Folia Microbiologica
https://doi.org/10.1007/s12223-026-01569-0

RESEARCH

Evaluation of the antimicrobial and antibiofilm activities of aztreonam-avibactam against the *Burkholderia cepacia* complex

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Received: 25 April 2026 / Accepted: 29 July 2026
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Abstract

Beta-lactamase production by *Burkholderia cepacia* complex (Bcc) leads to beta-lactam resistance and worsens patient prognosis. This study describes the activity of aztreonam, a monobactam, in combination with avibactam, a beta-lactamase inhibitor, against Bcc isolates. Broth microdilution and time-kill assays were conducted for planktonic cells. Additionally, broth microdilution was repeated using media supplemented with 5% and 10% bovine serum albumin to evaluate its impact on MICs. The effects of aztreonam/avibactam on biofilm formation and the metabolic activity of mature biofilms were determined in all biofilm-positive isolates. Additionally, the biofilm matrix of selected strains was analyzed in terms of viable bacterial count, total protein content, and extracellular DNA (eDNA) concentration, and the biofilm architecture was visualized using scanning electron microscopy (SEM). All the strains were determined to be resistant to aztreonam, and the MIC_{50} and MIC_{90} of aztreonam/avibactam were found to be 4/4 mg/L and 8/4 mg/L, respectively. Aztreonam/avibactam exhibited activity against planktonic form of Bcc, and decreased the aztreonam MIC values. Serum albumin activity was insignificant at low doses but affected the MIC more at high doses. Moderate to strong biofilm formation was observed in 25 of the 29 isolates, and aztreonam/avibactam showed strain- and concentration-dependent activity against Bcc biofilm formation and mature biofilms. The combination also reduced biofilm matrix components such as total protein and eDNA, and some strain-specific effects were observed at sub-inhibitory concentrations. These findings support the potential use of aztreonam/avibactam as a therapeutic option against Bcc infections by targeting both planktonic bacteria and key components of biofilm matrix.

Keywords *Burkholderia cepacia* complex · Aztreonam/avibactam · Biofilm

Introduction

The *Burkholderia cepacia* complex (Bcc) is an opportunistic pathogen found in various environments, including healthcare settings (Shaban et al. 2020). It primarily causes infections in patients with chronic or critical conditions and immunocompromised individuals, leading to severe infections such as meningitis, pneumonia, bloodstream infections, and urinary tract infections (Rhodes and Schweizer 2016; Kenna et al. 2017). Recent outbreak reports indicate a rising trend in healthcare-associated infections due to Bcc since January 2020, often linked to contaminated disinfectants, ultrasound gels, and medical products. These outbreaks highlight the global health burden posed by Bcc, especially in vulnerable patient populations (Shaban et al. 2020).

Compared with other non-fermentative Gram negative bacteria, Bcc exhibits remarkable intrinsic resistance to

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Published online: 10 August 2026

multiple antimicrobial classes, including cephalosporins, penicillins, aminoglycosides, polymyxins, and tetracyclines. However, some agents, such as ceftazidime, cefepime, imipenem, meropenem, and minocycline, may retain activity against specific Bcc isolates (Rhodes and Schweizer 2016; EUCAST 2023). The intrinsic multidrug resistance of Bcc is mediated by multiple mechanisms, including low outer membrane permeability, β-lactamase production, efflux pumps, and target modifications (Rhodes and Schweizer 2016; Gautam et al. 2020). Owing to its intrinsic resistance to numerous antibiotics, treatment options for Bcc are significantly limited. Given the considerable importance of managing Bcc infections, there is a lack of information about their microbiological epidemiology and clinical characteristics (Ibrahim et al. 2024).

Aztreonam is a member of the monobactam class antibiotics. Although aztreonam is more stable for hydrolysis by metallo-beta-lactamases (MBLs) than other beta-lactams are, most clinically relevant serine lactamases (ESBLs, *Klebsiella pneumoniae* carbapenemases (KPCs), AmpC β-lactamases) can also hydrolyze it (Ramsey and MacGowan 2016). Avibactam protects aztreonam from hydrolysis by serine β-lactamases. The aztreonam-avibactam combination can effectively inhibit serine lactamases and shows promise in combating multidrug-resistant bacterial pathogens (Sader et al. 2024b).

Bacterial biofilms are ecosystems consisting of microbial communities that adhere to a variety of living or nonliving surfaces and are surrounded by extracellular polymeric substances secreted by the bacteria (Sharma and Rajpurohit 2024). Bcc can evade host immune responses through biofilm formation and exhibit high levels of antibiotic resistance. These biofilms play an important role in the persistence and chronicity of Bcc-associated infections, particularly in individuals with cystic fibrosis, where they contribute to long-term colonization and progressive lung damage. Biofilm-associated growth not only protects bacterial cells from antibiotics and phagocytosis, but also facilitates adaptation to hostile environments (Murphy and Caraher 2015). The structural and functional integrity of Bcc biofilms depends on the secretion of extracellular polymeric substances (EPS) that form the biofilm matrix. This matrix is composed primarily of polysaccharides, proteins, and extracellular DNA (eDNA), all of which are critical for maintaining biofilm architecture, surface adhesion, and intercellular communication. Recent studies have highlighted the importance of anti-biofilm strategies, such as destroying DNA or inhibiting EPS biosynthesis, in the management of chronic Bcc infections (Messiaen et al. 2014).

Despite the growing concern over multidrug-resistant Bcc infections, particularly in cystic fibrosis and immunocompromised patients, there is a notable lack of data regarding the potential efficacy of aztreonam-avibactam against these pathogens. In this study, we aimed to address this gap by evaluating planktonic and biofilm-associated susceptibilities across diverse clinical isolates.

Materials and methods

Bacterial isolates

Twenty-nine clinical Bcc isolates were obtained from the bacterial archive of the Department of Medical Microbiology, Marmara University, Istanbul, Türkiye. These isolates were recovered initially from various clinical specimens, including endotracheal aspirates, sputum, and blood, between 2019 and 2023 (Supp. file 1). Ethical approval was obtained from the Ethics Committee of Marmara University, Institute of Health Sciences, Türkiye (19.11.2024-106). All the isolates were thawed from frozen stocks and reidentified using MALDI-TOF MS. The reference strain *Pseudomonas aeruginosa* ATCC® 27853 was used in this study as a control.

