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Research Paper

The role of extracellular polymers on *Staphylococcus epidermidis* biofilm biomass and metabolic activity

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Staphylococcus epidermidis is now well established as a major nosocomial pathogen, associated with indwelling medical devices. Its major virulence factor is related with the ability to adhere to indwelling medical devices and form biofilms. In this study, the biofilm matrix of four *S. epidermidis* clinical isolates was extracted and the polysaccharides and proteins content was quantified. The results were correlated with the total biofilm biomass (determined by crystal violet assay) and cellular metabolic activity (evaluated with XTT reduction assay). According to the results, the exopolymers studied play an important role not only on structure and biofilm biomass but also on cellular activity. Thus, the strain forming biofilms with the highest level of polysaccharides (*S. epidermidis* 1457) also formed thicker biofilms but with the lowest metabolic activity. The protein concentration also varied among strains, with the biofilm matrix of *S. epidermidis* 9142 presenting a higher concentration of proteins comparing to the remaining strains. This fact indicates the different levels of importance that matrix proteins can hold on biofilm composition among strains albeit overall, it is suggested that extracellular protein production it is not a determinative factor for biofilm total biomass.

Keywords: Biofilm / Extracellular matrix / Metabolic activity / Polysaccharides / Proteins

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Introduction

Staphylococcus epidermidis is a coagulase-negative *Staphylococcus* (CNS) that has emerged in the last years as one of the most important nosocomial and opportunistic pathogens. It is most commonly associated with infections originating from indwelling medical devices such as catheters and prostheses [1]. This is directly related with its capacity to adhere and form thick and multilayered biofilms on abiotic surfaces, constituted of colonizing bacteria and a self-excreted amorphous exopolymeric matrix in which they are embedded [2]. Generally, the *S. epidermidis* biofilm matrix comprises several extracellular polymeric substances (EPS) such as polysaccharides [3, 4], proteins [5–7], considerable amounts of extracellular teichoic acids [4, 8] and also extracellular DNA [9]. All these compounds seem to play an important role in the biofilm formation process and maintenance. The polysaccharide intercellular adhesin (PIA), a polymer of *N*-acetyl glucosamine has been described as crucial for the process of cell-to-cell adhesion and biofilm accumulation [10] and as an essential component of the extracellular matrix [11]. During the process of proliferation and accumulation as multilayered cell clusters, *S. epidermidis* also secretes a few exoenzymes, aiming to help in the invasiveness of host tissues and host defenses [12]. EPS provide mechanical stability to such structures, forming a three-dimensional, gel-like, highly hydrated and negatively charged environment in which the cells are immobilized [13, 14]. The extracellular matrix is extremely important for intercellular connection during surface colonization [15] and protection against the host immune system and resistance to antibiotics [16]. Besides, the matrix-enclosed microcolonies are separated by water channels that provide a nutrient flow system within the biofilm [17]. Moreover, depending on the

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environment in which the biofilm has developed, the biofilm matrix composition can vary greatly [18].

The present study aims to evaluate the role of two extracellular polymers, polysaccharides and proteins, on biofilm biomass and metabolic activity of clinical isolates of *S. epidermidis*.

Materials and methods

Bacterial strains and culture conditions

Four clinical isolates of *S. epidermidis* were used in this work: strain 9142, a known producer of the surface PIA [19]; strain 1457 (isolated from an infected central venous catheter) and strains IE214 and IE186 (both isolated from blood of patients with infective endocarditis). All strains were kindly provided by Dr. G. B. Pier, Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston.

All strains were grown for 18 ± 2 h, at 37 °C and 120 rpm in 30 ml of Tryptic Soy Broth (TSB; Merck, Darmstadt, Germany). Then, the cells were centrifuged (10,500 × g, 5 min, 4 °C), washed twice with a saline solution [0.9% NaCl (Merck) in distilled water] and sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) (22% amplitude, 10 s). The cellular suspensions were adjusted to a final concentration of approximately 1×10^9 cells ml^{-1}, determined by optical density at 640 nm, prior to be used in biofilm assays.

