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Article

Evaluating the Efficacy of Delafloxacin and Combination Therapy Against *Staphylococcus aureus* Biofilms: Insights into Activity in Acidic Environments

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Abstract: Background: *Staphylococcus aureus* is a major contributor to biofilm-associated infections, which complicate the treatment of chronic conditions like osteomyelitis and prosthetic infections. Biofilms, characterized by bacterial communities encased in a protective matrix, hinder the effectiveness of antimicrobial agents and contribute to persistent infections. **Objective:** This study aimed to evaluate the activity of delafloxacin, a novel fluoroquinolone, against biofilm-forming *S. aureus* strains, including methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) isolates. **Methods:** Delafloxacin's efficacy was compared with traditional antibiotics (vancomycin, linezolid, doxycycline, sulfamethoxazole/trimethoprim, and rifampicin) using minimal inhibitory concentration (MIC) assays, including testing at different pH levels to simulate acidic biofilm conditions. Synergistic interactions between delafloxacin and other antimicrobials were assessed using the checkerboard method. Biofilm eradication was tested through minimal biofilm eradication concentration (MBEC) assays, biomass quantification, and cell viability (MTT assay). **Results:** The results showed that all isolates were susceptible to vancomycin and linezolid, with varying resistance to other antibiotics. Delafloxacin demonstrated potent activity, with MIC reductions observed in acidic conditions. Synergistic effects were minimal, with only a few isolates showing synergy with vancomycin and doxycycline. Biofilm eradication tests revealed limited success in eradicating biofilms at breakpoint concentrations. However, rifampicin in combination with delafloxacin significantly reduced biofilm biomass in some isolates. No significant changes in cell viability were noted across treatments. **Conclusion:** This study highlights delafloxacin's potential against biofilm-associated infections, particularly in acidic environments. Despite the challenges in biofilm eradication, delafloxacin combined with rifampicin shows promise for improved treatment strategies.

Keywords: *Staphylococcus aureus*; biofilm; delafloxacin; rifampicin

1. Introduction

Staphylococcus aureus plays a significant role in biofilm production, contributing to its persistence and pathogenicity, especially in chronic infections [1]. Biofilms are structured communities of bacterial cells enclosed in a self-produced polymeric matrix that adhere to surfaces and protect *S. aureus* from antimicrobial agents. The matrix limits the penetration of antibiotics, and the slow growth rate of bacteria within the biofilm further enhances resistance [2].

The leading cause of chronic osteomyelitis and prosthetic infections is *S. aureus*. Its ability to form biofilms significantly contributes to the persistence and difficulty in treating these conditions [3]. Surgical removal of the infected prosthetic device and debridement of infected bone tissue are necessary to manage these infections followed by long-term antibiotic therapy [4].

Treatment typically involves a combination of antibiotics, including those that can penetrate biofilms and target dormant bacteria [5]. Prolonged antibiotic therapy is often required to fully

eradicate the infection [6]. The best antibiotics against *S. aureus* biofilm are rifampicin and daptomycin, although other antibiotics have variable activity [7,8]. Although rifampicin is highly effective against biofilm-embedded *S. aureus* due to its ability to penetrate biofilms and target dormant bacteria, it should not be used as monotherapy because resistance can develop quickly [9]. Daptomycin is effective against biofilms, especially when used in combination with other antibiotics, including rifampicin [10].

Fluoroquinolones can be effective against *S. aureus*. However, their effectiveness against biofilm-associated infections can vary [11]. They have good tissue penetration and can reach biofilm-embedded bacteria [12]. However, the biofilm matrix can still limit their penetration to some extent. The mechanism of action on DNA gyrase and topoisomerase IV is effective against actively growing bacteria but less so against dormant cells within biofilms. To improve efficacy, quinolones are often used in combination with other antibiotics. For instance, combining quinolones with rifampicin can enhance the overall antibacterial effect and help prevent resistance [12,13].

Delafloxacin is a newer fluoroquinolone with promising activity against Methicillin-resistant *S. aureus* (MRSA), including biofilm-associated infections [14]. Delafloxacin even seems to be more active than daptomycin against the MRSA strain at equipotent concentrations [15]. Delafloxacin can be used orally, making it a very attractive drug in the treatment of orthopedic infections that require prolonged treatment, an advantage over daptomycin, vancomycin and other anti-MRSA drugs. Furthermore, delafloxacin shows greater activity in acidic environments, a common condition in biofilm [16]. Considering these aspects and the recommendations for combined therapy in the treatment of infections associated with biofilm, the objective of this study was to evaluate the activity of delafloxacin combined with other drugs in a biofilm model.

The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance. The current state of the research field should be carefully reviewed and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the principal conclusions. As far as possible, please keep the introduction comprehensible to scientists outside your particular field of research. References should be numbered in order of appearance and indicated by a numeral or numerals in square brackets—e.g., [1] or [2,3], or [4–6]. See the end of the document for further details on references.

