ACTA MICROBIOLOGICA BULGARICA Volume 35 / 2 (2019)

Impact of Growth Conditions on Biofilm Formation by Model Gram-Negative and Gram-Positive Bacterial Strains

Dayana Borisova[1], Viktoriya Jordanova[2], Stoyanka Stoitsova[1], Tsvetelina Paunova-Krasteva[1*]

[1] Department of General Microbiology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences
[2] National Genetic Laboratory, University Hospital of Obstetrics and Gynecology "Maichin Dom", Medical University, Sofia

Abstract

Present-day medical microbiology faces the risks of biofilm-related infections. These are much more resistant to routinely applied antibacterial preparations, and are often the cause of recurrent infections. The search of novel anti-fouling preparations puts forward the elaboration of a reliable complex approach for selecting and estimating the successful agent. As a first step, screening procedures should include a fast methodology, and the crystal violet assay is widely applied for semi-quantitative estimation of biofilm biomass. However, the species- and strain-peculiar mechanisms of biofilm formation require optimisation of the experimental protocols with regards to the individual demands of model strains. This study aims to characterise the biofilm-forming behavior of a set of model Gram-positive and Gram-negative strains. The Gram-positive strains included in the experiments were *Staphylococcus aureus* ATCC 29213 and *Bacillus subtilis* 168. Gram-negative strains were *Pseudomonas aeruginosa* PAO1 and six *Escherichia coli* K-12 strains. The tested variables were growth medium, time of cultivation and growth temperature. Notably, these variables have different, sometimes even opposite effects on planktonic growth and biofilm development. The results provide information needed for the proper design of further screening experiments on these strains, for novel substances with putative anti-biofilm activities.

Keywords: Biofilm formation, Gram-positive and Gram-negative strains, effects of growth medium, temporal characteristics and temperature of cultivation

Резюме

Съвременната медицинска микробиология е изправена пред риска от инфекции, свързани с образуването на биофилми. Биофилмите са много по-резистентни към антибиотици и често са източник на рецидивиращи инфекции. Търсенето на нови анти-биофилмни препарати поставя въпроса за създаването на комплексна методология за селекция и оценка на подходящи вещества. Първите стъпки следва да включват бърза скрининг-методология и тестът "кристал виолет" е един широко прилаган полу-количествен подход за оценка на биофилмната биомаса. Независимо от това, че този тест е широко приет, поради видово- и щамовоспецифичните механизми на биофилм образуването се налага оптимизиране на експерименталните протоколи с оглед индивидуалните изисквания на моделните щамове. Цел на изследването е да характеризира биофилм-образуващите характеристики на набор от моделни Грам-положителни и Грам-отрицателни щамове. Включените в изследването Грам-положителни щамове са *Staphylococcus aureus* ATCC 29213 и *Bacillus subtilis* 168. Грам-отрицателните щамове са *Pseudomonas aeruginosa* PAO1 и шест щама *Escherichia coli* K-12. Изследвани са следните вариабилни: растежна среда, продължителност на култивирането и растежна температура. Тези фактори имат различен, понякога - дори противоположен ефект върху развитието на бактериите в течна среда и като биофилм. Рзултатите дават възможност за създаване на подходящ експериментален дизайн за осъществяване на скрининг-изследвания върху тази група моделни щамове с включването на нови вещества с очаквана анти-биофилмна активност.

* Corresponding author: pauny@abv.bg

Introduction

Bacterial biofilms represent cell communities embedded in extracellular polymer substances that grow attached to surfaces. Biofilm formation follows several stages: (1) reversible adherence of the bacteria to the surface; (2) loss of motility and irreversible attachment; (3) development of microcolonies; (4) differentiation of the mature biofilm; (5) detachment of motile bacteria from the biofilm and its dissemination to other loci (Souza dos Santos, 2018). Attachment is a complex process regulated by diverse characteristics of the growth medium, substratum, and cell surface (Donlan, 2002).

