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Impact of *N*-Acetylcysteine and Antibiotics Against Single and Dual Species Biofilms of *Pseudomonas aeruginosa* and *Achromobacter xylosoxidans*

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Abstract
Lungs of cystic fibrosis patients are often colonized or infected with organisms, such as *Pseudomonas aeruginosa* and other emerging pathogenic bacteria such as *Achromobacter xylosoxidans*. Further, it is well established that infections of the cystic fibrosis lung airways are caused by polymicrobial infections, although its composition and diversity may change throughout the patient's life. In the present study, we investigated the effects of *N*-acetylcysteine (NAC) and amikacin, aztreonam, ciprofloxacin, and tobramycin alone and in combination against single- and dual-species biofilms of *P. aeruginosa* and *A. xylosoxidans,* in vitro and in the *Caenorhabditis elegans* infection model. Results showed that tobramycin and ciprofloxacin were the most effective antibiotics, while aztreonam was the least effective antibiotic against both single- and dual-species biofilms of *P. aeruginosa* and *A. xylosoxidans*. However, NAC showed little effect on both single- and dual-species, even with a combination of antibiotics. Increased survival was observed in *C. elegans* when treated with NAC in combination with tobramycin or ciprofloxacin, compared to no treatment or NAC alone. Tobramycin and ciprofloxacin were found effective in biofilms, but more research is needed to better understand the effects of NAC and antibiotics against single- and dual-species biofilms.

Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This results in either no CFTR protein being made, or in a malformed CFTR protein that can't perform its key function in the cell. This transmembrane protein is responsible for transporting ions across the surface of epithelial cells; an inability of this protein to function leads to decreased volume of periciliary fluid in the airways and impaired mucus detachment [1, 2]. Consequently, the lungs of CF patients are highly susceptible to bacterial infections, as well as biofilms. A biofilm is an assemblage of surface-associated microbial cells, which is enclosed in an extracellular polymeric substance matrix. Bacteria in the CF airways mostly persist as biofilm structures and are more tolerant to antimicrobial agents, which causes to recurrent and chronic infections [3, 4].

The airways of patients with CF are highly complex and consist of polymicrobial infections that vary in their composition and diversity throughout a patient's lifetime [5]. These polymicrobial communities can make multispecies biofilms of microorganisms that can interact with each other to impact the course, treatment, and outcome of biofilm-related CF airway infections [6]. *Pseudomonas aeruginosa* has been reported as the predominant organism of established microbial communities in at least 50% of adult CF patients—these communities display high levels of antibiotic resistance and virulence. Additionally, chronic infections of *P. aeruginosa* are associated with contributing to morbidity and mortality in CF patients [5]. Although the current literature on bacterial competition in CF patients has mostly focused on major pathogens such as *P. aeruginosa* and *Staphylococcus aureus*, the CF lung microbiome may contain other emerging pathogenic bacteria such as *Achromobacter xylosoxidans* [4, 7].

Achromobacter xylosoxidans is becoming more common pathogen in CF patients. It can form biofilms and

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chronically colonize the respiratory tract; however, the competitive ability of *A. xylosoxidans* has rarely been studied [1]. Risk factors for the acquisition of *A. xylosoxidans* are not completely clear, but older age, increased disease burden, and chronic *P. aeruginosa* infections are common in patients who develop chronic *A. xylosoxidans* infections [8].

Because the therapeutic options are limited for biofilm-associated infections, especially in CF patients, it is urgent and necessary to explore novel strategies. *N*-acetylcysteine (NAC) has been introduced into clinical practice for the treatment of pulmonary and cardiovascular diseases, psychiatric disorders, infectious diseases, rheumatoid arthritis and plasma hyperlipoproteinemia, and has long been used for its mucolytic, antioxidant, and anti-inflammatory properties [9]. It is commonly administered in combination with antibiotics for the treatment of lower respiratory tract infections, especially in patients with chronic respiratory diseases characterized by abundant and/or intense mucus production. Additionally, an increasing amount of data has revealed that NAC may display antimicrobial and antibiofilm activity against various clinically important pathogens [10, 11]. Several in vitro studies have reported that NAC effectively inhibits the adhesion of bacteria to the epithelial cells and polymeric materials and also reduces the production of extracellular polysaccharide matrices while promoting the disruption of mature biofilms [12, 13]. It's mechanism of action is still not well known; however, the effect of NAC against biofilms can be analyzed under different aspects: (i) antibacterial properties; (ii) detachment of the biofilm; (iii) inhibiting bacterial adhesion and production of extracellular polysaccharides (EPS) [9].

