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Biofilm Biomass Differentially Modulates Neutrophil Oxidative Responses, Macrophage Cytokine Production, and Biofilm Persistence in Chronic Wound-Associated *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Abstract

Chronic wounds frequently contain polymicrobial biofilms that sustain inflammation and delay repair. This in vitro study examined clinical *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolates from five patients with chronic wounds under four conditions: sterile control, *S. aureus* biofilm, *P. aeruginosa* biofilm, and mixed-species biofilm. Mature biofilms were co-cultured with neutrophils or monocyte-derived macrophages from five healthy donors. Biofilm biomass was quantified by crystal violet staining, neutrophil reactive oxygen species (ROS) production by luminol-enhanced chemiluminescence, macrophage IL-1β, TNF-α, and IL-10 concentrations by ELISA, and biofilm viability by MTT assay. Mixed-species biofilms produced the greatest baseline biomass (OD_{570}, 2.733 ± 0.491) and the highest peak ROS (16,751 ± 4,937 RLU), ROS area under the curve, and cytokine concentrations. After macrophage co-culture, mixed-species biofilms retained the greatest residual biomass (OD_{570}, 1.780 ± 0.339) despite having the lowest biofilm viability (47.5 ± 9.1%; $P < 0.001$). Baseline biomass correlated with peak ROS (Spearman $\rho = 0.829$; $P < 0.001$) and predicted peak ROS in simple linear regression ($R^2 = 0.698$; $P < 0.001$). These findings indicate that greater biofilm biomass is associated with stronger innate immune activation and persistent residual biofilm. Because the biological sample comprised five wound isolates and five donors, the inferential findings should be regarded as exploratory pending validation in larger, independent cohorts.

Keywords: Innate Immunity, Reactive Oxygen Species, Mixed-Species Infection, Crystal Violet Assay, Interleukin-1 Beta, Tumor Necrosis Factor Alpha

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INTRODUCTION

Chronic wound problems result from prolonged inflammation and delayed wound healing.[1] Unlike acute wounds, they involve ongoing microbial colonization, disrupted blood vessel growth, extracellular matrix breakdown, and continuous tissue damage.[2] They are common in diabetic foot ulcers, venous leg ulcers, and decubitus ulcers, causing recurrent infections, prolonged treatment, amputations, and high costs. With rising rates of diabetes, vascular disease, and obesity and an aging population, managing these wounds is a clinical and public health priority that requires an understanding of their chronic nature.[3-5]

Among the factors that impede chronic wound healing, microbial biofilm formation is particularly influential. In these wounds, microorganisms form structured communities embedded in the extracellular polymeric substance (EPS) they produce, which provides stability, facilitates communication, limits antimicrobial penetration, and protects against antibiotics and immune responses.[6] Biofilms create persistent infections, maintain inflammation, and hinder tissue regeneration and remodeling. Their high resistance explains why biofilm-related infections are difficult to eradicate despite antimicrobial treatment.[7,8] *Staphylococcus aureus* and *Pseudomonas aeruginosa* effectively colonize damaged tissue, form biofilms, and evade immune defenses.[9] *S. aureus* adheres strongly and evades immune responses, whereas *P. aeruginosa* produces substances that promote biofilm maturation, bacterial adaptation, and tissue damage.[9,10] These organisms often coexist in chronic wounds, forming polymicrobial biofilms that are more complex, tolerant, and pathogenic than single-species biofilms.[11,12] These findings suggest that polymicrobial biofilms better reflect the biological environment of chronic wound infections.

The innate immune system constitutes the host's initial line of defense during wound healing. Neutrophils arrive quickly at infection sites, where pathogen recognition triggers an oxidative burst that produces reactive oxygen species (ROS).[13] Although essential for killing microbes, prolonged biofilm stimulation can cause excessive oxidative stress, damaging tissues, degrading the extracellular matrix, and delaying repair.[14] As inflammation progresses, macrophages regulate the immune response through bacterial clearance, cytokine release, and tissue remodeling. Pro-inflammatory mediators such as IL-1β and TNF-α, along with regulatory cytokines such as IL-10, affect this equilibrium.[5,15,16] Disruption leads to unresolved inflammation, poor bacterial clearance, and ongoing tissue damage, making neutrophil oxidative responses and macrophage cytokine production indicators of innate immune activity in chronic wound infections.

While many studies examine biofilm formation, oxidative stress, cytokines, or bacterial survival separately, the link between biofilm biomass and immune activation remains unclear.[17] Most research examines these processes separately, making it difficult to determine whether increased biofilm biomass influences neutrophil activation, macrophage responses, or biofilm persistence following immune exposure.[18] Polymicrobial biofilms comprising *S. aureus* and *P. aeruginosa* exhibit interactions that differ from those observed in monospecies biofilms, making this issue particularly significant. A combined analysis of biofilm structure and immune response is needed to understand the persistence of chronic wounds.

This study created an in vitro chronic wound model using clinical bacterial isolates, human neutrophils, and macrophages to investigate the relationship between biofilm biomass and immune responses. It evaluated biofilm density, neutrophil oxidative activity, macrophage cytokine production, residual biofilm, and viability. The hypothesis was that larger polymicrobial biofilms would provoke stronger immune responses and be more persistent than monospecies biofilms.

This integrated in vitro model was designed to clarify the relationships among biofilm burden, innate immune activation, and persistence, thereby providing a mechanistic basis for future chronic wound therapeutic studies.

MATERIALS AND METHODS

From April to June 2026, an in vitro study was conducted at the Institute of Tropical

Disease, Surabaya, Indonesia. Clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were obtained from wound specimens collected from five patients at Ns. OZ Wound Care Clinic, Surabaya. Four experimental conditions were evaluated: sterile control, *S. aureus* biofilm, *P. aeruginosa* biofilm, and mixed-species biofilm. The workflow included bacterial isolation, biofilm formation, immune cell co-culture, ROS measurement, cytokine quantification, and post-co-culture biofilm assessment (Figure 1). The clinical presentations of samples LK001–LK005 are shown in Figure 2. The Health Research Ethics Committee of Noor Huda Mustofa University approved the study (No. 3517/KEPK/UNIV-NHM/EC/IV/2026), and all participants provided written informed consent.

