# CELL-FREE SUPERNATANT OF STAPHYLOCOCCUS AUREUS CULTURE INCREASES *ANTIMICROBIALS SUSCEPTIBILITY* OF *PSEUDOMONAS AERUGINOSA*

*M.S. Fedorova, A.V. Mironova, A.R. Kayumov, E.Y. Trizna**

Kazan Federal University, 18 Kremlyovskaya St., Kazan, 420008, Russia

* Corresponding author: trizna91@mail.ru

**Abstract.** Along with the wide spread of bacterial antibiotic resistance over the world, the treatment efficiency of infectious disease is greatly affected by the mixed biofilm formation by pathogenic bacteria. *Staphylococcus aureus* and *Pseudomonas aeruginosa*, a frequent cause of nosocomial infections, exhibit both synergistic and antagonistic interactions in co-culture, leading to various changes in the metabolic profile of bacteria, which in turn affect their sensitivity to antimicrobials. Here we show that *S. aureus* cell-free culture liquid exhibits bacteriostatic properties and increases the efficacy of antimicrobials against *P. aeruginosa*. Thus, the MICs of amikacin, gentamicin, and ciprofloxacin decreased 2-4 fold in the presence of cell-free supernatant of *S. aureus* 24 h culture. Furthermore, the combination of the latter with antimicrobials increased the efficacy of amikacin up to 64-fold. Thus, the combined use of cell-free culture liquid of *S. aureus* with broad-spectrum antibiotics can be used to increase the effectiveness of antimicrobial therapy of *P. aeruginosa*.

**Keywords:** biofilm, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, culture liquid, antibiotics, antibiotic resistance, synergy.

## Introduction

Over the world, diseases caused by biofilms are difficult to treat since bacteria in biofilms exhibit increased resistance to antimicrobials (Ding *et al.*, 2021; Beaudoin *et al.*, 2017; Briaud *et al.*, 2019). To date, it is believed that rather multispecies than monobacterial biofilms are formed during the development of infection. The multispecies communities are characterized by a different metabolic profile and properties in contrast to their monospecific counterparts (Harrison *et al.*, 2020; Cendra *et al.*, 2019). These changes may affect the sensitivity of bacteria to antibiotics (Hall *et al.*, 2017; Uruén *et al.*, 2020; Luo *et al.*, 2021; Singh *et al.*, 2021). Since very few antimicrobial agents effective against infections associated with the formation of biofilms are available, the discovery of new therapeutic strategies to combat biofilms is a modern challenge in medicine (Simões *et al.*, 2021; Xuan *et al.*, 2021; Makabenta *et al.*, 2021). In patients with pneumonia, the most frequently isolated types of opportunistic pathogens are *Staphylococcus aureus* and *Pseudomonas aeruginosa*. These are the most common multidrug-resistant pathogens and exhibit both synergistic and antagonistic interactions in mixed biofilms (Behzadi *et al.*, 2021; Little *et al.*, 2021; Cheung *et al.*, 2021).

The antimicrobial peptides (AMPs), which can act individually and increase the activity of antibiotics, seem to be one of the alternatives to antimicrobial therapy (Grassi *et al.*, 2017; Portelinha *et al.*, 2021). *S. aureus* is able to produce AMPs (aureocins), which have high bactericidal activity and increase the activity of known antibiotics against various microorganisms (Ceotto *et al.*, 2012). We have shown previously that the sensitivity of *P. aeruginosa* to broad-spectrum antibiotics increases in the *S. aureus* - *P. aeruginosa* mixed community (Trizna *et al.*, 2020). In the present study, we show that *S. aureus* cell-free culture liquid exhibits bacteriostatic properties and increases the efficacy of antimicrobials against *P. aeruginosa*.