However, there is a lack of data about the effects of NAC both alone, and in combination with antibiotics, against dual-species biofilms. In this study, for the first time, we examined the effects of NAC and conventional antibiotics against single- and dual-species biofilms of *P. aeruginosa* and *A. xylosoxidans*.

Materials and Methods

Strains and Culture Conditions

Pseudomonas aeruginosa PA01 and *A. xylosoxidans* ATCC 27061 strains were subcultured from freezer stocks onto tryptic soy agar (TSA, Difco Sparks, MD, USA) and incubated at 37 °C overnight.

Caenorhabditis elegans N2 (glp-4; sek-1) was propagated under standard conditions, synchronized by hypochlorite bleaching, and cultured on nematode growth medium using *E. coli* OP50 as a food source [14].

Antimicrobial Agents

NAC, amikacin (200 µg/ml), aztreonam (500 µg/ml), ciprofloxacin (150 µg/ml), and tobramycin (200 µg/ml) were kindly provided by their manufacturers. Increasing concentrations of NAC (1000, 2500, 5000 and 10,000 µg/ml) were used. The antibiotic concentrations were used partly based on the concentrations that can be reached in serum or sputum, according to previous studies [6, 15]. Stock solutions of antimicrobial agents from dry powders were prepared in water and stored frozen at − 80 °C, and frozen solutions were used within 6 months.

Single- and Dual-Species Biofilm Formation

Biofilm formation was performed as described previously [6, 15]. Briefly, loops of overnight cultures of bacteria were transferred to brain heart infusion broth (Thermo Scientific, Oxoid, USA) and incubated at 37 °C overnight in an orbital shaker (75 rpm). After incubation cultures were centrifuged (about 3000 rpm, 5–10 min), washed twice with sterile phosphate-buffered saline (PBS) and resuspended in BHIB to a cellular density equivalent to 1×10^6 and 1×10^7 colony-forming units/ml (cfu/ml) for single- and dual-species biofilm, respectively. Biofilms were formed by pipetting 100 µl of the standardized cell suspension into selected wells of sterilized polystyrene flat-bottomed 96-well tissue culture microtiter plates (Greiner Bio-One, Kremsmuenster, Austria) and incubating for 24 h at 37 °C. Control wells, containing bacteria alone or medium alone, were also included.

Biofilm Antimicrobial Assay and cfu Counts

After incubation, the waste medium was aspirated gently, and nonadherent cells were removed by washing the biofilms three times with sterile PBS. Antibiotics were added to the washed biofilms and plates were incubated. 5000 and 10,000 µg/ml final concentrations of NAC were used in experiments examining the combination of NAC and antibiotics. After 24 h of treatment, biofilms were washed with PBS, cells were collected by sonication and vortexing and bacterial viability was monitored by cfu assay as described previously [6, 15]. Serial dilutions were made in sterile PBS and plated onto Pseudomonas isolation agar (Sigma Aldrich, St. Louis) (for *P. aeruginosa* enumeration) and CHROMagar Orientation medium (ChromAgar, Paris, France) (for *A. xylosoxidans* enumeration) using the drop plate method, and plates were incubated at 37 °C. Following 24 h of growth, colonies were counted and expressed as the number of cfu/ml.

