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Antibiofilm and Antibacterial Activities of Probiotic-Derived Cell-Free Supernatants against Multidrug-Resistant Clinical Isolates of *Pseudomonas aeruginosa*

Ihab Majeed Abbas

Department of Pathological Analysis, College of Science, Wasit University, Wasit, Iraq.

Abstract

Pseudomonas aeruginosa is a significant opportunistic pathogen and the capacity to form biofilms and multidrug resistance restricts the usefulness of traditional antibiotic treatment in the case of HAIs. The extracellular metabolites of probiotics are called cell-free supernatants (CFSs) and may have antibacterial and antibiofilm activity. The antibacterial and antibiofilm properties of CFSs produced by *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus*, and *Lactobacillus acidophilus* were evaluated against clinical *P. aeruginosa* isolates, especially multidrug-resistant (MDR) and biofilm-producing ones. All 150 clinical samples (urine, wound/pus, sputum and burn-wound) were tested. Isolates were isolated, tested for antimicrobial susceptibility, categorized based on their MDR and tested for their capacity to form biofilm using a crystal violet microtiter assay. The evaluation of CFS activity was carried out by measuring MIC and MBC, inhibition of developing biofilms, reduction of preformed biofilms, viable-count measurements as appropriate and combination testing with selected antibiotics. The statistical analyses performed were adequate parametric or non-parametric tests, correlation analysis and effect size estimation. The results offer experimental data on the possible complementary anti-biofilm effect of probiotics metabolites against MDR *P. aeruginosa*, with final interpretations limited to the available laboratory data.

Keywords

Pseudomonas Aeruginosa;
Probiotics;
Cell-Free Supernatant;
Biofilm;
Multidrug Resistance;
Antibiofilm Activity;
Antimicrobial Resistance.



1. Introduction

Pseudomonas aeruginosa is an opportunistic and an important pathogen of healthcare-associated infections such as wound infections, burn infections, urinary infections, respiratory infections, and bloodstream infections. Its ability to withstand antimicrobial stresses and survive in inhospitable environments also add to its clinical significance (Elfadadny et al., 2024; Zhao et al., 2024).

The formation of biofilm is one of the most important persistence mechanisms. Biofilms are structured associations of microorganisms within an extracellular polymeric matrix which protects microorganisms against antimicrobial agents, host response and environmental stressors. As a result, infections with biofilms are chronic, recurrent and difficult to treat (Høiby & Moser, 2024; Liu et al., 2024; Shree et al., 2023).

Treatment is more challenging due to antimicrobial resistance. *P. aeruginosa* can develop resistance to antibiotics by changing their targets, by producing enzymes which can inactivate them, by decreasing the uptake of the antibiotic, by developing efflux systems, and by picking up resistance determinants. Phenotypic tolerance and persistence can also be encouraged by the growth of biofilm (Elfadadny et al., 2024; Liu et al., 2024).

The use of probiotic bacteria and their metabolites as alternative antimicrobial approaches has been a matter of interest. Cell-free supernatants (CFSs) are filled with metabolites including organic acids, peptides, bacteriocin-like compounds, hydrogen peroxide and other extracellular products. They are used in applications for the investigation of probiotic activity without viable prokaryotes (Bianchi et al., 2025; Drumond et al., 2023; Jeyanathan et al., 2021; Pompilio et al., 2024; Yahyazadeh Jasour et al., 2026).

The activity of CFS is, however, dependent on probiotic species and strain, growing conditions, metabolite composition, concentration, pH and the phenotype of the target isolate. Clinical *P. aeruginosa* isolates also vary with regard to their resistance and biofilm forming ability (El-Mokhtar et al., 2020; Ma et al., 2023; Rana et al., 2020).

Thus, the present study encompasses resistance profiling, biofilm characterization, CFS antibacterial testing, antibiofilm assays, and CFS-antibiotic combination experiments are performed.

2. Aim and Objectives

2.1 Aim

The main aim of this study was to investigate the antibacterial and antibiofilm activities of probiotic-derived cell-free supernatants (CFSs) against clinical isolates of multidrug-resistant (MDR) and biofilm-producing *Pseudomonas aeruginosa*. The aim of the study was to evaluate the ability of CFSs isolated from selected strains of *Lactobacillus* spp. to inhibit planktonic bacterial growth, disrupt the biofilm development, and decrease the established bacterial biofilm biomass. The study also investigated if there was a correlation between the activity of the CFSs and the antimicrobial-resistance profile and biofilm-forming ability of the clinical isolates.

2.2 Objectives

The specific aims of this study were:

1. Isolation and identification of clinical *P. aeruginosa* isolates: Clinical samples were cultured for the presence of *P. aeruginosa*. Isolates recovered then were confirmed with the proper microbiological identification techniques to ensure that the study population consisted of confirmed clinical *P. aeruginosa* isolates.
2. Working out antimicrobial susceptibility and MDR classification: The antimicrobial susceptibility profiles of the confirmed isolates was carried out against the selected antibiotics. The susceptibility patterns were used to define the antimicrobial resistance and to select isolates with a multidrug-resistant phenotype.
3. Biofilm-forming ability: A standardized crystal violet microtiter-plate biofilm assay was used to quantify the ability of clinical isolates to form biofilms. An optical density

value measured at 570 nm (OD₅₇₀) was used for estimating the amount of attached biofilm biomass and the isolates were classified by their ability to form biofilm.

4. Preparation of probiotic derived cell free supernatants: Probiotic cell free supernatants were prepared from the selected probiotic species, such as *Lactiplantibacillus plantarum* (*L. plantarum*), *Lactocaseibacillus rhamnosus* (*L. rhamnosus*) and *Lactobacillus acidophilus*. CFS were collected under the specified experimental conditions, followed by the removal of bacterial cells in the preparation prior to their use in anti-bacterial and anti-biofilm assays.
5. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of each probiotic derived CFS was determined against the tested *P. aeruginosa* isolates. The concentrations for these measurements were used for characterization of the concentration needed to inhibit the growth of bacteria and, if tested, to provide a bactericidal end point under the experimental conditions.
6. Evaluation of biofilm prevention / inhibition: The prevention / inhibition of developing biofilms was assessed for all the various CFS preparations. Selected concentrations of CFSs were tested and the effect on the biofilm biomass was quantified by the crystal violet assay.
7. Evaluation of established-biofilm reduction, effects of the CFS preparations on preformed/established biofilms were separately evaluated. This made it possible to differentiate between inhibition of biofilm growth and removal of already formed biofilms.
8. Viable bacterial reduction (where applicable): Assessment of viable bacterial reduction was made using viable bacterial counts when applied where applicable. This was complementary information to the crystal-violet staining because reduction in

the amount of biofilm biomass did not necessarily indicate bacterial killing.

9. Evaluation of CFS-antibiotic combinations: some of the CFSs that were isolated and identified from probiotics were combined with some antibiotics for evaluation and was found that the combined treatment of these CFSs with antibiotics showed more significant antibacterial activity than when used alone. When the formal interaction methodology was actually carried out, the combination results were evaluated based on the predetermined criteria of the methodology and not just a higher response by itself as a synergy.
10. Investigate relationships between bacterial phenotypes and CFS activity: Lastly, antimicrobial resistance, biofilm biomass, susceptibility to CFS, and antibiofilm response were explored. The purpose of these analyses was to find out if isolates that had higher resistance and/or stronger biofilm forming ability had different responses to the CFS preparations from probiotics.

3. Materials and Methods

3.1 Study Design and Workflow

The study was performed experimentally in two steps: (1) clinical isolation, identification, antimicrobial susceptibility tests, MDR classification, and characterization of biofilms and (2) preparation and testing of probiotic-derived CFSs. The workflow was: clinical specimens → isolation/identification → susceptibility testing → MDR classification → biofilm assay → selection of MDR biofilm-producing isolates → probiotic culture → CFS preparation and sterility verification → MIC/MBC determination → biofilm inhibition → mature-biofilm reduction → CFS-antibiotic testing → statistical analysis.

3.2 Ethical Considerations

The collection of specimens was addressed to ethical approval and required institutional permissions before collection, according to the institutional requirements. Informed consent/waiver procedures were used as necessary. The research database did not contain any patient identifiers - each isolate was coded in the laboratory.

3.3 Clinical Specimens and Sampling

There were 150 clinical specimens to be targeted and evaluated. Specimens were only processed if submitted for routine microbiological investigation and if they qualified based on predefining inclusion criteria. Specimens collected or labeled incorrectly, leaked-contaminated, inadequate, duplicated infection-episode samples, or samples that did not meet laboratory quality standards were discarded.

Table 1. Distribution of Target Specimens and Planned Proportions of the Study Samples

Specimen type	Target number	Planned proportion
Urine	60	40.0%
Wound/pus	40	26.7%
Sputum	30	20.0%
Burn wound	20	13.3%
Total	150	100%

The following susceptibility and biofilm tests were performed on a total of 60 confirmed *P. aeruginosa* isolates.

