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EFFECT OF *Staphylococcus epidermidis* ON *Pseudomonas aeruginosa* BIOFILM IN MIXED-SPECIES CULTURE

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ABSTRACT

Staphylococcus epidermidis and *Pseudomonas aeruginosa*, are clinically relevant pathogens that often produce biofilms. To investigate the co-survivability of *S. epidermidis* and *P. aeruginosa* in mixed cultures biofilm and planktonic form, it is important to understand more about the interspecies interaction of both species. The interspecies interaction was analyzed using streak and drop agar plate assay, cell viability assay (CFU), spectrophotometry-based method, and microscopic analysis. The findings suggest that both cells and supernatant of *P. aeruginosa* inhibit the planktonic growth of *S. epidermidis*. The cell viability result shows that PAO1 biofilm cells were decreased by 88%, and SE biofilm cells were increased by 75% concerning their control. Opposite to the *P. aeruginosa*, the *S. epidermidis* biofilm and EPS matrix were found to increase in mixed culture biofilm, which was further confirmed by microscopic analysis. In contrast, differential agar media result shows that the reduction in the biofilm (CFU/ml) of *P. aeruginosa* is independent of *S. epidermidis* cells concentration. Finally, the effect of the supernatant on biofilm was investigated, and it found that *S. epidermidis* biofilm was enhanced while *P. aeruginosa* biofilm was reduced in the presence of partner bacterial supernatant, which indicated that *S. epidermidis* in biofilm mode could hinder the biofilm formation of *P. aeruginosa*. The outcomes show that the culture supernatant of *S. epidermidis* can be used to prevent *P. aeruginosa* associated biofilm infections.

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1 Introduction

Biofilm is the consortium of multiple microbial species enclosed with a tightly packed exopolysaccharide matrix known as mixed culture biofilm. This mixed microbial consortium competes for common nutritional resources for survival in the hostile environment (Rogers et al., 2010). It is expected that significant interaction takes place between coexisting microbial species. This coexisting interaction may be mutualistic or antagonistic (Cai et al., 2019). Earlier it was reported that different bacteria coexist in cystic fibrosis (CF), implant-related infection, and contact lenses that contribute to the pathogenesis of the disease (Morales et al., 2013; Baldan et al., 2014). As in the biofilm mode of growth, microorganisms of different species exist together and interact with each other agonistically. Therefore analysis of single-species biofilms will not be sufficient for the treatment of mixed culture biofilm-related infections (Hibbing et al., 2010; Baldan et al., 2014) as virulence factors produced by *S. epidermidis* (SE) is comparatively less than *S. aureus* and are human-friendly bacterium found in the skin microflora and sebaceous gland. It is reported as a harmless commensal bacterium, but it becomes virulent when it reaches inside the human body through the use of indwelling medical devices (Foster, 2020; Joshi et al., 2021). It can be responsible for causing nosocomial infection, blood infection, respiratory tract infection, urinary tract infection, and endocarditis. *S. epidermidis* is found resistant against several antibiotics like trimethoprim, penicillin, ciprofloxacin, clindamycin, levofloxacin, erythromycin, gentamicin, and mupirocin, which nowadays becomes a serious issue in hospitals (Chabi & Momtaz, 2019). It is believed that being present in the skin protects against the other pathogenic bacteria by competing with them (Otto, 2013). *P. aeruginosa* (PAO1) is the most prevalent and more virulent Gram-negative bacterium found in polymicrobial infections, including cystic fibrosis (CF) and diabetic foot and surgical infections. In 2017, *P. aeruginosa* evolved as one of the life-threatening pathogen (Thi et al., 2020). According to a WHO report, *P. aeruginosa* was listed as a pathogen of main concern for the development of a new treatment strategy (WHO 2017). *P. aeruginosa* possesses the intrinsic resistant ability to the existing antibiotics (Pang et al., 2019). The present study was carried out to understand how these bacterial species influence each other in mixed planktonic culture and biofilm communities.

2 Materials and Methods

2.1 Bacteria and growth conditions

This examination utilized the bacterial strain *P. aeruginosa* PAO1 and *S. epidermidis* 435 that were acquired from the MTCC, IMTECH, Chandigarh, India. Propidium Iodide (PI) and FITC-con A were purchased from Sigma Aldrich, India. *P. aeruginosa* was cultivated at pH 7 in Luria-Bertani (LB) medium, *S. epidermidis* strain was cultivated in Tryptic Soya Broth (TSB), and both were maintained at 37°C for 24 h. Mannitol salt agar (*S. epidermidis*) and pseudomonas isolation agar (*P. aeruginosa*) was used as selective media for the viable count.

