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Antibacterial Activity of Delafloxacin and Polymyxin B Against Planktonic and Biofilm-Associated Bacteria

Evaluation of Single and Combination Treatment Against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Master's thesis in Biotechnology

ANKITA PRIYA

DEPARTMENT OF LIFE SCIENCES

CHALMERS UNIVERSITY OF TECHNOLOGY
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Supervisor: Santosh Pandit, Department of Life Sciences
Examiner: Ivan Mijakovic, Department of Life Sciences

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Department of Life Sciences
Chalmers University of Technology
SE-412 96 Gothenburg
Telephone +46 31 772 1000

Cover: Schematic illustration of antibiotic treatment effects on planktonic and biofilm-associated *S. aureus* and *P. aeruginosa*. Created with BioRender.com.

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Department of Life Sciences
Chalmers University of Technology

Abstract

Biofilm-associated infections are difficult to treat because bacteria growing in biofilms show increased tolerance to antibiotics compared with planktonic cells. In chronic wound infections, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are commonly detected and can contribute to persistent infection. This thesis investigated the antibacterial activity of delafloxacin and polymyxin B against *S. aureus* and *P. aeruginosa* under planktonic and biofilm-associated conditions. The antibiotics were tested alone and in combination for MIC determination, time-kill assays, biofilm treatment assays, and scanning electron microscopy. The results showed that both antibiotics reduced bacterial viability, with stronger effects generally observed at higher concentrations. Planktonic cells were more affected than biofilm-associated cells, supporting the increased tolerance of bacteria growing in biofilms. Combination treatment reduced bacterial survival in several conditions and showed stronger effects than single-antibiotic treatments. SEM analysis showed dense bacterial attachment in untreated biofilms, while treated *S. aureus* and *P. aeruginosa* biofilms showed reduced bacterial coverage and altered biofilm structure. Overall, the findings suggest that delafloxacin and polymyxin B have antibacterial effects against both planktonic and biofilm-associated bacteria, but complete biofilm eradication was not achieved under the tested conditions.

Keywords: Biofilm, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, Delafloxacin, Polymyxin B, antibiotic tolerance

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Ankita Priya, Gothenburg, May 2026

Contents

1
Introduction

In this chapter, the background, aim, research questions, project scope, and disposition of the thesis are presented.

1.1 Project Background

Biofilm-associated infections are difficult to treat because bacteria growing in biofilms are more tolerant to antibiotics than free-living planktonic cells. Biofilms are structured bacterial communities attached to a surface and embedded in a self-produced extracellular matrix, which can protect the cells from antimicrobial treatment [1, 2]. This is clinically important in chronic wound infections, where delayed healing and treatment failure are common problems [3, 4].
Chronic wound infections are often polymicrobial. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are commonly detected in such infections and are both able to form biofilms [5, 6]. *S. aureus* is a Gram-positive opportunistic pathogen, while *P. aeruginosa* is a Gram-negative opportunistic pathogen known for high tolerance to antimicrobial treatment [7, 8]. Therefore, both species are relevant models for studying antibiotic effects under planktonic and biofilm-associated conditions.
Standard antibiotic susceptibility tests, such as minimum inhibitory concentration (MIC) determination, are usually performed using planktonic bacteria. However, these results do not always reflect the response of biofilm-associated cells, since biofilms can limit antibiotic penetration and contain slow-growing or tolerant bacterial populations [9, 2]. Therefore, additional assays are needed to evaluate antibiotic activity against biofilms.
Delafloxacin and polymyxin B were selected because they act through different antibacterial mechanisms. Delafloxacin is a fluoroquinolone antibiotic with activity against clinically important Gram-positive and Gram-negative bacteria, including *S. aureus* [10, 11]. It acts by interfering with bacterial DNA replication through inhibition of DNA gyrase and topoisomerase IV [11]. Polymyxin B is mainly active against Gram-negative bacteria and is relevant for studying *P. aeruginosa* because it targets the bacterial outer membrane [12, 13]. Since these antibiotics act through different mechanisms, they were tested both alone and in combination to compare their effects on bacterial survival and biofilm morphology.

1.2 Aim

The aim of this thesis was to evaluate the antibacterial activity of delafloxacin and polymyxin B against *S. aureus* and *P. aeruginosa* under planktonic and biofilm-associated conditions. The project also aimed to compare the effects of single-antibiotic treatment and combination treatment.

1.3 Research Questions

The following research questions were addressed in this thesis:

1. How effective are delafloxacin and polymyxin B against planktonic *S. aureus* and *P. aeruginosa*?
2. How do biofilm-associated cells respond to antibiotic treatment compared with planktonic cells?
3. Does the combination of delafloxacin and polymyxin B reduce bacterial survival more effectively than single-antibiotic treatment under the tested conditions?
4. How does antibiotic treatment affect biofilm morphology as observed by scanning electron microscopy?

1.4 Project Scope

This project was scoped to evaluate the antibacterial activity of delafloxacin and polymyxin B against single-species cultures of *S. aureus* and *P. aeruginosa*. The study focused on comparing planktonic and biofilm-associated bacterial responses to single and combination antibiotic treatments.
Although *S. aureus* and *P. aeruginosa* can coexist in polymicrobial wound infections, dual-species biofilms were not included in this thesis due to time constraints. Therefore, the results should be interpreted as observations from single-species in vitro biofilm models rather than as a direct representation of polymicrobial wound biofilms.

1.5 Disposition of the Thesis

This thesis is structured into six chapters. Chapter 1 introduces the project background, aim, research questions, and scope. Chapter 2 presents the theoretical background on biofilms, antibiotic tolerance, the bacterial species used in the study, and the antibiotics investigated. Chapter 3 describes the experimental methods. Chapter 4 presents the results from MIC determination, time-kill assays, biofilm treatment assays, and SEM analysis. Chapter 5 discusses the main findings, limitations, and future perspectives. Chapter 6 summarizes the conclusions of the thesis.

2
Theory

This chapter presents the theoretical background relevant to the project. It describes bacterial biofilms, antibiotic tolerance in biofilms, the clinical relevance of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, the interaction between these two species, and the antibiotics delafloxacin and polymyxin B. The chapter also introduces the experimental assays used to evaluate antibacterial activity.

2.1 Bacterial Biofilms

Bacteria can grow either as free-living planktonic cells or as surface-associated communities known as biofilms. A biofilm is a structured microbial community attached to a surface and embedded in a self-produced extracellular matrix. This matrix is mainly composed of polysaccharides, proteins, extracellular DNA, and other biomolecules that provide structural stability and protection to the bacterial community [1, 14].
Biofilm formation is generally described as a stepwise process involving initial attachment, irreversible attachment, microcolony formation, maturation, and dispersal, as illustrated in Figure 2.1 [15, 1]. During this process, bacterial cells change their behaviour compared with planktonic cells. These changes include altered metabolism, reduced growth rate, and increased ability to survive environmental stress. Because of this, biofilms are considered an important survival strategy for bacteria in both natural and clinical environments [1, 15].
The extracellular matrix plays an important role in biofilm function. It helps bacterial cells attach to surfaces, keeps cells close together, and protects the community from environmental stress and antimicrobial agents [14, 1]. However, the structure and composition of the matrix can vary depending on the bacterial species and environmental conditions.

2.2 Antibiotic Tolerance in Biofilms

Biofilm-associated bacteria are often more difficult to kill than planktonic bacteria. This does not always mean that the bacteria have developed genetic antibiotic resistance. Instead, biofilms can show increased antibiotic tolerance due to their physical structure and altered physiology [2, 16].
Several mechanisms contribute to antibiotic tolerance in biofilms. First, the extracellular matrix can reduce or delay antibiotic penetration into the biofilm [17]. Second, oxygen and nutrient gradients within the biofilm can create slow-growing

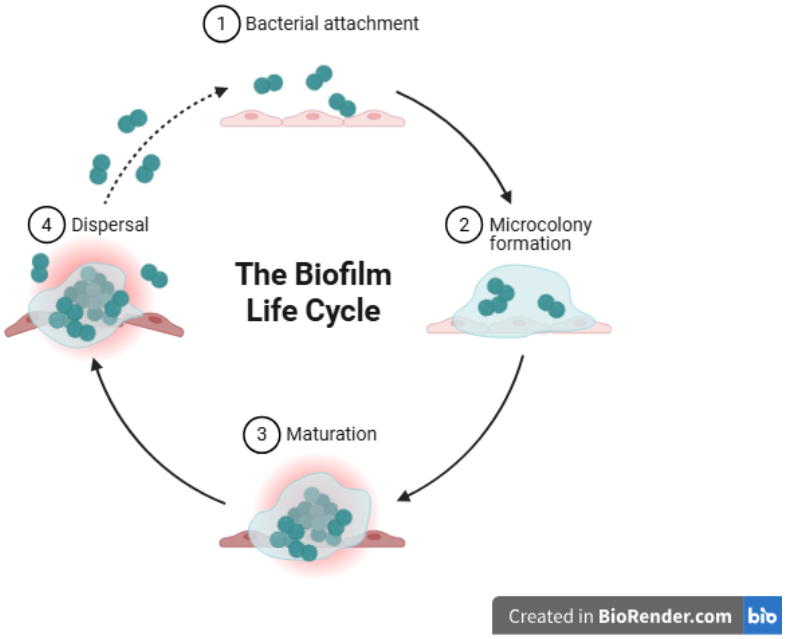


Figure 2.1: Schematic overview of the biofilm life cycle, including bacterial attachment, microcolony formation, maturation, and dispersal. Created with BioRender.com and adapted from [15, 1].

or metabolically less active bacterial populations, which are less affected by antibiotics that target active cellular processes [16, 2]. Third, some biofilm-associated cells may survive antibiotic exposure and contribute to regrowth after treatment [2]. These mechanisms together make biofilm-associated infections difficult to eradicate, as illustrated in Figure 2.2.
Because of this difference between planktonic and biofilm-associated bacteria, standard antibiotic susceptibility tests such as MIC may not fully predict how effective an antibiotic will be against biofilms [9]. Therefore, additional assays such as time-kill experiments, biofilm treatment assays, and microscopy-based analysis are useful for evaluating antibacterial effects under different growth conditions.

2.3 Biofilms in Chronic Wound Infections

Biofilms are highly relevant in chronic wound infections. Chronic wounds provide a moist and nutrient-rich environment that supports bacterial colonization and biofilm formation [3, 4]. Biofilm growth in wounds can delay healing by maintaining inflammation, protecting bacteria from treatment, and reducing the effectiveness of antimicrobial therapy [3].
Chronic wound infections are often polymicrobial, meaning that more than one bacterial species is present in the same wound environment [5]. Among the commonly detected species are *Staphylococcus aureus* and *Pseudomonas aeruginosa* [5, 6]. These bacteria are clinically important because both can form biofilms and contribute to persistent infection.