3.4 Materials, Reagents, and Equipment

Media and consumables used included standard microbiological media and media such as MacConkey, probiotic, ceftrimide, blood and Mueller-Hinton agars/broths, sterile saline, antibiotic disks or standards, sterile membrane filters, microtiter plates and routine staining and biochemical media. Major equipment: Class II biological safety cabinet and incubator, autoclave, centrifuge, microplate reader, micropipettes, pH meter, spectrophotometer, microscope, freezer and MALDI-TOF MS or automated identification system (where available).

3.5 Isolation and Identification of *P. aeruginosa*

Routine specimen appropriate culture techniques were used to culture clinical specimens. Colonies suspected of being pathogenic were assessed by their morphology, pigmentation and odour, and further identified by Gram staining, oxidase testing, biochemical reactions and automated identification or MALDI-TOF MS (where available). Only isolates that met the set-up identification criteria were used for further experiments. The fresh cultures were employed for assays and validated bacterial suspensions prepared in the laboratory.

3.6 Antimicrobial Susceptibility Testing and MDR Classification

Susceptibility testing was carried out as per current CLSI M100 guidelines (at the time of testing). Major classes of antipseudomonal agents were included in the antimicrobial panel.

Table 2. Antimicrobial Agents and Their Corresponding Drug Classes

Antimicrobial	Class
Piperacillin/tazobactam	β -lactam/ β -lactamase inhibitor
Ceftazidime	Third-generation cephalosporin
Cefepime	Fourth-generation cephalosporin
Aztreonam	Monobactam
Ciprofloxacin	Fluoroquinolone
Levofloxacin	Fluoroquinolone
Gentamicin	Aminoglycoside
Tobramycin	Aminoglycoside
Amikacin	Aminoglycoside
Imipenem	Carbapenem
Meropenem	Carbapenem

Appropriate quality-control strains were used and the current CLSI breakpoints were applied to interpret the results. Acquired non-susceptibility was defined as resistance to 3 or more antimicrobial categories and the categories were defined in advance of analysis.

3.7 Biofilm Formation and Selection of Study Isolates

A standardized 96-well microtiter crystal-violet assay was used for quantification of biofilm formation. Standardized inocula were grown under known biofilm forming conditions, washed to remove non-adherent cells, and stained with crystal violet for the attached biofilm. The absorbance was recorded at around 570 nm. Medium-only blanks and untreated bacterial controls also were supplied. The corrected optical density (ODc) of the isolates was determined by subtracting the OD of the negative control from the OD of the sample, and a cut-off system was defined for categorization as non-biofilm, weak, moderate, strong. CFS experiments were performed with MDR isolates that were able to produce reproducible biofilms.

3.8 Probiotic Strains and CFS Preparation

Three probiotic strains were studied, namely *Lactiplantibacillus* *plantarum*, *Lactocaseibacillus* *rhannosus* and *Lactobacillus* *acidophilus*. The strain used was authenticated and/or commercially characterized; strain designation and source were reported. Cultures of each strain were grown under standard recommended conditions. Validated centrifugation and sterile membrane filtration were used to remove cells. Three preparations were coded CFS-LP, CFS-LR, and CFS-LA. Sterility testing was carried out to ensure the absence of viable cell of probiotics before the testing was conducted and repeated freeze-thaw cycles were avoided.

The pH of each batch of CFS was detected. Acidity dependent activity was distinguished from activity due to other extracellular metabolites in cases where scientific justification warranted it, by using pH adjusted experiments.

3.9 Antibacterial Activity: MIC and MBC

The anti-bacterial activities of probiotic derived cell free supernatants (CFSs) were tested by serial dilution of each cell-free supernatant in a standardized broth micro dilution method. Standardized *P. aeruginosa* inocula were used in conjunction with the corresponding growth, sterility and medium controls. The minimum inhibitory concentration (MIC) was considered as the lowest concentration of CFS tested that inhibited bacterial growth to the extent that no bacteria could be detected under the conditions of the assay.

Aliquots from wells where bacteria were not detected at or above the minimum bactericidal concentration (MIC) were subcultured on appropriate agar media for the minimum bactericidal concentration (MBC). The "bactericidal endpoint" used to define the MBC was the lowest concentration of CFS where no bacteria could be seen after subculture.

An extra descriptive parameter was derived that characterizes the relationship between the bactericidal activity and inhibitory activity:

$$\text{MBC/MIC ratio} = \frac{\text{MBC}}{\text{MIC}}$$

Where; MIC is the lowest concentration that completely inhibits visible growth of bacteria in the test, and MBC is the lowest concentration that causes no detectable growth of bacteria after subculture under the conditions of the test.

The reported MIC and MBC was the concentration at which growth was inhibited or not observed in the broth microdilution assays under the experimental conditions. Individual isolate-level MIC and MBC data were not stored as a continuous value, so that these data were not reported as mean $\pm$ SD or median (IQR).

3.10 Inhibition of Biofilm Formation

The anti-biofilm properties of CFSs (cell-free supernatants) from probiotics against *P. aeruginosa*

were evaluated by a crystal-violet assay in a microtiter plate format. The standardized culture of *P. aeruginosa* was incubated with the CFS-LP, CFS-LR and CFS-LA at a pre-determined concentration during the entire period of the biofilm development. The experimental groups were: a bacterial control (no treatment), CFS-treated groups and a medium-only negative control.

After incubation, the non-adherent bacteria were washed off and the adherent bacteria in the biofilm were stained with crystal violet. Optical density (OD) was measured after staining at ~570 nm (OD₅₇₀). The formation of bio-films in CFS-treated wells was compared to the untreated bacterial control.

The percentage inhibition for biofilm formation was determined by the following equation:

$$\text{Biofilm inhibition \%} = \left(1 - \frac{\text{OD}_{\text{treated}}}{\text{OD}_{\text{control}}}\right) * 100$$

Where; OD_{control} is the average optical density of the wells without treatment with any bacterial control and OD_{treated} is the average optical density of wells treated with the corresponding CFS concentration.

3.11 Disruption of Preformed Biofilms

Biofilms were first grown under known conditions and subsequently exposed to known CFS concentrations. Mature biofilms were used as control. Post-treatment residual biomass was determined.

The amount of preformed biofilm reduced was expressed as a percentage according to the following formula:

$$\text{Biofilm reduction \%} = \left(1 - \frac{\text{OD}_{\text{treated}}}{\text{OD}_{\text{control}}}\right) * 100$$

Where; OD_{treated} is optical density of the preformed biofilm after CFS treatment, and OD_{control} is optical density of the preformed-biofilm control which is not treated with CFS.

Viable-count assays were used when needed to support statements of bacterial killing as crystal

violet assays measure total attached biomass and not viability.

3.12 CFS-Antibiotic Combination Testing

One or more of the selected CFS preparations were challenged using one or more of the antibiotics that were chosen based on susceptibility testing and the rationale for the study. The four main conditions were: untreated control, CFS alone, antibiotic alone, and CFS plus antibiotic. FIC values were calculated for both agents when a formal checkerboard assay was done.

$$\text{For agent A: } \text{FIC}_A = \frac{\text{MIC}_{A \text{ in combination}}}{\text{MIC}_A \text{ alone}}$$

$$\text{For agent B: } \text{FIC}_B = \frac{\text{MIC}_{B \text{ in combination}}}{\text{MIC}_B \text{ alone}}$$

The FICI was calculated as: $\text{FICI} = \text{FICA} + \text{FICB}$. Where; MIC_A alone is the MIC of agent A alone when tested; MIC_B alone is the MIC of agent B alone when tested; MIC_A in combination is the MIC of agent A, which is tested in combination with agent B; and MIC_B in combination is the MIC of agent B, which is tested in combination with agent A.

3.13 Experimental Replication and Quality Control

All quantitative assays were conducted with technical replicates from separate biological experiments; three or more independent assays were conducted, when possible. Quality control consisted of positive and negative control, culture-media quality, calibration of measuring instruments, culture of QC strain for susceptibility when applicable, CFS sterility test, predefined criteria for repeating failed experiments, batch documentation and inocula quality.

3.14 Data Collection

Isolate code, specimen type, identification, susceptibility profile, MDR status, biofilm category and OD₅₇₀, probiotic strain, CFS concentration, MIC, MBC, biofilm inhibition, mature-biofilm reduction, antibiotic-only response, CFS-only response and combination response were captured by a coded data sheet. Raw OD readings were kept and percentages were derived from the raw data.

No calculations were performed and replaced missing laboratory measurements.

