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Escherichia coli adhesion, biofilm development and antibiotic susceptibility on biomedical materials

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Abstract

The aim of this work was to test materials typically used in the construction of medical devices regarding their influence in the initial adhesion, biofilm development and antibiotic susceptibility of *Escherichia coli* biofilms. Adhesion and biofilm development was monitored in 12-well microtiter plates containing coupons of different biomedical materials – silicone (SIL), stainless steel (SS) and polyvinyl chloride (PVC) – and glass (GLA) as control. The susceptibility of biofilms to ciprofloxacin and ampicillin was assessed and the antibiotic effect in cell morphology was observed by scanning electron microscopy (SEM). The surface hydrophobicity of the bacterial strain and materials was also evaluated from contact angle measurements. Surface hydrophobicity was related with initial *E. coli* adhesion and subsequent biofilm development. Hydrophobic materials, such as SIL, SS and PVC, showed higher bacterial colonization than the hydrophilic glass. Silicone was the surface with the greatest number of adhered cells and the biofilms formed on this material were also less susceptible to both antibiotics. It was found that different antibiotics induced different levels of elongation on *E. coli* sessile cells. Results revealed that, by affecting the initial adhesion, the surface properties of a given material can modulate biofilm buildup and interfere with the outcome of antimicrobial therapy. These findings raise the possibility of fine-tuning surface properties as a strategy to reach higher therapeutic efficacy.

Keywords: *Escherichia coli*, surface hydrophobicity, bacterial adhesion, biofilm formation, antibiotic susceptibility

INTRODUCTION

Bacterial adhesion to biomedical surfaces is a complex process that is affected not only by biological features, but also by many physico-chemical factors, such as the surface properties of the device (chemical composition, charge, hydrophobicity, roughness and texture), the bacterial hydrophobicity and charge, and environmental factors (temperature, pH, fluid flow conditions, etc.).[1] From an overall physico-chemical point of view, adhesion can be mediated by non-specific interactions (long-range, distances > 150 nm), including Lifshitz-van der Waals forces, electrostatic forces, acid-base interactions and Brownian motion forces.[1,2] As soon as bacterial cells reach a surface, they will be attracted or repelled by it, depending on the sum of the different non-specific interactions.[3] Hydrophobic interactions are usually the strongest of all long-range non-covalent forces involved in bacterial attachment and can be defined as the attraction between apolar, or slightly polar, cells or other molecules, when immersed in an aqueous solution.[2] It has been demonstrated that hydrophobicity plays an important role in a wide range of microbial infections.[4]

Biofilm formation in medical devices generally comprises several steps. Firstly, there is the deposition of a conditioning film produced by the host on the foreign body.[5,6] It is followed by the approach and attachment of microorganisms, consisting in the first step in the pathogenesis of medical device-related infections.[7] The initial adhesion is reversible, involves hydrophobic and electrostatic forces, and is followed by irreversible attachment mediated by bacterial polysaccharides which anchor the organisms to the surface.[5] After that, cell growth, multiplication and dissemination occur.

Microbial biofilms are well-known for their high resistance to antibiotic and biocide treatments.[8] Bacteria within biofilms can tolerate the presence of high antibiotic concentrations (in the range that is therapeutically prescribed), making most of the device-

77 related infections difficult or impossible to eradicate.[9] Some factors that contribute to
78 biofilm resistance include physical or chemical diffusion barriers to antibiotic penetration
79 within the biofilm matrix, slow growth rate of biofilm cells due to nutrient limitation,
80 activation of the general stress response, and the presence of persister cells or antibiotic-
81 resistant small-colony variants.[10] Two antibiotics with distinct modes of action were used
82 on this study: ciprofloxacin and ampicillin. Ciprofloxacin, a broad-spectrum synthetic
83 antibiotic of the fluoroquinolone drug class, functions by inhibiting DNA gyrase and
84 topoisomerase IV (enzymes necessary to separate bacterial DNA strands), thereby
85 inhibiting cell division.[11] Ampicillin, a β-lactam antibiotic, inactivates the synthesis of
86 cross-linked peptidoglycan and also interferes with septum formation during cell
87 division.[12] Both antibiotics are among the most frequently prescribed antimicrobial agents
88 worldwide and can be used on the treatment of urinary tract infections[13-16] in which *E.*
89 *coli* is notably the main causative agent.[17]
90 Biomedical devices are currently made of different materials. Silicone polymers have
91 been further applied in urinary catheters, contact lenses, ophthalmologic implants, heart
92 valves, breast implants, blood pumps, tubing and adhesives.[18,19] Stainless steel 316 is used
93 in surgical instruments, as well as in orthopaedic, craniofacial and cardiovascular implant
94 devices.[20] PVC covers more than 25% of all plastic materials used in medical
95 applications, including intravenous fluid bags and tubing, blood and plasma bags, enteral
96 feeding and dialysis equipment, endotracheal tubes, short-term catheters and gloves.[21,22]
97 *E. coli* has become a common pathogen in predisposed hosts with indwelling medical
98 devices.[23] Its pathogenic strains are responsible for 70 to 95% of urinary tract infections
99 (UTIs), one of the most typical bacterial diseases. These infections are especially frequent
100 in cases of catheterization due to biofilm development on the indwelling urinary
101 catheters.[24,25] Catheter-associated UTI accounts for more than 1 million cases per year in

United States alone and involves an annual cost of caring for patients with this infection of approximately $2 billion.[26]

The main goal of this study was to assess the importance of the initial adhesion events on the development of *E. coli* biofilms in clinically relevant materials and to determine if the surface properties can affect the outcome of an antimicrobial therapy. Additionally, SEM visualization of the biofilms enabled the assessment of morphological changes in the bacterial cells resulting from the antibiotic treatment. A better understanding of these effects may provide clues for the fine-tuning of the surface properties of biomedical materials in order to mitigate bacterial adhesion and increase the efficiency of antimicrobial therapy.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Escherichia coli JM109(DE3) from Promega (USA) was used in this study because this strain has shown a good biofilm forming ability in both turbulent[27] and laminar[28] flow conditions. Its genotype is *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, λ^-, Δ(*lac-pro*AB), [F',*tra*D36, *pro*AB, *lac*I^qZΔM15], λ(DE3).

