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Research Article

Disrupting the Status Quo: Nitric Oxide's Role in Tackling *Pseudomonas aeruginosa* Infections

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I N F O

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A B S T R A C T

Background: Antibiotic resistance in gram-negative organisms, particularly *Pseudomonas aeruginosa*, presents a formidable challenge in healthcare settings due to its biofilm-forming ability. This study investigates the influence of nitric oxide (NO) on *P. aeruginosa* biofilms, with a focus on the expression of biofilm-related genes (pslA, pelA, and algD).

Method: 71 isolates of *P. aeruginosa* were obtained from clinical specimens and subjected to biofilm formation assays and exposure to NO using sodium nitroprusside (SNP) as a donor. Gene expression analysis was conducted using quantitative reverse transcription-PCR. A scanning electron microscope (SEM) was used to visualise biofilm morphology.

Results: SNP exposure disrupted biofilm formation in most isolates, although some showed resilience. SEM revealed significant biofilm disruption post-NO treatment. Gene expression analysis indicated varied responses to NO, with some isolates showing increased expression of biofilm-related genes while others exhibiting downregulation.

Conclusion: Albeit nitric oxide disrupted *P. aeruginosa* biofilms, its impact on gene expression varies among isolates. Understanding these dynamics could help develop targeted therapeutic strategies for biofilm-associated infections.

Keywords: *Pseudomonas Aeruginosa*, Biofilm, Nitric Oxide, Gene Expression, Antimicrobial Resistance

Introduction

Due to the increasing prevalence of antibiotic resistance within healthcare settings, doctors are encountering significant challenges in effectively treating infections caused by gram-negative organisms.[1] A multidrug-resistant opportunistic bacterium, *Pseudomonas aeruginosa*, poses a significant challenge in clinical settings due to its ability to form resilient biofilms, leading to chronic infections and treatment difficulties.[2,3] The key to the formation and maintenance of these biofilms are genes such as *pslA, pelA*, and *algD*, encoding crucial components of the extracellular matrix essential for biofilm architecture and stability.[4]

Research into antimicrobials explored alternative methods and treatments beyond traditional antibiotics. This included antimicrobial peptides with diverse structures and mechanisms, bacteriophages, inhibitors targeting virulence

factors, siderophores, and adjuncts like monoclonal antibodies. This exploration was prompted by alarming rates of antibiotic resistance across bacterial species.[5]

Data records indicate a notable resistance issue among *P. aeruginosa* strains towards fluoroquinolone antibiotics such as ciprofloxacin and levofloxacin, with approximately 20–30% exhibiting resistance.[6] Moreover, a study conducted by Al-Sheikhly et al. highlighted a concerning resistance rate of 70.5% to amikacin.[7] Similarly, findings from Al-Doory et al. and Al-Dulami et al. revealed a substantial 61.6% resistance rate to ceftazidime among their respective isolates. These studies collectively underscore the pressing challenge posed by antibiotic resistance in *P. aeruginosa* strains.[8,9] As well as, other studies found that the resistances for Ampicillin and Cefotaxime were 100% for each, and the lowest resistance were for Ciprofloxacin (6.66%) and Impineme (0.0%).[10]

Pseudomonas aeruginosa isolates from burn sources have been shown to have a high biofilm-forming capability and to be antibiotic resistant.[11]Another study demonstrated that the prevalence rate of *Pseudomonas aeruginosa* resistance to carbapenem has increased among immunocompromised burn patients due to the increased use of this class of antibiotics, particularly meropenem.[12] In a research conducted at (2018) that show cased of the *P. aeruginosa* carrying New Delhi Metallo-β-lactamase and resistant to carbapenem (imipenem and meropenem).[13] The transition from the biofilm growth mode to the free-swimming planktonic state is facilitated by sublethal levels of nitric oxide (NO), playing a crucial role in dispersing *P. aeruginosa* biofilms.[14] Furthermore, signalling pathways regulating various physiological processes, including biofilm control, gene expression, virulence, and cellular morphology, have been associated with NO.[15] However, the gaseous nature of NO presents challenges when applying it in biological systems, as high concentrations can be lethal to bacteria. Therefore, sodium nitroprusside (SNP) has been utilised as a NO donor to treat infections caused by antibiotic-resistant *P. aeruginosa*. Consequently, this treatment induces biofilm dispersion into planktonic motile cells, rendering them susceptible to antibiotics following exposure.[16]

