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ORIGINAL PAPERR

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Analysis of the bacterial biofilm formation in different models of the *in vitro* culture

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ABSTRACT

Introduction. Microtiter plate assay (MPA) remains one of workhorses of *in vitro* biofilm research but it requires optimization of experimental conditions to fulfill the biofilm formation requirements of different bacterial pathogens.

Aim. The aim was to determine the effect of TSB and RPMI1640 culture media and selected culture variables (O_2 vs. 5% CO_2, extended incubation time) on the biofilm production by bacteria commonly involved in biofilm-related infections: *Enterococcus faecalis* (EF), *Escherichia coli* (EC), *Staphylococcus aureus* (SA), *Pseudomonas aeruginosa* (PA), *Klebsiella pneumoniae* (KP).

Material and methods. The investigation was performed using the MPA with crystal violet.

Results. Statistically significant ($p<0.05$) increase in biofilm production between 24h and 72h time points was observed for EF (TSB o_2, RPMIo_2 and RPMIco_2), EC (TSBo_2), SA (TSBo_2, TSBco_2), KP (TSBo_2, TSBco_2), PA (RPMIco_2, TSBco_2). The TSB caused a significantly greater stimulation of biofilm production compared to RPM1640. It outcompeted RPMI1640 irrespective of the atmospheric conditions for SA and KP and under aerobic conditions for EF.

Conclusion. Although the TSB provided the most optimal conditions for biofilm production, the process was influenced by the strain type, atmospheric conditions and period of cultivation which limits the ability to design a single universal model of the *in vitro* biofilm investigation.

Keywords. *in vitro* biofilm, microtiter plate assay, RPMI 1640, tryptic soy broth

Introduction

The ubiquitous ability of potentially pathogenic microorganisms to live attached to biotic (tissues) and abiotic (medical implants) surfaces as sessile communities known as biofilms accompanied by their inherent tolerance to innate and adaptive host defences and antibiotic therapies have brought the biofilm-related infections to the forefront the most significant concerns of modern medicine.[1-3]

A microbial biofilm is defined as a "structured consortium of microbial cells surrounded by a self-produced polymer matrix".[3,4] The matrix is thought to play a key role in the protection of the biofilm-embedded bacteria from host defences and is partially involved in the restricted diffusion of antimicrobial agents into the biofilm.[1]

Moreover, biofilms are characterized by physiological and biochemical gradients. Consumption of oxygen and glucose originating in the surface layers of biofilms,

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leads to anaerobic nutrition-depleted niches with restricted metabolic activity in the depths of the aggregated structure which corresponds to a top-to-bottom gradient of decreasing antibiotic susceptibility. Antibiotic tolerance is also mediated by accumulation of metabolic waste products and extracellular signalling molecules due to a high cell density in the biofilm depths whereas horizontal resistance gene transfer within and beyond species borders facilitates the spread of antibiotic resistance. Finally, bacteria growing in biofilms can actively adapt to stress by turning on the stress-response genes antagonizing the deleterious effects of antibiotics and the host immune system.[1,3]

Much of the current knowledge about the biofilm-related infections is a result of investigation of surface-associated biofilms produced *in vitro*. The microtiter plate assay (MPA) remains one of the workhorses of the *in vitro* biofilm research. This method is a low-cost, high-throughput biofilm screening approach for the investigation of surface-attached biomass production in liquid media.[5,6] Although the biofilm production is involved in the majority of bacterial infections, it is influenced by different external parameters which still remain to be fully elucidated. Moreover, most of the studies, have investigated the biofilm-producing capabilities of single or mixed bacterial and fungal species.[2,7-10]

Aim

The aim of our study was to determine the effect of two different culture media (TSB and RPMI 1640) and selected culture variables (degree of aeration, incubation period) on the development of biofilms produced by five reference strains of bacterial species commonly involved in the biofilm-related infections. The studied microorganisms included *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*.

Material and methods

Bacterial strains and growth conditions

Five reference bacterial strains: *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* ATCC 700603 used in this study were obtained from the American Type Culture Collection (ATCC). All strains were cultivated on trypticasein soy infusion (TSI, Biocorp, Poland) or trypticasein soy agar (TSA, Biocorp, Poland) at 37°C.