The wound specimens were cultured on blood and MacConkey agar and then incubated aerobically at 37 °C for 18-24 hours. Pure isolates were identified based on colony morphology, Gram staining, and biochemical characterization and confirmed using MALDI-TOF MS. Bacterial suspensions were standardized to 0.5 McFarland (approximately 1 × 10^8 CFU/mL) before biofilm formation. A sterile, flat-bottom 96-well microplate was used to develop mature biofilms by incubating the bacterial suspensions. The crystal violet assay at OD_{570} was used to measure initial biofilm biomass.

Peripheral venous blood (20 mL) was collected from each of five healthy donors using heparin as an anticoagulant. Neutrophils were isolated by density-gradient centrifugation with Polymorphprep and used when viability was at least 95%. Mature biofilms were co-cultured with neutrophils (1 × 10^6 cells/well) for 24 hrs at 37 °C in 5% CO_2. ROS production was quantified by luminol-enhanced chemiluminescence using a Centro LB 960 luminometer and summarized as peak relative light units (RLU) and area under the response curve (AUC; RLU·min). Peripheral blood mononuclear cells were isolated using Ficoll-Paque PLUS; monocytes were differentiated for 7 days in RPMI-1640 containing 10% fetal bovine serum and macrophage colony-stimulating factor (50

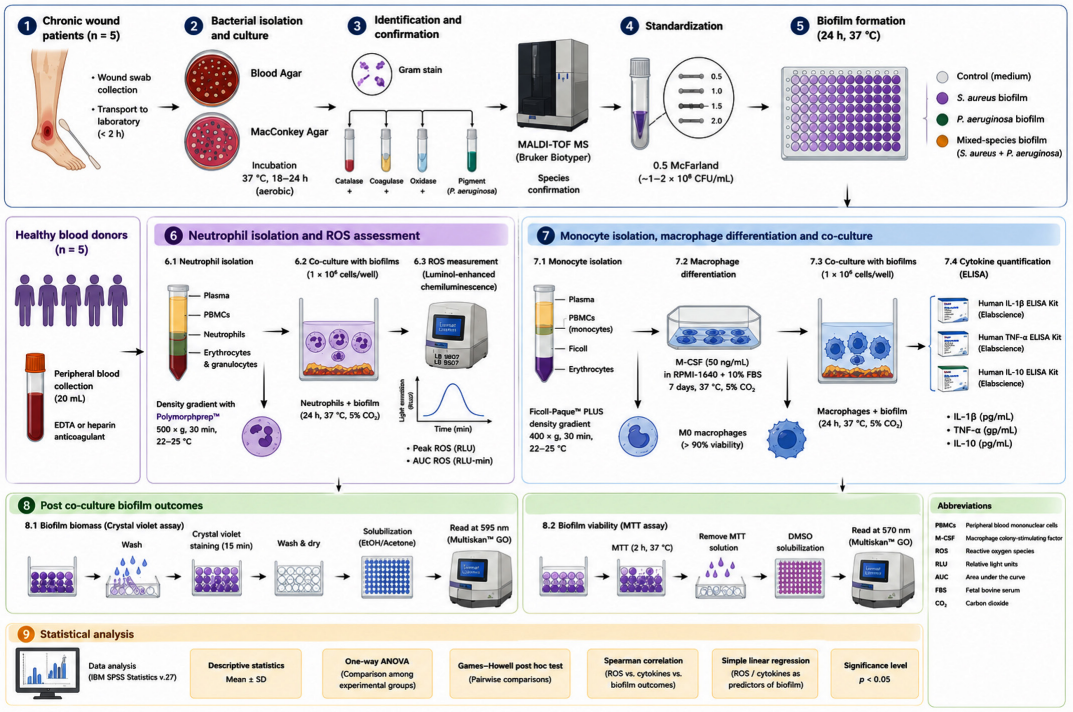

Figure 1. Experimental workflow from chronic-wound sampling and bacterial identification through biofilm formation, immune-cell co-culture, ROS and cytokine measurement, post-co-culture biofilm assessment, and statistical analysis

ng/mL), consistent with established monocyte-to-macrophage differentiation procedures.[19] Macrophages (1×10^6 cells/well) were co-cultured with mature biofilms for 24 hrs at 37 °C in 5% CO_2. Supernatants were collected, and IL-1β, TNF-α, and IL-10 concentrations were quantified by ELISA according to the manufacturers' instructions; cytokine measurements were performed under standardized handling conditions to minimize preanalytical instability.[20] Residual biofilm biomass was measured by crystal violet staining, an established microtiter-plate biofilm biomass method,[21] and biofilm was assessed by MTT reduction. Biomass reduction was calculated relative to baseline biomass. Each wound-isolate-donor-condition combination was assayed in triplicate, yielding 300 well-level observations (5 wound samples × 5 donors × 4 conditions × 3 technical replicates).

Statistical analyses were performed using IBM SPSS Statistics version 30.0 and GraphPad Prism version 10. Continuous variables are reported as mean ± standard deviation. Distributional assumptions were assessed using the Shapiro-Wilk test, and variance homogeneity was assessed using Levene's test. Group differences were evaluated using Welch's one-way ANOVA followed by Games-Howell pairwise comparisons. Associations were examined using Spearman's rank correlation. Simple linear regression was

Table 1. Baseline biofilm biomass across experimental groups

Experimental Group	OD_{570} (Mean ± SD)
Control	0.072 ± 0.028
S. aureus	1.524 ± 0.324
P. aeruginosa	2.114 ± 0.389
Mixed biofilm	2.733 ± 0.491

Table 2. Neutrophil oxidative responses

Experimental Group	Peak ROS (RLU)	AUC ROS (RLU·min)
Control	1,023.84 ± 327.96	31,137 ± 21,885
S. aureus	7,814 ± 2,241	223,380 ± 41,293
P. aeruginosa	12,004 ± 3,506	338,545 ± 51,255
Mixed biofilm	16,751 ± 4,937	487,335 ± 76,516



Figure 2. Clinical presentation of chronic-wound samples used for bacterial isolation: (A) LK001, sacral/gluteal pressure injury; (B) LK002, diabetic foot ulcer at a post-toe-amputation site; (C) LK003, dorsal-foot venous ulcer; (D) LK004, sacral/gluteal pressure injury associated with diabetes mellitus; and (E) LK005, plantar diabetic foot ulcer (Source: Photographs from the clinical documentation of Ns. OZ Wound Care Clinic, Surabaya, Indonesia)

used to assess whether baseline OD_{570} predicted peak ROS. Two-sided P-values <0.05 were considered statistically significant. Because triplicate wells were technical replicates nested within a design involving five wound isolates and five donors, inferential estimates were interpreted as exploratory rather than as population-level confirmation.

