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Bacterial Exofactors Modulate Biofilm Growth and Resistivity to Antimicrobial Drugs

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Abstract

Some bacteria have the ability to co-exist, proliferate and survive in a multicellular community, biofilm. Each participating bacteria can form its colonies and encases itself by a self-produced insoluble extracellular matrix substance (EPS). Microcolonies within biofilm are held together by interactions and bonding of the substances present in the EPS with their separation from the water channels. Similar to insoluble EPS, bacterial microcolonies release soluble exofactors that have direct impacts on the survivability, growth and antibacterial resistivity of other microcolonies made of single- or multi-species bacteria in the same biofilm. How the exofactors of microcolonies of one-type bacteria impact on microcolonies of other-type bacteria is still unclear. We studied about the role of exofactors released from *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa,* which are common biofilm-forming pathogenic bacteria. Exofactors facilitate to transform the microenvironment where bacteria can acquire alternative lifestyle with a long survival period and resistivity to certain antimicrobial drugs.

Keywords

Biofilm, Exofactors, Antimicrobial Drugs, Growth, Extracellular Matrix Substance, Microcolonies

1. Introduction

Aggregation of living microorganisms either on biotic or abiotic moist surfaces in a 3D structure with the support of insoluble extracellular polymeric substance (EPS) establishes the biofilm [1]. Micro-organisms in the biofilm community, besides insoluble substances, release the soluble factors, known as exofactors

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which are important to maintain and modulate the dynamic complex process in a biofilm so as to provide the microenvironment to co-exist all the micro-organisms in the community [1] [2]. Micro-organisms are coordinated in a way that they can survive in an unfavorable condition by adding several protective advantages [1] [3]. The bacteria acquire an alternate lifestyle in biofilm, where they establish their own microcolonies, encased by a self-produced EPS, indicating the significant growth [4] [5]. Bacterial biofilms can be pathogenic and can acquire resistivity to certain antibiotic drugs, depending on the bacteria types and other conditions [6] [7].

We included most common bacteria, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* that are associated with the biofilm formation and have impacts on health and diseases. *P. aeruginosa* is a gram-negative pathogen that establishes the biofilms with alginate, PEL and PSL exopolysaccharides, surface proteins and lectin-binding proteins [8]. *S. aureus* is a gram-positive pathogen that forms the biofilm with a polysaccharide intercellular adhesion (PIA) which is a polymer of N-acetyl glucosamine (PNAG) and some biofilm associated adhesive proteins (Bap). *E. coli,* another gram-negative bacterium, forms the biofilm with the help of certain proteins, for example, curli protein and type 1 fimbriae [1] [9]. These bacteria can make their own biofilms, however, the impacts of exofactors of one-type bacteria to other-type bacteria have not been studied in detail [6] [10]. This study delivers important information on how one-type bacteria impacts on another since, in multicellular biofilm, there is an association of more than one-type of bacteria. We cultured bacteria in tryptic soy broth for three days, starting from the 0.5 McFarland concentration. After sufficient growth with increased turbidity, we centrifuged the broth tubes and separated the supernatants. After confirmation of the bacteria absence in the supernatant through the negative subculture on blood agar, we used the cell-free supernatant to influence other-type bacteria for the growth and antibacterial resistivity [11] [12]. Because of the early phase of study, the proteomics and metabolomes of supernatants from all studied-bacteria are beyond the capacity of this study. The supernatant contains the exofactors, factors secreted by the bacteria during their physiologically and metabolically active stage, ranging from the exoenzymes (e.g., nucleases & proteases) to the metabolic end products, for example, lactic or pyruvic acid [12] [13] [14].

Exofactors or certain metabolites exhibit positive or negative impact on the bacteria [11] [15]. Since exofactors from different bacteria have different concentrations of certain common factors (e.g., catalase, oxidase, kinases, proteases) and some additional factors (e.g., hyaluronidases, pyruvic acid & mupirocin) that influence the growth and modify the bacterial susceptibility to antibacterial drugs by the affect on different components of the bacteria [12] [16]. Some metabolites, for example, amino acid-derived, have a positive influence on the bacterial growth activities [17], while some have negative impacts on bacterial growth [18]. We cultured the selected bacteria with the presence of cell-free su-

pernatant from one-type bacteria to see the effect of exofactors on growth and susceptibility to another-type bacteria. We therefore selected six antibiotics for susceptibility testing based on their mode of bacterial inhibition, namely, cefoxitin and ampicillin that inhibit cell wall synthesis, polymyxin B for cell membrane synthesis inhibition, levofloxacin to inhibit DNA synthesis, and erythromycin and clindamycin for protein synthesis inhibition [19] [20] [21] [22].