Biofilm formation

The assay of biofilm formation was performed as published previously[11] with some modifications. Formation of biofilms was carried out in 96-well microtiter plates (NUNC, Thermo Fisher Scientific Inc, Denmark). An overnight culture of bacteria (ca. 4 in McFarland standards) was diluted 1:100 in TSB additionally supplemented with 1% D-(+)-glucose and RPMI 1640 medium (Sigma Aldrich). Aliquots (200 µl) of diluted culture were inoculated into five wells each of the 96-well ster-

Table 1. The mean OD values for each of the tested reference strains, at individual time points of incubation, considering different culture conditions

Incubation time point	O_2											
	TBS						RPMI 1640					
	C Mean (SD)	EF Mean (SD)	EC Mean (SD)	SA Mean (SD)	PA Mean (SD)	KP Mean (SD)	C Mean (SD)	EF Mean (SD)	EC Mean (SD)	SA Mean (SD)	PA Mean (SD)	KP Mean (SD)
24 h	0.1375 (0.027)	0.7873 (0.061)	0.6454 (0.108)	0.4265 (0.211)	1.4340 (0.322)	0.9272 (0.373)	0.1372 (0.023)	0.2364 (0.079)	0.1674 (0.029)	0.3613 (0.201)	0.7701 (0.358)	0.2566 (0.062)
48 h	0.1383 (0.018)	1.3589 (0.605)	1.3312 (0.778)	1.5994 (0.519)	1.7791 (0.484)	2.1143 (0.304)	0.1596 (0.003)	0.4866 (0.117)	0.3294 (0.078)	0.494 (0.062)	1.2215 (0.533)	0.3631 (0.095)
72 h	0.1417 (0.018)	2.4769 (0.017)	2.0129 (0.519)	2.0194 (0.452)	2.0064 (0.453)	2.0249 (0.509)	0.2157 (0.062)	0.7054 (0.182)	0.7431 (0.216)	0.5078 (0.678)	1.0431 (0.545)	0.7461 (0.228)
Incubation time point	**CO_2**											
	TBS						**RPMI 1640**					
	C Mean (SD)	**EF Mean (SD)**	**EC Mean (SD)**	**SA Mean (SD)**	**PA Mean (SD)**	**KP Mean (SD)**	**C Mean (SD)**	**EF Mean (SD)**	**EC Mean (SD)**	**SA Mean (SD)**	**PA Mean (SD)**	**KP Mean (SD)**
24 h	0.1167 (0.015)	1.066 (0.545)	0.3244 (0.058)	0.6189 (0.172)	0.8665 (0.344)	1.2056 (0.788)	0.1396 (0.024)	0.237 (0.052)	0.1761 (0.040)	0.3844 (0.189)	1.7814 (0.282)	0.2162 (0.043)
48 h	0.1379 (0.004)	1.1395 0.261)	2.4619 (0.011)	2.4596 (1.131)	1.5764 (0.264)	2.3245 (0.242)	0.1482 (0.001)	0.2631 (0.059)	0.2793 (0.058)	0.3779 (0.125)	2.2371 (0.178)	0.2897 (0.099)
72 h	0.1487 (0.008)	0.9620 (0.218)	1.31 (0.049)	2.2261 (0.758)	1.8382 (0.455)	2.0418 (0.589)	0.1792 (0.010)	0.9963 (0.466)	0.662 (0.323)	0.5078 (0.226)	2.4252 (0.049)	0.4112 (0.267)

SD – standard deviation; C – control; EF – *Enterococcus faecalis*; EC – *Escherichia coli*; SA – *Staphylococcus aureus*; PA – *Pseudomonas aeruginosa*; KP – *Klebsiella pneumoniae*;

ile microtiter plate. The TSB and RPMI 1640 broths (200 µl) were used as negative controls (K). Biofilms were grown statically for 24, 48 and 72 h at 37°C in aerobic conditions as well as in the presence of 5% CO_2. The media were replenished after every 24h of growth. Following incubation, the wells were carefully washed twice with 0.9% NaCl, and dried for 1 h at 50°C. Biofilms in wells were stained with 0.1% crystal violet (CV; 200 µl) for 15 min in order to determine total biofilm biomass. After staining, the wells were washed by flushing the plate three times with 200 ml of distilled water to remove unbound CV and air-dried. The biofilm-bound dye was extracted with 200 µl of 70% (v/v) ethanol. The optical density (OD) was then determined at 570 nm using the microplate reader. Each experiment was performed in triplicate.

