Antibacterial and Anti-Biofilm Efficacy of Rifampicin-Minocycline Combination against *Stenotrophomonas maltophilia* Clinical Isolates

Wanutcha Billateh[1], Rosesathorn Soontarach[2,5], Rattanaruji Pomwised[3], Arnon Chukamnerd[2], Kamonnut Singkhamanan[1], Komwit Surachat[1,4] and Sarunyou Chusri[2,*]

[1]Department of Biomedical Sciences and Biomedical Engineering, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand
[2]Division of Infectious Diseases, Department of Internal Medicine, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand
[3]Division of Biological Science, Faculty of Science, Prince of Songkla University, Songkhla, Thailand [4]Translational Medicine Research Center, Faculty of Science, Prince of Songkla University, Songkhla, Thailand
[5]Center of Antimicrobial Biomaterial Innovation-Southeast Asia, Faculty of Science, Prince of Songkla University, Songkhla, Thailand
*Corresponding author, Email: sarunyouchusri@hotmail.com

Abstract

Stenotrophomonas maltophilia is an important hospital-acquired pathogen associated with a high mortality rate, especially in immunocompromised patients. The severity of *S. maltophilia* infections is largely attributed to its various antibiotic resistance mechanisms and ability to produce biofilms, which protect the bacteria and make treatment challenging. In this study, we aimed to investigate the effects of a rifampicin-minocycline combination against *S. maltophilia* and its biofilms. The minimum inhibitory concentrations (MICs) of rifampicin and minocycline were evaluated using a broth microdilution method and the minimum bactericidal concentrations (MBCs) were also determined accordingly. The synergistic effects of rifampicin and minocycline were explored using a checkerboard assay. In addition, a crystal violet assay was used to assess the biofilm-forming ability of *S. maltophilia* and the effect of the rifampicin-minocycline combination on biofilm eradication. The results showed that all isolates were resistant to rifampicin and 90% exhibited resistance to minocycline among 20 *S. maltophilia* clinical isolates. The synergistic effect of the rifampicin-minocycline combination was observed at 40% (8/20) of the isolates, while an additive effect was 60% (12/20). Almost all *S. maltophilia* produced strong biofilm, except isolate SM25, which produced a moderate biofilm. The high concentrations of the combination were more effective in eradicating *S. maltophilia* biofilms compared to rifampicin or minocycline alone. However, some isolates demonstrated that high concentrations did not completely eradicate biofilms. In conclusion, the rifampicin-minocycline combination has a synergistic effect against *S. maltophilia* isolates and helps eradicate bacterial biofilms. Additionally, further studies are necessary to explore the effectiveness of the rifampicin-minocycline combination in biofilm eradication.

Keywords: *Stenotrophomonas maltophilia, combination, rifampicin, minocycline, biofilm, synergistic effect*

1. Introduction

Stenotrophomonas maltophilia is increasingly recognized as an important opportunistic pathogen associated with hospital-acquired infections (Buchovec et al., 2022). This pathogen is commonly found in clinical and non-clinical environments (Brooke, 2012). Currently, *S. maltophilia* is considered an emerging pathogen with increased infection rates (Banar et al., 2023). Infections caused by *S. maltophilia* include bacteremia, pericarditis, urinary tract infections, and pneumonia (Zuravleff & Yu, 1982). In addition, cystic fibrosis patients with *S. maltophilia* infection have a higher mortality rate due to a lack of effective lung function (Barsky et al., 2017). Importantly, *S. maltophilia* possesses the ability to form biofilms on abiotic and biotic surfaces. Biofilm forming is a crucial virulence strategy used by many pathogenic bacteria to

survive under extreme conditions and defend against the host immune system and antibiotic treatments (Brooke, 2012; Pradhan et al., 2023).

