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ORIGINAL PAPER

Role of planktonic and sessile extracellular metabolic byproducts on *Pseudomonas aeruginosa* and *Escherichia coli* intra and interspecies relationships

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Abstract Bacterial species are found primarily as residents of complex surface-associated communities, known as biofilms. Although these structures prevail in nature, bacteria still exist in planktonic lifestyle and differ from those in morphology, physiology, and metabolism. This study aimed to investigate the influence of physiological states of *Pseudomonas aeruginosa* and *Escherichia coli* in cell-to-cell interactions. Filtered supernatants obtained under planktonic and biofilm cultures of each single species were supplemented with tryptic soy broth (TSB) and used as the growth media (conditioned media) to planktonic and sessile growth of both single- and two-species cultures. Planktonic bacterial growth was examined through OD_{640} measurement. One-day-old biofilms were evaluated in terms of biofilm biomass (CV), respiratory activity (XTT), and CFU number. Conditioned media obtained either in biofilm or in planktonic mode of life triggered a synergistic effect on planktonic growth, mainly for *E. coli* single cultures growing in *P. aeruginosa* supernatants. Biofilms grown in the presence of *P. aeruginosa* biofilms-derived metabolites presented less mass and activity. These events highlight that, when developed in biofilm, *P. aeruginosa* release signals or metabolites able to prejudice single and binary biofilm growth of others species and of their own species. However, products released by their planktonic counterparts did not impair biofilm growth or activity. *E. coli*, living as planktonic or sessile cultures, released signals and metabolites or removed un-beneficial compounds which promoted the growth and activity of all the species. Our findings revealed that inter and intraspecies behaviors depend on the involved bacteria and their adopted mode of life.

Keywords Interspecies signaling · Synergistic and antagonistic interactions · Planktonic growth · Biofilm growth

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Introduction

In nature, bacteria live by interacting and communicating with each other, regardless if they belong to the same (intraspecies) or different species (interspecies). One of the major mechanisms of cell–cell communication in bacteria involves the synthesis and release of chemical molecules called diffusible signal molecules [50]. These signals can be cell-density related (quorum sensing-QS) or produced by bacteria at different stages of growth. Primary and secondary metabolites are recognized to contribute to a wealth of interactions between organisms [10] and can include a variety of nutrients, toxic or neutral metabolic byproducts, antibiotics, and other signaling molecules. Such products are released and accumulated in the surrounding environment during bacterial growth [13] and can induce expression of certain genes and/or physiological changes in neighboring cells [14, 32]. The properties of these signals and the response elicited by them are important in ensuring bacterial survival and propagation in natural environments where hundreds of bacterial species

coexist [17]. Responses of bacteria to chemical signals are quite varied and can include synergistic and/or antagonistic effects. Most research into interspecies bacterial interactions has focused on the beneficial aspects of these relationships that may include coaggregation [18, 26, 31, 38, 43] and conjugation [15]. These positive interactions give advantages to microorganisms through the transfer of chemical signals, exchange of genetic information [33], growth promotion and increase of metabolic activity [42], and protection from adverse environmental conditions [23]. Positive interactions among competitors can even contribute to biodiversity [16]. However, not all interactions are beneficial, since antagonistic interactions play an important role in bacterial species predominance. Competition for substrate is considered to be the major evolutionary driving force in the microbial world [44]. Negative interactions can give rise to sporulation, suppression of respiration [12], growth inhibition through the production, for instance, of antimicrobial compounds, as antibiotics [35, 47]. For instance, lactic acid bacteria are known to produce some substances with antimicrobial activity, including antimicrobial peptides, which are able to inhibit foodborne pathogens and spoilage bacteria [7].

Most studies have reported cell-to-cell communication mediated by small diffusible molecules produced only by bacteria living in planktonic conditions. However, bacteria living in a biofilm differ from their planktonic counterparts, cohabiting as single cells, not only at the morphological level but also in their physiological and metabolic state [19]. These differences in the cell's physiological state are reflected by substantial changes in their gene expression pattern [3, 39, 40], and may even induce quite varied responses in other bacteria.

