SCREENING FOR ANTIBIOFILM EFFECT OF ANTIBACTERIAL PEPTIDE PAM-5 ON CLINICAL STRAIN OF MULTIDRUG-RESISTANT *Pseudomonas aeruginosa*

By

WONG LI CHIK

A project report submitted to the Department of Allied Health Sciences

Faulty of Science

Universiti Tunku Abdul Rahman

in partial fulfilment of the requirements for the degree of

Bachelor of Science (Hons) Biomedical Science

MAY 2022

ABSTRACT

SCREENING FOR ANTIBIOFILM EFFECT OF ANTIBACTERIAL PEPTIDE PAM-5 ON CLINICAL STRAIN OF MULTIDRUG-RESISTANT *Pseudomonas aeruginosa*

WONG LI CHIK

Biofilm represents a major resistant mechanism in many pathogenic bacteria. *Pseudomonas aeruginosa* (*P. aeruginosa*) is known as one of the notorious bacteria associated with biofilm-mediated antibiotic resistance, leading to prolonged hospitalization and treatment. In recent years, many studies have focused on the use of antimicrobial peptides (ABPs) as an alternative antibacterial agent against resistant bacteria, including biofilm-producing bacteria. A novel ABP named PAM-5 was previously reported for its promising antibacterial effect on a spectrum of planktonic pathogenic bacteria. However, the effect of PAM-5 on biofilm-grown bacteria has yet to be elucidated. Therefore, in this study, the ability of PAM-5 to inhibit biofilm formation as well as to eradicate of mature biofilm formed by a clinical isolate of multidrug resistant (MDR) *P. aeruginosa* (1894170) was evaluated. Using microtiter-based biofilm inhibition assay, a clinically isolated MDR *P. aeruginosa* was treated with PAM-5 at concentrations ranging from 4 µg/ml to 512 µg/ml. The amount of biofilm from the treated bacteria was quantified by crystal violet staining. For biofilm eradication, MDR *P. aeruginosa* was grown in microtiter

plate for 48 hours to establish mature biofilm, followed by peptide treatment at the same range of concentrations. Upon PAM-5 treatment, the biofilm mass was quantified by crystal violet staining while the metabolic activity of the biomass was investigated via MTT assay. From this study, PAM-5 was shown to inhibit > 50% MDR *P. aeruginosa* biofilm formation at 16 µg/ml and the inhibition effect increased in a dose-dependent manner. On the other hand, only 8 µg/ml of PAM-5 was required to eradicate >50% of the mature biofilm as well as the metabolic activity of the biofilm-embedded bacteria, in which PAM-5 was able to reduce the viable biofilm-embedded bacteria in a dose-dependent manner. In conclusion, PAM-5 could inhibit biofilm formation, eradicate mature biofilm, and kill biofilm embedded cells of clinical strain of MDR *P. aeruginosa*.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my project supervisor, Mr. Yuen Hawk Leong for the continuous support and guidance throughout the duration of my final year project. I truly appreciate for his patience, motivation, enthusiasm, and immense knowledge. His guidance and endless support helped me to solve the problems during my benchwork and thesis writing.

Furthermore, I would like to thank the lab officers and assistants, Mr. Saravanan, Ms. Hema, Ms. Fatin, Ms. Natrah, and Ms. Ain for their invaluable help and assistance throughout the duration of research benchwork.

Also, I would also like to grab this opportunity to thank my lab mate, Sze Hui for her companion and assistance throughout this project duration.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

WONG LI CHIK

APPROVAL SHEET

This project report entitled "**SCREENING FOR ANTI-BIOFILM EFFECT OF PAM-5 ON CLINICAL STRAIN OF MULTIDRUG-RESISTANT *Pseudomonas aeruginosa*"** was prepared by WONG LI CHIK and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Mr. Yuen Hawk Leong) Date:….1/6/2022...
Supervisor
Department of Allied Health Sciences
Faculty of Science
Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

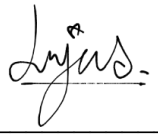
Date: _**30/5/2022**__

PERMISSION SHEET

It is hereby certified that **WONG LI CHIK** (ID No: **19ADB06362**) has completed this final year project entitled "SCREENING FOR ANTIBIOFILM EFFECT OF ANTIBACTERIAL PEPTIDE PAM-5 ON CLINICAL STRAIN OF MULTIDRUG-RESISTANT *Pseudomonas aeruginosa*" under the supervision of Mr. Yuen Hawk Leong from the Department of Allied Health Sciences, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,



(WONG LI CHIK)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVATIONS

ABPs	Antibacterial peptides
AIs	Autoinducers
APD	Antimicrobial peptide database
Arg/ R	Arginine
BHIB	Brain heart infusion broth
CF	Cystic fibrosis
CFUs	Colony forming units
CV	Crystal violet
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
eDNA	Extracellular DNA
EPS	Exopolysaccharide substances
ESBL	Extended-spectrum beta-lactamase
His/ H	Histidine
HNP-1	Human neutrophil defensin-1
Lys/ K	Lysine
MBCs	Minimal bactericidal concentrations

MDR	Multidrug resistant
MHB	Mueller Hilton broth
MICs	Minimum inhibitory concentrations
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBPs	Penicillin-binding protein
QS	Quorum sensing
Trp/ W	Tryptophan
TSB	Trypsin soy broth
VAP	Ventilator associated pneumonia
XDR	Extensively drug-resistant

CHAPTER 1

INTRODUCTION

Bacterial biofilms have been associated with resistance to host immune response and antimicrobial therapy, leading to failure in treatment of many bacterial infections and prolonged hospitalization in clinical setting (Lebeaux et al., 2014; Hrynyshyn et al., 2022). Bacterial biofilms are aggregates of bacterial communities which are usually formed on biotic or abiotic surfaces (Kragh et al., 2016; Zheng et al., 2021). Once settled on these surfaces, the bacteria begin to communicate via quorum sensing and secrete multiple substances to form layers of extracellular matrix, allowing them to survive under different hostile environment as well as the host's immune defence and antibiotic attack (Hathroubi et al., 2017; Abebe, 2020). Upon certain stages of development, the sessile bacteria from the biofilm will be dispersed from the original community structure and disseminate to other surfaces for new biofilm formation. Therefore, biofilm represents a major obstacle to antibiotic treatment in many chronic bacterial infections.

Pseudomonas aeruginosa (*P. aeruginosa*) is notorious for its high incidence and prevalence of antibiotic resistance. Being one of the members under the ESKAPE pathogens that represents *Enterobacter* species (E), *Staphylococcus aureus* (S), *Klebsiella pneumoniae* (K), *Acinetobacter baumannii* (A), *Pseudomonas aeruginosa* (P) and *Enterococcus faecium* (E), *P. aeruginosa* is able to compromise the efficacy of many antibiotics via intrinsic and acquired

resistance. Notably, *P aeruginosa* is also reported as one of the biofilm-forming bacteria (Thi et al., 2020; Tuon et al., 2022), which enables the bacterium to establish many chronic infections in its hosts such as cystic fibrosis (CF), malignant external otitis, endophthalmitis and pneumonia (Bodey et al., 1983; Gellatly and Hancock, 2013; Bush, 2022). As the result of barrier effects by the biofilm, *P. aeruginosa* is able to persist in its host despite antibiotic treatment. Therefore, it is crucial to explore novel antibacterial agents that are able to overcome biofilm-mediated resistance.

Antibacterial peptides (ABPs) are short peptides with bacteriostatic or/and bactericidal effects (Li et al., 2017). Over the last few decades since their discoveries, many new ABPs were isolated from living organisms or chemically synthesized for evaluation on their antibacterial properties (Dean et al., 2011; Huan et al., 2020; Ramazi et al., 2022). To date, more than 2000 ABPs with promising antibacterial effects were documented. However, only a handful of these peptides were reported for antibiofilm property. Therefore, continuous efforts are needed to explore more ABPs not only with direct bacteriostatic or bactericidal effects, but also possess the ability to inhibit biofilm formation and eradicate mature biofilm.

In the earlier studies, a novel ABP named PAM-5 was found to exert promising bactericidal effects towards a panel of Gram-negative bacteria, including *P. aeruginosa* (Chan, 2016, unpublished). However, these antibacterial findings were based on the action of PAM-5 towards planktonic bacteria, while its effect towards sessile bacteria in the biofilm community is yet to be elucidated.

Therefore, the objectives of this study are:

1. To screen for the ability of PAM-5 to inhibit biofilm formation by clinical strain of multidrug-resistant (MDR) *Pseudomonas aeruginosa* via crystal violet assay.
2. To screen for the ability of PAM-5 to eradicate matured biofilm by clinical strain of multidrug-resistant (MDR) *Pseudomonas aeruginosa* via crystal violet and MTT assays.

CHAPTER 2

LITERATURE REVIEW

2.1 Biofilm

2.1.1 Overview of Biofilm

Bacterial biofilm is defined as aggregates of bacteria of the same or different species that adhere to a surface and survive within layers of self-produced extracellular matrix (Sharma et al., 2019). Bacteria may attach to virtually any biotic or abiotic surfaces and build architecturally complex communities known as biofilms (Khatoon et al., 2018). Biofilm is regarded as the mode of growth that enable bacteria to survive under hostile conditions, as well as providing protection to the bacteria from environmental changes including temperature, humidity, pH and nutrient availability (Kean et al., 2018; Rizzato et al., 2019; Ghazay et al., 2021).

Within biofilms, bacteria grow in multicellular pattern which aggregate to form colonies that are embedded within layers of extracellular matrix (ECM) produced by the bacteria (Lopez et al., 2010; Secor et al., 2018; Armbruster and Parsek, 2018; Muhammad et al., 2020). The ECM, also known as exopolysaccharide substance (EPS), is a complex combination of polysaccharides, proteins, extracellular-DNA, and lipids (Annous et al., 2009; Costa et al., 2018). These components assist the bacteria to attach to a surface during initial biofilm establishment. Besides, these biofilm components also help to retain nutrients and other essential requirement for bacterial survival and

growth, as well as shielding the bacteria from host immune system and antimicrobial agents (Flemming et al., 2008; Reichhardt et al., 2014; Santos et al., 2018). Additionally, biofilm is also important for holding the bacterial community close together, hence facilitating cell-to-cell communication known as quorum sensing (QS). Furthermore, biofilm also facilitates genetic material exchange via horizontal gene transfer among the bacterial community (Flemming et al., 2016; Liu et al., 2017; Abe et al., 2020). These genetic exchanges allow the bacteria to acquire better survival criteria such as antibiotic resistance and adaptation to nutrient deprivation.

2.1.2 Biofilm Formation

The formation of biofilm consists of five sequential stages as shown in **Figure 2.1**. At the initial stage, individual planktonic bacteria deposit and adhere to an abiotic or biotic surface. This adherence is mediated by weak interactions such as hydrophobic interactions, electrostatic forces and van der Waals between the bacteria and the surface (Carniello et al., 2018; Muhammad et al., 2020). At this stage, the bacteria will decide whether to proceed to biofilm formation or detach from the surface and return to their planktonic phenotype, depending on the surface condition (Toyofuku et al., 2015). If the former is chosen, then the biofilm formation will proceed to stage two, where EPSs are produced by the loosely bound bacteria to consolidate their surface attachment. Under this stage, the EPSs will complex with any preconditioned and permissive surface, rendering the bacteria into irreversible attachment to the surface. As more planktonic bacteria are attaching to the surface along with additional EPS production, the bacteria begin to aggregate into multilayer cell clusters (Roy et

al., 2017). Then, the attached bacteria undergo certain physiological and structural changes, for instance, repression of synthesis of mobility structures and induction in the synthesis of adhesive structure (Karatan and Watnick, 2009; Berne et al., 2015).

During the third stage, bacteria which are encased within the ECM will undergo coordinated community growth and the aggregated bacteria become significantly more layered. These processes are controlled by chemical signals that intercommunicate the bacterial community which is known as quorum sensing (QS) (Rutherford and Bassler, 2012; Dincer et al., 2020). QS is one of the bacterial activities that are essential to regulate biofilm maturation, and it is regulated by chemical signalling molecules called autoinducers (AIs) (Bhardwaj et al., 2013; Solano et al, 2014).

At stage four, the fully matured biofilm reaches its maximum cell density and develops into spatially well-arranged, three-dimensional mushroom-like structures with liquid-filled channels to circulate nutrients, oxygen and other essential substances to different layers of the biofilm (Roy et al., 2017; Muhammad et al., 2020). At the last stage, some microcolonies of the matured biofilm will be dispersed from the main community as planktonic bacteria. These free form of bacteria will migrate and settle on a new surface to form new biofilms (Stoodley et al., 2002; Schachter, 2003). In short, biofilm formation is a cyclical process with the bacterial transition between planktonic and biofilm phenotype, and this cycle will continue to colonize more surfaces if there is no intervention to stop or prevent it.

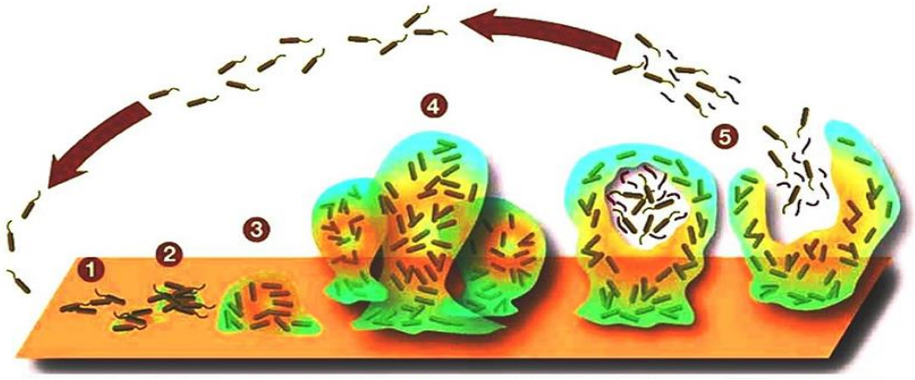


Figure 2.1: A model showing the typical bacterial biofilm developmental stages (Adapted from Bai et al., 2021). (1) Initial attachment of planktonic bacteria; (2) Monolayer formation and production of EPS; (3) Microcolonies formation; (4) Biofilm maturation; (5) Biofilm dispersion.

2.1.3 Adverse Impacts of Biofilm Towards Human

Biofilms have a wide range of effects on human since they are present naturally, medical and industrial environments (Hatt and Rather, 2008; Hall-Stoodley and Stoodley, 2009; Lopez et al., 2010). As mentioned earlier, biofilm could be formed on biotic and abiotic surfaces. In particular to the latter, the occurrence of biofilms on the surfaces of food and medical devices is always associated with serious infections resulting from foodborne diseases and nosocomial infections. The former is attributed to biofilms that are formed on food matrixes or food factory equipment, while the latter is always the consequence of biofilm formation on invasive medical devices. For instance, improperly handled fresh fish products may be colonized by biofilms arised from certain pathogenic bacteria such as *Listeria monocytogenes*, *Salmonella enterica* and *Aeromonas hydrophila* (Mizan et al.,2015). On the other hand, biofilm development in food equipment or food factories such as liquid pipelines, water tanks, reverse osmosis membranes, raw materials or packaging materials are also commonly reported (Camargo et al., 2017; Galié et al., 2018; Panebianco et al., 2022).

Direct or indirect food contamination by the above-mentioned biofilm colonization may cause bacterial infection in gastrointestinal tract or food intoxication by the toxin secreted by biofilms (Rossi et al., 2017; Bai et al., 2021).

The ability of many bacteria to develop and remain as biofilms is an advantage to the microorganisms because biofilm-grown bacteria are less susceptible to the bacteriostatic or bactericidal effect of many antibacterial agents (Stewart, 2015; Yamada and Kielian, 2018; Ciofu and Tolker-Nielsen, 2019). Additionally, biofilm also represents an evasion strategy by the bacteria against their host immune system. The presence of multiple layers of ECM may hinder the recognition of bacterial surface antigens by innate immune cells. For instance, *P. aeruginosa* has been shown to inhibit immune recognition by down-regulating the pathogen-associated molecular pattern expression during biofilm development (Rada, 2017), as well as blocking the direct access of antibodies, complement and other effector immune cells to the biofilm-embedded bacteria (Roy et al., 2017; Tuon et al., 2022).

More aggressively, some reports demonstrated that biofilm could interfere or even inactivate certain immune effector functions. For example, *P. aeruginosa* biofilm was found to be able to inactivate host complement proteins by increasing the secretion of alkaline proteases and elastases (Mulcahy et al., 2013). Additionally, under the QS regulation, certain species of biofilm-embedded bacteria were reported to secrete toxin that is cytotoxic towards immune cells. A study by Peschel and Otto (2013) showed that biofilm-

embedded *Staphylococcus aureus* (*S. aureus*) was able to produce toxins known as γ-haemolysins HlgAB and leukocidin GH that are able to lyse leukocytes. Apart from that, two other toxins, namely α-toxin and leukocidin AB, were also found in *S. aureus*-produced biofilms. These toxins were reported to inhibit macrophage phagocytosis and intracellular killing of phagocytosed bacteria, as well as direct killing of the innate immune cell (Koziel et al., 2014; Scherr et al., 2015). These findings implicated that biofilm-mediated infections may persist in host and could hardly be eradicated by host immune response, leading to chronic and systemic infections.

Moreover, EPS of biofilm matrix enables the embedded bacterial communities to remain in close proximity and potentially promote dissemination of antimicrobial resistance. EPS provides an ideal reservoir for cellular exchange of resistant plasmids among the bacterial community via horizontal gene transfer (Flemming et al., 2016). As a result, progeny bacteria that have acquired resistance towards different types of antibiotics might be produced within this biofilm microenvironment.

Biofilm-mediated antibiotic resistance is a major concern to public health as it reduces the efficacy of many conventional antibiotic treatments which leads to chronic bacterial infections. This biofilm-mediated complication was supported by a review paper from Yamada and Kielian (2018), who reported that biofilm is the primary cause of many chronic infections such as otitis media, chronic nosocomial infections, cystic fibrosis, and chronic wound infections.

2.1.4 Mechanism of Antibiotic Resistance Associated with Biofilm

Several studies have demonstrated that bacteria in biofilms have higher tolerance to antibiotics as compared to their planktonic counterpart (Mah, 2012; Sharma et al., 2019; Dincer et al., 2020; Sabino et al., 2022). The different capacity of antibiotic resistance is mainly attributed to the distinct resistant mechanisms between the two bacterial phenotypes. In fact, studies have indicated that the common resistant mechanisms by planktonic bacteria may not be employed by the biofilm-grown bacteria (Lata et al., 2015; Sharma et al., 2015; Sharma et al., 2016), and the latter possess several unique mechanisms that protect them from the deleterious effects of antibacterial agents.

As mentioned earlier, the EPSs which are produced by biofilm bacterial community serve as the major resistant mechanism to many antimicrobial agents, including conventional antibiotics. Together with other macromolecules, sugar and minerals, EPSs form layers of extracellular matrix and exopolysaccharides which serve as physical barrier against environmental desiccation and host immune attack (Sharma et al., 2019). Most importantly, in the clinical context, these ECM layers are able to restrict or block penetration of antibiotics to reach the bacteria in deeper layers (Dincer et al., 2020). Certain components of the EPS were found able to bind to certain antibiotics, thus reducing concentrations of the latter to reach the biofilm-embedded bacteria (Singh et al., 2021). For example, an anionic EPS component from *P. aeruginosa* named *Pel* exopolysaccharides was found able to bind and dissipate cationic antibiotics, thus reducing the antibiotic effective concentrations to the core bacteria (Colvin et al., 2011). Consequently, the minimum inhibitory

concentrations (MICs) or minimum bactericidal concentrations (MBCs) of the antibiotics against these bacteria are hardly achieved, allowing the continuous survival of the bacteria within the biofilm. Worse, the sublethal antibiotic concentrations may impose induction pressure that promote mutational mediated resistance among the bacterial community (Knudsen et al., 2016).

Next, the microenvironment within the biofilm may serve as another compromising factor towards antibiotic efficacy. The amount of oxygen and nutrient are at decreasing gradient from the surface or peripheral layers of biofilm to the inner or centre regions of the matrix (Penesyan et al., 2021). The anaerobic condition within the biofilm may impair the actions of some antibiotics that require active transport into bacteria, such as aminoglycosides (Ramirez and Tolmasky, 2010). Besides that, anaerobic condition along with nutrients deprivation may slow down bacterial growth and metabolic rate. As the bacteria are proliferating at extremely slow rate, their membrane permeability are relatively low, thus reducing the entry and intracellular accumulation of antibiotics that mainly act on intracellular targets such as fluroquinolones, tetracyclines and aminoglycosides (Blanco et al., 2016; Munita and Arias, 2016). Furthermore, metabolically inactive bacteria in the inner regions of biofilm are also less susceptible to those antibiotics that target bacterial biosynthetic activities such as DNA replication and protein synthesis (Uruén et al., 2020). This small population of dormant bacteria are also known as persistent cells, which are transformed from the active and rapidly growing bacteria under antibiotic pressure (Germain et al., 2015). Once the antibiotic treatment ceases or the antibiotic concentration depletes, these persistent cells

will revert back to their planktonic phenotype and disperse from the original biofilm to other parts of host for another biofilm development (Lebeaux et al., 2014).

Biofilm-associated resistance represents a major obstacle to treatment of many bacterial infections. Many pathogenic bacteria were found to employ this strategy to establish chronic infections by overcoming the host immune responses and antibiotic treatments. One of the notorious bacteria that are associated with biofilm-mediated resistance is *P. aeruginosa*.

2.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) is an aerobic Gram-negative bacillus which belongs to the *Pseudomonadaceae* family (Wu and Li, 2015; Planet, 2018). This bacterium is an opportunistic pathogen capable of causing chronic lung infection, ventilator associated pneumonia (VAP) and chronic wounds infections, particularly in immunocompromised individuals (Moradali et al., 2017; Azam and Khan, 2019). In recent years, the incidence of multidrug resistant (MDR) *P. aeruginosa* is on the rise (CDC, 2019). Some MDR strains of *P. aeruginosa* were found to resist almost all antibiotics, including fluoroquinolones, cephalosporins, carbapenems, and aminoglycosides (Ventola, 2015; Nguyen et al., 2018; Horcajada et al., 2019; Pang et al., 2019). These strains are regarded as extensively drug-resistant (XDR) *P. aeruginosa*.

The emergence and dissemination of MDR and XDR *P. aeruginosa* have become a major threat to health care settings as they reduce the efficacy of

treatment by many conventional antibiotics. Consequently, limited effective antibiotics are available to cure the bacterial infections, and patients under these infections are always associated with high morbidity and mortality (Morgan, 2016; Karaiskos et al., 2019; World Health Organisation, 2021).