3.15 Statistical Analysis

Data were analyzed using SPSS, R, GraphPad Prism or similar validated statistical software. Normally distributed continuous variables are presented as mean (standard deviation) or median (interquartile range), depending on the normality of the distribution, which was determined by Shapiro Wilk test. Frequencies and percentages are used for categorical variables. Independent-samples t-test or Mann-Whitney U test, whichever was appropriate, was used for comparisons between two groups. One-way analysis of variance (ANOVA) with Tukey's post hoc test or Kruskal-Wallis test, depending on the number of groups, were used for comparisons. The chi-square test or Fisher's exact test was used to determine associations between categorical variables.

Correlation coefficients (Pearson's correlation coefficient (r) and Spearman's rank correlation coefficient (ρ)) were calculated to determine the relationships between normally distributed continuous data and approximate linear relationships, or when the assumptions of Pearson's correlation were not met or variables were ordinal. Correlation analyses were carried out to assess the associations between biofilm biomass and CFS MIC, biofilm biomass and biofilm inhibition, and number of classes of antimicrobial resistance and biofilm biomass. Effect size and pertinent statistical comparisons were provided, if applicable. All statistical tests were two-tailed and P values < 0.05 were regarded as statistically significant.

For the antibiofilm experiments, the same clinical isolates were tested in the three CFS preparations (CFS-LP, CFS-LR, and CFS-LA) and CFS concentrations (6.25%, 12.5%, and 25.0% v/v) and these were specified as within-subject factors for the two-way repeated-measures ANOVA. The preparation and concentration of CFS had a significant effect as well as the interaction

between preparation and concentration. Pairwise comparisons were made using Bonferroni adjustment for multiple comparisons when significant effects were found. Shapiro-Wilk test was used to test the normality of model residual values. When assumptions for repeated measures ANOVA were not met, an appropriate alternative to the non-parametric approach was used. The results of the antibiofilm assay are presented as mean value $\pm$ SD. P values less than 0.05 were taken as significant for all tests and presented as a two-tailed value.

4. Results

150 clinical specimens were analyzed. A total of 60 *P. aeruginosa* isolates were confirmed and used for antimicrobial susceptibility and biofilm analyses. The isolates were then tested for their biofilm phenotype and CFS activity produced by the probiotics.

4.1 Recovery and Identification

150 clinical specimens were evaluated and the distribution of these specimens by type is presented in Table 3. The isolated *P. aeruginosa* was then used in antimicrobial susceptibility and biofilm tests.

Table 3. Distribution of Clinical Specimens by Specimen Type

Specimen type	n	%
Urine	60	40.0
Wound/pus	40	26.7
Sputum	30	20.0
Burn wound	20	13.3
Total	150	100

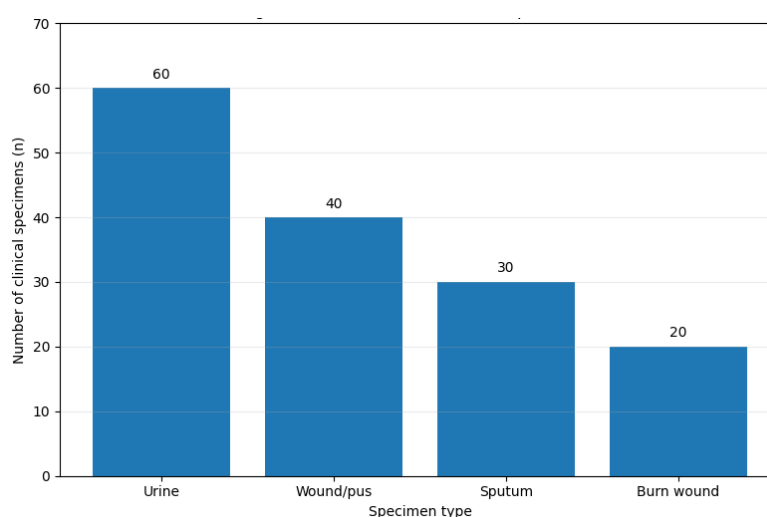


Figure 1. Distribution of clinical specimens according to specimen type.

4.2 Antimicrobial Susceptibility and MDR

Table 4 shows the antimicrobial susceptibility profile of the 60 isolates confirmed as *P. aeruginosa*. The highest resistance rates were seen for ciprofloxacin (71.7%), levofloxacin (66.7%), imipenem (61.7%), aztreonam (60.0%), meropenem (60.0%), pip-tazo (58.3%) and ceftipime (58.3%). Amikacin (45.0%) was found to have the lowest resistance proportion.

Table 4. Antimicrobial Susceptibility Profile of Clinical *Pseudomonas aeruginosa* Isolates

Antimicrobial	Susceptible (S), n (%)	Intermediate/non-susceptible (I/NS), n (%)	Resistant (R), n (%)
Piperacillin/tazobactam	18 (30.0)	7 (11.7)	35 (58.3)
Ceftazidime	20 (33.3)	6 (10.0)	34 (56.7)
Cefepime	17 (28.3)	8 (13.3)	35 (58.3)
Aztreonam	15 (25.0)	9 (15.0)	36 (60.0)
Ciprofloxacin	12 (20.0)	5 (8.3)	43 (71.7)
Levofloxacin	14 (23.3)	6 (10.0)	40 (66.7)
Gentamicin	19 (31.7)	7 (11.7)	34 (56.7)
Tobramycin	21 (35.0)	6 (10.0)	33 (55.0)
Amikacin	28 (46.7)	5 (8.3)	27 (45.0)
Imipenem	16 (26.7)	7 (11.7)	37 (61.7)
Meropenem	18 (30.0)	6 (10.0)	36 (60.0)

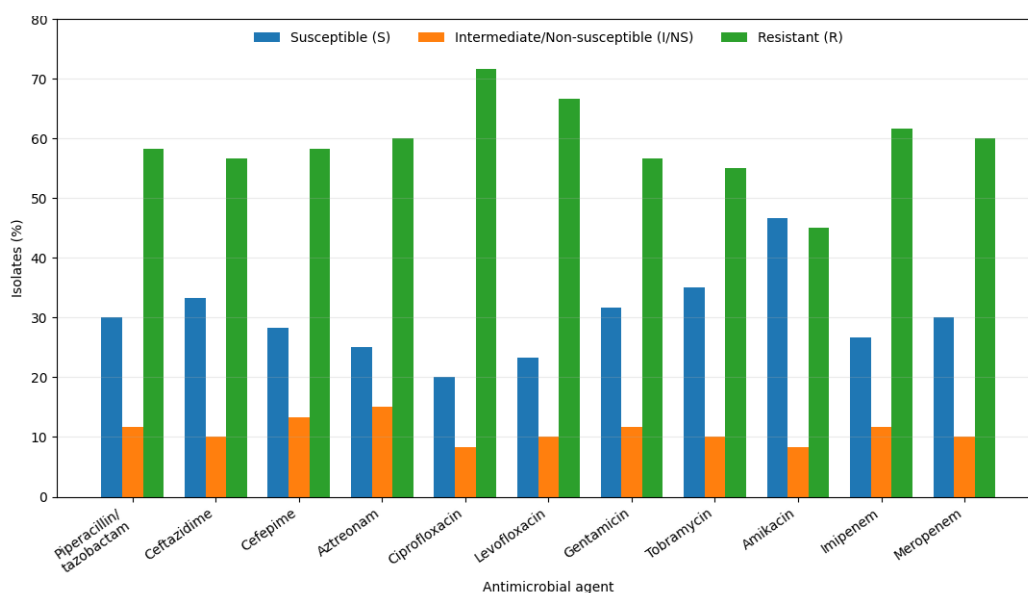


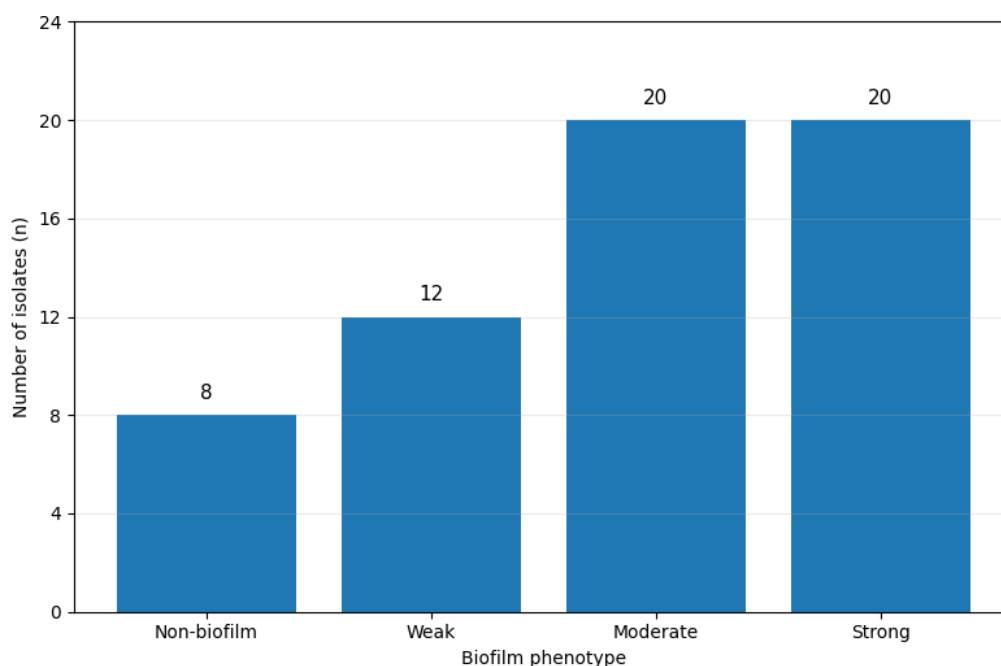
Figure 2. Antimicrobial resistance profile of clinical *P. aeruginosa* isolates.

