Review

Effectiveness or Understanding: Where Does the Key to Defeating Biofilm and Produce an Impactful Article Lie?

Silvana Alfei *, Gian Carlo Schito, Caterina Reggio, Guendalina Zuccari *

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Effectiveness or Understanding: Where Does the Key to Defeating Biofilm and Produce an Impactful Article Lie?

Silvana Alfei [1,*], Gian Carlo Schito [2], Caterina Reggio [3] and Guendalina Zuccari [1,3,*]

[1] Department of Pharmacy (DIFAR), University of Genoa, Viale Cembrano, 4, 16148 Genoa, Italy

[2] Department of Surgical Sciences and Integrated Diagnostics (DISC), University of Genoa, Viale Benedetto XV, 6, 16132 Genova, Italy

[3] Laboratory of Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Via G. Gaslini 5, 16147 Genoa, Italy

* Correspondence: alfei@difar.unige.it (S.A.); guendalina.zuccari@unige.it (G.Z.); Tel.: +39 010 355 2296 (S.A.)

Abstract

Biofilms (BFs) bacteria are dramatically intensifying tolerance to conventional antibiotics, no longer effective. Therefore, the research for new antibiofilm (ABF) compounds are noticeably increasing the studies proliferation rate. In this regard, intriguing questions should raise to be debated. To this end, the problematics of BF, mainly in medical setting, have been afforded here in an original way, examining the tension "between efficacy and understanding". Questions include: are BF mechanistic studies indispensable and strictly required especially at academic levels with poor economic support? When may a purely phenotypic approach still hold scientific value? Could be demonstrate empirical efficacy alone, sufficient for scientific relevance of the study? Do high costs, long times mechanistic insights, also associated to environmental issues, represent the necessary key to defeating BFs and the benchmark that determines the robustness and impact of ABF research? The state of the art of global challenge against BF, responsible for difficult-to-treat and even lethal chronic infection, has been provided. The available armamentarium of best functioning antibiotics/combinations has been discussed, while the correct way to investigate ABF mechanisms has been clarified. Among 102 studies on the ABF activity, considered, distributed in Tables and discussed, mechanistic investigations carried out correctly have been found in only 34 ones. Only efficacy screens, stopping at phenotypic descriptions, as reported in 68 out of 102 papers, are considered essential for discovering efficacious ABF compounds and are welcome by Editors and scientific community. Such approach represents the main trend of most recent literature and is strongly desirable for publication.

Keywords: antibiofilm agents; mechanistic insight; biofilm inhibition/disruption; research relevance; phenotypic efficacy; antimicrobial resistance

1. Introduction

Antibiotics are frequently over prescribed and misused among humans and animals or abused without medical indication [1]. Frequently, they are used by patients not completing or respecting entire antibiotic dosage and not strictly following the correct antibiotic regimen. Often, antibiotics are indicated for not stringently necessary prophylactic antibiotic therapies. All these incorrect behaviours strongly promote genetic mutation among micro-organisms, which develop resistance to multiple antibiotics (MAR) [2]. In this context, superbugs continuously emerge, thus constituting worrying microbial species, which have developed resistance even to all available antibiotics. Multidrug resistant (MDR) superbugs are responsible of intractable infections, thus causing long-term hospitalization, increased morbidity, mortality rate and economic loss [2]. Poor hygiene and scarce sanitation existing in certain geographic areas can further worsen an already critical scenario

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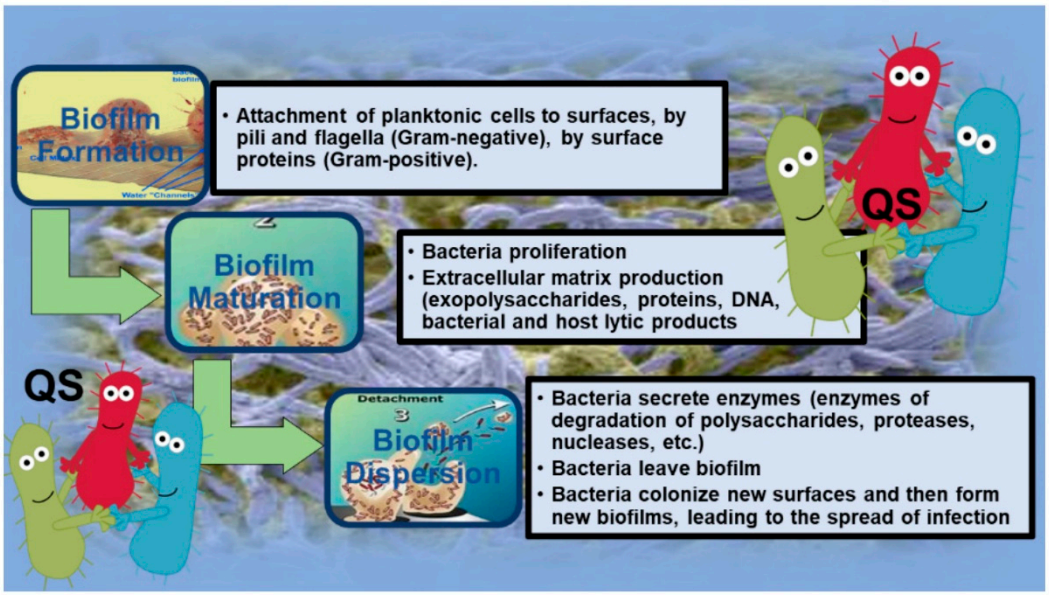
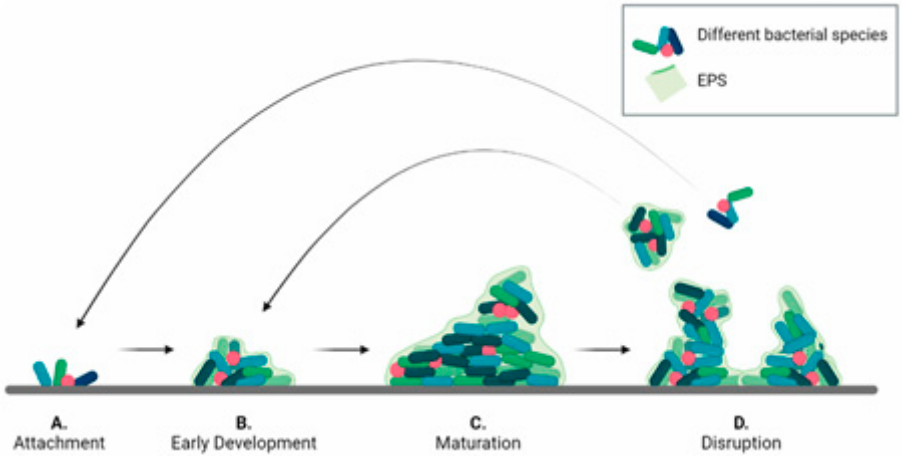
[3]. *Staphylococcus aureus* is an important human opportunistic pathogen, causing a wide variety of severe clinical infections, such as bacteraemia and pneumonia[4,5].

Its successful treatment remains a significant challenge, due to the emergence of the above-mentioned MAR strains, such as methicillin-resistant ones (MRSA), which are superbugs, which have previously demonstrated resistance also to other antibiotics [4]. Biofilm (BF) formation capacity is a further and almost intractable form of resistance and pathogenicity of *S. aureus* and other opportunistic Gram-positive *Staphylococci* such as *S. epidermidis*.

Generally, BFs can be developed by several microorganisms including Gram-positive (*Staphylococci*, *Enterococci* and competitive dental BF producing *Streptococcus mutans* and *Lactobacillus* spp) and Gram-negative (*P. aeruginosa, Escherichia coli, Acinetobacter baumannii* and *Klebsiella pneumoniae*) bacteria, fungi (*Candida* supp), molds and yeasts. Generally, BF is composed of a structured community of microbial cells, which first adhere to a living or inanimate surface (facilitated by its possible roughness) (Figure A, B). In Gram-negative species, adhesion occurs by the anchoring of planktonic (normal motile bacterial cells) to the surface by pili and bacterial flagella [6,7]. Differently, in Gram-positive species, anchoring occurs through surface proteins[8] (Figure 1A).



(A)



(B)

Figure 1. BF cycle developmental. Both images are published (Alfei et al and Yang et al) in open assess journals [9,10]under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/, accessed on 27 April 2026) and no needs permissions.

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Following adhesion, the bacteria begin to proliferate, forming microcolonies and produce the extracellular matrix (BF biomass), thus allowing the integrity of the BF[11–13]. BF bacteria embed/surround themselves with this external complex biomass, known as external polymeric substances (EPS), which consists of exopolysaccharides, nucleic acids (*e* DNA and *e* RNA), proteins, lipids, and other biomolecules, also including both bacterial and host lytic products[9,11–13]. Following the maturation of BF, the dispersion phase occurs, in which the secretion of enzymes, catalysing the degradation of polysaccharides (proteases, nucleases, etc.) and disintegration of the matrix is observed. This phase allows other bacteria to leave old BF, colonize new surfaces, and then form new BFs, leading to the spread of infection [11–13] (Figure 1). Collectively, EPSs constitutes a protective mechanism by which bacteria succeed in surviving even in harsh and stressing environments and in hosts treated with antibiotics [9]. Upon attachment to surfaces, bacteria pass from the planktonic (motile) form to the sessile ones, including immotile, dormient and persisters cells, with reduced metabolism. Closed inside EPSs, all bacterial cells collaborate, communicate by a complex system known as quorum sensing (QS) system, regulated by the expression of specific genes, which allow microorganisms to also reducing their need for nutrients and energy[9]. Sessile BF bacteria are extensively more resistant to clearance by immune responses and to treatment with antibiotics[14–16]. Collectively, bacterial BFs represent a major challenge in the management of chronic and recurrent infections [17], as their characteristic EPSs confers marked tolerance to antimicrobials and host immune responses, which can reach them only with great trouble or fail in reaching them [18]. The growing prevalence of MDR BF-producer bacterial strains urgently needs to identify novel compounds/strategies, possible not involving hosts immune system, with effective antibiofilm activities (ABFAs), to substitute traditional antibiotics, [19]. In this context, the identification and characterization of novel compounds with ABFAs represents a highly interesting area of research. Newly synthesized molecules—including small organic molecules, heterocyclic derivatives, quaternary compounds, functionalized nanomaterials, and organic-metal hybrids—have shown significant potential in preventing BF formation or disrupting mature structures [20–22].

Evaluating the ABFA of these compounds requires the use of standardized and reproducible methodologies. Among the most widely used, there are assays based on biomass quantification, such as the Congo red and crystal violet colorimetric tests, determination of cell viability within the BF, and microscopic analysis of three-dimensional structures of it [23]. Despite these approaches provide relevant information on new compounds efficacy, under certain controlled experimental conditions [24], they do not give complementary information on the mechanism of action of new agents.

Recent literature highlights a growing number of studies dedicated only to the synthesis and characterization of new ABF molecules, with promising results against clinically relevant pathogens such as *S. aureus, P. aeruginosa,* and *E. coli* [19,21].The comparative analysis of these compounds and their experimental protocols represent a fundamental step in identifying candidates with potential therapeutic applications or applicable as antimicrobial coatings preventing BF formation on medical devices, regardless their mechanisms of action [25]. In this regard, searching among published papers on ABF compounds, it was evidenced that others than the lack of mechanistic insights are the limitations of some published studies.

1.1. State of the Art About Biofilm (BF) Inhibition

In recent years, BF research has focused on two main areas, including understanding the mechanisms that confer tolerance and persistence to BF bacteria, as well as developing new agents capable of preventing BF formation or eradicating established BF structures. BF is now recognized as a major cause of treatment failure in medical device-associated infections and severe chronic infections, thanks to the protection provided by EPS to BF bacteria and the profound physiological changes occurring in bacteria within BF [18].

On a pharmacological level, several studies have shown that some classic antibiotics, such as rifampicin, possess marked antibacterial activity in specific clinical contexts, such as prosthetic joint infections [26]. Unfortunately, their efficacy is often limited by the emergence of untreatable

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resistance associated to BF formation, thus requiring the use of complex combinations [26]. Also, other conventional antibiotics, including cefazolin, are inadequate for the complete removal of the BF and to counteract the phenotypic behaviour of BF cells, which endow bacteria with high tolerance to antibiotics[27].

This has prompted a pressing need to discover and develop novel ABF agents and the search for new molecules, both natural and synthetic, with mechanisms of action aimed at EPS disruption, inhibition of QS, or impairment of initial adhesion [28–30].

Overall, the state of the art of BF inhibition research clearly indicates that the exploration of novel laboratory-synthesized compounds, including heterocyclic derivatives, quaternary salts (QASs and QPSs), nanomaterials (NMs), and hybrid systems (HSs), represents one of the most promising avenues to overcome the limitations of conventional therapies against BF-associated infections [18,19,21].

A significant area of research concerns the synthesis of new organic and hybrid compounds with antibiofilm activity (ABFA). New ammonium *bis*-quaternary salts (*b*-QASs) derived from natural products, sulphur-containing camphor derivatives and a series of compounds, based on 1,3-oxazole and isosteric analogues, were developed and subjected to *in vitro* BF inhibition assays, to evaluate their antibacterial effects and characterize their ABF profile against different ATCC or MDR strains of Gram-positive and Gram-negative species [19,21,23]. Apostol et al combined results from *in vitro* assays to *in silico* studies [23]. Collectively, at least in *in vitro* models, all authors demonstrated a significant reduction in BF biomass and in BF cells viability, by applying the synthesized molecules [19,21,23].

At the same time, interest in NMs and hybrid systems as ABF platforms has grown. Silver-bacteriocin nanoconjugates [22], copper-based nanocomposites obtained through “green” biosynthesis [20], cellulose-loaded nanoparticles (NPs) and borax [24] were nanotechnologically prepared. Such NMs demonstrated marked activity in reducing BF formation in common pathogens and enhanced ABF efficacy. Nanocomposites (NCs) by Kart et al. demonstrated also high potential for applications in wound healing [24].

The key role of natural molecules and complex extracts, as a source of novel ABF agents have been reviewed in several relevant articles [28,29]. The lactic fermentation of plant matrices, such as Scottish edible seaweed, leading to the enrichment of the metabolome in bioactive molecules with ABF activity, has been recently reported [25]. These results suggest that the combination of bioprocesses and synthetic chemistry can generate libraries of compounds with improved ABF properties.

As abovementioned, the assessment of ABFA is based on a series of consolidated assays, including biomass quantification by crystal violet or Congo Red staining, measurement of cell viability within the BF, determination of minimum BF eradication concentrations (MBEC), and microscopic analysis of three-dimensional structures [17,23]. Authors have emphasized the importance of standardized protocols to allow reliable comparisons between the efficacy of new ABF compounds [18] and the integration of these methods into the broader vision of BF prevention in the medical sector, where ABF strategies proposed by other scientists already exist [30].

2. Biofilm Models for Biofilm Studies

BFs are complex, structured microbial communities embedded in a self-produced extracellular matrix (EPS). Because BF cells behaviour differs dramatically, depending on environmental conditions, the scientific literature distinguishes several experimental and observational models of BF. These models differ in realism, controllability, and reproducibility. Choosing the appropriate model is essential for comparing studies and interpreting results. The following sections summarize the four major categories of BF models (BFMs) used in microbiology and biomedical research, as described in foundational reviews by Donlan [31] and Hall-Stoodley et al.[32].

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2.1. Biofilm Models Categories

As above-mentioned, different models for BF growth exist, usable for carrying out experiment on BF inhibition or tests of disaggregation of already mature structures. Table 1 summarizes the main available typologies of BF models (BFMs), which are classified in three macro categories, such as *in vitro*, *ex vivo*, *in vivo* and *in situ* models. Additionally, Table 2 collect similar models reporting also their sub-categories, as well as associated advantages and limitations.

Table 1. BFMs Classification Summary.

Model category	Terminology	Notes	Refs
In vitro	Dynamic BF	Formed under flow or shear conditions	[32]
	CDC BF reactor	Standardized dynamic system	
	Simulated ELF exposures	Controlled exposure to ELF fields	
Ex vivo	*Ex vivo* CF BFs	Grown on real CF sputum or tissues	[32–35]
In vivo	Murine lung infection model with *P. aeruginosa*	Formed in lung tissue	[32] [36] [37] [38]
	Chronic wound BFMs (mouse/rat)	Form in chronic or diabetic wounds	
	Comparative animal models for chronic wounds	Biological tissue models for comparisons	
	Medical device–associated BFMs (catheters, implants)	Implanted devices colonized by pathogens *in vivo*	
	Non-surface-associated biofilm infection models	Tissue-embedded models	
In situ	BFs on MD	To study BF on MDs directly in patients	[32] [39,40] [38]
	Environmental BFMs *	To study BF directly in environment	
	Dental BFs observed in the oral cavity	To study BF directly in mouth	

MDs = Medical devices; * pipes, industrial surfaces, tubes, cisterns, container, tanks.

Table 2. Major BFMs categories and sub-categories for studies on BF inhibition/disaggregation.

Models	**Advantages**	**Limitation**	**Refs.**
***In vitro* models**			
Microtiter plates (static)	⬆Reproducibility, full control of EP	⬇ PR vs biological environments	[32] [40] [36] [38]
Flow cells [D]			
CDC BF reactor [D]			
Drip-flow reactor [D]			
Microfluidic devices [D]			
***Ex vivo* models**	**Advantages**	**Limitation**	**Refs.**
Cystic fibrosis (CF) sputum	⬆ Realistic microenvironment	Biological variability Limited availability of samples	[32] [33–35]
Lung/skin tissue fragments			
Mucus/secretions from patients			
Explanted medical devices *			
***In vivo* models**	**Advantages**	**Limitation**	**Refs.**
Murine models of chronic infection	⬆ Physiological relevance	Ethical constraints ⬆Cost, technical complexity	[32] [36]

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Subcutaneous implant models			[37] [38]
Infected wound models			
Lung infection models for *P. aeruginosa*			
***In situ* models**	**Advantages**	**Limitation**	**Refs.**
BFs on medical devices in patients	Maximum real-world relevance	No control over environmental variables	[32] [39,40] [38]
Environmental BFs **			
Dental BF observed in the oral cavity			

[D] = Dynamic; * catheters; EP = experimental parameters; PR = physiological relevance; ⬆ = high, higher, highly; ⬇ = reduced, low, lower, limited; **pipes, industrial surfaces, tubes, cisterns, container, tanks.

2.1.1. In Vitro Models

BFs can be made grown *in vitro*, in fully artificial, highly controlled laboratory systems. These models, including both static and dynamic models, allow precise manipulation of nutrients, shear stress, temperature, and other variables, and are widely used to study BF physiology and antimicrobial tolerance [32,36,38,40].

The Calgary Biofilm Device (CBD) is a high-throughput *in vitro* static model designed to produce uniform, reproducible bacterial BFs on a 96-peg lid that fits into a standard microtiter plate. BFs grow on the pegs during incubation, allowing systematic testing of minimum biofilm eradication concentrations (MBECs) for antimicrobial agents.

Scientifically, the CBD enables:

- Formation of 96 equivalent biofilms, reducing variability across replicates[41]
- Quantitative assessment of antibiotic susceptibility, revealing that biofilm-associated bacteria often require 100–1000 × higher antibiotic concentrations than planktonic cells [41].
- Visualization and validation of biofilm structure using scanning electron microscopy and standard microbiological methods [41]. This model is widely used due to its standardization, scalability, and relevance for studying chronic, device-related infections dominated by biofilm physiology.

Examples of *in vitro* models which comprehend both static and dynamic systems are included in the first section of Table 1 and 2. The following Figure 2 shows the four main types of dynamic *in vitro* models, as reported in the review by Crivello et al[42].

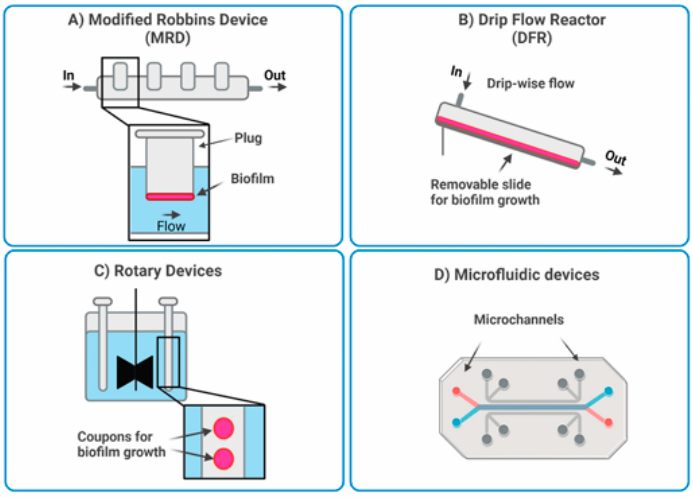

Figure 2. Summary of different dynamic models: (A) modified Robbins device (MRD), (B) drip flow reactor (DFR), (C) rotary devices, and (D) microfluidic devices. Image obtained with Biorender by Crivello et al [42].

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2.1.2. Ex Vivo Models

Ex vivo BFs are grown on real biological material, but outside the organism. These models preserve the biochemical and structural complexity of tissues or secretions and are particularly relevant for diseases such as cystic fibrosis, where sputum-based models closely mimic the in vivo environment[32–35].

Examples of these models have been included in the second section of Tables 1 and 2.

2.1.3. In Vivo Models

In vivo BFs form within a living organism, providing the closest approximation to natural infection dynamics. These models are essential for understanding host–pathogen interactions and the clinical impact of BFs [32,36–38]. Examples of these models have been included in the third section of Table 1 and in the classification summary of Table 2.

Brief Scientific Discussion of In Vivo BF Models and Their Sub-Classification

Understanding BF formation in vivo is fundamental for developing effective strategies, aimed at inhibiting and/or disrupting BFs highly resilient microbial communities, implicated in a wide range of chronic infections, which demonstrated significantly heightened tolerance to antimicrobials. Validated models that accurately reflect the complexity of BF-associated infections are essential in such understanding challenge. A variety of in vivo BF models have been developed to mimic the most clinically relevant infection environments. These models serve as indispensable tools to evaluate microbial dynamics, resistance to antimicrobials, and therapeutic efficacy.

The “Murine Lung Infection Model” with *Pseudomonas aeruginosa*

It is the one of the most widely studied *in vivo* BFMs, that uses a mouse lung infection model sustained by alginate-embedded *P. aeruginosa* [43]. In this system, bacteria are delivered into mouse lungs within alginate beads, enabling the formation of persistent BF structures that mimic the chronic infections observed in *P. aeruginosa* cystic fibrosis patients. This model reliably reproduces the microenvironmental gradients, immune exposures, and antibiotic penetration barriers characteristic of human disease[43]. The model has been used to study the evolution of antimicrobial resistance (AMR) under antibiotic pressure, as demonstrated in works, where repeated passages under ciprofloxacin exposure, resulted in rapid emergence of resistance-associated mutations and shifts in inflammatory cytokine profiles. These findings highlighted the complex interplay between host immunity, microbial adaptation, and antibiotic stress, that shapes BF behaviour *in vivo*[43].