Acridine Orange-Propidium Iodide (AO-PI) Staining and Fluorescent Microscopy

Bacterial cell viability was evaluated using the AO-PI double fluorescence staining assay. To observe the cell death of single- and dual-biofilm of *P. aeruginosa* and *A. xylosoxidans* after antibiotic and NAC treatment separately and together. Bacteria were cultured in 24 well tissue culture plate (1×10^6 and 1×10^7 cfu/ml for single- and dual-species biofilm, respectively). After 24 h of incubation with antibiotics with/without NAC, cells were stained with 1 µl of aqueous Acridine orange (AO)/Propidium iodide (PI) solution (1 mg/ml of AO in PBS; 1 mg/ml of PI in PBS) that were mixed in 1 ml PBS buffer for 5 min in dark. After AO/PI staining, cells were washed with PBS and changes in cells were observed under an inverted fluorescent microscope (Zeiss, Axio Observer Z1) [15, 16]. Quantitative measures of pixels were obtained by ImageJ software with live&dead quantification tool.

Caenorhabditis elegans Survival Assay

For the *Caenorhabditis elegans* survival assay, synchronized worms (L4 stage) were suspended in OGM medium (95% M9 buffer, 5% brain heart infusion broth, 10 µg/ml cholesterol) and added into 96-well plates with at least 20 worms per well [14]. Nematodes were infected with 25 µl of overnight culture (*P. aeruginosa* and *A. xylosoxidans*) adjusted to 2×10^9 CFU/ml in OGM medium and exposed to 25 µl of treatment [NAC alone (10 000 µg/ml, and in combination with tobramycin (200 µg/ml) or ciprofloxacin (150 µg/ml)]. Cytotoxicity wells with only antimicrobial agents and nematodes were added to the experiments. Uninfected nematodes in the OGM medium as well as infected but untreated nematodes were used as controls. The assay plates were incubated at 25 °C for up to 2 days and the number of viable and dead nematodes was assessed every 24 h. The fraction of dead worms was determined by counting the number of dead worms and the total number of worms in each well [14, 17].

Statistical Analysis

All experiments were performed in triplicate in two separate sets of experiments. All data were expressed as the mean ± standard deviations of two independent experiments. One-way ANOVA and Bonferroni's Multiple Comparison tests were used to compare the differences between single and dual species biofilms and P value of <0.05 was considered statistically significant.

Results

Effects of Antibiotics on Bacteria in Single- and Dual-Species Biofilms

Tobramycin and ciprofloxacin were found to be effective against *P. aeruginosa* and *A. xylosoxidans* in both single- and dual-species biofilms, whereas aztreonam showed the least antibiofilm activity. Amikacin also showed antibacterial activity against *P. aeruginosa* biofilms (Fig. 1).

Effects of NAC on Bacteria in Single- and Dual-Species Biofilms

The effects of increasing concentrations of NAC (1000, 2500, 5000 and 10,000 µg/ml) against single and dual-species biofilms were also investigated (data not shown). According to the results, NAC caused a 1-log reduction of both bacteria at different concentrations, except when at a concentration of 10,000 µg/ml against *A. xylosoxidans* in dual-species biofilms (Fig. 1).

Effects of Antibiotics and NAC Combinations Against Single and Dual-Species Biofilms

The susceptibility of the biofilms to the combinations of NAC (5000 and 10,000 µg/ml) with antibiotics was also investigated. There was no increase in antimicrobial activity seen against single-species *A. xylosoxidans* biofilms, except for NAC (5000 µg/ml) and aztreonam combination (Fig. 1a). The combination of NAC (5000 µg/ml) with ciprofloxacin, and the combination of NAC (5000 µg/ml) with tobramycin decreased the number of bacteria by more than 1-log in dual-species biofilms of *P. aeruginosa* and *A. xylosoxidans* (Fig. 1a and b). Surprisingly, there was no increase in antimicrobial activity with increasing concentration of NAC, against either single- or dual-species biofilms.

