UNIVERSITI PUTRA MALAYSIA

ELECTRON MICROSCOPY STUDIES ON THE EFFECT OF ANTIMICROBIAL PEPTIDES DERIVED FROM PEDIOCOCCUS ACIDILACTICI KP10 AND LACTOCOCCUS LACTIS GH1 ON VANCOMYCIN-RESISTANT ENTEROCOCCUS AND METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS

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A PROJECT PAPER SUBMITTED AS A PARTIAL REQUIREMENT FOR THE DEGREE OF THE BACHELOR OF SCIENCE (BIOMEDICAL SCIENCE)

DEPARTMENT OF BIOMEDICAL SCIENCES

FACULTY OF MEDICINE AND HEALTH SCIENCES

UNIVERSITI PUTRA MALAYSIA

SERDANG, SELANGOR

2020

ABSTRACT

ELECTRON MICROSCOPY STUDIES ON THE EFFECT OF ANTIMICROBIAL PEPTIDES DERIVED FROM *PEDIOCOCCUS ACIDILACTICI* KP10 AND *LACTOCOCCUS LACTIS* GH1 ON VANCOMYCIN-RESISTANT *ENTEROCOCCUS* AND METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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Introduction: The emergence and dissemination of drug-resistant bacteria among major Gram-positive and Gram-negative species has exposed the public to severe health threats. As a result, the efficacy of conventional antibiotics has decreased dramatically over time and an imperative solution is urgently required to look for a more effective therapeutics agent against infections caused by antibiotic-resistant bacteria. Recent studies reported that antimicrobial peptides (AMPs) with potential novel mechanisms of inhibition exhibit significant potency against bacteria. The AMPs for this study are isolated from *Pediococcus acidilactici* KP10 and *Lactococcus lactis* gh1 and they are tested against vancomycin-resistant *Enterococcus* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA). **Objective:** This study generally aims to determine the effect of AMPs isolated from *P.acidilactici* KP10 and *L.lactis* gh1 on VRE and MRSA through microscopic analysis. **Methodology:** The Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the AMPs were determined against VRE and MRSA using Nutrient Broth (NB) as the growth medium. The bacteria cells were then treated with MIC values of respective AMPs or left untreated as control. Subsequently, the effect of the AMPs against the bacterial cells in NB was examined using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). **Result:** Micrographs from SEM and TEM of MRSA and VRE bacterial cells treated with AMPs isolated from *P.acidilactici* KP10 and *L.lactis* gh1 displayed several distinguished and apparent signs of morphological and internal composition changes including rough surfaces, formation of blisters, pitted membranes and lysed cells. **Conclusion**: The growth of VRE and MRSA bacterial cells were inhibited by AMPs isolated from *P.acidilactici* KP10 and *L.lactis* gh1.

Keywords: antimicrobial peptides, AMPs, antibiotic-resistance, vancomycin-resistant, VRE, methicillin-resistant, MRSA

ABSTRAK

KAJIAN MIKROSKOPI ELEKTRON TERHADAP KESAN PEPTIDA ANTIMIKROBIAL YANG DIASINGKAN DARIPADA *PEDIOCOCCUS ACIDILACTICI* KP10 DAN *LACTOCOCCUS LACTIS* GH1 TERHADAP VANCOMYCIN-RESISTANT *ENTEROCCOUS* DAN METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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Pengenalan: Kemunculan dan penyebaran bakteria tahan dadah di kalangan spesies Gram-positif dan Gram-negatif utama telah mendedahkan orang ramai kepada ancaman kesihatan. Hasilnya, keberkesanan antibiotik konvensional telah menurun secara dramatik dan penyelesaian penting diperlukan untuk mencari ejen terapeutik yang lebih berkesan terhadap jangkitan yang disebabkan oleh bakteria tahan antibiotik. Kajian baru-baru ini melaporkan bahawa peptida antimikrobial (AMP) dengan mekanisme pencegahan baru mempamerkan potensi yang besar terhadap bakteria. AMP untuk kajian ini diasingkan daripada *Pediococcus acidilactici* KP10 dan *Lactococcus lactis* gh1 dan mereka diuji terhadap Enterococcus-resistant *vancomycin* (VRE) dan Methicillin-resistant *Staphylococcus aureus* (MRSA). **Objektif**: Kajian ini umumnya bertujuan untuk menentukan kesan AMP yang diasingkan dari *P.acidilactici* KP10 dan *L.lactis* gh1 pada VRE dan MRSA melalui analisis mikroskopik. **Metodologi**: Konsentrasi Penghalang Minimal (MIC) dan Konsentrasi Bakterisidal Minimal (MBC) daripada AMP terhadap VRE dan MRSA ditentukan menggunakan Nutrient Broth (NB) sebagai medium pertumbuhan. Sel-sel bakteria kemudian dirawat dengan nilai MIC dari masing-masing AMP atau tidak dirawat sebagai kawalan. Selanjutnya, kesan AMP terhadap sel-sel bakteria akan diperiksa menggunakan *scanning electron microscope* (SEM) dan *transmission electron microscope* (TEM). **Keputusan:** Mikrograf dari SEM dan TEM sel-sel bakteria MRSA dan VRE yang dirawat dengan AMPs dari *P.acidilactici KP10* dan *L.lactis* gh1 menunjukkan beberapa perubahan morfologi and komposisi dalaman yang jelas dan nyata termasuk permukaan yang kasar, pembentukan lepuh, kawah yang mendalam dan sel lisis. **Kesimpulan**: Pertumbuhan sel bakteria VRE dan MRSA telah dihalang oleh AMPs yang diasingkan dari *P.acidilactici* KP10 dan *L.lactis* gh1.

Kata kunci: peptide antimikrobial, AMPs, tahan dadah, vancomycin-resistant, VRE, methicillin-resistant, MRSA

ACKNOWLEDGEMENT

"In the name of Allah S.W.T., the Most Benevolent and Most Merciful"

I would like to express the highest level of gratitude and praise to the Almighty, for giving me the chances, determination and perseverance to accomplish this project accompanied with His guidance and endless grace and mercy throughout the journey of completing this research as a compulsory requirement for SBP 4990.

I would also like to express profound gratitude and special appreciation to my supervisor, Associate Professor Dr Mohd Nasir Mohd Desa and co-supervisor, Puan Nor Fadhilah Khairil Mokhtar for their ideas, support, patience, guidance and time as well as encouragement to me throughout the journey in these two semesters. The constant blessing, guidance and help given by them shall be extremely beneficial for me in the future.