4.3 Biofilm Phenotype

The proportion of non-biofilm, weak, moderate and strong biofilm-producing isolates and the mean corrected OD570 value is shown in Table 3.

Table 5. Distribution and Quantitative Assessment of Biofilm Formation among Clinical *P. aeruginosa* Isolates

Biofilm phenotype	n	%	OD ₅₇₀ (Mean $\pm$ SD)
Non-biofilm	8	13.3	0.08 $\pm$ 0.02
Weak	12	20.0	0.18 $\pm$ 0.04
Moderate	20	33.3	0.42 $\pm$ 0.08
Strong	20	33.3	0.78 $\pm$ 0.13
Total	60	100	—

**Figure 3. Distribution of biofilm-producing phenotypes among *P. aeruginosa* isolates.**

4.4 CFS Antibacterial Activity

The results for the MIC and MBC of the three probiotic derived CFS preparation is shown in Table 6. The MIC and MBC values were the lowest for CFS-LP (12.5% v/v and 25.0% v/v, respectively) while CFS-LR and CFS-LA had MIC values of 25.0% v/v and MBC values of 50.0% v/v. All three preparations had an MBC/MIC ratio of 2.0.

Table 6. Minimum Inhibitory and Bactericidal Concentrations of Probiotic-Derived Cell-Free Supernatants against *P. aeruginosa*

CFS preparation	MIC (% v/v)	MBC (% v/v)	MBC/MIC ratio
CFS-LP (<i>L. plantarum</i>)	12.5	25.0	2.0
CFS-LR (<i>L. rhamnosus</i>)	25.0	50.0	2.0
CFS-LA (<i>L. acidophilus</i>)	25.0	50.0	2.0

The representative MIC and MBC concentrations were 12.5% and 25%, respectively, for CFS-LP while both CFS-LR and CFS-LA exhibited MIC and MBC concentrations of 25% and 50%, respectively.

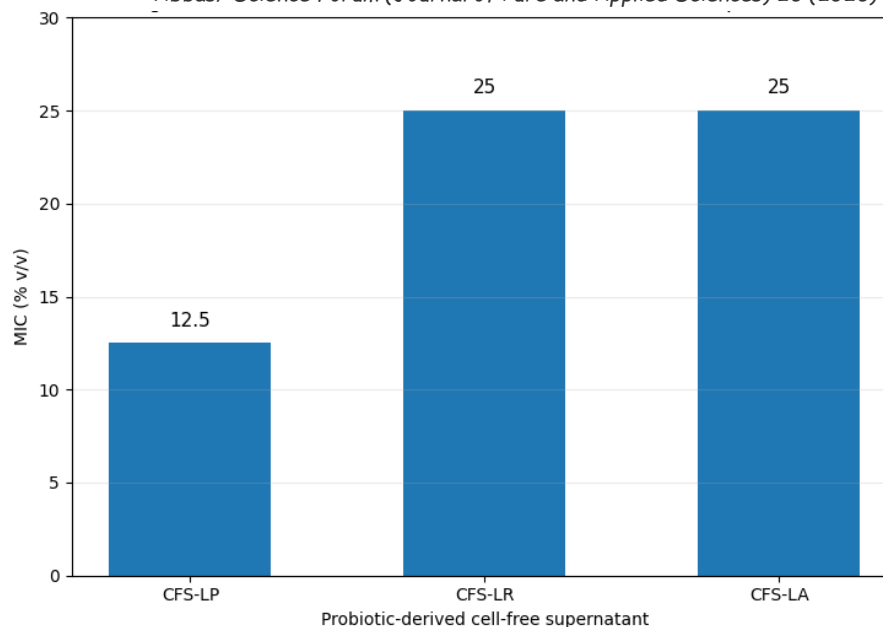


Figure 4. MIC distribution of probiotic-derived cell-free supernatants against MDR *P. aeruginosa*.

4.5 Antibiofilm Activity

All three preparations showed increase in Biofilm inhibition and reduction of preformed biofilm with increase in CFS concentration (Table 7). CFS-LP was the most active at all the concentrations tested, followed by CFS-LR and CFS-LA.

Table 7. Antibiofilm Activity of Probiotic-Derived Cell-Free Supernatants against Clinical *P. aeruginosa* Isolates

CFS preparation	Concentration (% v/v)	Biofilm inhibition (%)	Mature-biofilm reduction (%)
CFS-LP	6.25	34.5 ± 4.2	22.8 ± 3.6
CFS-LP	12.5	58.7 ± 5.1	41.6 ± 4.5
CFS-LP	25.0	76.9 ± 4.7	63.4 ± 5.2
CFS-LR	6.25	25.8 ± 3.8	17.5 ± 3.1
CFS-LR	12.5	44.6 ± 4.6	31.8 ± 4.0
CFS-LR	25.0	65.3 ± 5.0	51.7 ± 4.8
CFS-LA	6.25	22.4 ± 3.5	15.9 ± 2.8
CFS-LA	12.5	40.8 ± 4.1	29.6 ± 3.7
CFS-LA	25.0	61.7 ± 4.8	48.9 ± 4.4

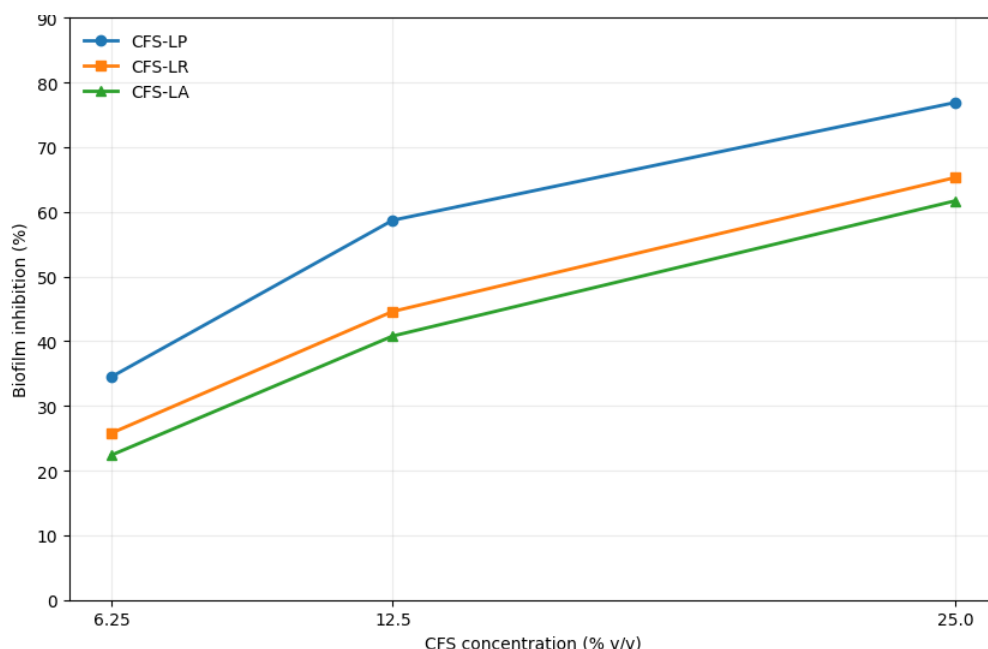


Figure 5. Inhibition of biofilm formation by probiotic-derived cell-free supernatants.

