Inhibitory Effect of Antibiotics and Probiotics on Multidrug-Resistant Pseudomonas aeruginosa Isolates Producing Some Virulence Factors

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Article's Information	Abstract
	Antimicrobial-resistant (AMR) bacterial infection is major challenges for global health systems. Pseudomonas aeruginosa is the most important clinical and epidemiological microorganisms. It is the most common cause of nosocomial infections among non-fermenting, Gram-negative bacteria and the most common cause of opportunistic infections in patients with diseases. P.aeruginosa sample were collected from two hospitals (Al-Kadhimiya and Abu Ghraib Hospital) from the beginning of November to the end of December 2023, and identified using standard microbiological methods. All isolates were evaluated for the production of virulence factors (biofilm formation and protease activity). Four isolates with the highest values of virulence factors were selected for further analysis of antibiotic and probiotic susceptibility using broth dilution test. The study results revealed the presence of 27 samples of Pseudomonas aeruginosa bacteria, which is equivalent to 22.5% of the total 120 samples examined were identified. All isolates produced varying amounts of biofilm and protease. Four most virulent isolates were selected for susceptibility testing. All four isolates showed susceptibility to the tested antibiotics and probiotics, and significant growth inhibition was observed. This study revealed the presence of P. aeruginosa isolates with different virulence potential in Baghdad hospitals. Highly virulent isolates showed susceptibility to the tested antibiotics and probiotics, suggesting the need for further investigation of alternative treatment strategies besides conventional antibiotics.

1. Introduction

Pseudomonas aeruginosa is ubiquitous in many natural environments, including soil and water, and is a major cause of human infections. This is due to its ability to survive and even thrive at different temperatures, in different nutrient sources, and among genetic species that are highly virulent and resistant to antibiotics, disinfectants, and other antimicrobial compounds [1]. Pseudomonas aeruginosa is an opportunistic human pathogen that can cause both acute and chronic infections. Urinary tract infections, lung infections, soft tissue infections, bone and joint infections, bacteremia, and various systemic diseases are examples of acute infections [2]. Pseudomonas aeruginosa tends to form biofilms, which are complex bacterial communities that adhere to a variety of surfaces, including metals, plastics, and medical implant materials, and tissues. Growth in biofilms promotes bacterial survival, once a biofilm is formed it is extremely difficult to destroy [3]. Due to the flexibility of P. aeruginosa and its strong inherent drug resistance, conventional antimicrobial treatments such as antibiotics are often fewer efficient and increase fatality [4].

2. Materials and Methods
2.1. Sampling
One hundred twenty clinical samples (urine, wounds, sputum and burns) were collected from patients at AL Kadhimiya Educational Hospital and Abu Ghraib Hospital in Baghdad. Pseudomonas aeruginosa diagnosis:

The sample swabs were cultured on nutrient agar, MacConkey agar, and cetrimate agars, and then incubated at 37°C under aerobic conditions for 24 h. The bacteria were characterized based on bacteriological and biochemical tests and further conformational characterization was performed using the Vitek 2 system [5].

2.2. Quantitative study of virulence components of P. aeruginosa
a. Assay of Biofilm Formation
The ability to form biofilm was quantified by a colorimetric microplate assay [6]. Bacterial colonies were developed overnight at 37°C for 24 h. In brain heart broth. Every bacterial suspension was added to a fresh dilution (1:100) of medium, and 150 µL of this dilution was used to antisepticize the medium in a 96-well flat-bottom polystyrene microtiter plate. After incubation at 37°C for 24 h (without shaking), the wells were gently washed three times with 200 µL of PBS . To spot the biofilm, 100 µL of 99% methanol was added. Thereafter 15 minutes, every solving was eliminated and the plate was air-dried. In the subsequent step, 150 µL of 1% crystal violet (CV) was added to all wells for 20 minutes. After the dye was removed, the bound CV was liberated by adding 150 µL of 33% acetic acid. Every absorbance of the decolorized solution was measured at 490 nm using an ELISA reader (Stat Fax-2100). Each test was performed in triplicate. Uni-nucleated culture medium was used as a control. Based on the optical density of the sample (ODi) and the average optical density of the negative control (ODc), the samples were classified according to Table 1.

