Original Article

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TANAFFOS

Galactooligosaccharides Suppress the Development of Biofilm in the Presence of Blood Polymorphonuclear Cells of Non-Small Cell Lung Cancer (NSCLC) Patients: A Pilot Study

Masoumeh Nomani [1], Babak Salimi [1], Mohammad Varahram [2], Hamidreza Jamaati [3], Esmaeil Mortaz [1,3,4,5]

[1] Clinical Tuberculosis and Epidemiology Research Center, National Research Institute of Tuberculosis and Lung Diseases(NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran, [2] Mycobacteriology Research Center, NRITLD, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [3] Chronic Respiratory Diseases Research Center, NRITLD, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [4] Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [5] Respiratory Immunology Research Center, NRITLD, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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Correspondence to: Mortaz E
Address: Clinical Tuberculosis and Epidemiology Research Center, National Research Institute of Tuberculosis and Lung Diseases(NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran
Email address: emortaz@gmail.com

Background: Bacterial biofilms pose a significant healthcare challenge due to their robust resistance to antibiotics and their involvement in various diseases, including cancer and infectious conditions. Biofilms hinder the antimicrobial activity of polymorphonuclear leukocytes (PMNs) by restricting their penetration through the extracellular matrix. With the rise of antimicrobial resistance, non-antibiotic strategies are gaining attention. Galacto-oligosaccharides (GOS) have been investigated for their potential to inhibit bacterial growth. This study investigates the impact of GOS on the biofilm development of GFP-labelled *Staphylococcus aureus* (GFP-SA) and *Pseudomonas aeruginosa* (GFP-PA) in the presence of blood PMNs from patients with non-small-cell lung cancer (NSCLC) and healthy controls.

Materials and Methods: Bacterial biofilms were investigated in the presence or absence of PMNs isolated from the blood of 12 NSCLC patients and 3 healthy controls (HC), with the inclusion of GOS. These PMNs were incubated with GFP-SA or GFP-PA biofilms and exposed to various concentrations of GOS (2.5%, 5%, 10%, 15%, and 20%) for 24 hours in a microplate setting. The progress of bacterial biofilm was quantified using the crystal violet assay.

Results: GOS itself, with bacteria at high concentrations (10%, 15%, and 20%), suppressed biofilm formation. PMNs of NSCLC patients show higher biofilm formation than in the absence of PMNs.

Conclusion: Based on our findings, PMNs in NSCLC patients may disrupt the natural balance of bacteria in the lungs, potentially leading to an overgrowth of biofilm-forming bacteria. GOS are known to influence immune function and may improve the ability of PMNs to fight infections. Consequently, it is proposed that GOS could enhance the ability of PMNs to phagocytose bacterial biofilms and counteract the negative effects of cancerous PMNs on biofilm formation. However, further research is needed to confirm whether GOS can effectively manage bacterial biofilms in lung cancer patients.

Keywords: Polymorphonuclear leukocytes (PMNs); Biofilm; Galacto-oligosaccharides (GOS); *Staphylococcus aureus*; *Pseudomonas aeruginosa*; NSCLC patients; Phagocytosis

INTRODUCTION

Bacterial biofilms are structured communities of microorganisms enclosed in a self-produced extracellular polymeric substance (EPS) composed primarily of polysaccharides, proteins, and extracellular DNA (1). This barrier-like structure significantly impedes the penetration of antimicrobial drugs and enhances bacterial survival against host immune responses (2, 3).

Biofilms contribute to approximately 80% of human infections and are increasingly recognized as important factors in cancer development (4, 5). The mechanisms linking biofilms to cancer include chronic inflammation leading to DNA damage, production of carcinogenic bacterial toxins, and metabolic alterations that parallel those observed in cancer cells (4,6). In patients with lung cancer, dysbiosis of the respiratory microbiota disrupts normal cytokine production and T cell function, potentially activating oncogenic pathways (7,8). Respiratory pathogens can effectively colonize the epithelial lining of the upper respiratory tract, proliferate on the mucosal surface, evade the host immune response, and transmit to susceptible hosts (9, 10).