Antimicrobial agents and media

All antibiotics used in this study, including aztreonam and avibactam, were purchased from Sigma Aldrich®. Stock solutions of antibiotics were prepared from dry powders by the suppliers' guidelines and stored at -80 °C for a maximum period of six months. Cation-adjusted Mueller–Hinton broth (CAMHB) was used for the minimum inhibitory concentrations (MICs) determination and time-kill assays, whereas Tryptic Soy Broth with 1% glucose (g-TSB) was used for biofilm assays. The bovine serum was obtained from A.B.T.™, diluted with CAMHB, and subsequently filtered through a 0.22-micrometer pore-size filter. Bovine serum albumin was dissolved in CAMHB at 50 and 100 mg/mL (5% and 10%, respectively).

MIC determination

MICs of antibiotics were determined using the broth microdilution technique outlined by the EUCAST against 29 Bcc isolates (EUCAST 2024). Serial dilutions of aztreonam were prepared in 96 well U-bottom microplates, and avibactam was added to all wells at a fixed final concentration of 4 mg/L. Bacterial suspensions were prepared from overnight cultures and adjusted to 0.5 McFarland standard prior to dilution. Bacterial suspensions were inoculated at a final concentration of 5×10^5 CFU/mL and the microplates were incubated at 37 °C for 18–24 h. The lowest concentration

that stops the visible growth of bacteria was considered the MIC.

To evaluate the effect of serum albumin on MIC values, Bcc bacterial suspensions were prepared in CAMHB supplemented with serum albumin to achieve a final concentration of either 5% or 10% serum albumin (Keepers et al. 2014).

All the experiments were conducted triplicate as independent biological replicates.

Time-kill experiments

Six Bcc isolates were selected for time-kill experiments according to their MIC results. Time-kill experiments were conducted in three independent biological replicates. Briefly, bacterial suspensions were adjusted to achieve an initial inoculum of approximately 10^6 CFU/mL. Aztreonam at 0.5×MIC (half of the MIC), 1×MIC, 2×MIC (twice the MIC), and 4×MIC (four times the MIC) in combination with a fixed final concentration of 4 mg/L avibactam was added to the bacterial inoculum in tubes. Tubes were incubated at 37 °C with shaking. Samples were collected at 0, 2, 4, 6, and 24 h, serially diluted, and plated on TSA for viable cell counting. Colonies were counted, and time–kill curves were generated using GraphPad Prism version 8.0 (San Diego, CA, USA). The lower detection limit (DL) was determined to be 1 $\log_{10}$ CFU/mL. The bactericidal effects were determined by a 3-log reduction in the initial inoculum, according to NCCLS guidelines (NCCLS 1999).

Determination of biofilm formation

The biofilm formation abilities of the Bcc isolates were determined by a crystal violet assay (O'Toole 2011). Inocula (10^6 CFU/mL-200 µL) were added to 6 wells for each isolate. After overnight incubation, biofilms were stained with crystal violet, and the bound dye was quantified at OD570 using a microplate reader. The negative control (c: media only) and experimental groups were compared, and the results were evaluated as follows (Xu et al. 2016): OD ≤ ODc: non-biofilm producer; ODc < OD ≤ 2xODc: Weak biofilm producer; 2xODc < OD ≤ 4xODc: Moderate biofilm producer; 4xODc < OD: Strong biofilm producer. The assay was performed in three independent biological replicates, with six technical wells for each isolate in each experiment.

Effect of aztreonam/avibactam on biofilm attachment and formation

Assays to determine the inhibition of biofilm attachment and formation were conducted according to previous procedures, with the following modifications (Overhage et al. 2008; Damar-Celik et al. 2021). A biofilm-producing overnight culture of Bcc clinical strains was diluted to provide 1×10^7 CFU/200 µL in 1% g-TSB. All Bcc strains were exposed to aztreonam at concentrations corresponding to the MIC, one-fifth of the MIC, and one-tenth of the MIC based on the MIC values of the aztreonam/avibactam combination. Avibactam was added to all wells at a fixed final concentration of 4 mg/L. The plates were incubated for 0, 2, 4, 6, and 24 h at 37 °C. The positive control was Bcc strains in 1% g-TSB alone. After incubation, the wells were washed with PBS three times. Crystal violet was used to dye the biofilm. The absorbance was measured at OD_{570}. The inhibition of biofilm attachment and formation was calculated by considering 100% biofilm formation as the positive control. The assay was performed in three independent biological replicates, with three technical wells for each isolate in each experiment.

Effect of aztreonam/avibactam on mature biofilms

The effects of the antibiotic on the metabolic activity of mature Bcc biofilms were evaluated according to a previously described procedure, with modifications (Oyardi et al. 2021). Aztreonam/avibactam was tested for antibiofilm activity against 25 moderate and strong biofilm-producing Bcc isolates. The mature biofilms (24 h) in the microtiter plates were gently washed with 200 µL of PBS three times. Aztreonam concentrations were prepared in g-TSB to obtain final concentrations of the MIC, twice the MIC, and four times the MIC based on the MIC values of the aztreonam/avibactam combination. The avibactam concentration was fixed at 4 mg/L in each well. Two hundred microliters of antibiotics were added to each well, and the plates were incubated at 37 °C for 24 h. After 24 h of treatment at 37 °C, biofilm metabolic activity was quantified by MTT reduction, and absorbance was measured at OD_{595} using a microplate reader (SpectraMax i3x). The percentage reduction in metabolic activity of mature biofilms was calculated relative to the untreated control group, which was considered as 100% metabolic activity. The assay was performed in three independent biological replicates, with three technical wells for each isolate in each experiment.

CFU/ml determination in biofilm

The impact of aztreonam/avibactam on biofilm formation was also assessed by quantifying viable bacterial cells within the biofilm structure (Hacioglu et al. 2020). Four Bcc strains (strain numbers: 20, 22, 23, and 24) were selected based on preliminary screening results, where two strains exhibited lower susceptibility and two strains

exhibited higher susceptibility to aztreonam/avibactam in terms of biofilm inhibition. Bacterial suspensions (10^6 CFU/mL) prepared in 1% TSB were treated with aztreonam/avibactam at MIC, 1/5×MIC, and 1/10×MIC concentrations, while untreated wells served as controls. After 24 h incubation at 37 °C, biofilm-associated cells were recovered by PBS washing and biofilm disruption using sonication in an ultrasonic water bath (Branson®, 40 kHz). Serial dilutions were plated onto TSA, and viable cell counts were determined after incubation at 37 °C for 24 h. The experiment was independently repeated three times.