2.2 Planktonic interaction studies

2.2.1 Interaction on an agar plate and liquid medium

Each strain's inhibitory effect was evaluated after 24 h growth by streak agar assay and drop agar assay. Briefly, 1% of OD_{600}0.5 cultures of both species PAO1 and SE was inoculated into fresh, sterilized LB broth media and kept in a shaking incubator for 24 h at 37°C. In streak agar plate assay, an equal amount of each culture (20µl fresh culture) was spread on half of the agar plates. In drop agar assay, 100µl of SE's 24 h grown culture as uniformly spread on the LB agar plate using a cotton swab. Then drops containing 10 µl of PAO1 culture were placed in the center of the agar surface, and all agar plates were incubated for 24 h at 37°C. The same step was repeated for spotting SE culture on the PAO1 lawn. To estimate the growth of PAO1 in the presence of SE cells, free supernatant, and *vice-versa*. PAO1 was grown in the presence of SE culture supernatant (0.5, 1.5, 2.5, 5, and 15%) in polystyrene 96-well microtiter plates (Tarson) at 37°C in a shaking incubator and at different time interval (0, 1, 2, 4, 6, 8 and 24 h) absorbance was measured (A_{600} nm) (Hoffman et al., 2006; Fugère et al., 2014).

2.2.2 Preparation of mixed culture consortia

Overnight grown cultures of SE and PAO1 were centrifuged at 4ºC and 1000rpm for 15 min, the pellet was rinsed with PBS (Phosphate buffer saline, pH 7.4), and desired OD_{600} was set as per experimental requirements. To access the interspecies interaction in mixed culture biofilm, varying cell concentration of SE 0.5, 0.6, 0.7, 0.8, and 0.9 OD that was corresponding to $5.5x10^9$, $6x10^9$, $7x10^9$, $8.5x10^9$, and $1.5x10^{10}$ CFU/ml respectively were diluted in TSB (1:100) and mixed with 1:100 diluted 0.5 OD_{600} culture of PAO1 ($5.5x10^9$ CFU/ml), and different mixed cultures were termed as M1, M2, M3, M4, M5 respectively (Iwase et al., 2010).

2.3 Mix culture biofilm studies

2.3.1 Co-survival study in a biofilm system

Briefly, for mixed culture biofilm,100µl standardized inoculums of PAO1 were inoculated in a 96-well polystyrene plate with 100µl of different cells concentration of SE. For control, 100µl of standardized inoculums of single species were inoculated in a polystyrene plate (96-well microtiter plate) with 100µl of sterile TSB medium. Then these plates were incubated for 24 h at 37°C in a static condition. The wells were washed with sterile PBS (three times), air-dried, and stained with 0.1% crystal violet (CV) for 15

min. Then, the plate was rinsed with distilled water and air-dried. And finally, in glacial acetic acid (30%), the stained biofilm was resuspended, and absorbance was recorded at 570nm (Fugère et al., 2014).

2.3.2 Microscopic Analysis

For the microscopic analysis petri-dishes (12mm, Borosil) was used and biofilm formed on the glass coverslip containing 2.5 ml of diluted inoculums of single culture with 2.5 ml of sterilized TSB for control biofilm and 2.5 ml diluted inoculums of PAO1 mixed with 2.5 ml of each cells concentration of SE for mixed cultures biofilm and incubated in static condition for 24 h. This was followed by discarding the free planktonic cells, washed with PBS (three times) and stained with 0.1% CV dye, removed extra stained again through washing without disturbing biofilm, then the biofilms coverslip were placed on a glass slide, dried, and observed under the light microscope and images were taken at 100X (Nithya et al., 2010).

2.3.3 Scanning Electron Microscopy (SEM)

A scanning electron microscopic study was carried to examine the cells of both bacteria in the mixed biofilm. Briefly, the mixed biofilm formed (M1-M5) on the coverslip as described above, and biofilms were fixed with paraformaldehyde (2%)-glutaraldehyde (2.5%) solution formed in 0.2M phosphate buffer (pH 7.4). Fixed biofilms on coverslips were dehydrated with ethanol series of 10, 30, 50, 70, and 100% for 10 min, and after that, each sample was put at room temperature overnight to dry completely. The resultant samples were sputtered with gold-palladium (20:80) and visualized under a Scanning electron microscope (Sigma) (Fischer et al., 2012).

2.3.4 Viable count assay

The biofilm formed on the coverslip surface was detached by the sonication procedure, as previously described by Kobayashi et al. (2009). The biofilm was sonicated in 1 ml of PBS (pH 7.4) for 5 minutes, followed by vortexing for 2 minutes. After sonication and vortexing, the suspension is subsequently diluted 10^5 times and inoculated to differential selective media (Mannitol salt agar for *S. epidermidis* and pseudomonas isolation agar for *P. aeruginosa*) and incubated for 24 h at 37°C.