Biofilms are a group of bacterial cells embedded in a self-produced matrix of extracellular polymeric substances (EPS). Biofilms act as a protective barrier for bacterial cells, making them more resistant to antibiotics than bacteria that do not form biofilms, as antibiotics cannot easily penetrate the biofilm (Wang et al., 2016). A previous study reported that biofilms produced by *S. maltophilia* are linked to approximately 65% of hospital-acquired infections (Flores-Treviño et al., 2019). In addition to biofilm formation, the increasing antibiotic resistance has been linked to various mechanisms, which may limit the number of available drugs for treatment (Giamarellos-Bourboulis et al., 2002). Minocycline is one of the antibiotics of choice to treat *S. maltophilia* infection because it has high susceptibility activity *in vitro* tests, but clinical data remain limited (Hand et al., 2016). Minocycline has good penetration, and the absorbent in tissue is safe to use because it does not impair liver or renal function. (Asadi et al., 2020; Yang et al., 2016) However, between 2000 and 2022, minocycline resistance rates in *S. maltophilia* were 1.4% (Dadashi et al., 2023). Rifampicin is one of the antibiotics used for treating tuberculosis (TB) and Gram-positive bacteria, especially when biofilms are prominent (Rothstein, 2016). Rifampicin has activity against *S. maltophilia*; however, the bacteria can quickly develop resistance when used as monotherapy (Betts et al., 2014). Previous studies have demonstrated that the combination of rifampicin and minocycline exhibits greater killing effects on Gram-positive bacteria than rifampicin or minocycline alone. Additionally, this combination exhibits a synergistic effect with no antagonism between the two antibiotics (Segreti et al., 1989). However, *in vitro* activity of rifampicin in combination with minocycline as an antibacterial and anti-biofilm agent remains limited in Gram-negative bacteria. Therefore, this study aimed to investigate the antibacterial and anti-biofilm activity of the rifampicin-minocycline combination against *S. maltophilia* clinical isolates from Songklanagarind Hospital, Songkhla, Thailand.

2. Objectives

To evaluate the antibacterial and anti-biofilm activity of the rifampicin-minocycline combination against *S. maltophilia* clinical isolates from Songklanagarind Hospital, Songkhla, Thailand.

3. Materials and Methods

3.1 Bacterial isolates

Twenty isolates of *S. maltophilia* were previously collected from patients in Songklanagarind Hospital, Songkhla, Thailand. Isolates were obtained from various specimens, including sputum (n = 12), pus (n = 2), bronchial wash (n = 2), urine (n = 1), hemoculture (n = 1), body fluid (n = 1), and tissue (n = 1). All isolates were cultured at 37 °C on Tryptic Soy Agar (TSA) and tested for purity and viability. Bacterial stocks were prepared in Mueller-Hinton Broth (MHB) and stored at -80 °C in 20% glycerol.

3.2 Antibiotic susceptibility testing

The minimum inhibitory concentrations (MICs) of rifampicin and minocycline were determined using broth microdilution, according to the Clinical and Laboratory Standards Institute guideline (CLSI, 2023). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as the reference strains for minocycline and rifampicin, respectively. Briefly, all isolates were grown to the log phase at 37 °C for 5 h with shaking at 150 rpm. The bacterial concentration was adjusted to 0.5 McFarland standard (1.5×10^8 CFU/ml) and diluted 1:100 in MHB (1.5×10^6 CFU/ml). One hundred microliters of bacteria were added to a 96-well microtiter plate containing 100 µL of 2-fold serial diluted antibiotic with MHB, resulting in a final bacterial concentration of 5×10^5 CFU/ml per well. The plates were incubated at 37 °C for 20 h, and bacterial inhibition was assessed using 20 µL of 0.01% resazurin per well. The MIC was expressed as the lowest concentration of the antibiotic that inhibited the growth of bacteria. After that, 10-µL aliquots from wells without bacterial growth were dropped onto Mueller Hinton Agar (MHA) and incubated at 37 °C for 24 hours. The minimum bactericidal concentration (MBC) was expressed as the lowest concentration of the antibiotic that kills the bacterial population, determined by the absence of bacterial growth.

3.3 Antibiotic combination study

The antibiotic combination study was performed using a checkerboard assay to investigate the synergistic effects. The initial bacterial inoculum was adjusted to 10^6 CFU/ml, and 100 µL of them were added to the wells containing 100 µL of antibiotic combinations with different concentrations. The plates were incubated at 37 °C for 20 hours, and bacterial inhibition was assessed using 0.01% resazurin. The experiment was performed in triplicate for two independent repeats. The degree of efficacy between antibiotics was defined in terms of the fractional inhibitory concentration index (FICI) (Elkhoumesy et al., 2017).

$$\text{FICI} = \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}}$$

The FICI for each combination was used to interpret as follow: synergy (FICI ≤ 0.5), additive (0.5 < FICI ≤1), indifference (1 < FICI ≤ 4), and antagonism (FICI > 4).