RESULTS

Baseline biofilm biomass

The crystal violet assay was utilized to measure baseline biofilm biomass across four groups: control, *S. aureus*, *P. aeruginosa*, and mixed species. The baseline biofilm biomass assessment revealed distinct differences among

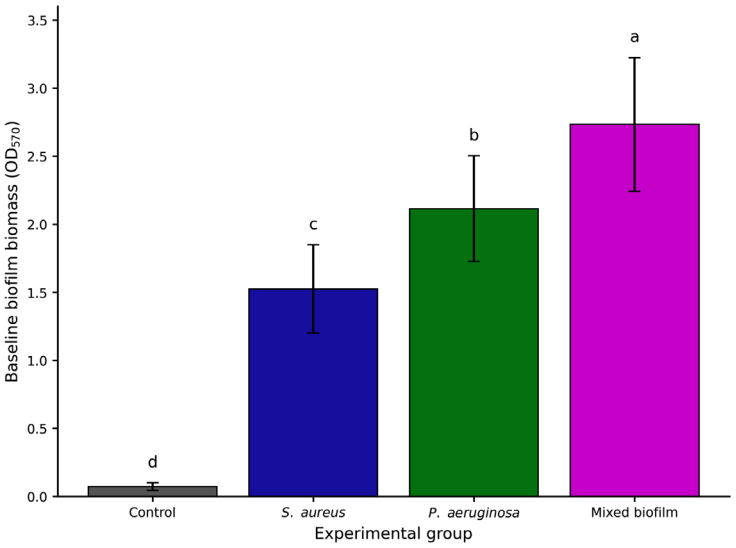

Figure 3. Baseline biofilm biomass measured by crystal violet staining at OD_{570}. Bars show mean ± SD. Different lowercase letters indicate significant pairwise differences by Games-Howell test (P < 0.05)

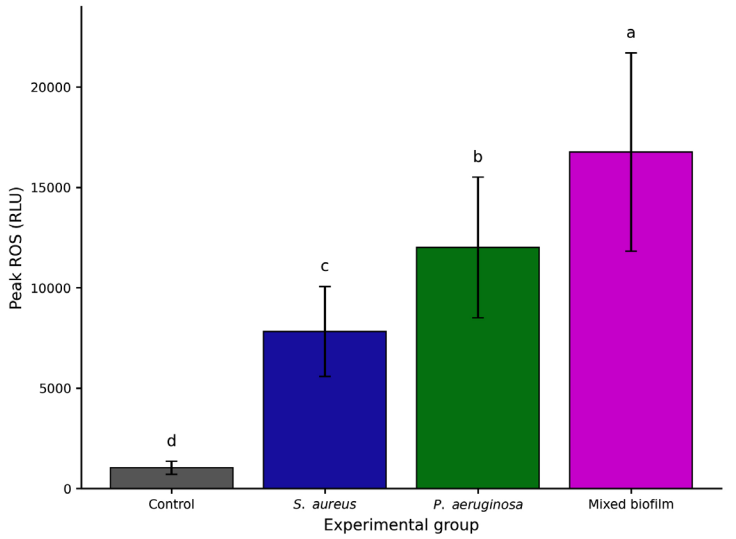

Figure 4. Peak neutrophil reactive oxygen species production after biofilm exposure. Bars show mean ± SD in relative light units (RLU). Different lowercase letters indicate significant pairwise differences by Games-Howell test (P < 0.05)

all experimental groups (Table 1; Figure 3). The control group had minimal biomass (0.072 ± 0.028), whereas *S. aureus* showed increased biomass (1.524 ± 0.324). *P. aeruginosa* had higher biomass (2.114 ± 0.389), and the mixed-species group had the highest biomass (2.733 ± 0.491).

Neutrophil oxidative responses

Neutrophil oxidative responses increased progressively across experimental conditions (Table 2; Figures 4 and 5). Peak ROS was 1,023.84 ± 327.96 RLU in the control group, 7,814 ± 2,241 RLU for *S. aureus*, 12,004 ± 3,506 RLU for *P. aeruginosa*, and 16,751 ± 4,937 RLU for the mixed-species biofilm. ROS AUC showed the same ordering: 31,137 ± 21,885, 223,380 ± 41,293, 338,545 ± 51,255, and 487,335 ± 76,516 RLU·min, respectively.