2.3.5 Effect of bacterial supernatant on interspecies biofilm

To analyze the effect of SE culture supernatant on PAO1 biofilm formation, PAO1culture was inoculated with various SE culture concentrations supernatant to the wells of polystyrene microtiter plates (0.5, 1.5, 2.5, 5, and 15%) and incubated for 24 h at 37°C. After incubation, the biofilm was stained with CV dye, as described above. The same procedure was followed to investigate the impact of the PAO1 supernatant on SE biofilm.

2.3.6 Fluorescent Microscopic analysis

For fluorescent microscopy analysis, PAO1 was developed on a glass coverslip in the absence or presence of cell-free supernatant SE435 (0 to 15%) in a static condition and *vice versa.* After 24 h of incubation, the biofilm formed was gently washed with PBS, fixed with paraformaldehyde, and total biofilm cells were stained with PI (10µg/100µl) (Kalia et al., 2020), and EPS was stained with FITC-ConA (Singh et al., 2017)(50µg/ml in PBS, pH 7.4) containing Ca^{2+} (0.1mM). By using an OLYMPUS BX51 fluorescent microscope, fluorescent microscopy was performed, and the images were taken at 20X.

2.4 Statistical Analysis

One-way ANOVA, "Tukey's Multiple Comparison Test," was used for statistical analyses. Error bars indicate the standard deviations for three measurements. A value at $p<0.05$ was considered statistically significant.

3 Results

3.1 *P. aeruginosa* inhibits *S. epidermidis* growth in planktonic form

The interaction between *P. aeruginosa* (PAO1) and *S. epidermidis* (SE) was analyzed on the agar plate (Figure 1). When equal inoculums of an overnight culture of both species were grown in equal half of the agar plate, it was reported that PAO1 inhibited the growth of SE. The clear inhibitory boundary was observed, as shown in Figure 1a. Zone of inhibition was found around the spot inoculated PAO1, and instead of lawn, spotted colonies of SE were observed, as shown in Figure 1b. On the other hand, SE did not show the zone of inhibition around the point inoculums on a lawn of PAO1 (Figure 1c). Furthermore, to test whether this inhibition was limited to the cell to cell contact, filter-sterilized supernatant of PAO1 were incubated with SE cells and *vice versa* (Figure 2). It was found that the growth of SE was inhibited by supernatant (0.5-15%) of PAO1 in a concentration-dependent manner, as shown in Figure 2a. However, the supernatant of SE did not affect PAO1 growth (Figure 2b). The maximum inhibition was observed at 15% supernatant concentration in 24 h. Net 0.7, 0.8, 1, 1.1, and 1.3 fold reduction in the growth of SE was observed at 0.5, 1.5, 2.5, 5, and 15% supernatant of PAO1, respectively.

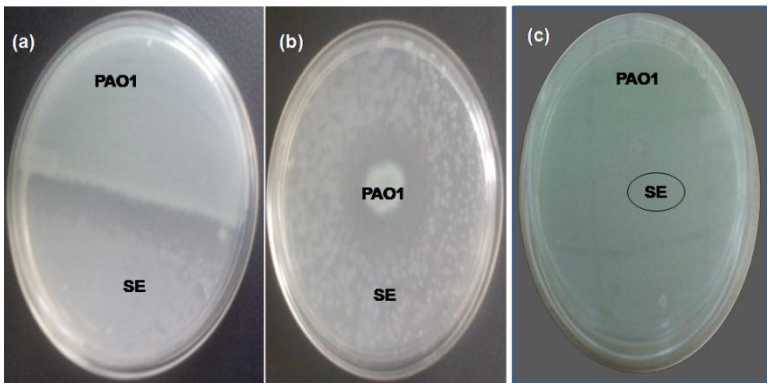

Figure 1 *S. epidermidis* growth inhibition by *P. aeruginosa* on the agar plate. (a) streak agar plate (b) *P. aeruginosa* drop agar plate (c) *S. epidermidis* drop agar plate.

