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Aspartic acid unveils as antibiofilm agent and tobramycin adjuvant against mucoid and small colony variants of *Pseudomonas aeruginosa* isolates *in vitro* within cystic fibrosis airway mucus

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ABSTRACT

Antibiotics are central to managing airway infections in cystic fibrosis (CF), yet current treatments often fail due to the presence of *Pseudomonas aeruginosa* biofilms, settling down the need for seeking therapies targeting biofilms. This study aimed to investigate the antibiofilm activity of aspartic acid and its potential as an adjuvant to tobramycin against *P. aeruginosa* biofilms formed by mucoid and small colony variant (SCV) tobramycin tolerant strain. We assessed the effect of aspartic acid on both surface-attached and suspended *P. aeruginosa* biofilms within CF artificial mucus and investigated the synergistic impact of combining it with non-lethal tobramycin concentrations. Our findings showed that aspartic acid inhibited planktonic *P. aeruginosa* without affecting its viability and prevented biofilm formation by hindering bacterial adhesion or interfering with EPS production, depending on the experimental conditions. In CF mucus, aspartic acid significantly reduced bacterial growth, with the highest inhibition observed when combined with tobramycin, showing notable effects against the mucoid and tolerant SCV strain. Despite these reductions, *P. aeruginosa* repopulated the mucus within 24 h of stress withdrawal. Additional strategies, including delayed tobramycin application and a second dose of co-application of aspartic acid and tobramycin were explored to address bacterial survival and recovery. Although none of the strategies eradicated *P. aeruginosa*, the second co-application resulted in slower bacterial recovery rates.

In conclusion, this study highlighted aspartic acid as an effective antibiofilm agent and demonstrated for the first time its potential as an adjuvant to tobramycin. The combined use of aspartic acid and tobramycin offers a promising advancement in CF therapeutics, particularly against *P. aeruginosa* biofilms formed by mucoid and SCV strains, mitigating their antibiotic resistance.

1. Introduction

Cystic fibrosis (CF) is an inherited disease primarily affecting the lungs. It stems from a defect in the CF conductance regulator gene, resulting in the buildup of thick and sticky mucus that can lead to several airway complications, making CF patients more susceptible to chronic infections [1]. *Pseudomonas aeruginosa* is chiefly responsible for chronic infections, contributing significantly to the decline of lung function, prolonged hospitalizations, and increased mortality among CF patients [2]. Consequently, effective management of CF involves targeting *P. aeruginosa* infections.

Current eradication protocols for *P. aeruginosa* in CF rely on the administration of inhaled and intravenous antibiotics, such as tobramycin, aztreonam, and colistin [3,4]. Tobramycin, a cornerstone in Gram-negative infection treatment, including *P. aeruginosa* infections, is widely used in CF therapeutics. It can be administered by inhalation (TOBI® Podhaler™), intravenously, and in combination with other antibiotics such as colistin, piperacillin, meropenem, and ceftazidime [5–7].

Despite these treatments, the failure rate of current antibiotic regimens, including tobramycin, remains high [8,9] and this is largely attributed to *P. aeruginosa's* diverse adaptation mechanisms that include enhanced antibiotic resistance, biofilm formation, overproduction of alginate (mucoid phenotype), slow growth rate (small colony variants, SCV), and loss of motility [10–15]. Biofilm formation is a key factor in long-term persistence and resistance of *P. aeruginosa,* making bacteria impervious to conventional therapies. Several mechanisms contribute to this impressive biofilm resistance, such as restricted antibiotic penetration through the biofilm matrix and/or the covalent binding of antibiotics to the matrix compounds, the presence of persisters, slow growth of bacteria due to nutritional constraints and restricted oxygen penetration, quorum sensing, and phenotypic and genotypic bacteria alterations against antimicrobials mechanisms [16,17].

In recent years, there has been a focused effort on examining antimicrobial resistance mechanisms of biofilms and aiding in the development of new strategies to overcome this resistance and restore the

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effectiveness of existing antibiotics [18–21]. Adjuvants hold significant promise as enhancers of antibiotics in combating biofilm-associated infections [19]. These compounds, although normally lacking antimicrobial activity, can enhance the antibiotic action when combined or co-administered by blocking the antibiotic resistance mechanisms [22, 23]. Adjuvants may also alter the bacterial lifestyle from biofilm to planktonic state, target biofilm antimicrobial resistance mechanisms, disrupt biofilm architecture and improve antibiotic penetration or prevent biofilm formation [24–26].

In our earlier investigation seeking ciprofloxacin adjuvants, aspartic acid showed great potential as an antibiotic adjuvant by restoring ciprofloxacin action against *P. aeruginosa* grown in an *in vitro* CF airway environment [20]. It was postulated that this adjuvant effect might be caused by its antibiofilm activity. In this study, we aimed to investigate the antibiofilm activity of aspartic acid against *P. aeruginosa* isolates from CF patients, representing the most clinically relevant phenotypes in CF, mucoid and SCV phenotype. Additionally, we aimed to evaluate its potential as an adjuvant to aminoglycosides for fighting *P. aeruginosa* infections. Given the widespread use of tobramycin in CF management for controlling *P. aeruginosa* infections [5–7] it stands as a prime candidate for adjuvant research.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Antibacterial and antibiofilm activity of aspartic acid was carried out against two common *P. aeruginosa* phenotypes isolated from CF lungs, specifically mucoid (PA-Muc) and SCV (PA-SCV), as well as a non-CF respiratory clinical isolate (PAI) kindly provided by a Portuguese Hospital. The CF strains were selected based on their distinct susceptibilities to tobramycin determined in this study, while the inclusion of non-CF strains was due to the potential transmission of *P. aeruginosa* from non-to CF individuals. Bacteria were routinely cultured on Tryptic Soy Broth (TSB, Liofilchem) or Tryptic Soy Agar (TSA, Liofilchem) at 37 °C. All strains were preserved in cryovials (Nalgene) at − 80 ± 2 °C to minimize putative adaptation to the laboratory environment. Prior to each experiment, bacterial cells were grown on TSA plates overnight at 37 °C.