Macrophage cytokine production

Macrophage inflammatory responses were determined by measuring IL-1β, TNF-α, and IL-10 concentrations following 24 hrs of biofilm co-culture. Cytokine concentrations differed significantly among the experimental groups (Table 3; Figure 6). The control group produced the lowest

Table 3. Macrophage cytokine production (pg/mL)

Group	IL-1β	TNF-α	IL-10
Control	25.0 ± 6.5	20.6 ± 4.7	36.4 ± 10.7
S. aureus	214.7 ± 52.4	180.1 ± 37.5	93.1 ± 26.7
P. aeruginosa	291.9 ± 56.7	248.7 ± 50.3	135.0 ± 33.2
Mixed biofilm	439.1 ± 96.1	371.1 ± 77.7	166.9 ± 48.6

Table 4. Post-co-culture biofilm viability and persistence

Group	OD_{570} Post	Biofilm viability (%)	Biomass Reduction (%)
Control	0.067 ± 0.026	96.9 ± 2.9	7.5 ± 1.5
S. aureus	1.219 ± 0.261	72.8 ± 7.0	20.0 ± 2.8
P. aeruginosa	1.586 ± 0.303	60.3 ± 7.2	25.0 ± 2.8
Mixed biofilm	1.780 ± 0.339	47.5 ± 9.1	34.9 ± 3.4

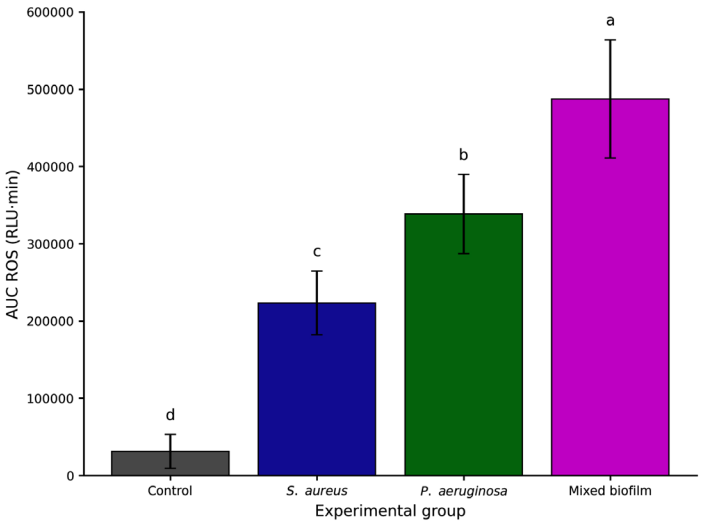

Figure 5. Area under the neutrophil reactive oxygen species response curve. Bars show mean ± SD in RLU·min. Different lowercase letters indicate significant pairwise differences by Games-Howell test ($P < 0.05$)

concentrations of all cytokines, including IL-1β (25.0 ± 6.5 pg/mL), TNF-α (20.6 ± 4.7 pg/mL), and IL-10 (36.4 ± 10.7 pg/mL). Higher cytokine concentrations were detected after exposure to monospecies biofilms. The mixed-species biofilm induced the highest cytokine levels: IL-1β, 439.1 ± 96.1 pg/mL; TNF-α, 371.1 ± 77.7 pg/mL; and IL-10, 166.9 ± 48.6 pg/mL; these differences were statistically significant (P < 0.001).

Post-co-culture biofilm viability and persistence

Quantitative changes in residual biofilm biomass, biofilm viability, and percentage reduction after macrophage co-cultivation revealed significant variation among the experimental groups (Table 4; Figure 7). The control group had the lowest residual biomass (0.067 ± 0.026), followed by *S. aureus* (1.219 ± 0.261) and *P. aeruginosa* (1.586 ± 0.303). The mixed-species biofilm had the highest residual biomass (1.780 ± 0.339). Biofilm viability declined from 96.9 ± 2.9% in the control group to 72.8 ± 7.0% in *S. aureus*, 60.3 ± 7.2% in *P. aeruginosa*, and 47.5 ± 9.1% in the mixed-species biofilm. Biomass reduction increased significantly from 7.5 ± 1.5% in the control group to 34.9 ± 3.4% in the mixed-species

Table 5. Spearman correlation analysis

Variable Pair	Spearman's ρ	P-value
OD_{570} vs Peak ROS	0.829	<0.001
OD_{570} vs IL-1β	0.861	<0.001
OD_{570} vs TNF-α	0.871	<0.001
Peak ROS vs IL-1β	0.91	<0.001
Peak ROS vs TNF-α	0.91	<0.001
Peak ROS vs IL-10	0.85	<0.001
Peak ROS vs Biofilm viability	-0.72	<0.001

Table 6. Linear regression predicting peak ROS

Parameter	Value
R	0.836
R^2	0.698
Adjusted R^2	0.696
β (standardized)	0.836
F (1,298)	690.344
P-value	<0.001

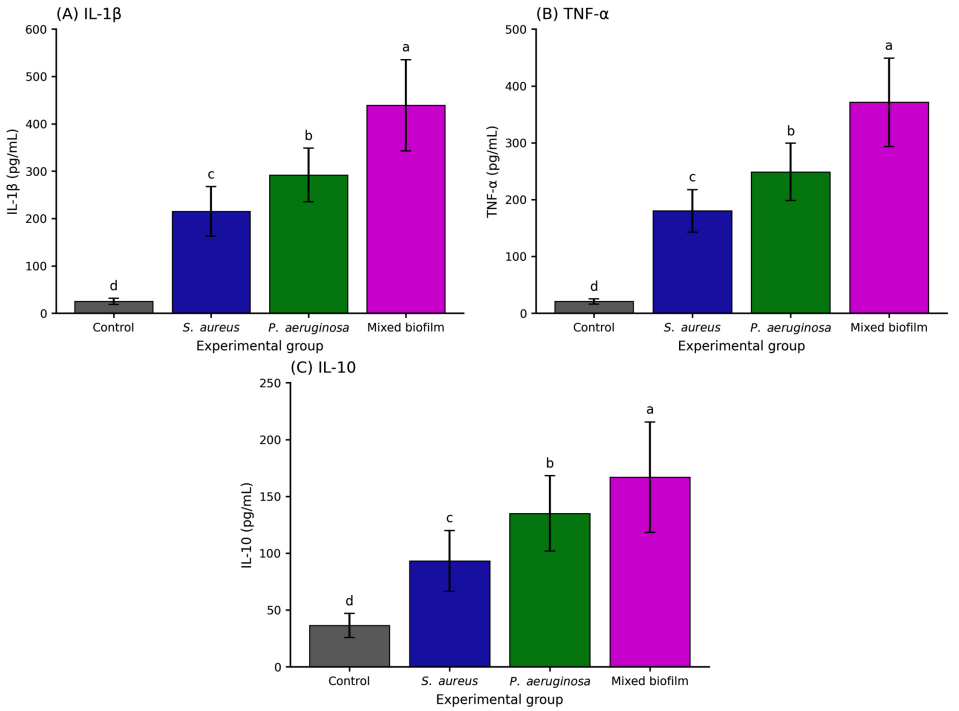

Figure 6. Macrophage cytokine production after 24 hrs of biofilm co-culture: (A) IL-1β, (B) TNF-α, and (C) IL-10. Bars show mean ± SD. Different lowercase letters indicate significant pairwise differences by Games-Howell test (P < 0.05)

biofilm, with the difference between groups being statistically significant (P < 0.001).