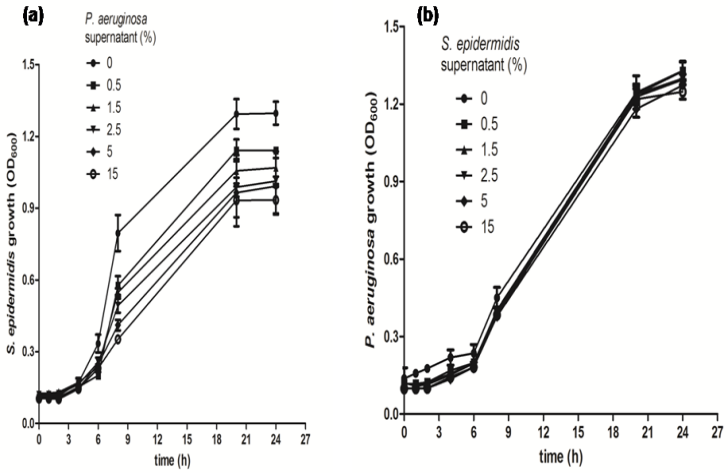

Figure 2(a) Effect of filter-sterilized *P. aeruginosa* supernatant on the growth of *S. epidermidis* and 2(b) indicates the effect of filter-sterilized *S. epidermidis* supernatant on the growth of *P. aeruginosa.* Error bars indicate the ± standard deviations of the measurements.

3.2 Co-survival study in a biofilm system

A mixed culture biofilm study was performed to understand the nature of interaction in biofilm mode. PAO1 presented higher biofilm than *S. epidermidis* ($OD_{570,}$ mean±SD:1.312±0.092; PAO1 vs. 0.645±0.257; SE1, 0.617±0.127; SE2, 0.927±0.091; SE3, 1.038±0.159SE4, 1.054±0.197SE5) as shown in Figure 3a. Moreover, the mixed culture biofilm level was significantly lower (net 1.4 fold) than PAO1 and similar to SE, which indicates that the presence of SE may affect the biofilm formation of PAO1. The increased biofilm biomass of mixed culture from M1-M5 indicates the enhanced persistence of SE in mixed culture.

3.3 Microscopic analysis of mixed culture biofilm

The microscopy was performed to analyze the interaction and variation in species-specific growth in mixed culture (Figure 3b). The single species culture biofilm demonstrated the abundant and dense colonization on glass coverslips under the light microscope and SEM. After 24 h, PAO1, single-species biofilm was seen as adherent and immersed within EPS on the glass coverslips. SE control biofilm was seen in a cluster, either in pairs, tetrad or clumps, although EPS was scarcely visible. However, the reduction of PAO1 and SE biofilm cells was seen in M1 and M2 mixed culture compared to the control single culture counterpart. This signifies that in M1-M2 mixed culture, biofilm PAO1 and SE has a mutually suppressive effect on each other. Furthermore, as seen in M3-M5 mixed culture, biofilm decreases the PAO1 cell number in contrast to its single control biofilm. While increasing EPS, uniform distribution, and increasing the accumulation of SE biofilm cells were seen as in M2-M5 mixed biofilm (Figure 3b).

