

Effect of Bacterial Biofilms on Topical Antimicrobial Therapy

Original Paper

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Abstract Chronic wounds are frequently complicated by bacterial biofilm formation, which significantly impairs healing and reduces the effectiveness of antimicrobial therapy. The aim of this study was to evaluate the efficacy of commonly used topical antimicrobial agents against multidrug-resistant wound pathogens in relation to the timing of their application and the stage of biofilm development.

Multidrug-resistant bacterial strains isolated from non-healing wounds were used to create an experimental collection, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, and *Acinetobacter baumannii*. Biofilm formation was assessed using the microtiter plate method according to Stepanović et al. The antimicrobial efficacy of 11 topical agents in various pharmaceutical forms was subsequently tested in a modified in vitro burn wound model based on Hammond et al., with three time intervals representing different stages of biofilm development (4, 6, and 24 hours after inoculation).

All tested strains demonstrated the ability to form biofilm, with a significant increase in intensity over time. In the early stages (4–6 hours), several agents, including octenidine-, iodine-, and silver-based preparations, exhibited strong bactericidal activity across most strains. However, when treatment was delayed to 24 hours, corresponding to mature biofilm formation, the efficacy of the majority of agents markedly decreased, often approaching the effect of sterile gauze. Only octenidine- and iodine-based agents retained partial activity against selected pathogens.

The findings confirm that the timing of topical antimicrobial application is a key determinant of therapeutic success. Early intervention significantly enhances antimicrobial efficacy, whereas delayed treatment in the presence of established biofilm leads to substantially reduced effectiveness. These results highlight the importance of prompt and proactive wound management and support the need for multimodal strategies combining antimicrobial therapy with biofilm disruption.

Keywords biofilm – topical antimicrobials – wound healing

INTRODUCTION

Chronic non-healing wounds represent a major global health burden, affecting millions of patients and generating significant socioeconomic costs exceeding 98 billion USD annually in the United States alone (Khan, 2024). The persistence of these wounds is often associated with bacterial colonization and infection, especially when microorganisms organize into complex biofilm structures. Biofilms are now recognized as a fundamental barrier to wound healing and one of the main reasons for the failure of both systemic and topical antimicrobial therapies (Schneider, 2025). A biofilm is defined as a structured community of microorganisms enclosed in a self-produced extracellular polymeric substance

(EPS) composed of polysaccharides, proteins, lipids, and nucleic acids (Kirketerp-Møller, 2011 and Fonseca, 2011). Biofilms are polymicrobial ecosystems, often involving *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus* spp., and *Candida* spp., whose synergistic interactions further enhance pathogenicity and persistence (Chegini, 2025). It is estimated that more than 70% of hard-to-heal wounds contain biofilm-associated bacteria (Khan, 2024), and biofilm-associated infections display up to a 1,000-fold increase in antimicrobial resistance compared to planktonic bacteria (Chegini, 2025). The alarming rise in multidrug-resistant (MDR) pathogens, notably *P. aeruginosa* and *S. aureus*, underscores the need for localized strategies that circumvent systemic antibiotic resistance (Chegini, 2025). Topical antiseptics and

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antimicrobial dressings provide the advantage of achieving high local concentrations with minimal systemic toxicity, yet their efficacy is frequently limited by the biofilm barrier (Khan, 2024). Local therapy strategies include well-known agents like silver or Polyhexamethylene biguanide (Muslim 2025, Watson, 2024). Other emerging systems include biopolymer-based composite films that combine natural matrices such as hydroxyethyl cellulose with inorganic nanoparticles and essential oils. For instance, films embedding zinc oxide nanoparticles and mesoporous silica loaded with cinnamon essential oil have demonstrated synergistic antimicrobial and antibiofilm effects against *S. aureus* and *E. coli* (Motelica, 2024). Additionally, natural compounds such as asiatic acid and ursolic acid derived from *Eucalyptus globulus* have exhibited promising antibiofilm activity against Gram-positive and Gram-negative bacteria (Mezzasalma, 2025). Given the multifactorial nature of chronic wound infection, optimal therapy requires a multimodal approach that combines mechanical or enzymatic debridement, effective antibiofilm and antimicrobial topical agents, and management of systemic risk factors. Clinical evidence increasingly supports the concept of “wound hygiene,” involving regular cleansing, biofilm disruption, and re-application of topical antiseptics within a structured regimen (Khan, 2024). However, despite growing preclinical and early clinical data, further research is essential to standardize testing methods, establish optimal application protocols, and integrate these advanced topical therapies into evidence-based wound care algorithms (Schneider, 2025).

