

Brief Report

Disaggregated *Helicobacter pylori* Biofilm Impairs Bactericidal Activity and Bacterial Phagocytosis by Human Neutrophils

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Abstract: *Helicobacter pylori* (*H. pylori*), a prevalent human pathogen affecting nearly half the global population, is a major contributor to chronic gastritis, peptic ulcer, and gastric cancer. *H. pylori* develops biofilms (BFs) allowing bacteria to evade the immune response. Differences in composition between planktonic and biofilm cells influence the host's immune response, yet the specific biofilm components modulating this response remain uncharacterized. Considering the above, this study evaluated the effect of in vitro-generated *H. pylori* BF on the antibacterial activity of neutrophils. This work utilized sonication to obtain disaggregated *H. pylori* BF (d-BF-*Hp*) to challenge human neutrophils, assessing their bactericidal and phagocytic activity against *Staphylococcus aureus*. *S. aureus* survival in the presence of neutrophils was enhanced by 10 µg/mL of d-BF-*Hp*'s protein. Conversely, *S. aureus* survival was significantly lower at 30 µg/mL compared to 10 µg/mL d-BF-*Hp*. Furthermore, 10 and 30 µg/mL of d-BF-*Hp* significantly reduced the neutrophil phagocytosis rate. Our findings suggest that d-BF-*Hp* components diminish neutrophil bactericidal activity, although this effect was not observed at higher d-BF-*Hp* concentrations. Increased d-BF-*Hp* concentrations proportionally reduced neutrophil phagocytic capacity. Future work should explore the mechanisms underlying the alteration of neutrophil microbicidal properties.

Keywords: bacterial infection; disaggregated biofilm; *Helicobacter pylori*; immune response; neutrophils; phagocytosis



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1. Introduction

Helicobacter pylori is a microaerophilic, Gram-negative, slow-growing, helical-shaped bacterium with abundant flagella [1]. *H. pylori* infection is a risk factor for chronic gastritis and peptic ulcer that affects 50% of the world's population [2]. The most important virulence factors identified in this bacterium are cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA). CagA is considered a marker of the most virulent strains of *H. pylori* and is associated with cell proliferation and inflammation in the gastric mucosa. Meanwhile, VacA causes the formation of cytoplasmic vacuoles and apoptosis in gastric cells [3]. *H. pylori* has the ability to develop biofilms (BFs), which are complex matrices formed by deposits of exopolysaccharides, amino acids, lipids, and extracellular DNA [4]. Through nuclear magnetic resonance analysis, it has been determined that the BF of *H. pylori* is formed predominantly by polysaccharides with 1,4-mannosyl linkages [5]. Immersed in the matrix are also heterogeneous bacterial cells, as well as pores and interstitial spaces that function as water channels for irrigation [6]. Moreover, cellular structures can be found, such as outer membrane vesicles, flagella, and pili [6]. BF protects microorganisms

against extreme environments and external agents [7]. Additionally, it has been shown that the BF of some bacterial pathogens can promote immune evasion processes, such as the production of the leukotoxin leucocidin AB present in the BF of *S. aureus*, which interferes with the phagocytic activity of macrophages and neutrophils [8,9]. BF formation is one of the challenges facing standard treatments for *H. pylori*, as it affects their eradication efficacy through its role in antibiotic resistance and immune tolerance. Despite recent interest in *H. pylori* infection and management, BFs are still understudied [10].

H. pylori colonization generates a significant infiltration of neutrophils in the gastric mucosa [11]. The pathophysiological relevance of the levels of this infiltration has traditionally been used to classify the severity of gastric tissue damage [12]. Neutrophils are immune cells that importantly control infectious processes using microbicidal processes such as phagocytosis, degranulation, and extracellular traps (NETs) to combat bacterial pathogens [13]. It has been demonstrated that neutrophils are attracted to BF through chemotactically active molecules such as lipopolysaccharides, peptidoglycans, microbial DNA, and other pathogen-associated molecular patterns (PAMPs) [14]. The interaction of the BF of *H. pylori* and neutrophils has been poorly studied, although it is known that neutrophil-activating protein (NAP) is upregulated in BF cells compared to planktonic cells [5]. The NAP molecule is released around the gastric epithelial monolayer, where it contributes to the inflammatory process by increasing the adhesion and activation of neutrophils [15]. To enhance the understanding of the immune evasion mechanisms of the bacteria, it is important to study the possible existence of *H. pylori* BF components that could interfere with the antibacterial activity of neutrophils. In this work, the effect of *H. pylori* BF generated in vitro and disaggregated by sonication on the antibacterial activity of neutrophils was studied using *S. aureus* as a prokaryotic model to evaluate this activity.