The experimental data demonstrate that exofactors from *S. aureus* and *E. coli* supported the growth of microcolonies of each other, however, exofactors from *S. aureus* inhibited the growth of *P. aeruginosa*. Exofactors also influenced the efficacies of antimicrobial drugs. For example, *E. coli* acquired the resistivity to erythromycin by the exofactors of *P. aeruginosa* while no effect from those of *S. aureus*. It reveals the assembly of different bacterial genera has significant impacts on pathogenesis through acquiring new functions in alternative lifestyle in biofilm.

2. Materials and Methods

Thermo Scientific™ blood agar (BA) plates (R01200), Remel™ tryptic soy broth (TSB) (R08943), and Mueller Hinton agar (R454082) were purchased from Fisher Scientific. Antibiotic discs of BD BBL ™ Sensi-Discs; polymyxin B (300 U, B31324), ampicillin (10 µg, B30705), erythromycin (15 µg, B30793), clindamycin (2 µg, B31213), levofloxacin (5 µg, B4331706), and cefoxitin (30 µg, B31591) were also purchased from Fisher Scientific.

Acclimatization of the bacteria for *in-vitro* culture

We selected three bacteria, namely *E. coli*, hemolytic *S. aureus*, and *P. aeruginosa*. They were thawed and cultured onto three blood agar plates and incubated at 37˚C for three days for the environmental acclimatization. They were then subcultured on blood agar and MacConkey agar using the four-quadrant streak technique and incubated at 37˚C for four days. Bacterial colonial characteristics on both blood agar and MacConkey agar were recorded. *S. aureus* did not grow on MacConkey agar, while *E. coli* produced pink-colored 1 - 3 mm colonies and *P. aeruginosa* produced colorless minute colonies. We further confirmed the bacteria with some biochemical tests, for example, catalase (positive), oxidase (negative), coagulase (positive) tests for *S. aureus*. IMViC, TSI, Urease, ONPG test, MUG test for *E. coli*, and an additional acetamide test for *P. aeruginosa*.

Standardization of the bacterial turbidity

After verification of the bacteria, we prepared bacterial suspension of each bacterium from blood agar with the concentration equivalent to 0.5 McFarland turbidity in 4 mL tryptic soy broth (TSB) tubes in triplicates, and incubated at 37˚C for three days. The growth of each TSB tube was recorded. *E. coli* produced the most turbid result, indicating that it grew the fastest, while *P. aeruginosa* produced the least turbid result, indicating its slowest growth. All the tubes were centrifuged at 5000 rpm for about 10 minutes, producing the sediment of the bacterial growth and clear supernatant. We inoculated clear supernatant on the

5% blood agar and there was no growth of bacteria after 24 hours of incubation, confirming that clear supernatant was out of the bacteria.

Culture of bacteria in presence of the supernatant

We prepared the bacterial suspension in a new 3 mL-TSB tube with the 0.5 McFarland turbidity as *E. coli* set-, *S. aureus* set- and *P. aeruginosa* set-in triplicates for each 0.5 mL and 1 mL group. 0.5 mL and 1 mL of collected clear supernatant were added into TSB tubes labeled as 0.5 mL and 1 mL, respectively. We collected supernatant from each bacterial growth after centrifugation, as described above. 0.5 mL and 1 mL of clear supernatant were added to the TSB tubes. Briefly, four tubes of *E. coli* with 0.5 McFarland turbidity were prepared in 3 mL TSB broth. 0.5 mL supernatant from *P. aeruginosa* was added into an *E. coli* tube and 1 mL to another. Likewise, 0.5 mL and 1 mL supernatant from *S. aureus* in other two tubes. All the tubes as described before were incubated for three days at 37˚C. The growth of all tubes was compared according to their turbidity.

Bacterial susceptibility to the antibacterial drugs

Bacterial susceptibility to certain antibiotics was performed in triplicates by the disc diffusion method. We selected six antibiotics based on their mode of bacterial inhibition. Polymyxin B (inhibits cell membrane synthesis), levofloxacin (inhibits DNA synthesis), cefoxitin and ampicillin (both inhibit cell wall synthesis), and erythromycin and clindamycin (both inhibit protein synthesis) were selected for this experiment. All the bacteria from the same stock were used to preparing the supernatant and for susceptibility testing in Mueller Hinton Media (MHM) plates.

3. Results

Bacterial growth rate in broth culture

Among the selected three types of bacteria, *E. coli* produced the maximum turbidity while *P. aeruginosa* produced the minimum turbidity with the intermediate turbidity of *S. aureus* in TSB broth in three days at 37˚C (**Figures 1(B)-(D)**). As a general rule, maximum turbidity indicates the higher growth rate with the release of more metabolites and exoenzymes or exosubastances.

Culture supernatant influences the bacterial growth

There was not much significant difference in the *E. coli* turbidity compared to its control growth when it was cultured with the less volume of culture supernatant (0.5 mL) from *P. aeruginosa* (PAS), but it increased in turbidity in presence of a higher volume of supernatant. It was an interesting that the *E. coli* turbidity increased significantly in the presence of more supernatant volume (1 mL) from *S. aureus* (SAS) (**Figure 2(A)** & **Figure 2(B)**).

