INTRODUCTION

The majority of studies on bacterial infections have concentrated on single species, but groups of co-infecting microorganisms are responsible for a lot of infections. Awareness of the significance of mixed infections is increasing nowadays, particularly in biofilms where numerous bacterial species interact, communicate, and compete with one another. The most prevalent bacterial pathogens, *P. aeruginosa* and *S. aureus*, have developed a complex network of evasion, counter-inhibition and subjugation in their fight for nourishment and space. Their interactions with each other, which are strain- and environment-specific, exhibit fierce competition that is typically associated with worse patient outcomes, as in the case of cystic fibrosis or wound infections. ¹

The primary cause of most chronic infections are biofilms, which are bacterial communities having distinct properties from free-living planktonic cells. Biofilms exhibit various characteristics including: differential efflux pumps’ expression, a self-produced extracellular polymeric matrix (EPS) outside the cells, an elevated antimicrobial resistance and the capacity to evade the host defense mechanisms ²-³.

Clinicians and researchers are prioritizing the prevention and management of biofilm in wounds, which is connected to the heightened awareness worldwide being paid to antimicrobial stewardship due to the rise in the prevalence of bacteria that are multidrug resistant ⁴. Most wounds have biofilms, which are known to contain a variety of bacterial species; their prevalence is thought to have a negative impact on wound healing. The diagnosis and management of biofilms in wounds are supported by a variety of guidelines, however in clinical practice, biofilm detection is challenging ⁴, ⁵. It is currently unclear how bacterial contact affects the progression and severity of co-infected wounds, from which *S. aureus* and *P. aeruginosa* are typically isolated ⁶.

Research has demonstrated a connection between *S. aureus* and *P. aeruginosa* in cystic fibrosis (CF), lung infection models ⁷. The interaction of 2 organisms in a chronically infected lesion is less well characterized. *S. aureus* and *P. aeruginosa* frequently co-colonize chronic wounds, and once a wound becomes infected with *P. aeruginosa*, it becomes extremely difficult to cure ⁶.

*P. aeruginosa* was found to be the primary pathogen in infections involving both *S. aureus* and *P. aeruginosa* in vitro co-culture ⁸, where it could inhibit *S. aureus* in both planktonic and biofilm forms ⁹. *P. aeruginosa’s* effect on *S. aureus* has received the majority of attention in investigations on these interactions ¹⁰,¹¹. For example, substances released by *P. aeruginosa* may impede the growth of *S. aureus*, affecting the sensitivity of *S. aureus* to antibiotics ¹². Exoproducts of *P. aeruginosa* have the ability to lyse *S. aureus* and take its iron

### ABSTRACT

**Background:** *Pseudomonas aeruginosa* and *Staphylococcus aureus* coexist in wounds causing severe illness. **Objectives:** This research was conducted to study the impact of *S. aureus* extract on *P. aeruginosa*. **Methodology:** Cell free extracts of four strains of *S. aureus* isolated from wounds were used to estimate their effect on biofilm formation, antibiotic resistance, and cytotoxicity of three clinical *P. aeruginosa* isolates from mixed wound infections. **Results:** Results disclosed that extracts of *S. aureus* significantly decreased biofilm formation and cytotoxicity in the tested *P. aeruginosa* isolates. Antibiotic resistance was assessed using three antibiotics; gentamicin, amikacin and imipenem. This is due to their higher resistance rates. Cell free extracts of *S. aureus* reduced antibiotic resistance of the tested *P. aeruginosa* isolates to aminoglycosides (Gentamicin and Amikacin) by (2-3) folds, while imipenem resistance was not changed. **Conclusion:** Based on this work’s findings, *S. aureus* could affect *P. aeruginosa* in mixed wound infections through reduction of biofilm formation, cell cytotoxicity and antibiotic susceptibility.
reserves for growth. Therefore, _P. aeruginosa_ exoproducst mediate the majority of reported interactions between these two species. Information on how _S. aureus_ affects _P. aeruginosa_ biofilm development and resistance to antibiotics are still limited. _S. aureus_ produces a vast array of virulence factors including adhesins, enzymes, polysaccharides and peptides that may have impact on other bacteria and the host.