As described earlier, *P. aeruginosa* is the most reported bacterium associated with biofilm formation, and many MDR and XDR *P. aeruginosa* are increasingly reported as biofilm producers (Mulcahy et al., 2013). Despite its intrinsic resistant mechanisms such as multidrug efflux pump (Blair and Piddock, 2009; Nikaido and Takatsuka, 2009), biofilm formation represents another major strategy for *P. aeruginosa* to establish infections in hosts and survive through host immune responses and antibiotic treatments (Oluyombo et al., 2019). A study had shown that *P. aeruginosa* that are embedded within biofilm could resist antibiotic treatment at the capacity of up to 1000 times more than their planktonic counterparts (Lewis, 2001). Consequently, patients who are suffering from infections by this bacterium, particularly nosocomial infections, may require prolonged hospitalization with regular changes of antibiotic treatment. Hence, there is indeed an urgent need to develop alternative antibacterial agents which are able to overcome the issue of biofilm-mediated resistance by this bacterium.

2.3 Antibacterial Peptides (ABPs)

2.3.1 Overview of Antibacterial Peptides

Antibacterial peptides (ABPs) are short peptides or polypeptides (5-100 amino acids in length) which possess bacteriostatic or bactericidal effects. These

peptides were initially discovered from frogs' skin as part of the innate immune defence against aquatic microbial infection (Mar and Michl, 1976). Subsequently, many more ABPs were further discovered and characterized from different organisms. These include defensin from rabbit leukocytes (Hirsch, 1956), cathelicidins from snakes (Wang et al., 2008), lactoferrin from cow milk (Groves et al., 1965), cancrin from sea amphibian (Lu et al., 2008), cecropin from *Drosophila* (Vilcinskas, 2013), nisin and gramicidin from *Lactococcus lactis* (Cao et al., 2018), as well as human beta-defensin 2 (hBD-2), cathelicidin LL-37 and histatin from human (Wang et al., 2014). Apart from natural sources, a substantial number of ABPs are synthetically made or modified from the natural ABPs. As a result of active exploration and development, to date, a total of 2316 ABPs have been documented in the Antimicrobial Peptide Database (APD) (https://aps.unmc.edu/) since the discovery of the first ABP in 1976. Apart from these, increasing number of novel ABPs are constantly added to the list, reflecting the significant potential of these bioactive compounds.

A thorough review on the documented ABPs shows that these peptides display remarkable structural and functional diversity (Yasir et al., 2018). Despite this, majority of the peptides share certain common characteristics. Firstly, most of the ABPs are cationic in nature with a net positive charge ranging from +2 to +11 (Pasupuleti et al., 2011). This cationicity is attributed to the presence of large proportion of positively charged amino acids in the peptide sequence, such as lysine (Lys, K), histidine (His, H) and arginine (Arg, R) (Kumar et al., 2018). Secondly, many ABPs possess an amphipathic structure, in which the peptides

consist of both hydrophobic and hydrophilic regions at different proportions or ratio (Lei et al., 2019). Collectively, both cationicity and amphipathicity of ABPs significantly contribute to their antibacterial effects, which will be further described later in this chapter.

As mentioned earlier, the substantial increase in the number of new ABPs added to the APD clearly reflects the research interest among biomedical scientists on these bioactive peptides. In fact, accumulating data from many studies on ABPs have prompted the idea that ABPs are better alternative antibacterial agent as compared to conventional antibiotics due to several reasons (Erdem Büyükkiraz and Kesmen, 2021; Rima et al., 2021; Zhang et al., 2021).

2.3.2 Advantages of Antibacterial Peptides

As mentioned earlier, the ability of many ABPs to exert inhibitory or bactericidal effects towards many drug- or multidrug-resistant bacteria could be attributed to several unique characteristics of these peptides, enabling them to work in ways that are distinct form many conventional antibiotics. Firstly, many ABPs were found to exhibit rapid killing effects towards their target bacteria. Unlike the slow-acting antibiotics which require hours or days to eliminate their target bacteria (Fair and Tor, 2014), several ABPs were found to eliminate the bacteria in minutes. For example, WR12, a short ABP which is solely composed of arginine (R) and tryptophan (W), was shown to kill methicillin-resistant *Staphylococcus aureus* (MRSA) completely within 30 minutes, as compared to vancomycin which took more than 24 hours to achieve the same effect (Mohamed et al., 2016). The rapid killing kinetics is one of the major

advantages for these bioactive compounds, as the bacteria might be deprived of the time needed to acquire resistance towards the peptides.

Secondly, many ABPs were found active against a broad spectrum of bacteria. A single ABP might be able to inhibit or kill bacteria from different genus or Gram categories. As exemplified by LL-37 (Pasupuleti et al., 2011), cathelicidin (Kościuczuk et al., 2012), as well as synthetic ABPs such as T9W (Zhu et al., 2015), KW-13 (Liu et al., 2015), and HJH-1 (Wang et al., 2018), most of these ABPs were shown to exhibit antibacterial effects towards a broad spectrum of bacteria. Most importantly, a huge proportion of these peptides were able to inhibit or kill many drug- or multidrug-resistant bacteria. For instance, colistin, an ABP isolated from a soil bacterium named *Paenibacillus polymyxa*, was shown to exert strong bactericidal effect towards several MDR nosocomial pathogens such as MRSA, MDR *P. aeruginosa* and carbapenem-resistant *E. coli* (Falagas et al., 2005; Poirel et al., 2017; MacNair et al., 2018). Similarly, Omega76, a 20-amino acid synthetic ABP derived from phage display selection, was demonstrated to eliminate ESBL-producing *K. pneumoniae,* carbapenem-resistant *P. aeruginosa* and tigecycline-resistant *A. baumannii* (Nagarajan et al., 2019). These promising findings indicate that ABPs may possess certain distinct characteristics from the conventional antibiotics that enable them to overcome the issue of antibiotic resistance.

Next, ABPs may exert their antibacterial effects by means of more than one mechanism. As mentioned earlier, numerous ABPs were shown active against broad spectrum of bacterial species. This indicates that the actions of these

peptides are not bacterial-specific and might be mediated via non-ligand specific interaction. Conventional antibiotics, on the other hand, exert their bacteriostatic or bactericidal effects by targeting a specific bacterial structure or component which require high affinity interactions between the two entities before the antibacterial effect could be extended. For example, tetracyclines, a class of antibiotics which include tetracycline, doxycycline and tigecycline, inhibit bacterial protein synthesis. These antibiotics bind to 30S ribosomal subunit, which blocks the attachment of aminoacyl-tRNA to mRNA, thus impeding the subsequent steps of translation. Fluoroquinolones, on the other hand, bind to subunits of bacterial DNA topoisomerase and interfere with DNA replication. Another class of antibiotics, namely beta-lactams, represents the major class of antibiotics which encompass penicillin, cephalosporins, carbapenems and monobactams. These antibiotics primarily kill their target bacteria by binding to penicillin-binding proteins (PBPs), thus interfering with transpeptidation during synthesis of bacterial cell wall. However, the bacteriostatic or bactericidal actions of these antibiotics could be easily impeded due to structural alteration of these bacterial components, which would decrease or impair the binding affinity of these antibiotics to the components (Kapoor et al., 2017; Peterson and Kaur, 2018; Botelho et al., 2019). Consequently, these mutated bacteria are rendered insusceptible to these antibiotics. Therefore, the specific actions of many conventional antibiotics on these alterable bacterial structures or easily mutated molecular targets set a major drawback to these antibacterial agents.

Conversely, the ligand non-specific action of many ABPs may represent an advantage to these novel antibacterial agents. Several findings have reported that an ABP may simultaneously or sequentially act on multiple bacterial structural component or metabolic activities. For example, an ABP derived from human neutrophils, namely human neutrophil defensin-1 (HNP-1), was demonstrated for its ability to permeabilize both outer and inner membranes of *E. coli*, followed by inhibition of DNA, RNA replication and protein synthesis (Le et al., 2017). Besides, this ABP was also shown able to bind to Lipid II and impair biosynthesis of bacterial cell well (Schneider et al., 2010; Malanovic and Lohner, 2016; Le et al., 2017). A hybrid peptide named DM3 was reported to exert rapid bactericidal effect by inhibiting DNA replication and transcription (Le et al., 2016), as well as impairing genes that are associated with amnio acid biosynthesis (Bouza and Burillo, 2010). Other ABPs that were reported to inhibit multiple bacterial structures and metabolic activities are Buforin II, microcin J25, indolicidin and magainin, which were comprehensively reviewed by Le et al. (2017). As these ABPs are able to act on multiple bacterial target sites rather than one defined target, any means of mutation-mediated structural alteration to a single component by the bacteria may not completely compromise the peptide efficacy, as the peptides are able to act on other alternative target(s). In addition, it is unlikely for the bacteria to modify all the peptide-targeted components simultaneously as it is too metabolically costly to the bacteria (Fjell et al., 2011). Therefore, this special feature of ABPs could possibly explain their ability to cause rapid killing as well as the low likelihood of bacterial resistance.

2.3.3 Antibiofilm Effects of Antibacterial Peptides

Interestingly, besides the above-mentioned antibacterial mechanisms, antibiofilm effect was also reported for several ABPs such as LL-37 (Wang et al., 2014), SMAP-29 (Blower et al., 2015), Pleurocidin (Tao et al., 2011), Octominin (Thulshan Jayathilaka et al., 2021) and Octopromycin (Rajapaksha et al., 2021). Apart from their direct bacteriostatic or bactericidal effects towards planktonic bacteria, these ABPs were shown to be able to impair biofilm formation as well as eradicate or degrade the established biofilm via different mechanisms. These include blocking surface adhesion by planktonic bacteria, interfering with bacterial quorum sensing, down regulating genes that are associated with biofilm formation as well as disintegrating the matrix of the well-formed biofilm. As biofilm is regarded as one of the factors associated with antibiotic-resistance, these promising findings significantly strengthen the optimism on the use of ABPs to fight against multidrug resistant bacteria.

However, as compared to the increasing numbers of ABPs with direct antibacterial effects towards planktonic bacteria, only a handful of ABPs endowed with antibiofilm effects were discovered or developed (Chung and Khanum, 2017; Di Somma et al., 2020). Therefore, more exploratory studies are needed to expand the number of antibiofilm ABPs to address the issue of biofilm-associated drug resistance.

2.3.4 Synthetic Antibacterial Peptide PAM-5

PAM-5 is a synthetic peptide that consists of 15 amino acids with the peptide sequence of K-W-K-W-R-P-L-K-R-K-L-V-L-R-M. Using phage display

peptide selection approach, the original prototype of PAM-5 was obtained from a peptide that was bound to a surface ligand of *P. aeruginosa* ATCC 27853 at the highest affinity during the biopanning selection procedures as depicted in **Figure 2.2**. Subsequently, this prototype peptide was further modified by amino acid substitution to yield the final peptide with the above-mentioned sequence, with the cationicity and percentage of hydrophobicity of +7 and 46%, respectively (Tan, 2014, unpublished). This rational modification rendered PAM-5 with antibacterial features when it was shown to exhibit bactericidal effects towards a range of bacteria which encompasses *P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae*, *S.* Typhi and *S. flexneri* (Chan, 2016, unpublished). More interestingly, PAM-5 was also active against several drug- and multidrug-resistant bacteria which included MDR- *P. aeruginosa*, ESBL-producing *E. coli* and carbapenem-resistant *K. pneumoniae* (Chan, 2016, unpublished).

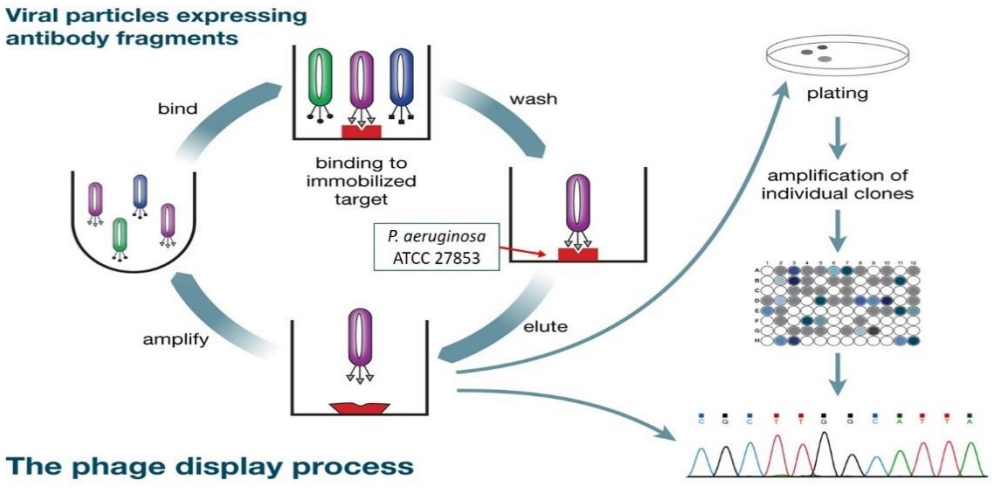


Figure 2.2: Selection of phage-displayed peptides via biopanning (Figure adapted from: New England Biolabs).

Nevertheless, the above-mentioned findings only represent the antibacterial potencies of PAM-5 towards planktonic bacteria, while the similar effect towards biofilm-embedded bacteria has not been elucidated. Since the ultimate objective of PAM-5 development is towards clinical application against bacterial infections, it is worth to study on its antibiofilm efficacy towards clinical isolates of *P. aeruginosa* which are commonly associated with multidrug resistance.

CHAPTER 3

MATERIALS AND METHODS

3.1 General Experimental Design

The antibiofilm effects of a synthetic ABP named PAM-5 on a clinical isolate of multidrug resistant (MDR) *Pseudomonas aeruginosa* 1894170 was screened via two microtiter plate-based assay, namely biofilm inhibition assay and biofilm eradication assay. For biofilm inhibition assay, MDR *P. aeruginosa* was treated with PAM-5 pre-coated in wells of a microtiter plate, followed by staining with crystal violet solution for biofilm mass quantification. For biofilm eradication assay, MDR *P. aeruginosa* was grown in microtiter plate for mature biofilm formation, followed by PAM-5 treatment. Subsequently, the amount of biofilm mass was quantified by crystal violet staining while the metabolic activity of the biofilm-embedded bacteria was measured using MTT stain. All the assays were independently triplicated to ensure data reproducibility.

3.2 Materials

3.2.1 Labware and Equipment

Refer to Appendix A.

3.2.2 Preparation of Buffers, Media, and Reagents

Refer to Appendix B.

3.2.3 Bacterial Strains

The bacterial strain used in this study was a clinical strain of *Pseudomonas aeruginosa* (*P. aeruginosa*) isolated from a patient in Gleneagles Medical Center, Penang. The bacterium was isolated from a contaminated granular setting on the patient's hand after two-weeks of hospitalization due to bacteraemia. Given a lab number as 1894170, this bacterium was a multidrug resistant strain which was found insusceptible to levofloxacin, moxifloxacin, doripenem, ertapenem, meropenem, ceftazidime, ceftriaxone, and cefepime. The bacterium was initially subcultured from the clinical isolation culture agar provided by the hospital laboratory to a Muller Hilton (MH) agar. For long term storage, the bacterium was grown in Luria Bertani (LB) broth, then preserved in 25% (v/v) glycerol solution and stored at -80°C. Before carrying out the antibiofilm assay, the bacterium was retrieved from the frozen glycerol stock and inoculated onto MH agar as the master culture plate via single colony streaking. After overnight incubation at 37°C, the master culture plate was stored at 4ºC for a maximum of seven days to ensure the freshness of the bacteria.

3.2.4 PAM-5 Synthesis

PAM-5 (KWKWRPLKRKLVLRM) was synthesized and purchased from Genscript (United States of America). The purity of the peptide was determined as 88.5% by the manufacturer via reverse-phase high performance liquid chromatography (HPLC). It was received in lyophilized form in a dry, tightly sealed tube with silica gel. The peptide was stored at -20°C.

3.2.5 PAM-5 Preparation

Prior to dissolving the peptide, it was equilibrated to room temperature for about half an hour. In order to prepare 500 µL of peptide stock solution at the concentration of 1,024 µg/mL, 1,024 µg of PAM-5 was weighted and dissolved in 100 µL of degassed, filtered-sterilized distilled water. The solubilized peptide was then topped up with 900 µL of degassed, filtered-sterilized phosphate-buffered saline (PBS, pH 7.4). Subsequently, the peptide stock solution at the concentration of 1,024 µg/ml was subjected to two-fold serial dilution to yield a range of peptide concentrations from 1,024 µg/mL to 8 µg/mL. According to the manufacturer recommendation, the diluted peptide solution was stored in silica vials at 4ºC and was used within seven days to ensure peptide efficacy. The overall method in PAM-5 preparation and dilution is shown in **Figure 3.1**.

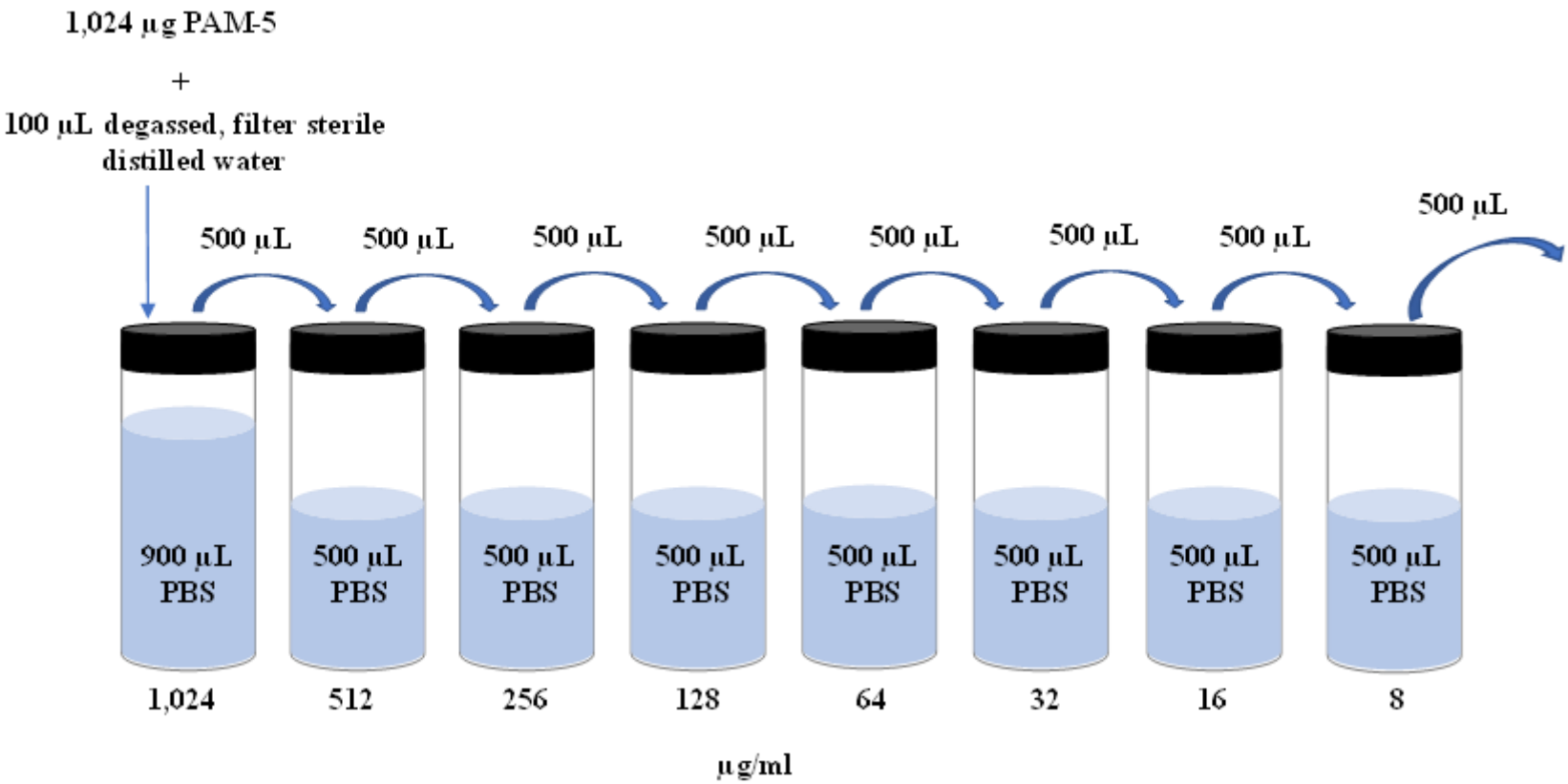


Figure 3.1: Illustration of PAM-5 dissolution and two-fold serial dilution to yield a series of peptide concentrations from 1,024 µg/mL to 8 µg/mL.

3.3 Methodology

3.3.1 Optimization of Biofilm Growth

Before carrying out the antibiofilm assays, the formation of biofilm was optimized by comparing the growth of biofilm in three different culture media, which were Muller Hilton Broth (MHB), Tryptic Soy Broth (TSB), and Brain Heart Infusion Broth (BHIB). Firstly, an overnight culture of the MDR *P. aeruginosa* was prepared under aseptic condition by inoculating 1 to 2 bacterial colonies from the master culture agar into 10 mL of each culture medium. These three broth cultures were incubated for 16 to 20 hours at 37°C with agitation of 200 rpm. After overnight incubation, 200 µL of the overnight culture was added to 19.8 mL of each respective fresh culture medium and mixed well. Subsequently, 100 µL of the diluted culture was filled into each well for a total of seven wells of a 96 well microtiter plate. In a parallel row, four wells were filled up with 100 µL of the respective fresh medium, which served as the sterility test. The layout of the microplate setup as described above is depicted in **Figure 3.2**. The microtiter plate was then incubated for 24 hours at 37 °C.

On the next day, the amount of biofilm formed in different broth media was determined by crystal violet (CV) assay. The planktonic cells in each well were discarded and the wells were rinsed twice with 200 µL of sterile PBS (pH 7.4). Then, the wells were stained with 100 µL of 0.5% (w/v) CV solution and incubated for 30 minutes at room temperature. Following that, excess CV stain from each well was removed, and the wells were washed thrice with 400 µL of sterile distilled water (dH_2O). Upon washing, the microtiter plate was blotted vigorously on paper towel to remove any residue water, followed by drying the

plate at 37°C for another 30 minutes. After drying, 100 µL of 33% (v/v) acetic acid was added into each well to solubilize the CV stain. The absorbance of solubilized CV was measured by a microplate reader (BMG Labtech FLUOstar Omega) at 595 nm to quantify and compare the amount of biofilms formed in different culture media. The culture medium which produced the highest average absorbance reading of CV was chosen as the medium for the subsequent biofilm inhibition and biofilm eradication assays, which will be described later.

