The Effect of Dual-Species Biofilms, Monosaccharide and D-Amino Acids on Pseudomonal Biofilm

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Abstract

Background: The major therapeutic failure in clinical settings is due to problematic biofilm-producing bacteria like *Pseudomonas aeruginosa*. This study aims to investigate the effect of dual-species biofilms, monosaccharide, and D-amino acids on pseudomonal biofilm. **Methodology:** A total of 130 patients with catheter-associated urinary tract and wound infections were involved in this study. Quantitative biofilm assay by alginate beads was performed. The dual-species biofilms have been done, and the effect of ciprofloxacin, monosaccharides, and D-amino acids on sessile cells was detected. **Conclusion:** This study suggested that the combined action of both monosaccharides (glucose and galactose) and the combination of each one with ciprofloxacin is the enhancing of *P. aeruginosa* biofilm and increases survival strategy against ciprofloxacin. In contrast, a noticeable biofilm reduction and marked synergistic action for ciprofloxacin have been determined in the combination of the two D-amino acid, alanine, and glycine in comparison to the effect of each one alone. Furthermore, the dual biofilm of *P. aeruginosa* with each of *K. pneumonia* and *E. coli*, markedly reduced pseudomonal biofilm, while co-culture with *S. aureus* revealed strong support of pseudomonal pathogenicity and increased its biofilm production.

Keywords: *Biofilm, monosaccharide, D-amino acids*

Introduction

Pseudomonas aeruginosa, a Gram-negative bacillus widely found in nature, is an opportunistic pathogen that can cause disease in immunosuppressed patients. [1] The organism is known to produce biofilm and cause severe infections. [2] A biofilm is generally considered to be a community of microbes enmeshed in an extracellular matrix (often a polysaccharide), typically attached to surfaces, that displays characteristics different from their planktonic counterparts. These unique properties of biofilms complicate their eradication and may contribute to the development of chronic disease. [3] Additionally, naturally occurring biofilms typically involve multiple species of bacteria. Polymicrobial growth necessitates interspecies interactions that involve some degree of intercellular communication and metabolic cooperation. The interactions within mixed-species biofilms have been characterized as cooperative, competitive, or neutral, nature based on the genetic background of the involved species. [4]

P. aeruginosa produces at least three extracellular polysaccharides with different chemical structures, functions, and biosynthetic pathways: alginate, Psl, and Pel. Pel is a glucose-rich structure and Psl consists of repeating D-glucose units. Some *P. aeruginosa* strains predominantly produce Psl, which is associated with matrix stability. [5] Factors that can trigger biofilm dissolution of the biofilm are D-amino acids, which are naturally produced by late stationary phase cultures. Incorporating D-amino acids within the cell wall modulates peptidoglycan synthesis and inhibits attachment to the biofilm matrix proteins. Thus, these amino acids may reduce intercellular adhesion,

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negatively affecting biofilm stability. [6]

Therefore, one of the aims of this study was to investigate the effect of monosaccharides and D-amino acids on pseudomonal biofilms, including their effects on biofilm inhibitory concentrations (BICs). Another aim of the study was to gain insights into inter-species interactions in dual species biofilms, such as survival strategies in the presence of antibiotics.

Patients and Methods

A total of 130 clinical specimens were obtained from patients with catheter-associated urinary tract and wound infections. The patients attended the Ramadi (Iraq) Teaching Hospital or private clinics in Ramadi between October 2019 and February 2020.

Microbiological diagnosis of study isolates

P. aeruginosa isolates were recovered following growth on selective media (2.2% nalidixic acid with cetrimide agar). All study isolates of *P. aeruginosa, S. aureus, K. pneumonia,* and *E. coli* were identified using the Vitek system (BioMérieux, Marcy-l'Etoile, France) and confirmed using biochemical tests. [7]

Detection of biofilm production

Bacterial biofilms were produced on alginate beads, following the method of Vásquez-Ponce *et al*., [8] Briefly, this method entails by cultured bacteria overnight in BHI broth, and added (600 µL) to the wells of a 48-well culture plate. Prepared alginate beads had been added to the wells and the plates were incubated overnight. The cultured beads were transferred to triplicate fresh wells containing BHI broth (600 µL) and the desired antibiotic, and the plates were incubated overnight, and then calculating by Mils Misra method.

Production was included in this study using alginate beads. It includes preparation of alginate beads, biofilm set up and formation on it, dislodging of sessile cells from beads and then counting bacteria by Miles Misra method the isolates' ability to form biofilms was first determined by a qualitative microtiter plate assay. This test involved the inoculation in the wells of a 96-well microtiter plate of a standardized bacterial inoculum. The methanol was added to each well for 15 minutes after overnight incubation and then the plate was drained, allowing dry air to be allowed. Before the well was rinsed out, crystal violet for 5 min was added to each well. In each well, acetic acid (160 µl) was added and an optical density at 570 and 630 nm for each well was read. Optical (OD) density was used to classify the isolate's capability to make a biofilm into weak (OD < 1,078), intermediate (OD = 1.078–2.156), or strong (OD > 2,156). [9]

Biofilm susceptibility test

Susceptibility testing was performed according to the Clinical Laboratory Standard Institute (CLSI) method. [10] Briefly, 600 µl of brain heart infusion (BHI) containing approximately 10^4 colony forming units (CFU)/mL was pipetted into each well of a 48-well plate. Alginate beads, prepared as described by Allison *et al*., [11] were also added (600 µL) to each well, and the plate was incubated (37º C, 150 rpm) overnight. Following the incubation, the media and non-adherent bacteria were removed and fresh media was added. In experiments investigating the effects of ciprofloxacin, the antibiotic was added to the media. For the experiments investigating the effects of added monosaccharides, the BHI broth was supplemented with glucose, fructose, or galactose at 20 µg/dL, or with combinations of glucose/fructose, glucose/galactose, or fructose/galactose, each at 10 µg/dL. For experiments involving D-amino acids (alanine or glycine), the BHI was supplemented with either amino acid (500 mM) The plates were incubated for 24 h (37º C, 150 rpm). Triplicate wells of beads were collected following 2, 4, 6, 8, 12, 12, and 24 h of incubation. Following this incubation, the samples were washed in distilled water and placed into a tube containing 2 mL of dissolving solution (5.3 g NaCO3 and 5.2 g citric acid in 100 ml). The beads were homogenized, and samples were diluted and the bacterial numbers determined by plating on BHI agar and determining the numbers of viable bacteria recovered. These tests were made according to the criteria mentioned by the Clinical Laboratory Standard Institute (CLSI). They were checked with the determination of the stability of growth in terms of CFU with the presence and absence of ciprofloxacin.

Effects of ciprofloxacin, monosaccharides, and D-amino acids

In some experiments the antimicrobial action of ciprofloxacin (MHOX; Prepared Media Laboratories, Tualatin, Oreg) was investigated against two *P.*

aeruginosa isolates. One isolate (#1) was challenged with 900 µg/mL of the antibiotic; the second isolate (#2) was challenged with 800 µg/mL.

Similarly, the effects of adding remint. Glucose, galactose, and fructose [BDH, England] were investigated. [12]

The effects of D-amino acids were also explored using 500 mM D-alanine and D-glycine, alone or in combination, on sessile and planktonic cells.