The aim of this study was to investigate if _S. aureus_ could affect _P. aeruginosa_ behaviors crucial to chronic infection, such as biofilm formation, antibiotic susceptibility and cytotoxic effect.

**METHODOLOGY**

**Bacterial isolates and Growth Medium**

The study has been approved by the institutional review board of Princess Nourah bint Abdulrahman University (PNU) (IRB Log Number: 21-0105), Riyadh, Saudi Arabia. Three _P. aeruginosa_ clinical isolates of and nine _S. aureus_ isolated from wound infections were used throughout this study. Tryptic soy broth (TSB) (Merck, Germany), mannitol salt agar (MSA) (Merck, Germany), and cetrimide agar (CA) (Merck, Germany) were used to cultivate both organisms. In nutrient broth (Oxoid), isolates were grown aerobically at 37°C. The bacterial strains were kept in stocks of 10% (V/V) glycerol at −80°C. Gentamicin, Amikacin, and Imipenem in Mueller Hinton broth (Oxoid, Basingstoke, UK). 

**Preparation of _S. aureus_ cell free extracts**

The 9 used cultures of _S. aureus_ were incubated in BHI medium at 37°C while being shaken at 150 rpm. After the culture biomass was adjusted to 10^8 CFU/mL, cell free extracts were created by centrifuging the sample at 10,000 rpm for 10 minutes at 4°C. The cell free extracts were next filtered through a 0.2 µm syringe filter and kept at −80°C until they were used.

**Mixed culture biofilm assay**

Microtiter plate assay was used to determine biofilm formation according to Fugère et al. For mixed culture biofilm, 100µl of the 3 tested Pseudomonas isolates were inoculated in a 96-well polystyrene plate with 100µl of cell free supernatants of the 9 used _S. aureus_ isolates. For control, 100µl of the three _P. aeruginosa_ of single species were inoculated in a polystyrene plate (96-well microtiter plate) with 100µl of sterile TSB medium. After incubation at 37°C for 24h, each well content was aspirated, and rinsed with 200 µl of PBS (pH 7.4) three times. To get rid of any non-adherent cells, the plates were shaken ferociously. The remaining attached bacteria were fixed for 15 minutes with 150 µl per well of absolute methanol before being stained for 20 minutes by 150 µl of crystal violet (1% w/v) per well. After that, 150 µl of 33% (v/v) glacial acetic acid per well was used to dissolve the bound dye to the adherent cells. The absorbance was interpreted at wavelength 492 nm using microplate reader (BIO RAD – xMark™ Microplate spectrophotometer).

**Determination of Minimum Biofilm Inhibitory Concentration (MBIC)**

The three tested _P. aeruginosa_ isolates recovered from single- and dual-species biofilms were evaluated for antimicrobial susceptibility by microbroth dilution method using CLSI 2018 guidelines. Due to high rates of resistance, aminoglycosides and carbapenems as gentamicin, amikacin, and imipenem were chosen. One hundred microliter inocula of both single and dual species of 1.5x10^8 CFU/mL TSB cultures were incubated for 24h at 37°C in 96-well microplates (Thermo Fisher Scientific, USA). After incubation, the wells were twice rinsed with sterile normal saline solution after the supernatant was removed. The wells containing the developed biofilms were then added 100 l of two-fold serial dilutions of the antibiotics Gentamicin, Amikacin, and Imipenem in Mueller Hinton broth (Oxoid, Basingstoke, UK). MBIC was measured following an 18-hour incubation period at 37°C as the lowest concentration of an antibiotic that shows no growth. MIC was also determined for _P. aeruginosa_ isolates as single species.