Despite extensive research, the impact of NO on *P. aeruginosa* biofilms remains poorly understood. While previous studies have highlighted the role of NO in microbial behaviour and biofilm dispersal,[14,17] there is a notable gap in the literature regarding its specific effects on the expression of biofilm-related genes like *pelA, pslA,* and *algD.* Understanding the interplay between NO signalling and biofilm gene expression could provide critical insights into biofilm physiology and uncover novel therapeutic strategies. Thus, this article aims to address this gap by conducting research on the influence of NO on *P. aeruginosa* biofilms, with a particular focus on elucidating its effects on the expression patterns of *pelA, pslA,* and *algD*. By bridging this knowledge gap, we can advance our understanding of biofilm regulation and develop targeted approaches for managing *P. aeruginosa* biofilm-associated infections.

Material and Method

Study Design

The present study is a cross-sectional study along with experimental aspect. Specifically, it is a cross-sectional study for the initial collection of specimens followed by experimental work to assess the response of *P. aeruginosa* to nitric oxide in terms of biofilm and gene expression.

Ethical Statement

This work has been approved by the College of Science Research Ethics Committee (ref. CSEC/0223/0151). All the participants were allowed to provide the researchers with the specimens. Informed consent according to the Declaration of Helsinki was obtained from all participants

Isolation and Identification of *Pseudomonas aeruginosa*

A total of 120 specimens representing a variety of infections were collected from patients aged 20–85 years who were admitted to different hospitals in Baghdad including Imam–Ali Hospital and Baghdad Medical City complex (Ghazi Al-Hariri Hospital, Baghdad Hospital, Burn and Wounds Hospital, and Teaching Laboratories) during the period from Februrary 2023 to May 2023. A total of 80 wound swabs were collected from patients suffering from wound and burn infections, 25 mid-stream urine specimens from patients with urinary tract infections, and 15 sputum specimens were collected from patients with respiratory infections. By using MacConkey agar, cetrimide agar, blood agar (HiMedia, India), gram stain, VITEK 2, and biochemical identification, a total of 71 *Pseudomonas aeruginosa* isolates were detected.

Biofilm Formation

A colourimetric microtiter plate assay was used to quantify the production of biofilms.[18] All isolates were cultured in brain heart infusion broth at 37 °C for 24 hours. Following incubation, 100 µL of bacterial growth was transferred into a 2 mL tube of normal saline, and the turbidity was adjusted to McFarland 0.5 standard. Subsequently, 180 µL of Luria-Bertani broth containing 1% glucose was added to sterile flat-bottomed 96-well polystyrene microtiter plates. Then, 20 µL of the prepared bacterial suspension was dispensed into three wells of the microtiter plates, while six wells containing bacteria-free Luria-Bertani broth were designated as negative controls. After incubating at 37 °C for 24 hours, all plates underwent gentle washing three times with distilled water and were then dried. Afterwards, 200 µL of methanol was added to each well, incubated at room temperature for 15 min, washed and left to dry. To stain the plates, 200 µL of 0.1% crystal violet solution

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was added to each well and incubated for 15 minutes at room temperature. For ten minutes, 200 µL of absolute ethanol was combined with glacial acetic acid (1:1 v/v) to resolubilise the dye. Using a microtiter plate reader (BioTek, USA), the optical density (OD) of each well was measured at 630 nm. Three standard deviations (SD) above the mean OD of the negative control was the definition of the cut-off OD (ODc). Based on their OD value, all isolates were divided into four categories: non-biofilm producer (OD ≤ ODc), weak biofilm producer (ODc < OD ≤ 2ODc), moderate biofilm producer (2ODc < OD ≤ 4ODc), and strong biofilm producer (OD > 4ODc).[19-21]

Exposure of *Pseudomonas aeruginosa* to Nitric Oxide Using Sodium Nitroprusside as Nitric Oxide Donor

