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NOVEL ANTI-BIOFILM AGENTS FROM UNEXPOLITED SOIL AND MARINE MICROORGANISMS

by
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ABSTRACT
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Title: Novel Anti-biofilm Agents from Unexploited Soil and Marine Microorganisms

Background: Biofilm formation, especially on indwelling medical devices, is highly problematic and represents a crucial problem in patient care. Biofilms are considered a protective environment for the bacterial communities, making those biofilm-forming microorganisms 500-5000 times more resistant to conventional antibiotics. Therefore, therapeutic interventions are needed not only to inhibit biofilm formation, but also to try and eradicate pre-formed biofilms. This gave rise to plenty of studies that resorted to plant-derived, soil-derived and marine-derived extracts as agents to combat these infections. Hence, we aimed at investigating the activity of unexploited Lebanese soil and marine microorganisms in inhibiting and/or eradicating biofilms of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, two potent biofilm-forming pathogens.

Materials and Methods: Water and soil samples were collected from four regions in Lebanon, Tabarja (Tbj), Beit Meri (BM), Mazrat Meshref (MM) and Zekrit (ZK). From the collected samples, secondary metabolites were extracted in 14 different production media to be further tested. First, they were tested for their antibacterial activity on a panel of ESKAPE pathogens, and then for their anti-biofilm activity using biofilm formation assays. The active extracts were then subjected to bio-guided fractionation and physiological, phenotypic and biochemical characterization. Finally, pure compounds were obtained after several rounds of purification and those were again tested for their ability to inhibit or eradicate *Pseudomonas aeruginosa* Pan14 and *Acinetobacter baumannii* DSM biofilms.

Results: The extracts generated by the isolates in the 14 different production media demonstrated minimal or no antibacterial activity on any of the pathogens, so we proceeded to test these bacterial extracts for their anti-biofilm activity.
Crude extracts derived from the medium C of both BM9 and TBJ13 significantly reduced the biofilm formation in *Pseudomonas aeruginosa* Pan14 o 24.1% and 11.8%, respectively. On the other hand, crude extracts derived from medium C of ZK31 reduced the biofilm formation in *Acinetobacter baumannii* DSM to -3.4%.
Therefore, pH and NaCl tolerance, API and Gram staining were performed for the three extracts to properly characterize them before proceeding with 16S sequencing for Tbj13 and BM9.
Medium C of the three isolates was then produced on a larger scale and subjected to liquid-liquid partitioning. The fractions obtained were further tested showing anti-

biofilm activity in Tbj13 C Hexane, BM9 C Chloroform + Hexane and BM9 C Ethyl acetate against *Pseudomonas aeruginosa* Pan14 and ZK31 C chloroform against *Acinetobacter baumannii* DSM.
Extracts showing the highest activity were purified using column chromatography, thin layer chromatography and high-performance liquid chromatography. All of this led us to obtain three pure compounds: Tbj13 C Hexane, BM9 C (C + H) G a and BM9 C (C + H) G b which significantly inhibit *Pseudomonas aeruginosa* Pan14 biofilm formation with a P value < 0.0001.

Conclusion: Targeted anti-biofilm therapy is crucial to effectively reduce biofilm-associated infections. This highlights the importance of this study and more importantly, of the natural products as a source of potentially active soil and marine-derived bioactive compounds. Due to the scarcity of anti-biofilm drugs and to the fact that most of the discovered agents have not succeeded until now, we aim to discover and develop anti-biofilm agents with proper bioavailability, pharmacokinetic properties, and most importantly to provide safe, non-toxic administration to infected individuals.

TABLE OF CONTENTS

ILLUSTRATIONS

Figure

TABLES

Table

CHAPTER I

LITERATURE REVIEW

A. Overview of Antimicrobial resistance (AMR)

Antimicrobial resistance has increased dramatically over the years, and it now represents one of the most alarming issues worldwide, hastened by the abuse and misuse of antibiotics (Ferri et al., 2017). It jeopardizes the effective treatment and/or prevention of the wide range of bacterial, viral, parasitic or fungal infections caused by resistant agents (Prestinaci et al., 2015).

Specifically, antibacterial resistance is the process by which bacteria overcome the effect of the antibiotics that are commonly used to inhibit the associated infections (Ferri et al., 2017). Some microorganisms that are usually sensitive to certain antibiotics might eventually develop resistance due to prolonged exposure to those agents (Giedraitienė et al., 2011). Additionally, some strains are becoming resistant to more than one antibiotic, a phenomenon known as multidrug resistance (Nikaido, 2009). There are three known mechanisms of antimicrobial resistance: intrinsic, adaptive, and acquired. Intrinsic resistance is the innate ability of a bacterial species to limit the activity of antimicrobials through inherent structural characteristics. An example of intrinsic resistance would be having a semipermeable outer membrane (OM) through which large antibiotics cannot penetrate. For instance, vancomycin cannot breach the OM of *Pseudomonas aeruginosa*. Additionally, *P. aeruginosa* expresses efflux pumps granting it resistance against several classes of antibiotics (Arzanlou et al., 2017; Fernández & Hancock, 2012). On the other hand, acquired resistance can be gained through horizontal transfer of resistance genes (Pang et al., 2019). This phenomenon is

observed in the case of some *P. aeruginosa* strains which acquire plasmids, one of the forms of horizontal gene transfer. Those plasmids confer resistance to this pathogen against penicillins and cephalosporins through encoding new ß-lactamases (Sacha et al., 2008). Finally, adaptive resistance is obtained due to certain environmental cues/pressures which lead to altered genes or protein expression. Unlike the first two, this type of AMR can be reversed if the trigger is removed (Fernández & Hancock, 2012). One of the most common examples of this adaptive resistance is biofilm formation which will be discussed in details along this review (Pang et al., 2019). A biofilm is a community of microbial cells that resist the capabilities of certain antibiotics, thus promoting their survival (Bernardes et al., 2015).

B. Overview of biofilm structure and formation

1. General features and characteristics of biofilm

Biofilms are one of the most widely distributed modes of life on Earth (Flemming et al., 2016). They are the predominant mode of growth for many bacteria in nature, industry, the human body, as well as in hospital settings (Donlan, 2002; Wei & Ma, 2013).

In the biofilm form, bacteria exhibit patterns of adaptive resistance to antibiotics, and to the host immune defenses, making them 500-5000 times more resistant to conventional treatment than planktonic cells. This allows them to thrive in unfavorable environments (Bernardes et al., 2015; Khatoon et al., 2018).

The biofilm is defined as a microbial community of cells that are lodged in an adhesive extracellular matrix (ECM) composed of extracellular polymeric substances (EPS), extracellular DNA (eDNA) and proteins (Bernardes et al., 2015). The presence

of this ECM allows them to be found adherent to each other and/or to surfaces, as seen in *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and some other bacteria (Flemming et al., 2016; Roy et al., 2018; Wei & Ma, 2013).

The ECM also plays an important role when it comes to the bacterial transition from the planktonic, free-living state to mature biofilms, because it encases the bacterial aggregates and provides protection (Ciofu & Tolker-Nielsen, 2019). Additionally, it enhances the capacity of bacteria to resist to several antibiotics, as it makes it difficult for those antimicrobial agents to penetrate the structure of the biofilm.

Another possible reason leading to the resistance is the presence of heterogeneous subpopulations of bacterial cells known as persister cells that spontaneously enter into a dormant state. This dormancy promotes the tolerance of the antibiotics by these cells since antimicrobial agents need mitotically or biochemically active bacteria to be able to target them (Bernardes et al., 2015; Lewis, 2010; Tassew et al., 2017).

2. Biofilm life cycle

Biofilm formation consists of a sequence of events that involves the transition from a planktonic state to a sessile state and this is almost uniform in several organisms (Clutterbuck et al., 2007; O'Toole, 2003; Rasamiravaka et al., 2015).

A. Initial adhesion/attachment

The first step in the biofilm formation process is defined by a reversible attachment of free-floating bacteria to biotic or abiotic surfaces. This contact can be mediated by the bacterial extracellular appendages which include type IV pili, flagella

and fimbriae with the help of certain surface proteins such as OmpA, fibronectin binding proteins, protein A, SasG, and biofilm associated proteins (BAP).

Physical forces also play an important role in the bacterial attachment, and those include hydrophobic interactions, van der Waal's and electrostatic interaction forces (Clutterbuck et al., 2007; Jamal et al., 2018; Rasamiravaka et al., 2015; Roy et al., 2018).

Some species are unable to use the previously mentioned interactions or forces to attach to surfaces or to each other, so they rely on cell-cell communication systems, most commonly quorum sensing (QS) (Roy et al., 2018).

B. Micro-colony formation and EPS production

After the initial contact with the surfaces, the reversible attachment turns irreversible and motile cells attach more firmly and become immobilized to the surfaces. This is highly driven by the formation of the ECM polysaccharides and associated proteins; bacteria start multiplying and forming layers one on top of the other, and further dividing to form micro-colonies. This is the initial step leading to the maturation of the biofilm which is demonstrated by the excretion of the EPS that surrounds and protects the formed micro-colonies (Høiby et al., 2011; Jamal et al., 2018; Sun et al., 2013).

C. Maturation and architecture

Under adequate growth conditions, the maturation stage starts. This step is driven by cell-cell communication, where chemical signaling molecules are secreted. Additionally, gene products, known as auto-inducers, are expressed to form a more

complex architecture of biofilm consisting of water channels that aid in transporting the needed nutrients and oxygen to the inside of the biofilm (Chung & Toh, 2014; Jamal et al., 2018).

D. Detachment/ dispersion of biofilm

The last step in the process consists of planktonic bacterial cells detaching from the mature biofilm to spread and form new biofilms on new surfaces (Høiby et al., 2011). This detachment is a natural process in most cases, and it is mediated by releasing saccharolytic enzymes. For example, *P. aeruginosa* produces the enzyme alginate lyase to degrade the EPS matrix and lead to subsequent detachment. This stage can be also mediated by the increased expression of proteins related to the flagellar motion of the bacteria helping them to detach and spread the infection to new locations (Jamal et al., 2018).

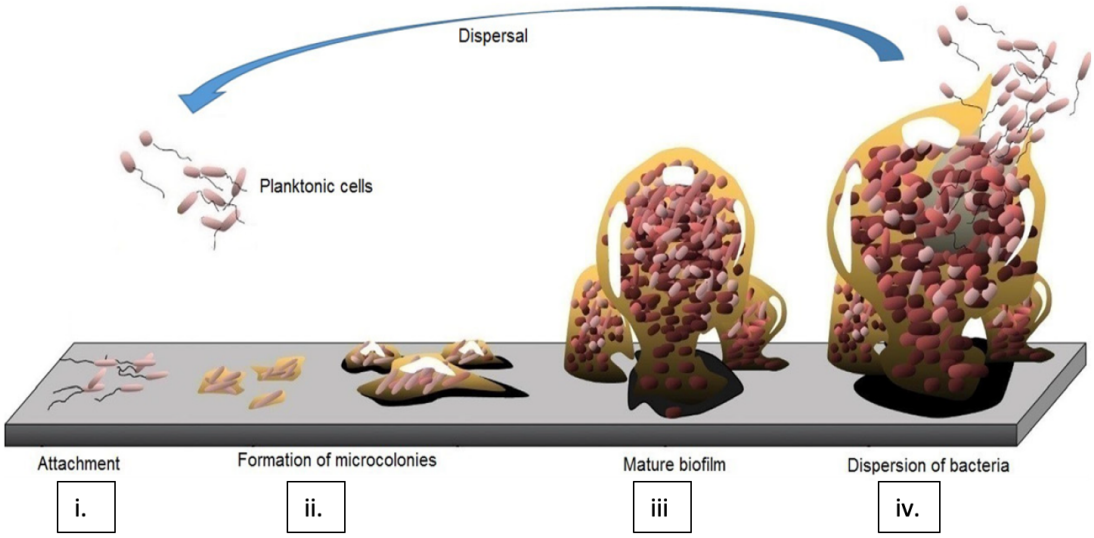

Figure 1. Biofilm life cycle (Chung & Toh, 2014) with modifications

C. Regulators involved in biofilm formation

The switch from planktonic cells to a sessile state is driven by several regulatory systems and genetic regulations. Those systems are important to better understand the biofilm formation process and to identify therapeutic alternatives to target bacterial infections. Of the systems that have been closely investigated, three will be discussed (Wei & Ma, 2013).

1. C-di-GMP

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is a soluble intracellular second messenger who is in charge of the switch between planktonic cells and sessile biofilm-associated forms. Specifically, high levels of c-di-GMP stimulate the biosynthesis of adhesins and exopolysaccharides and inhibit the motility of planktonic cells facilitating the transition to the biofilm state. Whereases, low levels of c-di-GMP decrease the production of adhesins and exopolysaccharides and enhance the bacterial motility, which leads to biofilm detachment and dispersal back to the planktonic state (Wolska et al., 2016).

The synthesis and degradation of c-di-GMP is controlled by 2 enzymes having opposite effects, the diguanylate cyclase (DGC) having a GGDEF domain and specific phosphodiesterases (PDE) with EAL or HD-GYP domains. These domains are responsible, respectively, for the formation and degradation of c-di-GMP (Jenal & Malone, 2006; Wei & Ma, 2013). Generally, Gram-negative bacteria are mostly characterized with these domains (Hengge, 2009).

2. Quorum sensing (QS)

QS is a cell-cell communication system that allows bacteria to control their gene expression and cellular proliferation in a cell-density related manner (Rutherford & Bassler, 2012; Vipin et al., 2019). QS typically controls the production, detection, and response to important extracellular virulence factors, known as autoinducers (AIs) (Favre-Bonté et al., 2003). Those AIs are produced by bacteria and accumulate in the extracellular environment, so when they surpass a certain threshold, they activate the QS system (Karatan & Watnick, 2009).

Both Gram-positive and Gram-negative bacteria use QS. Gram-negative bacteria usually use many autoinducers, and the most common class is acyl-homoserine lactones (AHLs) (Kai Papenfort1, 2014).

While gram-negative bacteria use different autoinducers, gram positive bacteria recruit post translationally modified signaling molecules known as autoinducing peptides (Bhatt, 2019).

3. Small non-coding RNA molecules (sRNA)

Small non-coding RNAs have been proven to be important modulators of gene expression and bacterial developmental processes such as biofilm formation (Chambers & Sauer, 2013). sRNAs participate in the biofilm formation process by controlling the bacterial switch from planktonic to sessile state (Ghaz-Jahanian et al., 2013). This occurs by two main mechanisms. The first one occurs by sRNA base pairing with target mRNA. Base pairing between sRNAs and mRNA targets can affect target gene expression by altering the ribosome binding sites, hence the mRNA translation and stability. This pairing can be categorized as cis or trans depending on the location of the

sRNAs relative to their target in the bacterial genome. Cis-encoded sRNAs are transcribed from the DNA directly opposite to their mRNA target. Those sRNAs share substantial complementarity with their targets. Whereas, sRNAs found elsewhere in the genome are encoded as trans and they share limited complementarity with their targets (Chambers & Sauer, 2013). The second mechanism is protein binding. By imitating the protein binding sequences present in multiple mRNAs, protein-binding sRNAs antagonize and sequester their cognate regulatory proteins (Ghaz-Jahanian et al., 2013). sRNAs are also involved in stress adaptation, microbial pathogenesis, and a variety of metabolic processes. The mentioned characteristics have made sRNAs an imperative tool in microbial engineering and synthetic biology (Wolska et al., 2016).

D. Biofilm-forming pathogens

It is now proven that the biofilm state is a characteristic of a variety of microorganisms (Jamal et al., 2018; Tasneem et al., 2018). Studies showed a direct correlation between biofilm formation and persistent infections, and this was proven in the case of cystic fibrosis patients whose lungs were constantly colonized with *P. aeruginosa*. Moreover, the link between infections and the growth of biofilm on medical devices was studied in a patient with recurrent *S. aureus* bloodstream infection (Lebeaux et al., 2014). Another pathogen also known to be associated with such infections is *Acinetobacter baumannii*. Infection with this pathogen can lead to pneumonia, skin and soft tissue infections, nosocomial meningitis, as well as medical device-associated infections (Eze et al., 2018). The pathogens related to these infections are the ones to be discussed in this section, along with their correlation to serious infection such as urinary tract infections.

1. Overview of urinary tract infections

Urinary tract infections (UTIs) are a serious health concern. They are considered the most commonly acquired bacterial infections accounting for 30-40% of nosocomial infections (Bagshaw & Laupland, 2006). The term UTI describes a microbial invasion, mostly bacterial, that can present itself in the urethra, bladder, ureters, or the kidneys (Foxman, 2014). Generally, this type of infection is caused by uropathogenic microbes which are organisms found in urine of infected individuals (Jepsen, 1987).

Of the common uropathogens associated with urinary tract infections, *P. aeruginosa* (Lamas Ferreiro et al., 2017), *A. baumannii* (Wong et al., 2017), and S. aureus (Onanuga & Awhowho, 2012) are closely associated with these infections. Additionally, *Escherichia coli*, as the predominant pathogen, *Staphylococcus saprophyticus, Klebsiella, Enterobacter, Proteus* species and few others are correlated to UTIs (Ronald, 2003).

2. Pseudomonas aeruginosa

P. aeruginosa is a Gram negative, aerobic, non-lactose fermenting, rod-shaped pathogen that is known to be among the most virulent of the opportunistic pathogens and a major cause of nosocomial infections (Silby et al., 2011).The majority of the cases of *P. aeruginosa* infections are associated with a weakened immune system of the host. This includes patients with AIDS, neutropenia and patients with other concurrent infections (Lyczak et al., 2000). It is mainly associated with chronic infection of the lungs of cystic fibrosis patients, as well as chronic urinary tract infections, ventilator-associated pneumonia, and chronic wounds (Ciofu & Tolker-Nielsen, 2019).

a. Biofilm formation of *P. aeruginosa*

P. aeruginosa produces clinically relevant biofilm which makes it the model organism for the study of this life form. The importance of the *P. aeruginosa* biofilm resides in its protective extracellular matrix. This matrix is composed of three exopolysaccharides, Psl, Pel, and alginate. It also contains eDNA, proteins, and bacterial extracellular appendages such as fimbriae, type IV pili (T4P), and flagella (Ma et al., 2009; Wei & Ma, 2013). The matrix formation takes place when the cells are at the stage of microcolony formation, which leads to the production of pellicles, also known as mature solid-surface associated (SSA) biofilms at the air-liquid interface (Friedman & Kolter, 2004). The major contributors in this step are the mannose rich Psl polysaccharide and the glucose rich Pel polysaccharide (Yang et al., 2011).