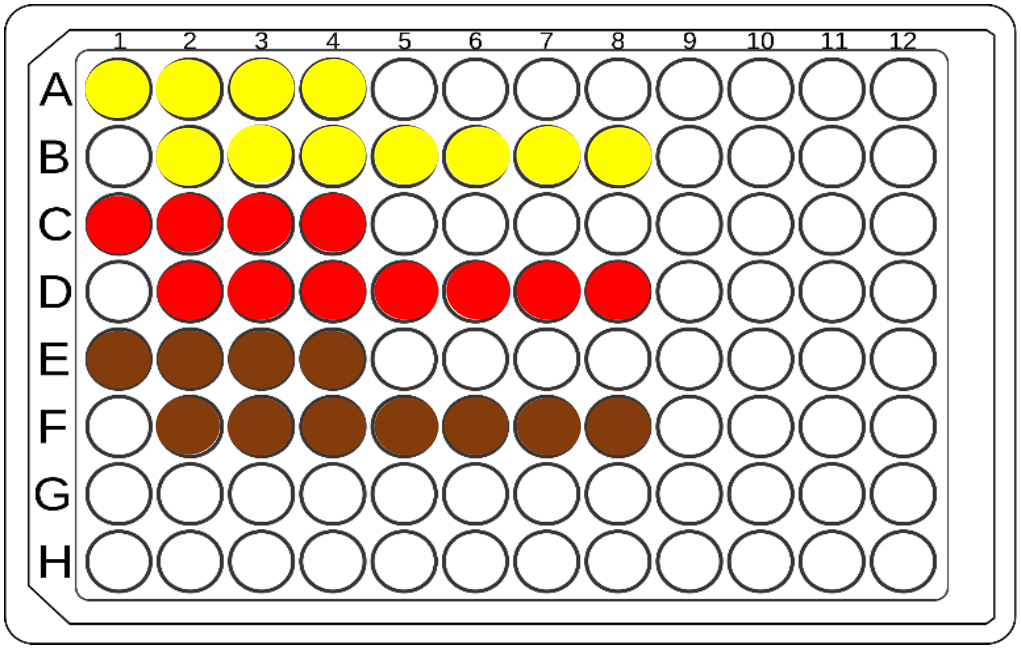


Figure 3.2: Layout of microtiter plate set up for optimization of biofilm growth using MHB (yellow colour), TSB (red colour), BHIB (brown colour). Well B2 to B8, D2 to D8 and F2 to F8 were filled with diluted culture grow in MHB, TSB and BHIB, respectively. Well A1 to A4, C1 to C4 and E1 to E4 were filled with the respective fresh medium, which served as the sterility control.

3.3.2 Biofilm Inhibition Assay

Based on the optimization which its result will be further reported in **Section 4.2**, BHI was chosen as the medium for biofilm growth. Using this broth, overnight liquid bacterial culture was prepared as described in **Section 3.3.1**. On the next day, 100 µL of PAM-5 of increasing concentrations (8 µg/mL to 1,024 µg/mL) was filled into wells of a 96 well-microtiter plate (each well for one concentration), followed by incubating the plate at 37°C for at least 4 hours. Then, the overnight culture was diluted into fresh BHI broth at the same dilution factor as described earlier. Subsequently, 100 µL of the diluted culture was added into each well which has been pre-coated with the peptide. On the other hand, 100 µL of bacterial suspension along with 100 µL of PBS were also added into separate wells without peptide coating, where these wells served as the negative control. Sterility control was also set up by filling up a few wells with fresh BHI broth. The layout of the microtiter plate setup as described above is depicted in **Figure 3.3**. Then, the microtiter plate was incubated for 24 hours at 37°C.

On the next day, the biofilm inhibition effect of PAM-5 was assessed using CV assay. The suspension in the wells was carefully removed with multichannel pipetter, and the wells were rinsed twice with 200 µL of sterile PBS (pH 7.4) to remove any residue planktonic bacteria and medium. Then, 100 µL of 0.5% (w/v) CV solution was added into each well and the plate was incubated in dark for 30 minutes at room temperature. After that, the CV solution in the wells was removed carefully by pipette aspiration, and the wells were washed thrice with 400 uL of dH_2O. After washing, the microtiter plate was blotted on paper towels

to remove the excess cells and dye completely. The microtiter plate was then dried in a 37°C incubator for 30 minutes. After that, 100 µL of 33 % (v/v) of acetic acid was added into each well to solubilize the CV stain. The absorbance of solubilized crystal violet in the wells was then measured by a microplate reader (BMG Labtech FLUOstar Omega) at the wavelength of 595 nm.

The percentage of biofilm inhibition was calculated using the formula as shown below.

Percentage of biofilm inhibition = $100 - [(\frac{A_{595\ nm}\ \text{treatment sample} - \text{Blank}}{A_{595\ nm}\ \text{negative control} - \text{Blank}}) \times 100]$

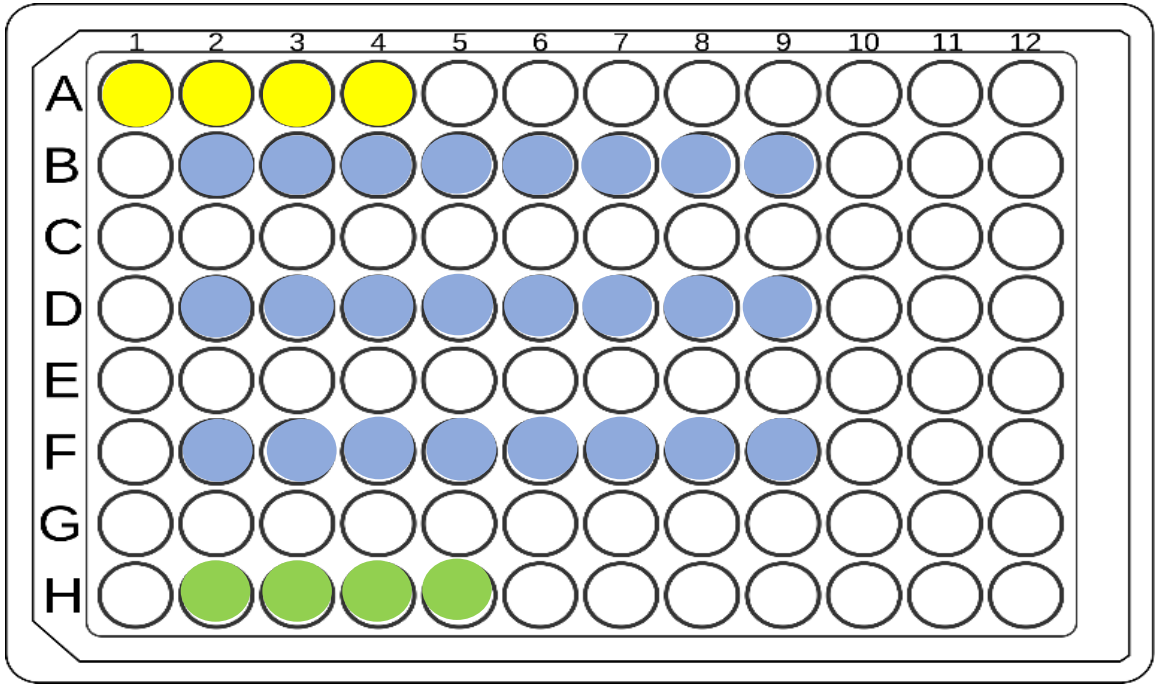


Figure 3.3: Layout of microtiter plate setup for biofilm inhibition assay. Wells A1 to A4 (yellow colour) which served as the sterility test were filled with fresh BHIB. Well B2 to B9, D2 to D9 and F2 to F9 (blue colour) were pre-coated with PAM-5 at concentrations ranging from 4 µg/mL to 512 µg/mL, respectively, followed by addition of MDR *P. aeruginosa* suspension. For negative control, H2 to H5 (green colour) were filled with the bacteria and PBS.

3.3.3 Determination of Planktonic Viability After Peptide Treatment

For the biofilm inhibition assay as described in **Section 3.3.2**, after overnight treatment of the bacteria and prior to CV staining, the turbidity of the content in each well was first visually examined and recorded. Then, 10 µL of the bacterial suspension from each well was inoculated and spread on MH agar, and the agar plates were incubated overnight at 37°C. Next, 100 µL of bacterial suspension from the remaining contents in the wells was aspirated and added into 900 µL of PBS in a microcentrifuge tube, followed by 10-fold serial dilution up to 10^{-5}. Next, 10 µL of aliquot from the 10^{-5}–diluted bacteria were inoculated onto MH agar plate by using spread plate method. The bacterial-inoculated agar plates were incubated overnight at 37°C. On the next day, the colonies on each MH agar plate were counted and the bacteria titer (CFU/mL) for each plate was calculated by using the formula as shown below.

$$\text{Bacteria titer (CFU/mL)} = \frac{\text{Number of colonies x Total dilution factor}}{\text{volume of culture plated (mL)}}$$

The protocols for the PAM-5-treated and non-treated bacteria as described above are depicted in **Figure 3.4**.

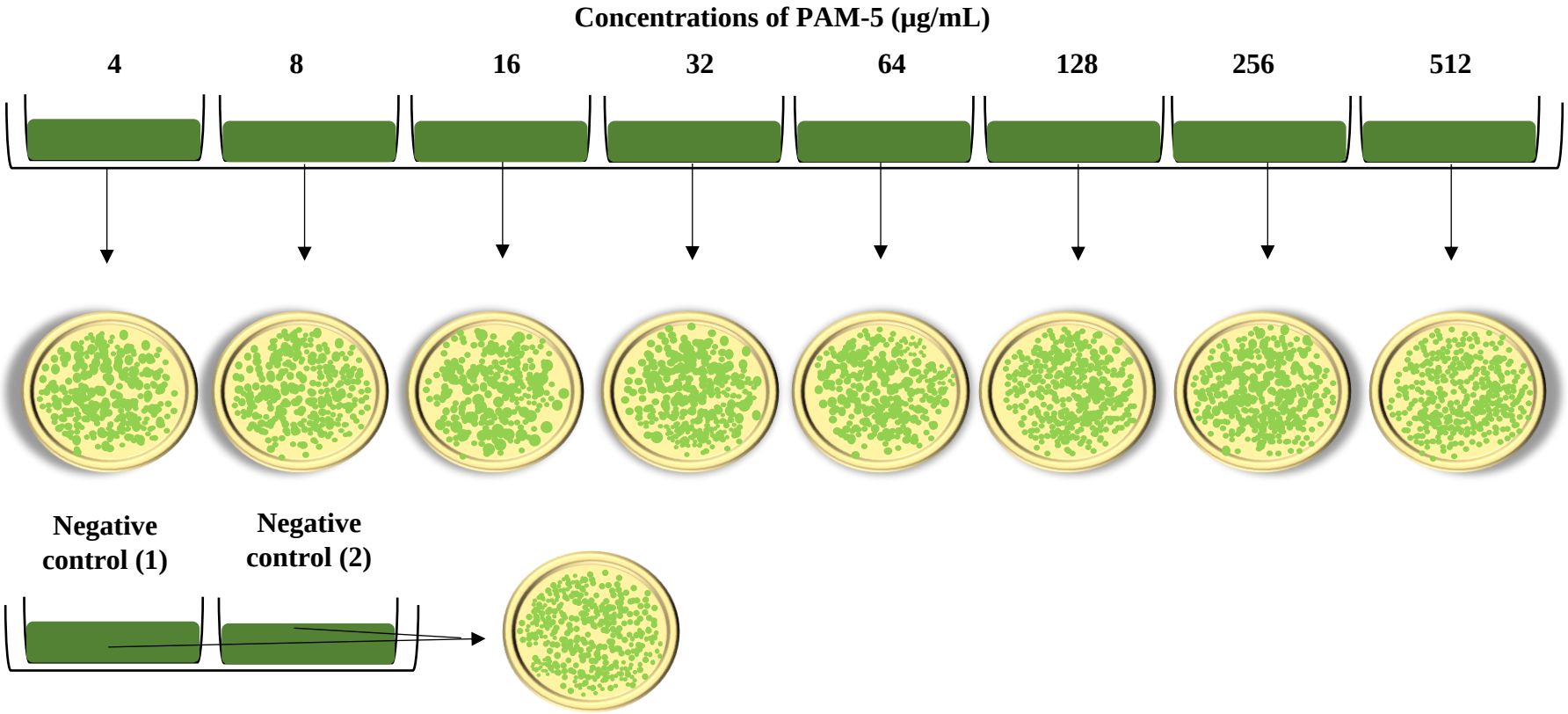


Figure 3.4: Illustration of the protocols in plating the planktonic bacterial after PAM-5 treatment to determine the bacterial viability.

3.3.4 Screening for Eradication Effect of PAM-5 on Matured Biofilm

By using BHI broth, overnight liquid culture of *P. aeruginosa* was prepared according to the protocol as describe in **Section 3.3.1**. After overnight incubation, 200 µl of the overnight bacterial culture was added into 19.8 mL of fresh BHI broth and mixed well. Then, 100 µL of the diluted culture was dispensed into wells of a 96 well-microtiter plate, which would be treated with PAM-5. A separate row of 4 wells was set up as negative control by filling 100 µL of the diluted bacterial suspension into the wells. Meanwhile, 100 µL of BHI broth was added into separate wells as the sterility control. The microtiter plate setup as described above is depicted in **Figure 3.5**. After loading, the microtiter plate was incubated for 48 hours at 37°C to form mature biofilm.

After the incubation, the planktonic cells were carefully removed from the wells via pipette aspiration, followed by washing the wells twice with 400 uL of sterile PBS (pH 7.4) to remove any residue planktonic bacteria. Subsequently, 100 µl of PAM-5 at concentrations ranging from 8 µg/mL to 512 µg/mL was added into the wells which were pre-grown with bacterial mature biofilm, in which each well was treated with one peptide concentration. On the other hand, the wells which served as the negative control were filled with 100 µL of PBS. After that, the microtiter plate was incubated for 24 h at 37°C.

On the next day, the suspension in the wells was carefully discarded by pipette aspiration, followed by rinsing the wells twice with 200 µL of PBS. After that, each well was filled with 100 µL of 0.5% (w/v) CV solution, and the microtiter plate was incubated in dark for 30 minutes at room temperature. Next, the excess

CV staining solution was removed carefully by pipette aspiration. The wells were washed thrice with 400 uL of dH_2O. After washing, the microtiter plate was blotted on paper towels to remove any residue solution, followed by drying the plate in 37°C incubator for 30 minutes. After drying the wells completely, 100 µL of 33% (v/v) acetic acid was added into each well to solubilize the CV stain in the wells. Thereafter, the absorbance of the solubilized CV stain in the wells was measured by microplate reader (BMG Labtech FLUOstar Omega) at the wavelength of 595 nm.

The percentage of mature biofilm eradication was calculated using the formula as shown below.

Percentage of biofilm eradication =

$$100 - \left[\left(\frac{A_{595\,nm}\ \text{treatment sample} - \text{Blank}}{A_{595\,nm}\ \text{negative control} - \text{Blank}}\right) \text{x } 100\right]$$

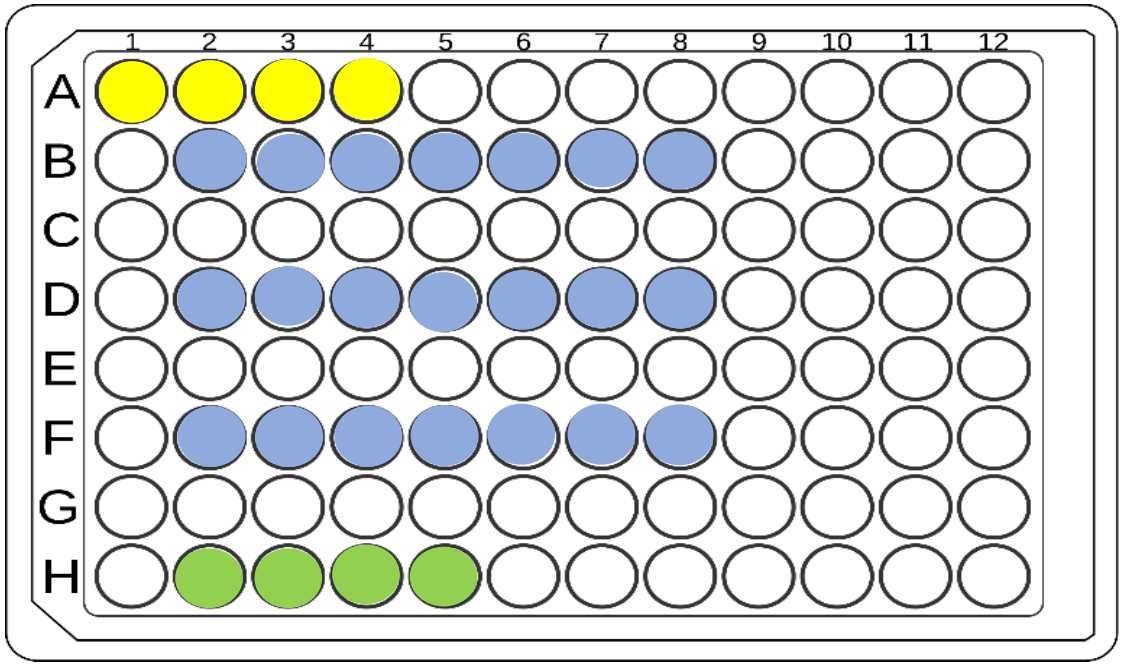


Figure 3.5: Layout of microtiter plate setting for mature biofilm eradication assay. The bacterial suspension was incubated in the wells for 48 hours to establish matured biofilm before PAM-5 treatment. After incubation, Well B2 to B8, D2 to D8 and F2 to F8 (blue colour) were treated with increasing concentration of PAM-5 ranging from 8 µg/mL to 512 µg/mL, respectively. Well H2 to H5 (green colour) which served as the negative control were loaded with PBS. As for sterility test, Well A1 to A4 (yellow colour) were filled with fresh BHIB for sterility control.

3.3.5 Evaluation of Biofilm Metabolic Activity

The ability of PAM-5 to disperse the mature biofilm and kill the biofilm-embeded bacteria was evaluated using MTT assay. The setup for this assay was similar to the protocols as described in **Section 3.3.4**, and the layout of the setup is shown in **Figure 3.6**. After 48 hours of incubation, the planktonic bacteria were removed and the wells were washed thrice with 400 µL of PBS. Subsequently, 100 µl of PAM-5 solution along with 50 µL of BHI broth were addded into the treatment sample wells. As for the negative control, biofilm-coated wells were added with 100 µL of sterile PBS and 50 µL of BHI broth. Sterility control was also set up by filling up a few wells with 50 µL of fresh BHI broth. Then, the microtiter plate was incubated for 24 hours at 37°C.

On the next day, the suspension in the wells were carefully removed by pipette aspiration and the wells were rinsed twice with 200 µL of sterile PBS. Then, 100 µL of freshly prepared 0.3% (w/v) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution and 30 µL of BHI broth were added into the wells. The microtiter plate was incubated for 3 hours in dark at 37°C. After that, the excess MTT solution was carefuly removed by pipette aspiration. Finally, 100 µL of dimethyl sulfoxide (DMSO) was added to each well to solubilize the purple formazan that was formed from the MTT stain, and the absorbance of the formazan in the wells was measured by microplate reader (BMG Labtech FLUOstar Omega) at the wavelength of 570 nm.

The percentage of reduction in the biofilm metabolic activity was calculated by the formula as shown below.

Percentage of metabolic activity reduction =

$$100 - \left[\left(\frac{A_{570\ nm}\ \text{treatment sample} - \text{Blank}}{A_{570\ nm}\ \text{negative control} - \text{Blank}}\right) \text{x } 100\right]$$

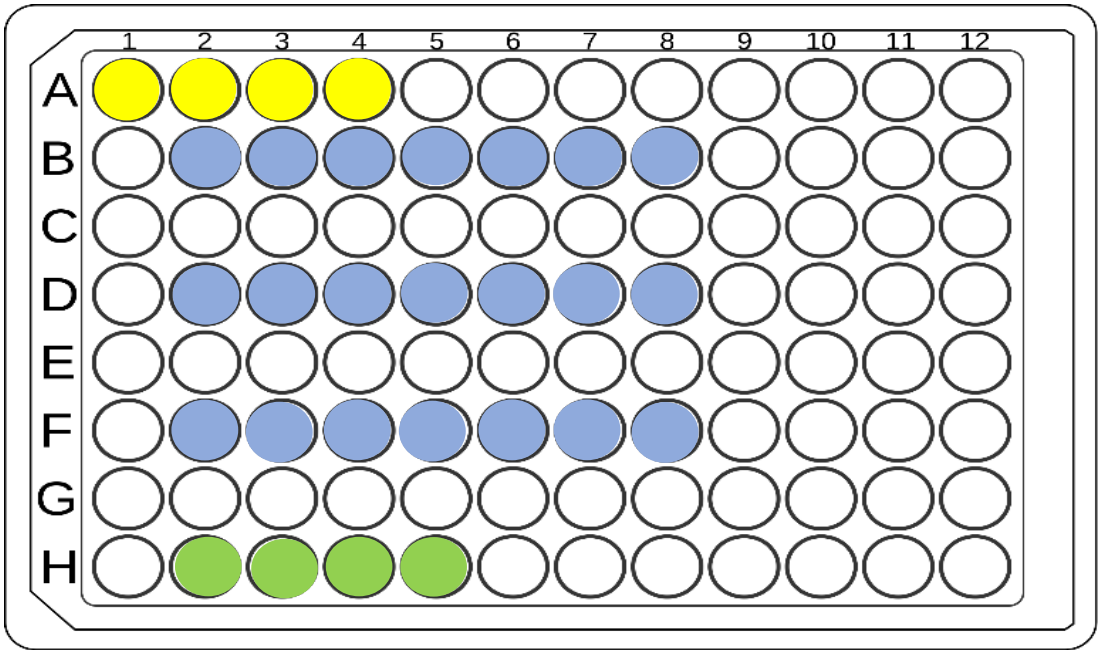


Figure 3.6: Layout of microtiter plate set up for MTT assay. Bacterial suspension was incubated in the wells for 48 hours to establish matured biofilm before PAM-5 treatment. After incubation, Well B2 to B8, D2 to D8 and F2 to F8 (blue colour) were added with increasing concentrations of PAM-5 ranging from 8 µg/mL to 512 µg/mL, respectively; while Well H2 to H5 (green colour) which served as the negative control, were added with PBS. Fresh BHI broth was filled in Well A1 to A4 (yellow colour) for sterility test.