The Chronic Wound Biofilm Models (Mouse/Rat)

Notably, BFs are implicated in up to 60% of chronic wounds, including diabetic foot ulcers, venous leg ulcers, and burn wounds. In animal chronic wound models, wounds are created surgically or chemically and subsequently inoculated with pathogens capable of forming mono- or polymicrobial BFs[44]. These models capture essential hallmarks of chronic wounds: impaired healing, persistent inflammation, and robust microbial communities embedded within protective matrices (EPS). A comprehensive review highlighted how these models support testing of various ABF approaches, including QS inhibitors, bacteriophage therapy, matrix-degrading enzymes, nanomaterials, and combination wound-care strategies[44]. Compared with *in vitro* systems, animal wound models incorporate host factors such as immune responses, tissue architecture, and wound exudate composition, making them valuable translational tools for evaluating novel therapeutics[44]. In Vivo Wound Healing and Biofilm Models have been used for broader comparative analyses of

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animal models used for wound research. They emphasize the variability in skin architecture, immune responses, and healing kinetics across species. These differences influence BF formation and therapeutic outcomes, underscoring the need for careful model selection, depending on the biological question under investigation. Recent evaluations of such models noted that chronic wound studies benefit from systems that account for microbiota–host interactions, including the use of humanized mouse strains. These insights help bridge the translational gap between preclinical models and human pathology, although limitations persist in replicating all aspects of human wound biology[45]. These comparative wound models are *in vivo* biological systems, not dynamic reactor-based or simulated ELF models.

The In Vivo Medical Device–Associated Biofilm Models

These BFMs include BFs which are grown *in vivo* on urinary catheters, prosthetic joints, vascular grafts, and cardiac devices, which are the leading cause of persistent healthcare-associated infections[46]. To study these conditions, animal models involving implanted medical materials have been developed. In such models, pathogens such as *S. aureus* or *P. aeruginosa* are introduced at the implant site, enabling BF formation on materials that closely mimic clinical settings[46]. These models are essential for evaluating antimicrobial biomaterials, antibiofilm coatings, and controlled drug-release systems, as well as host interactions with colonized surfaces. They maintain the architectural and immunological features of *in vivo* BFs but do not rely on hydrodynamic flow or reactor-based systems typically used in engineered models[46]. The use of these models has been described within broader reviews of clinical BFs research methodologies, which highlight both *in vivo* and *in vitro* strategies to study biofilm development and treatment responses[46].

The In Vivo Non-Surface-Associated Biofilm Infection Models

These BFNs encompass non-conventional BFs, which do not develop on surfaces but may occur in mucosal secretions or soft tissues without attachment to a hard surface[47]. Non-surface-associated BFMs have therefore emerged to study diseases such as chronic otitis media, cystic fibrosis airway infections, and deep soft-tissue infections. These infections consist of bacterial aggregates embedded in host-derived secretions or tissues rather than classical surface-attached BFs[47]. Reviews focusing on improved BFMs emphasize the importance of these systems for capturing the diversity and complexity of medical BFs, noting that they challenge existing definitions and require advanced approaches for accurate modelling and evaluation[47].

2.1.4. In Situ BFMs

In situ BFMs are studied directly in their natural environment, without removal or manipulation. This approach is crucial for understanding BFs development on medical devices or in environmental settings [32,38,39,40]. Examples of these models have been included in the fourth section of Tables 1 and 2.

Scientific Discussion of In Situ BFMs and Their Sub-Classification

In situ medical device associated BFMs allow to examine BF formation directly on indwelling or implanted medical devices under real-world physiological conditions, allowing researchers to observe microbial adhesion, EPS-matrix development, and resistance behaviours, without removing the device or altering its microenvironment[31]. These models capture how microorganisms irreversibly attach to catheter, prosthetic, or implant surfaces, producing EPS, as well as create structurally complex and highly drug-resistant communities (sessile bacteria), responsible for persistent device-associated infections [31]. BFs formation generally follows an already descripted multistep process, including an initial attachment, microcolony formation, maturation, and dispersion, regulated by environmental cues and QS signals, and involves pathogens such as *S. aureus*, *S. epidermidis*, *P. aeruginosa*, and *C. albicans*, which adopt sessile phenotypes that enhance their

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immune evasion and antimicrobial tolerance[48]. Real-time *in situ* monitoring strategies, such as optical fibre sensors embedded within device lumens, impedimetric and QCM-based sensors, enable early detection of bacterial attachment, quantification of BF biomass, and assessment of treatment efficacy, thus supporting timely intervention before clinical infection develops[49,50]. These models are vital for understanding the pathogenesis of chronic device-related infections, which account for a substantial proportion of healthcare-associated morbidity, and for guiding the development of ABF coatings, surface modifications, and novel therapeutic strategies aimed at preventing colonization and reducing device failure[51].

In situ environmental BFMs serve to directly study the microbial aggregates embedded in the self-produced EPS matrix, while they remain on their natural substrates, preserving structural integrity, hydration, and micro-environmental conditions[52]. This approach avoids artefacts associated with dehydration or sample manipulation, enabling accurate observation of native BF architecture and heterogeneity[52,53]. In this regard, confocal laser scanning microscopy (CLSM) allows intact *in situ* visualization without the structural distortions seen in traditional electron microscopy. Such models capture the authentic environmental adaptability of BFs, which respond dynamically to nutrient gradients, hydrodynamics, oxygen levels and other ecological drivers while retaining their characteristic EPS-mediated cohesion[53]. *In situ* models are crucial for understanding BFs' functional roles in natural systems, including pollutant degradation, metal immobilization, and regulation of biogeochemical processes such as carbon cycling, all of which depend on the complex ecological interactions preserved only under *in situ* conditions[53]. Furthermore, advanced *in situ* monitoring techniques—such as wettability-based film detection or surface-enhanced Raman scattering (SERS) for real-time molecular characterization—provide powerful tools for tracking BF development directly in environmental matrices[54].

Specifically, *in situ* dental BFMs enable the study of oral microbial communities directly within the natural oral environment, preserving the complex ecological, chemical, and mechanical conditions that shape dental plaque formation[55,56]. These models overcome the limitations of simplified *in vitro* systems by allowing BFs to develop on natural tooth surfaces or intraoral appliances worn by volunteers, thus maintaining authentic saliva exposure, pellicle formation, nutrient fluxes, and multispecies interactions fundamental to caries and periodontal disease development[55,56]. Quantitative and structural analyses, using microscopy and sequencing, have shown that early supragingival BFs, initially dominated by facultative anaerobes such as *Streptococci*, transit over time into more diverse matrix-embedded communities, enriched with anaerobic taxa like *Fusobacterium*, *Prevotella*, and *Porphyromonas*, reflecting natural successional dynamics that cannot be reproduced reliably *in vitro* [57]. Intraoral appliances, such as palatal, lingual, and buccal splints, serve as carriers for enamel or dentin slabs that permit controlled, reproducible biofilm growth while retaining the host's physiological influences[58]. Systematic reviews highlight that these appliances can support undisturbed BF formation and allow high-resolution microbiological analysis, particularly when coupled with next-generation sequencing[57]. Collectively, *in situ* dental BFMs are essential for accurately characterizing plaque ecology, BF maturation, and the evaluation of caries-preventive or antimicrobial strategies under conditions, that closely reflect the human oral cavity [56,58].

Critical Discussion by Authors

Although the classification of BFMs into *in vitro*, *ex vivo*, *in vivo*, and *in situ* categories provides a useful conceptual framework, each model carries intrinsic limitations that must be carefully considered, when interpreting experimental outcomes. *In vitro* systems, while highly reproducible and essential for mechanistic studies, often oversimplify the environmental and biochemical complexity of natural BFs, leading to discrepancies between laboratory findings and clinical behaviours[32,36,38,40]. Conversely, *ex vivo* models offer a more realistic microenvironment, particularly in diseases such as cystic fibrosis where sputum composition strongly influences microbial physiology[32–35]. However, their biological variability and limited standardization can

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complicate cross-study comparisons. *In vivo* models remain indispensable for understanding host–pathogen interactions, immune responses, and the clinical impact of BFs [32,36–38]. Yet, ethical constraints, interspecies differences, and the difficulty of controlling confounding variables limit their scalability and translational precision. *In situ* observations provide unparalleled ecological validity, but the absence of experimental control makes it challenging to establish causality or dissect specific mechanisms[32,38–40]. Also, the *in vivo* BFMs provide critical insights into microbial adaptation, antimicrobial resistance, and host–pathogen interactions within clinically relevant environments. They are indispensable for translational research aimed at developing effective ABF therapies. While they lack the hydrodynamic control and mechanistic simplicity of dynamic *in vitro* systems such as flow cells or CDC reactors. Their strength lies in faithfully replicating the complexities of real infections—including immune factors, tissue architecture, heterogeneous nutrient landscapes, and chronic inflammatory conditions. Future research benefits from integrating *in vivo*, dynamic *in vitro*, and systems-based approaches to obtain a comprehensive understanding of BF behaviour and treatment responses. A critical challenge across all model types is the lack of a single system that fully captures the structural, metabolic, and ecological heterogeneity of BFs. This evidence underscores the importance of integrating multiple complementary models within the same research program. A mechanistic insight obtained *in vitro* should ideally be validated *ex vivo* or *in vivo*, while *in situ* observations should inform the design of more controlled experiments. A robust and useful BF research should not lie in any single model, but in the strategic combination of diverse approaches, to build a coherent and clinically meaningful understanding of BF biology.

3. The Nine Best Available Antibiotics Against Biofilms

In this following section MICs denote minimum inhibitory concentration for planktonic cells, MBIC/MBEC mean minimum BF inhibitory/eradication concentration. Assay methods used include different modalities, such as Calgary device, CDC reactor, *in vivo* implant models etc., so absolute values are assay-dependent, and comparisons are most meaningful within a single study. MBECs are typically 10–1000 × MIC, and lack of standardized endpoints, which complicates cross-study comparisons. Table 3 collect the most active available antibiotics and/or their more active combinations against BF by *S. aureus* and *P. aeruginosa,* as strains representative for Gram-positive and Gram-negative species. Detailed information about such drugs is available in the subsequent sub-sections.

Table 3. Antibiotics against bacterial BFs: mechanisms, BF-active concentrations, comparison to planktonic MICs.

Drug/Class	PT/Mechanism	Pathogen(s) / Strains	BFM *	MBIC/MBEC/DE	Planktonic MIC (mg/L)
RIF*vs*.RIFM	RNA PI	SA, SE, CoNS	MBEC® (CTM)	RIFA MBEC < RIFAMMBEC	RIFA ≅ RIFAM
VAN+GEN	CWS+30S RI	MRSA & MSSA on PUS	CV/MTT, CTMs CFPM	VAN+GEN >70%MRSA (MBEC)	Single MICs modest
GEN+RIFM	30S RI±RNA PI	MRSA & MSSA	IV implant MBEC (24 h EXPO)	MIC_{60} 128-16 µg/mL	GENTA 0.25–1
DAP	MD	*Staphylococci* (CTM)	MBEC/MBIC across MSs	MBEC ≫ MIC	↓ MICs vs ↑ MBECs
CIP+MER	DNA · GI/topoIV+PBPs	PA (PAO/ΔmutS;/CW44)	CDC DBF; SELF EXPOs ↑ 120 h	Combo suppressed REG/RES	CIPRO/MERO 0.25–0.5/2–4

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TOB	30S RI	PA (MS, PAO1 + clinical)	CTM, CDC, BFA 24–72 h	MBEC ≤250 to ≥2000 µg/mL	≤1–2 SISO
COL+RIFM	OMD+RNA PI	PA (also COL-resistant)	HMM, EVCFM; DBFM	PP, COL+ RIFA active	≤4 variable on strains
AZI **	AV/QSM (las/rhl)	PA (PAO1, nfxB mutant)	MCTM MBIC °°°	$MBIC_{90}$, 256→4 (72h)	Standard resistant MIC
CAZAVI***	β-L+DBO β-LI	*P. aeruginosa* °°	MIC, CE+CEF MBEC	CEFTA MBEC 32–128×MICs	↻ to ≤4 mg/L

References related to Table rows are embedded in the following text. RIF = Rifabutin; RIFM = rifampicin; VAN = vancomycin; GEN = gentamicin; DAP = daptomycin; CIP = Ciprofloxacin; MER = meropenem; TOB = tobramycin; COL = colistin; AZI = azithromycin (sub-MIC effects); CE = cellulase; CEF = ceftazidime; CAZAVI = ceftazidime-avibactam * assay, media, exposure; ** sub-MIC effects; *** ± matrix enzyme; # from prosthetic joint infections; PJIs; PT = primary target; MBIC = minimal biofilm inhibition concentration; MBEC = minimal biofilm eradication concentrations; assay-dependent biofilm values (MBIC/MBEC) are reported with model details when available; CoNS = coagulase negative *S. epidermidis*; SA = *S. aureus*, SE = *S. epidermidis*; PA = *P. aeruginosa*; PUS = polyurethane surfaces; °rat femoral implant; MRSA = methicillin-resistant *S. aureus*; MSSA = methicillin susceptible *S. aureus*; °°clinical cohorts; isogenic mutants, enzyme adjunct study; MS = multiple strains; HY = hypermutable; CTM = Calgary type model; MCTM = modified CTM; CFPM = continuous-flow peristaltic model; °°° 1640 RPMI, 24–72 h; HMM = host-mimicking media; EV = *ex vivo*; CFM = cystic fibrosis models; DBF = dynamic biofilm; BFA = biofilm age; SELF = simulated extremely low frequency; IV = in vivo; EXPO = exposure; MSs = multiple studies; DE = detachment efficiency; REG = regrow; PP = poor penetration ; SISO = susceptible isolates; ↻ = restored; 30S R = 30S ribosome; β-LI = β-lactamase inhibitors; β-L = β-lactam; AV = anti-virulence; QSM = quorum sensing modulation; OMD = outer membrane disruption; DNA GI = DNA gyrase inhibitors; MD = membrane depolarisation; CWS = cell wall synthesis.

3.1. Rifamycins (Rifampicin, Rifabutin)

Rifampicin (also rifampin) has long been recognized as a cornerstone in the treatment of peri-prosthetic joint infections (PJIs) and other foreign-body materials, caused by *Staphylococcus* species, due to its unique BF-penetrating properties [26]. It is capable to inhibit staphylococcal Gram-positive BFs formations, by inhibiting RNA polymerase (transcription), thus inhibiting bacterial RNA synthesis, independently of bacterial division [26]. Its effectiveness is significantly influenced and reduced by BF maturity. Mature BF consists of a denser matrix that impairs antibiotic penetration and immune responses, harbours persister cells, and induce metabolic alterations, all promoting reduced killing [26]. A comparison between rifabutin and rifampicin against 132 staphylococcal clinical PJI isolates (51 *S. aureus*, 48 *S. epidermidis*, 33 CoNS) was reported by Thill et al [59]. Rifabutin MBEC medians were lower than those of rifampicin across all groups, despite similar or slightly higher MICs. Note that the study provides distribution rather than one value per strain, but trend is consistent [59]. Anyway, as for multiple antibiotics, MBEC were ≫ MIC. Collectively, rifampicin and daptomycin often showed the best ABFA among comparators, although complete eradication was uncommon and combinations were advised. Additionally, due to the high risk of resistance emergence in the presence of a high bacterial load, the combination therapy incorporating rifampicin, for implant-associated infections, has been reported to improve outcomes [26,60]. In this regard, Okae et al reported that in a rodent *in vivo* implant model with *S. aureus* (USA300 MRSA; UAMS-1 MSSA), addition of rifampicin (≈1.5 µg/mL) to gentamicin reduced gentamycin $MBEC_{60}$ by 8-fold (e.g., day-3 USA300: 1024 → 128 µg/mL), underlining the relevance of rifamycin-based combinations[61]. Among classic agents, rifamycin-containing combinations remain stand-outs for device infections sustained by staphylococcal species, but rifabutin may achieve lower MBECs than

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rifampicin *in vitro* [59]. Despite its established role, the use of rifampicin remains subject to debate, primarily due to inconsistencies in the literature regarding clinical efficacy, timing of initiation, choice of combination agent, and variability in outcomes across infection types [26].

Early experimental data by Tshefu in the 1980s already showed a marked decline in treatment success, when rifampicin initiation was delayed with a cure rate of tissue cage infections decreasing from 100% to 57% [62]. Furthermore, the timing of rifampicin initiation in clinical practice varies considerably. Beldman et al. advocated delaying rifampicin administration for at least five days after surgery, to reduce the risk of resistance by allowing the bacterial load to decrease [63].

After surgical exchange in chronic staphylococcal infections, rifampicin is maintained to target residual BF-like bacteria and prevent relapse or early recolonization [64,65].

In addition, rifampicin activity may be strain dependent. While earlier studies used a single laboratory strain, newer research indicates that ABFA varies across rifampicin-susceptible staphylococci [59]. The mechanisms behind this variation are incompletely understood and may include differences in BF matrix composition, gene regulation, and metabolic states of BF-embedded bacteria. However, the clinical impact of this variability remains to be fully elucidated.

Critical Appraisal of Clinical Evidence, Challenges, and Controversies

One of the first clinical evidence of rifampicin activity in staphylococcal implant-related infections was reported by Widmer et al. in patients undergoing debridement, antibiotics, and implant retention (DAIR) [66]. Zimmerli et al. conducted a randomized controlled trial (RCT) with a sample size of 33 patients, demonstrating a significantly higher cure rate in the rifampicin group (100% vs. 58%,P = 0.02), when combined with ciprofloxacin [64,65]. However, the study has several limitations including small sample size, the significant difference being observed only in the as-treated analysis, and the use of ciprofloxacin monotherapy in the control arm, which is now considered suboptimal for staphylococcal infection [64,65]. Subsequent prospective studies by Ascione et al and Lora-Tamayo et al corroborated the improved outcomes for *Staphylococcus* spp. in PJIs when rifampicin was used in combination with an appropriate surgical strategy[67,68].

Despite accumulating evidence, some studies have questioned the clinical benefit of rifampicin-based regimens. A multi-centre randomized controlled trial by Karlsen et, using 99 patients with PJI after hip and knee arthroplasties, administered with rifampicin in addition to cloxacillin or vancomycin in case of methicillin resistance, has not proven a statistically significant advantage by adding rifampicin to standard antibiotic treatment [69]. Anyway, both this trial by Kerlsen [69] and another study by Pushkin et al [70] which failed to demonstrate a significant advantage, revealed important limitations[69,70]. The study by Pushkin et al. was terminated prematurely after enrolling only 14 patients due to drug-drug interactions between fusidic acid and rifampicin. The study by Karlsen et al. was underpowered and suffered from protocol deviations, unclear surgical strategies, and lack of infectious diseases specialist involvement [69]. Concerns remain, regarding the choice of antimicrobial combination agents for rifampicin[71].

Rifampicin is recommended also for prosthetic valve endocarditis (PVE), despite the lack of supporting data from randomized controlled trials. Retrospective data show that a substantial proportion of patients with rifampicin-susceptible *Staphylococcus* spp. PVE were treated without rifampicin (79/180, 43.9%), and when rifampicin was used, its addition was not associated with improved outcomes[72]. This may be attributed to the limited evidence supporting its efficacy, derived primarily from early studies by Karchmer et al. on *Staphylococcus epidermidis* and Drinković et al., who failed to demonstrate a difference in *S. aureus v*alve sterilization rates[73,74]. Similarly, Shrestha et al. found no reduction in mortality or relapse risk [75]. These findings were reinforced by a recent systematic review and meta-analysis by Ryder et al., which summarized the available data and failed to establish a definitive benefit of rifampicin in PVE treatment[76].

In the context of other implant-related infections, such as prosthetic vascular graft infections (PVGI), cardiac implantable electronic device (CIED) infections, neurosurgical shunt infections, and spinal stimulators, rifampicin use remains largely extrapolated from PJI data [77–79].

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Dose optimization is also a matter of debate. While guidelines differ, the EVRIOS trial showed that 10 mg/kg daily rifampicin gave results like those of 20 mg/kg for treating *Staphylococcus* spp. implant-associated infections, with fewer serious adverse events[80].

Regardless all opposite and contrasting findings on the use of rifampicin-containing combinations, rifampicin remains a critical adjunct in the management of implant-associated infections, due to its ABFA and synergy in combination therapy. Medics should keep in mind that rifampicin efficacy is highly dependent on patient selection, timing of initiation, and appropriateness of the surgical and antimicrobial strategies employed. As previously specified, clinical benefits appear most pronounced in acute implant-related infections with immature BFs, such as PJIs managed with DAIR procedure, where rifampicin is used in combination with an effective antibiotic agent. Conversely, its role in chronic infections with mature BFs and without surgical exchange, as well as in PVE and other implant-associated infections, remains uncertain and is not consistently supported by evidence.

While observational data suggest improved outcomes with rifampicin, conflicting results from randomized controlled trials highlight the need for further well-designed studies to clarify its optimal use. In this context, the ongoing Dutch multi-center clinical trial RiCOTTA may provide valuable insights, as well as other initiatives such as the ROADMAP trial [81,82]. Until more conclusive evidence is available, its use must be tailored to clinical circumstances, ensuring that the benefits outweigh the risks, particularly in the context of resistance development and drug-drug interactions.

3.2. Vancomycin and its Possible Combinations

Vancomycin is often selected as reference antibiotic since usually MRSA and MRSE are susceptible to vancomycin. Staphylococci are considered not resistant to vancomycin when its MICs are 0.5-1.0 μg/mL (commonly found values) or < 2.00 μg/mL (according to EUCAST and CLSI) as reported in Table 4.

Table 4. Vancomycin MIC (μg/mL) values for susceptible *Staphylococci* (recent data).

Species	Typical MIC Range	Susceptibility Interpretation	Refs.
VSSA	0.5–1 μg/mL up to 2 μg/mL	Susceptible if MIC ≤ 2 μg/mL (CLSI/EUCAST)	[83]
MRSA *	≈1 μg/mL baseline MIC	Susceptible if MIC ≤ 2 μg/mL	[84]
CoNS **	MIC < 2 μg/mL up to ≤4 μg/mL	CLSI susceptible breakpoint: MIC ≤ 4 μg/mL	[85,86]

VSSA = vancomycin-susceptible *S. aureus*; * methicillin resistant *S. aureus* susceptible to vancomycin; ** coagulase negative *S. epidermidis* susceptible to vancomycin. .

In fact, a 2024 study of 100 *S. aureus* isolates observed that most clinically susceptible strains have MICs around 1 μg/mL, with the highest values (MIC = 2 μg/mL) associated to the risk of therapeutic failure (h-VISA phenotype), and not to loss of full susceptibility[83]. Also, a 2025 study on *in vitro* resistance selection indicated that MRSA ATCC 43300 strains exhibited baseline MICs for vancomycin typically around 1 μg/mL, prior to any selective pressure[84]. Higher MICs (MIC ≤ 4 μg/mL) can be found and are accepted as index of susceptibility to vancomycin (CLSI) for coagulate negative staphylococci (CoNS), and especially for *S. epidermidis* strains. In this regard, in a large cohort of CoNS bacteremia (2024), 69.3% of isolates had MICs < 2 μg/mL, while approximately 30.7% showed MICs ≥ 2 μg/mL, remaining within the "susceptible" range[85]. Also *S. epidermidis* was the species most frequently associated with MICs of 2 μg/mL, while remaining technically susceptible [86]. In this regard, MICs of B-TPP-QPs against these strains were like or lower than those of vancomycin, thus making possible a direct comparison between their antibiofilm capacity in the following sections. Concerning the molecular mechanisms supporting the ABF properties of vancomycin against *Staphylococci*, it has been reported that, despite rather complex, the ABF mechanism of vancomycin is based on few aspects. It was evidenced that vancomycin binds to peptidoglycan precursors, specifically to dipeptides D-ala-D-ala, in the reachable outer layers of

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young biofilm[87–89], thus preventing wall elongation[90,91] (Figure 3). Anyway, vancomycin, being a relatively large molecule, has difficulty penetrating the BF's polysaccharide matrix, thus functioning only at high concentrations (as observed)[92,93] or when the BF is still young [89,94,95], thus weakening the overall structure, reducing bacterial adhesion to the substrate (catheters, implants) and making the bacteria more vulnerable to other agents, such as gentamicin [96]. Also, smart delivering of vancomycin by proper carriers, such as polymethacrylate polymers (PMMA) can enhance its ABF properties[97,98]. Several studies show that vancomycin is most effective before BF is fully mature, by preventing bacterial division, impeding the production of the extracellular matrix and reducing the bacteria's ability to consolidate on the surface[94,99,100]. Anyway, also for vancomycin, for already developed BF, combinations with other antibiotics or alternative strategies (enzymes, matrix-disrupting agents, etc.) will be necessary[101–103].