2. Materials and Methods

2.1. Reagents and Culture Media

Reagents: Cyclodextrin (CCD) (Sigma-Aldrich, Inc., St. Louis, MO, USA), glucose (Chemical Technique, Mexico City, México), crystal violet (Karl S.A. de C.V., León, Gto., Mexico), Coomassie Blue (Sigma-Aldrich, Inc., St. Louis, MO, USA), Lymphoprep (Fresenius Kabi Norge AS, Halden, Norway), Histopaque-1119 (Sigma-Aldrich, Inc., St. Louis, MO, USA), Bovine Serum Albumin (BSA) (Sigma-Aldrich, Inc., St. Louis, MO, USA), Trypan blue (Sigma, Steinheim, Germany), and Wright-Giemsa (Golden Bell, Mexico City, Mexico).

Culture media: Brucella broth (BB) (Becton, Dickinson, Sparks, MD, USA), *H. pylori* selection antibiotics (Selective supplement DENT, Oxoid Ltd., Basingstoke, Hampshire, UK), Brain–Heart Infusion (BHI) broth and BHI agar (BHIA) (BD Bioxon, Becton Dickinson, México), Mannitol Salt Agar (MSA) (BD Bioxon, Becton Dickinson, Mexico), Blood Agar Base (BD Bioxon, Becton Dickinson, Cuautitlan Izcalli, Mexico, Mexico), and Roswell Park Memorial Institute (RPMI)-1640 medium with phenol red (Sigma-Aldrich, Inc., St. Louis, MO, USA).

2.2. Characterization of *H. pylori* BF Formation In Vitro

A clinical isolate of *H. pylori*, A97, which was previously obtained from a gastric biopsy, was used. Molecular analysis confirmed that this strain has two key virulence factors: the cytotoxin-associated gene (*cagA*+) and the vacuolating toxin (*vacA*) genotype s2/m2. Bacteria were cultured on Blood Agar Base supplemented with 5% defibrinated sheep blood, and incubated at 37 °C under microaerophilic conditions. For BF formation, BB supplemented with 0.1% CCD, 0.3% glucose, and 4 µL/mL of *H. pylori* selective antibiotics was dispensed into 24-well culture plates (Celltreat®, Ayer, MA, USA). Subsequently, 20 µL of bacterial suspensions, incubated for 72 h in BB and adjusted to 1.5×10^6 bacteria/mL

(McFarland turbidity standard), were added to each well to a final volume of 1 mL. The culture plates were then incubated at 37 °C in a microaerophilic environment for five days. To confirm that the culture conditions used allowed the formation of BF, this structure was identified from 3 wells of the culture plate, using the method described by O'Toole based on the staining of the BF matrix with crystal violet [16].

2.3. Preparation of Disaggregated *H. pylori* BF

The BF of *H. pylori* cultured in a 24-well plate was recovered by carefully removing the supernatant. The wells were washed with 200 µL of phosphate-buffered saline (PBS) [1.75 mM NaH₂PO₄, 8 mM Na₂HPO₄, and 75 mM NaCl, pH 6.5]. After the removal of the initial PBS, 200 µL of fresh PBS was added to the wells to facilitate BF removal by gentle scraping. The resuspended BF was collected in a sterile container and kept in an ice bath for subsequent disaggregation through sonication. Sonication was carried out using a Sonicor ultrasonic processor (UP 400A 50/60Hz cycles, Sonicor Instrument Co., West Babylon, NY, USA) with 5 pulses of 10 s at level 4. The disaggregated *H. pylori* BF (d-BF-*Hp*) obtained corresponds to a mixture of cellular debris together with components of the ultrasonic disintegration of the BF matrix and was used in the tests to assess the microbicidal activity of neutrophils.