Effects of Antibiotics and NAC Combinations Against Single- and Dual-Species Biofilm on Cell Death

A live/dead cell viability assay was applied to evaluate the antimicrobial activities of ciprofloxacin, tobramycin and amikacin separately, and combined with NAC against single- and dual-species biofilms of *A. xylosoxidans* and *P. aeruginosa*. As shown in Fig. 2, the control groups (single- and dual-species biofilms of *A. xylosoxidans* and *P. aeruginosa*) showed green fluorescence, indicating that the bacteria were still alive. In contrast, after being treated with ciprofloxacin, tobramycin and amikacin, most of the bacteria showed red fluorescence, suggesting that most of the bacteria were

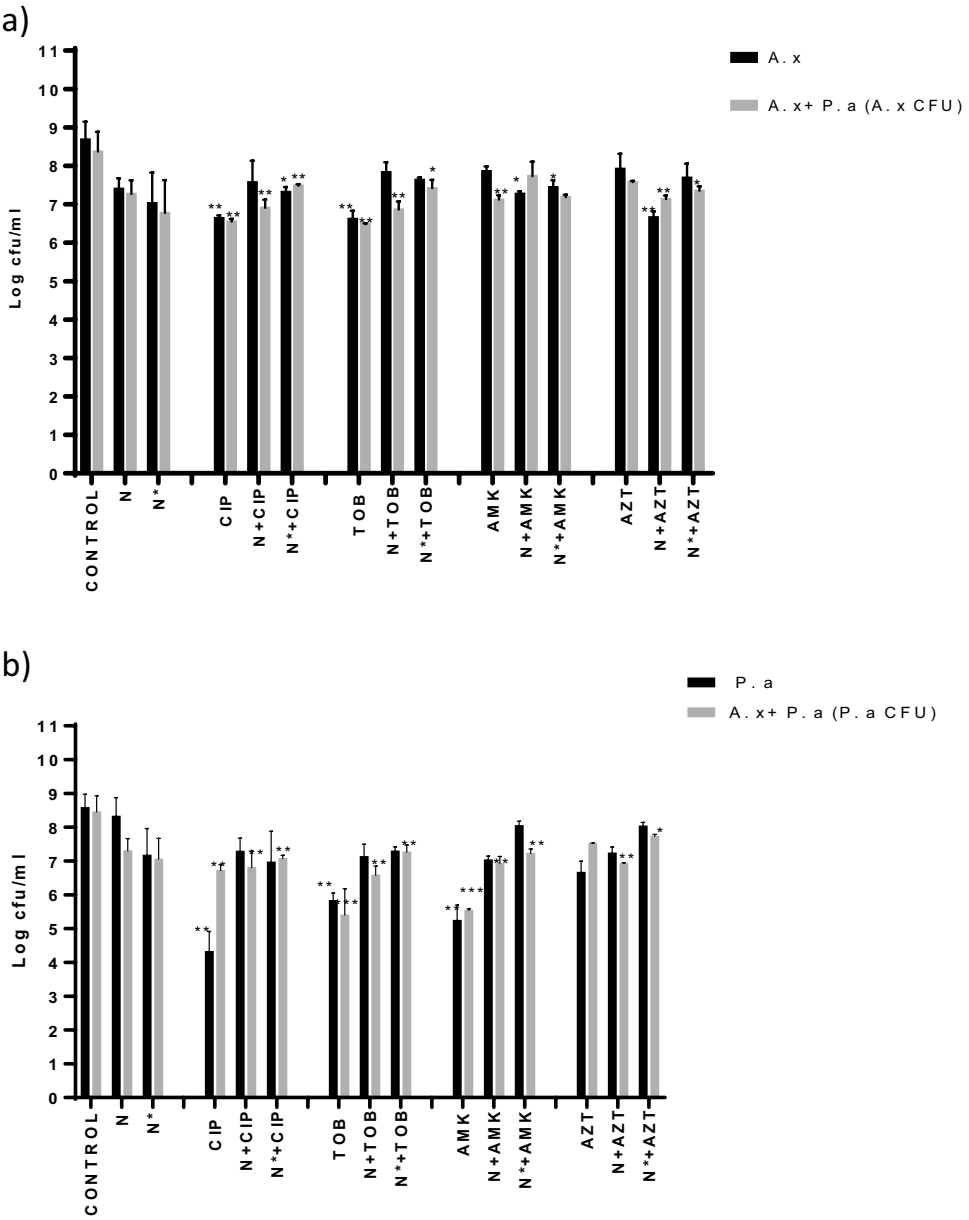

Fig. 1 Effects of antibiotics, NAC, and their combinations against single- and dual-species biofilms. Each experiment is representative of at least two independent tests and average number of cfu of the microorganisms recovered from biofilms was shown; the error bars indicate the standard deviations. **a** Effects of antibiotics and NAC combinations on *A. xylosoxidans* cells in single- and dual-species biofilms. Dual biofilms contain *P. aeruginosa* and *A. Xylosoxidans*. **b** Effects of antibiotics and NAC combinations on *Pseudomonas aeruginosa* cells in single- and dual-species biofilms. Dual biofilms contain *P. aeruginosa* and *A. xylosoxidans*. *A.x. Achromobacter xylosoxidans P.a. Pseudomonas aeruginosa*. *N N*-acetylcysteine (5000 µg/ml), *N** *N*-acetylcysteine (10,000 µg/ml) *CIP* ciprofloxacin, *TOB* tobramycin, *AMK* amikacin, *AZT* aztreonam.*, **It was found to be statistically significantly (*$P \leq 0.05$, **$P \leq 0.01$ vs control, ANOVA test followed by Bonferroni's multiple comparison tests)

killed. Furthermore, in response to the treatment of the combination of antibiotics with NAC, the proportion of dead cells increased in single- and dual-species biofilms of *A. xylosoxidans* and *P. aeruginosa*, in comparison to the response to the treatments of antibiotics and NAC separately (Fig. 2p).

Effect of the NAC Alone and in Antibiotic Combinations on Survival of Infected *Caenorhabditis elegans*

The in vivo effect of NAC alone, and in combination with tobramycin or ciprofloxacin, was evaluated by using a *C. elegans* model system. Infection of *C. elegans* with *P. aeruginosa*, *A. xylosoxidans* and dual species (*P. aeruginosa* + *A. xylosoxidans*) decreased the number of living nematodes after 24 h (Fig. 3).

Increased survival was observed after NAC and antibiotic treatment of *C. elegans* nematode infected with microorganisms compared to no treatment. The highest survival was observed at NAC and ciprofloxacin combinations. NAC and ciprofloxacin combinations were also found to be non-toxic to *C. elegans* (data not shown). However, no live nematodes were found in any of the wells at 48 h, except in uninfected controls.