Statistical analysis

Statistical analyses were performed using 2-tailed unpaired t-test (2 groups) or one-way ANOVA followed by Tukey's multiple comparisons post test. $P<0.05$ was considered statistically significant. All data are described as mean ± SD in the text.

Results

All tested bacterial strains adhered and developed into biofilms on the wells of the microtiter plates in both culture media (TSB and RPMI 1640) used in the study. However, the degree of the biofilm development reflected by the measurement of the OD was dependent on the type of the culture medium, incubation period and atmospheric conditions: aerobic vs. the increased CO_2 concentration.

It was noted that the biofilm formation progressed for 72 h and its maximum yield was found at this time point for the majority of strains. Nevertheless, we also observed a slight decrease (not reaching statistical significance) in the OD was observed at the 72 h compared to the 48 h time point in six models of the biofilm culture (Table 1).

Statistically significant ($p<0.05$) increase in the OD of the biofilm produced between the 24 h and 72 h time points was observed for *E. faecalis* incubated in TSB O_2, RPMI 1640 O_2 and RPMI 1640 CO_2, *E. coli* incubated in TSB O_2, *S. aureus* incubated in TSB O_2 and TSB CO_2, *K. pneumoniae* incubated in TSB O_2 and TSB CO_2, and for *P. aeruginosa* incubated in RPMI 1640 CO_2 and TSB CO_2 (Fig. 1).

Significant increase in the mean OD between the 24 h and 48 h time points was observed for *E. coli* incubated in TSB CO_2, and for *S. aureus* and *K. pneumoniae* incubated in TSB O_2 and TSB CO_2. Significant increase in the mean OD between the 48 h and 72 h time points was observed for *E. faecalis* incubated in TSB O_2 only.

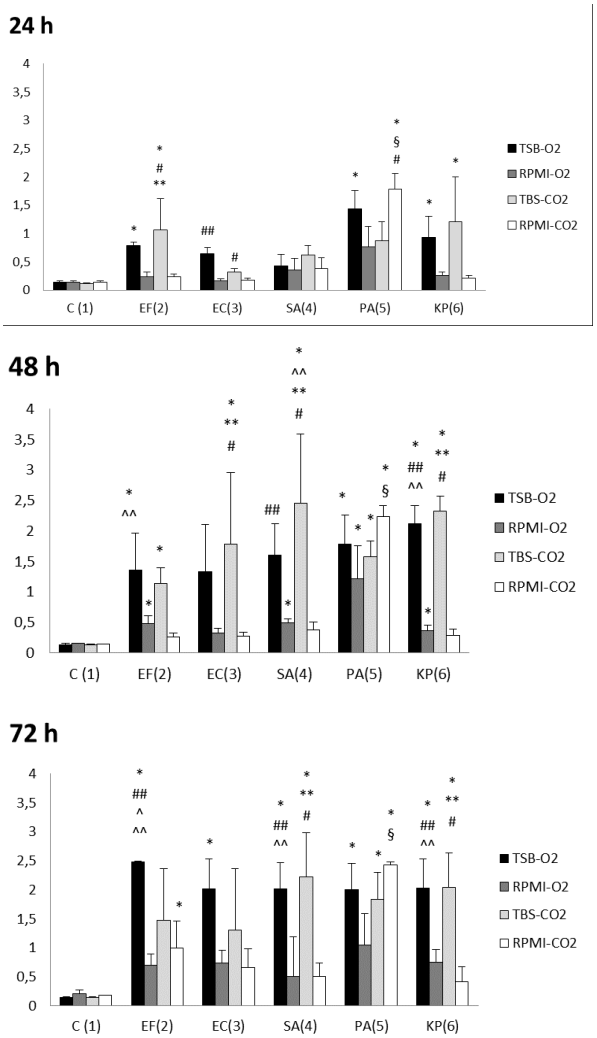

Fig. 1. Bacterial biofilms grown under different culture media and different oxygenic conditions, evaluated at 24 h, 48 h and 72 h time points. Data are mean values performed in triplicate in the same culture conditions. Significant differences ($p<0.05$) between different culture conditions within each tested bacterial species are indicated as follows:

*control vs bacterial strain;

** RPMI-O2 vs TSB-CO2

TSB-CO2 vs RPMI-CO2

TSB-O2 vs RPMI-O2

^ TSB-O2 vs TSB-CO2

^^ TSB-O2 vs RPMI-CO2

§ RPMI-O2 vs RPMI-CO2

The above results indicate that the extended (72 h) incubation results in the formation of the mature, well-established *in vitro* biofilm. On the other hand, as noted earlier, the production of the biofilm did not reach a steady increase throughout the extended incubation period for all tested strains, under all applied incubation conditions. This notion was striking for four out of the five tested strains incubated in TSB CO_2 whose biofilm OD decreased after the 48 h time point.

These observations lead to the conclusion that aerobic incubation stimulates the biofilm formation to the greatest extent. The majority of the strains (4 out of 5) reached the maximal biofilm producing capability at the 72 h time point under aerobic conditions in both culture media (Table 1).

On the other hand, all tested strains cultured in RPMI 1640 at the increased CO_2 reached the maximum biofilm yield (Table 1).

It should be noted, however, that the RPMI 1640 medium was inferior to the TSB in terms of the degree of the biofilm production reflected by its OD. The significantly greater ($p<0.05$) mean OD values at the 72 h time point (Fig. 1) were observed when the two media and the accompanying degree of aeration during culture were compared in the following bacterial species:

- TSB O_2 vs RPMI 1640 O_2 for *E. faecalis*, *S. aureus*, and *K. pneumoniae*
- TSB CO_2 vs RPMI 1640 CO_2 for *S. aureus* and *K. pneumoniae*
- TSB CO_2 vs RPMI 1640 O_2 for *S. aureus* and *K. pneumoniae*
- TSB O_2 vs RPMI 1640 CO_2 for *E. faecalis*, *S. aureus,* and *K. pneumoniae*

The obtained results indicate that for *S. aureus* and *K. pneumoniae* the TSB medium outcompeted RPMI 1640 irrespective of the atmospheric incubation conditions whereas for *E. faecalis* the TSB stimulated a greater biofilm production under aerobic conditions.

An interesting observation was made for *P. aeruginosa* which produced the most profuse biofilm during incubation in RPMI CO_2 (Table 1, Fig. 1). Nevertheless, this strain produced the biofilm under all applied conditions with the only statistical significance noted when the RPMI CO_2 incubation was compared to the RPMI O_2 (OD=2.42 vs. OD=1.04, respectively, at the 72h time point). This medium did not gain a significant advantage over the TSB.

Discussion

The principle of the MPA is to yield the biofilm growth on abiotic surfaces submerged in media and exposed to fluid dynamics of varying degrees. Although it is impossible to indiscriminately extrapolate conclusions drawn from the MPA research to the pathogenesis of the biofilm-related *in vivo* infections due to different growth environments for bacteria provided by urine, blood or other body fluids, missing immune response and inability to reflect complex oxygen and nutrient gradients found in infections compared to the *in vitro* studies, the MPA and other *in vitro* assays allow for the investigation of the *in vitro* biofilm with a stringent control of experimental parameters and simultaneous ability to change single variables.[2,3,4,6]

It has been known that environmental conditions including culture media and available nutrients can modulate microbial biofilm production and its function. They have a major impact on the biofilm growth and development and the metabolic activity of cells in maturing biofilms.[7,9] It has even been suggested that composition of the medium is the most important factor influencing the ability of bacteria to produce biofilm under *in vitro* conditions.[5]

The TSB medium is a commonly used enrichment medium containing enzymatic digests of casein and soybean (providing amino acids and other complex nitrogenous substances), glucose (as an energy source), sodium chloride (which maintains the osmotic equilibrium) and dibasic potassium phosphate (as a buffer to control pH). This medium in routinely used for the cultivation of a wide variety of microorganisms but it has also been frequently used in the biofilm investigation[5]. The RPMI 1640 medium, in turn, mimics the composition of human body fluids as it contains high concentration of amino acids, vitamins and inorganic salts[10]. It is commonly used in cell and tissue culture for growing of a variety of mammalian cell lines.