Since the initial thorough descriptions of the biofilms (Costerton *et al*., 1994), it has further been well-recognised that sessile communities may develop on both environmental surfaces and on, or inside other organisms, including the human body (indwelling devices and tissues) where they cause biofilm infections (Wang *et al*., 2015; Coughlan *et al*., 2016). The latter are difficult to overcome, and are related with the recurrency of bacterial diseases (VIjayakumar *et al*., 2016; Jamal *et al*., 2018).

Biofilms may be considered an adaptive mode of life where sessile bacteria, much better than their free-floating counterparts, survive environmental hazards: toxic substances, desiccation, etc., or, inside plant or animal hosts – the evolutionary evolved immunity deffences and/or antibacterial treatments (Jamal *et al*., 2018). Therefore the development of novel effective anti-biofilm strategies is a serious challenge for researchers (Costerton *et al*., 1994; Speranza *et al*., 2018). As a first step of the estimation of putative anti-biofilm effects of novel substances, laboratory screening experiments are indispensable and these most often include the routinely applied crystal violet (CV) test. This is a reliable and easy-to-perform semi-quantitative assay for biofilm biomass. In screening experiments aimed to identify anti-biofilm substances, the CV assay can be applied to different model Gram-positive and Gram-negative microorganisms. While the test itself is well-known and easy to apply, in question is the standardization and/or optimization of the conditions for the biofilm growth of the model microorganisms prior to the CV staining.

For estimation of antibiotic sensitivity of non-biofilm bacteria, the growth media of choice and the respective protocols recommended by ISO are Mueller-Hinton broth and agar. Mueller-Hinton media provide satisfactory growth of most non-fastidious pathogens, acceptable batch-to-batch reproducibility, low sulfonamide, trimethoprim, and tetracycline inhibitors and a large amount of data has been collected from antimicrobial susceptibility tests with this medium over several decades (https://www.iso.org/obp/ui/#iso:std:iso:ts:16782:ed-1:v1:en). Therefore these media are preferably applied also in screening protocols for novel anti-bacterials.

When choosing growth medium and growth conditions for experiments for anti-biofilm studies with biofilms the situation is however not so clear, mostly because of the complex nature of the sessile consortia. The necessity of careful elaboration of the experimental design in biofilm studies has been underlined by many researchers (Costerton *et al*., 1994; Vijayakumar *et al*., 2016; Speranza *et al*., 2018), still present-day biofilm experiments are far from a standardized approach. The problem is the variable response of bacterial species and strains to the conditions of biofilm growth. Such a variety of responses is most probably due to the adaptive, convergent role of the biofilm for bacterial survival and the involvement of variable molecular mechanisms in the sessile growth. Our experience is that the outcome of anti-fouling experiments may depend, among other factors, on the bacterial strain and the nature of the explored substances (Vacheva *et al*., 2011, 2012a; Borisova *et al*., 2018). Hence, for proper experimental design a preceding thorough examination is needed of the sessile behavior of the chosen model strains. In literature, various cultivation protocols were applied by different authors prior to applying the CV dying. There are data obtained as a result of the application of different minimal media (among which M63) or rich media (among which LB). The medium-of-choice for antibiotic sensitivity estimation of non-biofilm bacteria, MH, is not quite popular in biofilm studies and has been used more rarely (e.g. Vijayakumar *et al*., 2016).

Our team has lately been planning experiments for the identification of novel anti-biofilm substances of synthetic or natural origin. Bearing in mind the above considerations, the present study was undertaken with the aim to characterize the biofilm-formation behavior of a set of model Gram-positive and Gram-negative strains under variable experimental conditions. We have tested biofilm development in three media: M63, LB, and MH, and the impact of cultivation duration and growth temperature.