Consistent with the *E. coli* turbidity result, *S. aureus* turbidity was increased in the presence of higher volume (1 mL) of PAS compared to its less volume (0.5 mL). The *S. aureus* turbidity was increased by the culture supernatant of *E. coli* (ECS) volume from 0.5 mL or 1 mL (**Figure 2(A)** & **Figure 2(B)**).

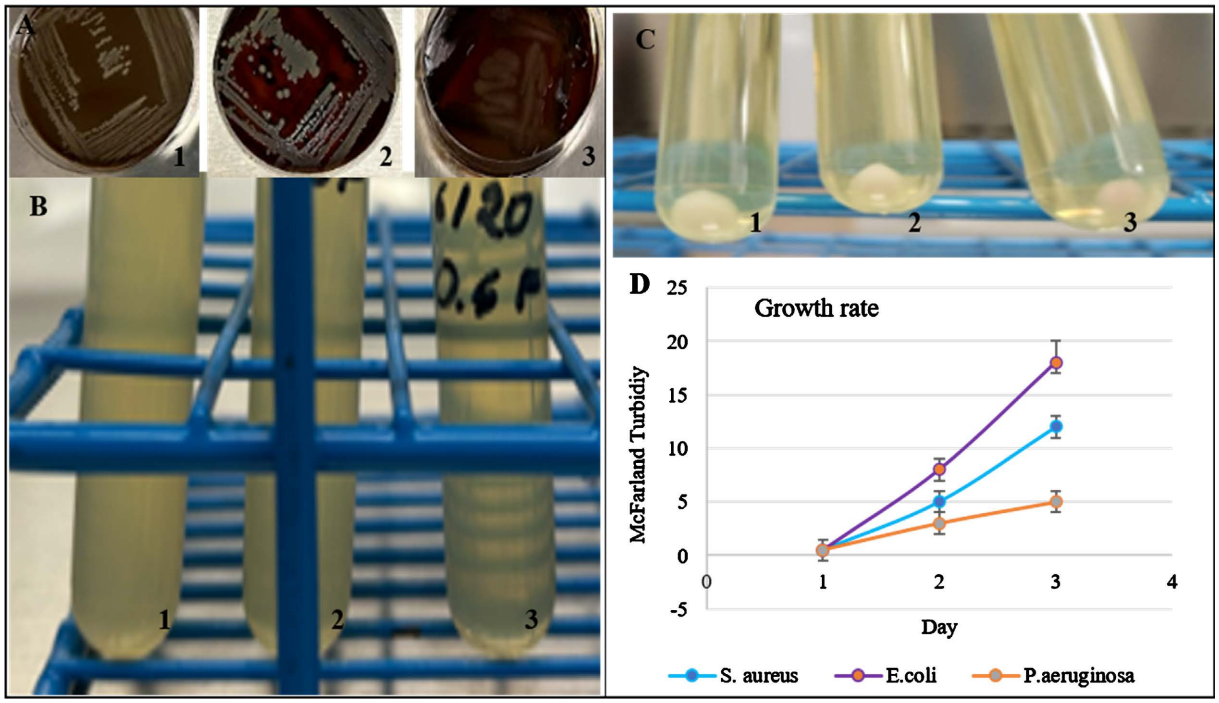

Figure 1. Bacterial growth of *E. coli*, *S. aureus* & *P. aeruginosa* on blood agar (BA) and tryptic soy broth (TSB) after 3-day culture. 0.2 mL of 0.5 McFarland turbidity of bacteria were inoculated on BA (A1)-(A3) and TSB (B) & (C). *E. coli* had shown most turbidity and *P. aeruginosa* showed least turbidity in TSB culture in 3-day culture (B1) & (B3) with most sediment of *E. coli* and least of *P. aeruginos*a (C1) & (C3). *S. aureus* turbidity was less compared to *E. coli* turbidity (B2) & (C2). Growth rate is proportional to the turbidity, where *E. coli* had higher growth rate compared to others and *P. aeruginosa* had lower growth rate with *S. aureus* in the middle of two organisms (C1)-(C3) and (D). Error bars in figure D represent the SD of the means of three independent experiments & $P < 0.01$.

In other hand, *P. aeruginosa* produced a higher turbidity when it was cultured with less supernatant volume (0.5 mL) of both ECS and SAS, implying that the growth of *P. aeruginosa* was suppressed by the presence of both *E. coli* and *P. aureus* (Figure 2(A) & Figure 2(B)).

Culture supernatant alters the bacterial susceptibility to antibiotics

The bacteria were incubated with the cultural supernatant for three days in TSB broth in the experiment group and without cultural supernatant in the control group. Bacterial suspension with the turbidity of 0.5 McFarland was prepared for the disc diffusion susceptibility testing.