2. Results

2.1. MIC Determination by Broth Microdilution and E-Test

Among the 12 *S. aureus* isolates, 10 were MRSA and 2 were MSSA. The MIC results are detailed in Table 1. All isolates were susceptible to vancomycin and linezolid. One isolate was resistant to doxycycline, while two isolates exhibited resistance to both SMX-TMP and rifampicin. Delafloxacin was tested using broth microdilution and E-test, and evaluated at different pH levels (7.4 and 5.5) (Table 1). In the E-test, 4 isolates were susceptible to delafloxacin, and the results were consistent with those obtained by broth microdilution. In 4 of the 12 isolates, the E-test showed a 1 dilution decrease in MIC compared to the broth microdilution at pH 7.4. At pH 5.5, there was a significant reduction in MIC compared to pH 7.4. The MIC decrease ranged from none (1 isolate), 1-fold (1 isolate), 2-fold (8 isolates), 3-fold (1 isolate), to 4-fold (1 isolate).

Table 1. Minimal inhibitory concentration (MIC) of several antibiotics, including delafloxacin with different pH of the medium, and E-test. The comparison of pH 7.4 with 5.5 is described as “number of dilutions” in times (1x, 2x, 3x and 4x).

Clinical Isolates	VAN	LNZ	SMX-TMP	DOX	RIF	DLX pH 7.4	DLX pH 5.5	DLX Etest	Dilutions pH 7.4 to 5.5
	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	
2	0.5 (S)	2.0 (S)	0.5 (S)	0.125 (S)	0.03 (S)	2.0 (R)	0.5 (R)	2.0 (R)	2x
3	0.5 (S)	2.0 (S)	1.0 (S)	0.125 (S)	0.03 (S)	2.0 (R)	0.5 (R)	2.0 (R)	2x
49	0.5 (S)	2.0 (S)	0.5 (S)	0.125 (S)	0.03 (S)	2.0 (R)	0.5 (R)	2.0 (R)	2x
41	0.5 (S)	1.0 (S)	32 (R)	4.0 (R)	2.0 (R)	0.25 (S)	0.03 (S)	0.125 (S)	3x
57	0.5 (S)	2.0 (S)	0.5 (S)	0.125 (S)	0.03 (S)	2.0 (R)	1.0 (R)	2.0 (R)	1x
71	0.5 (S)	4.0 (S)	0.5 (S)	0.125 (S)	0.03 (S)	2.0 (R)	0.5 (R)	2.0 (R)	2x
80	0.5 (S)	2.0 (S)	0.5 (S)	0.125 (S)	0.03 (S)	2.0 (R)	0.5 (R)	2.0 (R)	2x
102	0.5 (S)	4.0 (S)	0.5 (S)	0.25 (S)	0.03 (S)	2.0 (R)	0.5 (R)	2.0 (R)	2x
130	0.5 (S)	4.0 (S)	0.5 (S)	0.125 (S)	0.03 (S)	0.004 (S)	0.004 (S)	0.002 (S)	0
132	1.0 (S)	2.0 (S)	1.0 (S)	0.5 (S)	0.03 (S)	0.25 (S)	0.03 (S)	0.19 (S)	2x
136	0.5 (S)	2.0 (S)	32 (R)	1.0 (S)	2.0 (R)	0.25 (S)	0.015 (S)	0.125 (S)	4x
156	0.5 (S)	2.0 (S)	0.5 (S)	0.125 (S)	0.03 (S)	2.0 (R)	0.5 (R)	2.0 (R)	2x

*SMX-TMP = Sulfamethoxazole / trimethoprim; DOX - Doxycycline; RIF - Rifampicin; DLX - Delafloxacin; VAN - Vancomycin; LNZ – Linezolid; S - Susceptible; R - Resistant.

2.2. Synergism Test

The synergy between delafloxacin and other antimicrobials was assessed using the checkerboard method. Minimal synergy was observed among the tested isolates. Only one isolate showed synergy with vancomycin, and two isolates demonstrated synergy with doxycycline. Specifically, two isolates that were susceptible to doxycycline and the tested antibiotics (isolates 41 and 132) were identified. No antagonism was observed between the drug combinations, suggesting that these combinations may be additive or synergistic (Table 2).

Table 2. Analysis of synergism by checkerboard of different antibiotics associated with delafloxacin. In gray the results showing potential synergism. Σ FIC is the sum of the fractional inhibitory concentrations. Synergism was indicated by Σ FIC < 0.5, indifference by Σ FIC between 0.5 and 4.0, and antagonism by Σ FIC > 4.0.

	VAN	LNZ	SMX-TMP	DOX	RIF
Isolate	Σ CIF	Σ CIF	Σ CIF	Σ CIF	Σ CIF
2	1.5	1	1	1	1
3	0.75	1	1	1	1
49	1	1	1	1	1
41	0.5	1.25	1	1	1
57	1	1	1	0.4	1
71	1	1	1	1	1
80	1	1	1	1	1
102	1	1	1	1	1
130	1.25	1	2	1.5	2
132	1	1	2	0.5	1
136	1.25	1	1	1.5	1
156	1	1	1	1	1

*SMX-TMP = Sulfamethoxazole / trimethoprim; DOX - Doxycycline; RIF - Rifampicin; DLX - Delafloxacin; VAN - Vancomycin; LNZ - Linezolid.