Psl is an extracellular polysaccharide encoded by the polysaccharide synthesis locus. It is a key component at the early stage of biofilm formation, where it promotes strong adhesion, cell-cell and cell-surface interactions (Ma et al., 2009). In addition to Psl functioning as a structural scaffold, it functions as a signaling molecule for the succeeding steps of biofilm development. It also functions in protecting cells of the biofilm against antibiotics due to its acidic nature capable of binding to cationic antibiotics (Colvin et al., 2011; Wei & Ma, 2013).

Pel is synthesized by the products of the pel gene cluster (pelA-F). It is also required for the pellicle formation and cell-cell interaction. Similar to Psl, it interacts with the extracellular DNA protecting the biofilm cells against antibiotics, particularly aminoglycosides by the same mechanism as Psl (Colvin et al., 2011). The defined structure and biochemical composition of Pel is still unknown. But according to some studies, it is composed of cationic exopolysaccharides (Franklin et al., 2011).

Alginate is a negatively charged polymer composed of guluronic and mannuronic acid (Jennings et al., 2015). It is responsible for the typical mucoid phenotype in the lungs of cystic fibrosis patients, it also establishes stability for the matrix and protection of *P. aeruginosa* in harsh environments. Alginate plays a substantial role in the protection and stability of the biofilms as well as in water and nutrients retention. Similar to Pel and Psl, it mediates antibiotic resistance by directly binding to cationic antibiotics, making it a characteristic of chronic, persistent lung infections (Franklin et al., 2011; Wei & Ma, 2013).

b. Regulators involved in *P. aeruginosa* biofilm formation

QS, c-di-GMP and sRNAs apply in almost all biofilm-forming pathogens, but in a specific manner relating to each pathogen. For *P. aeruginosa*, QS systems play an important role in the biofilm formation process as well as in the production of virulence factors that aid in the disease-causing phenomenon (Lebeaux et al., 2014; Rutherford & Bassler, 2012). This pathogen has two widely studied QS systems. The las system possesses a transcriptional regulator, the lasR, and a synthase protein, the LasI which has a role in the synthesis of AHL signal molecule, N-(3-oxo-dodecanoyl)-L-homoserine lactone. The other QS system is based on the proteins RhlI and RhlR. RhlR is the transcriptional regulator, and RhlI synthase is required for the production of the AHL, N-butyryl-L-homoserine lactone (C4-HSL) (R. S. Smith & Iglewski, 2003).

Additionally, the production of rhamnolipids, responsible for the detachment and dispersal, is controlled by the QS system. Hence, the QS system is responsible for the dispersal step (Wolska et al., 2016).

On the other hand, there are four c-di-GMP effectors which are FleQ, PelD, Alg44, and PilZ. FleQ functions as an activator in flagellar biosynthesis, whereas the other three are known to regulate the production of exopolysaccharide components (Wei & Ma, 2013).

In addition, sRNAs play a substantial role in the regulation and production of *P. aeruginosa* biofilm where the two best known are rsmY and rsmZ (Ventre et al., 2006). Those two sRNAs are regulated by the GacA/GacS system network as shown in figure 2 (Ghaz-Jahanian et al., 2013). According to Chambers and Sauer (2013), those sRNAs contribute to the attachment of *P. aeruginosa* bacterial cells to abiotic surfaces, but elevated levels of these sRNAs may hamper subsequent biofilm development, especially rsmZ (Chambers & Sauer, 2013).

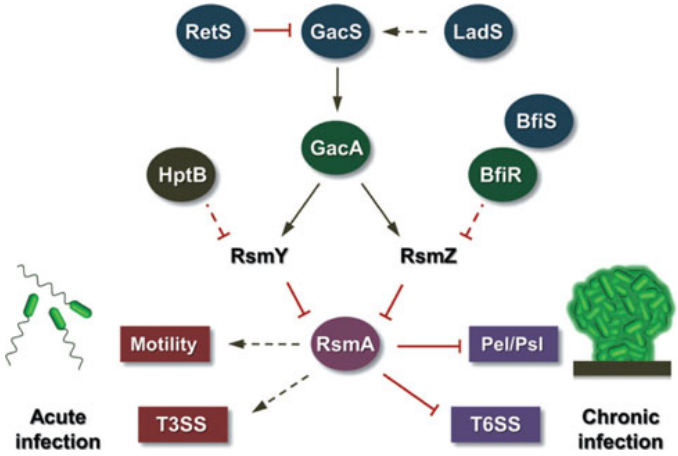

Figure 2. GasS/GacA system (Ghaz-Jahanian et al., 2013)

3. Acinetobacter baumannii

A. baumannii is a Gram-negative, non-fermenting, opportunistic, nosocomial pathogen. It has been an important target of study due to its involvement in a wide range of serious infections. These infections have been proven to be more problematic in compromised individuals, such as patients supported with ventilators which are at a

high risk of developing ventilator-associated pneumonia. In addition to compromised patients with urinary tract infections, bloodstream infections, wound, skin, and soft-tissue infections. This list goes on and its components are associated with high morbidity and mortality rates (Gaddy & Actis, 2009; Longo et al., 2014).

A. baumannii can survive unfavorable conditions such as desiccation, starvation, and antimicrobial treatment. The outstanding survival properties of this pathogen, its multi- and pan drug resistance, as well as its virulence are associated with its ability to form biofilm. It possesses the ability to colonize biotic surfaces such as epithelial cells and fungal filaments. It also thrives on abiotic surfaces like polystyrene, glass, and more importantly urinary catheters triggering catheter-associated urinary tract infections (CAUTIs) in ICU patients (Gaddy & Actis, 2009; Longo et al., 2014). The presence of dormant cells, a characteristic of *A. baumannii*, contributes to its ability to persist and form biofilms on the abiotic surfaces (Longo et al., 2014).

a. Biofilm formation of *A. baumannii*

A. baumannii biofilm formation is a well-orchestrated process, controlled by several environmental conditions, and virulence factors relating to its antibiotic resistance and ability to form biofilm on surfaces (Longo et al., 2014).

There is a variety of virulence factors related to the biofilm formation of this pathogen. The most important is the biofilm-associated protein (BAP), which is produced by *A. baumannii* and encoded by the bap gene. This protein is involved in cell-cell interaction and functions in the initiation step after *A. baumannii* attaches to a surface (Gaddy & Actis, 2009; Thummeepak et al., 2016). Another factor is the outer membrane protein (OmpA) which is an adhesion molecule encoded by the ompA gene. It plays a role in

the attachment to human epithelial cells and contributes to drug resistance (Eze et al., 2018; Thummeepak et al., 2016).

The majority of *A. baumannii* strains possess a CsuA/BABCDE pilus usher-chaperone assembly system which is regulated by a two-component system (bfmS/bfmR). This system allows the bacterial cells to adhere to abiotic surfaces and to form microcolonies leading to the development of the biofilm later on (Gaddy & Actis, 2009; Longo et al., 2014). The involvement of Csu pili and OmpA in important for the bacterial adhesion of this pathogen, since it lacks flagella (Qvortrup et al., 2019).
It was also shown that there was a positive correlation between the expression of the blaPER-1 broad-range β-lactamase gene and the adherence of bacterial cells to epithelial cells (Gaddy & Actis, 2009). *A. baumannii* biofilm maturation also depends on the production of poly-β-1,6-N-acetylglucosamine (PNAG), an exopolysaccharide matrix component encoded by a cluster of four genes (pgaABCD) and found in almost all strains (Longo et al., 2014).

b. Regulators involved in *A. baumannii* biofilm formation

The mechanisms used in regulating *A. baumannii* biofilm are less studied than for other microorganisms. However, QS has been shown to play an important role in regulating the biofilm formation. This pathogen has an AHL-based system composed of an AHL synthase, AbaI, and an AHL receptor, AbaR (Qvortrup et al., 2019). The components of this system are produced and transported by an AdeFGH efflux pump (Saipriya et al., 2020).

The correlation between the bfmS/bfmR system and the QS system shows that the upregulation of the bfmS and bfmR genes enhances the activity of the QS system,

hence the ability of *A. baumannii* to form biofilms on surfaces. In addition to the QS system being able to control the biofilm formation, it has also been known to regulate pili formation in this pathogen (Eze et al., 2018). Interestingly, *A. baumannii* has the ability to produce AidA which is a quorum quenching, or QS inhibitor (QSI) enzyme. The activation of this enzyme results in the inhibition of biofilm formation (Qvortrup et al., 2019).

Concerning c-di-GMP involvement in *A. baumannii* biofilm formation, not enough studies were conducted. However, its function is similar to that found in other Gram-negative organisms where high levels promote the biofilm formation. Whereas low levels lead to the enhancing the bacterial motility, inhibiting their attachment and thereby preventing the development of a mature biofilm (Wang & Wang, 2016).

E. Anti-biofilm strategies

Biofilm-associated infections cover a wide range of chronic, persistent infections that are difficult to treat due to the bacterial pathogenicity and ability to develop resistance to antibiotics (Ong et al., 2018; Roy et al., 2018). The common way to treat these infections is to remove the indwelling medical equipment and replace it. But this method is accompanied by difficulties regarding the cost of the procedure, as well as the state of a critically ill patient (Rodrigues, 2011). Therefore, alternative strategies must be found to prevent or treat these infections. This can be accomplished by better understanding and targeting the major phases of the biofilm development. Hence, three main strategies were suggested by targeting the following steps (Azman et al., 2019).

1. Adhesion to surfaces and subsequent bacterial colonization

The first anti-biofilm strategy targets the adhesion step of biofilm development. This can be achieved by altering the physiochemical properties of the surfaces making them anti-adhesive, or by coating them with antimicrobial agents to prohibit bacterial colonization (Azman et al., 2019; Ong et al., 2018). Generally surface coating with hydrophilic polymers is crucial to meddle in the interaction between bacteria and surfaces since microbial surfaces are hydrophobic. The two most common and effective examples used to coat polyurethane catheters are Poly-N-vinylpyrrolidone and hyaluronic acid. Despite their success in preventing bacterial adhesion to surfaces, those molecules possess limitations, due to their rapid dissolution or degradation by certain enzymes. This is exemplified by the case of hyaluronic acid being degraded by the hyaluronidase enzyme (Ong et al., 2018). Additionally, the effectiveness of the surface coating technique depends on the bacterial species (Rodrigues, 2011).

Other alternative strategies also proven to be effective, include silver nanoparticles, and small molecules such as rhodanines and chelating agents having an inhibiting effect on staphylococcal biofilm. However, the exact mechanism of action and killing activity of those molecules is still under development and is not very clear (Chung & Toh, 2014).

2. Signaling molecules

The biofilm development can be interrupted by disturbing the quorum sensing mechanism. Inhibiting the quorum sensing, known as quorum quenching (QQ) is the second adapted anti-biofilm strategy (Yada et al., 2015). QQ plays a role in diminishing or averting biofilm formation, in addition to reducing the bacterial virulence and

resistance (Pang et al., 2019). This approach can be achieved by the use of natural or synthetic quorum sensing inhibitors (Chung & Toh, 2014). For example, halogenated furanones produced by sea-weed plants function in inhibiting the AHL system resulting in defective biofilm formation (Givskov et al., 1996). Additionally, molecules like – L/ D-S- adenosyl homocysteine, S- adenosylcysteine and sinefungin stop the production of AHL (Yada et al., 2015).

However, the use of quorum sensing inhibitors is restrained since they have been associated with high toxicity levels. For instance, furanones are too toxic for humans which makes them unsuitable for treating human bacterial infections (Ong et al., 2018).

On another note, the second messenger c-di-GMP is involved in the dispersal of the biofilm to broaden and facilitate the transmission of infections. Targeting this messenger can also alter the biofilm architecture and dispersal. This can be done by focusing on the enzymes responsible for regulating c-di-GMP. Several molecules such as, LP 3134, LP 3135, LP 4010, and LP 1062 suppress the activity of the enzyme diguanylate cyclase (DGC) leading to the inhibition of biofilm dispersal in *P. aeruginosa* and *A. baumannii* (Sambanthamoorty et al., 2014).

3. EPS matrix aggregation

Since bacteria are highly protected and recalcitrant to antibiotics, an optimal way to access the bacterial cells is to target the EPS matrix. This strategy involves substances that can destroy the essential EPS matrix components and disrupt its integrity (Azman et al., 2019; Stewart, 2015). As this matrix is shattered, the benthonic bacterial cells become exposed to the host responses or to the antimicrobial agents,

hence increasing their susceptibility (Azman et al., 2019). For instance, polysaccharide lyases and DNases are two examples of enzymes that can disrupt the major components of the matrix (Stewart, 2015). Specifically, DNase I has the ability to break down the extracellular DNA (eDNA), while the glycoside hydrolase, Dispersin B can degrade the polymers of β-1-6 N-acetylglucosamine (PNAG) which is an essential polysaccharide in the biofilm formation of *A. baumannii* (Roy et al., 2018). However, it is best to administer the biofilm-dispersing enzymes in combination with antimicrobial agents for better efficiency and killing of bacteria lodged within the EPS (Darouiche et al., 2009).

F. Natural products as a source of anti-biofilm agents

Natural products, also known as secondary metabolites, have been studied over the years and gained a substantial role in the field of drug discovery. They have been used historically in curing a wide range of infectious diseases and illnesses (Dias et al., 2012). Those secondary metabolites can be obtained from a variety of organisms like bacteria, fungi, plants and invertebrates inhabiting terrestrial and marine niches (Melander et al., 2020). Metabolites produced by microorganisms participate in a wide spectrum of therapeutic goals, including cancer therapy, inflammatory diseases as well as biofilm infections (Melander et al., 2020).

Despite the presence of a plethora of antibiotics available to treat a variety of conditions, their abuse and misuse have led to developing antimicrobial resistance. This calls for the need to search for other approaches to treat the biofilm-associated infections (Song et al., 2017). Recent studies concerning biofilm and QS inhibition shed the light on the importance of natural products in this area, where various synthetic and natural peptides showed antibiofilm activity (Bernardes et al., 2015; Lu et al., 2019).

1. Plant-derived anti-biofilm agents

Plants rely on physical and chemical defenses to survive in nature, and this is accomplished by releasing defensive chemicals, also known as secondary metabolites. Those metabolites have been proven to have anti-biofilm activities (Song et al., 2017). By the most, their mode of action counts on targeting the EPS matrix. This leads to the repression of the cell adhesion and the blocking of the ECM formation or the QS system. Hence, this will result in a defective biofilm formation (Lu et al., 2019). Among the various plant derivatives, N-(heptylsulfanylacetyl) -L-homoserine, an extract from garlic, has been found to be a powerful QS inhibitor, interrupting the signaling in *P. aeruginosa* (Lu et al., 2019). This characteristic can also be observed in flavonoids, which are a family of plant-derived metabolites (Pang et al., 2019).

Another effective inhibitor of biofilm formation is Quercetin. It's a plant polyphenol found in many fruits and vegetables. It is capable of inhibiting biofilm formation by decreasing the expression of LasI, LasR, RhII and RhlR in the QS system. This leads to a disruption of the biofilm formation as well as inhibition of the various virulence factors (Ouyang et al., 2016).

Moreover, there is a wide variety of natural compounds that are extracted from plants and serving as potent anti-biofilm agents. Among those there are terpenes, essential oils, carotenoids, cinnamaldehyde, American cranberry extracts and many more (Bernardes et al., 2015; Chung & Toh, 2014; Pang et al., 2019; Song et al., 2017). However, not enough research has been done to establish the exact mechanism of action, also their dosage and pharmacodynamics still need to be established (Song et al., 2017).

2. Marine-derived anti-biofilm agents

a. Non-bacterial agents

In the marine environment, biofouling or marine growth is defined by the attachment of bacterial biofilms, tubeworms, mussels, barnacles, and algae. Previously used anti-biofouling agents caused toxicity which affected the marine life (Salta et al., 2013). Thus, researchers started the search for new sources of anti-biofilm agents from a plethora of marine organisms. Among the species studied, marine sponges (Phylum Porifera) were the most beneficial. Those sponges protect themselves by using certain chemicals which have been proven to have anti biofouling as well as anti-biofilm effects (Stowe et al., 2011). However, only two classes have anti-biofilm without anti-microbial activity. Those two classes include the pyrrole-imidazole alkaloids (PIA) and the terpenoids (Hertiani et al., 2010; Huigens et al., 2007).

Among the PIAs, bromoageleferin has been closely studied and shown to have anti-biofilm properties. Bromoageleferin belongs to the oroidin class of natural products which are characterized by a 2-aminoimidazole (2-AI) subunit believed to be responsible for the anti-biofilm activity (Huigens et al., 2007). Two analogs of bromoageleferin, CAGE (cis-bromoageleferin) and TAGE (trans-bromoageleferin), were assayed for their anti-biofilm activity on *P. aeruginosa*. Both analogs showed a potent inhibitory effect on the biofilm of PAO1 and PA14 strains. However, cytotoxicity was detected for those two derivatives which makes them unsuitable for clinical use (Huigens et al., 2007).

b. Bacterial agents

Nevertheless, microorganisms represent an important potential source to find therapeutic alternatives for biofilm-associated infections. The most common phyla are Actinobacteria or Actinomycetes which are Gram-positive, filamentous bacteria having antibacterial, antifungal, antiviral, anticancer as well as anti-biofilm activities. The majority of those agents are exclusively isolated from Streptomyces species (Bérdy, 2012). For instance, the marine strains of Actinobacteria, KP12, SW19 and CW17 showed significant biofilm inhibition against a wide range of pathogenic bacteria including *P. aeruginosa, S. aureus, Enterococcus faecalis, Vibrio cholera* and others (Azman et al., 2019). Additionally, butenolide is an anti-biofouling compound derived from *Streptomyces* species. This compound has an effective role in inhibiting the biofilm formation of both Gram-positive and Gram-negative bacteria, as well as eradicating already formed biofilms. It is not only considered a promising anti-biofilm agent, but also an antibiotic enhancer (Yin et al., 2019).

3. Soil-derived anti-biofilm agents

a. Bacterial agents

Microorganisms can be found in soil as single cells or an assembly of cells in a way that up to 10 billion microorganisms of various species can be found in one gram of soil (Daniel, 2004). Isolation of certain microorganisms and their derivatives, like *Streptomyces*, showed significant biofilm inhibition. A recent example would be the isolation of three peptidic metabolites, cahuitamycins A, B and C, from *Streptomyces gandocaensis* strain DHS334 (Park et al., 2016). Of those metabolites, only cahuitamycin C conveyed significant inhibition against *A. baumannii* biofilm (Park et

al., 2016). Additionally, an inhibitor belonging to the prodiginine group of antibiotics, streptorubin B, was discovered. It is derived from *Streptomyces* MC11024 and has been proven to have the ability to inhibit methicillin-resistant S. aureus (MRSA) N315 biofilm (Suzuki et al., 2015).