Quantification of biofilm matrix proteins

To quantify the protein content in the extracellular matrix of biofilms disrupted during the CFU assay, the Bradford protein assay (Thermo Scientific®) was performed (Nguyen et al. 2021). Biofilm suspensions obtained after ultrasonication were transferred into microcentrifuge tubes and centrifuged at 4000 rpm for 10 min to pellet the bacterial cells. The resulting supernatants, containing extracellular matrix components, were carefully collected and filtered through 0.22 µm filters to remove any remaining cellular debris. Biofilm suspensions obtained after disruption of mature biofilms were centrifuged, and the supernatants were collected for protein analysis. Protein concentrations were determined using a BSA standard curve, and absorbance was measured at 595 nm using a microplate reader. The assay was performed in three independent biological replicates.

Quantification of extracellular DNA (eDNA) in biofilm

The effect of different concentrations of aztreonam/avibactam on the amount of extracellular DNA (eDNA) in the biofilm matrix of Bcc strains was assessed (Nguyen et al. 2021). DNA was extracted from the biofilm matrix supernatants obtained during the CFU assay using a commercial DNA purification kit (GeneMATRIX Tissue & Bacterial DNA Purification Kit, EURx) according to the manufacturer's instructions. The extracted DNA concentration was quantified using a NanoDrop™ spectrophotometer (Thermo Scientific) by measuring absorbance at 260 nm. The assay was performed in three independent biological replicates.

SEM analysis of biofilm structure

SEM was employed to visualize the biofilm architecture of Bcc isolate and to assess the structural effects of aztreonam/avibactam at MIC concentration. For this purpose, 2 mL of bacterial suspension was added onto sterile glass coverslips placed in 6-well cell culture plates. Samples were incubated at 37 °C for 24 h under static conditions, either in the presence of aztreonam/avibactam at MIC or without antibiotic (control group). Biofilms were fixed by adding 2.5% glutaraldehyde and incubated at 4 °C for 20 min. After fixation, samples were washed three times with cold PBS and dehydrated by sequential passage through a graded ethanol series (30%, 50%, 70%, 90%, and 100%). To complete the dehydration process, hexamethyldisilazane (HMDS) was applied to each coverslip and allowed to air-dry for 15 min. Dried samples were sputter-coated with a thin layer of gold and examined using Hitachi brand High-Tech SU8700 model Field Emission Scanning Electron Microscope (FE-SEM; the Central Laboratory of Middle East Technical University (METU/ODTÜ)).

Results

Broth microdilution results

EUCAST stated that it is not possible to determine antibiotic MIC breakpoints against Bcc and that susceptibility tests are not reliable. However, it recommends that the broth microdilution method should be used as the most reliable method. Therefore, clinical cutoff values for the Bcc have not been published by the EUCAST. Accordingly, the percentages of resistance to aztreonam and aztreonam/avibactam were not determined in this study. The Bcc isolates tested had MIC values of 32 to >32 mg/L against aztreonam, whereas aztreonam/avibactam had MIC values in the range of 1/4–16/4 mg/L when avibactam was added at a fixed concentration of 4 mg/L (Table 1). To summarize, the MIC_{50} and MIC_{90} values for aztreonam were determined to be >32 mg/L, while

Table 1 MIC results (mg/L) of antibiotics with or without BSA

	Aztreonam	Aztreonam/Avibactam	Aztreonam/Avibactam + 5% BSA	Aztreonam/Avibactam + 10% BSA
MIC Range	≥32	1/4–16/4	4/4–32/4	4/4–32/4
MIC_{50}	>32	4/4	8/4	32/4
MIC_{90}	>32	8/4	16/4	32/4

these values for aztreonam/avibactam were determined to be 4/4 mg/L and 8/4 mg/L, respectively. The results revealed that avibactam significantly decreased the MIC of aztreonam.

When 5% BSA was added to aztreonam/avibactam, the MIC_{50} and MIC_{90} values were doubled. When 10% BSA was added, both the MIC_{50} and MIC_{90} values were 32/4 mg/L. In particular, high-dose BSA can affect MIC values.

Results of the time-kill experiment

The time-dependent killing test results revealed that aztreonam/avibactam activity varies depending on the strain. None of the concentrations tested at 2 h caused a decrease in the number of bacteria. MIC values generally prevented the increase in the initial bacterial count at all hours tested. The sub-MIC values did not significantly differ from those of the control group. The 4×MIC values showed at least a 3-log reduction compared with the initial bacterial count for strains 1 and 6 at the end of 24 h. However, only strain 1 exhibited a 3-log reduction at 2×MIC (Fig. 1).

DL: detection limit.

Biofilm experiment results

When the biofilm formation potential of the Bcc isolates was evaluated, two isolates were found to be biofilm negative, and two isolates were weakly biofilm positive. Nine of them were evaluated as moderate, and 16 of them were evaluated as strongly biofilm positive compared with the negative control (Supp. file 1). Biofilm studies

