles & Ghigo, 2012). At the basis of their formation and their resistance to the various effects of the external environment are the relationships between microorganisms, which include competition for nutrients from the composition of the medium for cultivating biofilms, antagonism and symbiosis. The structure of biofilms and the composition of the microbial community in them are determined by the fluctuation in the values of environmental factors (Moons et al., 2006). Changes in the values of environmental factors affect the physiological state of the cells in the structure of the forming biofilm. In the process of development,

conditions are created for contacts between bacterial cells and contacts of cells with the surface on surfaces that are different in nature and morphology. Changes in environmental conditions have an impact on the properties of the bacterial cell, expressed in a change in the level of gene regulation for the implementation of the process of biofilm formation or in the charge of the cell surface, as well as on the physicochemical characteristics of the substrate (García-Gonzalo et al., 2013).

The growth and development of microbial biofilms is accompanied by the biosynthesis of exocellular polysaccharides, which ensure intercellular contacts in them and their attachment to the surface of the substrate, protecting microbial cells from the influence of adverse environmental factors – the formation and secretion of surfactants produced by other microbial species, which stimulate the transition from attached state to plankton, toxic compounds, oxidative and acid stress (Kingston et al., 2013). The formation of biofilms and the composition of the matrix forming them depend on the characteristics of the growth medium and the bacterial surface, its course is affected by the pH-value and temperature of the environment (Hamon & Lazazzera, 2001).

The osmoticity of the medium affects the process of biofilm formation involving strains of two or more bacterial species, which is determined by the nature and concentration of the osmolyte (Kavamura & Melo, 2014; Ogasawara et al., 2010). The basis of the regulation of the formation of biofilms by *E. coli* strains due to the change of the osmoticity of the medium is the activity of the two-component regulatory system (Cpx, EnvZ/OmpR and Rcs) (Hou et al., 2014; Dudin et al., 2013). High osmoticity of the medium causes repression of the genes responsible for the synthesis of curli-fringes in *E. coli* strains in the process of biofilm formation (Dudin et al., 2013). Under these conditions of cell development of the species in a state attached to different surfaces, the regulatory protein CpxR (Hou et al., 2014) and the sensor kinase RcsC (Ogasawara et al., 2010) have a negative effect on the expression of the csgD gene for the biosynthesis of curli- fringes in the way of binding the receptor region of the positive regulator OmpR (Dudin et al., 2013). The biosynthesis of the regulatory protein CpxR is activated at high salt content in the biofilm culture medium, which suppresses the formation of fringes in E. coli strains (Jubelin et al., 2005). Replacement with sucrose leads to the inhibition of the activity of the csgD gene for the synthesis of curli-fringes, which takes place with the participation of another regulatory protein – H-NS (Dudin et al., 2013), which is a low-molecular-weight protein that binds the DNA molecule (Sakamoto et al., 2012).

The high osmotic value of the medium inhibits the expression of genes in *B. subtilis* strains responsible for the formation of exocellular polysaccharides, proteins that are part of the matrix of biofilms, and contributes to the reduction of the thickness of the pellicle in the formation of biofilms at the interface between the air and the surface layer of the liquid environment (Rubinstein et al., 2012). This process is determined by the reduction of KinD kinase activity, which leads to an increase in the level of the Spo0A factor (Aguilar et al., 2010) and inhibition of the sporulation process during the development of the species in a state attached to different surfaces (Rubinstein et al., 2012; Aguilar et al., 2010). The kinase KinD catalyzes the transfer of phosphate groups to the core factor Spo0A, which ensures the maintenance of a high intracellular level of Spo0A–P, necessary for the initiation of the processes of biosynthesis of exocellular polymers of the biofilm matrix and for inhibition of the initial stage of spore formation (Rubinstein et al., 2012; Aguilar et al., 2010). The concentration of NaCl in the culture medium of 5% stimulates the formation of surface-active compounds by *B. subtilis* strains (Jha et al., 2017), which play the role of signaling molecules, the accumulation of which in the intercellular space creates conditions for the process of formation of biofilms on the roots of crop plants as a result of the increase in KinC activity of the Spo0A regulatory pathway (Lopez et al., 2008; McLoon et al., 2011) and limits the spread of strains of the species *Podosphaera fusca* (Romero et al., 2007), of the phytobacterial species *Xanthomonas campestris* pv. *cucurbitae* and *Pectobacterium carotovorum* subsp. *carotovorum* in them (Zeriouh et al., 2011). The increase in its content is accompanied by the inhibition of surfactin synthesis by *B. subtilis* strains (Jha et al., 2017), which is influenced by the pH value of the medium (Simoes et al., 2007), affecting the electrostatic interaction forces between the bacterial cell and the surface layer of the substrate and the concomitant formation of biofilms. Most natural biofilms are polymicrobial in composition, but the mechanism remains unclear about how osmolytes regulates polymicrobial biofilm development and the biofilm matrix component and spores in their structures.

The main objective of the present work is to study the effects of the osmolality of the cultivating on the biofilm development and architecture of biofilms of the bacterium (*B. subtilis*) during their interactions with *Escherichia coli* K-12 1655 strain. Effects of the concentration of NaCl in the cultural medium on bacterial biofilm formation and the mechanisms were analyzed by the crystal violet staining method combined with cultivated microbial analysis and confocal laser scanning microscopy. Taken together, the results of this study demonstrate a close link between biofilm formation and osmolality regulation in *B. subtilis* and *E. coli* strains, allowing a better comprehension of how bacteria can cope

with unfavorable osmotic value under environmental conditions.

Material and Methods

Experimental Design

In the first part of this research, a dual-species model biofilm consisting of *Bacillus subtilis* and *Escherichia coli* was developed. In order to obtain a strongly adherent and mature model biofilm, different (incubation) conditions were altered, i.e., growth medium. The adherence of the biofilm at each of the different (incubation) conditions was quantified by means of crystal violet staining and subsequent optical density (OD) measurements. To determine the cell density/maturity of the biofilm, viable plate counts were used. General and selective media were applied to determine the total biofilm cell density and the contribution of each individual species to this total cell density.