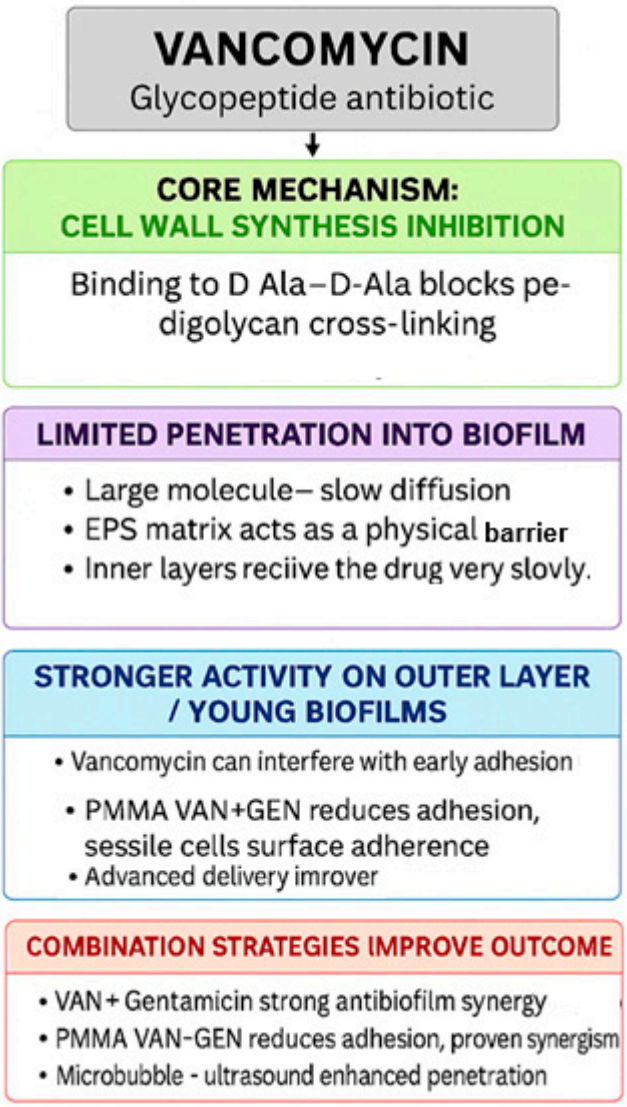

Figure 3. Mechanism and limitation of ABFA of vancomycin.

The combination VAN + gentamicin (GEN) is effective in inhibiting Staphylococcal BF formation on catheters. While vancomycin inhibits cell-wall synthesis in a time-dependent way, gentamicin impairs ribosome 30S in a concentration-dependent mode. MBEC values of vancomycin for *S. aureus* found in a few studies demonstrated a very large range of concentrations (1 >8000μg/mL) depending on BF age, treatment time, kind of medium and especially kind of strains, while MIC for the same strains were very low and in a narrower range (0.5-2 μg/mL)[104]. Borges et al observed that a

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combination of vancomycin 20–40 μg/mL + gentamicin 8 μg/mL was capable of a > 70% reduction in MRSA biofilm on polyurethane. Viability inhibition and complete eradication in MBEC assays in some conditions were observed while monotherapies were modest. Dynamic flow model confirmed superiority of the combination, respect to single antibiotics administrations. MICs for isolates were measured; synergy was observed at concentrations close to clinical lock levels [105]. Calgary and other BF models similarly showed that MBECs for vancomycin alone often far exceeding MICs (10–1000×MICs), underscoring the need for combinations or lock strategies[106–110]. Collectively, vancomycin associated to gentamicin exert a synergistic effect against *S. aureus* biofilms and thus being a clinically relevant combination for antibiotic-lock therapy scenarios [105].

3.3. Gentamicin ± Rifampicin

When rifampicin was discussed in Section 2.1., it was reported that gentamicin is often associated to it. Such combination is used to counteract *in vivo* implants infections due to BF formed by *S. aureus*. Both antibiotics are aminoglycosides, which act by inhibiting ribosomes (gentamicin) and blocking transcription (rifampicin). It was reported that gentamicin alone demonstrated an $MBEC_{100}$ of 256–1024 μg/mL (day 3–14), on *in vivo* implant *S. aureus* BF on steel screws (rat femur), while vancomycin or cefazolin demonstrated significantly higher values of 2048–4096 μg/mL [61]. Adding rifampicin (≈1.5 μg/mL), $MBEC_{60}$ of gentamicin was reduced by 8-fold. Planktonic MICs for MRSA/MSSA were much lower (typical gentamicin MIC 0.25–1 μg/mL), highlighting BF tolerance [61].

Collectively, gentamicin possesses the lowest *in vivo* MBECs among tested agents, and rifampicin improves performance—supporting combination therapy on implants [61].

3.4. Daptomycin

Daptomycin possesses a rapid bactericidal activity and the capability to inhibit Staphylococcal BFs by a calcium-dependent membrane depolarization mechanism. Methodology papers comparing MIC vs MBEC in staphylococci repeatedly found daptomycin among the most active agents in inhibiting BF but still requiring MBECs well above MIC and rarely achieving complete eradication alone [106–110]. In staphylococcal BFs (Calgary model), MBEC ≫ MIC were observed for daptomycin, vancomycin, gentamicin, etc. being daptomycin and rifampicin ranked best among other antibiotics, yet combinations remained advisable [106–110].

3.5. Ciprofloxacin ± Meropenem

This combination demonstrated to inhibit *P. aeruginosa* dynamic BFs, intended as biofilm grown under dynamic conditions, that is, in the presence of liquid movement, shear stress and continuous nutrient exchange. In these systems, BF grows more closely to real conditions (tissues, catheters, tubing) than "static" biofilms in plates (see BF models). While ciprofloxacin acts by inhibiting DNA gyrase/topo IV), meropenem causes PBP inhibition, so that their combination suppress resistance and improve killing bacteria in BF.

BFs by two hypermutable strains of *P. aeruginosa* (PAO ΔmutS, CW44) was grown in dynamic CDC biofilm reactor, a device designed and validated by the CDC (Centers for Disease Control and Prevention) to grow BFs under dynamic and reproducible conditions. At clinically relevant ELF concentrations monotherapy failed, regrowth was observed and MICs shift up to 8 μg/mL for ciprofloxacin and 64 μg/mL for meropenem. Conversely, combination treatments achieved synergistic killing of planktonic and BF populations and suppressed resistance over 120 h outperforming monotherapy[111].

3.6. Tobramycin

Tobramycin kills *P. aeruginosa* BF bacteria in a concentration dependent way based on inhibition of 30S ribosome unit. Quantitative data report that while MICs against different strains of *P.*

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aeruginosa were in the range 0.5-16 μg/mL and typical MICs for susceptible *P. aeruginosa* are ≤1–2 mg/L, depending on essay conditions, medium, BF age, treatment time and strains, MBECs on the same strains spanned in the range 2-2560 μg/mL, and 72-h biofilms required higher concentrations, thus underscoring the frequent 100–1000 × MICs gap to MBEC (https://pdf.benchchem.com/1172/Application_Notes_and_Protocols_Utilizing_Tobramycin_for_Biofilm_Research.pdf, accessed on February, 23 2026, accessed on 01 April 2026) [104]. Tobramycin can be sequestered at the BF periphery, thus limiting its penetration and reducing its ABFA. Penetration and killing potency can be improved *in vitro* adding divalent cations[112]. Synergy strategies with AgNPs or melittin hydrogels enhance killing of established BF cells in a strain-dependent mode[113].

3.7. Colistin

Colistin belong to the polymyxin family and is a cationic cyclic natural polypeptide active against Gram-negative bacteria including planktonic *P. aeruginosa*. Colistin acts disrupting *P. aeruginosa* outer membrane and targeting LPS. Poor penetrating capacity limits its ABF potency and in host-mimicking matrices and *ex vivo* cystic fibrosis (CF) BFs, i.e. BFs of CF pathogens grown on real biological samples taken from patients but studied outside the body (see BF models), much higher doses are needed to achieve killing compared with *in vitro* assays[114]. The combination example of colistin + rifampicin showed active killing against colistin-resistant and -susceptible *P. aeruginosa* biofilms formed dynamically, suggesting that membrane perturbation by colistin could facilitate rifampicin entry [115].

3.8. Azithromycin

Azithromycin (AZM) exerts *P. aeruginosa* BF modulation at sub-MIC concentrations antagonizing QS (las/rhl), reducing virulence factors, and impairing BF formation, with a media- and time-dependent way[116,117]. The effect of AZM on young and mature BFs of different strains of *P. aeruginosa* was investigated in the modified CBD by estimation of the minimal biofilm inhibitory concentration (MBIC). The AZM $MBIC_{90}$ in LB/RPMI1640 on young BFs treated for 24 hours was 16 vs 4 μg/mL for PAO1, 32 vs 8 μg/mL for ΔmutS, and 256 vs 16 μg/mL for ΔnfxB, while in mature BFs was 256 vs 2 μg/mL for PAO1 and ΔmutS and 16 vs1 μg/mL for ΔnfxB. The effect of AZM was improved when the treatment was prolonged to 72 h, supporting the intracellular accumulation of AZM. An increased susceptibility of *P. aeruginosa* BFs to AZM was observed in RPMI 1640 than in LB medium[118]. Also in clinical context, benefits in CF *P. aeruginosa* BF were observed at tissue level at sub-MIC concentrations, thus confirming an anti-QS/anti-virulence modulation mode. Anyway, still, azithromycin is not reliably bactericidal against established BF [116,117].

3.9. Ceftazidime-Avibactam (CZA)

Combination ceftadizime-avibactam (CZA), also known as CAZAVI, consists of a β-lactam antibiotic + a di-azabicyclo-octane β-lactamase inhibitor[9]. It inhibits BF by *P. aeruginosa* by improving activity against AmpC/extended spectrum β-lactamase (ESBL) producers (not metallo β-lactamase). Evidence relevant to MIC showed that CZA restored ceftazidime susceptibility (MICs ≤ 4 μg/mL) across diverse clinical isogenic mutants isolates of *P. aeruginosa* (91% overall), with developed combined key β-lactam resistance mechanisms[119]. Anyway, despite these findings support CZA inclusion in combination regimens, MBEC datasets are limited. Pairing ceftazidime with cellulase reduced MBEC to 32–128×MICs respect to higher factors observed for ceftazidime alone for *P. aeruginosa* BFs, illustrating a matrix-disruption + β-lactam strategy.

3.10. Possible Reported Antibiotics Combinations

It was established that combinations using rifamycin-based regimens, such as CST+RIF or with add of CIP+MEM, as well as enzymes/peptides for *Pseudomonas* BF outperform monotherapy [61,103,111,113,120]. Also, host-mimicking conditions (e.g., CF sputum media, epithelial lining fluid

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simulations) are more predictive and often raise MBEC vs standard media, as well as expose penetration limits [114]. Unfortunately, standardization remains limited. It is important, before comparing results across papers, making a verification of endpoint definitions (MBIC vs MBEC), growth substrate, and quantification methods is mandatory [121]. However, about *Staphylococci*, rifabutin vs rifampicin MBECs (clinical PJI isolates), consensus/Q&A on MBEC and relative performance of agents (rifampicin/daptomycin), VAN+GEN synergy in MRSA/MSSA polyurethane models, *in vivo* implant MBEC studies (gentamicin ± rifampicin) can be found in literature[59,61,105,122]. Similarly for *Pseudomonas*, CIP+MER in dynamic CBR (ELF-level exposures), tobramycin MBEC ranges and effect of BF age/media, tobramycin penetration limits, colistin penetration & CST+RIF synergy, azithromycin QS antagonism and improved MBICs in RPMI; CZA MIC restoration as well as cellulase-ceftazidime MBEC reduction, have been reported [104,111,114–120].

4. Are Intimate Molecular Mechanistic Insight Essential for Papers on Biofilm (BF) Worthy of Publication?

There are several unsolved challenges needing solutions, which limit more effective studies on BF, their rational designing, and which impede more rapid clinical translation of laboratory results. MBEC/MIC disparities of 10–1000 × and pitfalls needing for standardization (resazurin MBIC conditions), especially for Gram-positives strains [106,121,123] make difficult clinical translation of laboratory therapies. Additionally, the difficult interpretation of BF data, and their difference depending on BF age, exposure time, and flow vs static, alter outcomes materially [104,106]. Continuing the search for new antimicrobial agents with antibiofilm activity (ABFA), several studies have been developed, where structurally different new prepared compounds have been investigated to assess their ABF effects and to indagate also the molecular mechanisms, by which they exert such capacity. Valuable opinions of several experts in BF field underscore the necessity of molecular mechanism investigations for making a new study on BF eradication creditable for publication. On the other hand, several are the published studies found, especially in the last years, which investigated and discussed only the ABF capacity of new molecules, without disclosing any possible intimate mechanism supporting their activity. Which could be the major reasons?? This review was borne also to find rational answer/s to this question.

4.1. The Most Important Mechanism to Inhibit/Eradicate BF

4.1.1. Target/Impairment of QS and Virulence Pathways

Many active compounds disrupt BF formation by interfering with QS systems or suppressing the transcription of genes essential for BF maturation. Allicin, a sulphur compound from *Allium sativum*, inhibits early *P. aeruginosa* adhesion by suppressing extracellular polymeric substances (EPS), which are a complex mixture of biological polymers—mainly polysaccharides, proteins, lipids, and extracellular DNA (*e*DNA)—that microorganisms secrete to form the structural "matrix" of a biofilm. EPS synthesis in turn regulates and reduces the expression of QS-virulence determinants such as rhamnolipids and pyocyanin [124]. Similarly, ajoene, another garlic-derived molecule, blocks both AHL-dependent and secondary signalling pathways, by downregulating small regulatory RNAs (*rsmY, rsmZ*) in *P. aeruginosa* and RNAIII in *S. aureus*, resulting in strong antibiofilm (ABF) and anti-virulence (AV) effects [125].

Other plant metabolites, including carvacrol, inhibit the *lasI*/*lasR* system and prevent AHL synthesis, reducing *P. aeruginosa* surface colonization and disrupting communication required for BF maturation [126]

Flavonoids such as naringenin and quercetin also directly bind *LasR*, acting as non-AHL competitive inhibitors that attenuate downstream virulence and BF formation in Gram– pathogens including *P. aeruginosa*, *K. pneumoniae*, and *Yersinia enterocolitica* [127].

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Collectively, QS-disrupting molecules show strong potential in hindering initial adherence, microcolony formation, and the expression of pathogenic traits, positioning them as leading antibiofilm candidates.

4.1.2. Suppression of EPS Synthesis and Destabilization the BF Matrix

EPS matrix is a major barrier to antibiotics penetration. Several compounds act by degrading, destabilizing, or preventing EPS formation. Emodin, an anthraquinone from *Polygonum* and *Rheum* species, significantly decreases *e*DNA release and downregulates genes central to *S. aureus* matrix formation such as *cidA*, *icaA*, *agrA*, *sarA*, and *sortaseA* [128,138].

Aloe-emodin inhibits PIA production and reduces extracellular proteins, impairing BFs development on abiotic surfaces [129].

Hordenine inhibits alginate, rhamnolipids, and pyoverdine production via suppression of *lasI*, *lasR*, *rhlI*, and *rhlR*, substantially reducing *P. aeruginosa* BF integrity [130]

Crucially, enzymatic degradation strategies have shown strong efficacy: EPS-degrading enzyme cocktails (DNases, proteases, glycosidases) successfully eradicate established biofilms across species by physically dismantling matrix architecture [131].

4.1.3. Inhibition of Microbial Adhesion and Motility

Interference with early adhesion is another validated strategy to inhibit BF. Extracts from *Musa acuminata* inhibit *P. aeruginosa* adhesion and suppress early BF development, while syringopicroside, tested against *Streptococcus suis*, prevented cell–surface attachment [132]. Also, compounds such as zingerone disrupt motility phenotypes (swarming, twitching), directly impairing the physical behaviours necessary for microcolony formation in *P. aeruginosa* [133]

4.2. Experimental Needs and Advanced Technologies for Studying the Molecular Mechanisms of Biofilms (BFs)

Advanced studies of the mechanisms of BF formation and inhibition/disruption require a combination of molecular biology, biochemistry, omics analysis, and high-resolution imaging techniques[134]. Recent sources highlight how such investigations, if correctly carried out, has gained critical importance, offering insights into bacterial and microorganism communication, when in stressed conditions[135,136]. QS synchronizes collective bacterial behaviours across diverse chemical signals and target genes. It is regulated by autoinducers, which could be selectively targeted, thus offering innovative approaches to regulating QS, thus emphasizing the potential of quorum quenching and QS inhibitors to mitigate bacterial pathogenicity[135,136]. These ABF strategies have shown promise in aquaculture and plant resistance, disrupting QS pathways to combat infections[135]. QS also provides opportunities for developing biosensors for early disease detection and preventing BF formation, which is critical to overcoming antimicrobial resistance[135]. Collectively, indagating QS represent a mandatory step to indagate possible mechanism of BF inhibition/eradication of new compounds. Unfortunately, QS investigations could require expensive instrumentation, highly equipped laboratories, and highly specialized personnel. Recent research emphasizes that characterizing QS systems requires high-resolution mass spectrometry (LC-MS/MS, GC-MS) techniques, to identify and quantify autoinducers (AIs), AHLs, AI-2, and signal peptides[137]. To study the global regulation of gene expression mediated by QS, and to analyse the effect of inhibitors on the transcription of genes involved in virulence, EPS, and bacterial adhesion, RNA-seq, microarray, or qRT-PCR are necessary [138]. Additionally, such analyses are necessary to understand how QS interference modifies molecular pathways [138]. Luminescence bioassays (for *Vibrio* spp.), fluorescent reporters, enzymatic assays, and CRISPR/Cas gene editing are fundamental to evaluate the response of QS systems and to study autoinducers and autoinduction mechanisms [139]. LC-MS/MS systems can cost €200,000–€500,000, in addition to specialized reagents. RNA-seq requires advanced bioinformatics, sequencers, and dedicated software. Reporter-based assays

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require controlled incubators, fluorescence/luminescence readers, and specialized microbiological infrastructure. For the study of LPS (lipopolysaccharide) biosynthesis and modification, additional advanced instrumentation would be necessary. The characterization of LPS and its structural modifications requires advanced analytical chemistry and genetic techniques, including gel electrophoresis with silver staining, which is an advanced chromatography, and spectrometry for *core* oligosaccharide and *O*-antigen analysis[140]. As shown by a study on *Vibrio fischeri*, these techniques are crucial for linking mutations to BF phenotypic changes [140]. Whole-genome sequencing (WGS) is necessary to identify mutations in LPS biosynthesis genes and is used to detect the VF_0133 mutation associated with a BF-like phenotype [140]. Advanced microscopy techniques, such as confocal microscopy, scanning electron microscopy (SEM) are needed to examine the morphology of altered BF communities. In this regard, WGS requires sequencers costing €100,000–€250,000 plus software and bioinformatics personnel. Instrumentation for LPS and silver staining analysis requires advanced biochemistry laboratories and specialized reagents. The analysis of BF matrix, which is a complex multi-component extracellular material, consisting of DNA, RNA, proteins, lipids, saccharides and EPS, requires integrated multi-omics techniques (proteomics, metabolomics, genomics), to identify matrix components and the molecular pathways involved, and are essential for studies on the role of QS in global BF regulation[137]. Nuclease enzymes and degradation assays are necessary to determine the role of extracellular DNA (*e*DNA), while confocal laser scanning microscopy (CLEM) is needed to acquire 3D images of the BF and analyse the distribution of *e*DNA and EPS. Notably, proteomic/metabolomic platforms cost > €300,000, while confocal microscopy often exceeds €150,000–€300,000. For the study of adhesion factors and sessile phenotypic transformation are necessary other expansive instrumentation. Specifically, bacteria adhesion and the transition of cells from the planktonic (motile) to the sessile state, require precise analysis of phenotypic changes, using crystal violet microtiter assays and time-lapse imaging, also used in studies of mutants with BF-like phenotypes[140]. Transcriptomic analyses are necessary to assess the regulation of adhesion and EPS genes under QS or in the presence of inhibitors [138], while site-specific mutagenesis investigations are necessary to decipher the role of single genes in phenotypic transition (e.g., mutations in sypG-independent regulators) [140]. Mutagenesis and controlled culture platforms require fully equipped BSL2 laboratories, while advanced imaging assays require high-resolution microscopes and dedicated software. Collectively, molecular analysis of BF inhibitors acting on QS, LPS, EPS, *e*DNA, and adhesion factors, requires a suite of interdisciplinary techniques that include genomics, spectrometry, advanced biochemistry, and high-resolution imaging, available only in highly equipped and often very expensive laboratories. This is confirmed by recent studies on QS and gene expression regulated by molecular signals, which required MS, RNA-*seq* [137,138], on the analysis of LPS and mutations associated with BF-like phenotypes, which require WGS and spectrometry [140] and on studies on BF phenotypic regulation, requiring advanced imaging tools and complex structural assays [139]. Following, Table 5 collects the analytical techniques and instruments necessary to investigate molecular mechanisms of BF inhibition with a qualitative information concerning the associated collective costs.

Table 5. Techniques for studying the molecular mechanisms of biofilm (BF) inhibition (QS, LPS, EPS/eDNA, adhesion) and associated collective/qualitative costs.