Biofilm assays

Poly(methylmethacrylate) (PMMA) (Repsol, Brønderslen, Denmark), i.e., acrylic, was cut into 2 cm × 2 cm squares and used as substratum. Prior to use, the coupons were aseptically and individually washed with ultra-pure sterile water and a 70% ethanol solution, and left to dry overnight at 60 °C. Afterwards, each square was placed into a well of a 6-well tissue culture plate containing 4 ml of TSB, enriched with 0.25% of glucose (Merck). For every strain, an inoculum of 50 μl with 1×10^9 cells ml^{-1} was added per well. The plates were incubated for 8 d at 37 °C on an orbital shaker (120 rpm). The medium was replaced by fresh TSB + 0.25% glucose every 12 h (fed-batch mode).

All experiments were done in triplicate, in three independent occasions.

Crystal violet assay

The total biomass attached to the acrylic coupons was measured by methanol (Merck) fixation, crystal violet (Merck) staining and acid acetic (Merck) elution as previously described [20]. The eluted dye was removed from each well and placed in a 96-well microtiter plate and its absorbance determined by an ELISA reader (Bio-Tek Instruments Inc., Vermont, USA) at 570 nm.

Scanning Electron Microscopy (SEM)

The coupons with the biofilms were dehydrated by a 15 min immersion in increasing ethanol concentration solutions: 10, 25, 40, 50, 60, 70, 80, 90 and 100% (v/v), having then been placed in a sealed desiccator. Samples were then mounted on aluminium stubs with carbon tape, sputter coated with gold and observed with a Leica Cambridge S-360 scanning electron microscope (Leo, Cambridge, UK). In order to assess the extent of bacterial adhesion in each sample, three fields were used for image analysis. All photographs were taken using a magnification of ×500.

XTT reduction assay

The quantification of biofilm cellular activity was assessed through the XTT reduction assay as previously described [21], with some modifications. Accordingly, the coupons with biofilm were transferred to a new microtiter plate with each well containing 1 ml of XTT (200 mg l^{-1}) solution (Sigma, USA) plus PMS (20 mg l^{-1}) (Sigma). The microtiter plates were incubated under agitation (120 rpm) for 3 h at 37 °C, in the dark. Following that, each solution was centrifuged for 5 min at 9500 × *g* and the absorbance read at 490 nm.

Biofilm matrix extraction

The extraction of the biofilm extracellular matter was performed using the cation exchange Dowex resin (50 × 8, Na^+ form, 20–50 mesh Aldrich-Fluka 44445), according to the procedure described by Frølund *et al.* [22]. Prior to extraction, the Dowex resin was washed with the extraction buffer [2 mM Na_3PO_4 (Merck); 4 mM NaH_2PO_4 (Merck); 9 mM NaCl and 1 mM KCl (Merck); pH 7.0]. Then, the biofilms previously scrapped off the acrylic coupons were washed with phosphate buffer (0.01 M; pH 7.0) and centrifuged for 5 min, at 9000 × *g*. The extraction was performed using 2 g of washed Dowex resin and 10 ml of extraction buffer per g of biofilm and stirring for 2 h at 400 rpm and –4 °C. The extracellular polymers (supernatant) were obtained by centrifugation at 9000 × *g* for 20 min.

Proteins and polysaccharides quantification

The total protein content extracted from the matrix was determined by the colorimetric bicinchoninic acid (BCA) assay [23] (Bicinchoninic Acid Kit for Protein Determination, Sigma, USA) using bovine serum albumin

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(BSA) as standard. The extracted polysaccharides were quantified by the phenol-sulphuric acid method of Dubois *et al.* [24], using glucose as standard.

Biofilm dry-weight determination

Biofilms dry-weight (dw) was assessed as described by An and Friedman [25], with some modifications. Briefly, the coupons were removed from the tissue culture plate wells and dried for 24 h, at 80 °C. Next, the coupons were weighed and placed again at 80 °C for 2 h and, in order to verify the dry weight stability, weighed once more. Afterwards, the biofilms were scraped off the squares, which were cleaned with 70% of ethanol solution, left overnight at 80 °C and then weighed. Biofilm dry-weights were assessed by the difference between the weight of the coupon with and without the biomass attached.

Statistical analysis

The results from all the assays were compared by the one-way analysis of variance by applying the Levene's test of homogeneity of variances and the Tukey multiple comparisons test, using SPSS (Statistical Package for the Social Sciences Inc., Chicago). All tests were performed with a confidence level of 95%.

