AMERICAN UNIVERSITY OF BEIRUT

NOVEL ANTI-BIOFILM NATURAL PRODUCTS FROM MICROORGANISMS ISOLATED FROM LEBANON

by
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AN ABSTRACT OF THE THESIS OF

Reem Imad Wazni for Master of Science
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Title: Novel anti-biofilm natural products from microorganisms isolated from Lebanon

Background: Antimicrobial resistance (AMR) is rising at an alarming pace causing a major crisis threatening public health globally. Among various mechanisms of drug resistance, bacterial biofilms, which are layers of microbial cells attached to a surface and buried firmly in an exopolysaccharide matrix, is considered a predominant one. Biofilm associated bacteria are less sensitive to antibiotics than the planktonic cells. Indeed, resistance to gentamicin and ceftazidime in *Escherichia coli*, tazobactam, and colistin in *Klebsiella pneumonia*, and ciprofloxacin in *Pseudomonas aeruginosa* was related to biofilm formation.
Since it is becoming increasingly clear that biofilm formation is implicated in the spread of multidrug-resistance, and conventional antibiotics are being inadequate at eradicating biofilm-forming pathogens, it is highly urgent to discover novel molecules that exhibit strong antibiofilm activity and can be combined with antibacterial agents. Fortunately, soil and marine microorganisms are potential natural sources for the isolation of natural compounds with effective biological activities. However, scarce material is found in the literature, and little research is being done about screening for antibiofilm compounds. This study aims to isolate novel antibiofilm compounds produced by environmental bacteria from several regions in Lebanon.

Methods: Marine and soil samples were collected from three different areas in Lebanon. Serial dilutions were done on the samples for Actinomycetes isolation. Subsequently, 30µl of each dilution were plated on ISP3 and soil agar. After purification, pure colonies were stored in 50% glycerol at -80°C. Then, these selected environmental bacteria were cultivated in 14 different culture media in order to produce secondary metabolites. The latter was then extracted via acetone/methanol and then tested for their anti-biofilm activity against *Pseudomonas aeruginosa* (PAN14), *Staphylococcus aureus* (N315), *Acinetobacter baumannii* (DSM 30008 & T36). Screening of the crude extracts was done in microtiter plates via two assays. The first checks extracts' potency to inhibit biofilm occurrence, whereas the other evaluates its capacity to inhibit biofilm formation after the film is established.

Results: According to our findings, crude extracts derived from the marine sample TBJ13C and soil sample BM9C demonstrated a strong anti-biofilm activity against *P. Aeruginosa* (PAN14). Both extracts with their hexane and ethyl acetate fractions, respectively, showed highly effective inhibition of biofilm growth, as well as eradication of preformed ones in PAN14. Further, crude extracts produced by MM3, MM7, and MM9 also exhibited significant anti-biofilm activity in preventing biofilm formation, primarily in PAN14, as some had the ability to do so in two other models, namely, *Staphylococcus aureus* MRSA *and Acinetobacter baumannii* (T36).

Conclusion: Therefore, this process steps forward from a screening hit to phenotypic and biochemical characterization, anti-biofilm compound isolation, molecular characterization, and structure elucidation.

CONTENTS

ILLUSTRATIONS

TABLES

CHAPTER I

INTRODUCTION

"There is no time to wait. Unless the world acts urgently, antimicrobial resistance will have disastrous impact within a generation," said the Interagency Coordination Center of the World Health Organization (WHO) in a recently published report on antimicrobial resistance (AMR) [1]. AMR, a complex global challenge that has emerged as one of the main public health crisis, has been the focus of research carried out over the past two decades [2]. This growing issue of resistance has been placed at the top of the health challenges list of the 21st century, as it substantially contributed to a wide range of unsalvageable microbial infections that were once easy to treat [3][4]. According to the " Antibiotic Resistance Threats in the United States, 2019" report, recently released by the Center of Disease Control (CDC), more than 35,000 people are dying each year in the US as a result of antibiotic-resistant infections out of the approximated 2.8 million who are getting severely infected [5]. Further, failure to tackle this significant healthcare problem, the mortality rate is estimated to reach 10 million deaths per year as of 2050[1]. In order to combat this growing concern, it is highly crucial to discover novel classes of antimicrobial agents acting on previously unexploited targets; however, to date, researchers are continually struggling to identify new ones.

Antibiotics have evolved naturally over millions of years as a competitive survival strategy among microorganisms. Their introduction to human medicine in the 1940s is thought of as one of the most significant medical advances [6]. Undoubtedly, these wonder drugs were considered as a magic bullet in the war of infection treatment, as they selectively attack microbial pathogens without affecting the host, thus saving millions of lives [7]. Despite this success, antibiotic resistance subsequently evolved as a mechanism of adaptation to antibiotic exposure resulting in drug inefficiency [6]. Ever since, uncertainty has arisen, as most pathogenic microbes have become resistant to common conventional antimicrobial drugs, hence, increasing morbidity and mortality rates worldwide [8]. The majority of these pathogenic bacteria were seen to exist in biofilm form, a predominant life-mode of most bacterial species[9]. Moreover, it has been likely that the matrix of the biofilm acts as a protective screen that provides a mechanical and biochemical defense, therefore creating additional bacterial tolerance to antimicrobial agents[10]. In this regard, studies have reported biofilm formation as a potent drug-resistant mechanism, adding it to the known classical ones mainly including, non-judicial use of antibiotics, active drug efflux, target site modification, and drug inactivation [11].

More importantly, over several years of research, accumulated evidence demonstrated that the advent of biofilm formation added significantly to the impact of infectious diseases[12]. According to the National Institution of Health (NIH) estimates, 80 % of the chronic infections are associated with the biofilm mode of growth [13]. Since these infections tend to be localized, persistent, and recurrent, 17 million new biofilm-associated infections occur every year in the United States, resulting in the death of 550,000

people[14] [13]. What is certain the most, is that this type of infection is usually difficult to treat as bacteria organized in a biofilm can highly withstand the host's immune system, thus, preventing its function in eliminating biofilm-embedded cells[15]. Further, knowing that the currently available antibacterial agents are only designated to target planktonic bacteria, the treatment of these persistent infections has become significantly challenging [16]. Therefore, this surge in biofilm-associated infections coupled with the multi-drug resistance calls for the discovery of novel anti-biofilm compounds that can effectively regulate and eradicate biofilm formation.

Up to now, a plethora of potential anti-biofilm agents inspired by natural products have been identified; however, due to their high toxicity levels, none of these agents is available in the market to date [17]. In the Department of Experimental Pathology, Immunology and Microbiology at AUB, previous work dealt with assessment of a number of anti-biofilm products on biofilm formation was done[18][19][20][21]. In this study, we aim to discover new novel anti-biofilm compounds isolated from soil and marine samples, rich sources of antimicrobial metabolites, in hopes that they would be less toxic and exhibit a higher potency in inhibiting biofilm formation and growth as well as disrupting performed ones.

CHAPTER II

LITERATURE REVIEW

A. General features of biofilm

Biofilm can be found widely in nature. It is the predominant mode of growth for most bacteria in industry, aquaculture, the human body, as well as in-hospital niches [22]. A commonly used definition of bacterial biofilm is a highly structured community of sessile microbial cells, permanently bound to an interface or other cells, and firmly attached to a protective self-produced extracellular matrix (ECM) [23]. This adhesive matrix, which is mainly composed of exopolysaccharides (EPS), extracellular DNA (eDNA), and proteins, provides strength to the interaction of the microbial aggregates in the biofilm, thus acting as a stabilizing scaffold for the three-dimensional biofilm structure[24][25]. Indeed, the EPS matrix accounts for over 90% of the dry mass of a biofilm[24].

1. Characteristics of biofilm

In its biofilm form, bacteria show a highly elevated pattern of adaptive resistance to antibiotics, display resistance against the host immune system, and survive in unfavorable environmental conditions[24][26]. Besides the fact that the EPS matrix protects constituent cells from external aggressions, which is one possible reason for the increased resistance, some bacterial cells within the biofilm, often referred to as persister cells, exhibit antibiotic tolerance and significantly compromise the efficacy of currently available drugs[14] [27]. Subsequently, biofilms have been proved to be more than 1000-

fold resistant to treatment with conventional antibiotics, normally used to treat planktonic cells[28]. In a microbial biofilm, the phenotypic switch from a planktonic lifestyle to a sedentary biofilm phase requires the coordinated regulation of numerous environmental and genetic factors[29]. As the cell density increases, most biofilm cells are likely to encounter nutrient and oxygen limitation as well as higher levels of waste products and secondary metabolites[30]. Consequently, this nutritional restriction along with oxygen and electron acceptors reduction, can result in physiological and genetic heterogeneity of sessile cells[31]. Owing to these properties, this mode of growth poses great challenges and render biofilm as one of the primary sources of chronic and persistent infections[22].

B. Biofilm life cycle

Since biofilm formation was recognized as a complex developmental process and considered as an essential virulence factor for many pathogens, its components and mechanistic details have attracted considerable attention in research over the past decade[32]. Indeed, biofilm formation generally occurs in four necessary steps (Figure 1).

1. Adhesion/Attachment

The first step of this process involves the adherence and attachment of bacterial free-floating cells, to a biomaterial or epithelial surface [19][33]. Upon intercepting the surface, these cells are mediated by several forces, mainly including hydrophobic interactions, protein adhesion, electrostatic interactions, and Van der Waal forces [23][26]. Following that, microbial cells attach reversibly to the surface through appendages like

fimbriae, pili, and flagella, with the help of specific surface-associated proteins such as OmpA, fibronectin-binding proteins, protein A, and biofilm-associated protein (BAP) [23] [33].

2. Production of EPS/Formation of micro-colonies

Once motile cells excrete the extracellular polymeric substance (EPS), they become more firmly attached and subsequently irreversibly immobilized on surfaces [12]. Through particular chemical signaling within the EPS, a process of multiplication starts, as motile cells develop into micro-colonies[33].

3. Maturation of biofilm

Subsequently and in optimal growth conditions, aggregated cells develop into multi-layered clusters, and undergo maturation forming a complex architecture of biofilm with water channels equipped to aid the flow of oxygen and nutrients into the matrix[34]. Moreover, this step requires the communication of microbial cells with one another through auto-inducer signals, resulting in the expression of specific biofilm genes[12].

4. Detachment and dispersion

In the final stage of development, as the biofilm reaches a specific critical mass, detachment and dispersion of planktonic cells take place, where they become ready to colonize new areas and reinitiate the biofilm-lifecycle[23].

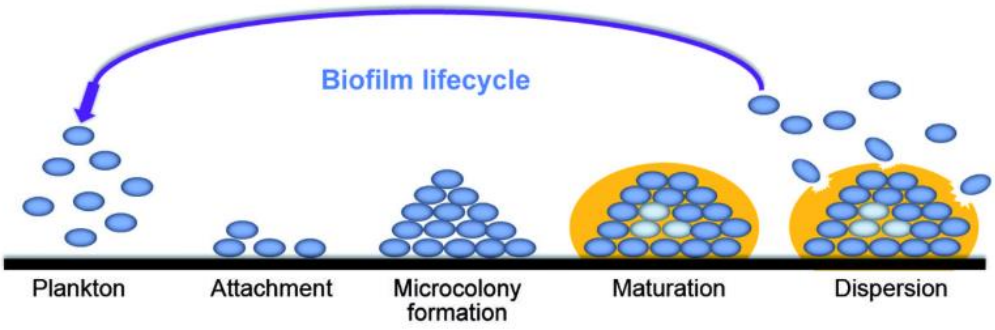


Figure 1. Biofilm development steps produced by microorganisms [31]

C. Regulators involved in biofilm formation

It is believed that a deeper understanding of the regulatory mechanisms involved in biofilm formation may ultimately provide insights into identifying alternative therapeutic interventions to control and prevent bacterial infections. From among the latter, quorum sensing (QS), bis-(3'-5')-cyclic di-guanosine monophosphate (c-di-GMP), and small RNAs (sRNAs) are the three primary regulators that have been the subject of intensive investigations, in research, in the past years [35].

1. Quorum sensing

Quorum sensing (QS), a cell-to-cell communication system, is an intercellular signal used as a specific language among sessile aggregates[35]. It is a cell density-dependent phenomenon, in which upon its increase, fluctuation in gene expressions occur, including those encoding production of virulence and pathogenesis factors[36]. This system has received much attention in research as it participates in the regulation of biofilm formation, particularly during the maturation and dispersal phases [35]. Typically in QS, bacteria produce, detect, and release small self- generated signal molecules called

auto-inducers (AIs). N-acyl homoserine lactone (AHL) and auto-inducing peptide (AIP) are two common auto-inducers used by Gram-negative and Gram-positive bacteria, respectively[37]. Further, autoinducer-2 (AI-2) is another signaling molecule, which is used by both types of bacteria for interspecies communication[13] [37].

2. C-di-GMP

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (C-di-GMP) is a ubiquitous intracellular second messenger that is primarily involved in the molecular transition between motile planktonic and sedentary-bacterial lifestyle[35]. According to recent discoveries, c-di-GMP plays a crucial role in stimulating the biosynthesis of adhesins and exopolysaccharide mediated biofilm formation, secretion of extracellular (eDNA), as well as controlling motility and virulence of planktonic cells [22]. Indeed, levels of c-di-GMP signaling in bacteria are regulated by the opposite activity of two enzymes, the diguanylate cyclases (DGCs) with a GGDEF domain and c-di-GMP phosphodiesterases (PDEs)[38]. These enzymes that respectively catalyze the formation and degradation of c-di-GMP have three protein domains named GGDEF, EAL, and HD-GYP, which are almost found in most bacterial species[39]. Further, c-di-GMP is known to be a central regulator of biofilm formation in almost all Gram-negative bacteria investigated to date and in some Gram-positive bacteria[40].

3. Small non-coding RNA molecules (sRNAs)

Small non-coding RNA (sRNAs) molecules are also vital regulators that have an imperative role in the bacterial biofilm formation process[41]. They significantly participate in the regulation of bacterial physiological behavior, where they control the switch between planktonic and sessile mode[41]. Small RNAs are ultimately advantageous for pathogens in adaptation and modification of the host immune response[41]. Besides, they are involved in virulence bacterial gene expression, adaptation to stress, as well as microbial pathogenicity [35]. Understanding the mechanisms that are adopted by such molecules is highly critical; however, studies pointing to the function of sRNA in the biofilm life cycle are limited, thereby, further research is required[42].

D. Mechanisms of biofilm-associated antimicrobial resistance

Knowledge of the genetic and molecular mechanisms of antibiotic resistance in biofilm formation is of great necessity for a rational design of anti-biofilm strategies. Since it seemed that the root of the antimicrobial resistance in biofilms is not related to the known familiar resistance mechanisms, four hypotheses have been proposed to explain the possible underlying mechanisms (Figure 2).

1. Failure of drug penetration

The first hypothesis involves the glycocalyx or exopolysaccharide matrix that may aid in the slow or incomplete penetration of antimicrobial agents [30][43]. The EPS matrix

mainly functions in regulating biofilm growth and support pathogenic bacteria to survive in extreme adverse host conditions [26] [39]. Its physical and chemical structure, as well as its high viscosity, has been significantly associated with the drug diffusion delay through the biofilm matrix[44]. According to some reported studies, one reason to explain this reduced penetration is the ionic binding between specific positively charged antimicrobial agents with negatively charged slime layer substances[45]. Although this hypothesis has been considered a vital resistance mechanism, it does not seem to be a predominant one, as recent evidence indicates that EPS is probably an efficient initial barrier to some types of agents such as small antimicrobial peptides but not to all antimicrobials [30][46].

2. Heterogeneous population and growth rate

Typically, biofilm communities consist of heterogeneous cellular populations that vary in their growth rates [44]. Within a biofilm, some internally localized cells are expected to experience nutrients and oxygen limitations or accumulation of deleterious metabolic products, which can hinder their multiplication [47]. Consequently, it has been shown that following a decreasing gradient in nutrients and oxygen, the cells in the periphery region of the biofilm are metabolically active and proliferate normally; however, inside niches, cells are inactive, slowly growing, and enter a dormant state[30]. More importantly, this physiological change minimizes the sensitivity of the dormant variants to antimicrobial agents that generally used to kill the metabolically active cells[47]. Further, under anaerobic conditions, bacterial cells inside biofilm express specific genes that result in increasing the level of resistance against antibiotics [30][48].

3. Neutralizing enzymes

While slow growth and heterogeneity may explain a great deal of the antimicrobial resistance properties of biofilms, other factors may also play a role. One such feature is the presence of neutralizing enzymes in the biofilm matrix that can degrade or inactivate trapped antimicrobial molecules[47]. Typically, these mainly proteinic enzymes accumulate in the EPS matrix and reduce the biofilm infiltration of antimicrobial agents, thereby preventing them from reaching their cellular targets[24]. Indeed, such enzymes confer resistance to antimicrobials by multiple biochemical mechanisms including hydrolysis or modification [47].

4. Biofilm-specific persister cells

The fourth hypothesis to explain the reduced biofilm susceptibility to biocides concerns the phenomenon of persistence by bacterial communities. Unlike the resistant cells, persister cells are dormant variants that do not grow in the presence of antibiotics[43]. Paradoxically, these cells exhibit a high tolerance to antibiotics, as they survive a lethal dose of antimicrobial agents that can normally kill a bulk of metabolically active bacterial cells [49]. Because persister cells can spontaneously shift out of their quiescent state and re-induce the growth of bacterial biofilm, once antibiotic concentration drops, they have been considered as the main culprit for reactivating chronic infections [49]. Moreover, this antibiotic tolerance was observed to occur in a majority of biofilm-associated pathogens, especially *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus*[50][51].

Even though the discovery of these cells is not recent, their study is still in its infancy[49]. Hence, a deeper understanding of their mechanism of formation is highly crucial to prevent biofilm-associated infections effectively [52].

According to several studies, the functioning of each of these mechanisms alone partially accounts for the increased antimicrobial recalcitrance in biofilm; however, the amalgamation of these defenses helps to ensure the survival of biofilm cells in the face of even the most aggressive antimicrobial treatment regimen[44].

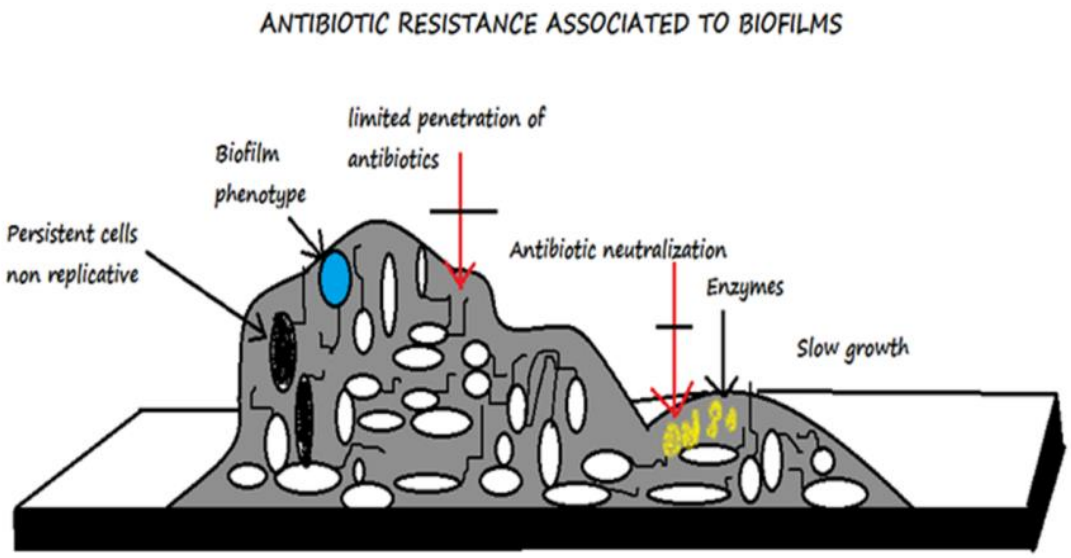


Figure 2. Antibiotic resistance associate to biofilm [44]

E. Biofilm-associated pathogens

It is now realized that nearly all bacterial species, including clinically relevant microorganisms, live in a biofilm state [35]. Indeed, 99% of bacteria in nature would be under this form[53]. By using multiple models of biofilm infections, several microbes that utilize biofilm formation were identified in the last three decades [32]. Amongst them,

Pseudomonas aeruginosa, Staphylococcus aureus, and *Acinetobacter baumannii* have been, and still to date, drawing considerable interest for researchers due to their involvement in the most complicated clinical infections[54][55][56].