Scope/Technique	Technologies/Instruments Required	Applications in the Study of BF Inhibition	Costs *	Refs.
Detection and quantification of QS AI	LC MS/MS, GC MS for AHL/AI 2/signal peptides, TSP	Direct measurement of QS modulation by INH, definition of DR, KP	High	[137,138,141]

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QS reporter systems (L/F)	Reporter strains (lux, GFP/RFP), L/F, controlled incubators	Screening of QS INHs, RR of A/I of signaling pathways	Medium	[141,142]
Transcriptomics (RNA seq/qRT)	PCR, RNA extraction, libraries, sequencer/thermal cycler, BIs	Evaluation of INHs effects on EPS, V/AD and QS pathway genes	High	[137,138]
LPS analysis (core/O antigen) gel	Ag staining, chromatography, MS for LPS profiles	Correlation LPS changes/BF phenotypes, response to INHs/mutations	High	[140]
Genomics (WGS, short/long reads) & TGM	WGS, variant analysis; mutagenesis (point substitutions)	Identify TG/P (QS, LPS, AD), link mutations to BF-like phenotypes	High	[140]
Confocal/SEM microscopy of BFs	LSC, SEM; specific staining (*e* DNA/EPS))	3D BF architecture; EPS, *e*DNA distribution, response to anti-QS treats	High	[137]
Adhesion/BF formation assays (CV)	Microtiter assays, absorbance reader; optional time-lapse imaging	BF biomass quant., comparison of WT, mutant, INH-treated cells	Low	[140]
Matrix proteomics/metabolomics	LC MS/MS HDI; protein/EPS/metabolite preparation	Identification of KMCs and INH target pathways (QS-dependent)	High	[137]
Screening of anti-QS phytocompounds	Extracts/purification, QS reporter assays, LC-MS (MI)	Candidates' selection for modulating QS, reducing BF Fo/V	Medium	[138,143]
Multi-species QS/BF functional studies	Controlled cultures, inter/intraspecific signal analysis, DA	Evaluation of the efficacy of QS inhibitors in MC **	Medium	[141,143]

* Indicative cost category (Low/Medium/High) based on technical complexity and need for specialized equipment and skills; **evaluate influence of QS inhibitors on BF dispersal; QS = quorum sensing; CV = crystal violet; L = luminescence; F = fluorescence; PCR = polymerase chain reaction; Bis = bioinformatics; Ag = silver; WGS = whole genome sequencing; TGM = target mutagenesis; AI = autoinducers; LSC = laser scanning confocal; HDI = high definition images; MCs = Mixed communities; Fo/V = formation/virulence; INH = inhibitor; DR = dose response; KP = kinetic profile; MI = molecular identification; DA = dispersal essay; TSP = target samples preparation; A/INH = activation/inhibition; RR = rapid readout; AD = adhesion; TG = target genes; P = proteins; WT = wild type; KMCs = key matrix components.

Summarizing Table 5 contents, experiments aimed at clarifying the molecular mechanisms of biofilm (BF) inhibition were mainly carried out by integrating QS systems biology, BF matrix analysis (EPS/eDNA), LPS biosynthesis, and adhesive/sessile phenotypes of bacteria. To this end, advanced analytical platforms and interdisciplinary expertise were required[137,138,140–144]. Candidate inhibitors of QS system were initially evaluated using reporter systems (lux/GFP) in model strains (*Vibrio*, *Staphylococcus*), with luminescence/fluorescence readings and dose-response curves. Active compounds were confirmed with LC MS/MS for the quantification of AHL/AI 2/signal peptides and with qRT PCR/RNA seq for the effect on the expression of QS-regulated genes (virulence, EPS, adhesion). LPS structural modifications were analysed with electrophoresis and silver staining, chromatography, and spectrometry, while WGS and reverse genetics assays associated variants in glycosyltransferase genes with variations in BF formation. Biomass quantity was measured with a crystal violet assay; 3D architecture, EPS and eDNA distribution, and response to inhibitors were examined with confocal microscopy/SEM and selective staining; where necessary,

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proteomics/metabolomics identified key components and target pathways. Surface adhesion assays and time-lapse imaging were combined with transcriptomics to map the regulatory networks that drive phenotypic transformation under QS control and/or inhibited by compounds of interest. Each experiment included negative/positive controls (e.g., QS-deficient strains or known inhibitors), at least three biological replicates, and two orthogonal confirmatory techniques (reporter + MS; WGS + phenotype). Data analysis included normalization, correction for multiple comparisons, and verification of reproducibility across platforms. The integration of sequencers, high-resolution mass spectrometry, and advanced microscopy requires the use of highly equipped core facilities and laboratories (BSL 2), with high costs for instrumentation, maintenance, and specialized personnel, as reflected in recent methods and reviews on QS and antibiofilm (ABF) strategies.

4.3. Practical Step-by-Step Minimal Experimental Workflow to Obtain Mechanistic Insight for BF Inhibition/Disruption

Step 1 should involve morphological and structural investigation on BF treated with new molecules by Raman, FTIR, SEM, CLSM, or AFM analyses, which are able to show possible BF architecture collapse, cell clustering changes, EPS shrinkage surface disruption [145]. Anyway, alone, these inspections do not provide molecular mechanisms. This limitation is emphasized in BF morphology studies [145]. An essential step 2 should involve the investigation of a possible ROS dependence of BF inhibition/eradication by carrying out ROS scavenger rescue experiments, using catalase, SOD and mannitol[146]. If scavengers reverse the ABF effect, causality can be claimed[146]. Step 3 should evaluate molecular markers of stress pathways causing the inhibition/dispersal of BF, by investigating genetic responses modulating QS, EPS synthesis, oxidative stress (OS), BF dispersal. Mechanistic reviews emphasize the necessity of molecular-level analysis of up- or down-regulation of key genes, including *katA*, *sodA*, *oxyR* and *soxRS* for OS, *lasR*, *rhlR*, *agr*, *icaA*, *bssS* and *pel*, *psl* for BF regulation, *algD*, *gtfB* and *gtfC* for EPS synthesis, as well as *bdlA* and *rbdA* genes, for BF dispersal, by qPCR or RT-PCR analyses [147–149]. EPS composition analysis, essential for investigating the molecular mechanisms of ABF molecules, should be step 4 [150]. Experiments such as EPS biochemical assays which measure changes in polysaccharides (e.g., Congo Red/phenol-sulfuric method), proteins (Bradford/BCA), extracellular DNA (PicoGreen) should be carried out [150]. Step 5 should involve the investigation of cell damage pathways by membrane integrity assays, by PI/Syto9 live–dead staining, lipid peroxidation assay (MDA measurement)[151,152], DNA damage assays (Comet assay, quantification of 8-oxo-dG)[153,154], protein oxidation markers (OxyBlot or carbonyl content assays). In step 6, the BF dispersal mechanism testing should be performed, investigating matrix degradation, signalling changes, nutrient shifts, ROS-induced detachment by measuring planktonic cell release after treatment and the impact on known dispersal genes (e.g., *bdlA*) [155,156]. By linking together structural, molecular, and functional evidence is possible to deduce the mechanism by which a new molecule under investigation can inhibit/disperse BF. The following Figure 4A and 4B shows two graphical versions of pipelines for investigations needed to establish the molecular mechanisms supporting the inhibition/eradication effects of a molecule under testing.

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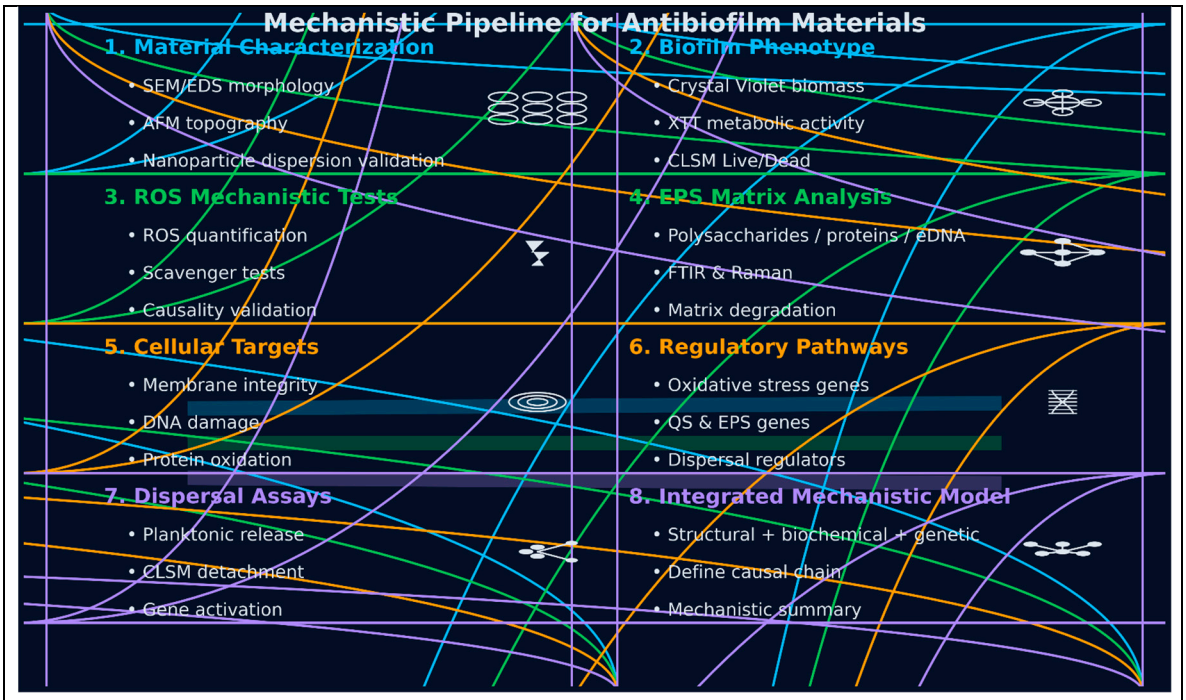
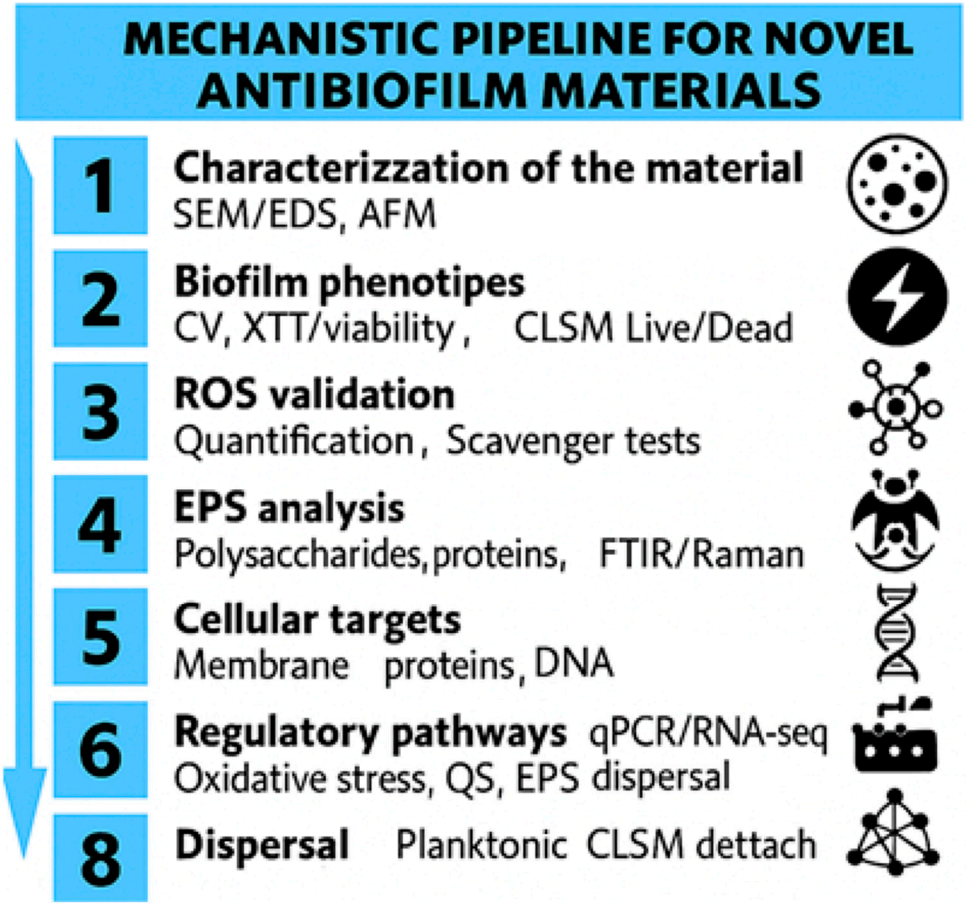


(A)



(B)

Figure 4. Two graphical versions (A, B) of eight-steps pipelines summarizing investigations needed to establish the molecular mechanisms supporting the inhibition/eradication effects of a molecule under testing.

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5. Mechanism Versus Not Mechanism Containing Published Papers

5.1. Relevant Published Studies on Biofilm (BF) Inhibition/Eradication Not Reporting Molecular Mechanisms Are Recent

Just being random research in Scopus and PubMed, without imposing a range time and crossing the keywords biofilm inhibition, biofilm eradications, no mechanisms, no mechanistic insights, we found several interesting contributes of the wanted type, in the last five years. Duda-Madei et al prepared sulphur derivatives of camphor and tested them to detect their antimicrobial and antibiofilm (ABF) potentials against eight strains of Gram-positive species including *S. aureus*, *S. epidermidis* and *E. faecalis* and eight strains of Gram-negative ones, including *E. coli*, *A. baumannii* and *P. aeruginosa* isolates[19]. Although the definition of MIC_{50} and MIC_{90} given by authors is not correct in microbiology, they were clear in notifying their intents. They measured these values intending them as the concentration of new compounds needed to inhibit 50 and 90% of bacteria of a considered isolate, as for IC_{50} values. In this context, compounds *rac*-thiocamphor (**1a**) and (S, S)-(+)-thiocamphor (**2a**), demonstrated very high MIC_{90} against *S. aureus* (128, 256 up to > 512 μg/mL), *S. epidermidis* (64, 256 up to > 512 μg/mL) and *E. faecalis* (128 up to > 512 μg/mL), and both compounds were incapable to inhibit BF by MRSA and *E. faecalis* at concentration tested (1/2 and 1/4 MIC_{90} (**1a**), 1/8 and 1/4 MIC_{90} (**2a**), vs MRSA, while 1/2 MIC and MIC of **1a** and **2a**, vs *E. faecalis* [19]. Compound **1a** exhibited the highest inhibition effect (22%) on the BF formation over 24 hours by *S. epidermidis* 275lp, at 1/16 MIC_{90}. **2a** demonstrated the highest inhibitions of 50% and 22% of BF produced by *S. aureus* ATCC 25923 and *S. epidermidis* S22 at MIC_{90} and ½ MIC_{90}, respectively. Both compounds **1** and **2a** demonstrated no antibacterial activity against all tested Gram-negative strains (MIC ≥ 512 μg/mL)[19]. Interesting experiments conducted with the use of the Bioflux system to assess **1a** and **2a** capacity to exhibit antimicrobial activity in the flow conditions (in contrast to stationary conditions) given negative results [19]. Also, *bis*-quaternary ammonium salts (*b*-QASs) **1**-**6**, based on natural (3,5-dibromo-4-hydroxy-phenyl)-acetic acid derivatives were synthesized, to assess *in vitro* their antibacterial (MICs = 25.0 to 200.0 μg/mL) and ABF activity against *S. aureus* and *P. aeruginosa*, including antibiotic-resistant strains at 100.0 and 200 μg/mL[21]. At 100.0 μg/mL concentration, the best antibacterials *b*-QASs **1**, **5** and **6** inhibited the BF formation by *S. aureus* ATCC25923 susceptible to cefotazime by 95%, while by about 65% and 95%, respectively at 4 × MIC and 2 × MIC, while they did not inhibit BF by MDR *S. aureus* strain. All three compounds inhibited the BF by MDR *P. aeruginosa* and PA01, resistant to both cefotazime and cefotriazone by 95% (MDR *P. aeruginosa*, **1**, 4 × MIC) and ≥ 90% (MDR *P. aeruginosa*, **5**, 4 × MIC and **6**, 2 × MIC and by > 95% (PA01, **1**, MIC, **5** and **6**, 2 × MIC)[21]. At the tested concentration of 100.0 μg/mL, only *b*-QASs **1** and **6** succeeded in inhibiting BF of *E. coli* ATCC 25922 and MDR *E. coli*, both resistant to cefotazime and cefotriazone, by 95% (ATCC, **6**, 4 × MIC) and by > 95% (ATCC, **1**, MIC). Inhibitions by 58 and 60% were observed when compounds were administered at ½ MIC (**1**) and MIC (**6**). At the doubled concentration of 200 μg/mL both **1**, **5** and **6** reduced the BF of all bacteria tested (except for that produced by MDR *S. aureus*) by > 95%, like reference antibiotics cefotazime and cefotriazone, having anyway significantly lower MICs[21]. Antimicrobial peptides (AMPs) have attracted great attention as a potent alternative to traditional antibiotics[157–159], as they exhibit broad-spectrum antimicrobial activity and are less affected by mechanisms underlying bacteria antibiotic resistance [160]. In fact, although extensive resistance to many classes of conventional antibiotics has emerged, the highly conserved and essential nature of the bacterial membrane on which AMPs acting by a-specific membrane disruptors causing cells death, would indicate a decreased potential for bacteria to be resistant to AMPs[161]. On the other hand, the susceptibility of AMPs to proteases and the emergence of bacterial strains resistant also to natural AMPs, such as polymyxins [162,163], and their too sharp cytotoxic profile, restricts their efficient pharmaceutical applications. To combat this challenge, Akhash et al synthesized a novel analogue peptide (mKLK) based on a D-form amidated of sapecin B-derived peptide (KLK), by replacing two lysine residues with two tryptophan and one leucine by lysine and inserting one alanine[161]. mKLK retained the original antibacterial activity potency of KLK, while a remarkable inhibitory activity

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against MRSA and MSSA BFs was seen in the *in vivo* murine model of catheter-associated BF infections [161]. Authors demonstrated that mKLK peptide outperformed original KLK in inhibiting their BF formation at sub-MIC concentrations. Despite molecular mechanism by which mKLK inhibited and eradicated mature BF of MRSA and MSSA bacteria was not investigated, the strong potential therapeutic advantage of using mKLK peptide for preventing BF-associated MRSA and MSSA infections was established, which is pivotal in BF research [161]. Also, quaternary and not quaternised ammonium salts (QASs and ASs) are among the most effective antimicrobial and in several case biocidal agents, that have been used for more than a century and today are extensively tested as ABF compounds [164–170]. However, due to the growing trend of bacterial resistance emergence and the residual high toxicity of synthesized QASs, research in this field remains under evaluated [171]. Recent studies have suggested that the introduction of the amide functional group into QASs structures can lead to soft variants that, while retaining the original antimicrobial properties, demonstrated a tuneable cytotoxicity[171]. In this regard, 3-amidoquinuclidine QAS derivatives developed by Odžak et al showed less cytotoxicity than commercially available QASs[171]. Anyway, by a careful consideration of reported results, only in sporadic cases, these new compounds exhibited MICs comparable or lower than those observed for commercial parents, namely cetyl pyridinium chloride (CPC) and benzyl dimethyl dodecyl ammonium bromide (BAB), against both Gram-positive and Gram-negative species. Anyway, some of these new compounds exhibited excellent potential to suppress BF formation (almost 100%) by *S. aureus* ATCC 25923 at 12.5 μg/mL, outperforming commercial compounds CPC (< 50%) and BAB (about 75%), taken as reference QASs, at same concentration [171]. Additionally, developed compounds showed low tendency to induce bacterial resistance[171]. Despite authors investigated several other aspects regarding their QASs, either mechanism of BF inhibition was not investigated, nor references antibiotics were used. Nonetheless, 3-amidoquinuclidine QASs could be considered as novel antimicrobial agents that pose a low threat to ecosystems and human health[171]. More recently, Godoy et al published a further work on inhibition of BF produced by *S. aureus* strains isolated from milk samples obtained from dairy areas in southern Chile from cows diagnosed with bovine mastitis (BM)[172]. Specifically, they synthesized chitosan nanoparticles (CNPs) and investigated their antibacterial potency by determining MICs (128 μg/mL), and MBCs (250 μg/mL) against different strains of *S. aureus* susceptible to both ampicillin and gentamicin. They also compared the inhibition zone diameter caused by CNPs with those caused by these two antibiotics, with promising results. Upon establishment that *S. aureus* M15 was the strongest BF producer of their collection, CNPs capability to inhibit its BF production was investigated at MIC, ½ MIC and 1/3 MIC revealing a 100%, > 50% and > 20% BF inhibition respectively[172]. Anyway, as for the previous studies above-mentioned, any molecular mechanism supporting these findings was investigated[172]. Authors limited to hypothesize that all CNPs effects depended on their membrane detrimental and disruptive activity, in turn depending on their high Z-potential (positive surface charge) of +55.4 ± 2.5 mV [172]. This work unequivocally remains a study of paramount importance, regardless the absence of molecular mechanism investigations. In fact, despite it has established a questionable ability of CNPs to inhibit planktonic bacterial proliferation, has evidenced that they can efficiently modulate BF formation by *S. aureus* sessile strains, depending on CNPs concentration. In this perspective, CNPs represent a possible alternative for the control, prevention, and/or treatment of clinically relevant BM pathogens, which affect dairy cattle, posing a significant challenge to the dairy industry, mainly due to the increasing antibiotic resistance. Apostol et al reported on the antimicrobial activity assessment of 49 compounds previously synthesized as derivatives of alanine or phenylalanine that incorporate a 4-(4-X-phenylsulfonyl)phenyl fragment (X = H, Cl, or Br), including 21 acyclic compounds (6 × *N*-acyl-*α*-amino acids, 1 × *N*-acyl-*α*-amino acid ester, and 14 × *N*-acyl-*α*-amino ketones) and 28 pentatomic heterocycles from the oxazole-based compound class (6 × 4*H*-1,3-oxazol-5-ones, 16 × 5-aryl-1,3-oxazoles, and 6 × ethyl 1,3-oxazol-5-yl carbonates)[23]. Both in silico and *in vitro* qualitative and quantitative assays were used to investigate the antimicrobial potential of these derivatives against planktonic and BF-embedded S. *epidermidis* 756, *B. subtilis* ATCC 6683, *E. coli* ATCC 25922 and *P.*

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aeruginosa ATCC 27853 susceptible to ciprofloxacin (MICs = 0.0012-0.15µg/mL). Some of the tested compounds showed promising MICs of 14, 28.1 and 56.2 µg/mL or higher MICs up to > 225 µg/mL depending on the type of strains. Their ABFA was first evaluated by assessing their minimal biofilm inhibitory concentration (MBIC) which was equal to 8 × MIC, 2 × MIC, MIC, 1/16 × MIC, 1/8 × MIC, ¼ × MIC and ½ × MIC, depending on their chemical scaffold and lipophilic character[23].

A further study where inhibition of BF and its eradication were investigated without studying the related molecular mechanisms, was that by Kart et al[24]. Authors loaded zinc oxide (ZnO), zinc oxide borax (ZnOBorax), zinc copper oxide ($ZnCuO_2$) NPs and borax ($Na_2B_4O_7 \times 10H_2O$), into bacterial cellulose (BC) obtaining BC-NPs [24]. BC-NPs were evaluated to assess their BF inhibition and degradation effects via the CV assay method using *Listeria innocua* ATCC 33090, *S. aureus* ATCC 29213, *P. aeruginosa* PAO1, *E. coli* ATCC 10536, *Streptococcus parasanguinis* ATCC 15909, *Bacillus cereus* RSKK 863, *Enterococcus hirae* ATCC 10541 and *C. albicans* ATCC 64548, as human pathogens [24]. Rather that the lack of mechanisms studies, the limitation of this study is represented by the difficulty in interpreting the effects of BC-NPs on BF. Their minimal concentrations needed to inhibit planktonic cells (which should be MICs) were not reported, thus rendering impossible to understand what fraction or multiple of such amount correspond to the concentrations needed to inhibit/degrade BF by NPs sheets [24]. In addition, bars of control are not visible in Figures 2 and 3 in the paper [24]. Anyway, according to data reported for BF inhibition (%) caused by the mysterious MIC related concentrations of NPs, it was evidenced that for BF produced by *S. aureus* ATCC 29213 (CV method) was 65.53% (BC–ZnONPs), 71.74% (BC–Borax), 66.60% (BC–ZnOBorax), 28.27% (BC–$ZnOCuO_2$). *E. coli* ATCC 10536 BF (CV) was inhibited by 38.44% (BC–ZnONPs) and 39.97% (BC–Borax), while that by *L. innocua* ATCC 33090 by 44,79% (BC–ZnONPs), 54,86% (BC–Borax) 65,14% (BC–$ZnCuO_2$). Concerning BF degradation, at 24 h (CV), BC–Borax degraded 58.98% of mature biofilm by *P. aeruginosa* PAO1, 62,40% of that by *L. innocua* and 54.26% of that by *S. parasanguinis*, while BC–ZnOBorax degraded 64.02% of BF by *S. aureus* and 52.93% of BF by *L. innocua* [24]. Collectively, BC–NPs effectively interfere with BF (to varying degrees depending on the strain and type of NPs) but provide no information on their ability to inhibit free-living bacterial growth. Readers do not know which sheets (if any) have actual planktonic antibacterial activity, nor whether the ABFA depends on direct toxicity, interference with adhesion, release of ions, structural modifications to the BF matrix.