## Materials and Methods

*Bacterial strains and growth conditions*

*S. aureus* ATCC 29213 (Museum Strain of the American Collection of Microorganisms), *P. aeruginosa* ATCC 27853 (Museum Strain of the American Collection of Microorganisms) were used in this study. Bacteria were grown in

flasks with a medium: flask volumes ratio of 1:7.5 with a shaking intensity of 200 rpm at 37 °C. To obtain biofilms, bacteria were grown for 48 hours under static conditions at 37 °C in Basal medium broth (BM) (glucose 5g, peptone 7g, $MgSO_4 \times 7H_2O$ 2.0g and $CaCl_2 \times 2H_2O$ 0.05g in 1.0 liter tap water) in plates with an initial density of bacterial culture of $1 \times 10^6$ CFU/ml.

*Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)*

MICs of antimicrobials were determined by microdilution approach in BM broth according to EUCAST recommendations (Leclercq *et al.*, 2013). Antibiotics were diluted with broth in a 96-well plastic plate (Eppendorf Cell Culture Plates) at concentrations of 0.25–512 μg/mL. The wells were inoculated with 200 μL of bacterial culture ($2–9 \times 10^5$ CFU/mL) in BM and incubated at 37°C under static conditions. The minimum inhibitory concentration was defined as the lowest concentration of antibiotic at which no bacterial growth was observed after 24 hours of incubation. Next, the minimum bactericidal concentration (MBC) was determined. For that, a 1000× culture dilution was made from wells with no visible growth and incubated for 24 hours in broth without any antimicrobials. The MBC was taken as the lowest concentration of the substance at which bacterial growth was completely absent at 24 hours of incubation.

*Determination of the permeability of the extracellular matrix of biofilms for antibacterial substances (Anderl et al., 2000)*

The mono- and polymicrobial biofilms were obtained on sterile nitrocellulose discs. The bacterial suspension with density of $3 \times 10^7$ CFU/ml in BM broth were dropped on discs which were placed on plates with LB agar and incubated for 48 hours at 37°C. Then disks were transferred onto a new LB agar plate containing an antibiotic at a concentration corresponding to 1×MBC for corresponding bacterium. A smaller membrane disk moistened with BM broth was placed on the disks with biofilms. Finally, 6-mm Whatman disks were laid out on the surface of upper membrane, allowing absorbing the antibiotic diffusing from the nutrient medium through biofilm. After 24 hours incubation at 37 °C, the Whatman disks were placed on new plates with bacterial culture spread on the surface of LB agar. After 24-hour incubation, the growth repression zones were measured. As a control, discs were placed on membranes with biofilms and kept in a medium without antibacterial substances. To assess the effect of antibiotics themselves on bacterial cultures, discs were incubated on sterile membranes without bacterial biofilms.

*Obtaining cell-free culture liquid*

*S. aureus* cells were grown for 24 hours in LB broth with a shaking intensity of 200 rpm at 37 ºC. Next, cells were removed by centrifugation for 15 minutes at 12000 rpm at 37 °C. The supernatant was filtered using sterile Minisart High Flow, 0.2 μm filters.

*Alamar-blue test*

To evaluate the bacterial viability, 100 μl of bacterial suspension was transferred into a 96-well plate. The biofilms were resuspended in 0.9% NaCl by mechanical scratching. The resazurin sodium salt (Sigma) solution was added to the culture liquid to a final concentration of 120 μM and incubated for 10 min. In the presence of a pink color, the bacteria were identified as viable. The blue color indicated death cells.

*CFUs count*

The CFUs count was assessed by drop plate assay (Herigstad *et al.*, 2001) with modifications (Baidamshina *et al.*, 2017). Briefly, a serial 10-fold dilutions of the bacterial suspension were prepared in 0.9% NaCl. For cells in biofilms, 0.9% NaCl was added to the wells and bacteria were suspended by scratching the well bottoms with subsequent treatment in an ultrasonic bath for 2 min to facilitate the disintegration of bacterial clumps. Then 5 μl from each dilution was dropped onto LB agar plate and incubated for 24 hours at 37 °C. CFUs were counted from dilutions containing 5–10 colonies.