I am also indebted to the staff member and postgraduate students of Microbiology Laboratory and Department of Biomedical Sciences, Universiti Putra Malaysia, especially Mr Muhammad Sabri Mohd Nawawi, Ms Nurul Hana Zainal, Ms Nurul Diana Dzaraly and Mr Abdul Rahman for every valuable information shared. Without their help and cooperation, this project would not be possible.

Finally, I would like to express a deep sense of gratitude, thankfulness and appreciation to my parents for their great role in my life and numerous sacrifices they have made for me. I also thank my siblings for their continuous moral support throughout my studies until the completion of this project.

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MRSA	Methicillin-resistant *Staphylococcus aureus*
VRE	Vancomycin-resistant *Enterococci*
AMP	Antimicrobial peptide
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
^{o}C	degree Celsius
µg	microgram
ml	millilitre
NB	Nutrient broth
NA	Nutrient agar
MSA	Mannitol salt agar
MRS	De Man, Rogosa and Sharpe
D-Ala-D-Ala	D-alanyl-D-alanine
D-Ala-D-Lac	D-alanyl-D-lactate
ATCC	American Type Culture Collection

CHAPTER 1

INTRODUCTION

1.1 Introduction

The emergence of antibiotic resistant pathogenic bacteria has increased dramatically over the last decade and is at an alarming rate (Aslam B et al., 2018). Two of the most epidemiologically crucial antibiotic-resistant organisms are Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Enterococcus* (VRE) which have been associated with significantly higher infection severity and death compared to infections caused by sensitive organisms of the same species (Cosgrove et al., 2001; Salgado and Farr, 2002).

Infections initiated by these resistant bacterial pathogens were also associated with higher hospital costs contributed by longer illness period, extra diagnosis test and the use of more expensive drugs (Li B and Webster TJ, 2018). This condition also indicates that we can no longer fully rely on the available antibiotics to manage the ever-increasing infectious diseases caused by the bacteria.

The decreasing effectiveness of conventional antibiotics acts as a stimulant for researchers to comprehend how antimicrobial peptides (AMPs) can be applied therapeutically. Hence, AMPs which have long been discussed as possible additions or alternatives to conventional antibiotics have been proposed for clinical use. However, there are still gaps in the area of understanding the fundamental principles of AMP's action which has significantly impaired the process of developing AMP into clinical products (Almeida & Pokorny, 2009). Although the efforts in converting nonclinical AMPs candidates into effective ones are not without challenges, the

discovery and advancement of alternative therapeutics peptides is believed to boost in the near future through recent development in basic principles of their mechanism of action and resistance pattern.

In general, AMPs are amphipathic molecules that are positively charged and contain a large quantity of hydrophobic residues. The number of positive net charges is associated with their antibacterial activity while the hydrophobicity is related with their haemolytic activity (Lei et al.,2019). These properties allow AMPs to interact with negatively charged cell membranes through electrostatic interaction and subsequently penetrate it (Bahar & Ren, 2013). Maintaining the membrane integrity is crucial for a variety of cell fundamental functions such as respiration, transport, peptidoglycan cross-linking, osmoregulation and biosynthesis of cell components. Disturbance inflicted on the membrane can result in metabolic dysfunction and even cell death either directly or indirectly (Pag et al., 2008). Previous studies had reported that there are multiple mechanisms in which AMPs exert their antibacterial action which includes the toroidal pore model, the barrel-stave model and carpet model (Som, Vemparala, Ivanov, & Tew, 2008).

The potential benefits of AMP as an antimicrobial agent are significant with a broad range of targets (Easton, Nijnik, Mayer, & Hancock, 2009). One of the AMPs involved in this study is *P.acidilactici* KP10, which can be obtained from a range of traditionally fermented products. It was found to exert antimicrobial activity against a wide range of gram-positive and gram-negative bacteria (Abbasiliasi et al., 2017). In a separate study conducted on *L.lactis* gh1 reported that *L.lactis* gh1 was effective against food-borne pathogens, namely, *Listeria monocytogenes* (Jawan et al., 2018).

Hence, this study was conducted to study the effect of AMPs derived from *P.acidilactici* KP10 and *L.lactis* gh1 on resistant strain pathogens, VRE and MRSA.

There are plenty of conventional methods to visualize the inhibitory effect of antimicrobial agents which includes disc diffusion, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and a few others (Bajpai et al., 2010). However, they are unable to give detailed information on the morphological changes or damages brought about by the antimicrobial agents. Therefore, it is imperative to employ the visual analysis of electron microscopes to evaluate the morphological damage of bacterial cells after treatment with specific antimicrobial agents. This study will investigate the effect exerted by the AMPs through visualization of scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

1.2 Problem statement

The emergence of antibiotic-resistant pathogens globally has led to severe health problems in the clinical setting. The condition is further worsened by slow discovery and development of antibiotics by pharmaceutical companies. It is also associated with rapid development and dissemination of resistance in pathogens that is facilitated by available antibiotics which employ the same mechanism of inhibition. This study serves as a recommendation for researchers to employ a different approach using antimicrobial peptides (AMPs) to counter the increasing number of infection cases caused by resistant bacterial pathogens.

1.3 Objectives

1.3.1 General objective

To determine the effects of antimicrobial peptides (AMPs) derived from *P.acidilactici* KP10 and *L.lactis* gh1 on VRE and MRSA through microscopic analysis.

1.3.2 Specific objectives

1.3.2.1 To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of AMPs isolated from *P.acidilactici* KP10 and *L.lactis* gh1 on VRE and MRSA

1.3.2.2 To study the morphological and internal compositions changes of VRE and MRSA after treatment with AMPs from *P.acidilactici* KP10 and *L.lactis* gh1

1.4 Hypothesis

i. Antimicrobial peptides (AMPs) from *P.acidilactici* KP10 and *L.lactis* gh1 could inhibit the growth of VRE and MRSA.

ii. Antimicrobial peptides (AMPs) from *P.acidilactici* KP10 and *L.lactis* gh1 can cause morphological and internal composition changes on VRE and MRSA.

1.5 Significance of the study

There is insufficient data reported to support the determination on the effect of AMPs derived from *P.acidilactici* KP10 and *L.lactis* gh1 against VRE and MRSA. Hence, this study is relevant to be conducted to provide more information and act as a recommendation to local researchers and clinicians to improve the situation caused by antibiotic-resistant pathogens.

CHAPTER 2

LITERATURE REVIEW

2.1 Antibiotic resistance

Antibiotic-resistant pathogenic bacteria are emerging at an alarming rate and impose a wide variety of significant challenges to human health globally (J. O'Neill, 2014). This condition has greatly compromised the efficacy of antibiotics as a valuable therapeutic. In general, resistance can occur naturally or through mutation. In bacteria, genes that are responsible for resistance against antibiotics can be inherited from relatives or nonrelatives through the aid of plasmid, a mobile genetic element. This process, known as horizontal gene transfer, enables different species of bacteria to acquire resistance (Read & Woods, 2014). It is also facilitated by the misuse of antibiotics, antibiotic load in the environment and nontherapeutic use of antibiotics as animal's growth promoter (Butye, 2003).