The protein concentration of the d-BF-*Hp* was quantified using the Bradford method, with BSA as the standard [17]. To ensure that the obtained d-BF-*Hp* was free from viable *H. pylori* cells or contamination with other bacteria, samples were cultured on blood agar (5%) and incubated at 37 °C under microaerophilic conditions for 24 h. Additionally, samples were cultured on BHIA and incubated at 37 °C for 24 h. The presence and integrity of the proteins in the d-BF-*Hp* were further assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The gel was stained with Coomassie Blue and the d-BF-*Hp* was stored at –20 °C until further use.

2.4. *Staphylococcus aureus*

A *S. aureus* isolate, previously obtained in the laboratory, was used, with its identification as catalase+, coagulase+, and thermonuclease+ confirmed in accordance with the Official Mexican Standard (NOM-210-SSA1-2014) [18]. For bacterial suspensions, the isolate was incubated for 24 h at 37 °C in 5 mL of BHI. After incubation, bacterial cells were harvested by centrifugation at 1409 × g for 5 min, and the pellet was resuspended in 5 mL of PBS. The optical density (OD) at 600 nm was measured in triplicate from a 1:3 dilution of this suspension using a spectrophotometer (Jenway 7305, Bibby Scientific, Stone, Staffs, UK). Bacterial concentration (bacteria/mL) was determined by interpolating the OD values against an OD curve generated from McFarland standards (1 to 10). Bacterial suspensions were then adjusted to 1 × 10⁶ bacteria/mL in PBS. This cell concentration was verified by quantifying colony-forming units (CFU) after seeding 100 µL of serial dilutions of *S. aureus* on MSA plates and incubating at 37 °C for 24 h.

2.5. Interaction of Human Neutrophils with Disaggregated *H. pylori* BF

The protocol for obtaining blood samples was approved by the Institutional Bioethics Committee of the Universidad Autónoma de Aguascalientes (office SG-1783-2023, project identification PIBB24-1) on 30 October 2023. All adult volunteers signed an informed consent prior to blood donation for research purposes. Neutrophils were purified using a discontinuous density gradient prepared with Lymphoprep and Histopaque-1119, as previously described [19]. Contaminating erythrocytes were lysed by osmotic shock in a 0.15 M NH₄Cl solution. Cells were washed twice with PBS and resuspended in 1 mL of supplemented RPMI-1640 medium containing phenol red. Cell quantification was performed using a Neubauer chamber, and neutrophil viability was determined by the

Trypan blue exclusion test. Only preparations with neutrophil purity and viability greater than 95% were used.

To test the effect of the d-BF-*Hp*, 1×10^6 neutrophils were used. These cells were stabilized in RPMI for 30 min at 37 °C. Subsequently, they were incubated with d-BF-*Hp* (10 and 30 µg of total protein/mL) for 30 min at 37 °C. Finally, *S. aureus* (1×10^6) was added to achieve a 1:1 multiplicity of infection (MOI) with neutrophils, and the mixture was incubated at 37 °C for 1 h. The cellular pellets were obtained by centrifugation at $300 \times g$ for 10 min at 4 °C to determine phagocytic cells, or at $8050 \times g$ for 7 min at 4 °C to determine CFU. All assays were performed in duplicate in independent trials.

2.6. Determination of Phagocytic Cells by Optical Microscopy

To evaluate the effect of d-BF-*Hp* on phagocytosis, the number of *S. aureus*-phagocytosing cells was quantified by optical microscopy. The cellular pellets obtained after interaction were gently resuspended in 30 µL of RPMI. Smears prepared on sterile, degreased slides were fixed with absolute methanol, stained with Wright-Giemsa, and examined under an optical microscope. Images were captured by a digital camera (Motic BA310, Hong Kong, China) adapted to the microscope and analyzed using the Motic Images Plus Program, version 2.0. For each experimental condition, 200 cells were counted on each slide to determine the percentage of neutrophils that bound to or engulfed bacteria. Control groups included neutrophils with or without d-BF-*Hp* or *S. aureus* bacteria.