Materials and Methods

Bacterial strains and growth media

The Gram-positive strains included in the study were *Staphylococcus aureus* ATCC 29213 and *Bacillus subtilis* 168 (Vasileva-Tonkova *et al*., 2011). Escherichia coli K-12 strains included W1655 F+, kindly provided by Prof. J. Gumpert, Institute of Molecular Biotechnology, Jena, Germany, strain 3110 (NCIPD, Sofia, Bulgaria) and strains 406, 409, 420 and 446 purchased from NBIMCC (Sofia, Bulgaria). More details on the K-12 strains are provided by Vacheva *et al*. (2012b). The *Pseudomonas aeruginosa* PAO1 strain was from the International Reference Panel (De Soyza *et al*., 2013).

The growth media applied were: Tryptic soy agar (TSA), Tryptic soy broth (TSB) and Mueller-Hinton broth (MH) were purchased from Sigma-Aldrich. LB broth (10 g tryptone, 10 g NaCl and 5 g yeast extract per litre) and M63 medium (0.02 M KH2PO4, 0.04 M K2HPO4, 0.02 M (NH4)2SO4, 0.1 mM MgSO4 and 0.04 M glucose) were prepared in the laboratory.

Estimation of bacterial growth and biofilm formation

The bacteria were maintained as frozen stocks in TSB supplemented with 20% glycerol. Before the experiments, they were streaked on TSA for selection of single colonies. The strains were further applied on slant agar and kept as source of inocula for no more than 20 days. To prepare the inocula, a loopful of each strain was inoculated in TSB and cultivated overnight at 37°C. The overnight cultures were dissolved 1:100 in the test media (MH, M63, or LB), vortexed, and 150 µl quotes were applied into the wells of a 96-well plate, 6 wells per sample. The plates were incubated for 24 h at 37°C. To estimate the bacterial growth in each well, the optical density (OD) was first estimated by ELISA reader at 620 nm wavelength. Then the non-adherent bacteria were removed, the wells were washed in 3 changes of PBS, and colored for 15 min with 0.1% aqueous solution of crystal violet (CV). The wells were rinsed extensively in several changes of PBS, the dye was solubilised with either a mixture of ethanol and acetone in proportion 4:1 for the Gram-positive strains, or 70% ethanol for the Gram-negative strains. The absorbance of the solubilised dye was measured at 570 nm.

Results and Discussion

Culture media may have different effect on bacterial growth and biofilm formation

Preliminary experiments on the growth dynamics of the tested strains inoculated on 96-well polystyrene plates have confirmed that on hour 24 of culture all strains in all media are in the stationary phase (data not shown).

For the purpose of the present study we measured the OD 620 of the wells as an indicator of the bacterial growth and then carried out the procedure for CV staining to measure biofilm in these same wells. On Fig. 1. is shown the comparison between non-biofilm and biofilm growth within the same experiments.

