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ORIGINAL ARTICLE

The Impact of *Staphylococcus aureus* on Biofilm, Antibiotic Resistance and Cytotoxicity *of Pseudomonas aeruginosa* Isolated from Mixed Wound Infections

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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.*

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 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

reserves for growth [13]. Therefore, *P. aeruginosa* exoproducts 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 [14].

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 antibiotics were provided by King Abdullah bin Abdulaziz University Hospital (KAAUH).

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.[15]. 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 [16]. 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 [17]. MIC was also determined for *P. aeruginosa* isolates as single species.

MTT cytotoxicity assay

The MTT assay, as described by Mishra et al.[18], 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% CO_2) 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% vitality control. The cytotoxicity was calculated as 100% against control cells [19].

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

S. aureus isolates	% of Biofilm Reduction		
	P1	P2	P3
S1	41%	74%	71%
S2	22%	61%	64%
S3	31%	74%	68%
S4	22%	39%	77%

P1, P2, and P3: the 3 tested *P. aeruginosa* isolates

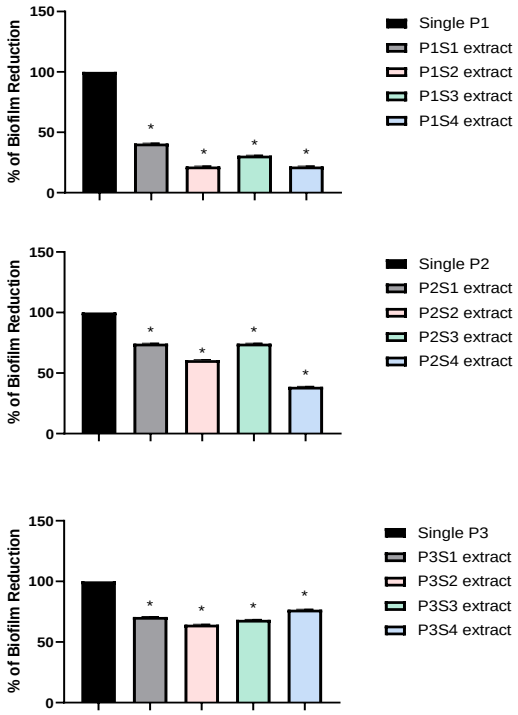

Fig. 1: Effect of four different *S. aureus* supernatants on biofilm of *P. aeruginosae* (P1, P2 & P3) in single and mixed cultures

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).

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

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

P1, P2, and P3: the 3 tested *P. aeruginosa* isolates

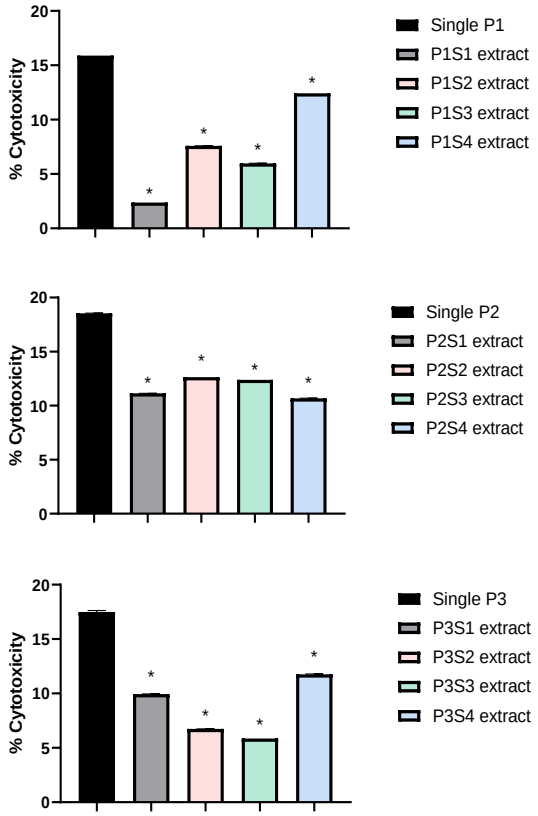

Fig. 2: Effect of four different *S. aureus* supernatants on cytotoxicity of *P. aeruginosae* (P1, P2 & P3) in single and mixed cultures.