Preparation of Sodium Nitroprusside Stock Solution

The conventional method for investigating NO signalling involves employing NO donors such as SNP, facilitating the controlled and temporary release of NO within biological systems.[12] In a study assessing the antimicrobial efficacy of NO, a stock solution for the Minimum Inhibitory Concentration (MIC) experiment was prepared using an 80 mg/mL concentration of SNP. This was achieved by dissolving 0.8 g of SNP in 10 mL of sterile distilled water, with the obtained SNP solution stored in darkness.[22,23]

Estimation of Minimum Inhibitory Concentration

The MIC of SNP was determined using the Resazurin-based turbidometric assay, following the protocol outlined by Teh et al.[24] In summary, a 96-well microtiter plate was utilised to prepare double serial dilutions of SNP (40, 20, 10, 5, 2.5, 1.250, 0.625, 0.312.5, 0.15625, and 0.078125 mg/mL) dissolved in Mueller-Hinton broth. Subsequently, 20 µL of bacterial suspension (adjusted to match the MacFarland 0.5 turbidity standard) was added to each well and thoroughly mixed. After an overnight incubation period at 37 °C, 5 µL of resazurin (6.75 mg/mL) was added to all wells and incubated for an additional 4 hours at 37 °C. Colour changes (from blue to pink) were observed and recorded, with the MIC defined as the lowest concentration before the observed colour change.

Effect of ½MIC of SNP on Biofilm Dispersion of *Pseudomonas aeruginosa*

To explore the impact of NO on biofilm formation, we employed the microtiter plate method. This experiment was conducted following the same protocol as described earlier up to the methanol fixation step. Next, 100 µL of freshly prepared Luria-Bertani broth was added to each well, along with 100 µL of SNP at a concentration corresponding to half the MIC. The plates were then incubated at 37 °C for an additional 24 hours. Subsequently, the remaining steps involving fixation, staining, and resolubilisation of the dye using glacial acetic acid were performed as previously described in the section on biofilm formation.

Examination of Specimens Using Scanning Electron Microscope

Separately, two pieces of polystyrene (each measuring 3 × 3 mm^2) were submerged in 2 mL of Luria-Bertani broth fortified with 1% glucose. A bacterial culture was introduced until it matched the 0.5 McFarland standard. Both containers were then subjected to an incubation period of 24 hours at 37 °C. Subsequently, the media was carefully discarded, and another 2 mL of freshly prepared media was added to both containers. However, one of the containers was supplemented with SNP at a concentration corresponding to half the MIC and incubated at 37 °C for 24 hours. Following this, both pieces were subjected to two washes with distilled water, followed by fixation with methanol for a duration of 10 minutes. Finally, the specimens were meticulously examined under the inspect F50 field emission scanning electron microscope (SEM) (FEI, Holland).

Gene Expression

RNA Extraction

RNA was extracted from the biofilm of *P. aeruginosa* according to the protocol described by the SV Total RNA Isolation System (Promega, USA). To evaluate the suitability of the extracted RNA for future applications, a Quantus Fluorometer (Promega, USA) was employed to measure its concentration. This assessment involved adding an aliquot of 100 µL of diluted Quantifluor dye for every 1 µL of extracted RNA. Subsequently, following a 5-minute incubation period at room temperature, the RNA concentration data were determined.

Quantitative Reverse Transcription-PCR

The gene expression levels of *pelA, pslA,* and *algD* were assessed, and the findings were adjusted for normalisation using the housekeeping gene, *fbp*. The components of the reaction mixture are shown in Table 1. Additionally, the protocol of the thermo-cycler was optimised following multiple attempts, and the resulting protocol is listed in Table 2.

Table 1.RT-qPCR Reaction Mix

Components	Volume (µL)
Luna Universal One-Step Reaction Mix (2X)	10
Luna WarmStart RT Enzyme Mix (20X)	1
Forward primer (10 µM)	0.8
Reverse primer (10 µM)	0.8
Template RNA	Variable
Nuclease-free water	Variable

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Table 2.Protocol for Quantitative Detection of Expression of *pslA, pelA and algD*

Cycle Step	Temperature (°C)	Time	Cycles
Reverse transcription	55	10 minutes	1
Initial denaturation	95	1 minute	1
Denaturation	95	10 seconds	40
Extension	52[a], 57[b]	30 seconds	

[a]*pslA* and *pelA*, [b]*algD*

Statistical Analysis

The experiments were conducted three times each, and the data were analysed using Statistical Package for Social Sciences (SPSS) 21.0. This software was utilised to assess the influence of parameters in the study and to compute the mean and standard deviation. A t-test was employed to assess the impact of NO on biofilm. Any differences with a p-value below 0.05 were deemed significant. A fold change of under 2 was considered insignificant, following the guidelines of Rasigade et al.[25] Nonparametric data were represented using median and interquartile range.