2.2. Determination of minimum inhibitory concentration (MIC)

The antibiotic susceptibility of *P. aeruginosa* strains was determined by MIC using the microdilution assay following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards) [27]. Overnight cultures of *P. aeruginosa* strains were washed and diluted with Mueller Hinton Broth (MHB) to 5×10^5 CFU/mL and transferred to 96-well plates. Further, bacterial strains were exposed to different concentrations of tobramycin (Sigma Aldrich) ranging from 0.125 to 64 mg/L at 37 °C, 120 rpm for 18–21 h in air conditions. MIC was determined by the minimum concentration of antibiotic required to inhibit 95 % of growth, measuring the optical density at 620 nm on a microtiter plate reader (EZ Read 800 plus, Biochrom). The clinical breakpoint for tobramycin for resistance was 16 mg/L as defined by CLSI [27]. MHB alone and bacterial cultures free of tobramycin were used as negative and positive controls, respectively. All tests were performed 5 times (independent biological assays) with 3 technical replicates.

2.3. Preparation of aspartic acid stock solution

L-aspartic acid (Sigma Aldrich) was dissolved in 2 M NaOH (Sigma-Aldrich) according to the manufacture's recommendations. A concentrated solution of aspartic acid was prepared and applied to bacterial cultures to obtain a final concentration of 20 mM, as described in the literature [20]. Aspartic acid stock solutions were freshly prepared before their application to culture.

2.4. Effect of tobramycin and aspartic acid on P. aeruginosa growth curve and cell viability

The effect of tobramycin and aspartic acid, alone or in combination, on the growth curve and cell viability of *P. aeruginosa* strains was assessed by inoculating 1×10^7 CFU/mL into TSB containing 1 or 2 mg/L of tobramycin with or without 20 mM of aspartic acid. Overnight *P. aeruginosa* inocula of each bacterial strain were washed twice in sterile water by centrifugation (9000 *g*, 7 min). *P. aeruginosa* cell suspensions were then diluted to a final concentration of 1×10^7 CFU/mL and 200 μL of cell suspension was transferred to sterile 96-well round-bottom culture plates (Orange Scientific). The plates were incubated at 37 °C for 24 h, in static culture conditions to resemble CF airway conditions. A growth curve was constructed by measuring the optical density (OD) at 620 nm overtime, until a maximum of 24 h of culture. Cell viability was determined, after 24 h growth, by colony forming units (CFU) counting. For this, the content of the plates was recovered, serially diluted and plated on TSA. Experiment controls of the activity of 2 M NaOH, the solvent of aspartic acid solutions, were conducted to discard its contribution to the antibacterial activity of aspartic acid (Figs. S1 and S2). All experiments were performed at least three times.

3. Antibiofilm assays

3.1. Assessment of tobramycin and aspartic acid effect on P. aeruginosa biofilm formation

Tobramycin and aspartic acid, alone or in combination, were tested as prophylactic agents (applied before *P. aeruginosa* growth and biofilm formation), and as antibiofilm agents (applied after the formation of 24 h-old biofilms). Their effects were analyzed by crystal violet staining method (CV) [28]. *P. aeruginosa* cell suspensions were washed and diluted as described previously. Flat bottom 96-well plates were incubated at 37 °C in static culture conditions for 24 h for biofilm formation, in the presence of 20 mM aspartic acid with or without 2 mg/L of tobramycin, following a prophylactic approach. After 24 h, the content of the plates was discarded and washed twice with sterile water to remove weakly attached cells and suspended cell products. Afterwards, 200 μL of methanol was added to each well and plates were allowed to stand for 15 min in order to fix the biofilm. Methanol was discarded and plates were left to dry at room temperature. Biofilms were then stained with 200 μL of 1 % CV for approximately 5 min and were washed twice with water. Finally, the amount of biofilm formed was quantified by solubilization of the CV in 200 μL of 33 % (v/v) acetic acid. OD was measured at 570 nm using a microtiter plate reader (Thermo Scientific Multiskan FC Microplate Photometer). Viability of the adhered bacteria was also determined by CFU counting. After washing biofilms, biofilm cells were detached by sonication using an ultrasonic bath (Sonic model SC-52, UK) operating at 50 kHz, for 10 min as previously optimized [29], and further serially diluted and plated on TSA to determine the number of culturable cells. For the antibiofilm approach, flat bottom 96-well plates were first incubated at 37 °C in static culture conditions for 24 h for biofilm formation and, subsequently, 20 mM of aspartic acid with and without 2 mg/L of tobramycin were applied to 24h-old biofilms. Their efficacy was evaluated after 24 h using the same procedures described above. All experiments were performed at least 3 times.

3.2. Assessment of antibiofilm activity by confocal laser scanning Microscopy (CLSM)

Biofilms were formed on plastic coverslips 13 mm (Thermo Scientific, USA) placed on 24-well polystyrene microtiter plates at 37 °C for 24 h, as described above. After 24 h, the biofilms were washed twice using 0.9 % (w/v) NaCl and cell viability was determined using a LIVE/

DEAD™ BacLight™ Bacterial Viability Kit (Invitrogen, Thermo Fisher Scientific), consisting of SYTO 9 and propidium iodide (PI). The biofilms were visualized using an Olympus™ Fluo-View FV1000 (Olympus) confocal laser scanning microscope using 10x and 40x objective. SYTO9 was detected using a filter with an excitation wavelength of 485 nm and an emission filter of 498 nm. PI was detected using a filter with an excitation wavelength of 536 nm and an emission filter of 617 nm. The experiments were performed three times.