Staphylococcus aureus and *Pseudomonas aeruginosa* are particularly problematic biofilm-forming pathogens in clinical settings. *S. aureus* biofilms on medical devices and host tissues contribute to persistent chronic infections through enhanced antibiotic resistance and immune evasion. Similarly, *P. aeruginosa* forms robust biofilms that compromise both antibiotic efficacy and host immune defenses (11, 12).

The interaction between immune cells and bacterial biofilms remains incompletely understood, yet it is crucial for developing effective therapeutic strategies (13). Polymorphonuclear neutrophils (PMNs), as primary responders to bacterial infections, play a complex role that may paradoxically enhance biofilm formation under certain conditions. Given the rising antimicrobial resistance, alternative therapeutic approaches are urgently needed. Galacto-oligosaccharides (GOS), non-digestible carbohydrates with demonstrated anti-inflammatory, immunomodulatory, and anti-adhesive properties, represent promising candidates for biofilm intervention (14, 15).

The objectives of this study were to: (1) evaluate the direct effect of GOS on bacterial biofilm formation, (2) assess how PMNs from NSCLC patients versus healthy controls influence biofilm development, and (3) determine whether GOS can counteract any PMN-mediated enhancement of biofilm formation. Overall, this study aims to establish GOS as a potential therapeutic intervention for preventing biofilm-associated infections in cancer patients, particularly by investigating its impact on both Gram-positive and Gram-negative bacterial biofilms on neutrophils from NSCLC patients.

MATERIALS AND METHODS

Ethical consideration

The study was approved by the Research Ethics Committee of the National Research Institute of Tuberculosis and Lung Diseases (IR.SBMU.NRITLD.REC.1402.081). Sample collection and processing were conducted in accordance with the Declaration of Helsinki, 2013.

Bacterial strains

Methicillin-resistant *Staphylococcus aureus* (SA) strain MW2 and *Pseudomonas aeruginosa* (PA) containing a GFP reporter construct (kindly provided by Prof. L. Koenderman, Utrecht University, The Netherlands) were used as previously described (16).

Galactooligosaccharides (GOS) preparation

Galacto-oligosaccharides (Vivinal GOS syrup, VGOS; 45% GOS in dry matter) were provided by FrieslandCampina Domo (Borculo, The Netherlands) and prepared as previously described (17). A comprehensive range of GOS concentrations (0.0625%, 0.125%, 0.25%, 0.5%, 1%, 2.5%, 5%, 10%, 15%, and 20%) was evaluated during initial optimization experiments to determine their effects on bacterial biofilm formation. Several of these concentrations did not produce statistically significant or biologically relevant outcomes and were consequently excluded from the final manuscript.

Isolation of PMN and evaluation of bacterial Lag Time

This research, conducted between August 2022 and April 2023, included 12 newly diagnosed, treatment-naïve patients with NSCLC and 3 healthy controls without chronic illnesses, allowing for the assessment of baseline PMN characteristics without confounding effects from therapy. The small cohort reflects the challenges of recruiting treatment-naïve NSCLC patients and the logistical demands of working with freshly isolated primary PMNs, which require immediate processing. Participants were between 40 and 70 years old. This study focused on PMNs to establish proof of concept for GOS-mediated inhibition of bacterial biofilms during the earliest innate immune response. PMNs were isolated from heparinized peripheral blood obtained from NSCLC patients and healthy controls using a modified Ficoll-Paque density gradient centrifugation protocol (18).

Briefly, whole blood was layered onto Ficoll-Paque and centrifuged at 400 × g for 30 minutes at 20°C without brake. After careful removal of the plasma and PBMC layers, the pellet was subjected to hypotonic lysis using ammonium chloride (NH_4Cl) solution (0.83% NH_4Cl in 0.01 M Tris-HCl, pH 7.2) for 5 minutes to lyse red blood cells selectively. The reaction was quenched by adding an equal volume of cold PBS, and PMNs were washed twice with cold PBS by centrifugation at 300 × g for 5 minutes at 4°C. Finally, PMNs were suspended in HEPES III buffer (HEPES, Gibco, Waltham, MA, USA) supplemented with 0.5% (w/v) bovine serum albumin, 1 mM $CaCl_2$, and 5 mM glucose, and counted using microscopy. PMN purity was assessed by light microscopy. Cell viability was determined by trypan blue exclusion, and only preparations with a viability of greater than 98% were used. PMNs were applied at a final concentration of 2×10^6 cells/ml, representing a physiologically relevant density for neutrophil–biofilm interactions. This concentration was validated in preliminary experiments to ensure that cell viability was maintained throughout the incubation period.