Bacterial Strains and Culture Media

In this research, *Bacillus subtilis* and *Escherichia coli*, both acquired from the BCCM/LMG bacteria collection of NBIMCC, NCIPD and the "Stephan Angeloff" Institute of Microbiology in Sofia, were used. Stock-cultures were stored at −80°C in Luria Bertani Broth (LB, NCIPD, Sofia), which were both supplemented with 20 (v/v) % glycerol (NCIPD, Sofia). For every experiment, a purity plate was prepared by spreading a loopful of stock-culture onto a LB agar plate (Plate Count Agar (NCIPD, Sofia). The purity plates for Bacillus subtilis and Escherichia coli were incubated for 24 h at 37°C.

Starting from the purity plates, pre-cultures were prepared by transferring one colony into an Erlenmeyer flask containing 20 mL of LB medium (LB, NCIPD, Sofia). *Bacillus subtilis* and *Escherichia coli* pre-cultures were incubated for 24 h at 37°C. Following this incubation period, stationary phase cultures with a cell density of ~10^9 CFU/mL were obtained.

Biofilm Development Conditions

The stationary phase pre-cultures were used to develop a 100-fold diluted inoculum with a cell density of ~107 CFU/mL. The investigated pre-culture ratios (*Bacillus subtilis* and *Escherichia coli*) were 1:1 and the growth media was Luria Bertani Broth (NCIPD, Sofia), which proved to be the optimal media for single-species and multispecies biofilm development by *Bacillus subtilis* and *Escherichia coli*, respectively.

To develop the biofilms, 1.2 mL of the inoculum was transferred to a small Petri dish made out of polystyrene (50 mm diameter, 9 mm height, Simport, Canada). After inoculation, Petri dishes were closed and gently shaken to make sure the inoculum covered the entire surface. Dependent on the applied (incubation) conditions, Petri dishes were incubated for 24 h at 20°C, which were the optimal temperatures for *Bacillus subtilis* and *Escherichia coli* single-species and multispecies biofilm formation, respectively.

Crystal Violet Assay

Bacterial biofilms were developed into 96-well microtiter plates (Greiner Bio-One, Kremsmünster, Austria) with 100 µL of bacteria in post-exponential growth phase in VNSS per well. In parallel, 100 µL of cell-free culture supernatant of another strain were collected in VNSS at the beginning of the stationary growth phase and were added on the bacterial biofilms (the addition of VNSS alone was used as a control). The final OD600 nm was 0.4 into each well. After 24 h of growth in static conditions and a temperature of 20°C, samples were washed thrice with NaCl (36 $g.l^{-1}$) and dried during 30 min at room temperature. Biofilms were stained during 15 min with 200 µL of Crystal Violet at 0.01% (w/v) and rinsed thrice with NaCl (36 $g.l^{-1}$) and dried for 10 min. The quantification of biofilm was evaluated by releasing the stain from the biofilm with absolute ethanol for 10 min at 20°C, at 120 rpm and measuring the absorbance of the Crystal Violet solution at 595 nm. The final OD595 nm of each sample was divided by the blank (i.e., VNSS medium only treated with Cristal Violet).

Quantification of colonies

Each specimen was individually placed in a centrifuge tube containing 4.5 mL of sterile physiological solution, and these tubes were vortexed for 1 min to detach the biofilms from the acrylic samples. After this, aliquots of 25 µL of serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were seeded in duplicate on Plate Count Agar (NCIPD, Sofia) and MacCocey Agar (NCIPD, Sofia) for the identification of *Bacillus subtilis* and *Escherichia coli*, respectively. Red and pink colonies grown on MacCocey Agar (NCIPD, Sofia) were presumptively identified as *Escherichia coli*. After incubation at 37°C for 24 h, the colony-forming unit per milliliter (CFU/ml) was determined and log-transformed (log10).

Multispecies Biofilm Formation

For the dual-species biofilm, bacteria in post-exponential growth phase were suspended in ASW and inoculated in 24 well plates (Corning Incorporated Costar®, New York, NY, United States) to a final OD600 nm of 0.3 (0.15 per strain). For the biofilms involving two bacterial strains, the final OD600 nm was 0.3 or 0.4 (0.1 per strain). Controls includ-

ed single species biofilms formed in the same concentrations and conditions than the multispecies biofilm. To study the influence of the osmolality of the medium, a series of experiments related to the cultivation of *Bacillus subtilis* and *Escherichia coli* strains in 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)) were carried out, to which NaCl was added so that its final concentration was 100 mM, 150 mM and 200 mM, respectively. For the purposes of the task, 18-hour cultures of *B. subtilis* 170, *B. subtilis* 168 and *E. coli* K-12 1655 strains were prepared in advance in a medium broth (BulBio Laboratory – Sofia). 50 µl of the liquid cultures were inoculated into 5 cm^3 of M63 liquid 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) to which NaCl had been added so that its final concentration was 100 mM, 150 mM, and 200 mM, respectively. The control sample did not contain NaCl. For each factor, the experiment was carried out in five test tubes, in the first one only 50 µl of the liquid culture of *B. subtilis* 170 strain was seeded, in the second – *B. subtilis* 168, in the third – *E.coli* K-12 1655, in the fourth tube inoculate 50 µl of the liquid culture of *B. subtilis* 170 strain and 50 µl of the liquid culture of *E. coli* K-12 1655 strain, in the fifth place inoculate 50 µl of the liquid culture of *B. subtilis* 168 strain and 50 µl of the liquid culture culture of *E. coli* K-12 1655 strain. Fresh cultures are distributed in 96-well plates. Each fresh culture was dispensed into 12 wells, placing 150 µl of the liquid fresh culture in each well. 150 µl of distilled water is dripped into the final unseeded wells. The first plate is placed at a temperature of 20°C. Cultivation of the biofilms on the plates was carried out for a duration of 24 h. After that, the plankton was separated from each well and washed three times with saline (0.85% NaCl), 150 µl of saline was placed in half of the wells, and the biofilm was peeled off using a knife previously burned and cooled in sterile saline of them, and for each variant of the experiment, the suspension is collected from 6 wells in one eppendorf.