A bacterial suspension was prepared by inoculation of 500 μL of a glycerol stock (kept at -80 ºC) in a total volume of 0.2 L of inoculation medium previously described by Teodósio et al.[29] This consisted of 5.5 g/L glucose, 2.5 g/L peptone, 1.25 g/L yeast extract in phosphate buffer (1.88 g/L KH_2PO_4 and 2.60 g/L Na_2HPO_4), pH 7.0. This culture was grown on a 1 L shake-flask, incubated overnight at 37 ºC with agitation. Subsequently, cells were harvested by centrifugation (at 3202 *g* for 10 min at 25 ºC) and suspended in Mueller-Hinton broth (Merck, Germany) to remove all traces of the overnight growth medium. Cells were again harvested by centrifugation (as described before) and

suspended in Mueller-Hinton broth in order to obtain an inoculum containing approximately 1 x 10^7 cells/mL.

Surface preparation

Coupons with dimensions of 1 x 1 cm from glass (GLA; Vidraria Lousada, Lda, Portugal), stainless steel 316 (SS; F. Ramada, Portugal), polyvinyl chloride (PVC; Neves & Neves, Lda, Portugal) and silicone (SIL; Neves & Neves, Lda, Portugal) were prepared. SS, PVC and SIL were selected because of their recurrent use in clinical applications and glass, a hydrophilic surface, was used in this study for comparative purposes.

All materials were immersed in a solution of 5% (v/v) commercial detergent (Sonasol Pril, Henkel Ibérica S.A.) and pre-warmed distilled water (37 ºC) for 30 min with gentle shaking. To remove any remaining detergent, coupons were rinsed 5 times in 2 mL of ultrapure water per coupon in ultrapure water and air-dried for 1 h.[17,30] Then they were immersed in 96% (v/v) ethanol for 30 min, except for PVC and SIL that were only immersed for 10 s.[31] After being rinsed with ultrapure water and air-dried again, SS and GLA coupons were autoclaved for 15 min at 121 ºC[30] whereas SIL and PVC coupons were autoclaved for 20 min at 70 ºC[17] to avoid damaging the coupons. The sterility of the SIL and PVC coupons was confirmed by the absence of bacterial growth in the surface after a 24 h incubation in Mueller-Hinton broth at 37 ºC.

Free energy of adhesion between bacteria and surfaces

The free energy of adhesion (ΔG_{iwl}^{TOT}) between the *E. coli* and all tested surfaces (SIL, SS, PVC and GLA) was assessed according to the procedure described by Simões et al.[32] Lawns of *E. coli* were prepared as described by Busscher et al.[33] in order to ascertain the bacterial surface hydrophobicity.

The contact angles of the bacteria and the surfaces were determined by the sessile drop method using a contact angle meter (OCA 15 Plus, Dataphysics, Germany). The surface tension components of the bacteria and the adhesion surfaces were obtained by measuring the contact angles with three pure liquids. These measurements were carried out at room temperature (25 ± 2 ºC) using water, formamide and α-bromonaphthalene (Sigma-Aldrich Co., Portugal) as reference liquids. The surface tension components of the reference liquids were obtained from literature.[34] Contact angle measurements were performed in three independent experiments. On each experiment, at least 25 determinations for each liquid, material and microorganism were made. Afterwards, the hydrophobicity of the bacteria and the surfaces was evaluated by the method of van Oss et al.[35-37] In this approach, the degree of hydrophobicity of a given material (i) is expressed as the free energy of interaction between two entities of that material immersed in water (w) - ΔG_{iwi}. If the interaction between the two entities is stronger than the interaction of each entity with water (ΔG_{iwi} < 0 mJ/m^2), the material is considered hydrophobic. Conversely, if ΔG_{iwi} > 0 mJ/m^2, the material is hydrophilic. ΔG_{iwi} was calculated from the surface tension components of the interacting entities, according to the equation:

$$\Delta G_{iwi} = -2\left(\sqrt{\gamma_i^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_i^+\gamma_w^-} + \sqrt{\gamma_i^-\gamma_w^+} - \sqrt{\gamma_i^+\gamma_i^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \quad (1)$$

where γ^{LW} accounts for the Lifshitz-van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ^{AB}), with $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$.

The surface tension components were estimated by the simultaneous resolution of three equations of the type:

$$(1+\cos\theta)\gamma_i^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_i^{LW}} + \sqrt{\gamma_s^{+}\gamma_i^{-}} + \sqrt{\gamma_s^{-}\gamma_i^{+}}\right) \quad (2)$$

where θ is the contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$.

When studying the interaction (free energy of adhesion) between substances i and I that are immersed or dissolved in water, the total interaction energy, ΔG_{iwI}^{TOT}, can be expressed as:

$$\Delta G_{iwI}^{TOT} = \gamma_{iI}^{LW} - \gamma_{iw}^{LW} - \gamma_{Iw}^{LW} + 2\left[\sqrt{\gamma_w^{+}}\left(\sqrt{\gamma_i^{-}} + \sqrt{\gamma_I^{-}} - \sqrt{\gamma_w^{-}}\right) + \sqrt{\gamma_w^{-}}\left(\sqrt{\gamma_i^{+}} + \sqrt{\gamma_I^{+}} - \sqrt{\gamma_w^{+}}\right) - \sqrt{\gamma_i^{+}\gamma_I^{-}} - \sqrt{\gamma_i^{-}\gamma_I^{+}}\right] \quad (3)$$

Thermodynamically, if $\Delta G_{iwI}^{TOT} < 0$ mJ/m^2 adhesion of the bacteria to the substratum is favourable, whereas adhesion is not favourable if $\Delta G_{iwI}^{TOT} > 0$ mJ/m^2.