2.7. Viability of *S. aureus*

The cellular pellets obtained by centrifugation at $8050 \times g$ at the end of the interaction were resuspended in 1 mL of sterile distilled water and mixed vigorously for 3 min to lyse the neutrophils and release intracellular bacteria. Dilutions (1×10^{-3}) of the samples were plated by streaking on MSA and incubated for 24 h at 37 °C to obtain CFU. The results were expressed as a percentage of the CFU obtained in each treatment. In addition to CFU quantification, a spot dilution test (in duplicate) was performed to evaluate bacterial growth in each dilution and to demonstrate bacterial viability. For this purpose, three 5 µL aliquots of each cellular suspension were spotted onto a Petri dish with MSA. The drops were allowed to dry to prevent displacement. Plates were incubated at 37 °C for 24 h [20].

2.8. Statistical Analysis

Data were analyzed with GraphPad Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, USA) and presented as the mean $\pm$ standard error of the mean (SEM) from triplicate experiments. One-way analysis of variance (ANOVA), followed by a Bonferroni test, was used for group comparisons [21]. Statistical significance was considered when $p < 0.05$.

3. Results

3.1. Disaggregated BF *H. pylori* Increase *S. aureus* Survival in Human Neutrophils

To evaluate the effect of d-BF-*Hp* on bacterial survival after phagocytosis in neutrophils, we assessed the CFU percentage of *S. aureus* within neutrophils following d-BF-*Hp* stimulation (Figure 1). As expected, the viability of *S. aureus* was significantly reduced by $53.5\% \pm 27.75$ when compared to the CFU of *S. aureus* in the absence of neutrophils. Notably, 10 µg/mL of d-BF-*Hp* enhanced the survival of *S. aureus* in the presence of neutrophils, as its viability showed a significant increase compared to *S. aureus* when exposed to neutrophils. However, in the presence of 30 µg/mL of d-BF-*Hp*, *S. aureus* survival was significantly lower than that observed with 10 µg/mL of d-BF-*Hp*. But, it is similar to the survival obtained when the bacteria are challenged only with neutrophils ($p > 0.05$).