DISCUSSION

Microorganisms are prevented from invading the underlying tissues and organs by the skin, which serves as an essential protective barrier. Any damage to the skin's integrity, including wounds, can let microbes get into the underlying tissues and possibly leads to an infection [20]. Multiple bacterial species cohabit in a single environment to form biofilms, which are common in nature. The relationships between these bacterial species or the roles they play in these multi-species biofilms are not well understood, yet. As a result, it is essential to research these interactions under circumstances that are similar to those seen in vivo. Even though co-culturing them in the lab has proven to be challenging, *S. aureus* and *P. aeruginosa* are regularly isolated from wounds and the lungs of CF patients.

This study focused on how *S. aureus* affected *P. aeruginosa* when co-cultured together. Here we studied the effect of cell free extracts of nine *S. aureus* clinical isolates on three clinical *P. aeruginosa* isolates. Both organisms were isolated from wounds. Nine *S. aureus* 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 co-inoculated 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. aeruginaosa* 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 OprD 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 co-culture 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 Pseudomonas isolates. 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

The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article none.

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REFERENCES

1. Hotterbeekx A, Kumar-Singh S, Goossens H, and Malhotra-Kumar S. In vivo and in vitro interactions between *Pseudomonas aeruginosa* and Staphylococcus spp. Frontiers in Cellular and Infection Microbiology. 2017; 7(106) https://doi.org/10.3389/fcimb.2017.00106
2. Bjarnsholt T, Alhede M, Alhede M, Eickhardt-Sørensen SR, Moser C, Kühl M, et al. The in vivo biofilm. Trends Microbiol. 2013. 21, 466–474. doi: 10.1016/j.tim.2013.06.002
3. Kostakioti M, Hadjifrangiskou M, and Hultgren SJ. Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the post antibiotic era. Cold Spring Harb. Perspect. Med. 2013. 3, 1–24. doi: 10.1101/ cshperspect. a010306
4. World Union of Wound Healing Societies. Position Document: Management of Biofilm. London: Wounds International. 2016.
5. Metcalf DG, and Bowler PG. Clinician perceptions of wound biofilm. Int Wound J 2016; .13:717–25.
6. Serra R, Grande R, Butrico L, Rossi A, Settimio UF, Caroleo B, Amato B, Gallelli L, and De Franciscis S. Chronic wound infections: the role of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Expert Rev Anti Infect Ther. 2015; 13:605–13.
7. Maliniak ML, Stecenko AA, and McCarty NA. A longitudinal analysis of chronic MRSA and *Pseudomonas aeruginosa* coinfection in cystic fibrosis: A single-center study. J Cyst Fibros. 2016; 15:350–6.
8. Seth AK, Geringer MR, Galiano RD, Leung KP, Mustoe TA, and Hong SJ. Quantitative comparison and analysis of species-specific wound biofilm virulence using an in vivo, rabbit-ear model. J. Am. Coll. Surg.2012. 215, 388–399. https://doi.org/10.1016/j.jamcollsurg.2012.05.028.
9. DeLeon S, Clinton A, Fowler H, Everett J, Horswill AR, and Rumbaugh KP. Synergistic interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an in vitro wound model. Infect. Immun. 2014 Nov;82(11):4718-28. https://doi.org/10.1128/iai.02198-14.
10. Billings N, Millan M, Caldara M, Rusconi R, Tarasova Y, Stocker R, and Ribbeck K. The extracellular matrix component Psl provides fastacting antibiotic defense in *Pseudomonas aeruginosa* biofilms. PLoS Pathog. 2013. 9:e1003526. http://dx.doi.org/10.1371/journal.ppat.1003526.
11. Filkins LM, Graber JA, Olson DG, Dolben EL, Lynd LR, Bhuju S, and O'Toole GA. Coculture of *Staphylococcus aureus* with *Pseudomonas aeruginosa* drives S. aureus towards fermentative metabolism and reduced viability in a cystic fibrosis model. J Bacteriol. 2015. 197:2252–2264. http:// dx.doi.org/10.1128/JB.00059-15.
12. Hoffman LR, Déziel E, D'Argenio DA, Lépine F, Emerson J, McNamara S, Gibson RL, Ramsey BW, and Miller SI. Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of Pseudomonas aeruginosa. Proc Natl Acad SciUSA. 2006. 103:19890 –19895. http:// dx.doi.org/10.1073/pnas.0606756104.
13. Mashburn LM, Jett AM, Akins DR, and Whiteley M. *Staphylococcus aureus* serves as an iron source for Pseudomonas aeruginosa during in vivo coculture. J Bacteriol. 2005. 187:554 –566. http://dx.doi.org/10.1128/ JB.187.2.554-566.
14. Foster TJ. Immune evasion by Staphylococci. Nat. Rev. Microbiol. 2005. 3, 948–958. https://doi.org/10.1038/nrmicro1289.
15. Fugère A, Séguin DL, Mitchell G, Déziel E, Dekimpe V, et al. Interspecific small molecule interactions between clinical isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* from adult cystic fibrosis patients. PLOS ONE. 2014. 9(1): e86705.
16. Clinical and Laboratory Standard Institute. 2018. M100 Performance Standards for Antimicrobial Susceptibility Testing 28th edn, (Clinical and Laboratory Standards Institute: Wayne, PA).
17. Salem AH, Elkhatib WF, and Noreddin A.M. "Pharmacodynamic assessment of vancomycin-rifampicin combination against methicillin resistant *Staphylococcus aureus* bioflm: A parametric