1. Pseudomonas aeruginosa

P. aeruginosa is a gram-negative, non-lactose fermenting, motile, and aerobic bacterium that has turned out to be among the most virulent of opportunistic human pathogens[54]. This organism is particularly notorious for causing devastating chronic infections in immunocompromised and hospitalized individuals, leading to severe symptoms and, in many cases, death [31][35]. It is mainly associated with chronic infections of lungs in cystic fibrosis patients, as well as chronic urinary tract infections, ventilator-associated pneumonia, and chronic wounds[57]. According to the CDC, 32,600 infections among hospitalized patients and 2,700 estimated deaths in the United States were caused by multidrug-resistant *Pseudomonas aeruginosa* in 2017[5]. Due to that, and to the fact that *P. aeruginosa* is an avid biofilm former, this pathogen has been the key subject of biofilm studies and has received the most attention as a model organism in research [32].

a. Matrices of *P. aeruginosa* Biofilms

In *P. aeruginosa*, biofilm formation can be initiated through the adhesive action of several components, including flagella, type IV pili, fimbria, extracellular DNA, and Psl polysaccharide[25][55]–[58]. During biofilm formation, the majority of these factors

facilitate the transport and attachment of the motile P. aeruginosa to surfaces[32]. For maintaining structural integrity and antibiotic resistance of biofilm, *P. aeruginosa* produces at least three exopolysaccharides: the mannose- rich Psl polysaccharide, the glucose-rich Pel polysaccharide, and alginate [54][35]. Further, these components, together with eDNA, determine the biofilm architecture[62].

i. Alginate (Alg)

Alginate, a linear polysaccharide composed of D-mannuronic acid and L-glucuronic acid residues, predominantly constitutes the EPS matrix of biofilms conferring a mucoid phenotype (Figure 3) [41]. In a biofilm, alginate plays an essential role in structural stability, protection, as well as in water and nutrients retention [63]. Since it mediates biofilm antibiotic resistance and suppresses host immune response, alginate was found to be a hallmark of *P. aeruginosa* chronic infection, particularly in the lung infections of CF patients [35].

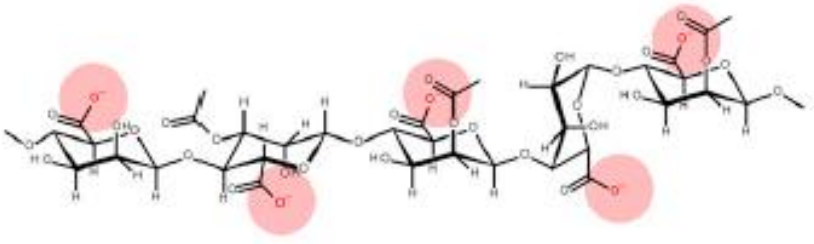


Figure 3. Structure of alginate polysaccharide [60]

ii. Psl and Pel

Since Psl and Pel are two main polysaccharides that comprise a major component of the biofilm matrix, they are potential targets for the control and possible eradication of biofilms formed by *P. aeruginosa*[64]. As it has been recently elucidated, Psl is a cell surface exopolysaccharide that appears to contain a repeating pentasaccharide composed of D-mannose, L-rhamnose, and D-glucose residues (Figure 4)[61]. It is a key element at the early stage of biofilm formation, where it promotes the initial surface adhesion and attachment process of sessile cells[61]. Besides its function as a structural scaffold, Psl can provide an instant protective role against anti-biofilm agents as well as a broad spectrum of conventional antibiotics, providing a survival advantage during pathogenesis[65].

Similar to Psl, Pel is a primary matrix structural polysaccharides in non-mucoid *P. aeruginosa* that initiates cell-cell interaction and maintain biofilm structure[62]. It interacts with extracellular DNA in the biofilm matrix and protects bacteria against antibiotics, particularly aminoglycosides[62]. Pel biochemical composition has not been clarified yet, but according to previous evidence, it is mainly composed of cationic exopolysaccharides[63].

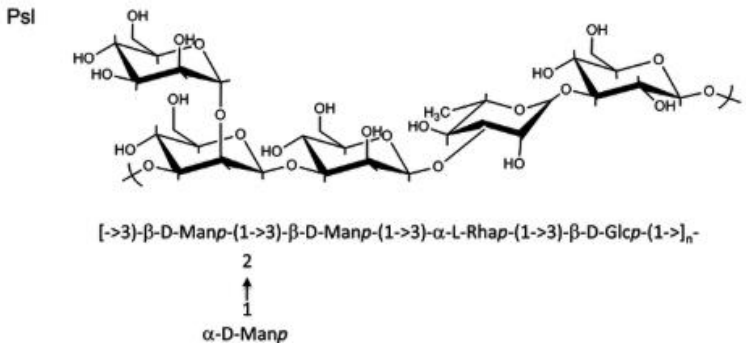


Figure 4. Structure of Psl polysaccharide [60]

b. Regulation of biofilm formation in *P. aeruginosa*

i. Quorum sensing (QS)

Recent findings have shown that the QS network, a complicated system in *P. aeruginosa,* is highly capable of countering adverse environmental changes[66]. It provides the pathogen with flexibility in the control of virulence gene expression as well as a variety of biological processes important for their growth and survival[66]. Generally, *P. aeruginosa* comprises two primary N-acyl homoserine lactone (AHL)-dependent QS based systems, the LasI/LasR, and RhlI/RhlR [67]. Besides that, it also contains a third intercellular system known as Pseudomonas quinolone signal (PQS) [67]. Both of the AHL systems comprise a gene encoding an auto-inducer, *lasI*, that is responsible for the synthesis of N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *rhlI* that is required for the production of N-butanoyl homoserine lactone [35]. In the PQS system, 2-heptyl-3-hydroxy-4-quinolone is known to be the sensing factor[68]. Further, one of the characteristics of these three systems is that they are interconnected in hierarchical order with LasR regulating the Rhl and Pqs systems (Figure 5) [67]. For the formation and persistence of *P. aeruginosa* biofilms, QS directs the production of essential biofilm factors [10]. Among these QS-regulated factors are rhamnolipids, the biosurfactant amphipathic glycolipids that are involved in maintaining the channels in a mushroom-shaped structure, along with the proper distribution of the nutrients and oxygen[69]. Indeed, QS regulates the production of the extracellular DNA that is associated with the stability of *P. aeruginosa,* as well as the antimicrobial resistance exhibited by the biofilms[66].

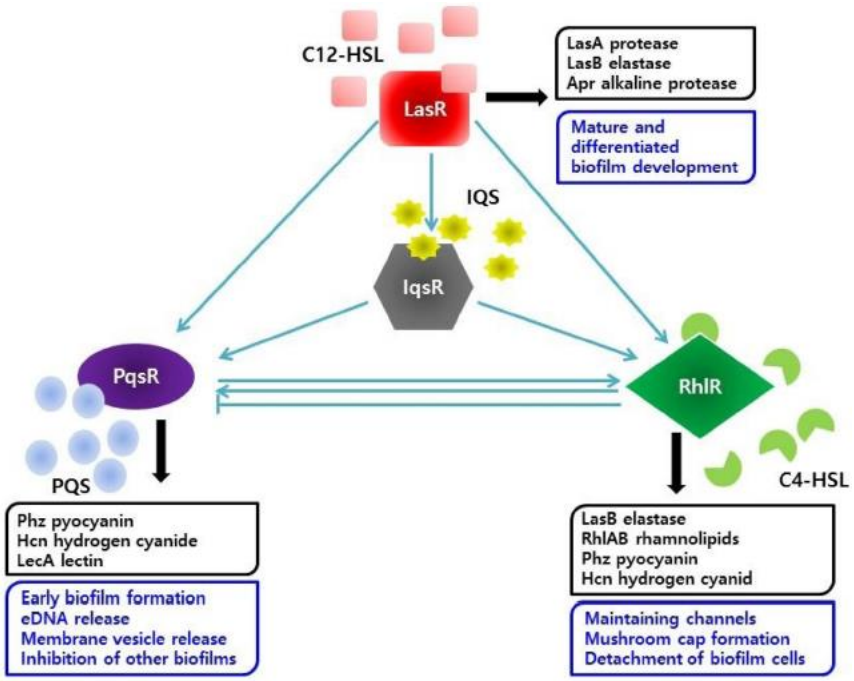


Figure 5. Interactions between quorum sensing systems of *P. aeruginosa* [145]

Blue arrows represent an activation effect. The blue perpendicular line represents an inhibitory effect. Black arrows represent virulence factor outputs (black box) and functions in biofilm development (blue box).

ii. C-di-GMP

Secondary messenger **(**c-di-GMP) is also an essential regulator that governs biofilm formation in *P .aeruginosa*. Typically, high intracellular levels of c-di-GMP positively regulate the synthesis of EPS matrix polysaccharides and adhesins, enabling bacteria to form biofilm[35][39]. However, low levels of this molecule downregulate the production of these components, enhance bacterial motility, and subsequently induce biofilm dispersal[57]. Principally, regulating the level of c-di-GMP signaling in bacteria is modulated by the enzymes DGCs with a GGDEF domain and PDEs (Figure 6) [57]. Since these two enzymes contain specific sensory domains, they are thought to enable the bacteria to respond to adverse environmental factors and adjust their production of biofilm matrix

components[38]. Further, in *P. aeruginosa*, four c-di-GMP effectors are present, including PelD, FimX, Alg44, and FleQ [35]. PelD, FimX, and Alg44 are known to regulate exopolysaccharide components, whereas FleQ regulates flagella biosynthesis[22].

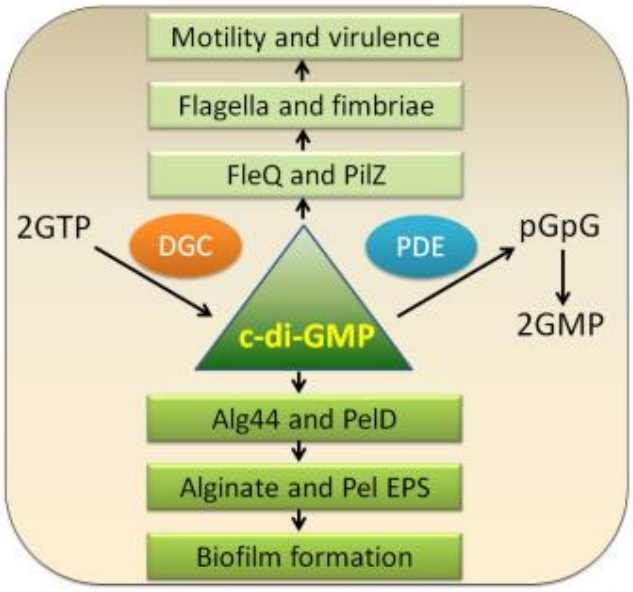


Figure 6. Schematic presentation of physiological functions of c-di-GMP [18]

iii. Small regulatory RNAs (sRNAs)

In addition to the AHL and PQS systems, *P. aeruginosa* controls its state and the production of multiple virulence factors via sRNA molecules[35]. Among the vast number of sRNAs expressed in *P. aeruginosa,* RsmY, and RsmZ are the best-known ones[35]. RsmY and RsmZ are mainly activated by the GacA/GacS system network via the phosphorylation of GacA by GacS (Figure 7) [69]. Of this regulatory pathway, RsmA, an effector protein, negatively controls biofilm formation and inhibits the transcription of proteins associated with Psl polysaccharide biosynthesis[66]. In *P. aeruginosa*, RsmY and RsmZ counteract RsmA translational repression activity, leading to Psl exopolysaccharide

production, and thereby initiating biofilm formation[42]. Indeed, RsmY and RsmZ were found to be involved in the switch from the planktonic to the sessile mode of growth[66].

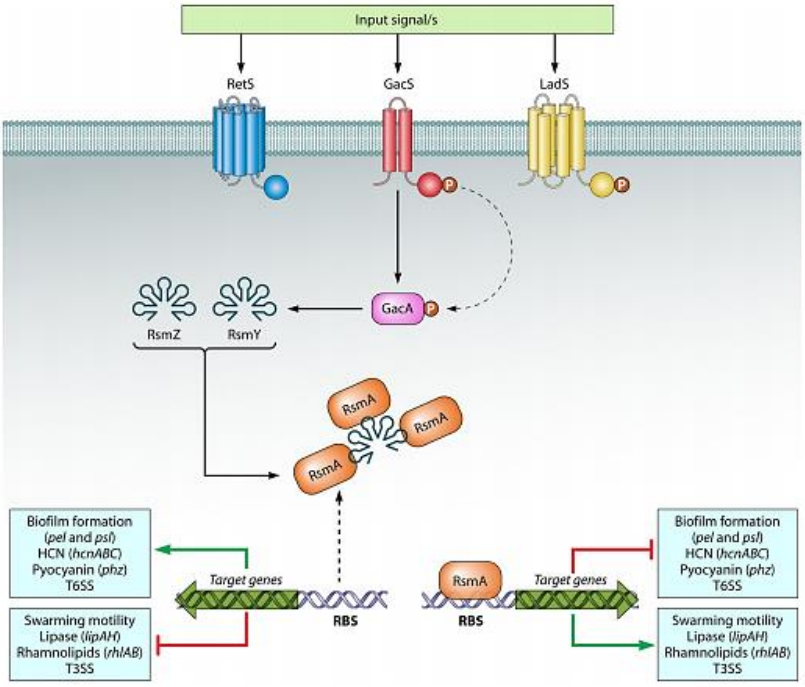


Figure 7. The GAC system network in *P. aeruginosa* [69]

2. Staphylococcus aureus

Staphylococcus aureus, including methicillin-resistant *S. aureus* (MRSA), is one of the most critical biofilm-forming pathogens that potentially cause life-threatening chronic infections [35][34]. Clinically, these ubiquitous bacterial species are the leading cause of nosocomial and hospital-acquired infections[70]. Further, it has the propensity to cause a long list of community-onset infections such as osteomyelitis, endocarditis, chronic wound infection, chronic rhinosinusitis, periodontitis, and ocular infections [35][70]. Since these biofilm-associated infections resist innate and adaptive immune defense mechanisms as well as classical antimicrobial agents, its treatment and prevention are limited to surgical

interventions, the addition of antimicrobial agents to indwelling-medical devices or administration of stringent antibiotic combination therapies[71]. Owing to the escalating involvement of *S. aureus* in biofilm formation, its exhibition of multiple-antibiotic resistance, and their tendency to transform from an acute infection to one that is persistent, chronic, and recurrent, this pathogen continues to receive substantial attention.

a. Genes associated with *S. aureus* biofilm matrix

In reality, the *S. aureus* biofilm life cycle follows the typical scheme of any other biofilm-forming pathogen. In its biofilm form, *S. aureus* is rooted within a glycocalyx or slime layer composed primarily of 80% teichoic acid as well as staphylococcal and host proteins[34]. The polysaccharide intercellular antigen (PIA), which is mainly composed of b-1,6-linked N-acetylglucosamine polymer and eDNA, exhibits an essential role in the formation of the matrix [34][72]. Its synthesis, export, and modification are mediated by the proteins encoded in the *ica* locus (*Ica*ADBC) [73][74]. Principally, the expression of these four genes is regulated by the global regulators SarA and sigmaB, and the negative regulator IcaR [55][75][76]. The latter is up or downregulated by the global regulator of the stress response (Spx), and the protein regulator of biofilm formation (Rbf), respectively[77][78]. Despite the *ica* gene locus's importance in biofilm development, biofilm formation by MRSA, a major human pathogen, was unaffected by *icaADBC* operon-deletion[79]. Interestingly, its biofilm formation was mediated through cell-to-cell aggregation and biofilm-associated protein (Bap) independent of PIA production[80]. Moreover, eDNA, the most lately identified and appreciated biofilm matrix component, has

also been indicated as a factor generally involved in the cell attachment stage and structural integrity, thus enhancing biofilm formation[81]. Other than these imperative components, in *S. aureus*, several surface proteins have also been implicated as essential factors in the attachment and biofilm matrix development including, *S. aureus* surface protein G (SasG), fibronectin-binding proteins (FnBPA and FnBPB), biofilm-associated protein (Bap), extracellular matrix binding protein (Embp), and staphylococcal protein A [80][82][83][84][85].

b. Regulation of Biofilm Matrix in *S.aureus*

i. Quorum Sensing

In staphylococci, the quorum sensing system is encoded by the accessory gene regulator (Agr) locus[31]. This locus contains *agrA*, *agrC*, *agrD*, and *agrB* genes, along with the intercellular effector of the system RNAIII [32]. Upon the detection of an autoinducing peptide (AIP), which is formed by the modification of AgrD by AgrB, Agr system activation takes place and subsequently stimulates the transcription of RNAIII (Figure 8) [35]. Indeed, *S. aureus* QS has been shown to regulate the virulence state of the cell[35]. It downregulates the genes of the cell wall-associated adherence proteins such as MSCRAMMs, as well as it positively regulates the synthesis of surfactants, including detergent-like peptides, nucleases, and proteases aiding in biofilm detachment[32]. Therefore, the activation of the Arg system results in switching from the biofilm mode of growth back to the planktonic state [32].

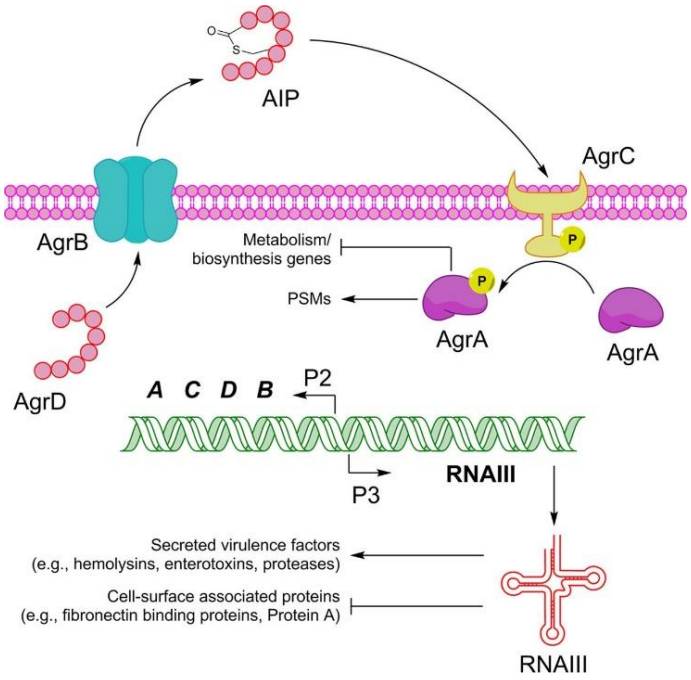


Figure 8. Schematic of the *S. aureus* accessory gene regulatory (Agr) system [146].

ii. C-di-GMP

In *S. aureus*, the C-di-GMP mechanism of action in biofilm regulation is still not well understood[86]. However, due to previous studies, it was reported that C-di-GMP could inhibit the cell-to-cell (intercellular) adhesive interactions and biofilm formation, thereby acting as a potential biofilm blocker[86].

iii. sRNAs

Small RNAs expression is modulated by RNAIII and contributes to *S. aureus* virulence[35]. In S. aureus, 250 sRNA genes were discovered; however, studies are still lagging, as their functions and mechanisms are mostly unknown[35].