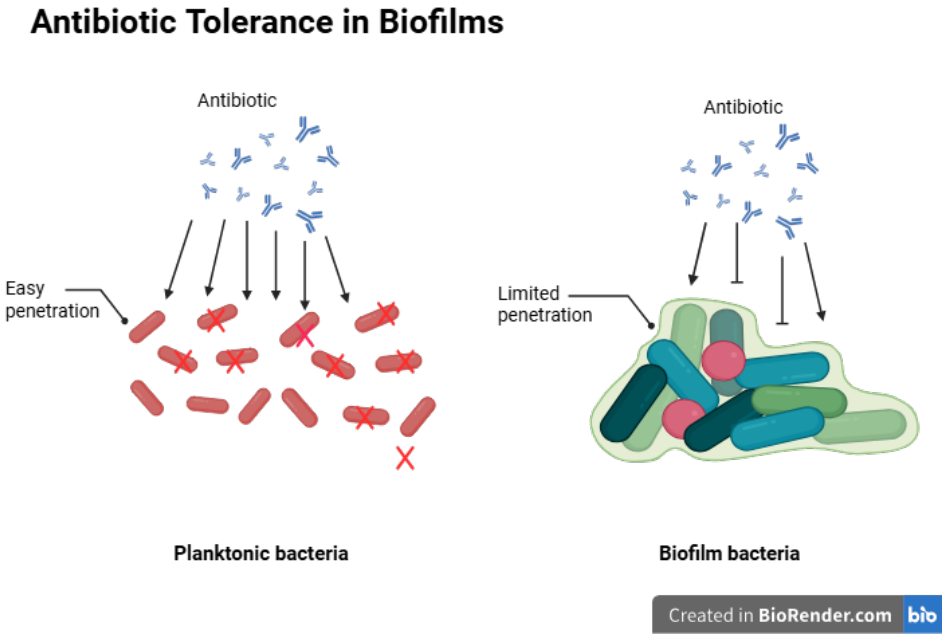


Figure 2.2: Schematic comparison of antibiotic action against planktonic and biofilm-associated bacteria. In planktonic populations, antibiotics readily reach bacterial cells, whereas biofilm-associated bacteria can exhibit increased tolerance due to limited antibiotic penetration and protective biofilm structure. Created with BioRender.com and adapted from [2, 16].

The spatial organization of bacteria in chronic wounds can vary. For example, *S. aureus* and *P. aeruginosa* may occupy different regions of the wound, and their distribution is not always random [6]. This is important because bacterial location and interaction can influence treatment response.

2.4 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive opportunistic pathogen associated with skin and soft tissue infections, wound infections, bloodstream infections, and implant-associated infections [7]. It is clinically important because of its ability to persist under stressful conditions and form biofilms on biological and non-biological surfaces [7].
S. aureus biofilm formation allows bacterial cells to attach to surfaces, grow as structured communities, and become less susceptible to antimicrobial treatment [7, 2]. In biofilms, the bacteria are protected by extracellular matrix components and may contain slow-growing subpopulations that survive antibiotic exposure [2]. This makes *S. aureus* biofilms clinically relevant and difficult to treat.

2.5 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen commonly associated with chronic infections, wound infections, and infections in immunocompromised patients [8, 2]. It is clinically important because it can form biofilms and has high tolerance to many antimicrobial treatments [8, 16].
P. aeruginosa is a strong biofilm-forming bacterium. Its biofilm matrix can contain polysaccharides such as alginate, Pel, and Psl, which contribute to biofilm stability and protection [8]. These matrix components support bacterial attachment, biofilm maturation, and survival under stressful conditions. Because of these properties, *P. aeruginosa* biofilms are often difficult to eradicate using conventional antibiotic treatment [2, 16].

2.6 Interaction Between *S. aureus* and *P. aeruginosa*

S. aureus and *P. aeruginosa* are frequently found together in chronic wound environments and other persistent infections [5, 6]. Their interaction can influence bacterial growth, biofilm formation, and antibiotic susceptibility [18, 19].
The interaction between these two organisms can be complex. In some conditions, *P. aeruginosa* can affect the growth of *S. aureus*, while in other conditions the two species can coexist in structured biofilms [18]. Recent studies also show that dual-species biofilms can have altered antibiotic tolerance compared with mono-species biofilms [19]. Therefore, understanding these bacterial interactions is important when studying wound-associated biofilms.
In this project, the final experimental work focused on mono-species biofilms of *S. aureus* and *P. aeruginosa*. However, the clinical relevance of these bacteria is partly linked to their frequent coexistence in polymicrobial wound infections [5, 6].

2.7 Delafloxacin

Delafloxacin is a fluoroquinolone antibiotic with activity against a range of Gram-positive and Gram-negative bacteria [10, 11]. Fluoroquinolones act by inhibiting bacterial DNA gyrase and topoisomerase IV, enzymes required for DNA replication and cell division [11]. Interference with these enzymes prevents normal bacterial DNA processing and can lead to bacterial cell death [11].
Delafloxacin is of interest because it has shown activity against clinically relevant pathogens, including *S. aureus* [10, 11]. In this project, delafloxacin was evaluated against *S. aureus* and *P. aeruginosa*, both alone and in combination with polymyxin B.

2.8 Polymyxin B

Polymyxin B is an antibiotic mainly active against Gram-negative bacteria. Its activity is associated with binding to lipopolysaccharides in the outer membrane of Gram-negative bacteria, leading to disruption of membrane integrity and bacterial cell death [12]. Because of this mechanism, polymyxin B is relevant for difficult-to-treat Gram-negative infections, including infections caused by *P. aeruginosa* [12].
Polymyxin B has also been studied against planktonic and biofilm-associated *P. aeruginosa* cells [13]. However, as with many antibiotics, its activity against biofilms can be reduced compared with planktonic cells [13, 2]. In this project, polymyxin B was evaluated against *S. aureus* and *P. aeruginosa*, both alone and in combination with delafloxacin.

2.9 Combination Antibiotic Treatment

Combination antibiotic treatment is used to improve antibacterial activity by targeting bacteria through more than one mechanism [20, 19]. In biofilm-associated infections, combination treatment may help improve bacterial killing, reduce regrowth, or affect different bacterial populations within the biofilm [20, 19].
For example, combining agents with different targets may improve treatment response compared with a single antibiotic [20]. However, the effect of combination treatment depends on the bacterial species, antibiotic concentrations, growth condition, and whether bacteria are planktonic or in a biofilm-associated state [19]. Therefore, combination effects need to be evaluated experimentally.
In this project, delafloxacin and polymyxin B were tested alone and in combination to compare their antibacterial effects against *S. aureus* and *P. aeruginosa*.

2.10 Experimental Assays Used in This Project

MIC assays were used to evaluate the inhibitory activity of the antibiotics against planktonic bacteria. MIC identifies the lowest antibiotic concentration that prevents visible bacterial growth after incubation and is commonly used as a standard measure of planktonic antibiotic susceptibility [9].
MBC assessment was performed after MIC testing by plating samples from wells showing no visible growth. This was used to observe whether bacterial cells were able to form colonies after antibiotic exposure.
Time-kill assays were used to evaluate bacterial survival over time after antibiotic treatment. Unlike MIC, which gives a single endpoint value, a time-kill assay shows how bacterial viability changes during the treatment period [20].
Biofilm treatment assays were used to evaluate antibiotic activity against surface-associated bacterial cells. Since biofilm-associated bacteria can respond differently from planktonic cells, this assay was important for comparing antibiotic activity under biofilm conditions [9, 2].
Scanning electron microscopy was used to observe biofilm morphology after treatment. SEM provides visual information about bacterial attachment, surface struc-

ture, and biofilm organization, allowing comparison between untreated and treated samples.

3

Methodology

This chapter describes the experimental methods used in this project, including bacterial culture preparation, viable count determination, MIC determination, MBC assessment, time-kill assays, biofilm formation and treatment, SEM sample preparation, and data analysis.

3.1 Bacterial Strains and Culture Conditions

Staphylococcus aureus (SA) and *Pseudomonas aeruginosa* (PA) were used in this study. The bacterial strains were stored at -80 °C until use. Before starting the experiments, the strains were refreshed on agar plates to obtain viable and actively growing cultures.

For refreshing, the bacterial stock tubes were taken from -80 °C storage. Using a sterile inoculation loop, bacterial material was collected from each stock tube and streaked onto LB agar plates. The bacteria were spread across the agar surface using a zig-zag streaking method to obtain isolated colonies. The plates were incubated at 37 °C for 24 h. After incubation, the refreshed bacterial plates were stored at 4 °C and used for preparation of overnight cultures.

Overnight cultures were prepared using colonies grown on agar plates. For each bacterial strain, 2–3 well-isolated colonies were picked aseptically using a sterile inoculation loop. Colonies of *S. aureus* were inoculated into 5 mL tryptic soy broth (TSB), while colonies of *P. aeruginosa* were inoculated into 5 mL Luria–Bertani (LB) broth. The cultures were incubated overnight at 37 °C with shaking at 180 rpm. These overnight cultures were used for preparation of bacterial suspensions in the following experiments.

3.2 CFU/mL Determination by Serial Dilution and Spread Plating

The viable bacterial concentration of overnight cultures was determined by ten-fold serial dilution followed by spread plating. This was performed to estimate the number of colony-forming units per millilitre (CFU/mL) present in the overnight cultures before further experimental use.

3.2.1 Preparation of Premixes

For each bacterial strain, premixes were prepared using different volumes of overnight culture. For *S. aureus*, 5 µL, 15 µL, and 25 µL of overnight culture were each added separately to 5 mL TSB. For *P. aeruginosa*, 5 µL, 15 µL, and 25 µL of overnight culture were each added separately to 5 mL LB broth. The premixes were mixed thoroughly by vortexing to ensure uniform bacterial distribution before serial dilution.

3.2.2 Serial Dilutions

For each premix, five sterile microcentrifuge tubes were prepared and labelled D1–D5. Each tube contained 900 µL sterile 0.89% saline solution. A volume of 100 µL from the premix was transferred into D1 and mixed thoroughly to obtain a 10^{-1} dilution. From D1, 100 µL was transferred into D2 to obtain a 10^{-2} dilution. The same procedure was continued sequentially until D5, resulting in dilutions from 10^{-1} to 10^{-5}.

3.2.3 Plating and Incubation

From selected dilutions, 100 µL was spread onto LB agar plates using a sterile T-spreader. Dilutions 10^{-3}, 10^{-4}, and 10^{-5} were plated to identify countable colony numbers. The plates were incubated at 37 °C for 18–24 h.

3.2.4 Colony Counting

After incubation, colonies were counted manually. Plates containing 30–300 colonies were selected for analysis, while plates with fewer than 30 colonies or more than 300 colonies were excluded. Across the premixes, the 10^{-4} dilution consistently gave countable colonies and was used for CFU/mL estimation.

