# SCREENING OF ANTIBACTERIAL SPECTRUM OF SYNTHETIC PEPTIDE PAM-5 ON SELECTED GRAM-POSITIVE BACTERIA

By

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## ABSTRACT

### SCREENING OF ANTIBACTERIAL SEPCTRUM OF SYNTHETIC PEPTIDE PAM-5 ON SELECTED GRAM-POSITIVE BACTERIA

**Ding Yi En**

Antibacterial peptides (ABPs) have been extensively studied as a potential alternative antibacterial agent against bacterial infections. PAM-5, a synthetic antibacterial peptide, was previous shown active against several strains of Gram-negative bacteria encompassing both reference and clinically isolated antibiotic-resistance strains. However, little is known about its activity against Gram-positive bacteria. Hence, the objective of this study was to screen for the potency of PAM-5 towards several Gram-positive bacteria using microbroth dilution assay. Briefly, selected Gram-positive bacteria encompassed reference strains of *Staphylococcus aureus, Enterococcus faecalis, Streptococcus pyogenes,* and a clinical isolate of *Streptococcus anginosus* were treated with different concentrations of PAM-5 followed by titer determination of the viable bacteria to determine the minimal bactericidal concentration of the peptide. The surface disruptive effect of PAM-5 on the bacteria was then observed by scanning electron microscopy (SEM). Besides, the potency of PAM-5 was also compared to polymyxin B and melittin. The findings demonstrated that PAM-5 possessed heterogeneous potencies against these bacteria, in which it was not active against *E. faecalis* (MBC > 256 µg/mL), poorly active against *S. aureus* (MBC = 256

μg/mL), moderately active against *S. pyogenes* (MBC = 64 μg/mL) and highly active against *S. anginosus* (MBC = 4 μg/mL). SEM analysis revealed that PAM-5 was unable to cause surface disruption to the treated bacteria. Apart from that, the potencies of PAM-5 against these Gram-positive bacteria were generally lower as compared to polymyxin B and melittin. In conclusion, apart from its potent effects against Gram-negative bacteria as shown in previous studies, PAM-5 exhibited limited spectrum of antibacterial activity against Gram-positive bacteria.

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## DECLARATION

I hereby declare that the project 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.

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DING YI EN

**APPROVAL SHEET**

The project report entitled **"SCREENING OF ANTIBACTERIAL SEPCTRUM OF SYNTHETIC PEPTIDE PAM-5 ON SELECTED GRAM-POSITIVE BACTERIA"** was prepared by DING YI EN 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:

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**PERMISSION SHEET**

It is hereby certified that DING YI EN (ID No: 15ADB05985) has completed this final year project entitled "SCREENING OF ANTIBACTERIAL SEPCTRUM OF SYNTHETIC PEPTIDE PAM-5 ON SELECTED GRAM-POSITIVE BACTERIA" under the supervision of Mr. Yuen Hawk Leong from the Department of Biomedical Science, 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,

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(DING YI EN)

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **A** | Alanine |
| **ABPs** | Antibacterial Peptides |
| **ATCC** | American Type Culture Collection |
| **BA** | Blood agar |
| **BHI** | Brain-Heart Infusion |
| **CFU** | Colony Forming Unit |
| **CL** | Cardiolipin |
| **CLSI** | Clinical and Laboratory Standard Institute |
| **CVCC** | China Veterinary Culture Collection Center |
| **Dab** | α,γ-diaminobutyric |
| **DNA** | Deoxyribonucleic acid |
| **F** | Phenylalanine |
| **G** | Glycine |
| **GAS** | Group A streptococcus |
| **GelE** | Gelatinase |
| **HNP** | Human Neutrophil Peptides |
| **I** | Isoleucine |
| **K** | Lysine |
| **L** | Leucine |
| **LB** | Luria-Bertani |
| **M** | Metionine |
| **MBC** | Minimum Bactericidal Concentration |

| | |
|---|---|
| **MH** | Mueller-Hinton |
| **MIC** | Minimum Inhibitory Concentration |
| **MprF** | Outer membrane modifier enzyme |
| **mRNA** | messenger Ribonucleic acid |
| **MSA** | Mennitol-Salt agar |
| **P** | Proline |
| **PBS** | Phosphate Buffer Saline |
| **PC** | Phosphatidylcholine |
| **PE** | Phosphatidylethanolamine |
| **PG** | Phosphatidylglycerol |
| **PS** | Phosphatidylserine |
| **Q** | Glutamine |
| **R** | Arginine |
| **RNA** | Ribonucleic acid |
| **rpm** | Rotation per minute |
| **S** | Serine |
| **SAG** | Streptococcus anginosus group |
| **Sak** | Staphylokinase |
| **SEM** | Scanning Electron Microscope |
| **SIC** | Streptococcal inhibitor of complement |
| **SM** | Sphingomyelin |
| **SpeB** | Streptococcal pyrogenic exotoxin B |
| **T** | Threonine |
| **TA** | Teichoic acid |
| **TSA** | Tryptic Soy agar |

| | |
|---|---|
| **V** | Valine |
| **VRE** | Vancomycin-resistant enterococci |
| **W** | Tryptophan |

# CHAPTER 1

## INTRODUCTION

Despite numerous new antibiotics being developed over the past few decades, the unwarranted use of these compounds has substantially reduced their efficacy due to the emergence and dissemination of drug and multidrug-resistant bacteria worldwide. The rapid dissemination of these multidrug-resistant bacteria has raised the concern about the sustainability of conventional antibiotics in combating infections caused by these bacteria. In fear of the possible post-antibiotic era, various studies have been conducted to search for novel and alternative therapeutics to overcome the crisis. Of all the promising antibacterial agents that are understudy, antibacterial peptides (ABPs) have gained considerable research attention in view of their advantages over the conventional antibiotics (Batoni et al., 2016).

Since the discovery of ABPs in 1938 (Lee, 2015), the peptides have been studied extensively with the hope that these biological entities could serve as an alternative or complementary medicine to conventional antibiotics against bacterial infections. Preceding studies thereafter further demonstrated that these short stretch of peptides which occurs naturally or synthetically, possess multiple antibacterial features. One of the prominent features of ABPs is the wide spectrum of bacterial targets that are usually not limited to a particular genus or species. Conjointly, evidences have also been documented that several natural

ABPs are active against a broad spectrum of bacterial targets, which encompasses both Gram-positive and Gram-negative bacteria (Liu et al., 2010). Nevertheless, very few synthetic ABPs possess this broad-spectrum feature as compared to natural peptides. In fact, many synthetic ABPs were found to be active against several bacteria, yet, the spectrum of bacterial target is usually restricted to either one of the Gram-stain categories.

Furthermore, it is widely accepted that majority of the well-studied ABPs exert its antibacterial effects through a series of membrane interactions. These initial interface interactions between the peptides and bacterial surface are important for the subsequent mechanisms of action which leads to bacteriostatic or bactericidal effects (Hollmann et al., 2018). As the bacterial membrane represents the primary contact site between the bacteria and ABPs, any additional structure or entities that prevent the direct contact of ABPs to the membrane might substantially reduce the potency or efficacy of the peptides (Malanovic and Lohner, 2016). In considerations to this, it is anticipated that the presence of a thick cell wall layer in Gram-positive bacteria may reduce the direct access of certain ABPs to the bacterial membrane, thus, reducing the potency of the peptides towards the bacteria. This anticipation could explain the findings on the limited spectrum of bacterial targets by many synthetic ABPs that are mostly active against Gram-negative bacteria only (Volzing et al., 2013).

In this study, a 15-mer synthetic peptide, namely PAM-5 was studied. Over the past few years, successive studies were carried out on PAM-5 to screen for its

antibacterial spectrum on selected pathogenic bacteria which include various reference and multi-drug resistant strains of Gram-negative bacteria such as *Pseudomonas aeruginosa, Escherichia coli,* and *Klebsiella pneumoniae* (Chan, 2016; Leong, 2018; Yong, 2018) (unpublished data). Further studies on PAM-5 by Phoon (2016) (unpublished data) also demonstrated that the bactericidal effects of this peptide are mainly mediated by membrane disrupting mechanisms on Gram-negative bacteria.

Limited study, however, was carried out to screen for the effects of PAM-5 on Gram-positive bacteria. Till date, the potency of PAM-5 was only screened against one Gram-positive bacteria *Staphylococcus aureus* (*S. aureus*) by Chan in 2016 (unpublished data), which revealed that PAM-5 can only exert its complete killing effect against this bacterium at a high minimum bactericidal concentration (MBC). Subsequently, an early conclusion was made based on this finding stating that PAM-5 is less effective against Gram-positive bacteria. Nonetheless, such conclusion was unjustifiable as it was made based on only single Gram-positive bacterium. Therefore, this study aimed to provide a more thorough comprehension on the antibacterial spectrum of PAM-5 on Gram-positive bacteria. The objectives of studies were:

1. To screen for the potency of PAM-5 on selected Gram-positive bacteria using microbroth dilution assay
2. To screen for surface-disruptive effects of PAM-5 on Gram-positive bacteria through scanning electron microscope (SEM) analysis.

3. To compare the potency of PAM-5 to melittin and polymyxin B on Gram-positive bacteria.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 The Antibiotic Resistance Crisis

Antibiotic discovery is one of the biggest turning points in the field of modern medicine, revolutionizing therapeutic paradigm for bacterial infections. Ever since the discovery of penicillin in 1928, the clinical application of antibiotics had contributed to significant reduction of morbidity and mortality rate of bacterial infections for a few decades (Gaynes, 2017). However, the optimism on these agents as the universal cure for all bacterial infections was seriously impinged by the increasing incidence and prevalence of antibiotic-resistant bacteria. As the consequence of abuse usage, antibiotics are slowly losing their efficacies against many bacteria which are able to acquire resistance towards these compounds rapidly via mutations. According to a report by the World Health Organization (WHO) in 2014, the crisis of antibiotic resistance has been classified as one of the most significant public health threats. Based on the report, the current annual death toll resulted from antibiotic-resistant bacteria is approximately 700, 000. If this medical issue is not given serious attention, the figure is estimated to surge to 10 million in 2050.

The major factors contributing to the high prevalence and incidence of antibiotic-resistance is mainly human-associated, attributed by misuse of the agent both in healthcare and agricultural sector. In fact, close monitoring studies have revealed

that the therapeutic efficacy of an antibiotic is not sustainable. Based on the analysis of antibiotic history, the introduction of a novel antibiotic is always followed by emergence of resistance to that antibiotic shortly thereafter (Davies and Davies, 2010; Podolsky, 2018).

More complicatedly, this situation is further exacerbated by the depletion of new antibiotic pipeline from pharmaceutical industries due to multifaceted factors. These include the lack of commercial interest by pharmaceutical companies in research and development of new antibiotics. As compared to other long-demanding drugs for chronic illness such as hypertension and diabetes mellitus, investment in antibiotic development does not seem to be profitably rewarding as the latter are usually prescribed for short term use (Aslam et al., 2018). In addition to that, the introduction of new antibiotic to the market is becoming more difficult due to tighter regulatory barriers established by the United States Food and Drug Administration (FDA). Consequently, these factors have reduced the supply of new antibiotic of novel mechanisms into the market, thus limiting the choices of effective antibiotics available to treat infections by drug-resistant bacteria (Lee, 2015).

Hence, on the verge of desperation, an alternative to antibiotics is urgently needed to combat the issue of antibiotic-resistance before the world is moving towards the post-antibiotic era.

## 2.2 Limitation of Antibiotics

Apart from the above-mentioned human-associated and other environmental factors, the intrinsic limitations of the antibiotic might serve as another drawback that reduces its antibacterial efficacy. One of these limitations is the high target specificity of theses therapeutic compounds. Antibiotics are generally categorized into different classes based on their mechanisms of action. For example, beta-lactam antibiotics such as penicillin, cephalosporin and carbapenems act by inhibiting cell wall synthesis through binding to transpeptidases (Fair and Tor, 2014). Aminoglycosides, which include gentamicin, amikacin and neomycin, is a class of antibiotics which acts by binding to 30S ribosomal subunit, resulting in inaccuracy of mRNA translation and failure of protein synthesis (Li et al., 2014; Arenz and Wilson, 2016). On the other hand, quinolones (e.g. cinoxacin, nalidixic acid, ciprofloxacin etc.) exert their antibacterial effect by interfering with DNA replication (Aldred et al., 2014). The action of each of these antibiotic classes is so specific that they would not cross-react on other bacterial target sites that are not specific. When a bacterium is exposed to an antibiotic at its sub-inhibitory concentration in a long run, independent mutation might cause certain simple alteration to these specific antibiotic-binding sites (Martinez and Baquero, 2000). In the combination of antibiotic specific mode of action and the genetic plasticity of bacteria, simple modification or mutation to these antibiotic target sites would reduce or prevent the binding of the antibiotics on these targets (Zhu et al., 2015). As such, this would increase the risk of inducible resistance.

Moreover, many antibiotics have been shown to possess slow inhibitory or killing kinetics where long durations that range from hours to days are usually required to suppress or kill the treated bacteria completely. Example of the antibiotics are vancomycin, ciprofloxacin and linezolid, which were shown to kill their target bacteria completely after 24 hours of treatment (Mohamed et al., 2016). In another study, a duration of 12 hours was needed by amikacin in order to achieve complete killing of its target bacteria (Mohamed et al., 2014). These slow antibacterial actions might provide survival advantage to those fast replicating bacteria with high mutational capacity in an infected host (Richardson, 2017). Under the antibiotic stress, the surviving bacteria might acquire inducible resistance that allows the bacteria to outcompete the antibacterial action of these slow acting antibiotics. Subsequently, strains of bacteria with the resistant capacity will dominate the infection and compromise the efficacy of those antibiotics.

In consideration to the above-mentioned limitations, it is an imminent need to explore for novel antibacterial agents which act differently than antibiotics. Apart from having potent antibacterial efficacy, these novel agents should possess special characteristics that are able to overcome the limitations of antibiotics so that the issue of inducible resistance will not ensue.

## 2.3 Antibacterial Peptides

### 2.3.1 Overview

Antibacterial peptides (ABPs) are short peptides composed of approximately 8 to 50 amino acid residues. As implied in the name, these peptides possess antibacterial effects towards bacteria either selectively or in a broad spectrum manner (Hancock and Falla, 1996; Nusslein et al., 2006). Majority of ABPs are cationic in nature with a net positive charge ranging from +2 to +9. Structural studies revealed that ABPs are generally present in different configurations such as α-helix, β-sheet, loop and extended structures (Brogden, 2005; Pushpanathan et al., 2013). Ever since the discovery of ABP in 1939, these peptides are widely studied for their biological active mechanisms and potency (Bahar and Ren, 2013). The multidimensional properties of ABPs have attracted considerable attention from scientists worldwide in view of their potential to become alternative antibacterial agent to antibiotics. As some of the earliest discovered ABPs such as cecropins, defensins, magainins and buforins were isolated from the immune system of living organisms, it is believed that these peptides might provide a thorough protection against bacteria (Park et al., 2000; Omardien et al., 2016).

### 2.3.2 Advantages of ABPs

Accumulating data from many studies have proposed that ABPs are promising candidate of alternative antibacterial agents as they possess certain advantages that allow them to work in ways better than conventional antibiotics.

Firstly, many ABPs are not or least harmful towards mammalian cells. Instead, they are selectively toxic to prokaryotic cells such as bacteria. This selective toxicity is attributed to the difference in the membrane structure between prokaryotic and eukaryotic cells. For prokaryotic cells, the surface membrane is composed of phospholipid bilayers which contain a high proportion of electronegative constituents such as phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidylserine (PS) (Giulio and Zhao, 2006; Ebenhan et al., 2014). On the other hand, the membranes of eukaryotic cells such as mammalian cells are mostly composed of zwitterionic phospholipid such as phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (SM), contributing to an overall neutrally charged membrane (Brender et al., 2012). These principal differences in phospholipid stoichiometry and architecture between the both membranes lead to the preferential binding and adsorption of cationic ABPs onto the anionic bacterial cell membrane driven by electrostatic interaction, which explain the selective toxicity towards prokaryotic cells (Seo et al., 2012; Malanovic and Lohner, 2016).

In contrast to the specific mode of action by antibiotics, ABPs elicit their antibacterial effects by targeting multiple cellular determinants on the surface and/or within bacterial cells. These actions include disruption of cellular membrane integrity, inhibition of cell wall, nucleic acid, and protein synthesis (Lee et al., 2016; Bechinger and Gorr, 2017; Kumar et al, 2018). As multiple sites of the bacteria are targeted simultaneously by these ABPs, it is metabolic costly for the bacteria to undergo several mutations concurrently to alter these target sites. Thus, the risk of inducible resistance by these bacteria is lower

towards the peptides than the conventional antibiotics (Munita and Arias, 2016; Wang et al., 2016).

One of the ABPs with multiple antibacterial mechanisms is bactenecin, in which the monomeric form of this peptide was shown to inhibit the synthesis of cell wall, protein and nucleic acid simultaneously (Lee et al., 2009). Apart from that, a human neutrophil peptide named α-defensin, was also found to be active against multiple targets such as protein translational process, RNA transcription and cell wall biosynthesis (Leeuw et al., 2012; Brook et al., 2016, Le et al., 2017). Mechanistic studies on indolicidin also showed that the peptide was able to inhibit DNA synthesis and induce filamentation in *Escherichia coli* followed by bacterial death (Fallat et al., 1996; Subbalakshmi and Sitaram, 1997). These multi-hit mechanisms are usually associated with bactericidal effect of the ABPs, which further reduces the risk of bacterial resistance to the peptides.

Furthermore, many ABPs were shown to exert rapid killing effects towards their target bacteria, in which complete bactericidal effect can be achieved within minutes of treatment (Mohamed et al., 2016). A study on a synthetic peptide named T9W had reported that it was able to kill *P. aeruginosa* ATCC 27853 completely within 5 minutes of treatment. Additionally, the peptide was also able to kill various strains of drug-resistance *P. aeruginosa* completely within 30 minutes of treatment. These rapid killing effects impose an additional strength to ABPs on the battle against bacteria as the latter is deprived of the time for adaptation and acquiring mutational-mediated resistance (Zhu et al., 2015).

Most importantly, many ABPs were shown to possess good antibacterial potency against a broad spectrum of bacteria from different families. In particular to certain host ABPs, where their antibacterial spectrum can even encompass bacterial species from both Gram-positive and Gram-negative categories. This broad spectrum of bacterial targets is attributed to the non-receptor-mediated actions of ABPs on their bacterial sites. According to the findings by Le et al. (2017), majority of the membrane-active ABPs execute their bactericidal actions via non-receptor-mediated binding to bacterial membrane, followed by membrane disruption and lysis of the bacteria. These non-specific actions allow an ABP to act on bacteria of different species, which is way much efficient than most of the antibiotics with specific mechanisms.

### 2.3.3 Previous Findings on the Spectrum of ABPs

In view of the advantages as described previously, ABPs have been extensively studied in various aspects for their potential clinical application. Numerous ABPs from the natural sources have been isolated and studied for their antibacterial potencies, spectrum of bacterial targets, mechanisms of action as well as potential toxicity to mammalian cells. One of the examples of natural ABPs is indolicidin that is isolated from bovine neutrophils. This short peptide was reported to have broad spectrum of antimicrobial activities against both Gram-positive and Gram-negative bacteria including *Pseudomonas aeruginosa, Salmonella typhimurium, Escherichia coli, Staphylococcus aureus* and *Staphylococcus epidermidis* (Hancock and Falla, 1996; Subbalakshmi and Sitaram, 1997).

A natural ABP isolated from human neutrophils, namely LL-37, was found to possess broad spectrum antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria (Turner et al., 1998; Shurko et al., 2018). Interestingly, the synthetic derivative of this natural ABP namely cathelicidin ChMAP-38 was shown to exert similar good potency to its paternal peptide. This peptide was reported to possess broad spectrum antibacterial effects against *Micrococcus luteus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and several strains of multidrug-resistance bacteria such as *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* (Panteleev et al., 2018). Conversely, most of the conventional antibiotics are narrow spectrum where they are only effective against certain bacterial family or Gram-category.