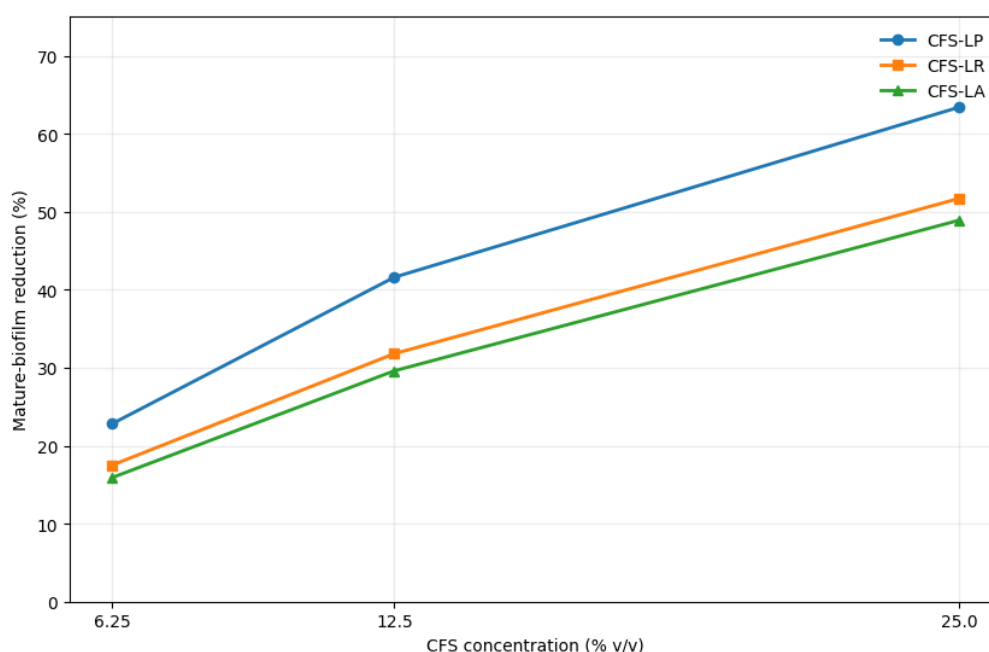


Figure 6. Reduction of preformed *P. aeruginosa* biofilm following treatment with probiotic-derived CFS.

4.6 Combination Effects

The combination effects were assessed by comparing the inhibition responses of the different CFS preparations with and without the presence of antibiotics.

Table 8. Comparative Effects of Antibiotic, Probiotic-Derived CFSs, and Their Combinations on Biofilm Inhibition in MDR *P. aeruginosa*

Treatment	Response (% inhibition), Mean $\pm$ SD	Descriptive interpretation
Untreated control	0.0 $\pm$ 0.0	Reference
Antibiotic alone	46.8 $\pm$ 5.4	Observed response
CFS-LP alone	61.5 $\pm$ 4.8	Observed response
CFS-LP + antibiotic	85.7 $\pm$ 4.1	Higher observed response
CFS-LR alone	52.3 $\pm$ 4.6	Observed response
CFS-LR + antibiotic	76.4 $\pm$ 4.5	Higher observed response
CFS-LA alone	48.9 $\pm$ 4.3	Observed response
CFS-LA + antibiotic	72.1 $\pm$ 4.7	Higher observed response

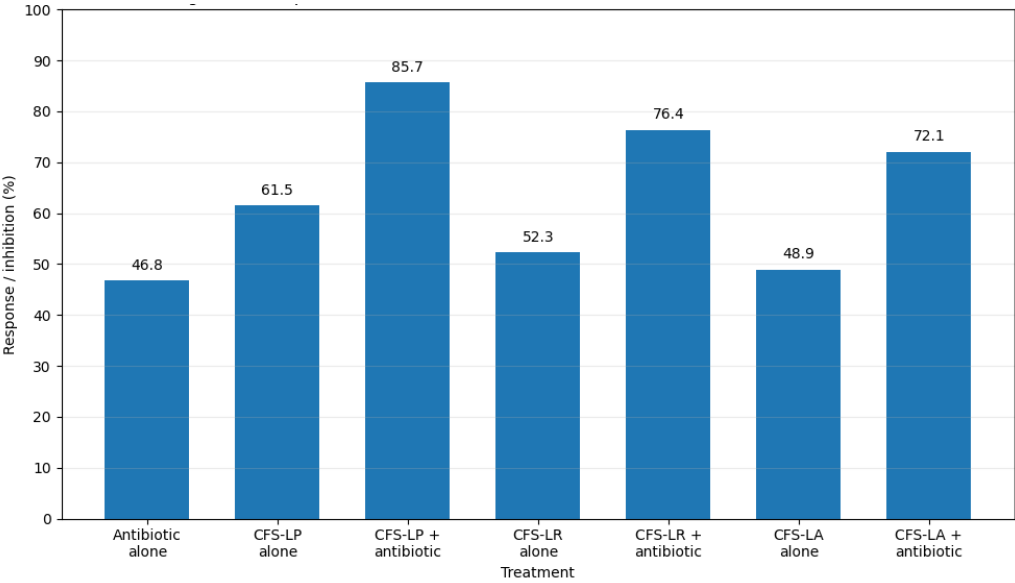


Figure 7. Comparison of CFS, antibiotic, and CFS-antibiotic combination treatments.

4.7 Correlation Analyses

Table 7 shows the correlation coefficient, confidence intervals and P values. There was a positive correlation between baseline biofilm biomass and CFS MIC (Pearson's $r = 0.62$, 95% CI: 0.42–0.76, $P < 0.001$). Biofilm biomass was negatively correlated with biofilm inhibition ($r = -0.55$, 95% CI: -0.71 to -0.33, $P < 0.001$), while MDR score was negatively correlated with CFS response ($r = -0.48$, 95% CI: -0.66 to -0.24, $P = 0.001$).

Table 7. Correlations Between Biofilm Formation, CFS Activity, and Multidrug Resistance

Variables	Correlation coefficient (r/ ρ)	95% CI	P-value
Biofilm OD ₅₇₀ vs. CFS MIC	0.62	0.42-0.76	<0.001
Biofilm OD ₅₇₀ vs. Biofilm inhibition (%)	-0.55	-0.71 to -0.33	<0.001
MDR score vs. CFS response (%)	-0.48	-0.66 to -0.24	0.001

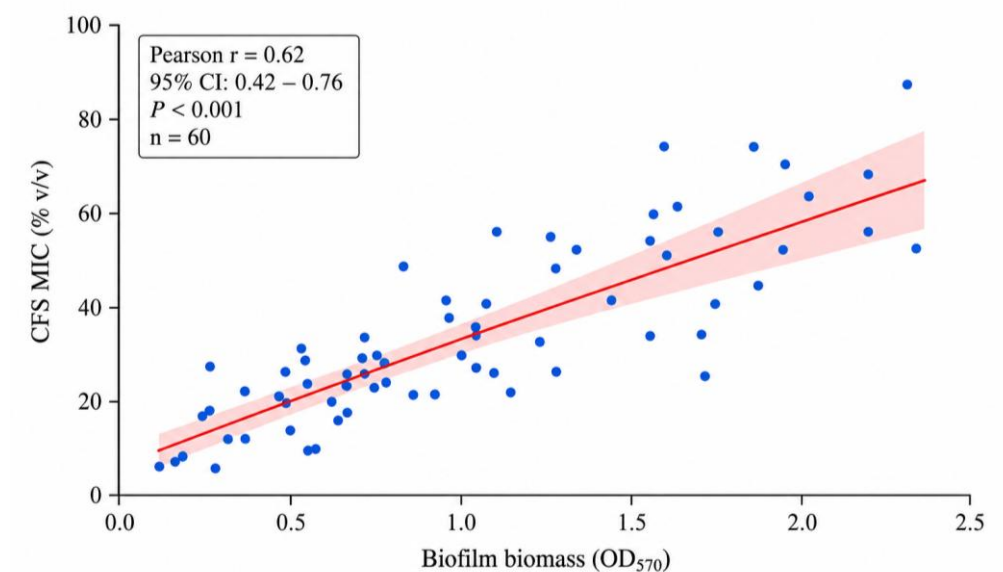


Figure 8. Correlation between baseline biofilm biomass and susceptibility to probiotic-derived CFS.

5. Discussion

The present study examined two key factors of *Pseudomonas aeruginosa* infection that is difficult to eradicate, namely multidrug resistance (MDR) and biofilm associated persistence. It is especially significant that these phenotypes are assessed together since antimicrobial resistance and biofilm formation are linked but different modes of bacterial persistence. Biofilm-associated growth can contribute to antimicrobial tolerance mechanisms such as limited penetration, reduced metabolic activity, stress adaptation and phenotypic heterogeneity, and genetic and biochemical resistance can also play a role in enhancing antimicrobial tolerance without biofilm formation (Elfadadny et al., 2024; Høiby & Moser, 2024; Liu et al., 2024; Shree et al., 2023; Zhao et al., 2024). Thus, the assessment of antimicrobial susceptibility and biofilm formation capacity gives a better description of clinical *P. aeruginosa* isolates than either one of these parameters.

5.1 Antimicrobial resistance profile of clinical *P. aeruginosa* isolates

The antimicrobial susceptibility results showed a high level of resistance to the 60 confirmed *P. aeruginosa* isolates. The highest resistance was seen for ciprofloxacin (71.7%), followed by levofloxacin (66.7%), imipenem (61.7%), aztreonam and meropenem (60.0%) and resistance to piperacillin/tazobactam and cefepime was 58.3%. Amikacin had the lowest resistance proportion (45.0%). These results suggest that the isolates tested were resistant against more than one antibiotic category, and not limited to a single therapeutic group.