MATERIALS AND METHODS

Topical antimicrobials play an important role in complex wound care, especially in non-healing wounds, which we encounter with increasing frequency in our daily clinical practice. For this reason, and because we currently lack a standardized microbiological testing method that would inform us about the effectiveness of various topical antimicrobial agents in individual patients, we decided to conduct a laboratory study to verify the effect of local treatment on biofilm-forming bacteria. Multidrug-resistant bacterial strains were isolated from non-healing wounds of patients treated at the University Hospital Bratislava and used to create an appropriate bacterial collection for laboratory research. Clinically important wound pathogens (*P. aeruginosa* – two strains, *S. aureus*, *E. coli*, *E. faecalis*, and *A. baumannii*) were selected from the bacterial collection and tested for biofilm production according to Stepanović et al. (2007). These five strains—biofilm producers—were used to evaluate the efficacy of topical antimicrobials commonly available for wound treatment. This evaluation was performed in the modified in vitro wound model according to Hammond et al. (2011).

1. Biofilm formation testing according to Stepanović et al.

Triplicates of wells on a polystyrene 96-well microtiter plate were inoculated with 200 µL of bacterial suspension of each

tested strain in LB (Luria–Bertani) broth with a density of 1×10^6 CFU/mL (colony-forming units per milliliter). In parallel, three microtiter plates were inoculated and incubated at 37 °C for 4, 6, and 24 hours, respectively. After incubation, the medium was aspirated, and the wells were washed three times with PBS (phosphate-buffered saline) to remove non-adherent cells. Subsequently, the biofilm was fixed with methanol and stained with 0.5% crystal violet. After decanting the dye, gently rinsing the wells, and drying, the bound dye was eluted with 99% ethanol. The optical density, corresponding to the amount of biofilm formed, was measured spectrophotometrically at 570 nm (MRX Microplate Reader, DYNEX Technologies, USA). The intensity of biofilm formation was evaluated semi-quantitatively according to the criteria described by Stepanović et al. (2007).

2. Testing the antimicrobial efficacy of topical agents using an in vitro burn wound model according to Hammond et al. (2011)

Three monobacterial contaminated burn wound models were prepared:

- Model A: wound 4 hours after bacterial inoculation
- Model B: wound 6 hours after bacterial inoculation
- Model C: wound 24 hours after bacterial inoculation

On all three models, the semi-quantitative efficacy of 11 topical antimicrobial agents commonly used in burn surgery was evaluated in various pharmaceutical forms.

Tested agents

1. Solutions:

- Acidum aceticum 1% (magistral preparation)
- Nitrofurantoin 0.2% (magistral preparation) – FUR 0.2%
- Betadine® (povidone-iodine 10%, EGIS Pharmaceuticals)
- Octenisept® (octenidine dihydrochloride 0.1%, phenoxyethanol 2%, Schülke & Mayr)
- Aquvitox®-D ($\leq 0.03\%$ HClO, $\leq 0.03\%$ NaOCl, 0.0001% ClO₂, Aquasystem)

2. Creams:

- Sulfadiazinum argentum 1% (Dermazin®, Sandoz Pharmaceuticals)—SS 1%

3. Combination:

- Acidum aceticum (1%) + Sulfadiazinum argentum (1%)—AA 1% + SS 1%

4. Impregnated dressings:

- Acticoat® (contains silver, Smith & Nephew)
- Aquacel® Ag (contains silver, ConvaTec)
- Ialugen® Plus (contains hyaluronic acid and Sulfadiazinum argentum, IBSA)
- Actilite® (contains medical-grade honey, Advancis Medical)

For clarity, the following abbreviations are used in tables: AA, acetic acid; FUR, nitrofurantoin; SS, silver sulfadiazine.