However, profound limitations were found in the subsequent studies of natural peptides as isolation and purification of these compounds are laborious, tedious and time consuming (Rai et al., 2016). Furthermore, although it is known that majority of the ABPs possess selective toxicity towards bacteria, certain natural peptides such as melittin and polymyxin B are cytotoxic to mammalian cells and haemolytic (Rady et al., 2017). Alternatively, successive studies on ABPs focus on the *de novo* prototype design and production of synthetic peptides using phage displayed peptide selection (Wu et al., 2016), truncated derivatives (Yan et al., 2003) or modification from the natural ABPs (Panteleev et al., 2018). An example of synthetic peptide derived from natural peptide is buforin II, an analogue to buforin I which can be isolated from Asian toad stomach. While the 39-amino acid buforin I possess weaker antimicrobial effects, buforin II with 21

amino acid residues has a stronger and broader spectrum of antibacterial activities on both Gram-positive and Gram-negative bacteria (Park et al., 2000). Apart from that, synthetic peptide KW-13 also demonstrated a broad spectrum antibacterial targets against clinical isolates of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (Liu et al., 2015). A recent study by Wang et al. (2018) showed that a cationic synthetic peptide HJH-1, an analogue to haemoglobin α-subunit of bovine erythrocytes P3 has a wide spectrum antibacterial effects on *Escherichia coli* ATCC 29522, *Staphylococcus aureus* ATCC 29213, and *Salmonella pullorum* CVCC 3533.

Although a wide range of ABPs is able to act against broad-spectrum of bacterial targets, some ABPs only selectively act on Gram-positive or Gram-negative bacteria. For instance, several ABPs were reported to act selectively against Gram-positive bacteria. These include Temporin-1SPb (Ashcroft et al., 2004), Bombinin H7 (Mangoni et al., 2000), Phylloseptin-H11 (Thompson et a., 2007) and Thuricin S (Chehimi et al., 2007). On the other hand, several ABPs which are selective against Gram-positive bacteria were also documented. These include Casecidin 15 (Birkemo et al., 2009), Japonicin-1CDYa (Jin et al., 2009), GLK-19 (Wang et al., 2009) and A3-APO (Szabo et al., 2010). Structural analysis revealed that these ABPs are short peptides consist of 13 – 19 amino acids, which might explain the relative narrow spectrum of these ABPs as compared to those naturally occurring ABPs which are relatively longer. Therefore, the variability in the antibacterial spectrum may be attributed to their structural differences.

## 2.4 Synthetic peptide PAM-5

PAM-5, a rationally designed synthetic peptide, consists of 15 amino acids with the sequence of K-W-K-W-R-P-L-K-R-K-L-V-L-R-M. This peptide was modified from a 12-mer phage-displayed peptide selected from a biopanning process against *Pseudomonas aeruginosa* (*P. aeruginosa*) (Gwee, 2012; Lee, 2015) (unpublished data).

In an earlier study by Lee (2015) (unpublished data), promising antibacterial effects was demonstrated by PAM-5 when it was initially screened for its potency towards *P. aeruginosa* ATCC 27853. Subsequently, the antibacterial spectra of this peptide was further tested on other Gram-negative bacteria encompassing both reference strains and clinically isolated drug-resistant strains (Chan, 2016; Leong, 2018; Yong, 2018) (unpublished data). As presented in **Table 2.1**, PAM-5 exhibited promising bactericidal effects towards all the tested Gram-negative bacteria, including certain drug and multidrug-resistant bacteria such as extended spectrum beta-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli* at different minimal bactericidal concentrations (MBCs) that ranged from 8 μg/mL to 32 μg/mL. However, despite the averagely good potency against the Gram-negative bacteria, PAM-5 was shown to be poorly active against *Staphylococcus aureus*, which was the only one Gram-positive bacterium included in **Table 2.1**. This finding could not stand alone to conclude that PAM-5 is ineffective against Gram-positive bacteria as the single bacterium may not reflect the overall susceptibility of this Gram category to PAM-5. Therefore, in this study, PAM-5 was screened for its potency towards additional

species and strains of Gram-positive bacteria to evaluate its spectral coverage for this bacterial category.

**Table 2.1** Minimum bactericidal concentrations (MBCs) of PAM-5 on selected Gram-positive and Gram-negative bacteria as screened previously by Chan (2016); Leong (2018); Yong (2018) (unpublished data).

| **Bacteria** | **Strains** | **MBC (μg/ml)** |
|---|---|---|
| *Pseudomonas aeruginosa* | ATCC 27853 | 8 |
| | Cefazolin-resistant (C.I.) | 16 |
| | Multidrug-resistant (C.I.) | 16 |
| *Escherichia coli* | ATCC 25922 | 8 |
| | Penicillin- & Cefazolin-resistant (C.I.) | 16 |
| | Extended spectrum beta lactamases producing (C.I.) | 32 |
| *Klebsiella pneumoniae* | ATCC 13883 | 32 |
| | Carbapenem resistant Enterobacteriaceae | 8 |
| | Extended spectrum β-lactamases producing (C.I.) | 16-32 |
| *Acinetobacter baumannii* | ATCC 19606 | 8 |
| *Acinetobacter junii* | Cefazolin- and cephalosporin (3rd gen.)-resistant | 16 |
| *Salmonella typhi* | C.I. | 16-32 |
| *Shigella flexneri* | C.I. | 32 |
| *Staphylococcus aureus* | ATCC 25923 | 96 |

*C.I. indicates clinical isolates

# CHAPTER 3

# MATERIALS AND METHODOLOGY

## 3.1 General Experimental Design

In this study, PAM-5 was tested for its potency against several species of Gram-positive pathogenic bacteria using microbroth dilution assay. In brief, the bacteria were treated with different concentrations of PAM-5 in a 96-well microtiter plate. After overnight incubation, the treated bacteria were inoculated onto agar media to examine the bacterial viability as well as to determine the minimum bactericidal concentration (MBC) of PAM-5 and other comparative ABPs. The assay for each bacteria was triplicated to ensure reproducibility of the results. Finally, the treated bacteria were subjected to scanning electron microscope (SEM) analysis to examine any structural or morphological changes on the surface of PAM-5 treated Gram-positive bacteria as compared to non-treated bacteria.

## 3.2 Materials

### 3.2.1 Glassware, Consumable and Equipment

Refer to Appendix A.

### 3.2.2 Preparation of Buffer and Media

Refer to Appendix B.

### 3.2.3 Bacterial Strains

A total of four bacterial strains were employed in this study. Three of them were obtained from the Department of Biomedical Science, Universiti Tunku Abdul Rahman, which included *Streptococcus pyogenes* (*S. pyogenes*) ATCC 19615, *Staphylococcus aureus* (*S. aureus*) ATCC 25923, and *Enterococcus faecalis* (*E. faecalis*) ATCC 19433. The remaining bacterium, which was a clinical isolate of *Streptococcus anginosus* (*S. anginosus*) 1360589, was obtained from the pathology laboratory from Gleneagles Medical Centre, Penang.

### 3.2.4 Preparation of Bacterial Glycerol Stock and Master Culture Plate

The bacteria were first enriched on enrichment media and/or selective media catered for each bacterium. *S. aureus* was inoculated onto Mannitol-Salt agar (MSA), *S. pyogenes* on blood agar (BA) while *E. faecalis* and *S. anginosus* were inoculated onto tryptic soy agar (TSA). After inoculation, the agar plates were incubated overnight at 37°C. On the next day, each bacterium was grown in its

respective enriching liquid media after ensuring the purity of the culture, *S. pyogenes*, *S. anginosus* and *E. faecalis* were grown in Brain-Heart Infusion (BHI) broth, while *S. aureus* was grown in Luria-Bertani (LB) broth. The bacteria were grown to their respective late-log phase. Upon that, the bacterial culture was centrifuged at 6, 000 x g to obtain the bacterial pellet. After discarding the supernatant, the pellet was washed by resuspending it with PBS (pH 7.4) followed by centrifugation at 6, 000 x g for 6 minutes. These washing steps were repeated at least twice. After the last washing, the bacterial pellet was resuspended with 1 mL of PBS (pH 7.4) and added with equivalent volume of 50% (v/v) glycerol to produce the bacterial stock in glycerol at the final concentration of 25% (v/v). Finally, 500 µL of bacterial suspension was transferred into each microcentrifuge tube. The tubes were kept in cryogenic-box and stored in -80°C freezer.

Prior to performing antibacterial assay, the bacteria were retrieved from the glycerol stock and inoculated onto selective/enrichment media aforementioned as a master culture plate. The cultures were incubated overnight at 37°C and retrieved on the following day. For short term storage, the master culture plates of *S. aureus*, *E. faecalis*, and *S. anginosus* were stored in 4°C, while *S. pyogenes* was stored in room temperature for a maximum of seven days to ensure the freshness of the bacteria.

### 3.2.5 PAM-5 Synthesis

PAM-5 (KWKWRPLKRKLVLRM) was synthesized and purchased from Bio Basic Inc. (Canada). The peptide was received in lyophilised form with a purity of 79.48% as determined by reverse-phase high-performance liquid chromatography. The molecular mass of the peptide was determined as 2038.63 Da through mass spectrometry. The peptide was stored at -20°C in a dry and tightly sealed, screw-capped vial supplemented with silica gels.

### 3.2.6 Preparation of PAM-5 Solution

Prior to dissolving the peptide, the peptide vial was allowed to equilibrate to room temperature for approximately one hour. Using an analytical balance, an amount of 1, 024 µg of PAM-5 was weighed in a silica vial. Then the peptide was dissolved with 100 µL of degassed, filtered-sterilized distilled water, followed by addition of 900 µL of degassed, filtered-sterilised phosphate buffer saline (PBS) to a final concentration of 1,024 µg/mL. After that, the peptide was two-fold serially diluted with degassed, filtered-sterilised PBS from the stock concentration of 1, 024 µg/mL to 4 µg/mL in silica vials. The diluted peptides were stored in 4°C for a maximum period of seven days to ensure the efficacy of peptides as recommended by the manufacturer.

### 3.2.7 Preparation of Polymyxin B Solution

Polymyxin B was purchased from Merck Millipore. It is a strong antibacterial peptide against many bacteria (Yu et. al., 2015). In this study, Polymyxin B was

used as comparative ABP to PAM-5 for the screening of antibacterial potency against the selected Gram-positive bacteria as mentioned in **Section 3.2.3**. The peptide solution was prepared using the similar procedure as described in the preparation of PAM-5 in **Section 3.2.6**.

### 3.2.8 Preparation of Melittin Solution

Apart from Polymyxin B, Melittin (Calbiochem®) was used as the second comparative ABP to PAM-5. At the initial stock concentration of 256 µg/mL, melittin was two-fold serially diluted from 256 µg/mL to 4 µg/mL with degassed, filtered-sterilised PBS in silica vials. The diluted peptides were stored in 4°C for a maximum period of seven days to ensure the peptide efficacy.

## 3.3 Methodology

### 3.3.1 Preparation of *Streptococcus pyogenes* Suspension for Antibacterial Assay

The antibacterial potency of PAM-5 against the selected Gram-positive pathogenic bacteria aforementioned in **Section 3.2.3** was determined by microbroth dilution assay. The procedures for this assay were adopted from Clinical and Laboratory Standard Institute (CLSI, 2018) with several modifications. The bacteria were grown at different conditions and media as described in the following sections.

An overnight bacterial suspension was prepared by inoculating two to three colonies of *S. pyogenes* from the master culture plate into 12 mL of Brain-Heart Infusion (BHI) broth. The culture was incubated at 37°C statically. On the following day, the absorbance of the overnight culture was measured to acquire the log phase of bacterial growth. The bacterial culture was then centrifuged at 10, 000 x g for 10 minutes at 4°C. After removing the supernatant, the bacteria pellet was washed by resuspending it with 2 mL of phosphate buffer saline (PBS, pH 7.4) followed by another round of centrifugation. These washing steps were repeated at least twice in order to remove the broth residues completely. Following the last wash, the bacterial pellet was re-suspended in 1 mL of PBS to become the crude bacteria.

In order to determine the titre of the bacteria, the crude bacteria suspension was subjected to 10-fold serial dilutions in BHI broth, followed by inoculating the diluted bacteria on tryptic soy agar (TSA). After overnight incubation at 37°C, the number of bacterial colony on the agar was counted, which provided the data to calculate the overall bacterial titre by using the equation as shown below:

$$\text{Bacterial titre (CFU/mL)} = \frac{\text{No.of colonies}}{\text{Volume inoculated X dilution factor}}$$

Bacteria which were diluted to the titre of $10^3$ CFU/mL was chosen as the inoculation titre for the antibacterial assay.

### 3.3.2 Preparation of *Staphylococcus aureus* Suspension for Antibacterial Assay

The preparation of *S. aureus* for the antibacterial assay was different from *S. pyogenes* in several aspects. An overnight bacterial suspension was prepared by inoculating two to three colonies of *S. aureus* from the master culture plate into 10 mL of Mueller-Hinton (MH) broth. The culture was incubated in a 37°C shaking incubator at the agitation of 200 rpm. After overnight incubation, 200 µL of the overnight culture was added into 20 mL of fresh MH broth, and allowed for further incubation at the same condition until it reaches its mid-log phase of bacterial growth, which was equivalent to the absorbance value that falls within the range of 0.500 – 0.600 at $OD_{600}$.

Once the mid-log phase was achieved, the bacterial culture was centrifuged at 6,500 x g at 4°C for 6 minutes. The bacteria pellet was washed twice by resuspending it with 2 mL of PBS (pH 7.4), followed by another round of centrifugation. Upon the last wash, the pellet was re-suspended in 1 mL of PBS.

Unlike *S. pyogenes*, during the titre determination, the suspension of *S. aureus* was subjected to 10-fold serial dilutions in PBS broth, followed by inoculating the diluted bacteria onto Mueller-Hinton (MH) agar. The number of colonies growing on the media were counted after overnight incubation at 37°C. The bacterial titre in crude bacteria was determined using the equation provided in **Section 3.3.1**. Bacteria with the dilution corresponding to the titre of $10^4$ CFU/mL was chosen for antibacterial assay.

### 3.3.3 Preparation of *Enterococcus faecalis* Suspension for Antibacterial Assay

The steps in preparation of *E. faecalis* bacterial suspension prior to antibacterial assay were similar to the steps as described in **Section 3.3.2.** However, instead of MH broth, Brain-heart infusion (BHI) broth was used in overnight culture and dilution of bacteria. After acquiring the crude bacterium, the bacterium was diluted to $10^3$ CFU/mL for the subsequent antibacterial assay.

### 3.3.4 Preparation of *Streptococcus anginosus* Suspension for Antibacterial Assay

Similar to the preparation of *E. faecalis*, BHI was used as the growing media for *S. anginosus* in the preparation of the bacterial suspension.

### 3.3.5 Antibacterial Assay

One hundred microliters of bacterial suspension with the inoculation titre of $10^3$ – $10^4$ CFU/mL was loaded into each well of a flat-bottomed 96-well microplate. The bacteria were then treated with 100 µL of PAM-5, each well with the peptide at the final concentration that ranged from 2 µg/mL to 256 µg/mL. Serving as the comparative ABPs for the antibacterial assay, both Polymyxin B and melittin were used to treat the bacteria in separate wells. For Polymyxin B, the final concentrations used to treat the bacteria ranged from 2 µg/mL to 256 µg/mL. On the other hand, the range of final concentrations of melittin used for the comparative study was 2 µg/mL to 64 µg/mL. Untreated bacteria in 100 µL of

PBS served as the negative control in this assay. The content in the wells was summarised in **Table 3.1** and **Figure 3.1**. After setting up the peptide treatment and negative control, the microtiter plate was subjected to 1-hour pre-incubation at 37°C prior to addition of enrichment broth into the treated bacteria. For *S. aureus*, the enrichment broth was MH broth, while BHI broth was used for *S. pyogenes, S. anginosus* and *E. faecalis.* The microtiter plate was then incubated at 37°C for approximately 16 to 18 hours.

On the next day, the content in the wells was visually inspected for the presence of turbidity as a sign of bacterial growth. Subsequently, 10 µL of the bacterial suspension from each well was inoculated onto the agar for gross view inspection on the antibacterial effect of PAM-5. Different inoculating media was used for different bacteria. For *S. aureus*, the treated and untreated bacteria were inoculated on MH agar; for *S. pyogenes,* the suitable inoculating media was blood agar, whereas tryptic soy agar (TSA) was used to grow the peptide-treated *S. anginosus* and *E. faecalis.*

Then, the turbid content in the wells of the microtiter plate that signified bacterial growth was serially diluted with PBS and inoculated onto semi-solid media as described above. The agar plates were incubated overnight at 37°C followed by colony counting to determine the titre of the viable bacteria. Minimum bactericidal concentration (MBC) is defined as the lowest concentration of peptide treatment which produce a 99.9% decrease of bacterial density (CLSI,

2012). **Figure 3.2** illustrates the determination of MBC in microbroth dilution assay.

**Table 3.1:** The content in each test and negative control well in microbroth dilution assay.

| Contents | Test Well | Negative Control Wells |
|---|---|---|
| Bacterial suspension (µL) | 100 | 100 |
| *ABPs (µL) | 100 | - |
| PBS (µL) | - | - |
| Enrichment broth (µL) | 50 | 50 |

*Antibacterial peptides (ABPs) interchange between PAM-5, polymyxin B and melittin in each assay.

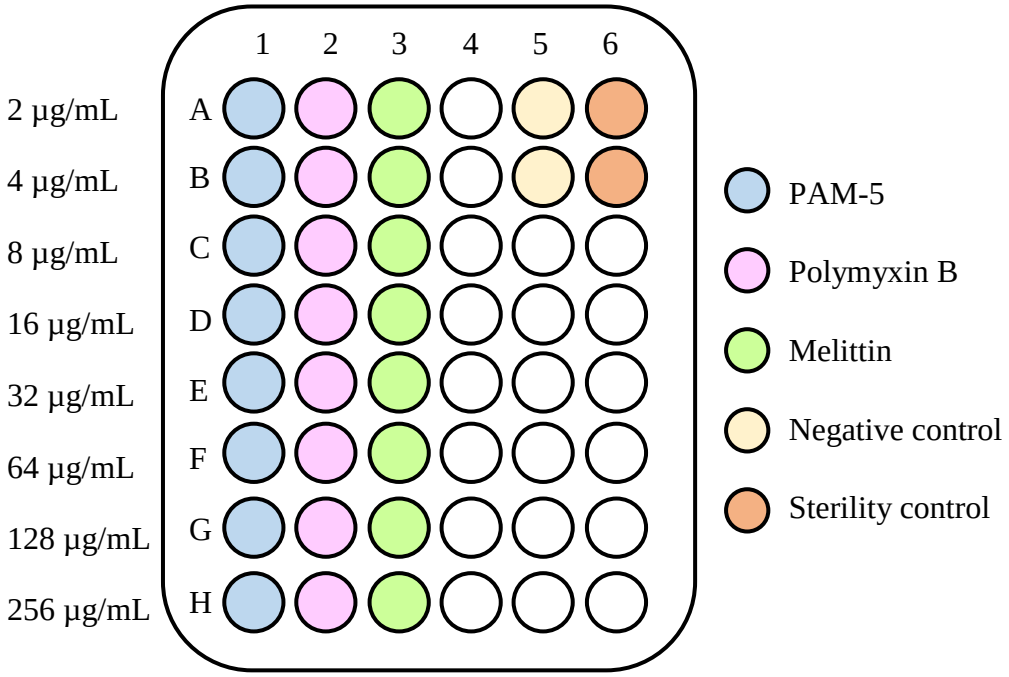


**Figure 3.1** Illustration for the summary of settings for antibacterial assay on 96-microtiter plate. Each coloured well was added with 100 µL of bacterial suspension treated with respective ABPs. Column 1) PAM-5; Column 2) Polymyxin B and Column 3) Melittin. Negative control was set up in well 5A and 5B while sterility control was set up in 6A and 6B.