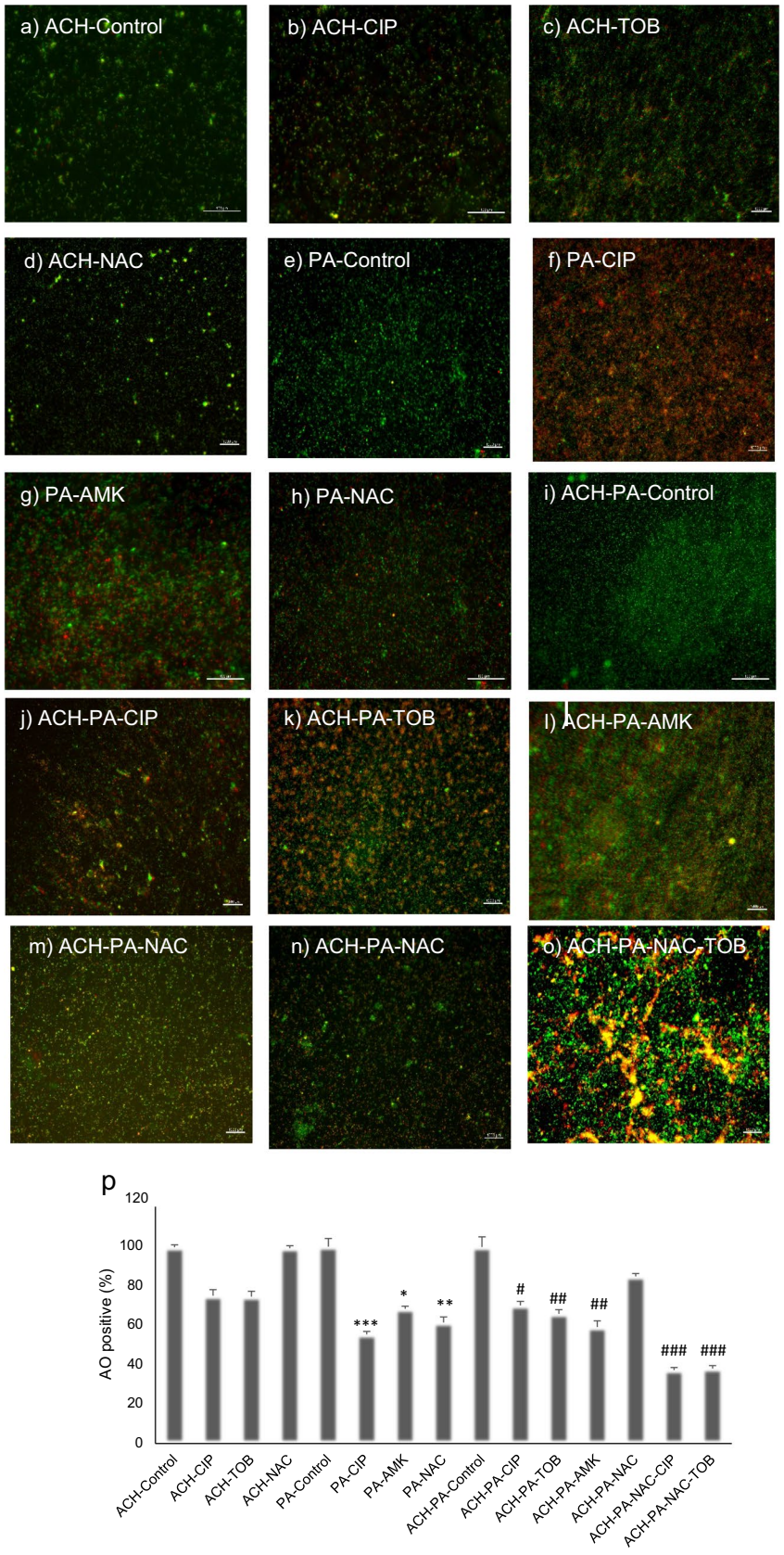

Fig. 2 Acridine orange/Propidium iodide (AO/PI) dual staining. Fluorescence microscopic images of single- and dual-species biofilms of *A. xylosoxidans* and *P. aeruginosa*, the live bacteria stained by AO displayed green and the dead bacteria dyed by PI exhibited red fluorescence (Magnification, ×200). **a**–**d** Images of single-species *A. xylosoxidans* biofilms **a** control **b** treated with ciprofloxacin **c** treated with tobramycin **d** treated with NAC, **e–h** Images of single *P. aeruginosa* biofilms **e** control **f** treated with ciprofloxacin **g** treated with amikacin **h** treated with NAC. **i–o** Images of dual-species biofilms of *P. aeruginosa* and *A. xylosoxidans* **i** control **j** treated with ciprofloxacin **k** treated with tobramycin **l** treated with amikacin **m** treated with NAC **n** treated with NAC and ciprofloxacin combinations **o** treated with NAC and tobramycin combinations, (NAC concentration, 10,000 µg/ml). **p** Graph showing percentage of live cell fluorescence intensity calculated by ImageJ software and statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Values are expressed as mean ± SEM ($n = 3$). Significant differences compared to PA control = *. Significant differences compared to ACH-PA control = #. *, #$P < 0.05$, **, ##$P < 0.01$, ***, ###$P < 0.001$