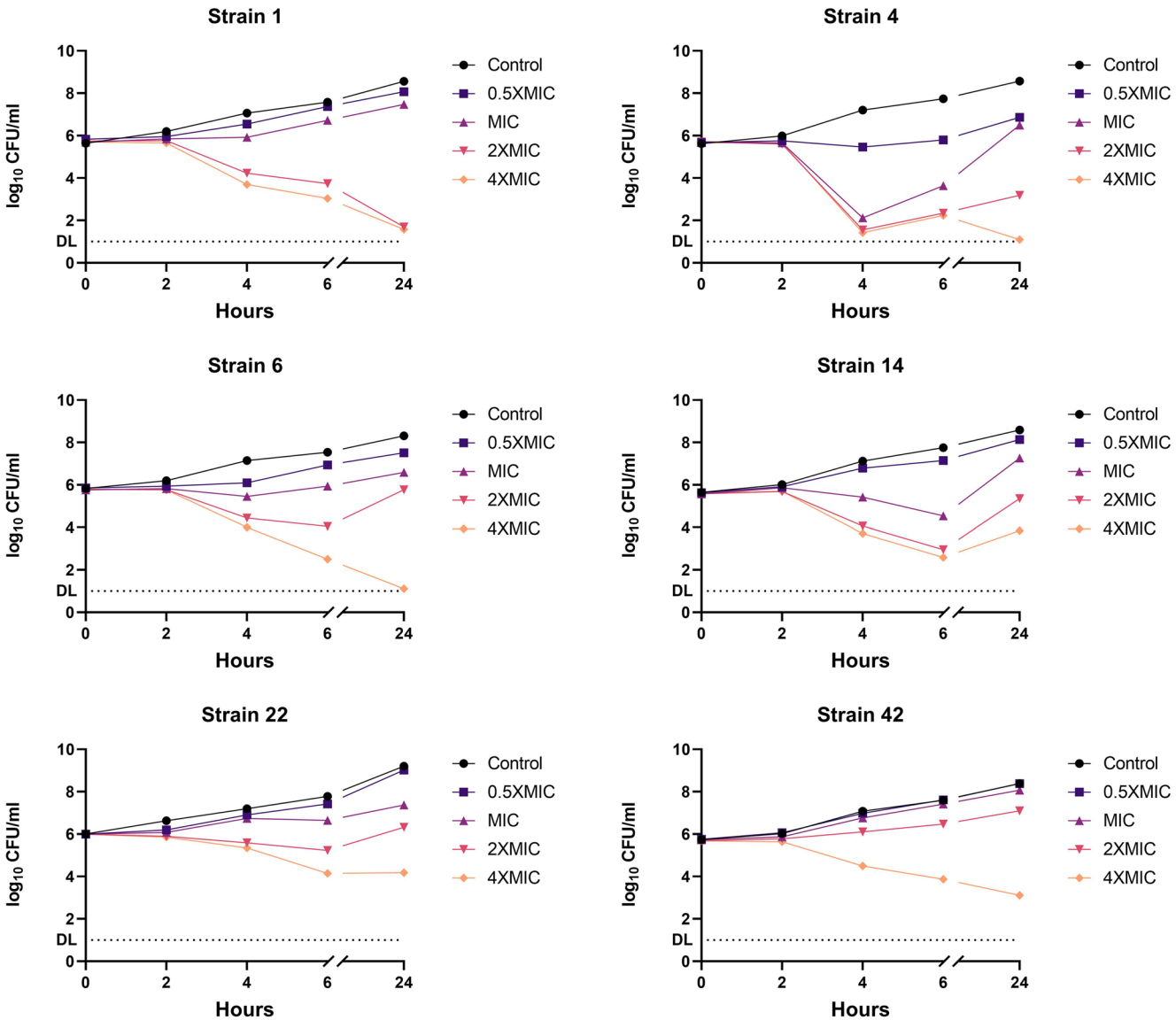


Fig. 1 Survival curves of Bcc isolates treated with aztreonam/avibactam over time

were continued with moderate and strong biofilm-positive isolates.

The biofilm inhibition potential of aztreonam/avibactam against biofilm-positive Bcc strains was investigated at the MIC and sub-MIC values (one-fifth of the MIC and one-tenth of the MIC). As presented in (Fig. 2), the values represent the percentage of biofilm inhibition compared to the untreated control group. Positive percentages indicate inhibition of biofilm formation, while negative percentages reflect an increase in biofilm biomass relative to the control. For instance, a value of + 50% represents 50% inhibition, whereas − 20% indicates that biofilm formation was 20% higher than the control under the given condition. As expected, biofilm inhibition varied depending on the strain. The biofilms were more inhibited as time progressed. Among the 25 isolates, the MIC

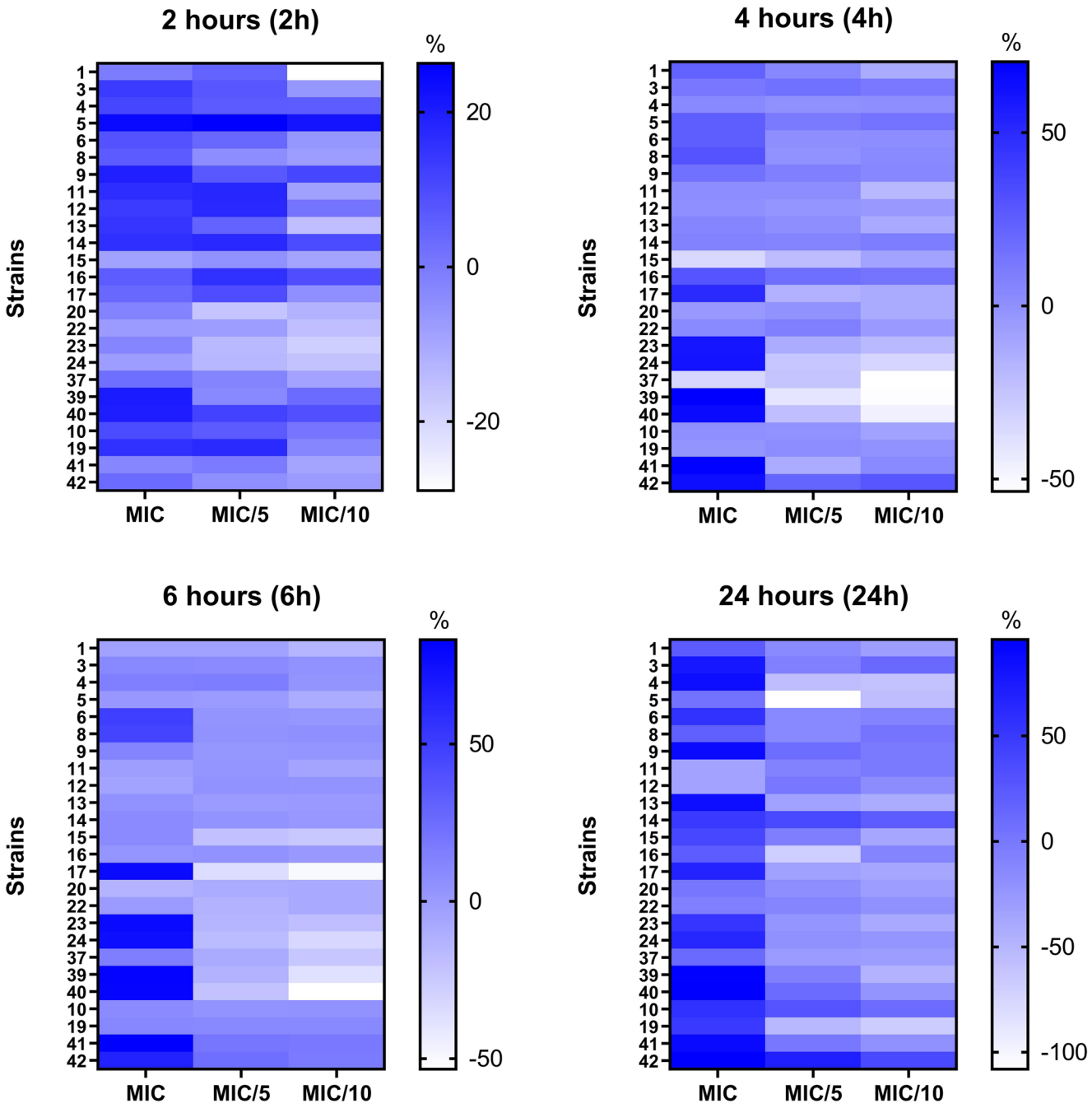


Fig. 2 Heat maps showing the biofilm inhibition percentages of Bcc strains treated with aztreonam/avibactam at MIC, one-fifth MIC, and one-tenth MIC at 2, 4, 6, and 24 h Positive values indicate inhibition compared to the untreated control, while negative values represent increased biofilm formation