Dual-species biofilms

P. aeruginosa was cultured with isolates of *Staphylococcus aureus, Klebsiella pneumoniae,* and *Escherichia coli*. Overnight cultures of the target bacterial species were re-suspended in BHI broth (1 mL) to a density of approximately 10^8 CFU/mL; 300 µl of two cultures l total bacterial cell volume from both bacteria in 1:1 ratio, then it was vortexing. *P. aeruginosa* was vortexing with *S. aureus, K. pneumonia* and *E. coli* (independently). Using 48 wells plate, 600 µl BHI broth containing 1/10 000 from co-culture was pipetting 600 µl into each well. Alginate beads were inserting to the wells after washing them in SDW using flamed wire and pliers. The plate was incubating overnight at 37ºC and 150 rpm. Triplicate beads were used for each mixed culture. Media and non-adherent cells were discarded after incubation. They were putting by the experiment in a new medium with ciprofloxacin and without ciprofloxacin. Then Incubate overnight at 37º C, 150 rpm. The beads were collecting at 2, 4, 6, 8, 12, and 24 h; and washed in 600 µl dH_2O in a 48 well plate before dissolving in a 15 ml falcon tube in two ml dissolving solution, the beads were smashing with a sterile loop stick. In order to differentiate between bacteria in the Miles Misra technique, biofilm production was estimated for each bacterium (alone and together), using selective media and antibiotics.

In dual-species biofilms between the studies isolate no. 2 and *S. aureus*, *P. aeruginosa* was inhibited by adding four µg/ml of amikacin to the culture media. Also, the isolate no. 2 was inhibited by exposing to 4 µg/ml of ceftazidime when it is co-cultured with *K. pneumonia*. Further, in dual culture with *E. coli*, the isolate no. 2 was inhibited by two µg/ml of gentamicin. The calculation of CFU for all isolates which were co-cultured with isolate no. 1 was done according to the following equation: CFU of dual-species biofilms culture minus CFU of isolate no. 1. All other above bacteria were inhibiting by cetrimide agar contains naldixic acid. [13]

Statistical Analysis

Data analyses were performed using the SPSS-23 (SPSS, Chicago, IL, USA) statistical package. Data are presented as simple measures of frequency, percentages, means, standard deviations, and ranges (minimum-maximum values). For quantitative data, differences between means were examined using t-tests for independent means or paired t-tests for differences between paired observations. An analysis of variance (ANOVA) was used to examine difference among more than two independent means. For qualitative data, the chi-square test was used to examine differences between percentages. P-values ≤ 0.05 were considered significant.

Results

The increase in the number of *P. aeruginosa* cells (isolates 1 and 2) attached to the alginate beads, under control conditions (BHI only, no additional antimicrobial or other additive) is shown in Figure 1. There was complete inhibition of growth when cultivating sessile cells of *P. aeruginosa* overnight on alginate beads when the BHI broth either 800 or 900 µg/mL ciprofloxacin for isolates no. 2 and 1, respectively (Figure 1). However, when the sessile cells are actively growing on the alginate beads and producing the biofilms, there is low-level resistance, and after four hours incubation, the ciprofloxacin concentration has a negligible effect on the sessile cells. The development of survival strategy against the selected ciprofloxacin concentration with both isolates only occurs between 4 and 8 hours growth. However, the survival of sessile cells decreased sharply and by 12 hours to isolate no. 2 and 24 hours to isolate no. 1 (figure 1).

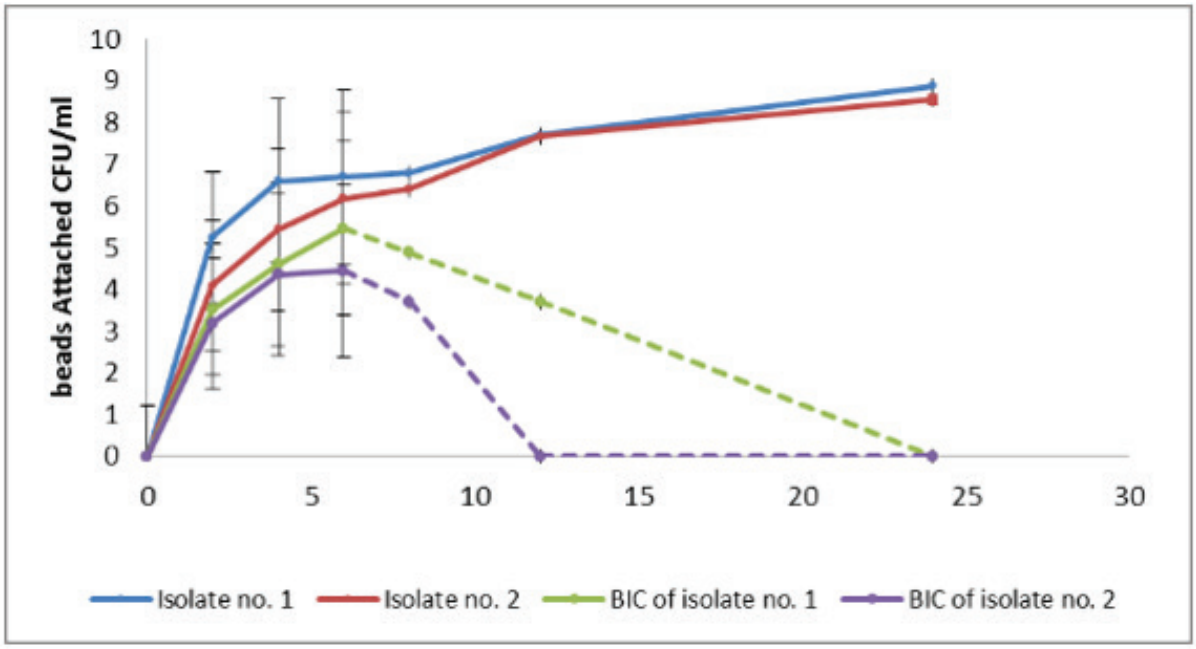

Figure 1. Development of biofilms on alginate beads of two isolates no. 1 and 2 for 24 h in logarithmic panel with no additive and with ciprofloxacin addition. Cultures were set up from overnight cultures as described in materials and methods, with triplicate beads or wells and sampled at the indicated time points. Beads were washed, dissolved, serially diluted and plated for CFU/ml counts. Mean CFU/ml counts are plotted against time of growth with error bars. Dashed line indicates the detection inhibit sessile cells.

The number of cells that adhered to the alginate beads was dependent on both concentrations of the supplements added and the bacterial isolate. The sessile *P. aeruginosa* cells exhibited copious growth when glucose or galactose was added to the media, although much more growth was discernible in the presence of glucose (Figures 2). Glucose supplementation resulted in more adherent cells than either fructose or galactose, and both isolates responded similarly to the added sugar (Figures 2). Both study isolates were yielded, enhancing biofilm production with the addition of glucose ($P<0.05$). Significant differences were detected in the biofilm production for both isolates with the presence of galactose ($P<0.05$) with one log increase, compared to sessile cells without any addition. No significant differences observed in the biofilm production with fructose supplementation compared to sessile cells without any addition (Figure 2).