3.2.5 CFU/mL Calculation

CFU/mL was calculated using the number of colonies, the dilution factor, and the plated volume. Since 100 µL was plated, the plated volume was 0.1 mL. The CFU/mL was calculated using Equation 3.1.

$$CFU/mL = \frac{\text{Number of colonies} \times \text{Dilution factor}}{0.1} \tag{3.1}$$

where the dilution factor represents the reciprocal of the dilution used for plating. The calculated CFU/mL values were used to estimate the bacterial concentration of the overnight cultures and to select suitable culture conditions for subsequent experiments.

3.3 Minimum Inhibitory Concentration (MIC) Determination

Minimum inhibitory concentration (MIC) was determined using the broth microdilution method in sterile 96-well plates. MIC testing was performed to determine the lowest concentration of antibiotic that inhibited visible bacterial growth of *S. aureus* and *P. aeruginosa* after incubation.

3.3.1 Preparation of Antibiotic Dilutions

Antibiotic stock solutions were prepared for delafloxacin and polymyxin B (PMB). Antibiotic dilutions were prepared in Mueller–Hinton (MH) broth. The required volume of antibiotic stock solution was calculated using Equation 3.2.

$$C_{stock} \times V_{stock} = C_{target} \times V_{final} \tag{3.2}$$

where C_{stock} is the concentration of the antibiotic stock solution, V_{stock} is the volume of stock solution required, C_{target} is the desired antibiotic concentration, and V_{final} is the final volume.
For each antibiotic, the first well of the 96-well plate was prepared by adding the required volume of antibiotic stock solution together with MH broth to obtain a total volume of 200 µL. The remaining wells in the same row were filled with 100 µL MH broth. A two-fold serial dilution was then performed by transferring 100 µL from the first well to the next well. The contents were mixed properly, and 100 µL was transferred sequentially across the row. This procedure was continued until the final well. After mixing the final well, 100 µL was discarded so that all wells contained an equal volume of 100 µL antibiotic dilution.
The antibiotic concentration decreased stepwise across the wells by two-fold dilution. The dilution range was selected to include concentrations above and below the expected MIC values for each antibiotic and bacterial strain.

3.3.2 Preparation of Bacterial Inoculum

Bacterial inoculum was prepared from overnight cultures of *S. aureus* and *P. aeruginosa*. For *P. aeruginosa*, 25 µL of overnight culture was added to 20 mL MH broth. For *S. aureus*, 50 µL of overnight culture was added to 20 mL MH broth. The bacterial suspensions were mixed thoroughly before inoculation into the antibiotic-containing wells.

3.3.3 Inoculation and Incubation

After preparation of antibiotic dilutions, 100 µL of the corresponding bacterial suspension was added to each well containing 100 µL antibiotic dilution. This resulted in a final volume of 200 µL per well. The bacterial inoculum was added starting from the lowest antibiotic concentration towards the highest antibiotic concentration to reduce carryover between wells.

For each assay, a growth control containing bacteria without antibiotic and a sterility control containing medium without bacteria were included. The plates were incubated at 37 °C for 24 h.

3.3.4 MIC Reading

After incubation, the wells were examined visually for bacterial growth. The MIC was defined as the lowest antibiotic concentration that completely inhibited visible bacterial growth compared with the positive growth control. Wells showing clear medium were considered inhibited, while wells showing turbidity were considered positive for bacterial growth.

3.4 Minimum Bactericidal Concentration (MBC) Determination

Minimum bactericidal concentration (MBC) was assessed after MIC reading to observe whether bacteria from wells with no visible growth were able to form colonies on antibiotic-free agar plates.
Aliquots were taken from wells showing no visible growth in the MIC assay, including the MIC well and wells with antibiotic concentrations above the MIC. From each selected well, 2 µL was spotted onto antibiotic-free LB agar plates. The spots were allowed to absorb into the agar surface before incubation.
The plates were incubated at 37 °C for 18–24 h. After incubation, the plates were examined for colony growth. The MBC was defined as the lowest antibiotic concentration at which no colony growth was observed on the agar plate.

3.5 Time-Kill Assay

Time-kill assays were performed to evaluate the antibacterial activity of delafloxacin and PMB, alone and in combination, against planktonic *S. aureus* and *P. aeruginosa*. The assay was used to determine changes in viable bacterial counts over time after antibiotic exposure.

3.5.1 Preparation of Bacterial Culture

Overnight cultures of *S. aureus* and *P. aeruginosa* were prepared as described previously. For each experimental condition, 100 µL of overnight culture was added to 5 mL fresh MH broth to prepare the working culture. The cultures were mixed properly before antibiotic addition.

3.5.2 Preparation of Antibiotic Treatments

For both *S. aureus* and *P. aeruginosa*, delafloxacin and PMB were tested alone and in combination. Antibiotic treatment concentrations were prepared as multiples of the MIC values determined previously. Delafloxacin alone and PMB alone were tested at

1×, 5×, 10×, and 20× MIC. For single-antibiotic treatment, the relevant antibiotic was added directly to the bacterial culture. For combination treatment, delafloxacin and PMB were added together at the corresponding combination concentrations. The final volume for each treatment condition was 5 mL.

3.5.3 Incubation and Sampling

For each condition, an untreated control without antibiotic was included. All cultures were incubated at 37 °C with shaking at 180 rpm. Samples were collected at 0, 2, 4, 8, and 24 h. At each time point, 100 µL culture was withdrawn aseptically for serial dilution and plating.

3.5.4 Serial Dilution and Plating

Serial dilutions were prepared using sterile 0.89% saline. For each sample, 100 µL culture was transferred into 900 µL saline to obtain a 10^{-1} dilution. This procedure was repeated sequentially to obtain further dilutions. For untreated control samples, dilutions were prepared up to 10^{-6}, while for antibiotic-treated samples, dilutions were prepared up to 10^{-5} depending on the expected reduction in bacterial count. From selected dilutions, 100 µL was spread onto agar plates using a sterile spreader. Plates were incubated at 37 °C for 18–24 h.

3.5.5 Colony Counting and Data Analysis

After incubation, colonies were counted from plates containing 30–300 colonies. CFU/mL values were calculated for each time point using the colony count, dilution factor, and plated volume. Time-kill curves were generated by plotting $\log_{10}$ CFU/mL against time to evaluate the bacterial killing effect of each antibiotic treatment.

3.6 Biofilm Formation and Antibiotic Treatment

Biofilm formation and antibiotic treatment were performed to evaluate the effect of delafloxacin and PMB on biofilm-associated bacterial cells. Biofilms were formed on sterile 10 mm cover glasses placed in 24-well plates. After biofilm formation, antibiotic treatment was performed, followed by recovery of biofilm-associated cells by sonication, serial dilution, and plating.

3.6.1 Biofilm Formation

Overnight cultures of *S. aureus* and *P. aeruginosa* were prepared as described previously. For biofilm formation, bacterial suspensions were prepared by inoculating overnight culture into fresh medium.
For *S. aureus*, 100 µL of overnight culture was added to 10 mL TSB and mixed thoroughly by vortexing. For *P. aeruginosa*, 100 µL of overnight culture was added to 10 mL LB broth and mixed thoroughly by vortexing.

Sterile 10 mm cover glasses were placed into the wells of a sterile 24-well plate. For each antibiotic condition, separate cover glasses were used. A volume of 1 mL of the bacterial suspension was added to each well containing a cover glass.
The plates were incubated at 37 °C for 24 h under static conditions to allow biofilm formation on the surface of the cover glasses.

3.6.2 Antibiotic Treatment of *S. aureus* Biofilm

After 24 h of biofilm formation, the medium was carefully removed from each well without disturbing the attached biofilm. Fresh TSB containing antibiotics was then added to the wells, and untreated biofilms were included as controls.
For *S. aureus* biofilm treatment, delafloxacin and PMB were tested alone and in combination. Antibiotic treatments were prepared as an MIC-based concentration series for the biofilm assay. Delafloxacin alone and PMB alone were tested at 50×, 100×, and 200× MIC. For the combination treatment, delafloxacin and PMB were added together at the corresponding concentration levels. The final treatment volume was 1 mL per well. After addition of antibiotic solutions, the plates were incubated again at 37 °C for 24 h under static conditions.

3.6.3 Antibiotic Treatment of *P. aeruginosa* Biofilm

After 24 h of biofilm formation, the medium was carefully removed from each well without disturbing the attached biofilm. Fresh LB broth containing antibiotics was then added to the wells, and untreated biofilms were included as controls.
For *P. aeruginosa* biofilm treatment, delafloxacin and PMB were tested alone and in combination. Antibiotic treatments were prepared as an MIC-based concentration series for the biofilm assay. Delafloxacin alone was tested at 10×, 25×, 50×, and 100× MIC. PMB alone was tested at 50×, 100×, and 200× MIC. The delafloxacin–PMB combination was tested at 10×, 25×, 50×, and 100× MIC. For the combination treatment, delafloxacin and PMB were added together at the corresponding concentration levels. The final treatment volume was 1 mL per well. After addition of antibiotic solutions, the plates were incubated again at 37 °C for 24 h under static conditions.

3.6.4 Biofilm Recovery

After antibiotic treatment, the antibiotic-containing medium was removed carefully from each well. The cover glasses containing biofilms were transferred into sterile 15 mL Falcon tubes containing 5 mL sterile 0.89% saline solution. The samples were sonicated using a probe sonicator to detach biofilm-associated cells from the cover glasses. After sonication, the tubes were vortexed to obtain a uniform bacterial suspension.

3.6.5 Serial Dilution and Plating of Biofilm Cells

Recovered biofilm suspensions were serially diluted using sterile 0.89% saline. For each sample, 100 µL of the recovered biofilm suspension was transferred into 900 µL

saline to obtain a 10^{-1} dilution. The dilution was mixed properly, and the same procedure was continued sequentially to obtain further dilutions.
For untreated control samples, higher dilutions were selected for plating, while for antibiotic-treated samples, lower dilutions were plated depending on the expected reduction in viable bacterial count. From selected dilutions, 100 µL was spread onto agar plates using a sterile spreader. Plates were incubated at 37 °C for 18–24 h.

3.6.6 Colony Counting and CFU Calculation

After incubation, colonies were counted manually from plates containing countable colonies. CFU/mL values were calculated using the colony count, dilution factor, and plated volume. Since each biofilm sample was recovered in 5 mL sterile 0.89% saline, CFU/biofilm was calculated by multiplying the CFU/mL value by the recovery volume. The effect of antibiotic treatment on biofilm viability was evaluated by comparing treated biofilms with untreated controls.