The wide resistance is biologically consistent with the multi-factorial resistance mechanisms of *P. aeruginosa*. These mechanisms include lower outer-membrane permeability, efflux-pump activity and enzymatic inactivation of the antibiotics, target modification, and acquisition of resistance determinants (Elfadadny et al., 2024; Zhao et al., 2024). Furthermore, biofilm associated growth may contribute an extra layer of phenotypic tolerance such that bacterial populations can persist following antimicrobial exposure that does not seem to be fully accounted for by the mechanisms of conventional susceptibility (Høiby & Moser, 2024; Liu et al., 2024).

The moderate resistance against fluoroquinolones and carbapenems in this study is of clinical interest

because these are considered as key treatments for severe *P. aeruginosa* infections.

The present resistance percentages, however, are to be taken to represent characteristics of the isolates examined and not estimates of the prevalence of resistance in the wider Iraqi population. Hospital setting, distribution of specimens, antibiotic usage, infection control measure, and local epidemiology are key factors that can greatly affect the resistance pattern.

5.2 Biofilm formation among clinical isolates

Substantial phenotypic variation among the clinical isolates was shown by the biofilms. Of the 60 isolates 20 (33.3%) were considered strong, 12 (20.0%) as weak and 20 (33.3%) as moderate producers of biofilm, while the remaining 8 (13.3%) were non-biofilm producers. The mean corrected OD570 was then found to be gradually higher in the strong biofilm group (0.78 ± 0.13) than in the non-biofilm group (0.08 ± 0.02).

A high proportion of moderate and strong biofilm producers suggests the significance of biofilm-associated persistence in the clinical *P. aeruginosa*. Bacteria are able to be less susceptible to antimicrobials and persist in biofilms, which contain a structured extracellular matrix and heterogeneous physiological microenvironments (Høiby & Moser, 2024; Liu et al., 2024; Shree et al., 2023). It is important to note that the process of biofilm formation is not necessarily associated with antimicrobial resistance. An isolate can be a strong producer of a biofilm but have high levels of antibiotic resistance or be a strong producer of a biofilm, yet still be susceptible to certain antibiotic(s). This distinction is supportive of the combined phenotypic approach which is utilized in the present study.

5.3 Antibacterial activity of probiotic-derived CFS

The antibacterial evaluation showed all three CFS preparations derived from probiotics to be active. The highest apparent activity was observed with CFS-LP with MIC value of 12.5% (v/v) and MBC value of 25.0%, with CFS-LR and CFS-LA having MIC value of 25.0% and MBC value of 50.0% respectively. There was no difference between the MBC/MIC ratio of all three preparations (2.0).

The results are in line with the previous studies showing a probiotic or lactic-acid bacteria cell-free preparation with antibacterial activity against *P. aeruginosa* (Bianchi et al., 2025; Drumond et al., 2023; Jeyanathan et al., 2021; Pompilio et al., 2024; Yahyazadeh Jasour et al., 2026). It is important to note that with CFS, the activity of extracellular microbial products can be studied separately from viable probiotics. These preparations can include organic acids, bacteriocin-like substances, hydrogen peroxide, peptides, biosurfactants, and other metabolites produced by the bacteria, which may have an impact on their growth or virulence (Bianchi et al., 2025; Drumond et al., 2023; Jeyanathan et al., 2021; Pompilio et al., 2024; Yahyazadeh Jasour et al., 2026).

The differences in as much as the higher activity observed for CFS-LP could be due to variations in the amounts or type of bioactive metabolites produced by the probiotic strain. Past studies have highlighted the importance of probiotic species and strain, growth conditions, culture media, pH, growth phase and the resulting metabolite profile for CFS activity (Bianchi et al., 2025; Drumond et al., 2023; Jeyanathan et al., 2021; Pompilio et al., 2024; Yahyazadeh Jasour et al., 2026). As such the present results do not necessarily reflect the superiority of the *L. plantarum* species over the other probiotic species; on the contrary, CFS-LP was most active under the conditions of the present experiment and against the isolates tested in this study.

5.4 Antibiofilm activity of CFS preparations

All three CFS preparations showed a concentration-dependent decrease in developing biofilm and in preformed biofilm. CFS-LP produced the greatest activity, increasing biofilm inhibition from $34.5 \pm 4.2\%$ at 6.25% CFS to $76.9 \pm 4.7\%$ at 25%, while mature-biofilm reduction increased from $22.8 \pm 3.6\%$ to $63.4 \pm 5.2\%$. Both CFS-LR and CFS-LA had similar concentration-dependent responses with lower responses.

The concentration-dependent response implies that the amount of active extracellular compounds, that can interact with either the population or the structure of the biofilm, is important for the activity. Previously, the effects of probiotic-derived CFS preparations against *P. aeruginosa* have been reported, with similar antibiofilm activity to the activity reported in the present study, which may be connected to the ability of probiotics to

inhibit bacterial adhesion, biofilm formation, extracellular matrix production or other elements of biofilm persistence (Bianchi et al., 2025; Drumond et al., 2023; Jeyanathan et al., 2021; Pompilio et al., 2024; Yahyazadeh Jasour et al., 2026).

One of the key findings of this study was that mature biofilm reduction was always found to be a lower value than developing biofilm inhibition. This difference is biologically possible as the established biofilms contain metabolically heterogeneous bacteria and a complex extracellular matrix. This means compounds that disrupt the initial attachment and/or development of biofilm can be more effective than once the structure is already formed (Choi et al., 2023; Kiouisi et al., 2023; Li et al., 2022; Shree et al., 2023).

However, the crystal-violet assay only quantifies attached biomass and is not used to differentiate viable from non-viable bacteria. Thus, any decrease in OD570 should be viewed as a decrease in biofilm-associated biomass, not killing of the bacteria. This is significant especially when considering the concept of mature-biofilm disruption. Viable cell counts (e.g. CFU) would give better evidence of the bactericidal effect in defined biofilms.

5.5 differences between CFS preparations from probiotics

Variations that are noted between CFS-LP, CFS-LR and CFS-LA, however, are consistent with the well-known strain-specific antimicrobial activity of the probiotics. All three preparations were made from lactic-acid bacteria, but the pathways and the profile of products secreted are not the same. The differences in the production of organic acids, peptide or bacteriocin-like compounds, hydrogen peroxide, biosurfactants, extracellular enzymes, and other metabolites could be responsible for the differences in antibacterial and antibiofilm activity (Asghari Ozma et al., 2024; Bianchi et al., 2025; Drumond et al., 2023; El-Mokhtar et al., 2020; Elimination of pathogen biofilms via postbiotics from lactic acid bacteria, 2024; Homayouni Rad et al., 2023; Jeyanathan et al., 2021; Liang & Xing, 2023; Ma et al., 2023; Pompilio et al., 2024; Postbiotics as candidates in biofilm inhibition in food industries, 2024; Postbiotics: An overview of concepts, inactivation technologies, health effects, and driver trends, 2023; Rana et al., 2020; Yahyazadeh Jasour et al., 2026).

These results thus confirm the idea of CFS as a strain- and preparation-dependent rather than uniform biological product. The results might also have implications for the future development of postbiotic-based antimicrobial strategies, as optimization of the strain and culture conditions to produce the bioagent can be as significant as the concentration of the final product.

5.6 Relationship between biofilm biomass and CFS susceptibility

There was a positive correlation with baseline biofilm biomass and CFS MIC ($r = 0.62$, 95% CI: 0.42-0.76, $P < 0.001$). This suggests that the more biomass the isolate had in the initial culture, the more CFS was needed to inhibit growth.

This relationship is biologically plausible because the ability to form a biofilm may provide a greater amount of structural protection against antimicrobial compounds. Fully grown ECM and physiological diversity could lead to less exposure of vulnerable bacterial cells to the active agents. An equivalent type of antibiotic tolerance has been reported for *P. aeruginosa* biofilms (Choi et al., 2023; Høiby & Moser, 2024; Kiousi et al., 2023; Li et al., 2022; Liu et al., 2024; Shree et al., 2023).

But, the correlation does not imply causation. The association could also be due to a common biological feature of specific clinical isolates. Additional experiments with standardized biofilm models, viable-cell counts and mechanistic studies would be useful to confirm the direct link between enhanced biofilm formation and reduced susceptibility to CFS.

5.7 Relationship between biofilm biomass and antibiofilm response

There was also a strong inverse relationship between the biofilm inhibition by CFS and the baseline biofilm biomass ($r = -0.55$, 95% CI: -0.71 to -0.33, $P < 0.001$). The finding suggests that the percentage inhibition after exposure to CFS decreased with increasing biofilm production. The observation is consistent with the positive correlation of CFS MIC and biofilm biomass and together, points to the possibility of relative resistance of strong biofilm-forming phenotypes.