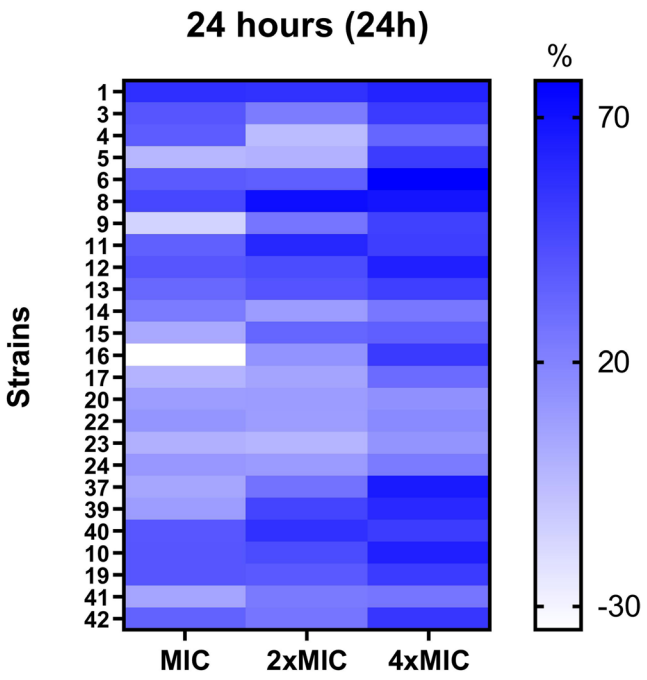


Fig. 3 Heat map showing the percentage reduction in metabolic activity of 24-hour mature Bcc biofilms treated with aztreonam/avibactam at MIC, twice the MIC, and four times the MIC

value of aztreonam/avibactam decreased biofilm formation by more than 50% at the end of 24 h for 13 isolates. Although aztreonam/avibactam generally reduced biofilm formation, increased biofilm biomass was observed at some sub-inhibitory concentrations in several isolates compared with untreated controls (Fig. 2).

After 24 h biofilms, i.e., mature biofilms, of moderate and strong biofilm-forming strains formed, the effectiveness of aztreonam/avibactam treatment on biofilm metabolic activity was tested. It was observed that the MIC concentration was not very effective, presumably because of the nature of the biofilm structure and reduced antimicrobial susceptibility of biofilm-associated cells. 4xMIC of aztreonam/avibactam reduced metabolic activity in mature biofilms by more than 50% in 64% ($n=16$) of the 25 tested strains. Only 4% ($n=1$) of the strains for MIC and 16% ($n=4$) for twice the MIC caused a greater than 50% reduction of metabolic activity (Fig. 3).

As shown in (Fig. 4), the number of viable bacteria within the biofilm was quantified for each strain following treatment with aztreonam/avibactam at MIC concentrations. No statistically significant difference was observed in strains 20 and 22 compared to untreated controls. However, a significant decrease in the number of biofilm-associated bacteria was detected in strains 23 and 24 ($p<0.05$).

The effect of different concentrations of aztreonam/avibactam (MIC, MIC/5, and MIC/10) on the total protein content of the biofilm matrix was evaluated in four different Bcc strains. The total protein concentrations in the EPS ranged approximately between 1300 and 1700 µg/mL, depending on the strain and treatment condition (Fig. 5). At the MIC concentration, total protein content within the biofilm tended to decrease in all strains; however, a statistically significant reduction was observed only in one strain. However, at lower concentrations (MIC/5 and MIC/10), the effects varied among the strains. Notably, in strain 20, a statistically significant increase in total protein content was observed at MIC/5 and MIC/10 concentrations ($p<0.05$). This finding suggests that sub-inhibitory concentrations may promote matrix-associated proteins in this strain. In the remaining strains, slight increases were also observed at lower concentrations, but these changes were not statistically significant.

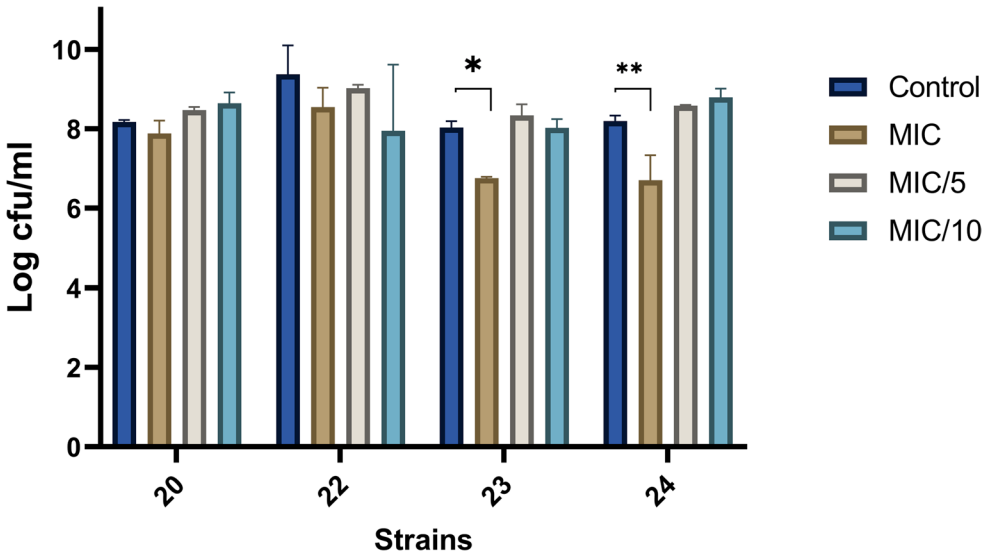


Fig. 4 Effect of different concentrations of aztreonam/avibactam on viable bacteria count in four *Burkholderia* strains' biofilms. Data represent mean ± standard deviation of three independent replicates. * $p<0.05$; ** $p<0.01$