Initial adhesion and biofilm assays

A total of 2 mL of cell suspension (1 x 10^7 cells/mL in Mueller-Hinton broth) was transferred into each well of a sterile 12-well polystyrene (PS), flat-bottomed microtiter plate (Orange Scientific, USA) containing the coupons of different materials. Initial adhesion and biofilm growth were allowed to occur by incubating the microtiter plates at 37 ºC without shaking. At different sampling times, 30 min for initial adhesion and 4, 8, 12, 16, 20 and 24 h for biofilm studies, coupons were removed from the microwells and quickly immersed on 2 mL of sterile saline (NaCl 0.85%) to remove the loosely attached

199 cells. Coupons were then vortexed in 10 mL of saline solution during 1 min[22] to suspend
200 and homogenize the biofilm cells. The extent of cell removal from the surface due to
201 vortexing was assessed and it was found to be greater than 95%.
202 For total cell counts, suspended biofilm cells were stained with 4'-6-diamidino-2-
203 phenylindole (DAPI), which stains both viable and non-viable cells.[38] Biofilm cells were
204 properly diluted, filtered through a Nucleopore, Track-Etch Membrane (Whatman Int.,
205 Ltd., USA) black polycarbonate membrane (pore size 0.2 μm) and stained with 1 mL of
206 DAPI reagent (0.5 mg/L) for 10 min in the dark.[38] Stained bacterial observation and
207 counting was performed using a Leica DM LB2 epifluorescence microscope connected
208 to a Leica DFC300 FX camera (Leica Microsystems Ltd., Switzerland). Cell numbers on
209 each membrane were estimated from counts of a minimum of 20 fields of view and the
210 final values were presented as log total cells/cm^2.
211 Planktonic culture densities were also determined for the same time points (30 min for
212 adhesion and 4, 8, 12, 16, 20 and 24 h for biofilm studies) by reading the optical density
213 (OD) at 610 nm using a microtiter plate reader (SpectraMax M2E, Molecular Devices,
214 UK).
215 To determine the initial adhesion, biofilm development and planktonic growth rates, three
216 independent experiments were performed for each surface, each of them with a triplicate
217 set of wells.
218
219 **Quantification of extracellular polymeric substances (EPS)**
220 The content of the main EPS found in biofilms (proteins and polysaccharides) formed on
221 the different materials was assessed after 24 h of biofilm growth. Matrix proteins and
222 polysaccharides from biofilms were separated from cells using Dowex resin (50 X 8, Na^+
223 form, 20-50 mesh; Fluka Chemika, Switzerland), according to the procedure described

by Simões et al.[39] Twelve coupons of each material were removed from the microtiter plate and the biofilms were suspended in 10 mL of extraction buffer (2 mM Na_3PO_4, 2 mM NaH_2PO_4, 9 mM NaCl and 1 mM KCl, pH 7). Then, 50 g of Dowex resin per g of volatile solids[39] were added to the biofilm suspension. The extraction took place for 4 h at 4 ºC (with stirring at 400 rpm), and ultimately the extracellular components (matrix) were separated from the cells through centrifugation (for 6 min at 3202 *g*). Total protein and polysaccharide content was assessed prior to extraction procedure. Protein (total and matrix) amount was determined for each material by the Bicinchoninic Acid Protein Assay Kit – BCA™ Protein Assay Kit (Thermo Fisher Scientific, USA) and polysaccharide (total and matrix) concentration was quantified by the phenol-sulphuric acid method of DuBois et al.[40] Protein and polysaccharide specific amounts were calculated taking into account the biofilm dry-weight assessed as described by Sousa et al.[41] Briefly, coupons obtained after 24 h of biofilm growth were removed from the plate wells and dried at 80 ºC for 24 h. The coupons were then weighed and the biofilms were scraped from the surface, which was then cleaned with ethanol, left overnight at 80 ºC and then weighed. Biofilm dry-weights were assessed by the difference between the weight of the coupon with and without the biomass attached.

Antibiotics and determination of biofilm minimum inhibitory concentration (biofilm MIC)

The antibiotics used in this study were ciprofloxacin (Sigma-Aldrich Co., Portugal) and ampicillin (AppliChem, Germany).

The biofilm MIC is defined as the lowest antibiotic concentration that inhibits visible growth[42] and was determined according to Takahashi et al.[43] with some modifications. Sterile 96-well polystyrene, flat-bottomed microtiter plate (Orange Scientific, USA) were

filled with 200 µL of cells at 1 x 10^7 cells/mL in Mueller-Hinton broth supplemented with each antibiotic at different concentrations. After 24 h of incubation at 37 ºC, the medium was removed and the wells were washed to remove non-adherent bacterial cells. The biofilms were suspended in saline solution by pipetting up and down and the OD was measured at 610 nm using a microtiter plate reader (SpectraMax M2E, Molecular Devices, UK). The biofilm MIC values were 0.08 µg/mL and 50 µg/mL for ciprofloxacin and ampicillin, respectively (data not shown).

Biofilm susceptibility

To assess the susceptibility of biofilms developed on all tested surfaces, a concentration corresponding to 5 × biofilm MIC of ciprofloxacin and ampicillin (0.4 µg/mL and 250 µg/mL, diluted in Mueller-Hinton medium, respectively) was added to the coupons after 24 h of incubation. The biofilm cells were exposed to antibiotics for 7.5 h and different coupons of each material were sampled every 1.5 h. The biofilms were suspended as previously described for initial adhesion and biofilm assays, filtered through a Nucleopore, Track-Etch Membrane (Whatman Int., Ltd., USA) black polycarbonate membrane (pore size 0.2 µm) and stained with the Live/Dead® (L/D) *Bac*Light™ Bacterial Viability kit (Invitrogen Life Technologies, Alfagene, Portugal) for 10 min in the dark.[27,44] Bacterial observation and counting of viable and non-viable bacteria was also performed as indicated for initial adhesion and biofilm assays. For viability assessment, live cells were divided by the total cell number (live plus dead cells) and the result was expressed in percentage of cell viability. In the specific case of glass, the percentage of total cell removal was also determined. One sample was analysed prior to antibiotic exposure (time 0 h) confirming that 100% of the cells were viable. Three

independent experiments were performed for each surface, each of them with a triplicate set of wells.

Scanning electron microscopy (SEM)

The morphological changes of *E. coli* biofilms formed on glass coupons and exposed to 6 h of antibiotic treatments were assessed by SEM. From the studied materials, glass was selected for SEM analysis since it had the lowest cell density after 24 h, which facilitates cell size determination. Prior to observation, biofilm samples were fixed and dehydrated as fully described by Gomes et al.[45] Coupons were then air-dried for 1 day in a desiccator and sputter-coated with a palladium-gold thin film[45] using the SPI Module Sputter Coater equipment for 120 s at 15 mA current. The biofilms were viewed with a SEM/EDS system (FEI Quanta 400FEG ESEM/EDAX Genesis X4M, FEI Company, USA) in high-vacuum mode at 15 kV to observe biofilm morphology. Twenty images were analysed in the absence of antibiotics and in the presence of each antibiotic studied (surfaces from three independent wells were analysed). Cell length was determined using the microscope software (xT Microscope Control, FEI Company, USA) by measuring 100 randomly selected cells in each condition.