4. *In vitro* growth of *P. aeruginosa* in an artificial CF airway environment

4.1. Artificial sputum medium preparation

Artificial sputum medium (ASM) was used to mimic the sputum of CF patients and it was prepared as described by Sriramulu et al. (2005) [30]. Briefly, 5 g/L of mucin from pig stomach (Sigma-Aldrich), 4 g/L of DNA from salmon sperm (Sigma-Aldrich), 5.9 mg/L of diethylene triamine pentaacetic acid (DTPA, Sigma-Aldrich), 5 g/L of NaCl, 2.2 g/L of KCl and 5 g/L of casoamino acids (MP Bimedica) were resuspended in water and the pH adjusted to 7 with Tris base. This ASM was then sterilized in an autoclave at 110 °C for 15 min and, after cooled, 5 mL of egg yolk emulsion (Fluka) was added.

4.2. Antipseudomonal activity of aspartic acid and tobramycin

The antipseudomonal activity of tobramycin and aspartic acid alone or combined in ASM was performed similarly to the TSB assays. Briefly, two mL of ASM were transferred to each well of a 24 well-plate (polystyrene, Orange, USA) and tobramycin and aspartic acid alone or combined were added. After 30 min, ASM was inoculated on the top with 5 μL of the bacterial cell suspensions, obtaining a final cellular concentration in each well of 1×10^7 CFU/mL. ASM cultures were incubated for 24 h at 37 °C aerobically in static culture conditions to resemble the reduced or absent cilia movements in CF lungs. Further, the content of the wells was collected aseptically and vigorously shaken for a few minutes using the vortex to detach the cells from possible small aggregates or adhered to mucin and reduce the number of aggregates that could underestimate CFU counts. The resulting cell suspensions were serially diluted and plated on TSA to determine the number of surviving culturable cells after the application of the compounds. Experiment controls of the activity of 2 M NaOH were also conducted to discard its contribution to the antipseudomonal activity of aspartic acid (Fig. S3).

To evaluate the activity at other growth stages, *P. aeruginosa* was first added to ASM as aforementioned and allowed to grow for 6 and 12 h. After, tobramycin and aspartic acid, alone or in combination, were added to ASM and their efficacy was evaluated after 24 h, by the determination of the number of surviving culturable cells. A similar methodology was followed to determine the antipseudomonal activity of two applications of tobramycin and aspartic acid (one *per* 24 h) and sequential application of the compounds, first aspartic acid and then tobramycin, with intervals 6 and 12 h. After 24 h of *P. aeruginosa* growth in ASM with one or two applications of tobramycin and/or aspartic acid, 2 mL of fresh ASM was added to cultures, and bacteria were allowed to grow for more 24 h before determining the number of culturable cells. All experiments were performed at least three times.

4.3. Statistical analysis

All graphs and statistical data analysis were performed using GraphPad Prism software package (GraphPad Software version 8.2.0). Means and standard deviation (SD) were calculated for all experimental conditions tested. Statistical analysis was carried out by two-way ANOVA with Tukey's multiple comparison and p values < 0.05 were considered significant.

5. Results

5.1. Antibacterial activity of tobramycin and aspartic acid

The antimicrobial activity of tobramycin, determined through the MIC against the three *P. aeruginosa* isolates, guided the selection of tobramycin concentrations to be combined with aspartic acid. PA-Muc and PAI were susceptible to tobramycin with a MIC of 0.5 mg/L, whereas PA-SCV was resistant to tobramycin with a MIC of 16 ≥ mg/L. Tobramycin concentrations of 1 and 2 mg/L, corresponding to 2- and 4-fold MIC of the susceptible strains, were selected for combination with aspartic acid to primarily prevent biofilm formation. Increased concentrations higher than the MIC were anticipated to be needed for an effective antibiofilm strategy based on our previous results [20,31]. Although PA-SCV was known to be tolerant to tobramycin and the selected concentrations were sub-MIC for this strain, we aimed to overcome or block its tolerance mechanisms using aspartic acid.

Growth curve analysis confirmed PA-SCV tolerance and PA-Muc and PAI susceptibility to the selected concentrations of tobramycin (Fig. 1). Additionally, the results demonstrated effective inhibition of the tolerant strain PA-SCV and reduced bacterial growth of the strains PA-Muc and PAI upon exposure to aspartic acid. PA-Muc and PAI displayed a delayed lag phase and protracted logarithmic phase compared to the control. Inhibition and reduction of bacterial growth by aspartic acid were not achieved by loss of planktonic cell viability as no significant differences were detected in the culturable number of bacteria compared to control (Figure S4). However, an additive and synergistic effect was observed for PA-SCV when aspartic acid was combined with 1 and 2 mg/L of tobramycin, respectively. It is noteworthy that aspartic acid seemed to antagonize tobramycin action against PA-Muc and PAI, highlighting the importance of the optimal combination of dosages. The synergistic interaction with aspartic acid and tobramycin prompted further investigation into its antibiofilm activity.

5.2. Effect of aspartic acid and tobramycin on biofilm biomass reduction and bacterial viability

The three *P. aeruginosa* strains exhibited robust biofilm-forming capabilities, yielding biofilms with comparable biomass levels (cells and matrix) (Fig. 2, control groups).

In the prophylactic approach (Fig. 2A, grey bars), tobramycin poorly reduced the biofilm biomass of PA-SCV, PA-Muc and PAI by 29, 27 and 38 %, respectively. Conversely, aspartic acid disturbed *P. aeruginosa* biofilm formation, resulting in a reduction of 75, 42 and 72 % in biofilm biomass for PA-SCV, PA-Muc and PAI, respectively, compared to the control groups. Notably, the greatest reduction in biofilm biomass was achieved with the combined application of aspartic acid and tobramycin. Specifically, at 2 mg/L of tobramycin and 20 mM of aspartic acid, the inhibition rates were approximately 83 and 85 % for PA-SCV and both PA-Muc and PAI, respectively, compared to the control group.