3.4 Statistical Analysis

Three independent experiments were carried out for biofilm inhibition, biofilm eradication, and biofilm metabolic activity. Each independent experiment was performed in triplicates. The mean values and standard deviations for the degree of biofilm inhibition, biofilm eradication and biofilm metabolic activity reduction were calculated. The statistical differences between the peptide-treated and non-treated biofilms were analyzed using one-way ANOVA test, and all values were considered significantly different if the p value was less than 0.05 ($p < 0.05$).

CHAPTER 4

RESULTS

4.1 Screening for Antibiofilm Effect of PAM-5 on Clinical Isolate *P. aeruginosa*

Before conducting the antibiofilm assays, different growth medium were tested to optimize the biofilm formation by the MDR *P. aeruginosa* 1894170. Subsequently, the antibiofilm effect of PAM-5 was screened through three different assays, namely biofilm formation inhibition assay, matured biofilm dispersal assay, and biofilm metabolic activity assay. The ability of PAM-5 to inhibit biofilm formation and disperse mature biofilm was evaluated by comparing the absorbance of crystal violet (CV) staining between PAM-5 treated bacteria or mature biofilm and negative control. On the other hand, the ability of PAM-5 to reduce the metabolic activity of biofilm embedded bacterial was determined by comparing the absorbance of formazan formed in peptide-treated and non-treated mature biofilm. For quantification of the antibiofilm effect of PAM-5, the biofilm inhibitory percentage, biofilm eradication percentage, and biofilm metabolic activity reduction percentage were evaluated.

4.2 Optimization of Biofilm Growth of Multidrug Resistant (MDR) *P. aeruginosa* 1894170

The optimization of biofilm growth was accessed using CV biomass staining method. MDR *P. aeruginosa* 1894170 was grown for 24 hours in three different media broth which were Muller Hilton Broth (MHB), Tryptic Soy Broth (TSB), and Brain Heart Infusion Broth (BHIB). The broth that promoted the greatest formation of biofilm was selected as the media broth for the subsequent assays.

The amount of biofilm formed by the clinical isolate in different broth media can be estimated and compared by CV staining followed by quantification through absorbance measurement. After solubilizing the CV that was remained in the wells as described in **Section 3.3.1**, the intensities of CV stains retained by the were visually inspected and compared.

As shown in **Figure 4.1**, highest purple colour intensity of solubilized CV stains were seen in wells from C1 to C7, which were filled with *P. aeruginosa* grown by BHIB, as compared to Well A1 to A7 and B1 to B7, which were loaded with the bacteria grown by MHB and TSB, respectively.

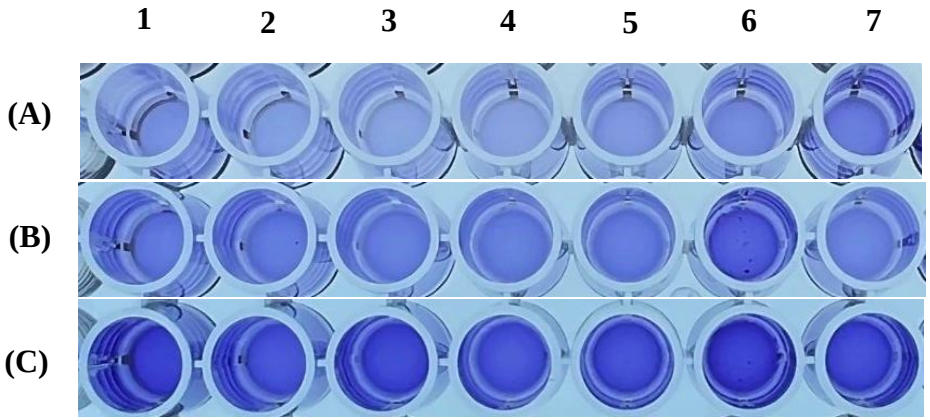


Figure 4.1: Gross view of solubilized crystal violet (CV) in the wells pre-grown with biofilm from *P. aeruginosa* after 24 hours cultured with: (A) MHB; (B) TSB; (C) BHIB.

Subsequently, the amount of biofilm mass in the wells was quantified by measuring the absorbance of solubilized CV stain. The mean absorbance of the biofilm CV solution from these wells were plotted and presented in a graph as shown in **Figure 4.2**. Based on the figure, among the three media, biofilm grown by BHIB produced the highest CV absorbance as compared to the biofilms grown by MHB and TSB. The mean CV absorbance produced by BHIB-grown biofilm was approximately 0.662, while the mean CV absorbance by MHB- and TSB-grown biofilm were 0.119 and 0.121, respectively. In other words, the mean CV absorbance from the BHIB-grown biofilm was significantly higher than the mean CV absorbance from the biofilm grown by the other two media ($p < 0.05$), in which the former was approximately 6 times higher than the latter two. Therefore, BHI broth was selected as the media broth for the antibiofilm assays.

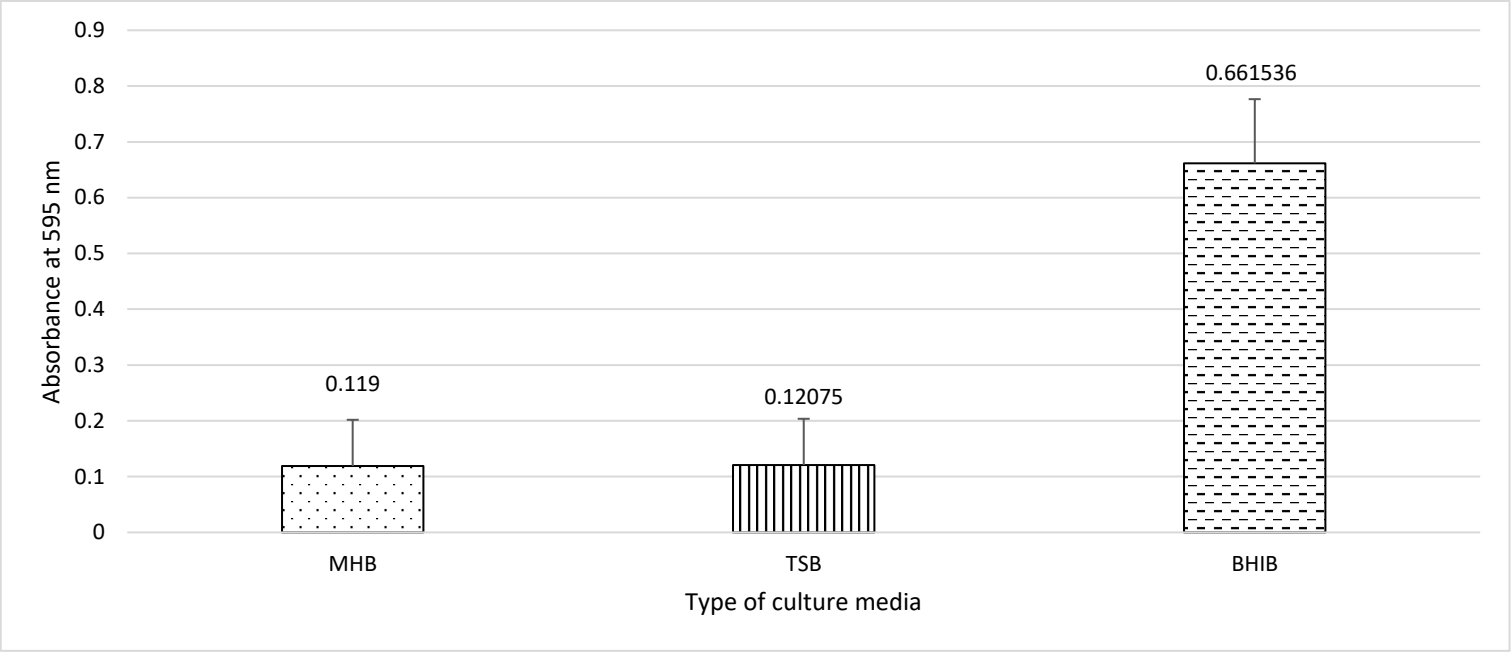


Figure 4.2: Absorbance of crystal violet staining of biofilm produced by MDR *P. aeruginosa* 1894170 grown in different media broth which were Mueller Hilton Broth (MHB), Tryptic Soy Broth (TSB) and Brain Heart Infusion Broth (BHIB).

4.3 Inhibition of Biofilm Formation

4.3.1 Effect of PAM-5 on Biofilm Formation by MDR *P. aeruginosa* 1894170

Triplicated biofilm inhibition assays revealed that PAM-5 was able to inhibit biofilm formation by MDR *P. aeruginosa* 1894170. **Figure 4.3** depicts the wells of a microtiter plate which were set up for this biofilm inhibition assay, in which Wells A1 to A8 were pre-coated with PAM-5 at concentrations ranging from 4 µg/mL to 512 µg/mL, followed by MDR *P. aeruginosa* loading. On the other hand, Wells B1 to B4 were filled with the bacterial suspension without peptide pre-coating. Clearly demonstrated from the figure, the CV intensities from the former were generally lower than the latter, which served as the negative control.

Moreover, there was an overall decreasing trend of CV intensities from Well A1 to Well A8. CV intensities from Wells A1 to A4 which were pre-coated with lower concentrations of PAM-5 (4 µg/mL to 32 µg/mL) were relatively higher than the wells from A5 to A8 (64 µg/mL to 512 µg/mL) after overnight incubation with the bacterium.

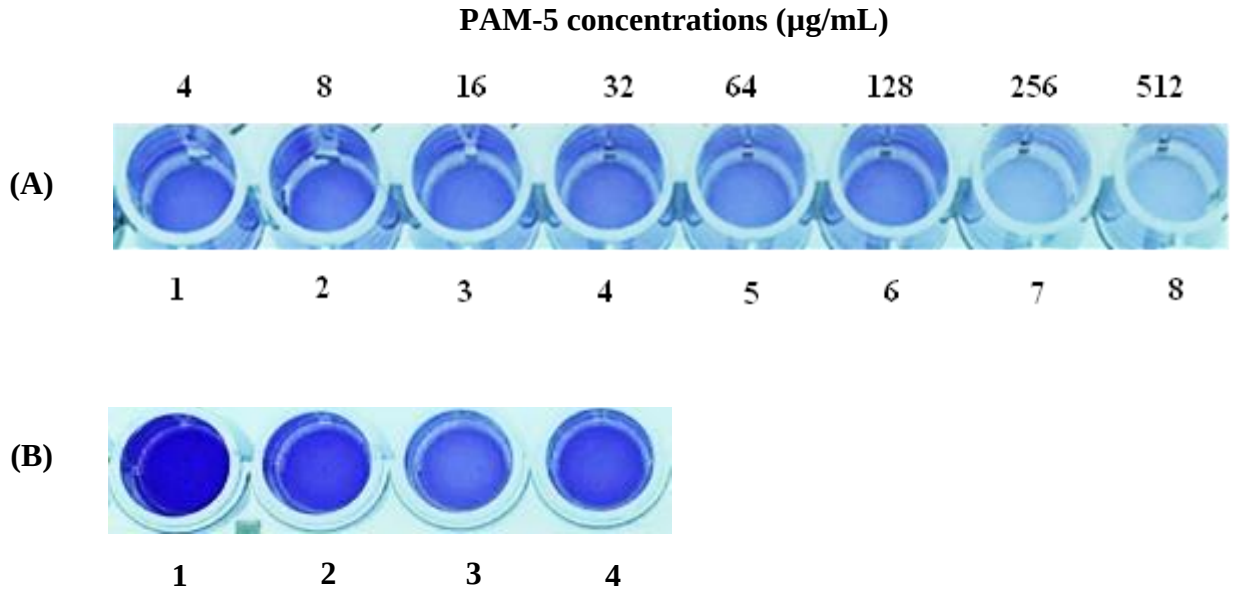


Figure 4.3: Crystal violet (CV) staining of MDR *P. aeruginosa* biomass after overnight incubating the bacterium (A) in wells pre-coated with PAM-5 at increasing concentrations from 4 µg/mL to 512 µg/mL (A1 to A8) and (B) in uncoated wells (B1 to B4) which served as the negative control.

The absorbances of the CV stains in the wells were measured by a microtiter plate reader, and the mean absorbances from the triplicate assays are presented in **Figure 4.4**. Clearly demonstrated from the figure, the absorbances of untreated bacteria which served as the negative control (N) were averagely higher at 0.685. In contrast, absorbance readings from all the wells filled with PAM-5-treated bacteria were significantly lower than the negative control ($p < 0.05$), regardless of the peptide concentrations. Even though the average CV absorbance was high for the bacteria treated with 4 µg/mL of PAM-5, it was still significantly lower than the negative control ($p < 0.05$). The absorbance readings produced by these peptide-treated bacteria were inversely proportional to the peptide concentrations, and the decreasing trend of CV absorbances as shown in this figure is even more apparent as compared to the one by visual observation as reported earlier. At the peptide concentration of 8 µg/mL, the CV absorbance from the biomass was almost 50% lesser than the negative control.

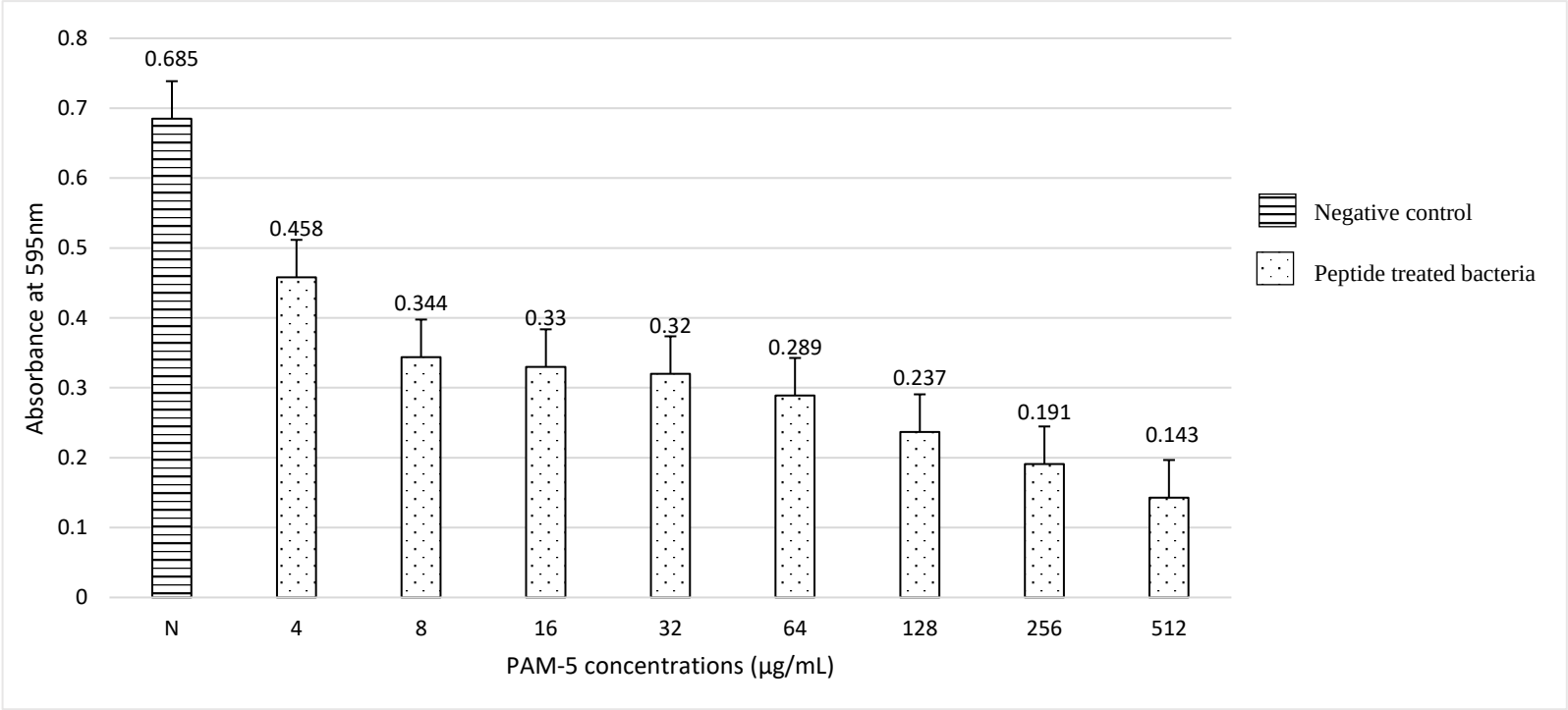


Figure 4.4: Absorbance of crystal violet staining of MDR *P. aeruginosa* 1894170 treated with pre-coated peptide at concentrations ranging from 4 µg/mL to 512 µg/mL (dotted). 'N' represents the absorbance of untreated bacteria which served as the negative control (light horizontal line). Differences between the peptide-treated group and the control were statistically significant ($p < 0.05$).

Using the equation as stated in **Section 3.3.2**, the percentages of biofilm inhibition were calculated, and the data is presented in **Figure 4.5**. Clearly seen from the graph, PAM-5 was shown to be able to inhibit biofilm formation by MDR *P. aeruginosa* 1894170 in a dose-dependent manner. At the concentration of 8 µg/mL, the peptide was able to inhibit almost 50% of the biofilm formation as compared to the untreated bacteria. Nevertheless, complete inhibition of biofilm formation was not achieved by the highest peptide concentration (512 µg/mL) as tested in this study. However, this peptide concentration could reduce the biofilm formation up to almost 80%.

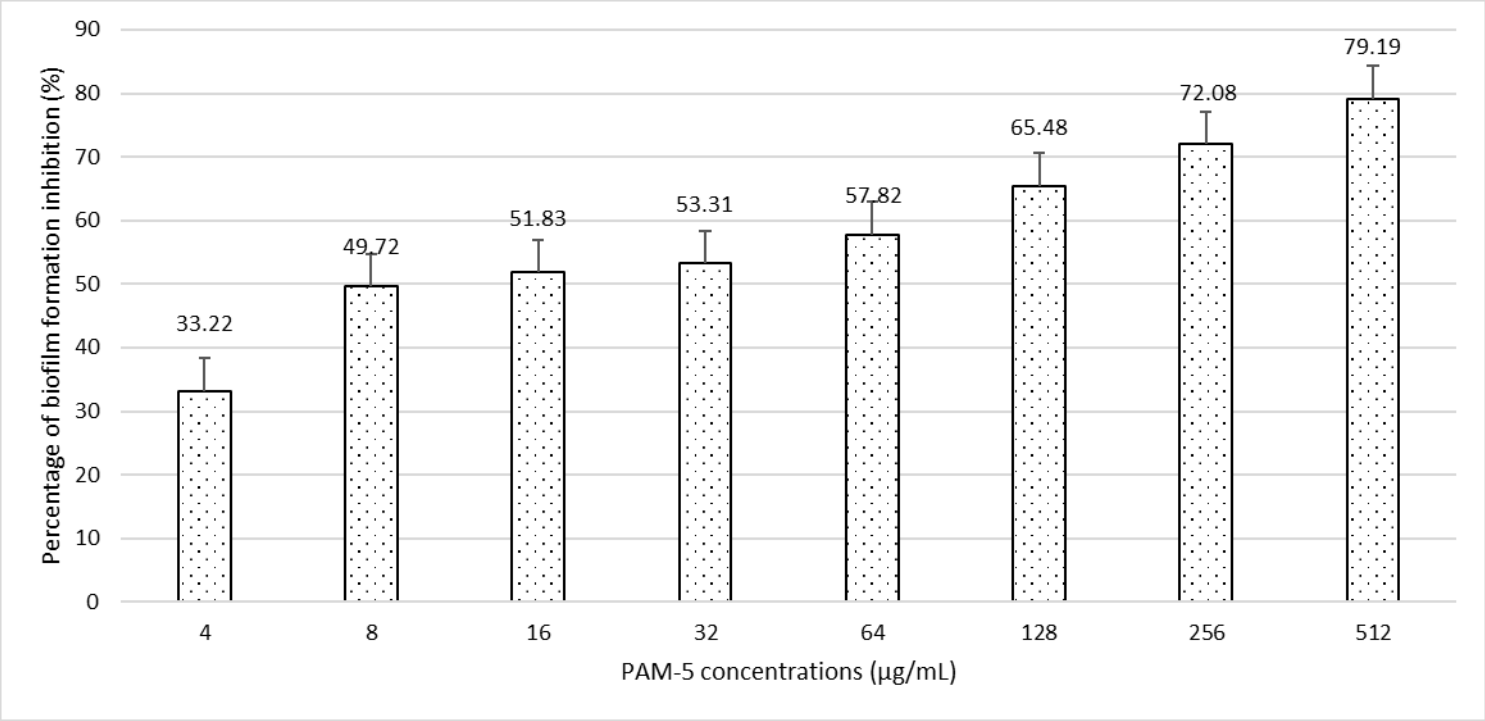


Figure 4.5: Percentage of inhibition towards biofilm formation by MDR *P. aeruginosa* 1894170 treated with PAM-5 at increasing concentrations ranging from 4 µg/mL to 512 µg/mL.

4.3.2 Determination of Planktonic Bacterial Viability After Peptide Treatment

In order to determine that the biofilm inhibition was solely due to inhibition of biofilm formation events but not by killing or reducing the planktonic bacteria by the peptide, the bacterial viability and titer after overnight peptide treatment was determined by using microbroth dilution assay and spread plate method. After performing the spread plate inoculation as described in **Section 3.3.3**, the appearance of the bacterial growth on the plates was recorded (**Figure 4.6**), and the titers for the bacteria treated with all peptide concentrations were determined based on the colonies on the titer plates as shown in **Figure 4.7**.

With reference to **Figure 4.6**, visual observation on the gross appearance of bacteria-inoculated media plates showed that the bacterial growth was not obviously impaired. Despite treatment with PAM-5 at concentrations ranging from 4 µg/mL to 512 µg/mL, MDR *P. aeruginosa* 1894170 was able to grow heavily on all the media plates (Plates A1 to A8) at the similar density as compared to the untreated bacteria (Plates B1 and B2). In addition, no observable difference was seen for the colony intensities between the bacteria treated with lower and higher concentrations of PAM-5.