In order to investigate whether bacteria lifestyle influences the type of signals and metabolites released into the surrounding environment, the effects of those products secreted by two important human-associated pathogen and commensal, *P. aeruginosa* and *E. coli*, living as free-floating cells and in biofilms, were analyzed. The role of such molecules was evaluated in cell suspensions and in biofilms of single and dual-species cultures formed by both strains.

Materials and methods

Bacterial strains and culture conditions

Pseudomonas aeruginosa ATCC 10145 (American Type Culture Collection) and *Escherichia coli* K12 MG1655 were used throughout this work. The strains were streaked and grown overnight at 37°C from a frozen stock on a nutrient agar plate (Tryptic soy agar, TSA, Merck, Portugal). Several colonies from each strain were used to inoculate a culture in 30 ml of tryptic soy broth (TSB, Merck, Portugal) that was incubated at 37°C, under agitation (130 rpm) for 12–18 h.

Preparation and collection of supernatants of *P. aeruginosa* and *E. coli*

Supernatants were obtained from planktonic (planktonic supernatants) and sessile cultures (biofilm supernatants) of each bacterium. In order to collect the planktonic supernatants, overnight cultures of *P. aeruginosa* and *E. coli* in TSB were centrifuged (7,000 × *g*; 4°C; 5 min) and the crude supernatant was separated from the cells, filtered (0.22-μm filter) and stored at −20°C for further use. For collecting the biofilm supernatants, cells reserved above were used to form single biofilms. Briefly, cells were ressuspended in fresh TSB and the OD_{640} was measured until reaching a final concentration of $\sim 10^7$ cells ml^{-1}. Both cultures were transferred to 96-well tissue culture plates (polystyrene, Orange Scientific, USA) (200 μl per well) and incubated at 37°C and 130 rpm, allowing biofilm formation for 24 h. After this time, supernatants were recovered, filtered (0.22-μm filter) and stored at −20°C. In order to test possible contamination, 10 μl of filtered supernatants (from planktonic and biofilm cultures) from each species were plated on TSB agar plates.

Bacterial growth in the presence of supernatants

Bacterial planktonic growth

Previous cultures of *P. aeruginosa* and *E. coli* were centrifuged (7,000 × *g*; 4°C; 5 min) and ressuspended in TSB, until reaching $\sim 10^7$ cells ml^{-1} (by OD_{640} measurement). For dual-species cultures, a combination of 50% of suspended inocula of each species was used. These cell suspensions were diluted 1:2 in each supernatant, collected previously, transferred to microtiter plates (200 μl culture per well), and incubated at 37°C with agitation of 130 rpm. Bacterial planktonic growth was followed by OD_{640} measurement at 1-h intervals, until reaching 24 h. In order to validate the influence of supernatants in planktonic growth, normal growth curves in the presence of only TSB medium were also examined and used as a control test. All the experiments were repeated three times.

Biofilm development and analysis

Biofilms were developed according to the modified microtiter plate test proposed by Stepanović et al. [45]. The bacterial suspensions prepared above, were diluted 1:2 in each supernatant and transferred, under aseptic conditions,

for a microtiter plate (200 µl per well). To promote biofilm formation, the plates were incubated aerobically on a horizontal shaker at 120 rpm, at 37°C. After 24 h, the content of each well was removed and the wells were washed twice with 250 µl of sterile water. Biofilms were also formed in the presence of TSB (control), in order to compare with those grown in supernatants. The plates were air dried for 20 min, and the remaining attached bacteria were analyzed in terms of biomass adhered on the surfaces of the microtiter plates and in terms of metabolic activity. The number of cultivable cells, by plate count agar, was also determined.