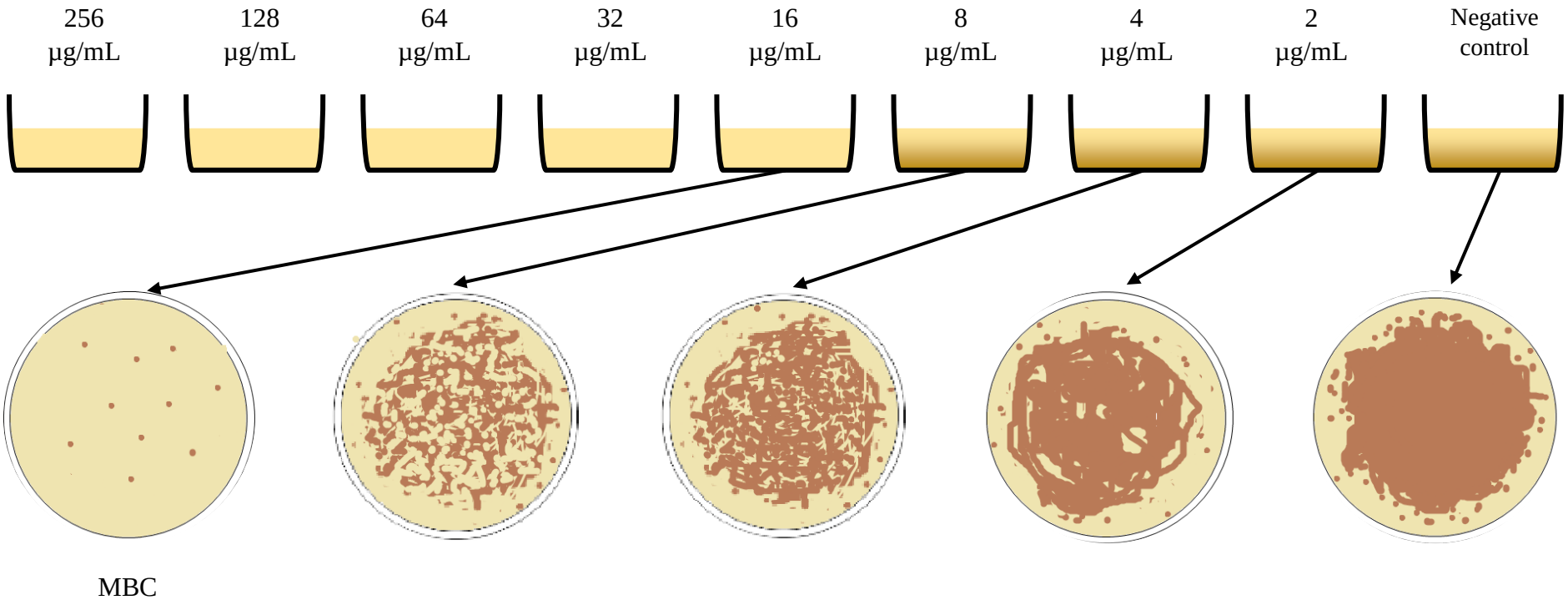


**Figure 3.2:** Illustration of MBC determination in microbroth dilution assay. Wells filled with bacteria treated with ABP at concentrations ranging from 2 µg/mL to 8 µg/mL are turbid as similar to the negative control. The MBC in this figure is 16 µg/mL (CLSI, 2018).

### 3.4 Scanning Electron Microscope (SEM) Analysis

In order to visualize the membrane-active effects of PAM-5 on Gram-positive bacteria, *Staphylococcus aureus* ATCC 25923 treated with PAM-5 was chosen for SEM analysis. In brief, the preparation of bacterial sample and treatment of the bacteria were carried out according to the protocols as described in **Section 3.3.2**. The titre of bacterial suspension for SEM analysis, however, was fixed at $10^7$ CFU/mL. One hundred microliters of the diluted bacterial suspension were loaded into a 1.5 mL micro-centrifuge tube, followed by treating the bacteria with 100 µL of PAM-5 at the concentration of 256 µg/mL. One hundred microliters of untreated bacteria were added with 100 µL of PBS to serve as the negative control.

The treated and untreated bacteria were incubated for an hour at 37°C. Subsequently, the bacterial samples were subjected to centrifugation at 6,000 x g for 6 minutes at 4°C. The supernatant was removed carefully with a pipette and the pellet was resuspended with 200 µL PBS. These washing were repeated twice. Next, 500 µL of 3% (v/v) glutaraldehyde in 0.1 M of PBS was added into the microcentrifuge tubes containing the pelleted bacteria. The tubes were then incubated at 4°C for approximately 18 hours. After that, the tubes were centrifuged at 4, 000 x g for 5 minutes at 4°C to remove the glutaraldehyde. The pellet was then washed with PBS. After washing, the samples were subjected to a series of dehydration by intermittent resuspension and centrifugation of bacteria samples in an increasing concentration of ethanol as shown in **Table 3.2.**

**Table 3.2:** Duration of centrifugation and concentration of ethanol used for dehydration of bacterial samples.

| Concentration of ethanol (v/v) | Duration of centrifugation (min) |
|---|---|
| 25% | 5 |
| 50% | 10 |
| 75% | 10 |
| 95% | 10 |
| 100% (performed in triplicates) | 10 |

After the serial dehydration by ethanol, further dehydration of the samples was performed using a freeze dryer (Scanvac COOLSAFE$^{TM}$) for approximately 18 hours. After the freeze drying, the samples were carefully transferred onto a carbon tape adhered to a copper stub, followed by immediate coating of the samples with platinum for around 50 seconds. Lastly, the copper stubs containing the samples were placed on a specimen holder of a scanning electron microscope (SEM) for viewing under different magnification. The morphological changes on the surface of treated and untreated bacteria were observed under magnification of 10,000X using the SEM (JSM-7610F).

# CHAPTER 4

# RESULTS

## 4.1 Antibacterial Effect of PAM-5 towards Selected Gram-positive Bacteria

The potency of antibacterial peptide PAM-5 was tested on several Gram-positive bacteria using microbroth dilution assay as described in **Section 3.3.2**. These bacteria included reference strains of *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 19433, *Streptococcus pyogenes* ATCC 19615 and a clinical isolate of *Streptococcus anginosus* 1360589. The potency of the peptide was determined by the value of minimum bactericidal concentration (MBC). Subsequently, the treated bacteria were serially diluted and inoculated onto agar media to assess the number of bacterial colonies growing on the media upon peptide treatment. These data were presented as the bacterial titre ($Log_{10}$ CFU/mL) after peptide treatment and were compared to the titre of untreated bacteria in order to evaluate the degree of inhibition by the peptide. These data were presented as the graphs as shown in the subsequent section. The comparison between the potency of PAM-5 with other well characterized ABPs, was carried out concurrently by treating the bacteria with polymyxin B and melittin in an independent set up. The titres of the ABP-treated bacteria as well as their corresponding MBCs were compared, which would provide the relative potencies between the ABPs.

### 4.1.1 Antibacterial Effect of PAM-5 towards *Staphylococcus aureus* ATCC 25923

**Figure 4.1 (a)** depicts the gross view of the growth of *Staphylococcus aureus* (*S. aureus*) ATCC 25923 after treating with different ABPs. Clearly shown in the figure, untreated *S. aureus* which served as the negative control grew heavily as a bacterial lawn on Plate W and Plate X. Similarly, the bacteria treated with PAM-5 at concentrations from 2 µg/mL to 128 µg/mL (Plate A to Plate G) were able to grow to the similar extent like the negative control, indicating the bacterium could survive the pressure of PAM-5 even at these high concentrations. However, under the treatment of PAM-5 at the highest concentration (256 µg/mL), *S. aureus* was almost eliminated, as indicated by the scanty bacterial colonies on the inoculating media (Plate H). This indicated that PAM-5 was only effective against this bacterium at the minimal bactericidal concentration (MBC) of 256 µg/mL.

Comparatively, polymyxin B was more active against *S. aureus* than PAM-5. With reference to **Figure 4.1 (a),** *S. aureus* could only survive the polymyxin B treatment at concentrations ranging from 2 µg/mL to 16 µg/mL, as shown by the heavy bacterial growth from Plate I to Plate L. The peptide was able to prevent the bacterial growth at 32 µg/mL, hence, this peptide concentration was defined as its MBC against this bacterium. Melittin, as compared to the other two peptides, demonstrated the greatest potency against *S. aureus*. Clearly demonstrated in **Figure 4.1 (b)**, melittin was able to achieve complete killing of *S. aureus* at MBC of two and four-fold lower than polymyxin B and PAM-5,

respectively. At this MBC (16 µg/mL), melittin was able to eliminate the bacteria by causing a titre reduction of approximately 7 $Log_{10}$ CFU/mL. Therefore, in terms of the action against *S. aureus*, PAM-5 was less potent as compared to polymyxin B and melittin.

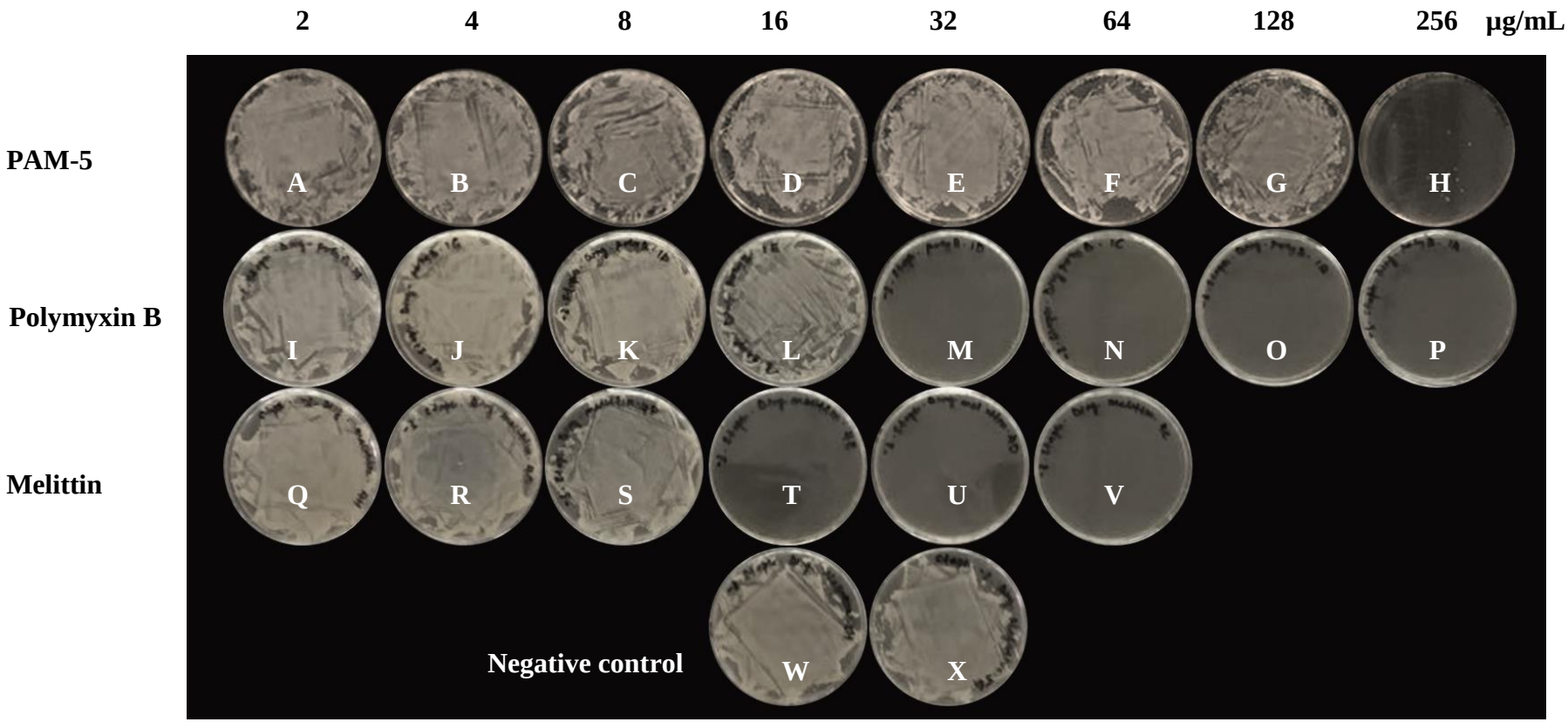


**Figure 4.1 (a):** Culture agar inoculated with *S. aureus* ATCC 25923 after treatment with different ABPs of different concentrations. The bacteria were treated with 2-fold increasing concentrations of the ABPs. Plate A to H were inoculated with PAM-5-treated bacteria, Plate I to P were inoculated with polymyxin B-treated bacteria, whereas Plate Q to V were inoculated with melittin-treated bacteria. Plate W and X are negative control which were inoculated with untreated bacteria. MBC of PAM-5, polymyxin B and melittin were determined as 256 µg/mL, 32 µg/mL and 16 µg/mL, respectively.

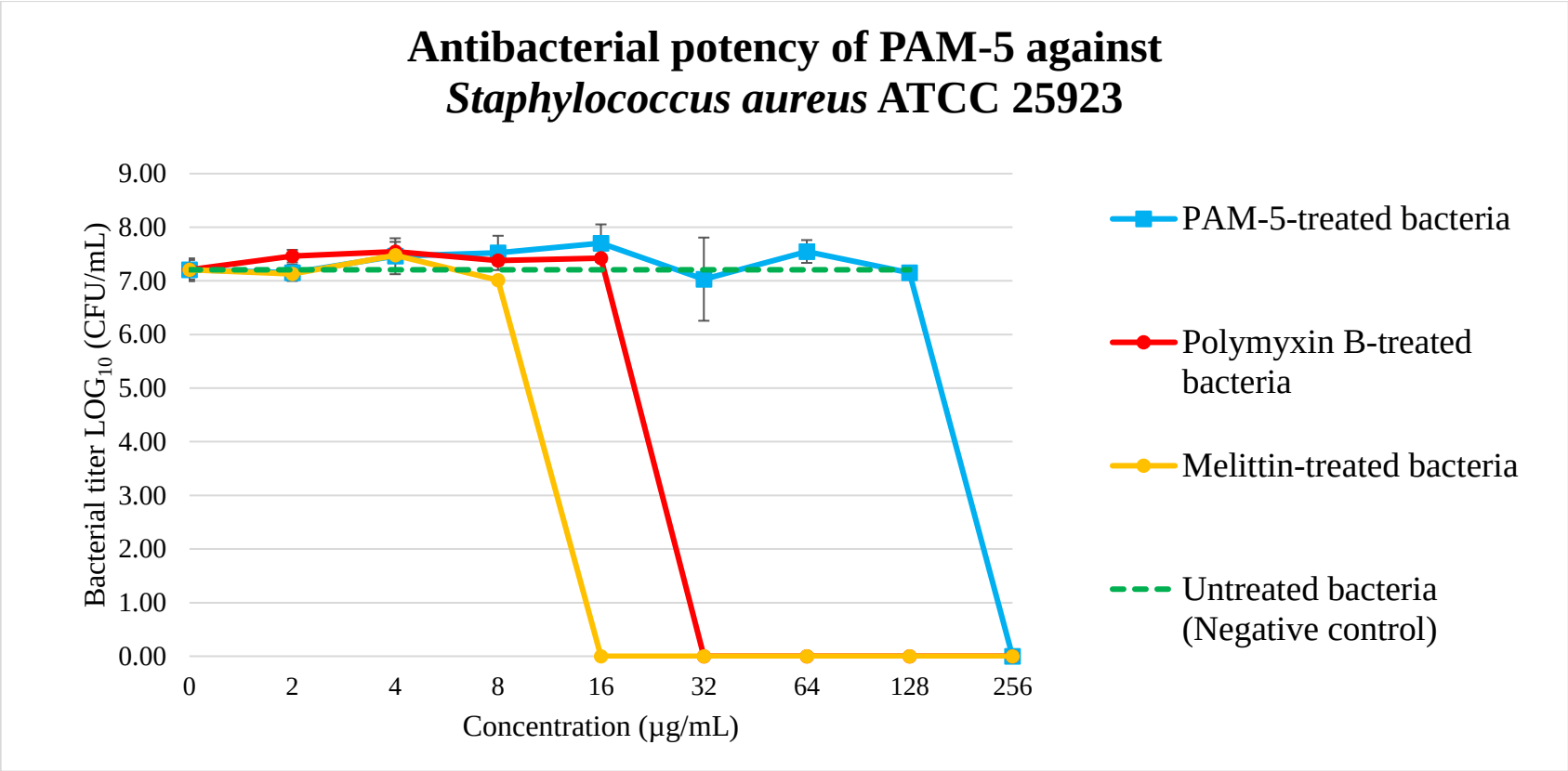


**Figure 4.1 (b):** Assessment of inhibitory effect of PAM-5, polymyxin B and melittin on *S. aureus* ATCC 25923 by microbroth dilution assay. Bacteria were treated with increasing concentrations of peptides and inoculated onto media after overnight incubation and serial dilution. The viability of the treated bacteria was determined by the bacterial titre in $Log_{10}$ CFU/mL.

### 4.1.2 Antibacterial Effect of PAM-5 towards *Enterococcus faecalis* ATCC 19433

While PAM-5 was found less active against *S. aureus,* it was totally non-active against *E. faecalis* ATCC 19433 at all tested concentration in this study. As demonstrated in **Figure 4.2 (a)**, despite treatment with PAM-5 at concentration as high as 256 µg/mL, the bacteria were able to survive and grew heavily on the inoculating media (Plate A to Plate H). With reference to the degree of inhibition as presented in **Figure 4.2 (b)**, no sign of antibacterial effect was seen. This was indicated by the relative similar titre between bacteria treated with PAM-5 and the negative control.

Despite being a more potent ABP than PAM-5 as demonstrated in previous studies, polymyxin B also failed to suppress the growth of *E. faecalis* at all tested concentrations although it was able to cause a slight reduction to the bacterial titre at 256 µg/mL. On the other hand, melittin demonstrated greater potency than PAM-5 and polymyxin B against *E. faecalis.* As shown in **Figure 4.2 (a)**, no bacterial colony was seen on Plate T which was inoculated with bacteria treated with 16 µg/mL of melittin. Upon that, no bacterial colony was observed on the subsequent media plates (Plate T to Plate V), indicating complete eradication of the bacteria by these higher concentrations of melittin. MBC of melittin was hence determined as 16 µg/mL. Based on these findings, PAM-5 was found to be less potent as compared to melittin against *E. faecalis.*

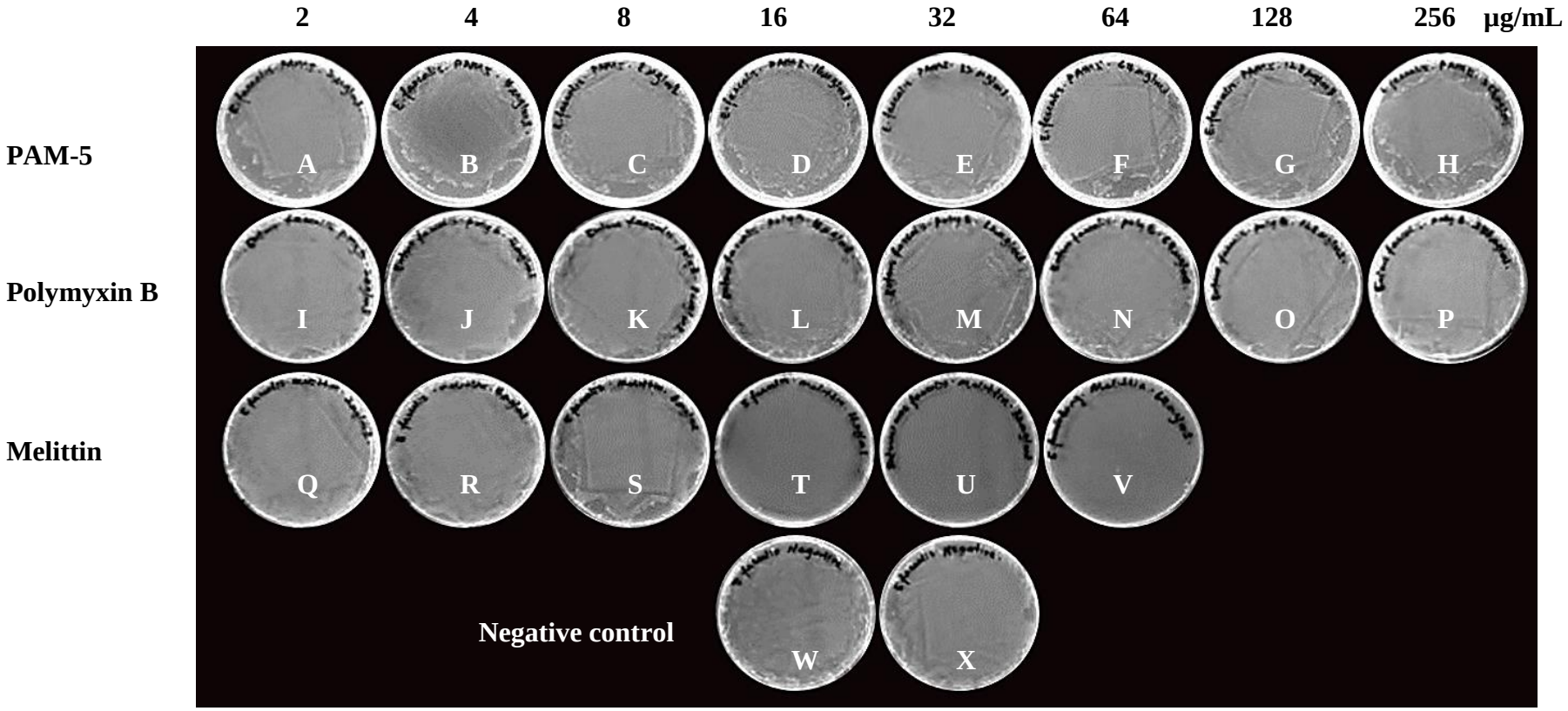


**Figure 4.2 (a):** Culture agar inoculated with *E. faecalis* ATCC 19433 after treatment with PAM-5 (first row Plate A to Plate H), polymyxin B (second row Plate I to Plate P) and melittin (third row Plate Q to Plate V) at increasing concentrations from left to right. Plate W and X were inoculated with untreated bacteria which served as the negative control. Both MBCs of PAM-5 and polymyxin B against *E. faecalis* were > 256 µg/mL, while MBC for melittin was determined as 16 µg/mL.