Table 1. Classification of bacterial biofilm formation by the microtiter plate method [7].

Mean OD490	Biofilm intensity
OD ≤ ODc	None producer
ODc < OD ≤ 2 * ODc	Weak
2ODc < OD ≤ 4 * ODc	Intermediate
4 * ODc < OD	Strong
* OD = optical density, ODc (Cut off value) = average OD of negative control + (3 *Standard Deviation).	

b. Assay of Protease Formation
Protease activity was determined as originally described as follows (8,9) .
1. Casein (0.8 ml, 0.5%, pH 8) was pre-incubated for 10 min in a water bath at 37 °C.
2. Thereupon, 0.2 ml of crude enzyme was supplementary to the substrate working solving and incubated for 20 moment.
3. Anteriorly adding the crude enzyme, the reaction was stopped by adding 3 mL of TCA (5%).
4. Twain the reaction and the blank were centrifuged at 5000 rpm for 20 min.
5. Every amount of TCA soluble product formed can be determined by calculating the absorbance of the supernatant at 280 nm.
6. Protease action was determined using the equalization:

$$Protease\ activity\ \frac{U}{MI} = \frac{\text{Absorbance at 280 nm}}{0.001 \times 20\,\text{min} \times 0.2\,\text{ml}}$$

c. Determination of Protein Concentration
The information you provided describes the initial steps for creating a standard curve in the Bradford protein assay using BSA. Below is a detailed description of what you have done [10].
1. Standard preparation: You prepared a series of BSA dilutions using different volumes of stock solution according to table 2. This created standards with different protein concentrations.

Table 2. Concentrations of the Bovine serum albumin solutions.

No. of Tube	V. of BSA/ml	Volume of D.W./ml	Final v/ml	Conc. (mg/ml)
1	0.0	1	0.0	0.0
2	0.1	0.9	0.1	0.1
3	0.2	0.8	0.2	0.2
4	0.3	0.7	0.3	0.3
5	0.4	0.6	0.4	0.4
6	0.5	0.5	0.5	0.5
7	0.6	0.4	0.6	0.6
8	0.7	0.3	0.7	0.7
9	0.8	0.2	0.8	0.8
10	0.9	0.1	0.9	0.9
11	1	0.0	1	1

2. Add Bradford reagent: Add a fixed volume (2.5 mL) of Bradford reagent (containing Coomassie Brilliant Blue G-250 dye) to each BSA standard and mix them.

3. Incubation: Incubate the mixture at room temperature for 2 minutes to allow the dye to bind to the protein.
4. Blank measurement: Prepare a blank containing only Tris-HCl buffer and Bradford reagent and measure its absorbance at 595 nm. Next Steps: Construct a standard curve between BSA concentration and the corresponding BSA absorbance as in figure 1.
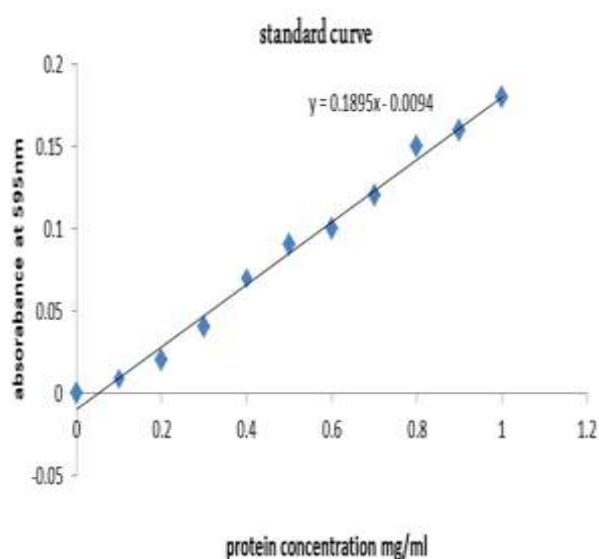
Figure 1: Standard curve of bovine serum albumin to determination of protein concentration by Bradford method

