European Review for Medical and Pharmacological Sciences 2022; 26: 1388-1397

Use of bacterial culture supernatants as anti-biofilm agents against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*

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Abstract. – OBJECTIVE: *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* are the most pervasive and challenging agents of bacterial nosocomial infections. Previous studies indicated that the microbial biofilms formed by these bacteria may play important roles in their pathogenesis and resistance to phagocytosis and antibiotics. The aim of this study was to explore the anti-biofilm activity of culture supernatants of *Salmonella enterica subsp. enteric serovar Typhimurium* SL1344 and *P. aeruginosa* PA01 against biofilms formed by *P. aeruginosa* PA01 and *K. pneumoniae* KR3167, respectively.

MATERIALS AND METHODS: Biofilm formation was quantified by crystal violet staining. A modified method was applied to separate planktonic and biofilm-forming cells. The viable cells in the planktonic and biofilm phases were quantitated by viable plate count. Dual-species interactions between *P. aeruginosa* PAO1 and *Salmonella enterica subsp. enterica serovar Typhimurium* SL1344 were investigated using different cell density ratios.

RESULTS: Biofilm formation of *P. aeruginosa* PA01 was significantly inhibited by the heat resistant components from the culture supernatants of *Salmonella enterica subsp. enterica serovar Typhimurium*. Biofilm formed by *K. pneumoniae* KR3167 was also inhibited by the culture supernatants of *P. aeruginosa* PA01. The supernatants obtained from planktonic cell caused greater biofilm reduction than those extracted from biofilm-forming cells.

CONCLUSIONS: This study is the first to report that sterile crude supernatants extracted from the cultures of *Salmonella enterica* and *P. aeruginosa* significantly inhibited biofilm formation of *P. aeruginosa* and *K. pneumoniae*, respectively. The active agents in the culture supernatants responsible for biofilm inhibition have not been determined yet. The culture supernatants of *Salmonella enterica* and *P. aeruginosa* should be further studied for their therapeutic potential to reduce biofilm formation produced by bacteria causing nosocomial infections.

Key Words:
Biofilm, Crude bacterial culture extract, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella*.

Introduction

Pseudomonas aeruginosa and *Klebsiella pneumoniae KR3167* are aggressive opportunistic pathogens that cause otitis media, bacteraemia, respiratory, urinary, and gastrointestinal tract infections. Reports suggest they are responsible for less than 20%[1] and 8%[2,3] of all hospital-acquired infections, respectively. It is generally difficult to control *P. aeruginosa* and *K. pneumoniae* infections with conventional antibiotics[4,5]. A microbial biofilm consists of communities of sessile cells attached to each other and/or a substratum and embedded in a mixture of polymers[6]. The ability of bacteria to attach to various surfaces and form biofilms is an important aspect of virulence, especially in respiratory infections. Biofilm-forming bacteria are widely distributed in the environment and can colonise specific sites. *Streptococcus viridans* causes endocarditic decay whilst *P. aeruginosa* inhabits the respiratory mucous membrane in cystic fibrosis (CF) patients[7]. Biofilms produce protective matrices that facilitate antibacterial resistance and evasion of the host immune response. Moreover, biofilms promote bacterial persistence and dissemination[8-10].

Biofilm-producing bacteria are resistant to the antibiotics tetracycline, ampicillin, gentamicin, and streptomycin, as well as to the oxidants iodine, ozone, and chlorine[11]. As a result, biofilm-associated infections are rapidly becoming untreatable and have substantially increased wound chronicity[12,13]. Moreover, 65-80% of all

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microbial infections may be associated with biofilms[8,14]. *P. aeruginosa* may also survive in biofilms in the lungs of CF patients[15,16].

The bacteria in biofilms are comparatively more resistant to multiple antibiotics than free cells. Consequently, novel therapeutic agents are required to control them[8]. One approach towards overcoming bacterial antibiotic resistance is to target quorum sensing (QS) systems or bacterial cell-to-cell communication rather than actually killing the bacteria. Blocking QS and hindering the ability of bacteria to communicate with each other may prevent the unified response triggered by QS[17]. Several synthetic and natural compounds have been administered to block QS systems in *P. aeruginosa*[18]. However, the few known compounds with intrinsic anti-QS activity are derived mainly from non-bacterial sources, such as halogenated furanones from the red alga *Delisea pulchra*[19], young seedlings and seedling exudates of legume *Medicago truncatula*[20], and southern Florida marine algae, which produce compounds that inhibit signals from the bacterial molecules N-acyl homoserine lactones (AHLs)[21]. One study[22] demonstrated that essential oils and plant extracts of peppermint (*Mentha × Piperita L.*), coriander (*Coriandrum sativum L.*), and anise (*Pimpinella anisum L.*) had activity against *S. aureus* and *E. coli* biofilms. Zhou et al[18] used hordenine as a novel QS inhibitor and anti-biofilm agent against *P. aeruginosa* PAO1.