Measuring biofilm biomass by CV is a quantitative method that does not distinguish between matrix and cells. Therefore, it was necessary to investigate how the antibiofilm activity of aspartic acid was distributed between these two components by estimating the number of the adhered cells and visualizing the biofilm matrix using CLSM. From culturable cell counting data, it was clear that *P. aeruginosa* adhered to surfaces upon exposure to aspartic acid, as a high number of cells were counted for all strains (Fig. 2A, green bars). An additive effect between aspartic acid and 2 mg/L of tobramycin was observed for PA-SCV, while antagonistic interactions were noted for PA-Muc and PAI, as the number of adhered cells was higher compared to the application of tobramycin alone. Taking together the data on bacterial viability and biofilm biomass quantification, the observed decrease in OD values (compared to the control group) suggested that bacteria adhered to surfaces without further EPS production. Although bacteria may adhere to surfaces in the presence of aspartic acid, they fail to produce EPS, which is crucial for

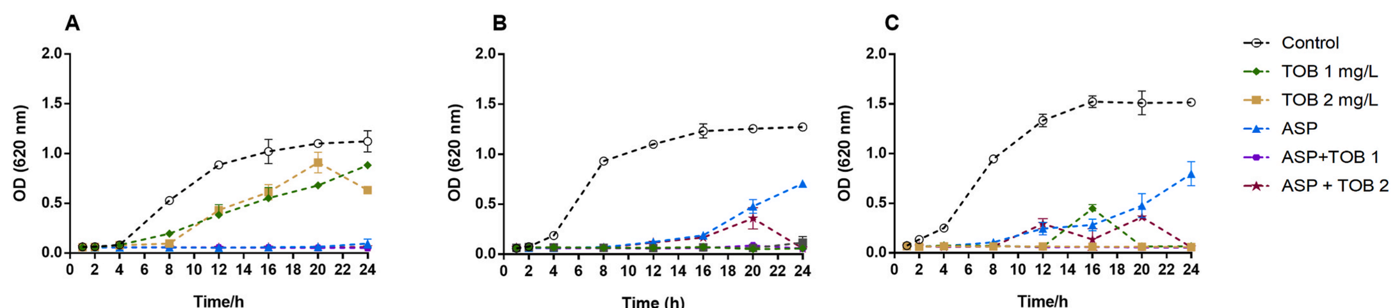

Fig. 1. Effect of 20 mM aspartic acid (ASP) either alone or in combination with 1 and 2 mg/L tobramycin (TOB) on planktonic cells. Growth curves of *P. aeruginosa* strains (A) PA-SCV, (B) PA-Muc and (C) PAI in TSB during 24 h were constructed by absorbance measurement at 620 nm. Experiments were repeatedly performed at least three times, each experiment with 8 technical replicates *per* condition tested.

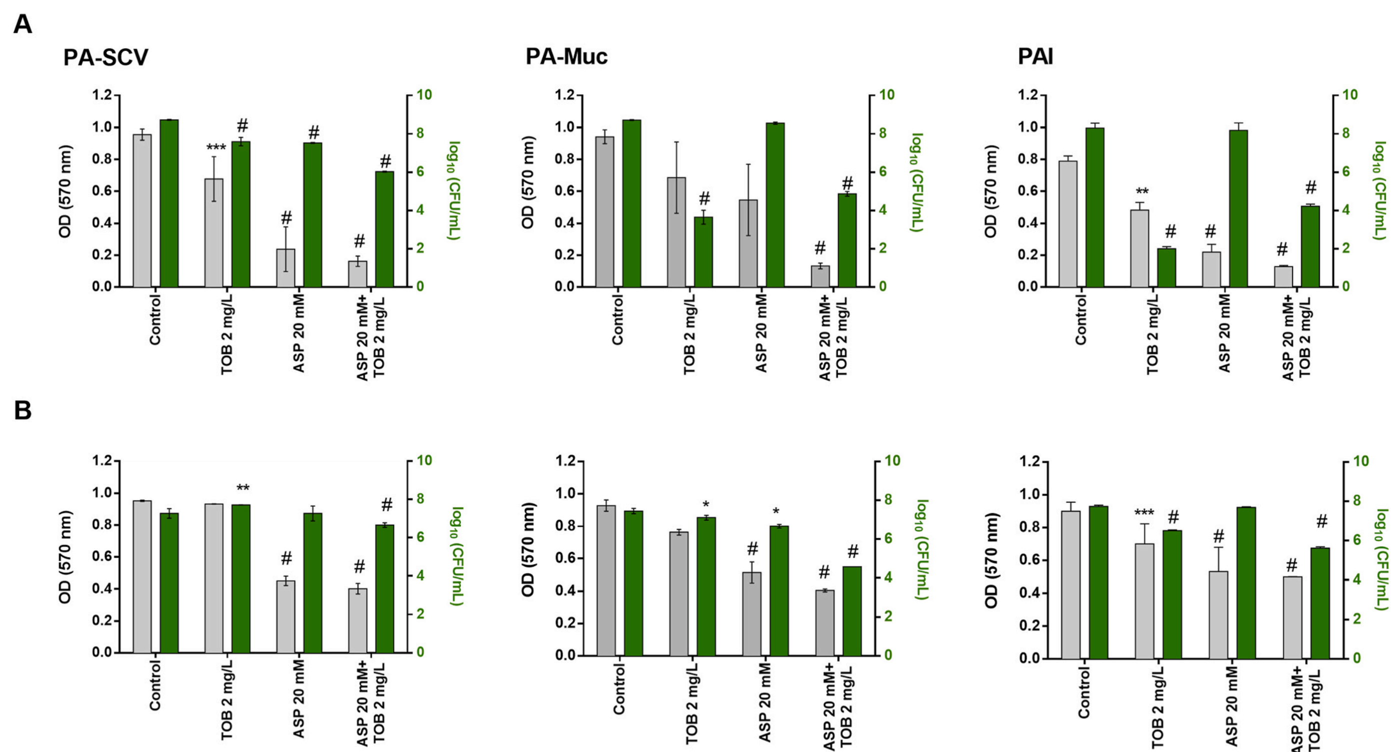

Fig. 2. Effect of 20 mM aspartic acid (ASP) either alone or in combination with 2 mg/L tobramycin (TOB), applied (A) before biofilm formation (prophylactic approach) and (B) after the formation of 24h-old biofilm (antibiofilm approach). The total amount of biofilm biomass (cells and matrix) was evaluated by CV method (biomass was quantified in terms of absorbance at 570 nm) and data are presented by grey bars. The total culturable adhered cells were determined after 24 h of the application of the compounds by CFU counting and data are presented by green bars. The bars and whisker represent mean values of absorbance or colony forming units ±SD. The differences in biofilm biomass and colony forming units after the application of the compounds alone or combined were compared to the control using two-way ANOVA followed by Tukey's multiple comparison *post hoc* test. Significant differences are indicated by asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, #$p < 0.0001$. Experiments were repeatedly performed at least three times. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

irreversible attachment and biofilm maturation.