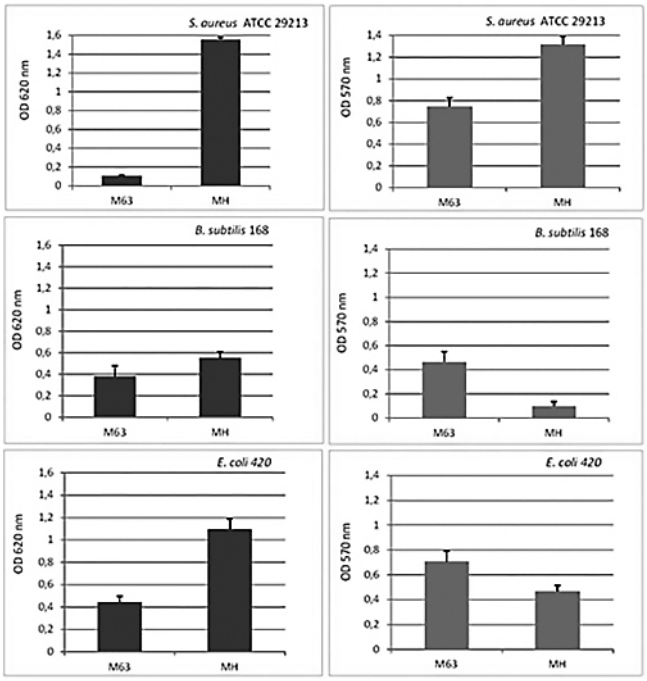

Fig. 1. Comparison of the effect of growth medium on bacterial growth (dark bars, absorbance at 620 nm) and biofilm formation (light bars, absorbance at 570 nm). The experiment was performed in 96-well plates, after cultivation for 24 hours at 37°C.

The results show that conditions that do not stimulate growth in broth may be promoting biofilm growth, and vice-versa. Thus *S. aureus* ATCC 29213 does not grow well in the liquid phase of M63 medium however it develops significant amount of biofilm in these same wells. While the MH broth culture promotes the planktonic growth of *B. subtilis* 168 compared to M63 medium, this strain produces more biofilm in the minimum salt medium than in MH. Likely is the situation with *E. coli* 420.

The development of biofilms in nutrient-limited media such as M63 has rarely been compared to that in rich media. Strain specificity of nutrient demands has been reported for *Campylobacter jejuni*

(Teh et al., 2016). The impact of the nutritional stress may be observed not only as changes of biofilm biomass. Thus, for *Burkholderia pseudomalei* it has been shown that growth of both plankton and biofilm under nutritional stress promotes the tolerance to antibiotics (Anutrakunchai et al., 2015, 2018). These results imply that the screening for anti-biofilm substances may benefit from parallel experiments performed under different nutritional conditions.

Culture media may have diverse effects on the dynamics of biofilm growth

The processes of bacterial biofilm development follow the same general pattern (Souza dos Santos *et al*., 2018). Initially, the motile bacteria approach the surface that is to be colonised and explore it by moving to and fro. This has been observed also in real time in our experiments by the application of TIRM to E. coli 420 (Velinov *et al*., 2011). This is the phase of the reversible adhesion. From one moment on some bacteria lose their flagella and attach irreversibly to the substratum. This is followed by attached growth of the bacteria starting with the formation of microcolonies, accummulation of biofilm biomass and, finally, mobilization of some cells and their detachment from the biofilm in search of novel niches. The latter process is little known, expectedly it is related with processes of degradation or loosening of the biofilm matrix (Huang *et al*., 2018).

While there is general agreement that biofilm development follows the above-outlined stages, it has to be taken into account that these processes exhibit complex spatio-temporal dynamics (Lee *et al*., 2019). The present experimental results show that the time course of biofilm growth may vary dependent on the growth medium. Fig. 2 shows the comparison between the time-courses of the biofilm development by *P. aeruginosa* PAO1 in three media, followed diurnally, on days 1, 2, and 3. It is clearly seen that in M63 medium the biofilm biomass increases between hours 24 and 72. Just the opposite, in LB the biofilm reaches its maximum value on hour 24 and this is followed by diminution of the biomass. Most probably, in the rich medium, once the biofilm has reached the state of significant growth, the bacteria sense some stimuli to become mobilized, leave the sessile community and explore new niches. This dynamics has to be considered when choosing the appropriate protocols for either inhibition of biofilm growth, or dispersal of pre-formed biofilms. And, finally, the data show that MH is promoting less than the other two media the sessile growth of this strain. In this medium, the biofilm reaches maximum development on day 1, followed by some diminution at later intervals.