d. Antibiotics Susceptibility Test
The test was performed by using a modified Kirby-Bauer method as follows [11]:
1. Collect a few colonies from an overnight culture plate with a sterile inoculating loop and emulsify in 5 ml of sterile saline until a turbidity equivalent to McFarland standard 0.5 is reached.
2. Dip a sterile swab into the vaccination tube and squeeze out the excess liquid from the tube wall.
3. Inoculate the surface of a Mueller-Hinton agar plate with the bacterial colonies. Wipe the entire surface of the plate, then rotate the plate 45° and swab the entire surface again. Finally, rotate the plate another 90° and unfold it again.
4. After a few minutes, place the seven antimicrobial discs listed in table 3 on the surface of the inoculated plate.
5. Incubate the plate at 37°C for 18-2 h. After incubation, examine the plate for the presence of a zone of bacterial growth inhibition around the antimicrobial discs.

e. MIC Determination by Broth Dilution
The minimum concentration of an antimicrobial agent that prevents visible growth of a specific bacterium. Broth dilution is a well-established method for determining MIC and is considered the "gold standard" for bacterial susceptibility testing to antibiotics and probiotics [12]. As follows:
1. Prepare antibiotic and probiotic dilutions: Antibiotic (amikacin) and probiotic (combination of Lactobacillus plantarum and Lactobacillus acidophilus) are serially dilutedat (1, 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024) µg/ml. They are prepared by adding different ratios to their stock solutions.
2. Inoculate broth: Take approximately 20 µL of the above dilutions using a micropipette and inoculate into a well of a microtiter plate. The well contains antibiotics dissolved in BHI at different concentrations.
3. Incubation and reading of MIC: Incubate the microtiter plate at 37°C for 18-20 h, after incubation, add 20 µL of Resazurin dye to all wells and incubate for 30 min to observe any color change. Using Resazurin dye to assess growth is a common method. A color change from blue to pink indicates active bacterial growth, while no color change indicates inhibition by the antibiotic

Table 3: Antibiotics used in this study.

Antibiotic	Disc content (µg)	Symbol of antibiotic
Piperacillin	(100 µg)	PRL
Piperacillin-tazobactam	(100/10 µg)	TZP
Meropenem	(10 mg)	MEM
Gentamicin	(10 µg)	GEN
Amikacin	(30 µg)	AMK
Ciprofloxacin	(5 µg)	CIP
Norfloxacin	(10 µg)	NOR
Levofloxacin	(5 µg)	LVX

3. Results and Discussion
3.1. Sample collection
A total of 120 samples (urine, wound swabs, and spu um) were collected and cultured on different media for diagnosis of P. aeruginosa. Of these, 27 isolates were confirmed as P. aeruginosa. These samples included (22.2%) wound swabs, followed by urinary tract infection samples (11.1%), burn wound swabs (55.5%), and sputum swabs (11.1%) as shown in table 4. The collected samples were cultured on cetrimide agar medium, which is considered to be a selective medium for P. aeruginosa, at 37°C for 24 h. 27 isolates showed positive growth on hexadecyl

trimethyl bromide agar and produced a green pigment. Multiple biochemical tests were performed to support and confirm the diagnosis. 27 isolates were positive for oxidase and catalase. Based on the biochemical test results, the Vitek 2 system was applied to all these isolates and the same results were obtained, confirming the identification as P. aeruginosa.

Table 4: Number and percentage of Pseudomonas aeruginosa isolates with specimens' source.

Source of Samples	Number of Specimens	Number of P. aeruginosa	Percentage P.aeruginosa
Burns infection	35	15	55.5%
Sputum infection	24	3	11.1%
UTI	34	3	11.1%
Wound infection	27	6	22.2%
Total	120	27	100%

3.2. Screening of Virulence factor Production from bacterial isolates

i. The quantitative screening of protease

The production of proteases and the estimation of their activity and specific activity, the results showed that all locally isolated P. aeruginosa protease producers had viral levels ranging from 900 u/mg to 320 u/mg specific activity as shown in table 2-1. Among these isolates, P. aeruginosa P24 isolated from burns was the most efficient in protease production. They pointed out that the differences in enzyme production among each isolate could be due to changes in the genetic code leading to changes in enzyme synthesis.