3.4 Screening of S. maltophilia biofilms

The biofilm-forming ability of *S. maltophilia* was assessed using the crystal violet assay as previously described (Kim et al., 2019). Isolates were randomly selected from those showing synergistic and additive effects. Briefly, bacterial culture was adjusted to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/ml) and then diluted 1:100 in Tryptic Soy Broth (TSB). Two hundred microliters of bacterial culture were added to the well and incubated at 37 °C for 24 hours. The cultures were removed and washed with 100 µL of 10 mM phosphate-buffered saline (PBS, pH 7.4). The contents of each well were removed and air-dried. The plates were stained with 200 µL of 0.01% crystal violet and kept at room temperature for 25 minutes. Excess cells were removed and washed with 100 µL of distilled water. After drying, 95% ethanol was added to the destained biofilm. The plates were measured by reading optical density (OD) at 492 nm with a microtiter plate reader. The experiment was performed in triplicate with two independent repeats. The well containing sterilized medium was used as an external control to ensure that biofilm formation was due to the bacteria rather than the medium. The ability to form biofilm of each isolate was classified as follows: no biofilm producer (OD≤0.05), weak biofilm producer (0.05<OD≤0.1), moderate biofilm producer (0.1<OD≤0.2), and strong biofilm producer (OD>2) (Samadi et al., 2018). The OD value of each isolate was calculated by subtracting the average OD of the control from the average OD of the test wells.

3.5 Effects of rifampicin-minocycline combination on S. maltophilia established biofilms

The overnight growth of bacterial culture was adjusted to a 0.5 McFarland standard and then diluted 1:100 in TSB. The well-containing TSB with bacterial culture served as a control. Two hundred microliters of bacterial culture were dispensed to each well of 96 well plates and incubated at 37 °C for 24 hours. The unattached cells were aspirated, and 200 µL of antibiotic alone (rifampicin and minocycline) and rifampicin-minocycline combination with different concentrations, including 1/2MIC, MIC, 2MIC, 4MIC, and 8MIC, were added to each well and incubated at 37 °C for 24 hours. The crystal violet assay was performed as described above, and experiments were performed in triplicate with three independent repeats. The OD value for each concentration was calculated by subtracting the average OD of the control from the average OD of the test wells.

3.6 Statistical analysis

Biofilm formation was analyzed using GraphPad Prism version 10.2. One-way ANOVA was used to determine statistical significance. A *p*-value of less than 0.05 when compared to the untreated control with antibiotic alone (rifampicin and minocycline and rifampicin-minocycline combination was considered statistically significant.

4. Results and Discussion

4.1 The results of antibiotic susceptibility testing

The MIC values of rifampicin and minocycline were determined according to CLSI standards. The results of MICs and MBCs are shown in Table 1. MIC values of rifampicin ranged from 4 µg/ml to 1024 µg/ml, while MIC values of minocycline ranged from 0.5 µg/ml to 8 µg/ml. The MBC values were 16 µg/ml to more than 1024 µg/ml for rifampicin and 32 µg/ml to 128 µg/ml for minocycline. MIC_{50} and MIC_{90} rifampicin were 8 µg/ml and 16 µg/ml, while minocycline showed MIC_{50} and MIC_{90} values of 2 µg/ml and 4 µg/ml, respectively. Table 2 shows the antibiotic susceptibility patterns of 20 *S. maltophilia* clinical isolates. All isolates were resistant to rifampicin, and 90% were resistant to minocycline. Rifampicin has been reported to exhibit activity against *S. maltophilia*, but resistance was 100%, similar to previous reports (Betts et al., 2014). The high resistance rate of rifampicin *in vitro* is probably due to its inability to permeate the outer membranes of Gram-negative bacteria (Drapeau et al., 2010). Meanwhile, minocycline is commonly recommended for the treatment of *S. maltophilia* infection because it has a high susceptibility rate (Wei et al., 2016). The increasing rate of minocycline resistance has been reported in current studies, even though the overall rate of resistance remains low (Dadashi et al., 2023). The mechanism contributing to minocycline resistance might involve the ABC efflux pump, which expels tetracycline antibiotics from the bacterial cell, leading to decreased antibiotic activity (Gil-Gil et al., 2020).