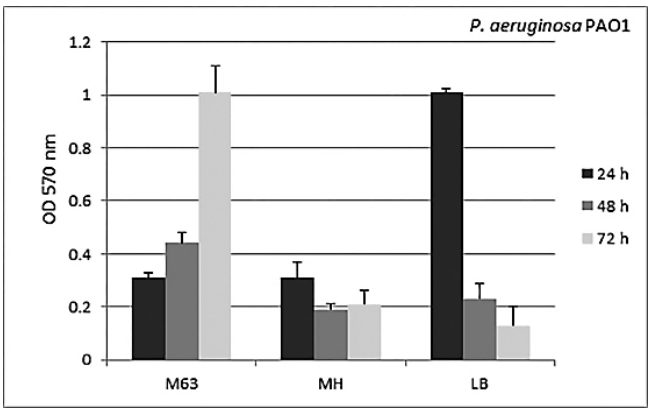

Fig. 2. Time-course of biofilm development by *P. aeruginosa* PAO1 in three growth media.

These time-course responses of *P. aeruginosa* PAO1 to the growth media characterize this strain but should not be extrapolated to other strains of this species. Thus, a vast variety of nutritional preferences between the three growth media have earlier been demonstrated for a big collection of clinical and environmental isolates of *P. aeruginosa* (Cullen *et al*., 2015).

The spatio-temporal dynamics of biofilm development and especially the mobilization and detachment of bacteria at later stages of the sessile-growth process have another important aspect: cells detached from biofilm may exert significant differences from cells grown as plankton. Thus, detached cells of *S. aureus* differed from plankton in their physico-chemical characteristics, cytotoxicity to He-La cells and production of virulence factors (Khelissa *et al*., 2017). Detached cells of *P. aeruginosa* had higher susceptibility to benzakonium chloride than cells grown as plankton (Khelissa *et al*., 2019). Therefore, future experiments aiming to find substances applicable for biofilm control should include also the effects on biofilms at the advanced stage of development, the detachment phase.

Strain-specific responses of biofilm growth to temperature

The set of *E. coli* K-12 strains used in the present study have been often included in experiments in our laboratory, with variable results between M63 and MH media (Vacheva *et al*., 2011, 2012 a, b) These studies have shown strain-specific responses to the growth medium.

In the present study we show that, in the same medium - MH, the biofilm-formation response to

growth temperature may also be strain-specific (Fig. 3). The duplicate set of experiments started from the same overnight inocula for each strain, diluted 1:100 in MH, and two identical plates were prepared and incubated at either 20° or 37°C. As expected, the OD 620 nm showed that in the liquid phase all strains grew better at 37°C (Fig 3A). But the biofilm developed in these same wells did not show such a strict pattern.

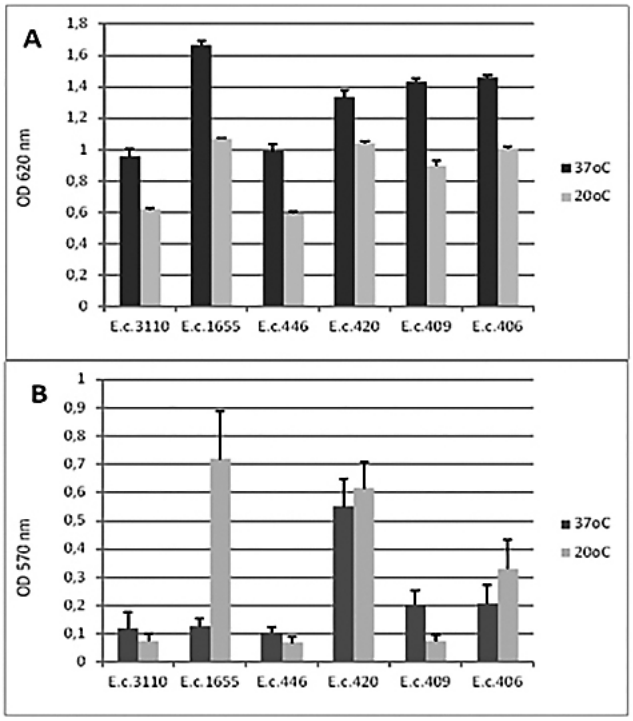

Fig. 3. Effect of cultivation temperature on the growth (A) and biofilm formation (B) of *E. coli* K-12 strains in MH broth.