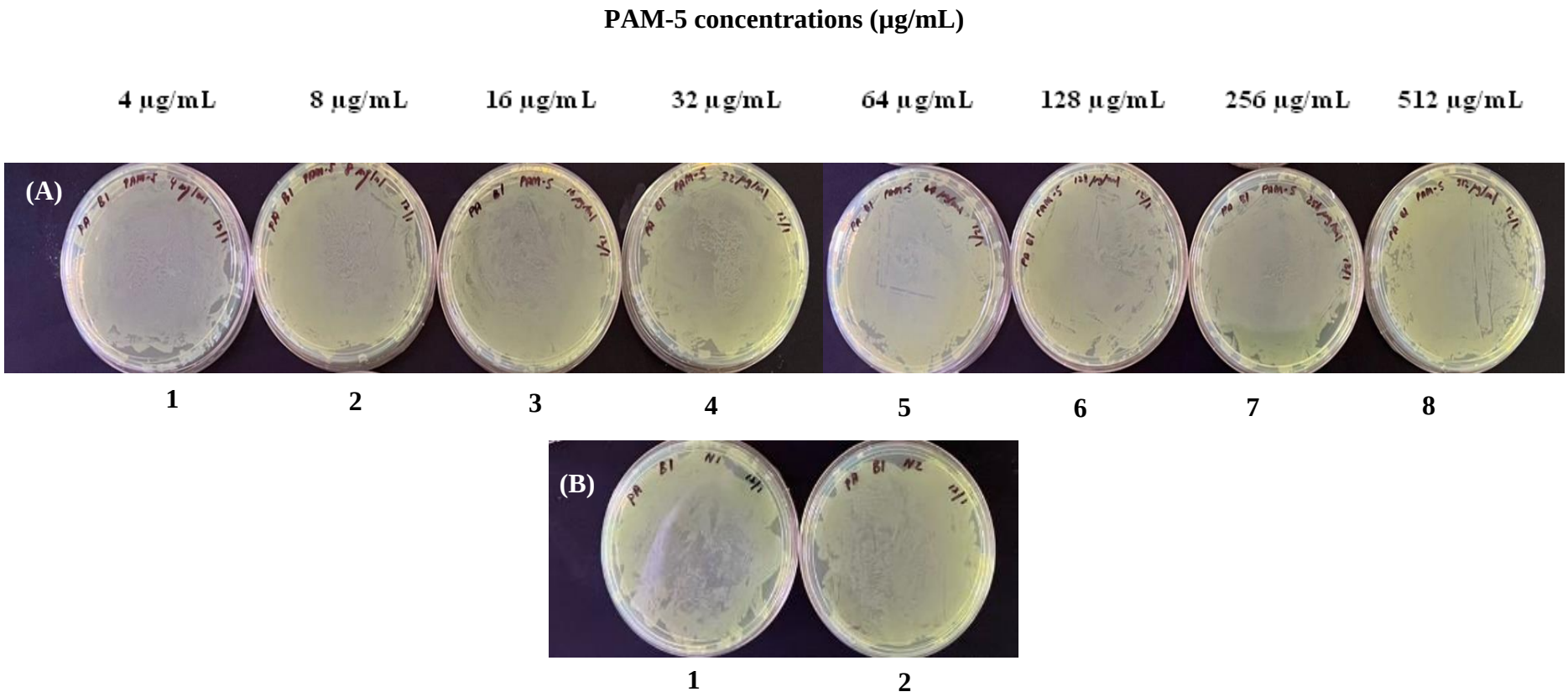


Figure 4.6: Gross view on the growth of MDR *P. aeruginosa* 1894170. (A) MH agar inoculated with *P. aeruginosa* suspension after treatment with PAM-5 at increasing concentrations from 4 µg/mL to 512 µg/mL. (B) MH agar inoculated with untreated bacterial suspension which served as the negative control.

Nevertheless, the gross view of the bacterial growth as reported earlier may not reveal the fine difference in titer between the treated and untreated bacteria. Therefore, the treated bacterial suspension was not only subjected to direct inoculation for gross view, but also for titer determination via microbroth dilution assay as described in **Section 3.3.3**.

As depicted in **Figure 4.7**, the aliquots derived from the bacterial suspensions which were treated with all peptide concentrations yielded almost similar bacterial titers. Although there were slight differences in the exact colony forming units (CFUs) between the bacteria treated with different peptide concentrations, but all of them were able to grow up to the titer of 8.0 Log_{10} CFU/mL, which were similar to the titer of untreated bacteria. However, the bacteria treated with the highest peptide concentration (512 µg/mL), produced slightly lower bacterial titer.

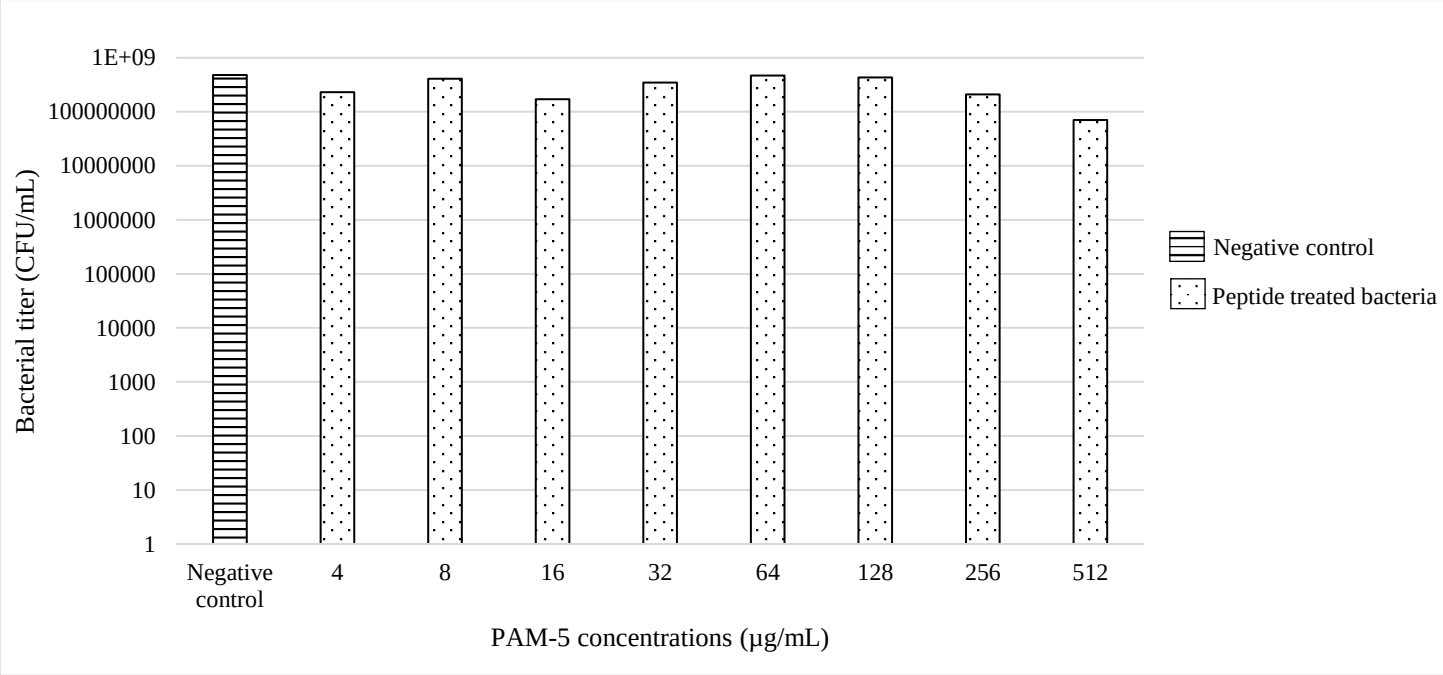


Figure 4.7: Titer (CFU/mL) of bacterial suspension treated with PAM-5 at concentrations ranging from 4 µg/mL to 512 µg/mL (dotted), and the untreated bacterial suspension which served as the negative control (light horizontal strips).

4.4 Effects of PAM-5 on Mature Biofilm Produced by MDR *P. aeruginosa* 1894170

In order to establish mature biofilm, MDR *P. aeruginosa* was incubated in the wells of microtiter plate for 48 hours prior to treatment with PAM-5. Following the treatment with PAM-5, the ability of the peptide to disperse mature biofilm was assessed by two different methods. Using one of the microtiter plates pre-grown with the biofilm, the amount of biofilm that still adhered to the wells after peptide treatment was quantified by CV staining and absorbance measurement. On the other hand, the second plate was subjected to MTT staining to quantify the amount of viable bacteria embedded within the biofilm after peptide treatment.

4.4.1 Biofilm Dispersal by PAM-5

Using the similar CV staining as described earlier, the amount of biofilm that was still adhered to the wells after PAM-5 treatment was quantified. With reference to **Figure 4.8**, which depicts one of the triplicated biofilm dispersal assays, the CV intensities of PAM-5-treated mature biofilms in Well A1 to Well A7 were obviously lower than the untreated biofilm (Wells B1 to B4) via visual comparison. The latter, which served as the negative control, produced relatively much higher CV intensities after the staining, indicating the high amount of biofilm retained in the wells.

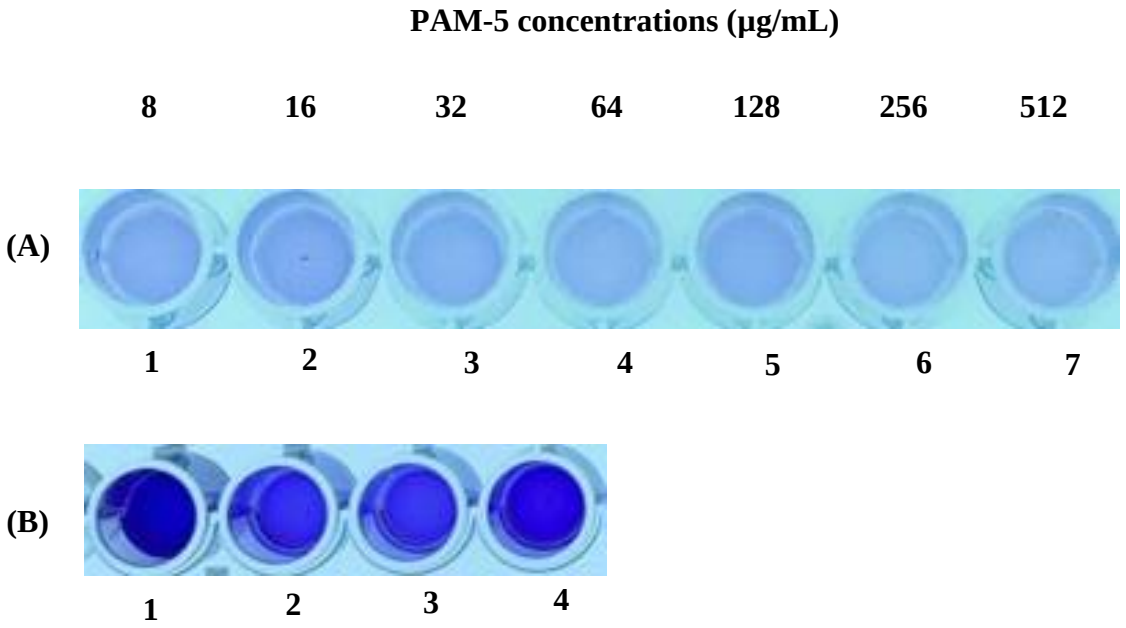


Figure 4.8: Gross view on crystal violet staining of mature biofilm after treatment with PAM-5. (A) Wells A1 to A7: mature biofilm treated with PAM-5 at increasing concentrations from 8 µg/mL to 512 µg/mL; (B) Wells B1 to B4: mature biofilm treated with PBS which served as the negative control.

The intensities of crystal violet solution in the wells were then quantified by measuring their absorbance and the data was presented as histogram as shown in **Figure 4.9**. Interestingly, there is a reasonable concordance between the visual observation as reported in **Section 4.4.1** and the absorbance trend as presented in **Figure 4.9**, where the low CV intensities as observed for the peptide-treated biofilms corresponded to the overall low CV absorbance value as seen in **Figure 4.9**. Even though under the lowest treatment concentration of 8 µg/mL, the peptide was still able to disperse the mature biofilm, as indicated by the significant reduction of the biomass staining (60.87% reduction) as compared to the untreated biofilm ($p < 0.05$). Following that, the CV absorbance from the peptide-treated biofilms was seen in a decreasing trend when the biofilms were treated with increasing peptide concentrations from 8 µg/mL to 512 µg/mL.

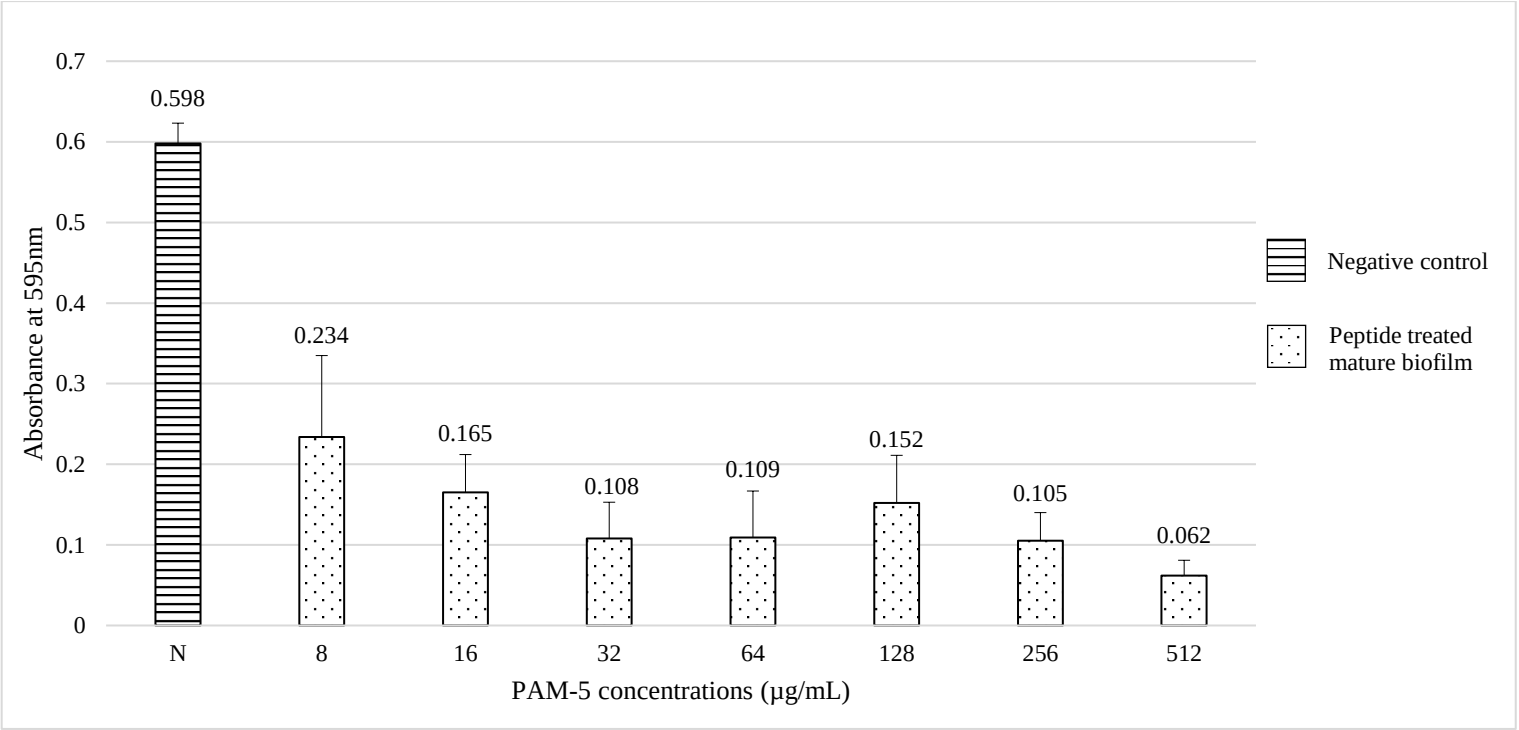


Figure 4.9: Absorbance of crystal violet staining of mature biofilm produced by MDR *P. aeruginosa* 1894170 after treatment with PAM-5 at concentrations ranging from 8 µg/mL to 512 µg/mL (dotted). **'N'** represents the absorbance of untreated biofilm which served as the negative control (light horizontal line). Differences between the peptide treated group and the control were statistically significant ($p < 0.05$).

The degree of biofilm dispersal by PAM-5 was depicted in **Figure 4.10**. Clearly shown by the graph, PAM-5 was able to disperse and eradicate mature biofilm produced by MDR *P. aeruginosa* 1894170 in a dose-dependent manner. As the concentrations of PAM-5 treatment increased, the percentages of biofilm eradication by the peptide also increased. Even though at the concentration as low as 8 µg/mL, PAM-5 was able to disperse and eradicate up to 60.82 % of the established biofilms. As the peptide concentration increased, more biofilms were eradicated by the peptide, where the degree of eradication ranged from 72.47% to 89.65%. Although complete eradication of mature biofilm was not achieved by the highest peptide concentration (512 µg/mL), but the minimum biofilm eradication concentration at 90% ($MBEC_{90}$) was almost achieved by this peptide concentration.

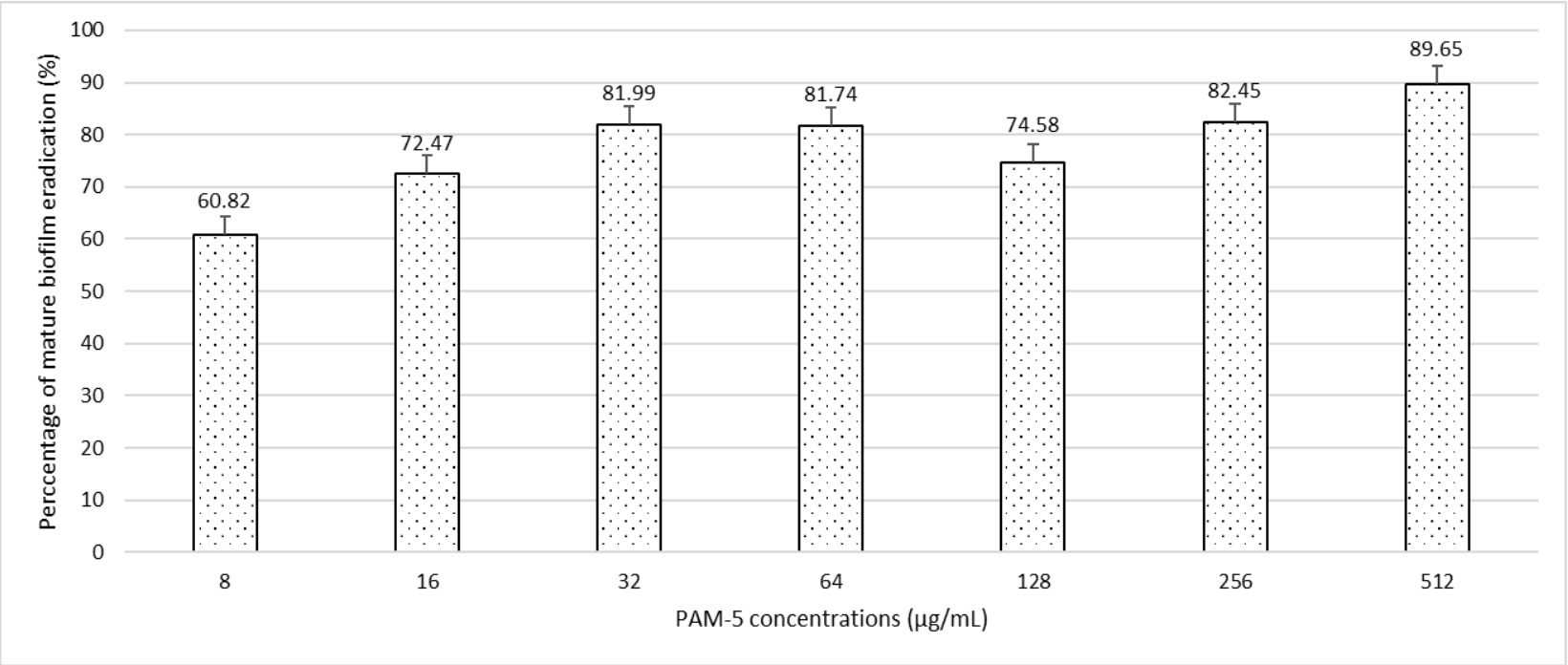


Figure 4.10: Percentages of PAM-5-mediated eradication of mature biofilms produced by MDR *P. aeruginosa* 1894170.

4.4.2 Effects of PAM-5 on the Viability of Biofilm-Embedded MDR *P. aeruginosa* 1894170

The ability of PAM-5 to disperse and eradicate mature biofilm as reported in **Section 4.4.1** provides a hint that this peptide may have reached the biofilm-embedded bacteria and killed them. Indeed, the findings from the bacterial viability assessment strongly supported this assumption.

Figure 4.11 shows the findings for one of the triplicated MTT assays as described in **Section 3.3.5**. Based on the setup as described earlier, Wells A1 to A7 were pre-grown with *P. aeruginosa* biofilms followed by PAM-5 treatment at increasing concentrations ranging from 8 µg/mL to 512 µg/mL. On the other hand, Wells B1 to B4 were filled with mature biofilm without PAM-5 treatment, which served as the negative control. As shown in the figure, the intensities of formazan in Wells A1 to A7 were generally lower than B1 to B4.

Nevertheless, there was no observable trend of decreasing formazan intensities from Wells A1 to A5, as the formazan intensities across these 5 wells were relatively similar. At higher peptide concentrations (256 µg/mL and 512 µg/mL), the formazan reduction became more apparent, where the formazan intensity for the biofilm treated with 512 µg/mL of PAM-5 (Well A7) was much lower than the biofilm treated with 256 µg/mL of the peptide (Well A6).