CLSM analysis of cultures treated prophylactically with aspartic acid showed that it significantly reduced bacterial adhesion to surfaces compared to the control group (Fig. 3). Similar results were obtained upon co-application of aspartic acid and tobramycin, likely due to the action of aspartic acid. Tobramycin alone allowed bacterial adhesion for PAI and biofilm formation by PA-SCV (Fig. 3).

Concerning the antibiofilm approach (Fig. 2B), aspartic acid alone demonstrated the greatest reductions in biofilm biomass, with inhibition rates of 57, 56 and 45 % for PA-SCV, PA-Muc and PAI, respectively. In terms of cell viability, an additive effect was observed when aspartic acid was combined with tobramycin, resulting in bacterial load reductions ranging from 0.6 to 3 log with limited relevance from a biological perspective. The reduction in biofilm biomass and cell viability observed with the antibiofilm approach was less pronounced compared to the prophylactic approach.

Overall, our findings indicated that aspartic acid exhibited an effective antibiofilm activity by inhibiting bacterial adhesion or interfering with the irreversible attachment by EPS production. Its mechanism of action appeared to depend on the surface material and growth conditions of biofilm formation.

5.3. Efficacy of aspartic acid and tobramycin combination against P. aeruginosa grown in artificial CF sputum medium

On the basis of significant bacteriostatic and antibiofilm activity of

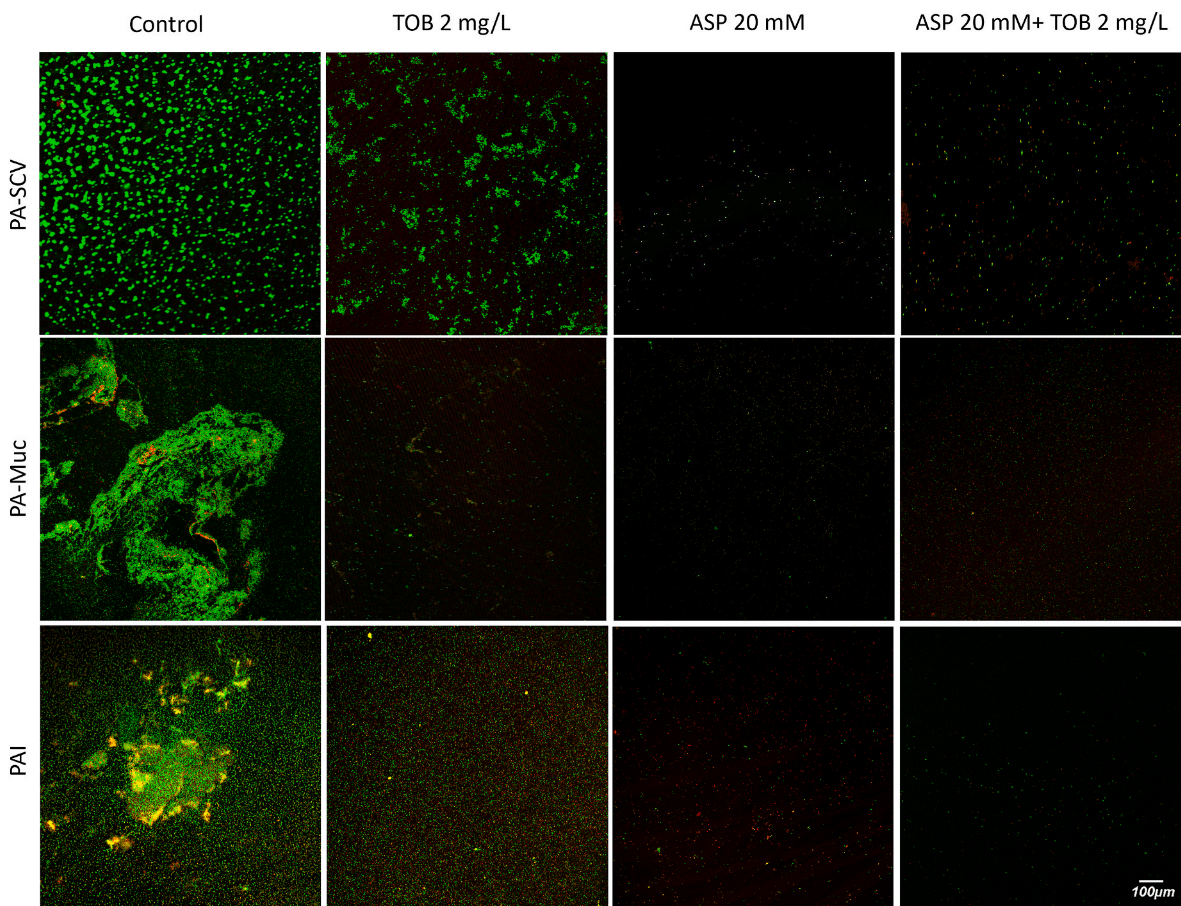

Fig. 3. Micrographs of *P. aeruginosa* biofilms on glass surfaces in the presence of 2 mg/L of tobramycin (TOB), 20 mM of aspartic acid (ASP), tobramycin with aspartic acid and growth controls. Cells were labeled with a Live/Dead staining. Live and dead cells are visualized in green and red, respectively. The images were acquired using an objective of 10x. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