CHAPTER II

MATERIALS AND METHODS

A. Source of bacterial isolates

The *P. aeruginosa* Pan14 used in this study is a strong biofilm former isolated from a deep tracheal aspirate of a patient with nosocomial infection.

Two *A. baumannii* isolates were involved in this study. The wild type isolate was *A. baumannii* DSM 30008, supplied by Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Germany. The other isolate, *A. baumannii* T36, was a strong biofilm former isolated from a urine sample of a patient in the AUBMC intensive care unit.

B. Collection of soil and marine samples

Soil samples were collected from three regions in Lebanon, Beit Meri (BM), Mazrat Meshref (MM), and Zekrit (ZK). The samples were then transported to the laboratory in sterile polyethylene bags. Marine samples were collected from the coastal region of Tabarja (Tbj) and transported to the laboratory in sterile glass bottles for further studies.

C. Bacterial isolation

The soil samples were dried for 7 days in a 37°C incubator. From each dry sample, 3g were dissolved in 100 mL of autoclaved distilled water and heated at 55°C for 30 minutes. Serial dilutions with a final volume of 1 mL were made out of the obtained bacterial solution with dilution factors of 1/5, 1/10, 1/100 and 1/1000. From

these dilutions, as well as from the original stock, 35 µL were inoculated on International Streptomyces Project-3 (ISP3) agar (20 g oat flakes, 18 g bacteriological agar, 2.5 mL of trace elements in 1L of distilled water with a pH of 7.8) and Soil agar (30 g from the dried soil sample, 18 g bacteriological agar, 10 g corn starch to 1L of distilled water, no pH adjustment). The water samples were also heated at 55°C for 30 minutes and then serially diluted in the same way described earlier. The water dilutions, as well as the non-diluted water sample, were plated on Seawater agar and Seawater ISP3 agar, which are the respective counterparts of soil agar and ISP3 but prepared with seawater instead of distilled water. Once the marine and soil samples were inoculated on the appropriate media, the plates were incubated at 28°C for 7-14 days. After bacterial growth starts to show on the plates, isolated colonies were streaked repetitively on ISP3 or Seawater ISP3 until pure cultures were obtained. The purified samples were then stored in 50% glycerol at -80°C. Environmental bacterial storage was achieved by adding 2 mL of autoclaved distilled water to the bacteria-containing agar plate and swabbing the colonies into the water. After the water has become turbid, 1 mL was transferred to Eppendorf tubes and centrifuged for 10 minutes at 4°C and 4000 rpm. Finally, the supernatant was removed, and the pellet was re-suspended in 1 mL of sterile 50% glycerol solution and ready to be stored at -80°C.

D. Secondary metabolites extraction

A starter culture was initiated by inoculating 35 µL of the stock solution into 5 mL of liquid ISP3 media. This inoculum, known as the first seed, was incubated for 48 hours in a shaker incubator at 28°C and 150 rpm. 1 mL of this seed was then transferred into 10 mL of fresh liquid ISP3 media, to obtain the second seed, which was left at the

same conditions for another 48 hours. Subsequently, 1 mL of the second seed was transferred to inoculate 50 mL of each of the 14 production media (Table 1), which served to exert pressure on the bacteria to release their secondary metabolites. The bacterial cultures were also kept at 150 rpm and 28°C for 7 days. At the end of the incubation period, 1 mL of sterile Amberlite XAD 16N (Sigma) resin solution was added to each of the 14 cultures to absorb the released secondary metabolites. These cultures, with the added resins, were left on a shaking platform at 150 rpm for 3-4 hours. The mixture was then centrifuged at 4°C and 4000 rpm for 20 minutes, the supernatant was discarded, and the secondary metabolites were then extracted. Their extraction from the resin containing pellet was achieved by adding a solution of 30 mL acetone/10 mL methanol. The solvents were left to evaporate under a fume hood at room temperature, and the crude extracts were dissolved in 1 mL of Dimethyl sulfoxide (DMSO) and stored at -20°C.

E. Screening for antibacterial activity on ESKAPE pathogens

To screen for the antibacterial activity of the crude extracts produced in the 14 different production media, broth micro-dilution (BMD) was performed. In a 96-well plate (Corning), 100 µL of Cation-adjusted Mueller Hinton Broth (MHCAB, Sigma) were pipetted in the wells of columns 2 to 12. Whereas in the first column, 195 µL of MHCAB and 5 µL of the crude extracts were added. The extracts were then two-fold serially diluted to obtain decreasing concentrations of the extracts from columns 1 to 11. Column 12 served as positive and negative controls. Once the dilutions were done, bacterial suspensions were prepared to be added to the assigned wells. This was done by inoculating few colonies of the bacteria grown overnight into MHCAB broth and

adjusting the turbidity to 0.5 McFarland. The bacterial suspension was then diluted to obtain a concentration of 5 x 10^6 CFU/mL. All wells of the 96-well plate, except for the negative control, were inoculated with 10 µL of the bacterial suspension to obtain a final concentration of 5 x 105 CFU/mL. Finally, the plate was covered and incubated overnight on a shaker at 37°C and 150 rpm.

The protocol and results were interpreted according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2020).

F. Screening for anti-biofilm activity

To assess the anti-biofilm activity of the crude extracts against clinically relevant biofilm-forming pathogens, two microtiter plate (MTP) assays were carried out. The first assay was done to test for the ability of the extracts to inhibit the formation of biofilm (IF). While the second assay evaluates their activity in eradicating pre-formed biofilms (PF). The two assays were used to visualize the potential of crude extracts against two biofilm-forming pathogens, *P. aeruginosa* Pan14 and *A. baumannii* DSM.

1. Pseudomonas aeruginosa Pan14

a. IF protocol

An overnight culture of Pan14 on Luria-Bertani (LB) agar was adjusted to 0.5 McFarland in LB broth and incubated overnight at 37°C. This culture was then diluted in LB broth with a dilution factor of 1/100. Seeding was done in a round-bottom 96-well plate (Costar® Inc). For the negative control, 100 µL of LB broth were added, while 100 µL of the diluted bacterial suspension were added to the positive control

wells and 100 µL bacterial suspension with 5 µL of treatment/crude extracts were added to the treated wells. The plate was incubated in a stationary incubator overnight at 37°C. After incubation, the wells were gently emptied to remove the planktonic cells and washed twice with distilled water. The adherent cells were stained with 1% crystal violet for 10 minutes at room temperature, washed twice with distilled water, and then left to air dry. To emulsify the stain, 200 µL of 95% ethanol were added to the stained wells and incubated for 15 minutes at room temperature. Finally, the contents of each well were briefly mixed by pipetting, and 125 µL were transferred to an optically clear flat-bottom 96-well plate (Costar® Inc). The absorbance at OD 595nm was measured using BIO-TEK ELx800 Automated Microplate Reader.

b. PF protocol

An overnight culture of Pan14 on LB agar was adjusted to 0.5 McFarland in LB broth and incubated overnight at 37°C. This culture was then diluted in LB broth with a dilution factor of 1/100. Seeding was done in a round-bottom 96-well plate. For the negative control, 100 µL of LB broth were added, while 100 µL of the diluted bacterial suspension were added to the positive control wells and to the wells to be treated. The plate was incubated in a stationary incubator overnight at 37°C. After incubation, 5 µL of the treatment/crude extracts were added to the wells to be treated, and the plate was incubated again overnight at 37°C. On the next day, the wells were gently emptied from the planktonic bacteria and washed with distilled water. The adherent cells were stained with 1% crystal violet for 10 minutes at room temperature, washed twice with distilled water, and then left to air-dry. To emulsify the stain, 200 µL of 95% ethanol were added to the stained wells and incubated for 15 minutes at room temperature. Finally, the

contents of each well were briefly mixed by pipetting and 125 µL were transferred to an optically clear flat-bottom 96-well plate. The absorbance at OD 595nm was measured using BIO-TEK ELx800 Automated Microplate Reader.

2. Acinetobacter baumannii DSM

a. IF protocol

An overnight culture of ACN DSM on Tryptic soy agar (TSA) was adjusted to 0.5 McFarland in Tryptic soy broth (TSB) + 0.25% glucose and incubated overnight at 37°C. This culture was then diluted in TSB + 0.25% glucose with a dilution factor of 1/40. Seeding was done in a round-bottom 96-well plate. For the negative control, 100 µL of TSB + 0.25% glucose broth were added, while 100 µL TSB + 0.25% glucose broth and an equal volume of bacterial suspension were added to the wells of positive control and the treated wells. Additionally, 5 µL of the crude extracts were added to the treated wells. The plate was incubated overnight at 37°C. After incubation, the cultures were gently removed and washed three times with phosphate-buffered saline (1X PBS, Lonza). The adherent cells were stained with 0.4% crystal violet for 15 minutes at room temperature, washed three times with distilled water, and then left to air dry. Afterward, the wells were filled with 250 µL of 95% ethanol for 15 minutes at room temperature. Finally, the contents of each well were briefly mixed by pipetting, and 125 µL were transferred to an optically clear flat-bottom 96-well plate. The absorbance at OD 595nm was measured using BIO-TEK ELx800 Automated Microplate Reader.

b. PF protocol

An overnight culture of ACN DSM on TSA was adjusted to 0.5 McFarland in TSB + 0.25% glucose and incubated overnight at 37°C. This culture was then diluted in TSB + 0.25% glucose with a dilution factor of 1/40. Seeding was done in a round-bottom 96-well plate. For the negative control, 100 µL of TSB + 0.25% glucose were added, while 100 µL TSB + 0.25% glucose and an equal volume of bacterial suspension were added to the wells of positive control and the treated wells. The plate was incubated overnight at 37°C. After incubation, 5 µL of the crude extracts were added to the wells to be treated, and the plate was incubated again overnight at 37°C. On the next day, the cultures were gently removed and washed three times with phosphate-buffered saline (1X PBS). The adherent cells were stained with 0.4% crystal violet for 15 minutes at room temperature, washed three times with distilled water and then left to air-dry. Afterward, the wells were filled with 250 µL of 95% ethanol for 15 minutes at room temperature. Finally, the contents of each well were briefly mixed by pipetting, and 125 µL were transferred to an optically clear flat-bottom 96-well plate . The absorbance at OD 595nm was measured using BIO-TEK ELx800 Automated Microplate Reader.

One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 8.0.0 for data analysis of the results obtained from the above-mentioned assays. Data were performed in triplicates and significance was symbolized as follows: **** for P value < 0.0001, *** for 0.0001 < P value < 0.001, ** for 0.001 < P value < 0.01 and * for 0.01 < P value < 0.05

G. Characterization of the environmental bacteria

1. Physiological characterization

a. pH tolerance

This parameter was evaluated on plates of media 5265 (10 g malt extract, 4 g yeast extract, 4 g glucose, and 5 g bacteriological agar to 1 L of distilled water) with pH levels of 3, 4, 5, 6, 7, 8, 9 and 10. Equal amounts of bacteria from the stock solution were inoculated on each of the different prepared pH media. The plates were incubated at 28°C for 7 to 10 days and visible growth was recorded.

b. NaCl tolerance

In order to perform this test, media 5339 (adding 10 g casein peptone, 5 g yeast extract, and 20 g bacteriological agar to 1 L of distilled water, pH=7) was prepared with 0, 2.5, 5, 7.5, and 10% of sodium chloride. Equal amounts of bacteria from the stock solution were inoculated on each of the different prepared media. The plates were incubated at 28°C for 7 to 10 days and visible growth was recorded.

2. Phenotypic and biochemical characterization

Phenotypic characterization of the bacterial strains was achieved by Gram staining and observing the bacterial morphology on ISP3 media. Their biochemical and enzymatic activity was studied by performing API 20E.

a. Gram stain

The bacterial colonies, grown overnight on ISP3 agar, were diluted in a drop of distilled water and heat-fixed on a microscope slide. The fixed smear was flooded with

crystal violet for 1 minute. Then, the slide was washed gently with tap water, and Gram's iodine was added and allowed to remain for 2 minutes. The iodine was rinsed off with tap water and followed by the careful addition of acetone, a decolorizing agent. Acetone was added drop by drop until the liquid running down the edge of the slide was clear. Finally, it was rinsed off and the counterstain, safranin, was added and left for 1 minute. The slide was washed with tap water to remove any excess stain and then blot-dried with an absorbent paper. Stained slides were observed under oil immersion using a bright field microscope (A. C. Smith & Hussey, 2005).

b. Analytical profile index (API)

This method was applied to determine the biochemical characteristics of the environmental bacteria, Tbj13, BM9 and ZK31. The three bacteria were streaked on ISP3 agar and incubated overnight at 28°C. Isolated colonies were collected on the second day and diluted in the solution provided with the kit until the density reached 0.5 McFarland. The wells of the strip provided were filled with the bacterial suspension prepared according to the instructions of the BioMérieux API 20E kit. The strip was then incubated overnight at 37°C and results were read on the second day by comparing the colors obtained to the provided leaflet.

Further genomic characterization was performed by extracting the DNA of those environmental bacteria and performing 16S ribosomal RNA (rRNA) sequencing.

H. Bio-active molecule purification

1. Upscale metabolite production

After small crude extracts showed anti-biofilm activity, an upscaled secondary metabolite production was generated. First and second seeds were prepared as described earlier but in larger amounts and were used to inoculated 6 to 10L of the selected medium. After an incubation period of 10-14 days in a shaker incubator at 150 rpm and 28°C, the secondary metabolites were extracted via acetone/methanol in a similar way to the small scale. The resulting extracts were then subjected to liquid-liquid extraction.

2. Liquid-liquid extraction

Liquid-liquid extraction, also known as solvent extraction or partitioning, is a chemical method used for the purification of biomolecules. It is employed whenever there are two immiscible or partially soluble liquid phases in contact with each other (Mazzola et al., 2008).

The extracts obtained after the upscale metabolite production were subjected to this protocol and four different fractions were collected: hexane, chloroform, ethyl acetate, and water/methanol. The obtained fractions were also tested for their anti-biofilm activity using the mentioned MTP assays. The fraction that presented the highest anti-biofilm activity was subjected to further compound segregation and purification.

3. Column chromatography and purification

Column chromatography is a technique that is used to separate a single chemical compound from a mixture dissolved in a fluid. It separates substances based on

differential adsorption of compounds (hydrophobicity or polarity) into the adsorbent as the compounds move at different levels through the column which allows them to be separated into fractions. In this case, column chromatography was performed on the active fractions for further investigation and analysis. This protocol was performed by loading the impure sample onto a column of adsorbent, mainly silica gel. After an organic solvent (s), known as the eluent was drained through the column, the components of the sample were separated from each other by partitioning between the stationary silica and the mobile eluent. Molecules were separated from each other due to their different polarities leading to them moving at different rates through the column. Consequently, the eluent was collected in fractions. To confirm that the separation of the components was successful, fractions were analyzed by thin-layer chromatography.

4. Thin layer chromatography (TLC)

Thin Layer Chromatography is a method used to separate, identify, and characterize non-volatile mixtures. This experiment was performed on a plastic sheet covered with a thin layer of silica gel. On the TLC plate, the fractions obtained from liquid-liquid extraction showing the highest activity were dissolved in 1 mL methanol and spotted. This was also done for the fractions obtained from the column chromatography. The TLC plate was placed in a chamber filled with a suitable solvent system. For both Tbj13 and BM9, the solvent system used was 90% dichloromethane (DCM)/10% methanol + 0.1% formic acid. After the solvent has risen within 1 cm from the top edge, the plate was removed, and the solvent front was marked immediately. The individual components were visualized under UV light as spots with different retention factors.

Whenever two or more fractions run in parallel showed a high similarity in their compounds, they were combined as one fraction. Combining the fractions together was achieved by simply mixing them in one vial. Since the solvent system used was methanol, a volatile solvent, the vials were left in the hood overnight to dry. Once all liquid has evaporated from the vials, the final mixtures were lyophilized, weighed, and dissolved in DMSO in a way to obtain a stock solution with a concentration of 5 mg/mL. These compounds were subsequently purified using high performance liquid chromatography (HPLC).

5. High performance liquid chromatography (HPLC)

HPLC is a technique in analytical chemistry used to separate, identify, and quantify individual compounds in a mixture. This technique is based on the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase (packing material of the column) where the molecules pass though the column based on their chemical structure.

After combining the fractions observed on the TLC plates and dissolving them in DMSO, further purification was achieved by HPLC. The samples were centrifuged, and the supernatant was collected in an HPLC vial and stored at -20°C. On the second day, the sample was injected into the HPLC column (HP 1100) along with the solvent system. For this experiment the solvents used were methanol and water. To both solvents, 0.1% formic acid was added to obtain more defined peaks. Next, the pump was turned on and the methods were prepared. Four methods were performed for each sample which differed by their way of separating the mixture. Finally, the methods were compared and the optimal one was adopted to start collecting the pure compounds. The

collection was achieved by observing the chromatogram obtained through a UV detector, in which each peak collected corresponded to a compound.

After the pure compounds were collected in HPLC vials, they were dried on a rotary evaporator. They were then lyophilized and weighed to be dissolved in DMSO with a concentration of 5 mg/mL.

Table 1. Production media components

Components (g)	V	Veg	A	B	C	INA	RA3	GPMY	V6	AF/MS	GYM	M8	NL2	COM
Potato starch								20						
Peptone		5	4				2		5					
Soluble starch	24	20										20	30	
Dextrose	1													
Meat extract	3	2	4						5			2		
Yeast extract	5	3	2				4	5	5	2	4	2	2.5	
Malt extract							10	5			10			
Soy-bean meal	5	2	2							6				
Glucose							10		20		4	10		25
Triptose	5													
Maltose			20											
Dextrin			10											
CaCO3		1		0.1	0.1	5				4		3	10	2
Glycerol				20		30	5	20						
Glycine				2.5	2.5							4		
Hydrolyzed casein									3					
NaCl				1	1	2			1.5	1				2
KH2PO4				1	1	2								0.15
FeSO4				0.1	0.1									
MgSO4.7H2O				0.1	0.1									
MgCl2.6H2O							2							
Tween 60					20									

Molasses													20	
Soy flour													15	25
Dried beer yeast														3
Ammonium sulfate														2
Soybean oil														3
pH	7.2	7	7	7	7	7.3	7.4	7.02	7.05	7.3	7	7	7.8	8.4

CHAPTER III

RESULTS

A. Antibacterial activity screening

Crude extracts derived from bacteria originating from the collected soil and marine samples were tested for their antibacterial activity on a panel of ESKAPE pathogens (*Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Escherichia coli*) by performing BMDs (Tables 2-6). The detection of inhibition was based on the absence of turbidity and/or cellular pellet in the well. The majority of the extracts generated by the isolates in the 14 different production media demonstrated minimal or no antibacterial activity on any of the pathogens, except for some extracts produced by BM9 which showed a slight inhibitory effect against Gram-positive bacteria (Table 2).