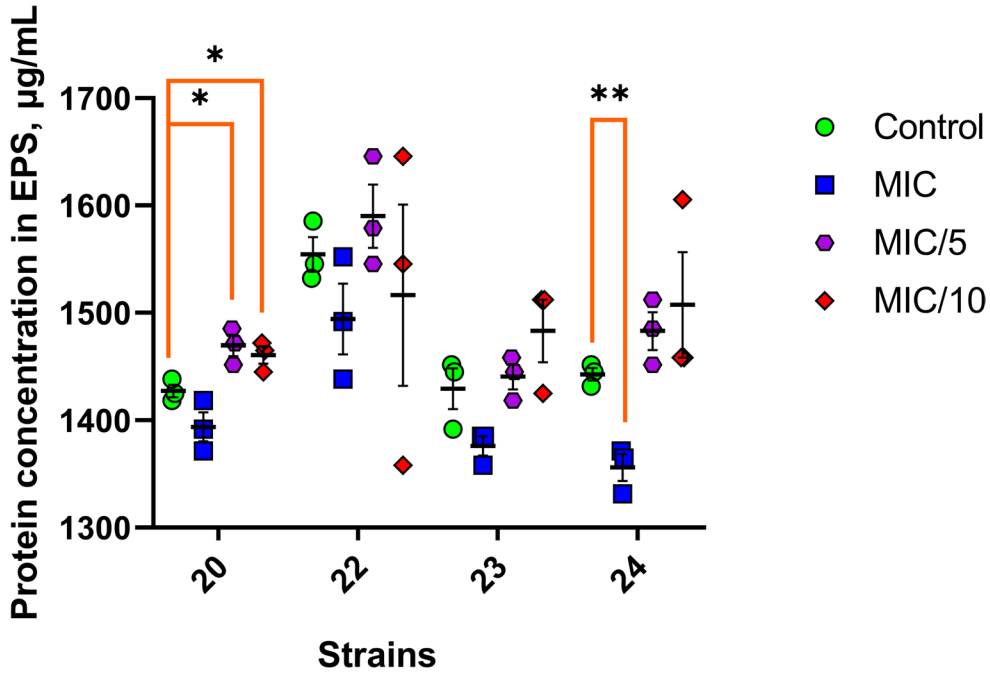


Fig. 5 Effect of different concentrations of aztreonam/avibactam on total protein content in biofilm matrix of Bcc strains. Bars represent mean ± SD of three independent experiments. Statistical significance compared to untreated control. * $p < 0.05$; ** $p < 0.01$

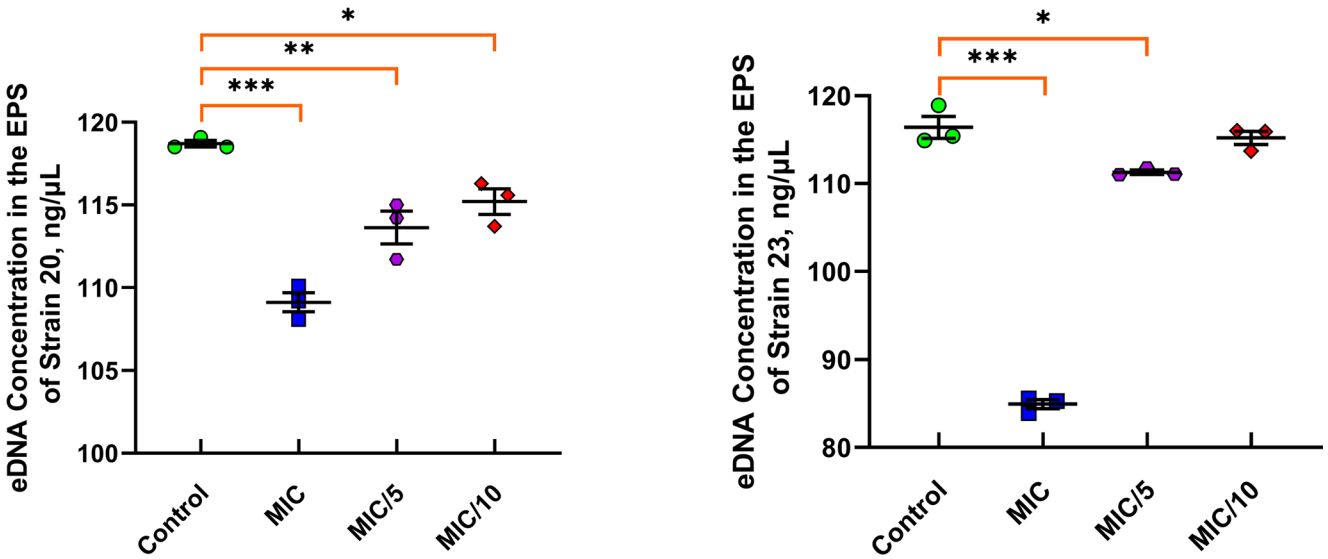


Fig. 6 Effect of different concentrations of aztreonam/avibactam on total extracellular DNA (eDNA) content in biofilm matrix of Bcc strains. Bars represent mean ± SD of three independent experiments. Statistical significance compared to untreated control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

The effect of aztreonam/avibactam at MIC, MIC/5, and MIC/10 concentrations on the eDNA levels within the EPS was assessed in two representative Bcc strains (strains 20 and 23), selected based on their differential responses to aztreonam/avibactam treatment. The eDNA concentrations ranged approximately from 80 to 120 ng/µL, depending on the strain and treatment (Fig. 6). In both strains, treatment with aztreonam/avibactam led to a consistent reduction in eDNA levels across all tested concentrations compared to the untreated control ($p < 0.001$). The most pronounced decrease was observed at the MIC concentration, but reductions at MIC/5 and MIC/10 were also notable. These findings suggest that aztreonam/avibactam may influence EPS-associated eDNA content in Bcc biofilms; however, this effect may vary among strains. Further studies involving a larger number of isolates are required to confirm these observations across Bcc populations.

According to SEM images, in the untreated control of strain 20 (Fig. 7A), a dense and mature biofilm structure