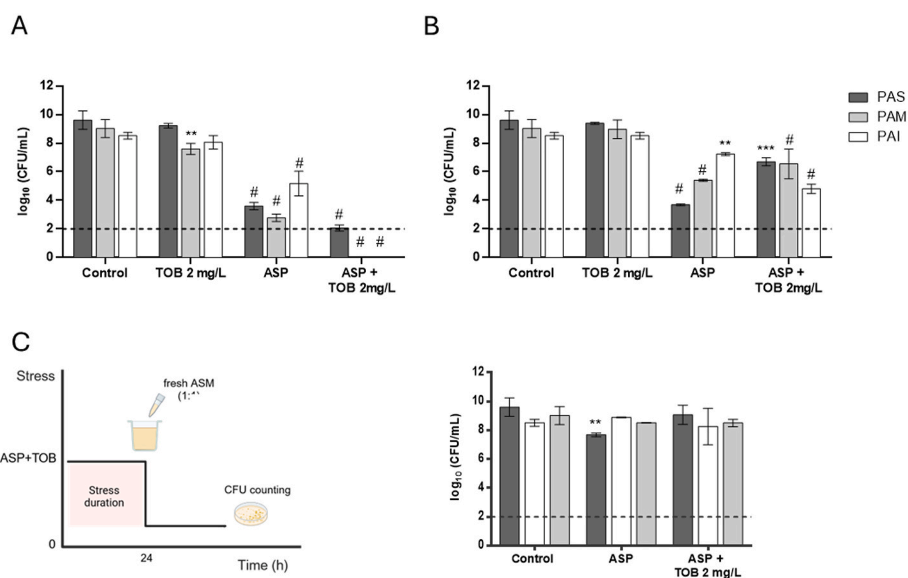

Fig. 4. Antipseudomonal activity of 20 mM aspartic acid (ASP) either alone or in combination with 2 mg/L tobramycin (TOB) (A) at 0h, and (B) after 24 h of three *P. aeruginosa* PA-SCV, PA-Muc and PAI growth in ASM. (C) Bacterial growth recovery after adding fresh ASM after 24 h of the application of 20 mM aspartic acid either alone or in combination with 2 mg/L tobramycin at 0 h. Values represent mean ± SD of, at least, 3 independent experiments. The differences in log10 CFU/mL of *P. aeruginosa* strains after the application of the aspartic acid, tobramycin or their combination were compared to the control using two-way ANOVA followed by Tukey's multiple comparison post hoc test. Significant differences are indicated by asterisks: *p < 0.05, **p < 0.01 ***p < 0.001, #p < 0.0001. CFU, colony-forming units; TOB, tobramycin; ASP, aspartic acid. The line indicates the detection limit. Figure partially created with BioRender.com on April 2024.

aspartic acid and its adjuvant effect on tobramycin, the investigation focused on its efficacy against *P. aeruginosa* grown in ASM. Incorporating ASM into our *in vitro* model has previously proven to be fundamental for assessing drug performance, as it recapitulates the *in vivo* conditions of CF airways [15,30]. Within ASM, both planktonic bacteria and biofilms are suspended, posing a challenge in separating and characterizing both growth states [15,20]. Therefore, in this study the assessment of the activity of aspartic acid on *P. aeruginosa* grown in ASM referred to its action on both planktonic and biofilm cells.

The results revealed that aspartic acid significantly reduced bacterial load in ASM for all strains (Fig. 4A), consistent with its previously observed antipseudomonal action. Notably, significant reductions were achieved in PA-Muc and the tolerant strain PA-SCV, with decreases ranging from 5 to 6 log compared to the control, noteworthy from a microbiological and clinical perspective. However, these reductions were restricted to the early stages of bacterial growth. When aspartic acid was applied after 6 and 12 h of growth, its effect on *P. aeruginosa* was not relevant (Fig. S5).

The highest antipseudomonal activity was obtained in the presence of aspartic acid combined with tobramycin which effectively inhibited the growth of *P. aeruginosa* strains. These results prompted a further investigation into the antipseudomonal activity of this combination of compounds against stationary planktonic bacteria and pre-established biofilms. For this purpose, aspartic acid and tobramycin were applied 24 h after *P. aeruginosa* growth in ASM (Fig. 4 B). Following this antibiofilm approach, the effectiveness of the combination of aspartic acid and tobramycin was lower compared to its prophylactic application, as was the effectiveness of aspartic acid alone. Additionally, tobramycin appeared to antagonize the activity of aspartic acid against 24-h-old *P. aeruginosa* populations in ASM.

Overall, these results suggested that aspartic acid exhibited potential as an adjuvant to tobramycin in ASM, particularly when applied at early stages of bacterial growth.

5.4. Study of P. aeruginosa resilience following aspartic acid and tobramycin treatment

Often, a small fraction of bacteria can survive to treatments, remaining undetectable by culturable-dependent methods because of technical limitations and/or by their viable but not culturable (VBNC) state. This bacterial survival poses a threat of recurrent infection. Therefore, we sought to determine the extent of bacterial inhibition after the treatment with aspartic acid and tobramycin and to investigate the timescale of a potential bacterial recovery in total abundance.

We monitored the number of culturable colony-forming units *per* mL every 24 h until four days following the initial application of 20 mM of aspartic acid alone and in combination with 2 mg/L of tobramycin. Notably, no culturable cells were detected for PA-Muc and PAI, while the number of culturable cells for PA-SCV remained approximately 1.6 $\times$ 10^2 CFU/mL. This non-growing bacterial behavior over four days might be attributed to the sustained presence of aspartic acid and tobramycin within ASM, impairing bacterial proliferation. To assess the potential for bacterial recovery, fresh ASM was added to *P. aeruginosa* cultures to hypothetically reduce stress (concentrations of aspartic acid with and without tobramycin) on bacteria and allow for the reinitiation of growth and repopulation of ASM. The results revealed a recovery of total bacterial numbers after 24h (Fig. 4C), indicating that a fraction of bacteria survived to aspartic acid treatment, either alone or combined with tobramycin. Additionally, these findings suggest that aspartic acid and tobramycin, when co-administrated in ASM, persisted for at least 24 h without degradation or inactivation and exerted an inhibitory effect on bacteria.