Biomass Biomass of single and dual-species biofilms were quantified by crystal violet (CV) staining method adapted from Stepanović et al. [45]. Briefly, the 24-h biofilms formed within the 96 wells were fixed with 250 µl of 98% methanol (Vaz Pereira, Portugal) per well for 15 min. Afterwards, the plates were emptied and left to dry for 20 min. Then, the fixed bacteria were stained for 5 min with 200 µl of crystal violet (CV) (Merck, Portugal) per well. Excess stain was rinsed off by placing the plate under running tap water. After the plates were air dried, the dye bound to the adherent cells was resuspended with 200 µl of 33% (v/v) glacial acetic acid (Merck, Portugal) per well. The optical density (OD) of the obtained solution was measured at 570 nm using a microtiter plate reader (Tecan, Model Sunrise-basic Tecan, Austria) and biofilm mass was presented as OD_{570} values. Control experiments to avoid false results were also performed in order to determine whether the tested media and the material of construction of the plates (polyester) could interact with CV.

Respiratory activity The respiratory activity of single and mixed biofilms was measured through the XTT colorimetric method, as described by Stevens and Olsen [46], with some modifications. Biofilms were washed as described before and 200 µl of a combined solution of XTT (Sigma-Aldrich) and PMS (phenazine methosulfate) (Sigma) was added to each well in order to obtain a final concentration of 150 µg ml^{-1} of XTT and 10 µg ml^{-1} of PMS. Afterwards, the plates were left to incubate for 3 h, at 150 rpm, in the dark. The biofilm activity was determined through measurement of the content of each well by optical density at 490 nm (OD_{490}) using a microtiter plate reader and the biofilm activity was presented as OD_{490}. Control tests, using culture medium, the supernatants supplemented in TSB and empty wells, were also carried out, in order to avoid misleading results.

Cell culturability In order to determine the number of CFUs, biofilms formed in the presence of TSB and in the supernatants were removed by sonication and were subsequently serially diluted. After plating the serial dilution on TSA, the plates were incubated at 37°C in an aerobic incubator for 18 h prior to enumeration. The number of cultivable bacterial cells was determined separately and expressed as CFU per cm^2.

Statistical analysis The data were analyzed using the Prism software package (GraphPad Software). One-way ANOVA tests were performed and $p < 0.01$ was considered significant.

Results

Influence of the supernatants (planktonic vs. biofilm) of *P. aeruginosa* and *E. coli* on bacterial planktonic growth

After the supernatants from planktonic and biofilm cultures of *P. aeruginosa* and *E. coli* strains have been collected, they were used as growth media on planktonic growth of single and mixed cultures formed by both species. The planktonic development of all cultures was analyzed every hour, by OD_{640} measurement. Figure 1 presents the planktonic growth curves from single cultures of *P. aeruginosa* and *E. coli* and of their mixed cultures grown in the presence of planktonic and biofilm supernatants from each strain. Cultures grown in TSB medium were used as controls and are represented by the darkest lines. As can be observed, no supernatant showed ability to affect the growth of both cultures involving *P. aeruginosa* strain, either in single (Fig. 1a) or in mixed cultures (Fig. 1c), since the respective growth curves followed a pattern similar to the growth curve control. These results suggest that extracellular metabolites released either from *P. aeruginosa* and *E. coli* in distinct mode of life, were unable to affect the growth profile of single and mixed cultures with *P. aeruginosa*. This showed that the relationship established between both strains, in mixed cultures, was not disturb by any supernatant (planktonic or biofilm) from each strain. Conversely, single *E. coli* planktonic growth was greatly stimulated when grown in the *P. aeruginosa* biofilm supernatant (Fig. 1b), showing a synergistic response face to signaling molecules released from *P. aeruginosa* biofilms.