3. Acinetobacter baumannii

Acinetobacter baumannii one of the most troublesome and opportunistic microorganisms, has emerged as a multi-drug resistant (MDR) pathogen posing a great threat for healthcare institutions globally [87]–[89]. This nosocomial pathogen, referred to as "Iraqibacter," has become a primary cause for concern in conflict zones, where a high incidence of problematic severe infections was noted among soldiers during the Iraq and Afghanistan war-zone medical facilities[88]. Unfortunately, this non-motile, aerobic, non-fermentative, and Gram-negative coccobacillus is notorious for its ability to survive in a variety of environmental conditions, particularly hospital environments and medical devices [85][87]. Its outstanding survival capability, along with its exceptional acquisition of antibiotic resistance, has been strongly associated with its ability to form biofilm [90]. Additionally, *A.baumanii* has been associated with a wide range of infections including bacteremia, ventilator-associated pneumonia (VAP), pneumonia, endocarditis, skin infections, wound infection, urinary tract infection, and meningitis as well as an exceedingly increased resistance to almost all known antibiotics [87]–[89]. Undoubtedly, this pathogen propelled the interest in the scientific community, as it created incomparable challenges and ignited epidemics that threatened public health, especially in immunocompromised individuals that have experienced a prolonged hospital stay[88]. According to the CDC AR threat report, the estimated cases of Carbapenem-resistant Acinetobacter in hospitalized patients has fluctuated between 11,700 and 8,500 cases from the year 2012 to 2017 in the United States, respectively [5]. Although the number of

infections significantly decreased, the optimal treatment for *A. baumannii* infections remains to be established.

a. Virulence determinants in *A. Baumannii*

Despite the intensive research that focused on the virulence potential of this emerging pathogen, little is still known about its true pathogenic potency[10]. In fact, *A. baumannii* harbors a group of biofilm-related virulence genes and proteins that are believed to influence biofilm formation such as outer membrane protein A (OmpA), biofilm-associated protein (Bap), beta-lactamase PER-1, the CsuA/BABCDE chaperone-usher pili assembly system, and poly-β-(1,6)-N-acetyl glucosamine (PNAG) [91].

OmpA, a major porin of *A. baumannii,* has been shown to contribute in antimicrobial resistance and biofilm formation of *A. baumannii*[91]. It's involved in the adhesion of *A.baumannii* on both abiotic and biotic surfaces, as it can attach and invade the host epithelial cells and subsequently translocate to the mitochondria, thereby inducing mitochondrial dysfunction [91][92]. Additionally, bap and the pili assembly system play a crucial role in controlling biofilm formation in *A. baumannii*[88]. Both factors have been determined to facilitate the initiation stage of biofilm formation as well as its maturation[92]. Furthermore, the presence and expression of the beta-lactamase blaPER-1 gene mainly participate in the biofilm formation and attachment of *A. baumannii* [88]. Lastly, poly-β-(1,6)-N-acetyl glucosamine (PNAG), which is encoded by *pga*ABCD gene cluster, has been observed to involve in biofilm formation, virulence, immune evasion, and antibiotic resistance[93].

b. Regulation of Biofilm Matrix in *A. baumannii*

i. Quorum sensing

QS system plays a critical role in *A. baumannii*'s physiology. This AHL-based system depends on a single LuxIR system (AbaI/R), where AbaI functions as the AHL synthase and AbaR as the AHL receptor (Figure 9) [10]. In this pathogen, AHL molecules control virulence factors, including biofilm formation and surface motility [56]. Their synthesis and transport are mainly regulated by an AdeFGH efflux pump[94]. Among the clinical isolates of *Acinetobacter* spp, it was found that biofilm formation is under the regulation of the auto-inducer synthase gene, *abaI* [95]. Further, studies provided some insights into the role of the *bfmS* and *bfmR* gene in *A. baumannii* pathogenesis by showing that the up-regulation of the expression of these genes influences *A.baummanii*'s ability to form biofilm on abiotic surfaces[88]. Interestingly, *A. baumannii* was also shown to produce a quorum quenching enzyme designated AidA [96]. This enzyme, upon its activation, may interfere with QS key steps and inhibit its regulated behavior, thereby blocking biofilm formation and preventing microbial infections[96]. Besides the little

information reported about the genes associated with the QS network in *A. baumannii*, the quorum quenching mechanism is also not well known, up to now [96].

ii. C-di-GMP

Few studies have reported the function of c-di-GMP signaling in the regulation of biofilm formation by *A. baumannii* [10]. In one of these studies, it was found that high concentrations of c-di-GMP promote biofilm formation in *A. baumannii*[56]. In contrast, low levels contribute to producing motility factors that inhibit bacterial adhesion and thereby prevent biofilm development[56]. Due to the lack of information regarding this regulator, the effect of c-di-GMP on *A. baumannii* biofilm requires more profound studies[56].

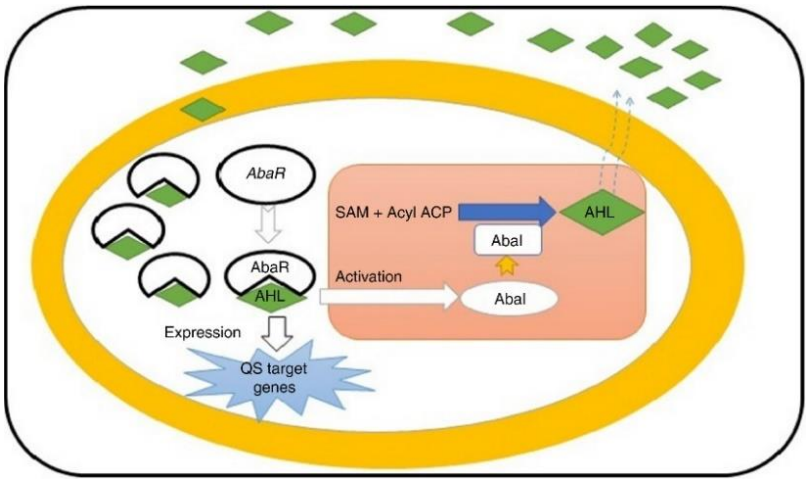


Figure 9. Illustration of a quorum-sensing mechanism in A. baumannii [147]

F. Infections and diseases associated with biofilm formation

Biofilm formation has been confirmed to be the root of the majority of persistent bacterial infections and a wide range of human diseases[24]. Indeed, this hallmark of chronic biofilm-based infection was introduced to medicine in the early 1970s, by the observation of *Pseudomonas Aeruginosa* aggregates, in the sputum of patients suffering from chronic lung infections [97]. Generally, biofilm-associated infections can be divided into two types: device-related and non-device related biofilm infections [33].

1. Device related biofilm infections

Microbial biofilms pose an extreme public health problem for persons requiring medical devices[98]. In the clinical setting, pathogenic bacteria can develop on the surfaces of indwelling implants, including central venous catheters, urinary catheters, pacemakers, mechanical heart valves, prosthetic joints, and intrauterine devices[24][25][33]. Because the host innate immune response is reduced in areas of the body in contact with foreign devices, these medical implants are particularly susceptible to biofilm formation[24]. In this condition, pathogens may originate from the patient's skin microflora, healthcare workers, or any other environmental sources, and attach on the surface of these devices, forming the infectious biofilm [12]. Through the contaminated indwelling medical devices, microorganisms gain access to human organs and tissues, causing severe infections such as urinary tract infection, bloodstream infection, prosthetic valve endocarditis, and nosocomial infections [12]. Clinically, a broad range of gram-positive and gram-negative microbial pathogens have been the cause of such infections[98]. Among them, *S. aureus* and *S.*

epidermidis are considered popular representatives, as they involve 40-50% of prosthetic heart valve infections, 50-70% of catheter biofilm infections, and 87% of bloodstream infections[26]. Currently, the only treatment used for medical device infections is the removal of the implant through surgical interventions; however, this strategy is traumatic and costly to patients [13] [24].

2. Non-device related biofilm infections

In humans, biofilm-associated infections are often chronic and opportunistic infections that affect most of the organs in the body, resulting in life-threatening conditions[12][33]. The outstanding hallmarks of chronic biofilm-based infections are extreme resistance to conventional and unconventional antibiotics, along with a radical capacity for evading the host defenses[33]. Particularly, microbial pathogens can form biofilm on both dead and living tissues, as well as on mucosal surfaces[13]. As recently documented, mucosal biofilms are involved in a plethora of human infections. For instance, one of its prototypes is the pulmonary infection caused by *P. aeruginosa*[99]. This opportunistic pathogen can colonize the airways once biofilm is formed, leading to chronic lung infection in cystic fibrosis patients[12][99]. In addition to chronic lung infection, native biofilm-associated infections also include chronic otitis media, native valve infectious endocarditis, chronic osteomyelitis, recurrent urinary tract infection, chronic wounds, dental caries, and periodontitis[12][25]. Since the only therapeutic option for clinicians is the administration of combinatorial antibiotics, which is often impractical, the treatment of such infections poses a significant challenge[24][99].

In this regard, to counter-attack the biofilm infections, there is an urgent need for in-depth research to better understand and regulate biofilms on indwelling medical devices, along with the development of new drugs with a broad-spectrum anti-biofilm activity that can eliminate recalcitrant biofilm.

G. Anti-biofilm strategies

Over the past years, diverse strategies have been adopted to combat biofilms[16]. However, due to their drawbacks, it is of fundamental importance to discover alternative approaches that can effectively prevent biofilm formation and eradicate mature ones [9][16]. Lately, three main strategies are being involved in research and are receiving considerable attention (Figure 10) [9].

1. Anti-adhesive / Antibacterial coating to surfaces

Starting with the first anti-biofilm strategy, it targets the adhesion stage of biofilm formation by inhibiting bacterial attachment and initiation on the surface [16]. This strategy can be approached in two ways, that is, by modifying physiochemical properties of surfaces to which microbes will attach, or incorporation of antimicrobial agents that will prohibit bacterial colonization onto the surface[9][100]. Since surface hydrophobicity plays a crucial role in the adhesion of microorganisms to abiotic and biotic surfaces, the prevention of bacterial adhesion can be achieved through surface coating with hydrophilic polymers [9]. For instance, coating polyurethane catheters by Poly-N-vinylpyrrolidone and hyaluronic acid, two hydrophilic polymers, can effectively prevent the bond of bacteria to

polymer surfaces[9]. Although these polymers are active, several drawbacks can limit their efficacy, such as their rapid dissolution and degradation by enzymes, or their ability to interact with body fluid components [16].

2. Interference with signaling pathways

Interference with signaling pathways, such as quorum sensing, is one of the main targets for many anti-biofilm agents[16]. This strategy, which seems to be promising, requires the use of anti-biofilm molecules or biofilm dissolving substance that can regulate the bacterial signaling genes expression, thereby inhibiting biofilm formation[9][100]. Such anti-biofilm molecules may be an enzyme, a peptide, an antibiotic, or polyphenols [16]. For instance, halogenated furanones, which are quorum sensing inhibitors, functions in inhibiting AHL-dependent gene expression, leading to the loss of exopolysaccharide production and defects in biofilm formation[53]. However, these inhibitors have shown to be reactive and toxic when treating bacteria- associated infections, thereby limiting them to external use only [9].

3. Disaggregation of EPS matrix

The third anti-biofilm strategy, known as enzymatic strategy, targets the disaggregation of the protective (EPS) matrix[16]. This strategy can be established by using dispersing enzymes that can destroy essential EPS matrix components, thereby affecting its integrity [16]. Once this highly protective biofilm matrix is disturbed, microbial aggregates become more exposed to antimicrobial agents as the subsequent penetration of such agents

becomes much easier [9]. According to recent studies, dispersin B, an enzyme produced by *Actinobacillus actinomycetemcomitans*, can degrade the polysaccharide intercellular adhesion molecules of a mature biofilm, produced by *Staphylococcus aureus* and *Staphylococcus epidermidis*[101]. Moreover, Dnase I and proteinase K are also two other matrix dispersing enzymes that show activity against pre-formed biofilm by effectively destroying the physical integrity of the biofilm[9]. Despite the effectiveness of such enzymes, several limitations reduce their practical usage, including enzyme specificity, as well as their ability to trigger human auto-immune response once these are recognized as foreign bodies[102].

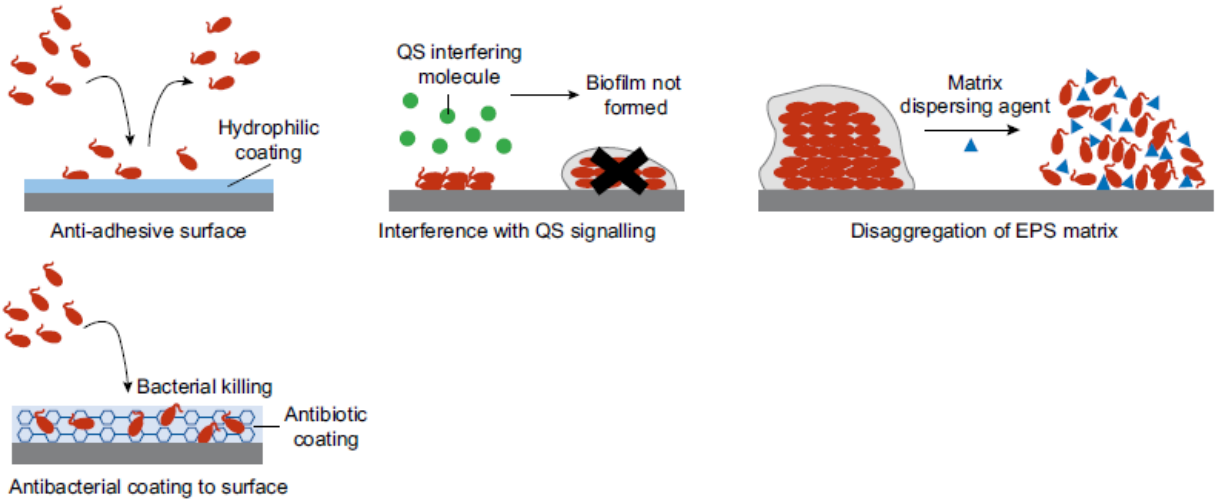


Figure 10. Anti-biofilm strategies to combat biofilms [16]

H. Anti-biofilm molecules mechanisms of action

A large body of evidence highlighted the importance of understanding anti-biofilms' mechanisms of action as a way to develop novel anti-biofilm compounds with

new pleiotropic modes[23]. In the current scenario, much attention has been directed towards anti-biofilm molecules that inhibit the biofilm-related cellular process, mainly targeting factors that regulate biofilm formation[23].

1. Quorum sensing inhibition

Since quorum sensing regulates the production of various virulence factors in most pathogens and is mainly responsible for the coordination of biofilm formation, it is considered as a potential target for antimicrobial agents[103]. Several anti-biofilm compounds were reported to have QS-inhibiting effects. Among these compounds is Azithromycin, which has been shown to interrupt the QS signal synthesis by reducing the integrations of AHLs in *P. aeruginosa*, thus inhibiting LasR-dependent gene expression and thereby preventing biofilm formation[36]. According to Parsek et al., SAM analogs, S-adenosyl-homocysteine (SAH), sinefugin, 5-methylthioadenosine (MTA), and butyryl-SAM are also anti-biofilm agents that can interfere with *P. aeruginosa* biofilm via blocking AHL-mediated QS [104]. Further, several anti-biofilm compounds derived from natural sources have been discovered functioning as competitive inhibitors of the QS signal. These inhibitors may include bergamottins, cyclic sulfur compounds, penicillic acid, furanone compounds, cinnamaldehyde, and hamamelitannin [100]. Many of these natural compounds with anti-QS activity were shown to increase susceptibilities of biofilm-bound bacterial cells to antibiotic treatment, especially in *P. aeruginosa*[100].

2. Inhibition of c-di-GMP signaling system

It has been increasingly clear that the enzymes involved in c-di-GMP play a pivotal role in regulating biofilm formation[23]. In this regard, several molecules have been reported as targets for these enzymes. Amongst them, LP 3134, LP 3145, LP 4010, and LP 1062 were discovered as small molecules that inhibit biofilm formation in *P. aeruginosa* and *A. baumannii* particularly[23]. These molecules were shown to inhibit diguanylate cyclase (DGC), leading to alterations in biofilm formation and its dispersion[23].

3. Cleavage of peptidoglycan

Tannic acid is one of the molecules that function in inhibiting the generation of biofilm by cleaving peptidoglycan, a layer present in the cell walls of most bacteria[105]. This polyphenolic compound prevents biofilm growth in *S. aureus*, by increasing the extracellular level of the immune-dominant Staphylococcal Antigen A (IsaA), a lysozyme-like enzyme that aids in the cleavage of the b-1,4 glycosidic bond between N-acetyl muramic acid (MurNAc) and N-acetyl glucosamine (GlcNAc)[105]. As the peptidoglycan is cleaved, the composition of proteins and teichoic acids present on the cell wall undergoes several modifications leading to the reduction in biofilm formation[105]. Additional to that, upon cleavage, some signaling molecules may be released, leading to the modulation of the biofilm-related gene expressions[105].

In addition to these major mechanisms, there are several targets followed by different anti-biofilm molecules. These anti-biofilm compounds with their functions are shown in figure (11).

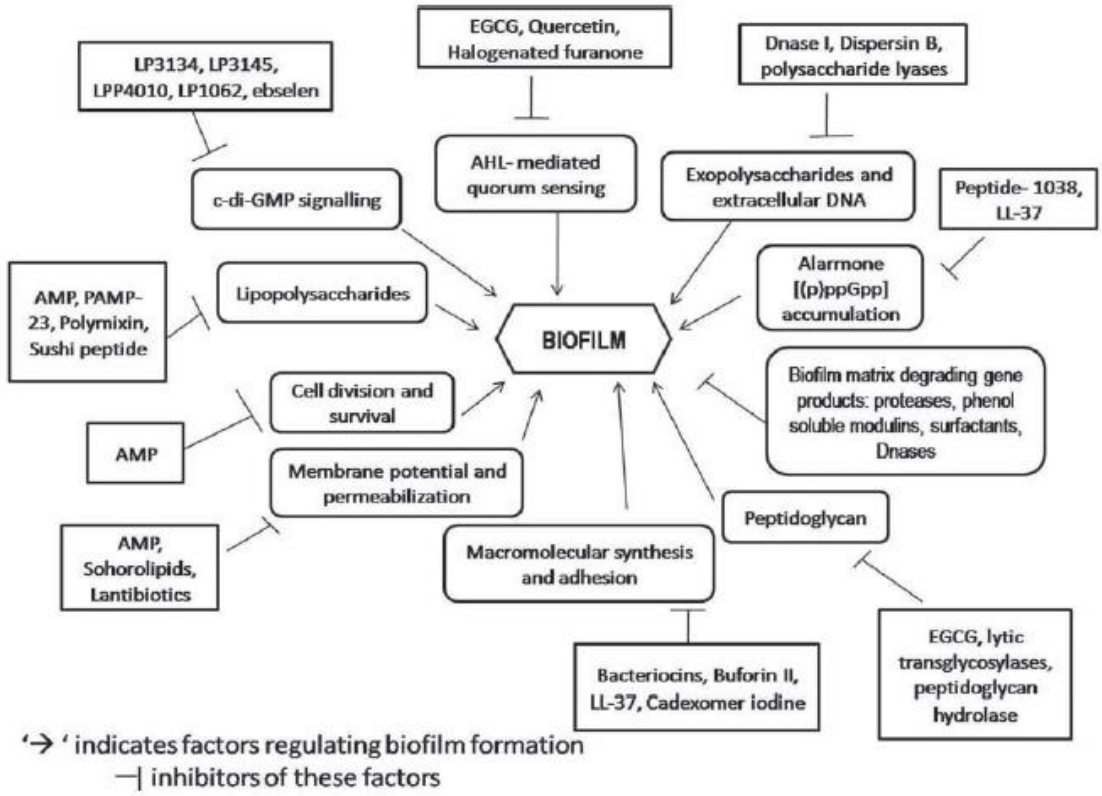


Figure 11. Schematic representation of overview of the targets of anti-biofilm molecules [23]

I. Natural derived anti-biofilm agents

Historically, natural products have been, and still to date, an invaluable source of inspiration in the discovery of novel compounds with enormous structural diversity and varied biological activities[106]. These privileged scaffolds that are incomparable to any available synthetic screening libraries are the origin of a majority of today's clinically used drugs, as they display one of the successful sources of potential drug leads [107]. Natural products, also known as secondary metabolites, are briefly defined as sets of small

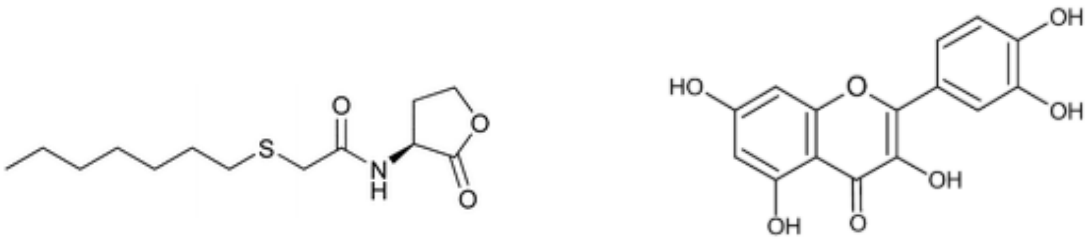
chemical molecules that mainly originate from the bacterial, fungal, plant, and marine animal sources[108].