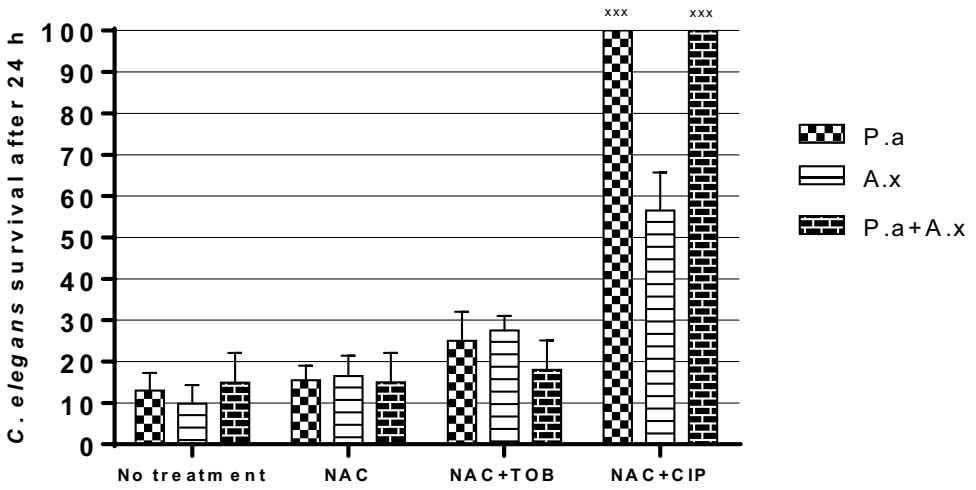

Fig. 3 Percent survival of infected *C. elegans* (average ± SD) after treatment with NAC alone, and in combination with tobramycin or ciprofloxacin. Compounds were tested two times in each assay, and each assay was repeated at least three times. The results are expressed as the percent survival after 24 h of infection and treatment was shown. Error bars indicate the standard deviations. *P.a Pseudomonas aeruginosa*. *A.x. Achromobacter xylosoxidans, P.a + A.x* Dual species of *P. aeruginosa* and *A. xylosoxidans. NAC N*-acetylcysteine, *TOB* tobramycin, *CIP* ciprofloxacin. ***It was found to be statistically significantly (***$P \leq 0.05$, vs no treatment, ANOVA test followed by Bonferroni's multiple comparison tests)

Discussion

Microorganisms attach to a surface and develop biofilms; these communities play a significant role in the persistence of bacterial infections. Bacteria within a biofilm can be 1000 times more resistant to antibiotics than planktonic bacteria—and unfortunately, no drugs that specifically target bacterial biofilms are in clinical use [12, 18]. While research on the activity of antibiotics against biofilms mainly focuses on single-species biofilms, many biofilm-related infections are associated with multiple species [6].

It has been well established that infections of CF lung airways are caused by polymicrobial infections, although the composition and diversity of these infections may change throughout the patient's lifetime [5]. In addition, patients colonized by *A. xylosoxidans* are frequently co-colonized by *P. aeruginosa* [19]. Therefore, in the present study, we investigated the effects of the conventional antibiotics' amikacin, aztreonam, ciprofloxacin, and tobramycin, both separately, and in combinations with mucolytic agent NAC, against single- and dual-species biofilms of *P. aeruginosa* and *A. xylosoxidans*.

According to our results, *P. aeruginosa* and *A. xylosoxidans* do not affect each other in dual-species biofilms. In accordance with these results, a study conducted by Vandeplassche et al. found that *P. aeruginosa* and *A. xylosoxidans* in multi-species biofilms reached the same density as in their single-species biofilms [4].

In our study, tobramycin and cipofloxacin were found to be the most effective against both single and dual-species biofilms of *P. aeruginosa* and *A. xylosoxidans*, while aztreonam showed the least antibiofilm activity. Increased survival was also observed in combination with tobramycin or ciprofloxacin treatment of *C. elegans* infected with *P. aeruginosa*, *A. xylosoxidans* and dual-species (*P. aeruginosa* + *A. xylosoxidans*) compared to no treatment or NAC alone. The highest nematode survival was observed in NAC and ciprofloxacin combinations. According to a limited number of studies, it has also been shown that ciprofloxacin is effective against single- and dual-species biofilms of *P. aeruginosa* and *A. xylosoxidans* [4, 20].