Matrix Components Staining

For the matrix staining, a static biofilm of 48 h was performed. Each biofilm was stained with DAPI at 5 µg/mL (Sigma-Aldrich, Darmstadt, Germany) and one of the following matrix dyes. Exopolysaccharides were stained with the Wheat Germ Agglutinin (WGA) associated with the Alexa FluorTM 555 conjugate (Thermo Fisher Scientific, Waltham, MA, United States) at 100 µg/mL to label N-acetyl-glucosamine. After 30 min of incubation of each probe, each coverslip was washed 3 times in PBS 1×. Finally, the coverslips were mounted with a drop of ProlongTM Diamond Antifade before observation with confocal laser scanning microscopy **Leica TCS SPE** at wavelength of 540 nm.

Data Extraction from Images and Statistics

At least three replicates and five pictures per replicate were performed and used for data extraction. The pictures have been acquired by epifluorescence microscopy or by CLSM. The percentages of recovery of epifluorescence microscopy were determined using an algorithmic method with RStudio 0.98.1025 (RStudio, Boston, MA, United States), where the brightness pixel was determined by threshold's definition of small area of picture around the pixel and against the background. Each picture has been divided in 36 pieces, that permitted empirically to render negligible the distortion of objective and the threshold was defined as two multiplied by percentile 5th of pixel values on a small area in which the pixel was measured. The biovolume, the average thickness and the evaluation of the maximum coverage in the CLSM pictures was determined with the COMSTAT software developed in MATLAB R2015a (MathWorks, Natick, MA, United States) as previously performed (Heydorn et al., 2000).

To test for statistically significant differences ($P < 0.05$) between two conditions a t-test was performed and between different time points, a two-way analysis of variance including the Bonferroni post-test were performed using SPSS 13.0 (IBM, Armonk, NY, United States).

Results and Discussion

The osmoticity of the medium regulates biofilm formation in a large number of bacterial species (Fig. 1). An increase in the sodium chloride content from 100 mM to 200 mM leads to a decrease in the growth of the biomass of the biofilm of *B. subtilis* 170 strain, while in *B. subtilis* 168 strain it maintains a relatively constant value. The values of the optical density at 570 nm after staining with a 1% solution of crystal violet decreased linearly with a change in the osmoticity of the medium in the range from 100 to 200 mM NaCl in the mixed biofilms of *B. subtilis* 170 and *E. coli* K-12 1655 ($r^2 = 0.9922$), *B. subtilis* 168 and *E. coli* K-12 1655 strains ($r^2 = 0.9774$).

At a sodium chloride content of 0.015 M in the culture medium, *B. subtilis* LMArzc17 and LMArzc 189 strains were distinguished by the maximum ability to form biofilms, the optical density after staining with crystal violet solution at 560 nm being 0.20 and 0.21 (Kavamura & Melo, 2014), which approximates the results of co-culturing *B. subtilis* 170 and *E.coli* K-12 1655, *B. subtilis* 168 and *E. coli* K-12 1655 strains to form biofilms in the present study. An increase in its concentration to 0.03 M was accompanied by a reduction in the ability to form biofilms of *B. subtilis* strains in the study by Kavamura & Melo (2014) with the exception

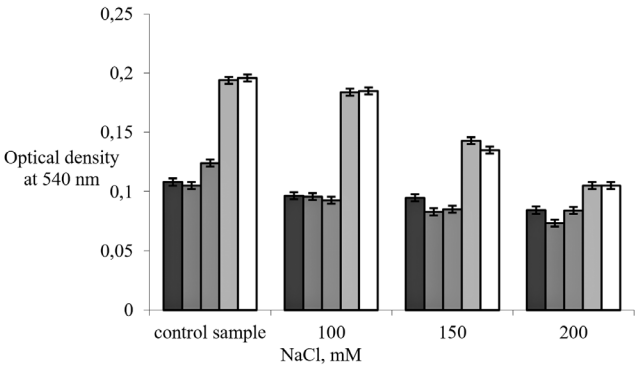

Fig. 1. The Influence of content of NaCl in the culture medium on the biofilm growth: ■*B. subtilis* 170; ■ *B. subtilis* 168; ■ *E. coli* 1655; ■ *B. subtilis* 170+ *E. coli* 1655; □ *B. subtilis* 168+ *E. coli* 1655. The growth of biofilms was carried out in 96-well plates for a time of 24 h at temperature of 20°C in a medium M63 with content of NaCl of 100 mM, 150 mM and 200 mM. The optical density was measured at 540 nm after staining of biofilms with 0.1 % crystal violet solution

Regression equations, described the change of the values of the optical density at 540 nm in an accordance to the content of NaCl in the medium for biofilm cultivation:

$y = -0.0061.x+0.1041$. $r^2 = 0.951$. $p < 0.05$ – *B. subtilis* 170

$y = -0.0112.x+0.1065$. $r^2 = 0.992$. $p < 0.05$ – *B. subtilis* 168

$y = 0.0792.e^{0.0481.x}$. $r^2 = 0.9405$. $p < 0.05$ – *E. coli* K-12 1655

$y = -0.0445.x + 0.2367$. $r^2 = 0.992$. $p < 0.05$ – *B. subtilis* 170-*E. coli* K-12 1655

$y = -0.0396.x + 0.2211$. $r^2 = 0.977$. $p < 0.05$ – *B. subtilis* 168-*E. coli* K-12 1655

of *B. subtilis* LMArzc189 strain, in which the optical density value at 560 nm is 0.25.