Despite the long list of currently available antimicrobial agents in the market, the treatment of biofilm-associated infections still represents a significant challenge of modern medicine[106]. Since bacterial resistance is undermining the efficacy of currently used antibiotics, the search for novel approaches to target pathogenic bacteria growing in biofilms is a big matter of concern[48]. To date, a plethora of potential anti-biofilm agents with unique structures have been identified, mainly derived from plants, medicinal herbs, marine sponges, and microorganisms[13]. Mostly, these anti-biofilms are non-proteinaceous drugs, that particularly interfere with the biofilm development via non-microbicidal mechanisms, or induces a phenotypic shift from biofilm to the planktonic stage[13].

1. Anti-biofilm compounds derived from plant extracts or medicinal herbs

Many plant-derived natural products have been identified to possess antimicrobial and anti-biofilm functions. Among the variety of molecules derived from these sources is the anti-biofilm agents N-(Heptylsulfanylacetyl)-l-homoserine lactone (Figure 12A), which is extracted from garlic, a rich source of many compounds with antimicrobial effects[17]. This compound is a potent QS inhibitor that functions in interrupting the signal by competitively inhibiting transcriptional regulators LuxR and LasR in *P. aeruginosa* [109]. Moreover, another effective inhibitor of biofilm formation and virulence factors in *P. aeruginosa* is quercetin(Figure 12B)[110]. It is a plant-based polyphenol that significantly

reduces LasI, LasR, RhlI, and RhlR expression levels involved in QS signaling[110]. Indeed, quercetin generally exists in most fruits, vegetables, and grains[17].



N-(heptylsulfanylacetyl)-l-homoserine lactone (A)

Quercetin (B)

Figure 12. N-(heptylsulfanylacetyl)-l-homoserine lactone (A) and Quercetin (B) structures [109][148].

Furthermore, several other natural products originating from herbal extracts shown in figure (13), were demonstrated to display different inhibitory effects on QS network and biofilm formation process in various bacterial species [103][111][112][113][114]. Indeed, some natural anti-biofilm agents have presented encouraging preclinical data for anti-biofilm efficacy, exhibiting a promising perspective for treating biofilm-associated infections. However, no anti-biofilm agents have been approved by the U.S. Food and Drug Administration to date [17].

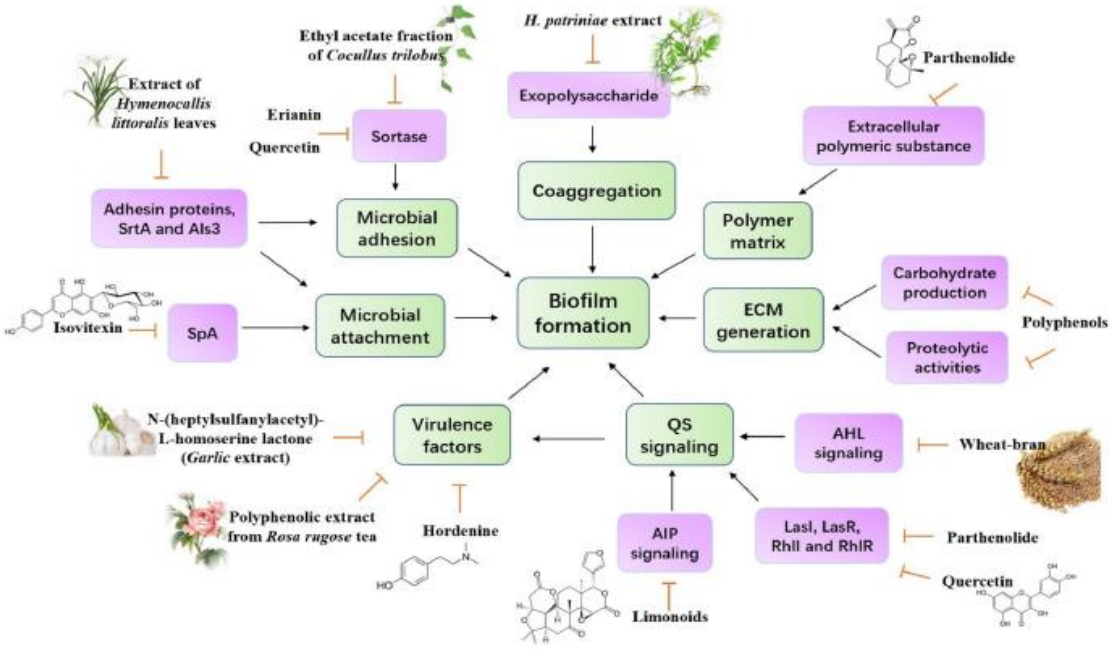
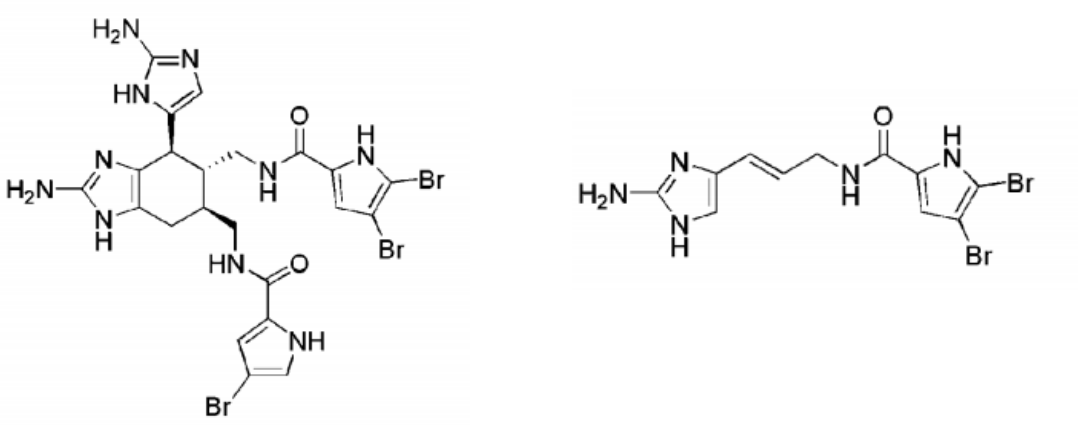


Figure 13. Anti-biofilm agents derived from natural plants and its potential mechanisms[17]

2. Non-Actinomycetes marine microorganisms derived anti-biofilm compounds

Marine microbes remain an unambiguous significant source of bioactive secondary metabolites that potentially own valuable anti-biofilm activities[115]. As estimated, more than 15,000 compounds have been discovered from marine sources over the past few decades, and new compounds are being added progressively[116]. Of all the species studied, marine sponges have been shown to possess abundant reserves of novel natural products [116]. These sponges have been the focus of much interest due to their close association with various microbial communities, including bacteria, archaea, microalgae, and fungi [116]. Indeed, approximately 5,300 different natural compounds are known from sponges and their associated microorganisms[117]. Among the diverse array of molecules

generated by these benthic organisms is pyrrole-imidazole alkaloids (PIA) [116]. As natural product, PIAs are found mainly as fused rings and halogenate derivatives, particularly in brominated forms[116]. Among their various potent biological functions, PIAs demonstrate a prodigious anti-biofilm activity, where it is believed that the conserved 2-aminoimidazole moiety of the PIAs, is responsible for this anti-biofilm property[116]. Bromoageleferin and oroidin (Figure 14) derived from *Agelas conifer* are two crucial members of pyrrole-2-aminoimidazole alkaloid that have shown to inhibit and disperse bacterial biofilm[118].



Bromoageliferin Oroidin

Figure 14. Structures of bromoageliferin and oroidin [118]

In fact, chemical libraries were generated by exploiting the 2-aminoimidazole moiety of bromoageliferin and oroidin, leading to the development of numerous molecules that revealed a biofilm inhibition activity against *P. aeruginosa*, *S.aureus, and A.baumannii* [118]. Of these molecules, CAGE and TAGE are two analogs of bromoageliferin (Figure 15), that were synthesized and assayed for their anti-biofilm activity on *Pseudomonas aeruginosa* biofilms[116]. Consequently, both derivatives were

able to inhibit the biofilm formation of two *P. aeruginosa* strains PAO1 and PA14, with the capability of TAGA to disperse the already formed ones[116]. However, due to their high level of toxicity, these molecules are not in clinical use to date[116].

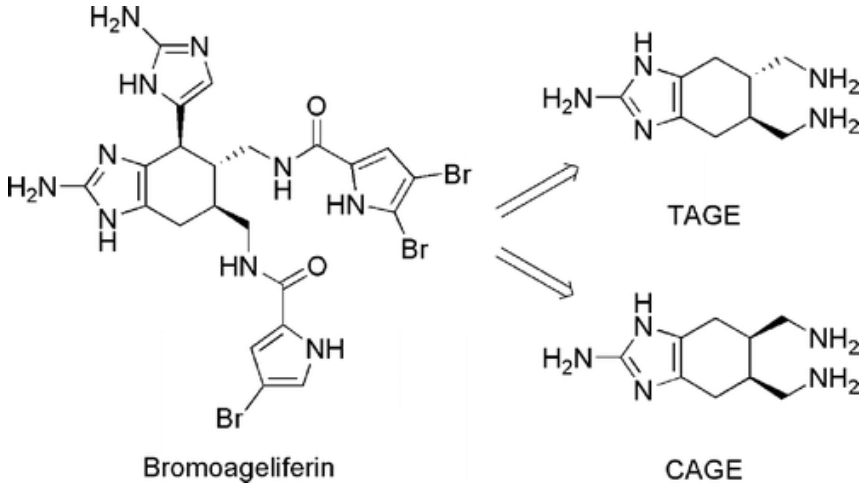


Figure 15. Bromoageliferin analogs [116]

3. Non-Actiomycetes Soil microorganisms derived anti-biofilm compounds

Bacteria that inhabit the soil are rich sources for the isolation of novel anti-biofilm compounds. As recently documented, *Bacillus* and *Paenibacillus* species were found to be potential sources for the isolation of active anti-biofilm metabolites with promising biological activities[119]. According to a study done by Alasil et al., a crude extract with its three identified compounds, derived from *Paenibacillus* sp. strain 139SI, were reported to exhibit a robust anti-biofilm activity against Gram-positive and Gram-negative pathogens[120]. These purified compounds were characterized to include amino acid antibiotics, phospholipase A2 inhibitor, and antibacterial agent[120]. As they compared their chemical structures to previously known bioactive compounds, they believed that the

three identified compounds could inhibit the cell-surface attachment, cell-cell interaction, and QS signaling[120]. Further, it's noteworthy to mention that these compounds showed no toxicity in murine models, thus considering them as promising alternative therapeutics to control biofilm-associated infections[120].

J. Actinobacteria / *Streptomyces* derived anti-biofilm compounds

1. Characteristics of Actinobacteria

The optimal choice of producer organism are bacteria that have a great potential for natural product biosynthesis [121]. The best example in this regard is Actinobacteria or Actinomycetes, the most dominant species in the bacterial domain[121]. These filamentous Gram-positive prokaryotes, which belong to the family Actinomycetaceae, are highly versatile natural product (NP) producers[122]. They are common soil inhabitants that are present in various ecological habitats, as well as in marine and freshwater ecosystems [123]. In fact, marine and soil microorganisms have been documented as potential producers for active anti-biofilm compounds[124]. Due to their vast genomes, along with the overabundance of factors that control their gene expression, Actinomycetes are considered to have a high metabolic diversity[125]. They produce perhaps the most unique and diverse compounds, as they have provided a wealth of bioactive secondary metabolites of medical interest, including antibiotics, antivirals, anticancer drugs, and immunosuppressive agents[126].

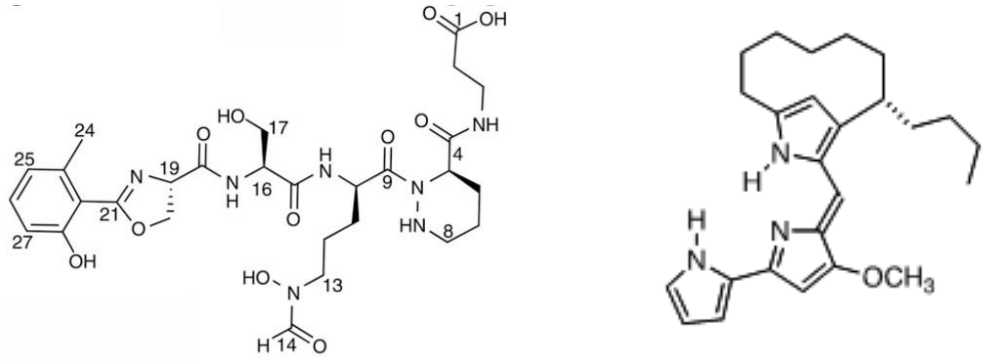
2. Characteristics of Streptomyces

The best-characterized genus of the Actinomycetaceae is *Streptomyces*, Gram-positive bacteria that belong to the family *Streptomycetaceae* [127]. They are distinguished by their complex developmental cycle, unusual filamentous organisms, and their reproduction lifestyle [127]. Mainly, *Streptomyces* are among the most numerous and most versatile soil microorganisms, where they constitute 50% of the total population of soil bacteria[128]. Due to their capacity to produce large varieties of bioactive secondary metabolites, with diverse and versatile biological activities, *Streptomyces* sp. have grasped considerable attention worldwide[129]. Following the discovery of streptothricin antibiotic in 1942 by Selman Waksman, and the discovery of streptomycin two years later, large antibiotics discovery efforts began in pharmaceutical companies [130]. Up to 2010, 40 % of 34,000 bioactive compounds were derived from actinomycetes, and approximately 80% of these actinomycetes compounds were produced by *Streptomyces* species, while the remaining 20% were isolated from non-*Streptomyces*[131]. Indeed, *Streptomyces* are the source of the majority of antibiotic classes in the market today, as researchers are continually screening for new bioactive compounds [132].

3. Anti-biofilm activity of soil actinobacterial compounds

To date, several bioactive compounds have been identified derived from various Actinobacteria strains. Recently, three peptide metabolites designated as cahuitamycins A, B, and C were discovered after purifying an organic extract isolated from *Streptomyces gandocaensis* strain DHS334, using high-performance liquid chromatography (HPLC)

[133]. Among these metabolites, cahuitamycins C (Figure 16A) was found to show a potent inhibitory effect on *Acinetobacter baumannii* biofilm formation process [133]. On the other hand, another biofilm inhibitor was discovered belonging to the prodiginine group of antibiotics, mainly known as streptorubin B (Figure 16B) [134]. This metabolite was extracted from *Streptomyces* sp. MC11024 and showed high inhibitory activity on biofilm formation by methicillin-resistant *S. aureus* (MRSA) [134]. In addition, ethyl acetate extract from *Streptomyces* sp. SBT343 was also found to display a significant antagonistic effect on *S. aureus* biofilm formation [16].



Cahuitamycin C (A) Streptorubin B (B)

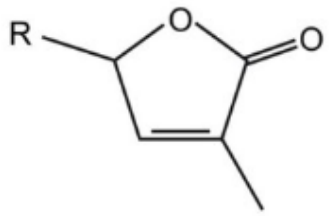
Figure 16. Cahuitamycin C (A) and Streptorubin B (B) structures [133][134]

4. Anti-biofilm activity of marine actinobacterial compounds

Although the majority of natural anti-biofilm agents have been found in soil-borne actinomycetes, perhaps because they dominate as antimicrobial agent producers, marine actinomycetes are also considered rich sources for anti-biofilm metabolites, according to

several reports[135]. However, scarce material is reported in the literature, as only a little attention has been given, particularly to marine microbial metabolites [107]. In one of these rare studies that were based on marine anti-biofilm inhibitors, the effect of bioactive crude extracts isolated from *Streptomyces* spp. (KP12, KP12, and CW17) was examined[135]. Consequently, these metabolites had revealed a high anti-biofilm activity against pathogenic biofilms, including *P. aeruginosa, E. coli, Vibrio parahaemoluticus, Streptococcus pneumonia, Staphylococcus aureus,* and *Enterococcus faecalis*[135]. They significantly inhibit biofilm formation by interrupting cell surface attachment as well as cell-cell interactions[135].

Moreover, butenolide (Figure 17) that was derived from marine *Streptomyces* sp. is another bioactive metabolite that was discovered with anti-biofilm activity[136]. This compound, structurally similar to brominated furanones, a previously identified non-toxic anti-biofilm metabolite, has established effective inhibition of biofilm formation, as well as eradication of pre-formed biofilm in different types of pathogens[136]. Hence it is considered as a promising anti-biofilm agent as well as an antibiotic enhancer[136].



Butenolide

Figure 17. Butenolide structure [136]

Accordingly, it is worth mentioning that soil and marine environments represent an unexploited reservoir of biodiversity able to synthesize a wide range of bioactive compounds, including anti-biofilm molecules[137]. Therefore, in the current scenario, research is focusing on driving the exploitation of soil and marine sources for the identification of novel and active anti-biofilm metabolites with new modes of action.

K. Combinatorial therapy

Since classical techniques in terms of either viability reduction or biofilm removal were shown to be ineffective in treating biofilm-associated infections, combinatorial therapy is receiving particular attention nowadays[138]. Indeed, considering the tremendous clinical significance of biofilm-related infections and their inherent recalcitrance to antibiotic treatment, a combinatorial strategy is the most practiced treatment in the clinical settings[138]. This strategy involves the use of anti-biofilm compounds in conjugation with conventional antibiotics as well as unconventional bioactive molecules [138]. Although anti-biofilm agents themselves do not kill the bacteria, they can make them more susceptible to traditional antibiotics as well as to the action of the host immune system[138]. Further, synergistic activity of anti-biofilm-based combinations can extend the antibiotic's spectrum of action, promote antibiotic intracellular uptake through membrane destabilization, and interfere with signaling molecules involved in biofilm formation[138]. A wide variety of bioactive agents combined with different conventional antibiotics have been studied, acting against distinct biofilm components[138].

In one of these studies on combinatorial therapies, the derivatives of 2-amino-imidazoles, that were recently reported to disperse bacterial biofilms, show a synergistic effect when combined with conventional antibiotics in treating *S. aureus* (MRSA) and *A. baumanni*[139]. Moreover, the combination of the anti-biofilm peptide IDR-1018, with the antibiotic Ciprofloxacin, resulted in the degradation of (p) ppGpp, a stress-related signaling nucleotide, in *P. aeruginosa*[140]. This combination potentiates the anti-biofilm activity by interfering with the stress-related signaling molecule, thus inhibiting biofilm formation[140]. Notably, anti-biofilm agent/antibiotic combinations appear to often reduce biofilm formation more effectively than when they are administered alone, highlighting the usefulness of anti-biofilm compounds in repurposing conventional antibiotics[138]. Further, the anti-biofilm agent likely maintains bacteria within their sensitive planktonic state, while the antibiotic eliminates the bacterial population [27]. In this regard, combinatorial therapy improves anti-biofilms activity as well as enhances antibiotic efficacy against multi-drug resistance strains, thereby facilitating biofilm control and eradication[138]. However, the mainstream approach in fighting against biofilm-related infections is now focused on the discovery of new novel anti-biofilm compounds.

CHAPTER III

MATERIALS AND METHODS

A. Collection of soil/marine samples

Two soil and one marine sample were collected from three different regions in Lebanon, Beit Mery, Mazrat Meshref, and Tabarja, respectively. The samples were transported to the laboratory in sterile polyethylene bags/glass bottles and stored for further study.