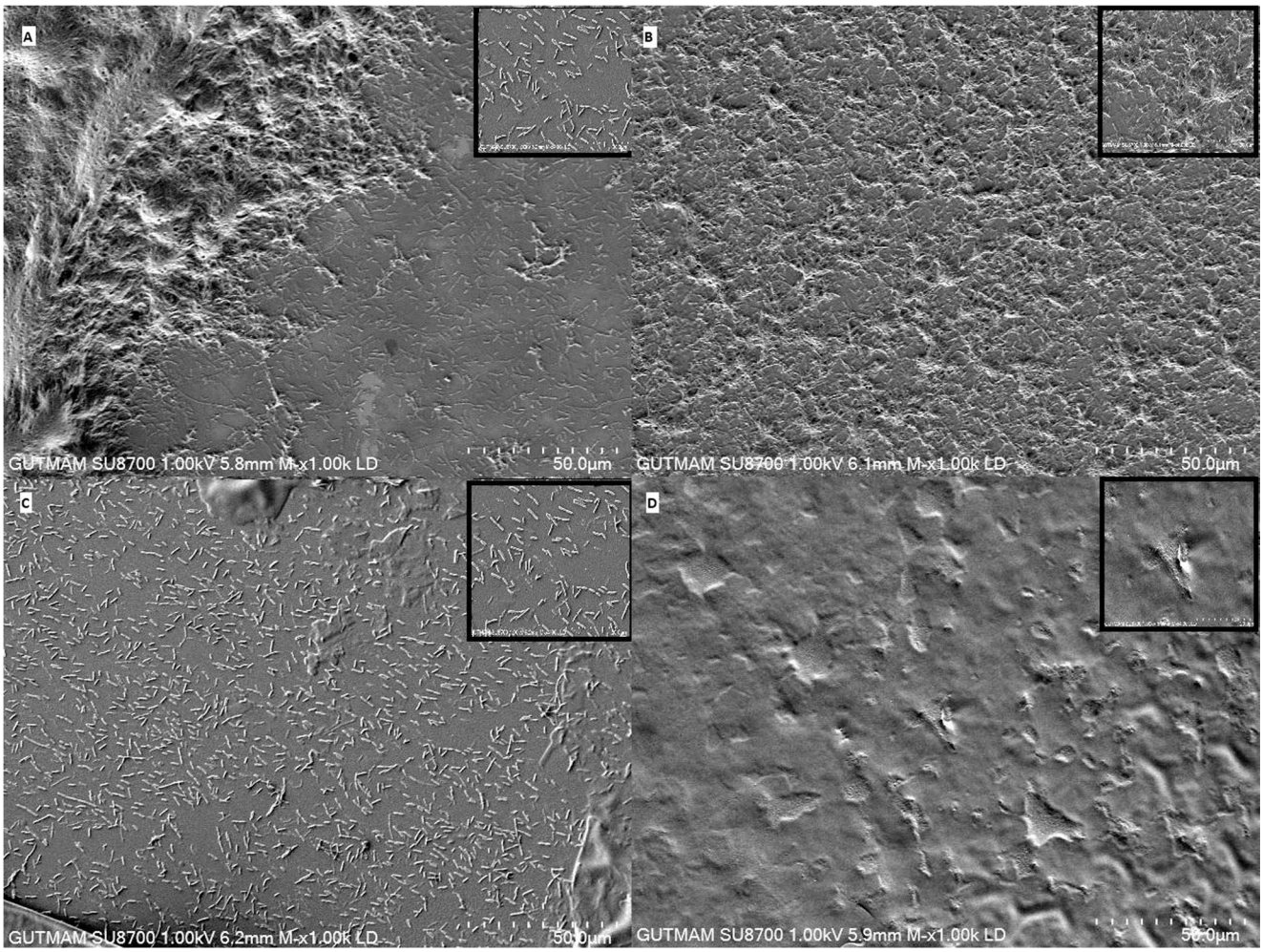


Fig. 7 SEM images of Bcc biofilms with and without aztreonam/avibactam treatment. (**A**) Untreated control of strain 20; (**B**) Strain 20 treated with aztreonam/avibactam at its MIC concentration; (**C**) Untreated control of strain 23; (**D**) Strain 23 treated with aztreonam/avibactam at its MIC concentration. All images were taken at 1000× magnification. Scale bar: 50 µm

with abundant extracellular matrix was observed, indicating robust biofilm formation. In contrast, treatment with aztreonam/avibactam at MIC concentration (Fig. 7B) led to a markedly altered biofilm architecture, lacking the complexity and density characteristic of mature biofilms. Similarly, strain 23 (Fig. 7C) exhibited a smoother but less compact biofilm in the untreated condition. However, following treatment (Fig. 7D), the surface appeared considerably cleaner, with reduced bacterial presence and matrix production. These observations suggest that aztreonam/avibactam treatment effectively inhibited biofilm development, particularly in strain 23, by impairing bacterial adhesion and extracellular matrix formation. These SEM findings are consistent with the quantitative results, reinforcing the inhibitory effect of aztreonam/avibactam on biofilm integrity.

Discussion

Bcc is a pathogen that affects respiratory tract infections in patients with conditions such as CF. It is a significant cause of mortality and morbidity due to its high antibiotic resistance. While the activity of aztreonam/avibactam against isolates of *Enterobacteriales* and *Stenotrophomonas maltophilia* has been studied extensively, the activity of this beta-lactam/beta-lactamase inhibitor combination against Bcc isolates has been poorly investigated. Our study has shown the importance of aztreonam/avibactam as a new alternative for the treatment of Bcc infections.

Multidrug-resistant microorganisms are significant healthcare problems and are currently the target of clinical and laboratory research. Aztreonam/avibactam has been shown to restore aztreonam activity and to be potent against carbapenem-resistant *Enterobacteriales* that are resistant to ceftazidime/avibactam, meropenem/vaborbactam, and/

or imipenem/relebactam (Sader et al. 2024a). Therefore, it has been approved by the European Commission for use in complicated intra-abdominal infections, hospital-acquired pneumonia, complicated urinary tract infections, and Gram-negative bacterial infections with limited treatment options (European Commission 2024).

Studies with ceftazidime/avibactam have shown that Bcc isolates are susceptible to this combination (Papp-Wallace et al. 2017; Van Dalem et al. 2018; Schaumburg et al. 2022). In addition, a study revealed that no synergism was observed when aztreonam was added to ceftazidime/avibactam (Schaumburg et al. 2022). For ceftazidime/avibactam-resistant isolates, it was determined that piperacillin/avibactam and the combination of piperacillin/tazobactam and ceftazidime/avibactam restored susceptibility (Zeiser et al. 2019). Combinations of aztreonam with clavulanic acid (CLA), sulbactam (SUL), tazobactam (TAZ), and avibactam (AVI) were made, and avibactam was determined to be one of the most effective inhibitors against some Bcc isolates. In the same study, the addition of avibactam led to at least a 4-fold reduction in MICs for some strains, although the response was highly strain-dependent (Everaert and Coenye 2016). These results highlight the complexity of resistance mechanisms in Bcc, which often extend beyond β-lactamase production to include efflux pumps and alterations in penicillin-binding proteins. Our findings support the potential utility of aztreonam/avibactam in the treatment of Bcc infections, taking into account the strain-to-strain variability and the importance of susceptibility testing. Recent genome-wide screen studies have further elucidated the complexity of antibiotic resistance in Bcc. Studies of Bcc clinical isolates have identified numerous envelope-associated determinants that influence resistance and susceptibility to β-lactams and other antibiotics. Notably, the results showed that avibactam potentiated the activity of aztreonam and meropenem more strongly than ceftazidime (Hogan et al. 2023).