ii. The Quantitative screening of biofilm

In this study, 27 isolates were evaluated using the microtiter plate method (MTP). In the MTP method, used 96-well polystyrene plates to detect biofilm production. The results in table 5 show that 19 (70%) P. aeruginosa of isolates produced moderate biofilm, which are almost consistent with the results reported El-Khashaab and are consistent with the reported results [14]. This resulted in 14 (40%) producing mediocre biofilm. 10 (15.8%) had weak biofilm production, which is almost consistent with the results reported [14,15].This showed that 9 (33.3%) produced weak biofilm, and in this study, 100% of the isolates produced biofilm.

Table 5: Percentage of P. aeruginosa producing biofilm according to the MTP method.

Biofilm	No. of isolates	Percentage (%)
Strong biofilm	0	0 %
Moderate biofilm	19	70 %
Weak biofilm	8	30%
Total	27	100 %

iii. Antibiotic susceptibility tests

Isolates of P. aeruginosa from different sources that exhibited the highest virulence were studied against various antibiotics using the disk diffusion method. This test found that four of the isolates were multidrug resistant (MDR) and "resistant to three or more antimicrobial classes." These MDR isolates exhibited resistance to more than one group of antimicrobial drugs: aminoglycosides, penicillins, beta-lactam combinations, fluoroquinolones, and carbapenems. These isolates exhibited different resistance capabilities to each antibiotic, as shown in table 6. In addition, the effects of antibiotics and probiotics on the most resistant P. aeruginosa isolates: P24 and P27 were examined.

Table 6: Antibiotic susceptibility tests for p.aeruinosa with diameter.

Antibiotic	P27	P24	P6	P19
Amikacin	R	R	R	R
Levofloxacin	R	R (9)	I (20)	R
Piperacillin	R	R	I (18)	R
Ciprofloxacin	R	R	S (26)	R
Piperacillin – tazobactam	R (10)	R (14)	S (24)	R (11)
Norfloxacin	R	R	S (32)	R
Gentamicin	R	R	R (6)	R
Meropenem	R (12)	R(15)	I (17)	I (16)

Determination of the Minimum Inhibitory Concentration (MIC) for antibiotic and probiotic. According to the resazurin reagent assay method, the results indicate that antibiotics (amikacin) and probiotics (a combination of Lactobacillus plantarum and Lactobacillus acidophilus) can inhibit the growth of Pseudomonas aeruginosa, thereby inhibiting the growth of Pseudomonas aeruginosa. The concentrations are shown in table 3.4.

Table 7: Minimum Inhibitory Concentration (MIC) for antibiotic and probiotic.

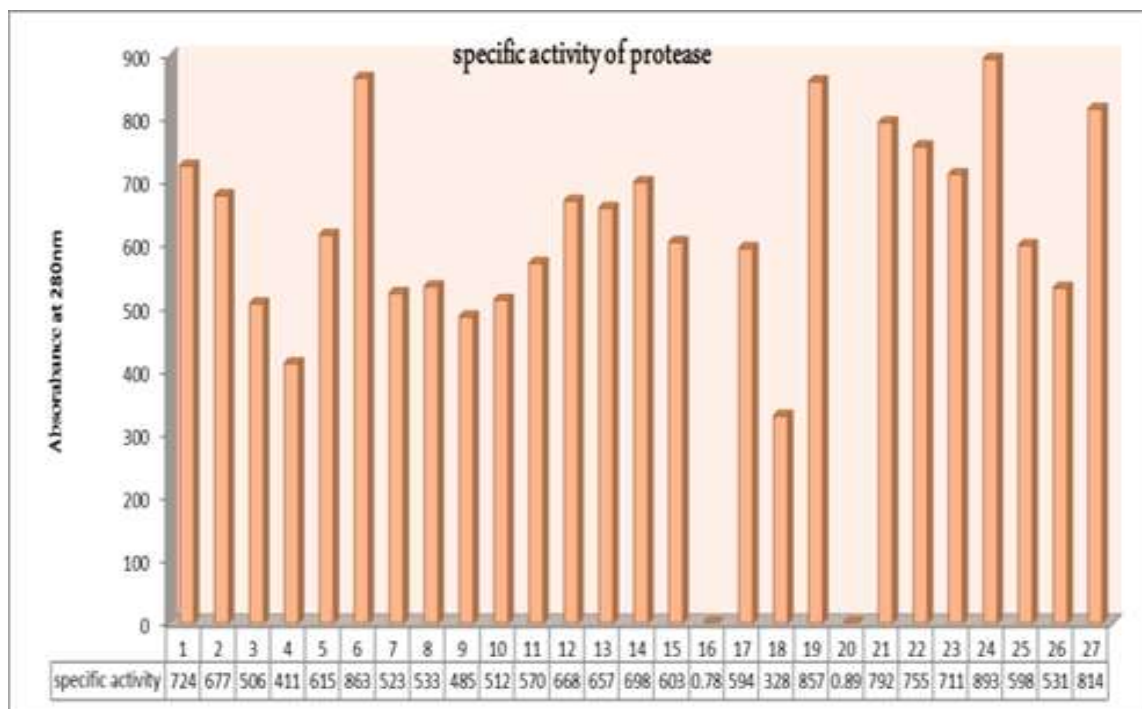| Isolate number	Antibiotic (Amikacin)	Probiotic
P24	512 µg/ml	512 µg/ml
P27	512 µg/ml	512 µg/ml