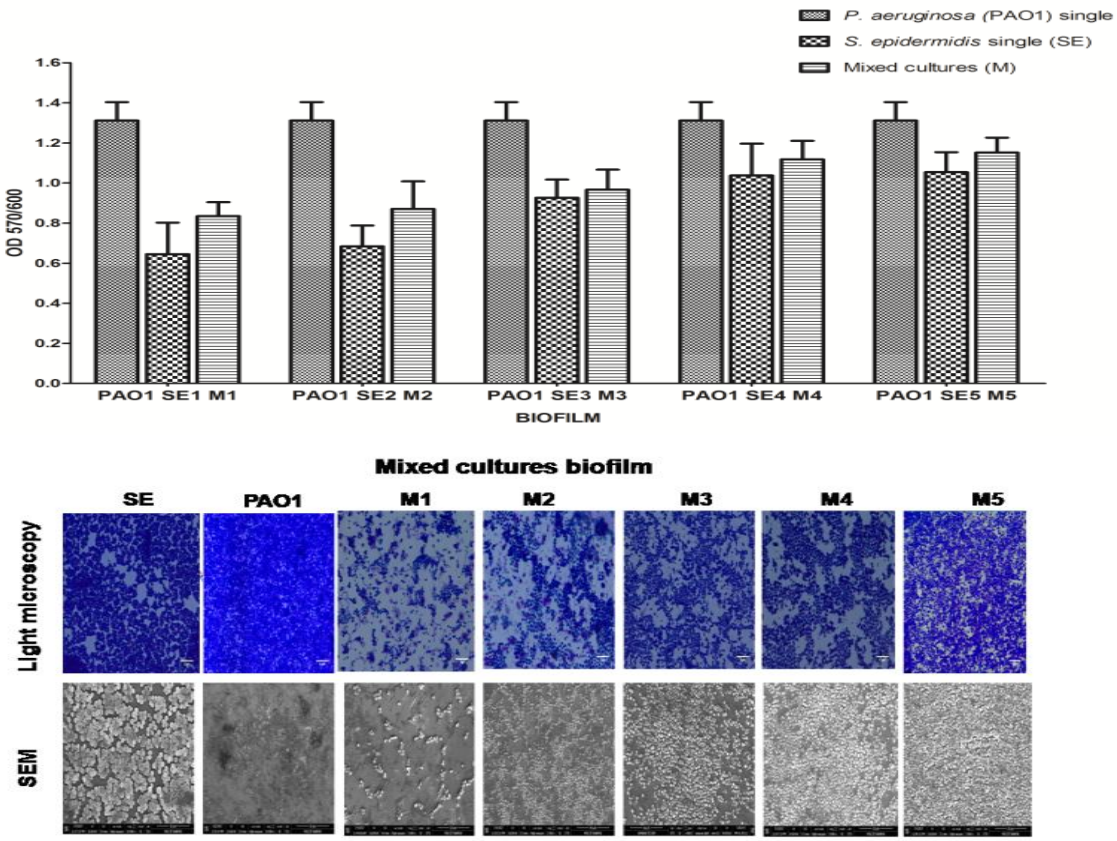

Figure 3(a) Quantification of single and mixed culture biofilm of *P. aeruginosa* and *S. epidermidis* by microtiter plate assay method. Where SE1, SE2, SE3, SE4, and SE5 indicate the different concentrations of *S. epidermidis*.M1-M5; mixed cultures biofilms of *P. aeruginosa* ($5.5x10^9$ CFU/ml) with different concentrations of *S. epidermidis*($5.5x10^9$, $6x10^9$, $7x10^9$, $8.5x10^9$, and $1.5x10^{10}$ CFU/ml). Error bars indicate the ± standard deviations of three measurements (b) Light microscopy (above lane) and scanning electron microscopy images (below lane) of mixed cultures biofilm of *P. aeruginous* and *S. epidermidis*. M1-M5; mixed cultures biofilms of *P. aeruginosa* with different concentrations of *S. epidermidis*.

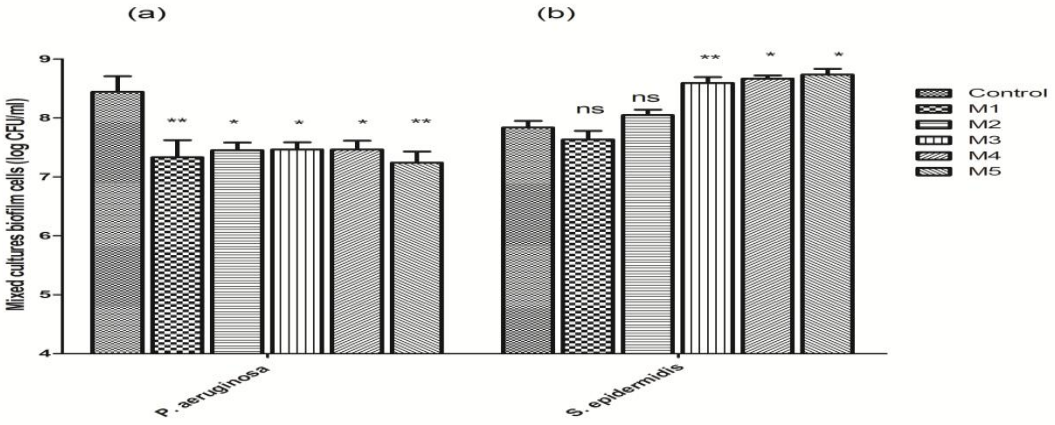

Figure 4 Biofilm cells of *S. epidermidis* and *P. aeruginosa* alone and in the mixed cultures (4a) Biofilm cells of *P. aeruginosa* in M1-M5 biofilm compared with control (alone). (4b) Biofilm cells of *S. epidermidis* in the M1-M5 biofilm with control. 100 µl standardized inoculums ($5.5x10^9$ CFU/ml) are used in M1. Error bars indicate the standard deviations for three measurements.*, P<0.05 compared with the control. **, P<0.001 when compared with the control.

3.4 Analysis of cell viability of biofilm cells

The biofilm viability was evaluated by the CFU count, as shown in Figure 4. When compared with the growth of single species biofilm, it was found that the viability of PAO1 and SE did not have a similar value in mixed biofilm. When compared to their CFU count of mixed culture biofilm, the viability (CFU) of biofilm formed by SE was higher than that of PAO1. When the viable count of SE was tested within mixed biofilms and compared with its single viability, its viability was observed to be significantly enhanced in M3-M5 mixed cultures biofilm and found similar in M1-M2 mixed biofilm (Figure 4b). On the other hand, the biofilm viability of PAO1 decreased in all sets of mixed biofilm (M1-M5), as shown in figure 4a. Biofilm cell count of SE was less than 8 log (CFU/ml), and PAO1 was noticed between 8 and 9 log (CFU/ml). The overall reduction in PAO1 biofilm cells was 88%, and the promotion of SE biofilm was 75% to their control.