2.3. Biofilm Test, Biomass, and MTT

For biofilm analysis, the four delafloxacin-sensitive isolates (41, 130, 132, and 136) were selected. In the MBEC (Minimum Biofilm Eradication Concentration) test, no complete eradication of biofilm was achieved even at antibiotic breakpoint concentrations. However, two isolates demonstrated significant anti-biofilm activity, with a two-log reduction in biofilm biomass when rifampicin was combined with delafloxacin (isolates 130 and 132). Additionally, isolate 132 showed anti-biofilm activity with the combination of delafloxacin and doxycycline (see Figures 1 and 2).

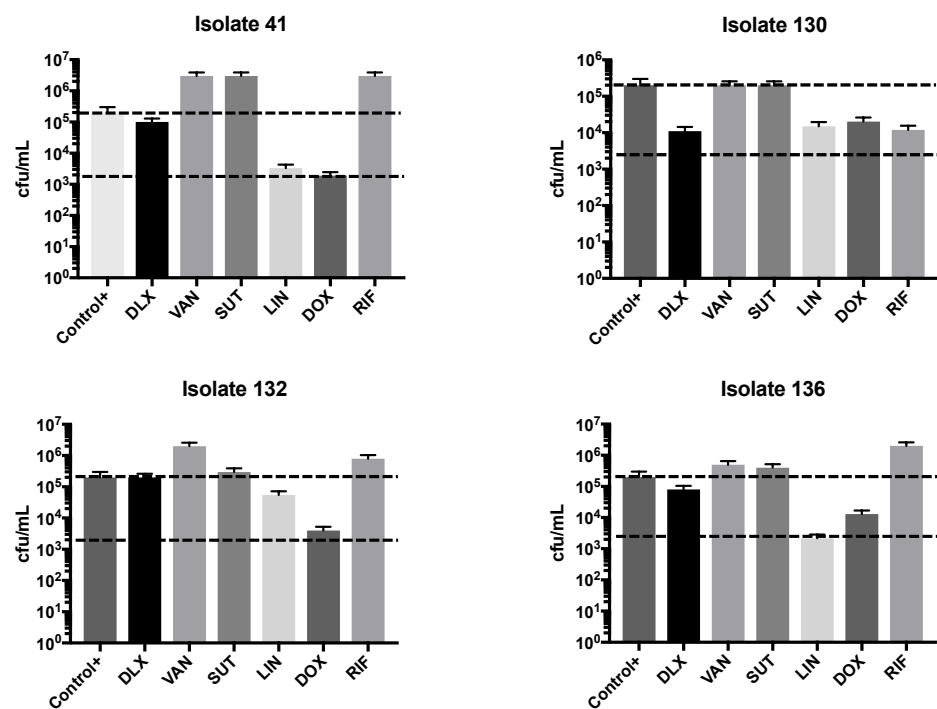


Figure 1. MBEC (Minimum Biofilm Eradication Concentration) test of antibiotics against MRSA susceptible to delafloxacin. The dashed line represents the 2 logs decrease in comparison with positive control. The number is the top represent the name of the isolate. SMX-TMP = Sulfamethoxazole / trimethoprim; DOX - Doxycycline; RIF - Rifampicin; DLX - Delafloxacin; VAN - Vancomycin; LN2 – Linezolid.