*Statistical analysis*

Experiments were performed in three biological replicates with three technical repetitions in each. The statistical significance of differences in determining the number of colonies by counting CFU from a series of dilutions was evaluated by the formula 10 log10[c], where c is the number of cells obtained, using Pearson's Chi-square test for homogeneity. Differences were considered significant at $p < 0.05$.

## Results

*The permeability of the extracellular matrix of S. aureus and P. aeruginosa mono- and dual species biofilms for antimicrobials*

The increased sensitivity of *P. aeruginosa* in a mixed *S. aureus–P. aeruginosa* community to broad-spectrum antibiotics has been shown previously (Trizna *et al.,* 2020). We asked whether this is a consequence of the change in the biochemical composition of the extracellular matrix of a mixed biofilm and consequent alteration in its permeability for antimicrobials. To test that, Ampicillin, vancomycin, amikacin and ciprofloxacin were added to LB agar at a concentration corresponding to respective 1×MBC (Table 1) and antibiotics diffusion through the biofilm has been assessed as described in Materials and Methods.

For the *S. aureus* and *P. aeruginosa* biofilms, no significant changes were found compared to the control, indicating a low permeability of antibiotics through the extracellular matrix of biofilms (Fig. 1). Of note, a significant inhibition of *S. aureus* growth has been observed around the discs incubated on the mixed *S. aureus-P. aeruginosa* biofilm, apparently, due to the synthesis of antimicrobial metabolites by *P. aeruginosa* in the mixed community. Thus, the change in the *P. aeruginosa* sensitivity in mixed *S. aureus - P. aeruginosa* biofilm is governed rather by the production of extracellular metabolites by *S. aureus* affecting *P. aeruginosa* than due to changes in the of the biofilm permeability.

*Effect of S. aureus cell-free culture liquid on P. aeruginosa susceptibility to antibiotics*

Some antibiotics are known to show synergy when combined with antimicrobial peptides (Grassi *et al.,* 2017). We assumed that the *S. aureus* secrets AMPs exhibiting synergistic effect with various antibiotics against *P. aeruginosa*. To test this assumption, the *S. aureus* cell-free culture liquid was diluted 4-fold with fresh LB medium in combination with various antibiotics in the concentrations of 0.5–512 μg/mL and seeded by *P. aeruginosa*. After 24h the residual viability of *P. aeruginosa* cells was assessed. In presence of 25% *S. aureus* cell-free culture liquid, the efficacy of aminoglycosides (Amikacin, Gentamicin) against *P. aeruginosa* increased 2-fold. Further, the effect of the *S. aureus* cell-free culture liquid on biofilm-embedded *P. aeruginosa* susceptibility to antibiotics was evaluated. For that, the cell-free culture liquid of *S. aureus* was diluted with fresh BM broth to final concentrations of 6% and 12% in combination with various antibiotics at concentrations of 0.25–128 μg/mL. As a control of the nutrients depletion, NaCl solution was added in the same proportions (6% and 12%). After 24h of incubation the residual viability of *P. aeruginosa* in biofilm was assessed (Table 2).

*Table 1*

**Minimum inhibitory and bactericidal concentrations of antibiotics, μg/mL**

| | ***P. aeruginosa*** | | ***S. aureus*** | |
|---|---|---|---|---|
| | MIC, μg/mL | MBC, μg/mL | MIC, μg/mL | MBC, μg/mL |
| Amikacin | 1 | 64 | 8 | 32 |
| Ciprofloxacin | 4 | 64 | 0.25 | 16 |
| Ampicillin | ND | ND | 0.5 | 64 |
| Vancomycin | ND | ND | 2 | 64 |