Statistical analysis

The adhesion, biofilm growth and susceptibility assays were compared using one-way analysis of variance (ANOVA) by applying Levene's test of homogeneity of variances and the Tukey multiple comparison tests using IBM SPSS Statistics software version 21. Paired *t*-test analysis was also performed when appropriate. All tests were used based on a confidence level of 95% (differences reported as significant for p values < 0.05).

RESULTS

Surface physico-chemical analysis

The surface hydrophobicity of the *E. coli* cells and of the GLA, SS, PVC and SIL surfaces was determined. It was found that the cell surface was hydrophilic since ΔG_{iwi} is positive (Table 1). From Table 1, it can be also observed that this *E. coli* strain had predominantly an electron donor surface (higher value of γ^-), with a very low electron acceptor character (γ^+). Concerning the material surfaces, GLA was hydrophilic ($\Delta G_{iwi} > 0$ mJ/m^2), whereas the remaining materials (SS, PVC and SIL) were hydrophobic ($\Delta G_{iwi} < 0$ mJ/m^2). From the hydrophobic materials, SIL was the most hydrophobic ($p < 0.05$), mainly due to its lower electron donor character, followed by PVC and SS.

In order to predict the probability of *E. coli* adhesion to the four tested materials, the free energy of interaction between the bacteria and the surfaces was calculated (Table 2). The results show that adhesion to SS, PVC and GLA was not thermodynamically favored ($\Delta G_{iwI}^{TOT} > 0$ mJ/m^2), whereas adhesion to SIL was favored ($\Delta G_{iwI}^{TOT} < 0$ mJ/m^2).

Bacterial adhesion and biofilm development

E. coli adhesion and biofilm growth on the four different materials is shown in Figure 1. Initial adhesion was determined after 30 min and significant differences ($p < 0.05$) were observed in all materials (Figure 1B). Furthermore, the extent of adhesion to silicone was approximately 5-fold higher than to glass, which was the material with fewer adhered cells after the initial period. Interestingly, glass was also the material showing the lowest number of adhered cells in the following data points concerning biofilm development ($p < 0.05$). Biofilm formation results (assayed in the period between 4 and 24 h) followed the initial adhesion trend for most of the time points (except for 4 and 8 h for PVC and SS) with statistically significant results obtained at 16 and 24 h ($p < 0.05$). Thus, with few

exceptions, the adhesion and biofilm development trend was: SIL > PVC > SS > GLA. It is noteworthy that for SS and PVC, there were no statistically significant differences in the cell density determined on both materials in the majority of time points.

Overall, the amount of biofilm formed in all the materials increased with time and so did the planktonic cell concentration (Figure 1A) that also increased with similar trends in all tested conditions.

The first 4 hours of experiment were those that showed the highest growth rate of planktonic (Figure 1A) and sessile cells (Figure 1B) since the optical density and the total number of adhered cells increased on average 16 and 7.5-fold, respectively, while in the remaining 4 h intervals, the OD and the biofilm only increased on average 1.3 and 1.5 fold.

Biofilm susceptibility assays

Figure 2 presents the susceptibility curves of *E. coli* biofilms formed on all materials to a concentration equivalent to 5 × biofilm MIC of the antibiotics tested, ciprofloxacin (Figure 2A) and ampicillin (Figure 2B). Regardless of the antibiotic used, biofilms formed on GLA, PVC and SS were more susceptible to the antimicrobial treatments than SIL since complete inactivation was attained after 7.5 h. A 7-log decrease was obtained on average for GLA, PVC and SS, whereas a 2-log reduction was obtained for cells adhered to SIL. Concerning the effect of ciprofloxacin (Figure 2A), the same reduction in biofilm viability was observed after a 3 h contact for all materials. From this moment onwards, the viability of biofilms obtained on GLA, PVC and SS markedly decreased and complete inactivation was attained at the end of the experiment. For the silicone surface, approximately 60% of the cells were still viable after the treatment. For ampicillin (Figure 2B), the decrease on biofilm viability in the first 4.5 h was more pronounced than with

348 ciprofloxacin for GLA, PVC and SS, but complete inactivation was also attained after
349 treatment for these surfaces whereas for SIL, 36% survived after exposure.
350 In order to evaluate the contribution of EPS to the biofilm susceptibility, the exopolymeric
351 matrix of all biofilms was extracted and quantified in terms of protein and polysaccharide
352 content. According to the results presented in Table 3, the amount of total and matrix
353 polymers (both proteins and polysaccharides) did not vary significantly ($p < 0.05$) with
354 the surface used.
355 Besides their effect on viability, the antimicrobial treatments promoted a significant
356 reduction on the total biofilm cell numbers. The results obtained for GLA (Figure 3) show
357 that between 1.5 and 3 h a reduction of 55% was obtained for ampicillin and a reduction
358 of 81% was obtained for ciprofloxacin after 4.5 h. Similar reduction profiles were
359 obtained for the remaining surfaces (data not shown).
360
361 **SEM analysis of biofilm morphology**
362 The morphological changes on the biofilm formed on glass coupons upon exposure to the
363 antibiotics were analysed by SEM (Figure 4). The micrographs of adherent cells subjected
364 to 5 × biofilm MIC of ciprofloxacin and ampicillin (Figures 4B and C) confirm that the
365 antibiotic treatment reduced the total number of initially attached cells, as seen on Figure
366 3. These images show that antibiotic-treated cells had filamentous forms when compared
367 to the control cells (not subjected to the treatments, Figure 4A). Moreover, biofilms
368 exposed to ampicillin had the most elongated shape. Determination of the cell length in
369 the higher magnification images (Figures 4D-F) resulted in a bar chart showing the size
370 distribution of biofilms cells exposed and not exposed to antibiotics (Figure 5). While
371 non-exposed cells had lengths ranging from 1.1 to 2.7 μm, those exposed to ciprofloxacin
372 measured between 2 and 7.1 μm (on average 2-fold longer), whereas cell lengths up to

9.1 μm were determined for ampicillin (on average 3.6-fold longer than the non-exposed cells). Also, a much narrower size distribution was found for the non-exposed cells.