Bacteria often release signals that detect and react to the signals of other bacterial species in co-culture. Cell-free supernatants from planktonic *K. pneumoniae* cultures exhibit anti-biofilm activity against most Gram-positive and certain Gram-negative bacteria[23]. Therefore, it may be possible to eradicate *K. pneumoniae* biofilms from medical devices and *P. aeruginosa* in the lungs of CF patients and on medical implants by using bacterial cell-free supernatants. A few studies[23,24] on the anti-biofilm activity of cell-free bacterial cultures against *K. pneumoniae* and *P. aeruginosa* have been reported. Hence, the purpose of this study was to explore the anti-biofilm activity of supernatants extracted from overnight cultures of *Salmonella enterica subsp. enteric serovar Typhimurium* SL1344 against *P. aeruginosa* PA01 biofilm and the activity of supernatants derived from overnight cultures of *P. aeruginosa* PA01 against *K. pneumoniae* KR3167 biofilm. A wide range of biological interactions has been observed among microbes in biofilms and in dual-specie models[24]. Medically and environmentally important biofilms may consist of various microbial species. Therefore, bacterial species interactions in a dual-species model comprising *Salmonella enterica* and *P. aeruginosa* were also evaluated.

Materials and Methods

This study was designed to investigate the effects of antibiofilm products (secondary metabolites) that are produced in overnight culture from and against the following Gram-negative bacteria; (*Klebsiella pneumoniae* KR3167, *Pseudomonas aeruginosa* PA01, and *Salmonella enterica subsp. enteric serovar Typhimurium* SL1344).

Bacterial Strains

The bacterial strains used in this study were provided by the University of Leicester, UK. The *K. pneumoniae* KR3167 was supplied by Dr. Kumar Rajakumar. The *P. aeruginosa* PA01 was provided by the laboratory of Dr. Peter Andrew. The *Salmonella enterica subsp. enteric serovar Typhimurium* SL1344 was furnished by the laboratory of Dr. Primrose Freestone. Lysogeny broth (LB) medium was used to grow the bacteria aerobically at 37ºC for ≤ 24 h.

Preparation of Bacterial Inocula for Phenotypic Characterization

All bacterial inocula were first normalized and standardised to OD_{600} = 0.1 to minimize errors and avoid variations caused by differences in inoculum size and cell density. For this purpose, standard inocula were initially prepared for all bacterial strains. After overnight incubation at 37°C, each strain was pelleted by centrifugation at 3,500 × g for 10 min and washed twice with phosphate-buffered saline (PBS). Each pellet was re-suspended in 1 mL PBS and adjusted to OD_{600} = 0.1 [10^5 colony-forming units (CFU) mL^{-1}]. Fresh LB medium was inoculated at a 1:100 ratio. The cells were diluted to 10^3 CFU mL^{-1} for the biofilm formation assay and analysis of the anti-biofilm efficacy of the secondary metabolites in the bacterial culture supernatants.

Determination of Biofilm Formation

Biofilm formation was determined by crystal violet staining as previously described[25,26]. This assay is quantitative and commonly used

to estimate biofilm formation in *Escherichia coli*[25], *Enterococcus faecalis*[26], *Staphylococcus aureus*[27], *Mycobacterium avium*[28], and *K. pneumoniae*[29]. Fresh LB medium was inoculated at 1:100 to achieve a cell density of 10^3 CFU mL^{-1} in 96-well flat-bottom polystyrene microplates. These were statically incubated overnight at 37°C. The biofilms were assessed by removing the planktonic cells from the microplates, followed by three separates washes with 200 µL PBS. The biofilm was dried at 25°C for 30 min, stained with 200 µL of 0.5% (v/v) crystal violet, and incubated at 25°C for 15 min. Excess crystal violet was removed by washing the biofilm three times with PBS. The stained biofilm in each well was dissolved in 200 µL ethanol: acetone (80:20) (v/v). Absorbances were measured at 595 nm in a microplate reader (Infinite F50; Tecan Group Ltd., Männedorf, Switzerland). Wells containing uninoculated medium served as controls to correct for the crystal violet that had bound to the plastic. The crystal violet biofilm staining assay permitted the enumeration of the planktonic and biofilm-forming cells in all bacterial strains[30].