**MTT cytotoxicity assay**

The MTT assay, as described by Mishra et al., was done to evaluate the cytotoxicity of both single and dual species on human liver carcinoma cells. In 96-well plates, 1×10^4 of HepG2 cells were inoculated in 0.1 mL of DMEM medium supplemented with 10% FBS and incubated in a humidified incubator (37 °C in 5% CO₂) for 24 h till reaching full confluence. As a control, 100 µl of a single _P. aeruginosa_ isolate was used, and 100 µl of (1:1) _P. aeruginosa_ and _S. aureus_ cell free extracts were added and cultured for 24 hours. After discarding the medium, 10 µl of tetrazolium dye (MTT) was incubated in a 1 mg/mL PBS solution. Each well received 100 µl of DMSO to solubilize the dark blue formazan crystals. Results were obtained at 570 nm using a microplate reader. MTT solution with DMSO served as a blank control for the microplate reading, while PBS-treated cells served as a 100% viability control. The cytotoxicity was calculated as 100% against control cells.

**Statistical Analysis**

GraphPad Prism was used to conduct the statistical analysis. The differences between the control and _S. aureus_-treated isolates were compared using a one-way ANOVA, and then a Tukey-Kramer post-hoc test was run. A P value of 0.05 or lower was determined statistically significant.
RESULTS

Bacterial isolates and Biofilm Formation Assay

Nine staphylococcus isolates were utilized to detect their effect on biofilm formation of Pseudomonas aeruginosa in mixed culture. Due to their significant impact on P. aeruginosa biofilm development in mixed culture, four S. aureus isolates were chosen to complete the investigation. It was observed that extracts of the four S. aureus isolates can reduce the biofilm formation of the three tested P. aeruginosa in mixed culture compared to the single species biofilm as shown in Figure (1). Tukey-Kramer as post-hoc test demonstrated that biofilm reduction caused by the four S. aureus extracts was significant correlated with the control single Pseudomonas biofilm (P < 0.0001). Percentages of biofilm reduction of the three P. aeruginosa isolates is shown in table (1).

Table 1: Percentages of biofilm reduction of P. aeruginosa caused by the 4 S. aureus extracts

<table>
<thead>
<tr>
<th>S. aureus isolates</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>41%</td>
<td>74%</td>
<td>71%</td>
</tr>
<tr>
<td>S2</td>
<td>22%</td>
<td>61%</td>
<td>64%</td>
</tr>
<tr>
<td>S3</td>
<td>31%</td>
<td>74%</td>
<td>68%</td>
</tr>
<tr>
<td>S4</td>
<td>22%</td>
<td>39%</td>
<td>77%</td>
</tr>
</tbody>
</table>

Table 2: Percentages of Cytotoxicity reduction of P. aeruginosa caused by the 4 S. aureus extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Cytotoxicity</th>
<th>% Cytotoxicity Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 single</td>
<td>15.89</td>
<td>Control</td>
</tr>
<tr>
<td>P1+S1</td>
<td>2.38</td>
<td>85%</td>
</tr>
<tr>
<td>P1+S2</td>
<td>7.58</td>
<td>52%</td>
</tr>
<tr>
<td>P1+S3</td>
<td>5.99</td>
<td>62%</td>
</tr>
<tr>
<td>P1+S4</td>
<td>12.43</td>
<td>22%</td>
</tr>
<tr>
<td>P2 single</td>
<td>18.55</td>
<td>Control</td>
</tr>
<tr>
<td>P2+S1</td>
<td>11.14</td>
<td>40%</td>
</tr>
<tr>
<td>P2+S2</td>
<td>12.64</td>
<td>32%</td>
</tr>
<tr>
<td>P2+S3</td>
<td>12.4</td>
<td>33%</td>
</tr>
<tr>
<td>P2+S4</td>
<td>10.68</td>
<td>42%</td>
</tr>
<tr>
<td>P3 single</td>
<td>17.62</td>
<td>Control</td>
</tr>
<tr>
<td>P3+S1</td>
<td>9.95</td>
<td>44%</td>
</tr>
<tr>
<td>P3+S2</td>
<td>6.73</td>
<td>62%</td>
</tr>
<tr>
<td>P3+S3</td>
<td>5.86</td>
<td>67%</td>
</tr>
<tr>
<td>P3+S4</td>
<td>11.725</td>
<td>33%</td>
</tr>
</tbody>
</table>

Determination of Minimum Biofilm Inhibitory Concentration (MBIC)

Susceptibilities to Gentamicin, Amikacin and Imipenem antibiotics were investigated in the tested P. aeruginosa isolates as single species and also recovered from the co-culture. Resistance to Gentamicin and Amikacin was declined, and its MIC decreased (2-3 times) for the biofilm coculture conditions compared to single culture. Regarding Imipenem, resistance of the tested isolates in coculture isolates showed no change compared to those of single culture.