Influence of biofilm supernatants of *P. aeruginosa* and *E. coli* on single and mixed biofilms growth and activity

The effect of the supernatants obtained under biofilm conditions from *P. aeruginosa* and *E. coli* strains on their

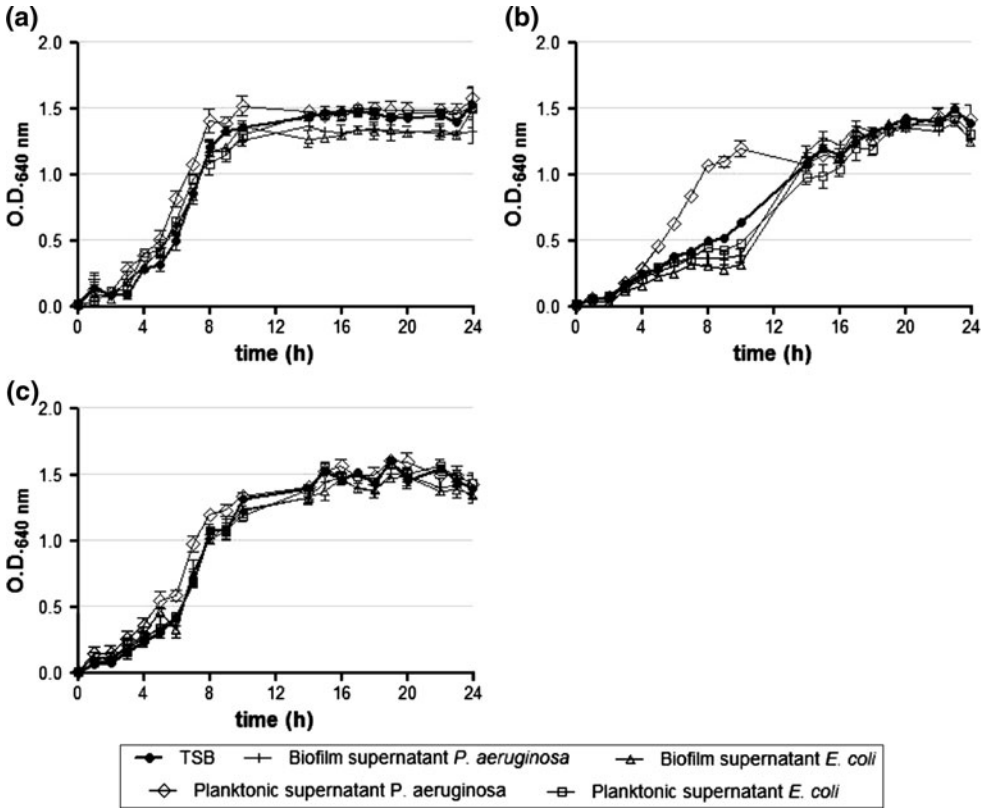

Fig. 1 Effect of the planktonic and biofilm supernatants on growth of planktonic single cultures of *P. aeruginosa* (**a**), *E. coli* k-12 (**b**), and in mixed cultures of those bacteria (**c**). Values are means of three separate assays, and the *bars* indicate SD

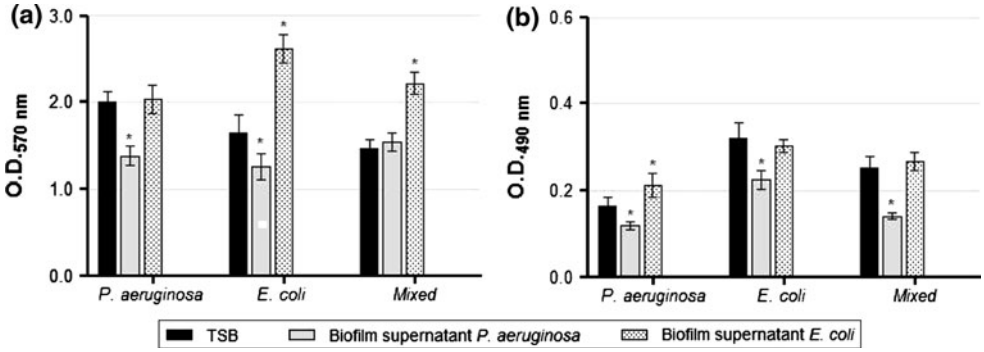

Fig. 2 Effect of biofilm supernatants from *P. aeruginosa* and *E. coli* on biofilm biomass (**a**) and respiratory activity (**b**) of their single and mixed biofilms. The values are means of three separate assays, and the *bars* indicate SD. * $p < 0.01$ (vs. TSB) in one-way ANOVA test