E. coli susceptibility:

The susceptibility of *E. coli* to polymyxin B did not change significantly from the zone of inhibition (ZOI) of 17 mm, only 1 - 2 mm reduced in ZOI had been observed in the presence of 0.5 mL of SAS and PAS. Bacterial resistivity to clindamycin remained unchanged by SAS and PAS. Interestingly, the ZOI was decreased from 20 mm to 5 mm to erythromycin by PAS even more, up to 0 mm, in higher volume of supernatant (1 mL) (Figure 3(A), and Figure 4(A) and Figure 4(C)). *E. coli* had no ZOI to ampicillin, indicating that *E. coli* is resistant to ampicillin regardless of SAS and PAS. The interesting result was obtained when ZOI was decreased from 35 mm to 20 mm of levofloxacin in the presence

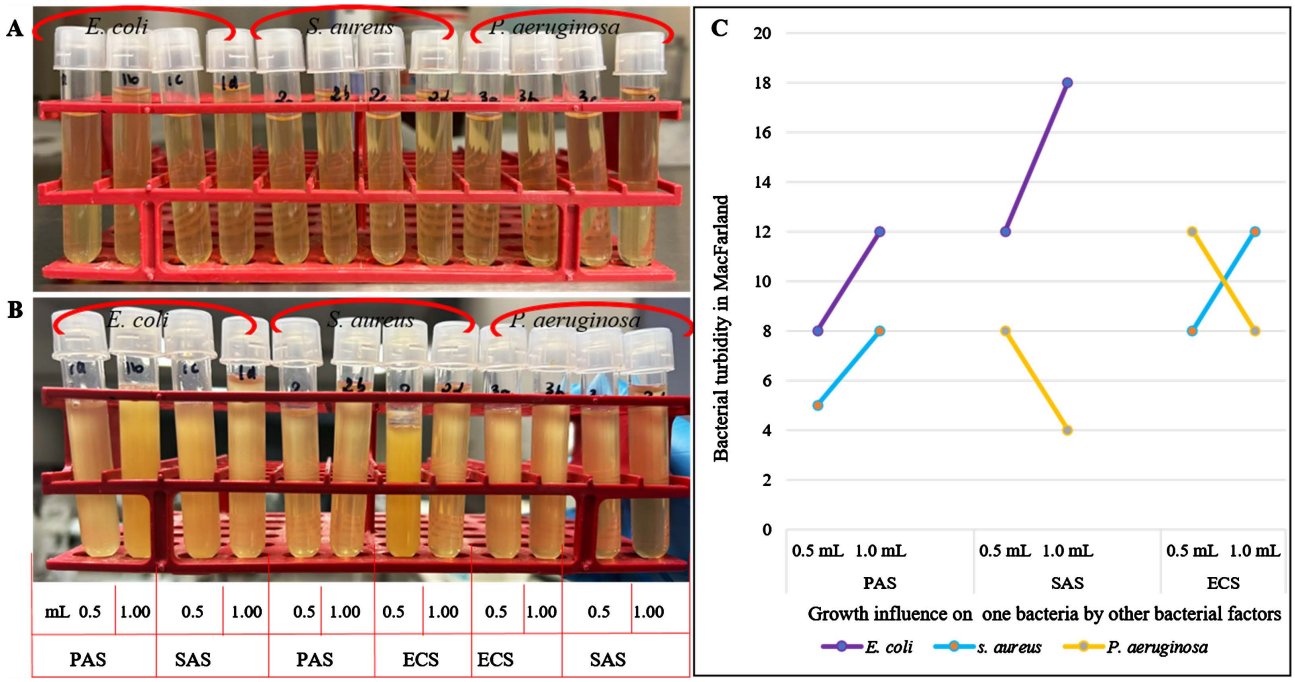

Figure 2. The growth of the one-type bacteria in TSB with the addition of the cell-free supernatant from other-type bacteria and analysis of the growth rate at 3-day of culture. 0.5 McFarland concentration of the cell suspension was prepared in 3 mL of TSB and 0.5 mL & 1 mL of cell-free supernatant was added at day one and incubated for three days at 37˚C in a normal incubator (A). *E. coli* was cultured with PAS and SAS, *S. aureus* with PAS and ECS, and *P. aeruginosa* with ECS and SAS. Bacterial turbidity was reported (B) and measured using the standard McFarland turbidity with the dilution with TSB and was analyzed (C). PAS supported the growth of both *E. coli* and *S. aureus*, SAS supported the growth of *E. coli*, not of *P. aeruginosa* and ECS supported the growth of *E. coli*, not of *P. aeruginosa*. PAS: cell-free supernatant from *P. aeruginosa* culture, SAS: cell-free supernatant from *S. aureus* culture, and ECS: cell-free supernatant from *E. coli* culture. $P < 0.01$.

of PAS. *E. coli* became slightly more resistant to cefoxitin (ZOI was 25 mm from 30 mm) in the presence of the 1 mL of SAS compared to 0.5 mL (ZOI was 28 mm from 30 mm) (Figure 3(A)).