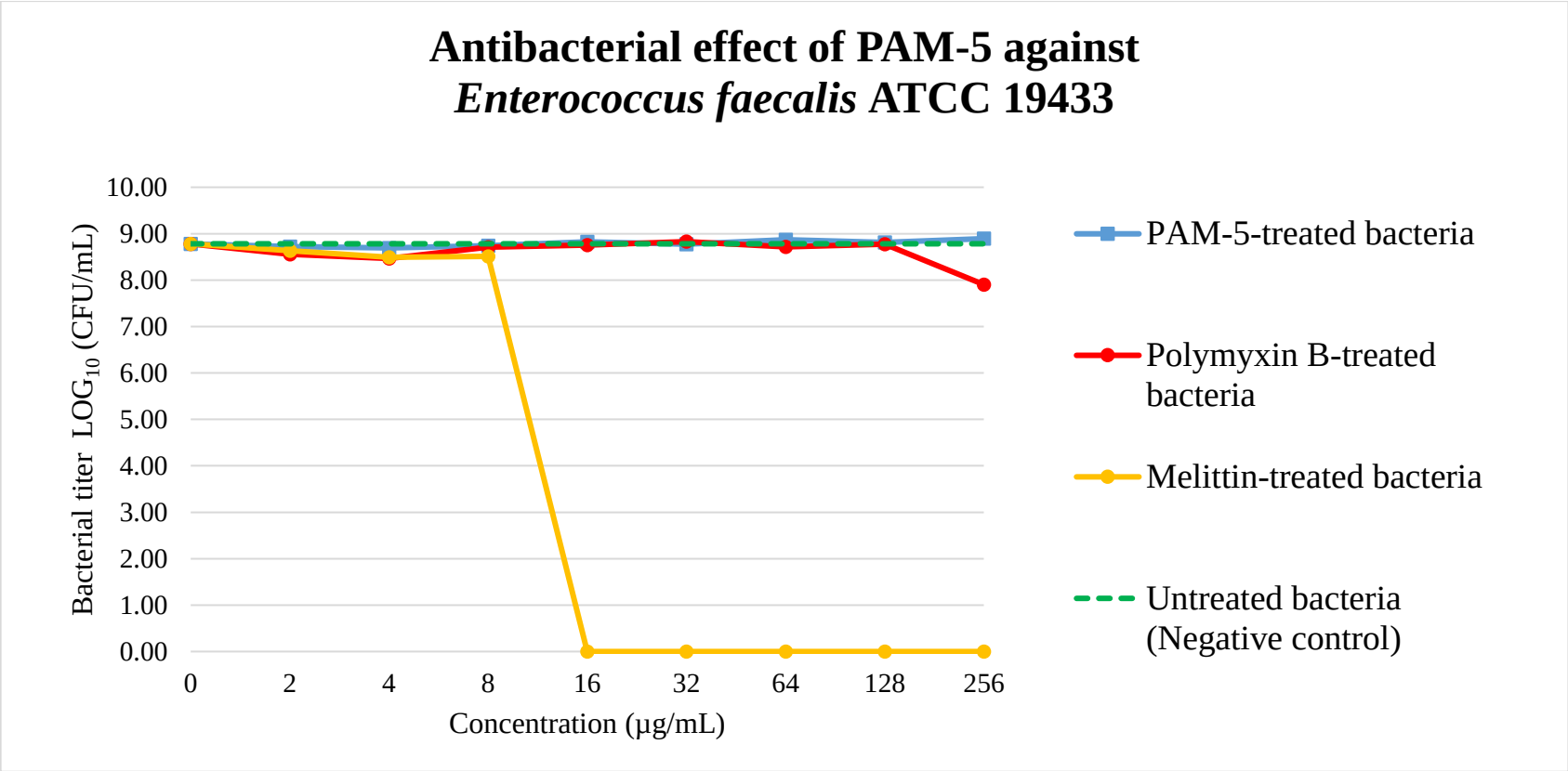


**Figure 4.2 (b):** Bacterial viability after treatment with PAM-5, polymyxin B and melittin. *Enterococcus faecalis* ATCC 19433 was incubated with increasing concentrations of ABPs followed by inoculation on media for titre determination. Untreated bacteria was set up as the negative control. The MBC for melittin against *E. faecalis* was 16 µg/mL while PAM-5 and polymyxin B were not active against this bacteria.

### 4.1.3 Antibacterial potency of PAM-5 towards *S. pyogenes* ATCC 19615

Despite the poor potencies of PAM-5 towards both *S. aureus* and *E. faecalis* as described previously, PAM-5 was shown to have better antibacterial effect on *S. pyogenes.* Clearly shown in **Figure 4.3 (a)**, the untreated bacteria which served as the negative control grew heavily as a lawn of bacteria on Plate W and Plate X whereby the lawn and other growing bacterial colonies were surrounded by notable zones of β-haemolysis. Comparatively, bacteria treated with PAM-5 at concentrations ranging from 2 µg/mL to 32 µg/mL (Plate A to Plate E) showed relatively similar extent of bacterial growth to that of the negative control (Plate W and Plate X). However, under the treatment of PAM-5 started from 64 µg/mL, *S. pyogenes* failed to grow as indicated by the absence of bacterial colonies on the inoculating media (Plate F to Plate H). This indicated that PAM-5 was effective against this bacterium at the minimum bactericidal concentration (MBC) of 64 µg/mL.

Comparatively, polymyxin B and melittin were found to be more effective against *S. pyogenes* as compared to PAM-5. With reference to **Figure 4.3 (a)**, *S. pyogenes* could survive the pressure of polymyxin B and melittin up to 4 µg/mL (Plate J) and 8 µg/mL (Plate Q), respectively. Above these concentrations, *S. pyogenes* was completely killed by the two ABPs. Therefore, the MBCs of polymyxin B and melittin against this bacterium were 8 µg/mL and 16 µg/mL, respectively. Both the peptides at these MBCs were able to reduce the bacterial titre by approximately 4.7 $Log_{10}$ CFU/mL as depicted in **Figure 4.3 (b)**. These

findings demonstrated that PAM-5 was less effective against *S. pyogenes* as compared to polymyxin B and melittin.

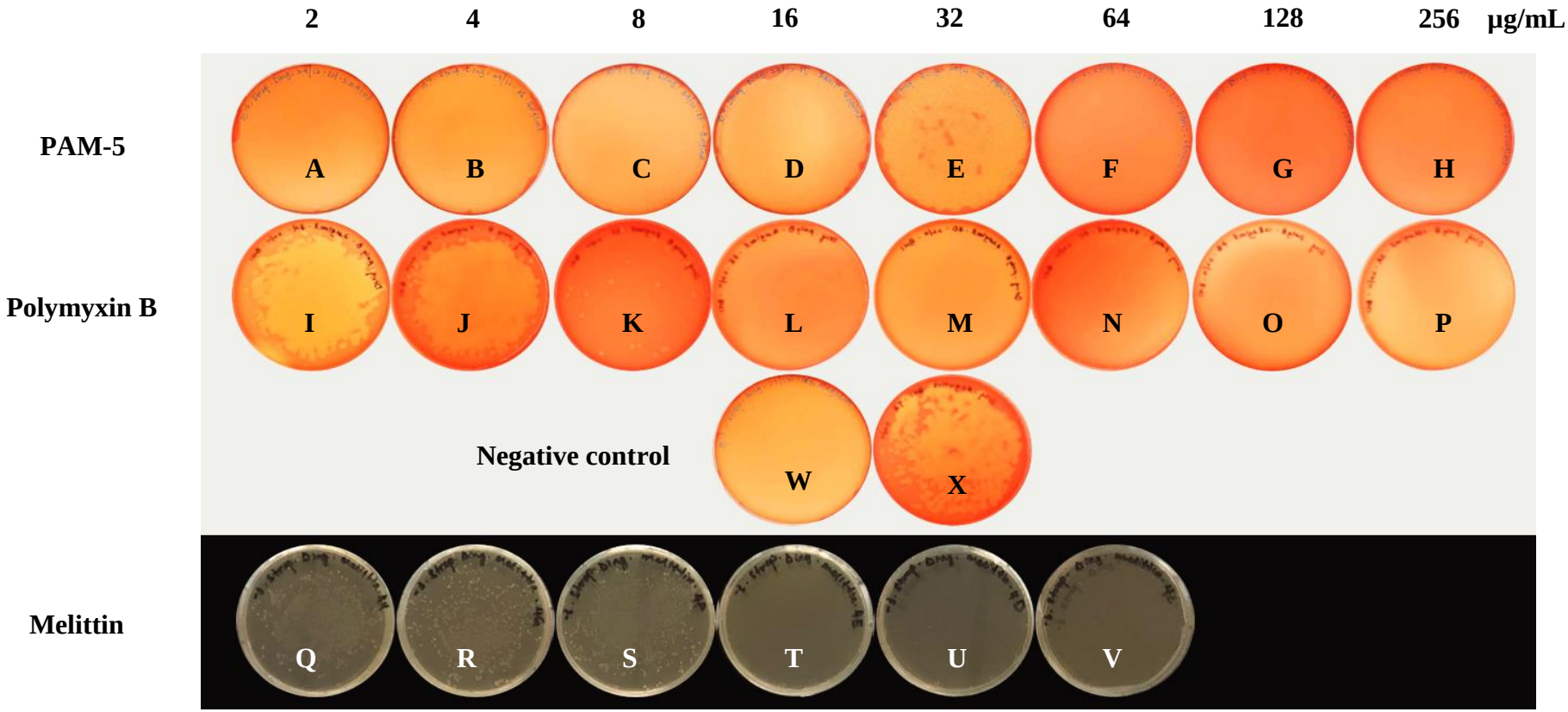


**Figure 4.3 (a):** Culture agar inoculated with *S. pyogenes* ATCC 19615. Plate A to H were inoculated with PAM-5-treated bacteria, Plate I to P were inoculated with polymyxin B-treated bacteria, while Plate Q to Plate V were inoculated with melittin-treated bacteria. The bacteria were treated with increasing concentrations of peptide from left to right (2 µg/mL to 256 µg/mL ) for PAM-5 and polymyxin B. The concentrations of melittin used for the treatment range from 2 µg/mL to 64 µg/mL. Plate W and X are negative control inoculated with untreated bacteria. MBCs of PAM-5, polymyxin B and melittin against *S. pyogenes* were determined as 64 µg/mL, 8 µg/mL and 16 µg/mL, respectively.

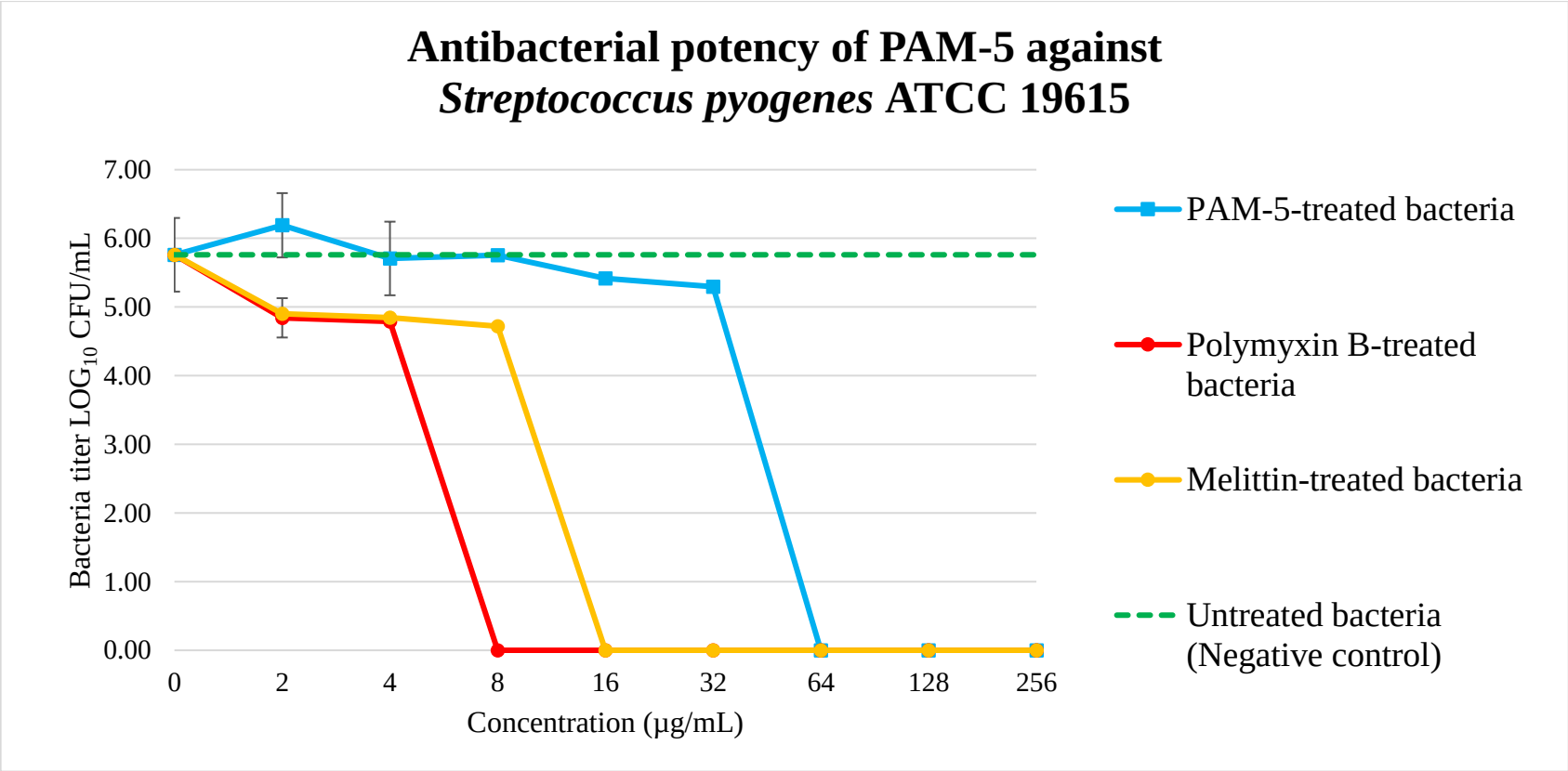


**Figure 4.3 (b):** Bacterial viability after treatment with PAM-5, polymyxin B and melittin. *Streptococcus pyogenes* ATCC 19615 was incubated with increasing concentrations of ABPs followed by inoculation on media for titre determination. Untreated bacteria was set up as negative control. The MBCs for PAM-5, polymyxin B and melittin against *E. faecalis* were 64 µg/mL, 16 µg/mL and 8 µg/mL, respectively.

### 4.1.4 Antibacterial Effect of PAM-5 towards Clinical Isolate of *Streptococcus anginosus* 1360589

Despite the poor potency on the Gram-positive bacteria as described previously, PAM-5 was found to possess good antibacterial effect on a clinical strain of *S. anginosus* 1360589. With reference to the gross view of the media inoculated with the treated bacteria [**Figure 4.4 (a)**], PAM-5 was able to kill *S. anginosus* completely starting from the concentration of 4 µg/mL to 256 µg/mL (Plate B to Plate H). At the MBC of 4 µg/mL, PAM-5 was able to eliminate the bacteria by approximately 8.5 $Log_{10}$ CFU/mL titre reduction [**Figure 4.4 (b)**]. This indicates that PAM-5 was effective against this bacteria even though at low concentrations.

However, PAM-5 was found to be less effective than polymyxin B as reflected by the lower MBC of the latter towards this bacterium (≤ 2 µg/mL). As shown in **Figure 4.4 (a)**, even at the lowest tested concentration of polymyxin B (2 µg/mL), the viability of the bacterium was completely supressed. Nevertheless, the MBC of polymyxin B against *S. anginosus* could not be determined as it could be lower than 2 µg/mL. On the other hand, melittin was found to possess lower potency against *S. aniginosus* as compared to the former two ABPs. As shown in **Figure 4.4 (a)**, complete killing of this bacterium can only be achieved at 32 µg/mL (Plate U), which was eight-fold higher than the MBC of PAM-5.