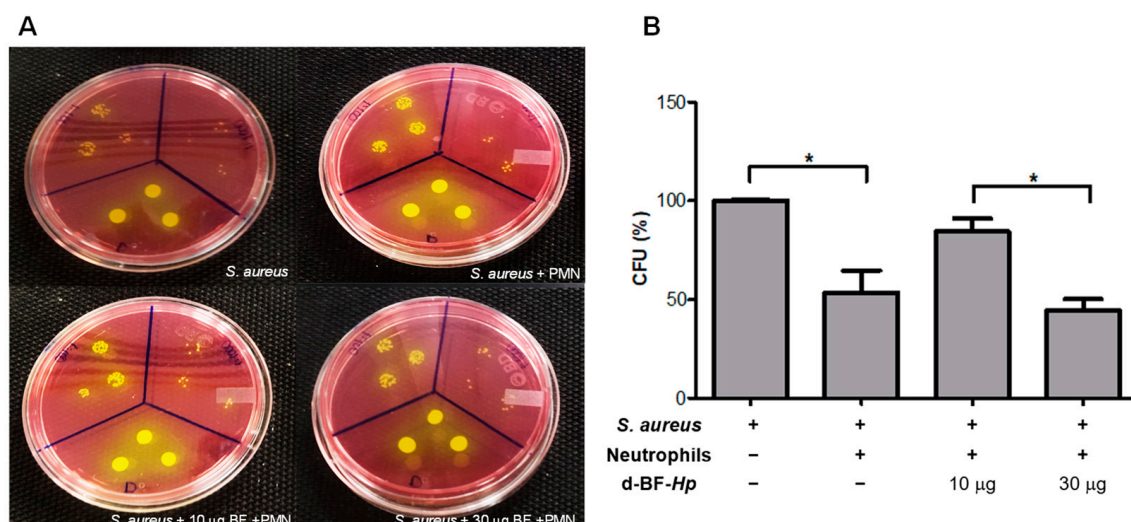


Figure 1. *Staphylococcus aureus* CFU percentage with human neutrophils. (A) Spot dilution test for viability control of *S. aureus* treated with *S. aureus* alone, *S. aureus* with neutrophils, *S. aureus* with neutrophils and 10 µg of d-BF-Hp, *S. aureus* with neutrophils and 30 µg of d-BF-Hp. (B) Effect of d-BF-Hp on *S. aureus* survival post-neutrophils phagocytosis. Mean comparisons are indicated (* $p < 0.05$).

3.2. Disaggregated *H. pylori* BF Reduces the Phagocytosis Rate of *S. aureus* by Neutrophils

To analyze the effect of d-BF-Hp on neutrophil phagocytosis, neutrophils were incubated with 10 and 30 µg/mL of d-BF-Hp and then co-cultured with *S. aureus* at a MOI of 1. Phagocytosis of *S. aureus* by neutrophils was observed in $31 \pm 6.9\%$ of the cells. However, in the presence of 10 and 30 µg/mL of d-BF-Hp, the percentage of phagocytic cells significantly reduced to $19.75 \pm 6.1\%$ and $15.63 \pm 6.43\%$, respectively, compared to the treatment without d-BF-Hp (Figure 2).

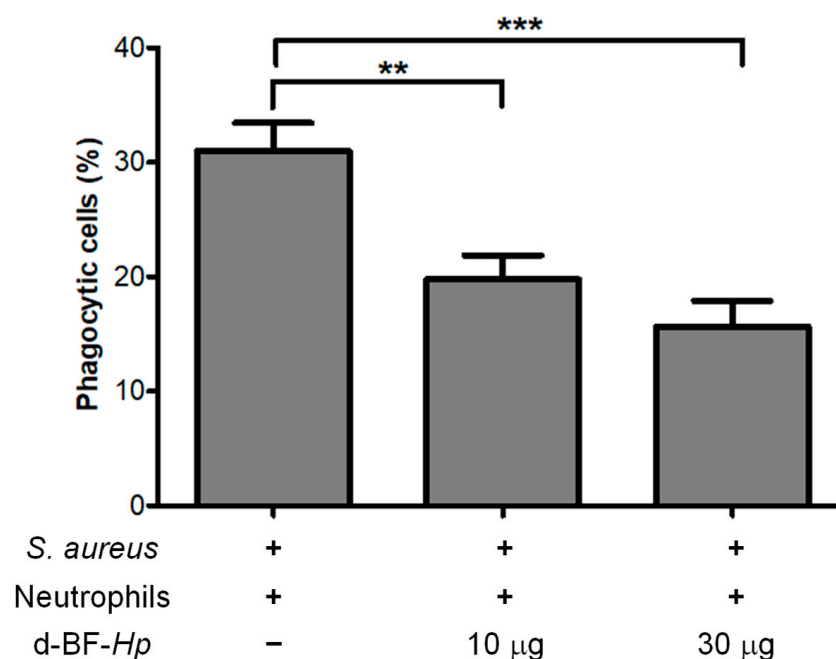


Figure 2. Effect of d-BF-Hp on neutrophil phagocytosis of *S. aureus*. Mean comparisons are indicated (** $p < 0.01$, *** $p < 0.001$).

4. Discussion

Helicobacter pylori infection elicits an immune response with immunomodulatory actions promoting the persistence of gastric colonization. This modulatory response involves the evasion of pattern recognition receptors, manipulation of immune cell activity, and downregulation of adaptive responses [22].

The ability of *H. pylori* to develop BF may favor the persistence of the infection, as BF impedes the effect of antibiotics and the function of the immune response [23]. Strains capable of forming BF exhibit a higher level of resistance as compared to those of strains incapable of developing these structures [4]. In this study, we sought to analyze in vitro the effect that *H. pylori* BF components may have on the microbicidal action of neutrophils, considering the use of a *S. aureus* strain as an indicator of antibacterial activity.