The application of the two media in our study revealed that TSB stimulated a significantly more profuse biofilm production compared to the RPMI 1640 for the strains tested as evidenced by the increase in the mean OD. We also observed a species/strain dependent advantage of the TSB over the RPMI 1640 at the maximum (72 h) time point. In case of *S. aureus* and *K. pneumoniae* the TSB medium outcompeted RPMI 1640 irrespective of the atmospheric incubation conditions whereas for *E. faecalis* the TSB stimulated a greater biofilm production following incubation under aerobic conditions.

P. aeruginosa, in turn, produced the most profuse biofilm following incubation in RPMI 1640 CO_2 ($p<0.05$ vs. RPMI 1640 O_2) as evidenced by the highest increase in the mean OD among the media and accompanying culture conditions used for this strain. However, this medium did not gain a statistically significant advantage over the TSB. The obtained result pointing at the unexpected most profuse biofilm production in RPMI CO_2 can be explained by the fact that *P. aeruginosa* is a versatile microorganism. It is able to grow both in oxic and hypoxic environments and to use both oxygen and nitrate as an electron acceptor for its heterotrophic respiration.[12]

The only bacterial strain for which none of the culture variables gained the upper hand was *E. coli*. This strain demonstrated the greatest biofilm OD following incubation in TSB O_2 but the difference between this value and other obtained results did not reach a statistical significance.

Our results are generally in line with previous literature data reporting that TSB, especially after supplementation with glucose[7,13] enhances and supports the biofilm formation. Some authors, however, noted a greater bio-

film-promoting ability of the brain-heart infusion (BHI) broth compared to the TSB for the clinical strains of *S. aureus*.[8] The authors noted that BHI is the source of proteins rich in leucine, proline, serine, and aspartic acid which may be essential for the production of bacterial adhesins such as staphylococcal fibronectin-binding protein and clumping factor.[8]

The RMPI 1640 was also examined for the biofilm production in several previous studies which brought conflicting results. Tan et al. revealed that the development of mixed biofilms with *Candida* species and *S. epidermidis* yielded the lowest biofilm formation when grown in RPMI 1640 medium compared to the other two media used in their study, namely TSB and BHI.[9] These authors also reported that the metabolic activity of biofilms produced by mixed biofilms of three *Candida* species and *S. epidermidis* was significantly reduced in RPMI 1640 compared to the TSB and BHI media. Wijesinghe et al. in turn, noted that the adherence of both *P. aeruginosa* and *S. aureus*, either in mono- or coculture, was optimal in RPMI 1640.[10] On the other hand, however, this medium performed worst in terms of support for growth. In their study, BHI medium was the one which fostered the maximal biofilm growth. The authors attempted to explain this observation taking into account the chemical composition of this medium and the ability of bacteria to metabolize different nutrients. Although RPMI is a rich medium containing high concentration of amino acids, vitamins and inorganic salts (which may initially induce the production of extracellular surface components promoting bacterial adhesion), its amino acid composition is higher than that of carbohydrates. During the period of rapid bacterial growth, carbohydrates are quickly used as the primary source of carbon followed by peptides, amino acids, nucleic acids, nucleotides and fatty acids. As the cells enter the stationary phase of growth, amino acid catabolism becomes predominant. As a result, ammonia is released into the medium and causes it to become basic. In order to maintain pH homeostasis in the cytoplasm bacteria must actively acquire protons from the basic medium environment which requires a high energy expenditure. In the next period, readily available nutrients are exhausted and bacteria must obtain nutrients from the dead bacteria which imposes another energy cost on the cell due to the necessity of conversion of nutrients originating from the bacterial debris into their constituent parts. The authors concluded that stress responses associated with the appearance of macromolecular agents in the medium could affect the viable cell mass and lead to its reduction in RPMI medium.[10]