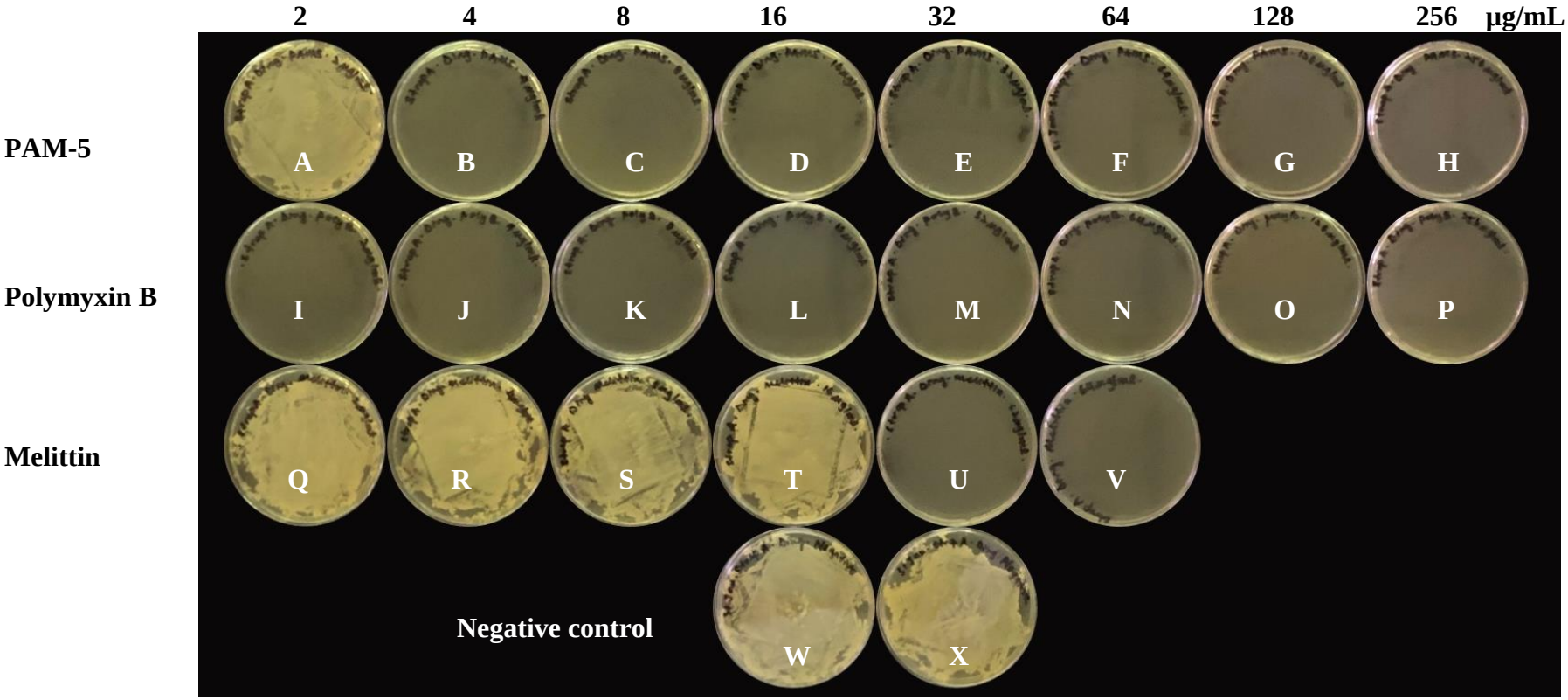


**Figure 4.4 (a):** Culture agar inoculated with *S. anginosus* 1360589 after treatment with different ABPs of different concentrations. The bacteria were treated with 2-fold increasing concentrations of the ABPs. Plate A to H were inoculated with PAM-5-treated bacteria, Plate I to P were inoculated with polymyxin B-treated bacteria, whereas Plate Q to V were inoculated with melittin-treated bacteria. Plate W and X are negative control inoculated with untreated bacteria. MBC of PAM-5, polymyxin B and melittin were determined as 4 µg/mL, ≤ 2 µg/mL and 32 µg/mL, respectively.

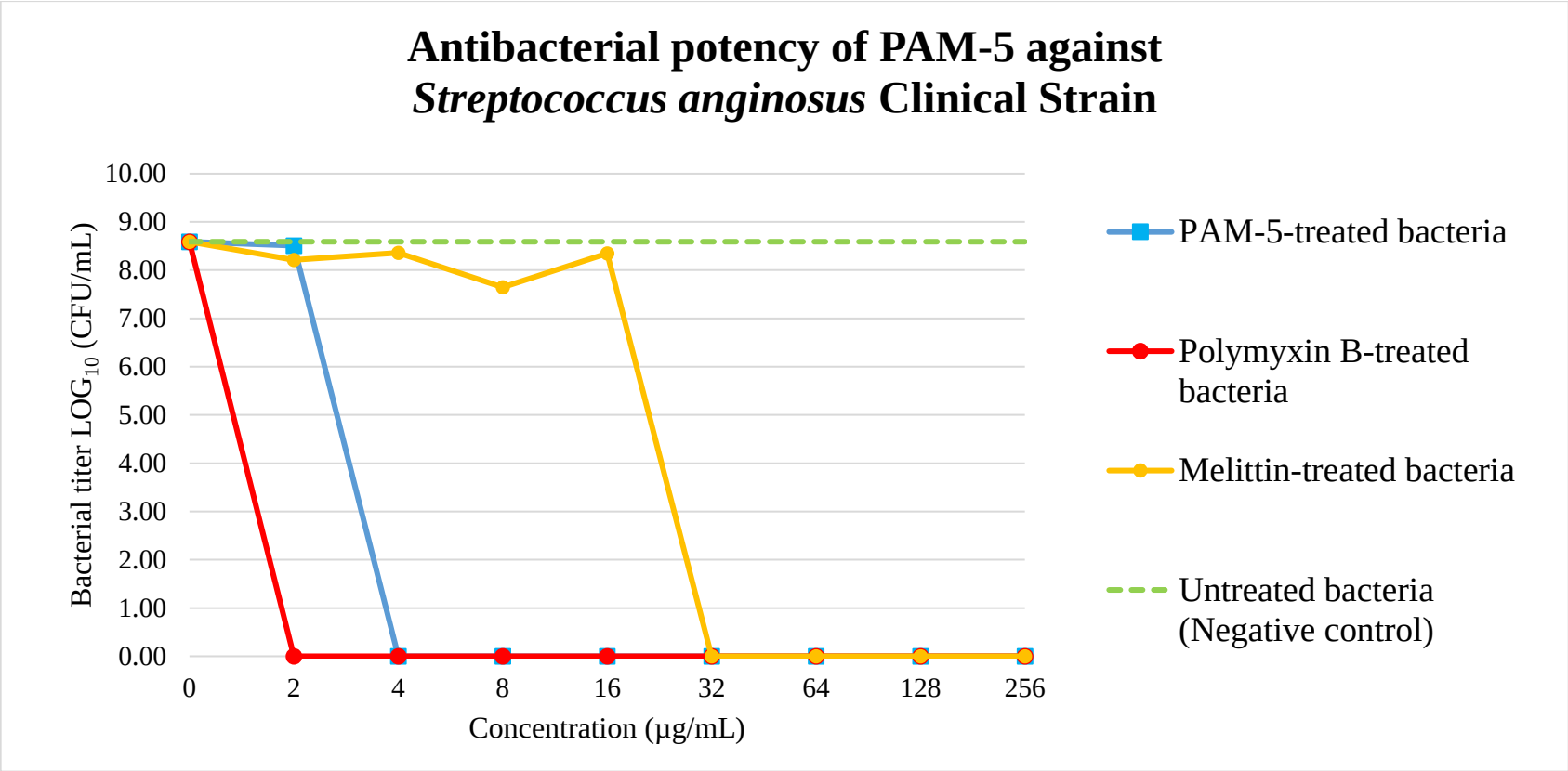


**Figure 4.4 (b):** Bacterial viability after treatment with PAM-5, polymyxin B and melittin. *Streptococcus anginosus* 1360589 was incubated with increasing concentrations of ABPs followed by inoculation on media for titre determination. Untreated bacteria was set up as the negative control. The MBC for PAM-5, polymyxin B and melittin against *E. faecalis* were 4 µg/mL, ≤ 2 µg/mL and 32 µg/mL, respectively.

**Table 4.1**: Summary on the antibacterial effects of PAM-5 and the comparative antibacterial peptides against various Gram-positive bacteria.

| **Bacterial species and Strain** | **MBC (µg/mL)** | | |
|---|---|---|---|
| | **PAM-5** | **Polymyxin B** | **Melittin** |
| *Staphylococcus aureus* ATCC 25923 | 256 | 32 | 16 |
| *Enterococcus faecalis* ATCC 19433 | > 256 | > 256 | 16 |
| *Streptococcus pyogenes* ATCC 19615 | 64 | 8 | 16 |
| *Streptococcus anginosus* 1360589 | 4 | ≤ 2 | 32 |

As described in the findings above, PAM-5 demonstrated heterogeneous potencies towards the four Gram-positive bacteria. Apart from that, PAM-5 was found to be less effective against these bacteria as compared to polymyxin B and melittin. The summary of potency of PAM-5 and the two comparative ABPs towards these bacteria is represented by their MBCs which are tabulated in **Table 4.1**.

## 4.2 Scanning Electron Microscope (SEM) Analysis on Surface Disruptive Effects of PAM-5 on *Staphylococcus aureus*

Scanning electron microscopy (SEM) was employed to provide a detailed insight of the effect caused by PAM-5 to the treated bacteria. Both PAM-5-treated and untreated *S. aureus* were observed under SEM for any structural and morphological difference.

As demonstrated in the micrograph in **Figure 4.5 (a)**, untreated *S. aureus* which served as the negative control was seen in spherical shape, which is characteristic of intact bacteria. As indicated by the arrow in the figure, the bacteria possessed smooth surface without any corrugation or blebbing which are the indicators of surface disruption. Comparatively, PAM-5 treated *S. aureus* was also structurally and morphologically similar to the untreated bacteria. Surface corrugation or blebbing was also not visible to most of the cocci treated by the peptide, indicating that PAM-5 was unable to disrupt the bacterial surface. In addition, the overall sizes of the treated and untreated bacteria were also similar.

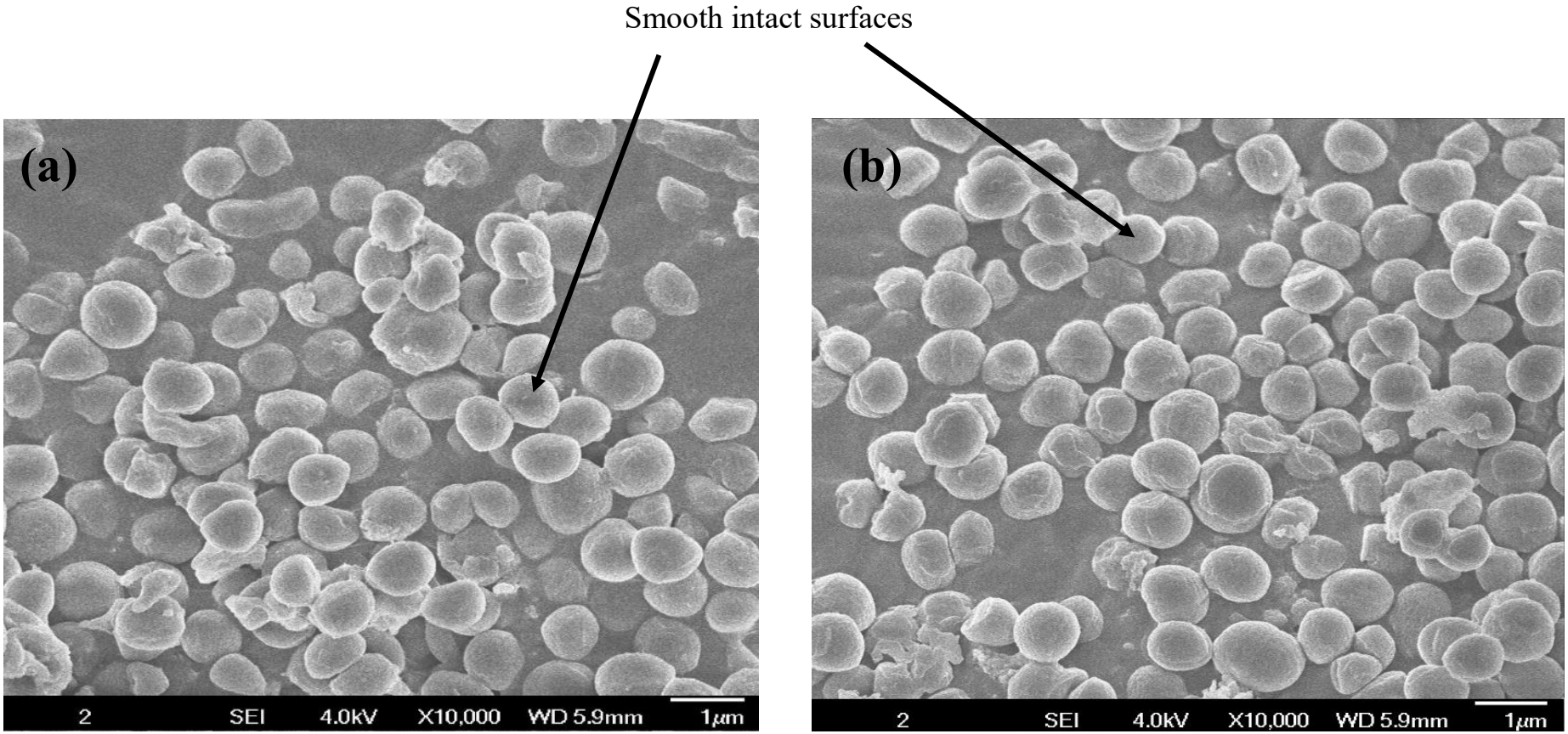


**Figure 4.5:** SEM micrograph of *S. aureus* ATCC 25923 at magnification of 10, 000X. (a) Bacteria in PBS was set up as the negative control; (b) PAM-5 treated bacteria.

# CHAPTER 5

# DISCUSSION

Over the decades, antibacterial peptides (ABPs) have been extensively studied in consideration to their potential application as alternative therapy against bacterial infections. Accumulating data have been suggesting that these compounds possess several advantages over conventional antibiotics such as rapid killing kinetics, multiple mechanisms of action, broad spectrum of bacterial targets, less toxicity and low likelihood of peptide resistance (Park et al., 2011; Zhu et al., 2015; Etebu and Arikekpar, 2016; Panteleev et al., 2018). These properties may allow ABPs to overcome the limiations of antibiotics and exert their bacteriostatic or bactericidal actions more effectively towards their target bacteria.

PAM-5, a 15-mer synthetic peptide, was shown to possess features of antibacterial peptides. Previously, this peptide was shown to exert rapid killing kinetics (Ng, 2018) (unpublished data), multiple killing mechanisms (Phoon, 2016; Tan, 2018) (unpublished data), and less toxicity towards mammalian cells (Lee, 2015) (unpublished data). However, the spectrum of bacterial targets by this peptide is yet to be fully defined. Despite the promising antibacterial potencies against several Gram-negative bacteria as reported in a few studies (Chan, 2016; Leong, 2018; Yong, 2018) (unpublished data) data on its effect against Gram-positive bacteria is very limited. So far, *Staphylococcus aureus*

ATCC 29523 was the only Gram-positive bacteria screened for the peptide potency, where the peptide was found less active against this bacterium (Chan, 2016) (unpublished data). Based on this finding, it was concluded that PAM-5 was not active against Gram-positive bacteria. However, such conclusion was unjustifiable if it is only inferred by the data of single Gram-positive bacterium. Hence, additional studies on the effect of PAM-5 on other species of Gram-positive bacteria should be included before concluding the spectrum of PAM-5. Therefore, in this study, several strains of Gram-positive pathogenic bacteria were added for the antibacterial screening in order to testify the previous conclusion. These bacteria encompassed the reference strain of *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 19433, *Streptococcus pyogenes* ATCC 19615 and a clinical strain of *Streptococcus* sp., namely *Streptococcus anginosus* 1360589.

## 5.1 Antibacterial Potency of PAM-5 towards *Staphylococcus aureus* ATCC 25923

*Staphylococcus aureus* (*S. aureus*) is one of the major human pathogens which is commonly isolated in clinical settings. Infections caused by this bacterium include furuncle, carbuncle, impetigo, scalded skin syndrome, infective endocarditis, toxic shock syndrome and etc (Tong et al., 2015; CDC, 2019). Most importantly, *S. aureus* represents one of the notorious members under the ESKAPE pathogens, which encompasses the names of six bacterial pathogens with high prevalence of antibiotic resistance (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii,*

*Pseudomonas aeruginosa* and *Enterobacter* spp.) (Maria-neto et al., 2015; Santajit and Indrawattna, 2016). In view of its clinical significance, as well as to ensure the data reproducibility from a previous study on the peptide efficacy towards this bacterium (Chan, 2016) (unpublished data), *S. aureus* was included as one of the Gram-positive target bacteria in this study.

As described in **Section 4.1.1**, PAM-5 was found to possess weak potency towards *S. aureus* ATCC 25923. This was indicated by the high peptide MBC (256 µg/mL) required to eliminate the bacteria completely. This MBC is relatively higher than the MBCs towards several Gram-negative bacteria as reported in a few studies previously (**Table 2.1**). Moreover, this finding validated the report by Chan (2016) (unpublished data), in which PAM-5 was less active against *S. aureus.*

The low potency of PAM-5 on *S. aureus* in this study corresponds to several other studies with the similar findings on the effect of ABPs towards Gram-positive bacteria. In a study by Sainath et al. (2013), a 12-mer synthetic ABP named EC5 failed to suppress the growth of *S. aureus* ATCC 25923 even though at a MIC of 256 µg/mL. In contrast, the ABP was able to inhibit or kill all the tested Gram-negative bacteria at relatively lower MICs or MBCs. M6, an ABP modified from a phage displayed peptide, also failed to inhibit *S. aureus* ATCC 25923 despite having promising antibacterial effects on Gram-negative bacteria (Pini et al., 2005). In other study, a 12-mer synthetic derivative from a porcine ABP named cathelicidin PAMP-36, was reported to be selectively potent against

Gram-negative bacteria only (Lv et al., 2014). The similarity of these findings was characterized by the high value of MBCs against the tested Gram-positive bacteria.

Several possible reasons might explain the low potency of PAM-5 on *S. aureus* as compared to other Gram-negative bacteria. Firstly, like other cationic ABPs, PAM-5 executes its antibacterial actions through membrane-active mechanisms. As reported in a study by Phoon (2016) (unpublished data), outer membrane disruption and inner membrane permeabilization were the two membrane-active mechanisms employed by PAM-5 to kill Gram-negative bacteria. These mechanisms are believed to be attributed to the cationic and amphipathic nature of the peptide, which allows it to interact with the anionic bacterial membrane via electrostatic interaction before initiating the membrane-disruptive mechanisms. However, in the presence of a thick layer of cell wall in Gram-positive bacteria, the direct access of the cationic ABPs to bacterial plasma membrane might be blocked or decreased, thus reducing the membrane-disrupting effects as occurred for Gram-negative bacteria. This barrier effect was reported in a study by Torcato et al. (2013) on an ABP named BP100. Based on their findings, higher amount of BP100 was required to kill Gram-positive bacteria as compared to Gram-negative bacteria. Detailed analysis revealed that the bacterial cell wall may reduce the effective concentration of the ABP to reach and accumulate on the plasma membrane.

Therefore, the peptidoglycan layers that form the bacterial cell wall may also serve as the barrier to PAM-5 from attacking the bacterial plasma membrane. Additional amount of PAM-5 might be required in order to compromise this barrier effect before killing the bacteria, as indicated by the high MBC of 256 μg/mL reported in this assay.

In addition to the barrier effect, the cell wall may confer resistance to cationic ABPs through minor modification to certain constituents in the peptidoglycan layers. Teichoic acid (TA) is the major component of cell wall that make up 60% of total mass for the structure (LaRock and Nizet, 2015; Joo et al., 2016). Due to its anionicity, TA appears to be a binding target by cationic ABPs before exerting their antibacterial activity to the bacteria. Nevertheless, *S. aureus* is able to modify its TAs through D-alanylation (Dlt) pathway, in which cationic D-alanine residues are added to the anionic TAs (Sorge et al., 2014). As the result of this modification, the surface molecules become less anionic, which reduces the potential electrostatic interaction with many cationic ABPs. The increase cationicity on the bacterial cell wall by this Dlt pathway contributes to the bacterial resistance to a broad range of cationic ABPs such as vancomycin, daptomycin, Polymyxin B, and cathelicidins (Ruzin et al., 2003; Nishi et al., 2004).

Additionally, *S. aureus* is able to secrete a protease known as aureolysin (Joo and Otto, 2015). Aureolysin is a zinc-dependent metalloprotease that has been demonstrated to cleave cationic peptides such as LL-37. Although the substrate

specificity for this protease is not well defined, mass-spectrometric analysis carried out by Sieprawska-Lupa et al. (2004) demonstrated that aureolysin was able to cause simultaneous hydrolysis of three peptide bonds in the C-terminal bactericidal domain of LL-37, resulting in the complete inactivation of the ABP. It is believed that the *S. aureus*-secreted protease might cause similar hydrolysis to PAM-5, thus compromising its antibacterial effect at lower peptide concentrations. Hence, higher concentrations of PAM-5 might be required to saturate the capacity of hydrolysis by this protease before a complete bactericidal effect is achieved.

Staphylokinase (Sak), a 136-mer protein secreted extracellularly by *S. aureus*, was found to serve as a sequester towards many cationic ABPs by preventing engagement of the peptides to the bacterial cell surface. A report had shown that Sak protein is able to form complex with human neutrophil peptides (HNP) such as α-defensins and LL-37, thus reducing their effective concentrations for antibacterial activity on the core bacteria (Braff et al., 2007). Although it was not clearly defined in this study, the similar sequestration might affect PAM-5, thus reducing its effective threshold concentration to initiate bactericidal action to the bacteria.