Strains 3110, 409 and 446 have previously been shown to form almost no biofilm at 20°C in M63 medium (Vacheva *et al*., 2012b). The present experiments performed in the MH medium confirmed this peculiarity for 3110 and 446. However at 37°C an increase of biofilm biomass was registered for strain 409 (Fig. 3B).

For their biofilm formation, strains 1655, 420 and 406 demonstrated preference for 20°C (Fig. 3B). Such a temperature preference has been shown earlier for many other K-12 strains though strain specific responses have also been observed (Mathlouthi *et al*., 2018). Investigations on other bacterial species have also focused on the effect of temperature on biofilm. Temperatures lower than 35°C promoted sessile growth of *Salmonella enterica* serovar Enteritidis (Iliadis *et al*., 2018) and *Acinetobacter baumanii* (De Silva *et al*., 2018). Cultivation at 37°C of *Streptococcus uberis* resulted in bigger biofilm biomass than at lower temperatures. Different data for the temperature preferences of *Listeria monocytogenes* have been reported by different laboratories (Nowak *et al*., 2015; Dhowlaghar *et al*., 2018). Growth temperature is an important factor not only because it affects biomass. It has been reported that growth temperature of *S. aureus* and *P. aeruginosa* influenced biofilm resistance for disinfectants (Lee *et al*., 2015; Abdallah *et al*., 2015a, b).

Summing up the present experimental data, it can be concluded that the species- and strain-peculiar responses to growth conditions have to be considered when preparing experimental designs directed to the identification of novel substances with putative application for biofilm control of the model Gram-positive and Gram-negative strains.

Acknowledgements: This study was part of the Bilateral Project between the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria and National Research Center, Cairo, Egypt – 2019 "Supression and destruction of biofilm formation using novel antibiofilm nanostructure materials: A multi-disciplinary approach". Dr. Ts. Paunova-Krasteva acknowledges the financial support of the Bulgarian Ministry of Education and Science under the National Research Programme "Young scientists and postdoctoral students" approved by PMC № 577/17.08.2018

References

Abdallah, M., C. Benoliel, P. Ferreira-Theret, D. Drider, P. Dhulster, N. E. Chihib (2015a). Effect of culture conditions on the resistance of *Pseudomonas aeruginosa* biofilms to disinfecting agents. *Biofouling* **31**: 49-59.

Abdallah, M., O. Khelissa, A. Ibrahim, C. Benoliel, L. Heliot, P. Dhulster, N. E. Chihib (2015b). Impact of growth temperature and surface type on the resistance of *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms to disinfectants. *Int. J. Food Microbiol*. **214**: 38-47.

Anuntrakunchai, C., R. W. Sermswan, S. Wongratanacheewin, A. Puknun, S. Taweechaisupapong (2015). Drug susceptibility and biofilm formation of *Burkholderia pseudomalei* in nutrient-limited condition. *Trop. Biomed*. **32**: 300-309.

Anutrakunchai, C., J. G. M. Bolscher, B. P. Krom, S. Kanthawong, S. Chareonsudjai, S. Taweechaisupapong (2018). Impact of nutritional stress on drug susceptibility and biofilm structures of *Burkholderia pseudomalei* and *Burkholderia thailandensis* grown in static and microfluidic systems. PLoS ONE **13**: e0194946.

Borisova, D., E. Haladjova, M. Kyulavska, P. Petrov, S. Pispas, S. Stoitsova, T. Paunova-Krasteva (2018). Application of cationic polymer micelles for the dispersal of bacterial biofilms. *Eng. Life Sci.* **18**: 943-948.