*Note:* ND – not determined

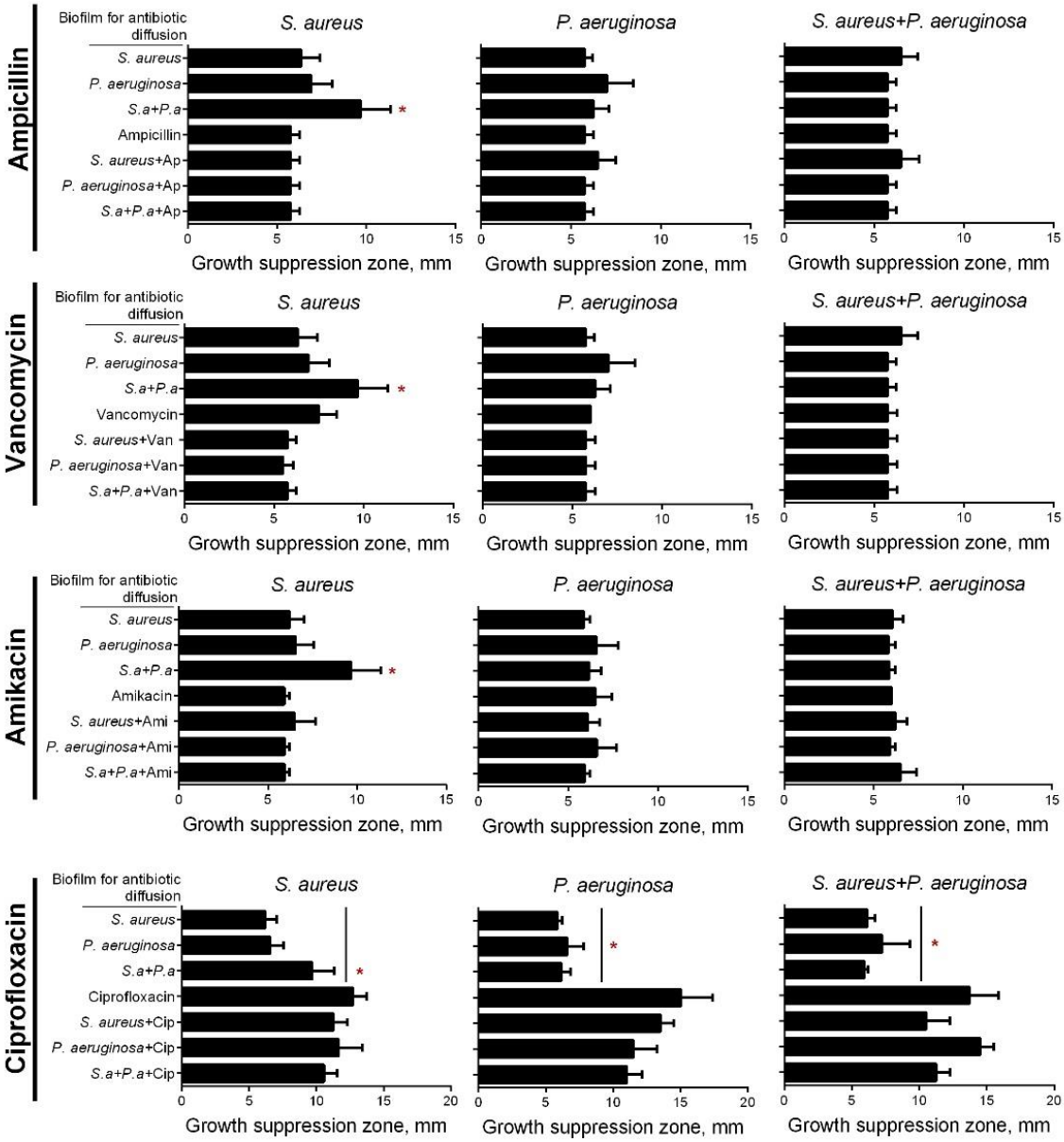


**Fig. 1.** Evaluation of the permeability of mono- and dimicrobial *S. aureus* - *P. aeruginosa* biofilms for antimicrobials

*Table 2*

**Synergism of cell-free culture liquid (CL) of *S. aureus* with antibiotics**

| Antibiotic | 6% NaCl | 6% CL | The effect increase, fold | 12% NaCl | 12% CL | The effect increase, fold |
|---|---|---|---|---|---|---|
| | MIC, μg/mL | | | MIC, μg/mL | | |
| Ciprofloxacin | 32 | 32 | 0 | 64 | 16 | 4x |
| Gentamicin | 64 | 16 | 4x | 64 | 32 | 2x |
| Amikacin | 128 | 2 | 64x | 64 | 4 | 16x |