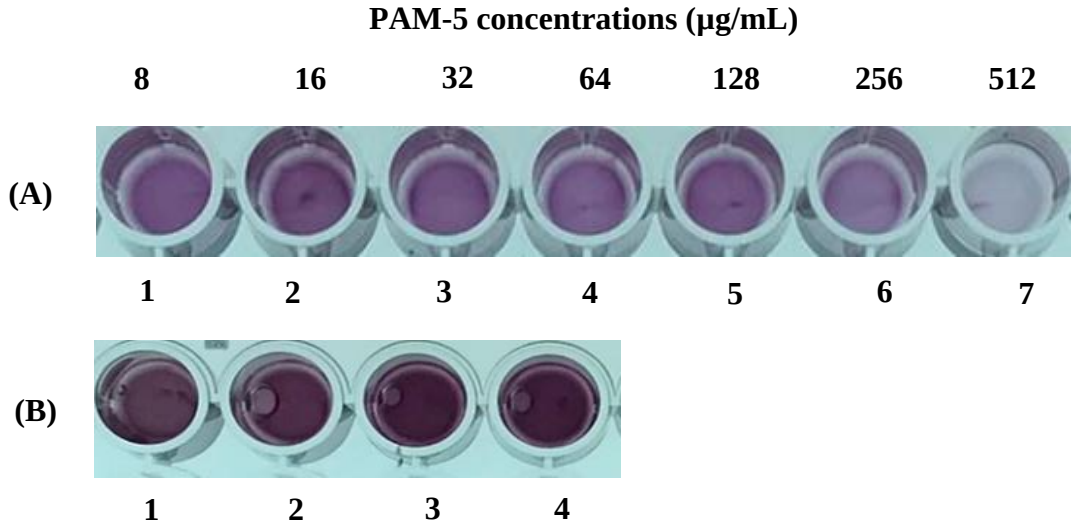


Figure 4.11: Gross view of formazan formation for one of the triplicated biofilm eradication assays on mature biofilm produced by MDR *P. aeruginosa* 1894170. (A) Well A1 to A7 are mature biofilms treated with PAM-5 at increasing concentrations from 8 µg/mL to 512 µg/mL, respectively; while (B) Well B1 to B4 are mature biofilms without peptide treatment which served as the negative control.

To quantify intensities of the solubilized formazan suspension, the absorbance of the well suspension was measured at 570 nm and the data of average absorbance of the formazan from the triplicated assays are presented in **Figure 4.12**. The overall absorbance of the formazan from the biofilms treated with all concentrations of PAM-5 (8 µg/mL to 512 µg/mL) was significantly lower than the untreated biofilm (N) ($p < 0.05$). Interestingly, measurement of the formazan absorbance could provide a clearer picture on the decreasing trend of formazan which was not apparently seen by visual observation as reported earlier. As show in the figure, as the concentrations of the peptide treatment increased, the amount of formazan formed by the treated biofilms became lesser. At the concentration of 512 µg/mL, the amount of formazan produced by the remaining viable bacteria in the well was about 10 times lesser than the untreated biofilm.

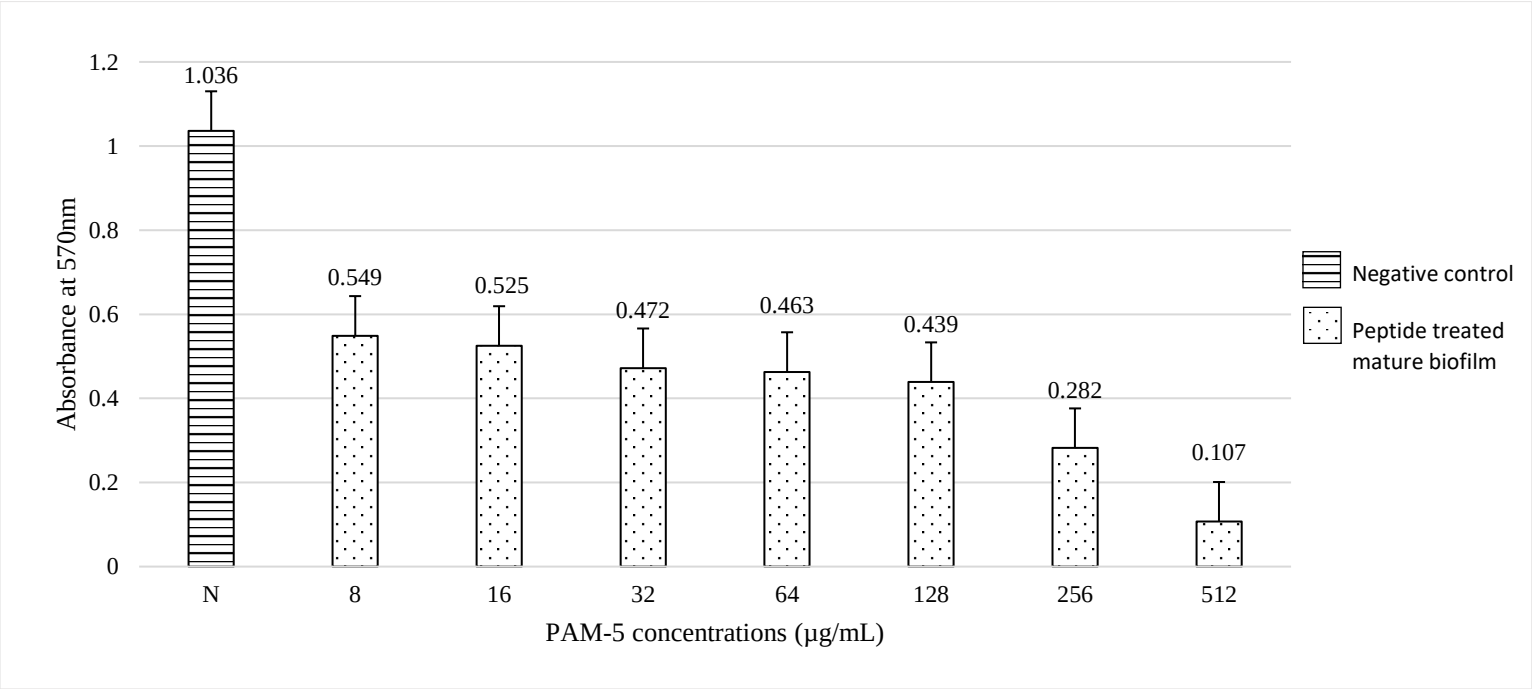


Figure 4.12: Absorbance of formazan formed by viable MDR *P. aeruginosa* 1894170 after treatment with PAM-5 at concentrations ranging from 8 µg/mL to 512 µg/mL (dotted). 'N' represents the absorbance of untreated biofilm which served as the negative control (light horizontal line). Differences between the peptide treated group and the control were statistically significant ($p < 0.05$).

The capacity and efficacy of PAM-5 to kill the biofilm-embedded bacteria could be reflected by the percentage of viability reduction to the bacterial community, which was calculated using the formula as stated in **Section 3.3.5**. Based on the data in **Figure 4.13**, PAM-5 was shown able to reduce the metabolic activity of biofilm-embedded MDR *P. aeruginosa* in a dose-dependent manner. As the concentrations of PAM-5 increased, its capacity to reduce the biofilm metabolic activity also increased. At the concentration of 512 μg/mL, PAM-5 was able to eliminate 90.24% of the viable bacteria embedded within the biofilm.

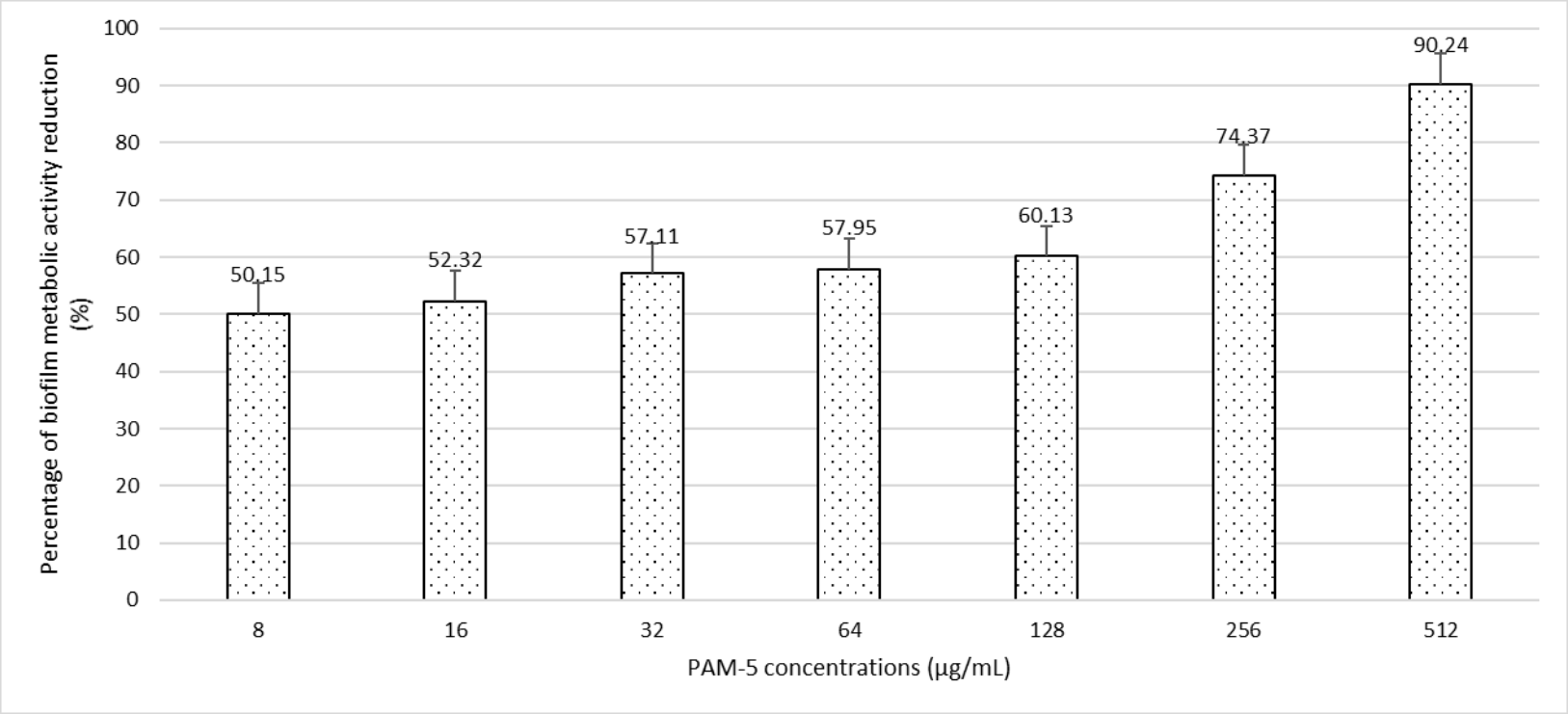


Figure 4.13: Percentages of reduction in biofilm metabolic activity by PAM-5 at various concentrations from 8 µg/mL to 512 µg/mL.

CHAPTER 5

DISCUSSION

Antibiotic resistance is one of the main problems that continuously threaten public health and clinical practice. Treatment of bacterial infections has become increasingly difficult due to the high prevalence and rapid dissemination of antibiotic resistant bacteria in healthcare setting. Apart from the various intrinsic and acquired resistant mechanisms which are well documented, biofilm represents another major cause of resistance which compromises the efficacy of many conventional antibiotics. In particular, antibiotic resistance by *Pseudomonas aeruginosa* (*P. aeruginosa*)-produced biofilm is a major obstacle to the treatment of chronic and nosocomial infections (Rasamiravaka et al., 2015; Ciofu and Tolker-Nielsen, 2019; Burmølle et al., 2010). Strategies focusing on targeting and disrupting biofilm matrix may be a promising approach to enhance bacterial susceptibility to antibacterial agents (Fulaz et al., 2019). Therefore, development of antibiofilm agents is urgently required to address this issue in clinical settings.

Over the last three decades, antibacterial peptides (ABPs) have gained increasing research attention in view of their potential as alternatives to antibiotics to fight against infections caused by antibiotic-resistant bacteria. Previously, a 15-mers synthetic peptide named PAM-5 was shown to exhibit antibacterial effects towards a spectrum of pathogenic bacteria (Chan, 2016, unpublished). However, these antibacterial findings were only based on its action towards planktonic

bacteria, while the potency of the peptide towards biofilm-embedded bacteria is yet to be elucidated. Thus, the antibiofilm effect of PAM-5 on clinical strain of multidrug-resistant *P. aeruginosa* was screened in this study.

5.1 Optimization of Biofilm Growth

In order to optimize biofilm growth for the antibiofilm assays, three different media, namely Mueller Hilton broth (MHB), Tryptic Soy Broth (TSB) and Brain Heart Infusion Broth (BHIB), were compared for their strength in promoting biofilm growth. The medium that grew the highest amount of biofilm as indicated by crystal violet (CV) staining was chosen as the growing medium for the subsequent antibiofilm assays.

As reported in **Section 4.2**, BHIB was found to enhance the highest amount of biofilm production by MDR *P. aeruginosa* as compared to the other two media. This might explain the use of BHIB for antibiofilm assays by many other studies (Karunanidhi et al., 2018; Torres et al., 2018; Mohammadi-Barzelighi et al., 2019; Chappell and Nair, 2020).

Biofilm production by *P. aeruginosa* is influenced by various factors such as temperature, incubation period, nutrient level and pH (Goller and Romeo, 2008; Ansari et al., 2017a; Ponomareva et al., 2018). According to Wijesinghe et al (2019), BHI medium contains higher levels of proteins rich in leucine, proline, serine, and aspartate that are required for bacterial surface adherence during the initial stage of biofilm formation and biofilm maturation of *P. aeruginosa*. In

addition, the presence of lipids such as choline and sphingosine in BHI broth may facilitate biofilm formation by preventing desiccation (Singh et al., 2017).

In general, the results obtained in this study indicated that BHI broth is the most conducive growth medium for *in vitro* biofilm formation by *P. aeruginosa*. Therefore, this medium was chosen as the growth medium for biofilm inhibition and biofilm eradication assays in this study.

5.2. Inhibitory Effect of PAM-5 on Biofilm Formation by Clinical Strain of MDR *P. aeruginosa* 1894170

As described in **Chapter 2**, biofilm formation begins with the adhesion of planktonic bacteria to biotic or abiotic surfaces to form microcolonies, followed by quorum sensing and production of EPS to establish mature biofilm. Thus, any means of inhibiting the initial step of biofilm formation, i.e. bacterial surface adherence, would stand a better chance to prevent the bacteria from gaining a foothold to establish mature biofilm on the surface. Therefore, in this study, PAM-5 was pre-coated on the well surfaces of the microtiter plate before addition of *P. aeruginosa* suspension. This was performed in order to assess the peptide's ability to inhibit the attachment of bacteria to the well surface. With reference to the findings as reported in **Section 4.3.1**, PAM-5 was shown to be able to reduce biofilm formation by MDR *P. aeruginosa* as indicated by the overall lower absorbance of crystal violet (CV) in the wells pre-coated with the peptide, as compared to the higher CV absorbance in the non-coated wells (negative control). Additionally, this biofilm inhibitory effect was exhibited by the peptide in a dose-dependent manner, suggesting the true biofilm inhibition effect exerted by the peptide. Along with the findings from the planktonic

bacterial viability assay as reported in **Section 4.3.2**, which demonstrated the high titer of planktonic bacteria despite the present of PAM-5 in the wells, it can be inferred that the peptide may possess certain ability to prevent surface adherence by MDR *P. aeruginosa* even though it was present abundantly in the well suspension.

Several studies have revealed or proposed the possible mechanism of actions exhibited by ABPs to inhibit biofilm formation. One of them is the ABP-mediated disruption of bacterial membrane, which may impair adherence of the defected planktonic bacteria to a surface. This action represents the major mechanism among those cationic ABPs, where they were found to bind to anionic bacterial membrane via electrostatic interactions (Bin Hafeez et al., 2021; Erdem Büyükkiraz and Kesmen, 2021; Ramazi et al., 2022). For instance, a proline-rich cationic ABP named Octopromycin was shown to cause complete loss of membrane integrity to *A. baumannii* (Rajapaksha et al., 2021). Interestingly, the peptide was also able to exert strong biofilm inhibition effect by reducing bacterial adherence to surfaces. EC1-17KV, a cationic ABP derivative from the edible sea-urchin *Echinus esculentus*, was reported for its ability to reduce the number of planktonic *P. aeruginosa* adhered to catheter surface in an animal study. Similarly, this anti-adherence property was also associated with its ability to destroy the bacterial membrane via Ca^{2+} and Mg^{2+} displacement from lipopolysaccharide (LPS), which is an important component to maintain bacterial membrane integrity (Ma et al., 2020). Correspondingly, PAM-5 was also reported for its ability to disrupt bacterial outer membrane (Phoon, 2016; Lim, 2021, unpublished) as well as permeabilizing bacterial inner

membrane (Phoon, 2016, unpublished), which could explain its ability to inhibit bacterial adherence and formation of biofilm on the well surfaces of the microtiter plate as found in this study.

Bacterial attachment to a surface is influenced by many factors, both from the bacterium itself and the surface. One of these factors is the hydrophobicity of the bacteria and the surface where the bacteria adhere to (Van Loosdrecht et al., 1990). The degree of hydrophobicity strongly influences the propensity of bacterial adhesion to a surface via hydrophobic interactions between the two entities (Giaouris et al., 2009). This could explain the high occurrence of biofilm on many abiotic and hydrophobic surfaces, including the polystyrene microtiter plate used for the antibiofilm assay in this study. It is noteworthy to know that adhesion of many bacteria to polystyrene surface is mainly mediated by hydrophobic but not electrostatic interaction, as polystyrene plates which were used in this study are neutrally charged (Curtis et al., 1983; van Loosdrecht et al., 1987; Mermut et al., 2006; Phillips et al., 2006). As reported in **Section 4.3.1**, surface coating of the wells with PAM-5 was shown able to reduce biofilm formation as compared to the uncoated wells (negative control). It is believed that the polystyrene hydrophobicity might be masked by the PAM-5 coating, thus reducing the hydrophobic binding between *P. aeruginosa* and the well surface. However, more studies are needed to testify this in molecular level.

Apart from impairing surface adherence, many ABPs with antibiofilm effects were demonstrated to interfere with bacterial quorum sensing (QS) associated with biofilm formation. As mentioned earlier in **Section 2.1.2**, QS plays several

important roles in the coordination of biofilm formation and maturation. Therefore, expression of QS-controlled genes that are associated with biofilm formation indeed represents a major target for biofilm intervention strategy. In a study by Overhage et al. (2008), a human ABP named human cathelicidin LL-37 was reported to decrease the expression of two biofilm-related genes named Rhl and Las, which are essential for initial bacterial attachment and growth on a solid surface. The QS attenuation is even more prominent in peptides rich in tryptophan (Trp). This was supported by a study which revealed that several Trp-containing ABPs were able to downregulate expressions of multiple regulatory genes that are essential for QS-mediated biofilm formation (Shang et al., 2021). Additionally, these peptides were also shown to downregulate gene expression for the synthesis of polysaccharides (eg, *Psl*, *Pel*, and alginate) that are required for biofilm stabilization in *P. aeruginosa*. Consequently, the maturation of biofilm is impaired. Interestingly, PAM-5 was shown to be able to bind to DNAs from several bacteria (Tan, 2018, unpublished) as well as possessing two Trp residues (K-W-K-W-R-P-L-K-R-K-L-V-L-R-M). The former may be associated with the peptide's ability to inhibit biofilm formation via gene interference, while the latter may render the peptide with the ability to downregulate gene expression for biofilm formation, as corresponded by the above-mentioned studies.

5.3 Effect of PAM-5 on Matured Biofilm Produced by Clinical Strain of MDR *P. aeruginosa* 1894170

The ability of ABPs to inhibit the early stages of biofilm development represents an important prophylactic strategy to prevent or minimize biofilm-mediated infection. However, the therapeutic value of ABPs which is reflected by their

ability to eradicate mature biofilm is equally important. This therapeutic feature is particularly needed to address the issue of biofilm colonization on human tissues that is commonly associated with chronic systemic infections and failure in antibiotic treatment (Kvich et al., 2020; Vestby et al., 2020). Therefore, apart from screening for its efficacy to impair biofilm formation, PAM-5 was also screened for its capability to eradicate mature biofilm established by MDR *P. aeruginosa*.

As reported in **Section 4.4.1**, PAM-5 was able to reduce mature biofilm mass in a dose-dependent manner. This is indicated by the overall lower amount of CV-stained biomass in the wells filled with mature biofilm treated with PAM-5, as compared to the untreated biofilm. To a certain extent, these findings provided a direct clue that PAM-5 was able to disintegrate and disperse mature biofilm that was established on a surface. Although the direct evidence on how exactly PAM-5 mediate biofilm dispersal is not available in this study, several possible mechanisms of biofilm degradation can be proposed in relation to the peptide characteristics. One of these characteristics is the peptide cationicity, which is believed to be an important attributing factor to biofilm degradation by targeting several essential elements in the biofilm matrix. During the stage of biofilm maturation, alginate and extracellular DNA (eDNA) are produced by *P. aeruginosa* to stabilize the matrix layers. The former is an anionic extracellular polysaccharide that enhances bacterial adhesion and aggregation on a surface (Somma et al., 2020; Das, 2021), while the latter is an essential factor that maintain structural integrity and stability of biofilms by several bacterial species, including *P. aeruginosa* (Wei and Ma, 2013; Das et al., 2014). It is speculated

that the cationic PAM-5 might interact with these anionic substances via electrostatic interaction, thus disrupting the alginate-mediated adhesion as well as the eDNA-mediated biofilm integrity. Consequently, as the concentrations of PAM-5 increased, bacterial surface adhesion and aggregation might become weaker, and more biofilm extracellular matrix (ECM) are disintegrated and dispersed. These could explain the decreasing amount of CV-stained biofilm mass as found in this study when the mature biofilms were treated with increasing concentrations of PAM-5.

Although there was no direct evidence to support this speculation, similar findings were also reported by other research groups that linked their cationic ABPs to biofilm degradation effect. For example, a cationic synthetic ABP named PI peptide was shown to be able to disrupt and disperse mature biofilm established by *Streptococcus mutans* (*S. mutans*) (Ansari et al., 2017b). According to the author, this biofilm dispersal effect might be attributed to the peptide cationicity that may disrupt the bonding between anionic eDNA and exopolysaccharides that are required for biofilm stability. Similarly, eradication of mature or preformed biofilm was also reported for three cathelicidin derived ABPs, namely SMAP-29, BMAP-27 and BMAP-28. These three ABPs are strongly cationic and were found able to bind to anionic extracellular polymeric substances (EPSs), thus reducing the available amount of the latter that is required to maintain stability of mature biofilm (Pompilio et al., 2011). Hepcidin 20, a cationic human liver-derived ABP, was shown to reduce extracellular matrix mass and polysaccharide intracellular adhesin (PIA) produced by *Staphylococcus epidermidis* (*S. epidermidis*). As a result, the architecture of the

bacterial biofilm was weakened (Brancatisano et al., 2014). Collectively, along with PAM-5 in this study, the cationicity of many ABPs may represent an important feature that enables the peptides to interfere with different anionic biofilm substances, thus reducing the stability and structural integrity in the biofilm architecture. Moreover, as certain biofilm adhesion factors are also targeted by some of these cationic ABPs, the adhesion of biofilm matrix and/or the biofilm-embedded bacteria to a surface might be weakened as well. Consequently, ABP-treated mature biofilm might gradually loose its adhesiveness to the surface where it was attached to, followed by detachment of some biofilm mass by the shear force during washing steps. This could explain the reduction of biofilm mass after PAM-5 treatment, followed by washing the biofilm-filled wells with PBS.