Preparation of Supernatant from Planktonic and Biofilm-Forming Cell Cultures

Biofilms were prepared in 12-well microplates as previously described[26,31]. A modified method was applied to separate planktonic and biofilm-forming cells[30]. The viable cells in the planktonic and biofilm phases were quantitated by viable plate count using the Miles method[32]. Cell-free sterile supernatants were prepared by inoculating a new LB medium with cells at a density equivalent to 10^3 CFU mL^{-1} supernatant. After overnight culture, the supernatants were sterilised by centrifugation at 3,500 × g for 10 min, filtered through a 2.5-µm acrodisc (Merck Millipore, Billerica, MA, USA), and stored at -20°C. To determine the bacterial supernatant activity and establish whether the signals were associated with proteinaceous or fatty acid secondary metabolites, each sterile supernatant was separated into two parts. The first part was inactivated by boiling for 2 min, whilst the second was used as a source of active secondary metabolites. The pH of each supernatant was measured with a Jenway pH meter model 3510 (Cole-Parmer, Vernon Hills, IL, USA) to rule out any possible effects of overnight cultures.

Determination of Supernatant Activity Against P. Aeruginosa PAO1 and K. pneumoniae KR3167 Biofilms

Sterile supernatants extracted from *Salmonella enterica subsp. enterica serovar Typhimurium* SL1344 overnight cultures were used against *P. aeruginosa* PAO1 biofilms. To assess the effects of these supernatants on biofilm formation, sterile supernatants from each bacterial culture were evaluated by mixing or diluting them two-fold in fresh LB medium (1/2, 1/4, 1/16, 1/32, 1/64, and 1/128) and inoculating them with *P. aeruginosa* PA01 at a 1:100 ratio. Biofilm formation in 96-well flat-bottom polystyrene plates was assessed by crystal violet staining as previously described [25,26]. All experiments were performed in triplicate. For each plate, two rows of wells were used as controls. One contained sterile uninoculated LB, and the other contained sterile supernatant. The viable bacteria were enumerated at the end of each biofilm formation assay as previously described[32]. Similar protocols were applied to determine the effects of the harvested *P. aeruginosa* PA01 supernatant on *K. pneumoniae* KR3167 biofilm formation. The supernatants of *P. aeruginosa* PA01 and *K. pneumoniae* KR3167 were used against their own growth to rule out the possibility of biofilm inhibition caused by low nutrient availability or inhibitory factors in the medium.

Biofilm of Mixed Culture Interactions

Dual-species interactions between *P. aeruginosa* PAO1 and *Salmonella enterica subsp. enterica serovar Typhimurium* SL1344 were investigated using different cell density ratios of *P. aeruginosa* PA01 (P) and *Salmonella enterica subsp. enterica serovar Typhimurium* SL1344 (S). Fresh LB medium was inoculated at 1:100 to achieve a cell density of 10^3 CFU mL^{-1}. The inoculum ratios (10 µL added to 1,000 µL LB medium) were as follows: (S) 5 µL:(P) 5 µL; (S) 2.5 µL:(P) 7.5 µL; and (S) 7.5 µL:(P) 2.5 µL. After overnight incubation at 37°C, biofilm was quantified by crystal violet staining as previously described.

Statistical Analysis

All assays were conducted at least in triplicate and repeated twice. Data were analysed by two-way ANOVA and Tukey's multiple-comparison test in GraphPad Prism v. 6 (GraphPad Software, La Jolla, CA, USA). Data were expressed as means ± standard error of the mean (SEM). For all analysis, p-value < 0.05 was considered

statistically significant. Significance is defined as *p-value< 0.05, **p-value< 0.01, ***p-value < 0.001, and ****p-value< 0.0001.

Results

Biofilm Formation Comparison

K. pneumoniae KR3167 formed the strongest biofilm followed by *P. aeruginosa* PA01, whereas *Salmonella enterica subsp. enterica serovar Typhimurium* formed the weakest biofilm; (OD_{595}: 0.939 ± 0.05107, 0.6267 ± 0.05029 and 0.1102 ± 0.01, respectively).