Heretofore, new antibiotics were steadily developed and introduced into the clinic. However, for the past two decades, only two new antibiotic classes have been introduced and neither of which have significant inhibitory effects on Gram-negative bacteria (Coates et al., 2011). Therefore, there is an increasing need to search for alternative approaches and develop novel therapeutic strategies to handle the situation.

2.2 Causes of antibiotic resistance

2.2.1 Overuse

Antibiotics are vastly used in primary care where an estimated amount of 80-90% of antibiotics are prescribed (Goossens et al, 2005). Most of these prescriptions were accounted for urinary tract and respiratory tract infections. Antibiotics are generally not recommended to cure viral infections such as coughs, colds and sore throat. However, surveys conducted in the UK and several other countries found that antibiotics were prescribed to half of all the patients regardless of the type of infection (Hawker JI et al, 2014). In countries where antibiotics are not regulated, such products are easily accessible and can be easily obtained without any prescription while in countries where antibiotics are regulated, it can be easily purchased online, all of which promotes overuse (Michael CA, Dominet-Howes D and Labbate M, 2014).

2.2.2 Unregulated agricultural use

As in the case with humans, antibiotics usage in agriculture are increasing substantially to manage infections caused by bacteria and as a growth booster for food production (Gross M, 2013). The use of such products on animals is believed to improve animal health and speed up the pace at which animals gain weight to generate more yield and better-quality products (Michael CA et al, 2014). These antibiotics were then transferred to humans when they ingest the food production of said agricultural practices. This occurs through the process of 1) death of sensitive bacteria by antibiotics used on animals, allowing opportunist antibiotic-resistance bacteria to thrive; 2) passing on of resistance bacteria to humans upon ingestion of

food products; 3) infections in humans with adverse health consequences (Golkar Z, Bagasra O, Pace DG, 2014).

This agricultural practice can further promote antibiotic resistance by affecting the environmental microbiome when the antibiotics were excreted by the animals and dispersed through fertilizer and groundwater (Barlett JG, Gilbert DN, Snellberg B, 2013). It can also interfere with the percentage of resistant versus susceptible microorganisms, subsequently altering the environmental ecology (Golkar Z, Bagasra O, Pace DG, 2014).

2.2.3 Lack of new antibiotics availability

One of the most effective approaches to combat antibiotic resistance is by developing new antibiotics with novel mechanisms of inhibition. However, regulatory and economic obstacles had significantly held back such antibiotic's development. Based on a study conducted in 2013, the antibiotic field had been neglected by 15 out of 18 of the largest pharmaceutical companies (Barlett JG, Gilbert DN, Snellberg B, 2013). This may be associated with the misconception of antibiotic development to be no longer considered as an economically sustainable investment as they are used for a short period of time, curative and do not yield great profit. Some pharmaceutical companies had also merged which caused the number of research teams and the diversity to decrease dramatically. Funding cuts attributed to the downturn of economy in certain countries also caused academia-conducted antibiotic research to be greatly cut back (Piddock LJ, 2012).

2.2 Mechanism of antibiotic resistance

Throughout evolution over millions of years, bacteria have developed complicated mechanisms of antibiotic resistance. This can be attained by various biochemical pathways. Certain bacteria are also known to be capable of possessing multiple resistance mechanisms at the same time to avoid killing by antibiotics. The four major classification of antibiotic resistance mechanisms includes modifications of the antibiotic, blocking from reaching the target, alteration of target sites and resistance attributed to the process of global cell adaptation (Munita and Arias, 2016).

2.2.1 Modification of antibiotic

Resistant bacteria could also produce enzymes capable of altering the chemical composition of antibiotics through the catalysation of acetylation, phosphorylation and adenylation. These alterations were subsequently reported to be affecting the synthesis of proteins at the ribosome level (Wilson, 2014). A wide variety of modifying enzymes have been identified but all biochemical reactions that they catalyse will eventually lead to reduced affinity towards the target.

The antibiotic can also be modified through direct destruction of antibiotic molecules. This is known to be the general mechanism employed in B-lactam resistance through the action of B-lactamase. These enzymes are known to make the antimicrobial agent ineffective by breaking up the amide bond of the B-lactam ring. For instance, the emergence of clinically relevant penicillin-resistant *S. aureus* infection was caused by a plasmid-encoded penicillinase that broke up the B-lactam ring bond. This condition was further worsened by the nature of penicillinase which

has a high rate of transmission between *S.aureus* strains, increasing the number of resistance bacteria (Bush, 2013).

Although efforts were taken to produce B-lactam compounds with less susceptibility to penicillinases, new plasmid-encoded B-lactamase arises each time. This is said to be attributed to the normal process of bacterial evolution. Currently, there are more than 1000 different B-lactamases identified through various studies (www.lahey.org/studies) and the number are increasing over time.

2.2.2 Prevention from reaching the antibiotic target

The target for most antibiotics used clinically is intracellular except for gram-negative bacteria which acts on the cell membrane. Therefore, in order to express antimicrobial effect on their respective targets, antibiotics need to pass through the outer and/or the inner membrane. Resistant bacteria have evolved to prevent this from occurring by reducing antimicrobial agents' uptake hence, decreasing the permeability. In gram-negative bacteria, the outer membrane serves as the first line of defence to prevent the entry of a wide variety of potentially toxic compounds, including antibiotics (Munita and Arias, 2016).

Certain bacteria have also developed the sophisticated machinery known as an "efflux pump" that can effectively remove toxic compounds and antimicrobial agents out of the cell. One of the earliest findings of said mechanism was the extrusion of tetracycline from *E.coli*'s cytoplasm back in the 1980s (McMurry, Petrucci and Levy, 1980). From then on, a number of efflux pumps classes have been identified and reported in both gram-negative and gram-positive bacteria, promoting their antimicrobial resistance property. To date, there are 5 major families of efflux pumps which includes the resistance-nodulation-cell-division family (RND), the

multidrug and toxic compound extrusion family (MATE), the major facilitator superfamily (MFS), the ATP-binding cassette family (ABC) and the small multidrug resistance family (SMR) (Piddock, 2006).

2.2.3 Alteration in target sites

Through this mechanism, the bacteria protect the target either by modifying it or preventing the binding of antibiotics to the binding site which subsequently lowers the affinity towards antibiotic molecules. The latter is a much more common mechanism employed by bacterial pathogens which can affect almost all families of antimicrobial molecules. This can be attained through enzymatic alteration of binding site, target site's genes point mutations or substitution of the initial target (Munita and Arias, 2016).