MTT toxicity assay

On HepG2 hepatocarcinomal cells, the single species of P. aeruginosa utilized has a more potent cytotoxic effect than the mixed culture. It was observed that cytotoxic effect of the tested P. aeruginosa was declined after treatment with S. aureus supernatants compared to the cytotoxicity of the single species as shown in Figure (2). Also, percentage of reduction of cytotoxicity compared to the control single species of the 3 Pseudomonas isolates was measured and reduction was significant with P value < 0.0001 as shown in table (2).
isolates were initially studied, but four *S. aureus* isolates were ultimately utilized because of their significant impact on *P. aeruginosa* biofilm formation in coculture. As in other research, *P. aeruginosa* completely outcompetes *S. aureus* when the two bacteria are coinoculated at a 1:1 ratio, hence in this study we studied the influence of *S. aureus* cell free extracts on *P. aeruginosa* biofilm development.\(^{11,21}\) We found that *P. aeruginosa* biofilm was significantly decreased in the tested three isolates by the effect of the 4 used *S. aureus* extracts when compared with the single culture. Also, the antibiotic susceptibility to aminoglycosides (gentamicin and amikacin) was decreased (2-3 fold) in the tested isolates compared to single Pseudomonas culture, while imipenem resistance remains the same. This might be explained by *S. aureus' influence on the quorum sensing and metabolism of the studied isolates of *P. aeruginosa*, as demonstrated by Dehbashi et al.\(^{22}\)

According to Dehbashi et al.\(^{22}\), the bacterial biomass must reach a certain level (quorum) in order for a biofilm to form. The two component systems (TCS) are activated once the QS systems detect the inducer chemicals, which changes the gene expression, pathogenicity, and biofilm formation. Similar to LasI/R, rhlI/R, and PQS in *P. aeruginosa*, the agr system in *S. aureus* plays a crucial role in QS. *Crc* regulates virulence factors mediated by QS, motility, antibiotic resistance, and carbon metabolism as a catabolite repression regulator. For example, *crc* mutants displayed sensitivity to rifampin, lactams, and aminoglycosides.\(^{23}\) Dehbashi et al.\(^{22}\) found that the MIC of amikacin decreased as the amount of *crc* expression was noticeably reduced. However, the hyper-activation of OpfD brought on by the inactivation of *crc* resulted in imipenem resistance.\(^{23}\) Orazi and O'Toole\(^{24}\) claim that *P. aeruginosa* and *S. aureus* interacting in coculture settings changes the former's metabolic pathway and causes it to shift to fermentative growth leading to less antibiotic resistance.

Additionally, we looked at how *S. aureus* extracts affected the cytotoxicity of *P. aeruginosa*. results showed that *P. aeruginosa* as single species exhibited the highest cytotoxicity when compared to dual species after mixing with the 4 *S. aureus* extracts. According to table (2), results revealed that cytotoxicity related to the single species (control) was reduced in isolate P1 from (85-22%) by the effect of the 4 *S. aureus* extracts, while % of cytotoxicity reduction in P2 isolate ranged from (42-32%) and reduction in P3 was from (67-33%). Reduction in cytotoxicity was significant in the 3 *P. aeruginosa* isolates related to the single species. Our findings are consistent with those of Yang et al.\(^{25}\), who showed that *S. aureus* significantly inhibited *P. aeruginosa* pathogenicity and biofilm development in an in vivo co-culture.
CONCLUSION

According to our previous findings, *S. aureus* cell free extracts have an effect on *P. aeruginosa* isolates from mixed wound infections. It is suggested in future work to study the effect of *S. aureus* quorum sensing molecules on metabolism and expression of virulence genes of *P. aeruginosa* from mixed wound infections.

Declarations:

Consent for publication

Not applicable.

Availability of data and material

Data are available upon request.

Competing interests

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REFERENCES