B. Bacterial isolation

After drying the soil samples at 37 °C for 5 to 7 days, 3g of each was heated in 100 mL water at 55° C for 30 minutes. Following that, serial dilutions were prepared (1:5, 1:10, 1:100, 1:1000) using autoclaved water with a final volume for each dilution equal to1 mL. Further, 30μL of each dilution was plated on International Streptomyces Project-3 (ISP3) agar, (10g oat flakes, 9g agar, and 2.5mL trace elements in 1L of distilled water) and on Soil agar (15g dried soil sample, 9g agar, and 5g corn starch with 250 mL distilled water). Similarly, the water sample was also heated at 55° C for 30 minutes and followed the same serial dilution steps as the soil samples. These dilutions were plated on Seawater agar and Seawater ISP3, which are the respective counterparts of Soil agar and ISP3 agar with the distilled water substituted with Seawater. The plates were left in an incubator at 28°C for 7 to 14 days. A few days later, the obtained colonies from the three samples were purified by

multiple rounds of subsequent streaking either on ISP3 or Seawater ISP3. Only the pure looking isolated colonies were stored in 50% glycerol at -80°C. Indeed, storing the bacteria requires adding 2 mL autoclaved water on the bacterial agar plate, swabbing the plate, and then collecting 1 mL of the spore/bacteria-containing liquid in Eppendorf tubes. These Eppendorf tubes were centrifuged for 10 mins at 4°C and 4 rpm. Subsequently, the supernatant was removed and the remaining pellet was resuspended in 1 ml of a sterile 50% glycerol solution and then stored at -80 °C.

C. Secondary metabolites extraction

For secondary metabolites extraction, a starter culture was initiated by inoculating 35µL of the spore stock into 5mL of liquid ISP3 and was then incubated for 2 days on a shaker incubator (150 rpm, 28°C). 1mL from the first seed was transferred into 10mL of fresh liquid ISP3 media and left at the same conditions for another 2 days. Subsequently, 1mL of this second seed culture was used to inoculate 50mL of each of the 14 different production media (Table 1) These cultures were also kept at 150rpm, 28°C for 7 days. At the end of the incubation period, 1mL of sterile Amberlite XAD 16N (Sigma) resin solution was added into each of the 14 cultures to absorb the secondary metabolites. It was left on a shaking platform at 150 rpm for 3-4 hours at room temperature. The mixture was then centrifuged (4000 rpm, 4°C, 20 minutes), the supernatant was discarded, and secondary metabolites were then extracted from the cell mass/resin pellet using 30mL acetone/10mL methanol. The solvent was left to evaporate at room temperature under a fume hood, and the crude extract was dissolved in 1mL DMSO and stored at -20°C.

D. Screening for antibacterial activity on ESKAPE pathogens

To screen for antibacterial activity of the crude extracts produced in different production media, broth micro-dilution (BMD) was performed. In a 96 well-plate, 100µL of Cation-adjusted Mueller Hinton Broth (MHCAB, Sigma) was pipetted in each well from columns 2 to 12. In the remaining column (1), 195µL of MHCAB was added into each well, followed by 5µL of crude extract (10µg/mL). These extracts then, using a multichannel pipette, simultaneously received a twofold serial dilution to obtain decreasing concentrations of the extract between columns 1 and 11. Column 12 received no extracts and served as a positive control. All 96 wells were then inoculated with 10µL of a bacterial solution*, then incubated in a shaking incubator at 37°C overnight. The number of wells where there was visual inhibition of growth was recorded.

*To prepare the bacterial inoculum, 2 to 3 colonies of an overnight appropriate bacterial culture were suspended in 2mL of MHCAB in order to obtain a difference in optical density (O.D.) between the clean broth and the bacterial suspension of 0.5 McFarland. Further, 750µL of the bacterial suspension was then diluted in 14.25mL of MHCAB to get a bacterial solution with a concentration of 5x10^5 CFU/mL.

E. Screening for anti-biofilm activity

To assess the antibiofilm activity of the crude extracts against clinically relevant pathogens, two microtiter plate (MTP) assays were carried out using 96-well flat-bottom polystyrene titer plates. One assay checks the extracts' potency to inhibit biofilm formation

(IF) while the other evaluates its ability to eradicate pre-formed biofilm (PF). The four pathogens that the crude extracts were tested on were selected based on their potential to form a robust biofilm.

1. Pseudomonas aeruginosa (PAN14)

a. IF Protocol:

- An overnight culture of PAN14 on LB agar was adjusted to 0.5 McF in LB broth then cultured overnight at 37°C.
- The overnight culture was diluted 1:50 in fresh LB broth.
- The 96-well round-bottom microtiter plate was then filled accordingly:
    - Negative control: 98 µL LB broth only
    - Positive control: 98 µL LB broth+ 50 µL bacteria.
    - Treated wells: 98 µL LB broth + 50 µL bacteria + 5 µL treatment.
- The plate was placed in a stationary incubator overnight at 37°C.
- The liquid in all the wells was then removed by pipetting.
- The plate was gently washed twice with distilled water, where each wash was done for 15 mins.
- 150 µL of 1 % crystal violet was added to each well, and the plate was then incubated at room temperature for 20min.
- After the crystal violet was removed, the plate was washed again with distilled water and left to dry for 1 hour at RT.
- 200 µL of 95 % ethanol was added to the wells and left for 20min.

- 120 µL from each well was transferred to a flat bottomed microtiter plate.
- The absorbance was then measured using BIO-TEK ELx800 Automated Microplate Reader at 630nm.

b. PF Protocol

- An overnight culture of PAN14 on LB agar was adjusted to 0.5 McF in LB broth then cultured overnight at 37°C.
- The overnight culture was diluted 1:50 in fresh LB broth.
- The 96-well round-bottom microtiter plate was then filled accordingly :
    - Negative control: 98 µL LB broth only.
    - Positive control or treated wells: 98 µL LB broth+ 50 µL bacteria.
- The plate was placed in a stationary incubator overnight at 37°C.
- The crude extracts were added (5 µL) for the treated wells.
- The plate was placed again in a stationary incubator overnight at 37°C.
- The liquid in all the wells was then removed by pipetting.
- The plate was gently washed twice with 0.9% NaCl, where each wash was done for 15 mins.
- The plate was left to dry for an hour at room temperature.
- 150 µL of 1 % crystal violet was added to each well, and the plate was then incubated at room temperature for 20min.
- After the crystal violet was then removed, the plate was washed again with 0.9%NaCl and left to dry for 1 hour at RT.

- 200 µL of 95 % ethanol was added to the wells and left for 20min.
- 120 µL from each well was transferred to a flat bottomed microtiter plate.
- The absorbance was then measured using BIO-TEK ELx800 Automated Microplate Reader at 630nm.

2. Acinetobacter baumannii (DSM & T36)

a. <u>IF protocol</u>

- An overnight culture of *A. baumannii* (DSM or T36) on TSA agar was adjusted to 0.5 McF in TSBG (tryptic soy broth with 0.25% glucose) then cultured overnight at 37°C.
- The overnight culture was diluted 1:40 in fresh TSB broth.
- The 96-well round-bottom microtiter plate was then filled accordingly:
    - Negative control: 200 µL TSB broth only.
    - Positive control: 200 µL of the bacterial suspension.
    - Treated wells: 200 µL of the bacterial suspension + 5 µL treatment.
- Free cells were removed, and biofilms were washed three times with 1x sterile phosphate-buffered saline (PBS).
- The wells were stained with 1% (w/v) crystal violet for 20 min at room temperature. After staining, the wells were washed twice with PBS to remove the unabsorbed crystal violet and air-dried at 37°C.
- Crystal violet was dissolved using ethanol/acetone (80, 20, v/v) for 20 min.

- The absorbance was then measured using BIO-TEK ELx800 Automated Microplate Reader at 595 nm.

b. PF protocol

- An overnight culture of *A. baumannii* (DSM or T36) on TSA agar was adjusted to 0.5 McF in TSBG (tryptic soy broth with 0.25% glucose) then cultured overnight at 37°C.
- The overnight culture was diluted 1:40 in fresh TSB broth.
- The 96-well round-bottom microtiter plate was then filled accordingly:
    - Negative control: 200 µL LB broth only.
    - Positive control or treated wells: 200 µL LB broth+ 50 µL bacteria.
- The plate was placed in a stationary incubator overnight at 37°C.
- The treatment was added (5 µL) for the treated well.
- The plate was then left overnight at 37°C
- Free cells were removed and biofilm were washed three times with 1x sterile phosphate-buffered saline (PBS).
- The wells were stained with 1% (w/v) crystal violet for 20 min at room temperature. After staining, the wells were washed twice with PBS to remove the unabsorbed crystal violet and air-dried at 37°C.
- Crystal violet was dissolved using ethanol/acetone (80, 20, v/v) for 20 min.
- The absorbance was then measured using BIO-TEK ELx800 Automated Microplate Reader at 595 nm.

Both the IF and PF protocols were adopted from Zeighami et al. (2019) with some modifications [91].

3. Staphylococcus aureus MRSA

a. IF Protocol

- An overnight culture of MRSA on LB agar was adjusted to 0.5 McF in TSBG (tryptic soy broth with 1% glucose) medium then cultured overnight at 37°C.
- The overnight culture was diluted 1:50 in fresh TSB with 1% glucose broth.
- The 96-well round-bottom microtiter plate was then filled accordingly:
    - Negative control: 200 µL TSBG broth only.
    - Positive control: 10 µL of the bacterial suspension + 190 µL of fresh TSBG medium.
    - Treated wells: 10 µL of the bacterial suspension + 190 µL of fresh TSBG medium + 5 µL treatment.
- The plate was placed in a stationary incubator at 37°C for 24–72h.
- The wells were carefully washed twice with 1× phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4·H2O, 1.4 mM KH2PO4, pH 7.4) to remove planktonic bacteria and allowed to dry overnight at 4°C.
- Then, biofilms were stained with 200 µl of 0.1% crystal violet (Merck, USA) for 5 min at room temperature.

- After staining, the wells were washed twice with distilled water to remove the unabsorbed crystal violet and air-dried at 37°C.
- Finally, adhered crystal violet was solubilized with 200 µl of ethanol and measured at 595 nm with a BIO-TEK ELx800 Automated Microplate Reader.

b. PF Protocol

- An overnight culture of MRSA on LB agar was adjusted to 0.5 McF in TSBG (tryptic soy broth with 1% glucose) medium then cultured overnight at 37°C.
- The overnight culture was diluted 1:50 in fresh TSB 1% glucose broth.
- The 96-well round-bottom microtiter plate was then filled accordingly:
    - Negative control: 200 µL TSBG broth only.
    - Positive control and treated wells: 10 µl of the bacterial suspension + 190 µl of fresh TSBG medium.
- The plate was placed in a stationary incubator overnight at 37°C.
- The treatment was added (5 µL) for the treated well.
- The plate was then left again in a stationary incubator overnight at 37°C.
- The wells were carefully washed twice with 1× phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4·H2O, 1.4 mM KH2PO4, pH 7.4) to remove planktonic bacteria and allowed to dry overnight at 4°C.
- Then, biofilms were stained with 200 µl of 0.1% crystal violet (Merck, USA) for 5 min at room temperature.

- After staining, the wells were washed twice with distilled water to remove the unabsorbed crystal violet and air-dried at 37°C.
- Finally, adhered crystal violet was solubilized with 200 µl of ethanol and measured at 595 nm BIO-TEK ELx800 Automated Microplate Reader.

Both the IF and PF protocols were adopted from Lade et al. (2019) with some modifications[141].

F. Bio-active molecule purification

1. Upscale metabolite production

An up-scaled secondary metabolite production was generated by inoculating 105µL of the spore stock into 15mL of liquid ISP3 and then incubated for 2 days on a shaker (150 rpm, 28°C). 10mL of the first seed was then transferred into 100mL of sterile liquid ISP3 and left at the same conditions for a couple of days. Further, the entirety of the second seed culture was used to inoculate 6L of the media whose extract showed the highest activity, including BM9 and TBJ13 which demonstrated significant activities in media C and media C in seawater, respectively. After one to two weeks of incubation on a shaker (150rpm,28°C), the secondary metabolites were extracted via acetone/methanol similar to small scale. The resulting crude extract was then divided into two parts. The first was dissolved in DMSO and tested again for their anti-biofilm activity to confirm previous results while the other was subjected to liquid-liquid partitioning.

2. Liquid/ Liquid partitioning

Liquid/liquid partitioning is a traditional technique usually performed in a separating funnel where the crude of interest is distributed between two immiscible solvents[142]. Briefly, this technique relies on the concept of "like dissolves like", where polar solvents such as chloroform dissolve polar solutes and non-polar solvents such as hexane dissolve non-polar solutes[142]. After the active crude extract was subjected to this protocol, four different fractions (water/methanol, hexane, chloroform, and ethyl acetate) were collected. These fractions were then tested for their anti-biofilm activity via BMD. The fraction which presented the highest anti-biofilm activity was subjected to further compound segregation.

3. Thin Layer Chromatography (TLC)

To determine the number and purity of the compounds in the extracts, TLC was done using a metal TLC plate whose surface was coated with a thin layer of silica gel. On the TLC plate, the fraction with the highest activity obtained from the liquid/liquid chromatography was spotted, after dissolving it with 1mL of methanol. The TLC plate was placed in a chamber filled with a suitable liquid solvent or a mixture of liquids. It was ensured that the spots were not below the solvent level to avoid being washed into the solvent. After the solvent has risen to the top of the plate, within 1 cm from the top, the plate was removed with forceps, and the solvent front was marked immediately with a pencil. Subsequently, the TLC plate was viewed under the UV light and analyzed depending on the migration and separation of the compounds.

4. Column chromatography and fractionation

Column chromatography, a technique widely used for the separation and purification of natural compounds, was done for further and more in-depth investigation of the most active fraction in the extracts. Briefly, the impure sample was loaded onto a column of adsorbant, mainly silica gel. After an organic solvent or a mixture of solvents (the eluent) were drained through the column, the components of the sample were separated from each other by partitioning between the stationary silica and the mobile eluent. Consequently, molecules with different polarities moved through the column at different rates, and the eluent was collected in fractions. To confirm that the separation of the components was successful, fractions were typically analyzed again by thin-layer chromatography.

G. DNA extraction of bacterial isolates

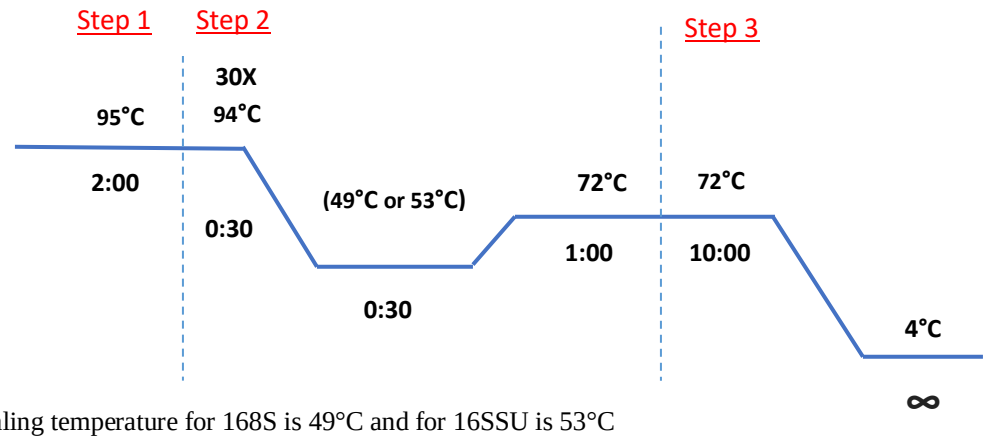
After 2 days of growing the bacteria in Tryptic soy broth (TSB) on a shaking incubator (28°C, 150 rpm), a cell pellet is collected by centrifugation at 4000rpm for 10 minutes at 4°C. Further, the pellet was washed by 10X PBS and centrifuged again for 5 mins at 4000 rpm. The washed pellet is then suspended in 180µL of 20mg/mL lysozyme solution (lysozymes (Sigma) dissolved in TE buffer (amresco; pH=8). Subsequently, the solution is incubated at 37°C for 60 minutes. 20µL of Proteinase K (Qiagen) is then added to the mixture and incubated at 56°C until the cells are completely lysed (no more than 3hours). Afterward, 200µL of AL buffer (Qiagen) is added to the solution, which is

incubated first at 70°C for 10minutes, then at 95°C for 15minutes to lyse any leftover spores. Then, the mix is centrifuged for 5 mins at 4000rpm at room temperature, the pellet containing cell debris is then discarded, and the supernatant is transferred into a new microcentrifuge tube. The rest of the protocol is performed according to a QIAmp DNA mini kit according to the manufacturer's instructions**.**

H. 16S rRNA gene amplification and sequencing

Amplification of 16S rRNA genes was performed by Polymerase chain reaction (PCR). After thawing all reagents on ice, 1 µL of the primers were diluted in 9 µL autoclaved water. 3 µL of the processed DNA extraction was placed in PCR tubes, with the following PCR mix components added and the final volume adjusted to 30 µL: 0.75 µL of each diluted primer (SSU-bact-27F: 5'-AGAGTTTGATCMTGGCTCAG-3', SSU-bact-519R: 5'-GNATTACCGCGGCKGCTG-3' & 8F: 5'AGAGTTTGATCCTGGCTCA-3', 1492R: 5'TACGGYTACCTTACGACTT-3') , 6 µL Taq polymerase (5X FIRE Pol Master Mix with 7.5 mM MgCl2, Solis Biodyne), and 19.5 µL water. Negative control was also prepared, which only consists of the PCR mix without the DNA extract to ensure that the other components of the reaction are not contaminated. The PCR tubes with the negative control were then placed in the BIO-RAD T100™ Thermal Cycler PCR machine for 2-3 hours (Figure 18). Once the PCR program ends, the quality of the PCR products was examined by agarose gel electrophoresis, where variable DNA fragments were separated according to their size and charge, resulting in clear defined bands. After the gel was placed under UV light, the DNA fragments that were already stained by DNA-binding dye (Gel

Pilot loading Dye 5x), glow, and their locations, along with their sizes, were determined. Further, the PCR products were purified/sequenced by macrogen, aligned using MEGA X program, and compared with similar 16S rDNA gene sequences using the BLAST search program.



* Annealing temperature for 168S is 49°C and for 16SSU is 53°C

Figure 18. PCR steps

I. Statistical analysis

Biofilm data were analyzed using the GraphPad Prism® 5.00 software (GraphPad Sofware Inc.). Data were expressed as the mean ± S.E.M. and analyzed with Student's t-test as indictated in the figures' legends. Significance was recorded using: * for p-value < 0.05, ** for p-value < 0.01 and *** for p-value < 0.001.

Table 1. Production media recipes

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	Components (g/L)	V	Veg	A	B	C	INA	Ra3	GPMY	V6	AF/MS	GYM	M8	NL2	COM
2	Potato starch								20						
3	Peptone		5	4				2		5					
4	Soluble starch	24	20										20	30	
5	Dextrose	1									20				
6	Meat extract	3	2	4						5			2		
7	Yeast extract	5	3	2				4	5	5	2	4	2	2.5	
8	Malt extract							10	5			10			
9	Soy-bean meal		2	2							6				
10	Glucose							10		20		4	10		25
11	Triptose	5													
12	Maltose			20											
13	Dextrin			10											
14	CaCO3		1		0.1	0.1	5				4		3	10	2
15	Glycerol				20		30	5	20						
16	Glycine				2.5	2.5							4		
17	Hydrolyzed casein									3					
18	NaCl				1	1	2			1.5	1				2
19	KH2PO4				1	1									0.15
20	FeSO4				0.1	0.1									
21	MgSO4.7H2O				0.1	0.1									
22	MgCl2.6H2O							2							
23	Tween 60					20									
24	Molasses													20	
25	Soy flour													15	25
26	Dried beer yeast														3
27	Ammonium Sulfate														2
28	Soybean Oil														3
29	pH	7.2	7	7	7	7	7.3	7.4	7.02	7.05	7.3	7	7	7.8	8.4

Table 2. Brand names of different materials used

Material Used	Brand Name
Potato Starch	Sigma-Aldrich
Peptone	DIFCO Laboratories
Soluble Starch	Merck
Dextrose	DIFCO™ Laboratories
Meat Extract	CONDA
Yeast Extract	USB
Malt Extract	Lab M, NEOGEN
Glucose	Sigma-Aldrich
Maltose	
Dextrin	
$CaCO_3$	Baker
Glycerol	Sigma-Aldrich
Glycine	Fisher
Hydrolyzed Casein	
NaCl	Merck
KH_2PO_4	Merck
$FeSO_4$	Sigma-Aldrich
Tween 60	Sigma-Aldrich
Soy Bean Flour	Commercial
Dried Beer Yeast	Commercial
$(NH_4)_2SO_4$	Fisher

Casein Peptone	
Bacteriological Agar	Lab M, NEOGEN
TSB	BD Phoenix™
96-Well Plates	Corning®
PCR Plates	BIO-RAD
Incubator (37ºC)	Thermo Scientific
Incubator (28ºC)	Amerex Instruments
Centrifuge	Thermo Scientific
Rotary Evaporator	Heidoplh

Chapter IV

RESULTS

A. Antibacterial activity screening

The crude extracts produced by different isolated strains, derived from either soil or marine samples, were evaluated for their antibacterial activity on a panel of ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species) pathogens[11]. After testing the extracts according to the assay specified earlier, the entire raw findings are displayed in the following tables (Table 3-5-6). Almost all extracts originating from all the strains grown in all 14 different production media demonstrated no antibacterial activity on any of the tested pathogens except for BM9, which showed a slight inhibitory effect against Gram-positive bacteria in different production media (Table 4). However, due to the low number of wells with inhibition, and to the fact that BM9-derived extracts exhibited an antibacterial effect only on Gram-positive pathogens only, which, in turn, are not our target in the study, this strain was not eligible to further investigations.