3.7 Scanning Electron Microscopy (SEM) Analysis

Scanning electron microscopy (SEM) was used to observe the surface morphology and structural organization of untreated and antibiotic-treated biofilms. Biofilms were formed on sterile 10 mm cover glasses as described previously and were processed for SEM after antibiotic treatment.
After treatment, the medium was carefully removed and the cover glasses were gently washed with sterile 0.89% saline to remove non-adherent cells and remaining medium. The biofilm samples were then fixed with 3% glutaraldehyde for 2 h to preserve bacterial cells and biofilm structure. After fixation, the fixative solution was removed, and the samples were washed gently before dehydration.
The samples were dehydrated using a graded ethanol series of 30%, 50%, 70%, 90%, and 100% ethanol for 10 min at each concentration. This gradual dehydration step was performed to remove water from the samples while minimizing structural damage to the biofilm. After dehydration, the samples were dried for 2 days before sputter coating.
The dried samples were sputter-coated with gold (5 nm) to make the sample surface conductive for SEM imaging. The coated samples were examined using SEM, and representative images were collected to compare bacterial attachment, surface morphology, and biofilm organization between untreated controls and antibiotic-treated samples.

3.8 Experimental Replicates

Time-kill assays and biofilm treatment assays were performed using three replicate measurements for each treatment condition. For biofilm treatment assays, three replicate cover glasses were used for each condition. Viable count data are presented as mean values with standard deviation. Error bars in the time-kill and

biofilm treatment figures represent standard deviation between the three replicate measurements. Viable counts were calculated as CFU/mL for planktonic time-kill assays and as CFU/biofilm for biofilm treatment assays.

4

Results

This chapter presents the results obtained from MIC determination, time-kill assays, biofilm treatment assays, and SEM analysis. The results are presented for *Staphylococcus aureus* and *Pseudomonas aeruginosa* after treatment with delafloxacin, polymyxin B (PMB), and the delafloxacin–PMB combination.

4.1 MIC Results

The MIC values of delafloxacin and PMB against *S. aureus* and *P. aeruginosa* are presented in Table 4.1. MIC values were determined for single-antibiotic treatments and for combination treatments. For the combination treatment, the MIC value of each antibiotic was recorded in the presence of the other antibiotic. These MIC values were used as the basis for preparing the concentration series used in the time-kill assays. Biofilm treatment assays were performed using higher treatment concentration series, as described in the Methodology chapter.

Table 4.1: MIC values of delafloxacin and PMB against *S. aureus* and *P. aeruginosa* when used alone and in combination. Values are expressed in µg/mL.

Bacterial strain	**Assay**	**Antibiotic**	**Partner**	**MIC**
S. aureus	Alone	Delafloxacin	None	0.42
S. aureus	Alone	PMB	None	0.15
S. aureus	Combination	Delafloxacin	PMB	0.019
S. aureus	Combination	PMB	Delafloxacin	0.015
P. aeruginosa	Alone	Delafloxacin	None	0.190
P. aeruginosa	Alone	PMB	None	0.190
P. aeruginosa	Combination	Delafloxacin	PMB	0.090
P. aeruginosa	Combination	PMB	Delafloxacin	0.090

As shown in Table 4.1, delafloxacin and PMB showed measurable inhibitory activity against both bacterial species. For *S. aureus*, the MIC values of both antibiotics were lower in the combination assay compared with the corresponding single-antibiotic conditions. For *P. aeruginosa*, delafloxacin and PMB showed similar MIC values when used alone, and both antibiotics showed lower MIC values in the combination assay.

MBC assessment was performed for selected conditions as part of the experimental workflow. However, the main quantitative comparison in this thesis was based on MIC values, time-kill assays, and biofilm treatment results. Since statistical

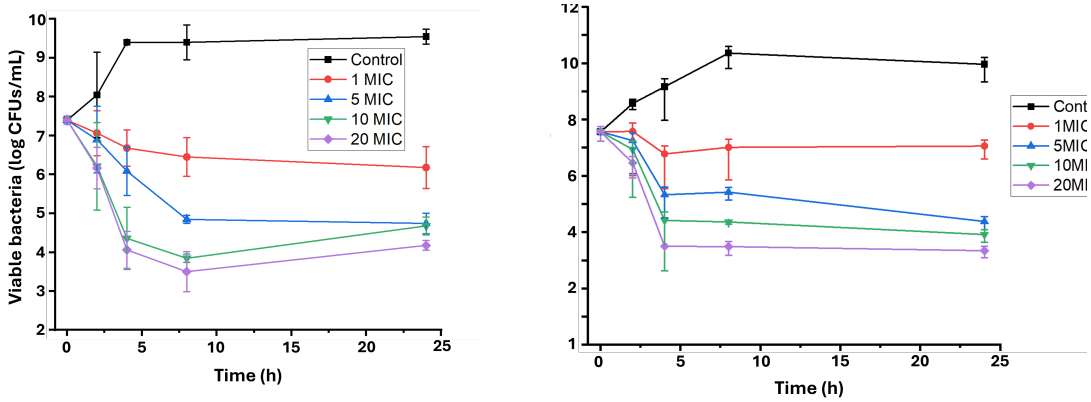
significance testing was not performed, differences between treatment groups were interpreted based on observed trends in mean values and standard deviations.

4.2 Time-Kill Assay Results

Time-kill assays were performed to evaluate the effect of delafloxacin, PMB, and their combination on planktonic bacterial viability over time. Viable counts were determined at 0, 2, 4, 8, and 24 h and expressed as CFU/mL. Antibiotic concentrations were prepared as multiples of the MIC values shown in Table 4.1. For single-antibiotic treatments, delafloxacin and PMB were tested at 1×, 5×, 10×, and 20× MIC. For combination treatments, both antibiotics were added together at the corresponding combination concentrations.

4.2.1 Time-Kill Assay of *S. aureus*

The time-kill results for *S. aureus* single-antibiotic treatments are shown in Figure 4.1, while the combination treatment is shown separately in Figure 4.2. The untreated *S. aureus* control showed bacterial growth over time in all three time-kill experiments. In contrast, antibiotic-treated samples generally showed lower viable counts compared with the untreated control.
Delafloxacin treatment reduced *S. aureus* viability compared with the untreated control. The reduction was more pronounced at higher concentrations, especially at 10× and 20× MIC (Figure 4.1a). PMB-treated samples also showed lower viable counts than the control, although the reduction was less pronounced than delafloxacin in some conditions (Figure 4.1b). The delafloxacin–PMB combination reduced *S. aureus* viability, with the lowest viable counts observed at the highest tested concentration (Figure 4.2).



(a) Delafloxacin (b) PMB

Figure 4.1: Time-kill curves of *S. aureus* treated with single antibiotics: (a) delafloxacin and (b) PMB. Viable bacterial counts were measured at 0, 2, 4, 8, and 24 h. Results are expressed as mean $\log_{10}$ CFU/mL with standard deviation from three replicate measurements.

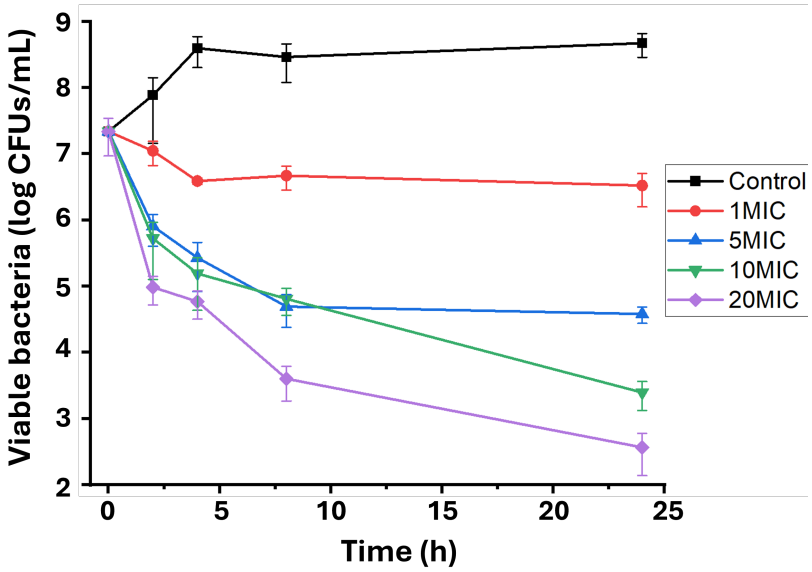
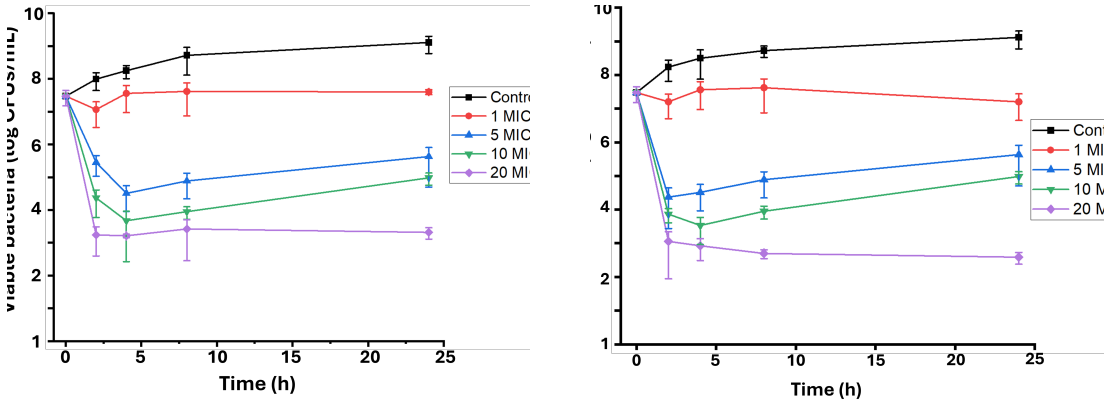


Figure 4.2: Time-kill curve of *S. aureus* treated with the delafloxacin–PMB combination. Viable bacterial counts were measured at 0, 2, 4, 8, and 24 h. Results are expressed as mean $\log_{10}$ CFU/mL with standard deviation from three replicate measurements.

4.2.2 Time-Kill Assay of *P. aeruginosa*

The time-kill results for *P. aeruginosa* single-antibiotic treatments are shown in Figure 4.3, while the combination treatment is shown separately in Figure 4.4. The untreated *P. aeruginosa* control showed bacterial growth over time. Antibiotic-treated samples generally showed lower viable counts than the untreated control. Delafloxacin treatment reduced viable counts compared with the untreated control, with a stronger reduction observed at higher concentrations (Figure 4.3a). PMB treatment also reduced *P. aeruginosa* viability, particularly at 5×, 10×, and 20× MIC (Figure 4.3b). The delafloxacin–PMB combination produced lower viable counts than the untreated control, with the lowest values observed at the highest tested concentration (Figure 4.4).



(a) Delafloxacin (b) PMB

Figure 4.3: Time-kill curves of *P. aeruginosa* treated with single antibiotics: (a) delafloxacin and (b) PMB. Viable bacterial counts were measured at 0, 2, 4, 8, and 24 h. Results are expressed as mean $\log_{10}$ CFU/mL with standard deviation from three replicate measurements.