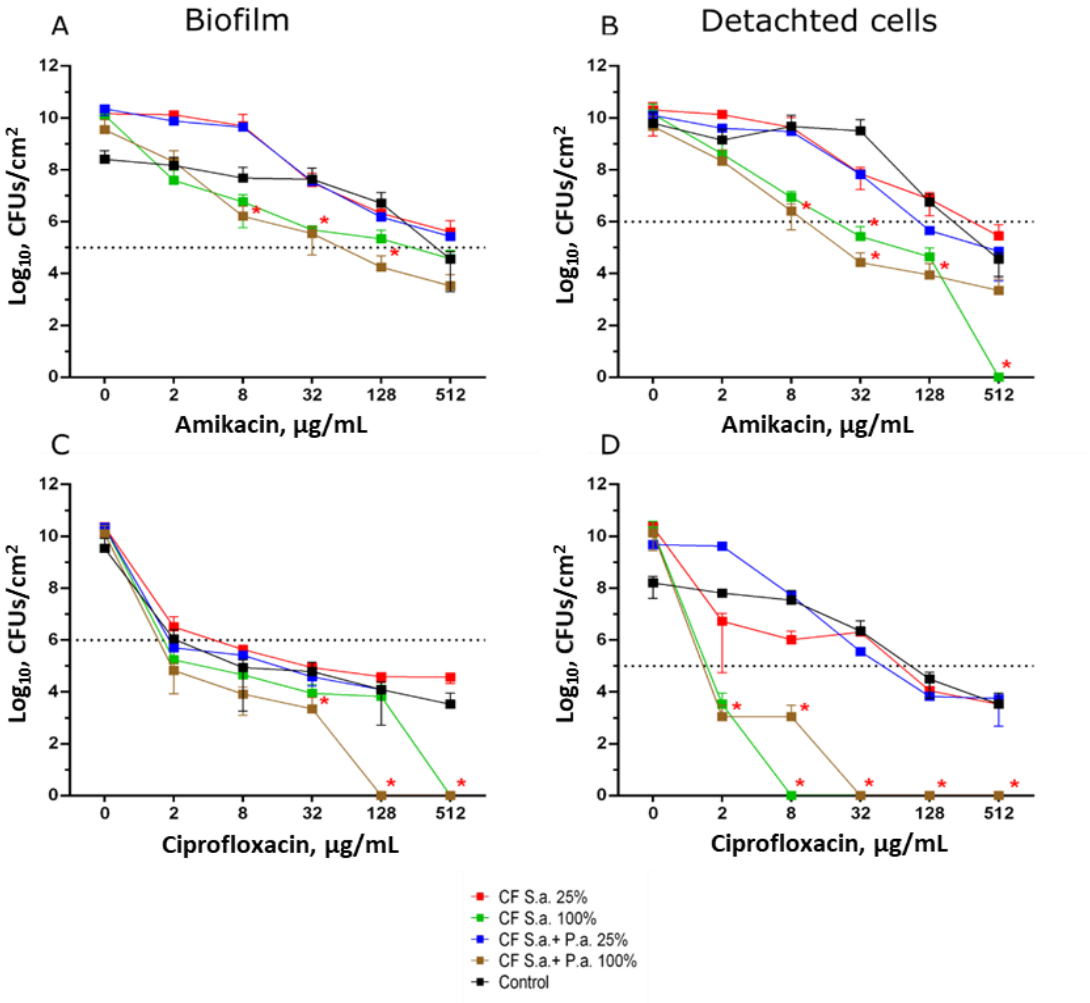


**Fig. 2.** The number of viable detached cells and biofilm-embedded cells *P. aeruginosa* in the presence of the culture liquid of *S. aureus*, *S. aureus* - *P. aeruginosa* and antibiotics. Viability was assessed by counting CFU by the serial dilution. Antimicrobials were added to 48-hour biofilms (A – 24-hour incubation with culture liquid and amikacin, cells in the biofilm; B – 24-hour incubation with culture liquid and amikacin, detached cells; C – 24-hour incubation with culture liquid and ciprofloxacin, cells in the biofilm; D – 24-hour incubation with culture fluid and ciprofloxacin, cells in the biofilm)