It has been demonstrated that the classical mucolytic drug, NAC, has antibacterial effects against several microorganisms. Additionally, previous studies have shown that NAC inhibits biofilm formation and disrupts mature biofilms of various bacteria, such as *P. aeruginosa* [21], *Acinetobacter baumannii* [22], *Escherichia coli* [12], *Burkholderia cenocepacia* [23], *S. aureus* [12], *Staphylococcus epidermidis* [24], etc. To the best of our knowledge, our study is the first to evaluate the antibiofilm activity of NAC combinations with amikacin, aztreonam, ciprofloxacin, and tobramycin against *P. aeruginosa* and *A. xylosoxidans* single- and dual-species biofilms. We showed that NAC reduced both single and dual-species biofilms of *P. aeruginosa* and *A. xylosoxidans* by at least 1-log, most at the concentration of 10,000 µg/ml. Additionally, there was no increase in antimicrobial activity observed with increasing concentrations of NAC, against either single- or dual-species biofilms of bacteria.

According to El-Feky et al. showed that NAC alone, or in combination with ciprofloxacin, inhibited biofilm production and disrupted preformed biofilms against various bacteria, including *P. aeruginosa* [12]. Zhao and Liu also investigated the inhibitory effects of NAC on biofilms produced by *P. aeruginosa*. They found that NAC could detach mature biofilms and that the combination of NAC and ciprofloxacin could significantly kill *P. aeruginosa* in biofilms [21]. Many other researchers have also shown that NAC is effective against biofilms, both alone and in combination with antibiotics, against different microorganisms [22–24]. However, no previous studies investigated NAC combinations with amikacin, aztreonam, and tobramycin against *P. aeruginosa,* and of course against *A. xylosoxidans*, have been published.

On the other hand, some researchers have shown that the combinations of NAC and various antibiotics do not increase the effects of antibiotics such as erythromycin [25]. Conversely, the combination with NAC affects the chemical instability of antibiotics such as imipenem, meropenem and ertapenem [26] and may also reduce the activities of antibiotics such as imipenem [27]; gentamicin and tobramycin [28]; ciprofloxacin and ofloxacin; streptomycin; kanamycin; spectinomycin; and erythromycin [29].

In our study, we did not see any effect other than a maximum 2-log reduction from combinations of NAC and aztreonam against single-species *A. xylosoxidans* biofilms and combinations of NAC and ciprofloxacin or tobramycin against dual-species biofilms of *P. aeruginosa* and *A. xylosoxidans*. In fact, antibiotics (except aztreonam) were found to be more effective against biofilms when used alone rather than in combination with NAC. Differences in the methodology or the NAC concentrations used in this study could be the reason our results differ from those of previous studies.

In the present study, immunofluorescence results also showed that ciprofloxacin and tobramycin,—with or without NAC—are the most effective at reducing the viability of both single- and dual-species biofilms of *P. aeruginosa* and *A. xylosoxidans.* Furthermore, these results not only confirmed the data we obtained from other experiments, but also correlated with them.

To our knowledge, this is the first study that investigates NAC in combination with conventional antibiotics against *P. aeruginosa* and *A. xylosoxidans* in single- and dual-species biofilms.

Conclusion

Consequently, among the antibiotics, tobramycin and ciprofloxacin were found to be the most effective against both single- and dual-species biofilms formed by the major pathogen in the cystic fibrosis population, *P. aeruginosa*, and the bacteria newly emerging worldwide, *A. xylosoxidans*. However, NAC showed little effect on both single- and dual-species biofilms of *P. aeruginosa* and *A. xylosoxidans*, even when in combination with antibiotics. Nevertheless, further investigations are needed to better understand the effects of NAC and conventional antibiotics against single- and dual-species biofilms.

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Data Availability Data and materials are available on request.

Code Availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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