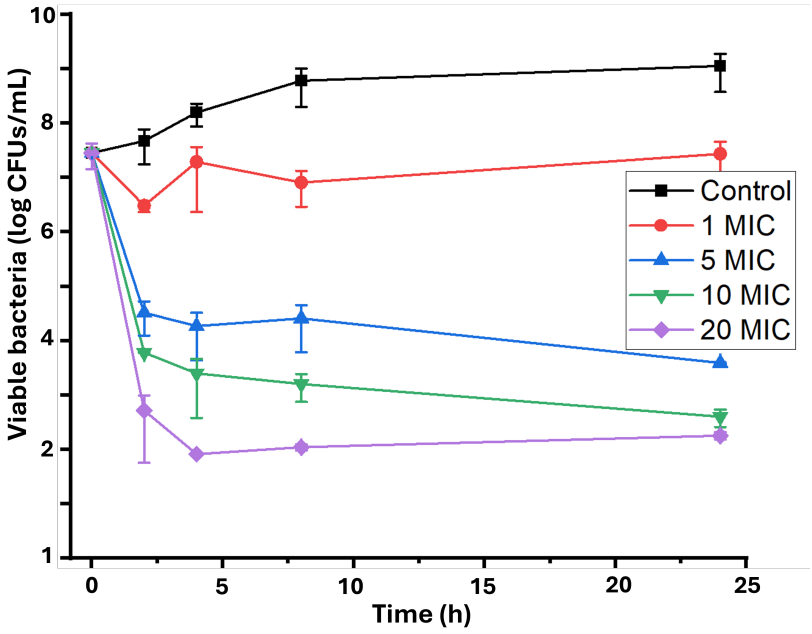


Figure 4.4: Time-kill curve of *P. aeruginosa* treated with the delafloxacin–PMB combination. Viable bacterial counts were measured at 0, 2, 4, 8, and 24 h. Results are expressed as mean $\log_{10}$ CFU/mL with standard deviation from three replicate measurements.

Overall, the time-kill assays showed that antibiotic-treated samples generally had lower CFU/mL values than untreated controls. For both *S. aureus* and *P. aeruginosa*, the reduction in bacterial viability appeared stronger at higher antibiotic concentrations. Since statistical testing was not performed, these results are interpreted as observed trends rather than statistically confirmed differences. Detailed

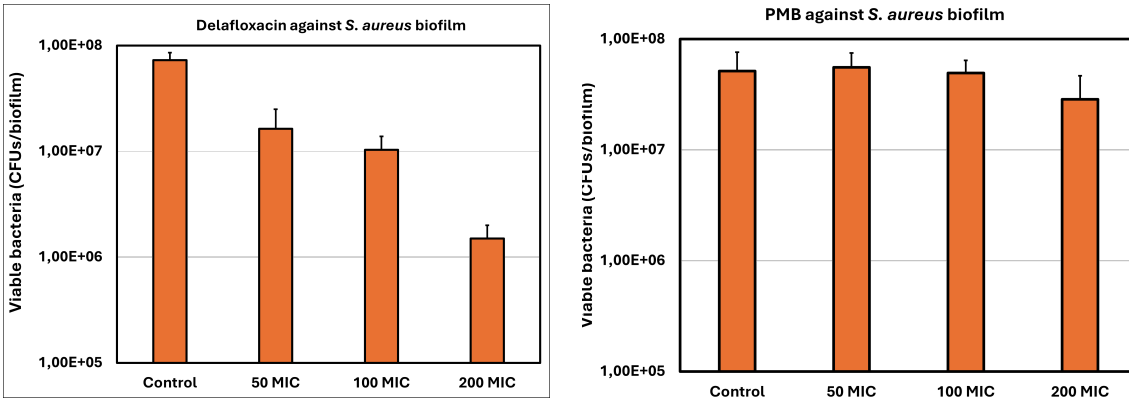
interpretation of the differences between single and combination treatments is presented in the Discussion chapter.

4.3 Biofilm Treatment Results

Biofilm treatment was performed to evaluate the effect of delafloxacin, PMB, and their combination on biofilm-associated *S. aureus* and *P. aeruginosa*. Biofilm viability was determined by recovering biofilm-associated cells, followed by serial dilution and plating. The results are expressed as CFU/biofilm. Biofilm treatments were performed using higher treatment concentration series than the planktonic time-kill assays.

4.3.1 Biofilm Treatment of *S. aureus*

The effect of delafloxacin and PMB single-antibiotic treatments on *S. aureus* biofilms is shown in Figure 4.5, while the combination treatment is shown separately in Figure 4.6. The untreated control biofilms showed high viable counts, confirming successful biofilm formation under the tested conditions.
Delafloxacin treatment reduced the number of viable biofilm-associated *S. aureus* cells compared with the untreated control, with the lowest viable counts observed at 200× MIC (Figure 4.5a). PMB treatment also reduced viable biofilm-associated cells compared with the untreated control, although the reduction was less pronounced than with delafloxacin (Figure 4.5b). The combination treatment produced a clear reduction in CFU/biofilm, with the lowest viable counts observed at the highest tested concentration (Figure 4.6).



(a) Delafloxacin (b) PMB

Figure 4.5: Viable counts of *S. aureus* biofilms after single-antibiotic treatment with (a) delafloxacin and (b) PMB. Results are expressed as mean CFU/biofilm with standard deviation from three replicate cover glasses.

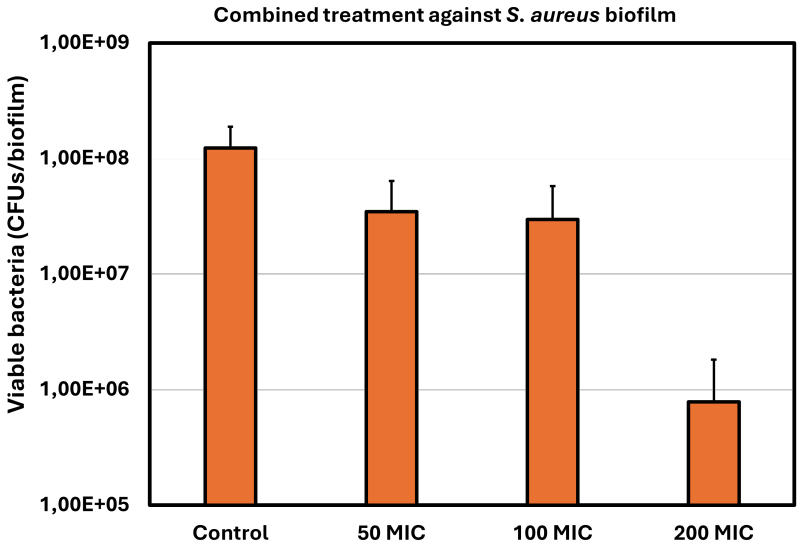
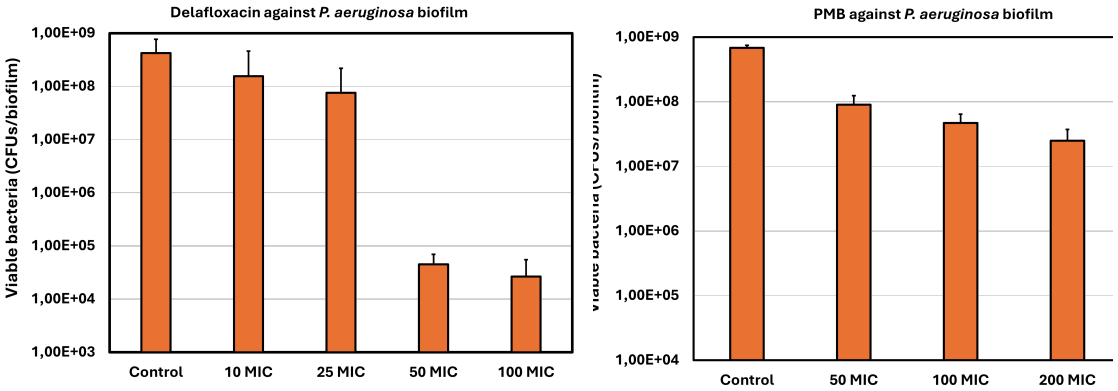


Figure 4.6: Viable counts of *S. aureus* biofilms after treatment with the delafloxacin–PMB combination. Results are expressed as mean CFU/biofilm with standard deviation from three replicate cover glasses.

4.3.2 Biofilm Treatment of *P. aeruginosa*

The effect of delafloxacin and PMB single-antibiotic treatments on *P. aeruginosa* biofilms is shown in Figure 4.7, while the combination treatment is shown separately in Figure 4.8. The untreated control biofilms showed high viable counts, indicating biofilm formation under untreated conditions.
Delafloxacin treatment reduced viable *P. aeruginosa* biofilm-associated cells compared with the untreated control, with lower viable counts observed at 50× and 100× MIC (Figure 4.7a). PMB treatment also reduced viable counts compared with the untreated control, although the reduction was less pronounced than the reduction observed with delafloxacin at higher concentrations (Figure 4.7b). The combination treatment produced lower CFU/biofilm values at higher treatment concentrations, with the lowest viable counts observed at the highest tested concentration (Figure 4.8).



(a) Delafloxacin (b) PMB

Figure 4.7: Viable counts of *P. aeruginosa* biofilms after single-antibiotic treatment with (a) delafloxacin and (b) PMB. Results are expressed as mean CFU/biofilm with standard deviation from three replicate cover glasses.

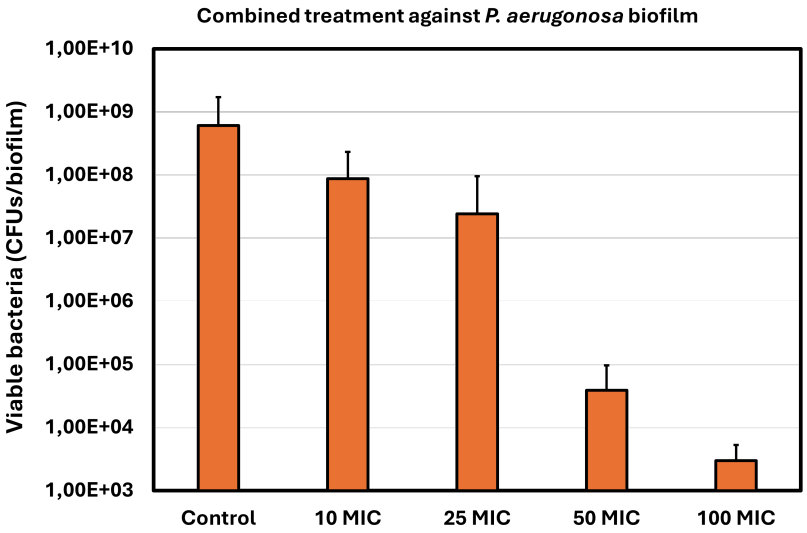


Figure 4.8: Viable counts of *P. aeruginosa* biofilms after treatment with the delafloxacin–PMB combination. Results are expressed as mean CFU/biofilm with standard deviation from three replicate cover glasses.