Correlation analysis

Spearman's rank correlation identified strong positive associations between baseline biofilm biomass and peak ROS, IL-1β, and TNF-α (Table 5). Peak ROS was also positively associated with IL-1β, TNF-α, and IL-10, whereas its association with biofilm viability was negative. All reported correlations were significant at P < 0.001.

Linear regression analysis

A simple linear regression was conducted to determine whether baseline biofilm biomass predicted neutrophil peak ROS production (Table 6). Baseline OD_{570} was a significant positive predictor of peak ROS (R = 0.836; R^2 = 0.698; adjusted R^2 = 0.696; F [1,298] = 690.344; P < 0.001), indicating that higher baseline biomass was associated with a greater neutrophil oxidative response.

DISCUSSION

Our data clearly indicate a significant increase in total biofilm accumulation during co-cultivation of *S. aureus* and *P. aeruginosa*, exceeding the biomass produced by either species alone. This finding points to polymicrobial cooperation as a driver of more mature, organized biofilms. Bacterial growth and enhanced production of extracellular polymeric substances increase biomass. By promoting bacterial adhesion and stabilizing the biofilm architecture, the EPS matrix restricts antimicrobial and immune-cell penetration, thereby favoring bacterial survival within infected tissue.[22,23]

This synergistic increase in dual-species biofilm production corroborates earlier documentation of host-pathogen dynamics, in which polymicrobial coordination accelerates matrix growth.[24] *S. aureus* aids attachment, whereas *P. aeruginosa* enhances maturation through exopolysaccharides, enzymes, pyocyanin, and quorum-sensing molecules.[25] These interactions create a complex, stable biofilm that is more resistant to stress.[26]

The findings support previous reports that polymicrobial biofilms have greater biomass, greater antimicrobial tolerance, and greater persistence than monospecies biofilms.[26] Similar patterns have been observed in chronic wound and respiratory infection models, in which interactions among multiple microbes produce thicker, more resistant biofilms.[24] Although biofilm formation varies with bacterial strain, growth conditions, and experimental models, evidence shows that cooperative interactions significantly increase biofilm accumulation. These results suggest that biofilm biomass not only reflects bacterial growth but also indicates the biological complexity of polymicrobial communities, which may contribute to persistent infections.

The study showed increased biofilm biomass alongside heightened neutrophil oxidative responses, linking biofilm load with innate immune

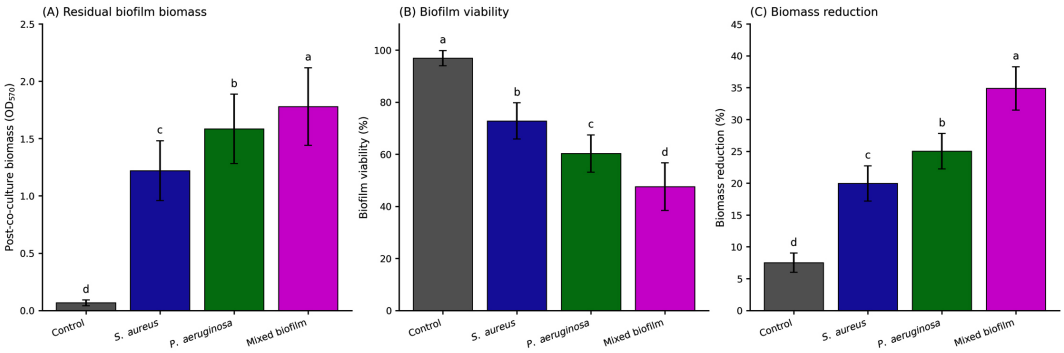

Figure 7. Post-co-culture biofilm outcomes: (A) residual biofilm biomass, (B) biofilm viability, and (C) biomass reduction. Bars show mean ± SD. Different lowercase letters indicate significant pairwise differences by Games-Howell test (P < 0.05)

activation. As frontline responders to bacterial invasion, neutrophils generate ROS through NADPH oxidase activation after detecting pathogens.[27] Although ROS are crucial for killing bacteria, prolonged exposure, particularly to mature biofilms, can cause excessive oxidative stress.[28,29] This may lead to tissue damage, extracellular matrix breakdown, impaired fibroblast growth, and delayed re-epithelialization, thereby sustaining inflammation in chronic wounds.

The stronger oxidative responses in mixed-species biofilms may be due to their complex structure and higher microbial load. Extracellular substances are abundant in mature biofilms of *S. aureus* and *P. aeruginosa*, protecting bacteria from phagocytosis and limiting immune-cell infiltration. This environment keeps neutrophils activated without effectively clearing bacteria, causing sustained oxidative bursts. *P. aeruginosa* also produces virulence factors such as pyocyanin, elastase, and rhamnolipids, which further affect neutrophil function and increase oxidative stress during chronic infection.[14]

These findings align with previous studies showing that polymicrobial biofilms provoke stronger inflammatory responses than monospecies biofilms because greater biofilm maturity prolongs host-pathogen interactions and enhances neutrophil activation.[30] Chronic wound models also indicate that persistent biofilms sustain continuous ROS production despite the presence of immune cells, promoting chronic inflammation rather than bacterial clearance.[11,31] These observations suggest that biofilm biomass influences neutrophil oxidative activity during wound infection. The increases in peak ROS and ROS AUC show that polymicrobial biofilms intensify and prolong neutrophil activation, highlighting their inflammatory potential.