In planktonic stage, the results obtained by adding monosaccharides to the culture medium indicated that the number of free swimming pseudomonal cells increased in two log with the addition of glucose, but with galactose and fructose the increase was negligible. Further, when ciprofloxacin was added the values of MICs were decreased (resistance decrease) with the addition of glucose, while the effect with the presence of galactose and fructose is negligible.

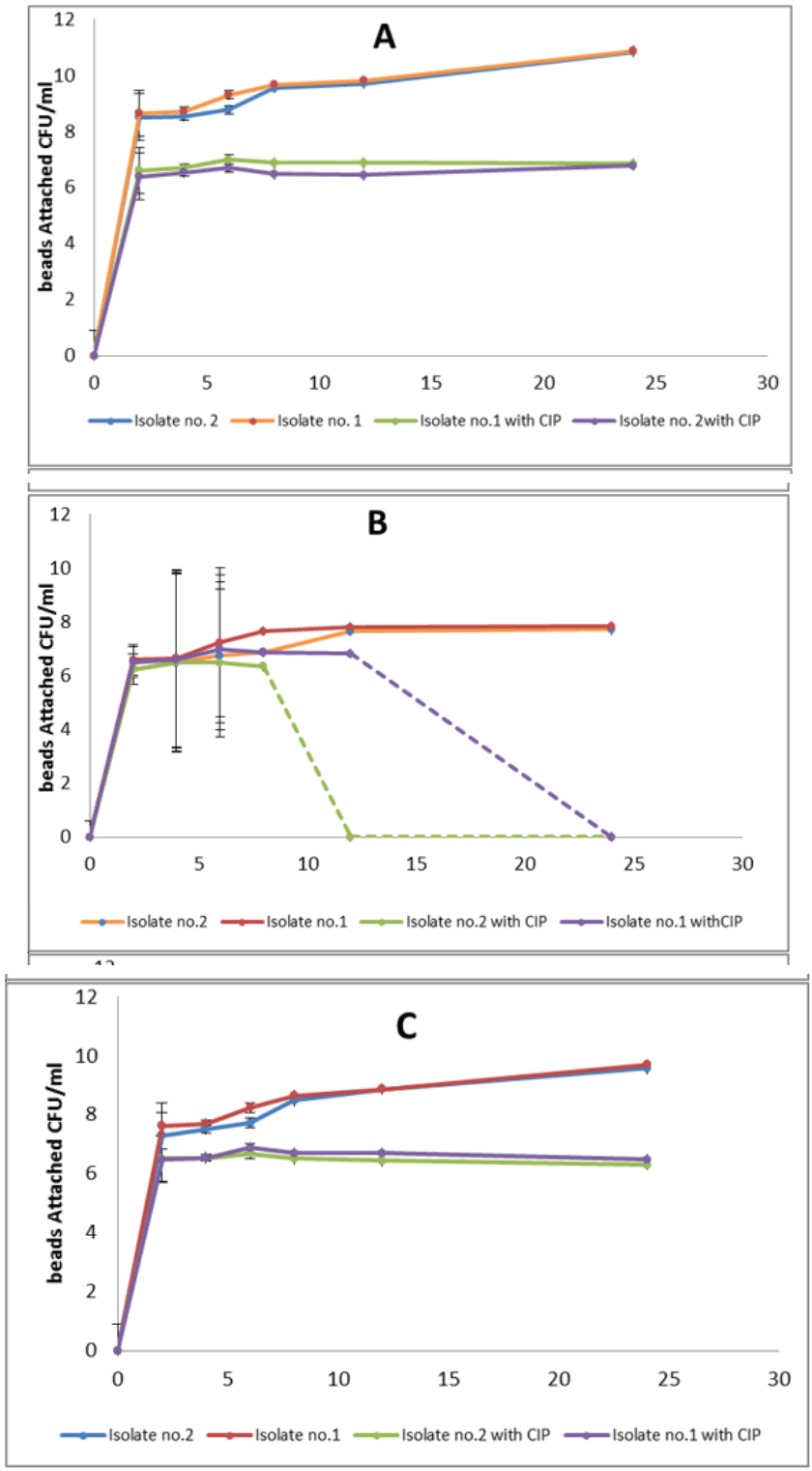

Figure 2. Development of biofilms on alginate beads of two isolates no. 2 and 1, with the addition of different monosaccharides to the culture media using logarithmic panel. Cultures were set up from overnight cultures as described in Materials and Methods, with triplicate beads or wells and sampled at the indicated time points. Beads were washed, dissolved, serially diluted and plated for CFU/ml counts. Mean CFU/ml counts are plotted against time of growth with error bars. Dashed line indicates the detection inhibit sessile cells A: glucose supplement, and glucose with ciprofloxacin supplements, B: fructose supplement, and fructose with ciprofloxacin supplements, C: galactose supplement, and galactosewith ciprofloxacin supplements.

The combined form of fructose with each glucose and galactose were supplementing to the culture media to determine the biofilm for producer isolates of *P. aeruginosa* during 24 h and compared with sessile cells of *P. aeruginosa* without any addition. The results revealed no significant differences for both isolates. The combined form of glucose and galactose was adding to media, which contains alginate beads to estimate the effect of combined action of glucose and galactose on the activity of sessile cells. They were showing significant differences ($P<0.05$) with one log increase in comparison with sessile cells of bacteria without addition (figure 3).

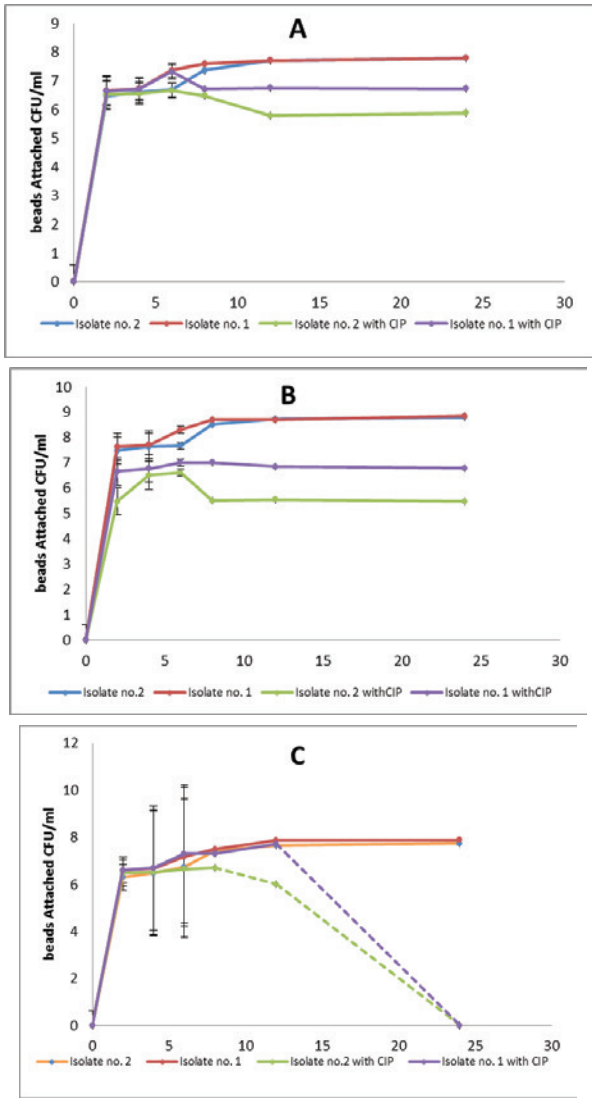

Figure 3. Development of biofilms on alginate beads of two isolates no. 2 and 1, with the addition of a different combined form of monosaccharides to the culture media. Cultures were set up from overnight cultures as described in materials and methods, with triplicate beads or wells and sampled at the indicated time points. Beads were washed, dissolved, serially diluted and plated for CFU/ml counts. Mean CFU/ml counts are plotted against time of growth with error bars. The dashed line indicates the detection inhibit sessile cells. A: glucose with fructose supplements, and glucose, fructose and ciprofloxacin supplements, B: glucose with galactose supplements, and glucose, galactose and ciprofloxacin supplements, C: galactose with fructose supplements, and galactose, fructose and ciprofloxacin supplements.