Thus, in the absence of notable antibacterial effects, we proceeded to test these bacterial extracts for their anti-biofilm activity.

Table 2. Table showing the number of wells with inhibition for crude extracts produced by strain BM9 in 14 different media against a panel of pathogenic bacteria

BM9	**Media**													
Bacteria	**V**	**Veg**	**A**	**B**	**C**	**INA**	**RA3**	**GPMY**	**V6**	**AF/MS**	**GYM**	**M8**	**COM**	**NL2**
***S. aureus* ATCC 29213**	0	0	3	2	0	2	0	3	0	3	0	0	0	0
***S. aureus* N315**	0	0	3	2	0	0	3	0	4	0	3	0	0	0
***E. feacalis* ATCC**	0	0	3	2	0	0	3	0	4	0	3	0	0	0

19433														
***A.baumannii* DSM 30008**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***K. pneumoniae* 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* Pan 14**	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 3. Table showing the number of wells with inhibition for crude extracts produced by strain Tbj13 in 14 different media against a panel of pathogenic bacteria

Tbj13	**Media**													
Bacteria	**V**	**Veg**	**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* 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
***A.baumannii* DSM 30008**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K. pneumon	0	0	0	0	0	0	0	0	0	0	0	0	0	0

***aie* ATCC 13883**														
***P. aeruginosa* ATCC 27853**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***P. aeruginosa* Pan 14**	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 showing the number of wells with inhibition for crude extracts produced by strain MM15 in 14 different media against a panel of pathogenic bacteria

MM15	**Media**													
Bacteria	**V**	**Veg**	**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* N315**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. feacalis* ATCC 19433**	2	0	0	1	0	0	1	1	1	0	0	0	0	0
***A.baumannii* DSM 30008**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***K. pneumoniae* ATCC 13883**	0	0	0	0	1	0	0	1	0	0	0	0	0	0
***P. aeruginosa* ATCC**	0	0	0	0	0	0	0	0	0	0	0	0	0	0

27853														
***P. aeruginosa* Pan 14**	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 showing the number of wells with inhibition for crude extracts produced by strain ZK21 in 14 different media against a panel of pathogenic bacteria

ZK21	**Media**													
Bacteria	**V**	**Veg**	**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	3	0	0	0	3	2
***S. aureus* N315**	0	0	0	1	0	0	0	1	3	0	0	0	2	0
***E. feacalis* ATCC 19433**	0	1	0	0	0	0	0	0	3	1	0	0	0	0
***A.baumannii* DSM 30008**	0	0	0	0	0	0	0	0	1	0	0	0	0	0
***K. pneumoniae* ATCC 13883**	0	0	0	0	0	0	0	0	0	1	0	0	1	0
***P. aeruginosa* ATCC 27853**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***P. aeruginosa* Pan 14**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. coli* ATCC**	0	0	0	0	0	0	0	0	0	0	0	0	0	0

25922														

Table 6. Table showing the number of wells with inhibition for crude extracts produced by strain ZK31 in 14 different media against a panel of pathogenic bacteria.

ZK31	**Media**													
Bacteria	**V**	**Veg**	**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* N315**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. feacalis* ATCC 19433**	0	0	2	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. pneumoniae* 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* Pan 14**	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 formation assays were done to test for the anti-biofilm activities of the extracts which showed no antibacterial activity against the bacterial pathogens. The two MTP assays, IF and PF, were conducted against *P. aeruginosa* Pan14 and *A. baumannii* DSM 30008.

Crude extracts derived from the medium C of both BM9 and TBJ13 significantly reduced the biofilm formation in *P. aeruginosa* Pan14 to 24.1% and 11.8%, respectively (Tables 7 and 8). The results proved a statistically significant decrease in Pan14 biofilm formation, with a $0.01 < \text{P-value} < 0.001$ for BM9 C and $0.001 < \text{P-value} < 0.0001$ for Tbj13 C (Figures 3 and 4).

The crude extracts of the two mentioned strains showed no significant inhibition or eradication of the biofilm of *A. baumannii* DSM. However, the analysis of those results was not presented here.

Table 7. Percentage of Pan14 biofilm formation treated with BM9-derived extracts produced in 14 different production media

BM9	**Media**						
	V	**Veg**	**A**	**B**	**C**	**INA**	**RA3**
Pan14	60.5%	32.9%	52.7%	179.3%	24.1%	88.4%	89.3%
IF	**GPMY**	**V6**	**AF/MS**	**GYM**	**M8**	**NL2**	**COM**
	76.3%	110%	73.9%	53.9%	49%	42.7%	55.4%

Table 8. Percentage of Pan14 biofilm formation treated with Tbj13-derived extracts produced in 14 different production media

Tbj13	Media						
	V	**Veg**	**A**	**B**	**C**	**INA**	**RA3**
Pan14	24.6%	42%	59%	93.5%	11.8%	52.6%	41.8%
IF	**GPMY**	**V6**	**AF/MS**	**GYM**	**M8**	**NL2**	**COM**
	64%	32.9%	32.4%	43.5%	32.7%	64.7%	38.4%

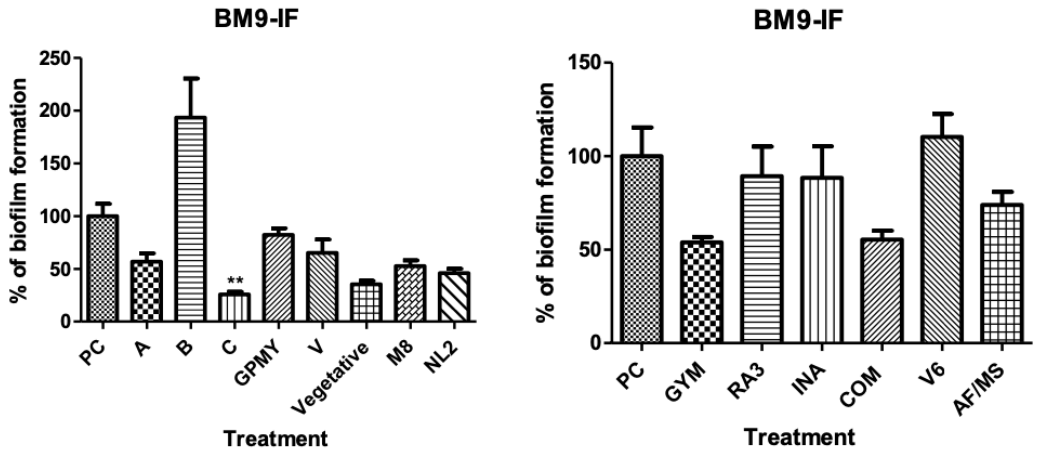

Figure 3. Percentage of Pan14 biofilm formation treated with BM9-derived extracts produced in 14 different production media. ** for 0.001 < P-value < 0.01

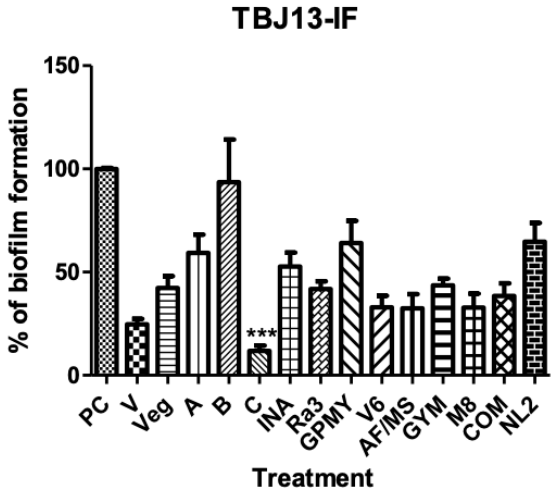

Figure 4. Percentage of Pan14 biofilm formation treated with Tbj13-derived extracts produced in 14 different production media. production media. *** for 0.0001 < P-value < 0.001

The same analysis was repeated for the crude extracts derived from ZK21, ZK31, and MM15. Crude extracts derived from medium C of ZK31 most significantly reduced the biofilm formation in *A. baumannii* DSM to -3.4% (Table 9). The results proved a statistically significant inhibition of ACN DSM biofilm formation, with a P-value of <0.0001 in medium C (Figure 5).

The 13 other media derived from ZK31 and the crude extracts of ZK21 and MM15 showed no or less significant effect on ACN DSM biofilm, as well as that of Pan14 (Figures 5-7).

On another note, some of the extracts of BM9 and ZK31 showed a possible enhancement of biofilm formation. For instance, table 7 and figure 3 showed that extracts produced in media B and V6 by BM9 enhance Pan14 biofilm formation. A similar activity was achieved by extracts produced in media V, GPMY, and GYM by ZK31 on the biofilm of ACN DSM (Table 9).

However, this was not the target of the project, so no analysis or further studies were done.

Table 9. Percentage of ACN DSM biofilm formation treated with ZK31-derived extracts produced in 14 different production media

ZK31	**Media**						
ACN DSM IF	**V**	**Veg**	**A**	**B**	**C**	**INA**	**RA3**
	104.6%	87.9%	80.2%	33.3%	-3.4%	57.6%	79.3%
	GPMY	**V6**	**AF/MS**	**GYM**	**M8**	**NL2**	**COM**
	102.9%	92.6%	35.3%	128.3%	71.5%	89.8%	90.5%

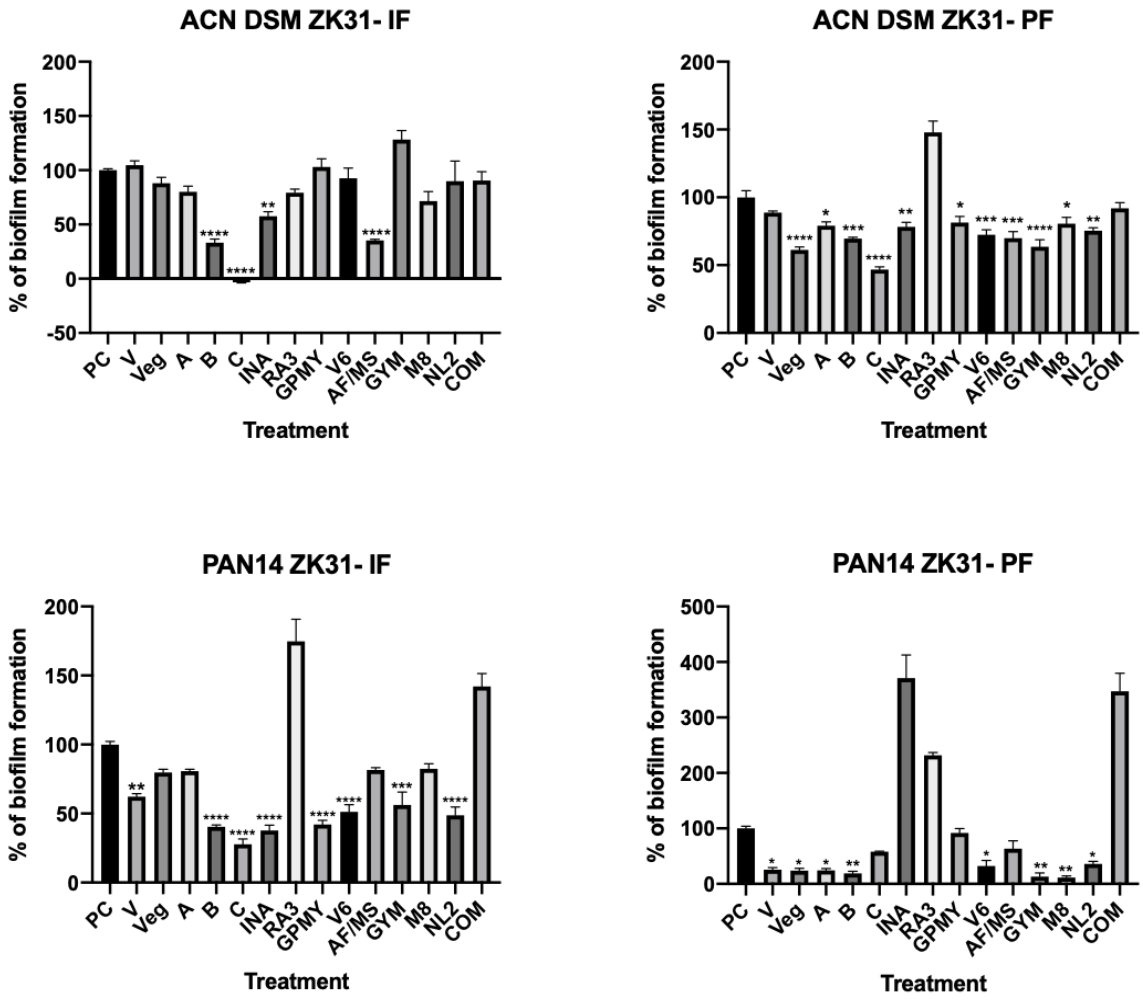

Figure 5. Percentage of biofilm formation in ACN DSM and Pan14 treated with ZK31-derived extracts produced in 14 different production media. **** for P-value < 0.0001, *** for 0.0001 < P-value < 0.001, ** for 0.001 < P-value < 0.01 and * 0.01 < P-value < 0.05

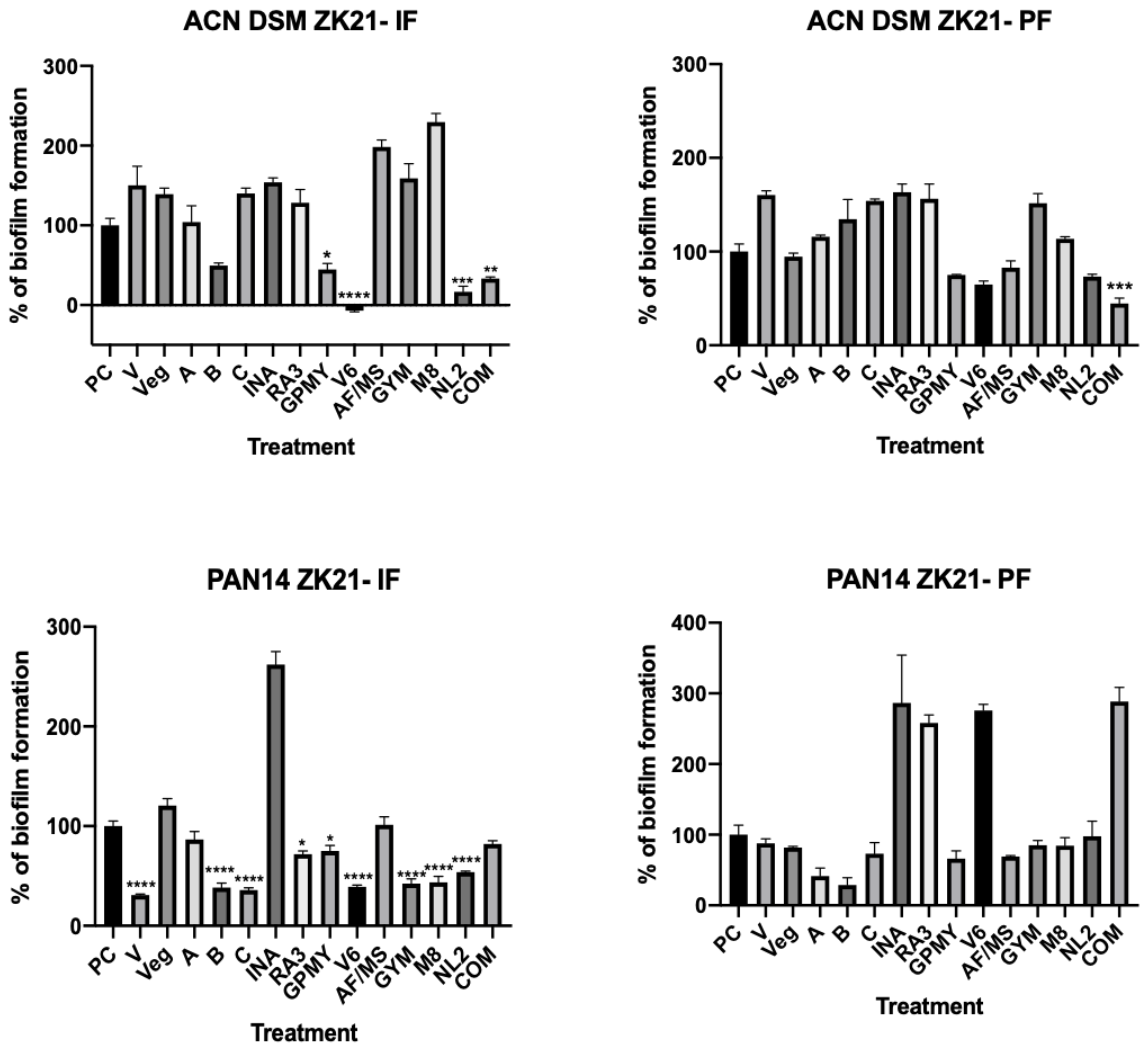

Figure 6. Percentage of biofilm formation in ACN DSM and Pan14 treated with ZK21-derived extracts produced in 14 different production media. **** for P-value < 0.0001, *** for 0.0001 < P-value < 0.001, ** for 0.001 < P-value < 0.01 and * 0.01 < P-value < 0.05

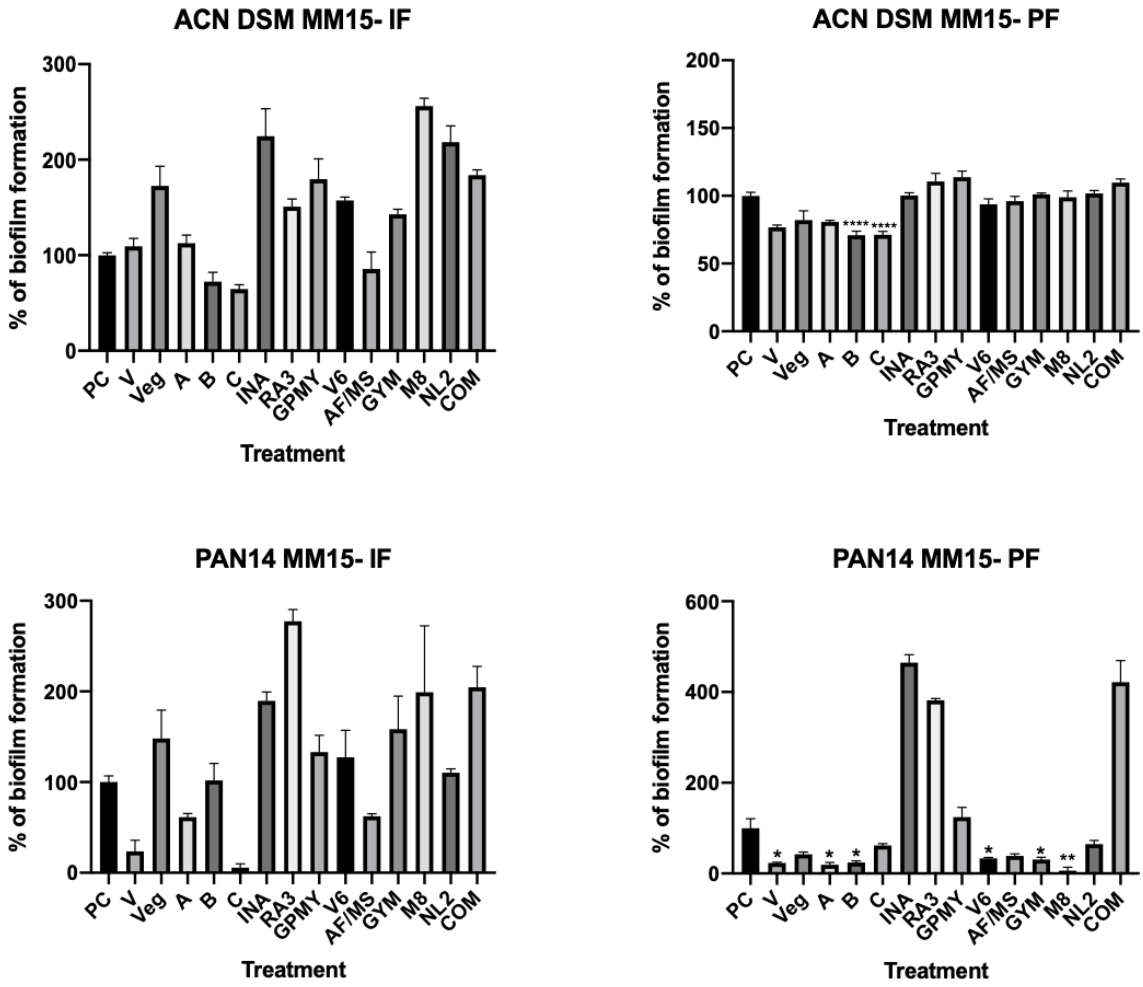

Figure 7. Percentage of biofilm formation in ACN DSM and Pan14 treated with MM15-derived extracts produced in 14 different production media. **** for P-value < 0.0001, ** for 0.001 < P-value < 0.01 and * 0.01 < P-value < 0.05

C. Characterization of the environmental bacteria

1. Physiological characterization

a. <u>pH tolerance</u>

Tbj13 showed growth on plates with a pH 5 and higher with the optimum being pH 6 (Figure 8).