3.5 Effect of bacterial supernatant on Biofilms

Further, to understand the mechanism of reduction in PAO1 biofilm cells, whether it is a contact or non-contact basis, the effect of PAO1 supernatant on SE and *vice versa* were evaluated, and it was observed that biofilm of PAO1 was diminished in the presence of supernatant of SE (Figure 5a). Overall 1.1 fold reduction in PAO1 was observed as compared to control. Further, the reduction of PAO1 biofilm in the presence of cell-free supernatant of SE on the coverslip in a static condition was also evaluated (Figure 5b). After the fixation of biofilm, total cells involves in biofilm formation were stain with PI and visualized under fluorescent microscopy. The micrograph of PAO1 treated samples showed a scattered appearance in comparison to the 24 h PAO1 control biofilm. The biofilm cells attached to the surface were scattered, and cell packs were rarely observable because of poor cohesiveness and subsequent adherence. The biofilm integrity in terms of EPS production was additionally limited within the tested samples. On the other side, SE biofilm was enhanced in the presence of PAO1 supernatant in a concentration-dependent manner (Figure 6). The cell-free supernatant of PAO1 invigorates SE biofilm in a concentration-dependent way with a general, 2.6 fold increment in biofilm production as compared with the sample without PAO1 supernatant (Figure 6b). Data of microscopic visualization of SE biofilm with and without PAO1 cell-free supernatant revealed that SE biofilm (red) and EPS (green) increases as the supernatant concentration increases (Figure 6a).

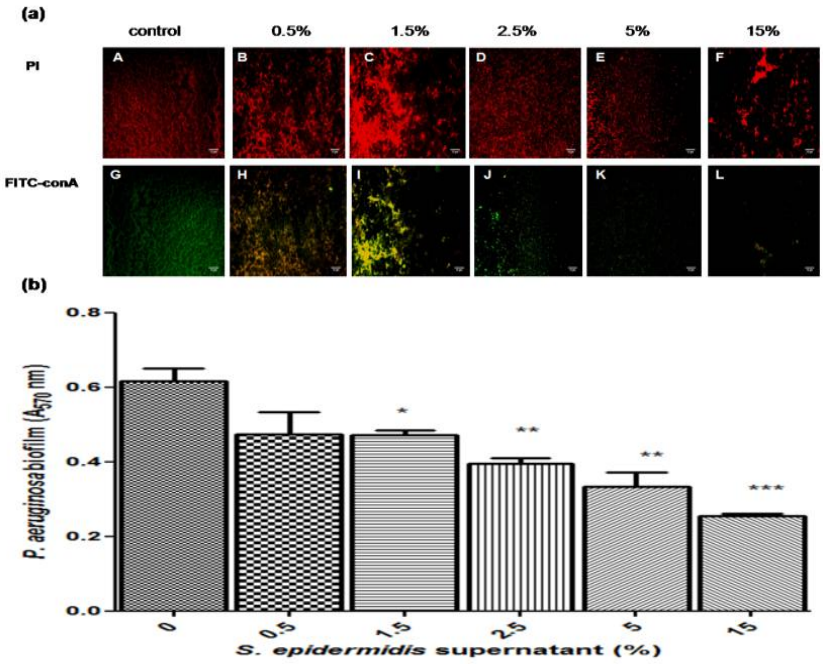

Figure 5 Inhibition of *P. aeruginosa* biofilm formation in the presence of cell-free culture supernatant of *S. epidermidis*. (5a); Microscopy images *P. aeruginosa* PAO1 biofilms in the presence of 0.5%, 1.5, 2.5%, 5%, and 15% (b, c, d, e, f, h, i,j, k, and l) and absence of (a, g) *S. epidermidis* 435 supernatant. Red fluorescent indicates the total cell involves biofilm, and green fluorescent, showing the EPS production in biofilm stained with FITC-ConA. (5b) Biofilm quantification of *P. aeruginosa* in the presence of 0.5%, 1.5, 2.5%, 5%, and 15% and without (zero %) culture supernatant of *S. epidermidis*. Error bars indicate the standard deviations for three measurements.*, P<0.05 compared with the control. **, P<0.001 when compared with the control. ***, P<0.0001 when compared with the control.