Testing procedure

Three cellulose discs were placed on a Petri dish containing Luria–Bertani (LB) agar. Each disc was inoculated with 10 µL of bacterial suspension (10^4 CFU/mL). The plates were incubated at 37 °C to obtain:

- Model A: 4-h incubation
- Model B: 6-h incubation
- Model C: 24-h incubation

Each disc was then covered with a sterile gauze square (5 × 5 cm) impregnated with the tested preparation (2 mL of solution, 1 g of cream or ointment) or with an impregnated dressing of the same size and weighted with a sterile glass plate. A sterile gauze square without any additive served as a control. All plates were subsequently incubated for 24 h at 37 °C. After incubation, each disc was transferred into a test tube containing 1 mL of LB broth, and bacteria were detached by vigorous vortexing for 6 minutes. From this suspension, four tenfold serial dilutions were prepared. From the original suspension and each dilution, 10 µL was plated on LB agar plates. After 20 hours of incubation at 37 °C, the number of CFUs was counted and expressed as CFU/disc. For a clear comparison of the antibacterial efficacy, a classification system for the effectiveness of tested topical agents was established based on the number of viable CFUs recovered.

RESULTS

Intensity of Biofilm Formation

All tested bacterial strains demonstrated the ability to form biofilm; however, the intensity varied among strains and across the observed time intervals (shown in Table 1). Four hours after inoculation, three strains (*A. baumannii* and both *P. aeruginosa* strains) produced biofilm only weakly. The remaining three strains formed weak biofilm only after 6 hours post-inoculation. After 24 hours, strong biofilm production was observed in *P. aeruginosa* (both strains) and *A. baumannii*, moderate biofilm formation in *E. coli* and *E. faecalis*, and weak biofilm production in *S. aureus*.

Efficacy of Topical Antimicrobial Agents

The efficacy of the tested topical agents differed depending on the type of preparation and on the microorganism inoculated into the burn wound model. In Model A, six agents showed excellent efficacy against all tested strains (Octenisept®, Sulfadiazinum argentum, Acidum aceticum + Sulfadiazinum argentum combination, Ialugen® Plus, Acticoat®, and Betadine®). In Model B, only two agents (Octenisept® and Ialugen® Plus) demonstrated excellent bactericidal activity

Table 1. Intensity of Biofilm Formation in Tested Wound Pathogens

Nr.	Identification	4 h	6 h	24 h
1	<i>P. aeruginosa</i> A	+	+	+++
2	<i>P. aeruginosa</i> B	+	+	+++
3	<i>S. aureus</i>	0	+	+
4	<i>E. faecalis</i>	0	+	++
5	<i>E. coli</i>	0	+	++
6	<i>A. baumannii</i>	+	+	+++

(0, no biofilm production; +, weak biofilm production; ++, moderate biofilm production; +++, strong biofilm production)

Table 2. Interpretation of Results for Efficacy of Topical Antimicrobial Agents

0	No antimicrobial activity CFU following treatment equals CFU in gauze control
+	Bacteriostatic effect The number of CFU after the action of the agent is lower than after the application of sterile gauze and at the same time higher than the initial inoculum
+++	Strong bactericidal effect The number of CFU after the action of the agent is lower than the initial inoculum

against all bacterial strains. In Model C, when antimicrobials were applied 24 hours after bacterial cultivation, when biofilm formation is highly probable, the efficacy of most agents was comparable to that of sterile gauze. None of the tested preparations was able to completely inhibit bacterial growth in all strains. The only exceptions were Octenisept® and Betadine®, which showed excellent activity against *S. aureus* and *E. coli*, and Aquacel® Ag, which was highly effective against *S. aureus*.

For clear and straightforward interpretation of the results, we created a visually appealing color scheme in which green corresponds to strong bactericidal efficacy of the agent, while red indicates situations where the given topical antimicrobial agent was ineffective in the respective model (Tables 2 and 3). In general, it was observed that the longer the interval between bacterial inoculation and the first application of the antimicrobial agent, the weaker the efficacy of the tested topical antimicrobials.

DISCUSSION

The results of our study confirm that the timing of topical antimicrobial application plays a crucial role in the effective suppression of bacterial growth in contaminated wounds. A clear pattern emerged across all experiments: the longer the bacterial growth was allowed to proceed before treatment, the weaker the antimicrobial effect observed. This finding correlates strongly with the dynamic process of biofilm maturation, which fundamentally alters microbial

Table 3. The Efficacy of Topical Antimicrobial Agents Tested on in Vitro Burn Wound Models Following Inoculation With Multidrug-resistant Bacteria