In the study conducted by Sader et al. using a broth microdilution assay against 219 Bcc isolates, the $MIC_{50/90}$s of aztreonam/avibactam were determined to be 4/16 mg/L (Sader 2022). Similarly, another study conducted with 150 Bcc and *B. gladioli* isolates reported MIC_{50}/MIC_{90} values of 4/16 mg/L for aztreonam/avibactam (Papp-Wallace et al. 2021). Additionally, a separate investigation documented $MIC_{50/90}$ values of 8/16 mg/L for the same combination (Huang et al. 2024). In comparison, our study yielded MIC_{50}/MIC_{90} values of 4/8 mg/L, demonstrating a high degree of consistency with previously published data. It was noted that the combination of aztreonam with avibactam resulted in a significant improvement in aztreonam MIC values against all Bcc strains, indicating its potential to enhance treatment outcomes against drug resistance. Despite the observed antimicrobial activity, we noted that in some strains (e.g., strains 4, 6, 14, and 22), viable bacterial counts increased over time at MIC, twice the MIC, and/or four times the MIC concentrations (Fig. 1). This regrowth suggests that specific subpopulations may survive initial antibiotic exposure and subsequently repopulate. Such behaviour can be attributed to adaptive tolerance mechanisms, including the activation of efflux pumps, induction of biofilm-related genes, or the presence of persister cells. Similar regrowth phenomena have been reported in previous studies as bacterial strategies to withstand antimicrobial stress (Brauner et al. 2016).

Sabour et al. (2022) reported that aztreonam has a moderate affinity for human serum albumin (HSA), indicating that aztreonam may bind to HSA through various interactions, which could lower its concentration in the bloodstream (Sabour et al. 2022). To explore this further, our study utilised bovine serum albumin (BSA) at different concentrations. BSA was selected because of its structural similarity to HSA, allowing us to assess its influence on the aztreonam/avibactam MIC (Ketrat et al. 2020). The results from a previous study revealed that HSA had minimal effects on the MIC of ceftazidime/avibactam, whereas the same study revealed that the MIC of ceftriaxone was significantly affected by the serum albumin (Keepers et al. 2014). Our study showed that aztreonam/avibactam was minimally affected by 5% BSA and highly affected by 10% BSA. The reduced efficacy of aztreonam/avibactam in the presence of 10% BSA may be explained by increased protein binding at higher albumin concentrations, leading to a lower free drug fraction. However, it should be noted that in vitro models assessing protein binding may yield variable results depending on multiple factors such as protein source, antibiotic concentration, pH, and medium composition (Zeitlinger et al. 2011). A limitation of our study is that these observations were made under in vitro conditions; therefore, in vivo studies are needed to validate the clinical relevance of protein binding effects on aztreonam/avibactam activity. Another limitation is that the potential alteration of aztreonam/avibactam MIC values in the presence of higher human serum albumin levels was not investigated. Future studies are needed to investigate this potential effect.

Biofilms are complex systems formed by microorganisms that aggregate to increase their resistance to antimicrobials, immune defenses, and environmental stresses. Since these complex structures are more resistant than the planktonic forms of microorganisms, they may be responsible for the majority of chronic infections (Bjarnsholt 2013). Our results on biofilm formation determined that 25 of the 29 Bcc isolates formed moderate and strong biofilms. In parallel with our results, the literature also confirmed that biofilm formation was an important virulence factor of Bcc (Schwab et al. 2002; Cunha et al. 2004). Bcc may contribute to persistent

and chronic infections through its ability to form biofilms in CF airways; therefore, Bcc infections in CF patients are difficult to manage (Høiby et al. 2017; Aiyer et al. 2021). Considering the efficacy of aztreonam/avibactam against Bcc planktonic cells, investigating its effects on both biofilm formation and mature biofilms of Bcc isolates is important. Our findings indicate that the antibiofilm efficacy of aztreonam/avibactam exhibited significant variability across different Bcc isolates. As anticipated, high concentrations were needed to achieve a substantial reduction in the metabolic activity of mature biofilms. Although aztreonam/avibactam treatment resulted in biofilm inhibition in most Bcc isolates, increased biofilm formation was observed at some sub-MIC concentrations. This phenomenon may be associated with bacterial stress responses triggered by exposure to sub-inhibitory antibiotic concentrations (Elawady et al. 2024). The need for higher antibiotic concentrations to affect established biofilms, together with the possibility that sub-MIC antibiotic exposure may enhance biofilm formation under certain conditions, highlights the importance of optimized dosing strategies for the effective management of biofilm-associated infections.

Our data also showed that aztreonam/avibactam effectively inhibited Bcc biofilms at MIC levels by reducing viable cell counts, protein and eDNA concentrations in biofilm matrix, and altering biofilm structure. However, sub-inhibitory concentrations (MIC/5 and MIC/10) in particular had a paradoxical effect in strain 20, where EPS protein levels increased despite suppressed eDNA. Sub-MIC levels of antibiotics may sometimes act as signaling cues, activating stress responses and enhancing biofilm formation through upregulation of EPS-associated genes (Ranieri et al. 2018). This phenomenon has been reported in many pathogens: sub-MIC levels of tetracycline and streptomycin can stimulate biofilm formation through upregulation of biofilm-associated genes, often in a dose-dependent manner (Liu et al. 2023). Conversely, eDNA suppression at all concentrations is consistent with reports indicating that certain sub-MIC exposures may still inhibit components critical for biofilm stability. Although these findings were obtained from a limited number of representative isolates, they highlight the potential complexity of sub-MIC antibiotic exposure, which may suppress certain biofilm-associated components while inducing adaptive changes in others. Therefore, maintaining effective therapeutic concentrations remains important for the management of biofilm-associated infections.

The intrinsic resistance mechanisms and strong biofilm-forming abilities of Bcc further limit treatment options. Our findings, although variable among strains, underscore the potential of aztreonam-avibactam as a therapeutic candidate and reveal the biological diversity in Bcc responses. The observed variability in antimicrobial susceptibility and biofilm inhibition across different strains highlights the need for future studies with larger isolate collections, deeper molecular analysis, and more controlled experimental conditions, including in vivo models.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12223-026-01569-0.

Author contributions All the authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by O.O., D.D., T.T., and B.A. The first draft of the manuscript was written by O.O., and all the authors commented on previous versions of the manuscript. All the authors read and approved the final manuscript.

Funding Open access funding provided by Northeastern University Library. This work was supported by the Gazi University Scientific Research Projects Unit (grant number: THD-2024-9649).

Data availability No datasets were generated or analysed during the current study.

Declarations

Ethical approval Was obtained from the Ethics Committee of Marmara University, Institute of Health Sciences, Türkiye (19.11.2024–106).

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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