Given the resilience of a bacterial population surviving aspartic acid and tobramycin treatment and its rapid repopulation within 24 h, we sought to optimize our approach to augment efficacy. Our focus turned to adjusting the timing of tobramycin application, aiming to impede bacterial survival while amplifying its efficacy. To this end, tobramycin was applied after 6 and 12 h after aspartic acid application to ASM. Both modes of sequential application of aspartic acid and tobramycin (6 and 12 h) yielded comparable results and resulted in a higher reduction in bacterial load compared to treatment with tobramycin alone (Fig. 5A). However, they did not surpass the efficacy achieved through co-administration (Fig. 4A).

Although co-administration proved to be the most effective mode of application method, it did not eradicate *P. aeruginosa* from ASM. Therefore, we hypothesized whether introducing a second application of aspartic acid and tobramycin within the co-administration regime, 24 h after the first application, could address this challenge and eradicate *P. aeruginosa*. Interestingly, the addition of a second dose of the combination achieved PA-SCV inhibition (Fig. 5B) that was not achieved with a single application (Fig. 4A). However, PA-SCV and PA-Muc were still able to repopulate ASM, but at a slow rate since low bacterial numbers were observed after 24h of stress reduction (Fig. 5C). This suggested that a second application of aspartic acid and tobramycin may confer benefits in combating bacterial persistence, but eradication was not achieved.

6. Discussion

In the light of the limited success of existing antimicrobial therapies, the search for compounds with antibiofilm properties to function as adjuvants to enhance antibiotic treatment outcomes has gained considerable attention [24–26]. In the present study, we sought to examine the antibiofilm activity of aspartic acid against *P. aeruginosa* and its ability to enhance the efficacy of aminoglycosides, a class of antibiotics of paramount clinical significance in CF and other infectious diseases.

Our comprehensive analysis, including growth curve assessments, biofilm biomass quantification, cell viability and microscopic observations have exemplified the multifaceted bacteriostatic, antibiofilm and adjuvant properties of aspartic acid against clinically relevant *P. aeruginosa* phenotypes including a mucoid strain, and a tobramycin tolerant SCV strain. Our findings indicated that aspartic acid holds a promise in impeding *P. aeruginosa* biofilm formation by hindering bacterial adhesion or interfering with EPS production (depending on the environmental conditions), thereby facilitating tobramycin to exert its antibacterial action. To our knowledge, this study represents the first report that supports the antibiofilm activity against *P. aeruginosa* and the adjuvant potential of aspartic acid to tobramycin, underscoring its potential significance in the therapeutic arsenal against *P. aeruginosa* biofilm-associated infections.

Prior studies have elucidated the aspartic acid capacity to prevent the establishment of biofilms in *Streptococcus mutans* and *Staphylococcus aureus* by reducing planktonic viability, bacteria attachment, and inhibiting bacterial aggregation [32–34]. Yang et al. (2015) have described that aspartic acid at higher concentrations ($>$10 mM) can inhibit the *S. aureus* planktonic growth by affecting bacterial viability and significantly reducing the rate of cell attachment [33]. In turn, Warraich et al. (2020) found that aspartic acid inhibited biofilm formation by interacting with positively charged *S. aureus* surface proteins, thereby preventing the association of eDNA with these proteins and the formation of a biofilm meshwork [32]. eDNA has a pivotal role in bacterial adhesion and aggregation, biofilm stability and antibiotic tolerance in several species [35]. Tong et al. (2014) observed antibiofilm activity of aspartic acid against *S. mutans,* but they did not investigate the mechanism of action. The authors speculated that the presence of aspartic acid may affect bacterial plasticity and regular metabolism [34]. In our study, we showed that aspartic acid did not reduce planktonic cell viability as indicated by the high number of culturable bacteria detected. Moreover, the number of bacteria adhering to surfaces was also high as shown by CFU counts. Therefore, we postulated that aspartic acid permits *P. aeruginosa* adhesion.