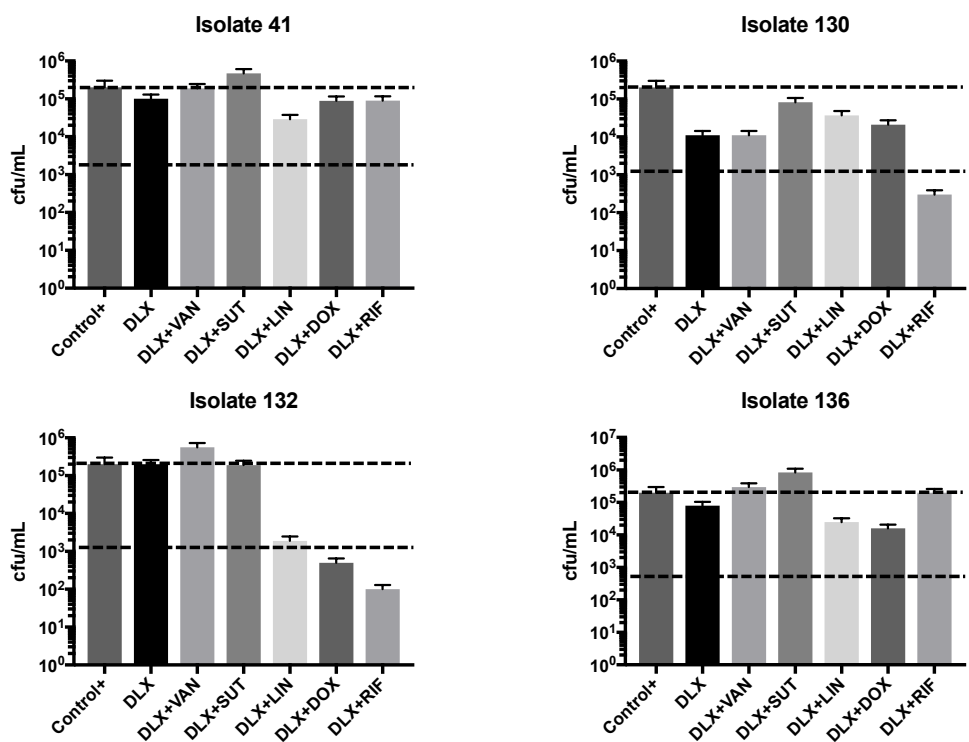


Figure 2. MBEC (Minimum Biofilm Eradication Concentration) test of antibiotics against MRSA susceptible to delafloxacin in combinations. The dashed line represents the 2 logs decrease in comparison with positive control. The number is the top represent the name of the isolate. SMX-TMP

= Sulfamethoxazole / trimethoprim; DOX - Doxycycline; RIF - Rifampicin; DLX - Delafloxacin; VAN - Vancomycin; LNZ - Linezolid.

The biomass assessment was conducted for each antibiotic individually as well as in combination with delafloxacin. Generally, there was a reduction in biomass with most single antibiotics, except for doxycycline with isolate 41, and rifampicin and SMX-TMP with isolate 132, where no reduction in biomass was observed. In the combinations of antibiotics with delafloxacin, no significant reduction in biomass was noted (see Figures 3 and 4).

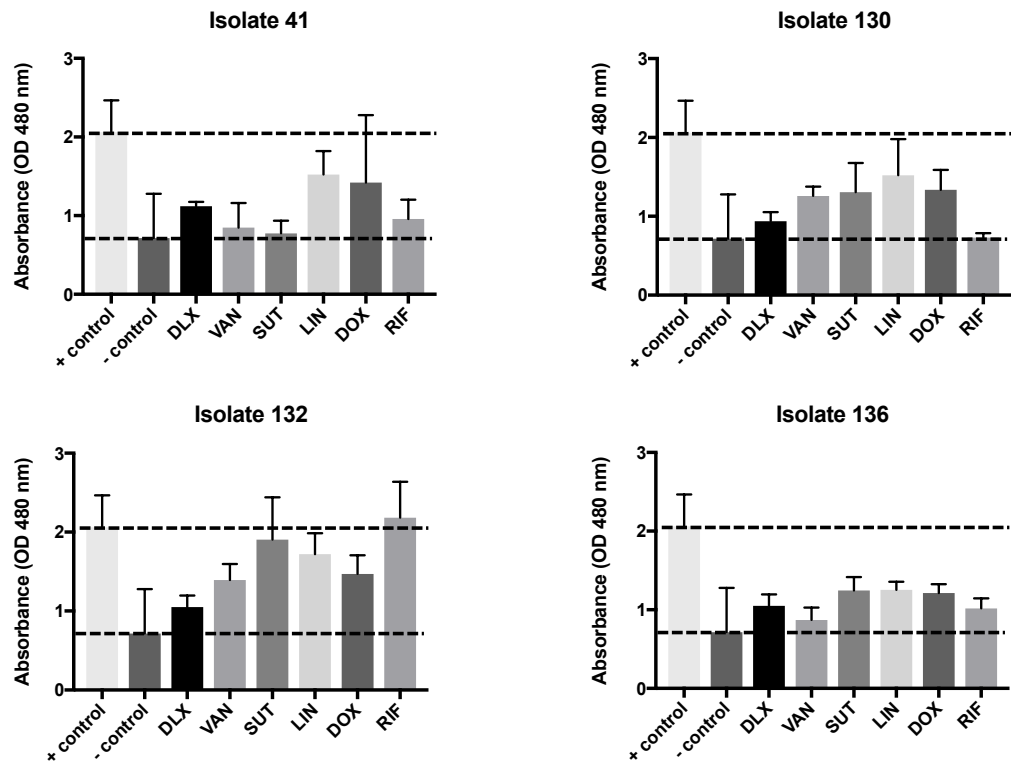


Figure 3. Biomass (by crystal violet) test of antibiotics against MRSA susceptible to delafloxacin. The dashed line represents the positive control and negative control. The number is the top represent the name of the isolate. SMX-TMP = Sulfamethoxazole / trimethoprim; DOX - Doxycycline; RIF - Rifampicin; DLX - Delafloxacin; VAN - Vancomycin; LNZ – Linezolid.