Biofilm formation and effects of GOS

To establish biofilms, 100 µl of bacterial suspension (90 µl RPMI 1640 supplemented with 10% FBS + 10 µl bacteria) was added to each well of 96-well flat-bottom polystyrene plates (black, clear-bottom; TC Surface, Thermo Fisher, USA). The plates were sealed and incubated overnight at 37°C. After incubation, adherent biofilms were rinsed three times with 200 µl of sterile PBS to remove non-adherent bacteria.

Fresh media (RPMI + 10% FBS), varying concentrations of GOS were then added to the wells in triplicate and incubated at 37°C for 24 hours. Each plate included a negative control (RPMI medium without GOS or bacteria) and a positive control (bacteria only). The wells were subsequently rinsed with PBS, and 125 µL of 1% crystal violet was added to each well, followed by incubation at room temperature for 15 minutes. To stabilize the crystal violet, 200 µl of an ethanol-acetone solution was added to each well. After 15 minutes, the optical density (OD) of each well was measured using an ELISA reader (Hiperion Microplate Reader).

Assessment of the bacterial viability in biofilms following GOS treatment

Bacterial viability within biofilms was assessed using a modified MTT colorimetric assay (MITOCIB, Iran) with modifications to ascertain the viability of microbial cells, as described by Walencka et al. (2006) (19). After biofilm formation, 10 µl of 0.5% MTT solution was added to each well and incubated at 37°C for 2 hours. The supernatant was then removed, and 100 µl of DMSO (MITOCIB, Iran) was added to dissolve the formazan crystals. Plates were further incubated at room temperature for 15 minutes. In this assay, the tetrazolium salt is reduced by bacteria with an active electron transport system to form a water-soluble purple formazan product. The color intensity of the soluble formazan was measured at 570 nm.

Bacterial biofilm, GOS, and PMNs culturing

To evaluate biofilm development and the effect of GOS on biofilms generated by *S. aureus* (SA) and *P. aeruginosa* (PA) in the presence of blood PMNs, experiments were conducted as previously described. In brief, bacteria (SA-GFP, PA-GFP) were cultured in RPMI 1640 supplemented with 10% FBS for 24 hours. On the following day, PMNs were isolated from heparinized peripheral blood of NSCLC patients and healthy controls using Ficoll-Paque

and suspended in RPMI medium supplemented with 10% FBS. The bacterial culture supernatant was then removed, and wells were rinsed three times with PBS to eliminate non-adherent bacteria. Subsequently, 100 µl of isolated human PMNs (2×10^6 cells/ml) was added to the wells along with varying concentrations of GOS (2.5%, 5%, 10%, 15%, and 20%). PMNs were co-cultured with bacterial biofilms and GOS and incubated at 37°C for 24 hours in a FLUOstar plate reader.

Determination of biofilm formation

After incubation of the biofilm with PMNs and GOS, the supernatant was removed, and the wells were rinsed with PBS. Next, 125 µl of 1% crystal violet (CV) was added to each well, and the plate was incubated at room temperature for 15 minutes. The wells were then rinsed with PBS to remove excess CV and air-dried at room temperature. To fix the crystal violet, each well was treated with 200 µl of an ethanol-acetone solution. The optical density (OD) at 570 nm was measured after 15 minutes (20, 21). Biofilm formation was quantified using the equation: BF = AB / CW, where BF represents biofilm development, AB is the OD_{570} of stained adhered bacteria, and CW is the OD_{570} of stained control wells containing bacteria-free media. Biofilm strength was categorized as follows: ≥6 (strong), 4–5.99 (medium), 2–3.99 (weak), and <2 (negative) (22, 23).