The appearance of insertions in *nhaR* gene is accompanied by a fourfold decrease in the biomass of biofilms formed by *E. coli* MG1655 strain and a tenfold decrease in its value in the original *E. coli* MG1655 strain, when the concentration of sodium chloride in the medium reaches 0.6 M (Geiger et al., 2017), which may explain the lack of statistically significant differences in optical density values at 560 nm, when *E. coli* K-12 1655 strain was cultivated in a medium with osmolality ranging from 100 mM to 200 mM NaCl ($p > 0.05$). The process of biophil formation by *E. coli* strains is influenced by the activity of the two-component regulatory system OmpR/EnvZ, which increases at high osmotic values of the medium above 0.6 M. Mutations in *ompR234* gene lead to the biosynthesis of the OmpR234 protein, which binds to the promoter region of *csgD* gene, responsible for the formation of curli-fringes, which promotes the adhesion of the strain on the surface layer of the substrate and its development into biofilms (Park et al., 2002). Point mutations in *ompR* gene do not result in loss of motility in *E. coli* strain according to the study by Geiger et al. (2017).

Increase in the number of cell population in the biofilm structure of *E. coli* K-12 1655 strain from $(1\pm0.2).10^3$ cfu/ml to $(12.53\pm0.21).10^3$ cfu/ml at a concentration of NaCl of 100 or 200 mM is probably associated with the activation of the transcription of *pga* operon, which is responsible for the biosynthesis of the proteins necessary for the formation of polyacetylglucosamine, which is part of the matrix. Its co-development with *B. subtilis* 170 and *B. subtilis* 168 strains does not determine its dominant role in the process of formation of biofilm structures. The number of colonies of *B. subtilis* strains in the biofilm structure kept a relatively constant value at NaCl content in the range of 150 mM to 200 mM, which increased when interacting with *E. coli* 1655 K-12 strain, but were generally more low compared to the control sample ($p < 0.05$). At a sodium chloride content of 200 mM in the medium, no cells of *E. coli* K-12 1655 strain were detected in biofilms in its association with strains of *B. subtilis* species, which is an indication of a transition from competitive relationships to antagonism under the conditions of increasing osmoticity of the medium (Tables 1 and 2). The population size of *B. subtilis* 170 strain decreased to a value of $13.5\pm0.3.10^3$ cfu/ml in the structure of mixed biofilms and approached its value in monospecies biofilms ($p > 0.05$), while for *B. subtilis* 168 strain it acquires a value of $12.93\pm0.47.10^3$ cfu/ml as a result of the increase in salt content in the medium for cultivating biofilms (Tables 1 and 2).

Table 1. The impact of NaCl in the cultural medium on colony forming units of *B.subtilis* 170 and *E.coli* 1655 strains in the structures of single-species and dual-species biofilms

№	NaCl, mM	Colony forming units in biofilms of *B. subtilis* 170 cfu/cm³	Colony forming units in biofilms of *E. coli* K-12 1655, cfu/cm³	Colony forming units in dual-species biofilms, cfu/cm³	
				B. subtilis 170	*E. coli* 1655
1.	0	$(0.7\pm0.00).10^6$	$(0.4\pm0.01).10^6$	$(92.6\pm0.66).10^6$	$(12.03\pm0.05).10^6$
2.	100	$36.5\pm0.62.10^3$	$1\pm0.2.10^3$	$116.43\pm0.97.10^3$	$31.56\pm0.32.10^3$
3.	150	$10.26\pm.10^3$	$0.22\pm.10^3$	$39.46\pm0.72.10^3$	$21.13\pm0.21.10^3$
4.	200	$10.3\pm0.26.10^3$	$12.53\pm0.21.10^3$	$13.5\pm0.3.10^3$	0

Table 2. The impact of NaCl in the cultural medium on colony forming units of *B.subtilis* 168 and *E.coli* 1655 strains in the structures of single-species and dual-species biofilms

№	NaCl, mM	Colony forming units in biofilms of *B. subtilis* 168, cfu/cm^3	Colony forming units in biofilms of *E. coli* K-12 1655, cfu/cm^3	Colony forming units in dual-species biofilms, cfu/cm^3	
				B. subtilis 168	*E. coli* 1655
1.	0	$(0.53\pm0.06).10^6$	$(5.1\pm0.10).10^6$	$(10.7\pm0.25).10^6$	$(10.36\pm0.32).10^6$
2.	100	$10.13\pm0.15.10^3$	$1\pm0.2.10^3$	$113\pm0.9.10^3$	$37.13\pm0.15.10^3$
3.	150	$10.34\pm0.25.10^3$	$2.26\pm0.25.10^3$	$37.9\pm0.89.10^3$	$20.3\pm0.2.10^3$
4.	200	$10.23\pm0.20.10^3$	$12.53\pm0.21.10^3$	$12.93\pm0.47.10^3$	$1.3\pm0.25.10^3$

In the study by Rivardo et al. (2009), *B. subtilis* V9T14 strain is characterized by the ability to form biosurfactants of lipoprotein nature at a content of sodium chloride in the medium of 10%, which exhibits anti-adhesion activity against *Escherichia coli* CFT073 and *Staphylococcus aureus* ATCC 29213 strains in the process of biofilm formation. The formation of biosurfactants at a sodium chloride content of 7% by *B. subtilis* strains is a mechanism for suppressing the development of *Escherichia coli*, *Staphilococcus aureus*, *Proteus mirabilis* strains to the formation of biofilms (Jha et al., 2016). Lipoprotein biosurfactants formed by *B. subtilis* strains are characterized by the property of causing damage to the cell membrane structure of their competing species (Mnif & Ghribi, 2015). They have a negative effect on the adhesion of microbial species, which prevents colonization on the surface of the substrate (Ramey et al., 2004). The biosurfactants formed by *B.subtilis* UMAF6614 strains provide its competitive advantage in biofilm formation (Zeriouh et al., 2014), which determines their dominant role in the structure of biofilms in the interaction with *Escherichia coli* K-12 1655 strain in the present study.