Ciprofloxacin was adding to the media, which contains glucose, fructose, and galactose, the combined form of glucose with each fructose and galactose, and combined form of fructose with galactose. For the study, isolates no. 1 and 2, there were significant differences ($P<0.05$) when ciprofloxacin added to culture media containing glucose. the effect of ciprofloxacin on the sessile cells in the presence of glucose is limited and it will becomes more effective and reduced sharply the no. of sessile cells when the glucose is drawing (in absence of glucose) (figure 1); ciprofloxacin did not eradicate the sessile cells with glucose presence. Furthermore, galactose enhanced survival of sessile cells with ciprofloxacin presence, but less than that occurred with glucose addition, as well as with the combined form of glucose and galactose addition with one log increase ($P<0.05$) compared with BIC of ciprofloxacin in the same time. Other additions when compared with BIC of ciprofloxacin statistically showing no significant differences when added ciprofloxacin to the culture media that containing fructose or combined form of fructose with each glucose and galactose during 24 h, which means that there was no noticeable increase or reduce in *P. aeruginosa* sessile cells (figure 4).

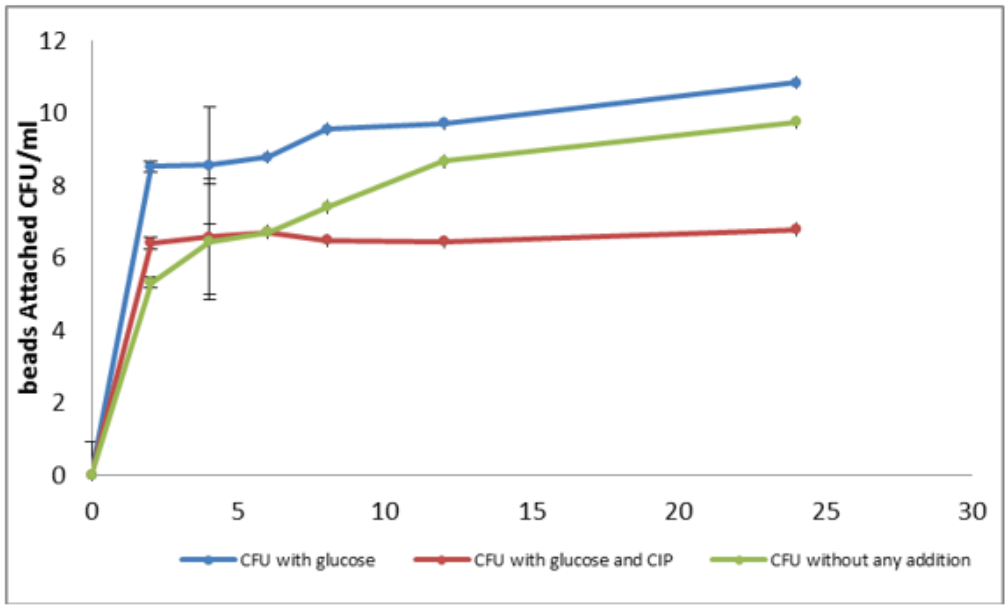

Figure 4. Development of biofilms on alginate beads of isolate no.2 without any addition, with adding glucose and with adding glucose with the ciprofloxacin. Cultures were set up from overnight cultures as described in Materials and Methods, with triplicate beads or wells and sampled at the indicated time points. Beads were washed, dissolved, serially diluted and plated for CFU/ml counts. Mean CFU/ml counts are plotted against time of growth with error bars.

Our initial experiments showed that D-gly, D-ala, and combined form of this amino acids, decreased biofilms formed by *P. aeruginosa*. However, the inhibitory action of the combined form of D-gly and D-ala was significantly ($P<0.05$) higher than D-gly and D-ala alone, while D-ala did not effect on sessile cells of isolate no.1. Hence, these amino acids were employed in experiments on account of its efficacy in combined action with ciprofloxacin. A positive correlation between these amino acids and ciprofloxacin was obtained from CFU count for both isolates. From the correlation lines, the number of adherent cells was calculated in the presence of D-gly, D-ala, and combined form of these D-amino acids supplementary. Rates of biofilm formation were obtained from the slopes of regression lines. Our results showed that a marked decrease in sessile cells when adding ciprofloxacin to the media containing D-amino acids. At 12 h, the BIC was observed with synergistic action between D-gly and ciprofloxacin for both isolates, and it was more potent with isolate no. 2. Also, the synergistic action between ciprofloxacin and combined form of D-gly with D-ala was observed. Synergistic

action also observed between D-ala and ciprofloxacin against isolate no. 2 (figure 5). The planktonic cells affected on amino acids. Planktonic cells showed a marked decrease in number with the addition of combined form of D-gly and D-ala, in comparison with D-gly alone. No observed effect appeared with the addition of the D-ala. Further, when adding the ciprofloxacin, the results showed synergistic reaction with the amino acids.