The cell-free culture liquid of *S. aureus* significantly increased the efficiency of the antibiotics. Thus, ciprofloxacin together with 12% culture liquid led to the death of *P. aeruginosa* at a concentration 4 times lower than the combination with saline. The effectiveness of gentamicin in combination with 6% culture liquid also increased 4 times. The maximum effect was observed when using the culture liquid with amikacin, where the efficacy of the antibiotic increased up to 64 times.

The mutual action of the cell-free culture liquid of *S. aureus* with broad-spectrum antibiotics against *P. aeruginosa* cells was also quantified by CFUs count. For that, 48 hours P. aeruginosa biofilms were established in 24-well plates, washed and filled with 25–100% culture liquid of *S. aureus* or a of *S. aureus* – *P. aeruginosa* mixed culture and antibiotics were added at concentrations equal to their 0.03 – 8 × MBC (Table 1). After a 24-hour incubation, the viability of detached cells and biofilm-embedded cells of *P. aeruginosa* was assessed by drop plate assay (Fig. 2).

The culture liquid itself of either *S. aureus* or *S. aureus* – *P. aeruginosa* did not decrease

CFUs count, suggesting that antimicrobial metabolites have rather bacteriostatic than bactericidal effect. The addition of amikacin with culture liquid increased the efficacy of antimicrobial. Thus, in presence of the latter, the decrease in CFUs count by 3 orders of magnitude was observed at concentrations of amikacin 4-16-fold lower compared to solely antimicrobial (Fig. 2).

When ciprofloxacin was added in 100% culture liquid of either *S. aureus* or *S. aureus* – *P. aeruginosa*, a decrease in the viability of detached *P. aeruginosa* cells by three orders of magnitude was observed at an antibiotic concentration of 2 μg/mL (which corresponds to 0.03×MBC of antibiotics), while the introduction of ciprofloxacin alone led to a similar effect only at an antibiotic concentration of 8×MBC. In biofilm, the complete cell death of *P. aeruginosa* was observed when the culture liquid of *S. aureus* – *P. aeruginosa* was introduced together with ciprofloxacin at a concentration of 2×MBC, while solely antibiotic led to a decrease in CFUs by three orders of magnitude at 8×MBC (Fig. 2).

## Discussion

Over the world, diseases caused by *Pseudomonas aeruginosa* in increased tolerance to antimicrobials raises significantly, suggesting that the development of novel approaches for treatment challenging. The antimicrobial peptides (AMPs), which can act individually and increase the activity of antibiotics, seem to be one of the alternatives to antimicrobial therapy (Grassi *et al.*, 2017 Portelinha *et al.*, 2021). *S. aureus* is able to produce AMPs (aureocins) with high bactericidal activity and increasing the activity of various antibiotics (Ceotto *et al.*, 2012). Thus, the sensitivity of *P. aeruginosa* to broad-spectrum antibiotics increases in the *S. aureus* – *P. aeruginosa* mixed community (Trizna *et al.*, 2020). Here we show that *S. aureus* produces extracellular metabolites which exhibit bacteriostatic properties and and can be used as enhancers of antimicrobials against *P. aeruginosa*.