response surface analysis," Journal of Pharmacy and Pharmacology. 2011. 63(1), 73–79.

18. Mishra N, Rana K, Seelam SD, Kumar R, Pandey V, Salimath BP, and Agsar D. Characterization and Cytotoxicity of Pseudomonas Mediated Rhamnolipids Against Breast Cancer MDA-MB-231 Cell Line. Front Bioeng Biotechnol. 2021 Nov 30; 9:761266. doi: 10.3389/fbioe.2021.761266. PMID: 34950641; PMCID: PMC8691732.
19. Byrappa S, Harsha Raj M, Kungyal T, Kudva N, and Narayana U. Synthesis and Biological Evaluation of Novel Isoxazolines Linked via Piperazine to 2- Benzoisothiazoles as Potent Apoptotic Agents. Eur. J. Med. Chem. 2017. 126, 218–224. doi:10.1016/j.ejmech.2016.09.094
20. Dong YH, Zhang XF, and Zhang LH. The global regulator Crc plays a multifaceted role in modulation of type III secretion system in *Pseudomonas aeruginosa*. Microbiology Open. 2013. 2, 161–172.
21. Baldan R, Cigana C, Testa F, Bianconi I, De Simone M, Pellin D, et al. Adaptation of *Pseudomonas aeruginosa* in cystic fibrosis airways influences virulence of *Staphylococcus aureus* in vitro and murine models of co-infection. PLoS One. 2014. 9: e89614. doi: 10.1371/journal.pone.0089614
22. Dehbashi S, Pourmand MR, Alikhani MY, Asl SS, and Arabestani MR. The effect of *Staphylococcus aureus* on the antibiotic resistance and pathogenicity of *Pseudomonas aeruginosa* based on crc gene as a metabolism regulator: An in vitro wound model study. Infect Genet Evol. 2020 Nov; 85:104509. doi: 10.1016/j.meegid.2020.104509. Epub 2020 Aug 21. PMID: 32835876.
23. Linares JF, Moreno R, Fajardo A, Martinez-Solano L, Escalante R, Rojo F, and Martinez JL. The global regulator *Crc* modulates metabolism, susceptibility to antibiotics and virulence in *Pseudomonas aeruginosa*. Environ. Microbiol. 2010. 12, 3196–3212.
24. Orazi G, and O'Toole GA. *Pseudomonas aeruginosa* Alters *Staphylococcus aureus* sensitivity to vancomycin in a biofilm model of cystic fibrosis infection. mBio. 2017. 8:e00873-00817. https://doi.org/10.1128/mBio.00873-17
25. Yang L, Liu Y, Markussen T, Hoiby N, Tolker-Nielsen T, and Molin S. Pattern differentiation in co-culture biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. FEMS Immunol Med Microbiol. 2011. 62:339–347. https://doi.org/10.1111/j.1574-695X.2011.00820.x