Effect of Supernatants on Biofilm Formation

Several dilutions (1/16, 1/32, and 1/64) of supernatants from an overnight culture of *P. aeruginosa* PA01 significantly reduced biofilm formation produced by *K. pneumoniae* KR3167 (OD_{595}: 0.38 ± 0.02, 0.46 ± 0.04, 0.59 ± 0.05, respectively; p-value < 0.0001) compared to the positive control *K. pneumoniae* KR3167 in LB medium alone, (OD_{595}: 0.93 ± 0.05), (Figure 1). On the other hand, the extracted supernatants from overnight culture of *K. pneumoniae* KR3167 failed to inhibit biofilm formed by *P. aeruginosa* PA01. Similarly, *Salmonella enterica subsp. enterica serovar Typhimurium* SL1344 had no significant effect on biofilm formed by *K. pneumoniae* KR3167 (data not shown). Furthermore, several dilutions (1/4, 1/8, 1/16, 1/32, and 1/64) obtained from the supernatants of *Salmonella enterica subsp. enterica serovar Typhimurium* SL1344 significantly decreased biofilm formation of *P. aeruginosa* PA01 (Figure 2). However, *P. aeruginosa* PA01 did not affect the biofilm formation of *Salmonella enterica subsp. enterica serovar Typhimurium* SL1344 (data not shown).

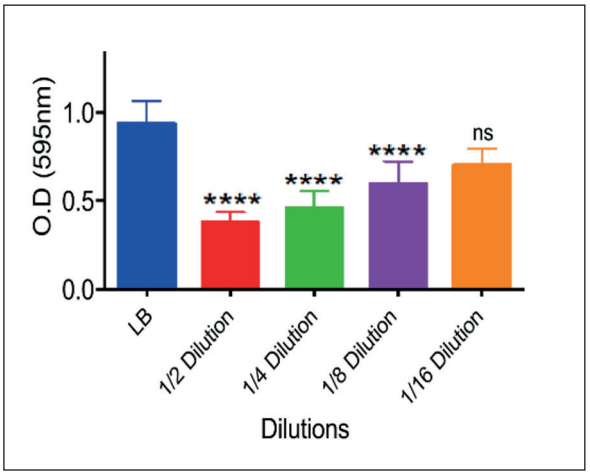

Figure 1. Effects of sterile supernatants harvested from *P. aeruginosa* PA01 on biofilm formed by *K. pneumoniae* KR3167. Biofilm formation was determined by crystal staining. Strains were grown in LB under static conditions in 96-well polystyrene microplates for 24 h. Biofilms were stained with 1% (v/v) crystal violet for 30 min. Data show the mean ± SEM of three independent triplicate experiments (n = 9) with significance evaluated using one-way ANOVA and Tukey's multiple comparisons test (****p-value ≤ 0.0001).

To investigate the heat tolerance and nature of the antibiofilm molecules, the supernatants of overnight bacterial cultures were exposed to boiling. Various dilutions of boiled and non-boiled filter-sterilized supernatants of *Salmonella enterica subsp. enterica serovar Typhimurium* SL1344 were equally able to cause a significant reduction of biofilm formed by *P. aeruginosa* PA0. Boiled supernatant dilutions (1/4, 1/8, 1/16, 1/32, and 1/64) reduced biofilm formation of *P. aeruginosa* PA01 (OD_{595}: 0.22 ± 0.01, 0.23 ± 0.01, 0.26 ± 0.01, 0.34 ± 0.02, 0.41 ± 0.02, and 0.59 ± 0.04, respectively; p-value < 0.0001), (Figure 2). The same dilutions of non-boiled supernatants significantly reduced biofilm formation of *P. aeruginosa* PA01 (OD_{595}: 0.22 ± 0.01, 0.3 ± 0.01, 0.34 ± 0.01, 0.37 ± 0.008, 0.43 ± 0.01, and 0.56 ± 0.03, respectively; p-value < 0.0001), (Figure 2). To examine the impact of supernatants on growth, the same dilutions of boiled and non-boiled supernatants of *Salmonella enterica subsp. enterica serovar Typhimurium* SL1344 did not affect the growth of *P. aeruginosa* PA01 (data not shown). Therefore, *S. enterica* exclusively inhibited the attachment of *P. aeruginosa* to the surfaces of polystyrene microplates.