MODEL A	<i>P. aeruginosa</i> 1	<i>P. aeruginosa</i> 2	<i>S. aureus</i>	<i>E. coli</i>	<i>E. faecalis</i>	<i>A. baumannii</i>
AA1%	+++	+++	+	0	+	+++
FUR	0	0	+++	0	+++	0
Aqvitox	+++	0	0	0	0	+++
Octenisept	+++	+++	+++	+++	+++	+++
SS1%	+++	+++	+++	+++	+++	+++
AA1%+SS1%	+++	+++	+++	+++	+++	+++
lalugen plus	+++	+++	+++	+++	+++	+++
Acticoat	+++	+++	+++	+++	+++	+++
Aquacel Ag	+++	+++	+++	0	0	+
Actilite	0	0	+++	0	0	+++
Betadine	+++	+++	+++	+++	+++	+++
MODEL B	<i>P. aeruginosa</i> 1	<i>P. aeruginosa</i> 2	<i>S. aureus</i>	<i>E. coli</i>	<i>E. faecalis</i>	<i>A. baumannii</i>
AA1%	+++	+++	+	0	+	+++
FUR0,2%	0	0	+++	0	+++	0
Aqvitox-D	0	0	0	0	0	0
Octenisept	+++	+++	+++	+++	+++	+++
SS1%	+++	+++	+++	+++	+++	0
AA1%+SS1%	+++	+++	+++	+++	+	+++
lalugen plus	+++	+++	+++	+++	+++	+++
Acticoat	+++	+++	+++	+++	+	+++
Aquacel Ag	+++	+++	+++	0	+	+
Actilite	0	0	+++	0	0	+
Betadine	+++	+	+++	+++	+++	+++
MODEL C	<i>P. aeruginosa</i> 1	<i>P. aeruginosa</i> 2	<i>S. aureus</i>	<i>E. coli</i>	<i>E. faecalis</i>	<i>A. baumannii</i>
AA1%	+	0	0	0	0	+
FUR0,2%	0	0	0	0	0	0
Aqvitox-D	0	0	0	0	0	0
Octenisept	+	+	+++	+++	0	+
SS1%	0	0	0	0	0	0
AA1%+SS1%	0	+	0	0	0	+
lalugen plus	0	0	0	0	0	+
Acticoat	0	0	0	0	0	0
Aquacel Ag	+	+	+++	0	0	0
Actilite	0	0	0	0	0	+
Betadine	0	0	+++	+++	0	+

susceptibility to antimicrobial agents. In our biofilm formation assay, all strains were capable of producing biofilm, but the intensity distinctly increased over time, with *P. aeruginosa* and *A. baumannii* demonstrating strong biofilm formation already at 24 hours. This reflects clinical observations that these pathogens are among the most problematic in chronic wounds due to their ability to rapidly generate structured,

protective biofilm communities (Khan, 2024; Schneider, 2025).

In the in vitro wound model, this phenomenon resulted in a marked decline in antimicrobial efficacy between Models A and C. While several agents—including Octenisept®, Betadine®, silver-based dressings, and silver sulfadiazine—showed excellent performance when applied 4–6 hours after

inoculation, nearly all preparations lost activity in the 24-hour model, with results approaching those of sterile gauze application. A similar time-dependent loss of efficacy was reported by Roche et al., who demonstrated significantly reduced activity of silver-containing therapies when applied 24 hours after MRSA contamination compared with early (4 h) treatment (Roche, 2012). The convergence of our findings with published data further supports the interpretation that biofilm maturation fundamentally limits the capacity of topical antimicrobial agents to eradicate bacteria, even when these agents are known to be potent in planktonic conditions. This phenomenon aligns closely with current understanding of biofilm biology. Biofilm formation begins within minutes to hours after bacterial attachment, and as the extracellular polymeric substance (EPS) develops, diffusion barriers, altered metabolic states, and quorum sensing all contribute to dramatic increases in antimicrobial tolerance—up to 1000-fold compared with planktonic bacteria (Chegini, 2025). Findings from the available literature further demonstrate that once biofilm is established, topical antimicrobials rarely achieve complete eradication. For example, Mezzasalma et al. showed that although plant-derived compounds such as asiatic and ursolic acid exhibit notable antibiofilm effects, none were able to fully inhibit biofilm formation across all tested strains (Mezzasalma, 2025). Similarly, Thomas et al. observed that an N-chlorotaurine gel—despite stability and activity against *S. aureus*—was ineffective against *P. aeruginosa* biofilms in a chronic wound model (Thomas, 2025). These findings reiterate that biofilms represent a robust defensive phenotype, particularly in Gram-negative pathogens, and that complete eradication is rarely achieved using topical agents alone.