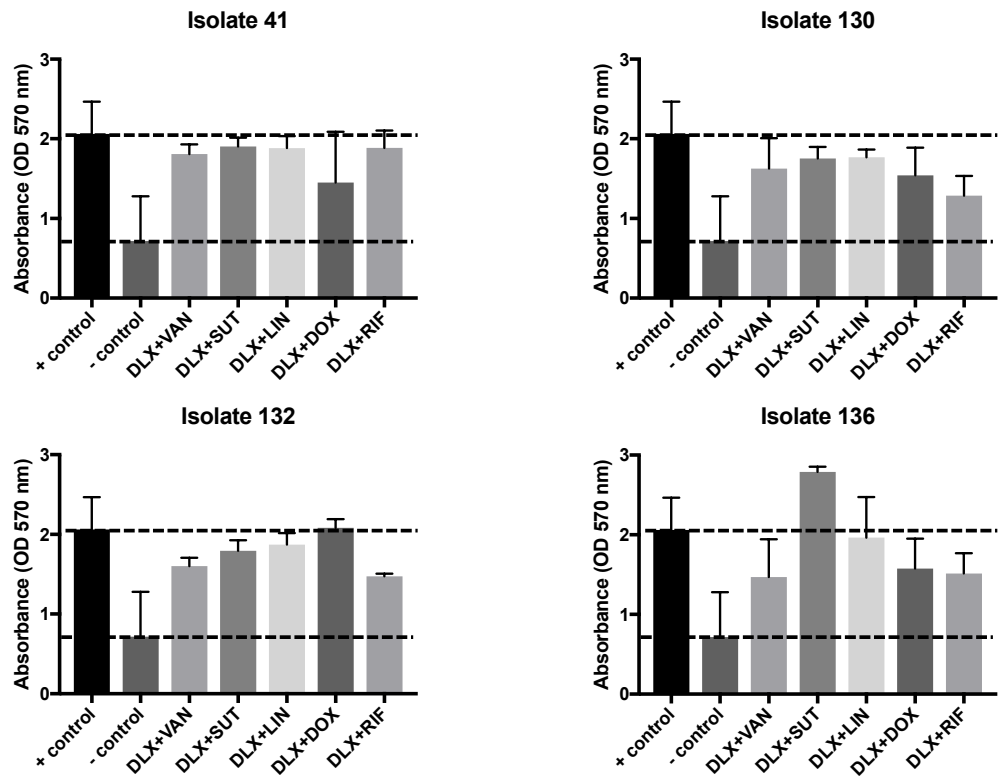


Figure 4. Biomass (by crystal violet) test of antibiotics against MRSA susceptible to delafloxacin in combinations. The dashed line represents the positive control and negative control. The number is the top represent the name of the isolate. SMX-TMP = Sulfamethoxazole / trimethoprim; DOX - Doxycycline; RIF - Rifampicin; DLX - Delafloxacin; VAN - Vancomycin; LINZ - Linezolid

Cell viability, assessed through the MTT assay, did not show any significant changes in biofilm cell viability. Viability remained consistent across all individual antibiotics as well as their combinations with delafloxacin (see Figures 5 and 6).