Effects of Supernatants Extracted from Planktonic and Biofilm Former Cells

To assess the origin of antibiofilm activity seen on the previous experiments, supernatants were extracted from planktonic and biofilm former cells and were evaluated against the biofilm formed by the same bacterial species. Since *K. pneumoniae* formed the largest amount of biofilm compared to *P. aeruginosa* and *S. enterica* in this study, supernatants of *K. pneumoniae* KR3167 overnight culture were examined against its own biofilm. The results showed that the supernatants of *K. pneumoniae* KR3167 harvested from the planktonic cell stationary phase significantly reduced biofilm formation at three successive dilutions (1/16, 1/32, and 1/64) (OD_{595}: 0.212 ± 0.016, 0.483 ± 0.014, and 0.747 ± 0.015, respectively,

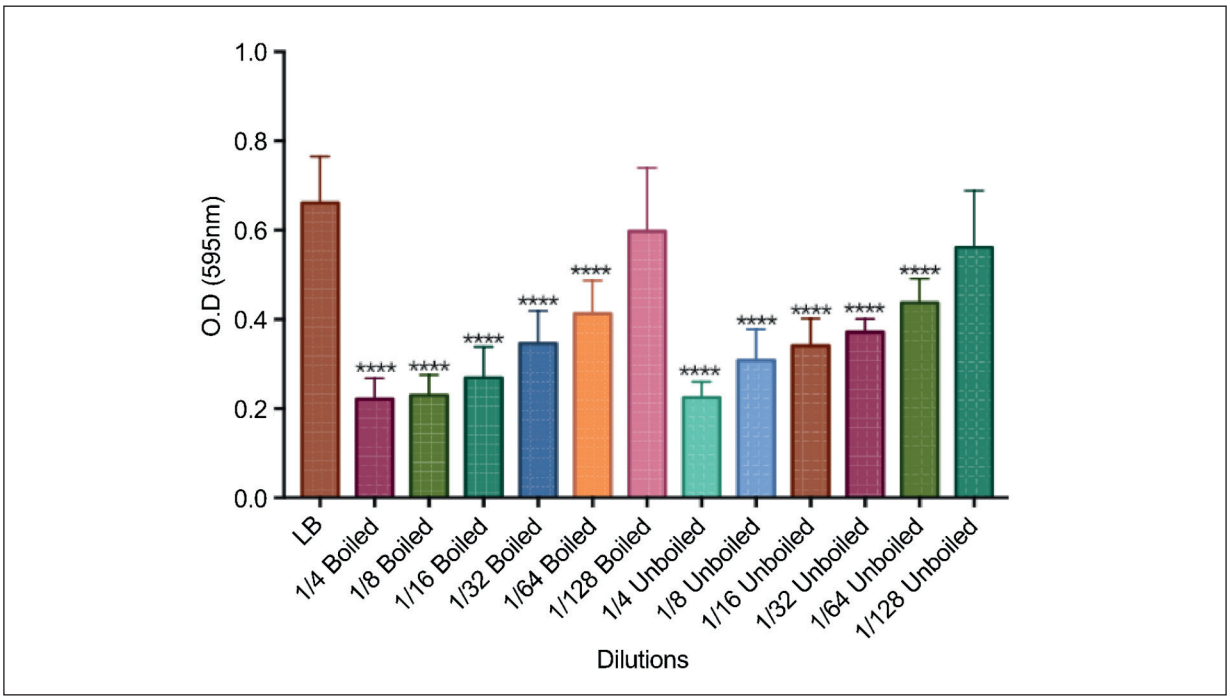

Figure 2. Biofilm formation by *P. aeruginosa* PA01 in response to supernatant extracted from planktonic cells of *Salmonella enterica subsp. enterica serovar Typhimurium* SL1344. Strains were grown in LB alone and in LB mixed with boiled or non-boiled supernatants under static conditions. Biofilms in 96-well polystyrene microplates were stained with 1% (v/v) crystal violet for 30 min. Data show the mean ± SEM of three independent triplicate experiments (n = 9) and significance was evaluated using the one-way ANOVA and Tukey's multiple comparisons test (****p-value ≤ 0.0001).

p-value < 0.0001), (Figure 3). On the other hand, the supernatants extracted from biofilm-forming cells also significantly reduced biofilm formation but exclusively at dilutions 1/16 and 1/32 (OD_{595}: 0.325 ± 0.042, and 0.629 ± 0.066, respectively, p-value < 0.0001) while no biofilm inhibition was