Comparatively, melittin was more potent against *S. aureus* as indicated by the lower MBC required to kill the bacteria completely. In a study conducted by Nguyen and Vogel (2016), Sak protein was found to bind weakly to melittin due to steric hindrance caused by the bulging aromatic side chain of Sak protein.

Additionally, the cationic amino acids that are present in Sak protein and melittin were found to cause certain level of repulsion between the two substances, thus reducing melittin sequestration by Sak protein. This might explain the lower MBC of melittin against *S. aureus.*

In overall, the presence of the above-mentioned intrinsic resistant mechanisms and barrier effect might explain the reduced susceptibility of *S. aureus* to PAM-5. These assumptions were further supported by the SEM observation on the bacteria after treatment with PAM-5. As described in the analysis, no observable structural and morphological differences were seen between PAM-5 treated and untreated bacteria. This indicated that PAM-5 may not able to cause the similar extent of surface or membrane disruption as occurred to the Gram-negative bacteria as reported in a previous study (Phoon, 2016) (unpublished data).

## 5.2 Antibacterial Potency of PAM-5 towards *Enterococcus faecalis* ATCC 19433

*Enterococcus faecalis* (*E. faecalis*) is a Gram-positive bacterium which is commonly found as commensal flora in the gastrointestinal tracts of humans and other mammals. However, beyond the boundary of its commensal habitat, *E. faecalis* may cause life-threatening infections such as infectious endocarditis, septicaemia and meningitis (Halkai et al., 2012). Moreover, this bacterium is also commonly associated with antibiotic-resistance in nosocomial environment (Banla et al., 2018). In particular, vancomycin-resistant enterococci (VRE) appears to be one of the most complicated bacterial infections that is difficult to

be treated by antibiotics (Raja et al., 2005). Under this concern, *E. faecalis* was selected as one of the target bacteria for this screening.

Despite the potent antibacterial effects of PAM-5 on Gram-negative bacteria (Chan, 2016; Leong, 2018; Yong, 2018) (unpublished data) as well as the weak potency against *S. aureus* as described earlier, this peptide was not active against *E. faecalis* even at the highest tested concentration (256 µg/mL) in this study. Interestingly, similar ineffectiveness was also observed for polymyxin B which was shown to be a more potent ABP than PAM-5. These findings indicate that *E. faecalis* may not only possess the similar evasive resistant mechanisms as described for *S. aureus*, but it may exert more aggressive strategies to confront the antibacterial agents, rendering them to become inactive. This assumption is supported by several reports that revealed the production of ABP-degrading proteases by *E. faecalis* that are able to compromise the efficacy of a number of ABPs. One of the most extensively studied proteases produced by this bacterium is gelatinase (GelE). In an *in vitro* study by Schmidtchen et al. (2002), this protease was shown to cleave an ABP secreted by human neutrophil, namely LL-37, resulting in the loss of antimicrobial activity of the peptide. This finding proposed that *E. faecalis* might utilize this protease to overcome the bactericidal effect of host ABPs, thus allowing it to establish infection in its host.

However, other studies on GelE demonstrated that this protease selectively cleave ABPs with certain motifs or amino acids. In a study by Nesuta et al. (2017), GelE was found to act on certain preferential cleavage sites within its

targeted ABPs. Using a synthetic analogue of an ABP derived from bee venom, namely HYL-20, GelE was shown to compromise the peptide by cleaving the peptide bond between the amino acids Lysine and Isoleucine (K – I), as well as Lysine and Leucine (K – L). It is believed that the cleavage of these peptide bonds may fragmentise the peptide into shorter peptide components which are insufficient to exert any antibacterial activity.

In a similar finding by Makinen and Makinen (1994), the *E. faecalis*-secreted protease is able to inactivate many ABPs by targeting peptide bonds between two specific amino acid residues. These cleavage sites are usually formed by two groups of amino acids of defined characteristics. As depicted in **Figure 5.1**, the cleavage site recognized by GelE is located between P1 and P1', where P1 can be formed by any basic or hydrophobic amino acids, while P1' is represented by any of the following hydrophobic amino acids: leucine (L), isoleucine (I), phenylalanine (F), or alanine (A) (Barrett et al., 2012). An ABP that harbours peptide bond formed between these two categories of residues may be the target of degradation by this protease. These findings would explain the ineffectiveness of PAM-5 and polymyxin B against *E. faecalis* in this study, where the cleavage sites of GelE are also present within these peptides as shown in **Figure 5.1**.

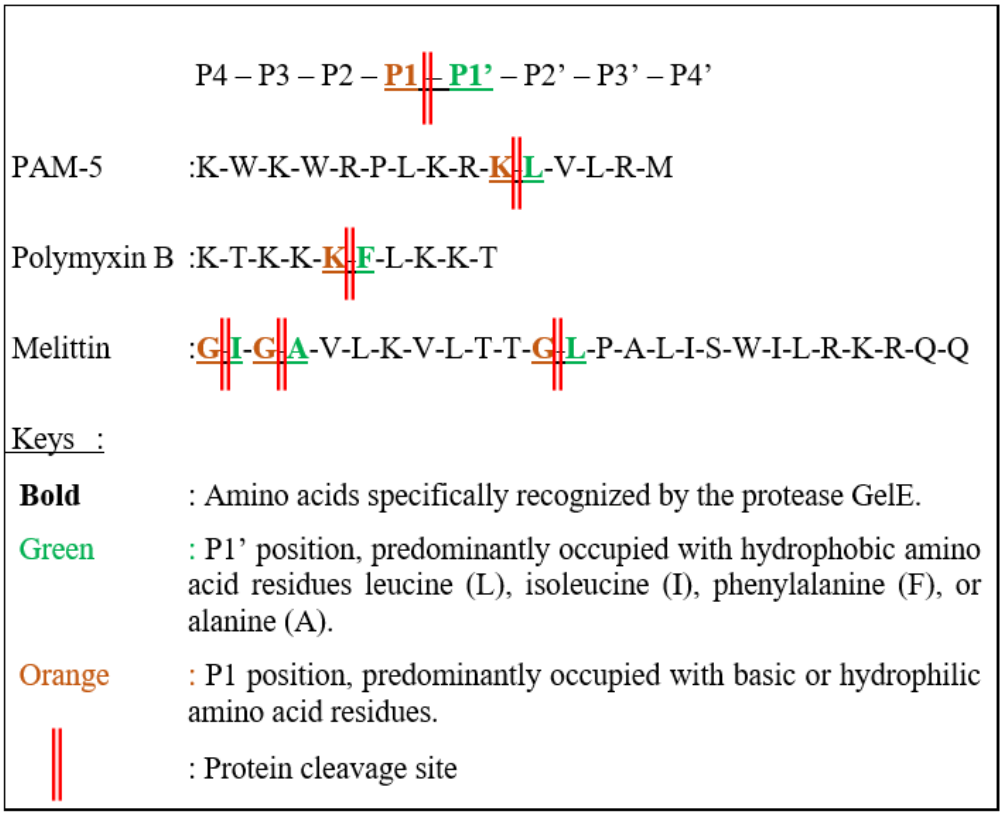


**Figure 5.1**: Proposed protein recognition and cleavage site of GelE secreted by *E. faecalis* on PAM-5, polymyxin B and melittin.

Unlike PAM-5 and polymyxin B, melittin was able to eliminate *E. faecalis* at a relatively lower MBC (16 µg/mL). Despite possessing the cleavage sites for GelE as shown in the figure above, its antibacterial potency was not completely compromised. Assuming that the protease was able to cleave this peptide at the cleavage sites as proposed above, the peptide fragments resulted from these cleavage may retain certain amino acids or motifs that contributed to antibacterial effects of the fragments. Two truncated studies on the 26-amino acid melittin showed that a remaining peptide fragment from the amino acid 12 to 26 was able to exert antibacterial effects at moderately high efficacy (Subbalakshmi et al., 1999; Yan et al., 2003). This indicated that the proteolytic cleavage does not necessarily cause complete inactivation to the peptide. Hence, in this study, it is speculated that the antibacterial activity of the melittin is

preserved even after proteolytic cleavage by the *E. faecalis*-secreted protease. The peptide fragments at the sequence of 4 – 12 (A-V-L-K-V-L-T-T-G) and 13 – 26 (L-P-A-L-I-S-W-I-K-R-K-R-Q-Q) after the cleavage carries the amino acid lysine, tryptophan and arginine that might retain its microbicidal effects, which allowed the effective killing of *E. faecalis.* Multiple studies have shown that the presence of the positively-charged amino acids such as tryptophan, arginine, and lysine in an ABP is essential to its antimicrobial activities as these residues are crucial in the initial interaction between the peptides and the bacterial cell membrane (Chan et al., 2006; Jindal et al., 2014). Therefore, it is assumed that melittin might retain its antibacterial activity despite cleavage by the bacterial protease as long as the essential motifs or amino acids for antibacterial effect are not affected. This could explain the relatively better potency of this ABP against *E. faecalis* as compared to PAM-5 and polymyxin B.

Next, ABP evasion by *E. faecalis* can also occur through cell wall modification. Similar to the mechanism as described for *S. aureus*, Dlt pathway is also present in *E. faecalis.* This pathway causes the increase in net charge of the cell wall which reduces the potential binding by many cationic ABPs. (Benachour et al., 2012). In the membrane of this bacterium, the presence of an outer membrane modifier enzyme named MprF may also contributes to its resistance to certain ABPs. As polymyxin B carries five positive charges, it is generally believed that polymyxin B kills bacteria through membrane lysis which begins with initial interaction of the peptide cationic side chain named α,γ-diaminobutyric (Dab) to the anionic components of the bacterial membrane (Yu et al., 2015). The aminoacylation of phosphatidylglycerol with amino acids lysine, alanine and

arginine catalysed by MprF causes the overall increase in positive charge to the outer membrane. This greatly reduces the initial electrostatic attraction of cationic ABPs to the bacterial membrane, thus reducing the bacterial susceptibility to polymyxin B (Bao et al., 2012).

With the aforementioned resistance mechanisms such as cell wall modification by Dlt and MprF pathway coupling with the selective actions of protease GelE secreted by *E. faecalis*, the decrease of peptide potency of PAM-5 and polymyxin B but not melittin could be explained.

## 5.3 Antibacterial Potency of PAM-5 towards *Streptococcus pyogenes* ATCC 19615

*Streptococcus pyogenes* (*S. pyogenes*) is classified under group A streptococcus (GAS) based on the Lancefield classification. It is one of the most common pathogenic Gram-positive bacteria which can cause serious infections such as cellulitis, necrotizing fasciitis, post-streptococcal glomerulonephritis, septicaemia and toxic shock syndrome (Sakurai et al., 2003; Ferretti et al., 2016). Being a notorious pathogen, it is worth to know its susceptibility to PAM-5.

As compared to the potency against *S. aureus* and *E. faecalis,* PAM-5 was more potent against *S. pyogenes* where the bacterium was completely eradicated at a lower MBC (64 µg/mL). However, this MBC is still far away from the range of MBCs characterized for an ideal ABP. According to Hancock and Chapple (1999), the best ABPs possess bactericidal effects towards a wide range of

bacteria at minimal inhibitory concentrations (MICs) ranging from 1 µg/mL to 8 µg/mL. As MIC was used under this defining criteria, it is generally accepted that the value of MBC for an antibacterial agent is rationally higher than its MIC. Thus, the anticipated range of MBCs for an ideal ABP would be around 2 µg/mL to 16 µg/mL. Based on this defining criteria, PAM-5 is considered moderately potent against *S. pyogenes*. However, PAM-5 was less potent than polymyxin B and melittin against *S. pyogenes* as indicated by the lower MBCs required by the latter two ABPs to eliminate the bacteria completely. Clearly indicated by these observations, it is assumed that *S. pyogenes* may possess similar evasion strategies as described for *S. aureus* and *E. faecalis*. In addition, it may equipped with its exclusive resistant mechanism which allows the bacterium to sustain through the stress imposed by the ABPs.

Several compounds produced by *S. pyogenes* may serve as its virulent and resistant factors towards ABPs. One of the compounds, namely streptococcal pyrogenic exotoxin B (SpeB), is a protease that degrades cationic ABPs extracellularly. SpeB has been shown to degrade many host antimicrobial peptides including LL-37 and β-defensins (Byberg et al, 2004). Detailed studies on SpeB had revealed that this protease degrades ABPs by targeting certain amino acids within the peptides. As demonstrated in **Figure 5.2**, SpeB has substrate specificity towards cationic amino acids at position P1, especially lysine (Caroll and Musser, 2011). Another substrate that is essential for protease-peptide recognition is the hydrophobic amino acids located at P2 position, in which valine (V) or isoleucine (I) is the preferential substrate. These findings indicate that SpeB produced by *S. pyogenes* might be able to degrade ABPs that

are majorly consist of hydrophobic and cationic amino acids which are crucial for their antibacterial activities. As such, melittin and PAM-5 which were used in this study might be the possible targets for SpeB, in which the possible cleavage sites of SpeB on both melittin and PAM-5 are shown in **Figure 5.2**.

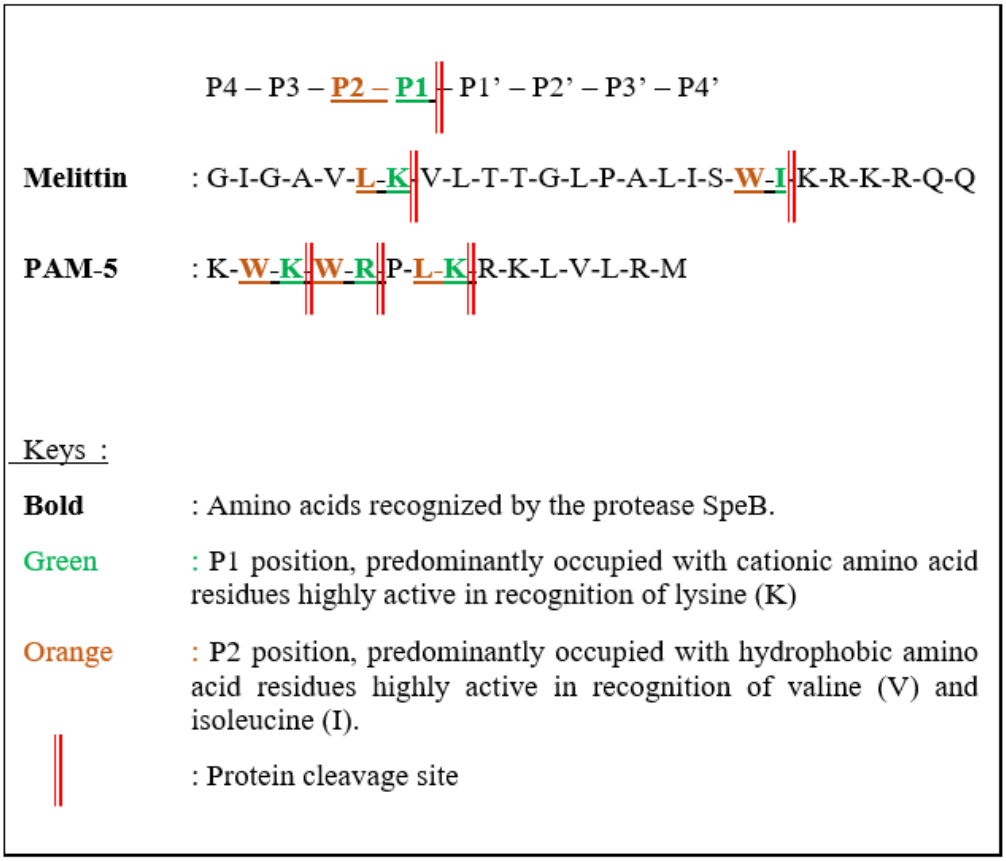


**Figure 5.2**: Proposed protein recognition and cleavage site of SpeB secreted by *S. pyogenes* on PAM-5 and melittin.

Apart from secreting extracellular protease, *S. pyogenes* also produces and secretes a sequester protein known as streptococcal inhibitor of complement (SIC) (Akesson et al., 1996; Nawrocki et al., 2014). According to Frick et al., (2003), this hydrophilic compound is able to interfere and sequester a variety of

natural ABPs such as α-defensins and LL-37, thus neutralizing their bactericidal effects.

Furthermore, like other Gram-positive bacteria, *S. pyogenes* has distinct bacterial surface structures as compared to Gram-negative bacteria. For instance, *S. pyogenes* is able to synthesize surface associated polysaccharides. As mentioned earlier, *S. pyogenes* is categorized under group A streptococcus (GAS). This group of streptococcus is characterized by the presence of hyaluronic acid capsule and surface M protein which contribute to the bacterial resistance to host immune defence elements such as antibody, opsonisation, complement system as well as antibacterial peptides produced by neutrophils (Dale et al., 1996; Cole et al., 2010). As PAM-5 is a membrane-active ABP, the presence of the bacterial capsule might reduce its direct interaction and accumulation on the bacterial plasma membrane, thus reducing its antibacterial effect. Consequently, higher amount of PAM-5 might be required to overcome the barrier effect in order to kill the bacteria, which might explain the higher MBC of the peptide against *S. pyogenes* in this study.

As such, the above mentioned resistant mechanisms by *S. pyogenes* might explain the reduced potency of both PAM-5 and melittin towards this bacteria. Consequently, higher amount of the ABPs would be required to kill the bacterium completely, thus resulting in moderately high MBCs of the peptides towards *S. pyogenes.*

## 5.4 Antibacterial Potency of PAM-5 towards *Streptococcus anginosus* 1960589

*Streptococcus anginosus,* which is under the Lancefield classification of streptococcus anginosus group (SAG), was previously known as *Streptococcus milleri.* It is a Gram-positive bacterium that is normally found in the upper respiratory tract as normal microbiota. However, under certain conditions, this bacterium may cause infections of different severity from mild skin infections to serious abscess formation (Obszanska et al., 2015). As this bacterium is rarely isolated from clinical samples, its susceptibility to ABPs would be an interesting topic to be investigated.

In contrast to the poor or low potencies of PAM-5 towards other Gram-positive bacteria as reported earlier, this peptide exhibited greater potency towards *S. anginosus.* This was indicated by the much lower MBC of this peptide required to eliminate the bacteria completely (4 µg/mL). Comparatively, both polymyxin B and melittin were able to eradicate this bacterium at MBCs that were not exceeding 32 µg/mL. These findings indicated that *S. anginosus* is more susceptible to the bactericidal effects of these ABPs.

Unlike other more virulent streptococcal species such as *S. pneumoniae* and *S. pyogenes, S. anginosus* possess lesser virulent factors, which categorize it as less virulent "viridan streptococci'. One of the well-studied virulent factors in streptococcus is exopolysaccharide capsule. As described for *S. pyogenes*

previously, this outer protective layer may serve as a barrier that prevent or reduce the direct access of ABP to bacterial plasma membrane. However, this virulent factor is rarely found in *S. anginosus* (Whitworth, 1990). According to a report by Kanamori et al. (2004), no capsule was found from the clinical isolates of *S. anginosus* from patients with pulmonary infections. The absence of capsule in this bacteria might be one of the possible explanations to the bacterial susceptibility to PAM-5 in this study. Without this barrier, the cationic PAM-5 might be able to reach the cell wall or even the plasma membrane of the bacterium in sufficiently higher concentration to exert its antibacterial effect.