Phagocytosis of both planktonic and biofilm bacteria

Phagocytosis by PMNs was performed as previously described (17). In brief, isolated PMNs were co-cultured with GFP-SA and GFP-PA biofilms in 96-well plates. The plates were then incubated in a FLUOstar Optima plate reader for 60 hours, as described previously.

Data analysis

Results are presented as mean ± SEM, and all experiments were performed in triplicate. Statistical significance was evaluated using Kruskal–Wallis one-way analysis of variance followed by Dunn's post hoc test, with analyses conducted in GraphPad Prism (version 8.00). Two-way ANOVA was applied for experiments involving two categorical variables. Statistical significance was considered at $p \leq 0.05$ and $p \leq 0.01$.

RESULTS

Bacterial biofilm formation

Bacteria were subjected to various concentrations of GOS to evaluate its effect on SA and PA biofilm formation. Compared to SA biofilm alone, biofilm formation was decreased by 20% and 15% with GOS treatment ($P \leq 0.01$ and $P \leq 0.05$, respectively; Figure 1A). The addition of 20% GOS substantially reduced PA biofilm formation ($P \leq 0.05$; Figure 1B). Furthermore, the survival rate of *M. haemolytica* under laboratory conditions was significantly reduced at elevated GOS levels (8% and 16%), as reported by Cai et al. (2022) (14).

MTT assay of bacteria

The findings indicated a reduction in bacterial viability within the biofilm upon the addition of GOS at concentrations of 20%, 15%, and 10% ($P \leq 0.001$, $P \leq 0.01$, and $P \leq 0.05$, respectively; Figure 2A). Furthermore, GOS at 20% and 15% significantly reduced the viability of PA within the biofilm ($P \leq 0.01$ and $P \leq 0.05$, respectively; Figure 2B).

Effect of GOS on the co-culture of bacterial biofilm in the presence of PMN

We investigated the impact of GOS on biofilm development in the presence of PMNs isolated from NSCLC patients. In the presence of NSCLC PMNs, the addition of GOS decreased SA biofilm formation ($P \leq 0.05$; Figure 3A). Furthermore, HC PMNs subjected to the same treatment showed even greater reductions in SA biofilm formation compared to SA biofilm alone ($P \leq 0.05$ and $P \leq 0.01$, respectively; Figure 4A). The level of biofilm formation in NSCLC PMNs remained significantly higher than that in HC PMNs (Figure 3A). In contrast, incubation of NSCLC PMNs with PA and GOS did not substantially affect PA biofilm formation (Figure 3B). Moreover, when HC PMNs were incubated with PA and GOS, biofilm formation was significantly reduced ($P \leq 0.05$; Figure 3B).

PMN-mediated phagocytosis of biofilm and planktonic *S. aureus* and *P. aeruginosa*

This study aimed to investigate interactions between PMNs from NSCLC patients and bacteria in both biofilm and planktonic (free-floating) forms. Figure 4 illustrates a reduced lag time in response to biofilms compared to free-floating *Staphylococcus aureus* ($P \leq 0.05$ for NSCLC and $P \leq 0.001$ for healthy controls (HC); Figure 4A). A similar trend was observed with *Pseudomonas aeruginosa* (PA), as PMNs from both groups exhibited a shorter lag time when exposed to PA biofilms compared to planktonic PA ($P \leq 0.05$ for NSCLC and $P \leq 0.01$ for HC; Figure 4B). Collectively, the capacity of PMNs to contain or maintain bacteria within a biofilm state is markedly lower than their ability to control free-floating (planktonic) bacteria. This reduced effectiveness may reflect the inherent protective mechanisms of biofilms, such as the extracellular polymeric matrix and altered bacterial metabolic states, which hinder phagocytic activity and antimicrobial responses of PMNs.