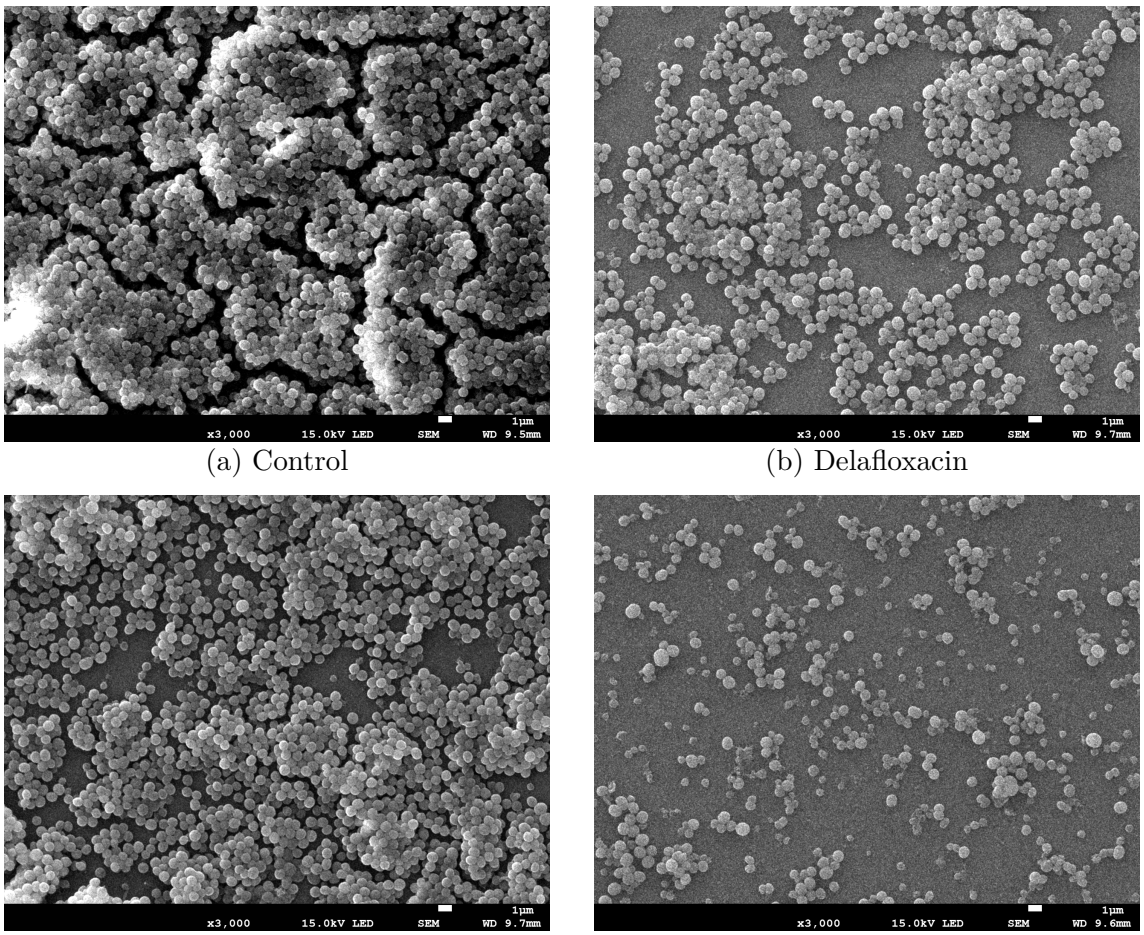
Overall, antibiotic treatment reduced the number of viable biofilm-associated cells compared with untreated controls. For *S. aureus*, delafloxacin and the combination treatment showed greater reductions than PMB alone. For *P. aeruginosa*, delafloxacin and the combination treatment produced lower viable counts at higher treatment concentrations, while PMB alone also reduced biofilm viability but to a lesser extent. Since statistical significance testing was not performed, these differences are described as observed trends. The interpretation of these treatment effects is discussed further in the Discussion chapter.

4.4 Scanning Electron Microscopy Analysis

Scanning electron microscopy (SEM) was used to examine morphological changes in untreated and antibiotic-treated biofilms. The SEM images were compared based on bacterial surface coverage, cell arrangement, aggregation, and visible biofilm structure. The analysis was qualitative and focused on visible differences between untreated control biofilms and biofilms treated with delafloxacin, PMB, and the delafloxacin–PMB combination for both *S. aureus* and *P. aeruginosa*. In this section, the terms cocci and rods refer to the typical spherical morphology of *S. aureus* cells and rod-shaped morphology of *P. aeruginosa* cells, respectively.

4.4.1 SEM Analysis of *S. aureus* Biofilms

The untreated *S. aureus* control biofilm showed dense surface colonization with many cocci attached to the surface (Figure 4.9a). The cells were closely packed and appeared as clustered aggregates, indicating strong bacterial attachment and biofilm formation under untreated conditions. The bacterial coverage was relatively continuous across the examined surface.
After delafloxacin treatment, the *S. aureus* biofilm showed reduced bacterial coverage compared with the untreated control (Figure 4.9b). The cocci appeared less densely packed, and fewer compact clusters were visible on the surface. This indicated a visible reduction in attached biofilm-associated cells after treatment.
PMB-treated *S. aureus* biofilms still showed bacterial aggregation on the surface, although the biofilm appeared less dense than the untreated control (Figure 4.9c). Attached cocci were still visible, suggesting that PMB treatment did not remove the biofilm-associated cells completely under the tested condition.
The delafloxacin–PMB combination treatment showed reduced surface coverage compared with the untreated control (Figure 4.9d). The bacterial cells appeared more scattered, and the dense clustered structure seen in the control biofilm was less evident. However, attached cells were still present, showing that complete removal of the *S. aureus* biofilm was not observed.



(a) Control (b) Delafloxacin

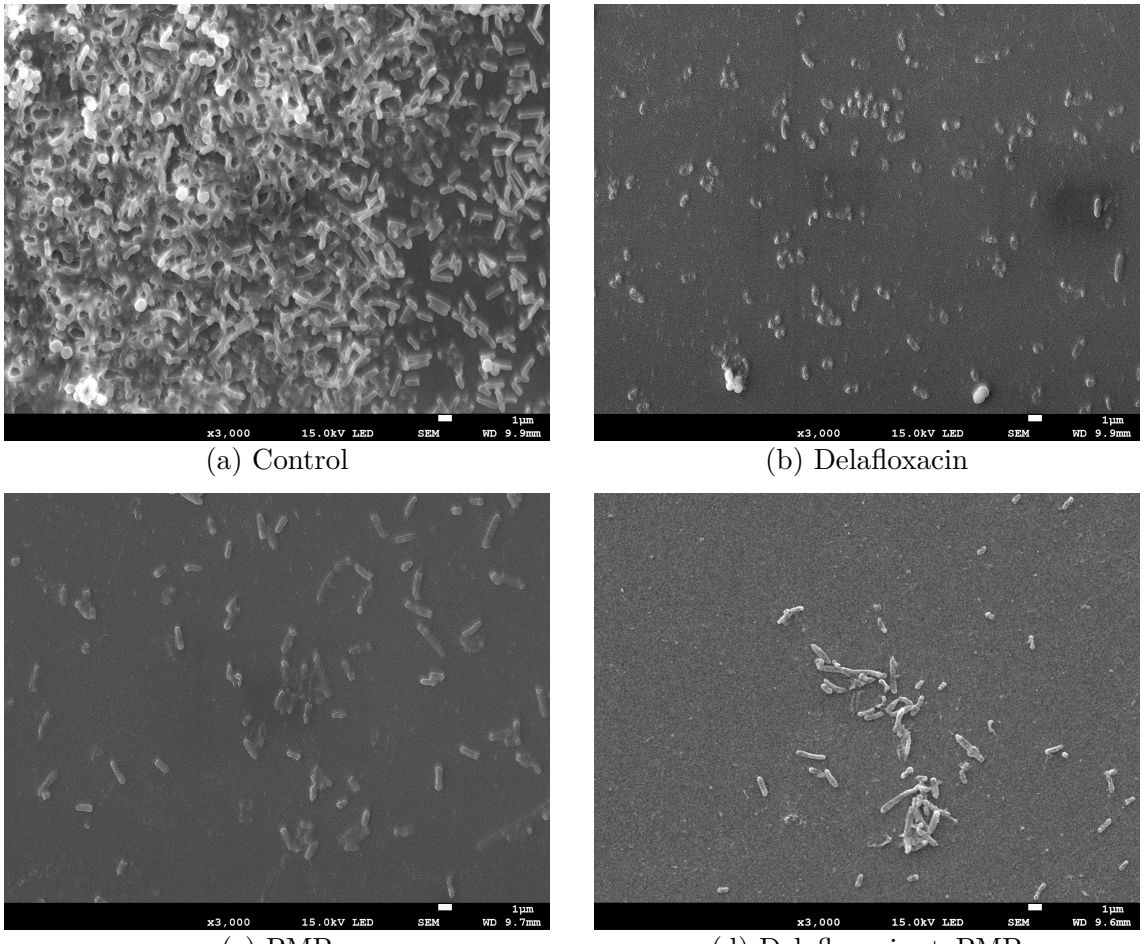
(c) PMB (d) Delafloxacin + PMB

Figure 4.9: Representative SEM images of *S. aureus* biofilms under untreated and antibiotic-treated conditions. The untreated control showed dense attachment of clustered cocci, while treated biofilms showed reduced surface coverage, less compact aggregation, and altered biofilm morphology.

4.4.2 SEM Analysis of *P. aeruginosa* Biofilms

The untreated *P. aeruginosa* control biofilm showed dense surface coverage with many rod-shaped bacterial cells attached to the surface (Figure 4.10a). The cells were distributed across the examined area and appeared closely associated with the surface, indicating successful biofilm formation under untreated conditions.
After delafloxacin treatment, the *P. aeruginosa* biofilm showed reduced bacterial attachment compared with the untreated control (Figure 4.10b). Fewer rod-shaped cells were visible, and the remaining cells appeared more scattered across the surface. The apparent differences in cell shape and brightness in the SEM images may be influenced by imaging perspective, sample preparation, and surface coating, so these observations were interpreted qualitatively rather than as direct evidence of a specific structural change.
PMB-treated *P. aeruginosa* biofilms also showed reduced surface coverage compared with the untreated control (Figure 4.10c). In the examined SEM field, fewer rod-

shaped cells were visible and the remaining cells appeared scattered across the surface. However, because SEM provides qualitative information from selected surface areas, this observation should be interpreted together with the CFU-based biofilm results, where PMB showed a smaller reduction in viable counts compared with delafloxacin and the combination treatment.
The delafloxacin–PMB combination treatment showed the most pronounced visible reduction in bacterial surface coverage among the examined SEM images (Figure 4.10d). Only scattered rod-shaped cells remained on the surface, and the dense bacterial coverage observed in the untreated control was no longer visible. The brighter appearance of some areas in the control and combination images may be related to differences in surface topography, coating, or imaging conditions rather than only to biological differences. Therefore, these SEM results were interpreted together with the quantitative CFU/biofilm data.