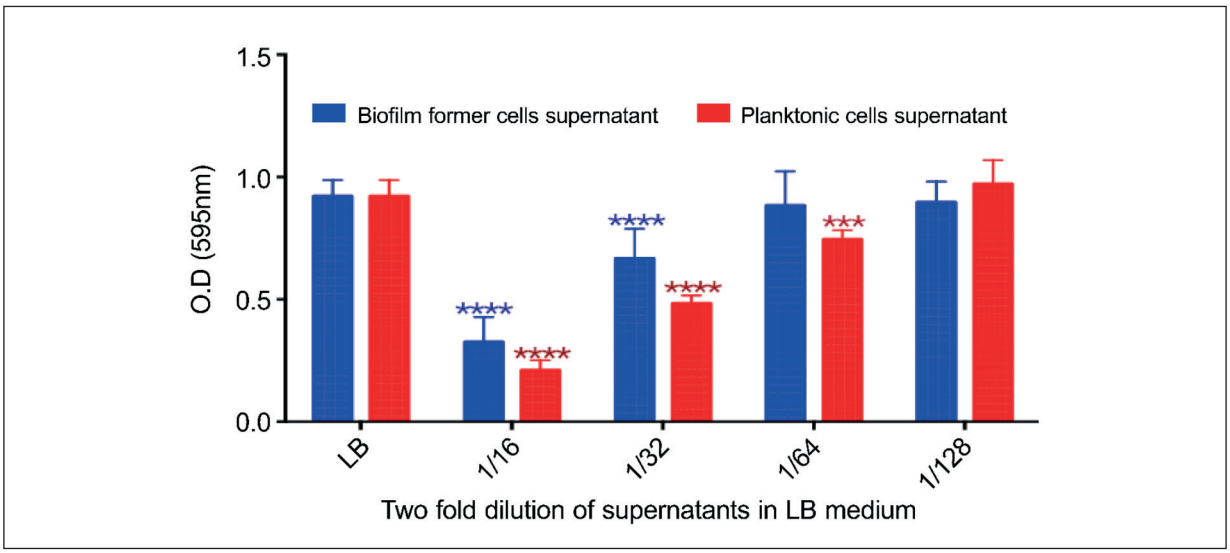

Figure 3. *K. pneumoniae* KR3167 biofilm formation in response to supernatant extracted from planktonic and biofilm-forming *K. pneumoniae* KR3167. Strains were grown on LB under static condition in 96-well polystyrene microplates for 24 h. Biofilm was stained with 1% (v/v) crystal violet for 30 min. Data show the mean ± SEM of three independent triplicate experiments (n = 9), evaluated by one-way ANOVA and Tukey's multiple comparisons test (****p-value ≤ 0.0001).

seen at dilution 1/64 (Figure 3). Thus, the supernatants obtained from planktonic cells caused greater biofilm reduction than the supernatants extracted from biofilm-forming cells of *K. pneumoniae* KR3167. In addition, the supernatants of *P. aeruginosa* PA01 and *K. pneumoniae* KR3167 had no significant impact (p-value > 0.05) on their own growth (data not shown). Hence, the observed inhibition of bacterial biofilm is independent of toxic waste accumulation and/or nutrient depletion in the culture medium.

Effects of Interspecies Interactions in Mixed Biofilms

An earlier study[33] on interspecies interactions reported that diffusible signal factors from *Stenotrophomonas maltophilia* promoted biofilm formation in *P. aeruginosa*. To observe the effects of *S. enterica* on biofilm formation of *P. aeruginosa* PA01 in mixed culture, interspecies interactions between *P. aeruginosa* PA01 and *S. enterica subsp. enterica serovar Typhimurium* SL1344 were investigated. After culturing at different cell densities, relative to *P. aeruginosa* in a monospecies biofilm (0.571 ± 0.053), mixed-species interactions between *P. aeruginosa* PA01 and *S. enterica subsp. Enterica serovars Typhimurium* SL1344 demonstrated that the former significantly decreased biofilm formation (0.25 ± 0.04, 0.26 ± 0.038, and 0.27 ± 0.045, respectively; p-value < 0.0001) (Figure 4).