The resistance of *B. subtilis* strains to osmotic stress is determined by the activation of the extracytoplasmic σ-factor, which increases its activity when the NaCl content increases in the range from 200 to 300 mM (Weir et al., 1991). The presence of this survival mechanism of adverse environmental factors determines the relative preservation of the number of colonies in the structure of monospecies biofilms of *B. subtilis* 170 and *B. subtilis* 168 strains ($p>0.05$), which increase significantly in mixed biofilms and exceed the number of colonies of *E. coli* K-12 1655 strain ($p<0.05$).

Under stress conditions, the biosynthesis of bacteriocin from *B.subtilis* 168 strain is initiated (Luo & Helmann, 2012), which appears to be the result of the repression of the AbrB factor by Spo0A (Kobayashi, 2007) and the activation of the σ^n-factor (Weir et al., 1991). As a result of these regulatory mechanisms, the activity of the two-component regulatory system, consisting of the histindin kinase SpaK and the regulatory protein SpaR, is increased. Histidine kinase undergoes autoinduction by increasing the activity of the quorum-sensing system or by other signaling mechanisms, as a result of which the process of transfer of phosphate groups to the main regulator and expression of the genes responsible for the biosynthesis of lantibiotics occur (Geiger et al., 2017). Subtelin synthesized by *B. subtilis* CAU131 (KCCM 10257) strain exerts an inhibitory effect on the development of *Staphylococcus epidermidis* KCTC 35494, *Escherichia coli* ATCC 43984 O157:H7, *Escherichia coli* DH5α, *Escherichia coli* KCCM11750 strains according to the study of Park et al. (2002). *Escherichia coli*, *Salmonella pullorum*, *Pseudomonas aeruginosa*, *Pasteurella multocida*, *Clostridium perfringens*, *Micrococcus luteus*, *Streptococcus bovis* and *Staphylococcus aureus* strains are sensitive to the effects of the bacteriocin secreted by *Bacillus subtilis* LFB112 strain (Xie et al., 2009). Data from previous studies on the biosynthesis of antimicrobial peptides and their influence on competing species explains the dominant role of *B. subtilis* 168 strain in the structure of biofilms formed in its association with *E. coli* K-12 1655 strain in the conducted study, but its number is lower compared to the control sample. Inhibition of lantibiotic biosynthesis (Luo & Helmann, 2012) and surfactin (Rivaldo et al., 2009), as well as increasing the activity of the regulator AbrB (Strauch et al., 2007), which represses genes for matrix formation (Strauch et al., 2007) is in agreement with the decrease in population numbers of *B. subtilis* strains in the structure of mixed biofilms when reaching a NaCl content of up to 200 mM in the culture medium in the present study.

Changing the osmotic value of the culture medium in the range from 100 mM to 200 mM NaCl changes the character of the surface of the biofilms, and the coefficient accounting for the irregularities in the surface layer of the structures changes in the range from 0.25±0.23 to 0 .29±0.26 for the co-culture of *B. subtilis* 170 and *E. coli* K-12 1655 strains, while for the co-culture of *B. subtilis* 168 and *E. coli* K-12 1655 strains it fluctuates in the range of 0.22±0.32 to 0.28±0.25, which significantly exceeds the value of the control sample ($p<0.05$). The increase in NaCl content in the

Table 3. Morphometric features of dual-species biofilms of *Bacillus subtilis* 170 and *Escherichia coli* K-12 1655. *Bacillus subtilis* 168 and *Escherichia coli* K-12 1655 strains. depending on the concentration of NaCl in medium

NaCl. mM	Mean thickness. μm*	Coefficient of unevenness	Relative area. μm^{2**}	Relationship area/volume. $\mu m^2.\mu m^{-3***}$
Dual-species biofilms of *Bacillus subtilis* 170 and *Escherichia coli* K-12 1655 strains				
0	10.35±0.41	0.03±0.02	0.99±0.02	0.096±0.003
100	5.19±0.94	0.29±0.26	0.68±0.04	0.144±0.010
150	4.31±0.55	0.27±0.26	0.44±0.03	0.181±0.011
200	3.52±0.56	0.25±0.23	0.19±0.02	0.219±0.016
Dual-species biofilms of *Bacillus subtilis* 168 and *Escherichia coli* K-12 1655 strains				
0	10.46±0.42	0.02±0.03	0.97±0.01	0.095±0.002
100	5.28±0.15	0.22±0.32	0.61±0.03	0.146±0.003
150	4.79±0.12	0.28±0.25	0.58±0.02	0.190±0.024
200	2.34±0.33	0.16±0.09	0.45±0.02	0.390±0.061

*y = -0.835.x + 6.01.r^2 = 0.999.p < 0.05 – *B. subtilis* 170-*E. coli* K-12 1655; y = -2.729.x + 12.93.r^2 = 0.991. p < 0.05 – *B. subtilis* 168-*E. coli* K-12 1655
**y = -0.264.x + 1.235.r^2 = 0.996.p < 0.05 – *B. subtilis* 170-*E. coli* K-12 1655; y = -0.169.x+1.1.r^2 = 0.965. p < 0.05 – *B. subtilis* 168-*E. coli* K-12 1655
***y = 0.0424.x + 0.0525.r^2 = 0.991.p < 0.05 – *B. subtilis* 170-*E. coli* K-12 1655;y = 0.1029.x-0.027.r^2= 0.972. p < 0.05 – *B. subtilis* 168-*E. coli* K-12 1655