The present data support the notion that growth media significantly influence the ability of bacteria to form biofilms. However, biofilm formation and adherence, as Hancock et al. noted following investigation of *E. coli* and *K. pneumoniae* biofilm formation, are not accomplished by the same mechanisms in different media.[2] The authors examined biofilm formation in two different minimal lab media (ABTG and MOPS), pooled human urine and LB medium. Their study revealed that production of a good biofilm in one medium does not predict an equally good biofilm forming ability in another growth medium, and strains that outperformed others in one medium do not necessarily do so in another growth medium. It is therefore conceivable, that available environmental resources influence the expression of different biofilm-promoting genes and utilization of different strategies involved in adherence (including surface proteins, adhesive factors, cell surface hydrophobicity) by microorganisms. Similarily, Hood and Zottola who studied four foodborne microorganisms (*Salmonella typhimurium, Listeria monocytogenes, E. coli* O157:H7, *Pseudomonas fragi* and *P. fluorescens*) concluded that the medium which produced the highest level of adherent cells was different for each microorganism.[14] Moreover, it was reported that some microorganisms may demonstrate enhanced adhesive abilities when the nutrients are lacking, while others can exhibit high adhesion rates even under basic growth conditions.[15]

According to the available data, incubation time also plays a crucial role in the biofilm development. It promotes the accumulation of greater amounts of the extracellular matrix substances. Most studies have used an incubation period limited to 24-48 hours which may not reflect actual kinetics of the biofilm growth and maturation.[8,9] In our study, a significant increase in the mean OD between the 24 h and 48 h time points was observed in only certain strains and was associated with the growth medium and atmospheric conditions during incubation. This increase was noted for *E. coli* incubated in TSB CO_2, and for *S. aureus* and *K. pneumoniae* incubated in TSB O_2 and TSB CO_2 which, again, indicates the supporting role of TSB for the production of bacterial biofilm under *in vitro* conditions.

Tan et al. in turn, observed increased biofilm biomass from 24 h to 48 h for all tested strains (the mixtures of *Candida* spp. and *S. epidermidis*) cultured in RPMI, TSB and BHI and additionally concluded that changing the culture media after 24 h of growth (which was also done in our study) had a positive effect on the increase in the biofilm biomass.[9]

Senevirante et al. who investigated the effect of culture media and nutrients on the biofilm production by laboratory and clinical strains of *E. faecalis* reported that 72 h of growth is required to achieve robust, mature biofilms, evidenced by *in vitro* and microscopic observation of analysed enterococcal strains. This tendency was observed for all the strains studied and was irrelevant of the cultured medium used (BHI, TSB and "Pg broth").[7]

Our study has brought more diverse results. It was noted that the biofilm formation progressed for 72 h and its maximum yield was observed at this time point for the majority of strains. The TSB O_2 incubation provided conditions that led to the significant increase in the OD of the biofilm between the 24 h and 72 h time points for all tested bacterial species with the exception of *P. aeruginosa*. The significant increase in the biofilm OD biofilm produced by this strain was noted following incubation under increased CO_2 concentration only, irrespective of the medium used. It could also be observed that the kinetics of the biofilm biomass formation between the 48 h and 72 h time points occurred more slowly compared to the increase between the 24 h and 48 h time points. This increase reached statistical significance only for *E. faecalis* incubated in TSB O_2 whereas significant increase in the OD was noted for *E. coli* (incubated in TSB CO_2), *S. aureus* (incubated in TSB O_2 and TSB CO_2), and *K. pneumoniae* (incubated in TSB O_2 and TSB CO_2) when the 24 h and 48 h time points were compared. Moreover, some strains demonstrated a slight decrease in the biofilm OD between the 48 h and 72 h time points with the most striking example of TSB CO_2 incubation which was associated with this decline in four out of five strains.

The obtained results indicate that the *in vitro* biofilm formation is dependent on the experimental design to a significant extent. Although the TSB medium provides the most optimal conditions for the biofilm production, this process is additionally influenced by atmospheric conditions during incubation and the period of the cultivation. The study also revealed the *in vitro* biofilm production capabilities are not only dependent on the external culture conditions but they are also influenced by the type of the bacterial strain tested. This, in turn, in spite of all experimental advantages of the MPA assay, limits the ability to design a single universal model of the *in vitro* biofilm investigation.

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