DISCUSSION

Initial adhesion and biofilm development

The first aim of this study was to assess if the initial adhesion of *E. coli* was related to the surface properties of the tested materials. The results indicated that the highest level of *E. coli* adhesion occurred when the hydrophobic SIL (ΔG_{iwi} < 0 mJ/m^2) was used as substratum, followed by PVC and SS (ΔG_{iwi} < 0 mJ/m^2) with less hydrophobic character. In contrast, the hydrophilic glass (ΔG_{iwi} > 0 mJ/m^2) was the less colonized surface. These findings suggest that the substratum hydrophobicity is a major factor in the initial adhesion of bacteria, with hydrophobic materials promoting adhesion. This increased adhesion to hydrophobic surfaces has been reported by independent groups for both Gram-negative[46-48] and Gram-positive bacteria.[49,50] Previous studies[31,51,52] have shown that bacterial adhesion can be correlated with surface hydrophobicity, but although increasing adhesion was obtained with increasing hydrophobicity, a weak correlation (r^2 = 0.78) between these two variables was found in this study.

According to the thermodynamic approach which considers the physico-chemical interactions, adhesion is favorable only when the resulting free energy is negative. Using this approach, it was concluded that adhesion is thermodynamically less favorable for glass (positive value of ΔG_{iwI}^{TOT}) and more favorable for silicone (negative value of ΔG_{iwI}^{TOT}). The adhesion tests of 30 min for GLA and SIL were in agreement with the thermodynamic approach since cells adhere to a lesser and a greater extent to GLA and SIL, respectively. Silicone surface is considerably prone to colonization by *E. coli*,[53-55] despite it is widespread use in biomedical devices.

After establishing an association between surface hydrophobicity and initial adhesion, the second objective of this work was to evaluate if the buildup of biofilm was correlated with initial adhesion. This would establish a link between surface properties and biofilm development. It has been shown that in some clinical situations, like the development of *E. coli* biofilms in urinary catheters, biofilms are completely mature after 24 h.[13] In the present work, a direct relationship was found between the amount of mature biofilm formed and the extent of the initial adhesion, as previously reported by Busscher & van Der Mei[56] for flow conditions and Simões LC et al.[1] for static conditions.

Unlike adhesion and biofilm maturation, the physico-chemical properties of materials appear to have negligible impact on *E. coli* planktonic growth.

Biofilm susceptibility

After establishing a link between the surface properties and biofilm formation, a third goal of this work was to assess if biofilms formed in different materials had different susceptibilities to antibiotic treatment. The results showed that biofilms growing in silicone were less susceptible when compared to those obtained on the other materials. It is widely known that the extracellular matrix is extremely important for protection against antibiotic effects[10,41] and therefore the exopolymeric matrix of all biofilms was quantified in order to see if significant differences could be found among the biofilms formed in different materials. The results showed that the EPS composition was not a major factor affecting biofilm sensitivity to the antibiotics. We speculate that the biofilms developed on silicone were less susceptible due to their higher cell density (number of cells per unit area). The spatial arrangement of a higher number of cells may create concentration gradients (of nutrients, antibiotic and oxygen) within the structure of the biofilms, a phenomenon described by Stewart & Costerton,[57] contributing to the decreased biofilm

susceptibility to antibiotics.[58,59] It has already been reported that the relative efficacy of some antimicrobial agents declines with the density of cells exposed.[10,60-62] In the work of Mah & O'Toole,[10] penetration of hydrogen peroxide was more difficult in thicker biofilms grown on glass slides (average cell density of $4x10^7$ cells/cm^2) than in a thin biofilm-covered bead (average cell density of $3x10^3$ cells/cm^2). Hence, it can be concluded that the surface properties affected the antibiotic susceptibility of biofilms[63-66] by influencing the amount of cells attached to the substratum after 24 h. Gristina et al.[63] were the first authors to suggest that the degree of colonization and antibiotic resistance are related to the biomaterial and may be altered by biomaterial-induced phenotypic changes rather than by a barrier effect of exopolysaccharides. Similarly, Webb et al.[64] found that the surface-adherent mode of bacterial growth determines the antibiotic resistance of biofilms. On the other hand, Arciola et al.[65] concluded that some materials can lead to the selection of variant adhesive bacteria with increased antibiotic resistance among the whole contaminant bacterial population.

Lastly, a filamentous morphology of biofilm cells exposed to antibiotics was visualized by SEM. It is well documented that antibiotics can affect bacteria in ways other than the expected bactericidal or bacteriostatic action, in particular they can induce morphological changes.[67-70] A common response of Gram-negative bacilli to the effects of β-lactam antibiotics is an abnormal elongation of the individual cells, with subsequent formation of long filamentous forms.[68] This type of aberrant morphological change is the outcome of the selective binding of β-lactams to cellular surface protein components responsible for cell wall septum formation and separation of two divided organisms.[67] *E. coli* cell filamentation as a result of exposure to ciprofloxacin was also observed by some investigators[71-73] and it was associated with the induction of SOS response.[69] In the present study, it was demonstrated for the first time that biofilm cells exposed to

ciprofloxacin had smaller sizes when compared with those in contact with ampicillin (belonging to a different class of antibiotics).

This work revealed that the surface properties of a given material can influence the initial adhesion of bacterial cells which in turn may affect the development of mature biofilms and consequently the efficiency of antibiotic treatment. Thus, these results suggest that modification of the surface properties (such as hydrophobicity) of materials that are used for the construction of biomedical devices may be used as a strategy to increase the efficacy of antimicrobial therapy.

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Table and Figure captions

Table I. Contact angles with water (θ_w), formamide (θ_F) and α-bromonaphthalene (θ_B), surface tension parameters and free energy of interaction (ΔG_{iwi}) between two entities of a given material (i) (surface or bacteria) when immersed in water (w). Values are means ± SDs of three independent experiments

Table II. Free energy of adhesion (ΔG_{iwI}^{TOT}) between *E. coli* and the different surfaces when immersed in water (w)

Table III. Characteristics of the *E. coli* biofilm formed on different materials after 24 hours of growth

Figure 1. Planktonic growth curves (A) and number of adhered cells (B) of *E. coli* on different materials along time: GLA (···●··· and ▬), SS (···○··· and ▬), PVC (···▽··· and ▬) and SIL (···△··· and ▬). Initial adhesion corresponds to the time point of 0.5 h, while 4, 8, 12, 16, 20 and 24 h refer to biofilm development. In panel B, for each time point, letters were assigned in alphabetic order from the lowest to the highest value (from a to d). These assignments were made as long as statistically significant differences exist between materials (for a confidence level greater than 95%, $p < 0.05$). The means ± SDs for three independent experiments are illustrated.