In addition, bacterial doubling time might play another role which might influence the bacterial susceptibility to an antibacterial agent. As compared to the doubling time of *S. aureus*, *S. pyogenes* and *E. faecalis*, in which their doubling times are averaged at 30 mins, 40 mins and 48 mins, respectively (Domingue et al., 1996; Vebo et al., 2010; Gera and Mclver, 2014), the duration needed by *S. anginosus* to double its number is around 150 – 195 mins (Stinson et al., 2003). This longer doubling time might render the bacterium susceptible to fast-acting antibacterial agent. On the other hand, PAM-5 was shown to kill its target bacteria relatively faster as compared to conventional antibiotics. In a study on the killing kinetic of PAM-5, Ng (2018) (unpublished data) demonstrated that PAM-5 was able to eliminate non-capsulated bacteria (eg. *Escherichia coli* and *Pseudomonas aeruginosa*) completely within 10 minutes of treatment. However, it took a longer duration to kill *Klebsiella pneumoniae*, which is an encapsulated bacterium. These findings indicated that the presence of bacterial capsule may serve as a barrier to ABPs. Since *S. anginosus* is a non-

capsulated bacterium, it is assumed that PAM-5 may exert rapid killing towards this bacterium. Therefore, the prolonged doubling time of *S. anginosus* coupled with the rapid killing kinetics of PAM-5 might significantly contribute to the complete killing of this bacterium before achieving its doubling time, which in turn explains the relatively low MBC of PAM-5 towards this bacterium.

## 5.5 Implications of This Study

Clearly demonstrated from the findings, PAM-5 is not utterly non-active against Gram-positive bacteria as concluded in a previous finding (Chan, 2016) (unpublished data). Instead, PAM-5 has selective action against certain Gram-positive bacteria, especially slow growing bacteria. This implies that PAM-5 might be a potential candidate of antibacterial agent against infections caused by certain Gram-positive bacteria, especially *S. anginosus.*

## 5.6 Limitations of this Study and Proposed Future Studies

In this study, although the potencies of PAM-5 against the four Gram-positive bacteria were determined, a strong conclusion on the overall potency of this peptide towards Gram-positive bacteria could not be made. As the bacteria selected for this study only represented three genus from the Gram-positive category (*Staphylococcus* sp., *Enterococcus* sp. and *Streptococcus* sp.), the potencies of PAM-5 towards these limited number of bacteria may not provide a general overview on the susceptibility of Gram-positive bacteria to this peptide. As compared to Gram-negative bacteria, it is less common to obtain clinical

isolate of Gram-positive bacteria in healthcare setting, thus explaining the limited number of clinical Gram-positive bacteria screened in this study. Therefore, additional member of Gram-positive bacteria from different genus should be included for the antibacterial screening in future study in order to have a better definition on the antibacterial spectrum of PAM-5.

In this study, the surface-disruptive study by SEM was only carried out for one Gram-positive bacteria (*S. aureus*), which may not reflect the similar observations on other Gram-positive bacteria. Therefore, future studies may consider to include more Gram-positive bacteria to allow a more representative study on the surface-disruptive effects of PAM-5 on Gram-positive bacteria.

On the other hand, the susceptibility of PAM-5 to various bacterial proteases can be investigated by studying the peptide cleavage sites of the enzymes through mass-spectrometry. The data obtained from these findings might explain the low potency of PAM-5 towards those protease-secreting Gram-positive bacteria, as well as the relative difference in the potencies between PAM-5, polymyxin B and melittin.

# CHAPTER 6

# CONCLUSION

Based on the findings in this study, PAM-5 was not entirely non-active against all tested Gram-positive bacteria. Based on the range of peptide concentrations tested, PAM-5 was not active against *Enterococcus faecalis* ATCC 19433 (MBC > 256 µg/mL). The peptide also demonstrated low potency towards *Staphylococcus aureus* ATCC 25923 in which complete killing of the bacteria can be achieved only at high MBC of 256 µg/mL. In contrast, PAM-5 demonstrated moderate potency towards *Streptococcus pyogenes* ATCC 19615 with the MBC of 64 µg/mL. Finally, PAM-5 was very active against a clinical isolate of *Streptococcus anginosus* 1360589 at the MBC of 4 µg/mL. Overall, as compared to the potencies against Gram-negative in the previous studies, PAM-5 was less active against Gram-positive bacteria. In view of the comparison between PAM-5, polymyxin B and melittin, PAM-5 was generally less potent than the latter two well characterized ABPs towards the four Gram-positive bacteria.

In conclusion, PAM-5 exhibited heterogeneous potencies against different Gram-positive bacteria as screened in this study. However, additional members of Gram-positive bacteria should be added for future screening to validate or strengthen this conclusion.

## REFERENCES


Akesson P., Sjoholm, A. G. and Bjorck, L., 1996. Protein SIC, a novel extracellular protein of *Streptococcus pyogenes* interfering with complement function. *The Journal of Biological Chemistry,* 271 (2), pp. 1081 – 1088.

Aldred, K. J., Kerns, R. J. and Osheroff, N., 2014. Mechanism of quinolone action and resistance. *Biochemistry,* 53 (10), pp. 1565 – 1574.

Arenz, S. and Wilson, D. N., 2016. Bacterial protein synthesis as a target for antibiotic inhibition. *Cold Spring Harbour Perspect in Medicine,* 2016, pp. 1 – 15.

Ashcroft, J.W., Qureshi, A., Rayner, J. C., Kotelnikova, S. V. and Lennon, D., 2004. Antimicrobial peptides: A paradigm for novel chemotherapeutic agents. *Caribbean Journal of Science,* 11(13), pp. 211 – 217.

Aslam, B., et al., 2018. Antibiotic resistance: A rundown of a global crisis. *Infection and Drug Resistance,* 11, pp. 1645 – 1658.

Bahar, A. A. and Ren, D., 2013. Antimicrobial peptides. *Pharmaceuticals,* 6 (12), pp. 1543 – 1575.

Banla I. L. et al., 2018. Modulators of *Enterococcus faecalis* cell envelope integrity and antimicrobial resistance influence stable colonization of the mammalian gastrointestinal tract. *Infection and Immunity,* 86 (1), pp. 1 – 15.

Bao, Y. Y. et al., 2012. Role of mprF1 and mprF2 in the pathogenesis of *Enterococcus faecalis. PLoS ONE,* 7 (6), pp. 1 – 10.

Barrett, A. J., Woessner, F. and Rawling, N. D., 2012. *Handbook of proteolytic enzymes, volume 1*. Amsterdam: Elsevier.

Batoni, G., Maisetta, G. and Esin, S., 2016. Antimicrobial peptides and their interaction with biofilms of medically relevant bacteria. *Biochemica et Biophysica Acta,* 1858(5), pp. 1044 – 1060.

Bechinger, B. and Gorr, S. U., 2017. Antimicrobial peptides: mechanisms of action and resistance. *Journal of Dental Research,* 96 (3), pp. 254 – 260.

Benachour, A. et al., 2012. The lysozyme-induced peptidoglycan n-acetylglucosamine deacetylase pgda (ef1843) is required for *Enterococcus faecalis* virulence. *Journal of Bacteriology,* 194 (22), pp. 6066 – 6073.

Birkemo, G. A., Sullivian, O., Ross, R. P. and Hill, C., 2009. Antimicrobial activity of two peptides casecidin 15 and 17, found naturally in bovine colostrum. *Journal of Applied Microbiology,* 106 (1), pp. 233 – 240.

Braff, M. H., Jones, A., Skerett, S. J. and Rubens, C. E., 2007. *Staphylococcus aureus* exploits cathelicidin antimicrobial peptides produced during early pneumonia to promote staphylokinase-dependent fibrinolysis. *Journal of Infectious Disease,* 195 (9), pp. 1365 – 1372.

Brender, J. R., McHenry, A. J. and Ramammorthy, A., 2012. Does cholesterol play a role in the bacterial selectivity of antimicrobial peptides? *Frontiers in Immunology,* 3 (195), pp. 1 – 4.

Brogden, K. A., 2005. Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nature Review Microbiology,* 3, pp. 238 – 250.

Brook, M. et al., 2016. Neutrophil-derived alpha defensins control inflammation by inhibiting macrophage mRNA translation. *PNAS,* 113 (16), pp. 4350 – 4355.

Byberg, P., Rasmussen, M., Pawel-Rammingen, U. and Bjorck, L., 2004. SpeB modulates fibronectin-dependent internalization of *Streptococcus pyogenes* by efficient proteolysis of cell-wall-anchored protein F1. *Microbiology,* 2004 (150), pp. 1559 – 1569.

Caroll, R. K. and Musser, J. M., 2011. From transcription to activation: how group A streptococcus, the flesh-eating pathogen, regulates SpeB cysteine protease production. *Molecular Microbiology,* 81(3), pp. 588 – 601.

Centre for Disease Control and Prevention, 2019. *Staphylococcus aureus in healthcare settings.* [Online] Available at https://www.cdc.gov/hai/organisms/staph.html [Accessed 2 April 2019].

Chan, D. I., Prenner, E. J., and Vogel, H. J., 2006. Tryptophan- and arginine-rich antimicrobial peptides: Structures and mechanisms of action. *Biochimica et Biophysica Acta,* 1758 (2006), pp. 1184 – 1202.

Chan, S. Y., 2016, Screening of broad-spectrum antibacterial effects of synthetic peptide PAM-5 against selected pathogenic bacteria, Bachelor Degree. University Tunku Abdul Rahman. Available at: http://eprints.utar.edu.my.libezp2.utar.edu.my/2273/1/BM-2016-1202490-1.pdf [Accessed: 14 October 2018].

Chehimi, S. et al., 2007. Purification and partial amino acid sequence of thuricin S, a new anti-Listeria bacteriocin from *Bacillus thuringiensis. Canadian Journal of Microbiology*, 53 (2), pp. 284 – 290.

Clinical Laboratory Standard Institute, 2018. [Online] Available at: https://clsi.org/media/1930/m100ed28_sample.pdf [Accessed: 20 October 2018].

Cole, J. N. et al., 2010. M protein and hyaluronic acid capsule are essential for *in vivo* selection of covRS mutations characteristic of invasive serotype M1T1 group A streptococcus. *Molecular Biology*, 1 (4), pp. 1 – 8.

Dale, J. B., Simmons, M., Chiang, E. C. and Chiang, E. Y., 1996. Recombinant, octavalent group A streptococcal M protein vaccine. *Vaccine,* 14 (10), pp. 944 – 948.

Davies, J. and Davies, D., 2010. Origins and evolutions of antibiotic resistance. *Microbiology and Molecular Biology Reviews,* 74 (3), pp. 417 – 433.

Domingue, G., Costerton, J. W. and Brown, M. R. W., 1996. Bacterial doubling time modulates the effects of opsonisation and available iron upon interactions between *Staphylococcus aureus* and human neutrophils. *FEMS Immunology and Medical Microbiology,* 16 (1996), pp. 223 - 228.

Ebenhan, T., Gheysens, O., Kruger, H. G., Zeevaart, J. R. and Sathekge, M. M., 2014. Antimicrobial peptides: their role as infection-selection tracers for molecular imaging. *Biomed Research International*, 2014, pp. 1 – 15.

Etebu, E. and Arikekpar, I., 2016. Antibiotics: Classification and mechanisms of action with emphasis on molecular perspectives. *International Journal of Applied Microbiology and Biotechnology Research*, 4, pp. 90 – 101.

Fair, R. J. and Tor, Y., 2014. Antibiotics and bacterial resistance in the 21st century. *Perspective in Medicinal Chemistry*, 6, pp. 25 – 64.

Fallat, T. J., Karunaratne, D. N. and Hancock, R. E. W., 1996. Mode of action of the antimicrobial peptide indolicidin. *The Journal of Bioloigical Chemistry*, 271 (32), pp. 19298 – 19303.

Ferretti, J. J. et al., 2016. *Streptococcus pyogenes: basic biology to clinical manifestation.* [e-book] Oklahoma City: Inicersity of Oklahoma Health Sciences. Available at: Google books <books.google.com> [Accessed 3 April 2019].

Frick, I. M., Akesson, P., Rasmussen, M., Schmidtchen, A. and Bjorck, L., 2003. SIC, a secreted protein of *Streptococcus pyogenes* that inactivates antibacterial peptides. *The Journal of Biological Chemistry*, 278 (19), pp. 16561 – 16566.

Gaynes, R., 2017. The discovery of penicillin – New insight after more than 75 years of clinical use. *Emerging Infectious Disease*, 23(5), pp. 849 – 853.

Gera, K. and Mclver, K. S., 2014. Laboratory growth and maintenance of *Streptococcus pyogenes* (The Group A Streptococcus, GAS). *Current Protocols in Microbiology*, 30, pp. 1 – 14.

Giulio, A. D. and Zhao, H. X., 2006. Antimicrobial peptides: basic mechanisms of action and emerging pharmacological interest. *Asian Journal of Biochemistry*, 1(1), pp. 28 – 40.

Gwee, C. P., 2012. Screening and isolation of antibacterial peptide against *Pseudomonas aeruginosa* by using 12-mer phage-displayed peptide library. Bachelor Degree. University Tunku Abdul Rahman. Available at: http://eprints.utar.edu.my.libezp2.utar.edu.my [Accessed: 14 October 2018].

Halkai, R., Hegde, M. N. and Halkai, K., 2012. *Enterococcus faecalis* can survive extreme challenges – overview. *Nitte University Journal of Health Science,* 2(3), pp. 1 – 5.

Hancock, R. E. W. and Chapple, D. S., 1999. Peptide antibiotics. *Antimicrobial agents and chemotherapy,* 43(6), pp. 1317 – 1323.

Hancock, R. E. W. and Falla, T. J., 1996. Antimicrobial peptides: Broad-spectrum antibiotics from nature. *Clinical Microbiology and Infection,* 1 (4), pp. 226 – 229.

Hollmann, A., Martinez, M., Maturana, P., Semorile, L. C. and Maffia, P. C., 2018. Antimicrobial peptides: Interaction with model and biological membranes and synergism with chemical antibiotics. *Frontiers in Chemistry,* 6 (204), pp. 1 – 13.

Jin, L. L. et al., 2009. Identification and characterization of a novel antimicrobial polypeptide from the skin secretion of Chinese frog (*Rana chensinesis*). *International Journal of Antimicrobial Agents,* 33 (6), pp, 538 – 542.

Jindal, M. H., Mohd Yusof, M. Y., and Sekaran, S. D., 2014. Net charge, hydrophobicity and specific amino acids contribute to the activity of antimicrobial peptides. *The Journal of Health and Translational Medicines,* 17 (1), pp. 1 – 7.

Joo, H. S. and Otto, M., 2015. Mechanisms of resistance to antimicrobial peptides in staphylococci. *Biochimica et Biophysica Acta,* 1848 (2015), pp. 3055 – 3061.

Joo, H. S., Fu. C. J. and Otto, M., 2016. Bacterial strategies of resistance to antimicrobial peptides. *The Royal Society,* 317, pp. 1 – 11.

Kanamori, S., Kusano, N., Shinzato, T., and Saito, A., 2004. The role of capsule of the *Streptococcus milleri* group in its pathogenicity. *Journal of Infections and Chemotherapy,* 2004 (10), pp. 105 – 109.

Kumar, P., Kizhakkedathu, J. N. and Straus, S. K., 2018. Antimicrobial peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility *in vivo*. *Biomolecules,* 8 (4), pp. 1 – 24.

LaRock, C. N. and Nizet, V., 2015. Cationic antimicrobial peptide resistance mechanisms of streptococcal pathogens. *Biochemica et Biophysica Acta,* 1848 (2015), pp. 3047 – 3054.

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

Lee, J. Y. et al., 2009. Different modes of action of homodimeric bactenecin and monomeric bactenecin, a chathelicidin derived antibacterial peptide. *BMB reports,* 42 (9), pp. 586 – 592.

Lee, T. J., 2015. Screening for toxicity effect of antibacterial peptides, PAM-5 and PAM-6, on eukaryotic cells. Bachelor Degree. University Tunku Abdul Rahman. Available at: http://eprints.utar.edu.my.libezp2.utar.edu.my [Accessed: 14 October 2018].

Lee, T. S., Hall, K. N. and Aguilar, M. I., 2016. Antimicrobial peptide structure and mechanism of action: a focus on the role of membrane structure. *Current Topics in Medicinal Chemistry,* 16 (1), pp. 25 – 39.

Lee, V. C., 2015. The antibiotic resistance crisis, part 1: causes and threats. *Pharmacy and Therapeutics,* 40 (4), pp. 277 – 283.

Leeuw, E. D. et al., 2012. Functional interaction of human neutrophil peptide-1 with the cell wall precursor Lipid II. *FEBS letters,* 584 (8), pp. 1543 – 1548.

Leong, Y. L., 2018. Screening of antibacterial potency of antibacterial peptide PAM-5 against selected multidrug resistant pathogenic bacteria, Bachelor Degree. University Tunku Abdul Rahman. Available at: http://eprints.utar.edu.my.libezp2.utar.edu.my [Accessed: 14 October 2018].

Li, G. Q., Burkhardt, D., Gross, C. and Weissman, J. S., 2014. Quantifying absolute protein synthesis rates reveals principles underlying allocation of cellular resources. *Cell,* 157 (3), pp. 624 – 635.

Liu, S. P., Zhou, L., Lakshiminarayanan, R. and Beueran, R.W., 2010. Multivalent antimicrobial peptides as therapeutics: Design principles and structural diversities. *International Journal of Peptide Research and Therapeutics,* 16 (3), pp. 199 – 213.

Liu, W. P., Chen, Y. H., Ming, X. and Kong, Y., 2015. Design and synthesis of a novel cationic peptide with potent and broad-spectrum antimicrobial activity. *Biomed Research International,* 2015, pp. 1 – 7.

Lv, et al., 2014. Antimicrobial properties and membrane-active mechansisms of potential α-helical antimicrobial derived from cathelicidin PAMP-36. *PLoS ONE,* 4 (1), pp. 1 – 12.

Makinen P. L., and Makinen, K. K., 1994. The *Enterococcus faecalis* extracellular metalloendopeptidase (EC 3.4.24.30; coccolysin) inactivates human endothelin at bonds involving hydrophobic amino acids residues. *Biochemical and Biophysical Research Commincations,* 200 (2), pp. 981 – 985.

Malanovic, N. and Lohner, K., 2016. Antimicrobial peptides targeting gram-positive bacteria. *Pharmaceuticals,* 9 (59), pp. 1 – 33.

Mangoni, M. L. et al., 2000. Structure-function relationships in bombinins H, antimicrobial peptides rom Bombina skin secretions. *Peptides,* 21 (11), pp, 1673 – 1679.

Maria-Neto, S., Almeida, K. C., Macedo, M. L. R. and Franco, O. L., 2015. Understanding bacterial resistance to antimicrobial peptides: from the surface to deep inside. *Biochemica et Biophysica Acta,* 2015, pp. 1 – 11.

Martinez, J. L. and Baquero, F., 2000. Mutation frequencies and antibiotic resistance. *Antimicrobial agents and Chemotherapy,* 44 (7), pp. 1771 – 1777.

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 (29707), pp. 1 – 14.

Mohamed, M. F., Hammac, G. K., Guptill, L. and Saleem, M. N., 2014. Antibacterial activity of novel cationic peptides against clinical isolates of multi-drug resistant *Staphylococcus psedintermedius* from infected dogs. *PLoS ONE,* 9 (12), pp. 1 – 20.

Munita, J. M. and Arias, C. A., 2016. Mechanisms of antibiotic resistance. *Microbiology Spectrum,* 4 (2), pp. 1 – 37.

Nawrocki, K. L., Crispell, E. K. and McBride, S. M., 2014. Antimicrobial peptide resistance mechanisms of Gram-Positive Bacteria. *Antibiotics,* 2014 (3), pp. 461 – 492.

Nesuta, O. et al., 2017. How proteases from *Enterococcus faecalis* contribute to its resistance to short α-helical antimicrobial peptides. *Pathogen and Disease,* 75 (7), pp. 1 – 12.

Ng, W. N., 2018, Study of Kinetic Killing of Antibacterial Peptide PAM-5 On *Escherichia coli* and *Pseudomonas aeruginosa,* Bachelor Degree. University Tunku Abdul Rahman. Available at: http://eprints.utar.edu.my.libezp2.utar.edu.my [Accessed: 14 October 2018].

Nguyen, L. T. and Vogel, H. J., 2016. Staphylokinase has distinct modes of interaction with antimicrobial peptides, modulating its plasminogen-activation properties. *Scientific Reports,* 6 (31817), pp. 1 – 13.