2.2.4 Resistance caused by global cell adaptive processes

Upon entry in the body of hosts, bacterial pathogens have to fight for nutrients and evade rival organisms attacking them. The host's immune system is also constantly attacking them in its effort to remove pathogens from the body. Hence, it is crucial for these pathogens to develop specific mechanisms in order to cope and adapt to the stressors and pressure in hostile environments. Through years of evolution, they have devised the mechanisms that are able to maintain membrane homeostasis and cell wall synthesis, both of which are important targets of antibiotics (Munita and Arias, 2016).

2.3 Antimicrobial peptides (AMPs)

The decreasing effectiveness of antibiotics has increased considerable interest on another alternative source of novel antimicrobial agent which is antimicrobial peptides (AMPs). They are proteinaceous compounds that can cause deleterious effect to bacteria other than the producing strain (V.Karthikeyan and S.W. Santhosh, 2009). AMPs have been identified through numerous studies to be a considerable replacement for available antibiotics. They are also known to be effective against a broad range of microorganisms at low micromolar concentration, including those that have developed resistance towards conventional antibiotics (Pereira,2006).

AMPs are typically short peptides with amino acids ranging from 12 to 50, contain large proportions of hydrophobic amino acids, positively charged under physiological condition and are able to rapidly kill invading microorganisms (Yeaman and Yount, 2003 and Brogden KA, 2005). Upon contact with negatively charged membranes, the positively charged peptides will rearrange themselves into amphipathic conformation (Hyun Kim et al, 2013). The cationic charge of the peptides is also responsible for their aggregation in close proximity to surfaces of gram-negative bacteria's outer membrane and gram-positive bacteria's cell wall, both of which are negatively charged.

Unlike conventional antibiotics, AMPs employ distinct mechanisms which act directly on bacterial membranes, damaging its structure or altering its permeability (Teixeira et al., 2012 and Hancock and Rozek, 2002). A study conducted reported that the porous 40-80-nm-thick mesh of gram-positive bacteria's cell wall allows many AMPs to pass easily (Malanovic and Lohner, 2016). As for gram-negative bacteria, their outer membrane employs a charge-exchange

mechanism which is also known as self-promoted uptake hypothesis that allows the crossing of some AMPs. Through this mechanism, Ca^{2+} and Mg^{2+} on lipopolysaccharides are competed against by the cationic peptides (Anunthawan et al., 2015). Both mechanisms destabilize the membrane which result in the disruption of membrane-associated protein complexes and subsequently the efflux of the cells vital metabolites, ions and components (Nguyen et al., 2011; Wilmes et al, 2011).

Some bacteria employ another antibacterial action of AMPs mechanism which is by acting on other macromolecules inside the cell (Hyun Kim et al, 2013). Once they gain entry inside the cell, they interact with internal targets using the same mechanism used for cell membrane, permeating and destroying them (Malanovic and Lohner, 2016; Koprivnjak and Peschel, 2011). The synthesis of important cell components and metabolic processes can also be interfered (Hale and Hancock, 2007; Brogden, 2005 and Le et al., 2017). Because of this property, development of resistance towards AMPs are thought to be improbable, unless there is a complete physiology alteration of the cell (Gordon et al., 2005 and Gaspar et al., 2013).

AMPs mechanism of action have been studied extensively in previous studies and although there is still indefinite amount of the number of mechanisms, the membrane perforation mode were the toroidal pore model, the barrel-stave model, the carpet model and aggregated channel model as shown in Figure 2.3 (Jakel et al., 2012 and Brogden, 2005). On the other hand, the non-membrane perforation mode suggests that AMP binds to bacterial cell membrane surfaces and disrupts the normal physiological functions such as protein synthesis which subsequently result in cell death (Zanetti, 2003).

The toroidal-pore model predicts that the AMPs form clusters outside the cell before penetrating the cell membrane and alter the bacterial phospholipid monolayer to create a hole of 1 to 2 nm diameter (Jakel et al., 2012). The barrel stave model, on the other hand hypothesized that the AMPs attach to the surface of membrane, forming pore structure which eventually leads to the extrusion of bacterial cell contents. In the carpet model, AMPs cause the disintegration of cell membranes by altering the surface tension of bacterial membranes (Jakel et al., 2012). As for the aggregated channel model, AMPs are predicted to form peptide-lipid polymers upon binding to the phospholipid molecules on the surface of the cell membrane, which will also result in insertion into the cell (Park & Hahm, 2005). All mechanisms described above will ultimately lead to death of cells.

The regulation of AMP expression and where it is expressed plays a dominant role in their mechanism of action. AMPs are commonly found at human body sites which are normally exposed to microbes. Although it became apparent that some of the AMPs are expressed constitutively, the fact remains that the majority are induced during infection, inflammation or injury (Zhang & Gallo, 2016). Since antibiotic-resistance is triggered by a diversity of mechanisms except for the cellular action possessed by antimicrobial peptides, this unique feature has driven a considerable research effort focused on the potential of AMPs and their mechanism as novel antimicrobial therapies.