Last year, Dutta et al. published a paper where BF inhibition and its eradication capacity, as well as cytotoxicity to human keratinocytes were investigated for new nanomaterials[22]. Concerning the assessment of possible molecular mechanism supporting the ABFA of new nanomaterials (NMs), only morphological appearance (SEM, confocal) of BF and dosage of some BF constituents after treatment have been investigated, which are not sufficient to claim mechanistic insight for the new ABF NMs. These observations may suggest possible modes of action, but they do not constitute a molecular mechanism on their own. Morphological BF imaging alone is descriptive, not mechanistic, while dosing BF constituent is a functional readout, not a mechanism (see previous Section 4). Anyway, in this study respect to other previously cited, the quantification of ROS hyperproduction in BF bacteria after BF inhibition associated to the verification of ABF effects reversal upon catalase administration, may constitute a molecular mechanism, establishing that NMs have a ROS-mediated ABFA [22]. Anyway, no investigation regarding the effects of NMs on QS and other genes sustaining BF inhibition/maturation were indagated. Genes involved in cells adhesion, QS, biofilm formation, maturation and dispersal were not investigated.

This do not invalidate the relevance of this study, where authors focused on biogenically synthesizing bacteriocin-capped silver nanoparticles (bac-AgNPs) and on determining their antibacterial, bactericidal and ABFA against two nosocomial severe disease-causing bacteria[22]. Specifically, *P. aeruginosa* ATCC 10145 and *S. aureus* ATCC 23235, were evaluated and results indicated that MBCs of bac-AgNPs was 0.017 µg/mL[22]. BF by *S. aureus* was reduced by the 81.8%, while that by *P. aeruginosa* by the 78.4% at a concentration of 1.7 µg/mL (100 × MBC) demonstrating significantly higher ABF potency than that of tetracycline at 1 mg/mL[22]. SEM, FTIR and confocal analyses showed that bac-AgNPs disrupted the EPS matrix within the bacterial BF. Other analysis

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confirmed that bac-AgNPs reduced the BF mass and the amount of some BF constituents, as well as induced ROS generation and death of bacterial cells. Overall, bac-AgNPs were found as non-cytotoxic against human HaCaT cell line, at microbiologically active concentrations, approving the candidacy of these particles as an effective therapeutic strategy to treat BF-associated nosocomial infections[22].

Another case of study on new ABF NMs reporting any kind of investigation on their molecular mechanisms of action was published just this year, by Ali et al [20]. The authors presented an eco-friendly approach to optimizing the extraction of bioactive compounds from false turkey-tail (*Stereum ostrea*) mushroom biomass and their application in the green synthesis of free (*f*-CuNPs) and immobilized (*im*-CuNPs) Cu (II) nanocomposites (NCs) for potential ABF applications[20]. The ABFA of optimized water extract of *f*-CuNPs and *im*-CuNPs was assessed *in vitro* at 5 mg/mL (5000 µg/mL), without first ascertaining their MICs, using CV broth assays against BF-by *S. aureus* and *E. coli* [20]. Gentamicin, selected as reference antibiotic, having MIC of 0.25-1.00 and 0.5-2.00 µg/mL [173,174] against susceptible *S. aureus* and *E. coli* respectively, was tested at 1 µg/mL, a concentration 5000-fold lower than that of NMs (4 × MIC or MIC on *S. aureus*, 2 × MIC and 1/2 × MIC against *E. coli*) [20]. Mediocre results and low inhibition, even lower than that of simple extracts (71% and 37% for *S. aureus* and *E. coli* respectively) and of copper NPs previously reported, were observed for both free and immobilized CuNPs. They inhibited the BF by *S. aureus* and *E. coli* by 23 and 30%, respectively, as well as by 42% and 14%, indicating a higher susceptibility of *S. aureus* to *im*-CuNPs, while *E. coli* was more susceptible to free ones[20]. The higher inhibition was that exerted by gentamicin (71 and 84% BF inhibition against sessile *S. aureus* and *E. coli*), despite administered at a concentration 5000-fold lower than NPs. In addition to the low impact that developed CuNPs could have in fighting BF, other limitations consist in the absence of MICs determinations and the experimental evidence of the biodegradability and not cytotoxicity of mushroom-derived NPs, claimed without foundations[20]. Anyway, despite not scientifically robust, the integrated methodology proposed by authors demonstrates to be a sustainable alternative to conventional chemical syntheses in biomedical and environmental sectors. Also, despite no molecular mechanism for the ABFA of CuNCs was investigated and demonstrated, but only assumptions for possible mechanisms were made by authors, based on possible previously reported mechanism of action for copper NPs, this work advanced green nanotechnology application for BF-related challenges[20].

This year again, Rondilla and co-authors investigated the ABF behaviour of two edible Scottish brown seaweeds (*Alaria esculenta* and *Laminaria digitata*) extracts, previously subjected to lactic acid fermentation at reduced pH = 4.5 [25]. Fermented extracts presented high presence of acid lactic bacteria (ALB), and no presence of pathogens, while a metabolite diversification was observed with an enhanced production of oxygenated unsaturated fatty acids [25]. As for their biological profile, authors demonstrated that fermented seaweed extracts inhibited the BF formation by ciprofloxacin resistant ATCC 43300 MRSA, with enhanced effects observed for fermented *L. digitata*. Fermented extracts were essayed at 100 µg/mL (MICs not assessed), while ciprofloxacin, used as reference antibiotic, was tested at 50 µM (~16.6 µg/mL) concentration (MIC/sub-MIC values). ABFA (%) at administered concentration of *L. digitata* extracts under fermentation, was 60.4% (1 day fermentation) up to 84.5% (7 days fermentation). Conversely, a consistent higher potency (83.6-89.5% inhibition), irrespective of fermentation duration was detected for *A. esculenta* extracts, establishing for a natural abundance of ABF metabolites in this extract. Anyway, also against MDR ciprofloxacin-resistant MRSA strains, ciprofloxacin inhibited MRSA BF by 102.8% at MIC/sub-MIC concentration, thus remaining the most valuable ABF weapon. Although this is another study where ABF mechanisms were not investigated, the lack of MICs determinations for extracts, represent the major limitation to reliable comparison of their ABFAs with those of ciprofloxacin and other ABF compounds. Anyway, findings by Rondilla et al demonstrated the feasibility of lactic acid fermentation to valorise edible seaweeds into safe, metabolite-enriched functional foods with potential health benefits. Table 6 under Figure 5 groups the papers above mentioned and discussed, while Figure 5 summarizes them divided for substances classes.

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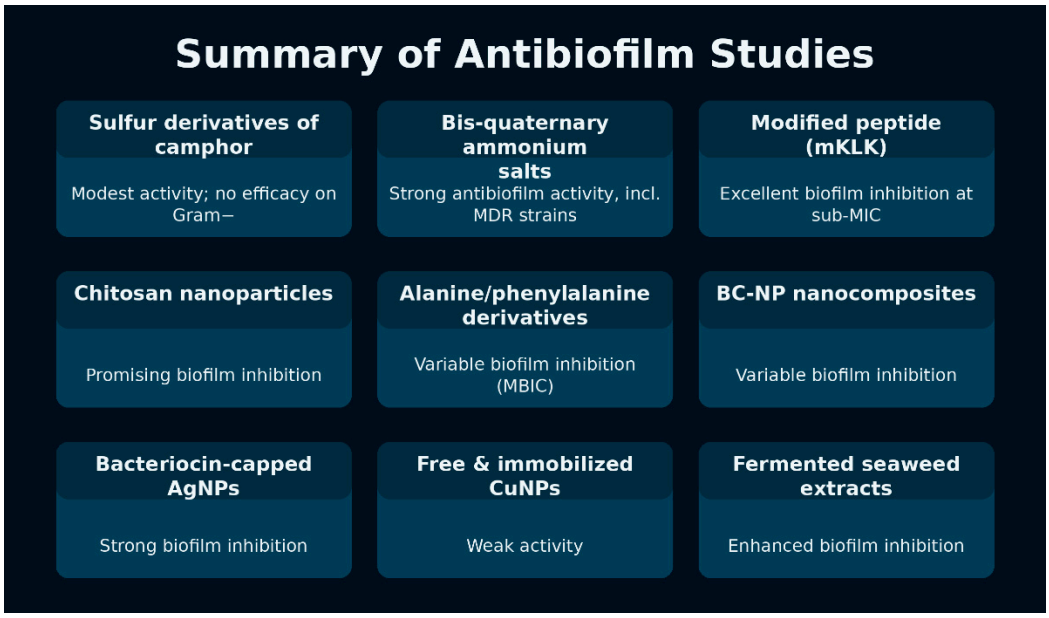

Figure 5. Schematic summary of the major collections of the last five-years studies above discussed.

Table 6. Scientific papers published over the years (2020-2026) reporting ABF studies where molecular mechanisms of action of compounds under test were not investigated.

Compound	Bacteria	Main Findings	Limitations	MI	Refs
1a, **2a**	*S. aureus*, *S. epidermidis* *E. faecalis* Multiple Gram-negative	MIC_{s90} = 64–>512 μg/mL, NI BF (MRSA, *E. faecalis*) BF LI (*S. epidermidis* and *S. aureus*) No activity vs Gram-negative, Negative BioFlux	Incorrect $MIC_{50/90}$ definition, modest activity	**No**	[19]
b-QASs	*S. aureus* (S and MDR) *P. aeruginosa*, *E. coli*	BF inhibition >95% on MDR strains	High MICs; variable effectiveness.	**No**	[21].
m-KLK	MRSA, MSSA	Strong BF inhibition in murine catheter model superior to parent peptide, effective at sub-MIC	N.C.	**No**	[161]
3-AQC QASs	Gram+ and Gram− panels	BF inhibition on *S. aureus* > 95% Reduced cytotoxicity vs commercial QASs	Antibacterial activity often inferior to CPC/BAB	**No**	[171]
CNPs	*S. aureus* strains from BM	BF inhibited 100%, >50%, >20% (MIC, ½ MIC, ⅓ MIC)	MIC interpretation questionable	**No**	[172]
ALA, PHALA, OXA	*S. epidermidis*, *B. subtilis* *E. coli*, *P. aeruginosa*	Some promising MICs; MBIC ranging ½–8×MIC	Activity correlated with physicochemical features.	**No**	[23]
BC-NPs	*S. aureus*, *P. aeruginosa* *E. coli*, *C. albicans*	BF inhibition and degradation	MICs not determined, dose–activity unclear Missing controls, data difficult to interpret	**No**	[24]
bac-AgNPs)	*S. aureus*, *P. aeruginosa*	Strong BF inhibition (78–82%), EPS disruption	ROS proven but no gene-level analyses	**P**	[22]

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f-CuNPs, *im*-CuNPs)	*S. aureus*, *E. coli*	Weak BF inhibition (≤ 42%); inferior to extracts	No MICs, weak activity, claims of biodegradability Non-toxicity not experimentally validated	**No**	[20]
FSWE	MRSA ATCC 43300	BF inhibition 60.4-89.5	MICs not determined	**No**	[25]

S = susceptible; BM = bovine mastitis BC NPs = ZnO, ZnOborax, $ZnCuO_2$, borax nanoparticles (NPs); bac-AgNPs = bacteriocin capped AgNPs; *f*-CuNPs, *im*-CuNPs = free and immobilized CuNPs; FSWE = fermented seaweed extracts; ALA = Alanine; PHALA = phenylalanine derivatives; OXA = oxazole compounds; P = partial; CNPs = Chitosan nanoparticles (CNPs); 3-AQC = Amido quinuclidine; *b*-QASs = *Bis*-quaternary ammonium salts; 1a, 1b = sulphur derivatives of camphor; LI = low inhibition; NI = no inhibition; BF = biofilm.

5.2. Relevant Published Studies on Biofilm (BF) Inhibition/Eradication Reporting Molecular Mechanisms

As above-mentioned, BF-associated infections represent one of the most resilient microbial states encountered in clinical practice, largely due to the complex interplay between its extracellular matrix components (EMCs), QS systems, stress responses, and metabolic dormancy. Over the past decade, a wide range of experimental studies have sought to identify molecules capable of inhibiting or eradicating bacterial and fungal BFs. The collected evidence across plant-derived phytochemicals, antimicrobial peptides, QS inhibitors, enzymatic degraders, and natural extracts, revealed also clear mechanistic patterns, especially against clinically relevant pathogens such as *P. aeruginosa*, *S. aureus* (including MRSA), *K. pneumoniae*, *E. coli* and *Candida* species. This time, the first research was made using both Scopus and PubMed databases, researching using keywords like those used previously, changing "no mechanisms" with "with mechanisms of actions", and adding QS system. Research was stopped to 32 publications. No range of years was imposed and works in the range 2011-2025 were found. The following Table 7 collects the found papers on BF inhibition/eradication, also containing the related mechanisms of action. A common characteristic of compounds in Tables 6 and 7 is that almost all found papers, regardless of the presence or not of mechanistic investigations correctly carried out, contained *in vitro* investigations.

Table 7. Studies on BF inhibition and/or eradication published in the years-range (2011-2026) containing mechanistic insights.

Model/Species	Molecular Mechanism	Key Findings	Refs.
P. aeruginosa	QS ↓ (lasI/lasR, rhlI/rhlR)+EPS pathway ↓↓↓ (pelA, pslA)	AgNPs+colistin ↓BF, ↓MIC/MBIC by ↑QS DR	[175]
P. aeruginosa CRPA, MDR	↓GacS/GacA, rsmYZ CTR of BF/EPS	GSSG/ARF ⚯ GacS, ↓BF; +macrolides ↑ABFA	[176]
P. aeruginosa	QS ↓ (pqs, rhl), ↓EPS	Allicin ↓ attachment, EPS and virulence factors	[124]
P. aeruginosa; S. aureus	QS RNA ↓↓↓ (rsmY/Z, rnaIII)	Ajoene ↓ BF formation & virulence	[125]
P. aeruginosa	QS ↓ targeting lasI/lasR	Carvacrol ↓↓ AHL synthesis	[126]
S. aureus	↓eDNA, DR of cidA, icaA, agrA, sarA	Emodin ↓ biofilm matrix and virulence	[177]
Candida spp.	CK2 ↓; ↓hyphal formation	Emodin ↓↓↓ fungal BFs	[128]
S. aureus	↓Extracellular proteins, ↓PIA	Aloe-emodin ↓ early biofilm development	[129]
P. aeruginosa	↓AHL, ↓elastase/pyocyanin/rhamnolipid	Hordenine ↓↓↓ QS and motility	[130]
MRSA	Membrane disruption; ↓staphyloxanthin	Pulverulentone A ↓ and destabilizes BFs	[178]

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Mixed species (BF enzymes)	EPS ↓↓↓ (glycoside-, prote-, DN-ases)	Enzymatic cocktails ↓↓↓ mature BFs	[131]
P. aeruginosa	Biofilm matrix degradation	Host defences peptides ↓↓↓ established BFs	[179]
S. aureus BFs *in vivo*	DNABII disruption	Antibody-mediated ↓↓↓ of BFs	[180,181]
Helicobacter pylori	EPS + eDNA matrix involvement	Biofilm growth ↑ clarithromycin resistance	[182]
P. aeruginosa	QS blockade via citrus flavonoids	Naringenin ↓ BF + virulence	[183,184]
P. aeruginosa	Anti-QS, ↓swarming/twitching	Zingerone ↓ multiple QS-regulated traits	[133]
P. aeruginosa	Matrix disruption	Musa acuminata extract ↓ BF	[132]
Streptococcus suis	Adhesion inhibition	Syringopicroside ↓ BF formation	[185]
S. aureus	Antimicrobial phenolics	Xanthohumol ↓ BF	[186]
P. aeruginosa, S. aureus	Membrane disruption	Lemongrass EO ↓ dual-species BF	[187]
S. aureus, Listeria, Gram–negative	Anti-QS	Eugenol ↓ violacein, Listeria & Klebsiella BF	[188]
K. pneumoniae, P. aeruginosa	Anti-QS	Quercetin ↓ adhesion and QS	[127]
P. aeruginosa	QS inhibition	Citrus limonoids regulate LuxO and ↓ BF	[189]
S.A., S. aureus, M. luteus, E. coli, P. aeruginosa C. albicans, C. glabrata	BF disruption	Clove oil ↓ biomass >80%	[190]
E. coli, P. aeruginosa, K. pneumoniae	EPS reduction	Cranberry polyphenols ↓ adhesion	[191]
MRSA	EPS disruption	Neem extracts ↓ MRSA BF	[192]
P. aeruginosa	Membrane disruption + ↓ EPS	Acacia nilotica extract ↓ BF	[193]
Pectobacterium Brasiliense	Cell membrane disruption	Honey (Manuka, Sidr) ↓ BF	[194]
P. aeruginosa UCBBB-PA14	c-di-GMP disruption	Citrus peel extracts ↓ BF	[195]
Acinetobacter Baumannii	By curcumin ABF activity	Curcumin ↓ adhesion and QS	[196]

S.A. = Syzygium aromaticum; CRPA = colistin-resistant *P. aeruginosa*; BF = biofilm; DR = down regulation; ABF = antibiofilm; ↓ = inhibition; reduction, decrease (also verbs); ↓↓↓ = fully disruption, fully eradication, strong inhibition, strongly inhibited; *M. = Micrococcus*; ↑ = improve.; ⚯ = bind, link; CTR = control.

The most part of studies reported in Table 7 was publicised after 2018 (2019-2025), even if a good production, accounting for 3 and 4 studies, belongs to years 2015 and 2017. Among recent ones, the study of Rosly et al further evidenced how BF presence can inactivate available antibiotic demonstrating that development of BF significantly augmented the recalcitrance of *H. pylori* against clarithromycin [182]. Additionally, a series of natural compounds by Wen et al and Varrier et al, such as naringenin, effectively inhibited BF formation and virulence of *S. aureus* and *P. aeruginosa* [183,184], thus underscoring the necessity of deepen the discussion on ABF effects of certain phytochemicals, including mechanistic considerations.

5.2.1. ABFA of Multi-Target Phytochemicals

Many essential oils and plant extracts exhibited multi-mechanistic ABF effects—simultaneously affecting membrane integrity, QS signalling, and EPS synthesis. Lemongrass oil targets both *P. aeruginosa* and *S. aureus*, disrupting membrane stability and suppressing adhesion [187]. Eugenol inhibits QS-regulated violacein production and reduces BFs in *Listeria*, *Pseudomonas*, and *Klebsiella*

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[188]. Cranberry polyphenols interfere with adhesion and EPS accumulation in *E. coli*, *P. aeruginosa*, and *K. pneumoniae* [191].

Clove oil, neem extracts, and Acacia nilotica extracts show potent inhibition against MRSA and Gram-negative pathogens of urinary tract (UT), causing intractable urinary tract infections (UTI) [190,192,193]. These multi-functional phytochemicals demonstrated that membrane disruption and QS interference often synergize to impair BF stability.

Natural Antimicrobial Peptides (AMPs) and Host-Derived Agents

Host defence peptides (HDPs), such as those included in Table 8, exhibited potent ABFA through membrane permeabilization and immunomodulatory effects. Evidence showed that AMPs eradicate mature *P. aeruginosa* BFs, outperforming many conventional antibiotics [179]. Their broad spectrum and low propensity for resistance make AMPs highly promising candidates for BF-targeted therapeutics.

Table 8. Host defense peptides (HDPs) with their well-established or investigated therapeutic effects.

HDP Family	Peptide	Primary Therapeutic / Biological Effects
α-Defensins	HNP-1 to HNP-4	Broad-spectrum antibacterial, antiviral; neutrophil-mediated innate immunity
	HD-5, HD-6	Antibacterial, antifungal; gut mucosal defense
β-Defensins	hBD-1	Constitutive antimicrobial protection (skin, epithelia)
	hBD-2	Antibacterial, antifungal; inflammation-induced epithelial defense
	hBD-3	Potent antibacterial (incl. MDR bacteria), antiviral; immunomodulatory
	hBD-4	Antibacterial; epithelial immune signaling
θ-Defensins	RTD-1, RTD-2	Broad antimicrobial; anti-inflammatory; activity against resistant pathogens
Cathelicidins	LL-37 (human)	Antibacterial, antiviral, antibiofilm; wound healing; immune modulation
	CRAMP (mouse)	Antimicrobial; inflammation regulation (experimental model)
	SMAP-29	Potent antibacterial; membrane disruption
Histatins	Histatin-1	Wound healing; oral epithelial repair
	Histatin-3	Antifungal; oral mucosal defense
	Histatin-5	Strong antifungal (Candida spp.)
Dermcidin-derived peptides	DCD-1, DCD-1L	Antibacterial in sweat; skin innate defense
C-type lectin–derived peptides	RegIIIα	Antibacterial (Gram-positive); gut barrier protection
	RegIIIγ	Antibacterial; mucosal immunity in gut
Other HDPs	Lactoferricin	Antibacterial, antiviral, antifungal; immune modulation
	Lysozyme-derived peptides	Antibacterial; cell wall degradation
	Azurocidin (CAP37)	Antimicrobial; chemotaxis; immune signaling

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	Cathepsin G peptides	Antimicrobial; inflammation modulation

Notes: Effects are described at a mechanistic/experimental level (antimicrobial, immunomodulatory, etc.), not as approved clinical indications.

Many HDPs show multiple overlapping effects: antimicrobial activity, immune modulation, wound healing, and antibiofilm properties. LL-37, hBD-3, RTD-1) are being explored as templates for novel anti-infective or anti-inflammatory therapies, especially in the context of antibiotic resistance.

Natural Extracts Active Against Fungal Biofilms

Emodin also demonstrated strong ABF effects against fungi, such as *C. albicans*, *C. tropicalis*, and *C. krusei* by inhibiting hyphal development—an essential step in fungal BF formation—and competitively inhibiting CK2 kinase activity [128]**.** Honey-based therapeutics (Manuka, Sidr, clover honeys) showed multi-species ABFA against MRSA, MSSA, and *P. aeruginosa*, primarily through osmotic stress, ROS generation, and matrix disruption [194].

Clinical Implications and Translational Relevance

Multiple studies emphasized that BF inhibition often correlates with decreased virulence, antibiotic sensitization, or reduced persistence *in vivo.* QS inhibitors (e.g., ajoene, naringenin), EPS disruptors (e.g., emodin, enzymes), and AMPs represent, mechanistically, diverse but convergent routes toward reducing BF-associated tolerance. Moreover, extracts, such as citrus peel fractions reduce intracellular c-di-GMP, a second messenger central to BF lifestyle transitions in *P. aeruginosa*, highlighting a targeted molecular avenue for future therapeutics [195]. Altogether, the experimental evidence establishes a clear mechanistic framework for the articles of this series included in Table 7. Effective ABF agents tend to inhibit QS, impair adhesion/motility, suppress EPS production, disrupt matrix architecture, or permeabilize microbial membranes (Figure 6). These modes of action, observed across dozens of independent studies, form the biochemical backbone of modern ABF strategies [185,186,189,196].