Nishi, H., Komatsuzawa, H., Fujiwara, T., McCallum, N., and Sugai, M., 2004. Reduced content of lysyl-phosphatidylglycerol in the cytoplasmic membrane affects susceptibility of moenomycin, as well as vancomycin, gentamycin and antimicrobial peptides in *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy,* 48 (12), pp. 4800 – 4807.

Nusslein, K., Arnt, L., Rennie, J., Owens, C. and Tew, G. N., 2006. Broad-spectrum antibacterial activity by a novel abiogenic peptide mimic. *Microbiology,* 152, pp. 1913 – 1918.

Obszanska, K. et al., 2015. *Streptococcus anginosus (milleri)* group strains isolated in Poland (1996-2012) and their antibiotic resistance patterns. *Polish Journal of Microbiology,* 65 (1), pp. 33 – 41.

Omardien, S., Brul, S. and Zaat, S.A., 2016. Antimicrobial activity of cationic antimicrobial peptides against gram-positives: current progress made in understanding the mode of action and the response of bacteria. *Frontiers in Cell and Developmental Biology,* 4 (111), pp. 1 – 16.

Panteleev, P. V. et al., 2018. Combined antibacterial effects of goat cathelicidins with different mechanisms of action. *Frontiers in Microbiology,* 9 (2983), pp 1 – 19.

Panteleev, P. V., 2018. Novel antimicrobial peptides from the Arctic polychaeta *Nicomache minor* provide new molecular insight into biological role of the BRICHOS domain. *Marine Drugs,* 16 (11), pp. 1 – 22.

Park, C. B. et al., 2000. Structure-activity analysis of Buforin II, a histone H2A-derived antimicrobial peptide: The proline hinge is responsible for the cell-penetrating ability of buforin II. *Proceedings of the National Academy of Sciences of the United States of America,* 97 (15), pp 8245 – 8250.

Park, S. C., Park, Y. Y. and Hahm, K. S., 2011. The role of antimicrobial peptides in preventing multidrug-resistant bacterial infections and biofilm formation. *International Journal of Molecular Sciences,* 12 (9), 5971 – 5992.

Phoon, E. W. Y., 2016, Screening of membrane-active mechanism of antibacterial peptide PAM-5 against *Pseudomonas aeruginosa,* Bachelor Degree. University Tunku Abdul Rahman. Available at: http://eprints.utar.edu.my.libezp2.utar.edu.my [Accessed: 14 October 2018].

Pini, A. et al., 2005. Antimicrobial activity of novel dendrimeric peptides obtained by phage display selection and rational modification. *Microbial agents and Chemotherapy,* 49 (7), pp. 2665 – 2672.

Podolsky, S. H., 2018. The evolving response to antibiotic resistance (1945 – 2018). *Palgrave Communications*, 4 (124), pp. 1 – 8.

Pushpanathan, M., Gunasekaran, P. and Rajendhran, J., 2013. Antimicrobial peptides: versatile biological properties. *International Journal of Peptides,* 2013, pp. 1 – 15.

Rady, I., Siddiqui, I. A., Rady, M. and Mukhtar, H., 2017. Melittin, a major peptide component of bee venom, and its conjugates in cancer therapy. *Cancer Letter,* 28 (402), pp. 16 – 31.

Rai, M., Pandit, R., Gaikwad, S. and Kovics, G., 2016. Antimicrobial peptides as natural bio-preservative to enhance the shelf-life of food. *Journal of Food Science and Technology,* 53(9), pp. 3381 – 3394.

Raja, N. S., Karunakaran, R., Ngeow, Y. F. and Awang, R., 2005. Community-acquired vancomycin-resistant *Enterococcus faecium:* a case report from Malaysia. *Journal of Medical Microbiology,* 54, pp. 901 – 903.

Richardson, L. A., 2017. Understanding and overcoming antibiotic resistance. *PLoS ONE,* 15 (8), pp. 1 – 5.

Ruzin, A. et al., 2003. Inactivation of mprF affects vancomycin susceptibility in *Staphylococcus aureus. Biochimica et Biophysica Acta,* 1621 (2003), pp. 117 – 121.

Sainath, S. R., Mohan, K. V. K. and Atreya, C. D., 2013. A peptide derived from phade display library exhibits antibacterial activity against *E. coli* and *Pseudomonas aeruginosa. PLoS ONE,* 8 (2), pp. 1 – 11.

Sakurai, A. et al., 2003. *Streptococcus pyogenes* infection induces septic arthritis with increased production of the receptor activator of the NF-kB ligand. *Infection and Immunity,* 71 (10), pp. 6019 – 6026.

Santajit, S. and Indrawattna, N., 2016. Machanisms of Antimicrobial resistance in ESKAPE pathogens. *BioMed Research International,* 2016, pp. 1 – 8.

Schmidtchen, A., Frick, I. M., Andersson, E., Tapper, H., and Bjorck, L., 2002. Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Molecular Microbiology,* 46(1), pp. 157 – 168.

Seo, M. D., Won, H. S., Kim, J. H., Ochir, T. M. and Lee, B. J., 2012. Antimicrobial peptides for therapeutic applications: a review. *Molecules,* 2012 (17), pp. 12276 – 12286.

Shurko, J. F., Gelega, R. S., Li, C. X. and Lee, G. C., 2018. Evaluation of LL-37 antimicrobial peptide derivatives alone and in combination with vancomycin against *S. aureus*. *The Journal of Antibiotics,* 71, pp. 971 – 974.

Sieprawska-Lupa, M. et al., 2004. Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus*-derived proteinases. *American Society for Microbiology,* 48 (12), pp. 4673 – 4679.

Sorge, N. M. et al., 2014. The classical Lancefield antigen of Group A *Streptococcus* is a virulence determination with implications for vaccine design. *Cell Host & Microbe,* 15 (6), pp. 729 – 740.

Stinson, M. W., Alder, S. and Kumar, S., 2003. Invasion and killing of human endothelial cells by viridans group streptococci. *Infections and Immunity*, 71 (5), pp. 2365 – 2372.

Subbalakshmi, C., Nagaraj, R., and Sitaram, N., 1999. Biological activities of C-terminal 15-residue synthetic fragment of melittin: design of an analogue with improved antibacterial activity. *Federation of European Biochemical Societies*, 448 (1999), pp. 62 – 66.

Subbalakshmi, C. and Sitaram, N., 1997. Mechanism of antimicrobial action of indolicidin. *FEMS Microbiology Letters*, 160 (1998), pp. 91 – 96.

Szabo, D. et al., 2010. The designer proline-rich antibacterial peptide A3-APO is effective against systemic *Escherichia coli* infections in different mouse models. *International Journal of Antimicrobial Agents*, 35 (4), pp. 357 – 361.

Tan, K. S., 2018, Screening for Bacterial DNA-Binding Ability of Antibacterial Peptide PAM-5, Bachelor Degree. University Tunku Abdul Rahman. Available at: http://eprints.utar.edu.my.libezp2.utar.edu.my [Accessed: 14 October 2018].

Thompson, A. H., Bjourson, A. J., Orr, D. F., Shaw, C. and McClean, S. 2007. A combined mass spectrometric and cDNA sequencing approach to the isolation and characterization of novel antimicrobial peptides from the skin secretions of *Phyllomedusa hypochondrialis azurea, Peptides,* 28 (7), pp. 1131 – 1143.

Tong, Y. C. S., Davis, J. S., Eichenberger, E., Holland, T. L. and Fowler, V. G., 2015. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clinical Microbiology Reviews,* 28(3), pp. 603 – 661.

Torcato, I. M. et al., 2013. Design and characterization of novel antimicrobial peptides, R-BP100 and RW-BP100, with activity against Gram-negative and Gram-positive bacteria. *Biochemica et Biophysica Acta,* 1828, pp. 944 – 955.

Turner, J., Cho, Y., Dinh, N. N., Waring, A. J. and Lehrer, R. I., 1998. Activities of LL-37, a cathelicidin-associated antimicrobial peptide of human neutrophils. *Antimicrobial Agents and Chemotherapy,* 42, pp. 2206 – 2214.

Vebo, H. C., Solheim, M., Snipen, L., Nes, I. and Brede, D. A., 2010. Comparative genomic analysis of pathogenic and probiotic *Enterococcus faecalis* isolates, and their transcriptional responses to growth in human urine. *PLoS ONE,* 5 (8), pp. 1 – 14.

Volzing, K., Borrero, J., Sadowsky, M. J. and Kaznessis, Y., 2013. Antimicrobial peptides targeting gram-negative pathogens, produced and delivered by lactic acid bacteria. *ACS Synthetic Biology,* 2 (11), pp. 643 – 650.

Wang, G., Li, X. and Wang, Z., 2009. APD-2: the updated antimicrobial peptide database peptide database and its application in peptide design. *Nucleic acids Research,* 37, pp. 933 – 937.

Wang, Q. et al., 2018. HJH-1, a broad-spectrum antimicrobial activity and low cytotoxicity antimicrobial peptide. *Molecules,* 23 (8), pp. 1 – 15.

Wang, S., Zeng, X. F., Yang, Q. and Qiao, S. Y., 2016. Antimicrobial peptides as potential alternative to antibiotics in food animal industry. *International Journal of Molecular Sciences,* 17 (603), pp. 1 – 12.

Whitworth, J. M., 1990. Lancefield group F and related Streptococci. *Journal of Medical Microbiology,* 33 (1990) pp. 135 – 151.

World Health Organization, 2014. *United Nations meetings on antimicrobial resistance.* [Online] Available at: https://www.who.int/bulletin/volumes/94/9/16-020916/en/?fbclid=IwAR07eE9s5p6S_JXKhO9pNX_meanWzhTbtRWNddTmbTyFh1B3eXLelP2q36w [Accessed: 20 October 2018].

Wu, C. H., Liu, I. J., Lu, R. M. and Wu, H. C., 2016. Advancement and applications of peptide pahge display technology in biomedical science. *Journal of Biomedical Science,* 23 (8), pp. 1 – 14.

Yan, H. S., Li, S. Z., Sun, X. J., Mi, H. F. and He, B. L., 2003. Individual substitution analogues of Mel(12-26) melittin's C-terminal 15-residue peptide: their antimicrobial and haemolytic actions. *FEBS Letters,* 2003 (554), pp. 100 – 104.

Yong, K. Y., 2018. Screening of antibacterial potency of antibacterial peptide pam-5 against selected multidrug resistant pathogenic bacteria, Bachelor Degree. University Tunku Abdul Rahman. Available at: http://eprints.utar.edu.my.libezp2.utar.edu.my [Accessed: 14 October 2018].

Yu, Z. L., Qin, W. R., Lin, J. X., Fang, S. S., and Qiu, J. P., 2015. Antibacterial mechanisms of polymyxin and bacterial resistance. *BioMed Research International,* 2015, pp. 1 – 11.

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), 3008 – 3017.

## APPENDIX A

## LIST OF LABWARE AND EQUIPMENTS

| Lab-ware/Equipment | Manufacturers |
|---|---|
| 15 mL centrifuge tube | Greiner, Germany |
| 50 mL centrifuge tube | Axvgen® Scientific, USA |
| 96-well microtiter plate, transparent, flat-bottomed | Greiner CELLSTAR®, Germany, NEST®, China |
| Biosafety-Cabinet Level-2 | TELSTAR, Philipines |
| Bunsen burner | Champingaz, France |
| Centrifuge machine | Eppendorf 5430 R, Germany |
| Incubator | Memmert, Germany |
| Measuring cylinder | GQ, Malaysia |
| Microcentrifuge tube | Axvgen® Scientific, USA |
| Micropipette set | Eppendorf Research® plus, Germany |
| Micropipette tip | Axvgen® Scientific, USA |
| Petri dish | NEST®, China |
| Schott bottle | DURAN®, Germany |
| Spectrophotometer | Thermo Scientific Genesys 20, Malaysia |
| Vortex mixer | VELP® Scientific, Europe |
| Freeze dryer | Scanvac COOLSAFE™, Denmark |
| Auto Fine Coater | JEOL (JFC-1600), USA |

# APPENDIX B

## PREPARATION OF BUFFERS AND MEDIA

### Preparation of Brain-Heart Infusion (BHI) broth

About 7.4 g of BHI broth powder (Himedia, India) was dissolved in 200 mL of distilled water and autoclaved at 121°C, 15 psi for 15 minutes.

### Preparation of Mueller-Hinton (MH) broth

About 4.2 g of MH broth powder (Liofilchem) was dissolved in 200 mL of distilled water and autoclaved at 121°C, 15 psi for 15 minutes.

### Preparation of Luria-Bertani (LB) broth

LB broth was prepared by measuring 8 g of LB broth powder (Merck Millipore). The powder was subsequently dissolved in 200 mL of distilled water and autoclaved at 121°C, 15 psi for 15 minutes.

### Preparation of Mueller-Hinton (MH) agar

Around 26.6 g of MH agar powder (Merck Millipore) was measured and dissolved with 700 mL of distilled water which was subsequently autoclaved at 121°C, 15 psi for 15 minutes. The medium was then poured into petri dishes and

allowed to sit for 15 minutes for solidification. The plates were then kept in 4°C refrigerator.

**Preparation of Mannitol Salt Agar (MSA)**

To prepare Mannitol-Salt agar (Himedia, India), 22 g of Mannitol-Salt agar powder (Himedia) was dissolved in 200 mL of distilled water and autoclaved at 121°C, 15 psi for 15 minutes. The medium was poured into petri dishes and kept in 4°C refrigerator after solidification.

**Preparation of Tryptic Soy Agar (TSA)**

Approximately 20 g of Tryptic soy agar powder were dissolved in 500 mL of distilled water. The media was then autoclaved at 121°C, 15 psi for 15 minutes. The medium was set aside for cooling before pouring onto petri dishes. Agar plates were then kept in refrigerator at 4°C.

**Preparation of Blood agar (BA)**

Approximately 20 g of Tryptic soy agar powder were dissolved in 500 mL of distilled water. The media was then autoclaved at 121°C at 15 psi for 15 minutes. Subsequently, media was set aside for cooling and 30 mL of human blood was added into the media and swirled to allow even distribution of blood. The media was then poured into petri dishes and then kept in refrigerator at 4°C after solidification.

**Preparation of Phosphate Buffer Saline (PBS)**

Phosphate buffer saline was prepared by adding 5 tablets of PBS tablets Merck Milipore into 500 mL of distilled water. The mixture was swirled continuously until the tablets dissolved completely in distilled water. pH of the solution were then adjusted to pH of 7.4 using 1M of sodium Hydroxide (NaOH) and 1M of Hydrochloric acid (HCl). The solution was then autoclaved at 121°C at 15 psi for 15 minutes.

**Preparation of Glutaraldehyde (3%) in PBS**

3% of glutaraldehyde was prepared adding 6 mL of 25% glutaraldehyde (Sigma-Aldrich Co., LLC) to 12.5 mL of 0.1 M PBS. The solution was then topped with distilled water to bring to a final volume of 50 mL.

## APPENDIX C

**Table A:** Bacterial titre of *S. aureus* ATCC 25923 ($Log_{10}$ CFU/mL) after treated with each antibacterial peptide at increasing two-fold concentration.

| **Concentration (µg/mL)** | **0** | **2** | **4** | **8** | **16** | **32** | **64** | **128** | **256** |
|---|---|---|---|---|---|---|---|---|---|
| **PAM-5** | 7.21 | 7.15 | 7.46 | 7.52 | 7.70 | 7.03 | 7.55 | 7.15 | 0.00 |
| **Polymyxin B** | 7.21 | 7.46 | 7.55 | 7.38 | 7.42 | 0.00 | 0.00 | 0.00 | 0.00 |
| **Melittin** | 7.21 | 7.13 | 7.48 | 7.02 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **Negative Control** | | | | | 7.21 | | | | |

**Table B:** Bacterial titre of *E. faecalis* ATCC 19433 ($Log_{10}$ CFU/mL) after treated with each antibacterial peptide at increasing two-fold concentration.

| **Concentration (µg/mL)** | **0** | **2** | **4** | **8** | **16** | **32** | **64** | **128** | **256** |
|---|---|---|---|---|---|---|---|---|---|
| **PAM-5** | 8.78 | 8.72 | 8.69 | 8.74 | 8.83 | 8.77 | 8.87 | 8.81 | 8.89 |
| **Polymyxin B** | 8.78 | 8.55 | 8.47 | 8.71 | 8.75 | 8.83 | 8.72 | 8.78 | 7.90 |
| **Melittin** | 8.78 | 8.63 | 8.49 | 8.51 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **Negative Control** | | | | | 8.78 | | | | |

**Table C:** Bacterial titre of *S. pyogenes* ATCC 19615 ($Log_{10}$ CFU/mL) after treated with each antibacterial peptide at increasing two-fold concentration.

| **Concentration (µg/mL)** | **0** | **2** | **4** | **8** | **16** | **32** | **64** | **128** | **256** |
|---|---|---|---|---|---|---|---|---|---|
| **PAM-5** | 5.76 | 6.19 | 5.71 | 5.76 | 5.42 | 5.29 | 0.00 | 0.00 | 0.00 |
| **Polymyxin B** | 5.76 | 4.84 | 4.79 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **Melittin** | 5.76 | 4.90 | 4.85 | 4.72 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **Negative Control** | | | | | 5.76 | | | | |

**Table D:** Bacterial titre of *S. anginosus* 1360589 ($Log_{10}$ CFU/mL) after treated with each antibacterial peptide at increasing two-fold concentration.

| **Concentration (µg/mL)** | **0** | **2** | **4** | **8** | **16** | **32** | **64** | **128** | **256** |
|---|---|---|---|---|---|---|---|---|---|
| **PAM-5** | 8.59 | 8.51 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **Polymyxin B** | 8.59 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **Melittin** | 8.59 | 8.21 | 8.36 | 7.64 | 8.35 | 0.00 | 0.00 | 0.00 | 0.00 |
| **Negative Control** | | | | | 8.59 | | | | |

## APPENDIX D

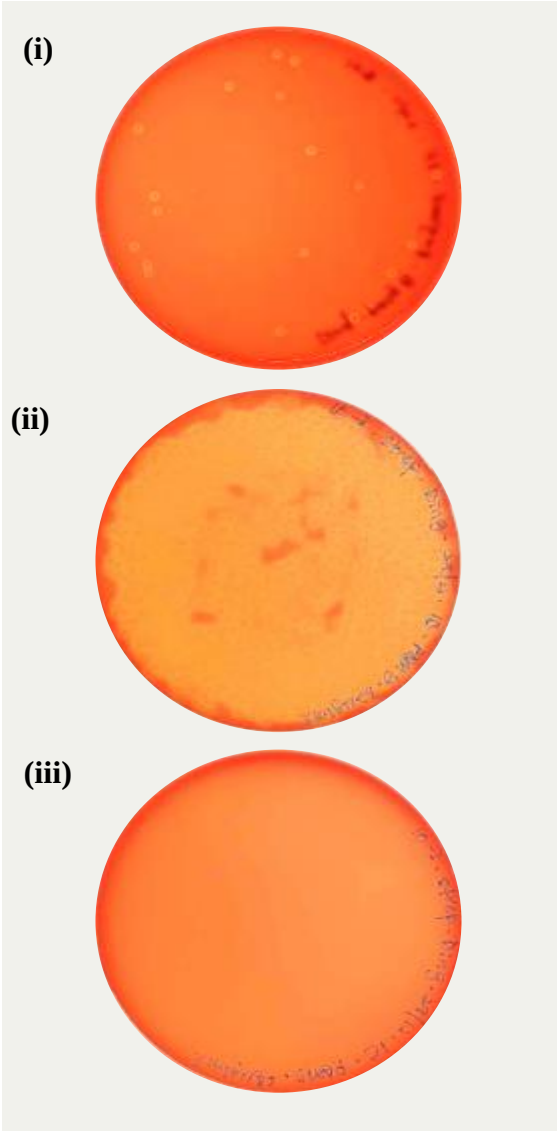


**Figure A:** *Streptococcus pyogenes* on blood agar showing zones of beta-haemolysis. (i) blood agar with scanty bacterial colonies; (ii) bacterial lawn surrounded with visible zone of β-haemolysis; (iii) Blood agar plate without bacteria growth.