Results

Fig. 1 presents the amount of biofilm formed on acrylic coupons, by the 4 strains studied, determined by the crystal violet assay. It can be observed that strain 1457 was the highest biofilm producer immediately followed by 9142, then IE214 and, at a lower extent, by strain IE186 ($p < 0.05$). However, overall, all strains can be considered as being good biofilm producers. Scanning electron microscope (SEM) images of the biofilms are presented in Fig. 2. The images reveal the thickness of biofilms, particularly high for strains 9142 and 1457. Biofilms of strains IE186 and IE214 seem to be thinner having fewer bacterial layers.

The results of the XTT reduction assay, indicative of the metabolic activity of cells within the biofilm, are presented in Fig. 3. Accordingly, strain IE186 showed the highest cellular metabolic activity ($p < 0.05$), followed by strains 9142, IE214, and at last 1457 ($p < 0.05$).

Afterwards, the exopolymeric matrix of all biofilms was extracted and quantified in terms of polysaccharides and proteins content, in order to evaluate the contribution of these extracellular polymers to the total biofilm biomass and biofilm metabolic activity. According to the results presented in Fig. 4, the amount of quantified polymers of the biofilm matrix varies significantly ($p < 0.05$) with the strain. Concerning polysaccharides composition, strain 1457 was the one that produced larger amounts of these molecules, immediately followed by strain IE214, and differing significantly from strains 9142 and IE186 ($p < 0.05$). Regarding the presence of proteins in the extracellular matrix, *S. epidermidis* 9142 was the strain with the highest content ($p < 0.05$), followed by strain IE214 and strains IE186 and 1457, these two with very similar values. This shows that, depending on the strain, proteins might constitute an important part of the biofilm matrix. It should be noted that polysaccharides and proteins were determined against different standards. Consequently, the concentrations determined are not absolute values. Thus, comparing in relative terms, the ratio of polysaccharides/proteins (Table 1) determined for the biofilm matrix of strain 1457 was 4.3 times greater compared to the same ratio in strain 9142 and approximately 2 times greater than in strains IE214 and IE186.

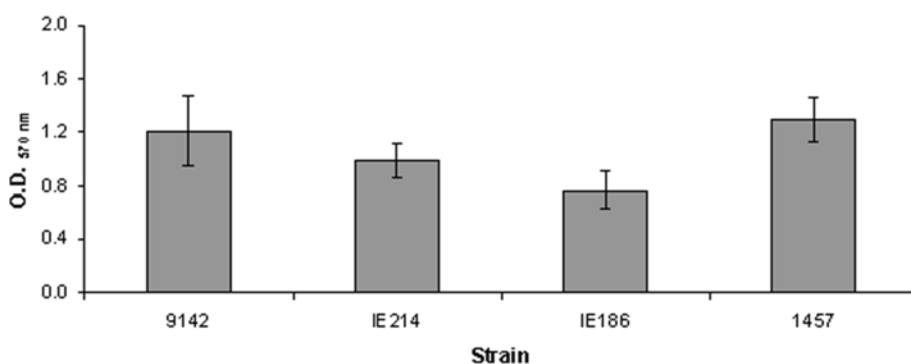

Figure 1. Biofilm biomass, expressed as crystal violet optical density (O.D.$_{570nm}$), of the four *S. epidermidis* strains studied (9142, IE214, IE186 and 1457), established over an 8 d-period.

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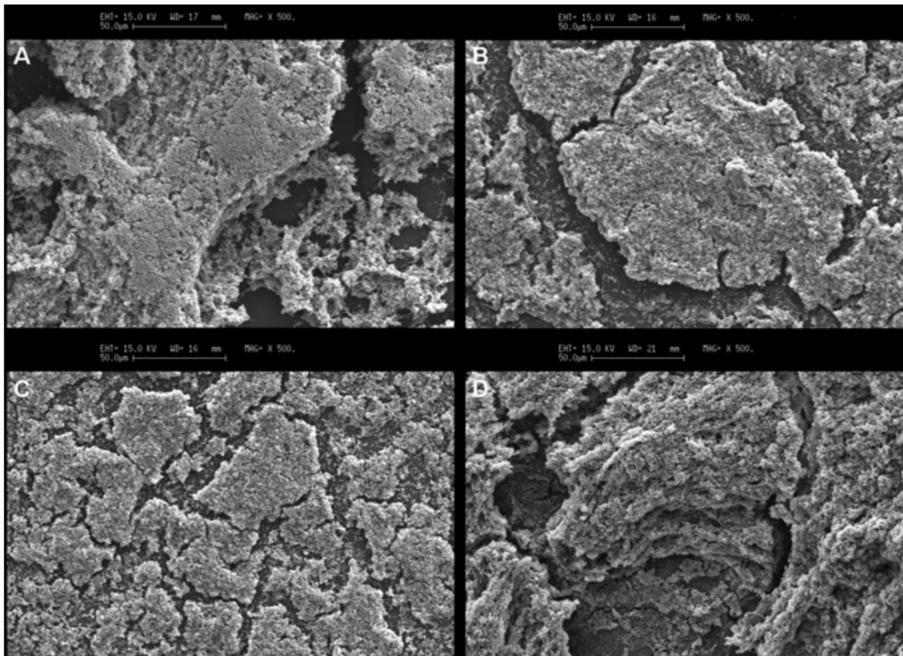