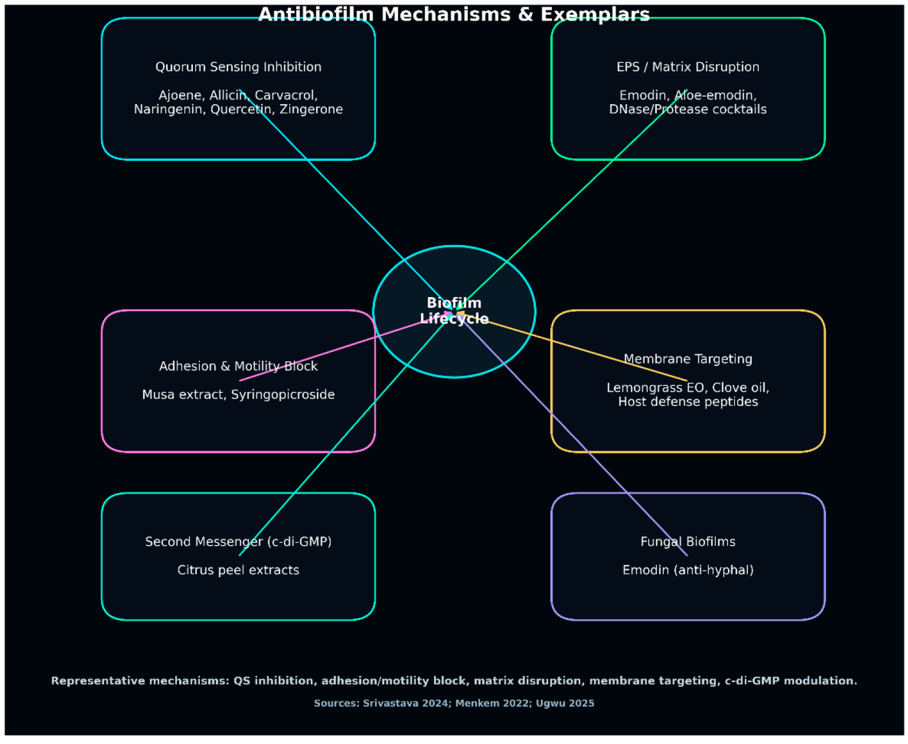

Figure 6. Representative mechanisms of action found for ABF compounds developed in the last years, separated for categories.

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5.3. Relevant Published Studies on Biofilm (BF) Inhibition/Eradication Not Reporting Molecular Mechanisms over Years

The additional Table 9 summarizes a miscellanea of papers reporting on the ABFA of some classes of synthetic and natural compounds without insights on their mechanisms of action found using Scopus and PubMed databases without imposing any range of years and adopting all the keywords used till now simultaneously, including both "no mechanisms" and "with mechanisms of actions". Information from the two databases were crossed and duplicate papers were removed. No paper containing correctly carried out mechanistic insights was found. No author of these papers investigated genes changings (QS), as observable in Table 9.

Table 9. Additional papers on the ABFA of some important categories of compounds not containing mechanistic insights.

Bacteria	ABFA	Model	Limitations	MI	Refs.
CHALCONES AND DERIVATIVES					
S. aureus MDR	40–80% inhibition	CV °	No *in vivo*	NO *	[197]
S. aureus, E. coli	↓9Log$_{10}$ at 128 μg/mL		No MA	NO	[198]
S. mutans	MBIC 500 μg/mL $^{½ MIC}$, 58-60%		NDT	NO	[199]
MRSA/MSSA	↓8/7Log$_{10}$ at 19.8 μg/mL		No MA	NO	[200]
S. aureus	MBIC 4–16 μg/mL		No *in vivo*	NO	[201]
NTHi	MBIC$_{50}$ = 16 μg/mL (71.35 μM)			NO	[202]
CYNAMMALDEHIDE and TRITERPENOIDS					
MRSA	~50% inhibition	CV °	No *in vivo*	NO	[203]
P. aeruginosa, S. pyogenes, E. coli	Up to 99.9% inhibition	CV °	No *in vivo*	NO	[204]
MRSA	66-67% inhibition, 76-85% removal	CV °	*In vitro+in vivo*	NO	[205]
S. aureus + *S. Enteritidis*	21–75, 21-77, 13-81% inhibition	CV ° on MBFs	No *in vivo*	NO	[206]
MRSA	30–70% inhibition	CV °	No *in vivo*	NO	[207]
FURANE DERIVATIVES					
P. aeruginosa, S. aureus, S. enterica, C. albicans	40–90% inhibition	CV °	*In vitro+in vivo*	NO	[208]
BENZAZOLES/BENZOTHIAZOLES					
S. aureus	MBIC 8–64 μg/mL	CV °	No *in vivo*	NO	[209]
S. aureus, E. coli	>80–90% inhibition		No *in vivo*	NO	[210]
ANTIMICROBIAL PEPTIDES					
MRSA, MDR strains	Up to 100% inhibition @	CV ° and CoMIC	No *in vivo*	NO	[211]
S. aureus biofilms	MBIC 2–4 μg/mL, 75-90% BFMR	CV °		NO	[212]
INDOLE DERIVATIVES					
P. aeruginosa, A. baumannii	45–82% inhibition	CV °	No *in vivo*	NO	[213]
SILVER NANOPARTICLES (AgNPs)					
S. aureus	Strong BFF eradication	CV ° + *in vivo*	LTD	NO	[214]
ESKAPE (see text)	40-83% inhibition, 30-73% eradication	CV °	No *in vivo*		[215]
MDR *K. pneumoniae*	50–100% inhibition				[216]
Carbapenem-resistant *K. pneumoniae*	MBIC 16–64 μg/mL				[217]
MDR *K. pneumoniae*	Reduced EPS + biofilm				[218]
OTHER COMPOUNDS/STRATEGIES					
S. aureus persisters	Biofilm eradication	*In vivo*	LSD	NO	[219]

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MRSA	40–70% inhibition	CV °	No *in vivo*	NO	[220]

* Only docking experiments showed that compounds **3b, 3k, and 3r** exhibited favourable binding energies and strong interactions with the SauPBP2a active site; MBFs = mixed biofilms; @ depending on concentrations; @ depending on strain species; ABFA = antibiofilm activity; BFMR = biofilm mass reduction; CoMIC = real-time ABF assay; CV = crystal violet method; MI = mechanistic insights; LTD = limited toxicity data; LSD = limited stability data; NTD = no cytotoxicity data; NTHi = non-typeable haemophilus influenzae.

As in the previous Tables 6 and 7, Table 9 highlighted that a very wide range of chemical scaffolds and biological strategies have been explored to counteract BF development in clinically relevant pathogens such as *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, and other multidrug-resistant (MDR) organisms. Small-molecule inhibitors—including chalcones, cinnamaldehyde derivatives, furanones, benzazoles, and indole-based compounds—have shown promising activity *in vitro*. Natural products and their derivatives, such as terpenoids and triterpenoids, also exhibited ABF potential, often through assumed but not demonstrated membrane perturbation or interference with QS. In parallel, antimicrobial peptides AMPs and NMs, particularly silver nanoparticles (AgNPs), have emerged as potent disruptors of BF structure and functions. More recently, innovative biological approaches such as bacteriophage–NPs hybrids and ClpP-activating molecules have demonstrated the ability to eradicate persister cells and degrade mature BFs. Despite this progress, significant gaps remain, which are other than the lack of mechanistic insights. Many studies (or even all) rely exclusively on static *in vitro* models or do not include cytotoxicity studies on eukaryotic cells, as well as *in vivo* confirmations. A systematic comparison of these diverse ABF strategies is therefore essential to identify common trends, strengths, and limitations, and to guide the development of clinically translatable ABF therapies.

5.3.1. Chalcones and Derivatives

Chalcones are versatile ABF scaffolds, constituting one of the most extensively investigated classes for this application. Substituted chalcones reported by Amengor et al. (2025) inhibited MDR *S. aureus* biofilms by 40–80% [197], being compounds **3b, 3k,** and **3r** the most active ones, which exerted ABF inhibition % in the high range 78-97% against ciprofloxacin which displayed ABF% of about 80% [197]. No mechanistic investigations were carried out, but only non-verified docking studies showed favourable binding energies and strong interactions with the SauPBP2a active site[197]. Recently, Col et al was reported that some chalcone analogous, such as a series of compounds (12-15) featuring fluoro and trifluoromethyl groups on the B ring, possesses potent antimicrobial activity[221]. Compounds **13** and **14** demonstrated low MICs = 15.6 and 7.81 μg/mL, respectively, against *S. aureus*, no investigations concerning their potential ABFA was carried out [221]. Cationic chalcones developed by da Chu et al. (2018) exhibited strong antibacterial activity and antibiofilm properties (100% inhibition). Specifically, twenty-nine cationic chalcone analogous (**5a-5ac**) were designed and synthesized. Representative compounds **5a** (MIC = 1 μg/mL against *S. aureus*, 0.5 μg/mL against MRSA) and **5g** (MIC = 0.5 μg/mL against *S. aureus*, 0.25 μg/mL against MRSA showed good bactericidal activity against both Gram-positive and Gram-negative bacteria, including the drug-resistant species, including KPC and NMD producers[198]. The cationic chalcone derivatives were shown to be stable under plasma conditions. The compounds did not allow bacteria to develop resistance and exhibited negligible toxicity toward mammalian cells. Additionally compound **5g** and **5a** caused a 9 $\log_{10}$ CFU reduction in viability of *S. aureus* and *E coli* respectively when administered at 128 μg/mL[198]. Garcia et al synthetised 17 chalcone derivatives, among which compound **5f** was able to inhibit the adhesion of MRSA and MSSA to human keratinocytes at sub-MICs concentration, to reduce their biofilm formation, as well as acted on preformed biofilm in concentration-dependent mode[200]. Scanning electron microscopy analyses confirmed severe perturbations caused by **5f** on MSSA and MRSA biofilm architecture. Specifically, **5f** was more efficient than vancomycin in reducing BF formation causing 8 and 7 Log_{10} CFU/ml reduction of BF formation by MSSA and MRSA respectively, at concentration 10 × MICs (19 and 78 μg/mL) [200].

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Conversely, in acting against mature BF, at same concentration of MIC and 10 × MIC, **5f** was better performant than vancomycin at MIC, but at 10 × MIC, vancomycin was better than **5f**. Anyway, **5f** caused 7.5 and 6.5 Log_{10} CFU/ml reduction of mature BF constructed by MSSA and MRSA respectively [200]. The acute toxicity assay, using *Galleria mellonella* larvae, indicated a low toxic effect of **5f** after 72 h, displaying lethality of 20% and 30% at both MIC (7.8 μg mL^{-1}) and 10 × MIC (78.0 μg mL^{-1}) concentrations, respectively[200]. A new series of 7 chalcones derivatives, containing the 3-furan-2-yl-1-*p*-aryl-propenone structure, and containing imine moieties (**1-7**) were synthesized and characterized using spectral analysis, by Abdula. The synthesized derivatives were screened *in vitro* against several bacterial species, including *A. baumannii, K. pneumonia, P. aeruginosa (*Gram-negative bacteria), and *S. aureus* (Gram-positive bacteria) to study the effect of different imine moieties on the activity of (*E*)-1-(4-aminophenyl)-3-(furan-2-yl)prop-2-en-1-one, which represent the potent hit against different bacterial species. The synthesized compounds were found to exhibit modest to vigorous activity, especially compounds **1**, **4**, and **6-7**. The MICs of compounds **1** and **6** against *A. baumannii* and *S. aureus* were determined. The anti-biofilm activity of the potent discovered compounds (**1**, **4**, **6**, and **7**) against *A. baumannii* and *S. aureus* were also determined, observing a mediocre inhibition of 30% (1 against both bacteria), 48 and 66%, 34 and 44% and 48% (**4**, **6** and **7**, against *A baumannii* and *S. aureus*, respectively)[202]. No mechanistic insight was performed, but only a docking study of the best discovered hits against the active site of glucosamine-6-phosphate synthase, the antimicrobial target enzyme was achieved to explore the interactions of the synthesized hits inside the enzyme residues, was performed[202]. Sashidhara et al synthesized a series of novel hybrids possessing chalcone and thiazole moieties and evaluated for their antibacterial activities. Collectively, this class of hybrids exhibited potency against *S. aureus*, and in particular, compound **27** exhibited potent inhibitory activity with respect to other synthesized hybrids. Furthermore, the haemolytic and toxicity data demonstrated that the compound **27** was nonhemolytic and nontoxic to mammalian cells. The *in vivo* studies utilizing a *S. aureus* septicaemia model demonstrated that compound **27** was as potent as vancomycin. Anyway, despite studies were carried out *in vivo*, its ABFA was not reported, leaving unveiled this potential activity[222]. Even more recently, Hetamian et al. knowing that *Streptococcus mutans*, caused dental BF formation (caries) by producing Gtf enzymes, which are related to the adhesion of *S. mutans* to tooth surfaces and bacterial cell aggregation, and that chalcones inhibit this path, evaluated the inhibitory effects of six chalcone derivatives on GtfC, the prevention of BF formation by *S. mutans*, and the assessment of the cytotoxicity of these derivatives[199]. No mechanistic insight was evaluated but only molecular docking was employed, to predict interactions between chalcone derivatives (**5a**, **b**, **d**, **h**, **NME2**, and **BA**) and GtfC and assess the possibility of the existence of an already known mechanism of action. The effectiveness of chalcone derivatives in inhibiting *S. mutans* BF formation was not virtually assessed using a colorimetric method (VC), with dye absorbance measured by an ELISA reader[199]. The same method was applied for cytotoxicity assessment. All chalcone derivatives inhibited the growth of *S. mutans*, *S. sanguinis*, and *S. salivarius* at MICs = 1000 μg/mL, while exhibited ABF properties against *S. mutans* at sub-MICs concentrations. Compounds **5a**, **b**, **h**, and **BA** successfully inhibited (>50%) the *S. mutans* BF, at ½ MIC, while at ¼ MICs inhibition was > 50% only for compounds **5a** and **5h** Conversely, at 1/8 MIC, inhibition by all compounds was in the range 2-30%. All compounds demonstrated low cytotoxicity on HepG2 cells. The results indicate that, except for compound AB (75% inhibition), compounds **5a** and **5b** exhibited the strongest ABF effect (58.2 and 60% inhibition respectively), among the six chalcone derivatives, showing effectiveness at lower concentrations in all assays, including MBIC tests[199].

Finally, Duangkamol et al synthesised a series of natural and synthetic chalcones with various chemical substituents, which evaluated *in vitro* for their ABFAs against strong BF-forming strains of non-typeable haemophilus influenzae (NTHi), an important human respiratory pathogen, frequently causes biofilm infections[201]. Of the tested chalcones, 3-hydroxychalcone (chalcone **8**) exhibited the most potent inhibitory activity, being its $MBIC_{50}$ = 16 μg/mL (71.35 μM), or approximately sixfold more active than the reference drug, azithromycin ($MBIC_{50}$ 419.68 μM). The inhibitory activity of

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chalcone **8**, which is a chemically modified chalcone, appeared to be superior to those of the natural chalcones tested. Significantly, chalcone **8** inhibited biofilm formation by all studied NTHi strains, indicating that the ABFAs of this compound occur across multiple strong-BF forming NTHi isolates of different clinical origins. According to antimicrobial and growth curve assays, chalcone **8** at concentrations that decreased BF formation did not affect growth of NTHi, suggesting the BF inhibitory effect of chalcone **8** was non-antimicrobial. These findings indicate that 3-hydroxychalcone (chalcone 8) has powerful antibiofilm activity and suggest the potential application of chalcone 8 as a new therapeutic agent for control of NTHi BF-associated infections, thus confirming further the relevance of paper non assessing any mechanism of action, as well[201].

5.3.2. Cinnamaldehyde and Analogous

Cinnamaldehyde and terpenoids are natural compounds with broad biological activity, which demonstrated robust antibiofilm activity across Gram-positive and Gram-negative pathogens. Being cinnamaldehyde a compound widely studied for its antimicrobial effects and for its capacity to inhibit QS, Rossi and Heuertz (2017), tested it as pure molecule against MRSA BF (clinical MRSA isolates from area hospital laboratories), using microplate assay for quantitative spectrophotometric evaluation of CV-stained BF adherent to microwells; and viable bacterial count assay for colony forming unit (CFU/ml). Despite no mechanistic investigation, results indicated that cinnamaldehyde inhibited 50% MRSA biofilm formation in a concentration-dependent manner with significance ($p<0.01$) at 50 and 100 μM. Colony counts of MRSA were also significantly ($p<0.01$) reduced in a concentration-dependent manner. This paper bases its relevance not so much on the mechanism of antibiofilm action of cinnamaldehyde, which is already known, but on having demonstrated that its pure nucleus has an even though limited antibiofilm effect, which can be however significantly increased by appropriate chemical modifications. In the year 2018, Firmino et al assessed the activities of essential oils derived from the trunk bark of *Cinnamomum zeylanicum* (EOCz) and *Cinnamomum cassia* (EOCc), where the major component of EOCz and EOCc was cinnamaldehyde, as well as cinnamaldehyde on bacterial biofilms of clinical interest [204]. Antibiofilm activity was assessed by quantifying the biomass and determining the number of viable cells. BF biomasses were reduced by up to 99.9%, but *Streptococcus pyogenes*, *P. aeruginosa*, and *E. coli* BFs were sensitive to all the concentrations and substances analysed. Collectively, 2000 μg/mL of cinnamaldehyde reduced the number of viable cells by 5.74 Log CFU/mL[204]. Despite Firmino et al did not investigate mechanism of action of cinnamaldehyde, the relevance of this work consists in having described substances with potential use against infections caused by bacterial BFs[204]. Anyway, cinnamaldehyde nano-emulsion formulations, decorated or not with rhamnolipid (Cin-RHL-NE and Cin-NE) developed by Yin et al. (2024) demonstrated MICs against *S. aureus* about two-fold lower than those of free cinnamaldehyde, and *in vivo* efficacy, underscoring the importance of formulation strategies, and their higher capability to counteract BF associated infections, due to adjunctive unique physical, chemical and biological properties[205]. The ability of cinnamaldehyde nano-emulsion decorated with to penetrate MRSA BF structures and eliminate BFs was assessed both *in vitro* and *in vivo*, without insights in mechanistic processes, thus further confirming that also papers lacking them are worthy of publication.

The efficacy of Cin-RHL-NE in the mouse skin wound healing model was superior to other formulation [205]. Crystal violet staining demonstrated that both Cin-RHL-NE and Cin-NE inhibited MRSA BF formation in a concentration-dependent manner, with effective concentrations ranging from 128 to 1.024 μg/mL [205]. Specifically, at a concentration of 256 μg/mL, both Cin-RHL-NE and Cin-NE significantly inhibited MRSA USA300 BF growth, with reductions of 66.9 and 65.5%, respectively[205], while Cin alone resulted in a much lesser inhibition, with only a 24.8% reduction in growth[205]. Also, the scavenging effect of Cin-RHL-NE and Cin-NE on mature BF of MRSA showed their highest removal rate of 84.6% and 76.3% at 1024 μg/mL[205]. Against mixed-species BFs, we found the study of Zhang et al. (2014). Authors reported the statistically significant 13–81% inhibition of mixed BF formation by two food-borne pathogens, exhibited by some food additives, although mechanistic data were

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lacking[206]. Specifically, food additives such as citral, cinnamaldehyde, and tea polyphenols (MICs = 0.8, 0.4 and 300-600 μg/mL) were tested on mixed BFs produced by foodborne *S. aureus* and *Salmonella* serotype Enteritidis, at sub-MIC up to MIC concentrations. Results demonstrated that citral, cinnamaldehyde, and tea polyphenols were able to significantly inhibit mixed BF formation already at sub-MIC concentrations and by 74, 77 and 81% at MIC [206]. Food additives inhibited the adhesion of mixed bacteria on stainless steel chips and increased the sensitivity of the mixed BF to disinfectants. This study, despite lacking mechanistic insights provides a scientific basis for the application of natural food additives to control BF formation of foodborne bacteria, thus being anyway relevant and publishable[206]. Pentacyclic triterpenoids α-amyrin, betulinic acid (BA) and botulin aldehyde were evaluated by Chung et al. (2022) to assess their effects in the reduction of BF biomass pre-formed by MRSA and the metabolic activity of the bacterial cells in the BF. Authors observed from moderate-to-strong inhibition (30-70%), likely through membrane disruption, but no mechanistic investigation was carried out[207]. These findings highlight the potential of natural compounds as ABF agents, though toxicological and mechanistic studies remain insufficient. Very recently, to meet the urgent need for novel antibacterial agents that are active also against worrying superbugs, and their BF, natural pentacyclic triterpenoids, including betulin (BET), BA and ursolic acid (UA), have been chemically modified, achieving compounds **1**–**7**. Among these, compounds **4**-**7** demonstrated potent antibacterial effects (MICs = 2-16 μg/mL) against Gram-positive MRSA, MRSE, as well as against vancomycin-resistant *Enterococcus faecalis* and *E. faecium* (VRE). Their effects were probably due to the insertion of a triphenyl phosphonium (TPP) group and were higher than those reported so far for other BET, BA and UA derivatives, especially considering the complex pattern of resistance of the isolates used in the study and their clinical source. New experiments are currently underway with the best performing compounds 5 and 7 (MICs = 2 μg/mL) about their possible ABF activity, time-killing curves and cytotoxicity on eukaryotic cells.

Synthetic analogues described by Topa et al and Chu et al (2018) showed moderate (max 60%) inhibition against *S. aureus* and *E. coli* [198,223].

5.3.3. Furanones

The library of 37 novel furanones synthesized by Gómez et al. (2022) mimicking those natural of marine origin, were evaluated as BF inhibitors in several opportunistic human pathogens including *S. enterica*, *S. aureus*, *E. coli*, *S. maltophilia*, *P. aeruginosa* and *C. albicans*[208]. They exhibited potent inhibition (40–90%) against *P. aeruginosa* and *S. aureus* and *C. Albicans* [208]. As important results, compound 2 and 27 reduced BF by 75 and 60% respectively, compound 24 reduced that of PAO1 and PAR7244 by 70 and 44% respectively, while compounds 33 and 34 reduced BF by P. aeruginosa by 52 and 51% respectively. Compounds 8 and 10 reduced BF by S. enterica by 41 and 44%, while concerning C. albicans, compounds 2, 3 and 34 reduced its BF by 90%, while compound 15, by 75%[208]. Despite authors assert that their furanones displayed ABF effects primarily through QS disruption, there is no experiment in this study evaluating QS function, but such mechanism of action was claimed, based on their alignment with the established role of halogenated furanones as QS inhibitors[208]. The most potent compounds were subjected to further analysis by confocal laser-scanning microscopy for their effects on *P. aeruginosa* and *C. albicans* BF individually, in addition to mixed polymicrobial biofilms. Lastly, we investigated the impact of a promising candidate on survival rates *in vivo* using a *Galleria mellonella* model[208].