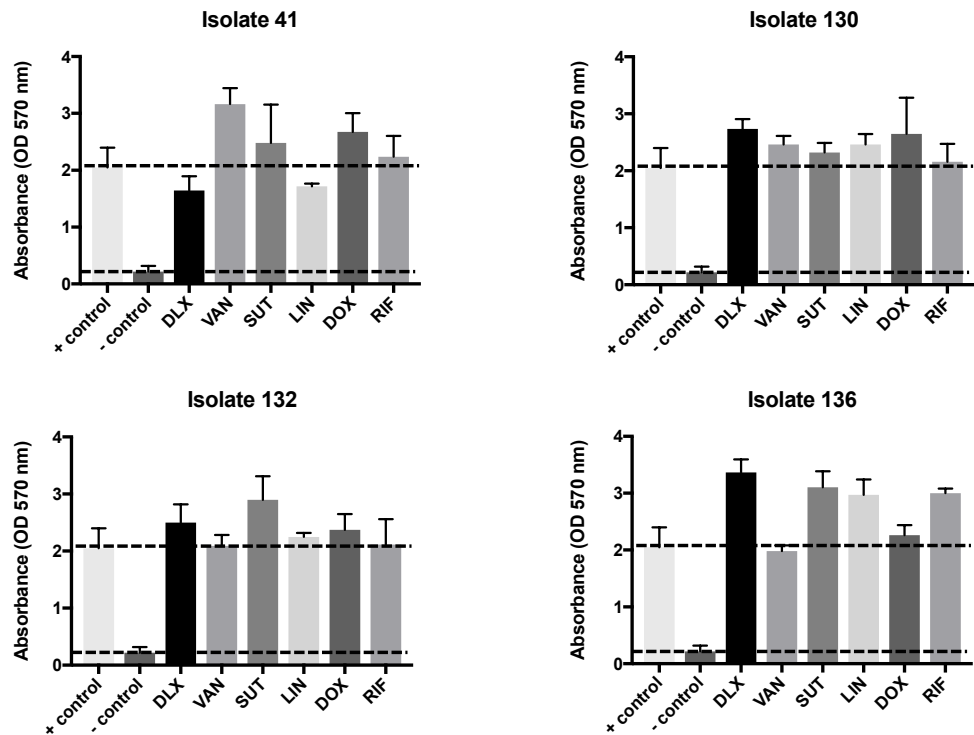


Figure 5. MTT in biofilm with antibiotics against MRSA susceptible to delafloxacin. The dashed line represents the positive control and negative control. The number is the top represent the name of the isolate. SMX-TMP = Sulfamethoxazole / trimethoprim; DOX - Doxycycline; RIF - Rifampicin; DLX - Delafloxacin; VAN - Vancomycin; LNZ - Linezolid.

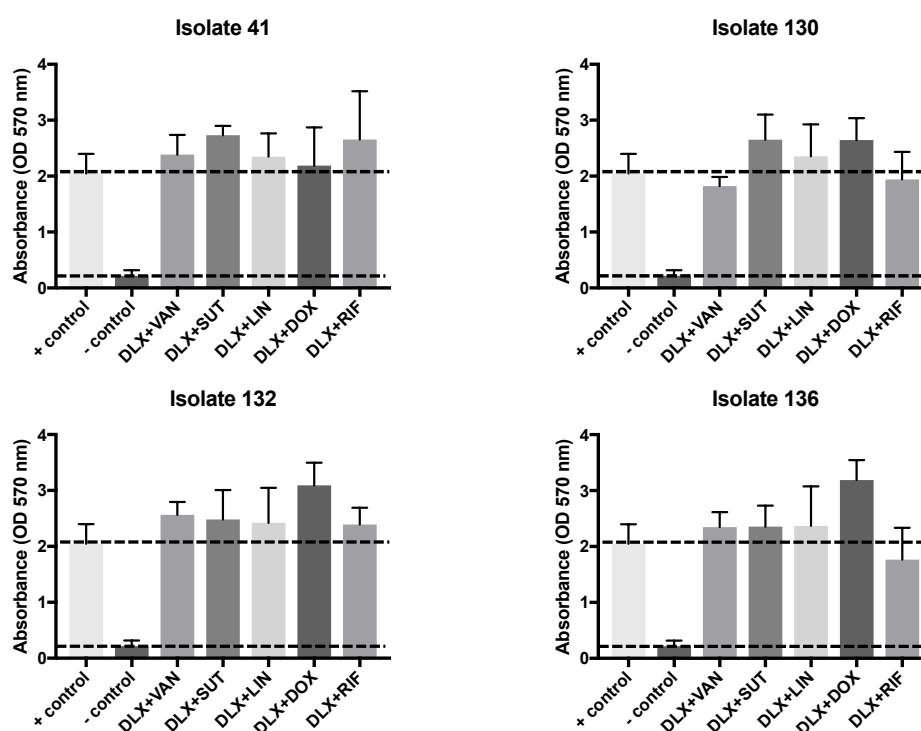


Figure 6. MTT in biofilm with antibiotics against MRSA susceptible to delafloxacin in combinations. The dashed line represents the positive control and negative control. The number is the top represent the name of the isolate. SMX-TMP = Sulfamethoxazole / trimethoprim; DOX - Doxycycline; RIF - Rifampicin; DLX - Delafloxacin; VAN - Vancomycin; LNZ - Linezolid.

3. Discussion

Staphylococcus aureus is renowned for its ability to form biofilms, a trait that significantly contributes to its pathogenicity, particularly in chronic infections such as osteomyelitis and prosthetic infections [2,5,17–19]. The biofilm structure, a complex community of bacterial cells embedded in a self-produced matrix, offers robust protection against both host immune responses and antimicrobial agents [6]. This matrix impedes the penetration of antibiotics and the slow growth of bacteria within the biofilm further exacerbates resistance, making these infections particularly challenging to treat [8,20,21].

The persistence of *S. aureus* biofilms in medical devices and bone tissues often necessitates surgical intervention coupled with prolonged antibiotic therapy [3,22–26]. Despite the use of advanced antibiotics and the removal of infected devices, the treatment of biofilm-associated infections remains difficult due to the inherent resistance mechanisms of biofilm-embedded bacteria [15,27–29].

The management of biofilm-associated infections often involves a combination of antibiotics. Rifampicin and daptomycin are frequently used due to their efficacy against biofilm-embedded bacteria [8,30–33]. Rifampicin, in particular, is known for its ability to penetrate biofilms and target dormant bacteria effectively. However, its use as monotherapy is limited due to the rapid development of resistance [9,34,35]. Daptomycin, another potent antimicrobial, is effective against biofilms, especially when used in combination with rifampicin, enhancing its overall efficacy [3].