The biofilm-diffusion test revealed that the change in the *P. aeruginosa* sensitivity in mixed *S. aureus* – *P. aeruginosa* biofilm is governed by the production of extracellular metabolites by *S. aureus* affecting *P. aeruginosa*. Indeed, in presence of 25% *S. aureus* cell-free culture liquid, the efficacy of aminoglycosides (Amikacin, Gentamicin) against *P. aeruginosa* increased 2 fold. In the case of biofilm-embedded *P. aeruginosa*, ciprofloxacin together with 12% culture liquid led to the death of *P. aeruginosa* at a concentration 4 times lower than the combination with saline. In combination with 6% culture liquid, the effectiveness of gentamicin increased 4 times and the MBC of amikacin decreased 64 times (Table 2, Fig. 2).

Taken together, these data confirm that *S. aureus* produces antimicrobial metabolites which increase the efficacy of antimicrobials against *P. aeruginosa* cells in both planktonic and biofilm-embedded form. These metabolites can serve as a promising approach to improve the antimicrobial therapy of infections associated with the formation of *P. aeruginosa* biofilms on various surfaces, while their identification remains challenging.

## Acknowledgments

This research was funded by Russian Science Foundation (grant N 20-64-47014 to A.K.) and performed in frames of Kazan Federal University Strategic Academic Leadership Program (PRIORITY-2030).

## References

ANDERL J.N., FRANKLIN M.J. & STEWART P.S. (2000): Role of antibiotic penetration limitation in Klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin. *Antimicrobial agents and chemotherapy* **44**(7), 1818–1824.

BAIDAMSHINA D.R., TRIZNA E.Y., HOLYAVKA M.G., BOGACHEV M.I., ARTYUKHOV V.G., AKHATOVA F.S., ... & KAYUMOV A.R. (2017): Targeting microbial biofilms using Ficin, a nonspecific plant protease. *Scientific reports* **7**(1), 1–12.

BEAUDOIN T., YAU Y.C.W., STAPLETON P.J., GONG Y., WANG P.W., GUTTMAN D.S. & WATERS V. (2017): Staphylococcus aureus interaction with Pseudomonas aeruginosa biofilm enhances tobramycin resistance. *NPJ biofilms and microbiomes* **3**(1), 1–9.

BEHZADI P., BARÁTH Z. & GAJDÁCS M. (2021): It's not easy being green: a narrative review on the microbiology, virulence and therapeutic prospects of multidrug-resistant Pseudomonas aeruginosa. *Antibiotics* **10**(1), 42.

BRIAUD P., CAMUS L., BASTIEN S., DOLÉANS-JORDHEIM A., VANDENESCH F. & MOREAU K. (2019): Coexistence with Pseudomonas aeruginosa alters Staphylococcus aureus transcriptome, antibiotic resistance and internalization into epithelial cells. *Scientific reports* **9**(1), 1–14.

CHEUNG G.Y., BAE J.S. & OTTO M. (2021): Pathogenicity and virulence of Staphylococcus aureus. *Virulence* **12**(1), 547–569.

CENDRA M.D.M., BLANCO-CABRA N., PEDRAZ L. & TORRENTS E. (2019): Optimal environmental and culture conditions allow the in vitro coexistence of Pseudomonas aeruginosa and Staphylococcus aureus in stable biofilms. *Scientific reports* **9**(1), 1–17.

CEOTTO H., DA SILVA DIAS R.C., DOS SANTOS NASCIMENTO J., DE PAIVA BRITO M.A.V., GIAMBIAGI-DEMARVAL M. & DO CARMO DE FREIRE BASTOS M. (2012): Aureocin A70 production is disseminated amongst genetically unrelated Staphylococcus aureus involved in bovine mastitis. *Letters in applied microbiology* **54**(5), 455–461.

DING L., WANG J., CAI S., SMYTH H. & CUI Z. (2021): Pulmonary biofilm-based chronic infections and inhaled treatment strategies. *International Journal of Pharmaceutics* **604**, 120768.

GRASSI L., MAISETTA G., ESIN S. & BATONI G. (2017): Combination strategies to enhance the efficacy of antimicrobial peptides against bacterial biofilms. *Frontiers in Microbiology* **8**, 2409.

HALL C.W. & MAH T.F. (2017): Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS microbiology reviews* **41**(3), 276–301.