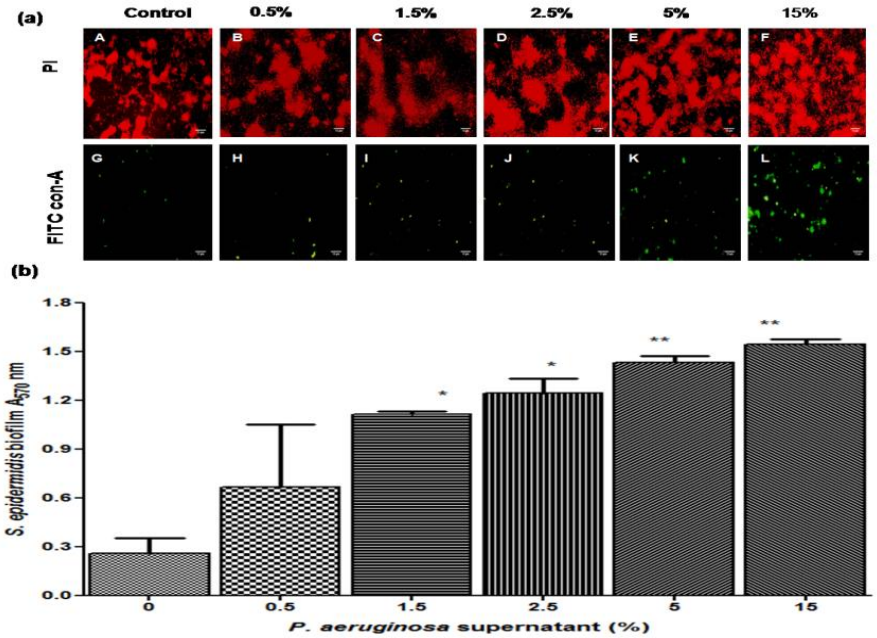

Figure 6 Biofilm formation of *S. epidermidis*in the presence of cell-free culture supernatant of *P. aeruginosa*. (6a) Microscopic images *S. epidermidis* biofilms formed with 0.5%, 1.5, 2.5%, 5%, and 15% (b, c, d, e, f, h, I, j, k, and l) and without (a, g) *P. aeruginosa* supernatant. Red fluorescence (a-f) indicates the total cell involves biofilm and green fluorescent, showing the EPS production in biofilm stained with FITC-ConA (g-l). (6b) *S. epidermidis* biofilm quantificationin the presence of 0.5%, 1.5, 2.5%, 5%, and 15% and without (zero %) culture supernatant of *P. aeruginosa*. Error bars indicate the standard deviations for three measurements.*, P<0.05 compared with the control. **, P<0.001 when compared with the control.

4 Discussion and Conclusion

The present study has evaluated the nature, and inter-bacterial interaction between PAO1 and SE435 in the planktonic and biofilm community, particularly emphasizes whose survivability enhanced to their planktonic counterparts. The agar plate assay, including both spot inoculation and equal half, streaked plates, zone of inhibition shows that the growth of SE was inhibited by PAO1. Additionally, the growth of SE was also inhibited by a culture supernatant of PAO1 (found net 0.7, 0.8, 1, 1.1, and 1.3 fold reduction). The antagonism behavior of bacterium could be due to the diffusible exoproducts released by PAO1and probably due to competition for nutrients and space in the same medium (Qin et al.,2009; Keown et al., 2020). Further analysis was carried out in the biofilm mode of growth. When both bacteria were cultivated alone, it was observed that, unlike SE biofilm, biofilm biomass of PAO1 (alone) was found net 2.8 fold higher, which may be due to increasing EPS production during biofilm formation. In mixed culture biofilm, it was observed that biofilm biomass of mixed-species culture was similar to SE and lower than PAO1 individually, which suggests that perhaps SE persists in the mixed culture and affects the EPS production (Holt et al., 2017; Woods et al., 2019).

The study was further confirmed by microscopic analysis. Microscopic analysis was performed to analyze the interaction of PAO1 and SE in mixed-species biofilm formation, and it was observed that SE was more abundant than PAO1 in all sets of mixed cultures. The mixed cultures SEM images of SE and PAO1 biofilms showed that the enhanced EPS matrix of SE in comparison with PAO1. Light microscopy and SEM confirmed that the PAO1 showed scanty architecture in mixed-species biofilm instead of dense colonization as in single species. This confirmed that the dominance of SE over PAO1 in mixed-culture biofilms.

Moreover, the formerly mixed cultures (M1-M2) were also less dense and non-uniform than that of controls and revealed few layers of cells, profuse cellular debris, together with degrading and morphological alter PAO1 cells. The result is consistent with the findings of Bandara et al. (2010), who reported that both *P. aeruginosa* and *Candida* spp. mutually affect each other in dual-species biofilm than single control biofilm. Further, the present investigation gave insights that complicated communications can occur between species inside the biofilm. Furthermore, the subsequent dynamic relies on various factors, for example, the microbial consortia, cell-cell communication, and the surface of attachment. SEM and light microscopy show that the total number of cells involves in biofilm formation, while only viable cells

involve in biofilms were measured by CFU count using selective or differential agar media. Hence, with the SE cells concentration, a few scattered and scanty biofilm of PAO1 was confirmed by the CFU count. Besides, the current study also evaluated the SE biofilm viable cells in all sets of mixed cultures.