The optimal conditions for the growth of BM9 were pH 7 and 8, although it was able to survive at pH 5 and higher. Some morphological modifications were observed at highly basic pH as a way to tolerate these unfavorable conditions (Figure 9).

Similarly, ZK31 showed the ability to grow on the plates of pH 5 to 10, with optimal growth at pH 7 (Figure 10).

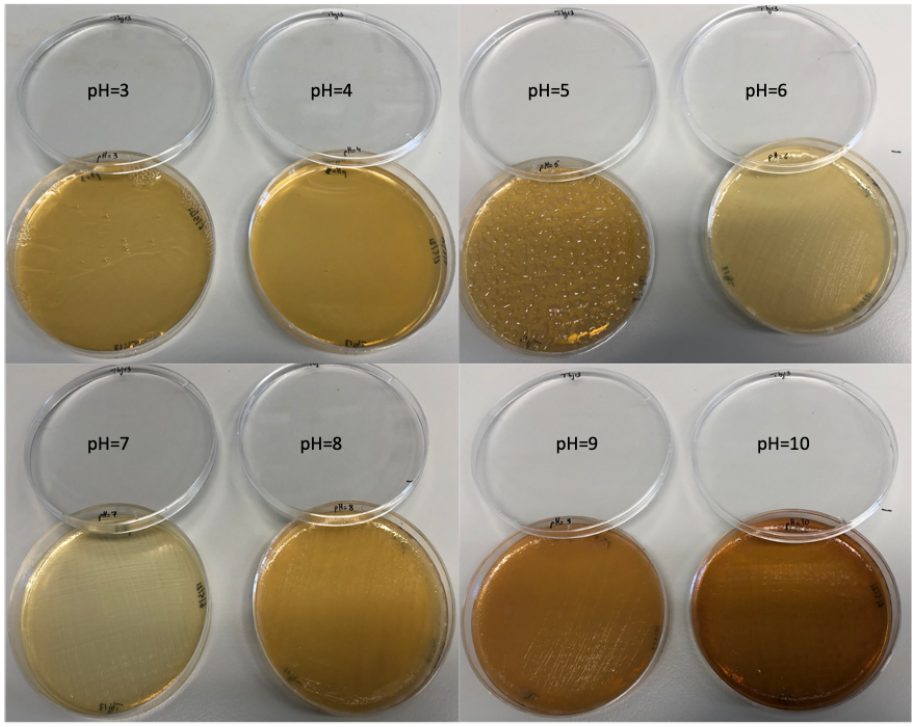

Figure 8. Tolerance of Tbj13 to varying pH conditions

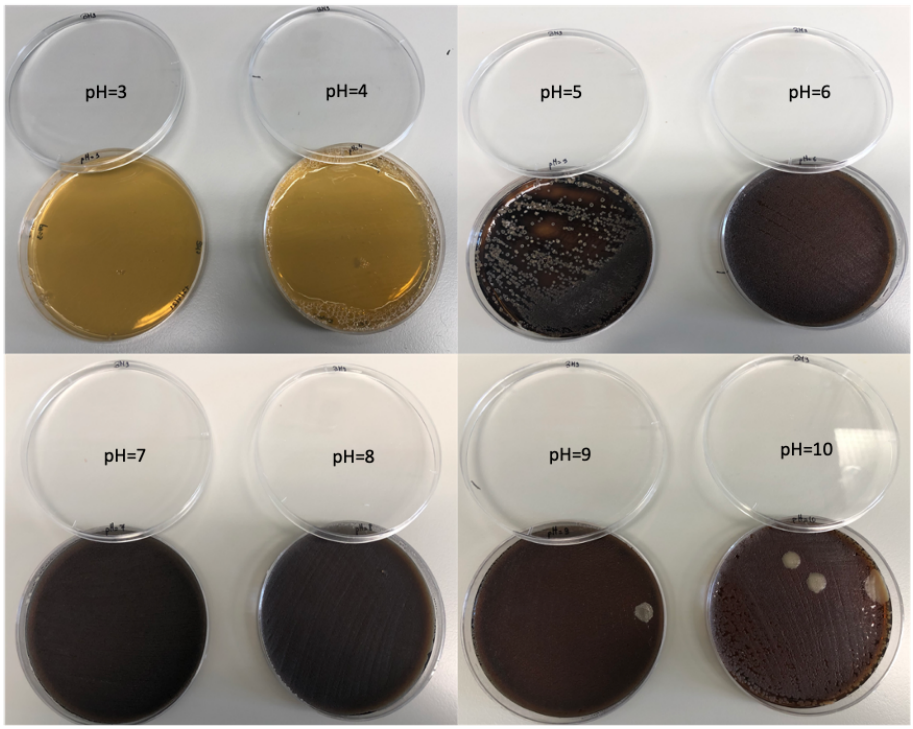

Figure 9. Tolerance of BM9 to varying pH conditions

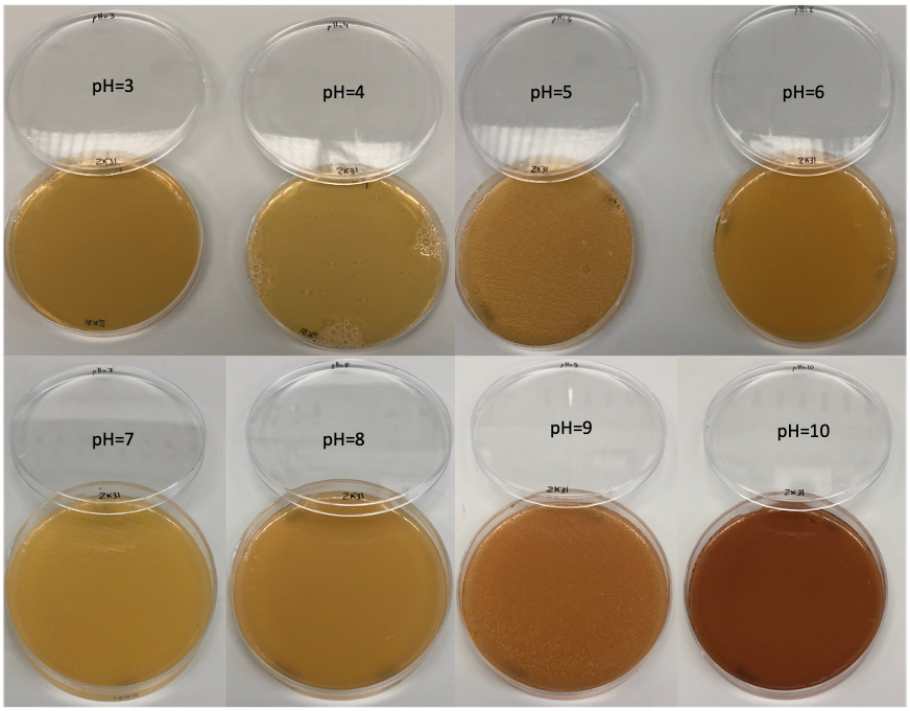

Figure 10. Tolerance of ZK31 to varying pH conditions

b. NaCl tolerance

Tbj13 was subjected to varying concentrations of NaCl, and it showed tolerance to all of them (Figure 11A). On the other hand, BM9 was able to tolerate up to 2.5% NaCl, with a lack of growth on higher concentrations (Figure 11B). Figure 11C showed that ZK31 had a higher tolerance than BM9, where it grew in the presence of NaCl up to a concentration of 7.5%.

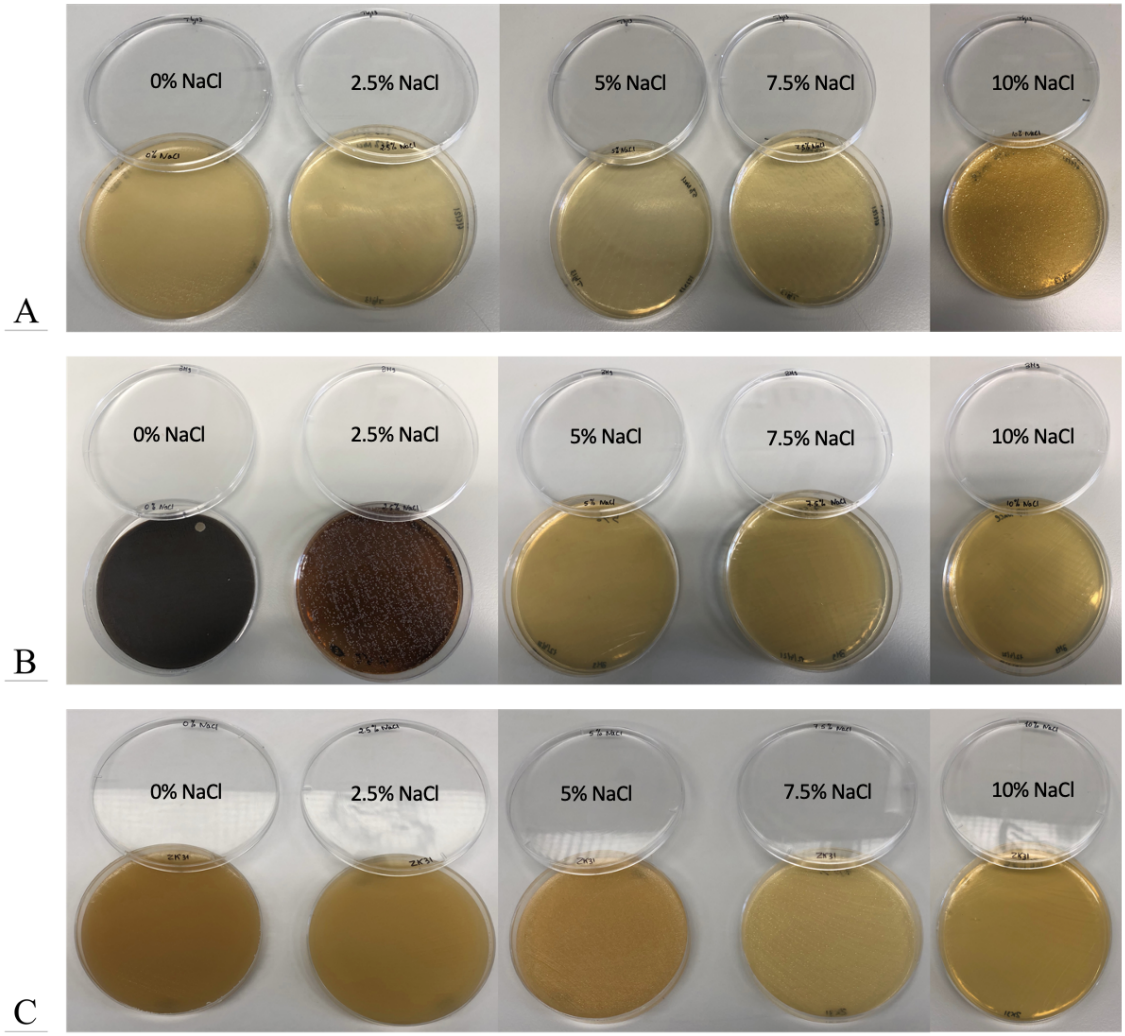

Figure 11. Tolerance of (A) Tbj13, (B) BM9, and (C) ZK31 to varying NaCl concentrations

2. Phenotypic and biochemical characterization

a. Gram stain

Under a bright field microscope, Tbj13 (Figure 12A) and ZK31 (Figure 12C) appeared as Gram-negative bacilli, whereas BM9 (Figure 12B) showed Gram-positive bacilli.

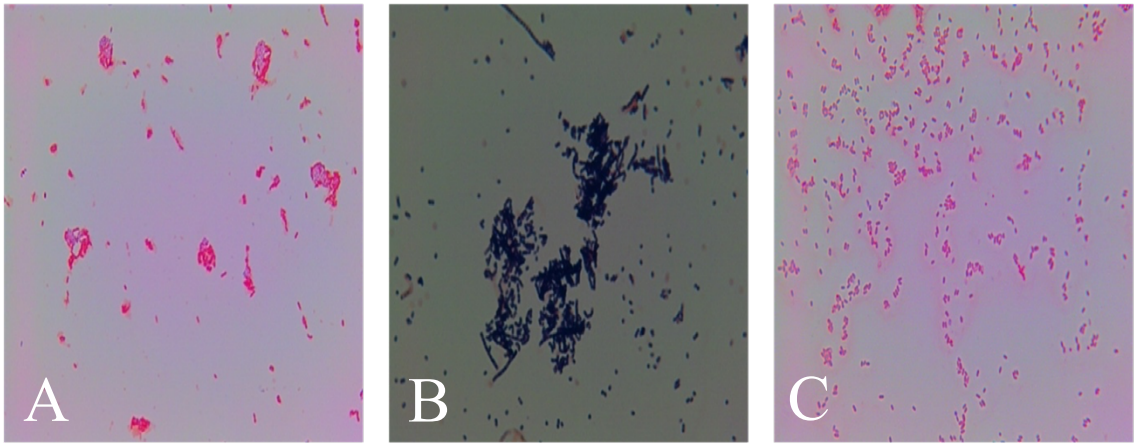

Figure 12. Gram stain results under bright microscope for (A) Tbj13, (B) BM9 and (C) ZK31.

b. API

The reactions tested for in the API 20E kit include beta-galactosidase (ONPG), arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), citrate utilization (CIT), H2S production (H2S), urea hydrolysis (URE), deaminase (TDA), indole production (IND), acetoin production (VP), gelatinase (GEL) and fermentation/oxidation of glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), rhamnose (RHA), sucrose (SAC), melibiose (MEL), amygdalin (AMY) and arabinose (ARA). Additionally, the oxidase (OX) test was done and included in the analysis.

The results of these reactions are presented in tables 10, 11, and 12 for Tbj13, BM9, and ZK31, respectively.

Table 10. API 20E test results for Tbj13 strain

	API 20E tests						
	ONPG	**ADH**	**LDC**	**ODC**	**CIT**	**H2S**	**URE**
	-	-	-	-	+	-	+
Tbj13	**TDA**	**IND**	**VP**	**GEL**	**GLU**	**MAN**	**INO**
	-	-	-	-	-	-	-
	SOR	**RHA**	**SAC**	**MEL**	**AMY**	**ARA**	**OX**
	-	-	-	-	-	-	-

Table 11. API 20E test results for BM9 strain

	API 20E tests						
	ONPG	**ADH**	**LDC**	**ODC**	**CIT**	**H2S**	**URE**
	-	-	-	-	-	-	-
BM9	**TDA**	**IND**	**VP**	**GEL**	**GLU**	**MAN**	**INO**
	-	-	-	+	-	-	-
	SOR	**RHA**	**SAC**	**MEL**	**AMY**	**ARA**	**OX**
	-	-	-	-	-	-	-

Table 12. API 20E test results for ZK31 strain

	API 20E tests						
	ONPG	**ADH**	**LDC**	**ODC**	**CIT**	**H2S**	**URE**
	-	+	-	-	+	-	-
ZK31	**TDA**	**IND**	**VP**	**GEL**	**GLU**	**MAN**	**INO**
	-	-	+	+	-	-	-
	SOR	**RHA**	**SAC**	**MEL**	**AMY**	**ARA**	**OX**
	-	-	+	-	-	-	+

c. Morphology of environmental bacteria on ISP3 agar

For more characterization, the three isolates were subcultured on the ISP3 agar to observe their morphology. Tbj13 (Figure 13A) grew as a smooth surface indistinguishable from the agar due to its light color. Similarly, ZK31 (Figure 13C) had a color like that of Tbj13 but appeared to be more mucoid and showed growth of isolated colonies. Unlike the first two, BM9 (Figure 13B) changed the agar color to a light orange and had the ability to produce white spores.