Figure 2. SEM photomicrographs of a general view of the mature biofilms (8 days old) of *S. epidermidis* 9142 (A), IE214 (B), IE186 (C) and 1457 (D) formed on acrylic coupons. Magnification ×500, bar = 50 µm.

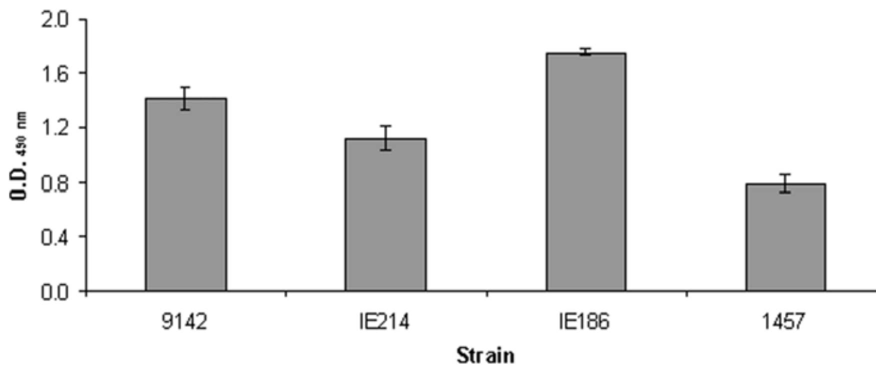

Figure 3. Cellular activity, measured by XTT reduction assay (O.D.$_{490nm}$) from biofilms of the four *S. epidermidis* clinical isolates studied: 9142, IE214, IE186 and 1457.

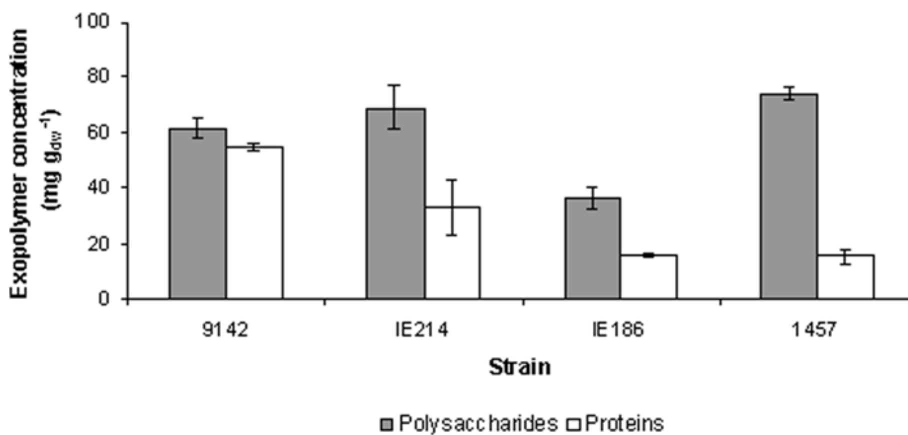

Figure 4. Concentration of polysaccharides (glucose as standard) and proteins (BSA as standard) extracted (mg $g_{dw^{-1}}$) by Dowex resin method from biofilms of *S. epidermidis* strains 9142, IE214, IE186 and 1457.

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Table 1. Ratio of polysaccharides/proteins (mg mg^{-1}) obtained in the biofilm matrix of the *S. epidermidis* strains studied.