single and binary biofilms growth and activity are presented in Fig. 2. Although *P. aeruginosa* and *E. coli* are both Gram-negative bacteria, they trigger different effects both in biofilms growth and activity. As shown in Fig. 2a, biofilm supernatant of *P. aeruginosa* inhibited its own growth, as well as *E. coli* single biofilm ($p < 0.01$). However, no effect was observed on the formation of mixed biofilms formed by both strains grown in the presence of this supernatant in relation to the medium control. As can be seen by Fig. 2b, conditioned media produced during *P. aeruginosa* growth also showed the ability to disturb cell viability, including for dual-species biofilms, resulting in an antagonistic response by all sessile cultures. Concerning the biofilm supernatant of *E. coli*, signaling molecules produced by this species resulted in biofilms with equal or even more mass than those grown in TSB, mainly for itself (reaching an increase of about 60% on biomass) (Fig. 2a). It was also noticed a marked increase in dual-species growth ($p < 0.01$) caused by this supernatant, suggesting that modifications of the medium by *E. coli* living in biofilm, either by production of metabolites, signals, or removal of un-beneficial compounds, benefits its relationship with *P. aeruginosa*, promoting the development of mixed biofilm. However, when compared to control, those signals revealed not cause significant deviations in biofilms metabolic activity. Thus, in a general point of view, the biofilm supernatant of *E. coli* demonstrated a positive impact in all biofilms, mainly on biomass accumulation rather than respiratory activity.

Influence of planktonic supernatants of *P. aeruginosa* and *E. coli* on single and mixed biofilms growth and activity

In Fig. 3 it is possible to observe the effect caused by planktonic supernant of *P. aeruginosa* and *E. coli* in mono- and dual-species biofilm growth and respiratory activity. Unlike biofilm supernatants, these results demonstrated that metabolites released by each bacterium under planktonic conditions did not show antagonistic effect regarding the growth and activity of biofilms. Planktonic supernatant from *P. aeruginosa* showed ability to maintain the biomass accumulation in single biofilms, instead of reducing it, as observed in the presence of its biofilm supernatant. However, in the case of mixed biofilms, signal molecules produced by *P. aeruginosa* in planktonic lifestyle caused the stimulation on their growth ($p < 0.01$). Concerning the role of those products delivered from *P. aeruginosa* in planktonic state, on biofilms activity, no particular effect was detected, resulting in biofilms with similar activity as those grown in TSB. Nevertheless, biofilms grown in planktonic supernatant of *E. coli* had a synergistic response, resulting in biofilms with more biomass ($p < 0.01$). Similar results were detected on respiratory activity of all biofilms grown in that supernatant, resulting in more active biofilms, mainly for those formed by itself. The growth of mixed biofilm involving *P. aeruginosa* and *E. coli* was also promoted by extracellular metabolites from planktonic cultures of *E. coli*, suggesting that interactions between the species forming mixed biofilm were reinforced either by metabolites, signals released from E. coli in planktonic lifestyle or removal of un-beneficial compounds, resulting in more robust and more active biofilms.