Table 1 Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of rifampicin and minocycline against 20 clinical isolates of *Stenotrophomonas maltophilia*

Antibiotics	MIC (µg/ml)			MBC (µg/ml)
	Range	MIC_{50}	MIC_{90}	
MNC	0.5 to 8	2	4	32 to 128
RIF	4 to 1024	8	16	16 to >1024

MNC, minocycline; RIF, rifampicin

Table 2 Antibiotic susceptibility patterns of 20 *Stenotrophomonas maltophilia* clinical isolates

Antibiotics	Susceptible, n(%)	Resistant, n(%)
MNC	2(10)	18(90)
RIF	-	20(100)

MNC, minocycline; RIF, rifampicin, synergy (FICI $\leq$ 0.5), additive (0.5 < FICI $\leq$1), indifference (1 < FICI $\leq$ 4), and antagonism (FICI > 4).

4.2 The results of antibiotic combination assay

The effects of rifampicin combined with minocycline are shown in Tables 3 and 4. Eight isolates (40%) showed synergistic effects, and 12 isolates (60%) showed additive effects. None of the indifferent or antagonistic effects were observed in the isolates. Generally, rifampicin is an antibiotic effective against both Gram-positive and Gram-negative bacteria; however, it is not recommended for use as a monotherapy due to its rapid resistance development during the therapy (Forrest, & Tamura, 2010). Combining antibiotics, such as rifampicin with colistin, has shown higher activity against multidrug-resistant *S. maltophilia* compared to monotherapy. A previous study reported that *in vitro* minocycline activity could prevent rifampicin resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) (Muder et al., 1994). Additionally, rifampicin-minocycline has a synergistic effect against multidrug-resistant (MDR) and extensive drug-resistant (XDR) *Pseudomonas aeruginosa* in both *in vitro* and *in vivo* studies (Lyu et al., 2017). We hypothesized that minocycline may enhance the permeability of rifampicin through the outer membrane, resulting in the synergistic effect of this combination.

Table 3 Fractional inhibitory concentration index (FICI) of rifampicin-based combination with minocycline against 20 clinical isolates of *Stenotrophomonas maltophilia*.

Isolate code	FIC_{MNC}	FIC_{RIF}	FICI of RIF/MNC
SM3	0.25	0.50	0.75

SM10	0.50	0.13	0.63
SM11	0.50	0.25	0.75
SM14	0.25	0.13	*0.38
SM16	0.50	0.13	0.63
SM20	0.25	0.50	0.75
SM21	0.13	0.50	0.63
SM24	0.25	0.25	*0.5
SM25	0.13	0.25	*0.38
SM27	0.50	0.03	0.53
SM28	0.25	0.25	*0.50
SM30	0.25	0.06	*0.31
SM32	0.13	0.25	*0.38
SM34	0.50	0.25	0.75
SM36	0.02	0.50	0.52
SM38	0.50	0.02	0.52
SM40	0.25	0.13	*0.38
SM41	0.25	0.50	0.75
SM45	0.03	0.50	0.53
SM49	0.25	0.25	*0.50

MNC, minocycline; RIF, rifampicin; * synergy

Table 4 Interpretation of rifampicin combination with minocycline percentages in 20 *Stenotrophomonas maltophilia* clinical isolates

Antibiotics	Synergy, n(%)	Additive, n(%)	Indifference, n(%)	Antagonism, n(%)
RIF/MNC	8(40)	12(60)	-	-

MNC, minocycline; RIF, rifampicin, synergy (FICI ≤ 0.5), additive (0.5 < FICI ≤1), indifference (1 < FICI ≤ 4), and antagonism (FICI > 4).

4.3 The results of biofilm formation in S. maltophilia

Eight isolates of the rifampicin-minocycline combination were used to explore the biofilm formation (4 isolates showed a synergistic effect, and the other 4 isolates showed an additive effect). The abilities of the eight isolates to produce biofilm are shown in Tables 5 and 6. Seven isolates (88%) were strong biofilm producers, while another (13%) isolate was a moderate biofilm producer. Our findings were discordant with other studies. In Iran, biofilm-producing *S. maltophilia* were mostly isolated from patients with bloodstream infections. Among them, the highest percentage were moderate biofilm producers (60.22%), followed by strong (19.35%) and weak (20.43%) biofilm producers (Sameni et al., 2023). Another study demonstrated that 24 isolates were isolated from the sputum of patients in Guangzhou, China. They reported that 14 (58%), 8 (33%), and 2 (9%) isolates were moderate, strong, and weak biofilm producers, respectively. Moreover, non-biofilm producers were not observed (Zhuo et al., 2014).