Results and Discussion

Isolation and Identification of *Pseudomonas aeruginosa*

Based on the outcomes of cell growth across various media, gram staining, and biochemical examinations, a collective count of *P. aeruginosa* isolates was identified. These were derived from 35 wound and burn specimens, 25 urine specimens, and 10 sputum specimens, all of which were subsequently validated using the VITEK compact 2 system.

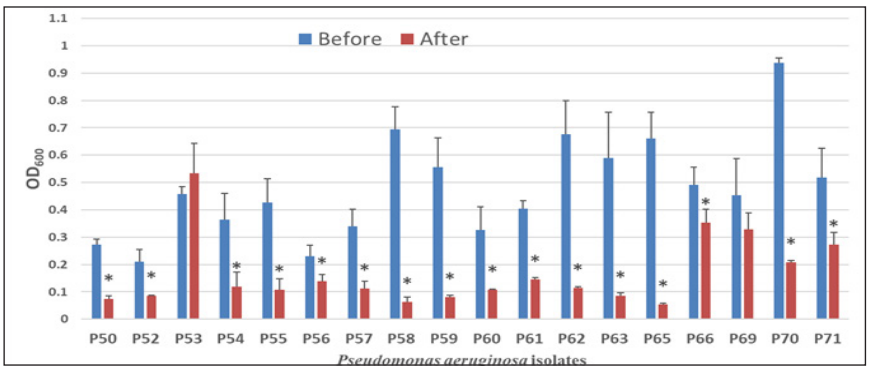
Formation of Biofilm

Among the 71 isolates tested, only 12 (16.90%), 3 (4.23%), and 18 (25.35%) displayed weak, moderate, and strong biofilm formation capabilities, respectively, while only 38 (53.52%) exhibited weak biofilm formation. Interestingly, the distribution of biofilm-forming isolates differed across specimen sources. Specifically, 28.57% of isolates from burn and wound specimens, 26.92% from urine, and 40.00% from sputum exhibited moderate to strong biofilm formation. These findings suggest a potential correlation between specimen source and biofilm-forming ability in *P. aeruginosa* strains.

Such variations in biofilm formation capabilities may stem from various factors, including differences in bacterial genotypes, environmental conditions, and host factors. Previous studies have highlighted the role of specific genetic determinants and regulatory pathways in modulating *P. aeruginosa* biofilm formation,[26–29] for instance, *P. aeruginosa* isolates from respiratory specimens may encounter different selective pressures and nutrient availability compared to those from urinary or wound sources, potentially influencing their biofilm formation potential.[30,31] Additionally, environmental cues, such as nutrient availability and oxygen levels, can influence biofilm development.[32] Moreover, host factors, such as immune responses and the presence of underlying medical conditions, may contribute to variations in biofilm formation among clinical isolates.[28] In a study concluded that bacteria have ability to produce biofilm in about nineteen (57.6%) isolates while fourteen isolates (42.4%) nonbiofilm producers.[33]

Effect of ½MIC of Nitric Oxide on Biofilm Formation of *Pseudomonas aeruginosa*

In this study, the impact of NO on the biofilm of 18 tested isolates, which exhibited moderate and strong biofilm formation, was investigated. The isolates were exposed to SNP at ½MIC, and their biofilm-forming capacity was compared with that at 0 concentration of SNP. The results depicted in Figure 1 unveiled a discernible pattern: across all tested isolates, there was a notable distinction in biofilm formation capacity before and after exposure to ½MIC of SNP. However, it's intriguing to note that isolates PA53 and PA69 showed a degree of resilience to this effect, displaying less susceptibility compared to the others.