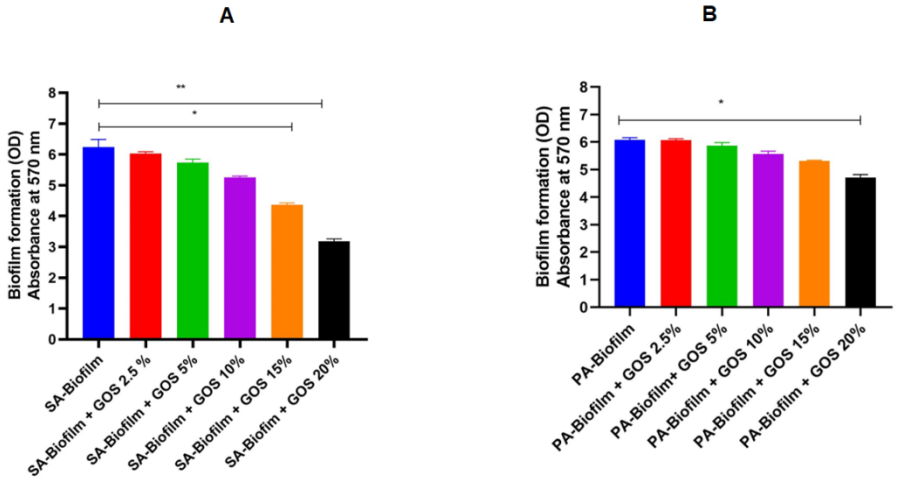

Figure 1. Impact of GOS on bacterial biofilm formation (A) Graphical analysis of biofilm of SA-biofilm in the presence of GOS (2.5%, 5%, 10%, 15%, and 20%) (Samples compared to SA-biofilm). (B) Graphical analysis of biofilm of PA-biofilm in the presence of GOS (2.5%, 5%, 10%, 15%, and 20%) (Samples compared to PA-biofilm). Results are presented as mean ± SEM. *P ≤ 0.05, **P ≤ 0.01, show significance between groups.
SA: *Staphylococcus aureus*; PA: *Pseudomonas aeruginosa*; GOS: Galacto-oligosaccharides

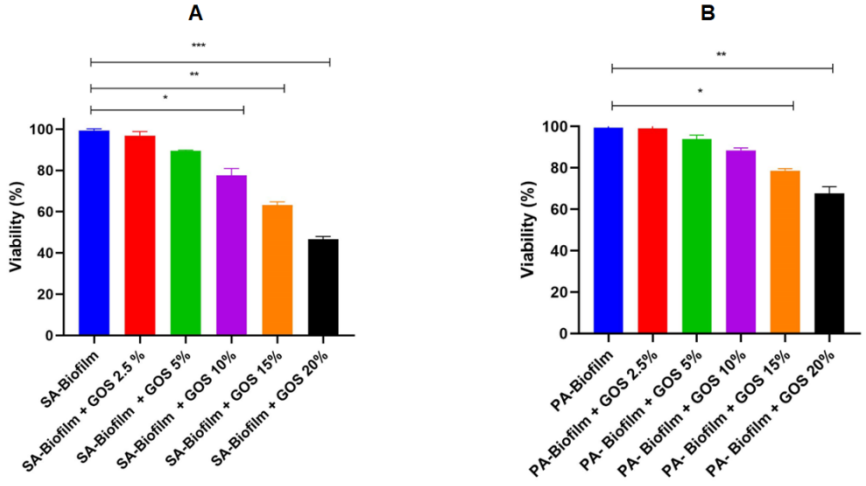

Figure 2. Effect of GOS on bacterial viability in biofilm (A) Graphical representation of the viability of SA-biofilm in the presence of GOS (2.5%, 5%, 10%, 15%, and 20%) (Samples compared to SA-biofilm). (B) Graphical representation of the viability of PA-biofilm in the presence of GOS (2.5%, 5%, 10%, 15%, and 20%) (Samples compared to PA-biofilm). Results are presented as mean ± SEM. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 show significance between groups.
SA: *Staphylococcus aureus*; PA: *Pseudomonas aeruginosa*; GOS: Galacto-oligosaccharides