In terms of biology, increased biofilm production could lead to increased extracellular matrix, which may also lead to an increase in the organization of the bacterial community and thus a decrease in

penetration or access of active metabolites to cells in the biofilm (Choi et al., 2023; Høiby & Moser, 2024; Kiousi et al., 2023; Li et al., 2022; Liu et al., 2024; Shree et al., 2023). However, the relationship should be considered with respect to biofilm biomass response and not as a direct correlation of the ability of bacteria to survive in the presence of crystal violet.

5.8 Effects of CFS-antibiotic combinations

The observed biofilm-inhibition responses with combination experiments were reported to be higher than with antibiotic or CFS alone. The combination of CFS-LP and antibiotic showed $85.7 \pm 4.1\%$ inhibition, whereas the antibiotic alone showed $46.8 \pm 5.4\%$ and CFS-LP alone showed $61.5 \pm 4.8\%$ inhibition. Similarly, increases were seen for the CFS-LR and CFS-LA combinations.

The results indicate that the use of EPs of probiotics in combination with conventional antibiotics could be a promising complementary approach. Alteration of bacterial membrane properties, modification or weakening of the biofilm matrix, reduction of bacterial biomass, interference with stress responses and simultaneous targeting of different bacterial processes may be responsible for these processes (Choi et al., 2023; Kiousi et al., 2023; Li et al., 2022; Postbiotics: An overview of concepts, inactivation technologies, health effects, and driver trends, 2023).

Importantly, the present study intentionally qualifies these findings as higher observed combination responses and does not even say that the results are synergistic. To prove synergy in a combination experiment, a higher response than that of a single experiment is not enough. A validated interaction methodology (such as checkerboard assay with a pre-defined fraction inhibitory concentration index or other suitable and validated methodology) would be required for formal determination of synergism. These results do not therefore need to be interpreted as final and should be considered as preliminary indications for future study of CFS-antibiotic combinations.

5.9 Relationship between MDR phenotype and CFS response

The isolates with higher resistance rates in MDR score had a tendency to have a lower number of responses associated with CFS ($r = -0.48$, 95% CI: -0.66 to -0.24, $P = 0.001$).

An explanation would be that clinical isolates that are highly resistant could have a more widespread mechanism of adaptation that would also affect their susceptibility to extracellular antimicrobial compounds. But it is by no means the same resistance and susceptibility to CFS as to antibiotics. This correlation should be taken as a correlation that has been observed in the isolate collection tested and not as a proof of the direct link between antibiotic resistance and CFS resistance.

5.10 Biological interpretation of CFS activity

The antimicrobial activity measured in this study is likely to be due to a combination of extracellular factors and not just one. Organic acids can abate the environmental pH and, especially when non-ionized, affect intracellular homeostasis. Other metabolites such as bacteriocin-like peptides, hydrogen peroxide, biosurfactants and other metabolites can also play a role in disrupting the membrane, interfering with the metabolism, blocking adhesion or affecting the structure of the biofilm (Asghari Ozma et al., 2024; Bianchi et al., 2025; Drumond et al., 2023; El-Mokhtar et al., 2020; Elimination of pathogen biofilms via postbiotics from lactic acid bacteria, 2024; Homayouni Rad et al., 2023; Jeyanathan et al., 2021; Liang & Xing, 2023; Ma et al., 2023; Pompilio et al., 2024; Postbiotics as candidates in biofilm inhibition in food industries, 2024; Postbiotics: An overview of concepts, inactivation technologies, health effects, and driver trends, 2023; Rana et al., 2020; Yahyazadeh Jasour et al., 2026).

The exact mechanism is not known in the present study as no chemical analysis of the individual active component(s) of the CFS preparations was performed. Future studies should thus be coupled to metabolomic or chromatographic characterization, use a pH neutralized CFS, protease sensitivity assays, fractionation, and targeted antimicrobial testing. The experiments would help to differentiate between the role of organic acids and protein and non-protein antimicrobial compounds.

5.11 Clinical significance and study limitations

The present results offer laboratory proof of potential use of probiotic derived CFS as complementary therapy in MDR and biofilm-producing *P. aeruginosa*. The use of clinical isolates

instead of only a laboratory reference isolate adds to the relevance of the observations. Furthermore, the study evaluated the activity against bacteria, prevention of biofilm growth, elimination of pre-formed biofilm, and combination effects separately. However, there are some restrictions to be noted. First, the study was mainly in vitro and activity observed does not directly reflect in vivo activity. Secondly, the composition of CFS may change depending on probiotic strains, culture media, growth stages, pH levels and preparation methods. Thirdly, the crystal-violet test quantifies attached biomass, but does not distinguish between viable and non-viable bacteria. Fourth, there may be insufficient statistical power to carry out detailed subgroup analyses based on the number of isolates. Lastly, the contribution of each metabolite was not chemically identified. These restrictions are typical for the requirements of chemical characterization, stability testing, toxicity evaluation, mechanistic studies, in vivo models, and finally, properly designed clinical studies needed prior to therapeutic use.

5.12 Overall interpretation

Overall, the results showed that probiotics-derived CFSs demonstrated a measurable anti-bacterial and anti-biofilm effect against the *P. aeruginosa* clinical isolates tested with CFS-LP demonstrating the highest anti-bacterial and anti-biofilm capacity under the experimental conditions. The ability of the CFS-antibiotic combinations to inhibit the growth of biofilm, to reduce the preformed biofilm, and to elicit greater responses as a function of concentration suggest that probiotic extracellular products may be useful as adjunctive antimicrobial agents.

The conclusions in this work must be kept in the scope of the experimental model and are not meant as proof of clinical efficacy, or of clear synergism, until further mechanistic and in vivo evidence of these results. This conservative interpretation is also in line with the statement of the study at the end which says the results are applicable only to tested isolates and laboratory conditions.

6. Strengths of the Study

An important advantage of this study is the use of clinical isolates of *Pseudomonas aeruginosa*, rather than a laboratory reference strain. The participation of clinical isolates helps to reflect the

antimicrobial resistance, biofilm formation capability and sensitivity to the cell-free product (CFSs) of probiotics in laboratory settings that mimic clinical conditions. This study involved 60 confirmed *P. aeruginosa* isolates which were collected from different types of clinical specimens to give a wide representation of the population of bacteria studied.

A second strength is the ability to include an integrated assessment of antimicrobial resistance and biofilm formation. The study was not a single pathogen resistance study, but rather represented a tri-fold methodology that characterized antimicrobial susceptibility, MDR status, and quantitative biofilm production. This is useful because both resistance and biofilm formation are bacterial traits that are associated but not synonymous, and a combined assessment of these traits gives a better understanding of the persistence of clinical *P. aeruginosa* strains.

Another good point is that the study was conducted on both planktonic and biofilm-associated antibacterial activity. Both MIC and MBC studies were used to investigate the CFS preparations and their capacity to inhibit developing biofilm and reduce established biofilm was also investigated. This is a wider assessment of CFS activity since inhibiting the growth of planktonic bacteria does not necessarily mean an effective inhibition of an established biofilm.

The study also differentiated between the prevention of biofilm formation and reduction of preformed biofilm. This is a significant methodological advantage as the structure and physiological properties of developing and mature biofilms are very different. To assess both endpoints, therefore, gives more detailed information whether the CFS preparation mainly affects the early biofilm growth or whether it also has an impact on an established biofilm structure.

Finally, three different probiotic based CFS preparations (CFS-LP, CFS-LR, CFS-LA) were compared and their responses were examined in conjunction with antibiotics. This comparative design enabled the identification of differences in antibacterial and antibiofilm activity of the different preparations, and a preliminary indication of the complementary activity of the preparations with conventional antimicrobial therapy.

7. Conclusion

This study aimed to develop a focused experimental setup for the assessment of the antibacterial and antibiofilm activity of cell-free supernatants (CFSs) produced by probiotics against clinical multidrug-resistant (MDR) and biofilm-producing *Pseudomonas aeruginosa* isolates. Clinical specimens were analyzed and confirmed isolates were analyzed for antimicrobial susceptibility testing, MDR classification and quantitative biofilm assessment. Bactericidal activity of CFS preparations was determined by using the MIC or MBC method and their antibiofilm activity was evaluated by determining the inhibition of biofilm and reduction of preformed biofilms.

The responses obtained from preparations of CFS alone and in combination with antibiotics were also compared. The baseline biofilm biomass, CFS susceptibility, biofilm inhibition, and MDR-associated response were significantly correlated by the correlation analysis. The results showed that CFSs isolated from probiotics have measurable antibiofilm and antibacterial effects under the conditions tested, and especially CFS-LP. The effects observed, however, were drawn upon within the constraints of the experimental design and conclusions were limited to the laboratory results and isolates tested.