Fluoroquinolones, including delafloxacin, represent another class of antibiotics with potential efficacy against *S. aureus*. Their mechanism of action involves inhibition of DNA gyrase and

topoisomerase IV, enzymes crucial for bacterial DNA replication and repair. Although fluoroquinolones have demonstrated good tissue penetration and some ability to reach biofilm-embedded bacteria, their effectiveness against dormant cells within biofilms is limited [8]. As a result, fluoroquinolones are often used in combination with other agents to improve treatment outcomes [11,13].

Delafloxacin, a newer fluoroquinolone, has shown promising activity against MRSA, including biofilm-associated infections [14]. It has demonstrated superior activity compared to daptomycin against MRSA at equipotent concentrations, making it a valuable option for treating resistant strains [36,37]. Additionally, delafloxacin's oral availability presents a significant advantage over other anti-MRSA agents like daptomycin and vancomycin, which are typically administered intravenously. This oral formulation is particularly beneficial for treating orthopedic infections that require prolonged therapy [38,39].

The unique advantage of delafloxacin lies in its enhanced activity in acidic environments, which is a common condition in biofilms [15,16]. This characteristic suggests that delafloxacin could be more effective in disrupting biofilm matrices and targeting embedded bacteria than other fluoroquinolones.

In this study, the MIC of delafloxacin was assessed alongside other antibiotics using the broth microdilution method and the Etest gradient strip method. All isolates were susceptible to vancomycin and linezolid. However, resistance to doxycycline, SMX-TMP, and rifampicin was observed in some isolates. Delafloxacin showed consistent results across both methods, confirming its effectiveness against the tested strains [40–42].

Interestingly, the MIC of delafloxacin decreased significantly at a lower pH of 5.5 compared to the neutral pH of 7.4. This finding highlights delafloxacin's enhanced activity in acidic environments, a common feature of biofilm-associated infections [43,44]. The observed decrease in MIC, ranging from none to four-fold reductions, underscores delafloxacin's potential effectiveness in acidic biofilm conditions.

The checkerboard method was employed to assess the synergistic effects of delafloxacin in combination with other antibiotics. Minimal synergy was observed overall. Synergy was noted with vancomycin in one isolate and with doxycycline in two isolates, suggesting that while delafloxacin has potential in combination therapies, the extent of synergy varies among different antibiotic pairs [45]. No antagonistic effects were detected, indicating that the combinations of delafloxacin with other drugs could be additive or synergistic, which is valuable information for designing effective treatment regimens.

Biofilm disruption was evaluated using various methods, including the MBEC, crystal violet staining, and the MTT assay. The MBEC test demonstrated that complete eradication of biofilm was not achieved even at breakpoint concentrations of the antibiotics. However, significant anti-biofilm activity was observed when rifampicin was combined with delafloxacin, resulting in a two-log reduction in biofilm biomass for two isolates. This result aligns with the known effectiveness of rifampicin against biofilms and suggests that combining it with delafloxacin can enhance biofilm disruption [9,11,24].

The crystal violet staining method, used to quantify biomass, revealed a reduction in biomass with most single antibiotics. However, no significant reduction in biomass was noted when delafloxacin was combined with other antibiotics. This finding suggests that while delafloxacin alone or in combination can impact biofilm biomass, the effect might not be as pronounced when compared to other antimicrobial agents [46].

Cell viability within biofilms was assessed using the MTT assay. The results showed no significant changes in cell viability across individual antibiotics and their combinations with delafloxacin. This indicates that while some antibiotics, like rifampicin, show effectiveness in reducing biofilm biomass, they may not significantly impact the overall viability of the biofilm cells. This discrepancy highlights the complexity of biofilm-associated infections and the need for comprehensive evaluation of both biomass reduction and cell viability to fully assess treatment efficacy [47].

This study underscores the potential of delafloxacin as an effective treatment option for MRSA biofilm-associated infections. Its enhanced activity in acidic environments and oral availability make it a promising candidate for treating chronic infections, particularly in orthopedic settings. The combination of delafloxacin with rifampicin shows promise in disrupting biofilms, though further research is needed to optimize treatment regimens and fully understand the mechanisms of synergy and resistance. The findings emphasize the importance of continued research into new antimicrobial agents and combination therapies to combat biofilm-associated infections effectively. By expanding our understanding of how different antibiotics interact and impact biofilm structures, we can develop more effective strategies for managing these challenging infections.

4. Materials and Methods

4.1. Isolates

A total of twelve *S. aureus* strains were selected from a high-complexity hospital (Curitiba, Paraná, Brazil), ten MRSA and two MSSA. These isolates were randomly chosen from blood cultures obtained from different patients over a span of three months.