Table 5 Biofilm-forming abilities of eight *Stenotrophomonas maltophilia* clinical isolates

Isolate code	Biofilm formation
SM3	Strong
SM11	Strong
SM20	Strong
SM25	Moderate
SM30	Strong
SM32	Strong
SM40	Strong
SM41	Strong

Table 6 Percentage of biofilm-forming ability of eight *Stenotrophomonas maltophilia* clinical isolates

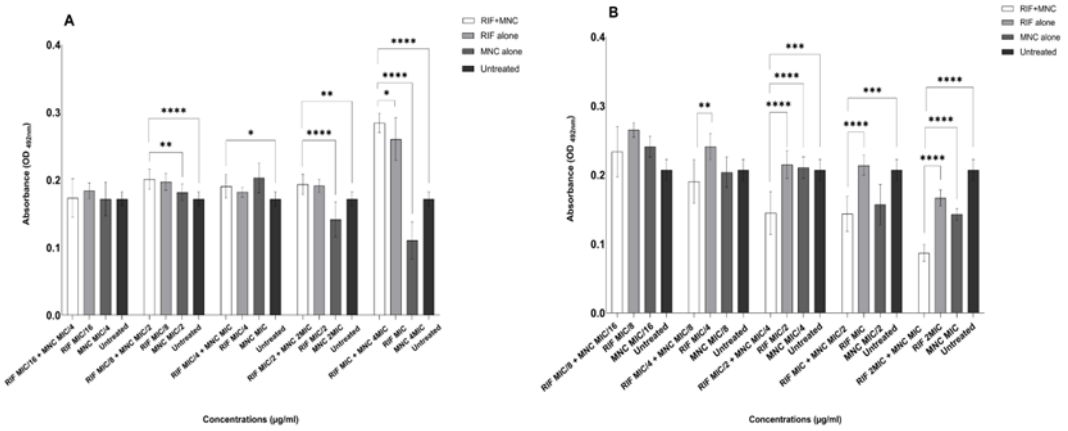
Strong biofilm producer, n(%)	Moderate biofilm producer, n(%)
7(88)	1(13)

4.4 The effects of the rifampicin-minocycline combination on S. maltophilia established biofilms

The anti-biofilm activities of the rifampicin-minocycline combination were studied in 7 biofilm producers and 1 moderate biofilm producer using a crystal violet assay. The combination was compared with untreated control, rifampicin alone, and minocycline alone. The concentrations used in this study were referenced from the FICI for the combination in each isolate. We could not specify a fixed concentration because it is based on the FICI of each isolate, which results in different concentrations for each figure.

Figures 1 (B, D, F, G, and H) showed that using a high combination concentration significantly decreased the biofilm of *S. maltophilia* compared to the untreated control ($P \leq 0.0001$). However, Figure 1E showed a statistically significant decrease at a high concentration ($P \leq 0.05$) compared to the untreated group. Figures 1B and 1H demonstrated that the high concentration of the combination exhibited higher activity compared to rifampicin and minocycline alone ($P \leq 0.0001$), except that in Figure 1H, the high concentration of the combination exhibited higher activity compared to rifampicin alone ($P \leq 0.01$). Figure 1D showed that the combination was significant compared with minocycline alone ($P \leq 0.05$), while Figures 1E and 1F showed a significant effect compared with rifampicin alone ($P \leq 0.05$ and $P \leq 0.0001$). However, using a higher concentration had no additional effect on reducing established biofilm. As seen in Figures 1A and 1C, the rifampicin-minocycline combination might be effective in eradicating biofilms in these isolates.

Minocycline has shown strong anti-biofilm activity against Gram-positive bacteria (Wu et al., 2013). However, the combination of rifampicin and minocycline enhances activity against biofilm-producing bacteria because rifampicin can penetrate the biofilm and kill bacteria inside (Ferreira et al., 2024; Tang et al., 2013). It has been reported that when rifampicin is used alone at sub-MIC levels (MIC/2 and MIC/4), it increases biofilm production in *S. aureus*. However, no reports have indicated that sub-MIC levels of minocycline promote biofilm formation (Lima-e-Silva et al., 2017).