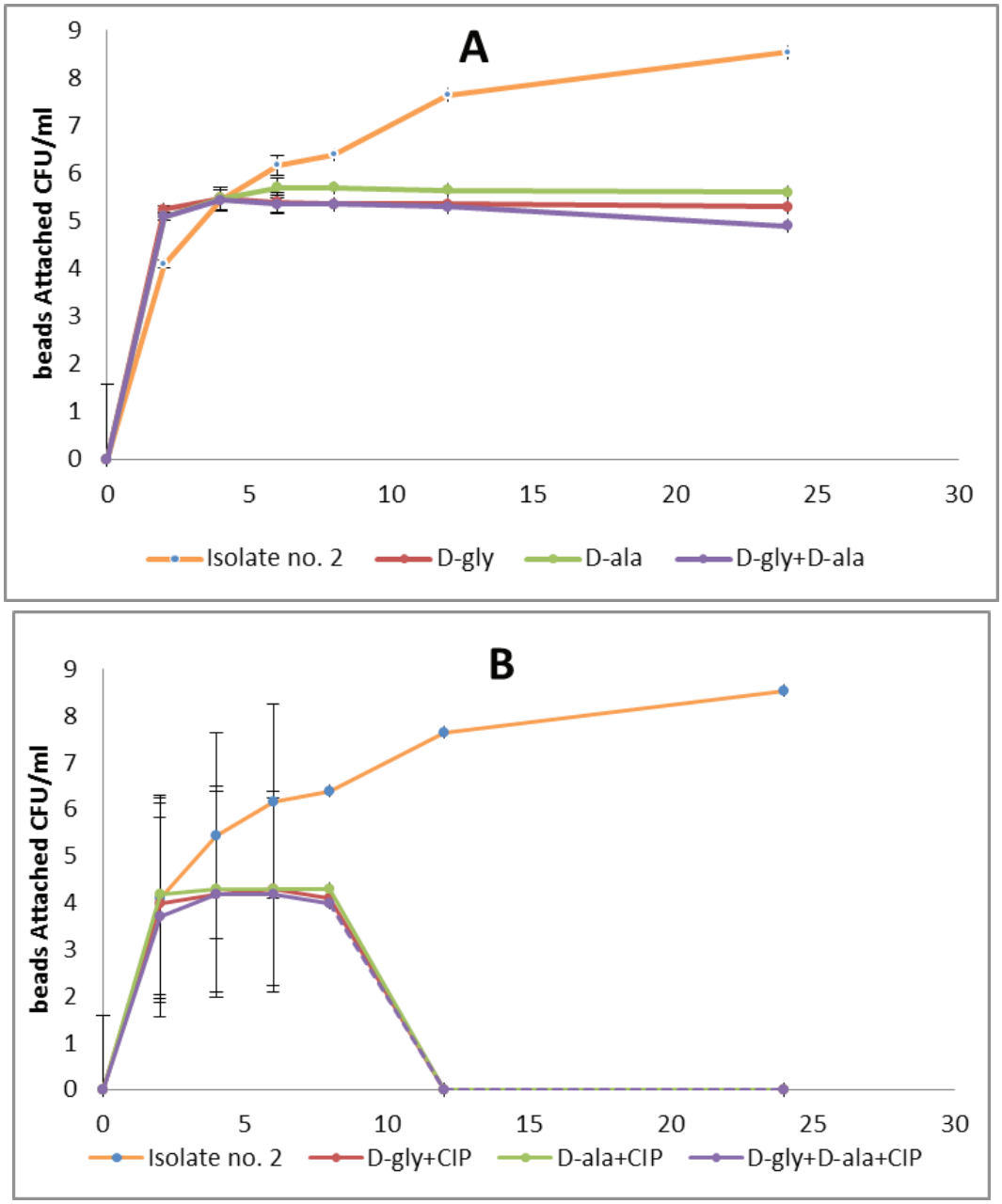

Figure 5. Development of biofilms on alginate beads for isolate no.2 versus addition some D-amino acids and ciprofloxacin to the media. Cultures were set up from overnight cultures as described in Materials and Methods, with triplicate beads or wells and sampled at the indicated time points. Beads were washed, dissolved, serially diluted and plated for CFU/ml counts. Mean CFU/ml counts are plotted against time of growth with error bars. The dashed line indicates the detection inhibit of sessile cells A: Sessile cells level without additions, and sessile cells level with added D-amino acids, B: Sessile cells level without any addition, D-amino acids, and ciprofloxacin addition.

Dual-species biofilms occur via a combination of bacterial auto/co-aggregation and attachment to a substratum. As dual-species biofilms under laboratory conditions, two isolates of *P. aeruginosa* with *S. aureus, K. pneumonia*, and *E. coli* were cultured for 24 h. They observed in 2, 4, 6, 8, 12, and 24 h. When estimated, the sessile cells compared to the single-species biofilm of *P. aeruginosa* and *S. aureus*, for the same sessile cells number of time. *P. aeruginosa* was increased of sessile cells in co-culture with *S. aureus*, while gave decreased in number in co-culture with *K. Pneumonia* and *E. coli*. However, changes were calculated relative to single-species biofilms for each bacterium. Dual-species biofilm composition analysis of sessile cells and calculation of competitive relative indicated that *S. aureus* reduced at each time point with less competitive relative indices than *P. aeruginosa*. The relative competitors are derived from sessile cell number and more significant competitive indexes, therefore mean higher bacterial numbers. This suggests that it was advantageous for *P. aeruginosa* to be part of a dual-species biofilm in terms of synergistic growth with *S. aureus*. The reduction of sessile cells of *S. aureus* after co-culture was significant differences ($P<0.05$) with one log increase (figure 6).

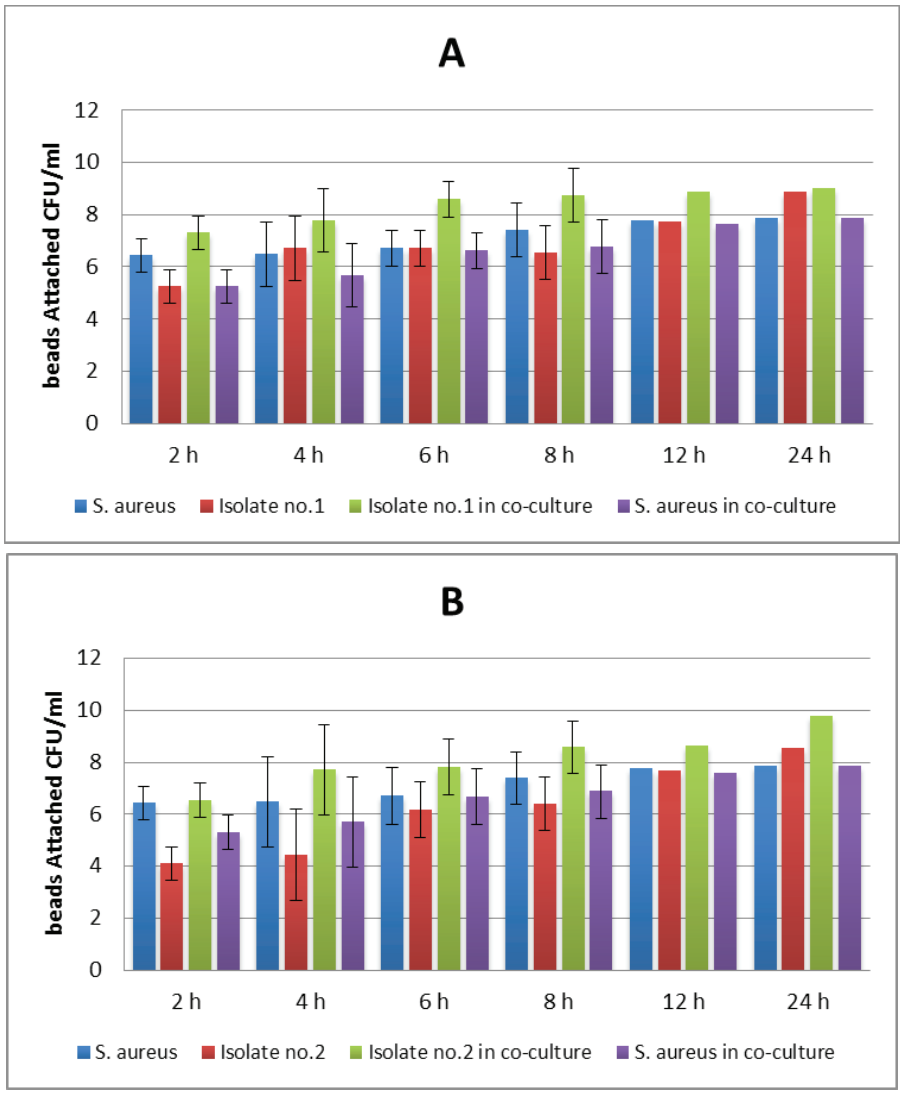

Figure 6. Development of biofilms on alginate beads for two isolates no.1 and 2 when co-cultured with *S. aureus*. Cultures were set up from overnight cultures as described in Materials and Methods, with triplicate beads or wells and sampled at the indicated time points. Beads were washed, dissolved, serially diluted and plated for CFU/ml counts. Mean CFU/ml counts are plotted against time of growth with error bars. , A: isolate no. 1, B: isolate no.2.