Note: Asterikes indicate statistically significant differences

Figure 1.Impact of Nitric Oxide on *Pseudomonas aeruginosa* Biofilm Formation

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The observed variability in the response of *P. aeruginosa* isolates to exposure to ½MIC of SNP raises intriguing questions about underlying mechanisms and potential implications. While the majority of tested isolates demonstrated a significant alteration ($p < 0.05$) in biofilm formation capacity following exposure, the relative resistance of isolates PA53 and PA69 warrants further investigation.

This phenomenon could be attributed to several factors, including genetic variations, phenotypic differences, and adaptive responses. Previous studies have highlighted the role of genetic determinants and regulatory pathways in mediating *P. aeruginosa* biofilm formation and its susceptibility to external stimuli.[34,35] Additionally, phenotypic heterogeneity within *P. aeruginosa* populations may contribute to differential responses to antimicrobial agents and environmental stressors.[36]

The resilience exhibited by isolates PA53 and PA69 could potentially stem from adaptive mechanisms that confer enhanced biofilm stability or reduced sensitivity to nitric oxide-mediated dispersal, for instance, mutations in genes associated with biofilm formation or nitric oxide detoxification pathways could confer a survival advantage under these conditions.[12,14]

Furthermore, the clinical implications of these findings warrant consideration. Understanding the factors that influence *P. aeruginosa* biofilm formation and dispersal is crucial for developing effective strategies for combating biofilm-associated infections. Targeting pathways involved in biofilm regulation and nitric oxide signalling may offer promising therapeutic avenues for managing *P. aeruginosa* infections.[16]

Figure 2 illustrates a scanning electron microscopy (SEM) image, depicting the presence of biofilms adhered to the polystyrene surface. Prior to NO treatment, the biofilms appeared mature and fully formed, suggesting extensive biofilm formation. However, upon exposure to NO, a noticeable disruption in the biofilm structure was observed, with only sparse patches of cell aggregates remaining. Comparing our SEM findings with other studies on the effects of NO treatment on *P. aeruginosa* biofilms reveals consistency with previous research, for instance, a study by Tortella Fuentes et al. investigated the impact of NO-releasing nanoparticles on *P. aeruginosa* biofilms and observed a similar disruption in biofilm architecture following NO treatment. The SEM images from their study showed a significant reduction in biofilm biomass and disruption of cell aggregates, consistent with our observations.[37]

Similarly, Fleming et al. explored the use of NO-releasing coatings on medical devices to prevent biofilm formation. Their SEM analysis revealed a marked reduction in biofilm thickness and coverage on NO-releasing surfaces compared to control surfaces. These findings corroborate our results, suggesting the effectiveness of NO in destabilising *P. aeruginosa* biofilms.[38]

Moreover, a study by Chua et al. investigated the role of nitrite as a precursor of NO in disrupting *P. aeruginosa* biofilms. Their SEM images showed a significant reduction in biofilm biomass and disruption of biofilm structure following nitrite treatment, similar to the effects observed in our study with direct NO exposure.[34]

Overall, the consistency between our SEM findings and those of other studies underscores the robustness of NO-mediated biofilm disruption in P. aeruginosa. These findings support the potential of NO-based strategies as promising approaches for combating biofilm-associated infections and improving clinical outcomes.

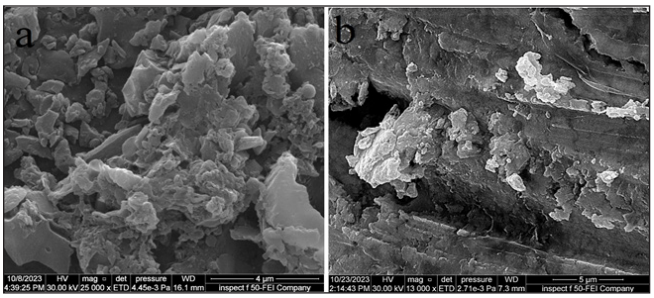

Figure 2.Electron Micrograph of *Pseudomonas aeruginosa* Biofilm (A) After and (B) Before Exposure to Nitric Oxide

Impact of Nitric Oxide on the Expression of Biofilm-Associated Genes (*pslA*, *pelA*, and *algD*) in *Pseudomonas aeruginosa*

The findings presented in Table 3 unveil a spectrum of responses among different isolates of *P. aeruginosa* when subjected to an experimental condition, likely NO. By examining the fold changes in gene expression levels of key biofilm-related genes (pslA, pelA, and algD), we gain insights into the intricate regulatory dynamics governing biofilm formation in these isolates in response to NO exposure.