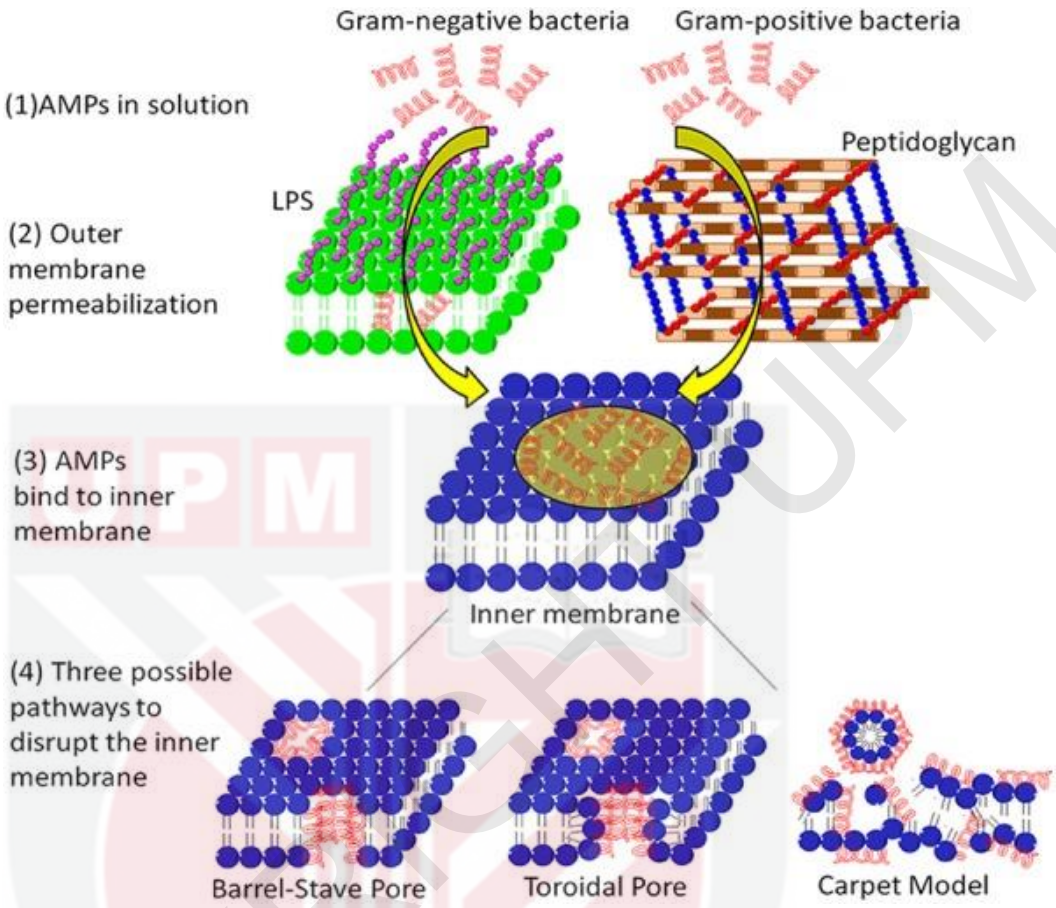

Figure 2.3 shows the illustrative diagram of action mechanisms of AMPs on eukaryotic and bacteria cell membrane, reference of Li (2017).

2.4 Vancomycin-resistant *Enterococcus*

Enterococci initially belonged to the class of enteric gram-positive cocci before it was introduced in the genus Streptococcus (Murray, 1990). However, due to genetic differences, enterococci have been put into their own genus, *Enterococcus* (Schleifer & Kilpper-Balz, 1984). Even though there were more than a dozen species reported, only two are responsible for most of human infections which are *E.faecium* and *E.faecalis*. The latter was then reported to be the primary species which represented >90% of all clinical isolates (Moellering, 1992).

For decades, Enterococci have been widely recognized to be the cause of endocarditis and hospital-acquired infections (Cetinkaya et al., 2000). Even so, they are generally not considered a highly virulent bacterial pathogen. However, through years of evolution, they have acquired resistance towards conventional antibiotics. This problem has created a global alarm because resistance to many available conventional antimicrobial agents makes treating enterococcal infections difficult. Vancomycin-resistant *Enterococcus* (VRE) were first identified in the mid-1980s and have spread rapidly since then to become a major health threat all over the world (Cetinkaya et al., 2000).

VRE possesses the biological properties that enable them to develop resistance to most of conventional antibiotics. VRE has been recovered in various countries after their early recovery from patients in the UK and France. These countries include Malaysia, Spain, the United States, Belgium, Denmark, Italy, Germany, The Netherlands, Canada, Australia and Sweden (Woodfor, Johnson and Morrison, 1995).

There are 5 types of phenotype that are responsible for resistance development towards vancomycin which are VanA, VanB, VanC, VanD and VanE (Fines, Perichon, Reynolds, Sahm, & Courvalin, 1999 and Arthur & Courvalin, 1993). VanA and VanB were hypothesized to be newly acquired clusters of genes that were initially absent in enterococci and were commonly related to *E.faecalis* and *E.faecium*. Additionally, VanA gene clusters found on the transposon Tn*1546* were also associated with resistance towards high concentrations of vancomycin (Arthur & Courvalin, 1993). In contrast, VanB gene clusters were found to be resistant to a range of concentrations (Evers & Courvalin, 1996).

VanA and VanB enterococci were believed to attain resistance through a unique mechanism in which the bacteria's cell wall precursors were produced by an alternative biosynthetic pathway that was aided by a cluster of genes. This mechanism subsequently lowers the binding affinity of vancomycin (Leclercq & Courvalin, 1997). Instead of the normal peptidoglycan (PG) with termini D-Ala-D-Ala, VRE ends with the depsipeptide D-Ala-D-Lac. As a result, vancomycin binds at 0.001 times the affinity of binding towards PG precursor (Bugg et al., 1991)

2.4 Methicillin-resistant *Staphylococcus aureus*

Staphylococcus aureus is a gram positive coccal bacteria that occurs singly, in pairs, tetrads and distinctive irregular "grape like" patterns (Stapleton and Taylor, 2002). In the pre-antibiotic era, *Staphylococcus aureus* bacteria was responsible for 80% of the deaths in patients (Skinner D and Keefer CS, 1941). As a result, in the early 1940s, penicillin G was introduced, and it managed to improve prognosis significantly. However, resistant strains were acknowledged just two years later

(Rammelkamp CH, Maxon T 1942). By the 1950's, they were commonly reported in hospitals.

Hence, in response to the spread of this penicillin resistant bacteria, an alternative B-lactam antibiotic named methicillin was developed and introduced into the clinic in 1959. B-lactam antimicrobial drugs act by targeting and inhibiting bacterial cell wall biosynthesis. However, in 1961, a case of methicillin-resistant had been reported (Bæk et al., 2014). Resistance to this mechanism allows the biosynthesis of cell walls to continue even in the presence of antibiotics (Herathge, George, & Rowley, 2011). The term methicillin resistance has persisted although methicillin is no longer used in clinical settings. The term also indicates resistance to virtually all B lactams except cephalosporin B lactams.

MRSA has also been noted to acquire resistance to new agents, notably vancomycin which was considered as one of the last treatment choices for MRSA infections and further complicating treatment (Liu c et al, 2011). *S. aureus* could also asymptomatically colonize mucosal surfaces and skin of healthy individuals. These bacteria which can survive well in the environment is often transmitted via airborne route and direct contact. Asymptomatic carriers are usually unaware of the infection, although they may develop the symptoms of minor skin infections. They also have a higher tendency to develop infection and were noted to be a significant source of distribution of *S.aureus* in the community (Henry and Frank, 2009). Because of their opportunist property, the bacteria can develop into a more serious infection such as toxic shock syndrome which can be lethal. (Stapleton & Taylor, 2002).

Moreover, MRSA is known for its ability to develop resistance towards virtually every antibiotic that has been developed. The mechanism that facilitates the

development of resistance includes horizontal transfer from outside sources to genes of the microorganisms, mutation of chromosome and antibiotic selection (Henry and Frank, 2009). Antibiotic-resistant strain often causes infections in epidemic waves commenced by one or several successful clones and MRSA is prominently highlighted during these epidemics (Henry and Frank, 2009). For the past decades, infections caused by antibiotic-resistant strains particularly by MRSA strain has increased drastically and caused severe burden to the healthcare and community settings globally.