Quantification of *S. aureus* CFU obtained from neutrophil cultures stimulated with d-BP-*Hp* allowed the evaluation of the effect of BF components on bacterial survival. The increased viability of *S. aureus* observed in neutrophils stimulated with the lowest concentration of BF components used in the study (10 µg/mL) indicates that elements of this bacterial structure may interfere with the bactericidal action of neutrophils. There are no previous reports like this study of the activity on neutrophils with *H. pylori* BF. In other bacteria, such as *Pseudomonas aeruginosa*, it has been shown that some quorum-sensing molecules associated with BF may function as inhibitors of reactive oxygen species production in neutrophils [24]. However, it is striking that in our study, the increase in *H. pylori* BF components (30 µg/mL) has almost no effect on the bactericidal activity of neutrophils. This phenomenon could be related to an overstimulation of neutrophils caused by the enrichment of PAMPs due to the increased concentration that could increase bactericidal activity, through mechanisms such as the formation of NETs [25].

The finding that components of *H. pylori* BP reduce the bactericidal activity of neutrophils may be a mechanism contributing to the chronic persistence of the bacteria. Since in vivo, it has been shown that *H. pylori* develops associated with a dense BF that adheres to the surface of the gastric mucosa [26]. BF components could diffuse from the luminal space to internal sites of the gastric mucosa, crossing the epithelial barrier, and then control the bactericidal activity of infiltrated neutrophils. It has been shown that molecules produced in bacterial BFs can generate alterations in the permeability of epithelial cells, as is the case with quorum-sensing molecules of the N-acyl homoserine lactone family that can modify the organization of intercellular junction proteins such as occludin, E-cadherin, and zonula occludens-1 [27].

In the phagocytosis analysis, a decrease in the phagocytic activity of neutrophils was identified depending on the increase in d-BF-*Hp*. This decrease could be related to the masking of PAMPs by BF polysaccharides, generating the inhibition of opsonization processes, thus altering the phagocytic capacity of neutrophils [28]. However, it is feasible to consider the possibility that within the components of the BF of *H. pylori*, factors are present that could directly alter the neutrophilic phagocytic capacity. These types of alterations have been documented in the BF of bacteria such as *S. aureus*, in which the presence of protein factors such as alpha-toxin (Hla) and leukotoxin leukocidin AB, has been identified as factors that cause dysfunction in the phagocytic response of macrophages and neutrophils [8,9].

Future studies could focus on characterizing the *H. pylori* BF components related to the alteration of the microbicidal activity of neutrophils, including the mechanism associated with this process. However, it is crucial to consider the compositional variations that bacterial BF presents, since it is known that the same strain presents different components depending on the environmental conditions in which it develops [29]. Furthermore, the

characterization of such components will facilitate the design of rapid methods for the detection of infections of *H. pylori* in advanced stages, not only in the initial stages [30].

5. Conclusions

In this in vitro study, it was observed that the components of the d-BF-*Hp*, including structural elements, cellular derivatives, and signaling molecules, among others, altered the bactericidal and phagocytic capacity of neutrophils against *S. aureus*. This suggests that some d-BF-*Hp* components can directly or indirectly modify the microbicidal actions of immune cells. Such a mechanism could favor immune evasion by contributing to *H. pylori*'s persistence during gastric colonization. Further research is needed to identify the precise d-BF-*Hp* components that are responsible for altering neutrophil microbicidal activity.

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Institutional Review Board Statement: The protocol was approved by the Institutional Bioethics Committee of the Universidad Autónoma de Aguascalientes approved the protocol for obtaining blood samples (office SG-1783-2023, project identification PIBB24-1) on [30 October 2023].

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data may be shared and should be requested from the corresponding author.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

BB	Brucella broth
BF	Biofilm
BHI	Brain–heart infusion broth
BHIA	Brain–heart infusion agar
CFU	Colony-forming Units
d-BF- <i>Hp</i>	Disaggregated <i>H. pylori</i> biofilm
MSA	Mannitol Salt Agar
NETs	Neutrophil Extracellular Traps
PAMPs	Pathogen-associated Molecular Patterns
RPMI	Roswell Park Memorial Institute 1640

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