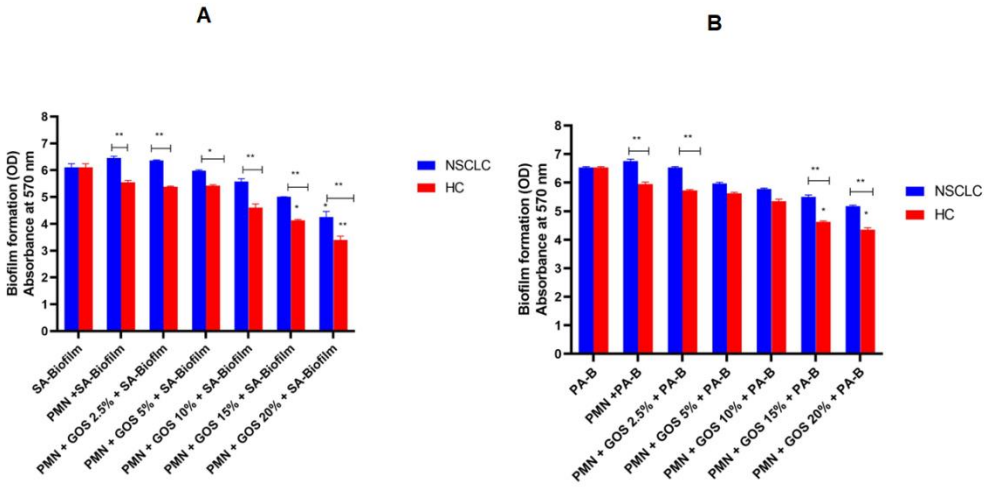

Figure 3. Effect of GOS on bacterial biofilm formation by PMN (NSCLC and HC) (A) Graphical analysis of SA-biofilm formation in the presence of PMN and GOS (2.5%, 5%, 10%, 15%, and 20%) (Samples compared to Samples compared to SA-biofilm). (B) Graphical analysis of PA-biofilm formation in the presence of PMN and GOS (2.5%, 5%, 10%, 15%, and 20%) (Samples compared to PA-biofilm). Results are presented as mean ± SEM. *P ≤ 0.05, **P ≤ 0.01, show significance between groups.
SA: *Staphylococcus aureus*; PA: *Pseudomonas aeruginosa*; GOS: Galacto-oligosaccharides; NSCLC: Non-small-cell lung cancer; HC: Healthy individual; PMN: polymorphonuclear

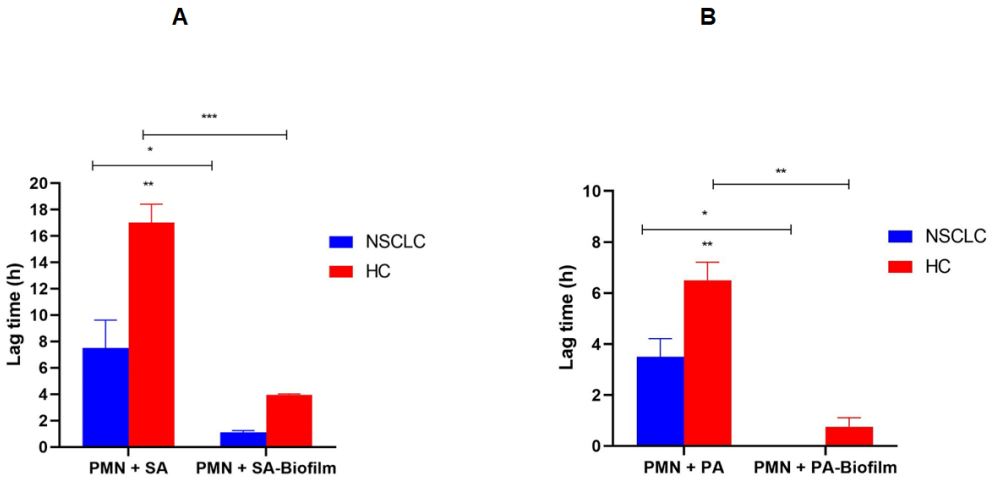

Figure 4. Assessment of phagocytosis in SA and PA biofilm and planktonic cells: (A) Graphical assessment of lag time involving PMN and GFP-SA, as well as PMN and SA-biofilm. (B) Graphical assessment of lag time involving PMN and GFP-PA, as well as PMN and PA-biofilm. Results are presented as mean ± SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 show significance between groups.
SA: *Staphylococcus aureus*; PA: *Pseudomonas aeruginosa*; NSCLC: Non-small-cell lung cancer; HC: Healthy individual; PMN: Polymorphonuclear.