Figure 2. Time-course of cell viability for 24-hour biofilms formed on different materials after exposure to 5 × biofilm MIC of ciprofloxacin (A) and ampicillin (B). GLA (···●···), SS (···○···), PVC (···▽···) and SIL (···△···). Results are presented as a percentage of initial viability determined by Live/Dead staining. Statistical analysis for a confidence level greater than 95% ($p < 0.05$) are pointed as: a – glass is different from the other materials, b – silicone is different from the other materials. The means ± SDs for three independent experiments are presented.

694 Figure 3. Evolution of glass attached cells from 24-hour biofilms after exposure to 5 ×
695 biofilm MIC of ciprofloxacin (–●–) and ampicillin (–○–). Results are expressed as a
696 percentage of remaining attached cells determined by Live/Dead staining considering the
697 sum of viable and non-viable cells. The means ± SDs for three independent experiments
698 are illustrated.
699 Figure 4. Scanning electron micrographs of 24-hour biofilms formed on glass surfaces:
700 (A) and (D) - not exposed to antibiotics; (B) and (E) - after 6 h of exposure to 5 × biofilm
701 MIC of ciprofloxacin; (C) and (F) - after 6 h of exposure to 5 × biofilm MIC of ampicillin.
702 Micrographs (D), (E) and (F) are high-magnification images (magnification: 5000×; bars
703 = 20 μm) of (A), (B) and (C) (magnification: 1000×; bars = 100 μm), respectively.
704 Figure 5. Cell length distribution of 24-hour biofilms formed on glass surfaces: (■) - not
705 exposed to antibiotics; (■) - after 6 h of exposure to 5 × biofilm MIC of ciprofloxacin; (
706 ■) - after 6 h of exposure to 5 × biofilm MIC of ampicillin. The arrows represent the
707 average cell length determined from SEM micrographs for each experimental condition.
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709 **TABLE I. Contact angles with water (θ_w), formamide (θ_F) and α-**
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712 **immersed in water (w). Values are means ± SDs of three independent experiments**
713

	Contact angle (°)			Surface tension parameters (mJ/m^2)			Hydrophobicity (mJ/m^2)
	θ_w	θ_F	θ_B	γ^{LW}	γ^+	γ^-	(ΔG_{iwi})
Surface							
SIL	115.4 ± 0.4	105.9 ± 0.3	78.4 ± 0.4	16.0	0.0	1.8	-75.0
SS	67.0 ± 1.7	60.4 ± 0.4	39.3 ± 0.5	34.9	0.0	21.7	-10.9
PVC	79.3 ± 0.9	79.4 ± 0.6	40.3 ± 0.5	34.4	0.0	20.9	-12.3
GLA	47.0 ± 0.4	49.1 ± 0.5	63.4 ± 0.9	23.2	1.7	40.2	19.3
Bacteria							
E. coli	28.3 ± 0.3	38.4 ± 0.4	47.0 ± 0.4	35.3	0.1	59.0	46.0

714
715

716 **TABLE II. Free energy of adhesion (ΔG_{iwI}^{TOT}) between *E. coli* and the different**

717 **surfaces when immersed in water (w)**

718

	ΔG_{iwI}^{TOT} (mJ/m^2)			
	SIL	SS	PVC	GLA
E. coli	-5.8	20.5	19.8	32.1

719

720

721 **TABLE III. Characteristics of the *E. coli* biofilm formed on different materials after**

722 **24 hours of growth**

723

Biofilm characteristics	Material			
	GLA	SS	PVC	SIL
Cellular density (cells/cm^2)	$9.29x10^7 \pm 1.07$	$1.42x10^8 \pm 1.15$	$2.02x10^8 \pm 1.15$	$2.71x10^8 \pm 1.18$
Total proteins ($mg/g_{biofilm}$)	74.1 ± 11.0	75.2 ± 2.43	107.6 ± 18.3	97.1 ± 23.3
Matrix proteins ($mg/g_{biofilm}$)	64.7 ± 8.21	58.2 ± 4.86	81.1 ± 5.64	78.7 ± 18.3
Total polysaccharides ($mg/g_{biofilm}$)	36.1 ± 14.1	42.3 ± 17.2	58.2 ± 11.6	56.5 ± 13.3
Matrix polysaccharides ($mg/g_{biofilm}$)	27.3 ± 10.1	29.4 ± 4.78	45.3 ± 11.9	37.8 ± 13.5

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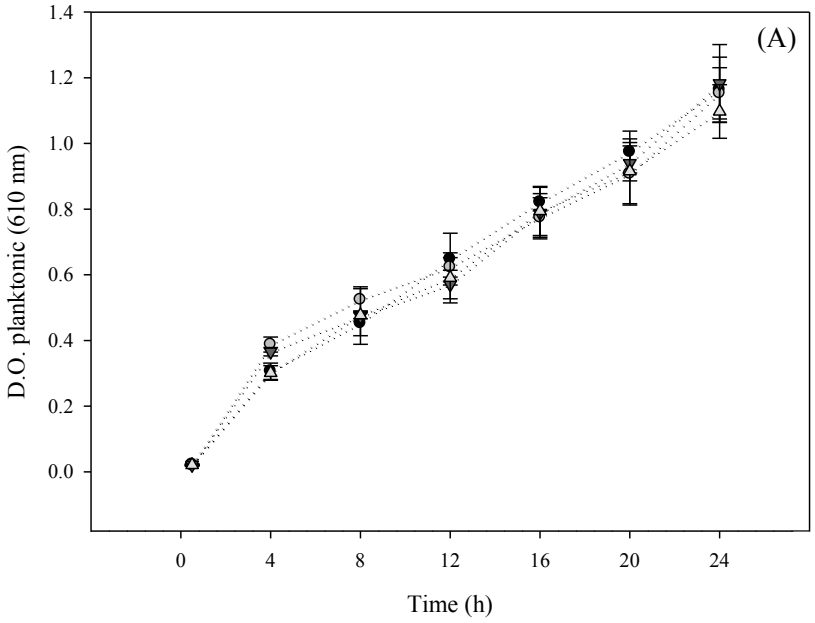