In this context, the viability of both species PAO1 and SE in M1-M5 mixed biofilm was compared with single biofilm viable cells. It was observed that the viability of SE in M3-M5 was significantly higher than the control biofilm (single culture), as shown in figure 5a. While similar biofilm cells were observed in M1-M2 (7.5 log (CFU/ml) ± 0.22). The possible explanation of enhanced survival in M3-M5 is that there may be an increased amount of *S. epidermidis* that allows the organism to out-compete the *P. aeruginosa* as compared to M1-M2. Although the population of *P. aeruginosa* PA14, either planktonic or biofilm, is not influenced by the presence of *S. aureus* (Filkins et al., 2015). At the same time, the outcomes of the current study demonstrate that the inhibitory activity of PAO1 against SE was only in planktonic form and the scanty existence of PAO1 as well as the promotion of SE biofilm in mixed culture biofilm. Similarly, Dehbashi et al., (2021), finding show that in co-culture biofilm state viability of *P. aeruginosa* bacteria decreased significantly with the presence of *S. aureus.*

Further, to understand the mechanism of reduction in PAO1 biofilm cells, whether it is contact or non-contact basis. The increase and decrease in the effect of SE and PAO1 supernatant, respectively, was investigated, and it was observed that SE supernatant reduced the PAO1 biofilm formation in mixed biofilm, and it was due to the secretion of extracellular products of SE. The proposed mechanism either interferes with the adhesion process of PAO1 or due to competition for surface attachment and nutrients within the same environment. On the other hand, the culture supernatant of PAO1 showed a positive impact on SE biofilm, as confirmed by the CV assay. Similarly, protease containing SE supernatant decreases the biofilm biomass of *S. aureus* (Vandecandelaere et al., 2014).

Similar results were obtained by fluorescent microscopy, and data confirmed that supernatant of SE interferes with the PAO1 biofilm formation and EPS production. On the other side, the SE-treated biofilm was enhanced in the presence of cell-free supernatant of PAO1 as compared to untreated biofilm. Moreover, a reduced biofilm of PAO1 was probably due to the presence of exogenous products such as proteases, nucleases, PNAG/PIA, and Phenol-soluble modulins (PSMs) functioning as surfactants in the supernatant of SE, which might interfere with adhering properties and pathogenicity of PAO1. Similarly, Kumari et al., (2019) findings also report the significant reduction of *Rhizopus arrhizus* spores and pre-existing hyphae with the exposure of phenol soluble modulins (PSMs) and *S. epidermidis*. Likely, Esp a serine protease delivered by *S. epidermidis* (Gram-positive commensal bacterium) prevents *S. aureus* biofilm formation, nasal colonization, and disperses preformed *S. aureus* biofilm (Iwase et al., 2010; Mitchell et al., 2010; Fugère et al., 2014).

Conversely, Lopes et al. (2011) demonstrated that metabolites present in *P. aeruginosa* supernatant stimulated the planktonic growth of *E. coli* and inhibited their biofilm formation. The overall data revealed that the survivability of *S. epidermidis* was higher in biofilm than in planktonic mixed and change the behavior of *S. epidermidis* from planktonic to biofilm form. This study supported that, in polymicrobial consortia, the pathogenicity and gene expression of pathogens can be altered by the presence of other bacterial species (Duan et al., 2003; Alves et al., 2018; Castro et al., 2019). The present study is important as it not only explores the nature of interspecies interaction but is also the first time we are reporting that biofilm inhibitory activity exhibited by the culture supernatant of SE435 is probably one of the clues to reduce or prevent the PAO1 associated biofilm infections.

In conclusion, this study analyses the interaction of *P. aeruginosa* with a mixed set of *S. epidermidis* in both planktonic and biofilm conditions. The findings show that the three mixed sets (M3, M4, and M5) can significantly reduced the *P. aeruginosa* biofilm growth. Additionally, in the case of competitive interaction where extracellular production of mix set M3, M4, and M5; increases the inhibitory activity of *S. epidermidis* against *P. aeruginosa*. Hence, the cell density controls the production of inhibitory molecules of *S. epidermidis* in mixed-species biofilm by cell density-dependent quorum sensing (cell to cell communication mechanism). Future research will involve identifying and developing potential inhibitors against resistant biofilm *in vitro* and *in vivo*.

Declaration of Competing Interest

The authors have declared that no conflict of interest exists.

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