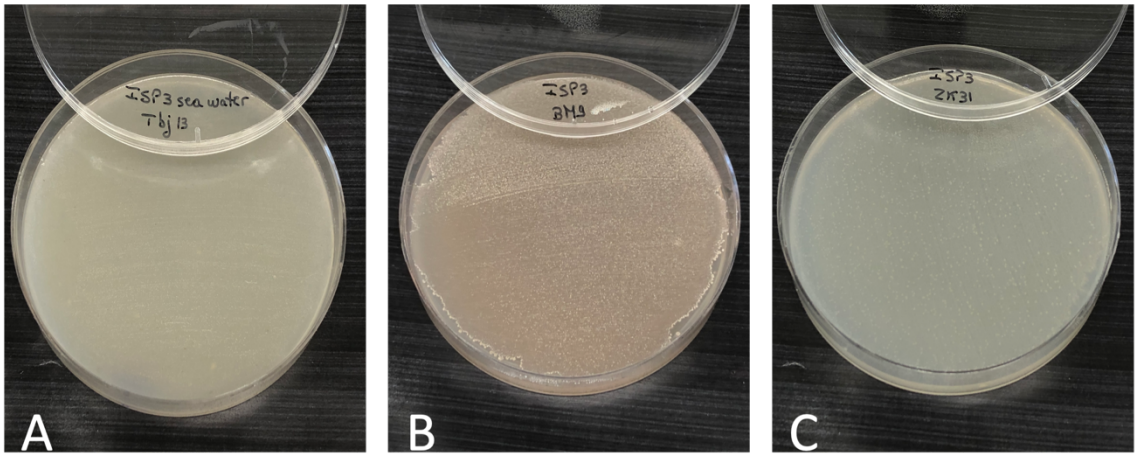

Figure 13. Morphology of (A) Tbj13 on ISP3 seawater, (B) BM9 and (C) ZK31 on ISP3

3. Genomic characterization

The sequencing of the 16S ribosomal RNA (16S rRNA) was previously conducted at macrogen for effective bacterial identification. The results showed that Tbj13 and BM9 belonged to *Halomonas* species and *Streptomyces* species respectively, as shown in tables 13 and 14.

Table 13. Description and Percent identity of BM9 after 16S sequencing and BLAST

	Description	Per. Ident
BM9	*Streptomyces galilaeus* strain JCM 4757 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%

Table 14. Description and Percent identity of Tbj13 after 16S sequencing and BLAST

	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%

D. Testing and purification of upscaled fractions

1. Anti-biofilm activity screening

The extracts produced in medium C by Tbj13, BM9, and ZK31 were subjected to further experiments since they showed significant activities in inhibiting biofilm formation. Upscale of those extracts was done in medium C and this was followed by liquid-liquid partitioning. Three fractions, chloroform, hexane, and ethyl acetate were obtained and tested. For Tbj13 C, as seen in table 15, the highest activity in eliminating the already formed biofilm of Pan14 resided in the hexane fraction. Tbj13 C Hexane reduced the biofilm formation of Pan14 to 1.3% and showed statistical significance with $0.01 < \text{P-value} < 0.05$ (Figure 14).

As for BM9 C, the ethyl acetate fraction reduced the biofilm formation in Pan14 to 11% and eliminated the pre-formed biofilm to reach 2%. Similarly, the other two fractions demonstrated extreme antibiofilm activities, but with lower impact and percentages (Table 16). The results proved a statistically significant inhibition in Pan14 biofilm formation, as well as a significant eradication with a $0.0001 < \text{P-value} < 0.001$ for BM9 C ethyl acetate (Figure 15).

Further, among the tested fractions of ZK31 C, the chloroform fraction exhibited the highest activity in reducing the formation of biofilm in ACN DSM to reach 1.7% (Table 17). Interestingly, all three fractions of ZK31 C exhibited significant inhibition against the biofilm of a clinical *A. baumannii* isolate, ACN T36, which made these fractions the subject of further investigations (Figure 16). However, the chloroform fraction led to inhibiting the biofilm down to 8.5% (Table 17) and showed a significance of $\text{P-value} < 0.0001$ for both isolates (Figures 16 and 17).

Table 15. Percentage of Pan 14 biofilm formation treated with Tbj13C-derived fractions

Tbj13 C		**Fractions**		
Pan 14	IF	**Hexane**	**Chloroform**	**Ethyl acetate**
		13%	18%	38%
	PF	1.3%	2.8%	4%

Table 16. Percentage of Pan14 biofilm formation treated with BM9C-derived fractions

BM9 C		**Fractions**		
Pan 14	IF	**Hexane**	**Chloroform**	**Ethyl acetate**
		13%	37%	11%
	PF	2.8%	2.1%	2%

Table 17. Percentage of ACN DSM and ACN T36 biofilm formation treated with ZK31C-derived fractions

ZK31 C		**Fractions**		
ACN DSM	IF	**Hexane**	**Chloroform**	**Ethyl acetate**
		51.3%	1.7%	110.9%
	PF	137.3%	144.6%	136.8%
ACN T36	IF	**Hexane**	**Chloroform**	**Ethyl acetate**
		40.3%	8.5%	64.6%
	PF	103.6%	76.9%	134.6%

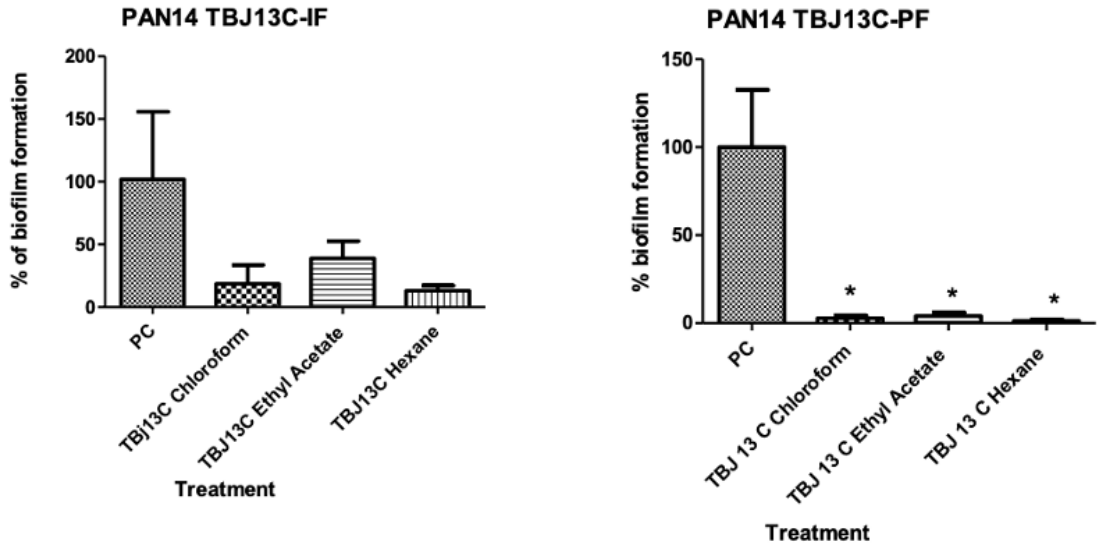

Figure 14. Percentage of Pan14 biofilm formation treated with Tbj13 C-derived fractions. * for 0.01 < P-value < 0.05

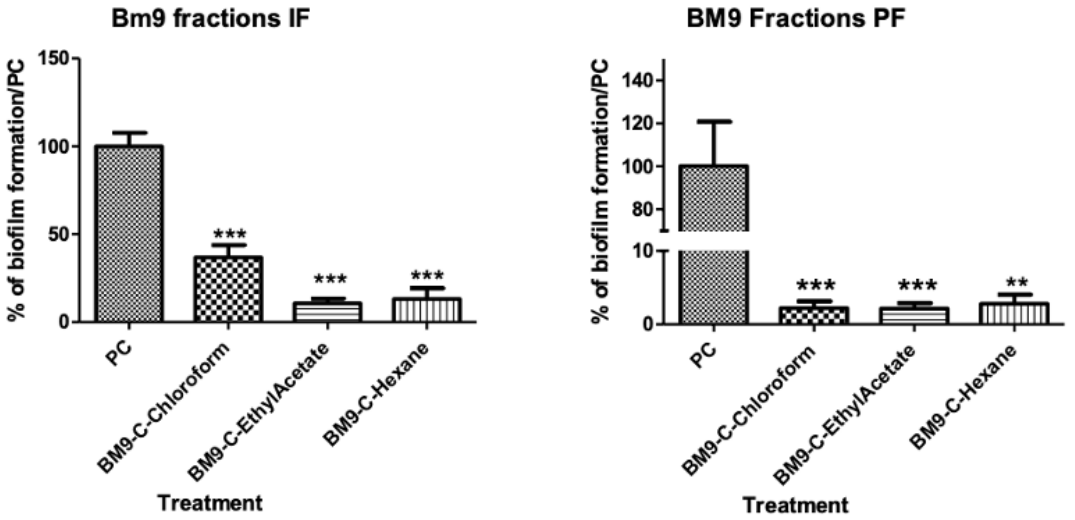

Figure 15. Percentage of Pan14 biofilm formation treated with BM9 C-derived fractions. *** for 0.0001 < P-value < 0.001 and ** for 0.001 < P-value < 0.01

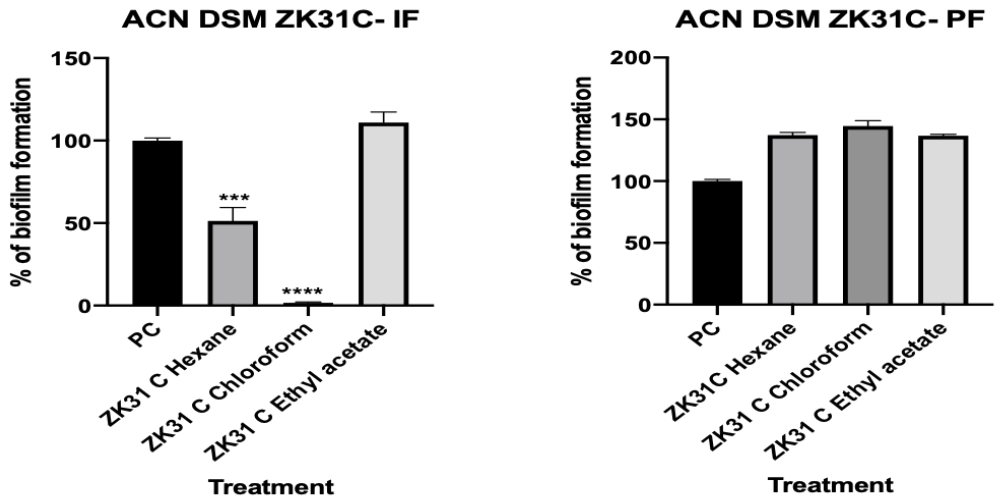

Figure 16. Percentage of ACN DSM biofilm formation treated with ZK31 C-derived fractions. **** for P-value < 0.0001 and *** for 0.0001 < P-value < 0.001

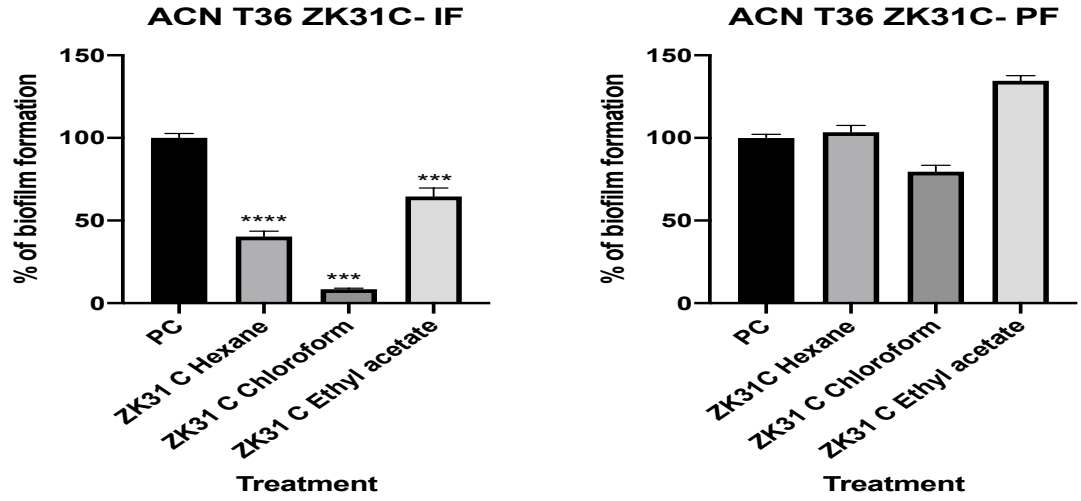

Figure 17. Percentage of ACN T36 biofilm formation treated with ZK31 C-derived fractions. *** for 0.0001 < P-value < 0.001

2. Column chromatography

Column chromatography was performed following the liquid-liquid partitioning on the previously obtained active fractions. Additionally, TLCs were done prior to column chromatography, to reveal the components of each fraction and any possible

similarity between them. For Tbj13, the separation of the active hexane fraction of medium C through the column yielded 4 fractions, A, B, C, and D. However, for BM9, the chloroform and hexane fractions of medium C showed high similarity which resulted in combining them to proceed with one active fraction, known as chloroform + hexane (C+H). Hence, column chromatography was performed on BM9 medium C (C+H) and yielded 7 fractions, A, B, C, D, E, F, and G. The two MTP assays were conducted against *P. aeruginosa* Pan14 using the obtained fractions. The results are seen in figures 18 and 19.

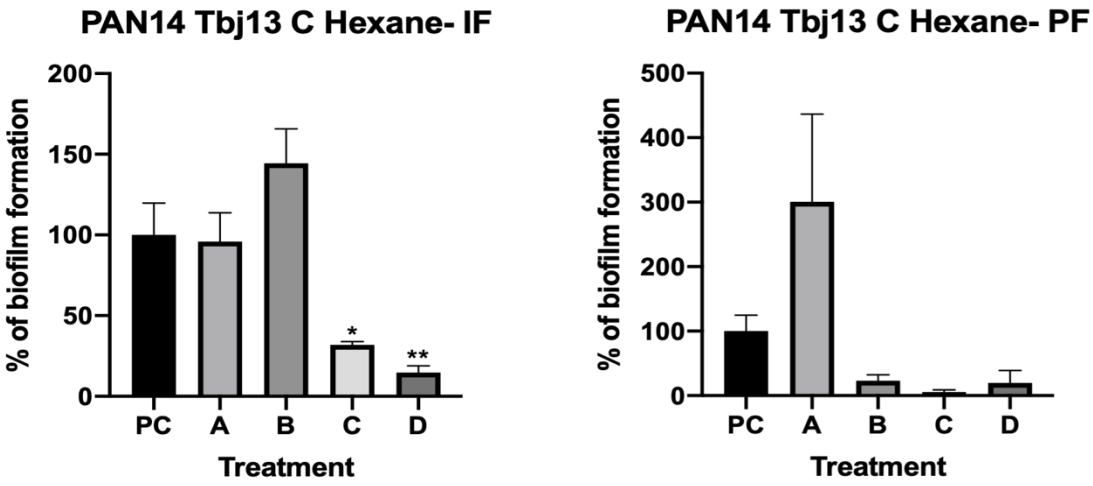

Figure 18. Percentage of Pan14 biofilm formation treated with Tbj13 C Hexane-derived column chromatography fractions. * for 0.01 < P-value < 0.05 and ** for 0.001 < P-value < 0.01

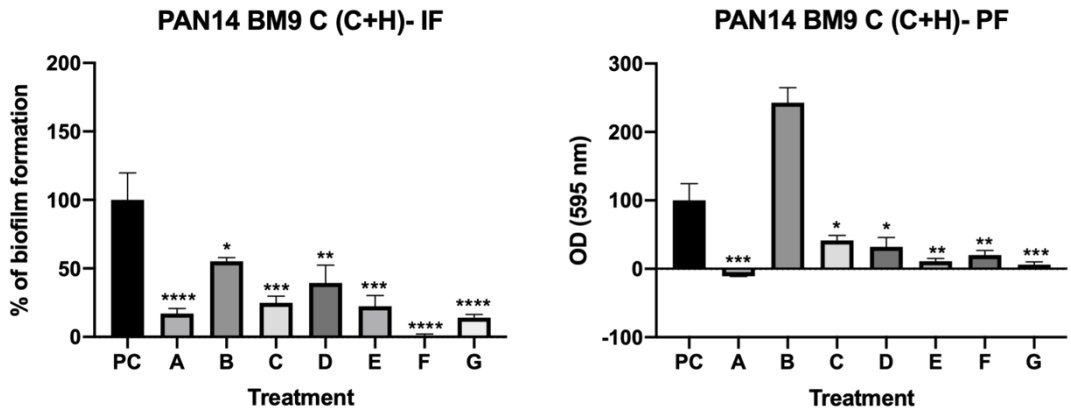

Figure 19. Percentage of Pan14 biofilm formation treated with BM9 C (C+H)-derived column chromatography fractions. **** for P-value < 0.0001, *** for 0.0001 < P-value < 0.001, ** for 0.001 < P-value < 0.01 and * 0.01 < P-value < 0.05

3. Thin layer chromatography (TLC)

Following column chromatography, the fractions of Tbj13 C Hexane and BM9 C (C+H) were spotted on TLC plates. As shown in figure 20, all 4 fractions of Tbj13 C Hexane presented very similar compounds, which allowed us to combine them as one fraction to be further purified. For BM9, as seen in figure 21, fractions A to F of BM9 C (C+H) share similar compounds, yielding one fraction as well. However, fraction G of BM9 C (C+H), harboring the highest biofilm inhibitory activity, was left as a separate fraction.