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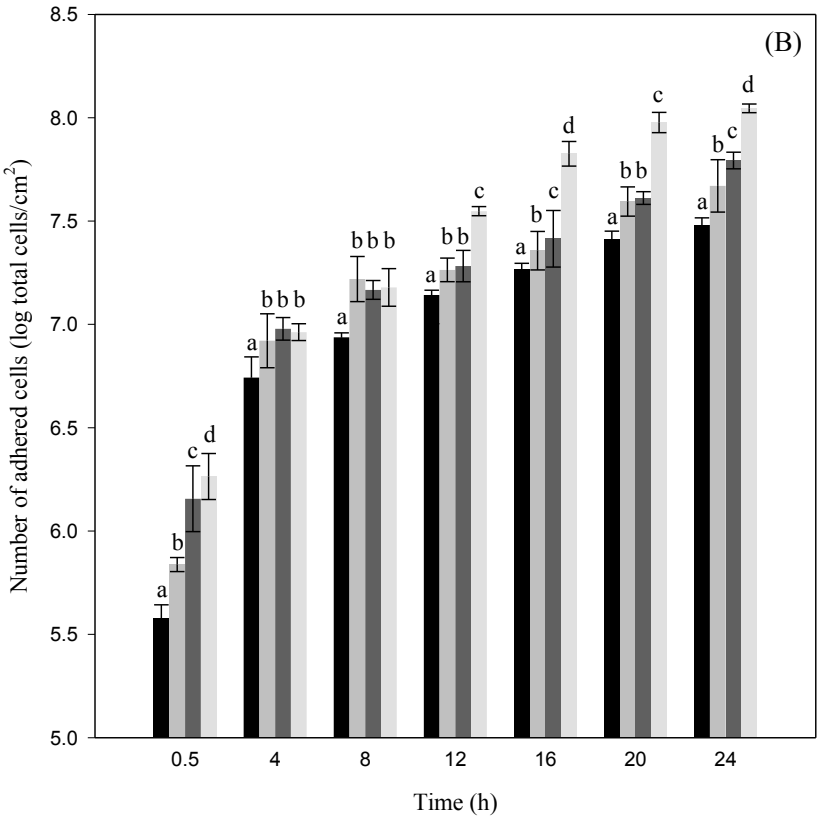

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729 **FIGURE 1.** Planktonic growth curves (A) and number of adhered cells (B) of *E. coli* on

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732 while 4, 8, 12, 16, 20 and 24 h refer to biofilm development. In panel b, for each time
733 point, letters were assigned in alphabetic order from the lowest to the highest value (from
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735 between materials (for a confidence level greater than 95%, $p < 0.05$). The means ± SDs
736 for three independent experiments are illustrated.
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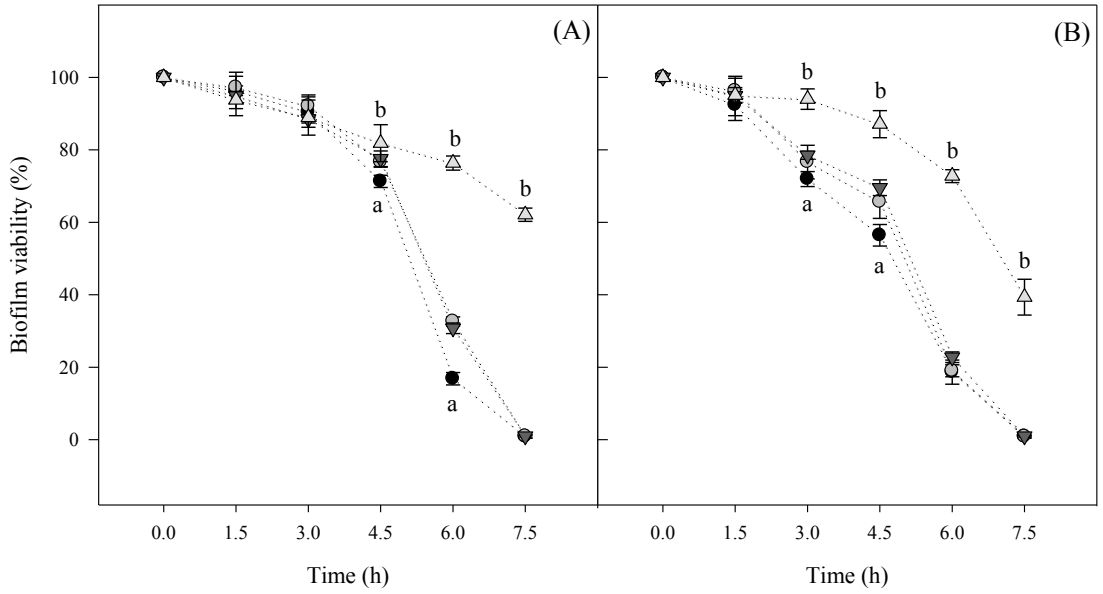

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740 **FIGURE 2.** Time-course of cell viability for 24-hour biofilms formed on different
741 materials after exposure to 5 × biofilm MIC of ciprofloxacin (A) and ampicillin (B). GLA
742 (···●···), SS (···○···), PVC (···▼···) and SIL (···△···). Results are presented as a percentage of
743 initial viability determined by Live/Dead staining. Statistical analysis for a confidence
744 level greater than 95% ($p < 0.05$) are pointed as: a – glass is different from the other
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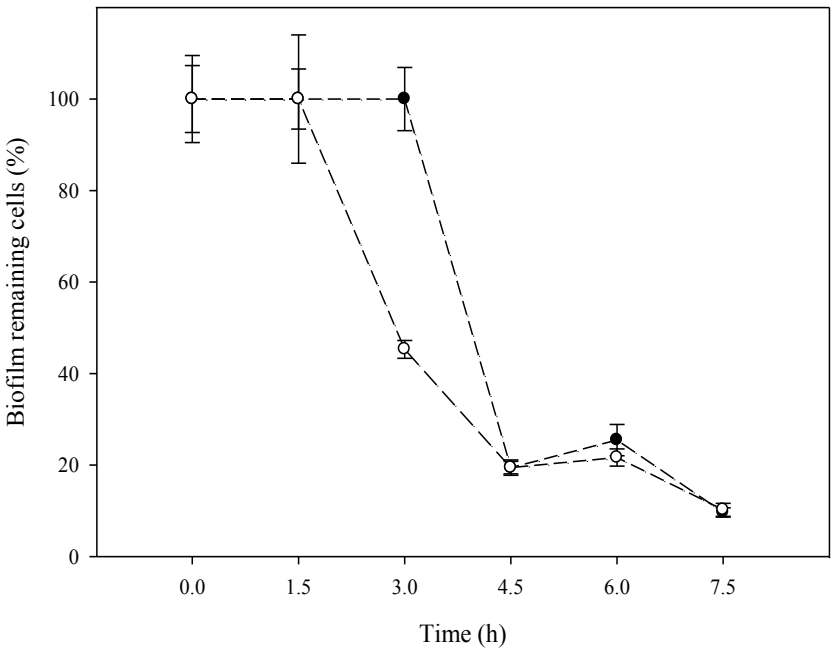