Interestingly, the antibiofilm effect of PAM-5 is not limited to biofilm inhibition and eradication only, as it was followed by killing of the biofilm-embedded bacteria. As the mature biofilm was disrupted and dispersed by PAM-5, it is anticipated that the barrier effect by the biofilm matrix would be compromised, thus enabling direct access of the ABP to the detached or sessile bacteria. With reference to a few previous studies, where PAM-5 was regarded as a bactericidal ABP which kills its target bacteria via membrane disruption (Phoon, 2016; Lim 2021, unpublished), it is anticipated that this peptide could kill the sessile *P. aeruginosa*. This anticipation is supported by the general decrement in the formazan intensities from the *P. aeruginosa* biofilms when they were treated with increasing concentrations of PAM-5 (as reported in **Section 4.4.2**). Although complete eradication of the sessile bacteria could not be achieved by

the range of PAM-5 concentrations used in this study (as indicated by **Section 4.4.2**), but more than 90% of the sessile bacteria was killed by the peptide at the highest tested concentration (512 μg/mL). With reference to the trend of metabolic activity reduction as presented in **Figure 4.13**, it is possible that complete killing of the sessile bacteria could be achieved if higher concentration of PAM-5 is tested.

5.4 Implications of Studies

The promising findings on the antibiofilm effect of PAM-5 as found in this study has raised the optimism on the therapeutic potential of this peptide in clinical application. The ability of PAM-5 to inhibit biofilm formation may highlight its potential prophylactic use in invasive or implanted medical devices such as catheter, granular or orthopedic implants, where the peptide could be coated on the surface of these devices to reduce the risk of biofilm formation. On the other hand, the ability of PAM-5 to eradicate mature biofilm as well as killing the sessile *P. aeruginosa* may potentiate its therapeutic use in treating biofilm-mediated infections, particularly cystic fibrosis (CF) that is always associated with biofilms produced by *P. aeruginosa*. Last but not least, these findings could also lead to the proposal of synergistic use of conventional antibiotics and PAM-5 to treat infections caused by multidrug resistant bacteria and biofilm.

5.5 Limitations and Future Studies

As mentioned in the discussion, the biofilm eradication effect of PAM-5 was only screened for the range of concentrations from 4 μg/mL to 512 μg/mL. Although both the crystal violet (CV) and MTT assays demonstrated biofilm

dispersal and metabolic reduction rate of 89.65% and 90.24%, respectively, the ability of the peptide to cause complete biofilm degradation and bacterial killing is yet to be determined by the range of concentrations used in this study. Therefore, in future study, the tested peptide concentration could be increased to a few folds higher, such as 1,024 µg/mL and 2,048 µg/mL to determine if the peptide could achieve total biofilm eradication.

Although the antibiofilm effects (biofilm inhibition and biofilm eradication) of PAM-5 are assumed to be associated with its hydrophobicity and cationicity, detailed experimental studies to validate the roles of these peptide features to the antibiofilm effects are yet to be carried out in this study. Therefore, in future study, peptide modification by amino acid substitution to PAM-5 could be carried out to compare the strength of these antibiofilm effects based on different peptide cationicity and hydrophobicity.

The biofilm inhibition and eradication effects of PAM-5 were determined based on crystal violet staining and MTT assay, which only provided the relative comparison in the amount of biofilm mass between the peptide-treated and untreated biofilm. These findings do not provide the visual data on the morphological and structural changes to the peptide-treated biofilm mass and embedded bacteria. Thus, future studies may consider to include inverted and scanning electron microscopy to study the effects of PAM-5 on different stages of biofilm development in more details.

Although it was not included in this study, it is worth to know the impact of PAM-5 to the expression of biofilm-associated genes such as *lasA*, *lasB*, *rhlA*, *rhlB*, *pelA*, *algD*, *psl*, *oprD*, *oprM*, *mexA*, and *mexB*, in which some of them were mentioned in the discussion earlier. Therefore, future studies may consider including real-time quantitative PCR analysis to investigate the transcription levels of these genes in the bacteria upon PAM-5 treatment. The data from these analyses may provide detailed insights on the mechanisms of antibiofilm by PAM-5.

The findings from this study demonstrated the antibiofilm effect of PAM-5 on only one bacterium (*P. aeruginosa*), which is insufficient to yield a strong conclusion that PAM-5 is an ideal antibiofilm peptide. Therefore, more bacterial species from both reference and clinical isolated strains should be included in future studies to determine if PAM-5 possess the similar strength of antibiofilm effects on these bacteria as well.

CHAPTER 6

CONCLUSION

In this study, the antibacterial peptide PAM-5 was shown to exhibit antibiofilm effects on clinical strain of MDR *Pseudomonas aeruginosa* 1894170. As demonstrated in this study, PAM-5 was able to inhibit biofilm formation, disperse mature biofilm and kill the biofilm-embedded bacteria. However, these findings only provide a preliminary perspective on the antibiofilm effects of PAM-5 on the tested bacterium and further detailed studies in the mechanisms of antibiofilm by PAM-5 are required. Despite the needs for future studies, current findings proposed that PAM-5 is a potent antibiofilm peptide against clinical strain of MDR *P. aeruginosa*. This suggests that PAM-5 may be a potential alternative antibacterial agent to combat biofilm formation in clinical settings.

REFERENCES

Abe, K. et al., 2020. Biofilms: hot spots of horizontal gene transfer (HGT) in aquatic environments, with a focus on a new HGT mechanism. *FEMS Microbiology Ecology*, 96(5), pp.1-11.

Abebe, G.M., 2020. The role of bacterial biofilm in antibiotic resistance and food contamination. *International Journal of Microbiology*, 2020, pp.1–10.

Annous, B.A. et al., 2009. Scientific status summary. *Journal of Food Science*, 74(1), pp.1-14.

Ansari, F.A., Jafri, H., Ahmad, I. and Abulreesh, H.H., 2017a. Factors affecting biofilm formation in *in vitro* and in the rhizosphere. *Biofilms in Plant and Soil Health*, pp.275–290.

Ansari, J.M. et al., 2017b. Anti-biofilm activity of a self-aggregating peptide against *Streptococcus mutans*. *Frontiers in Microbiology*, 8, pp.1-12.

Armbruster, C.R. and Parsek, M.R., 2018. New insight into the early stages of biofilm formation. *Proceedings of the National Academy of Sciences*, 115(17), pp.4317–4319.

Azam, M.W. and Khan, A.U., 2019. Updates on the pathogenicity status of *Pseudomonas aeruginosa*. *Drug Discovery Today*, 24(1), pp.350–359.

Bai, X. et al., 2021. Bacterial biofilms and their implications in pathogenesis and food safety. *Foods*, 10(9), p.2117.

Berne, C. et al., 2015. Adhesins involved in attachment to abiotic surfaces by Gram-negative bacteria. *Microbiology Spectrum*, 3(4), pp.1-45.

Bhardwaj, A.K. et al., 2013. Bacterial quorum sensing inhibitors: attractive alternatives for control of infectious pathogens showing multiple drug resistance. *Recent Patents on Anti-Infective Drug Discovery*, 8(1), pp.68–83.

Bin Hafeez, A., Jiang, X., Bergen, P.J. and Zhu, Y., 2021. Antimicrobial peptides: an update on classifications and databases. *International Journal of Molecular Sciences*, 22(21), p.11691.

Blair, J.M. and Piddock, L.J., 2009. Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an Update. *Current Opinion in Microbiology*, 12(5), pp.512–519.

Blanco, P. et al., 2016. Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants. *Microorganisms*, 4(1), p.14.

Blower, R.J., Barksdale, S.M. and van Hoek, M.L., 2015. Snake cathelicidin NA-CATH and smaller helical antimicrobial peptides are effective against *Burkholderia thailandensis*. *PLOS Neglected Tropical Diseases*, 9(7), p.e0003862.

Bodey, G.P., Bolivar, R., Fainstein, V. and Jadeja, L., 1983. Infections caused by *Pseudomonas aeruginosa*. *Clinical Infectious Diseases*, 5(2), pp.279–313.

Botelho, J., Grosso, F. and Peixe, L., 2019. Antibiotic resistance in *Pseudomonas aeruginosa* – mechanisms, epidemiology and evolution. *Drug Resistance Updates*, 44, p.100640.

Bouza, E. and Burillo, A., 2010. Oritavancin: a novel lipoglycopeptide active against Gram-positive pathogens including multiresistant strains. *International Journal of Antimicrobial Agents*, 36(5), pp.401–407.

Brancatisano, F.L. et al., 2014. Inhibitory effect of the human liver-derived antimicrobial peptide hepcidin 20 on biofilms of polysaccharide intercellular adhesin (PIA)-positive and PIA-negative strains of *Staphylococcus epidermidis*. *Biofouling*, 30(4), pp.435–446.

Burmølle, M. et al., 2010. Biofilms in chronic infections – a Matter of opportunity – monospecies biofilms in multispecies infections. *FEMS Immunology & Medical Microbiology*, 59(3), pp.324–336.

Bush, L.M., 2022. *Pseudomonas and Related Infections - Infectious Diseases* [Online]. MSD Manual Professional Edition. Available at: https://www.msdmanuals.com/professional/infectious-diseases/gram-negative-bacilli/pseudomonas-and-related-infections [Accessed: 20 April 2022].

Camargo, A. C., Woodward, J. J., Call, D. R., and Nero, L. A., 2017. *Listeria monocytogenes* in food-processing facilities, food contamination, and human listeriosis: the Brazilian Scenario. *Foodborne Pathog. Dis*. 14, pp.623–636.

Cao, J. et al., 2018. Yeast-based synthetic biology platform for antimicrobial peptide production. *ACS Synthetic Biology*, 7(3), pp.896–902.

Carniello, V. et al., 2018. Physico-chemistry from initial bacterial adhesion to surface-programmed biofilm growth. *Advances in Colloid and Interface Science*, 261, pp.1–14.

CDC, 2019. *Biggest Threats and Data* [Online]. Centers for disease control and prevention. Available at: https://www.cdc.gov/drugresistance/biggest-threats.html [Accessed: 15 March 2022].

Chan, S.Y., 2016. Screening of broad-spectrum antibacterial effects of synthetic peptide PAM-5 against selected pathogenic bacteria. Bachelor's Degree. Universiti Tunku Abdul Rahman, pp. 1-94.

Chappell, T.C. and Nair, N.U., 2020. Engineered lactobacilli display anti-biofilm and growth suppressing activities against *Pseudomonas aeruginosa*. *Npj Biofilms and Microbiomes*, 6(1). doi:10.1038/s41522-020-00156-6.

Chung, P.Y. and Khanum, R., 2017. Antimicrobial peptides as potential anti-biofilm agents against multidrug-resistant bacteria. *Journal of Microbiology, Immunology and Infection*, 50(4), pp.405–410.

Ciofu, O. and Tolker-Nielsen, T., 2019. Tolerance and resistance of *Pseudomonas aeruginosa* biofilms to antimicrobial agents—how *P. aeruginosa* can escape antibiotics. *Frontiers in Microbiology*, 10, pp.1-15

Colvin, K.M. et al., 2011. The pel polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. *PLoS Pathogens*, 7(1), p.e1001264.

Costa, O.Y.A. et al., 2018. Microbial extracellular polymeric substances: ecological function and impact on soil aggregation. *Frontiers in Microbiology*, 9, pp.1-14.

Curtis, A.S., Forrester, J.V., McInnes, C. and Lawrie, F., 1983. Adhesion of cells to polystyrene surfaces. *Journal of Cell Biology*, 97(5), pp.1500–1506.

Das, T. et al., 2014. Influence of calcium in extracellular DNA mediated bacterial aggregation and biofilm formation. *PLoS ONE*, 9(3), p.e91935.
Das, T., 2021. *Pseudomonas aeruginosa secreted biomolecules and their diverse functions in biofilm formation and virulence* [Online]. *www.intechopen.com*. IntechOpen. Available at: https://www.intechopen.com/chapters/75844 [Accessed: 21 April 2022].

Dean, S.N., Bishop, B.M. and van Hoek, M.L., 2011. Natural and synthetic cathelicidin peptides with anti-microbial and anti-biofilm activity against *Staphylococcus aureus*. *BMC Microbiology*, 11(1), p.114.

Di Somma, A., Moretta, A., Canè, C., Cirillo, A. and Duilio, A., 2020. Antimicrobial and antibiofilm peptides. *Biomolecules*, 10(4), p.652.

Dincer, S., Uslu, F.M. and Delik, A., 2020. *Antibiotic Resistance in Biofilm* [Online]. *www.intechopen.com*. IntechOpen. Available at: https://www.intechopen.com/chapters/72109 [Accessed: 26 September 2021].

Ding, Y. E., 2019. Screening of antibacterial spectrum of synthetic peptide PAM-5 on selected Gram-positive bacteria. Bachelor's Degree. Universiti Tunku Abdul Rahman, pp.1-89.

Erdem Büyükkiraz, M. and Kesmen, Z., 2021. Antimicrobial peptides (AMPs): a promising class of antimicrobial compounds. *Journal of Applied Microbiology*, 132(3), pp.1-24.

Fair, R.J. and Tor, Y., 2014. Antibiotics and bacterial resistance in the 21st century. *Perspectives in Medicinal Chemistry*, 6(6), p.PMC.S14459.

Falagas, M.E., Kasiakou, S.K. and Saravolatz, L.D., 2005. Colistin: the revival of polymyxins for the management of multidrug-resistant Gram-negative bacterial infections. *Clinical Infectious Diseases*, 40(9), pp.1333–1341.

Fjell, C.D., Hiss, J.A., Hancock, R.E.W. and Schneider, G., 2011. Designing antimicrobial peptides: form follows function. *Nature Reviews Drug Discovery*, 11(1), pp.37–51.

Flemming, H.-C. et al., 2016. Biofilms: an emergent form of bacterial life. *Nature reviews. Microbiology*, 14(9), pp.563–75.

Flemming, K. et al., 2008. High *in vitro* antimicrobial activity of synthetic antimicrobial peptidomimetics against Staphylococcal biofilms. *Journal of Antimicrobial Chemotherapy*, 63(1), pp.136–145.

Fulaz, S., Vitale, S., Quinn, L. and Casey, E., 2019. Nanoparticle–biofilm interactions: the role of the EPS matrix. *Trends in Microbiology*, 27(11), pp.915–926.

Galié, S. et al., 2018. Biofilms in the food industry: health aspects and control methods. *Frontiers in Microbiology*, 9, pp.1-18.

Gellatly, S.L. and Hancock, R.E.W., 2013. *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. *Pathogens and Disease*, 67(3), pp.159–173.

Germain, E., Roghanian, M., Gerdes, K. and Maisonneuve, E., 2015. Stochastic induction of persister cells by HipA through (p)ppGpp-mediated activation of mRNA endonucleases. *Proceedings of the National Academy of Sciences*, 112(16), pp.5171–5176.

Ghazay Fahad, A. and Mamdouh, B., 2021. Factors influencing bacterial biofilm formation and development. *American Journal of Biomedical Science & Research*, 12(6), pp.1-10.

Giaouris, E., Chapot-Chartier, M.-P. and Briandet, R., 2009. Surface physicochemical analysis of natural *Lactococcus lactis* strains reveals the existence of hydrophobic and low charged strains with altered adhesive properties. *International Journal of Food Microbiology*, 131(1), pp.2–9.

Goller, C.C. and Romeo, T., 2008. Environmental influences on biofilm development. *Current Topics in Microbiology and Immunology*, 322, pp.37–66.

Groves, M.L., Peterson, R.F. and Kiddy, C.A., 1965. Polymorphism in the red protein isolated from milk of individual cows. *Nature*, 207(5000), pp.1007–1008.

Hall-Stoodley, L. and Stoodley, P., 2009. Evolving concepts in biofilm infections. *Cellular Microbiology*, 11(7), pp.1034–1043.

Hathroubi, S., Mekni, M.A., Domenico, P., Nguyen, D. and Jacques, M., 2017. Biofilms: microbial shelters against antibiotics. *Microbial Drug Resistance*, 23(2), pp.147–156.

Hatt, J.K. and Rather, P.N., 2008. Role of bacterial biofilms in urinary tract infections. *Current Topics in Microbiology and Immunology*, 322, pp.163–192.

Hirsch, J.G., 1956. Phagocytin: a bacterial substance from polymorphonuclear leucocytes. *The Journal of Experimental Medicine*, 103(5), pp.589–611.

Horcajada, J.P. et al., 2019. Epidemiology and treatment of multidrug-resistant and extensively drug-resistant *Pseudomonas aeruginosa* infections. *Clinical Microbiology Reviews*, 32(4), pp.1-52.

Hrynyshyn, A., Simões, M. and Borges, A., 2022. Biofilms in surgical site infections: recent advances and novel prevention and eradication strategies. *Antibiotics*, 11(1), p.69.

Huan, Y., Kong, Q., Mou, H. and Yi, H., 2020. Antimicrobial peptides: classification, design, application and research progress in multiple fields. *Frontiers in Microbiology*, 11, pp.1-21.

Kapoor, G., Saigal, S. and Elongavan, A., 2017. Action and resistance mechanisms of antibiotics: a guide for clinicians. *Journal of Anaesthesiology Clinical Pharmacology*, 33(3), p.300.

Karaiskos, I., Lagou, S., Pontikis, K., Rapti, V. and Poulakou, G., 2019. The 'old' and the 'new' antibiotics for mdr gram-negative pathogens: for whom, when, and how. *Frontiers in Public Health*, 7, p.151.

Karatan, E. and Watnick, P., 2009. Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiology and Molecular Biology Reviews: MMBR*, 73(2), pp.310–347.

Karunanidhi, A. et al., 2018. Antibacterial and antibiofilm activities of nonpolar extracts of *Allium stipitatum* regel. against multidrug resistant bacteria. *BioMed Research International*, 2018, p.9845075.

Kean, R. et al., 2018. Gaining insights from candida biofilm heterogeneity: one size does not fit all. *Journal of Fungi (Basel, Switzerland)*, 4(1), pp.1-20.

Khatoon, Z. et al., 2018. Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. *Heliyon*, 4(12), p.e01067.

Knudsen, G.M., Fromberg, A., Ng, Y. and Gram, L., 2016. Sublethal concentrations of antibiotics cause shift to anaerobic metabolism in *Listeria monocytogenes* and induce phenotypes linked to antibiotic tolerance. *Frontiers in Microbiology*, 7, pp.1-15.

Kościuczuk, E.M. et al., 2012. Cathelicidins: family of antimicrobial peptides. A review. *Molecular Biology Reports*, 39(12), pp.10957–10970.

Koziel, J. et al., 2014. The janus face of a-toxin: a potent mediator of cytoprotection in staphylococci-infected macrophages. *Journal of Innate Immunity*, 7(2), pp.187–198.

Kragh, K.N. et al., 2016. Role of multicellular aggregates in biofilm formation. *MBio*, 7(2), pp. e00237-16.

Kumar, P., Kizhakkedathu, J. and Straus, S., 2018. Antimicrobial peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility *in vivo*. *Biomolecules*, 8(1), p.4.

Kvich, L., Burmølle, M., Bjarnsholt, T. and Lichtenberg, M., 2020. Do mixed-species biofilms dominate in chronic infections? –need for *in situ* visualization of bacterial organization. *Frontiers in Cellular and Infection Microbiology*, 10, pp.1-12.

Lata, M. et al., 2015. Proteomic analysis of ofloxacin-mono resistant *Mycobacterium tuberculosis* isolates. *Journal of Proteomics*, 127, pp.114–121.

Le, C.-F., Fang, C.-M. and Sekaran, S.D., 2017. Intracellular targeting mechanisms by antimicrobial peptides. *Antimicrobial Agents and Chemotherapy*, 61(4), pp.1-16.

Le, C.-F., Gudimella, R., Razali, R., Manikam, R. and Sekaran, S.D., 2016. Transcriptome analysis of *Streptococcus pneumoniae* treated with the designed antimicrobial peptides, DM3. *Scientific Reports*, 6(1), pp.1-9.

Lebeaux, D., Ghigo, J.-M., and 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), pp.510–543.

Lei, J. et al., 2019. The antimicrobial peptides and their potential clinical applications. *American Journal of Translational Research*, 11(7), pp.3919–3931.

Lewis, K., 2001. Riddle of biofilm resistance. *Antimicrobial Agents and Chemotherapy*, 45(4), pp.999–1007.

Li et al., 2017. Membrane active antimicrobial peptides: translating mechanistic insights to design. *Frontiers in Neuroscience*, 11, pp.1-18.

Lim, S., 2021. Screening for membrane-disruptive effect of novel antibacterial peptide PAM-5 on *Klebsiella pneumoniae* and *Escherichia coli* via scanning electron microscope. Bachelor's Degree. Universiti Tunku Abdul Rahman, pp. 1-56.

Liu, J. et al., 2017. Coupling between distant biofilms and emergence of nutrient time-sharing. *Science (New York, N.Y.)*, 356(6338), pp.638–642.

Liu, W.-P. et al., 2015. Design and synthesis of a novel cationic peptide with potent and broad-spectrum antimicrobial activity. *BioMed Research International*, 2015, p.578764.

Lopez, D. et al., 2010. Biofilms. *Cold Spring Harbor Perspectives in Biology*, 2(7).

Lu, Y. et al., 2008. The first antimicrobial peptide from sea amphibian. *Molecular Immunology*, 45(3), pp.678–681.

Ma, L. et al., 2020. Antimicrobial and antibiofilm activity of the EeCentroin 1 derived peptide EC1-17KV via membrane disruption. *EBioMedicine*, 55, p.102775.

MacNair, C.R. et al., 2018. Overcoming mcr-1 mediated colistin resistance with colistin in combination with other antibiotics. *Nature Communications*, 9(1), pp.1-8.

Mah, T.-F., 2012. Biofilm-specific antibiotic resistance. *Future Microbiology*, 7(9), pp.1061–1072.

Malanovic, N. and Lohner, K., 2016. Antimicrobial peptides targeting gram-positive bacteria. *Pharmaceuticals*, 9(3), p.59.

Mar, A. and Michl, H., 1976. A study of the high molecular weight hemolysin from the skin secretion of the ambhibian *Bombina variegata*. *Toxicon*, 14(3), pp.191–195.