Discussion

Various studies[18,22-24] have reported the anti-biofilm activity of certain bacterial supernatants and medicinal plant extracts against pathogenic bacteria. However, this study has found, for the first time, that sterile crude supernatants were extracted from overnight cultures of *Salmonella enterica subsp. enteric serovar Typhimurium* SL1344 and *P. aeruginosa* PA01 substantially inhibit biofilm formation of *P. aeruginosa* PA01 and *K. pneumoniae* KR3167, respectively. *P. aeruginosa* and *K. pneumoniae* are opportunistic Gram-negative pathogens associated with lung inflammation in CF and ventilator-related pneumonias[34-36]. Approximately 80-95% of all patients with CF die of respiratory failure as a result of chronic bacterial infection and inflammation in the airway[35]. Several studies[36-40] reported that the pathogenesis of *P. aeruginosa* and *K. pneumoniae* depends on their ability to resist antibiotics, evade the immune system, and form biofilms. Biofilm is vital to the persistence of *K. pneumoniae* and *P. aeruginosa* in CF airways[36-41].

This study revealed that the extracted supernatants of *P. aeruginosa* PA01 significantly reduced biofilm formation produced by *K. pneumoniae* KR3167. The ability of *P. aeruginosa* to suppress biofilm formed by other bacteria may explain why this bacterium is a predominant lung pathogen in CF patients[35]. An earlier study reported that *P. fluorescens* and *P. aeruginosa* attenuated *Candida albicans* adhesion[42]. With regards to *K. pneumoniae,* this observation is in line with another study that demonstrated that whole cells, acid, and neutral supernatants of *Lactobacillus fermentum* CRL 1058 inhibited biofilm formation of *K. pneumoniae*[43]. The mechanism behind anti-biofilm activity of *P. aeruginosa* supernatants could be related to its inhibition of cGMP signaling which is essential for biofilm formation in *K. pneumoniae*[36]. Furthermore, the supernatants may also repress the genes regulating biofilm formation since *P. aeruginosa* supernatants have

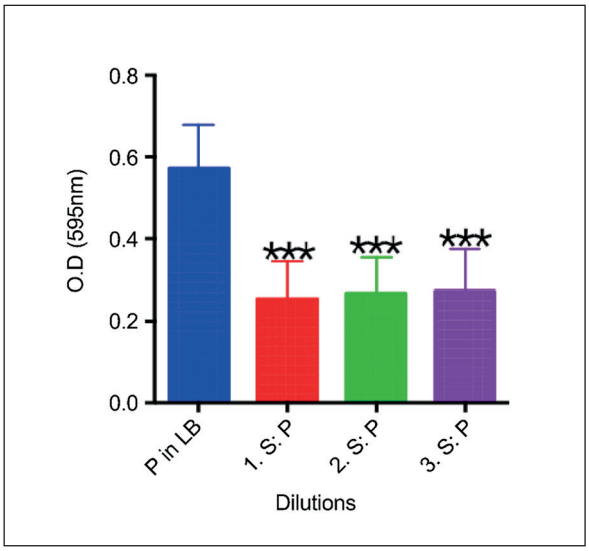

Figure 4. Biofilm formation in monocultures and mixed cultures with interactions between *P. aeruginosa* PA01, and *Salmonella enterica subsp. Enterica serovars Typhimurium* SL1344 in mixed biofilms. Strains were grown in LB under static conditions in 96-well polystyrene microplates for 24 h. Biofilms were stained with 1% (v/v) crystal violet for 30 min. LB was inoculated with 10 µL inoculum:1,000 µL LB **1.** *Salmonella* (S) 5 µL:5 µL (P) *Pseudomonas*. **2.** *Salmonella* (S) 7.5 µL:(P) 2.5 µL *Pseudomonas*. **3.** *Salmonella* (S) 2.5 µL:7.5 µL (P) *Pseudomonas*. Data represent the mean ± SEM of three independent triplicate experiments (n = 9) and significance was determined using one-way ANOVA and Tukey's multiple comparisons test (****p-value ≤ 0.0001).

been shown to downregulate the genes associated with adhesion and biofilm formation of *C. albicans*[42].