The study showed that polymicrobial biofilms activate the innate immune response, leading to increased neutrophil oxidative responses and macrophage cytokine production. Compared with monospecies biofilms, exposure to mixed-species biofilms led to increased secretion of the pro-inflammatory cytokines IL-1β and TNF-α, as well as IL-10.[32] This profile indicates that mature biofilms promote both inflammation and immune regulation. IL-1β and TNF-α are crucial for antibacterial defense but may cause prolonged inflammation and tissue damage and hinder regeneration when persistently produced.[33] The rise in IL-10 might represent a regulatory attempt to limit excessive inflammation; however, it appears inadequate to restore immune balance in the presence of persistent biofilms.

Mixed-species biofilms enhance cytokine production because of their increased microbial load and complexity. Mature biofilms release molecules such as lipopolysaccharide, lipoteichoic acid, peptidoglycan fragments, and extracellular DNA, activating pattern-recognition receptors on macrophages.[22] This activates Toll-like receptor signaling and the NF-κB pathway, leading to cytokine production, while inflammasomes promote the maturation of IL-1β.[34] Ongoing microbial exposure maintains macrophage activation, driving chronic wound inflammation.

The findings agree with prior studies showing that polymicrobial biofilms trigger stronger macrophage activation and cytokine secretion than monospecies biofilms.[35] Chronic wound studies indicate that increased biofilm maturity is associated with prolonged inflammation and poor immune resolution.[36] Although IL-10 increases alongside pro-inflammatory cytokines, it usually cannot counteract sustained immune activation when biofilms persist. Overall, biofilm biomass affects both microbial structure and the extent and duration of macrophage-driven inflammation, contributing to chronic inflammation in nonhealing wounds.

The study showed that although macrophage co-culture reduced biofilm biomass and viability, polymicrobial biofilms remained more persistent than monospecies biofilms. Residual biomass was highest in the mixed-species group, indicating that macrophages could not fully eliminate mature biofilms. This finding suggests that established biofilms have protective mechanisms that enable bacterial survival despite immune attack.[37] Therefore, macrophage activation alone is insufficient to eradicate mature polymicrobial biofilms once they have formed.

The greater persistence of mixed-species biofilms can plausibly be attributed to the protective EPS layer and cooperative interactions between *S. aureus* and *P. aeruginosa*.[26] As a physical barrier, EPS restricts macrophage infiltration, impedes antimicrobial diffusion,

and dampens phagocytic activity. Interspecies communication via quorum sensing coordinates gene expression involved in stress responses, nutrient utilization, and biofilm stability. These mechanisms enhance microbial survival and reduce immune clearance.[15,22]

The findings align with studies showing that mature polymicrobial biofilms are more resistant to immune clearance than monospecies biofilms because of their complex structure and extracellular matrix.[22] These biofilms enable bacterial persistence despite inflammation, thereby prolonging infection and delaying healing. Reductions in biofilm biomass do not indicate complete bacterial removal; residual biofilm after macrophage exposure is a key feature of chronic wounds and may help explain recurrent infections despite immune responses.

Correlation and linear regression analyses strengthened the study by showing that baseline biofilm biomass was associated with innate immune activation. Positive correlations between biofilm biomass, neutrophil oxidative responses, and macrophage cytokine production indicate that greater biofilm biomass is associated with stronger inflammatory responses. The regression model identified baseline biofilm biomass as a key predictor of peak ROS production, suggesting that biofilm burden influences neutrophil oxidative activity during chronic wound infection. These findings quantitatively demonstrate the relationship between biofilm development and host immune responses.

This predictive relationship is biologically plausible because larger biofilms contain greater amounts of bacterial biomass, extracellular substances, microbial metabolites, and pathogen-associated molecular patterns that continually stimulate innate immune cells. Persistent exposure to these components causes sustained activation of neutrophils and macrophages, leading to prolonged production of reactive oxygen species and cytokines.[38,39] Therefore, biofilm biomass is not only a structural marker of microbial colonization but also an indicator of the level of host inflammatory activation.

Similar associations between biofilm burden and inflammatory responses have been reported in experimental models of chronic biofilm-associated infections, in which increasing biofilm maturity was accompanied by enhanced oxidative stress and cytokine production. However, relatively few studies have quantitatively evaluated these relationships using both correlation and regression analyses within a single experimental model. By integrating structural biofilm measurements with functional immune parameters, the present study provides additional evidence that biofilm biomass is closely linked to the magnitude of innate immune activation and may therefore serve as a useful indicator for evaluating biofilm-associated inflammation in chronic wounds.

This study shows that biofilm biomass is linked to innate immune activation and resistance to immune clearance. Integrating biofilm biomass, neutrophil responses, macrophage cytokines, and post-co-culture biofilm viability within a single model highlights interactions between microbes and immune cells in chronic wounds. These findings suggest that biofilm persistence results from both microbial growth and ongoing interactions between biofilm structure and host inflammation.

The findings indicate that chronic wound infection management may require both biofilm disruption and control of damaging inflammation. Recent evidence emphasizes that biofilm-associated immune evasion, dysregulated ROS production, and incomplete biofilm removal can jointly sustain chronic infection.[40-42] Combining biofilm-disrupting strategies with appropriately selected antimicrobials and host-directed approaches warrants evaluation in larger translational models.

Several limitations affect the interpretation of these findings. First, the model was conducted in vitro and therefore did not reproduce vascular supply, tissue architecture, resident microbiota, or systemic host influences present in chronic wounds. Second, the biological sample comprised only five wound-derived isolate sets and five healthy donors. Although each combination was tested in triplicate, technical replicates do not increase the number of independent patients or donors; Welch's ANOVA, correlation, and regression estimates should therefore be considered exploratory, and their precision may be overstated if within-isolate or within-donor dependence is ignored. Third, the model focused on *S. aureus* and *P. aeruginosa*, whereas chronic

wounds may contain anaerobic bacteria, fungi, and more complex communities. Fourth, immune assessment was limited to neutrophil ROS and three macrophage-derived cytokines; neutrophil extracellular traps, macrophage polarization, complement, and adaptive immunity were not examined. Larger studies using mixed-effects analyses, more diverse isolates, and in vivo or tissue-based models are needed to validate these associations.