Influence of supernatants (planktonic vs. biofilm) of *P. aeruginosa* and *E. coli* on cultivable cells

In order to complement biofilms growth and activity experiments, the number of cultivable cells within biofilms grown in the presence of supernatants from different lifestyle was also evaluated. In Fig. 4 it is possible to observe the cell numbers, in log, in single and mixed biofilms grown in supernatants from sessile and planktonic cultures of each species. The results obtained from these experiment showed a slight reduction in CFU number for biofilms grown in the presence of all supernatants, when compared to the control. However, reductions in cell number capable of growing in solid medium were <1 log CFU cm^{-2}, as can be observed in Fig. 4. The decrease was more evident for biofilms growing in the presence of supernatants resulting from sessile cultures, being more noticeable for *E. coli* biofilms-derived metabolites ($p < 0.01$). In fact, the number of cells within *P. aeruginosa* single biofilms decreased from 7.05 log CFU cm^{-2} (growing in TSB) to 6.44 log

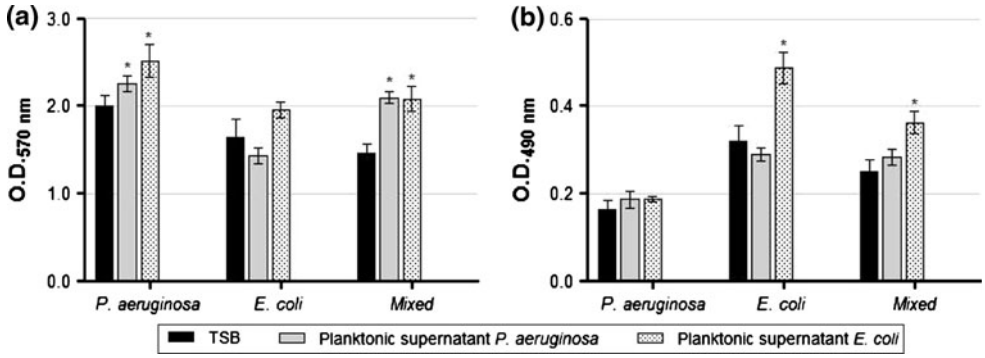

Fig. 3 Effect of planktonic supernatants from *P. aeruginosa* and *E. coli* on biofilm biomass (**a**) and respiratory activity (**b**) of their single and mixed biofilms. The values are means of three separate assays, and the *bars* indicate SD. * $p < 0.01$ (vs. TSB) in one-way ANOVA test

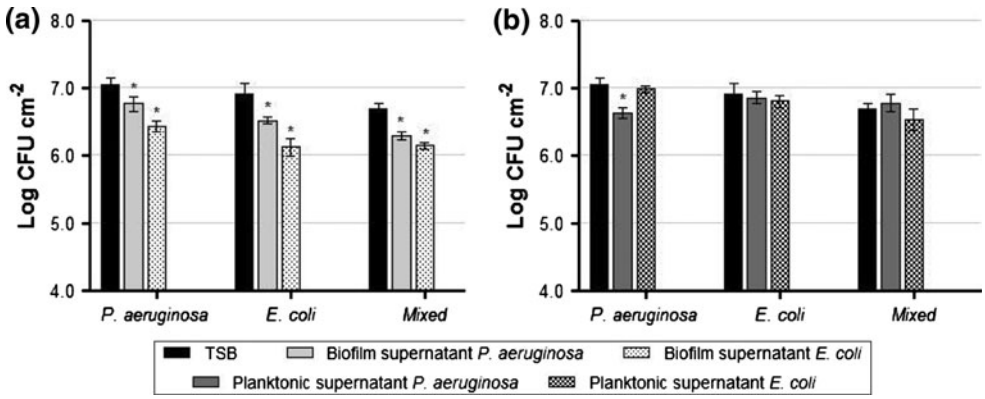

Fig. 4 Effect of biofilm (**a**) and planktonic supernatants (**b**) from *P. aeruginosa* and *E. coli* on CFU number within their single and mixed biofilms. The values are means of three separate assays, and the *bars* indicate SD. * $p < 0.01$ (vs. TSB) in one-way ANOVA test

CFU cm^{-2} (growing in TSB supplemented with *E. coli* biofilm supernatant). Cells from single *E. coli* biofilm presented a reduction from 6.92 to 6.12 log CFU cm^{-2}, when co-cultured in its own biofilm supernatant. With *P. aeruginosa* as a partner, the *E. coli* numbers still showed a small decrease, reducing from 6.69 to 6.14 log CFU cm^{-2}. Since biofilms growing in the presence of signaling molecules obtained from *E. coli* still remaining active, but with a reduced CFU number, this could indicate a loss of capability of cells to grow on agar.