Strain	Polysaccharides/Proteins (mg mg^{-1})
9142	1.1
IE214	2.1
IE186	2.4
1457	4.9

Discussion

In this work, the biofilm formation capability of the four strains assayed was evaluated through crystal violet staining, which allows the quantification of the total biomass attached to the acrylic coupons. The metabolic activity of biofilms was as well evaluated through XTT reduction assays. Then, polysaccharides and proteins of the biofilm extracellular matrix were quantified in order to evaluate their effect in biofilms total biomass and metabolic activity. Comparing the results obtained, in terms of biofilm biomass (Fig. 1) and biofilm activity (Fig. 2), it seems that even a small increase in biofilm biomass can lead to a decrease in the metabolic activity of cells. This is probably due to the fact that most of the total biofilm biomass of the strongest biofilm producing strains is composed of higher proportions of EPS (Fig. 4). As a result, the metabolically active cells within the biofilm become more diluted, their concentration diminishes, and the resultant colorimetric signal of the XTT reduction assay is weaker, thus justifying the lower levels of metabolic activity. Therefore, XTT reduction assay can not be seen as a method to quantify biofilm formation, as previously suggested [26] since it measures metabolic activity, which relies on the localization of the cell inside the biofilm. This assay should be used as a complementary method to the crystal violet assay in order to allow the most accurate possible characterization of biofilms.

The method chosen for EPS extraction can strongly influence the yield and chemical composition of extracellular polymers, and also can lead to different degrees of cellular lysis and consequent contamination of exopolymers with intracellular components [22, 27]. Several methods have been reported for extracting exopolymers from activated sludge flocs, some of them also assayed in biofilms [28]. These include high-speed centrifugation, steaming, ultrasonication and the use of chemical agents such as: NaOH or EDTA [29], Tris/HCl buffer [30] phosphate buffer and heat [31], formaldehyde [32] cation exchange Dowex resin [22] and glutaraldehyde [33]. In the present work, Dowex extraction was the method chosen due to the fact that is a very smooth method, with no significant cell lysis for up 2 h of extraction [28, 34]. In fact, in the work of Azeredo *et al.* [35] very small amounts of ATP, used as an indicator of cell lysis, were measured even after 4 h of extraction. Thus, concerning biofilm matrix composition, in terms of polysaccharides and proteins content, extracted by the Dowex resin method, the results obtained revealed different polysaccharides/proteins ratios among the different extracellular matrices studied (Fig. 4; Table 1). Therefore, it seems clear that the amount of these extracellular polymers vary significantly according to the strain [36]. It must be noted that *S. epidermidis* 1457 was the strain that produced the larger amount of biofilm and strain IE186 was the lowest biofilm producer (Fig. 1), and these were also the highest and the lowest polysaccharides producers, respectively. This suggests a certain degree of correlation between exopolysaccharides production and total amount of biomass formed. These results are in accordance with the work of Arslan and Özkardeş [37], who state that the production of an extracellular matrix among a majority of clinical CNS isolates is associated with the strains ability to produce thicker biofilms, thus suggesting a role of slime in pathogenesis. Comparing the results obtained, in terms of exopolysaccharides production (Fig. 4), and biofilm activity, determined by XTT reduction assays (Fig. 3), it seems clear that a strong production of exopolysaccharides can lead to a decrease in the metabolic activity of cells. It has been suggested that the biofilm matrix itself could constitute a barrier to the penetration and diffusion of the nutrients, oxygen and even antibiotics [38–41]. However, other studies suggest that diffusion of nutrients and oxygen through the biofilm matrix is almost equivalent to water [42]. This makes sense taking into account that water is considered to be the major component of the biofilm matrix – up to 97% [43]. Thus, the present results obtained are most probably due to the fact that in the biofilms with larger quantities of EPS the amount of metabolically active cells is less concentrated, leading to lower levels of total biofilm activity, measured by the XTT reduction assay. Besides, biofilms exhibit considerable structural, chemical and biological heterogeneity, with concentration gradients of nutrients, oxygen and signalling compounds. As a result, cells within a biofilm are in a wide range of physiological states [44]. This can be explained by the fact that nutrients and oxygen, e.g., are consumed by cells in the upper layers of the biofilm, leading to a decrease in their concentration with increasing depth into the biofilm and distance from the nutrient source [44]. Thus, on thicker biofilms, the concentration of

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nutrients that will reach the basal cell layers will be minimal, due to their previous consumption by cells in the outer layers, while in thinner biofilms, higher amounts of nutrients will reach these deeper cell layers. Consequently, the metabolic activity of cells in different locations within the biofilm will be necessary different, which will ultimately result in different total metabolic rates between biofilms with different thickness. This is corroborated by the present results that showed that *S. epidermidis* 1457 and IE214, both with substantial exopolymeric matrices, were the strains that revealed the lowest levels of metabolic activity. Also, in a previous work [45], the glucose uptake determined for an 8-d biofilm of strain IE214 was lower compared to the glucose uptake of *S. epidermidis* 9142 and *S. epidermidis* IE186 biofilms. Therefore, the higher amounts of polysaccharides detected in the extracellular matrix of strain IE214 may be responsible by the low concentrations of nutrients, such as glucose, that reach bacteria within the biofilm, reducing their uptake and, as a consequence, decreasing the levels of metabolic activity, expressed by the XTT results of the present work.