S. aureus susceptibility:

The addition of ECS increased the resistivity of *S. aureus* to polymyxin B (ZOI changed from 16 mm to 10 mm by 1 mL and 15 mm by 0.5 mL with the similar result from 1 mL PAS). There was a significant conversion of *S. aureus* from susceptible to resistant to clindamycin with ZOI result of 30 mm to 0 mm after addition of 1 mL PAS. There was not a significant change in clindamycin susceptibility by ECS (30 mm to 26 mm). *S. aureus* was resistant to both erythromycin and ampicillin, and remained the same in susceptibility testing after culture in the presence of ECS and PAS and hence we could not rule out the efficacy of their cultural supernatants on bacteria resistivity (Figure 3(B), and Figure 4(A) and Figure 4(B)). However, there was a significant increase in the resistivity of the bacteria in the presence of PAS compared to ECS to levofloxacin (ZOI changed from 30 mm to 20 mm by 1 mL and to 25 mm by 0.5 mL). In addition, there was no significant change in susceptibility in the presence of ECS and PAS of cefoxitin (ZOI changed from 28 mm to 32 mm by 1 ml and to 30 mm by 0.5 mL) (Figure 3(B)).

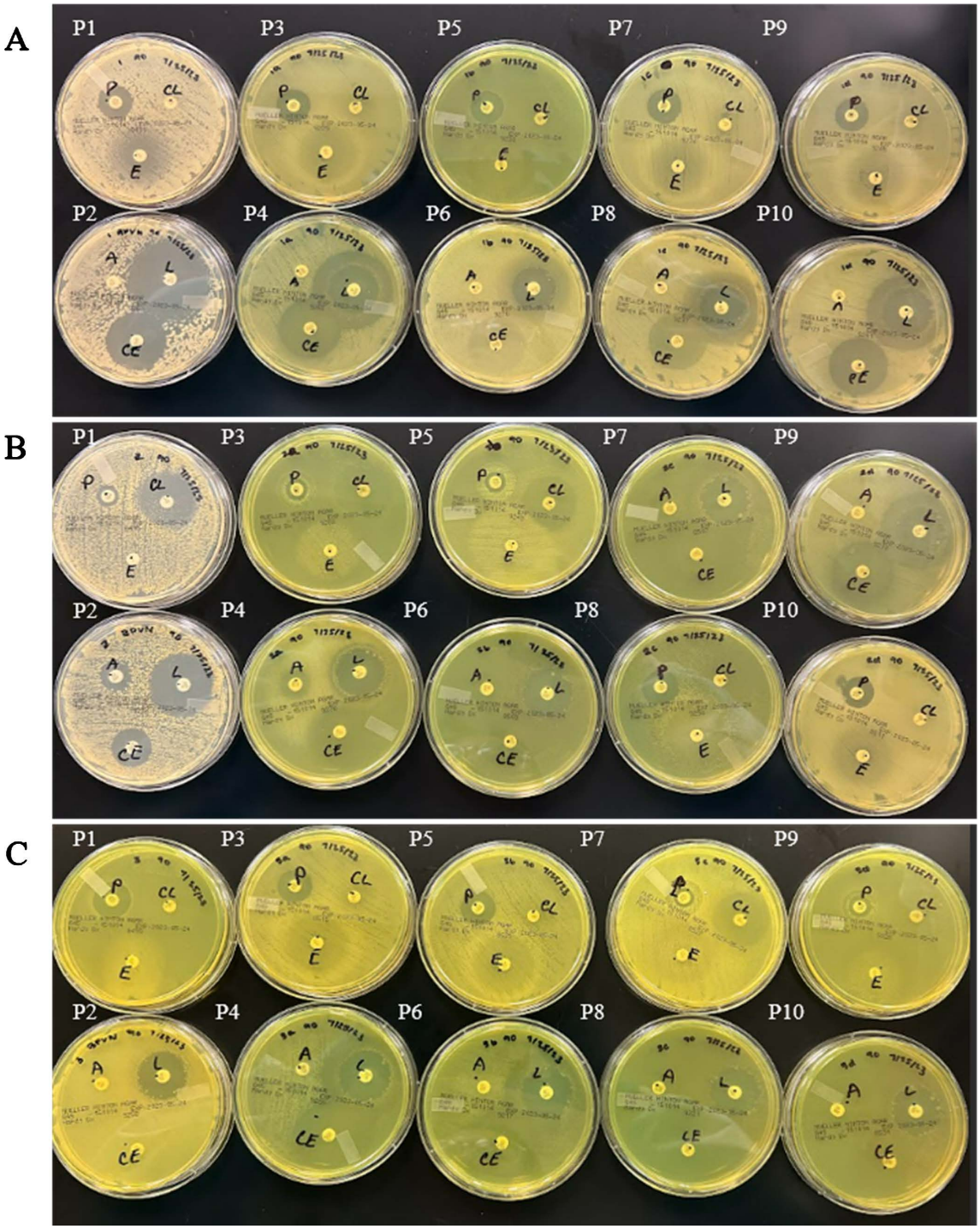

Figure 3. Antibiotic susceptibility testing of *E. coli*, *S. aureus* and *P. aeruginosa* to antibacterial drugs after bacterial culture in presence of cell-free supernatant. After 3-day growth of the bacteria with the cell-free supernatant, bacteria were inoculated in Muller-Hinton agar (MHA) for antibiotic susceptibility testing