culture medium of the biofilms in the study was accompanied by a linear decrease in the average thickness, which varied from 5.19±0.94 μm to 3.52±0.56 μm in the co-culture of *B. subtilis* 170 and *E. coli* K-12 1655 strains and from 5.28±0.15 μm to 2.34±0.33 μm in the co-culture of *B. subtilis* 168 and *E. coli* K-12 1655 strains. Their values were significantly lower than the control sample, characterized by the absence of sodium chloride in the medium, which is attributed to the inhibition of polyacetylglucosamine biosynthesis by both strains in the process of biofilm formation. Inhibition of the biosynthesis of exocellular polysaccharides, which are included in the composition of the matrix of biofilms and ensure their spread on the surface of the substrate, explain the decrease in the values of the relative area of spread and an increase in the ratio of area to volume, which follows a linear relationship for both pairs of strains when changing the osmoticity of the medium in the present study. At a concentration of sodium chloride of 200 mM, their values were, respectively, 0.19±0.02 μm^2 and 0.219±0.016 $\mu m^2.\mu m^{-3}$ in the biofilms of *Bacillus subtilis* 170 and *Escherichia coli* K-12 1655 strains, while the biofilms formed by the co-cultivation of the co-culture of *B. subtilis* 168 and *E. coli* K-12 1655 strains are characterized by a relative area of 0.45±0.02 μm^2 and an area-to-volume ratio of 0.390±0.061 $\mu m^2.\mu m^{-3}$, when the osmolaliity of the medium is reached of 200 mM (Table 3; Figures 2 and 3).

According to the study by Steil et al. (2003), and Kobayashi (2007), the high osmolality of the medium causes inhibition of the activity of the genes responsible for the biosynthesis of the exocellular polysaccharides of the composition of the matrix of the biofilms of *Bacillus subtilis* strains, and in the study of Hoffmann et al. (2002) the increase in the salt content is accompanied by the reduction of the synthesis of the Hag protein, which is the main component of the fringes, taking part in the implementation of flagellar motility. Their growth at a high salt content of 0.12 M NaCl leads to the repression of genes, whose activity is determined by the two-component regulatory system DegS/DegU, by means of the kinase KinD (Hoffmann et al., 2002). The expression of *epasA-O* operon in *B. subtilis* 168 strain, responsible for the formation of the exocellular polysaccharides of the biofilms, is proportional to the increase in the activity of the low molecular weight secretory protein YuaB (Verhamme et al., 2009), determined by the level of the regulatory protein AbrB (Kovács & Kuipers, 2011), which decreases upon activation of the DegS/DegU system due to the phosphorylation of the SpoOA regulator by the increase in KinD kinase activity and the increase in the intracellular level of SpoOA ~P (Verhamme et al., 2009). This regulatory mechanism can explain the linear decrease in the value of the following thickness of the formed biofilms as a result of the co-cultivation of *B. subtilis* 170 and *E. coli* K-12 1655, *B. subtilis* 168 and *E. coli* K -12 1655 strains and the inversely proportional increase in the magnitude of the ratio of the area of the structures to their volume when changing the NaCl content in the range from 100 mM to 200 mM in the present study. In *E. coli* K-12 strains, this effect is explained by the negative influence of the regulatory protein RpoS on the transcription of *yjb* and *wca* operons under conditions of varying sodium chloride content up to 0.7 M in the culture medium, which are responsible for the biosynthesis of the exocellular polysaccharides (Ionescu & Belkin, 2009).

The increase in the osmoticity of the medium creates conditions for the inhibition of *tapA-sipW-tasA* operon in

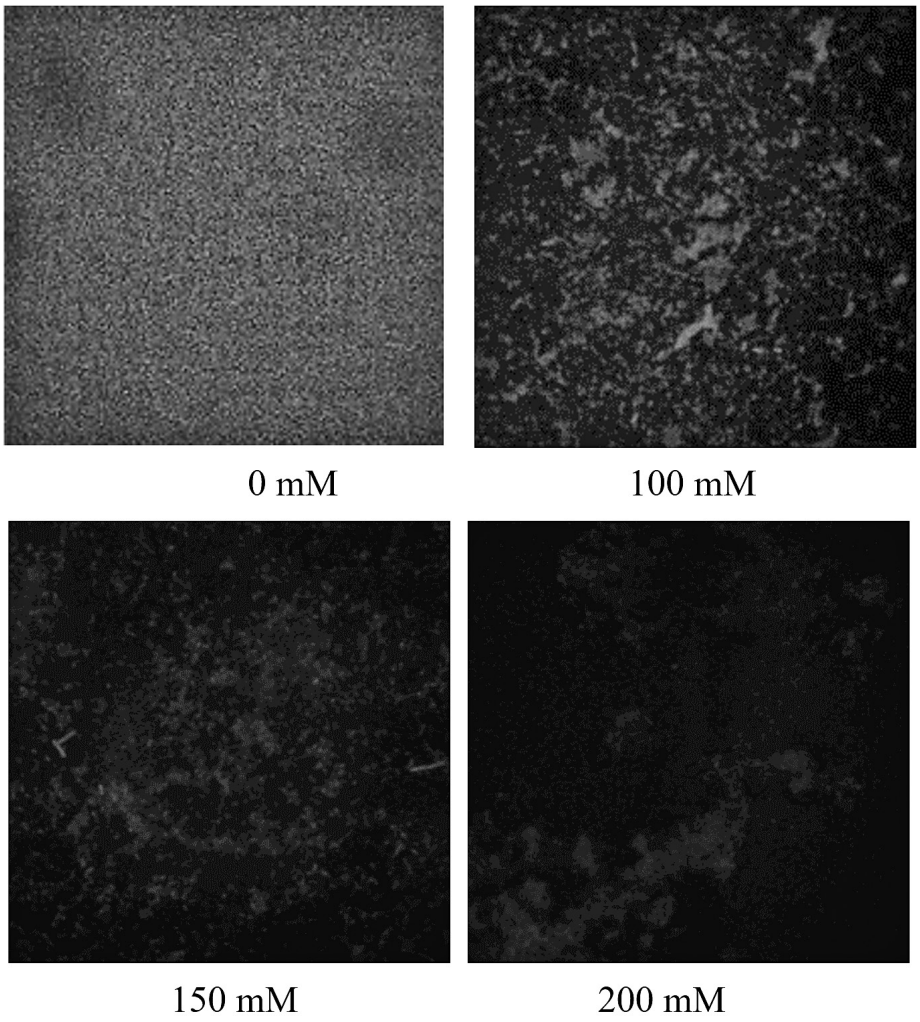

Fig. 2. Microscope study of biofilms of *Bacillus subtilis* 170 and *Escherichia coli* K-12 1655 strains at different concentration of NaCl in the cultural medium. Biofilm growth was carried out on the cover glasses for a time of 24 h at temperature of 20°C. The staining was carried out by using of complex fluorescence dye Live Bacterial Gram Stain Kit (Biotum), but the observations were carried out by using of confocal laser scanning microscopy Leica TCS SPE at wavelength of 540 nm