HARRISON F., ALLAN R.N. & MADDOCKS S.E. (2020): Polymicrobial Biofilms in Chronic Infectious Disease. *Frontiers in Cellular and Infection Microbiology*, 816.

HERIGSTAD B., HAMILTON M. & HEERSINK J. (2001): How to optimize the drop plate method for enumerating bacteria. *Journal of microbiological methods* **44**(2), 121–129.

LECLERCQ R., CANTÓN R., BROWN D.F., GISKE C.G., HEISIG P., MACGOWAN A.P., ... & KAHLMETER G. (2013): EUCAST expert rules in antimicrobial susceptibility testing. *Clinical Microbiology and Infection* **19**(2), 141–160.

LUO A., WANG F., SUN D., LIU X. & XIN B. (2021): Formation, development, and cross-species interactions in biofilms. *Frontiers in Microbiology* V. **12**, 1–15.

LITTLE W., BLACK C. & SMITH A. C. (2021): Clinical Implications of Polymicrobial Synergism Effects on Antimicrobial Susceptibility. *Pathogens* **10**(2), 144.

MAKABENTA J.M.V., NABAWY A., LI C.H., SCHMIDT-MALAN S., PATEL R. & ROTELLO V.M. (2021): Nanomaterial-based therapeutics for antibiotic-resistant bacterial infections. *Nature Reviews Microbiology* **19**(1), 23–36.

NEWSTEAD L.L., VARJONEN K., NUTTALL T. & PATERSON G.K. (2020): Staphylococcal-produced bacteriocins and antimicrobial peptides: Their potential as alternative treatments for Staphylococcus aureus infections. *Antibiotics* **9**(2), 40.

PORTELINHA J., DUAY S.S., YU S.I., HEILEMANN K., LIBARDO M.D.J., JULIANO S.A., ... & ANGELES-BOZA A.M. (2021): Antimicrobial peptides and copper (II) ions: Novel therapeutic opportunities. *Chemical Reviews* **121**(4), 2648–2712.

SIMÕES M., PEREIRA A.R., SIMÕES L.C., CAGIDE F. & BORGES F. (2021): Biofilm control by ionic liquids. *Drug Discovery Today* **26**(6), 1340–1346.

SINGH S., DATTA S., NARAYANAN K.B. & RAJNISH K.N. (2021): Bacterial exo-polysaccharides in biofilms: role in antimicrobial resistance and treatments. *Journal of Genetic Engineering and Biotechnology* **19**(1), 1–19.

TRIZNA E.Y., YARULLINA M.N., BAIDAMSHINA D.R., MIRONOVA A.V., AKHATOVA F.S., ROZHINA E.V., ... & KAYUMOV A.R. (2020): Bidirectional alterations in antibiotics susceptibility in Staphylococcus aureus — Pseudomonas aeruginosa dual-species biofilm. *Scientific reports* **10**(1), 1–18.

URUÉN C., CHOPO-ESCUIN G., TOMMASSEN J., MAINAR-JAIME R.C. & ARENAS J. (2020): Biofilms as promoters of bacterial antibiotic resistance and tolerance. *Antibiotics* **10**(1), 3.

VO T.D., SPAHN C., HEILEMANN M. & BODE H.B. (2021): Microbial Cationic Peptides as a Natural Defense Mechanism against Insect Antimicrobial Peptides. *ACS Chemical Biology* **16**(3), 447–451.

XUAN T.F., WANG Z.Q., LIU J., YU H.T., LIN Q.W., CHEN W.M. & LIN J. (2021): Design and Synthesis of Novel c-di-GMP G-Quadruplex Inducers as Bacterial Biofilm Inhibitors. *Journal of Medicinal Chemistry* **64**(15), 11074–11089.

YUAN L., HANSEN M.F., RØDER H.L., WANG N., BURMØLLE M. & HE G. (2020): Mixed-species biofilms in the food industry: current knowledge and novel control strategies. *Critical Reviews in Food Science and Nutrition* **60**(13), 2277–2293.