2.5 Scanning Electron Microscopy (SEM)

Essential information such as the condition, structure and type of the cell can be unveiled through the morphology study of cells. Exposure to various physical and chemical stimuli can bring about morphological alteration to the cell surface, including swelling, shrinking, blebbing, smoothing, etc. Remarkably, SEM have been widely used for the ultrastructural analysis of cells to facilitate the process of gathering valuable information about cell's structure. The first commercialization of the scanning electron microscopy (SEM) instrumentation occurred in the early 60s. Since then, SEM has advanced to become a powerful scientific tool for studying the ultrastructure of bio cellular molecules (Moropoulou et al., 2019). SEM is known to be able to display morphological structure of organisms, namely in diagnosis of infectious diseases.

However, SEM usage in routine microbiology and diagnosis was limited by the laborious specimen preparation (Beniac, Siemens, Wright, & Booth, 2014 and Beniac, Hiebert, Siemens, Corbett, & Booth, 2015). To overcome this, a number of high-quality polycarbonate filters were developed for the collection and surface

observation of various bacterial and virus species. Specimen preparation for SEM also requires a conducting surface that is needed for magnification greater than 1000 x. This is so that the charging for small organic particles such as bacteria and virus can be reduced, hence producing images with adequate contrast. This can be achieved through critical point drying but there is a risk of specimens cracking and shrinking to half the original size. Additionally, SEM also specifically requires specimens to be in a dehydrated condition to ensure best imaging performance. If wet specimens are with the microscope, the high vacuum condition will dry out the specimens at a very fast rate which can lead to reduced microscope performance, contrast and resolution.

Alternatively, "wet SEM", environmental SEM and cryo-techniques have been developed for the imaging of hydrated specimens (Beniac, Siemens, Wright, & Booth, 2014, Thiberge et al., 2004, Bergmans, Moisiadis, Van Meerbeek, Quirynen, & Lambrechts, 2005 and Hayles, Stokes, Phifer, & Findlay, 2007). This progress relies on technological improvements which allows conditions that sustain the sample in vacuum through the coupled techniques of improved differential pumping capabilities and of detectors (Thiberge et al., 2004).

2.6 Transmission Electron Microscopy (TEM)

Ever since its discovery and first use in the early 1940s, TEM continues to be an important technology in cell biology and has proven valuable to provide direct nano-structural information of cellular components. For microbes such as bacteria and virus, Transmission Electron Microscopy (TEM) are more frequently employed for analysing the ultrastructural of cells due to its ability to provide details about internal composition in addition to surface analysis (Golding, Lamboo, Beniac, & Booth, 2016). In general, TEM has the same principle of operation with a light microscope. However, instead of light, TEM utilized electrons. Since the wavelength of electrons are 100 000-fold shorter compared to visible light, the images produced by TEM will have much greater resolution compared to those obtained from even the highest -resolution light microscopes. Because of this, TEM is considered as a very powerful tool that can reveal the finest details of internal structures (Williams & Carter, 1996).

The application of TEM in cell biology requires the imaging of plastic-embedded stained cells by electron beam passage that goes through the specimens and will eventually be absorbed and scattered (Winey et al., 2014). Despite its power, TEM also has limitations which includes a limited number of cells imageable in detail, the possibility of fixation and staining artefacts formation and the compulsory use of fixed cells. The techniques for sample preparation of TEM are also laborious, costly and may require the expertise of experts to obtain successful results. Therefore, experimental design is the key.

CHAPTER 3

METHODOLOGY

3.1 Materials and Reagents

Nutrient Agar (NA) was used for the culture of VRE while Mannitol Salt Agar was used for the culture of MRSA. As for the bacteria producing the AMPs, *P.acidilactici* KP10 and *L.lactis* gh1 were cultured on M17 and MRS broth respectively. Both M17 and MRS broth, together with Nutrient broth (NB) were used for MIC and MBC determination which were carried out in 96-well microplates. Glutaraldehyde, Sodium Cacodylate Buffer, Osmium Tetroxide, a series of acetone (35%, 50%, 75%, 95% and 100%) were used in the early steps of sample preparation for SEM and TEM. However, for SEM processing, in addition to the materials and reagents mentioned, albumin coated aluminium foil, double sided tape and gold coating were used. As for TEM processing, in addition to the materials and reagents mentioned, acetone and resin mixture, beam capsules, toluidine blue, uranyl acetate stain and lead stain were used.

3.2 Organisms

The two resistant bacteria used in this study, MRSA (=ATCC 700699) and VRE (=ATCC 700221) were obtained from the American Type Culture Collection (ATCC). Inoculates of MRSA and VRE were prepared using the colony suspension method where colonies were picked from cultures that were previously allowed to grow on nutrient agar for 24 h at 37ºC and transferred to nutrient broth followed by incubation for 24 h at 37ºC.

Both isolates of producing antimicrobial peptides, *P.acidilactici* KP10 and *L.lactis* gh1 used in this study were kindly given to us by Professor Arbakariya Ariff, Dean of Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia. *P.acidilactici* KP10 were cultured in M17 broth while *L.lactis* gh1 were cultured in MRS broth.

3.3 Protein extraction and purification

AMPs from *P.acidilactici* KP10 and *L.lactis* gh1 were extracted and purified from the cell free culture supernatant of the bacteria using ammonia sulphate precipitation method according to Duong-Ly and Gabelli (2014) with some modifications. Firstly, *P. acidilactici* KP10 and *L.lactis* gh1 were cultured in M17 broth and MRS broth respectively for 24 h at 37^{o}C to induce the expression of protein of interest. Then, 1000 mL of cultured bacteria suspension were aliquoted into 50 mL falcon tubes and centrifuged at 10 000 x g at 4^{o}C for 15 min. All cell free supernatants were combined into a sterile bottle and 567g $(NH_4)_2SO_4$ was added until they were fully dissolved with the aid of a magnetic stirrer. The solvents were subsequently aliquoted into 50 mL tubes and incubated overnight at 4^{o}C (upregulated). Following incubation, the tubes were centrifuged at 10 000 x g at 4^{o}C for 15 min. Supernatants were removed from the precipitate and replaced with distilled water. The tubes were stored overnight at 4^{o}C. Finally, the content of all tubes was mixed into one large beaker for inoculum preparation.