Table 3.Alterations in the Expression Levels of Biofilm-Associated Genes

Isolate Code	Fold Change		
	pslA	*pelA*	*algD*
P50	2.517	4.316	0.0441
P53	0.0384	0.0412	0.0473
P58	4.2870	1.7411	3.7321
P61	0.0291	0.1767	0.2679
P65	0.0179	1.1486	0.0014
P69	0.5743	0.8122	0.0059
P70	0.0883	4.2870	0.6597

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Isolate P50 emerges as particularly noteworthy, displaying a notable increase in the expression of both *pslA* and *pelA* genes, indicative of a bolstered biofilm formation capacity. Conversely, the significant decrease in *algD* expression points towards a concurrent suppression of alginate biosynthesis, underscoring the complexity of regulatory networks modulated by NO within *P. aeruginosa* biofilms.

In stark contrast, isolate P53 demonstrates a stark downregulation across all three biofilm-related genes, hinting at a pronounced inhibitory effect of NO on biofilm formation pathways within this isolate. This finding implies a potential role for NO as a disruptor of biofilm development in certain *P. aeruginosa* strains.

The response of isolate P58 presents a multifaceted scenario, characterised by a substantial increase in *pslA* and *algD* gene expressions, alongside moderate enhancement of *pelA* expression. This intricate interplay suggests a nuanced regulatory mechanism involving both positive and negative influences of NO on biofilm-related gene expression in this particular isolate.

Isolates P61, P65, P69, and P70 collectively exhibit varying degrees of downregulation in biofilm-related gene expression post-NO treatment, suggesting a general trend towards suppression of biofilm-associated genetic activity in response to NO exposure, albeit with some variations in magnitude.

These findings underscore the heterogeneous and intricate responses of *P. aeruginosa* isolates to NO treatment, shedding light on the multifaceted regulatory mechanisms governing biofilm formation in this opportunistic pathogen. Further exploration into the specific molecular pathways and mechanisms underlying these observed gene expression changes is warranted to better understand the implications for biofilm formation, antimicrobial resistance, and pathogenicity in *P. aeruginosa.*

While research specifically investigating the impact of NO on the gene expression of *pslA, pelA*, and *algD* genes in *P. aeruginosa* is lacking, qualitative and quantitative assays have been employed to assess polysaccharide mass within biofilms post-NO exposure. Utilising fluorescence imaging, Congo red specific binding assays, and exopolysaccharide extraction following various durations of NO exposure, significant reductions in polysaccharide mass were observed at 6 h, 8 h, and 18 h, thereby facilitating biofilm dispersion.[39]

Furthermore, NO was found to decrease intracellular c-di-GMP levels and enhance phosphodiesterase activity in cell-free extracts, indicating potential post-translational regulatory mechanisms.[15] Given that c-di-GMP plays a pivotal role in regulating Pel biosynthesis post-translationally and governs Psl formation transcriptionally and translationally,[40] it is speculated that the altered matrix structure observed under the influence of NO may be attributed to changes in exopolysaccharides. [41]

In the context of *P. aeruginosa* dispersal response, NO treatment has been shown to reduce c-di-GMP levels by activating phosphodiesterases, leading to the cleavage of c-di-GMP into guanosine triphosphate (GTP). This GTP molecule can subsequently be transformed into guanine tetraphosphates by GTP diphosphokinase, a process implicated in biofilm dispersal.[42]

Conclusion

In this study, variations in *P. aeruginosa* biofilm formation and response to NO treatment were investigated. Different specimen sources showed varied biofilm-forming abilities, suggesting diverse influences. NO exposure led to significant changes in biofilm formation, with some isolates showing resilience. Analysis of gene expression revealed intricate regulatory dynamics. Consistency between findings and other studies supports NO's efficacy in disrupting *P. aeruginosa* biofilms, offering potential therapeutic strategies. Overall, this research enhances our understanding of biofilm biology and NO's role, helping in the development of new approaches to combat infections.

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Conflict of Interest: None

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