Costerton, J. W., Z. Lewandowski, D. DeBeer, D. Caldwell,

D. Korber, G. James (1994). Biofilms, the customized microniche. *J. Bacteriol*. **176**: 2137-2142.

Coughlan, L. M., P. D. Cotter, C. Hill, A. Alvarez-Ordonez (2016). New weapons to fight old enemies: Novel strategies for the (bio)control of bacterial biofilms in the food industry. *Front. Microbiol*. doi: 10.3389/fmicb.2016.01641

Cullen, L, R. Weiser, T. Olszak, R. F. Maldonado, A. S. Moreira, L. Slachmuylders, G. Brackman, Ts. Paunova-Krasteva, P. Zarnowiec, G. Czerwonka, J. Reilly, P. Drevinek, W. Kaca, O. Melter, A. de Soyza, A. Perry, C. Winstanley, S. R. Stoitsova, R. Lavigne, E. Mahenthiralingam, I. Sá-Correia, T.Coenye, Z. Drulis-Kawa, D. Augustyniak, M. A. Valvano, S. McClean (2015). Phenotypic characterization of an international *Pseudomonas aeruginosa* reference panel: Strains of cystic fibrosis origin show less in vivo virulence than non-CF strains. *Microbiology* **161**: 1961-1977.

De Silva, P. M., P. Chong, D. M. Fernando, G. Westmacott, A. Kumar (2018). Effect of incubation temperature on antibiotic resistance and virulence factors of *Acinetobacter baumanii* ATCC 17978. *Antimicrob. Agents Chemother.* **62**: e01514-17.

De Soyza, A., A. Hall, E. Mahenthiralingam, P. Drevinek, W. Kaca, Z. Drulis-Kawa, S. Stoitsova, V. Toth, T. Coenye, J., Zlosnik, J. Burns, I. Correia, D. De Vos, J. P. Pirnay, T. Kidd, D. Reid, J. Manos, J. Klockgether, L. Wiehlmann, B. Tummler, S. McClean, C. Winstanley (2013). Developing an international *Pseudomonas aeruginosa* reference panel. *MicrobiologyOpen* **2**: 1010-1013.

Dhowlaghar, N., P. A. Abeysundara, R. Nannapaneni, M. W. Schilling, S. Chang, W. H. Cheng, C. S. Sharma (2018). Growth and biofilm formation by *Listeria monocytogenes* in catfish mucus extract on four food contact surfaces at 22 and 10oC and their reduction by commercial disinfectants. *J. Food Prot*. **81**: 59-67.

Donlan, R. M. (2002). Biofilms: Microbial life on sirfaces. *Emerg. Infect. Dis*. **8**: 881-890.

Huang, K., Q. Yu, H. Ren, J. Geng, K. Xu, Y. Zhang, L. Ding (2018). Towards physicochemical and biological effects on detachment and activity recovery of aging biofilms by enzyme and surfactant treatments. *Bioresour. Technol.* **247**: 319-326.

Iliadis, I., A. Daskalopoulou, M. Simoes, E. Giaouris (2018). Integrated combined effects of temperature, pH and sodium chloride concentration on biofilm formation by *Salmonella enterica* ser. Enteritidis and Typhimurium under low nutrient food-related conditions. *Food Res. Int*. **107**: 10-18.

Jamal, M., W. Ahmad, S. Andleeb, F. Jalil, M. Imran. M. A. Nawaz, T. Hussain, M. Ali, M. Rafiq, M.A. Kamil (2018). Bacterial biofilm and associated infections. *J. Chinese Med. Assoc*. **81**: 7-11.

Khelissa, S. O., M. Abdallah, C. Jama, N. E. Chihib (2019). Actively detached *Pseudomonas aeruginisa* biofilm cell susceptibility to benzalkonium chloride and associated resistance mechanism. *Arch. Microbiol*. **201**: 747-755.

Khelissa, S., C. Jama, M. Abdallah, R. Boukherroub, C. Faille, N. E. Chihib (2017). Effect of incubation duration, growth temperature, and abiotic surface type on cell surface properties, adhesion and pathogenicity of biofilm-detached *Staphylococcus aureus* cells. *ABM Express* **7**: 191, doi.org/10.1186/s13568-077-0492-0.