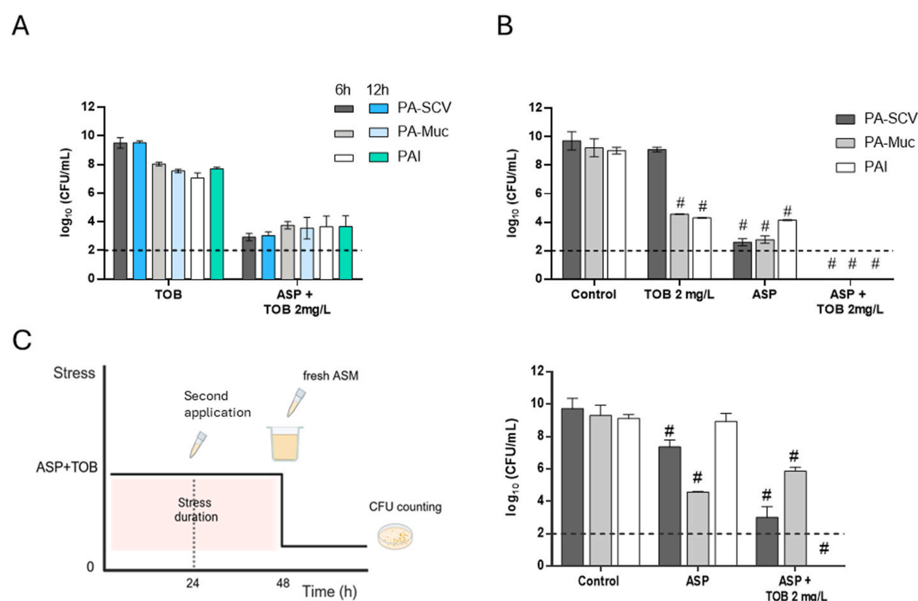

Fig. 5. (A) Antipseudomonal activity of 2 mg/L of tobramycin (TOB) at 6 and 12h after bacterial growth either alone or in combination with 20 mM of aspartic acid (ASP) against three *P. aeruginosa* PA-SCV, PA-Muc and PAI grown in ASM. (B) Antipseudomonal activity of two applications of 20 mM aspartic acid either alone or in combination with 2 mg/L tobramycin at 0 and 24 h against three *P. aeruginosa* strains grown in ASM. (C) Effect of stress reduction by adding fresh ASM after 24 h of the second application of 20 mM aspartic acid either alone or in combination with 2 mg/L tobramycin. Values represent mean ± SD of, at least, 3 independent experiments. The differences in log10 CFU/mL of *P. aeruginosa* strains after the application of the aspartic acid, tobramycin or their combination were compared to the control using two-way ANOVA followed by Tukey's multiple comparison post hoc test. Significant differences are indicated by asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, #$p < 0.0001$. CFU, colony-forming units; TOB, tobramycin; ASP, aspartic acid. The line indicates the detection limit. Figure partially created with BioRender.com on April 2024.

The efficacy of aspartic acid in different bacterial growth and biofilm formation conditions was evaluated, recognizing the potential impact of environmental factors on treatment outcomes [36]. For instance, biofilms in CF disease are suspended within airway mucus which made them distinct from the surface-attached biofilms [37,38]. Mucus poses a significant challenge due to its narrow mesh limiting drug performance in terms of distribution and long-residence in the mucus, making biofilm infections notoriously difficult to prevent and treat [39]. Consequently, we utilized ASM to simulate the CF lung environment, acknowledging its microbiological and physicochemical complexities, which often undermine conventional antimicrobial therapies. Validating our results in ASM underscores the relevance and translational potential of our findings of aspartic acid as an antibiofilm agent and as an antibiotic adjuvant in addressing the persistent challenges of biofilm-associated infections in CF patients.

In ASM, aspartic acid presented a significant biological action on its own, still leaving room for improvements in tobramycin action. Notably, aspartic acid significantly impaired the growth of a mucoid and SCV tobramycin tolerant strain, introducing a promising therapeutic strategy for CF. It broadens the scope of treatment options available for managing challenging mucoid and SCV *P. aeruginosa* infections in CF, which are often resistant to antibiotics. Chronic infections with mucoid *P. aeruginosa* are associated with a decline in lung function and increased morbidity and mortality in CF patients [40]. Mucoid biofilms increased resistance to antibiotics, complicating treatment options and leading to the need for prolonged and high-dose antibiotic therapies. Moreover, the reduction in SCV growth can directly impact the overall bacterial load in the lungs of CF patients, which is crucial for slowing disease progression. Effective management of SCVs can lead to fewer exacerbations and a better quality of life for CF patients [41].

Combination therapy offers several advantages over single-agent use, such as synergistic or additive activities among drugs, reduced individual drug concentration, and a reduced risk of resistant variant emergence [42]. Some studies have combined aspartic acid with other molecules with quite interesting results. For instance, Tong et al. (2014) described a synergistic action between aspartic acid and other amino acids such as glutamic acid and cysteine resulting in the inhibition of *S. mutans* biofilm formation [34]. The combination of aspartic acid with glycine and silver nanoparticles was also proved to be efficient in the inhibition of *Candida albicans* cell adhesion and mature biofilm formation [43]. A synergistic effect between aspartic acid and ciprofloxacin was elucidated in other studies where a reduction of *S. aureus* and *P. aeruginosa* load was observed using non-lethal concentrations of antibiotic [20,32]. In our study, aspartic acid also rendered non-lethal concentrations of tobramycin effective when both compounds were co-applied against *P. aeruginosa* grown in ASM. The combination of aspartic acid with tobramycin yielded meaningful benefits against mucoid and SCV *P. aeruginosa* suspended biofilms and importantly, the inhibitory effect of the combined therapy lasted at least 4 days using a single application. This finding may indicate that both agents have a suitable distribution and long-residence in the mucus.

Bacteria within biofilms can transiently survive under the lethal effect of treatments without being detected using culture-dependent methods [44,45]. After treatment ends or drug concentration drops, surviving bacteria can repopulate and originate a 'new' biofilm, contributing to biofilm resistance and recurrent infections. Although the clinical relevance of our results, analysis of *P. aeruginosa* recovery after aspartic acid and tobramycin withdrawal with fresh mucus demonstrated a rapid recovery of total bacterial numbers in ASM. Therefore, we attempted to optimize the application of both agents to achieve bacterial eradication. Delayed application of tobramycin after 6 and 12 h of aspartic acid did not improve treatment outcomes, but a second application of aspartic acid and tobramycin inhibited the growth of *P. aeruginosa* SCV tobramycin tolerant strain. Although none of these strategies eradicated *P. aeruginosa* in mucus, we verified limited bacterial recovery of mucoid and tobramycin-tolerant and SCV strains after 24h of the second dose of aspartic acid and tobramycin. In future experiments, sustained delivery of aspartic acid and tobramycin in CF mucus should be addressed to deeply explore aspartic acid as antibiofilm agent and antibiotic adjuvant.

Overall, the ability of aspartic acid to significantly inhibit the bacterial adhesion of mucoid and SCV tobramycin tolerant strains or interfere with EPS production highlights its potential as antibiofilm agent. Our results revealed that tobramycin combined with aspartic acid hinders bacterial growth and the formation of biofilms in abiotic surfaces and mucus, which tobramycin could not achieve alone. Therefore, aspartic acid has the potential to enhance the efficacy of existing antibiotics, such as tobramycin, making it easier to manage resistant infections and potentially reducing lung damage, slowing disease progression, and improving quality of life.

CRediT authorship contribution statement

Rosana Monteiro: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Ana Margarida Sousa:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Maria Olívia Pereira:** Writing – review & editing, Supervision.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2024.100252.

Data availability

Data will be made available on request.

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