3.4 Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

3.4.1 Preparation of inoculum:

The mixture containing precipitate was diluted 1:100 in normal saline solution (Inoculum 100 µL: Normal saline solution 10 ml) ~ approximately 10^6 CFU/ml

3.4.2 Preparation of test medium:

A sterile 96 well plate was labelled and prepared under aseptic conditions. A volume of 50 µL of Mueller Hinton Broth (MHB) was added into wells in Column 1 to 11. As for wells in column 12, 100 µL of MHB was added. Subsequently, 50 µL of the sample was added into well A, B, C and D. Serial dilution was performed using multichannel pipette with 50 µL of the solution from column 1 transferred into the well in column 2. The dilution procedure was repeated up to column 10. After mixing, 50 µL of the solution from column 10 was discarded. Column 11 acts as positive growth control while column 12 as sterility control of medium.

3.4.3 Inoculation of the test plates:

50 µL of the inoculum was added into each well except wells in Column 12 (sterility control). The test plates were then incubated for 12 h.

3.4.4 Reading Plate

The lowest concentration of the test material that inhibited the growth of organisms hence, preventing turbidity in colour is recorded as the MIC. Upon incubation, columns with clear medium and no turbidity were scored as MIC value.

In contrast, MBC was determined by directly plating the content of wells with concentration higher than MIC value. Its value is indicated by the lowest concentration that results in no colony growth from directly plated contents of the wells.

3.5 Sample preparation for electron microscope

VRE and MRSA were grown in their respective growth medium to an OD500 of 0.35. The main culture was then subdivided into 50 mL aliquots. With respective antimicrobial peptides, subcultures were treated for 12 h or left untreated as control. The cells were then harvested by centrifugation and supernatants were decanted to obtain pallets. The samples were allowed to fix by the addition of 2.5% glutaraldehyde for 4-6 h at 4^{o}C. Further processing for SEM and TEM slightly differs.

3.5.1 Scanning Electron Microscope

For SEM, following fixation, samples were washed with 0.1 M sodium cacodylate buffer for 3 changes of 10 min each with supernatant decanted in each step. Then, 1% osmium tetroxide was added into each sample for 2 h at 4ºC for post-fixation. Samples were washed again with 0.1 M sodium cacodylate buffer for 3 changes of 10 min each. Subsequently, the samples were dehydrated in ascending grades of acetone dilution (35%, 50%, 75%, 95% and 100%). After dehydration, the samples were pipetted onto aluminium foil of 1 cm diameter coated with albumin and were left to dry in the critical point dryer for 1 ½ h. Finally, they were mounted onto the stub using double sided tape and sputter coated with gold coating before they were examined under the SEM. SEM imaging was performed using JSM-IT100 InTouchScope™ SEM.

3.5.2 Transmission Electron Microscope

As for TEM, following fixation, animal serum was added to the samples and they were allowed to clot. The clotted samples were subsequently diced into 1 mm^3 size and fixed in 2.5% Glutaraldehyde for 1-2 h at 4ºC. The next few steps (washing, post-fixation, washing & dehydration) were carried out similar to SEM. Following dehydration, samples were infiltrated with an increasing concentration of acetone and resin mixture and embedded into a beam capsule filled with resin. A glass knife and ultramicrotome were utilized to cut 1 µm thick sections of the sample. The thick sections were then placed onto a glass slide, stained with toluidine blue and examined under a light microscope. The areas of interest were selected and cut for ultrathin sections. The silver sections were subsequently selected and picked up

using a grid. Finally, the sections were stained with uranyl acetate for 15 min (washed with double distilled water) and lead stain for 10 min (washed with double distilled water) before they were viewed under TEM.

CHAPTER 4

RESULT

4.1 Minimum Inhibitory Concentration (MIC)

Table 4.1.1 MIC of AMPs from *L.lactis* gh1 against VRE and MRSA

Types of Bacteria	MIC at Well no.	MIC (μg/ml)	MIC values (μg/ml)
VRE	4	1.47662	1.47662
	4	1.47662	
	4	1.47662	
MRSA	3	2.95325	2.95325
	3	2.95325	
	3	2.95325	

In Table 4.1.1, the MIC of AMPs from *L.lactis* gh1 against VRE was recorded at well no. 4 with average MIC values of 1.47662 μg/ml. As for the reaction against MRSA, MIC was recorded at well no. 3 with average MIC values of 2.95325 μg/ml. The corresponding result shows that MIC values were lower for VRE which means that it is more susceptible to the AMPs compared to MRSA.

Table 4.1.2 MIC of AMPs from *P.acidilactici* KP10 against VRE and MRSA

Types of Bacteria	MIC at Well no.	MIC (μg/ml)	MIC values (μg/ml)
VRE	6	0.34688	0. 34688
	6	0. 34688	
	6	0. 34688	
MRSA	1	11.10006	11.10006
	1	11.10006	
	1	11.10006	

In Table 4.1.2, the MIC of AMPs from *P.acidilactici* KP10 against VRE was recorded at well no. 6 with average MIC values of 0.34688 μg/ml. As for the reaction against MRSA, MIC was recorded at well no. 1 with average MIC values of 11.10006 μg/ml. The corresponding result shows that MIC values were lower for VRE which means that it is more susceptible to the AMPs compared to MRSA.

4.2 Minimum Bactericidal Concentration (MBC)

Table 4.2.1 MBC of AMPs from *L.lactis* gh1 against VRE and MRSA

Types of Bacteria	MBC at Well no.	MBC (μg/ml)	MBC values (μg/ml)
VRE	3	2.95325	2.95325
	3	2.95325	
	3	2.95325	
MRSA	2	5.09649	5.09649
	2	5.09649	
	2	5.09649	

In Table 4.2.1, the MBC of AMPs from *L.lactis* gh1 against VRE was recorded at well no. 3 with average MBC values of 2.95325 μg/ml. As for the reaction against MRSA, MBC was recorded at well no. 2 with average value of 5.09649 μg/ml. The corresponding result shows that MIC values were lower for VRE which means that it is more susceptible to the AMPs compared to MRSA.

Table 4.2.2 MBC of AMPs from *P.acidilactici* KP10 against VRE and MRSA

Types of Bacteria	MBC at Well no.	MBC (μg/ml)	MBC values (μg/ml)
VRE	5	0.69375	0.69375
	5	0.69375	
	5	0.69375	
MRSA	1	11.10006	11.10006
	1	11.10006	
	1	11.10006	

In Table 4.2.2, the MBC of AMPs from *P.acidilactici* KP10 against VRE was recorded at well no. 5 with average MBC values of 0.69375 μg/ml. As for the reaction against MRSA, MBC was recorded at well no. 1 with average value of 11.10006 μg/ml. The corresponding result shows that MIC values were lower for VRE which means that it is more susceptible to the AMPs compared to MRSA.