(a) Control (b) Delafloxacin

(c) PMB (d) Delafloxacin + PMB

Figure 4.10: Representative SEM images of *P. aeruginosa* biofilms under untreated and antibiotic-treated conditions. The untreated control showed dense attachment of rod-shaped cells, while treated biofilms showed reduced surface coverage and altered bacterial distribution. The delafloxacin–PMB combination showed the most pronounced visible reduction in attached cells.

5

Discussion

This chapter discusses the main findings of the study in relation to the antibacterial activity of delafloxacin, polymyxin B (PMB), and their combination against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The results are discussed based on MIC values, time-kill assays, biofilm treatment assays, and SEM observations.

5.1 Overview of Main Findings

The aim of this study was to evaluate the antibacterial effect of delafloxacin and PMB, alone and in combination, against planktonic and biofilm-associated *S. aureus* and *P. aeruginosa*. Overall, the results showed that both antibiotics reduced bacterial viability under the tested conditions, although the strength of the effect differed depending on bacterial species, antibiotic treatment, concentration, and growth state.

The MIC results showed that delafloxacin and PMB had inhibitory activity against both bacterial species. In the combination assay, lower MIC values were observed compared with the corresponding single-antibiotic conditions. This indicates that the presence of the second antibiotic influenced the inhibitory response. However, because formal synergy testing was not performed, the combination effect should be interpreted as improved activity under the tested conditions rather than confirmed synergy.

The time-kill assays showed that antibiotic-treated planktonic cultures generally had lower viable bacterial counts than untreated controls. In several treatment groups, higher antibiotic concentrations resulted in stronger reductions in CFU/mL. The biofilm treatment assays showed that biofilm-associated cells remained recoverable after treatment, even when higher antibiotic concentration series were used. This supports the increased antibiotic tolerance usually associated with biofilm growth.

Statistical significance testing was not performed in this study. Therefore, the differences between treatment groups were interpreted based on observed trends in mean CFU values and standard deviations rather than formal statistical comparisons.

5.2 Antibacterial Activity Based on MIC Results

MIC determination provided an initial comparison of the inhibitory activity of delafloxacin and PMB against the tested bacterial species. For *S. aureus*, both antibiotics showed measurable inhibitory activity when used alone. In the combination

assay, the MIC values of both delafloxacin and PMB were lower than their corresponding single-antibiotic MIC values.
For *P. aeruginosa*, delafloxacin and PMB showed similar MIC values when used alone. The combination assay again showed lower MIC values for both antibiotics compared with the single-antibiotic conditions. This suggests that the combination affected bacterial growth inhibition more strongly than either antibiotic alone under the MIC assay conditions.
Although the MIC results are useful for comparing inhibitory activity, they only represent planktonic growth inhibition at a fixed endpoint. MIC values alone do not show whether bacteria are killed over time, nor do they show how bacteria respond when growing as surface-associated biofilms. Therefore, time-kill assays and biofilm treatment assays were needed to further evaluate the antibacterial effects of the treatments.

5.3 Time-Dependent Killing of Planktonic Cells

Time-kill assays were performed to examine changes in bacterial viability over time after antibiotic exposure. In untreated control cultures, bacterial counts generally increased or remained high over time, showing that the bacteria were able to grow under the assay conditions. In contrast, antibiotic-treated samples showed lower CFU/mL values compared with untreated controls.
For *S. aureus*, delafloxacin reduced viable counts in a concentration-dependent manner. PMB also reduced *S. aureus* viability compared with the untreated control, although the extent of reduction differed between concentrations. The delafloxacin–PMB combination also reduced bacterial viability, with stronger effects generally observed at higher concentrations.
For *P. aeruginosa*, delafloxacin and PMB both reduced viable counts compared with the untreated control. The combination treatment also reduced bacterial viability, especially at higher concentrations. These results show that the tested antibiotics affected planktonic cells of both bacterial species, but the extent of killing varied between treatment conditions.
In some time-kill curves, viable counts increased again after an initial reduction, for example in some lower or intermediate concentration treatments. This recovery may indicate that a fraction of the bacterial population survived early antibiotic exposure and was able to regrow during the later incubation period. Possible explanations include survival of more tolerant subpopulations, reduced antibiotic activity over time, or adaptation of surviving cells during prolonged exposure. Since no additional mechanistic experiment was performed, this interpretation should be considered as a possible explanation rather than a confirmed mechanism.
Overall, the time-kill results supported the MIC findings by showing that antibiotic exposure reduced bacterial survival over time. The concentration-dependent reduction observed in several treatment groups indicates that higher antibiotic levels produced stronger antibacterial effects under planktonic conditions. However, because statistical testing was not performed, these differences should be interpreted as observed trends rather than statistically confirmed differences.

5.4 Effect of Antibiotic Treatment on Biofilms

Biofilm-associated bacteria are more difficult to eliminate than planktonic bacteria because the biofilm mode of growth provides protection against antimicrobial treatment. This protection can be related to reduced antibiotic penetration, slower bacterial growth, altered metabolism, and the presence of extracellular matrix material [2, 16]. Therefore, the biofilm treatment assay was important for evaluating antibiotic activity under a more tolerant growth condition.

This pattern was also observed in the present study. In the time-kill assays, planktonic *S. aureus* and *P. aeruginosa* showed reductions in viable counts after antibiotic exposure, especially at higher concentrations. In the biofilm treatment assays, viable biofilm-associated cells were also reduced, but they still remained recoverable after treatment. This shows that treatment reduced bacterial survival in both growth states, but biofilm-associated cells were not completely eliminated under the tested conditions.

These findings are consistent with previous studies showing that biofilm-associated bacteria are generally more tolerant to antibiotic treatment than planktonic cells. Ceri et al. showed that biofilm susceptibility testing can give different results from standard planktonic susceptibility testing, supporting the need to evaluate antibiotics under biofilm conditions [9]. Hall and Mah also described that biofilm tolerance can result from several mechanisms, including reduced penetration, altered metabolism, and survival of tolerant subpopulations [2]. Therefore, the incomplete eradication of biofilm-associated cells observed in this study agrees with the general understanding that biofilms are difficult to eliminate using antibiotic treatment alone.

For *S. aureus* biofilms, delafloxacin reduced the number of viable biofilm-associated cells compared with the untreated control. The reduction was stronger at higher treatment concentrations, showing a concentration-dependent effect. PMB treatment also reduced viable counts, but the reduction was less pronounced compared with delafloxacin and the combination treatment under the tested conditions.

The weaker effect of PMB against *S. aureus* may be related to its main antibacterial target. PMB mainly acts on the outer membrane of Gram-negative bacteria by interacting with lipopolysaccharides [12]. Since *S. aureus* is Gram-positive and lacks the same Gram-negative outer membrane structure, PMB would be expected to have less direct activity against *S. aureus* than against Gram-negative bacteria such as *P. aeruginosa*. This may explain why PMB appeared less effective than delafloxacin against *S. aureus* biofilms in this study.

The delafloxacin–PMB combination reduced viable *S. aureus* biofilm-associated cells, particularly at higher concentrations. However, complete eradication was not observed. This shows that although the combination treatment improved the reduction of viable biofilm-associated cells, *S. aureus* biofilms remained difficult to eliminate.

For *P. aeruginosa* biofilms, delafloxacin treatment strongly reduced viable counts at higher concentrations. PMB also reduced biofilm-associated cells, although the reduction was more moderate compared with delafloxacin and the combination treatment. The delafloxacin–PMB combination showed a marked reduction in CFU/biofilm, especially at higher concentrations.

The observed PMB effect against *P. aeruginosa* is consistent with its known activity against Gram-negative bacteria and with previous work showing interaction between PMB and *P. aeruginosa* planktonic and biofilm-associated cells [12, 13]. However, in the present study, PMB alone did not completely eliminate *P. aeruginosa* biofilms. This is also consistent with the general concept that biofilm-associated cells can remain tolerant to antibiotics even when the antibiotic is active against the planktonic form [2, 16].
The biofilm results show an important difference between planktonic and biofilm-associated bacteria. While both planktonic and biofilm-associated cells were affected by antibiotic treatment, biofilm-associated bacteria remained recoverable after treatment despite the use of higher concentration series in the biofilm assays. This supports the importance of testing antibiotics under both planktonic and biofilm-associated conditions, since planktonic susceptibility alone may not fully predict biofilm treatment response [9, 2].

5.5 SEM-Based Observation of Biofilm Morphology

SEM analysis was used to qualitatively examine the morphology and surface structure of untreated and antibiotic-treated biofilms. The SEM images supported the CFU-based biofilm results by showing visible differences in bacterial attachment, surface coverage, aggregation, and biofilm organization after antibiotic treatment.
For *S. aureus*, the untreated control biofilm showed dense surface colonization with clustered cocci attached to the surface. This morphology indicated strong bacterial attachment and biofilm formation under untreated conditions. After antibiotic treatment, the biofilm morphology changed from dense and compact surface coverage to a less organized structure with fewer visible attached cells. Delafloxacin-treated biofilms showed reduced bacterial coverage and less compact clustering compared with the untreated control. PMB-treated biofilms still showed visible coccal aggregation, suggesting that PMB did not alter the *S. aureus* biofilm morphology as strongly as delafloxacin under the tested conditions. The combination-treated biofilm showed reduced surface coverage and more scattered cells compared with the control, although attached cells were still present.
For *P. aeruginosa*, the untreated control biofilm showed dense surface coverage with many attached rod-shaped cells. After antibiotic treatment, the number of visible attached rods was reduced and the remaining cells appeared more scattered across the surface. Delafloxacin-treated biofilms showed reduced bacterial attachment compared with the untreated control. PMB-treated biofilms also showed reduced surface coverage in the examined SEM field, with fewer visible rod-shaped cells compared with the untreated control.
However, the SEM observation for PMB should be interpreted carefully because SEM is qualitative and represents selected surface areas, whereas CFU/biofilm results measure viable cells recovered from the whole cover glass. Therefore, the SEM and CFU data together suggest that PMB affected the visible surface morphology of *P. aeruginosa* biofilms, but viable biofilm-associated cells still remained after

treatment.
The combination-treated *P. aeruginosa* biofilm showed the most pronounced visible reduction in surface coverage, with only scattered rod-shaped cells remaining. This does not mean that the cells were completely undetectable or fully removed. Instead, the SEM images showed visibly fewer attached cells in the examined areas. This observation was consistent with the CFU-based biofilm treatment results, where the combination treatment reduced viable *P. aeruginosa* biofilm-associated cells. Together, the SEM observations suggest that antibiotic treatment changed biofilm morphology by reducing surface coverage, altering bacterial distribution, and decreasing compact biofilm organization.
Overall, SEM observations supported the biofilm treatment results by showing visible morphological differences after antibiotic exposure. However, SEM is qualitative and represents limited examined areas of the biofilm surface. Therefore, SEM observations should be interpreted together with the quantitative CFU/biofilm data.