Conversely, sessile cells of *P. aeruginosa* were observed when co-incubated with *K. pneumonia and E. coli.* Sessile cells of both isolates no. 1 and 2, which were one log reduce (P<0.05) compared to the sessile cells alone, while sessile cells of *K. pneumonia* with both isolates of *P. aeruginosa* no. 1 and 2 were one log increase (P<0.05) compared to sessile cells of *K. pneumonia* alone. Statistically, *E. coli* gave no significant differences compared to *E. coli* alone (figure 7).

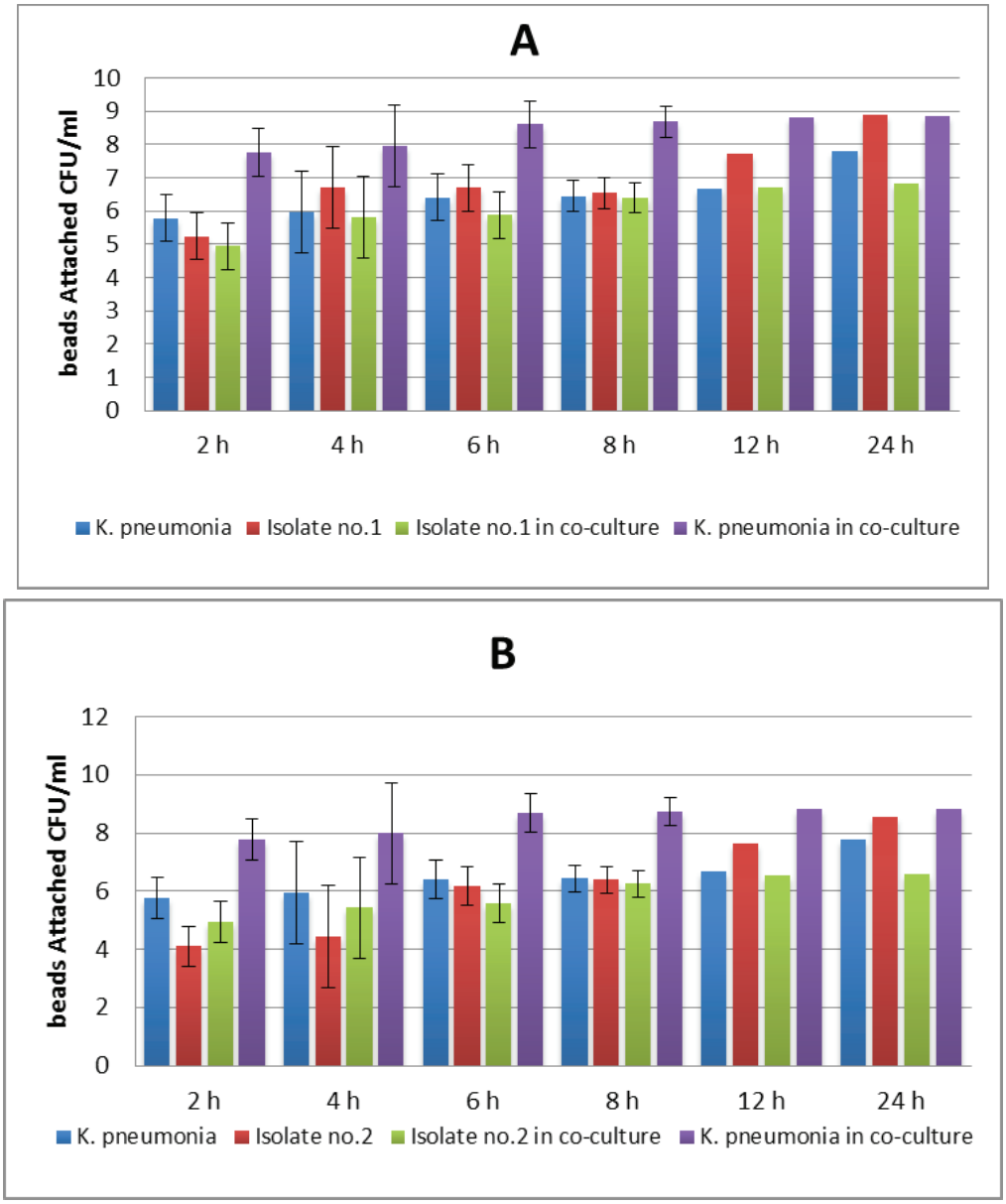

Figure 7. Development of biofilms on alginate beads for two isolates no.1 and 2 when co-cultured with *K. pneumonia*. Cultures were set up from overnight cultures as described in Materials and Methods, with triplicate beads or wells and sampled at the indicated time points. Beads were washed, dissolved, serially diluted and plated for CFU/ml counts. Mean CFU/ml counts are plotted against time of growth with error bars. , A: isolate no. 1, B: isolate no.2. A: isolate no. 2, B: isolate no. 1.

P. aeruginosa was cultured in the presence of *S. aureus, K. pneumonia*, and *E. coli* when grown on alginate beads. We were hypothesizing that exposure of *P. aeruginosa* to *S. aureus*, *K. pneumonia*, and *E. coli* might alter the BIC of *P. aeruginosa* to ciprofloxacin. To test this hypothesis, it was selecting a methicillin-resistance *S. aureus*, *K. pneumonia*, and *E. coli*. BIC was determined in 24 h for both isolates of *P. aeruginosa* with *S. aureus*. It was 900 µg/ml ciprofloxacin for isolates no. 1, and 800 µg/ml ciprofloxacin for isolate no. 2. After dual-species biofilms culture, they were become 800 µg/ml to isolate no. 1, whilst isolate no. 2 still 800 µg/ml. *S. aureus* with isolate no.1 before and after dual-species biofilms. BIC was still 1000 µg/ml. *S. aureus* with isolate no.2 before dual-species biofilms, BIC was 1000 µg/ml, while after dual-species biofilms culture, BIC has become 800 µg/ml (figure 6).

Dual species biofilms with ciprofloxacin addition, *P. aeruginosa with K. pneumonia* when had co-cultured on alginate beads, BIC was 900 µg/ml ciprofloxacin for isolate no. 1, while BIC of *P. aeruginosa* isolate no. 2 was 800 µg/ml. After co-incubation with *K. pneumonia* on alginate beads, BIC becomes 100 µg/ ml at four hours of isolate no. 2. Isolate no. 1 becomes 200 µg/ ml at the same time. *K. pneumonia* before co-cultured, BIC was 100 µg/ ml, while after co-cultured, it becomes 700 µg/ ml with isolate no. 2. With isolate no. 1 BIC of *K. pneumonia* becomes 300 µg/ ml (figure 7).