Bacillus subtilis strain for the biosynthesis of the TapA and TasA proteins from the composition of the protein component of the biofilm matrix (Rubinstein et al., 2010) and gene region *epasA- O*, the increase in whose activity promotes the biosynthesis of the main polysaccharides of the mitric composition of biofilms (Nagorska et al., 2008; Rubinstein et al., 2010), which is under the negative control of the regulatory protein SinR (Nagorska et al., 2008). The two-component regulatory system in *E. coli* strains, consisting of the regulatory protein CpxR and the histidine kinase CpxA, increases its activity at high salinity of the medium (Dudin et al., 2013), thereby repressing the maintenance of curled fringes in medium containing of sodium chloride of 100 mM (Ogasawara et al., 2010). An increase in its content to 200 mM is accompanied by activation of *pga* operon, responsible for the synthesis of poly-N-acetylglucosamine (Powers et al., 2015). These regulatory processes are apparently difficult in the co-development of *E. coli* K-12 1655 strain with *Bacillus subtilis* 170 and *Bacillus subtilis* 168 strains, due to which the strong decrease in the average diameter and relative volume of the structure of the mixed biofilms upon reaching a sodium chloride concentration of 200 mM was attributed to a decrease in the number of the cell population in them, while in *Bacillus subtilis* strains this regularity is due to the increase in the activity of the regulatory protein SinR, which leads to the repression of the genes responsible for the formation of the matrix of biofilms. The biosynthesis of the repressor protein SinR is accompanied by the activation of the histidine kinase KinD, which takes part in the processes of transfer of phosphate groups to the main regulator of spore formation SpoOA and the development of *Bacillus subtilis* strains to biofilms, which causes a high level of repression for the genes responsible for the biosynthesis of the main components of the matrix composition (Nagorska et al.,

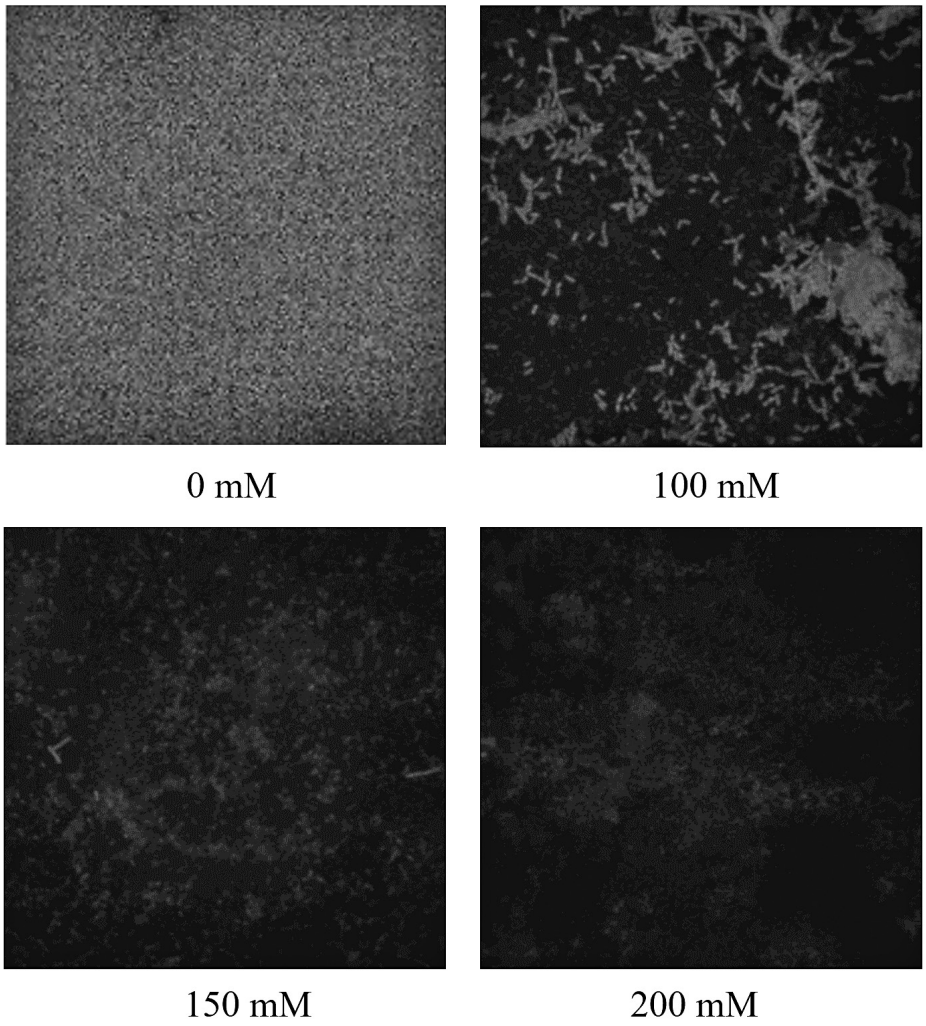

Fig. 3. Microscope study of biofilms of *Bacillus subtilis* 168 and *Escherichia coli* K-12 1655 strains at different concentration of NaCl in the cultural medium. Biofilm growth was carried out on the cover glasses for a time of 24 h at temperature of 20°C. The staining was carried out by using of complex fluorescence dye Live Bacterial Gram Stain Kit (Biotum), but the observations were carried out by using of confocal laser scanning microscopy Leica TCS SPE at wave length of 540 nm

2008; Rubinstein et al., 2012), which may also account for the reduction in the relative area of spread of the structures formed in the studied pairs of strains in the present study.