4.2. Minimal Inhibitory Concentration (MIC)

The antimicrobial susceptibility of the isolates to delafloxacin (DLX), vancomycin, linezolid, sulfamethoxazole/trimethoprim (SMX-TMP), doxycycline (DOX), and rifampicin (RIF) was assessed using the broth microdilution method, following CLSI M7-A9 guidelines [48]. The MIC was determined as the lowest concentration that prevented visible growth. To verify the MIC for delafloxacin, the Etest gradient strip method (Liofilchem, Waltham, MA) was used, with interpretation based on the point where the inhibition zone intersected the Etest scale. MIC interpretations were aligned with EUCAST 2024 guidelines [49]. Delafloxacin susceptibility was also tested at different pH levels. The experiments were conducted at pH 7.4, as recommended by CLSI M7-A9, and at pH 5.5 to evaluate the strains' behavior in an acidic environment.

4.3. Synergism Test

The checkerboard method was employed to evaluate the synergy between delafloxacin and other antimicrobials [50]. In this assay, one antibiotic was serially diluted along the ordinate, while the second antibiotic was diluted along the abscissa. A concentration of 10⁵ CFU/mL was used for the strains [51]. The MIC was defined as the lowest concentration showing no turbidity or growth. Synergism was quantified using the sum of the fractional inhibitory concentrations (Σ FIC), calculated as Σ FIC = FIC A + FIC B, where FIC A is the MIC of antimicrobial A in combination divided by its MIC when used alone, and FIC B is the MIC of antimicrobial B in combination divided by its MIC when used alone. Synergism was indicated by Σ FIC < 0.5, indifference by Σ FIC between 0.5 and 4.0, and antagonism by Σ FIC > 4.0 [52].

4.4. Biofilm Test, Biomass, and MTT

Based on results of the synergism observed between the proposed antimicrobials against *S. aureus* strains, the antimicrobials were tested both individually and in combination using in vitro biofilm models. For the analysis, the concentrations were determined according to the sensitivity breakpoints established by EUCAST 2024. In a 96-well microplate, samples were added at a concentration of 10⁶ CFU/mL in TSB broth and incubated at 37°C for 24 hours. After incubation, the liquid was removed and replaced with 100 μ L of TSB broth plus 100 μ L of antimicrobial solution, either individually or in combination. The plates were incubated at 37°C for an additional 24 hours. Then, three different methods were used, which included the minimal biofilm eradication concentration (MBEC), biomass (crystal violet), and cell viability (MTT).

4.5. MBEC

After the antibiotic treatment and washing step, 200 μ L of PBS (pH 7.5) was added to each well, and the 96-well microplates were sonicated in an ultrasonic bath for 5 min at 37 °C and 40 kHz (Sanders Medical, Santa Rita do Sapucaí, Minas Gerais, Brazil) to disrupt the biofilm matrix cells. Serial dilutions were made using sterile saline, which was poured onto TSA plates. The plates were then incubated for 24 h at 36 °C. The CFU count was determined and the results were calculated as CFU/mL, where MBEC is the minimum concentration in milligram per liter of each antifungal capable of reducing biofilm cells by 2 log₁₀ [53].

4.6. Estimation of Metabolic Activity by the MTT Reduction Assay

The biofilms were incubated in a 96-well microplate filled with 200 μ L MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1 mg/mL at 36 °C for 2 h. After incubation, MTT was aspirated, and the wells were washed thrice using PBS (pH 7.5). The resulting product (formazan) formed inside the cells was dissolved in 200 μ L of isopropanol and homogenized. Next, 100 μ L of this solution was transferred to a new 96-well microplate. Absorbance was measured using a Versa-Max microplate ELISA reader adjusted to a wavelength of 570 nm [54]. Biofilm classification was based on optical density (OD) according to a previous criteria [55]. The results of the MTT assay were expressed as a percentage relative to the positive control (PC), which was considered 100%.

4.7. Biomass

For the crystal violet technique, after incubating the microplate, all the liquid content was removed and 200 μ L of methanol peroxide was added to fix the remaining bacteria at the bottom of the wells. After 20 minutes, the content was discarded and allowed to dry at room temperature. Then, 200 μ L of 1% crystal violet was added for 5 minutes. After this time, the plate was washed with deionized water, and then 200 μ L of 33% glacial acetic acid was added to resuspend the biofilm formed at the bottom of the wells. Optical density readings were taken using an automated ELISA reader at a wavelength of 480 nm.

4.8. Statistical Analysis

All tests were performed in triplicates. Qualitative data are described as percentages, and quantitative data are expressed as medians with 10% and 90% percentiles. To compare the results among antibiotics, the Mann-Whitney U test was used to compare medians (one by one). Statistical significance was set at $p < 0.05$. The Shapiro-Wilk normality test showed a non-normal distribution of data, justifying the usage of a non-parametric test.

Author Contributions: Conceptualization, F.T. and J.M.; methodology, A.A. and G.O.; formal analysis, F.T. and P.S. ; investigation, A.A.; resources, J.M.; writing—original draft preparation, X.X.; writing—review and editing, F.T.; visualization, J.M. All authors have read and agreed to the published version of the manuscript.

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