with commonly used antibacterial drugs, namely, polymyxin B, clindamycin, erythromycin, ampicillin, levofloxacin and cefoxitin. *E. coli* susceptibility testing without the cell-free supernatant (control) (A), P1 to P2, in presence of 0.5 mL & 1.0 mL of PAS (A), P3 to P6, and in presence of 0.5 mL & 1.0 mL of SAS (A), P7 to P10. *S. aureus* susceptibility testing without the cell-free supernatant (control) (B), P1 to P2, in presence of 0.5 mL & 1.0 mL of PAS (B), P3 to P6), and in presence of 0.5 mL & 1.0 mL of ECS (B), P7 to P10. *P. aeruginosa* susceptibility testing without the cell-free supernatant (control) (C), P1 to P2, in presence of 0.5 mL & 1.0 mL of ECS (A), P3 to P6, and in presence of 0.5 mL & 1.0 mL of SAS (A), P7 to P10. P: polymyxin B, CL: clindamycin, E: erythromycin, A: ampicillin, L: levofloxacin, and CE: cefoxitin.

P. aeruginosa susceptibility:

P. aeruginosa susceptibility to polymyxin B remained unchanged, but it was decreased to clindamycin (ZOI changed from 15 mm to 5 mm by 0.5 mL and from 15 mm to 0 mm by 1.0 mL) by SAS (Figure 3(C), and Figure 4(A) and Figure 4(D)). However, ECS had not much effect on clindamycin susceptibility of *P. aeruginosa*. *It* was resistant to erythromycin and ampicillin regardless of SAS and ECS. Unlike *E. coli* and *S. aureus* susceptibility to levofloxacin, *P. aeruginosa* remained the same susceptibility to levofloxacin with no change in ZOI even after being cultured in the presence of ECS and SAS. Interestingly, *P. aeruginosa*