The number of spores in the structure of mixed biofilms formed by the association of *B. subtilis* 170 and *E. coli* K-12 1655, *B. subtilis* 168 and *E. coli* K-12 1655 strains increases exponentially when the osmoticity of the medium changes from 100 up to 200 mM, while in the structure of the monospecies biofilms their reduction was found under the same cultivation conditions. The number of spores in the structure of the mixed biofilms ranges from $1.89.10^2$ cfu/ml in the co-culture of *B. subtilis* 170 and *E. coli* K-12 1655 strains and $1.7.10^2$ cfu/ml in the co-cultivation of *B. subtilis* 168 and *E. coli* K-12 1655 strains at the sodium chloride concentration of 100 mM, and the change in its value to 200 mM was accompanied by a statistically significant increase of spores in the coated biofilms up to $4.5.10^2$ cfu/ml in the co-culture of *B. subtilis* 170 and *E. coli* K-12 1655 strains and $4.62.10^2$ cfu/cm3 for the co-culture of *B. subtilis* 168 and *E. coli* K-12 1655 strains. Their size is significantly greater than in monospecies biofilms, in the structure of which values of $0.4.10^2$ cfu/ml were found in *B. subtilis* 170 strain and $0.46.10^2$ cfu/ml in *B. subtilis* 168 strain (Table 4).

The decrease in the number of spores in the structure of biofilms of *B. subtilis* 170 and *B. subtilis* 168 strains as a result of the increase in the osmoticity of the medium is in agreement with the study of Nagler et al. (2014), according to which the increase in the content of sodium chloride in the medium to a value of 0.54 M ensures the inhibition of the process of maturation of spores in the structure of biofilms and heterogeneity in their population, determined by the differences in the structure of receptor sites of maturation. The presence of L-alanine in them determines their high affinity for Na^+ from the composition of the medium compared to

Table 4. The impact of NaCl in the cultural medium on spore forming units of *B.subtilis* 170. *B. subtilis* 168 and *E. coli* 1655 strains in the structures of single-species and dual-species biofilms

№	NaCl, mM	Spore forming units in biofilms of *B. subtilis* 170, cfu/cm^3	Spore forming units in biofilms of *B. subtilis* 168, cfu/cm^3	Spore forming units in dual-species biofilms, cfu/cm^3	
				B. subtilis 170 + *E. coli* 1655	*B. subtilis* 168 + *E. coli* 1655
1.	0	(6.66±0.5).10^3	(10±0.1).10^3	(2.54±0.47).10^3	(2.66±0.5).10^3
2.	100	(0.2±0.05).10^2	(2.4±0.02).10^2	(1.12±0.44).10^2	(1.13±0.30).10^2
3.	150	(4.66±0.21).10^2	(4.74±0.12).10^2	(0.56±0.57).10^2	(0.53±0.46).10^2
4.	200	(5.01±0.29).10^2	(6.12±0.17).10^2	(0.36±026).10^2	(0.30±0.07).10^2

- $y = 2.435.\ln(x) + 1.982$. $r^2 = 0.974$. $p < 0.05$ – *B. subtilis* 170-*E. coli* K-12 1655
- $y = 2.729.\ln(x) + 1.810$. $r^2 = 0.971$. $p < 0.05$ – *B. subtilis* 168-*E. coli* K-12 1655

residues of pyridine-2,6-dicarboxylic acids (Nagler et al., 2014). Another mechanism consists in the maintenance of the low intracellular level of the phosphorylated form of the main regulator SpoOA due to an increase in KinD kinase activity under the conditions of high osmotic medium (Aguilar et al., 2010).

The association of *B. subtilis* 170 and *B. subtilis* 168 strains with *E. coli* K-12 1655 strain in the process of static cultivation of biofilms leads to the establishment of an opposite trend, expressed in an increase in the number of spores in the formed structures, which is associated with the metabolic features of the strains under conditions of high osmolality of the environment. The formation of colicins by *E. coli* strains during the formation of biofilms (Rendueles & Ghigo, 2014), which creates a condition for the activation of the regulatory protein AbrB in *B. subtilis* strains (Strauch et al., 2007), as a result of which reaches the phosphorylation of the factor responsible for sporulation SpoOA~P by transferring phosphate groups and inducing the process of spore formation during biofilm formation (Strauch et al., 2007). According to Chai et al. (2012), the increase in the intracellular level of Spo0A~P leads to the repression of the genes for the regulator SinI and the activation of the genes for spore formation under the conditions of an increasing value of the osmotic value of the medium, as a result of which a significant increase in the number of spores in the structure of biofilms at a concentration of sodium chloride above 100 mM in the conducted study compared to the control sample and monospecies biofilms.

Conclusions

At cultivation in the cultural medium without NaCl, the bacterial growth rates of single-species biofilms of *Bacillus subtilis* strains and biofilms as a result of their interaction with *Escherichia coli* strains were similar and determines the mutualism between two strains in the structure of biofilms, but the temperature of the cultivating of the concentration of NaCl in the cultural medium over of 100 mM lead to the impeded growth and affect adversely the process of biofilm formation by the participation of *B. subtilis* 170 and *E. coli* K-12 1655, *B. subtilis* 168 and *E. coli* K-12 1655 strains, as a result of which a decrease in the value of the optical density, average thickness and relative spreading area and an inversely proportional increase in the ratio of the spreading area of the structures to their volume, but inhibits spore formation. Many studies on the osmolality regulation by soil-drawing bacteria of Bacillus subtilis strains have been performed in shaken cultures.

The importance of the interplay between extracellular matrix production and efficient osmolality regulation might have been overlooked. Considering that static growth in a biofilm is a highly relevant growing state for many soil bacteria, more research on the osmolality regulation by model soil bacteria in static cultures is required to draw a more comprehensive picture of the role of biofilm production during osmotic stress.

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