Figure 1. Result specific activity of protease

iv. Fighting Antibiotic Resistance in Pseudomonas aeruginosa: Exploring the Potential of Probiotics

The results of experiments investigating the potential synergistic effects of antibiotics and probiotics against antibiotic-resistant strains of Pseudomonas aeruginosa are shown in figure 2. The text focuses specifically on two isolate: P27 and P24.

i. **Isolate-P27:** Treatment with antibiotics alone produced a zone of inhibition with diameter 14 mm. Treatment with probiotics alone produced a zone of inhibition with diameter 13 mm. The combination of antibiotics and probiotics produced a synergistic effect, producing a zone of inhibition of 18 mm.

ii. **Isolate - P24:** Treatment with antibiotics alone produced a zone of inhibition with diameter 18 mm. Treatment with probiotics alone produced a zone of inhibition with diameter 12 mm. The combination of antibiotics and probiotics showed a significant synergistic effect, producing a zone of inhibition with diameter 28 mm.

Probiotics' potential effectiveness is suggested through the results observed. They can either be used as an adjunctive therapy or an alternative approach for antibiotic-resistant strains of P. aeruginosa, which underscores the promise of probiotics in fighting antibiotic resistance given the synergy effects seen with strains P27 and P24.

probiotics in fighting antibiotic resistance given the synergy effects seen with strains P27 and P24. This finding is supported by Mehboudi's et al which confirmed the impact of probiotic cell-free metabolites on multidrug-resistant (MDR) P. aeruginosa strains indicating that probiotic metabolites demonstrated antimicrobial activity against MDR P.aeruginosa while also down regulating expression of antibiotic resistance genes [16]. Hutchison's 2021 et al. version highlighted the use of probiotics in treating diarrhea [17]. It featured studies on antibiotic-associated diarrhea (AAD) which is a common complication of antibiotic therapy and can result in P. aeruginosa infection. The meta-analysis concluded that probiotics can substantially reduce the duration of AAD, thus hinting at the positive effect they might have on preventing P. aeruginosa colonization while under antibiotic treatment.

Figure 2. Antimicrobial Effect of probiotic And Antibiotic On P. aeruginosa Isolate , Using Well Diffusion Method

4. Conclusions

This study demonstrates the potential of probiotics, particularly Lactobacillus plantarum and Lactobacillus acidophilus, as adjunctive therapy for antibiotic-resistant Pseudomonas aeruginosa infections. Despite the virulence of the isolates, the probiotic mixture exhibited a synergistic effect with amikacin, suggesting a promising therapeutic approach. And given the observed synergy with amikacin, investigating the potential benefits of combining probiotic supplementation with other antibiotics or antimicrobial agents could be explored.

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