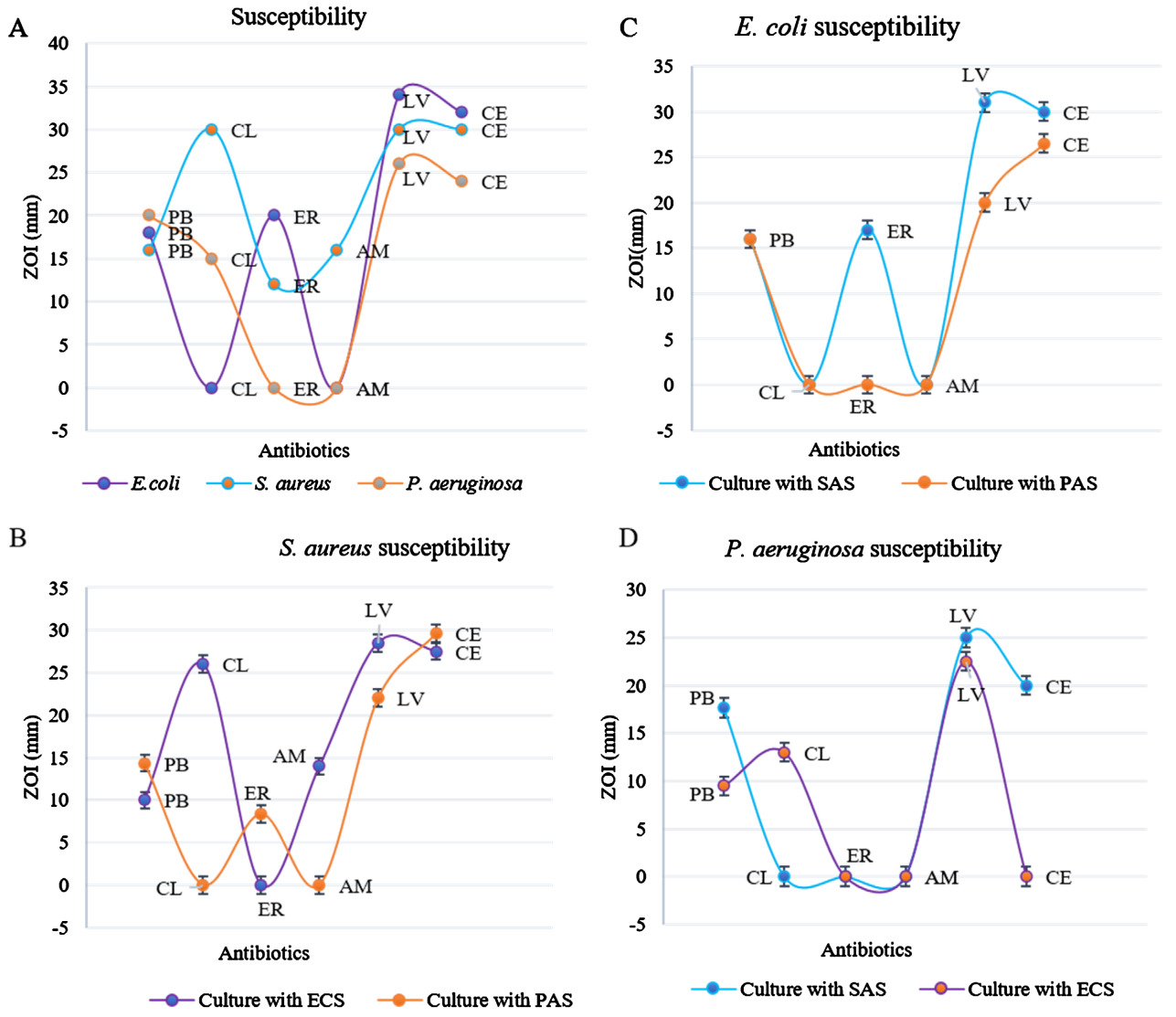

Figure 4. Comparison of the antibiotic susceptibilities of *E. coli*, *S. aureus* and *P. aeruginosa* in presence and absence of cell-free supernatants to antibacterial drugs, namely, polymyxin B, clindamycin, erythromycin, ampicillin, levofloxacin and cefoxitin. Zone of inhibition was recorded in absence of cell-free supernatant (A). S. aureus susceptibility testing in presence of PAS and ECS (B), *E. coli* susceptibility testing in presence of PAS and SAS (*C*), and *P. aeruginosa* susceptibility in presence of SAS and ECS (D). PB: polymyxin B, CL: clindamycin, ER: erythromycin, AM: ampicillin, LV: levofloxacin, and CE: cefoxitin. SAS: cell-free supernatant from *S. aureus* culture, and ECS: cell-free supernatant from *E. coli* culture. ZOI: zone of inhibition. Error bars in figures represent the SD of the means of three independent experiments & $P < 0.01$.

transformed to resistant to cefoxitin in the presence of ECS (ZOI from 24 mm to 0 mm), however, no change in presence of SAS (**Figure 4(A)** and **Figure 4(D)**).

4. Discussion

Biofilm provides the opportunity for the microorganisms to assume an alternate temporary multicellular lifestyle where aggregate facilitates survival, growth and antimicrobial resistivity in adverse conditions. Within the biofilm, the bacteria are encased in a self-produced insoluble EPS, forming a 3D scaffold with the support of various saccharide-binding proteins, pili, flagella, adhesive proteins and extracellular DNA (eDNA) [23] [24] [25]. The various enzymes (e.g., protease, amylase, xylanase, deaminase, decarboxylase, lipase, lechithinase, hippurase, catalase, oxidase, nitrate reductase, coagulase, kinase, phosphatase, etc.) and metabolites (intermediate to end products of carbohydrates, proteins, fats and minerals after the metabolism) exhibit the bacterial exofactors [26] [27] [28]. The enzymes not only support for the EPS modification in response to the microenvironment but also influence on metabolic activities of bacteria [29] [30]. For example, *E. coli* metabolizes the tryptophan by tryptophanase and releases indole, inhibiting its own growth. However, *P. aeruginosa* degrades the indole and promotes *E. coli* biofilm formation [31]. Similarly, bacterial metabolites modulate bacterial growth and antimicrobial resistivity depending upon the nature and properties of the bacteria [17] [32]

We selected *E. coli*, *S. aureus* and *P. aeruginosa* to know how the cell-free supernatant from one-type bacteria (e.g., *E. coli*) affects other-type bacteria for growth and antibiotic susceptibility. Tryptic soy broth (TSB) was selected for the turbidity study through the McFarland turbidity standard (**Figure 1(A)** to **Figure 1(D)**). The growth reports of all the bacteria at the same incubation condition (37˚C for three days in 3 mL of TSB) have shown that *E. coli* had the higher growth rate compared to *S. aureus* and *P. aeruginosa* with the least growth of *P. aeruginosa* (**Figures 1(B)-(D)**). McFarland turbidity was measured after the dilution and reported with the turbidity of *E. coli* was more than 15, *S. aureus* more than 10 and *P. aeruginosa* had 5 after three days of culture in TSB.