Discussion

Interactions between microorganisms are well-known phenomena that allow a colony or a group of organisms to behave in a co-ordinated fashion to regulate processes contributing to virulence, antibiotic production, biofilm formation, and other developmental programs. Even though bacteria are more prevalent in nature living as communities, these structures are morphologically and physiologically differentiated from free-living bacteria, which may be reflected in the use of different signaling pathways. However, studies reporting cell-to-cell communication have only been directed to the effect of molecules produced by individuals in biofilms and the role of signaling molecules by planktonic bacteria still less well understood. The main objective of the current work was to compare the performance of signals or of metabolic by-products, produced in different physiological status of two major human-associated pathogens and commensal, *P. aeruginosa* and *E. coli*, in respect to their single and mixed planktonic growth and in biofilms.

Data obtained from planktonic growth experiments showed that the growth of *P. aeruginosa* single cultures and in combination with *E. coli* was not affected by the metabolites or signals molecules released by both strains. Moreover, both cultures presented a similar growth profile, suggesting that *E. coli* did not interfere with dual-species growth. Besides that, the use of *P. aeruginosa* planktonic supernatant as growth medium triggered acceleration on growth of *E. coli*, suggesting that metabolites secreted by *P. aeruginosa* in planktonic mode of life gave rise to a synergistic effect on *E. coli* monospecies culture. Based on these results, it is possible to state the ability of *P. aeruginosa* to promote the growth of other planktonic species. According to Shank and Kolter [42], many microorganisms can grow better in combination with others or in the presence of their partner's diffusible compounds. Conversely, Qin et al. [34] demonstrated that *P. aeruginosa* supernatant dramatically reduced *Staphylococcus epidermidis* growth in planktonic cultures. According to some authors [8, 22], *P. aeruginosa* possesses significant antibiotic activity against Gram-positive bacteria. The present study reinforces that statement since *E. coli* growth was favored by *P. aeruginosa*, revealing a synergistic response between both Gram-negative bacteria.

For this study, it was also proposed to evaluate the role of the signaling molecules released by bacteria in planktonic and sessile growth against single and mixed biofilms. Regarding the effect of biofilm supernatants on biofilm growth and activity, our findings revealed that extracellular molecules derived from single *P. aeruginosa* and *E. coli* populations showed distinct results on such parameters. In fact, signaling molecules confer to bacterial population the ability to instigate a collective behavioral change to environmental challenges [2]. According to some authors [9], *P. aeruginosa* is an opportunistic pathogen, which regulates an arsenal of extracellular virulence factors, using a complex hierarchical QS cascade involving AHL molecules. QS-regulated phenotypes can include, for example, the synthesis of antimicrobial compounds, such as antibiotics or rhamnolipid surfactants, which are believed to be involved in detachment of cells from the biofilm [4, 41] and may even interfere with biofilm formation of the producing species or others on its proximity. Thus, our data showed that the production of QS molecules do not always provided direct benefit to the producing bacteria, as hypothesized by some authors [11, 36, 37]. In *E. coli*, AHLs from other bacteria are sensed through the LuxR homologue (SdiA). This species does not synthesize such signals [27, 48], but those are recognized to reduce its biofilm formation [1, 20, 28]. However, the signals and metabolites released from *P. aeruginosa* biofilms did not disturb the growth of mixed biofilms. This may indicate that *P. aeruginosa* and *E. coli* form a mutual cooperation, protecting each other from hostile environmental conditions and therefore being more difficult its disruption. This synergistic interaction between both bacteria can be attributed to a strategy used by *E. coli* to interfere with *P. aeruginosa* signal molecules or metabolites. It is widely accepted that disruption or interference of AHL QS diminishes AHL-dependent expression of virulence determinants in producing bacteria [49]. Another explanation focuses on a kind of pact that could have been done between *E. coli* and *P. aeruginosa*, where the first provides nutrients and other substances to the second bacterium, while this strain does not disrupt the relationship between them.