This study shows that biofilm biomass is closely linked to immune activation and persistence in chronic wounds caused by *S. aureus* and *P. aeruginosa*. Combining biofilm structure analysis with immune response measurements offers a comprehensive view of host–biofilm interactions beyond individual parameters. The findings suggest that biofilm biomass contributes to inflammation and microbial persistence, implying that therapies should target both biofilm disruption and immune modulation to improve chronic wound treatment.

CONCLUSION

Research indicates that biofilms composed of both *Staphylococcus aureus* and *Pseudomonas aeruginosa* produce more biomass than biofilms consisting of a single species. They also elicit stronger neutrophil oxidative responses, higher macrophage cytokine production, and greater persistence after exposure to immune cells. Analyses reveal that baseline biofilm biomass is associated with immune activation and predicts neutrophil responses, indicating that biofilm biomass is an important factor in host-biofilm interactions during chronic wound infection.

These findings provide insight into the mechanisms underlying chronic wound persistence by integrating biofilm structure and immune responses within a single model. Targeting biofilm architecture and inflammation may help manage chronic wound infections. To confirm these results and investigate their potential clinical applications, additional research using sophisticated in vivo models is required.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

OZ conceptualised, designed the study and secured research funding. ADWW applied methodology and supervised the study and provided intellectual input. RR and DF collected chronic wound specimens and recruited participants. OZ and ADWW conducted laboratory experiments. NA and HNC performed statistical analysis. BG, NA and OZ contributed to the interpretation of the results. OZ wrtoe the manuscript. ADWW, BG and HNC reviewed and revised the manuscript. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This study was approved by The Health Research Ethics Committee, Noor Huda Mustofa University, Bangkalan, Indonesia (Ethical Approval No. 3517/KEPK/UNIV-NHM/EC/IV/2026).

INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

REFERENCES

1. Zhao R, Liang H, Clarke E, Jackson C, Xue M. Inflammation in chronic wounds. *Int J Mol Sci.* 2016;17(12):2085. doi: 10.3390/ijms17122085
2. Cullen B. The role of oxidized regenerated cellulose/collagen in chronic wound repair. Part 2. *Ostomy Wound Manage*. 2002;48(6 Suppl):8-13.
3. Armstrong DG, Boulton AJM, Bus SA. Diabetic foot ulcers and their recurrence. *N Engl J Med.* 2017;376(24):2367-2375. doi: 10.1056/NEJMra1615439
4. McDermott K, Fang M, Boulton AJM, Selvin E, Hicks CW. Etiology, epidemiology, and disparities in the burden of diabetic foot ulcers. *Diabetes Care.* 2023;46(1):209-221. doi: 10.2337/dci22-0043
5. Theodorakopoulos G, Armstrong DG. Biofilm in diabetic foot ulcers: a systematic narrative review. *Int Wound J.* 2025;22(12):e70795. doi: 10.1111/iwj.70795
6. Flemming HC, van Hullebusch ED, Little BJ, et al. Microbial extracellular polymeric substances in the environment, technology and medicine. *Nat Rev Microbiol.* 2025;23(2):87-105. doi: 10.1038/s41579-024-01098-y
7. Mah TF. Biofilm-specific antibiotic resistance. *Future Microbiol.* 2012;7(9):1061-1072. doi: 10.2217/fmb.12.76
8. Yu X, Zhang Y, Yao Y, Cho WC, Shang A. Biofilm-mediated immune dysregulation in chronic pulmonary diseases: mechanisms and clinical implications. *Front Microbiol*. 2026;16:1736384. doi: 10.3389/fmicb.2025.1736384
9. Landa G, Clarhaut J, Buyck J, Mendoza G, Arruebo M, Tewes F. Impact of mixed *Staphylococcus aureus-Pseudomonas aeruginosa* biofilm on susceptibility to antimicrobial treatments in a 3D in vitro model. *Sci Rep.* 2024;14(1):29217. doi: 10.1038/s41598-024-79573-y
10. Howden BP, Giulieri SG, Wong Fok Lung T, et al. *Staphylococcus aureus* host interactions and adaptation. *Nat Rev Microbiol.* 2023;21(6):380-395. doi: 10.1038/s41579-023-00852-y
11. Versey Z, da Cruz Nizer WS, Russell E, et al. Biofilm-innate immune interface: contribution to chronic wound formation. *Front Immunol*. 2021;12:648554. doi: 10.3389/fimmu.2021.648554
12. Brandquist ND, Kielian T. Immune dysfunction during *S. aureus* biofilm-associated implant infections: opportunities for novel therapeutic strategies. *NPJ Biofilms Microbiomes.* 2025;11(1):108. doi: 10.1038/s41522-025-00782-y
13. Othman A, Sekheri M, Filep JG. Roles of neutrophil granule proteins in orchestrating inflammation and immunity. *FEBS J.* 2022;289(14):3932-3953. doi: 10.1111/febs.15803
14. da Cruz Nizer WS, Adams ME, Allison KN, et al. Oxidative stress responses in biofilms. *Biofilm*. 2024;7:100203. doi: 10.1016/j.bioflm.2024.100203
15. Zhao A, Sun J, Liu Y. Understanding bacterial biofilms: from definition to treatment strategies. *Front Cell Infect Microbiol.* 2023;13:1137947. doi: 10.3389/fcimb.2023.1137947
16. Seng P, Drancourt M, Gouriet F, et al. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin Infect Dis.* 2009;49(4):543-551. doi: 10.1086/600885
17. Bhattacharya M, Berends ETM, Zheng X, et al. Leukocidins and the nuclease Nuc prevent neutrophil-mediated killing of *Staphylococcus aureus* biofilms. *Infect Immun.* 2020;88(10):e00372-20. doi: 10.1128/IAI.00372-20
18. Seebach E, Elschner T, Kraus FV, Souto-Carneiro M, Kubatzky KF. Bacterial and metabolic factors of staphylococcal planktonic and biofilm environments differentially regulate macrophage immune activation. *Inflammation.* 2023;46(4):1512-1530. doi: 10.1007/s10753-023-01824-3
19. Rozner AE, Dambaeva SV, Drenzek JG, Durning M, Golos TG. Generation of macrophages from peripheral blood monocytes in the rhesus monkey. *J Immunol Methods.* 2009;351(1-2):36-40. doi: 10.1016/j.jim.2009.09.013
20. Ozbey G, Gorczynski R, Erin N. Stability of cytokines in supernatants of stimulated mouse immune cells. *Eur Cytokine Netw.* 2014;25(2):30-34. doi: 10.1684/ecn.2014.0353
21. Kragh KN, Alhede M, Kvich L, Bjarnsholt T. Into the well-a close look at the complex structures of a microtiter biofilm and the crystal violet assay. *Biofilm.* 2019;1:100006. doi: 10.1016/j.bioflm.2019.100006
22. Almatroudi A. Biofilm resilience: molecular mechanisms driving antibiotic resistance in clinical contexts. *Biology*. 2025;14(2):165. doi: 10.3390/biology14020165
23. Mayorga-Ramos A, Carrera-Pacheco SE, Barba-Ostria C, Guaman LP. Bacteriophage-mediated approaches for biofilm control. *Front Cell Infect* Microbiol. 2024;14:1428637. doi: 10.3389/fcimb.2024.1428637
24. Pouget C, Dunyach-Remy C, Magnan C, Pantel A, Sotto A, Lavigne JP. Polymicrobial biofilm organization of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in a chronic wound environment. *Int J Mol Sci*. 2022;23(18):10761. doi: 10.3390/ijms231810761
25. Murray EJ, Dubern JF, Chan WC, Chhabra SR, Williams P. A *Pseudomonas aeruginosa* PQS quorum-sensing system inhibitor with anti-staphylococcal activity sensitizes polymicrobial biofilms to tobramycin. *Cell Chem Biol.* 2022;29(7):1187-1199.e6. doi: 10.1016/j.chembiol.2022.02.007
26. Vestweber PK, Wachter J, Planz V, Jung N, Windbergs M. 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*. 2024;19(5):e0304491. doi: 10.1371/journal.pone.0304491
27. Manoharan RR, Prasad A, Pospisil P, Kzhyshkowska J. ROS signaling in innate immunity via oxidative protein modifications. *Front Immunol.* 2024;15:1359600. doi: 10.3389/fimmu.2024.1359600
28. Vaishampayan A, Grohmann E. Antimicrobials functioning through ROS-mediated mechanisms: current insights. *Microorganisms*. 2021;10(1):61. doi: 10.3390/microorganisms10010061
29. Mammari N, Lamouroux E, Boudier A, Duval RE. Current knowledge on the oxidative-stress-mediated