P. aeruginosa biofilm cell viability increased in 24 h when these microbes were co-culture with *E. coli* on alginate beads. With ciprofloxacin addition, BIC of *P. aeruginosa* isolates no. 2 becomes 500 µg/ml. Before co-cultured, BIC was 800 µg/ml. Isolate no. 1, BIC was 900 µg/ml, after co-cultured with *E. coli* BIC becomes 700 µg/ml. BIC of *E. coli* was describing before and after co-culture. It was 100 µg/ml ciprofloxacin before dual-species biofilms culture. *E. coli* when co-cultured with isolate no. 1; BIC becomes 200 µg/ml, while when co-cultured with isolate no. 2; BIC becomes 600 µg/ml (figure 8).

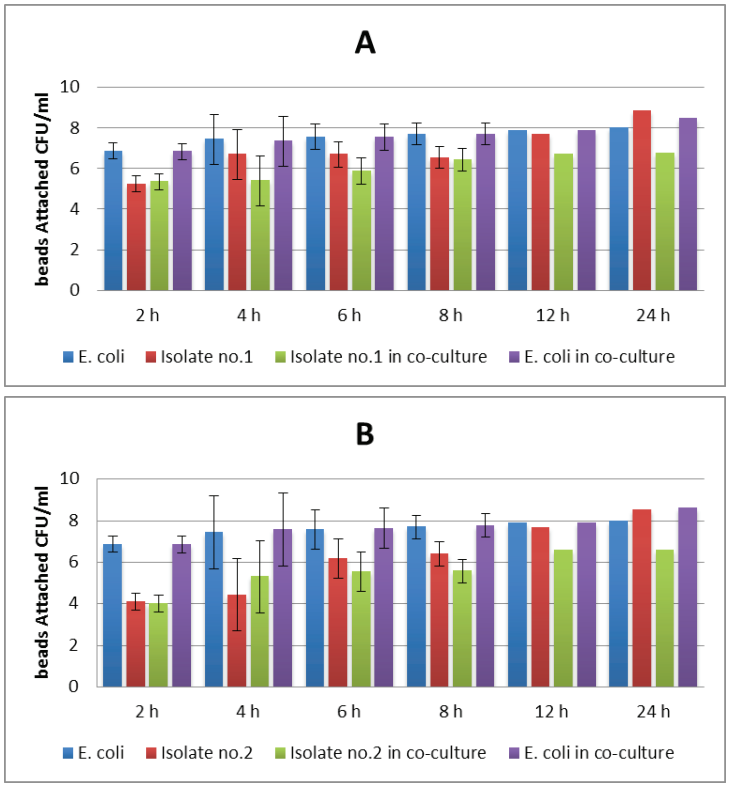

Figure 8. Development of biofilms on alginate beads for two isolates no.1 and 2 when co-cultured with *E. coli*. Cultures were set up from overnight cultures as described in Materials and Methods, with triplicate beads or wells and sampled at the indicated time points. Beads were washed, dissolved, serially diluted and plated for CFU/ml counts. Mean CFU/ml counts are plotted against time of growth with error bars. , A: isolate no. 1, B: isolate no.2.

Discussion

The impact of sugars and other factors on biofilm formation can vary depending on the type of bacteria present. [14] Up to our simple knowledge, this study has been done for the 1st time in our country. Our research suggested that the fructose and combined form of fructose with each glucose and galactose were at least stop the effect of these two types of sugars when this combined form tested with ciprofloxacin against pseudomonal biofilm. Further, this research suggested that the glucose and galactose affect markedly on sessile cells of *P. aeruginosa*, and it is in agreement with those observed by Rasamiravaka. [15] who concluded that the glucose could influence growth of biofilm. Geerlings and associates, [16] going to that the urine samples with glucose concentrations between 100 and 1000 mg/dL, were responsible for enhancing bacterial growth, while Khangholi and Jamalli, [14] revealed that the bacterial biofilm was not influenced with the presence of sugar. The interpretation of the above statements is may be due to that the *P. aeruginosa* had generated at least three polysaccharides (alginate, pel, and psl), which are essential for biofilm structure stability and the qualitative composition in the biofilm matrix of their polysaccharides, primarily alginate, Psl, and Pel, varies in the *P. aeruginosa*. The Pel polysaccharide is predominantly a glucose-rich matrix, whereas the Psl consists of D-mannose, L-rhamnose, glucose, and repeated pentasaccharides. In the early stages of biofilm formation, Pel and Psl may serve as a primary structure ground for biofilm development. [15] This may also be interpreted as what's has happened in diabetic patients in vivo who were more likely to get an infection from the urinary tract due to elevated levels of blood sugar; the high concentration of sugar provides to pathogenic bacteria a suitable growth environment. [17]

Our findings showed that the galactose enhancing biofilm formation after 24 hours. This result is in line with Khangholi *et* al., [14] who showed that galactose enhances biofilm formation. This may be due to the PSL production provides a benefit at the group level to cells growing in biofilms [18], and Psl is a galactose-rich exopolysaccharide. [19] The study results revealed that the glucose and galactose and combined form with each other, influencing survival strategy against ciprofloxacin. This result is in line with Allison *et al.,* [11] who concluded that after the addition of particular carbon metabolites to persister cells, the cells return into a state in contradiction of which antibiotics. [20] In contrast, Flume *et al.,* [21] reported that adjunctive sugar therapy might be particularly useful in treating *P. aeruginosa* pulmonary infections with increased intrinsic resistance to antibiotic and multidrug-resistant isolates. This interpretation may be due to that the sugars in the early stage can promote the interaction of a wide range of bacterial adhesions to form bacterial clusters, such as type IV pili. The sugars can help to bind or absorb antibiotics. Eventually, in clusters, sugar can alt the metabolic status of bacteria without raising the proliferation of bacteria, making them more antibiotic susceptibility.

The free swimming pseudomonal cells increased with the addition of glucose. Further, when ciprofloxacin was added the values of MICs were decreased with the addition of glucose. Our results were in line with Paranjape and Shashidhar, [22] who concluded that the glucose increases the respiration which in turn increases the metabolism and cell division rate. Furthermore, the addition of glucose could increase the susceptibility of persister cells to ciprofloxacin only. In general, the bacterial susceptibility can be increased by combining the antibiotics with glucose.

The planktonic cells affected on amino acids. Planktonic cells showed a marked decrease in number with the addition of combined form of D-gly and D-ala, in comparison with D-gly alone. Our initial experiments showed that D-gly, D-ala, and combined form of these amino acids, reduced biofilms formed by *P. aeruginosa*, relative to control. The combined form of D-gly and D-ala was more potent against biofilm production than those of control. These results are in agreement with Hammes *et al*., [23] who suggested that the amount of incorporated D-gly or D-ala can decrease sessile cells of bacteria. This interpreted may be due to D-gly, which is integrated into peptidoglycan. D-gly could replace L-alanine in position one and D-ala residues in positions 4 and 5 of the peptide subunit. The Replacing of L-ala in position one with UDP-MurNAc: l-Ala ligases, which suggest a ligase inhibition of peptides with uridine diphosphate-muramic acid accumulation. Modified peptidoglycan precursors are indicated to be produced by weak substrates of some of the enzymes in peptidoglycan synthesis, D-gly effect on sessile cells CFU/ml. Their investigations had shown

that D-gly interferes with several steps of peptidoglycan synthesis. Kao *et al*., [24] reported that the D-ala did not deter the development of *P. aeruginosa* biofilm, but delaying in biofilm formation.