Our results also highlight differences in the spectrum of activity of tested agents, with Octenisept® and Betadine® retaining partial efficacy even in Model C against select bacteria (*S. aureus*, *E. coli*). This is consistent with known characteristics of iodine-based and octenidine-based preparations, which exhibit rapid, non-specific mechanisms of action able to partially overcome biofilm-associated tolerance (Kessler, 2025). However, even these agents were unable to fully inhibit growth across all strains in the mature biofilm environment, further emphasizing the resilience of biofilm-mediated infections.

Recent research has increasingly focused on novel strategies to disrupt biofilm structures or potentiate the effects of antimicrobials. Several studies from our dataset demonstrate promising avenues: hydrogels enriched with plant extracts (Lethongkam, 2025), composite films incorporating zinc oxide nanoparticles and essential oils (Motelica, 2024), and enzyme-based debridement systems targeting matrix polysaccharides (Schneider et al., 2025). These interventions showed significant reductions of biofilm biomass—often 3–7 log units—in ex vivo or in vitro chronic wound models,

particularly when applied in combination with physical disruption methods such as mechanical or enzymatic debridement. The study by Schneider et al. is especially relevant, demonstrating that biofilm dispersal enzymes dramatically improve the effect of subsequent antimicrobial therapy and surgical debridement, providing a compelling model for integrated wound care.

Collectively, these insights suggest that topical antimicrobials alone are insufficient when applied to wounds with established biofilms, whether in vitro, ex vivo, or in vivo. Instead, successful management of chronic or heavily contaminated wounds requires a multimodal approach: early and repeated cleansing, timely antimicrobial application, mechanical or enzymatic debridement, and ongoing biofilm management within a structured wound hygiene protocol. Our data strongly support this approach and further indicate that timing of intervention is one of the most critical determinants of treatment success.

Translating our experimental results into clinical practice, it is reasonable to assume that delayed application of topical antimicrobials—for example, 24 hours after microbial contamination of a burn wound—is unlikely to sufficiently suppress bacterial proliferation or prevent infection. By this stage, biofilm maturation likely impedes diffusion and neutralizes active agents. These findings underscore the necessity of early and proactive wound management, particularly in burn surgery, where rapid colonization by biofilm-forming pathogens is well documented.

CONCLUSIONS

Our study demonstrates that the timing of topical antimicrobial application is a critical determinant of its efficacy in contaminated wound models. The results clearly show that early intervention—within the first hours following bacterial contamination—results in significantly higher antimicrobial activity, whereas delayed application, particularly after 24 hours, is associated with a substantial decline in efficacy. This reduction strongly correlates with the process of biofilm maturation, which represents a key barrier to successful antimicrobial therapy.

All tested bacterial strains could form biofilm, with a marked increase in biofilm intensity over time. This finding supports the concept that even initially planktonic bacterial populations rapidly transition into structured biofilm communities, which exhibit significantly increased tolerance to antimicrobial agents. In the in vitro burn wound model, this was reflected by the progressive loss of effectiveness of most tested topical agents, with several preparations showing activity comparable to sterile gauze in the mature biofilm stage.

Among the evaluated agents, octenidine- and iodine-based preparations demonstrated the most consistent

antimicrobial activity across different time points, although even these agents were unable to fully eradicate bacteria in the established biofilm environment. These findings highlight the inherent limitations of topical antimicrobial therapy when used as a standalone intervention in wounds with developed biofilm.

From a clinical perspective, our results emphasize the necessity of early and proactive wound management. Delayed initiation of topical antimicrobial therapy may be insufficient to prevent bacterial proliferation and infection due to the protective nature of biofilm. Therefore, effective treatment strategies should incorporate timely antimicrobial application combined with regular wound cleansing, biofilm disruption, and, when necessary, mechanical or enzymatic debridement as part of a structured wound hygiene approach. In conclusion, the successful management of contaminated and chronic wounds requires not only the appropriate selection of antimicrobial agents but also the precise timing of their application and integration into a multimodal therapeutic strategy targeting both planktonic bacteria and biofilm-associated communities.

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Not applicable

AUTHORS' CONTRIBUTIONS

MD arranged the general structure of the manuscript and was a major contributor. MD and AP collected swab samples for bacterial strain collection, and MH performed the microbial testing and created the visual documentation. MV discussed and evaluated the results. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

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COMPETING INTERESTS

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