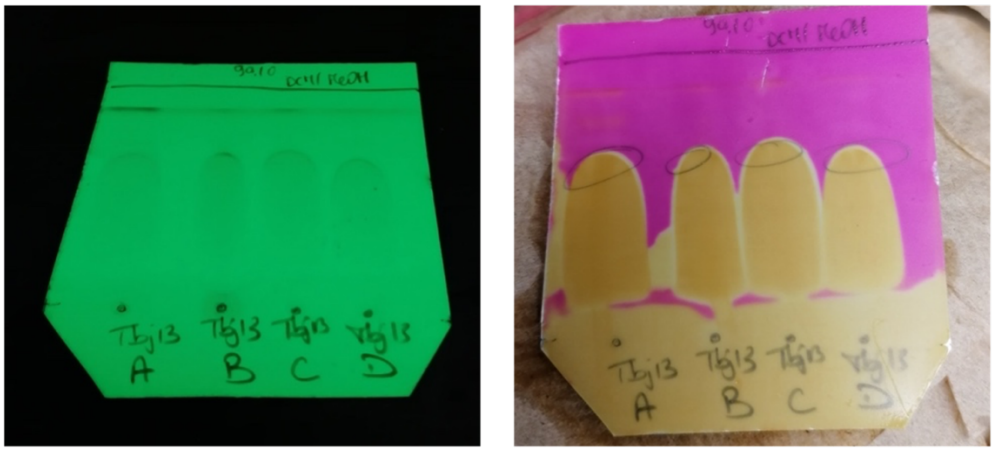

Figure 20. TLC results of Tbj13 C post-column fractions observed under UV (left) and after adding permanganate (right)

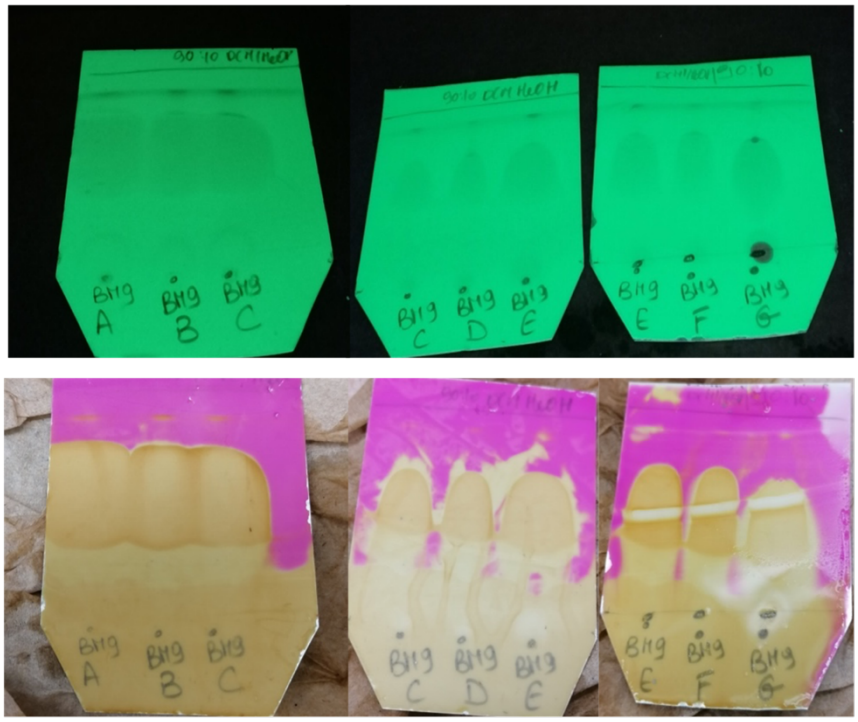

Figure 21. TLC results of BM9 C post-column fractions observed under UV (upper figure) and after adding permanganate (lower figure)

4. High-Performance Liquid Chromatography (HPLC)

After combining and collecting the active fractions of Tbj13 C Hexane and BM9 C (C+H) G, HPLC was performed for more purification in order to reach pure compounds.

After being prepared and loaded in the HPLC vial, Tbj13 C Hexane showed crystallization, which led to an unsuccessful run. However, based on previous work done in our laboratory, preliminary Nuclear Magnetic Resonance (NMR) data suggested Tbj13 to be a pure compound, but with an undeclared structure and formula. Conversely, the HPLC run was done on BM9 C (C+H) G yielded a chromatogram allowing its separation into two pure compounds, a, and b (Figure 22). Each peak observed in the chromatogram referred to a pure compound. BM9 C (C+H) G a

corresponded to the peak collected between 1.8 and 2, whereas BM9 C (C+H) G b was the one collected between 2.8 and 4.

Anti-biofilm testing using the two MTP assays was performed using the three pure compounds, Tbj13 C Hexane, BM9 C (C+H) G a, and BM9 C (C+H) G b against *P. aeruginosa* Pan14. Figure 23 showed that the biofilm formation of *P. aeruginosa* was decreased to 26.3% by Tbj13 C Hexane, 4.6% by BM9 C (C+H) G a, and 21.7% by BM9 C (C+H) G b. The three compounds showed highly significant activity with a P-value < 0.0001 (Figure 23).

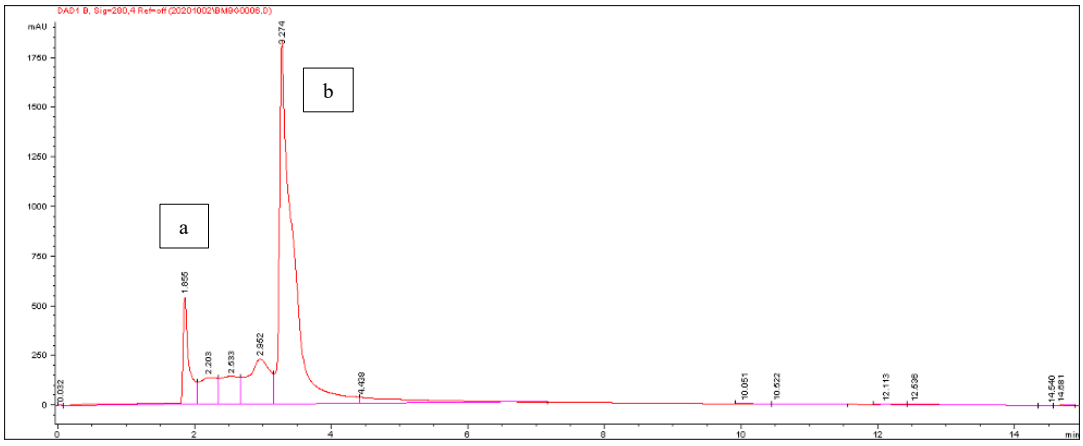

Figure 22. Chromatogram of BM9 C (C+H) G showing two pure compounds a and b

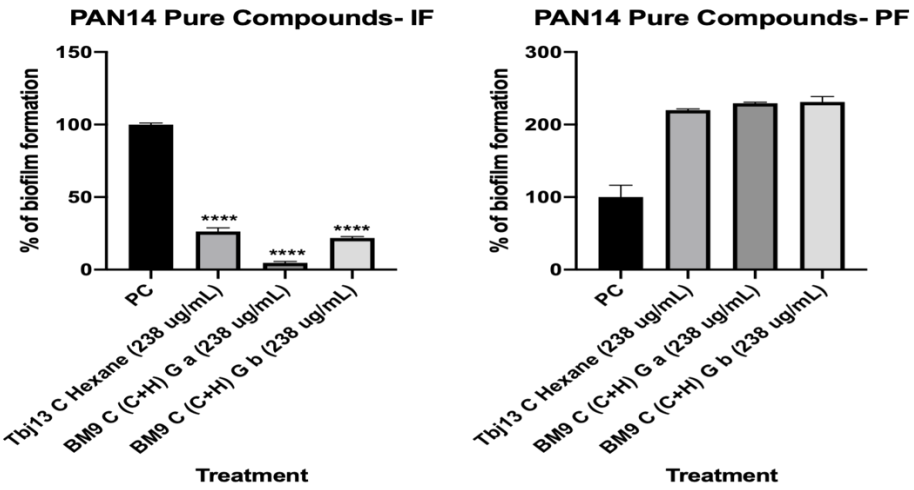

Figure 23. Percentage of Pan14 biofilm formation treated with the collected pure compounds. **** for P-value < 0.0001

CHAPTER IV

DISCUSSION

Biofilms are widely found in nature, yet they are considered highly problematic when it comes to human health (Tasneem et al., 2018). It was previously estimated that 65% of all bacterial infections were biofilm-associated. Moreover, these biofilms are linked to indwelling medical devices, such long-term installed urinary catheters. Hence, they can cause severe infections within the hospital setting (Jamal et al., 2018). Additionally, biofilms correlate to biofilm-forming microbial pathogens found in natural resources such as drinking water, or in food and dairy industries which threatens the public health sector (Tasneem et al., 2018). What exacerbates this issue is the ability of these biofilms to withstand the effect of antimicrobial agents giving rise to challenges in treating them (Lebeaux et al., 2014).

Consequently, due to the widespread existence of biofilms in several settings, coupled with their resistance to clinically utilized antimicrobials, plenty of studies aimed to discover anti-biofilm agents over the past two decades (Song et al., 2017).

Natural products contribute significantly to the drug discovery field. They have been studied for years for their role in treating many illnesses, and more specifically infectious diseases (Dias et al., 2012). These studies along with the limited biofilm-specific therapies led to investigating many natural products as potential novel anti-biofilm agents (Melander et al., 2020). However, up until now, many of these agents have not been able to surpass phases II and III of clinical trials nor have they been approved by the Food and Drug Administration (FDA) (Raj & Thomas, 2021). Additionally, some the discovered compounds that have proven to be effective in

inhibiting biofilm formation, were associated with cytotoxicity which made them unsuitable for clinical use, such as the two analogs of a marine sponge, *cis*-bromoageleferin and *trans*-bromoageleferin (Huigens et al., 2007), as well as halogenated furanones derived from sea-weed plants which played a role as quorum sensing inhibitors (Ong et al., 2018).

All of these findings paved the way for our study which aimed to investigate the effect of natural products derived from Lebanese soil and marine microorganisms against biofilm-forming pathogens. Previous studies showed that soil-derived *Streptomyces* species and marine-derived *Halomonas* species represent potential active secondary metabolites (Azman et al., 2019; Kayanadath et al., 2019). Similarly, our 16S sequencing results showed that the compounds obtained belonged to the mentioned genuses which supports the significant inhibitory activities obtained. Furthermore, we purified from the extracts of these bacteria three pure compounds, which had the ability to inhibit the biofilm formation of *P. aeruginosa* Pan14, the model microorganism for studying biofilm-associated infections (Wei & Ma, 2013). However, our study was limited to the discovery of the pure compounds and their characteristics, without studying their exact mechanism of action.

Mishra et al showed that targeting any of the different stages of the complicated biofilm life cycle can be considered a successful strategy. Targeting the first two steps, (1) the initial attachment to surfaces and/or (2) the EPS production and development of the biofilm structure, was proven to be highly effective since those are the most critical stages in the biofilm development process (Mishra et al., 2020).

Waturangi et al found that *Streptomyces* species isolates were the most effective agents in inhibiting *P. aeruginosa* biofilm formation. They showed the ability to

interfere with extracytoplasmic proteins involved in the initial bacterial attachment to abiotic surfaces (Waturangi et al., 2016). These findings may suggest the possible mechanism of action behind the anti-biofilm activity of the two soil-derived *Streptomyces* compounds, BM9 C (C+H) G a and BM9 C (C+H) G b, in our study.

Similarly, marine-derived microorganisms such as *Halomonas* species showed significant inhibition of *P. aeruginosa* biofilm by targeting the rhamnolipids which are associated with the dispersal step of the biofilm (Kayanadath et al., 2019). This mechanism of action may be the reason behind the anti-biofilm activity of Tbj13 C Hexane.

Moreover, another mechanism of action of anti-biofilm agents is interfering with the EPS production. This occurs through inhibiting the genes encoding for it, leading to the disruption of the structure of the biofilm and hampering its maturation process (Wei & Ma, 2013).

Besides the major steps of the biofilm life cycle, targeting the regulators and signaling molecules involved in the biofilm development process can also affect its formation and structure. For instance, quorum quenching has been proven as an effective strategy in averting the formation of biofilms. It relies on the inhibition of quorum sensing, an essential regulatory system in *P. aeruginosa* and *A. baumannii* biofilm formation (Pang et al., 2019). This aspect can help in correlating to the activity of ZK31 C Chloroform that showed significant inhibition of the *A. baumannii* biofilm. However, this compound still needs further purification and characterization in order to identify its clear target and mechanism of action.

Another regulatory system that can be targeted is the second messenger c-di-GMP. This strategy can impede the biofilm architecture and subsequent maturation (Sambanthamoorty et al., 2014).

As anti-biofilm agents, the compounds obtained can achieve their role by targeting the specific AHL systems as a quorum quenching mechanism. They may also restrict the activity of the DGC and PDE enzymes that regulate the levels of c-di-GMP in bacteria.

In conclusion, targeted anti-biofilm therapy is crucial to effectively reduce biofilm-associated infections. This highlights the importance of this study and more importantly, of the natural products as a source of potentially active soil and marine-derived bioactive compounds. Due to the scarcity of anti-biofilm drugs and to the fact that most of the discovered agents have not succeeded until now, we aim to discover and develop anti-biofilm agents with proper bioavailability, pharmacokinetic properties, and most importantly to provide safe, non-toxic administration to infected individuals.

A. Future perspectives

As the results presented show, three pure compounds with a significant inhibitory effect were discovered. Regarding the compounds inhibiting *P. aeruginosa* biofilm, further experiments are required to reveal their exact target and mechanism of action. This can be achieved by performing RT-PCR to study the changes in gene expression of the regulators of biofilm formation under the influence of our compounds. The active pure compounds need to have their structures elucidated by NMR, as well as their molecular mass and formula obtained by Liquid Chromatography-Mass Spectrometry (LC-MS). Moreover, the compounds need to be tested for any possible

cytotoxicity in vitro, and subsequently in vivo. Finally, after determining all of their characteristics and mode of action, these compounds will be tested in combination with antimicrobial agents to degrade the biofilm and control the spread of the planktonic bacteria dispersed.

The same steps mentioned above, in addition to purification steps, will be conducted for the active compound, ZK31 C Chloroform to study its activity on the biofilm of *A. baumannii*.

BIBLIOGRAPHY

Arzanlou, M., Chai, W. C., & Venter, H. (2017). Intrinsic, adaptive and acquired antimicrobial resistance in Gram-negative bacteria. *Essays in Biochemistry*, *61*(1), 49–59. https://doi.org/10.1042/EBC20160063

Azman, A. S., Mawang, C. I., Khairat, J. E., & AbuBakar, S. (2019). Actinobacteria—a promising natural source of anti-biofilm agents. *International Microbiology*, *22*(4), 403–409. https://doi.org/10.1007/s10123-019-00066-4

Bagshaw, S. M., & Laupland, K. B. (2006). Epidemiology of intensive care unit-acquired urinary tract infections. *Current Opinion in Infectious Diseases*, *19*(1), 67–71. https://doi.org/10.1097/01.qco.0000200292.37909.e0

Bérdy, J. (2012). Thoughts and facts about antibiotics: Where we are now and where we are heading. *Journal of Antibiotics*, *65*(8), 385–395. https://doi.org/10.1038/ja.2012.27

Bernardes, E. van T., Lewenza, S., & Reckseidler-Zenteno, S. (2015). Current Research Approaches to Traget Biofilm Infections. *Postdoc Journal*, *3*, 36–49.

Bhatt, V. S. (2019). Quorum sensing mechanisms in gram positive bacteria. *Implication of Quorum Sensing System in Biofilm Formation and Virulence*, 297–311. https://doi.org/10.1007/978-981-13-2429-1_20

Chambers, J. R., & Sauer, K. (2013). Small RNAs and their rolein biofilm formation. *Trends Microbiol.*, *23*(1), 1–22. https://doi.org/10.1016/j.tim.2012.10.008.Small

Chung, P. Y., & Toh, Y. S. (2014). Anti-biofilm agents: Recent breakthrough against multi-drug resistant Staphylococcus aureus. *Pathogens and Disease*, *70*(3), 231–239. https://doi.org/10.1111/2049-632X.12141

Ciofu, O., & Tolker-Nielsen, T. (2019). Tolerance and resistance of pseudomonas

aeruginosabiofilms to antimicrobial agents-how P. aeruginosaCan escape antibiotics. *Frontiers in Microbiology*, *10*(MAY). https://doi.org/10.3389/fmicb.2019.00913

Clutterbuck, A. L., Woods, E. J., Knottenbelt, D. C., Clegg, P. D., Cochrane, C. A., & Percival, S. L. (2007). Biofilms and their relevance to veterinary medicine. *Veterinary Microbiology*, *121*(1–2), 1–17. https://doi.org/10.1016/j.vetmic.2006.12.029

Colvin, K. M., Gordon, V. D., Murakami, K., Borlee, B. R., Wozniak, D. J., Wong, G. C. L., & Parsek, M. R. (2011). The pel polysaccharide can serve a structural and protective role in the biofilm matrix of Pseudomonas aeruginosa. *PLoS Pathogens*, *7*(1). https://doi.org/10.1371/journal.ppat.1001264

Daniel, R. (2004). The soil metagenome - A rich resource for the discovery of novel natural products. *Current Opinion in Biotechnology*, *15*(3), 199–204. https://doi.org/10.1016/j.copbio.2004.04.005

Darouiche, R. O., Mansouri, M. D., Gawande, P. V., & Madhyastha, S. (2009). Antimicrobial and antibiofilm efficacy of triclosan and DispersinB® combination. *Journal of Antimicrobial Chemotherapy*, *64*(1), 88–93. https://doi.org/10.1093/jac/dkp158

Dias, D. A., Urban, S., & Roessner, U. (2012). A Historical overview of natural products in drug discovery. *Metabolites*, *2*(2), 303–336. https://doi.org/10.3390/metabo2020303

Donlan, R. M. (2002). Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases*, *8*(9), 881–890. https://doi.org/10.3201/eid0809.020063

Eze, E. C., Chenia, H. Y., & El Zowalaty, M. E. (2018). Acinetobacter baumannii

biofilms: Effects of physicochemical factors, virulence, antibiotic resistance determinants, gene regulation, and future antimicrobial treatments. *Infection and Drug Resistance*, *11*, 2277–2299. https://doi.org/10.2147/IDR.S169894

Favre-Bonté, S., Köhler, T., & Van Delden, C. (2003). Biofilm formation by Pseudomonas aeruginosa: Role of the C4-HSL cell-to-cell signal and inhibition by azithromycin. *Journal of Antimicrobial Chemotherapy*, *52*(4), 598–604. https://doi.org/10.1093/jac/dkg397

Fernández, L., & Hancock, R. E. W. (2012). Adaptive and mutational resistance: Role of porins and efflux pumps in drug resistance. *Clinical Microbiology Reviews*, *25*(4), 661–681. https://doi.org/10.1128/CMR.00043-12

Ferri, M., Ranucci, E., Romagnoli, P., & Giaccone, V. (2017). Antimicrobial resistance: A global emerging threat to public health systems. *Critical Reviews in Food Science and Nutrition*, *57*(13), 2857–2876. https://doi.org/10.1080/10408398.2015.1077192

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S. (2016). Biofilms: An emergent form of bacterial life. *Nature Reviews Microbiology*, *14*(9), 563–575. https://doi.org/10.1038/nrmicro.2016.94

Foxman, B. (2014). Urinary tract infection syndromes. Occurrence, recurrence, bacteriology, risk factors, and disease burden. *Infectious Disease Clinics of North America*, *28*(1), 1–13. https://doi.org/10.1016/j.idc.2013.09.003

Franklin, M. J., Nivens, D. E., Weadge, J. T., & Lynne Howell, P. (2011). Biosynthesis of the pseudomonas aeruginosa extracellular polysaccharides, alginate, Pel, and Psl. *Frontiers in Microbiology*, *2*(AUG), 1–16. https://doi.org/10.3389/fmicb.2011.00167

Friedman, L., & Kolter, R. (2004). Two genetic loci produce distinct carbohydrate-rich structural components of the Pseudomonas aeruginosa biofilm matrix. *Journal of Bacteriology*, *186*(14), 4457–4465. https://doi.org/10.1128/JB.186.14.4457-4465.2004

Gaddy, J. A., & Actis, L. A. (2009). Regulation of Acinetobacter baumannii biofilm formation. *Future Microbiol.*, *23*(1), 1–9.