5.3.4. Benzazoles and Benzothiazoles

Bezazoles and benzothiazoles are emerging synthetic ABF agents. Benzazole derivatives reported by Aktekin et al. (2024) demonstrated MBIC values ranging from 8–64 μg/mL or inhibition levels of 30–70%. Specifically, di-heterocyclic benzazole structures **16** and **17** by Aktekin et al, demonstrated potent antibacterial activity (MIC = 7.8 μg/mL) against *S. aureus*, along with significant ABF activity[209]. Noteworthy, the capability of compound **17** to inhibit BF formation by at least 50% was observed from sub-MIC (3.90 μg/mL) concentration. Furthermore, both compounds exhibited

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the potential to inhibit preformed biofilm by at least 50% at the MIC concentration (7.81 μg/mL), without cytotoxic effects in HFF-1 cells[209]. Also, more recently (2025), the compounds containing benzimidazolium salts proposed by Yildirim et al, exhibited several biological activities, including antibacterial, antiviral, antifungal, anti-inflammatory, and anticancer effects[210]. The synthesized compounds demonstrated antibacterial activity against *S. aureus*, *E. faecalis*, *P. aeruginosa*, *E. coli* and antibiotic-resistant bacteria such as MRSA and MDR *E. coli*. Particularly, compounds **1** and **3** exhibited significant antibacterial activity against MRSA and MDR *E. coli*, with MIC values of 15.62 and 7.81 μg/mL, respectively. Compounds **1–6** showed also significant, dose-dependent inhibitory activity against MRSA and MDR *E. coli* BFs, showing over 80-90% BF inhibition at 16-128 × MICs (1000 μg/mL) concentrations[210]. Both studies proposed mechanisms supported by docking analyses, but without experimental validation. Anyway, these compounds represent promising synthetic scaffolds, which give relevance to the study regardless the absence of insights investigations. but further mechanistic and *in vivo* studies are required.

5.3.5. Antimicrobial Peptides: Potent but Under-Validated Candidates

Antimicrobial peptides, (AMPs) are generally more effective than antibiotics in counteract infections sustained by MDR bacteria, due to their positive superficial charge, which supports their ability to kill microorganisms using different metabolic pathways, but especially their a-specific mode acting as membrane disruptors [224,225]. Novel four AMPs developed by Polat et al. (2024) (NET1-4), combining two amino acid forms (D- and L-), inhibited MRSA and MDR biofilms by 50–90%, while lipopeptidic humimycin analogous studied by Rahman et al. (2025) achieved MBIC values as low as 2–8 μg/mL. Specifically, the strategy of mixing the D- and L- forms of amino acids, implemented by Polat et al, augmented the antibacterial potency of normal L-amino acids by 30% (MICs = 2-64 μg/mL against MRSA and 0.5-2 μg/mL against VRE), while cytotoxicity (IC_{50}) and haemolytic toxicity (HC_{50}) values remained within the safe range[211]. ABF activities of these peptides were investigated technically simulating the exact situation, by a real-time observable biofilm model and a new detection method based on it (CoMIC method) and by conventional CV method. Results revealed that NET1 peptide with D-leucine amino acid in its structure and the NET3 peptide with D-arginine amino acid in its structure are effective in inhibiting biofilm[211]. Specifically NET 1-4 caused BF inhibition by 100% at concentrations 4, 128, 4 and 128 μg/mL, while NET 1 and 2 caused BF inhibition by > 75 and 63%, at 2 and 64 μg/mL respectively. NET 3 caused >96% BF inhibition at 2 μg/mL and > 85% at only 1 μG/mL. Finally, compound NET 4 reduced BF by > 97% at concentration 64 μg/mL[211]. Humimycin structural analogous **2**-**6**, against *S. aureus* ATCC 29213, demonstrated MIC values from 2 to 16 μg/mL, with analogous **6** showing the strongest activity. In ABF assays, analogous **5** and **6** exhibited MBIC of 4 μg/mL and 2 μg/mL, respectively, up to 16-fold lower than the parental compound[212]. Treatment of mature biofilms with analogous **3–6** at four times the MIC led to biomass reductions ranging from 74.8 % to 89.8 %[212]. Resistance development was not observed after 10 serial passages under sub-MIC treatments[212]. These findings highlight the therapeutic potential of synthetic humimycin analogous as precision antibiofilm agents against *S. aureus* infections[212]. Despite no mechanistic insight was carried out and the absence of in vivo studies and concerns regarding stability and immunogenicity remain major barriers to clinical translation, these works were considered worthy of publication and relevant.

5.3.6. Indole Derivatives: Anti-Virulence Strategies

Marine indole derivatives described by Golberg et al. (2025) inhibited Gram-negative BFs by 40–80%, largely through suppression of virulence pathways rather than direct bactericidal activity[213]. Specifically, synthesized 1,1′-bisindole (NN) and 2,3-dihydro-2,2′-bisindole (DIV) attenuated BFs production by *P. aeruginosa* and *A. baumannii* alone and in combination with tobramycin resulted in significant BF inhibition, particularly in the eradication of mature BF by *P. aeruginosa*[213]. Reduction observed after 18 hours treatments at 37°C with 50 μM concentrations of both compounds varied depending on the treatment and on the bacterial strain. *P. aeruginosa* was inhibited by 45% by DIV

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and by 82% by NN, while *A. baumannii* was inhibited by 56% by DIV and by 72% by NN[213]. Overall, NN was more effective at reducing *P. aeruginosa* BF density, while both the NN and DIV treatments had smaller effects on the density of BF by *A. baumannii.* Both treatments caused only negligible bacterial cell mortality in *P. aeruginosa* and had no effect on the numbers of dead cells in the *A. baumannii* BF, which did not differ from the control[213]. A mature *P. aeruginosa* biofilm treated with 50 μM NN compound and in parallel with various antimicrobial agents at 20 μg/mL tobramycin by assessing MICs. For tobramycin, an antibiotic commonly prescribed to CF patients with *P. aeruginosa* infections, the lowest MIC value was observed for NN. Treatment of the BF with NN alone reduced the BF by 81%, but it did not completely eradicate it. On the other hand, when the mature BF was treated with both tobramycin and NN, despite an increase in its bio-volume, 50% of BF was eradicated, and the bacterial cells died. NN, however, was able to restore the efficacy of tobramycin without affecting the BF dispersal stage. Both *bis*-indole derivatives, suppressed bacterial virulence factors, reduced bacterial adhesion, and improved survival rates in infected *Caenorhabditis elegans* and human lung epithelial cell models[213]. Although authors found that NN repressed or upregulated 307 genes when compared to untreated *P. aeruginosa*, no mechanism of action directly correlated with indoles ABF action was reported. Anyway, these indole molecules, acting in resistance-quenching are potentially important candidates for inclusion in treatment protocols and the study was retained relevant and worthy of publication. The use of compounds that prevent the BF from accumulating the high cell densities critical to its structural and functional maintenance represents significant progress in the management of bacterial persistence. Therefore, a possible clinical implementation of these innovative compounds holds a promising future.

5.3.7. Silver Nanoparticles: Strong Activity with Safety Concerns

AgNP-based strategies consistently demonstrated strong ABF activity. Phage–AgNP hybrids developed by Szymczak et al. (2024) eradicated *S. aureus* biofilms *in vitro* and *in vivo*[214], AgNP–antibiotic combinations studied by Mishra et al. (2024) inhibited ESKAPE biofilms by 50–95%[215], AgNPs biologically synthesized by Elsaid et al (2023) using aqueous *E. camaldulensis* leaf extracts significantly (>50%) inhibited BF by 50 *K. pneumoniae* isolates [216], biogenic AgNPs from Wozeak et al. (2026) [217]and Siddique et al. (2020) [218]showed significant activity against carbapenem-resistant *K. pneumoniae*. All studies were without mechanistic insights. Two potential approaches for managing superbugs infections difficult to treat due to the presence of their BF, involves either the use of natural killers, namely lytic bacteriophages or metal nanoparticles with antimicrobial properties [214]. In this regard, Szymczak et al treated E. coli BFs grown for 24 hours; 6 h with T7WT and T7Ag-XII phage, AgNPs, or a combination of T7Ag-XII phage with AgNPs. BF inhibition was evaluated when bacterial BF was treated with T7 (two types T7WT -wild type- and T7Ag-VII (displaying the RFEHPAVPRTEM peptide)) concentrations in the range $10^6/10^4$-10^9 PFU (plaque forming unit)/mL, or AgNPs concentrations in the range 0.1-1000 μg/mL and subjected to analyses of optical density (OD_{600} and OD_{959}) and fluorescence, biofilm biomass, and bacterial viability. Authors demonstrated that T7 phage armed with AgNPs exhibited greater efficacy in terms of controlling bacterial BF, compared with phage or NPs alone. Recombinant AgNP-binding phage could effectively eradicate bacterial BF, even when used at low concentrations, remaining not toxic to eukaryotic cells., when used at concentrations that could eradicate bacterial biofilm[214]. Specifically, the initial analysis of the ABF activities of T7 (two types) and/or AgNPs, based on changes in optical density values of BF-forming cultures, showed that after treatment with either type of T7 phage (wt or Ag-XII), the optical density at 600 nm (OD_{600}) values decreased significantly by approximately 50%. There were no differences according to T7 phage type (wt or Ag-XII) or concentration. When BFs were treated with AgNPs alone, there were no decreases in OD600 values. Notably, the ABF activities of recombinant T7Ag-XII phage (at a concentration of 1×10^9 plaque-forming units [pfu]/mL) combined with various concentrations of AgNPs were significantly higher than the activities of T7WT phage alone or AgNPs alone [214] Lower concentrations of T7Ag-XII phages (1×10^8 [pfu]/mL) combined with decreasing concentrations of AgNPs did not significantly reduce OD600 values in

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comparison with either type of T7 phage. Subsequently, assessments of biofilm biomass and and viability were conducted, evidencing that treatment with either type of T7 phage (WT or Ag-XII) alone resulted in a significant reduction in biofilm biomass, with no statistically significant differences in efficacy between different phage titers used. The use of AgNPs alone seems to be as effective in biomass reduction as the use of T7WT phage alone. Importantly, the combined use of T7Ag-XII phage with AgNPs led to a significantly greater reduction of biofilm biomass, compared with the use of T7WT phage alone or AgNPs alone[214]. Moreover, the reduction of biofilm biomass was greater when using the combined treatment than when using T7WT phage alone or AgNPs alone, even when the T7Ag-XII phage and AgNP concentrations were reduced. The study of Mishara et al explored the combination of AgNPs with antibiotics (SACs) to create new antimicrobial agents effective against multiple antibiotic resistant (MAR) ESKAPE microorganisms[215]. SACs reduced concentrations of antibiotics needed for having the desired antibacterial/bactericidal effect by 32-fold, were bactericidal and retarded bacterial regrowth [215]. MIC of different antibiotics in SACs against *E. faecalis, K. pneumoniae, S. aureus, P. aeruginosa, A. baumannii* and *Enterobacter* supp, were in the range 0.25-4.0 μg/mL, while those of AgNPs were 0.1-3.7 μg/mL[215]. Synergy was estimated in 7 out of 9 cases. BF inhibition was in the range 40-83%. Specifically, BF by *S. aureus* was inhibited by 83%, by *K. pneumoniae*, by 65%, by *E. faecalis, P. aeruginosa* and *Enterobacter* supp, by 45%, while that by *A. baumannii*, by only 40%. Eradication of BF mature was instead in the range 30-73%. Despite, as many other authors, Mishra et al claimed to have carried out mechanistic studies concerning the BF inhibition, by demonstrating a certain loss of bacterial cell membrane integrity, and lack of cytoplasmic protein to confirm lysis, in this work no real mechanistic investigation was carried out[215]. Anyway, this paper by Mishra et al is of paramount relevance since in a scenario where MDR *K. pneumoniae* is a rising problem and the rate of BF formation in these isolates is high, AgNPs proposed in the study could be a solution to this problem, resulting antimicrobial against MAR ESKAPE microorganisms[215]. Elsaid et al biosynthesized AgNPs using *E. camaldulensis* leaf extract and essayed them against 50 MDR *K pneumoniae* isolates from [216]. MDR *K. pneumoniae* clinical isolates, which were resistant to most of tested antibiotics and BF-forming ability was detected in 56% of strains (28 out of 50), isolated from blood (32%), swab (14%), urine (18%) and sputum (36) of infected patients [216]. MICs of AgNPs were in the range 15.6-125 μg/mL, AgNPs significantly reduced the BF formation as 26 out of 28 isolates became non-biofilm producers and 2 out of 28 became weak producers[215]. Specifically, the MIC which was observed in 94% of bacteria tested (50) was 62.5 μg/mL, while BF inhibition at MIC was = 100% in one case, ≥ 90% in 7 cases, ≥ 80% in 6 cases, ≥ 70% in 8 cases, ≥ 60% in 5 cases and > 50% in one case. Conversely, although in our opinion the assertion of authors is questionable, they claimed that AgNPs showed minimal cytotoxic concentration and viability was up to 100 and 99.4% in normal human lung fibroblast cells (MRC-5) cell lines treated with AgNPs at conc. of 2 and 3.9 μg/mL respectively[216]. Despite, no real mechanistic investigation was carried out, this paper by Elsaid et al is of paramount relevance since in a scenario where MDR *K. pneumoniae* is a rising problem and the rate of BF formation in these isolates is high, silver nanoparticles proposed in the study could be a solution to this problem. The major limitation of AgNPs by Elsaid et al is their substantial cytotoxicity on eukaryotic cells, being the reported concentration safe for cells strongly lower MICs. In this regard, the IC_{50} of AgNPs versus MRC-5 cells was 46.9 μg/, that their selectivity index (SI) for MDR *K. pneumoniae* isolates, rather than human fibroblasts (MRC-5) were < 1 in the 96% of isolates and > 1 only in the 4%. To counteract the increasing resistance of *K. pneumoniae* to carbapenems and its BF, the antimicrobial and ABF activity of biogenic silver nanoparticles (Bio-AgNPs), synthesized using *Trichilia catigua* extract was assesses by Wozeak et al [217]. Very good MICs (0.49 to 15.62 μg/mL) were determined, susceptibility assays were performed, time-kill analysis, flow cytometry, and electron microscopy were carried out [217]. Eradication of BFs required 16-64 × MICs concentrations (7-29, 250-1000 μg/mL). Time-kill assays showed a concentration-dependent bactericidal effect, with 4 × MICs concentrations rapidly eliminating viable cells [217]. Despite membrane destabilization, increased fluidity at lower concentrations, and extensive structural damage at higher concentrations were observed and claimed

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as the major mechanism of action for bactericidal activity of Bio-AgNPs, no mechanistic insights was carried out concerning their capacity to eradicate BF. Anyway, Bio-AgNPs by authors unequivocally demonstrated potent antimicrobial and ABF effects against MDR *K. pneumoniae*, thus supporting the potential application of Bio-AgNPs as a therapeutic alternative for BF-associated infections, without any need of additional and cost-effective experiments[217]. This study, as such, highlights the bactericidal and ABF potential of Bio-AgNPs) as an effective tool against MDR *K. pneumoniae*, with efficacy against both free-living cells and structured BF [217]. Also, the AgNPs chemically synthesized by Siddique et al demonstrated antibacterial activity against MDR *K. pneumoniae* strains[218]. Trying a sort of mechanism of action of AgNPs in inhibiting BF, the effect of AgNPs on production on extracellular polymeric substances (EPS) constituting it was evaluated, but QS pathway or markers were not indagated, and a real mechanism was not proposed [218]. The cytotoxic potential of AgNPs on HeLa cell lines was also determined for evaluating their possible clinical application. AgNPs exhibited an MIC of 62.5-125 μg/mL (as reported in the paper of Elsaid et al), while their MBCs were 250 and 500 μg/mL[218]. The production of extracellular polymeric substance decreased after AgNP treatment while cellular protein leakage increased due to higher rates of cellular membrane disruption by AgNPs. The percentage BF inhibition was evaluated to be 64% for *K. pneumoniae* strain MF953600 and 86% for MF953599 at AgNP concentration of 100 μg/mL. AgNPs were evaluated to be minimally cytotoxic and safe at concentrations of 15-120 μg/mL[218]. Although real mechanistic insights were not carried out but only the membrane damage and reduction of EPS were observed, the data evaluated by this study provided evidence of AgNPs being quite safe antibacterial and ABF compounds against MDR *K. pneumoniae*, which is of paramount importance in a scenario where carbapenems often fail.

5.3.9. Innovative Strategies

Already in 2013, the ClpP activator acyldepsipeptide antibiotic (ADEB-4), providing an a-specific protease y degrading over 400 proteins, was shown by Conlon et al. capable to kill *S. aureus* persister BFs cells *in vitro*, forcing cells to self-digest. When ADEB-4 was administered in combination with rifampicin, produced complete eradication of *S. aureus* BF *in vitro* and *in vivo*, using a mouse model of a chronic infection. Such findings indicate a general principle for killing dormant cells—activation and corruption of a target, rather than conventional BF inhibition. Eradication of BF in an animal model by activating a protease suggests a realistic path towards developing therapies to treat chronic infections[219]. Dysregulating proteolytic machinery was an early example representing a unique approach targeting bacterial persistence[219]. Later in the year 2021, andrographolide sulfonate (AS) evaluated by Zhang et al. inhibited MRSA biofilms by 40–70%, likely through membrane and metabolic interference [226]. Specifically, AS is an active component of the traditional herbal medicine *Andrographis paniculata*, which was tested at three enormous doses (6,250 (1/8 MIC), 12,500 (1/4 MIC, and 25,000 (1/2 MIC) mg/mL), being MIC of AS 50 mg/mL, against MRSA BF *in vitro*, observing BF higher permeability and 60-70% inhibition. But more important for this review, real-time PCR and metabonomic studies were used to explore the mechanisms of the AS ABA, which were mediated by the inhibition of the expression of QS system regulatory genes, microbial surface components–recognizing adhesion matrix genes, intercellular adhesion genes, and a gene related to cellular eDNA release, and by the downregulation of five BF-related metabolites, including anthranilic acid, *D*-lactic acid, kynurenine, *L*-homo citrulline, and sebacic acid [226]. This study provided valuable evidence for the activity of AS against MRSA and its BFs and extended the methods to combat MRSA chronic infection [226]. These papers on innovative ABF strategies, containing correctly carried out mechanistic investigations, have been already inserted in Table 7. Anyway, additional papers were found and were included in Tables 10 and 11. Specifically, Table 10 collects 45 papers on innovative approaches developed to inhibit/eradicate BF, with pro and limitations, among which, no paper contained mechanisms investigations. Other papers on innovative ABF strategies divided for typology (2024–2026), found in relevant reviews on the typology or in single articles, containing or not containing mechanistic insights, correctly performed

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are included in Table 11. Such papers were found using Scopus and PubMed datasets, as usual, using all keywords used till now, but imposing a restricted range of years (2020-2026).

Table 10. Recent (2020-2026) innovative strategies for the prevention and eradication of BF in medical settings, not containing mechanisms investigations.

Type of Innovation	ABAMs	Device/Context	Type/Efficacy Evaluation	Advantages	Limitations	Ref
Nanomaterials (AgNPs)	NOM	UCs	⇓⇓ BF in *E. coli* *	Broad antimicrobial spectrum	Risky, uncontrolled Ag release	[227]
ECS (DNase I via AC-EPD)	NOM	TDs	⇓ BFB of *S. aureus*/*E. coli* *	⇑ Targeting, simple coating	⇓ Stability, ⇑ cost	[228]
Enzymatic [b]	NOM	Silicone catheters	⇑⇑ ABFA (*S. aureus*) *	⇑ Specificity (*Staphylococci)*	Narrow spectrum AMA	[229]
PT (ozone, O_3)	NOM	R/V or pathways	⇓ BF of *P. aeruginosa* *	Antibiotic-free, rapid AMA	Safety challenges	[230]
AMPs	NOM	CVCS/PSTY	⇓ *S. aureus* BF *	Novel class of molecules	⇓ Stability, ⇑ cost, haemolysis risk	[231]
Nano-porphyrin (PDT)	NOM	GMDs	⇓⇓⇓⇓ *S. aureus* BF *	Synergistic action, ⇑AMA	ELS, ⇓Clinical translation	[232]
P/PA (CFS)	NOM	Implantable/ODs	⇓ Surface colonisation *	Natural origin, ⇓ cytotoxicity	Variable composition	[233]
PCCs (Ag/TiO_x)	NOM	GMDs	BF ⇓ (all species) *	Long-term AMA	Cytotoxicity # related to metal release	[234]
Hydrophilic coat/AgNPs	NOM	GMDs	⇓ BA and growth *	Simple application to MDs	⇓Durability under real-use conditions	[235]
MNCs/CUR (nano-CS)	NOM	Medical implants	⇓⇓ BF *	Natural origin, biocompatible	Scalability and dosage control #	[236]
HAMP (CM-10K14K)	NOM	Foley catheters	⇓⇓ BF (*E. coli*) *	BF-targeted peptide design	⇓Stability/activity *in vivo*	[237]
PDA/uhPDMA	NOM	Catheters	Minimal/no BA *	Biocompatible, durable SUCs	Lack of clinical validation data	[238]
MMs ("Aqua Sperm")	NOM ***	Tubing/Flowlines	Effective BF removal *	⇑ Innovative physical removal	Early POC, complex implementation	[239]
SLIPS	NOM	Tubing/GMDs	⇓⇓ Biofouling *	Smart SUCs, ⇑-lasting effect	Regulatory# for triclosan use	[240]
FOTyr-AMP (NO donor)	NOM	GMDs/Implants	⇓⇓ BFB *, **	NO activity	Dose control and peptide stability #	[241]
Biosurfactants: LIPO/RLIP	NOM	Implant mimicking	⇓ Multispecies adhesion *	Natural, metabolically derived	Variable composition, ⇓ scalability	[242]]
RK22	NOM	GMDAs	⇓ BF development *, **	Targeted ABF peptide	⇓Peptide stability/production ylides	[243]
Ni–Cu–Zn NSFs	NOM	GMDAs	⇓ CFU count and BFB *	Durable magnetic material	Cytotoxicity # due to metal release	[244]

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AgCTSs	NOM	Implants/SIs	⇓⇓ BF *	Well-established SUCs	Ag release/ biocompatibility #	[245]
AgAK	NOM	DMs	⇓ MC by *S. mutans* *	Synergistic effects	Durability vs. cytotoxicity #	[246]]
PT with Er:YAG laser	NOM ****	ODs	POC, CSD	CFM, rapid action	Special equipment/safety control #	[247]
NSMs e.g., 1,8-cineole	NOM	UCs/PSs	⇓ CFU and BFB *	⇓⇓ Cost, available compounds	Variable efficacy; lack of *in vivo* data	[248]
PNMs (*Allium* extracts)	NOM	GMDC	⇓ Surface colonisation *	Natural origin, ⇓ cytotoxicity	Variable composition, normalization #	[249]
PPCs	NOM	MD surfaces	⇓ BF (all species) *	High chemical diversity, ⇓cost	⇓⇓Stability, no translational studies	[250]
LYSO enzymes	NOM	Catheters/SIM	⇓⇓ BA and BFB *	BIOS, possible SIMM	⇓⇓Enzyme stability, ⇑ production cost	[251]
NABFFs	NOM	Medical catheters	⇓⇓ MC *	Easy coating, compatible	⇓⇓Coating durability in practical use	[252]
DMPEI@PVC	NOM ***	PVC catheters	⇓⇓ in BA *	Simple application to PDs	Cytotoxic cations/regulatory #	[253]
PCCs (Ag@TiO_2/Ag@N–TiO_2)	NOM	UCs	⇓⇓ BF *	⇑-term AMA, ⇑ stability	Ag release dependent on UV/Vis light	[254]
MCHI	NOM ***	SIs/Catheters	⇓ MC *	Biocompatible, intrinsic AMA	⇓ ⇑-term/mechanical stability/durability	[255]
$ZnCl_2$@silica	NOM	Silica nasal splints	⇓ BF formation *, **	⇓Cost, local ion delivery	Irritation/dosage/leaching #	[256]
GLYC	NOM	Latex catheters	⇓ BA and BFB *	NACs; simple coating process	Variable comp, standardisation #	[257]
NZ/E catalytic system	NOM	Medical implants	⇓ Multispecies BF *	⇑CAT, OK for SIMM	⇓ CAT control/biosafety	[258]
MMs: U-µrobot)	NOM ***	UCs	Effective BF removal *	⇑Innovative; DIRCO	Complex implementation/regulatory #	[259]
Nano-SiO_2@RES@GSH	NOM	Foley catheters	⇓ BA and BFB *	Smart surface modification	Loading stability/release kinetics#	[260]
NEs (*Illicium verum*)	NOM	Urethral catheters	⇓ CFU count and BFB *	⇓Cost, available NAC	Variable potency, no *in vivo* validation	[261]
NSMs MDS YH7	NOM	Medical implants	⇑⇑ Inhibition of BF *	Dual redox–ABFA	Early POC, lack of toxicological profile	[262]
MA@CHI	NOM	Ti6Al4V Dis	⇓ MC *	Biocompatible, simple coating	⇓Long-term stability in oral cavity	[263]
PAICs	NOM	Catheters/SIs	Eradication of MRSA BF *	Precise activation	ELS required/phototoxicity #	[264]
Ag_2S@H–CeO_2 NCs	NOM	WTAs	⇓⇓ BF reduction *	Synergistic effects, ⇑⇑stability	Control of ion/metal release required	[265]