In the presence of the cell-free supernatant, the growth rate of bacteria had been changed and was not in the similar pattern as shown in **Figure 2(A)-(C)**. We noticed that the volume of the supernatant also fluctuated the growth rate of the bacteria. It is because of the different concentration and percentile of metabolites in 0.5 mL and 1.0 mL cell-free supernatant of each bacterial culture. Supernatant of *P. aeruginosa* (PAS) improved the growth of both *E. coli* and *S. aureus*. It might be because of the degradation of the inhibiting factors and enhancement of the growth by secreting the growth enhancing factors ([31]. In contrast to PAS, SAS (cell-free supernatant from *S. aureus* culture) turned down the growth of *P. aeruginosa*, only promoting the growth of *E. coli* (**Figure 2(B)** & **Figure 2(C)**). Interestingly, the result reported by *S. aureus* with the ECS (cell-free supernatant from *E. coli* culture) had shown an increase in the turbid-

ity, however; it provided the negative impact on growth of *P. aeruginosa* (**Figure 2(B)** & **Figure 2(C)**). Therefore, exofactors of PAS appeared to be growth enhancers to *E. coli* and *S. aureus*, while SAS and ECS appeared to support the growth of *E. coli* and *S. aureus* respectively (**Figure 2(B)** & **Figure 2(C)**), which was a similar result from previous study performed by *Culotti et al.* [31]. However, SAS and ECS did not support for the growth of *P. aeruginosa*. Results provided the information that *E. coli* can get more benefitted *from S. aureus* in a multispecies biofilm. However, they were not supportive of the growth of *P. aeruginosa*, indicating their antibacterial activities to *P. aeruginosa*. Instead, *P. aeruginosa* supported their growth.

Susceptibility experiments were performed to know how the exofactors in cell-free supernatants fluctuate the bacterial response to the antimicrobial drugs compared to the controls (cultures without cell-free supernatants) (**Figure 4(A)**). *E. coli* was resistant to clindamycin and ampicillin, *S. aureus* to erythromycin and *P. aeruginosa* to ampicillin and erythromycin, which were the similar results from previous studies (**Figure 4(A)** and **Figure 4(C)**) [33] [34] [35]. They were susceptible to polymyxin B, levofloxacin, and cefoxitin. Results had shown that there was not any significant change on levofloxacin and cefoxitin susceptibility by *E. coli* and *P. aureus* after their growth in the presence of SAS, PAS and ECS except by *P. aeruginosa*, which developed resistivity after its growth with ECS (**Figure 3** and **Figure 4**). It provided the significant information that ECS did not support for the growth of *P. aeruginosa*, but it increased its resistivity to cefoxitin and polymyxin B. *S. aureus* developed resistivity to erythromycin by ECS. SAS, however, did not have a significant impact on bacterial resistivity to the drugs. Instead, it seemed to increase *P. aeruginosa* resistivity to the clindamycin (**Figure 3** and **Figure 4**).

Overall, this study provides the information about the role of exofactors in transforming the properties of bacterial microcolonies in biofilm to adapt the new environment with the impact on growth and antibacterial susceptibility results. It delivers the message that co-existence of different bacterial species in biofilm alters the potentiality of the pathogenic bacteria with the outcomes of more severe in pathogenicity and antibacterial resistivity, challenging to cure patient, compared to the single-species biofilm. On the other hand, the co-existence of the different bacterial genera in biofilm might be supportive of suppressing the one-type bacteria by another-type bacteria, as we got the result where SAS inhibited the growth of *P. aeruginosa*.

5. Conclusion

The treatment of infections, especially those associated with biofilm, continues to be a significant challenge. The biofilm from single species of the bacteria has already shown resistance to many antibacterial drugs, which are commonly recommended. The biofilm from different bacterial genera or multispecies have broadened their resistivity to antibacterial drugs, creating severe health prob-

lems. In certain circumstances, biofilm can control the overgrowth of the certain bacteria, which might be resistant to multi-drugs. However, the probability is very minimal since the bacteria will acquire resistance rapidly in their alternate lifestyle in biofilm. To minimize the multi-species or multi-genera biofilm formation, it is essential to apply the appropriate strategy of sterilization to surgical instruments or catheters together with appropriate usage of antibacterial drugs in a synergistic approach to target all the microcolonies of single- or multispecies in biofilm. Further work is necessary to reveal the exofactors present in the supernatant of different biofilm-making bacteria which precisely play a significant role directly or indirectly through various signaling pathways to increase or decrease the growth as well as their impact on anti-bacterial drug responses by other biofilm-associated bacteria.

Acknowledgements

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Contributions

G. R. conceived the idea of the study. V.N & B. P. performed the experiments. G.R., V.N. and B.P analyzed the data. V.N. and B.P. contributed equally.

Ethical Approval

Not required.

Use of AI Tools Declaration

Authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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