The exopolysaccharide PIA has been described as one of the main molecules responsible for *S. epidermidis* biofilm formation [10, 46]. In a previous work [19], hemagglutination data, that reflect the level of expression of PIA, was presented and according to it, a considerable portion of the polysaccharides of the *S. epidermidis* IE214 biofilm matrix herein determined seems to correspond to PIA (hemagglutination titer of 1:16). Therefore, in an opposite way, the biofilm matrix of 9142 (hemagglutination titer of 1:2) appears to be comprised of larger amounts of other polysaccharides besides PIA.

In what concerns to proteins produced in the biofilm extracellular matrix, it seems that, depending on the strain, proteins might constitute an important part of the biofilm matrix, which is the case of *S. epidermidis* 9142. However, no direct relation was found between the proteins concentration determined and the amount of biofilm formed (Fig. 1). The proteins detected in the biofilm matrix are probably some cell wall associated proteins released to the extracellular matrix but also secreted enzymes with proteolytic activity whose function is to help in the invasiveness into host tissues [47].

The importance of the extracellular matrix to the biofilm recalcitrance is well known [48–50]. According to some studies [38–40] the biofilm matrix can act as a barrier to the penetration of antibiotics. In the work of Cerca *et al.* [51], the inhibition of biofilm formation of 3 *S. epidermidis* strains on acrylic, in the presence of sub-MICs of 3 antibiotics and 3 combinations of antibiotics, was tested. In 4 of the 6 situations studied, strain 9142 was more resistant to the antibiotics effect than strain IE186. Thus, the results of the present work, that showed the higher proportion of EPS of *S. epidermidis* 9142, compared to strain IE186, might be related to its resistance to antibiotics. *S. epidermidis* 9142 biofilm cells are immersed in a thicker matrix which provides the bacterial population protection from the host defense mechanisms and antimicrobial agents. Matrix polymers seem, thus, to largely contribute towards biofilm defense, a fact that, according to the present results, appears to be partially related with the amount of exopolymers that each strain has the ability to produce. However, the failure of antimicrobial agents to penetrate the biofilm matrix can not be seen as the sole reason of biofilms recalcitrance [48]. A number of studies have demonstrated that reductions in the diffusion coefficients of antibiotics within biofilms are insufficient to account solely for the observed changes in susceptibility [48, 52] given that antimicrobial agent access is supported, such as in the case of nutrients and oxygen, by the presence of water channels in the biofilm structure. Thus, the different susceptibilities of *S. epidermidis* 9142 and IE186 to antibiotics can probably be due to factors that are intrinsic to each individual strain. Depending on the chemical nature of both the antimicrobial agent and the matrix material, phenomena of drug adsorption or neutralization [48] can be favoured in 9142 biofilms. The expression of surface antigens [53] as well as the possible existence of certain percentage of persister cells, which are extremely tolerant to antibiotics [54], can also help to explain the lower susceptibility to antibiotics by this strain. A better capacity of horizontal transfer of resistance genes and recombination potential between the biofilm embedded bacteria has, as well, been referred as one of the reasons of the success of some *S. epidermidis* strains as pathogens in hospitals [55]. Besides, *S. epidermidis* 9142 showed lower levels of metabolic activity, a fact that has also been pointed as related to biofilm antibiotics resistance [56].

Concluding remarks

This work provided a reliable approach to lead to a better understanding of exopolymers influence in what concerns to biofilm total biomass and cells metabolism. Overall, the results herein presented suggest that the production of extracellular polymers plays a role in biofilm total biomass as well as in cellular metabolic activity and is strain dependent. The strain producing the highest amount of polysaccharides (*S. epidermidis*

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1457) formed a biofilm with the highest quantity of total biomass and the lowest level of metabolic activity. No relation was found between extracellular proteins production and biofilm total biomass and metabolic activity.

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