Ghaz-Jahanian, M. A., Khodaparastan, F., Berenjian, A., & Jafarizadeh-Malmiri, H. (2013). Influence of small RNAs on biofilm formation process in bacteria. *Molecular Biotechnology*, *55*(3), 288–297. https://doi.org/10.1007/s12033-013-9700-6

Giedraitienė, A., Vitkauskienė, A., Naginienė, R., & Pavilonis, A. (2011). Correspondence to Antibiotic Resistance Mechanisms of Clinically Important Bacteria. *REVIEW Medicina (Kaunas)*, *47*(3), 137–183.

Givskov, M., De Nys, R., Manefield, M., Gram, L., Maximilien, R., Eberl, L., Molin, Sø., Steinberg, P. D., & Kjelleberg, S. (1996). Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. *Journal of Bacteriology*, *178*(22), 6618–6622. https://doi.org/10.1128/jb.178.22.6618-6622.1996

Hengge, R. (2009). Principles of c-di-GMP signalling in bacteria. *Nature Reviews Microbiology*, *7*(4), 263–273. https://doi.org/10.1038/nrmicro2109

Hertiani, T., Edrada-Ebel, R. A., Ortlepp, S., van Soest, R. W. M., de Voogd, N. J., Wray, V., Hentschel, U., Kozytska, S., Müller, W. E. G., & Proksch, P. (2010). From anti-fouling to biofilm inhibition: New cytotoxic secondary metabolites from two Indonesian Agelas sponges. *Bioorganic and Medicinal Chemistry*, *18*(3), 1297–1311. https://doi.org/10.1016/j.bmc.2009.12.028

Høiby, N., Ciofu, O., Johansen, H. K., Song, Z. J., Moser, C., Jensen, P. Ø., Molin, S., Givskov, M., Tolker-Nielsen, T., & Bjarnsholt, T. (2011). The clinical impact of bacterial biofilms. *International Journal of Oral Science*, *3*(2), 55–65. https://doi.org/10.4248/IJOS11026

Huigens, R. W., Richards, J. J., Parise, G., Ballard, T. E., Zeng, W., Deora, R., & Melander, C. (2007). Inhibition of Pseudomonas aeruginosa biofilm formation with bromoageliferin analogues. *Journal of the American Chemical Society*, *129*(22), 6966–6967. https://doi.org/10.1021/ja069017t

Jamal, M., Ahmad, W., Andleeb, S., Jalil, F., Imran, M., Nawaz, M. A., Hussain, T., Ali, M., Rafiq, M., & Kamil, M. A. (2018). Bacterial biofilm and associated infections. *Journal of the Chinese Medical Association*, *81*(1), 7–11. https://doi.org/10.1016/j.jcma.2017.07.012

Jenal, U., & Malone, J. (2006). Mechanisms of cyclic-di-GMP signaling in bacteria. *Annual Review of Genetics*, *40*, 385–407. https://doi.org/10.1146/annurev.genet.40.110405.090423

Jennings, L. K., Storek, K. M., Ledvina, H. E., Coulon, C., Marmont, L. S., Sadovskaya, I., Secor, P. R., Tseng, B. S., Scian, M., Filloux, A., Wozniak, D. J., Howell, P. L., & Parsek, M. R. (2015). Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the Pseudomonas aeruginosa biofilm matrix. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(36), 11353–11358. https://doi.org/10.1073/pnas.1503058112

Jepsen, O. B. (1987). Urinary tract infections. An overview. *Chemioterapia*, *6*(3), 179–183. https://doi.org/10.1016/s0002-9629(15)40208-3

Kai Papenfort1, and B. B. (2014). Responsabilidad Social Empresarial Para Pequeños

Hoteles De La Ciudad De Cartagena *. *SABER, CIENCIA Y Libertad*, *9*(2), 91–108. https://doi.org/10.1038/nrmicro.2016.89.Quorum-Sensing

Karatan, E., & Watnick, P. (2009). Signals, Regulatory Networks, and Materials That Build and Break Bacterial Biofilms. *Microbiology and Molecular Biology Reviews*, *73*(2), 310–347. https://doi.org/10.1128/mmbr.00041-08

Kayanadath, S., Nathan, V. K., & Ammini, P. (2019). Anti-Biofilm Activity of Biosurfactant Derived from Halomonas sp., a Lipolytic Marine Bacterium from the Bay of Bengal. *Microbiology (Russian Federation)*, *88*(5), 585–599. https://doi.org/10.1134/S0026261719050072

Khatoon, Z., McTiernan, C. D., Suuronen, E. J., Mah, T. F., & Alarcon, E. I. (2018). Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. *Heliyon*, *4*(12), e01067. https://doi.org/10.1016/j.heliyon.2018.e01067

Lamas Ferreiro, J. L., Álvarez Otero, J., González Gónzalez, L., Novoa Lamazares, L., Arca Blanco, A., Bermúdez Sanjurjo, J. R., Rodríguez Conde, I., Fernández Soneira, M., & de la Fuente Aguado, J. (2017). Pseudomonas aeruginosa urinary tract infections in hospitalized patients : Mortality and prognostic factors. *Plos One*, 1–13.

Lebeaux, D., Ghigo, J.-M., & Beloin, C. (2014). Biofilm-Related Infections: Bridging the Gap between Clinical Management and Fundamental Aspects of Recalcitrance toward Antibiotics. *Microbiology and Molecular Biology Reviews*, *78*(3), 510–543. https://doi.org/10.1128/mmbr.00013-14

Lewis, K. (2010). Persister cells. *Annual Review of Microbiology*, *64*, 357–372. https://doi.org/10.1146/annurev.micro.112408.134306

Longo, F., Vuotto, C., & Donelli, G. (2014). Biofilm formation in Acinetobacter baumannii. *New Microbiologica*, *37*(2), 119–127.

Lu, L., Hu, W., Tian, Z., Yuan, D., Yi, G., Zhou, Y., Cheng, Q., Zhu, J., & Li, M. (2019). Developing natural products as potential anti-biofilm agents. *Chinese Medicine (United Kingdom)*, *14*(1), 1–17. https://doi.org/10.1186/s13020-019-0232-2

Lyczak, J. B., Cannon, C. L., & Pier, G. B. (2000). Establishment of Pseudomonas aeruginosa infection: Lessons from a versatile opportunist. *Microbes and Infection*, *2*(9), 1051–1060. https://doi.org/10.1016/S1286-4579(00)01259-4

Ma, L., Conover, M., Lu, H., Parsek, M. R., Bayles, K., & Wozniak, D. J. (2009). Assembly and development of the Pseudomonas aeruginosa biofilm matrix. *PLoS Pathogens*, *5*(3). https://doi.org/10.1371/journal.ppat.1000354

Mazzola, P. G., Lopes, A. M., Hasmann, F. A., Jozala, A. F., Penna, T. C., Magalhaes, P. O., Rangel-Yagui, C. O., & Jr, A. P. (2008). Liquid–liquid extraction of biomolecules: an overview and update of the main techniques. *Journal of Chemical Technology & Biotechnology*, *83*(May).

Melander, R. J., Basak, A. K., & Melander, C. (2020). Natural products as inspiration for the development of bacterial antibiofilm agents. *Natural Product Reports*, *37*(11), 1454–1477. https://doi.org/10.1039/d0np00022a

Mishra, R., Panda, A. K., De Mandal, S., Shakeel, M., Bisht, S. S., & Khan, J. (2020). Natural Anti-biofilm Agents: Strategies to Control Biofilm-Forming Pathogens. *Frontiers in Microbiology*, *11*(October). https://doi.org/10.3389/fmicb.2020.566325

Nikaido, H. (2009). Multidrug resistance in bacteria. *Annual Review of Biochemistry*,

78, 119–146. https://doi.org/10.1146/annurev.biochem.78.082907.145923

O'Toole, G. A. (2003). To build a biofilm. *Journal of Bacteriology*, *185*(9), 2687–2689. https://doi.org/10.1128/JB.185.9.2687-2689.2003

Onanuga, A., & Awhowho, G. O. (2012). Antimicrobial resistance of Staphylococcus aureus strains from patients with urinary tract infections in Yenagoa, Nigeria. *Journal of Pharmacy and Bioallied Sciences*, *4*(3), 226–230. https://doi.org/10.4103/0975-7406.99058

Ong, K. S., Mawang, C. I., Daniel-Jambun, D., Lim, Y. Y., & Lee, S. M. (2018). Current anti-biofilm strategies and potential of antioxidants in biofilm control. *Expert Review of Anti-Infective Therapy*, *16*(11), 855–864. https://doi.org/10.1080/14787210.2018.1535898

Ouyang, J., Sun, F., Feng, W., Sun, Y., Qiu, X., Xiong, L., Liu, Y., & Chen, Y. (2016). Quercetin is an effective inhibitor of quorum sensing, biofilm formation and virulence factors in Pseudomonas aeruginosa. *Journal of Applied Microbiology*, *120*(4), 966–974. https://doi.org/10.1111/jam.13073

Pang, Z., Raudonis, R., Glick, B. R., Lin, T. J., & Cheng, Z. (2019). Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies. *Biotechnology Advances*, *37*(1), 177–192. https://doi.org/10.1016/j.biotechadv.2018.11.013

Park, S. R., Tripathi, A., Wu, J., Schultz, P. J., Yim, I., McQuade, T. J., Yu, F., Arevang, C. J., Mensah, A. Y., Tamayo-Castillo, G., Xi, C., & Sherman, D. H. (2016). Discovery of cahuitamycins as biofilm inhibitors derived from a convergent biosynthetic pathway. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms10710

Prestinaci, F., Pezzotti, P., & Pantosti, A. (2015). Antimicrobial resistance: A global multifaceted phenomenon. *Pathogens and Global Health*, *109*(7), 309–318. https://doi.org/10.1179/2047773215Y.0000000030

Qvortrup, K., Hultqvist, L. D., Nilsson, M., Jakobsen, T. H., Jansen, C. U., Uhd, J., Andersen, J. B., Nielsen, T. E., Givskov, M., & Tolker-Nielsen, T. (2019). Small Molecule Anti-biofilm Agents Developed on the Basis of Mechanistic Understanding of Biofilm Formation. *Frontiers in Chemistry*, *7*(November). https://doi.org/10.3389/fchem.2019.00742

Raj, S. S., & Thomas, N. G. (2021). *Anti biofilm agents – Nature the cure*. *9*(1), 25–32.

Rasamiravaka, T., Labtani, Q., Duez, P., & El Jaziri, M. (2015). The formation of biofilms by pseudomonas aeruginosa: A review of the natural and synthetic compounds interfering with control mechanisms. *BioMed Research International*, *2015*. https://doi.org/10.1155/2015/759348

Rodrigues, L. R. (2011). Inhibition of bacterial adhesion on medical devices. *Advances in Experimental Medicine and Biology*, *715*, 351–367. https://doi.org/10.1007/978-94-007-0940-9_22

Ronald, A. (2003). The etiology of urinary tract infection: Traditional and emerging pathogens. *Disease-a-Month*, *49*(2), 71–82. https://doi.org/10.1016/S0011-5029(03)90001-0

Roy, R., Tiwari, M., Donelli, G., & Tiwari, V. (2018). Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. *Virulence*, *9*(1), 522–554. https://doi.org/10.1080/21505594.2017.1313372

Rutherford, S. T., & Bassler, B. L. (2012). Bacterial quorum sensing: Its role in virulence and possibilities for its control. *Cold Spring Harbor Perspectives in*

Medicine, *2*(11), 1–25. https://doi.org/10.1101/cshperspect.a012427

Sacha, P., Wieczorek, P., Hauschild, T., Olszañska, D., & Tryniszewska, E. (2008). *a novel mechanism resistance to β -lactam antibiotics*. *46*(2), 137–142. https://doi.org/10.2478/v10042-008-0020-9

Saipriya, K., Swathi, C. H., Ratnakar, K. S., & Sritharan, V. (2020). Quorum-sensing system in Acinetobacter baumannii: a potential target for new drug development. *Journal of Applied Microbiology*, *128*(1), 15–27. https://doi.org/10.1111/jam.14330

Salta, M., Wharton, J. A., Dennington, S. P., Stoodley, P., & Stokes, K. R. (2013). Anti-biofilm performance of three natural products against initial bacterial attachment. *International Journal of Molecular Sciences*, *14*(11), 21757–21780. https://doi.org/10.3390/ijms141121757

Sambanthamoorty, K., Luo, C., Pattabiraman, N., Feng, X., Koestler, B., Waters, C. M., & Palys, T. (2014). Identification of small molecules inhibiting diguanylate cyclases to control bacterial biofilm development. *Biofouling*, *30*(1), 17–28. https://doi.org/10.1080/08927014.2013.832224.Identification

Silby, M. W., Winstanley, C., Godfrey, S. A. C., Levy, S. B., & Jackson, R. W. (2011). Pseudomonas genomes: Diverse and adaptable. *FEMS Microbiology Reviews*, *35*(4), 652–680. https://doi.org/10.1111/j.1574-6976.2011.00269.x

Smith, A. C., & Hussey, M. A. (2005). Gram stain protocols. *American Society for Microbiology*, *1*(September 2005), 14.

Smith, R. S., & Iglewski, B. H. (2003). Pseudomonas aeruginosa quorum sensing as a potential antimicrobial target. *The Journal of Clinical Investigation*, *112*(10), 1–6. https://doi.org/10.1172/JCI200320364.Quorum

Song, X., Xia, Y.-X., He, Z.-D., & Zhang, H.-J. (2017). A Review of Natural Products with Anti-Biofilm Activity. *Current Organic Chemistry*, *22*(8), 789–817. https://doi.org/10.2174/1385272821666170620110041

Stewart, P. S. (2015). Prospects for anti-biofilm pharmaceuticals. *Pharmaceuticals*, *8*(3), 504–511. https://doi.org/10.3390/ph8030504

Stowe, S. D., Richards, J. J., Tucker, A. T., Thompson, R., Melander, C., & Cavanagh, J. (2011). Anti-biofilm compounds derived from marine sponges. *Marine Drugs*, *9*(10), 2010–2035. https://doi.org/10.3390/md9102010

Sun, F., Qu, F., Ling, Y., Mao, P., Xia, P., Chen, H., & Zhou, D. (2013). Biofilm-associated infections: Antibiotic resistance and novel therapeutic strategies. *Future Microbiology*, *8*(7), 877–886. https://doi.org/10.2217/fmb.13.58

Suzuki, N., Ohtaguro, N., Yoshida, Y., Hirai, M., Matsuo, H., Yamada, Y., Imamura, N., & Tsuchiya, T. (2015). A compound inhibits biofilm formation of staphylococcus aureus from streptomyces. *Biological and Pharmaceutical Bulletin*, *38*(6), 889–892. https://doi.org/10.1248/bpb.b15-00053

Tasneem, U., Yasin, N., Nisa, I., Shah, F., Rasheed, U., Momin, F., Zaman, S., & Qasim, M. (2018). Biofilm producing bacteria: A serious threat to public health in developing countries. *Journal of Food Science and Nutrition*, *01*(02). https://doi.org/10.35841/food-science.1.2.25-31

Tassew, D. D., Mechesso, A. F., Park, N. H., Song, J. B., Shur, J. W., & Park, S. C. (2017). Biofilm formation and determination of minimum biofilm eradication concentration of antibiotics in mycoplasma hyopneumoniae. *Journal of Veterinary Medical Science*, *79*(10), 1716–1720. https://doi.org/10.1292/jvms.17-0279

Thummeepak, R., Kongthai, P., Leungtongkam, U., & Sitthisak, S. (2016). Distribution

of virulence genes involved in biofilm formation in multi-drug resistant acinetobacter Baumannii clinical isolates. *International Microbiology*, *19*(2), 121–129. https://doi.org/10.2436/20.1501.01.270

Ventre, I., Goodman, A. L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., Bleves, S., Lazdunski, A., Lory, S., & Filloux, A. (2006). Multiple sensors control reciprocal expression of Pseudomonas aeruginosa regulatory RNA and virulence genes. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(1), 171–176. https://doi.org/10.1073/pnas.0507407103

Vipin, C., Mujeeburahiman, M., Ashwini, P., Arun, A. B., & Rekha, P. D. (2019). Anti-biofilm and cytoprotective activities of quercetin against Pseudomonas aeruginosa isolates. *Letters in Applied Microbiology*, *68*(5), 464–471. https://doi.org/10.1111/lam.13129

Wang, L., & Wang, L. (2016). Acinetobacter baumannii biofilm resistance mechanisms and prevention and control of progress. *Discussion of Clinical Cases*, *3*(2), 22. https://doi.org/10.14725/dcc.v3n2p22

Waturangi, D. E., Rahayu, B. S., Lalu, K. Y., & Mulyono, N. (2016). *Malaysian Journal of Microbiology against Gram-negative and Gram-positive bacteria*. *12*(4), 291–299.

Wei, Q., & Ma, L. Z. (2013). Biofilm matrix and its regulation in Pseudomonas aeruginosa. *International Journal of Molecular Sciences*, *14*(10), 20983–21005. https://doi.org/10.3390/ijms141020983

Wolska, K. I., Grudniak, A. M., Rudnicka, Z., & Markowska, K. (2016). Genetic control of bacterial biofilms. *Journal of Applied Genetics*, *57*(2), 225–238. https://doi.org/10.1007/s13353-015-0309-2

Wong, D., Nielsen, T. B., Bonomo, R. A., Pantapalangkoor, P., Luna, B., & Spellberg, B. (2017). Clinical and pathophysiological overview of Acinetobacter infections: A century of challenges. *Clinical Microbiology Reviews*, *30*(1), 409–447. https://doi.org/10.1128/CMR.00058-16

Yada, S., Kamalesh, B., Sonwane, S., Guptha, I., & Swetha, R. K. (2015). Quorum sensing inhibition, relevance to periodontics. *Journal of International Oral Health : JIOH*, *7*(1), 67–69. http://www.ncbi.nlm.nih.gov/pubmed/25709373%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4336667

Yang, L., Hu, Y., Liu, Y., Zhang, J., Ulstrup, J., & Molin, S. (2011). Distinct roles of extracellular polymeric substances in Pseudomonas aeruginosa biofilm development. *Environmental Microbiology*, *13*(7), 1705–1717. https://doi.org/10.1111/j.1462-2920.2011.02503.x

Yin, Q., Liang, J., Zhang, W., Zhang, L., Hu, Z. L., Zhang, Y., & Xu, Y. (2019). Butenolide, a Marine-Derived Broad-Spectrum Antibiofilm Agent Against Both Gram-Positive and Gram-Negative Pathogenic Bacteria. *Marine Biotechnology*, *21*(1), 88–98. https://doi.org/10.1007/s10126-018-9861-1