Table 3. Table depicting the number of wells with inhibition for crude extracts produced by strains TBJ13, MM3, MM5, MM6, MM7, and MM18 in 14 different media against a panel of

TBJ13 & MM strains	Media													
Bacteria	V	Vegetative	A	B	C	INA	RA3	GPMY	V6	AF/MS	GYM	M8	COM	NL2
S. aureus ATCC 29213	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. aureus Newman	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. aureus N315	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. feacalis ATCC 19433	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K. pneumonaie DSM	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A.baumannii DSM 30008	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K. pneumonaie ATCC 13883	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. aeruginosa ATCC 27853	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. aeruginosa mexAB	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. coli ATCC 25922	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. Table depicting the number of wells with inhibition for crude extracts produced by strain BM9 in 14 different media against a panel of ESKAPE pathogens.

BM 9	Media													
Bacteria	V	Vegetative	A	B	C	INA	RA3	GPMY	V6	AF/MS	GYM	M8	COM	NL2
S. aureus ATCC 29213	0	0	4	3	0	1	3	0	4	0	0	0	0	0
S. aureus Newman	0	0	4	3	0	1	3	0	3	0	2	0	0	0
S. aureus N315	0	0	4	3	0	1	3	0	3	3	3	0	0	0
E. feacalis ATCC 19433	0	0	3	2	0	0	3	0	4	0	3	0	0	0
K. pneumonaie DSM	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A.baumannii DSM 30008	0	0	0	0	0	0	0	0	0	0	0	0	0	0

***K. pneumonaie* ATCC 13883**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***P. aeruginosa* ATCC 27853**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***P. aeruginosa* mexAB**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. coli* ATCC 25922**	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 5. Table depicting the number of wells with inhibition for crude extracts produced by strain MM9 in 14 different media against Gram-positive pathogens.

MM 9	**Media**													
Bacteria	**V**	**Vegetative**	**A**	**B**	**C**	**INA**	**RA3**	**GPMY**	**V6**	**AF/MS**	**GYM**	**M8**	**COM**	**NL2**
***S. aureus* ATCC 29213**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***S. aureus* Newman**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***S. aureus* N315**	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 6. Table depicting the number of wells with inhibition for crude extracts produced by strain MM15 in 14 different media against a panel of ESKAPE pathogens.

MM 15	**Media**													
Bacteria	**V**	**Vegetative**	**A**	**B**	**C**	**INA**	**RA3**	**GPMY**	**V6**	**AF/MS**	**GYM**	**M8**	**COM**	**NL2**
***S. aureus* ATCC 29213**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***S. aureus* Newman**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***S. aureus* N315**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A.baumannii* DSM 30008**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***P. aeruginosa* mexAB**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. coli* ATCC 25922**	0	0	0	0	0	0	0	0	0	0	0	0	0	0

B. Anti-biofilm activity screening

Biofilm inhibition assays were conducted on the crude extract produced by five strains that already showed null to weak antibacterial activities, in order to evaluate their capability of inhibiting biofilm growth and/or eradicate preformed biofilm. The screening was based on the two protocols mentioned before and was done against four biofilm-forming pathogens, namely, *Pseudomonas aeruginosa* (PAN14), *Acinetobacter Baumannii* (DSM and T36), *and Staphylococcus aureus* MRSA. As demonstrated in the tables and bar graphs below, all the extracts were able to decrease biofilm formation or disperse preformed ones in PAN14 within a variable pattern. Some strains were capable of doing so in other pathogens, including ACN T36 and MRSA. It's worth mentioning that DMSO was tested alone as a control, and the results showed no antibiofilm activity.

Crude extracts derived from BM9 and TBJ13 were shown to significantly reduce biofilm formation in PAN14, particularly in media C, to 24.1% and 11.8% respectively (Tables 7 and 8). The results presented an extremely statistically significant decrease ($P < 0.01$ for BM9 and $P < 0.0001$ for TBJ13) of biofilm formation in media C, as shown in figures 19 and 20.

Table 7. Percentage of biofilm formation in PAN14 treated with BM9-derived extracts produced in 14 different production media.

BM9	**Media**						
Bacteria	V	Veg	A	B	C	INA	RA3
PAN14	60.5%	32.9%	52.7%	179.3%	24.1%	88.39%	89.3%
	GPMY	V6	AF/MS	GYM	M8	COM	NL2
	76.3%	110%	73.9%	53.9%	49%	55.4%	42.7%

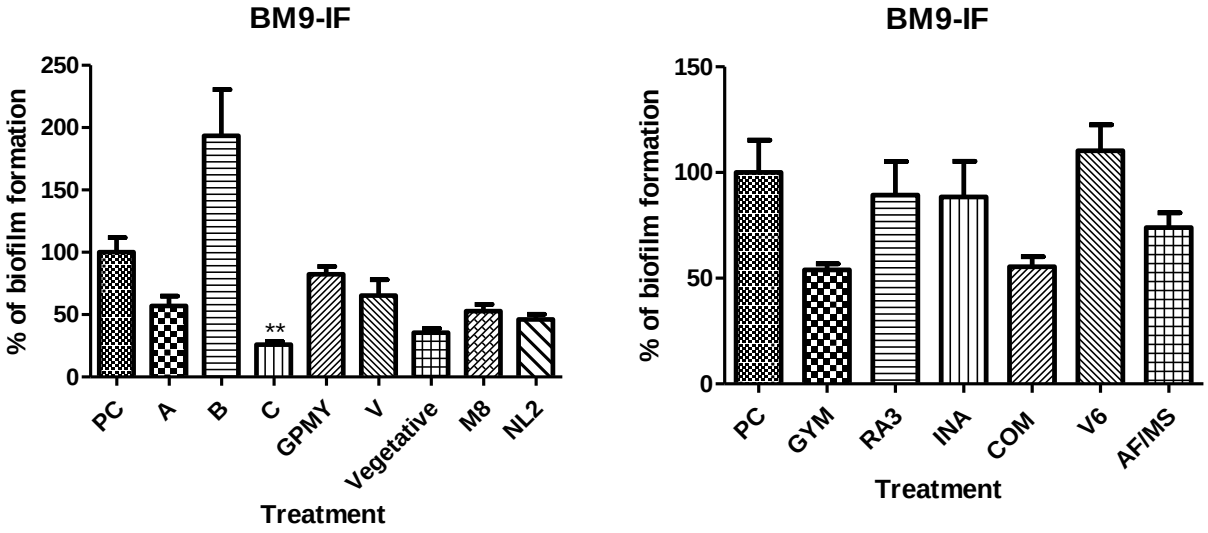


Figure 19. Percentage of biofilm formation in PAN14 treated with BM9-derived extracts produced in 14 different production media

Table 8. Percentage of biofilm formation in PAN14 treated with TBJ 13-derived extracts produced in 14 different production media.

TBJ13	Media						
Bacteria	V	Veg	A	B	C	INA	RA3
PAN14	24.6%	42%	59%	93.5%	11.8%	52.6%	41.8%
	GPMY	V6	AF/MS	GYM	M8	COM	NL2
	64%	32.9%	32.4%	43.5%	32.7%	38.4%	64.7%

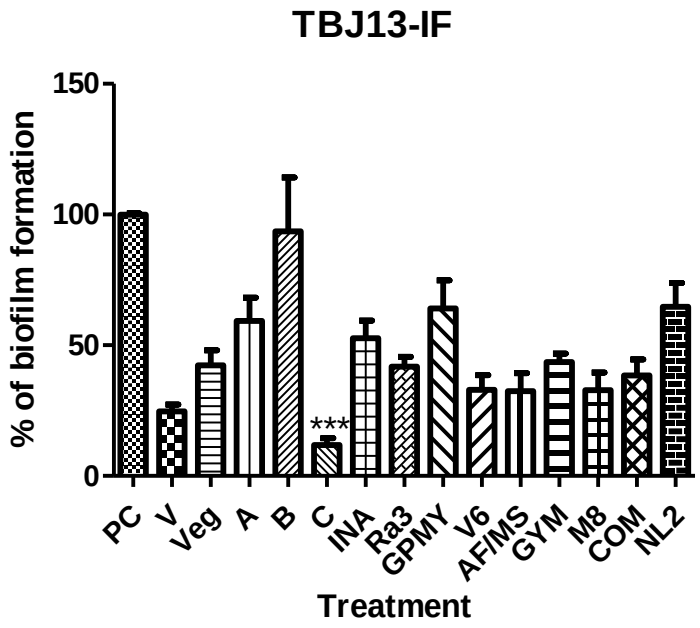


Figure 20. **Percentage of biofilm formation in PAN14 treated with TBJ13-derived extracts produced in 14 different production media**

In the same regard, extracts produced by MM3 strain were able to reduce PAN14 biofilm development, especially those produced in media C to 11.8% and media M8 to 10% as seen in Table 13. Additionally, MM3-derived extracts were capable of decreasing biofilm growth in ACN strain T36, as shown intensely by those produced by media GYM and GPMY to 11% and 12%, respectively (Table 9). Extracts from MM7 and MM9, two other soil-derived strains, also had a significant effect, particularly on PAN14 biofilms. MM7 extract produced in media B was able to reduce biofilm formation to 11% (Table 11), whereas MM9 extract produced in media NL2 was able to do so more efficiently (8%) as seen in Table 16. Besides, MM9 extract produced in media M8 was capable of decreasing biofilm formation in MRSA pathogen to 3% (Table 12). More importantly, it's worth mentioning that MM9 extracts produced in media V6 and Vegetative were also capable of

eliminating preformed biofilm in PAN14 to reach 8 and 9%, respectively. However, the extracts of this strain do not affect the biofilm formation of ACN DSM and T36, as seen in Table (12). Although the crude extracts of the mentioned strains decreased biofilm formation, they also enhanced biofilm formation in different production media, as mainly seen in MM4 strain. In contrast to the other strains, MM4 derived-extract had no significant effect on reducing biofilm formation in PAN14 and ACN T36 in any of the production media (Table 10).

Table 9. Percentage of biofilm formation in PAN14 and ACN T36 treated with MM3-derived extracts produced in 14 different production media.

MM 3		Media													
IF	Bacteria	V	Veg	A	B	C	INA	RA3	GPMY	V6	AF/MS	GYM	M8	COM	NL2
	PAN14	28.5%	36%	-	29%	11.8%	17.9%	48.5%	44.9%	46%	29.5%	26%	10%	147%	43%
	ACNT36	39%	44.7%	102.9%	26.9%	50%	70.8%	23.7%	12%	33.5%	40.5%	11%	17%	17%	33.5%
PF	Bacteria	V	Veg	A	B	C	INA	RA3	GPMY	V6	AF/MS	GYM	M8	COM	NL2
	PAN14	14%	32.5%	51.7%	35%	28%	97.8%	-	73%	122%	178%	18%	84%	61%	124%
	ACNT36	170%	-	317%	313%	-	-	76.8%	-	113%	703%	222%	-	104%	-

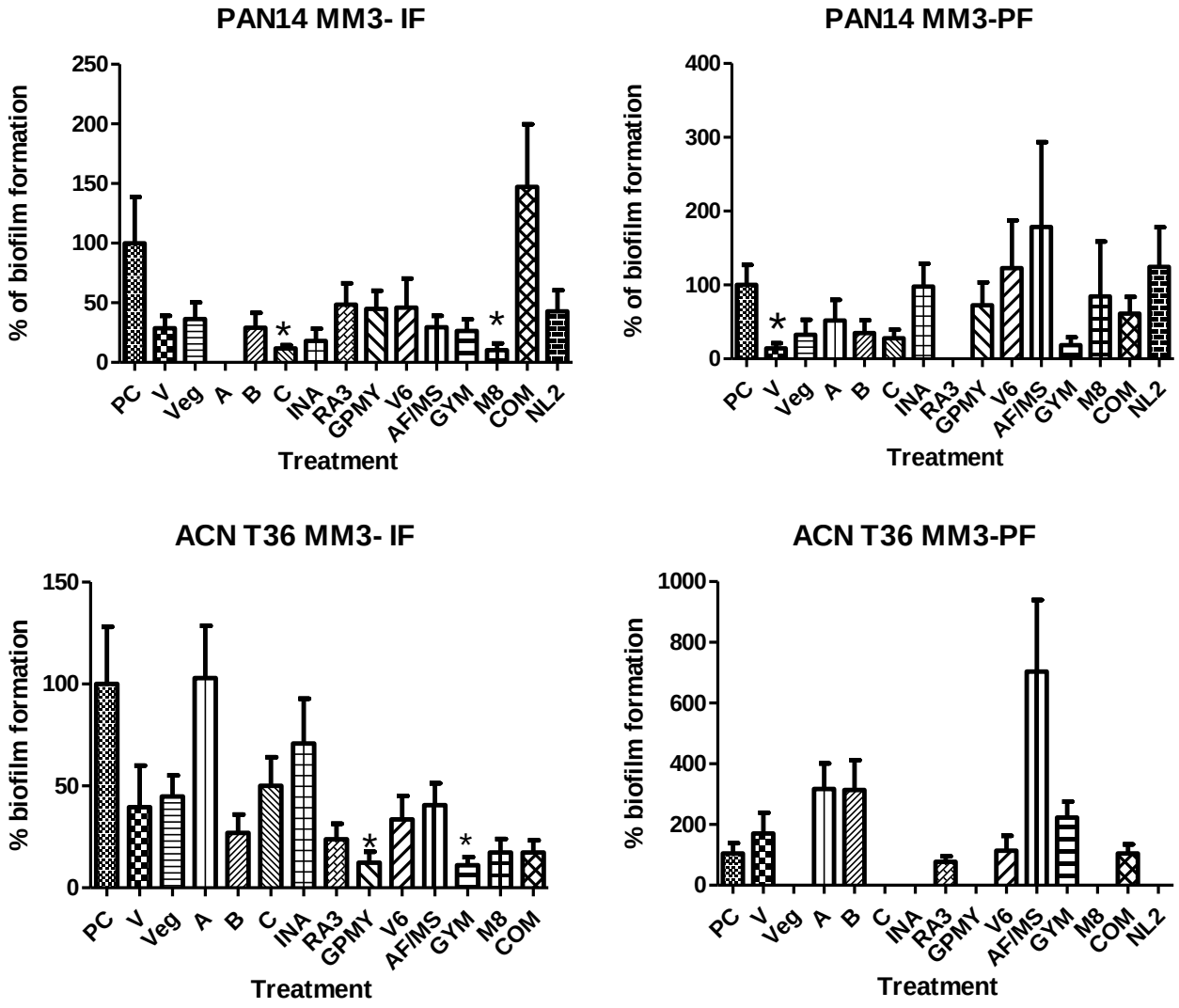


Figure 21. Percentage of biofilm formation in PAN14 and ACN T36 treated with MM3-derived extracts produced in 14 different production media.

Table 10. Percentage of biofilm formation in PAN14 and ACN T36 treated with MM4-derived extracts produced in 14 different production media.

MM 4		Media													
IF	Bacteria	V	Veg	A	B	C	INA	RA3	GPMY	V6	AF/MS	GYM	M8	COM	NL2
	PAN14	143%	57.8%	180.6%	46%	28%	53%	72%	71%	447%	-	33%	465%	-	62%
	ACNT36	99%	37%	75%	26%	25%	43.5%	18%	44.9%	40%	23.9%	44%	37%	116%	41.6%
PF	Bacteria	V	Veg	A	B	C	INA	RA3	GPMY	V6	AF/MS	GYM	M8	COM	NL2
	PAN14	17%	43.5%	11%	15%	5%	332%	-	12.4%	30.8%	29%	16.8%	20%	156%	241%
	ACNT36	103%	-	350%	115%	64%	170%	103%	92%	179%	203%	77.7%	135%	-	289%

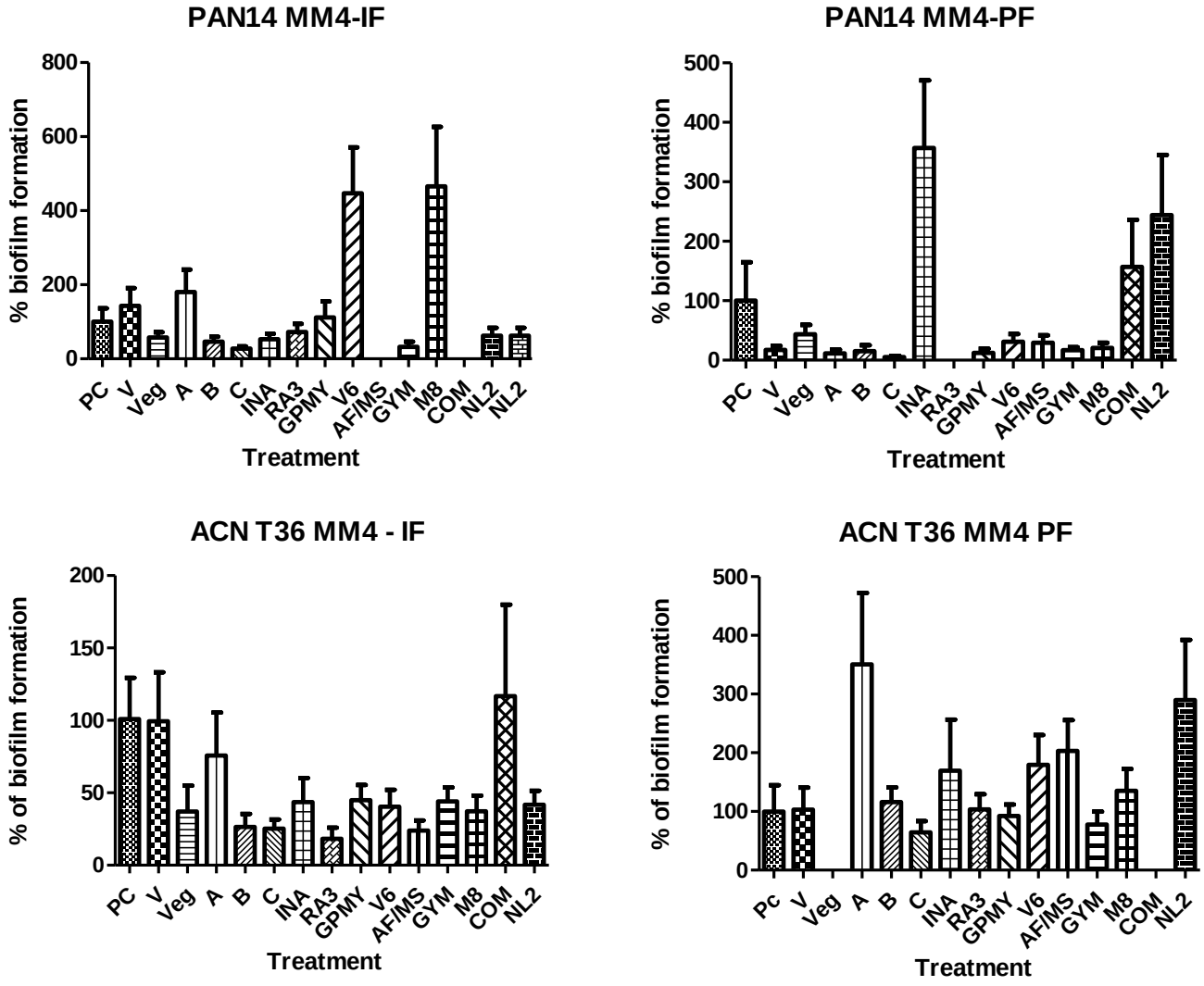


Figure 22. Percentage of biofilm formation in PAN14 and ACN T36 treated with MM4-derived extracts produced in 14 different production media.