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GPH@AgNP@HCs	NOM	Catheters/implants	⇓ BA and CFU count *	⇑⇑ Surface-based AMA	Cytotoxicity and metal ion release #	[266]
PDMS@CAPS	NOM	PDMS-MDs	⇓ MC *	Simple, ⇓ cost PADD	⇑-term stability/biocompatibility #	[267]
ESCMs	NOM	SSs/MFPs	POC; ⇑⇑ BF removal *	CFM, ODCM	Power supply, electrical safety #	[268]
UA@$BiVO_4$/MCF@Cs	NOM	Coatings for MD	⇑⇑ ABFA *	⇑ Remotely activated system	Ultrasound equipment, heat generation #	[269]
RuSa1 BAF	NOM	GMDC	⇓ CFU and BFB *	⇑Specificity to target bacteria	⇓Host range; phage resistance	[270]
EBAFs	NOM	GMDC	⇑ ABFA *	⇑Delivery and phage viability	Standardisation/⇑ scale/immunogenic #	[271]

[a] Lysozyme-like lysostaphin immobilised on silicone; BIOS = biologic selectivity; PDs = polymeric devices; CAT = catalytic activity; NACs = natural compounds; PADD = polymer additive; NOM = no mechanism of action for BF eradication, inhibition (QSI, ABF genes, gene for BF formation inhibition, etc.); ODs = orthopaedical devices; SIM = surgical implants; DIs = dental implants; SIs = surgical instruments; WTAs = wound treatment applications; SSs = sterilisation systems; MFPs = medical fluid pathways; MDs = medical devices; GMDC = General medical device context; QSI = quorum sensing interaction; CSIs = cationic surface interactions; BACE = bactericidal effects; PTT = photothermal therapy; MD = membrane damage/disruption; AMA = antimicrobial activity; SEM = surface energy modification; AAE = anti-adhesion effect; SD = surface decontamination;* *In vitro;* ** *In vivo;* *** mechanical, **** mechanical and/or thermal;#Problems, challenges, risk, risky for, issues; BA = bacterial adhesion; CSD = clear surface decontamination; POC = proof of concept; PT = physical technology; AMPs = Antimicrobial peptides (AMPs); PDT = photodynamic therapy; P/PA = post/probiotic approach; CFS = cell-free supernatant; PCC = photocatalytic coatings (Ag/TiOx); HPC = hydrophilic coatings with AgNPs; MNCs = magnetic nanocarriers; HAMPs = hybrid antimicrobial peptide; PDA = Polydopamine; uhPDMA = Ultra-Hydrophilic Poly(dimethylacrylamide); MMs = micromotors; AS = Aqua Sperm; SLIPS = surface with controlled triclosan release; FOTyr-AMP = nitric oxide (NO) donor AMP; LIPO/RLIPs = lipopeptides/rhamnolipids biosurfactants; RK22 = anti-biofilm (ABF) peptide; NSFs = Ni–Cu–Zn nano-ferrites; AgCTSs = Silver-coated titanium surfaces; AgAK = bio-nanocomposite "alginate/kaolin/Ag"; NSMs = natural small molecule (e.g., 1,8-cineole); PNM = plant-derived natural metabolites (Allium extracts); PPCs = plant-derived phenolic compounds; LYSO = lysozyme (hen egg white/recombinant human; NABFFs = nanomaterial-based antibiofilm formulation; DMPEI/PVC = N,N-dodecyl, methyl poly ethylenimine coating on polyvinyl chloride (PVC); Ag@TiO_2/Ag@N–TiO_2 = photocatalytic coatings (PCCs); MCHIBP = modified chitosan biopolymer; ZnCl2/silica = zinc salt incorporated into silicone; GLYC = biosurfactant (glycolipid from *Candida* sp.); NZ/E = nanozyme/enzyme-based catalytic system; GSH = reduced glutathione; FN-SiO_2 = functionalised nano silica; RES = resveratrol; NEs = natural extracts (Illicium verum); NSMs = novel small molecules; MDS = maleimido@diselenide; MA@CHI = methacrylic-anhydride–modified-chitosan (biopolymer); PAIRC = photoactive iridium complexes; NCs = nanocomposite (Ag_2S@H–CeO_2); GPH@AgNP@HCs = graphene–AgNP hybrid coating; PDMS@CAPS = polydimethylsiloxane (PDMS) functionalised with capsaicin (CAPS); ESCMs = electro–self-cleaning membranes; UA@$BiVO_4$@MFCC = ultrasonically activated $BiVO_4$/mesoporous carbon foam (MCF) composite; RuSa1 = BAF = bacteriophage; CUR = curcumin; EBAFs = encapsulated bacteriophages; TBL = targeted bacterial lysis; ⇓ reduction; ⇓⇓ high, strong (SR), marketed reduction (MR); ⇓⇓⇓ very strong reduction (VSR); BF = biofilm; BFB = biofilm biomass; ABF = antibiofilm; ABFA = ABF activity; ⇑ high, higher; improved, enhanced⇑⇑ = strong, marketed; ⇑⇑⇑ very strong; MC = microbial colonisation; BFP =biofilm penetration; USs = ultrasounds; ROS = reactive oxygen species; BFD = biofilm destruction/degradation; EL = Electrochemical; RC = redox cycling; TDs = titanium discs as implant surface model; SCs = silicone catheters; R/V = respirators/ventilator; CVCSs = central venous catheter surfaces; PSTYSs = polystyrene substrates; PSs = polymer surfaces; GMDs = general medical devices; GMDAs =GMD applications; DMs =dental materials; SIMM = surface immobilisation, SUCs = surfaces

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coatings; CFM = chemical-free method; ODCM = on-demand cleaning method; ELS = external light source; DIRCO = direction controllable.

Table 11. Other papers on innovative ABF strategies divided for typology (2024–2026), found in relevant reviews on the typology or in single articles, containing (bold) or not containing mechanistic insights, correctly performed.

Type of Innovation	Mechanism of Action (Brief)	Device/Context	Efficacy	Advantages	Limitations	Refs.
Liposomal NDSs	⇑ BFP, targeted release	General BFAI	⇑AMEs *	HBC, HCR	Variable stability, ⇑ production cost	[272]
Nano-emulsions	⇑ BFD/BFP	Chronic bacterial infections	BFMR *	EF, ⇑ delivery	Physical instability possible	
Nanogels	⇑LC, controlled release	BF on medical devices	⇑⇑⇑ BFMR *	⇑ Carrier versatility	Potential cytotoxicity	
Polymers/dendrimers	EPS matrix disruption; ⇑ BFP	Multispecies biofilms	*In vitro*	CV, tuneable activity	⇑Synthetic cost	
anti-CD54@Cur-DA NPs	⇑BFP, QS interference	ABF, BF infections	⇑⇑⇑ ABFA**	⇑ AME**	Risk of cytotoxicity	
MOFs	⇑Surface area, prolonged release	Polymicrobial infections	*In vitro*	Customizable	⇓Stability in physiological fluids	
CMVs	Biomimetic delivery, ⇑ targeting	MDRI	*In vitro*	"Stealth" BP	Complex production	
BZnNPs	Nitro-oxidative stress; BFD	Medical device surfaces	80% BF inhibition *	GS, ⇑ efficacy	⇓Stability data	[273]
BZnOPB	LIDBE	⇑Shelf-life, RBC, for FPI	97.5%/76.2% RBC *	SA vs MPs	Toxicity evaluation needed	[274]
BPh therapy	Targeted lysis of BFs	Catheters, PSs, IDs	93.75% recovery	⇑⇑ Specific; safe	⇓ Host range, resistance possible	[275]
EBPh	⇑Phage stability, ⇑BFP	Medical implants	⇑ ABA *	⇑⇑ Activity/delivery	Standardization and scaling challenges	[275]
CRISPRi	QS pathways, gene *fimH*	*E. coli*/recalcitrant BFs	52-76% BF inhib. *	⇑⇑ ABFA in UT	Delivery into UTI infections	[276]
NPs as CRISPR carriers	⇑Cellular uptake; protected CRISPR	BF on devices	*In vitro*	Synergistic effect	NP cytotoxicity must be controlled	
AuNPs deliver CRISPR	3.5-fold ⇑ Editing efficiency	GDAI	*In vitro*	⇑⇑ EE	⇑ Cost of gold nanomaterials	
Co-D: CRISPR/AB/AMPs	Synergistic elimination of BF	MDRI	⇑⇑ BFMR *	DMT	Formulation stability challenges	

Cur = Curcumin; PAMAM = Cur loaded amino-ended poly(amidoamine) dendrimer; Anti-CD54 = antigen CD54; DA = 2,3-dimethyl maleic anhydride; biotin-PEG-Plys= biotin modified with poly(ethylene glycol)-polylysine); anti-CD54@Cur-DA NPs = Cur-DA NPs modified with anti-CD54; GETR = gene editing targeted resistance; AMEs = antimicrobial effects; RBCs = reduced bacterial contamination; EF = easy formulation; HCR = highly controlled release; HBC = high biocompatibility; CV = chemical versatility; BP = biological profile; GS = green synthesis; FPI = food packaging industry; SA = selective activity; MPs = multiple pathogens; NDSs = Nano delivery systems; PSs = prosthetic surfaces; IDs = indwelling devices; GDAI = general device-associated infections; BFAI = biofilm-associate infections; MDRI = multidrug resistant infections; Co-D = co-delivering; * *in vitro* studies; ** *in vitro* and *in vivo* studies; LIDBIE = light irradiation dependent bacterial inactivation efficiencies; LC = loading capacity; BF = biofilm; BFP = biofilm penetration; BFD = biofilm disruption; BFMR = biofilm mass

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reduction; ABFA antibiofilm activity; ⇑ = hight, higher, improved, enhanced (more arrows means more intensity); SiO_2 NPs = silica bioxide nanoparticles; NPs = nanoparticles, AgNPs = silver NNPs; MOFs = metal-organic frameworks; CMVs = cell membrane vesicles; BZnNPs = biogenic Zinc NPs; EPS = extracellular polymeric substance; QS = quorum sensing; AMPs = antimicrobial peptides; BZnOPB = biogenic ZnOn/polyhydroxy butirrate NPs; EBPh = encapsulated bacteriophages; AB = antibiotic; EE = editing efficiency; DMT = dual mechanism therapeutic; ⇓ = limited, low, reduced, narrow.; UT = urinary tract; UTI = urinary tract infections; CRISPri = a gene specific editing tool to control UTI. In bold paper where mechanisms for biofilm inhibition have been indagated.

5.6. Overall Interpretation

Across all compound classes, two major limitations consistently emerge:

1. Predominance of *in vitro* static models, with limited evaluation of mature BFs or dynamic systems.
2. Scarcity of *in vivo* and toxicity studies, which hinders clinical translation of suggested compounds.

Concerning the scarce mechanistic validation, is not an actual problem in our opinion but only a side aspect of research on BF. If mechanisms investigation are existent, and correctly carried out, they can bring further relevance to studies, but incessant research should not be sacrificed to additional and not always performable or highly difficult to be carried out correctly, mechanistic insight. Works, also without ABF mechanisms investigations should/must go on be carried out to produce an increasing number of devices potentially usable to counteract BF-sustained infections not differently treatable. Surely, if docking or even indirect assays are performed, they need *in vitro* or *in vivo* confirmations. Future research should instead prioritize the production of standardized BF models, and comprehensive *in vivo* assessments to bridge the gap between laboratory findings and therapeutic application.

Collectively, information collected in Tables 6, 7, 8, and the followings Table 9 and 10 on innovative ABF strategies, established that, for fortune (in our opinion), there is a strong excess of papers (92 out of 126), whose authors preferred discovering as numerous as possible new smart materials with efficient ABA and low cytotoxicity at the concentration needed, rather than indagate the reason for their functioning which were elucidated only in 34 out of 126 articles considered.

5.7. Principal Component Analysis (PCA) on Results Reported in Tables 6, 7 and 9-11

What About PCA?

PCA is a multivariate chemometric technique frequently used to process datasets collecting very numerous correlated data (variables), most of which containing trivial information, to reduce them to a small number of non-correlated orthogonal variables (principal components, PC), providing the most important information [277,278] To carry out PCA, data must be arranged in matrices of n columns × n rows as described [279]. The matrices are processed by PCA. PCA results can be represented as score plots, loadings and biplots. In the score plots, scores are the new coordinates of the processed samples in the new space of the non-correlated PCs, where each sample assumes a position (score), depending on its category/typology associated to selected variables [280]. In loadings arrows redirected versus specific variables can be observed, and in biplots, arrows redirected versus specific variables can be observed in samples area (representing their score plot) positioned at certain scores. When all data are comparable (number of papers in terms of mechanistic investigation or typology for year), as in our case, it is possible to merge data from different matrices in an overall matrix containing all data. Additionally, it is possible using those of a matrix, called external dataset (EDS) and uploading them in the space of already processed data, deriving from a principal dataset (PDS). Samples located close to each other share similar characteristics, while those placed far apart could differ for different characteristics [280]. Note that, in PCA, principal components (PC1, PC2, PC3, etc.) do not have a fixed meaning valid for all datasets. PCs take on different meanings depending on the data type (in our case, number of papers for year and for typology), variables, and

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correlations. PCs are directions of maximum variance (intended as explained variance %, as shown in the following Figures 7–9), constructed specifically for that dataset. PCA works by finding new non-correlated coordinates (PCs), represented as x and y axes in the score plot. Considering PC1 (x axes) vs. PC2 (y axes), PC1 represents the direction along which the data variance is maximum, and PC2 represents that with maximum variance orthogonal to PC1, and so on. This means that each PC is a linear combination of the original variables specific to that dataset. Since often, each dataset has different correlations, different scales, different variances, and different structures, its PCs will be different and will have different meanings, and PCs will not represent the same "phenomenon" in different datasets.

A large matrix of 108 rows (ABF compounds reported in 126 papers) × 17 columns (years in the range 2011-2026) for a total of 1836 measurable non-orthogonal variables, was constructed, to be the principal dataset (PDS). A smaller matrix of 2 rows (paper containing mechanistic insight (34) and papers without them (92)) × 17 columns (years in the range 2011-2026) for a total of 34 measurable non-orthogonal variables, was constructed, to be the secondary, as well as external dataset (EDS), which was loaded in the space of PDS. The systems were simplified by exploiting multivariate analysis, named principal component analysis (PCA), using CAT (Chemometric Agile Tool, which is freely available online at https://www.gruppochemiometria.it/index.php/software/19-download-the-r-based-chemometric-software, accessed on 30 April 2026). When possible, before PCA, the data were scaled and centered. The results were reported as score plots of PC1 vs. PC2 (Figure 7), loading (Figure 8) and biplots (Figure 9).

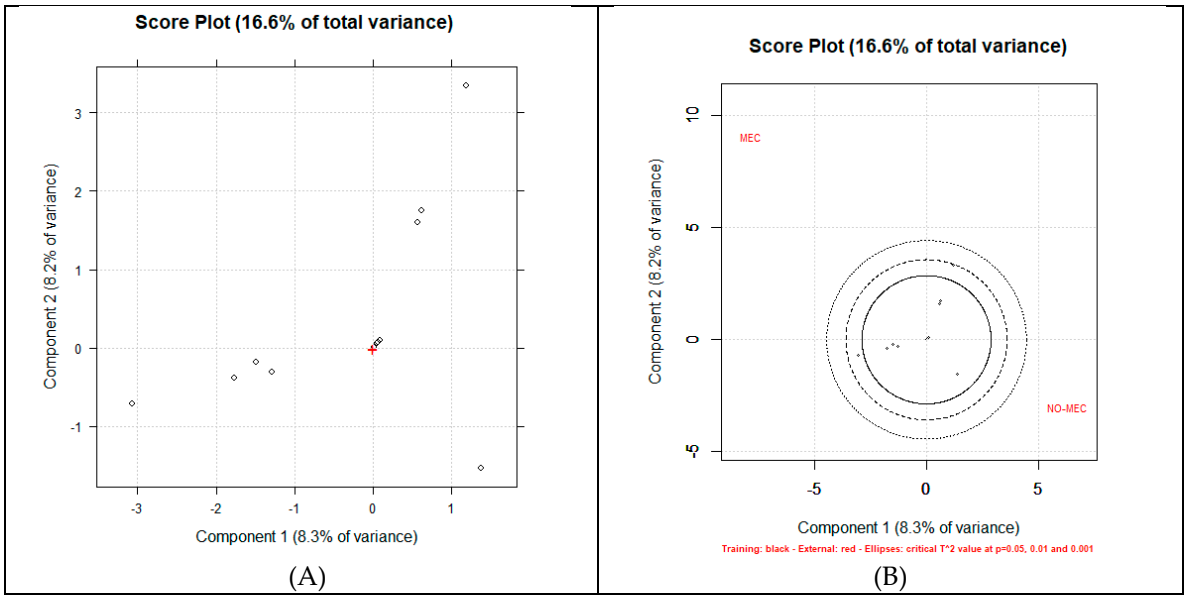

Figure 7. (A) Score plot of PC1 versus PC2 obtained processing PDS; (B) score plot of PC1 versus PC2 obtained processing EDS with PCA and uploading it in the space of score plot of PC1 versus PC2 of PDS.

In Figure 7A, the score plot of PC1 (explaining for the 8.3% of the total variance of 16.65%, explained by PC1 + PC2) was plotted versus PC2, which explained for 8.2% of variance of 16.65%. Since the total variance of the system was explained by numerous couples of PCs, despite 16.65% could seem a minimal value, it was the higher among variance explained by other couples of PCs. Empty circles represent papers, which often were overlapped because published the same year. The score plot of EDS processed by PCA and loaded within the space of PDS one, is instead observable in Figure 7B and evidenced that while on PC1, MEC papers were located at negative scores and NOMEC ones were clustered at positive scores, on PC2 it was the contrary. Can be observed that, despite overlapped papers, which affect the actual number of papers belonging to that space of score plot, especially on PC1, papers belonging to NOMEC area (positive scores) are more numerous than those appearing in the MEC one (negative scores), by at least two times.

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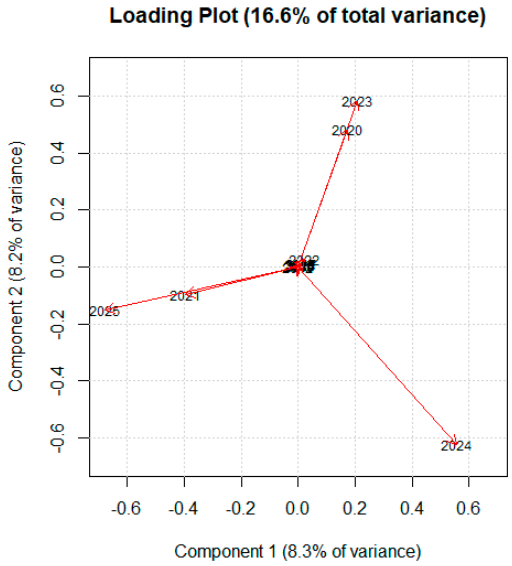

Figure 8. Loading plot of PC1 vs PC2.

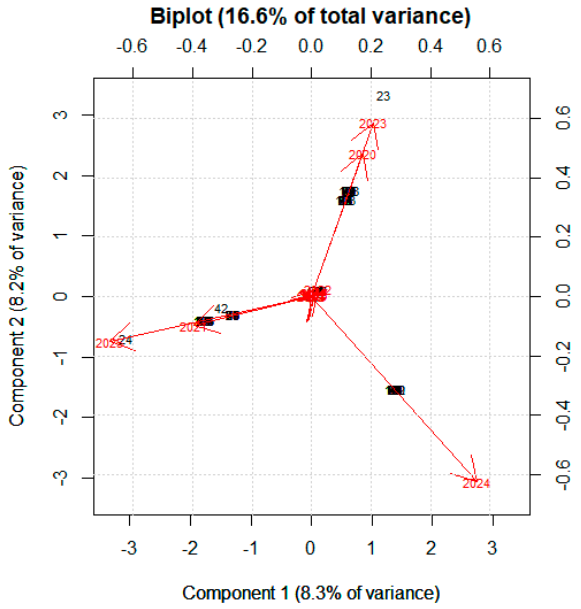

Figure 9. Biplot of PC1 vs PC2.

Figures 8 and 9 show the loadings, in this case representing the directions of certain publication years assumed inside the score plot of PC1 vs. PC2, and the biplot presenting both loadings and papers locations. Both images evidenced that years having direction towards negative score areas on both PC1 and PC2 where NOMEC positioned were recent (2020-2025), thus highlighting that the recent trend in research for new ABF compounds is that of not assessing mechanistic insights.

6. Conclusions

With this review, an all-round updated stated of the art of the research against BF has been provided. For doing this, the most used BF models for research at different levels have been presented and discussed, the current condition of available armamentarium to inhibit/eradicate young and/or mature BF was showed and correct methods to investigate ABF mechanisms have been displayed

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with discussion also on expensive instrumentation needed. Anyway, the *core* of this study consists of the several published papers, reported in different Tables and critically discussed, to understand how scientists, researcher, authors and Editors attribute importance to mechanistic insights, in the research on BF. Collectively, it was evidenced that, while mechanistic understanding could significantly elevate the relevance, reproducibility, and long-term impact of ABF research, they could be not universally mandatory for academic research, often not supported by external funds. As consequences, especially in recent years papers where mechanistic insights are missing are predominant and are anyway welcome.

Despite mechanistic inexperience may hinder reproducibility, comparability across studies, and rational optimization of promising leads, thus leading to the development of targeted compounds, while mechanistic clarity can transform empirical observations into actionable therapeutic concepts, we discovered that limits of ABF research are other than ABF mechanisms. As strenuous supporters of ABF research mainly directed to rapid discovery of new molecules active against BF, thus allowing faster advancements, rather than to understanding, we are glad to have found and provided a further confirmation that the residual gap existing between the relevance attributed to ABF papers reporting also mechanisms of action and that of those reporting not, should be definitely filled.

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