In order to gain a better understanding of the interactions between different microbial species in this complex environment, the analysis of these various communities is crucial. Recognizing these relationships will lead to the detection of unique goals and therapeutics that were likely to be missed otherwise. *P. aeruginosa* biofilm monospecies inoculated with *S. aureus, K. Pneumoniae*, and *E. coli* as a secondary colonizer showed significant increases and decreases in the number of sessile cells in contrast with single species *P. aeruginosa* biofilm. We are hypothesized that the dual culture between *P. aeruginosa* and *S. aureus* exhibits an increase of sessile cells of *P. aeruginosa*. Alves *et al*., [25] showed that the biomass rises detected between *P. aeruginosa* monospecies and a dual-species biofilm relative to single-species *P. aeruginosa* biofilms at the same period. Our findings showed a huge, statistically significant difference ($P \leq 0.05$) between *P. aeruginosa* monospecies and dual-species *S. aureus* Biofilm. *S. aureus* was using in biofilm growth tests as a promoter colonizer and improved *P. aeruginosa* attachment. *S. aureus* contributes to the *P. aeruginosa* biofilms formed for 24 h has a secondary colonizer and results in a decrease in the total biofilm biomass of *S. aureus*; this effect is not reciprocal. Somehow disrupts by *S. aureus* for *P. aeruginosa* biofilm, perhaps by dispersion or direct bactericidal action. Also, secreted components extracted from *S. aureus* biofilm repeated this effect, which indicates that the dispersal of biofilm is dependent on a secreted element. *S. aureus* is known to create nucleases that prevent or interfere with the production of biofilm in several bacteria pathogens, including *P. aeruginosa*. The results of this study were showing clear interactions between *S. aureus* and *P. aeruginosa* that are both competitive and reciprocal in pathogenicity and colonization for each organism.

The interpretation of bacterial-bacterial interactions may be due to an association between the SpA factor and *P. aeruginosa* Psl, the *S. aureus* element (*S. aureus*-specific staphylococcal protein A) SpA, had shown to inhibit the formation of biofilm as measured. Their inability to add SpA to type 4 pili in the case of *P. aeruginosa* was attributed when Psl was not present, and therefore their attachment was reduced. [26] Our finding revealed that the co-incubation between *P. aeruginosa* and *S. aureus* did not change survival against ciprofloxacin, while Orazi and O'Toole, [27] had shown that interspecies bacterial interactions alter antibiotic tolerance in unpredictable ways. Specifically, they found that *P. aeruginosa* protects biofilm and planktonic populations of *S. aureus* from an antibiotic. This interpretation may be due to the high-affinity quinone oxidation of *P. aeruginosa* inhibitor and siderophores, which is defending *S. aureus* biofilms from antibiotic therapy.

Natural biofilms had often were found within both the ecosystem and the host as multi-species populations that differ significantly from monospecies biofilm systems in their composition, structure, and survival strategies against antimicrobial resistance. *K. pneumonia* and *P. aeruginosa* were two bacteria that were often found together within biofilm-mediated chronic wound infections. The two species that are active in biofilms that are often found together, *P. aeruginosa* and the *K. pneumoniae.* [28.]In our results, *P. aeruginosa* showed one log reduction of sessile cells when co-cultured with *K. pneumonia*, while Goncalves *et al*., [29] showed that the anti-biofilm activity of *K. pneumoniae* supernatant against pseudomonal biofilm was indicated no significant effect on biofilm production. The interpretation may be due to The capsular polysaccharide of *K. pneumoniae* is implied in surface adhesion, spacing and order of bacteria in the initial step of biofilm formation, and is required for late biofilm maturation step. Though the initial adhesion of bacteria on the surface constitutes a crucial step in the formation of biofilm, the spread of bacteria on the surface is also another essential factor in the formation of a biofilm, especially in a multispecies highly competitive environment. Some Gram-negative rods such as the highly motile *P. aeruginosa* produce exopolysaccharides that promote their own surface movement during the early stages of biofilm formation. *K. pneumoniae* is a nonmotile Gram-negative rod and may have developed different social strategies leading to surface exclusion of competitors by large capsular polysaccharide production and therefore allowing successful surface colonization. [30]

When *P. aeruginosa* co-cultured with *E. coli* as it was occurred in our research, in addition to *K. pneumonia, P.*

aeruginosa sessile cells were reduced in number. This result is compatible with those observed by Culotti and Packman, [31] who highlighted the relationship between *P. aeruginosa* and *E. coli*. *P. aeruginosa* introduction triggered a growth response that allowed *E. coli* to developed biofilm. *E. coli* was continuously deposited in pre-established *P. aeruginosa* biofilms and also in nutrient agar reduced colonized the interiors of *P. aeruginosa* clusters. *P. aeruginosa* grew significantly lower under the same nutritional and flight conditions in mixed cultivations than in monocultures. In contrast, following the introduction of *E. coli*, *P. aeruginosa* biofilm biomass decreased. *E. coli'*s strong antagonism to *P. aeruginosa* is suggested by the reduction of *P. aeruginosa* biomass in dual biofilms. Extracellular indole accumulation had already documented in order to reduce the development of *E. coli* biofilm by impairing cell motility. Indole, which is an *E. coli* metabolite formed by tryptophanase from the amino acid breaks down by *P aeruginosa*, and can probably improve *E. coli* biofilms through the elimination of the growth inhibition induced by extracellular indole. *E. coli* strains that were indole-producing and indole-deficient, however, similarly increased in biofilms. The indole-deficient *E. coli* grew poorly in monoculture, but after the introduction of *P. aeruginosa* was able to form extensive biofilms. The studies with the indole producing strains *E. coli* observed similar growth patterns. This suggests that indole can play a significant role in *E. coli–P. aeruginosa* biofilm's growth behavior. [31]

Conclusion

The study suggested that the findings concerning the impact of monosaccharide and D-amino acids on *P. aeruginosa* biofilm. There were significant differences between the numbers of sessile cells before and after adding monosaccharide as well as in amino acids (with and without ciprofloxacin), glucose and galactose, and the combination of each one with ciprofloxacin is the enhancing of *P. aeruginosa* biofilm and increases survival strategy against ciprofloxacin. In contrast, a noticeable biofilm reduction and marked synergistic action for ciprofloxacin have been determined in the combination of the two D-amino acid, alanine, and glycine in comparison to the effect of each one alone. Furthermore, pseudomonal dual-species biofilms culture with *S. aureus* gave a raised number of sessile cells with the reduction of BIC. In co-culture with *K. pneumonia* and *E. coli*, sessile cells of *P. aeruginosa* were reduced in number and made Pseudomonal biofilm less resistant to ciprofloxacin.

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