Table 11. Percentage of biofilm formation in PAN14 and ACN DSM treated with MM7-derived extracts produced in 14 different production media.

MM 7		Media													
IF	Bacteria	V	Veg	A	B	C	INA	RA3	GPMY	V6	AF/MS	GYM	M8	COM	NL2
	PAN14	12.4%	18%	16%	11%	115%	64%	18%	13%	12%	27%	27%	19%	24%	30%
	ACNDSM	91%	72%	114%	86%	47.5%	200%	145%	138%	133%	150%	119%	326%	243%	173%
PF	Bacteria	V	Veg	A	B	C	INA	RA3	GPMY	V6	AF/MS	GYM	M8	COM	NL2
	PAN14	24%	18.9%	28%	39%	23%	-	120%	131%	-	15%	11%	12%	9.7%	40%
	ACNDSM	84.5%	83%	131%	64.5%	68%	-	68%	82%	53%	93%	76%	314%	59%	163%

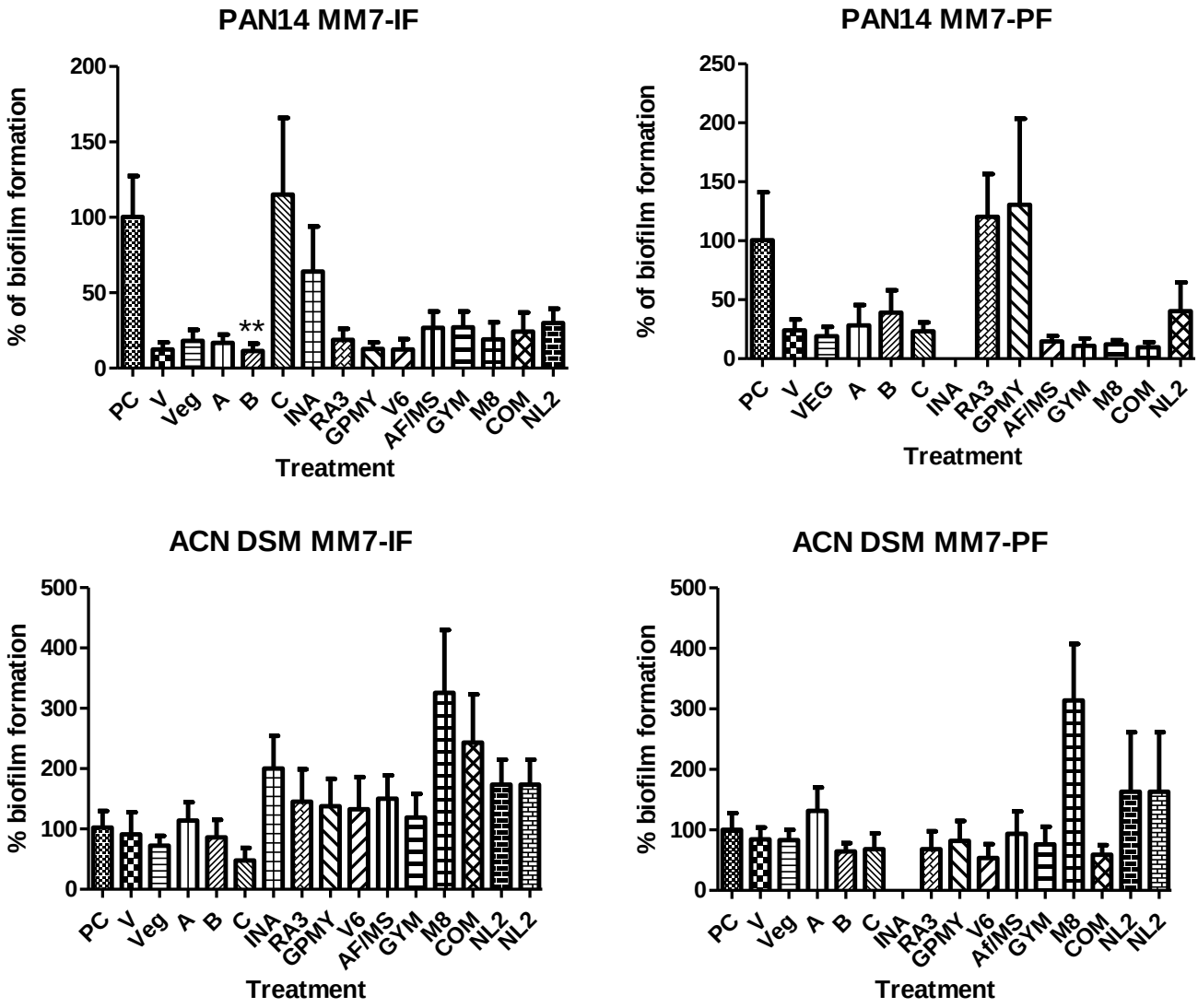
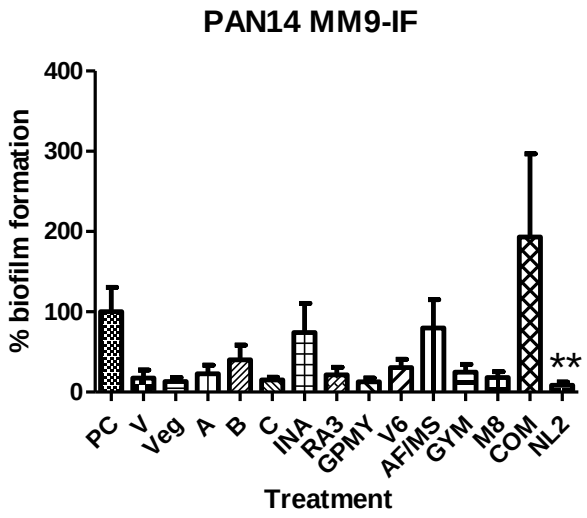
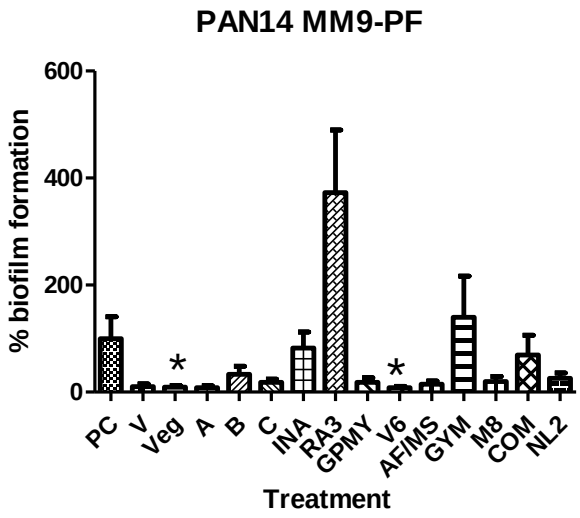


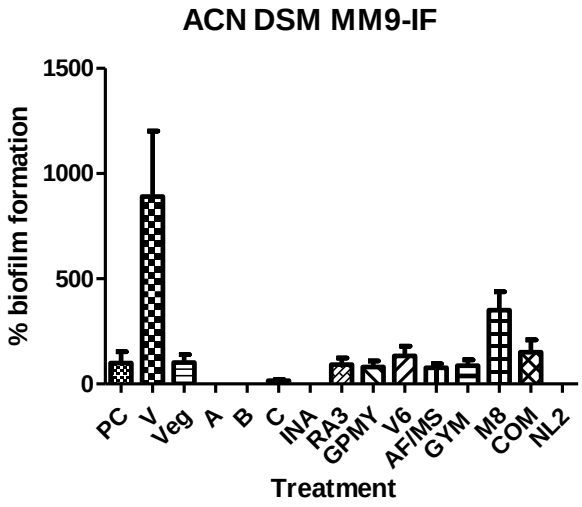
Figure 23. Percentage of biofilm formation in PAN14 and ACN DSM treated with MM7-derived extracts produced in 14 different production media.

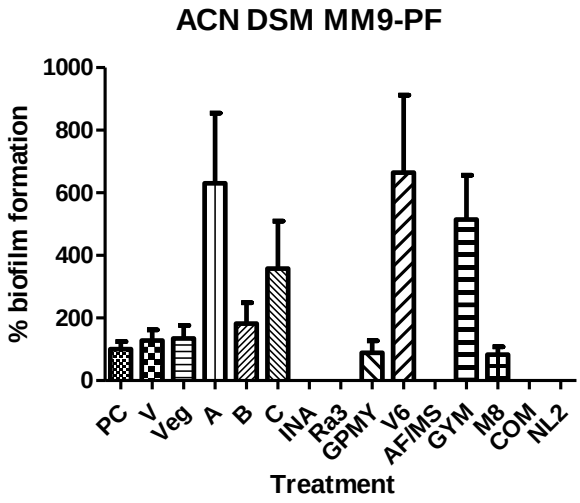
Table 12. Percentage of biofilm formation in PAN14, ACN T36, ACN DSM, MRSA treated with MM9-derived extracts produced in 14 different production media.

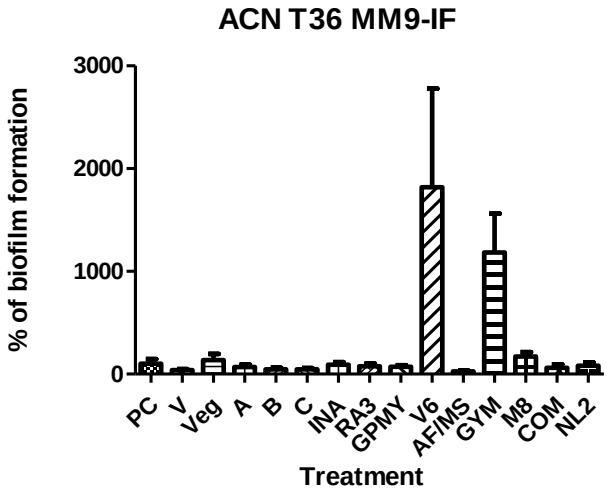
MM 9		Media													
IF	Bacteria	V	Veg	A	B	C	INA	RA3	GPMY	V6	AF/MS	GYM	M8	COM	NL2
	PAN14	17.5%	13%	22.7%	40%	15%	74%	21.4%	12.6%	30%	80%	25%	18%	193%	8%
	ACNDSM	890%	102%	-	-	15%	-	91.9%	80.7%	133%	76%	87%	351%	150%	-
	ACNT36	37.5%	135%	69%	45%	46%	92%	75%	70.6%	1818%	26%	1183%	171%	61%	82%
	MRSA	40%	51.4%	60%	33.3%	10%	70%	72%	58%	52%	21%	331%	3%	-	18%
PF	Bacteria	V	Veg	A	B	C	INA	RA3	GPMY	V6	AF/MS	GYM	M8	COM	NL2
	PAN14	10%	9%	8%	33%	18.4%	82%	372%	18%	8%	15%	139%	20%	69%	26%
	ACNDSM	128%	135%	630%	182%	358%	-	-	88.5%	664%	-	515%	83%	-	-
	ACNT36	82%	46%	-	91%	46%	-	68%	31%	90%	97%	61%	41%	48%	24%
	MRSA	153%	82.5%	127%	200%	-	67%	-	150%	289%	61%	-	-	-	-

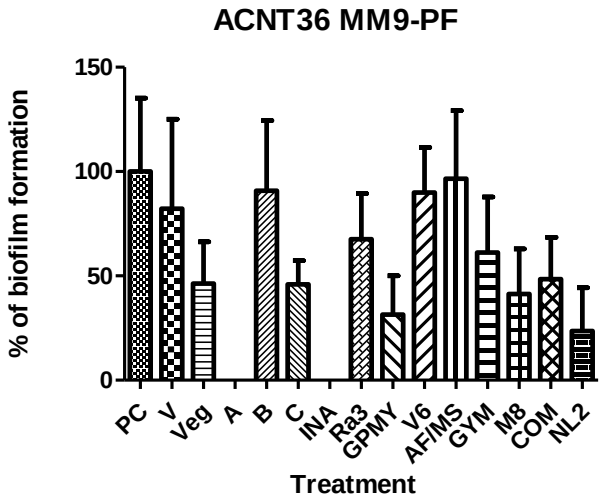





ACN DSM MM9-IF
% biofilm formation
1500
1000
500
0
PC V Veg A B C INA RA3 GPMY V6 AF/MS GYM M8 COM NL2
Treatment


ACN DSM MM9-PF
% biofilm formation
1000
800
600
400
200
0
PC V Veg A B C INA Ra3 GPMY V6 AF/MS GYM M8 COM NL2
Treatment


ACN T36 MM9-IF
% of biofilm formation
3000
2000
1000
0
PC V Veg A B C INA RA3 GPMY V6 AF/MS GYM M8 COM NL2
Treatment


ACNT36 MM9-PF
% of biofilm formation
150
100
50
0
PC V Veg A B C INA Ra3 GPMY V6 AF/MS GYM M8 COM NL2
Treatment

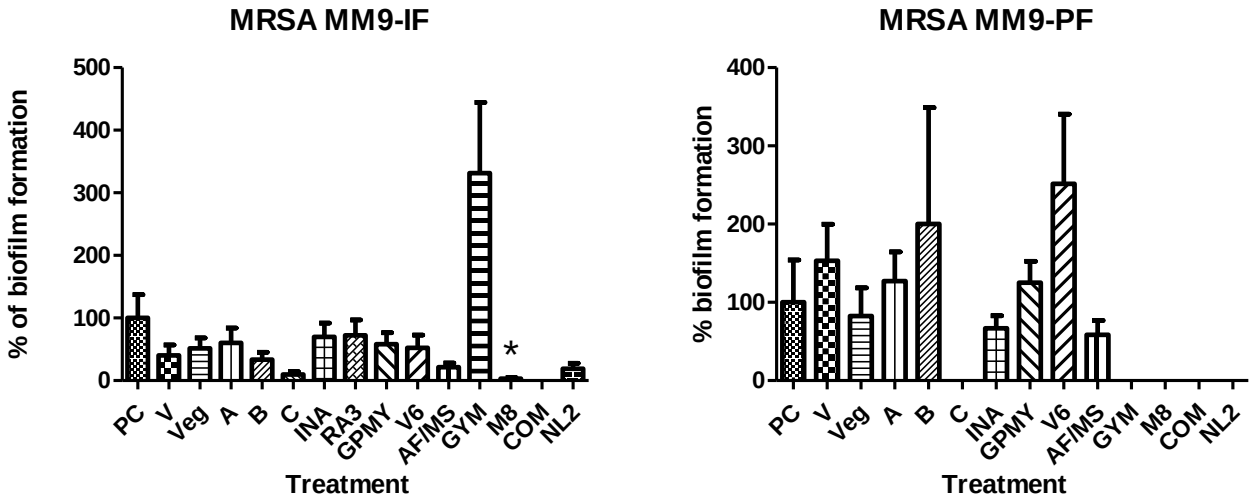


Figure 24. Percentage of biofilm formation in PAN14, ACN DSM, ACN T36 and MRSA treated by MM9-derived extract produced in 14 different production media.

C. Anti-biofilm activity of upscaled fractions

BM9 and TBJ13 were subjected to further investigations as their extracts produced in media C showed significant activities in reducing biofilm growth. Therefore, the extracts of these strains were upscaled in media C, and subjected to liquid/liquid partitioning, resulting in three active fractions. As shown in table 13, the highest overall significance belongs to the BM9C ethyl acetate fraction, which achieved the highest biofilm inhibition activity in reducing biofilm formation to 11% and eliminating preformed ones to reach 2 %. Similarly, the other two fractions also demonstrated extreme antibiofilm activities, but with lesser impact and percentages.

Table 13. Percentage of biofilm formation in PAN14 treated by BM9C-dervied

BM9 C		Media		
Bacteria		Chloroform	Ethyl Acetate	Hexane
PAN14	IF	37%	11%	13%
	PF	2.1%	2%	2.8%

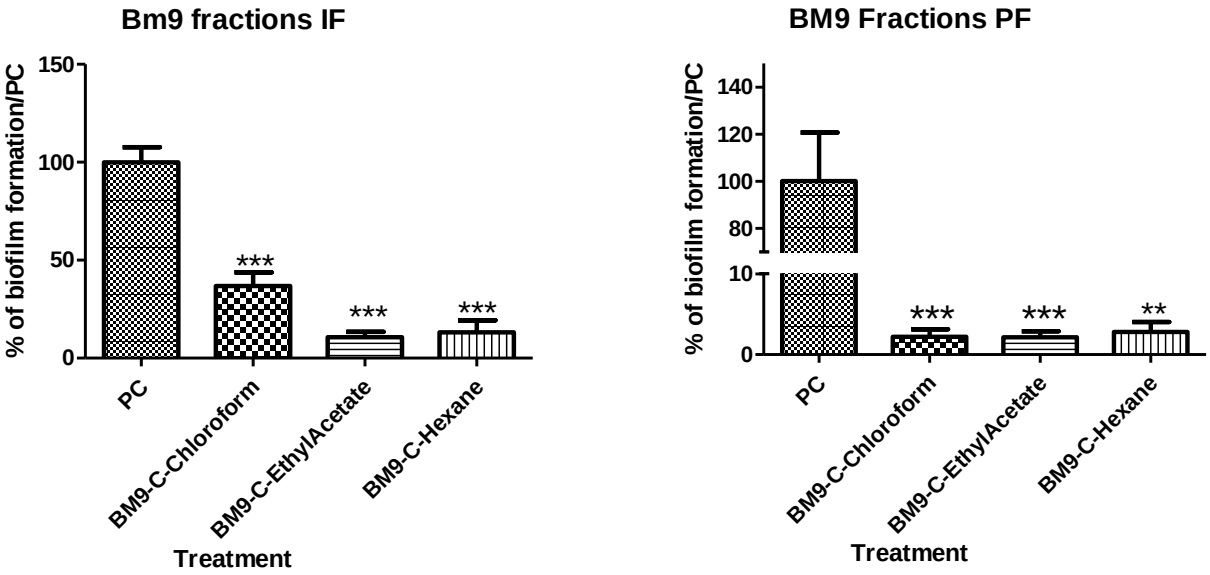


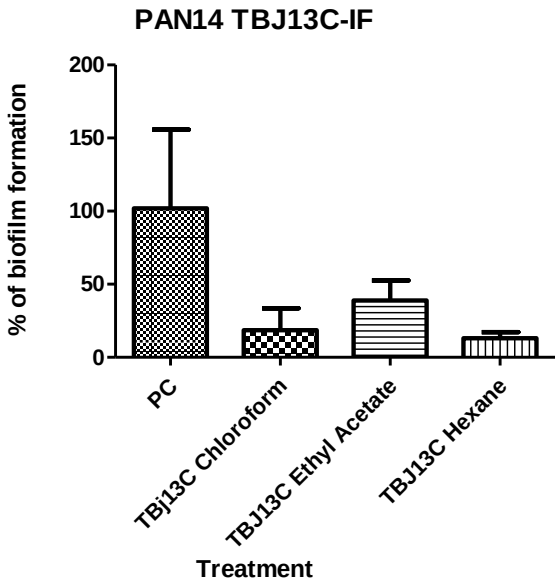
Figure 25. Percentage of biofilm formation in PAN14 treated by BM9C-derived extract.