Lee, J. H., Y. G. Kim, K. Lee, S. C. Kim, J. Lee (2015). Temperature-dependent control of *Staphylococcus aureus* biofilms and virulence by thermoresponsive oligi(N-vinylcaprolactam). *Biotechnol. Bioeng*. **112**: 716-724.

Lee, L. M., G. Rosenberg, S. M. Rubinstein (2019). A sequence of developmental events occurs underneath growing *Bacillus subtilis* pelicles. *Front. Microbiol*. **10**: 842, doi: 10.3389/fmicb.2019.00842

Mathlouthi, A., A., E. Pennacchietti, D. De Biase (2018). Effect of temperature, pH and plasmids on in vitro biofilm formation in *Escherichia coli. Acta Naturae* **39**: 129-132.

Nowak, J., C. D. Cruz, J. Palmer, G. I. Fletcher, S. Flint (2015). Biofilm formation of the *L. monocytogenes* strain 15G01 is influenced by changes in environmental conditions. *J. Microbiol. Methods* **119**: 189-195.

Souza dos Santos, A. L., A. C. M. Galdino, T. Pereira de Mello, L. de Souza Ramos, M. H. Branquinha, A. M. Bolognese, J. C. Neto, M. Roudbary (2018). What are the advantages of living in a community? A microbial biofilm perspective! *Mem. Inst. Oswaldo Cruz*. **11**: e180212.

Speranza, B., A. Liso, M. R. Corbo (2018). Use of design in experiments to optimize the production of microbial probiotic biofilms. *Peer J*. **6**: e4826.

Teh, A. H., S. Lee, G. A. Dykes (2016). The influence of prior modes of growth, temperature, medium, and substrate surface on biofilm formation by antibiotic-resistant *Campylobacter jejunii. Curr. Microbiol*. **73**: 859-866.

Vacheva, A., B. Mustafa, J. Staneva, M. Marhova, S. Kostadinova, M. Todorova, R. Ivanova, S. Stoitsova (2011). Effects of extracts from medicinal plants on biofilm formation by *Escherichia coli* urinary tract isolates. *Biotechnol. Biotech. Eq*. **25**: 92-97.

Vacheva, A., R. Georgieva, S. Danova, R. Ivanova, M. Marhova, S. Kostadinova, K. Vasileva, M. Bivolarska, S. Stoitsova (2012a). Modulation of *Escherichia coli* biofilm growth by cell-free spent cultures from lactobacilli. *Central Eur. J. Biol*. **7**: 219-229.

Vacheva., A, R. Ivanova, T. S. Stoitsova (2012b). Released products of pathogenic bacteria stimulate biofilm formation by *Escherichia coli* K-12 strains. *Antonie Van Leeuwenhoek* **102**: 105-119.

Vasileva-Tonkova, E., A. Sotirova, D. Galabova (2011). The effect of rhamnolipid biosurfactant produced by *Pseudomonas fluorescens* on model bacterial strains and isolates from industrial wastewater. *Curr. Microbiol*. **62**: 427–433.

Velinov, T., Y. Asenovska, D. Marinkova, L. Yotova, S. Stoitsova, M. Bivolarska, L. Stavitskaya (2011). Total internal reflection imaging of microorganism adhesin using an oil immersion objective. *Colloids Surf B Biointerfaces* **88**: 407-412.

Vijayakumar, S., S. Rajenderan, S. Laishram, S. S. Anandan, V. Balaji, I. Biswas (2016). Biofilm formation and motility depend on the nature of the *Acinetobacter baumanii* clinical isolates. *Front. Public Health*. **4**: doi: 10.3389/fpubh.2016.00105.

Wang, L., F. Daming, W. Chen, E. M. Terentjev (2015). Bacterial growth, detachment and cell size control on polyethilene terephthalate surfaces. *Sci. Rep*. **5**: doi:10.1038/srep15159.