Mermut, O. et al., 2006. *In situ* adsorption studies of a 14-amino acid leucine-lysine peptide onto hydrophobic polystyrene and hydrophilic silica surfaces using quartz crystal microbalance, atomic force microscopy, and sum frequency generation vibrational spectroscopy. *Journal of the American Chemical Society*, 128(11), pp.3598–3607.

Mizan, Md.F.R., Jahid, I.K. and Ha, S.-D., 2015. Microbial biofilms in seafood: a food-hygiene challenge. *Food Microbiology*, 49, pp.41–55.

Mohamed, M.F., Abdelkhalek, A. and Seleem, M.N., 2016. Evaluation of short synthetic antimicrobial peptides for treatment of drug-resistant and intracellular *Staphylococcus aureus*. *Scientific Reports*, 6(1), p.29707.

Mohammadi-Barzelighi, H. et al., 2019. Analysis of antibacterial and antibiofilm activity of purified recombinant azurin from *Pseudomonas aeruginosa*. *Iranian Journal of Microbiology*, 11(2), pp.166–176.

Moradali, M.F., Ghods, S. and Rehm, B.H.A., 2017. *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. *Frontiers in Cellular and Infection Microbiology*, 7, pp.517–537.

Morgan, D., 2016. New antibiotics: what do we need? *International Journal of Infectious Diseases*, 45, p.51.

Muhammad, M.H. et al., 2020. Beyond risk: bacterial biofilms and their regulating approaches. *Frontiers in Microbiology*, 11, pp.1-20.

Mulcahy, L.R., Isabella, V.M. and Lewis, K., 2013. *Pseudomonas aeruginosa* Biofilms in disease. *Microbial Ecology*, 68(1), pp.1–12.

Munita, J.M. and Arias, C.A., 2016. Mechanisms of antibiotic resistance. *Virulence Mechanisms of Bacterial Pathogens, Fifth Edition*, 4(2), pp.481–511.

Nagarajan, D. et al., 2019. Ω76: a designed antimicrobial peptide to combat carbapenem- and tigecycline-resistant *Acinetobacter baumannii*. *Science Advances*, 5(7), pp.1–19.

Nguyen, L., Garcia, J., Gruenberg, K. and MacDougall, C., 2018. Multidrug-resistant pseudomonas infections: hard to treat, but hope on the horizon? *Current Infectious Disease Reports*, 20(8), pp.1-10.

Nikaido, H. and Takatsuka, Y., 2009. Mechanisms of RND multidrug efflux pumps. *Biochimica Et Biophysica Acta (BBA) - Proteins and Proteomics*, 1794(5), pp.769–781.

Oluyombo, O., Penfold, C.N. and Diggle, S.P., 2019. Competition in biofilms between cystic fibrosis isolates of *Pseudomonas aeruginosa* is shaped by r-pyocins. *MBio*, 10(1), pp.e01828-18.

Overhage, J. et al., 2008. Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infection and Immunity*, 76(9), pp.4176–4182.

Panebianco, F., Rubiola, S. and Di Ciccio, P.A., 2022. The use of ozone as an eco-friendly strategy against microbial biofilm in dairy manufacturing plants: a review. *Microorganisms*, 10(1), p.162.

Pang, Z., Raudonis, R., Glick, B.R., Lin, T.-J. and Cheng, Z., 2019. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnology Advances*, 37(1), pp.177–192.

Pasupuleti, M., Schmidtchen, A. and Malmsten, M., 2011. Antimicrobial peptides: key components of the innate immune system. *Critical Reviews in Biotechnology*, 32(2), pp.143–171.

Penesyan, A., Paulsen, I.T., Kjelleberg, S. and Gillings, M.R., 2021. Three faces of biofilms: a microbial lifestyle, a nascent multicellular organism, and an incubator for diversity. *Npj Biofilms and Microbiomes*, 7(1), pp.1-9.

Peschel, A. and Otto, M., 2013. Erratum: phenol-soluble modulins and Staphylococcal infection. *Nature Reviews Microbiology*, 11(11), pp.814–814.

Peterson, E. and Kaur, P., 2018. Antibiotic resistance mechanisms in bacteria: relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. *Frontiers in Microbiology*, 9, pp.1-21.

Phillips, D.C. et al., 2006. Side chain, chain length, and sequence effects on amphiphilic peptide adsorption at hydrophobic and hydrophilic surfaces studied by sum-frequency generation vibrational spectroscopy and quartz crystal microbalance. *The Journal of Physical Chemistry C*, 111(1), pp.255–261.

Phoon, W.Y., 2016. Screening of Membrane-Active Mechanism of Antimicrobial Peptide, PAM-5, Against *Pseudomonas aeruginosa*. Bachelor's Degree. University Tunku Abdul Rahman, pp. 1 - 63.

Planet, P.J., 2018. *155 - Pseudomonas Aeruginosa* [Online]. ScienceDirect. Available at: https://www.sciencedirect.com/science/article/pii/B9780323401814001559 [Accessed: 18 March 2022].

Poirel, L., Jayol, A. and Nordmann, P., 2017. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clinical Microbiology Reviews*, 30(2), pp.557–596.

Pompilio, A. et al., 2011. Antibacterial and anti-biofilm effects of cathelicidin peptides against pathogens isolated from cystic fibrosis patients. *Peptides*, 32(9), pp.1807–1814.

Ponomareva, A.L., Buzoleva, L.S. and Bogatyrenko, E.A., 2018. Abiotic environmental factors affecting the formation of microbial biofilms. *Biology Bulletin*, 45(5), pp.490–496.

Rada, B., 2017. Interactions between neutrophils and *Pseudomonas aeruginosa* in cystic fibrosis. *Pathogens*, 6(1), p.10.

Rajapaksha, D.C. et al., 2021. Octopromycin: antibacterial and antibiofilm functions of a novel peptide derived from octopus minor against multidrug-resistant *Acinetobacter baumannii*. *Fish & Shellfish Immunology*, 117, pp.82–94.

Ramazi, S., Mohammadi, N., Allahverdi, A., Khalili, E. and Abdolmaleki, P., 2022. A review on antimicrobial peptides databases and the computational tools. *Database*, 2022, pp.1-17.

Ramirez, M.S. and Tolmasky, M.E., 2010. Aminoglycoside modifying enzymes. *Drug Resistance Updates*, 13(6), pp.151–171.

Rasamiravaka, T., Labtani, Q., Duez, P. and 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, pp.1–17.

Reichhardt, C. et al., 2014. Structure and function of bacterial biofilms by solid-state NMR. *Biophysical Journal*, 106(2), p.192a.

Rima, M. et al., 2021. Antimicrobial peptides: a potent alternative to antibiotics. *Antibiotics (Basel, Switzerland)*, 10(9), p.1095.

Rizzato, C. et al., 2019. Potential role of biofilm formation in the development of digestive tract cancer with special reference to *Helicobacter pylori* Infection. *Frontiers in Microbiology*, 10, pp.1-21.

Rossi, E. et al., 2017. 'It's a gut feeling' –*Escherichia coli* biofilm formation in the gastrointestinal tract environment. *Critical Reviews in Microbiology*, 44(1), pp.1–30.

Roy, R., Tiwari, M., Donelli, G. and Tiwari, V., 2017. Strategies for combating bacterial biofilms: a focus on anti-biofilm agents and their mechanisms of action. *Virulence*, 9(1), pp.522–554.

Rutherford, S.T. and Bassler, B.L., 2012. Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harbor Perspectives in Medicine*, 2(11), pp. a012427–a012427.

Sabino, H.A.C. et al., 2022. Biofilm and planktonic antibiotic resistance in patients with acute exacerbation of chronic rhinosinusitis. *Frontiers in Cellular and Infection Microbiology*, 11, p.813076.

Santos, A.L.S. et al., 2018. What are the advantages of living in a community? a microbial biofilm perspective! *Memórias Do Instituto Oswaldo Cruz*, 113(9), pp.1-7.

Schachter, B. 2003. Slimy business—the biotechnology of biofilms. *Nature Biotechnology*, 21(4), pp.361–365.

Scherr, T.D. et al., 2015. *Staphylococcus aureus* biofilms induce macrophage dysfunction through leukocidin AB and alpha-toxin. *MBio*, 6(4), pp.1-13.

Schneider, T. et al., 2010. Plectasin, a fungal defensin, targets the bacterial cell wall precursor lipid II. *Science*, 328(5982), pp.1168–1172.

Secor, P.R. et al., 2018. Entropically driven aggregation of bacteria by host polymers promotes antibiotic tolerance in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences*, 115(42), pp.10780–10785.

Shang et al., 2021. Trp-containing antibacterial peptides impair quorum sensing and biofilm development in multidrug-resistant *Pseudomonas aeruginosa* and exhibit synergistic effects with antibiotics. *Frontiers in Microbiology*, 12, pp.1-16.

Sharma, D. et al., 2015. Comparative proteomic analysis of aminoglycosides resistant and susceptible *Mycobacterium tuberculosis* clinical isolates for exploring potential drug targets. *PLOS ONE*, 10(10), pp.1-18.

Sharma, D. et al., 2016. Cytosolic proteome profiling of aminoglycosides resistant *Mycobacterium tuberculosis* clinical isolates using MALDI-TOF/MS. *Frontiers in Microbiology*, 7, pp.1-13.

Sharma, D., Misba, L. and Khan, A.U., 2019. Antibiotics versus biofilm: an emerging battleground in microbial communities. *Antimicrobial Resistance & Infection Control*, 8(1), pp.1-10.

Singh, A.K. et al., 2017. Standardization and classification of *in vitro* biofilm formation by clinical isolates of *Staphylococcus aureus*. *Journal of Global Infectious Diseases*, 9(3), pp.93–101.

Singh, S., Datta, S., Narayanan, K.B. and Rajnish, K.N., 2021. Bacterial exo-polysaccharides in biofilms: role in antimicrobial resistance and treatments. *Journal of Genetic Engineering and Biotechnology*, 19(1), pp.1-19.

Solano, C. et al., 2014. Biofilm dispersion and quorum sensing. *Current Opinion in Microbiology*, 18, pp.96–104.

Somma, A.D., Moretta, A., Canè, C., Cirillo, A. and Duilio, A., 2020. *Inhibition of Bacterial Biofilm Formation* [Online]. *www.intechopen.com*. IntechOpen. Available at: https://www.intechopen.com/chapters/70594#:~:text=Biofilm%20formation%20requires%20three%20different [Accessed: 21 April 2022].

Stewart, P.S., 2015. Antimicrobial tolerance in biofilms. *Microbiology Spectrum*, 3(3), pp.1-30.

Stoodley, P., Sauer, K., Davies, D.G. and Costerton, J.W., 2002. Biofilms as complex differentiated communities. *Annual Review of Microbiology*, 56(1), pp.187–209.

Tan, E. L., 2014. Evaluation of antibacterial property of short modified antibacterial peptides derived from phage display library against *Pseudomonas aeruginosa*. Bachelor's Degree. Universiti Tunku Abdul Rahman, pp. 1-60.

Tan, K. X., 2018. Screening for bacterial DNA-binding ability of antibacterial peptide, PAM-5. Bachelor's Degree. Universiti Tunku Abdul Rahman, pp. 1-89.

Tao, R. et al., 2011. Antimicrobial and antibiofilm activity of pleurocidin against cariogenic microorganisms. *Peptides*, 32(8), pp.1748–1754.

Thi, M.T.T., Wibowo, D. and Rehm, B.H.A., 2020. *Pseudomonas aeruginosa* biofilms. *International Journal of Molecular Sciences*, 21(22), p.8671.

Thulshan Jayathilaka, E.H.T. et al., 2021. Octominin: an antibacterial and anti-biofilm peptide for controlling the multidrug resistance and pathogenic *Streptococcus parauberis*. *Fish & Shellfish Immunology*, 110, pp.23–34

Torres, N.S. et al., 2018. Antimicrobial and antibiofilm activity of synergistic combinations of a commercially available small compound library with colistin against *Pseudomonas aeruginosa*. *Frontiers in Microbiology*, 9, pp.1-12.

Toyofuku, M. et al., 2015. Environmental factors that shape biofilm formation. *Bioscience, Biotechnology, and Biochemistry*, 80(1), pp.7–12.

Tuon, F.F., Dantas, L.R., Suss, P.H. and Tasca Ribeiro, V.S., 2022. Pathogenesis of the *Pseudomonas aeruginosa* Biofilm: a review. *Pathogens*, 11(3), p.300.

Uruén, C. et al., 2020. Biofilms as promoters of bacterial antibiotic resistance and tolerance. *Antibiotics*, 10(1), p.3.

Van Loosdrecht, M.C., Lyklema, J., Norde, W., Schraa, G. and Zehnder, A.J., 1987. The role of bacterial cell wall hydrophobicity in adhesion. *Applied and Environmental Microbiology*, 53(8), pp.1893–7.

Van Loosdrecht, M.C.M., Norde, W. and Zehnder, A.J.B., 1990. Physical chemical description of bacterial adhesion. *Journal of Biomaterials Applications*, 5(2), pp.91–106.

Ventola, C.L., 2015. The antibiotic resistance crisis: part 1: causes and threats. *P & T : A Peer-Reviewed Journal for Formulary Management*, 40(4), pp.277–83.

Vestby, L.K., Grønseth, T., Simm, R. and Nesse, L.L., 2020. Bacterial biofilm and its role in the pathogenesis of disease. *Antibiotics*, 9(2), p.59.

Vilcinskas, A., 2013. Evolutionary plasticity of insect immunity. *Journal of Insect Physiology*, 59(2), pp.123–129.

Wang, G., 2014. Human antimicrobial peptides and proteins. *Pharmaceuticals*, 7(5), pp.545–594.

Wang, G., Mishra, B., Epand, R.F. and Epand, R.M., 2014. High-quality 3D structures shine light on antibacterial, anti-biofilm and antiviral activities of human cathelicidin LL-37 and its fragments. *Biochimica Et Biophysica Acta (BBA) - Biomembranes*, 1838(9), pp.2160–2172.

Wang, Q. et al., 2018. HJH-1, a broad-spectrum antimicrobial activity and low cytotoxicity antimicrobial peptide. *Molecules: a Journal of Synthetic Chemistry and Natural Product Chemistry*, 23(8), p.2026.

Wang, Y.et al., 2008. Snake cathelicidin from *Bungarus fasciatus* is a potent peptide antibiotics. *PloS One*, 3(9), p.e3217.

Wei, Q. and Ma, L., 2013. Biofilm matrix and its regulation in *Pseudomonas aeruginosa*. *International Journal of Molecular Sciences*, 14(10), pp.20983–21005.

Wijesinghe, G. et al., 2019. Influence of laboratory culture media on *in vitro* growth, adhesion, and biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Medical Principles and Practice*, 28(1), pp.28–35.

World Health Organisation, 2021. *Antimicrobial Resistance* [Online]. Who.int. Available at: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance [Accessed: 28 December 2021].

Wu, M. and Li, X., 2015. *Chapter 87 - Klebsiella pneumoniae and Pseudomonas aeruginosa* [Online]. ScienceDirect. Available at: https://www.sciencedirect.com/science/article/pii/B9780123971692000871 [Accessed: 15 March 2022].

Yamada, Kelsey J. and Kielian, T., 2018. Biofilm-leukocyte cross-talk: impact on immune polarization and immunometabolism. *Journal of Innate Immunity*, 11(3), pp.280–288.

Yasir, M., Willcox, M. and Dutta, D., 2018. Action of antimicrobial peptides against bacterial biofilms. *Materials*, 11(12), p.2468.

Zhang, Q.-Y. et al., 2021. Antimicrobial peptides: mechanism of action, activity and clinical potential. *Military Medical Research*, 8, p.48.

Zheng, S. et al., 2021. Implication of surface properties, bacterial motility, and hydrodynamic conditions on bacterial surface sensing and their initial adhesion. *Frontiers in Bioengineering and Biotechnology*, 9, pp.1-22.

Zhu, X. et al., 2015. Bactericidal efficiency and modes of action of the novel antimicrobial peptide T9W against *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, 59(6), pp.3008–3017.

APPENDIX A

LIST OF LABWARE AND EQUIPMENT

Labware/Equipment	**Manufacturers**
Incubator	Memmert, Germany
Analytical balance	METTLER TOLEDO, USA
Microplate reader	BMG Labtech FLUOstar Omega, Australia
Biosafety Cabinet Level-2	ESCO, Singapore
Vortex mixer	Stuart, United States
Pipettor sets	Eppendorf Research® plus, Germany
96 well Flat Bottom Microplates,	Greiner CELLSTAR®, Germany
50 mL falcon tube, sterile	Nest Scientific USA Inc.
Petri dishes	JET BIOFIL®, Guangzhou, China
1.5 mL Microcentrifuge tube	DispoZ, FC-BIOS Sdn Bhd
Micropipette tips	Nest Scientific USA Inc.

APPENDIX B

PREPARATION OF BUFFERS, REAGENTS, AND MEDIA

Preparation of 50% glycerol solution

A volume of 50 mL absolute glycerol was dissolved into 50 mL of sterile distilled water to the final concentration of 50% (v/v) with total volume of 100 mL. The 50 % glycerol solution was the autoclaved at 121°C and 15 psi for 20 minutes.

Preparation of Mueller-Hinton (MH) broth

An amount of 4.2 g MH broth powder was dissolved in 200 mL of distilled water and the medium was autoclaved at 121°C and 15 psi for 20 minutes.

Preparation of Brain Heart Infusion (BHI) broth

An amount of 14.8 g BHI broth powder was dissolved in 400 mL of distilled water and the medium was autoclaved at 121°C and 15 psi for 20 minutes.

Preparation of Mueller-Hilton (MH) agar

The Difco™ MH agar powder was weighed at 15.2 g and dissolved in 400 mL of distilled water. The dissolved agar powder was then autoclaved at 121°C and 15 psi for 20 minutes. After incubation, the medium was stored inside incubator at 70°C and cool down under flowing tap water before poring onto petri dish. Then, the medium was poured into sterile petri dishes and the poured agar was allowed to solidify before storing them in 4°C.

Preparation of phosphate buffered saline (PBS) solution (pH 7.4)

Four PBS tablets (MP Medicals, LLC) were dissolved in approximately 380 mL of distilled water. Then, the pH of the solution was adjusted to pH 7.4 by using 1 molar of sodium hydroxide solution and measured with pH meter. After adjusted the pH to 7.4, the volume of PBS solution was topped up to 400 mL with distilled water and the PBS solution was then taken for autoclaved at 121°C and 15 psi for 20 minutes.

Preparation of 0.5% (w/v) crystal violet solution

An amount of 0.2 g crystal violet powder was dissolved in 10 ml of 99.98% methanol. 30 mL of sterile distilled water was added to the dissolved crystal violet hence the final volume of the crystal violet solution was 40 mL.

Preparation of 33% (v/v) acetic acid solution

A volume of 13 mL glacial acetic acid was added into 27 mL of distilled water and the acetic acid solution was at 121°C and 15 psi for 20 minutes.

Preparation of 0.3% (w/v) MTT solution

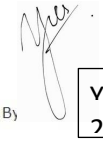
Three mg of MTT powder was dissolved in 1 ml of sterile degassed PBS (pH 7.4). The MTT solution was taken for sonication and vortex to completely dissolve the MTT powder. Then, the MTT solution was filter sterile through 0.22 µm syringe filter.

Document Viewer

Turnitin Originality Report

Processed on: 22-Apr-2022 01:39 +08
ID: 1816529769
Word Count: 13902
Submitted: 1

BM_FYP_THESIS_TURNITIN_WONG LI CHIK_SUB2 By Wong



Yuen Hawk
22/04/2

Similarity Index
7%

Similarity by Source
Internet Sources: 4%
Publications: 5%
Student Papers: N/A

include quoted | include bibliography | excluding matches < 8 words | mode: quickview (classic) report | Change mode | print | refresh | download

<1% match (publications)
Kye-Hwan Byun, Sang Ha Han, Min Woo Choi, Si Hong Park, Sang-Do Ha, "Effect of sublethal concentrations of bactericidal antibiotics on mutation frequency and stress response of Listeria monocytogenes", Food Research International, 2022

<1% match (publications)
Krishnan Ganesh Prasath, Rajaiah Alexpandi, Rayar Parasuraman, Murugesan Pavithra et al, "Anti-inflammatory potential of myristic acid and palmitic acid synergism against systemic candidiasis in Danio rerio (Zebrafish)", Biomedicine & Pharmacotherapy, 2021

<1% match (publications)
MASATOSHI TANAKA, "Analysis of Quinolone Resistance Mechanisms in a Sparfloxacin-Resistant Clinical Isolate of Neisseria gonorrhoeae", Sexually Transmitted Diseases, 10/1998

<1% match (Internet from 11-Apr-2022)
https://www.aimspress.com/fileOther/HTML-OLD/microbiology/microbiol-02-00412.htm

<1% match (publications)
Ramesh Salini, Shunmugiah Karutha Pandian, "Interference of quorum sensing in urinary pathogen Serratia marcescens by Anethum graveolens", Pathogens and Disease, 2015

<1% match (publications)
Dejing Shang, Xue Han, Wanying Du, Zhiru Kou, Fengquan Jiang, "Trp-Containing Antibacterial Peptides Impair Quorum Sensing and Biofilm Development in Multidrug-Resistant Pseudomonas aeruginosa and Exhibit Synergistic Effects With Antibiotics", Frontiers in Microbiology, 2021

Universiti Tunku Abdul Rahman			
Form Title : Supervisor's Comments on Originality Report Generated by Turnitin for Submission of Final Year Project Report (for Undergraduate Programmes)			
Form Number: FM-IAD-005	Rev No.: 1	Effective Date: 3/10/2019	Page No.: 1of 1

FACULTY OF SCIENCE

Full Name(s) of Candidate(s)	WONG LI CHIK
ID Number(s)	19ADB06362
Programme / Course	Biomedical Science
Title of Final Year Project	Screening for Anti-biofilm Effect of Antibacterial Peptide PAM-5 on Clinical Strain of Multidrug-Resistant *Pseudomonas aeruginosa*

Similarity	**Supervisor's Comments (Compulsory if parameters of originality exceed the limits approved by UTAR)**
Overall similarity index: 7 **%** **Similarity by source** Internet Sources: 4 % Publications: 5 % Student Papers: N/A %	
Number of individual sources listed of more than 3% similarity: 0	

Parameters of originality required and limits approved by UTAR are as follows:

(i) Overall similarity index is 20% and below, and

(ii) Matching of individual sources listed must be less than 3% each, and

(iii) Matching texts in continuous block must not exceed 8 words

Note: Parameters (i) – (ii) shall exclude quotes, bibliography and text matches which are less than 8 words.