Conversely to the effect provided by signal molecules or metabolites from *P. aeruginosa*, extracellular metabolites released by *E. coli* biofilms showed benefit in *P. aeruginosa* and *E. coli* single and mixed biofilms growth. In environmental and clinical settings, this becomes a serious problem, since these structures are prevalent and less susceptible to environmental stresses, giving rise to chronic

infections that are notoriously difficult to eradicate [24, 25].

Although historically interspecies interactions have focused on growth interactions, the truth is that a variety of other phenotypic outcomes are possible to occur. Thus, the metabolic activity of biofilms developed in supernatants was also evaluated. Our findings revealed that the signals and metabolites produced by *P. aeruginosa* consortia or the removal of un-beneficial compounds triggered an inhibition in all biofilms activity, either in single or in binary mode of growth. As mentioned above, *P. aeruginosa* produces a myriad of metabolites, many of which with an observable bioactivity, such as antibacterial or phytotoxic activity [21], which may have been associated with disturbance on biomass and metabolic activity in single biofilms.

Concerning the role of planktonic supernatants in biofilms, results showed that unlike the effect produced by biofilm supernatants, mainly those from *P. aeruginosa*, any antagonistic responses were elicited by planktonic supernatants in biofilms biomass and activity. It is well recognized that bacteria in planktonic forms have unnatural and free access to nutrients, multiply rapidly and often are highly motile [30]. Growing freely in culture medium, there is no needs to bacteria establish antagonistic interactions and affect their and other species growth. Conversely, sessile bacteria growing on surfaces have nutrient limitations and so may grow more slowly and have restricted mobility [6]. To increase their chances of survival, bacteria compete with each other and start to release toxins and other antimicrobial compounds, which are able to interfere with their neighbors. These different signaling pathways used by bacteria in different mode of growth could be in the basis of distinct responses trigger in other bacteria. In this study, it was observed that metabolites secreted by *E. coli*, either in planktonic or in sessile life, always trigger positive interactions in *P. aeruginosa*, resulting in biofilms with more mass and activity.

In order to complement the previous results, we also evaluated the CFU number within biofilms growing in supernatants. Although not significant (<1 log CFU cm^{-2}), we found a decrease in cell number within biofilms growing in all supernatants, being more evident using biofilm supernatants as growth media, particularly from *E. coli*. Since biofilms growing in this supernatant presented metabolic activity, but a decline in cell number, it may be a sign of loss of the culturability feature of the bacterial cells. Many factors can be pointed out to justify the non-culturability of bacteria, namely lethal/sub-lethal injury of cells, adaptation and differentiation among others [5, 29]. This could even be related with a survival strategy used for bacteria that are introduced into a new environment. Roszak and Colwell [37] coined the term "viable but nonculturable" for characterize those bacteria.

Conclusions

Bacteria are affected by the environment in which they live and the variety of other microbial species present. Interactions between microorganisms are well-known phenomena and represent a powerful selective force which has led to the evolution of a variety of effective strategies for colonizing and growing on surfaces. This work has focused on the influence of bacterial physiological state in cell-to-cell interactions. Bacteria living in distinct lifestyle lead to various and different behaviors on interspecies relationships, which suggests that the signals and metabolites produced in biofilm status are dissimilar from those delivered by their planktonic counterparts. Since most studies have been focused on interspecies communication mediated by individuals in biofilms, this study represents a novel area of interest, because although biofilm lifestyle takes prevalence in nature, bacteria in free suspension still exist and may even establish relationships between them.

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