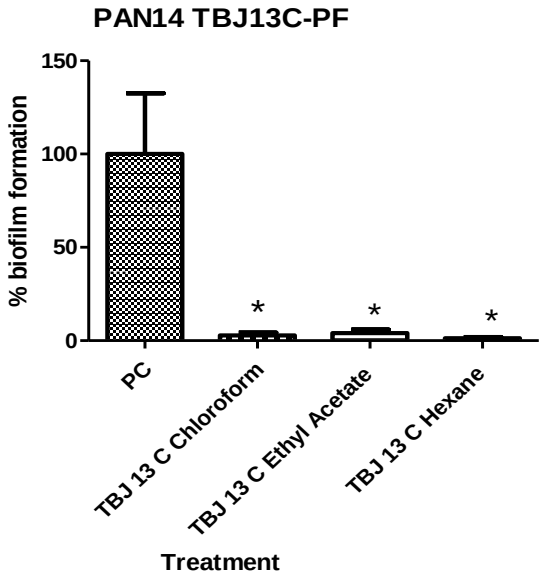
Further, among the tested fractions of TBJ13C, the hexane fraction exhibited the highest activity in eliminating already established biofilm in both PAN14 and ACN DSM to reach 1.3% and 8%, respectively (Table 14). Likewise, TBJ13C ethyl acetate and

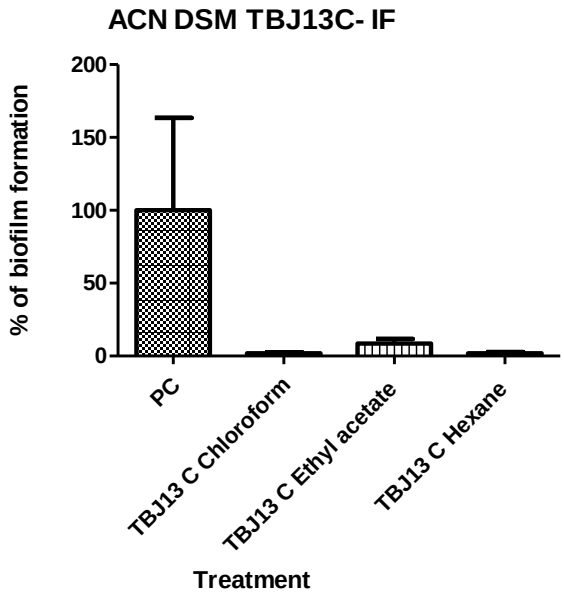
chloroform fractions also demonstrated remarkable antibiofilm activities against PAN14, however, with lower percentages. On the other hand, the chloroform fraction alone showed significant anti-biofilm activity in eradicating ACN DSM biofilms, as well as decreasing biofilm formation in MRSA to 5.7%.

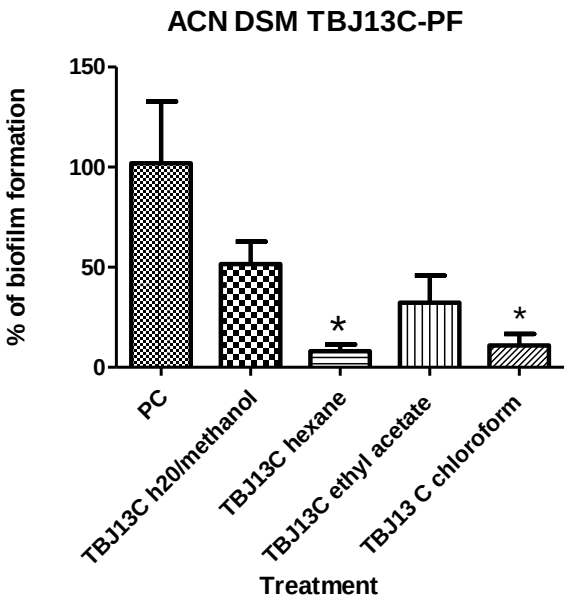
Table 14. Percentage of biofilm formation in PAN14, ACN DSM, ACN T36, and MRSA treated by TBJ13C-derived extract.

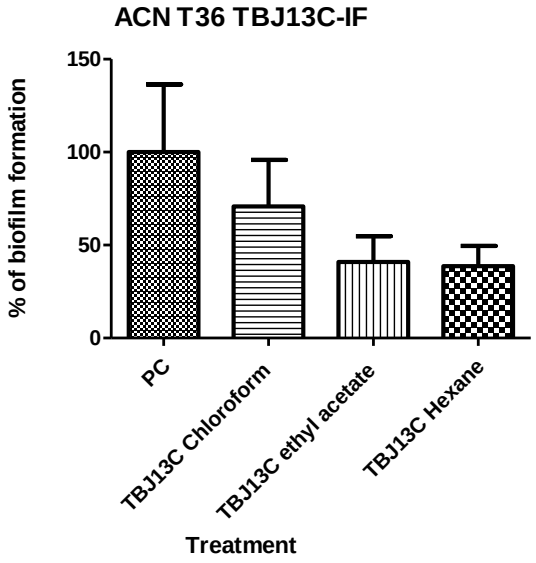
TBJ13 C		Media		
IF	Bacteria	Chloroform	Ethyl acetate	Hexane
	PAN14	18%	38%	13%
	ACNDSM	1.9%	8.5%	1.9%
	ACNT36	70.8%	40%	38.7%
	MRSA	5.7%	10.4%	-
PF	Bacteria	Chloroform	Ethyl acetate	Hexane
	PAN14	2.8%	4%	1.3%
	ACNDSM	10.8%	32%	8%
	ACNT36	46%	40.5%	70%
	MRSA	177%	791%	-

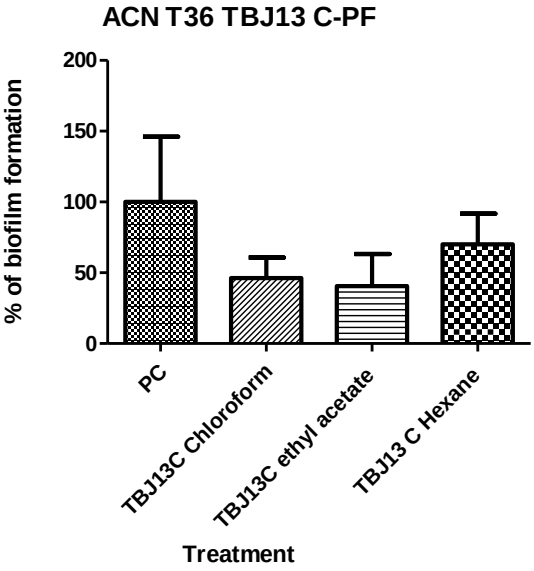





ACN DSM TBJ13C- IF
% of biofilm formation
200
150
100
50
0
PC
TBJ13 C Chloroform
TBJ13 C Ethyl acetate
TBJ13 C Hexane
Treatment


ACN DSM TBJ13C-PF
% of biofilm formation
150
100
50
0
*
*
PC
TBJ13C h20/methanol
TBJ13C hexane
TBJ13C ethyl acetate
TBJ13 C chloroform
Treatment


ACN T36 TBJ13C-IF
% of biofilm formation
150
100
50
0
PC
TBJ13C Chloroform
TBJ13C ethyl acetate
TBJ13C Hexane
Treatment


ACN T36 TBJ13 C-PF
% of biofilm formation
200
150
100
50
0
PC
TBJ13C Chloroform
TBJ13C ethyl acetate
TBJ13 C Hexane
Treatment

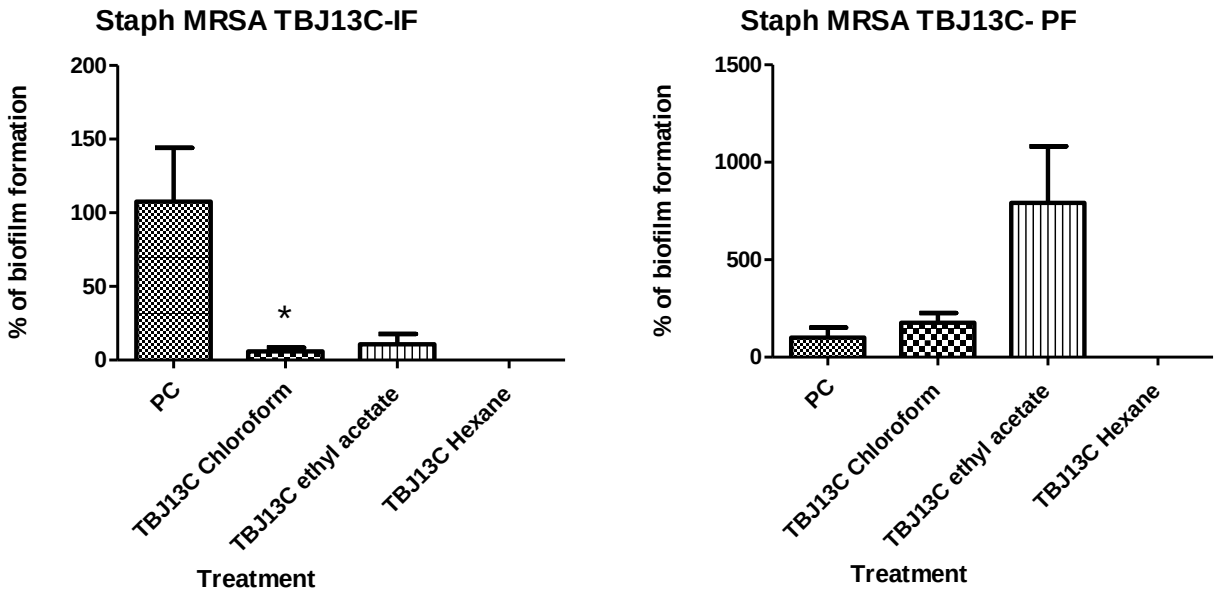


Figure 26. Percentage of biofilm formation in PAN14, ACN DSM, ACN T36, and MRSA treated by TBJ13C-derived extract.

D. Genomic Characterization

1. DNA extraction

The above- purified strains that were tested for their antibiofilm activities were further subjected to genomic DNA extraction analysis using a specific kit protocol. Since a ration (A260/280) of 1.8 represents a pure DNA sample, and the results obtained were > 1.8 (Table 15), the extracted DNA from the samples was considered of high purity.

Table 15. DNA extraction of MM3, MM4, MM7, and MM9 strains

Sample Name	Concentration(ng/µL)	A260/280	A260/230
MM3	160.563	1.96	2.17
MM4	985.389	2.18	2.82
MM7	333.412	2.14	2.41
MM9	648.057	2.05	2.18

2. 16S rRNA Sequencing

The sequencing of the 16S ribosomal RNA (16S rRNA) gene is recognized to be effective for bacterial identification [143]. After obtaining the 16S rRNA gene sequences of the unknown MM3, MM4, MM7, MM9, BM9, and TBJ13 strains, the sequencing data were assembled, edited, and compared with GenBank sequences by using the basic local alignment search tool (BLAST). The first five hits of every strain are represented in the below table, along with their percent identity. According to recent studies, sequence identity ≥ 99% is used to identify isolates of the same species. In this regard, since the percent identity of MM3, MM4, MM7, BM9, and TBJ13 strains ranges between 99.09% and 99.78%, these strains are thereby of known species and genus. However, MM9 strain, having a percent identity ranging between 98.7–99%, might be a new novel species.

Table 16. Description and Percent identity of MM3, MM4, MM7, MM9, BM9, and TBJ13 according to BLAST. *https://blast.ncbi.nlm.nih.gov/Blast.cgi*

	Description	Per. Ident
MM3	Streptomyces viridochromogenes strain NBRC 13347 16S ribosomal RNA, partial sequence	99.09%
	Streptomyces viridochromogenes strain NBRC 3113 16S ribosomal RNA, partial sequence	99.09%
	Streptomyces albogriseolus strain NBRC 3709 16S ribosomal RNA, partial sequence	99.09%
	Streptomyces albogriseolus strain NBRC 3413 16S ribosomal RNA, partial sequence	99.09%
	Streptomyces viridodiastaticus strain NBRC 13106 16S ribosomal RNA, partial sequence	99.09%

	Description	Per. Ident
MM4	Streptomyces cellulosae strain NBRC 13027 16S ribosomal RNA, partial sequence	99.32%
	Streptomyces cellulosae strain NRRL B-2889 16S ribosomal RNA, partial sequence	99.32%
	Streptomyces minutiscleroticus strain NBRC 13361 16S ribosomal RNA, partial sequence	98.87%
	Streptomyces minutiscleroticus strain NBRC 13000 16S ribosomal RNA, partial sequence	98.87%
	Streptomyces pseudogriseolus strain NRRL B-3288 16S ribosomal RNA, partial sequence	98.87%

	Description	Per. Ident
MM7	Bacillus proteolyticus strain MCCC 1A00365 16S ribosomal RNA, partial sequence	99.79%
	Bacillus wiedmannii strain FSL W8-0169 16S ribosomal RNA, partial sequence	99.79%
	Bacillus cereus strain IAM 12605 16S ribosomal RNA, partial sequence	99.58%
	Bacillus tropicus strain MCCC 1A01406 16S ribosomal RNA, partial sequence	99.58%
	Bacillus paramycoides strain MCCC 1A04098 16S ribosomal RNA, partial sequence	99.58%

	Description	Per. Ident
MM9	Streptomyces lienomycini strain NBRC 15425 16S ribosomal RNA, partial sequence	98.90%
	Streptomyces aurantiogriseus strain CSSP525 16S ribosomal RNA, partial sequence	98.90%
	Streptomyces lienomycini strain LMG 20091 16S ribosomal RNA, partial sequence	98.90%
	Streptomyces albogriseolus strain DSM 40003 16S ribosomal RNA, partial sequence	98.68%
	Streptomyces violaceorubidus strain LMG 20319 16S ribosomal RNA, partial sequence	98.46%

	Description	Per. Ident
BM9	Streptomyces galilaeus strain JCM 4757 16S ribosomal RNA, partial sequence	99.55%
	Streptomyces galilaeus strain NBRC 13400 16S ribosomal RNA, partial sequence	99.55%
	Streptomyces bobili strain NBRC 16166 16S ribosomal RNA, partial sequence	99.32%
	Streptomyces bobili strain NBRC 13199 16S ribosomal RNA, partial sequence	99.32%
	Streptomyces rhizophilus strain JR-41 16S ribosomal RNA, partial sequence	98.65%

		Description	Per. Ident
TBJ13	☑	Halomonas sp. strain MBL0129 16S ribosomal RNA gene, partial sequence	99.88%
	☑	Halomonas sp. strain 201707CJKOP-34 16S ribosomal RNA gene, partial sequence	99.88%
	☑	Halomonas sp. YLB-10 16S ribosomal RNA gene, partial sequence	99.88%
	☑	Halomonas sp. strain SR56 16S ribosomal RNA gene, partial sequence	99.88%
	☑	Halomonas sp. ZJ2214 16S ribosomal RNA gene, partial sequence	99.88%

CHAPTER V

DISCUSSION

Over the past three decades, research carried out suggests that biofilms are either extremely difficult or impossible to eradicate from the human body[33]. Up to date, health care institutions are struggling in diagnosing and treating infections caused by biofilm-forming pathogens, as these kinds of infections show high resistance to conventional drugs. Due to this situation, the discovery and development of anti-biofilm compounds and/or biofilm eradication agents is an urgent medical need.

As the results demonstrate the absence of antibacterial activity of the strains-derived extracts on almost all ESKAPE pathogens, these findings emphasize that the crude extracts may be either susceptible to microorganisms other than bacteria including fungi, parasites, viruses, or target specific microbial sessile cells that live inside the bacteria known as biofilm, thereby acting as anti-biofilm inhibitors. In our study, this was the case, where the crude extracts derived from different strains showed anti-biofilm activity by reducing biofilm growth and/or dispersing already established ones in the four selected biofilm-forming pathogens previously mentioned. Generally, studies have shown that *Streptomyces* and *Bacillus,* which are soil microorganisms, along with *Halomonas* species, marine microorganism, are potential sources of active secondary metabolites [16][119][144]. Since our extracts are derived from these three species, as shown in table 16, the report of the strains-derived extracts having anti-biofilm activities is not surprising.

Based on our findings, all strains-derived extracts were capable of inhibiting biofilm formation in different production media in *P. aeruginosa* strain (PAN14), one of the most commonly studied non-mucoid laboratory strains that exhibit a strong biofilm- forming capability[145]. Additionally, some strains, such as MM3 was also capable of inhibiting biofilm formation in ACN strain T36, a multidrug-resistant clinical pathogen that extremely forms robust biofilm (Table 9). Interestingly, its noteworthy that crude extracts of MM9 exerted dual actions in preventing and eradicating PAN14 biofilm in different production media (Table 12), indicating that different molecules in the extract can target different stages of biofilm formation. Further, as BM9C and TBJ13C strains were fractionated, they demonstrated significant activity in inhibiting PAN14 biofilm formation, as well as they showed good potential in eradicating its pre-formed biofilm, in ethyl acetate and hexane fractions, respectively (Tables 13 & 14).

Analyzing these results, molecules in these extracts and fractions seems to inhibit or interfere with specific biofilm formation processes including i) initial steps of biofilm formation; ii) EPS matrix formation; iii) chemical pathways required for bacteria to maintain the biofilm mode of existence, such as quorum sensing; iv) c-di-GMP signaling, particularly in Gram-negative bacteria [27]. Since the first step in the biofilm formation process is the attachment or adhesion of a cell to a surface, blocking this initial step is one of the suggested targets of the active crude extract's compounds, which, in turn, will reduce the chances of further development and establishment of biofilm in pathogens, and thereby prevent biofilm-associated infections[27]. Not only molecules in the crude extracts can target the early stages of biofilm formation, but also they may inhibit its late stages. For

instance, compounds in the active extracts may inhibit the expression of different genes of the EPS matrix, or interfere with its components, leading to the disruption of the biofilm architecture during the maturation process[22]. Besides, quorum sensing, an essential regulator of biofilm formation in *P. aeruginosa*, *A. baumannii,* and *S. aureus* pathogens could also be one of the possible novel targets of the active molecules to control biofilm-associated infections in human[10][35]. The active molecules can disrupt this intercellular communication signal by inhibiting the expression of its specific genes in every pathogen, thus acting as QS inhibitors. For instance, QS disruption can reduce the production of Pel exopolysaccharide in PAN14, and may lead to biofilm dispersal in *S. aureus* pathogen [55][145]. Another regulatory system that may be targeted by the crude extract's compounds is the c-di-GMP, a universal positive regulator of biofilm formation, mainly in *P. aeruginosa* pathogen[69]. As antibiofilm inhibitors, molecules in the active crude extracts may target DGCs and PDEs enzymes, in a way that can reduce the c-di-GMP level in bacteria. Therefore, the active molecules can interfere with cyclic di-GMP metabolism by either overexpressing DGC or inactivating PDE, which, in turn, will impede c-di-GMP biosynthesis, and thereby induce biofilm dispersal[69].

In conclusion, marine and soil microorganisms are well-known sources that potentially produce a myriad of novel bioactive compounds. Since most of the discovered anti-biofilm agents derived from these two sources are either in the initial stage of investigation, or have terribly failed to enter the preclinical test, our goal in this study is to develop these novel anti-biofilm compounds that not only do need to be efficacious but also

must have appropriate bioavailability, pharmacokinetic properties, as well as lack of toxicity.

Future perspectives

As our work illustrates that BM9C ethyl acetate and TBJ13C hexane fractions exhibit potent anti-biofilm activity, further assays will be arranged to determine how specific, and broadly active these soil and marine-derived metabolites are. Accordingly, our future work will be focused on separating the different molecules of BM9C ethyl acetate fraction, as well as TBJ13C hexane fraction, using flash chromatography and/or high-performance liquid chromatography (HPLC) technique. The attained chromatography fractions will then be tested by BMD, where the compounds that possess the highest anti-biofilm activities will have their structures elucidated by Nuclear Magnetic Resonance (NMR). Moreover, phenotypical and biochemical characterizations of these two strains will be performed using a scanning electron microscope (SEM), gram staining technique, biochemical (API 20E) profiling, as well as NaCL and PH growth characterization. Consequently, the active compound will then be tested in-vivo, aiming to have low toxicity levels, as well as further investigations, will be done to determine their mechanism of action. The active extracts of MM3, MM7, and MM9 strains in different production media will follow the same steps as of BM9 and TBJ13 in order to screen and purify any active anti-biofilm compounds.

Therefore, as a result of the complete lack of selective anti-biofilm drugs in the clinic, efforts will be targeted at fine-tuning these anti-biofilm molecules in the hope that

they can effectively minimize and eradicate biofilm-related infections, and thereby have a great potential in future applications as stand-alone biofilm treatment.

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