5.6 Comparison Between *S. aureus* and *P. aeruginosa*

The two bacterial species showed different responses to antibiotic treatment. These differences may be related to differences in cell envelope structure, biofilm organization, and species-specific antibiotic susceptibility. *S. aureus* is Gram-positive, while *P. aeruginosa* is Gram-negative, and these structural differences can influence how bacteria respond to antimicrobial agents.
For *S. aureus*, delafloxacin reduced planktonic and biofilm-associated cells, while PMB showed a weaker effect in the biofilm treatment assay. This difference may be related to the mechanism of PMB. PMB mainly targets lipopolysaccharides in the outer membrane of Gram-negative bacteria [12]. Since *S. aureus* is Gram-positive and does not have the same Gram-negative outer membrane structure, PMB would be expected to have less direct activity against *S. aureus* than against *P. aeruginosa*. In contrast, delafloxacin targets bacterial DNA replication through inhibition of DNA gyrase and topoisomerase IV, which may explain why delafloxacin showed activity against both bacterial species [11].
For *P. aeruginosa*, delafloxacin showed strong activity in the biofilm treatment assay at higher concentrations. PMB also reduced viable counts, but the effect was more moderate than delafloxacin and the combination treatment in the biofilm assay. In the SEM observations, PMB-treated biofilms showed reduced surface coverage in the examined field, while CFU/biofilm results showed that viable cells still remained at higher levels than in delafloxacin and combination treatments. The combination treatment produced a strong reduction in the CFU-based biofilm results and showed reduced surface coverage in the SEM observations.
The results highlight that antibiotic activity depends not only on the bacterial species but also on whether cells are planktonic or biofilm-associated. This supports the need to evaluate antibacterial treatment under both growth conditions.

5.7 Limitations of the Study

There were several limitations in this study. First, biofilm experiments can show biological variability due to differences in initial attachment, biofilm thickness, and recovery efficiency during sonication and vortexing. Although replicate measurements were used, variation in CFU/biofilm values was observed.
Second, statistical significance testing was not performed. Therefore, the observed differences between treatment groups cannot be described as statistically significant. The results were interpreted based on trends in mean CFU values and standard deviations, and the conclusions should be understood within this limitation.
Third, the study focused on selected antibiotic concentrations based on MIC values. Testing additional intermediate concentrations could provide a more detailed understanding of dose-dependent effects, especially for biofilm-associated cells.
Fourth, SEM analysis was used as a qualitative method to observe biofilm morphology and surface structure. Since SEM images represent limited examined areas of the biofilm surface, they should be interpreted together with the quantitative CFU/biofilm results.
Finally, although the combination treatment showed improved antibacterial activity in several assays, a formal synergy analysis was not performed. Therefore, the combination effect should be described as improved or enhanced activity rather than confirmed synergy.

5.8 Future Perspectives

Future work could include dual-species biofilm experiments with *S. aureus* and *P. aeruginosa* to better represent polymicrobial wound-associated biofilms. Since these two species are often found together in chronic wound infections, a dual-species model would allow evaluation of how bacterial interaction affects biofilm formation, antibiotic tolerance, and response to delafloxacin–PMB combination treatment.
Additional SEM imaging from independent biological replicates could also be performed to strengthen the comparison between quantitative CFU results and visual biofilm morphology. This would help determine whether the observed surface changes are consistent across different biofilm samples and treatment conditions.
Confocal microscopy with live/dead staining could be included to evaluate biofilm viability and structure in more detail. This would provide complementary information to SEM, since SEM shows surface morphology but does not directly distinguish live and dead cells.
A formal synergy analysis, such as a checkerboard assay and fractional inhibitory concentration index (FICI) calculation, could be performed to determine whether the delafloxacin–PMB combination is synergistic, additive, or indifferent.
Further work could also evaluate longer biofilm maturation times, additional antibiotic concentrations, and repeated treatment exposure. These experiments would help determine whether the antibiotic combination is effective against more mature and treatment-tolerant biofilms.

6
Conclusion

This thesis evaluated the antibacterial activity of delafloxacin and polymyxin B against *Staphylococcus aureus* and *Pseudomonas aeruginosa* under planktonic and biofilm-associated conditions. The results showed that both antibiotics reduced bacterial viability, with stronger effects generally observed at higher concentrations. The response depended on the bacterial species, antibiotic treatment, and growth condition.

Planktonic cells were generally more affected by antibiotic exposure than biofilm-associated cells. In the biofilm treatment assays, viable bacteria remained recoverable after treatment, showing that biofilm-associated cells were more tolerant and more difficult to eliminate under the tested conditions. The combination treatment reduced bacterial survival in several conditions and showed stronger effects than single-antibiotic treatments, especially at higher concentrations. However, because formal synergy testing was not performed, the combination effect should be interpreted as improved antibacterial activity rather than confirmed synergy.

SEM analysis supported the CFU-based results by showing visible differences between untreated and treated biofilms. Untreated biofilms showed dense bacterial attachment, while treated samples showed reduced bacterial coverage and altered biofilm structure. The combination treatment showed visible changes in biofilm structure, particularly for *P. aeruginosa*.

Overall, the findings suggest that delafloxacin and polymyxin B have antibacterial activity against both planktonic and biofilm-associated bacteria, but complete biofilm eradication was not achieved under the tested conditions. Future studies including additional independent biological replicates, statistical significance testing, confocal microscopy, dual-species biofilm models, and formal synergy analysis would strengthen the evaluation of this antibiotic combination.

Bibliography

[1] Hans-Curt Flemming, Jost Wingender, Ulrich Szewzyk, Peter Steinberg, Scott A. Rice, and Staffan Kjelleberg. Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology*, 14(9):563–575, 2016.

[2] Clayton W. Hall and Thien-Fah Mah. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiology Reviews*, 41(3):276–301, 2017.

[3] Steven L. Percival, Sara M. McCarty, and Benjamin Lipsky. Biofilms and wounds: an overview of the evidence. *Advances in Wound Care*, 4(7):373–381, 2015.

[4] M. Malone, T. Bjarnsholt, A. J. McBain, G. A. James, P. Stoodley, D. Leaper, M. Tachi, G. Schultz, T. Swanson, and R. D. Wolcott. The prevalence of biofilms in chronic wounds: a systematic review and meta-analysis of published data. *Journal of Wound Care*, 26(1):20–25, 2017.

[5] Kristine Gjødsbøl, Jens Jørgen Christensen, Tonny Karlsmark, Bo Jørgensen, Bjarke M. Klein, and Karen A. Krogfelt. Multiple bacterial species reside in chronic wounds: a longitudinal study. *International Wound Journal*, 3(3):225–231, 2006.

[6] Mustafa Fazli, Thomas Bjarnsholt, Klaus Kirketerp-Møller, Bo Jørgensen, Anders Schou Andersen, Karen A. Krogfelt, Michael Givskov, and Tim Tolker-Nielsen. Nonrandom distribution of pseudomonas aeruginosa and staphylococcus aureus in chronic wounds. *Journal of Clinical Microbiology*, 47(12):4084–4089, 2009.

[7] Derek E. Moormeier and Kenneth W. Bayles. Staphylococcus aureus biofilm: a complex developmental organism. *Molecular Microbiology*, 104(3):365–376, 2017.

[8] Cynthia Ryder, Matthew Byrd, and Daniel J. Wozniak. Role of polysaccharides in pseudomonas aeruginosa biofilm development. *Current Opinion in Microbiology*, 10(6):644–648, 2007.

[9] H. Ceri, M. E. Olson, C. Stremick, R. R. Read, D. Morck, and A. Buret. The calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *Journal of Clinical Microbiology*, 37(6):1771–1776, 1999.

[10] M. A. Pfaller, H. S. Sader, P. R. Rhomberg, and R. K. Flamm. In vitro activity of delafloxacin against contemporary bacterial pathogens from the united states and europe, 2014. *Antimicrobial Agents and Chemotherapy*, 61(4), 2017.

[11] Benjamin T. Mogle, Jason M. Steele, Sarah J. Thomas, Kevin H. Bohan, and William D. Kufel. Clinical review of delafloxacin: a novel anionic fluoroquinolone. *Journal of Antimicrobial Chemotherapy*, 73(6):1439–1451, 2018.

[12] Sarita S. Mohapatra, Swayam Prava Dwibedy, and Ipsita Padhy. Polymyxins, the last-resort antibiotics: Mode of action, resistance emergence, and potential solutions. *Journal of Biosciences*, 46(3), 2021.

[13] M. R. Lima, G. F. Ferreira, W. R. Nunes Neto, J. M. Monteiro, Á. R. C. Santos, P. B. Tavares, Â. M. L. Denadai, and M. R. Q. Bomfim. Evaluation of the interaction between polymyxin b and pseudomonas aeruginosa biofilm and planktonic cells: Reactive oxygen species induction and zeta potential. *Antibiotics*, 8(2):49, 2019.

[14] Hans-Curt Flemming and Jost Wingender. The biofilm matrix. *Nature Reviews Microbiology*, 8(9):623–633, 2010.

[15] Karin Sauer, Paul Stoodley, Darla M. Goeres, Luanne Hall-Stoodley, Mette Burmølle, Philip S. Stewart, and Thomas Bjarnsholt. The biofilm life cycle: expanding the conceptual model of biofilm formation. *Nature Reviews Microbiology*, 20(10):608–620, 2022.

[16] Niels Høiby, Thomas Bjarnsholt, Michael Givskov, Søren Molin, and Oana Ciofu. Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents*, 35(4):322–332, 2010.

[17] Boo Shan Tseng, Wei Zhang, Joe J. Harrison, Tam P. Quach, Jisun Lee Song, Jon Penterman, Pradeep K. Singh, David L. Chopp, Aaron I. Packman, and Matthew R. Parsek. The extracellular matrix protects pseudomonas aeruginosa biofilms by limiting the penetration of tobramycin. *Environmental Microbiology*, 15(10):2865–2878, 2013.

[18] Maria del Mar Cendra, Núria Blanco-Cabra, Lucas Pedraz, and Eduard Torrents. Optimal environmental and culture conditions allow the in vitro coexistence of pseudomonas aeruginosa and staphylococcus aureus in stable biofilms. *Scientific Reports*, 9:16284, 2019.

[19] Pia Katharina Vestweber, Jana Wächter, Viktoria Planz, Nathalie Jung, and Maike Windbergs. The interplay of pseudomonas aeruginosa and staphylococcus aureus in dual-species biofilms impacts development, antibiotic resistance and virulence of biofilms in in vitro wound infection models. *PLOS ONE*, 19(5):e0304491, 2024.

[20] Emel Mataraci and Sibel Dosler. In vitro activities of antibiotics and antimicrobial cationic peptides alone and in combination against methicillin-resistant staphylococcus aureus biofilms. *Antimicrobial Agents and Chemotherapy*, 56(12):6366–6371, 2012.

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CHALMERS UNIVERSITY OF TECHNOLOGY
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