References

- Asghari Ozma, M., Moaddab, S. R., Hosseini, H., Khodadadi, E., Ghotaslou, R., Asgharzadeh, M., et al. (2024). A critical review of novel antibiotic resistance prevention approaches with a focus on postbiotics. *Critical Reviews in Food Science and Nutrition*, 64(26), 9637-9655. <https://doi.org/10.1080/10408398.2023.2214818>
- Bianchi, M., Kaya, E., Logiudice, V., Maisetta, G., Curtis, A., Kavanagh, K., et al. (2025). Biotherapeutic potential of different fractions of cell-free supernatants from *Lactocaseibacillus rhamnosus* against *Pseudomonas aeruginosa*. *Frontiers in Cellular and Infection Microbiology*, 15, 1608897. <https://doi.org/10.3389/fcimb.2025.1608897>
- Choi, V., Rohn, J. L., Stoodley, P., Carugo, D., & Stride, E. (2023). Drug delivery strategies for antibiofilm therapy. *Nature Reviews Microbiology*, 21(9), 555-572. <https://doi.org/10.1038/s41579-023-00905-2>

Drumond, M. M., Tapia-Costa, A. P., Neumann, E., Nunes, Á. C., Barbosa, J. W., Kassuha, D. E., et al. (2023). Cell-free supernatant of probiotic bacteria exerted antibiofilm and antibacterial activities against *Pseudomonas aeruginosa*: A novel biotic therapy. *Frontiers in Pharmacology*, 14, 1152588. <https://doi.org/10.3389/fphar.2023.1152588>

Elfadadny, A., Ragab, R. F., AlHarbi, M., et al. (2024). Antimicrobial resistance of *Pseudomonas aeruginosa*: Navigating clinical impacts, current resistance trends, and innovations in breaking therapies. *Frontiers in Microbiology*, 15, 1374466. <https://doi.org/10.3389/fmicb.2024.1374466>

El-Mokhtar, M. A., Hassanein, K. M., Ahmed, A. S., Gad, G. F. M., Amin, M. M., & Hassanein, O. F. E. (2020). Antagonistic activities of cell-free supernatants of lactobacilli against extended-spectrum β -lactamase producing *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. *Infection and Drug Resistance*, 13, 543-552. <https://doi.org/10.2147/IDR.S235603>

Elimination of pathogen biofilms via postbiotics from lactic acid bacteria: A promising method in food and biomedicine. (2024). *Microorganisms*, 12(4), 704. <https://doi.org/10.3390/microorganisms12040704>

Gomes, W. R. S., dos Santos Souza, G., Ferreira, J. I. D., et al. (2026). Beyond probiotics: *Lactocaseibacillus rhamnosus* as a multi-target antagonist of *Pseudomonas aeruginosa* infections. *Folia Microbiologica*. <https://doi.org/10.1007/s12223-026-01573-4>

Højby, N., & Moser, C. (2024). *Pseudomonas aeruginosa* in the frontline of the greatest challenge of biofilm infection—Its tolerance to antibiotics. *Microorganisms*, 12(11), 2115. <https://doi.org/10.3390/microorganisms12112115>

Homayouni Rad, A., Pourjafar, H., & Mirzakhani, E. (2023). A comprehensive review of the application of probiotics and postbiotics in oral health. *Frontiers in Cellular and Infection Microbiology*, 13, 1120995. <https://doi.org/10.3389/fcimb.2023.1120995>

Jeyanathan, A., Ramallete, R., Blunn, G., Gibbs, H., Pumilia, C. A., Meckmongkol, T., et al. (2021). *Lactobacillus* cell-free supernatant as a novel bioagent and biosurfactant against *Pseudomonas aeruginosa* in the prevention and treatment of orthopedic implant infection. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, 109(10), 1634-1643. <https://doi.org/10.1002/jbm.b.34821>

Kılıç, T., & Bali, E. B. (2023). Biofilm control strategies in the light of biofilm-forming microorganisms. *World Journal of Microbiology and Biotechnology*, 39, 131. <https://doi.org/10.1007/s11274-023-03584-6>

Kiousi, D. E., Efstathiou, C., Tzampazlis, V., Plessas, S., Panopoulou, M., Koffa, M., et al. (2023). Genetic and phenotypic assessment of the antimicrobial activity of three potential probiotic lactobacilli against human enteropathogenic bacteria. *Frontiers in Cellular and Infection Microbiology*, 13, 1127256. <https://doi.org/10.3389/fcimb.2023.1127256>

Li, X., Wang, Q., Hu, X., & Liu, W. (2022). Current status of probiotics as supplements in the prevention and treatment of infectious diseases. *Frontiers in Cellular and Infection Microbiology*, 12, 789063. <https://doi.org/10.3389/fcimb.2022.789063>

Liang, B., & Xing, D. (2023). The current and future perspectives of postbiotics. *Probiotics and Antimicrobial Proteins*. <https://doi.org/10.1007/s12602-023-10045-x>

Liu, H. Y., Prentice, E. L., & Webber, M. A. (2024). Mechanisms of antimicrobial resistance in biofilms. *npj Antimicrobials and Resistance*, 2, 27. <https://doi.org/10.1038/s44259-024-00046-3>

Ma, L., Tu, H., & Chen, T. (2023). Postbiotics in human health: A narrative review. *Nutrients*, 15(2), 291. <https://doi.org/10.3390/nu15020291>

Pompilio, A., Kaya, E., Lupetti, V., Catelli, E., Bianchi, M., Maisetta, G., et al. (2024). Cell-free supernatants from *Lactobacillus* strains exert antibacterial, antibiofilm, and antivirulence activity against *Pseudomonas aeruginosa* from cystic fibrosis patients. *Microbes and Infection*, 26(4), 105301. <https://doi.org/10.1016/j.micinf.2024.105301>

Postbiotics as candidates in biofilm inhibition in food industries. (2024). *Letters in Applied Microbiology*, 77(4), ova069. <https://doi.org/10.1093/lambio/ova069>

Postbiotics of the *Lactiplantibacillus plantarum* EIR/IF-1 strain show antimicrobial activity against oral microorganisms with pH adaptation capability. (n.d.). *Microbiology Research*, 14(3), 1442-1456. <https://doi.org/10.3390/microbiolres14030098>

Postbiotics: An overview of concepts, inactivation technologies, health effects, and driver trends. (2023). *Trends in Food Science & Technology*, 138, 199-214. <https://doi.org/10.1016/j.tifs.2023.06.009>

Rana, S., Bhawal, S., Kumari, A., Kapila, S., & Kapila, R. (2020). pH-dependent inhibition of AHL-mediated quorum sensing by cell-free supernatant of lactic acid bacteria in *Pseudomonas aeruginosa* PAO1. *Microbial Pathogenesis*, 142, 104105. <https://doi.org/10.1016/j.micpath.2020.104105>

Shree, P., Singh, C. K., & Sodhi, K. K. (2023). Biofilms: Understanding the structure and contribution towards bacterial resistance in antibiotics. *Medicine in Microecology*, 16, 100084. <https://doi.org/10.1016/j.medmic.2023.100084>

Sornsene, P., Chatatikun, M., Mitsuwan, W., Kongpol, K., Kooltheat, N., Sohbenalee, S., et al. (2021). Lyophilized cell-free supernatants of *Lactobacillus* isolates exhibited antibiofilm, antioxidant, and reduces nitric oxide activity in LPS-stimulated RAW 264.7 cells. *PeerJ*, 9, e12586. <https://doi.org/10.7717/peerj.12586>

Toushik, S. H., Park, J. H., Kim, K., Ashrafudoulla, M., Ulrich, M. S. I., Mizan, M. F. R., et al. (2022). Antibiofilm efficacy of *Leuconostoc mesenteroides* J.27-derived postbiotic and food-grade essential oils against *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, and *Escherichia coli* alone and in combination, and their application as a green preservative in the seafood industry. *Food Research International*, 156, 111163. <https://doi.org/10.1016/j.foodres.2022.111163>

Tunçer, S., & Karaçam, S. (2020). Cell-free supernatant of *Streptococcus salivarius* M18 impairs the pathogenic properties of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. *Archives of Microbiology*, 202(10), 2825-2840. <https://doi.org/10.1007/s00203-020-02005-8>

Yahyazadeh Jasour, S., Mohammadhosseinzadeh, F., & Arefian, E. (2026). Antimicrobial and antibiofilm effects of *Lactobacillus* cell-free supernatants against standard and antibiotic-resistant *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Archives of Microbiology*, 208(8), 424. <https://doi.org/10.1007/s00203-026-04836-3>

Zahedi, P., Larypoor, M., & Razavi, M. R. (2026). Investigating the effect of exopolysaccharide of bacteria isolated from traditional dairy on *Pseudomonas aeruginosa* biofilm. *The Microbe*, 10, 100643. <https://doi.org/10.1016/j.microb.2025.100643>

Zhao, Y., Xu, H., Wang, H., Wang, P., Chen, S., et al. (2024). Multidrug resistance in *Pseudomonas aeruginosa*: Genetic control mechanisms and therapeutic advances. *Molecular Biomedicine*, 5, 62. <https://doi.org/10.1186/s43556-024-00221-y>