antimicrobial properties of metal-based nanoparticles. *Microorganisms.* 2022;10(2):437. doi: 10.3390/microorganisms10020437

30. Yadav MK, Chae SW, Go YY, Im GJ, Song JJ. In vitro multi-species biofilms of methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* and their host interaction during in vivo colonization of an otitis media rat model. *Front Cell Infect Microbiol.* 2017;7:125. doi: 10.3389/fcimb.2017.00125
31. Raziyeva K, Kim Y, Zharkinbekov Z, Kassymbek K, Jimi S, Saparov A. Immunology of acute and chronic wound healing. *Biomolecules*. 2021;11(5):700. doi: 10.3390/biom11050700
32. Kaya E, Batoni G, Di Luca M, et al. Planktonic and biofilm-associated *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* elicit differential human peripheral blood cell responses. *Microorganisms.* 2021;9(9):1846. doi: 10.3390/microorganisms9091846
33. Garlanda C, Di Ceglie I, Jaillon S. IL-1 family cytokines in inflammation and immunity. *Cell Mol Immunol.* 2025;22(11):1345-1362. doi: 10.1038/s41423-025-01358-8
34. Chan AH, Schroder K. Inflammasome signaling and regulation of interleukin-1 family cytokines. *J Exp Med.* 2020;217(1):e20190314. doi: 10.1084/jem.20190314
35. Choi E, Murray B, Choi S. Biofilm and cancer: interactions and future directions for cancer therapy. *Int J Mol Sci.* 2023;24(16):12836. doi: 10.3390/ijms241612836
36. Shen AZ, Taha M, Ghannoum M, Tyring SK. Biofilms and chronic wounds: pathogenesis and treatment options. *J Clin Med.* 2025;14(21):7784. doi: 10.3390/jcm14217784
37. Vidakovic L, Mikhaleva S, Jeckel H, et al. Biofilm formation on human immune cells is a multicellular predation strategy of *Vibrio cholerae*. *Cell*. 2023;186(12):2690-2704.e20. doi: 10.1016/j.cell.2023.05.008
38. Marrocco A, Ortiz LA. Role of metabolic reprogramming in pro-inflammatory cytokine secretion from LPS- or silica-activated macrophages. *Front Immunol.* 2022;13:936167. doi: 10.3389/fimmu.2022.936167
39. de Assis SIS, Amendola LS, Okamoto MM, et al. The prolonged activation of the p65 subunit of the NF-kappa-B nuclear factor sustains the persistent effect of advanced glycation end products on inflammatory sensitization in macrophages. *Int J Mol Sci.* 2024;25(5):2713. doi: 10.3390/ijms25052713
40. Sahu A, Ruhal R. Immune system dynamics in response to *Pseudomonas aeruginosa* biofilms. *NPJ Biofilms Microbiomes.* 2025;11(1):104. doi: 10.1038/s41522-025-00738-2
41. Hunt M, Torres M, Bachar-Wikstrom E, et al. Cellular and molecular roles of reactive oxygen species in wound healing. *Commun Biol.* 2024;7(1):1534. doi: 10.1038/s42003-024-07219-w
42. Liu Y, Long S, Wang H, Wang Y. Biofilm therapy for chronic wounds. *Int Wound J*. 2024;21(2):e14667. doi: 10.1111/iwj.14667