Importantly, *Salmonella enterica subsp. Enterica serovars Typhimurium* SL1344 supernatants were able to significantly reduce biofilm formation of *P. aeruginosa* PA01. Interestingly, agents with efficacy against *C. albicans* biofilm were detected in the supernatants of *S. enterica serovar Typhimurium*[44]. Previous investigations[13,45,46] stated that aqueous extracts of certain medicinal plants have anti-biofilm and anti-QS efficacy against *P. aeruginosa*. In addition, an old study reported that biofilm reduction appeared to be dependent on the blockage of QS, which is important for *P. aeruginosa* pathogenesis in CF patients[47]. This study showed that biofilm reduction was significantly greater in dual cultures of *S. enterica serovar Typhimurium* and *P. aeruginosa* PA01 than it was in a culture of *P. aeruginosa* PA01 alone. Hence, strong interspecies interactions prevented biofilm development in *P. aeruginosa*. A study[48] on mixed bacterial species interactions concluded that *Stenotrophomonas maltophilia* significantly inhibited biofilm formation by *Escherichia coli*. In the supernatants of single or mixed bacterial cultures, one species releases anti-QS signals to possibly block the signals that regulate the communication of the second species. AHLs and autoinducer-2 (AI-2) signals may participate in bacterial QS. In a biofilm co-culture model, unidirectional communication between *P. aeruginosa* and *B. cepacian* was observed. The latter recognised the AHLs from *P. aeruginosa*, but the reverse was not the case[34,48]. In contrast, AI-2 bi-directionally communicated between *E. coli* and *Vibrio harveyi*[49]. Biofilm reduction seen by both boiled and non-boiled supernatants suggests that the inhibitory signals do not arise from structural proteins. Davies and Marques[50] discovered that *P. aeruginosa* produces the unsaturated fatty acid, *cis*-2-decenoic acid, during growth and that this substance reduces biofilm formation in a wide range of bacteria. Other studies demonstrated that *Stenotrophomonas maltophilia* produces DSF fatty acids that modulate biofilm formation by *P. aeruginosa* and *E. coli*[33,48]. The putative biofilm inhibitors in certain bacterial supernatants might be lipopolysaccharides, exopolysaccharides, or other lipid molecules[42].

The present study also demonstrated that supernatants of planktonic cell caused greater biofilm reduction than those supernatants extracted from biofilm-forming cells. This could be due to physiological differences between sessile and planktonic cells of bacterial species. Heffernan et al[51] suggested that the biofilm performance and planktonic cells of *Pseudomonas fluorescens* DSM 8341 are variable in utilising fluoroacetate, glycolate, and degradation of xenobiotics. Further differences have been reported in protein profiles of biofilm and planktonic cells of *P. aeruginosa* in both stationary and exponential growth phases[52].

Since Maldonado et al[43] reported that the high lactic acid levels in *Lactobacillus fermentum* CRL1058 supernatant inhibited *Klebsiella* proliferation in mixed cultures. However, this study eliminated the possible influences of the bactericidal effects of supernatants and the changes in media pH on biofilm formation by the culture supernatants and/or interactions between bacterial strains. Moreover, the lack of growth-inhibiting metabolites in the *P. aeruginosa* and *S. enterica serovar Typhimurium* supernatants suggests the presence of certain novel agents displaying efficacious properties against *K. pneumoniae* and *P. aeruginosa* biofilms. Signal mimicry and degradation interrupt bacterial QS, which, in turn, inhibits downstream virulence and downregulates biofilm genes[53]. Biofilm production may also be suppressed either by a secreted metabolite or alterations in nutrient and/or macromolecule levels[42]. Nutrient competition and metabolism play essential roles in interspecies interactions[48]. QS controls microbial communication by modulating virulence factors and biofilm formation[54,55]. One approach towards overcoming antibiotic resistance is to target bacterial QS systems rather than killing the bacteria themselves[17]. For these reasons, the finding of this study could aid in identifying a novel agent that can target bacterial abilities to communicate and to form biofilm through interfering with their QS system.

Conclusions

This study has revealed that a crude bacterial culture supernatants alone were sufficient to suppress the biofilm formation of well-known pathogenic bacteria like *P. aeruginosa* and *K. pneumoniae*. The impact of such microbial entities on other organisms forming a biofilm on biological surfaces or indwelling medical devices might be of great prophylactic and therapeutic values. The novel molecules within supernatants of *P. aeruginosa* and *S. enterica* could be utilized to control *K. pneumoniae* and *P. aeruginosa* colonisation in

CF patients. Further investigations are required to identify the anti-biofilm molecules, which may potentially be anti-QS signals. It is also essential to elucidate the molecular mechanisms by which superannuants of certain bacterial species can repress or downregulate genes encoding for another bacterial biofilm formation.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgments

The author would like to thank Dr. Hastyar Najmuldeen for his endless support during this study. The author would like to thank Deanship of Scientific Research at Majmaah University for supporting this work under Project Number No. R-2022-63.

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