DISCUSSION

Biofilms are known to impair host immune responses, creating a favorable environment for chronic infections and, potentially, cancer progression (24, 25). In our study, we observed that galacto-oligosaccharides significantly reduced biofilm formation in both Gram-positive and Gram-negative bacteria. Using the MTT assay, we demonstrated that high concentrations of GOS suppressed bacterial viability in biofilms, consistent with earlier reports that *S. aureus* can reduce MTT activity (19, 24).

Previous studies have suggested that non-digestible oligosaccharides (NDOs), including GOS, possess beneficial biological properties due to their biocompatibility and ability to degrade naturally (14, 15). GOS have also been reported to inhibit bacterial adhesion and reduce biofilm formation by different pathogens (*E.*

coli, *Mannheimia haemolytica*, and *Bacteroides fragilis*) in vitro (14,15, 26). Our findings support these observations, showing that GOS inhibited *S. aureus* biofilm formation in NSCLC patients, although no effect was seen against *P. aeruginosa* biofilms.

Interestingly, our results revealed that PMNs from NSCLC patients enhanced biofilm formation of both *S. aureus* and *P. aeruginosa* compared to bacteria cultured without PMNs. Moreover, biofilm formation was more pronounced in patients with NSCLC than in healthy controls. These findings suggest that immune cells may inadvertently support biofilm development, aligning with earlier reports that cellular components from neutrophils can serve as a structural matrix for *P. aeruginosa* biofilms (27, 28).

Consistent with previous studies, we also observed that PMNs exhibited reduced phagocytic activity against biofilms compared to planktonic bacteria (28, 29). This supports the concept that biofilms provide partial protection against host immune defenses (11). While biofilms resist immune clearance, the resulting immune response may contribute to collateral tissue injury, further complicating infection outcomes (11). In addition, biofilms are not completely immune to neutrophil activity; however, cellular components released from necrotic neutrophils can provide a biological matrix that facilitates the formation of *Pseudomonas aeruginosa* biofilms when infections persist (27). Thus, neutrophils may unintentionally favor the development of more resistant bacterial strains, thereby reinforcing the structural and environmental stability of biofilms (28).

The development of innovative strategies to prevent and treat biofilm formation has attracted increasing attention. Future research should focus on these approaches, as they may provide more effective biofilm inhibitors than conventional therapies (30). Nevertheless, the mechanisms by which the human immune system responds to bacterial biofilms remain poorly understood. Managing biofilm-mediated infections is particularly challenging due to their structural complexity and the growing problem of antibiotic resistance. Overall, our findings highlight two key points: (I) GOS exhibit inhibitory effects on *S. aureus* biofilms in NSCLC patients, suggesting their potential as adjunctive agents to control biofilm-associated infections, and (II) PMNs may paradoxically enhance biofilm formation, emphasizing the need for deeper investigation into host–biofilm interactions. Given the challenges of treating biofilm-mediated infections and the rise of antibiotic resistance, further studies should explore biofilm–immune interactions in more detail and evaluate novel therapeutic approaches, including prebiotics such as GOS, for managing biofilm-related complications (26, 30).

Limitations of the study

This study is limited by its small sample size, focus on only two bacterial species, and lack of mechanistic assays to clarify GOS activity. In vitro conditions may not fully capture in vivo host–microbe interactions in NSCLC patients. Larger studies including diverse pathogens and mechanistic analyses are needed to validate and extend these findings.

CONCLUSION

This study demonstrates that PMNs promote biofilm formation in patients with NSCLC. GOS selectively inhibited *Staphylococcus aureus* biofilms, but not *Pseudomonas aeruginosa*, and at high concentrations reduced bacterial viability, indicating direct anti-biofilm activity. Neutrophils exhibited limited ability to clear biofilms compared with planktonic bacteria, underscoring the challenge of biofilm-associated infections in cancer. Future studies are needed to clarify the mechanisms of GOS activity, explore species-specific effects, and evaluate its potential therapeutic role in vivo.

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