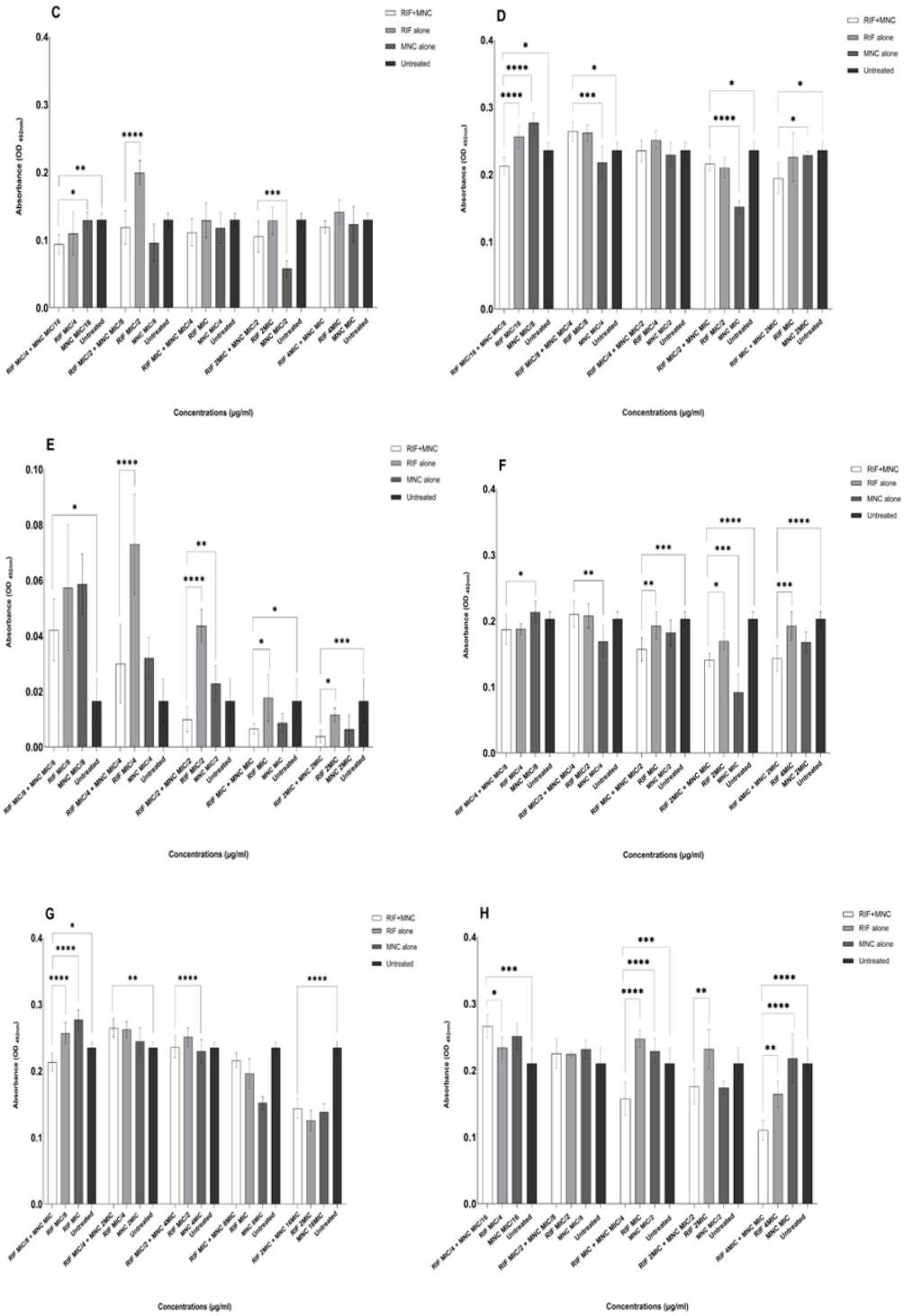


Figure 1 Effects of rifampicin in combination with different concentrations on *S. maltophilia* established biofilms. Isolates 32 (A), 40 (B), 41 (C), 30 (D), 25 (E), 3 (F), 11 (G), and 20 (H) were tested. The combination significantly reduced the established biofilm compared to the untreated control, rifampicin alone, and minocycline alone (*$P \leq 0.05$, **$P \leq 0.01$, *** $P \leq 0.001$, and ****$P \leq 0.0001$). Error bars represent the standard deviation.

5. Conclusion

The rifampicin-minocycline combination has a synergistic effect against *S. maltophilia* isolates, with no antagonism observed. Importantly, rifampicin-minocycline is more effective in eradicating bacterial biofilms than rifampicin or minocycline alone. However, further studies are necessary to evaluate the effectiveness of the rifampicin-minocycline combination in biofilm eradication and to assess its in vivo activity against *S. maltophilia*, ensuring that the concentrations used are appropriate for the human body while considering potential toxicity.

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7. Reference

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