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750 **FIGURE 3.** Evolution of glass attached cells from 24-hour biofilms after exposure to 5
751 × biofilm MIC of ciprofloxacin (–●–) and ampicillin (–○–). Results are expressed as a
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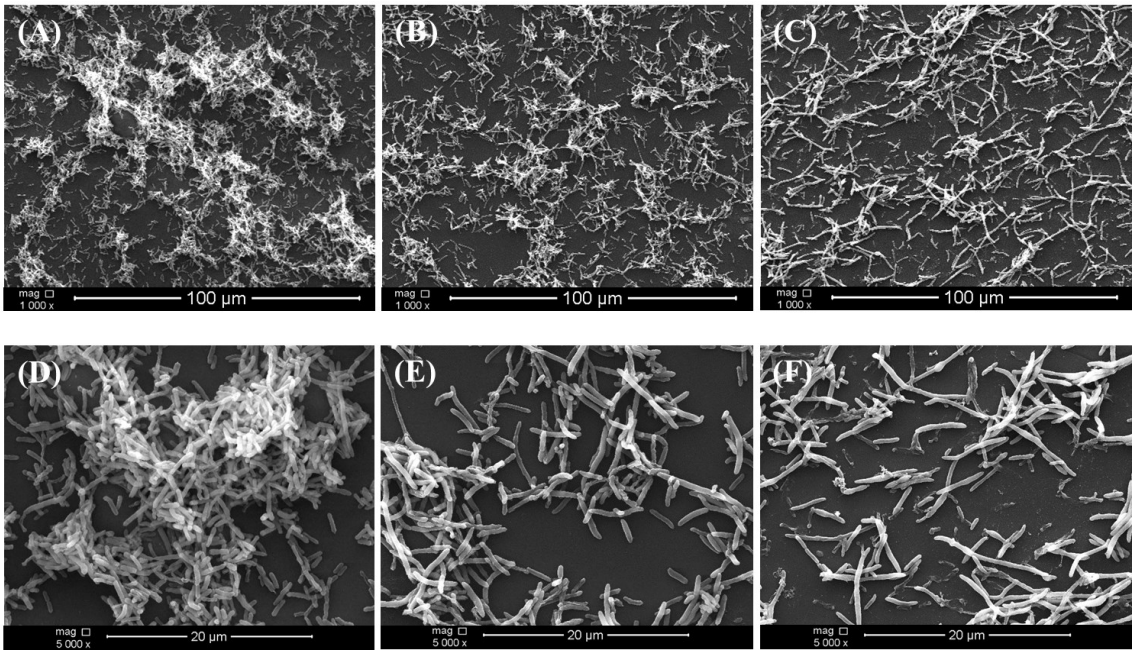
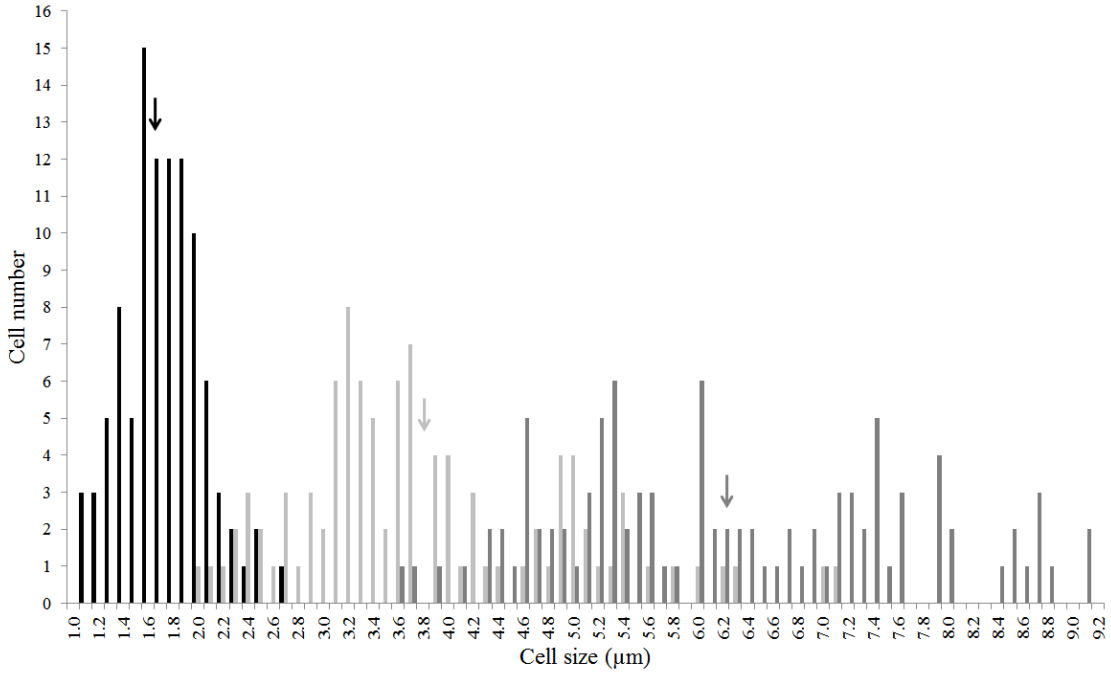


FIGURE 4. Scanning electron micrographs of 24-hour biofilms formed on glass surfaces: (A) and (D) - not exposed to antibiotics; (B) and (E) - after 6 h of exposure to 5 × biofilm MIC of ciprofloxacin; (C) and (F) - after 6 h of exposure to 5 × biofilm MIC of ampicillin. Micrographs (D), (E) and (F) are high-magnification images (magnification: 5000×; bars = 20 µm) of (A), (B) and (C) (magnification: 1000×; bars = 100 µm), respectively.



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765

766 **FIGURE 5.** Cell length distribution of 24-hour biofilms formed on glass surfaces: (■) -

767 not exposed to antibiotics; (■) - after 6 h of exposure to 5 × biofilm MIC of ciprofloxacin;

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