4.3 Ultrastructure of untreated MRSA and MRSA treated with AMPs derived from *P.acidilactici* KP10 and *L.lactis* gh1

4.3.1 SEM image of untreated MRSA

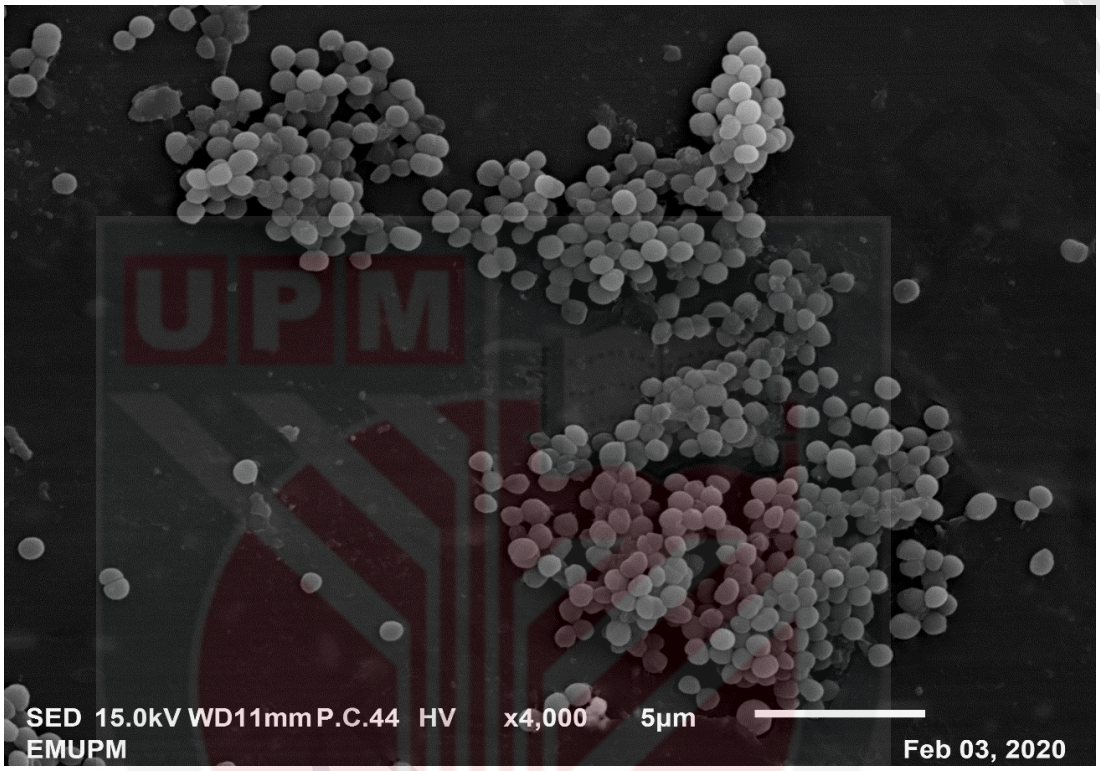

Figure 4.3.1 : Untreated MRSA

Figure 4.3.1 shows the untreated bacteria exhibited smooth and intact membranes surfaces with structure integrity.

4.3.2 SEM image of MRSA treated with AMPs from *P.acidilactici* KP10

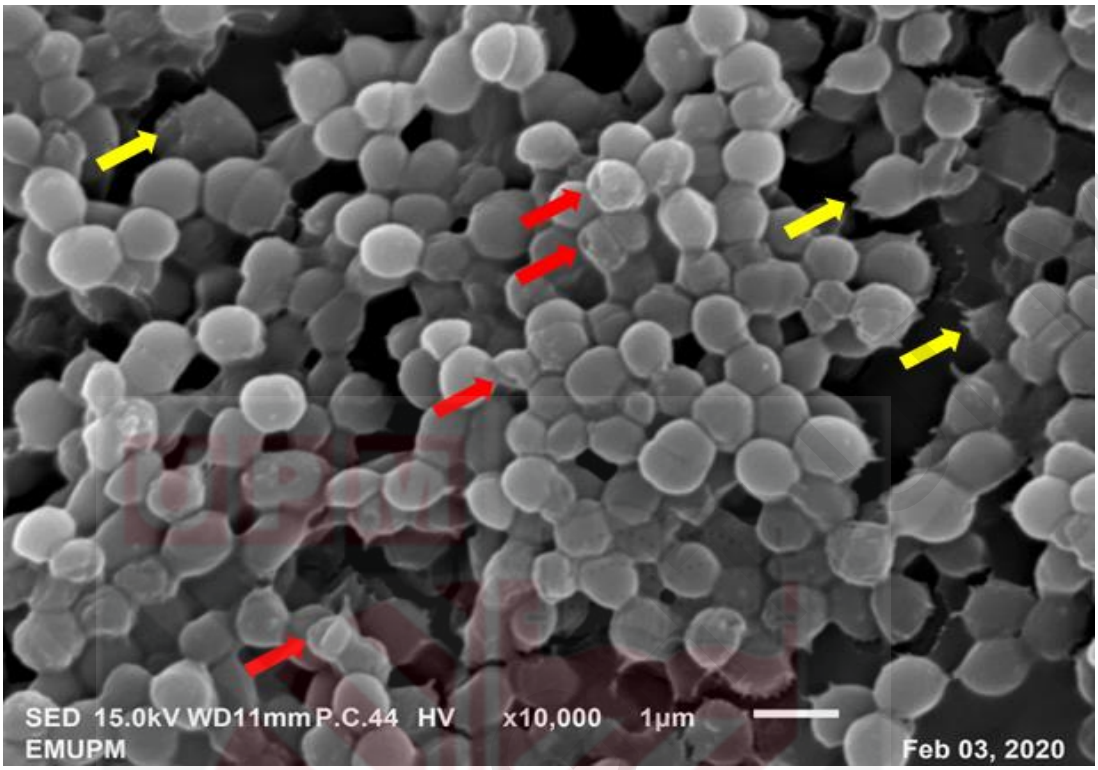

Figure 4.3.2: MRSA treated with AMPs from *P.acidilactici* KP10

Figure 4.3.2 shows the SEM image demonstrating the antibacterial effect of AMPs derived from *P.acidilactici* KP10 against MRSA. The surface of MRSA bacteria cells showed several damages following 12 h incubation. They appeared to be aggregated with dimples and blisters. There were also spheroplast (yellow arrow) which were hypothesized through previous studies to be the result of cell wall deficient. Occurrence of pitted membrane (red arrow) indicates that the AMPs might have attached and penetrated the bacteria's cell membranes leading to their disruption. Scale bars correspond to 1µm.

4.3.3 SEM image of MRSA treated with AMPs from *L.lactis* gh1

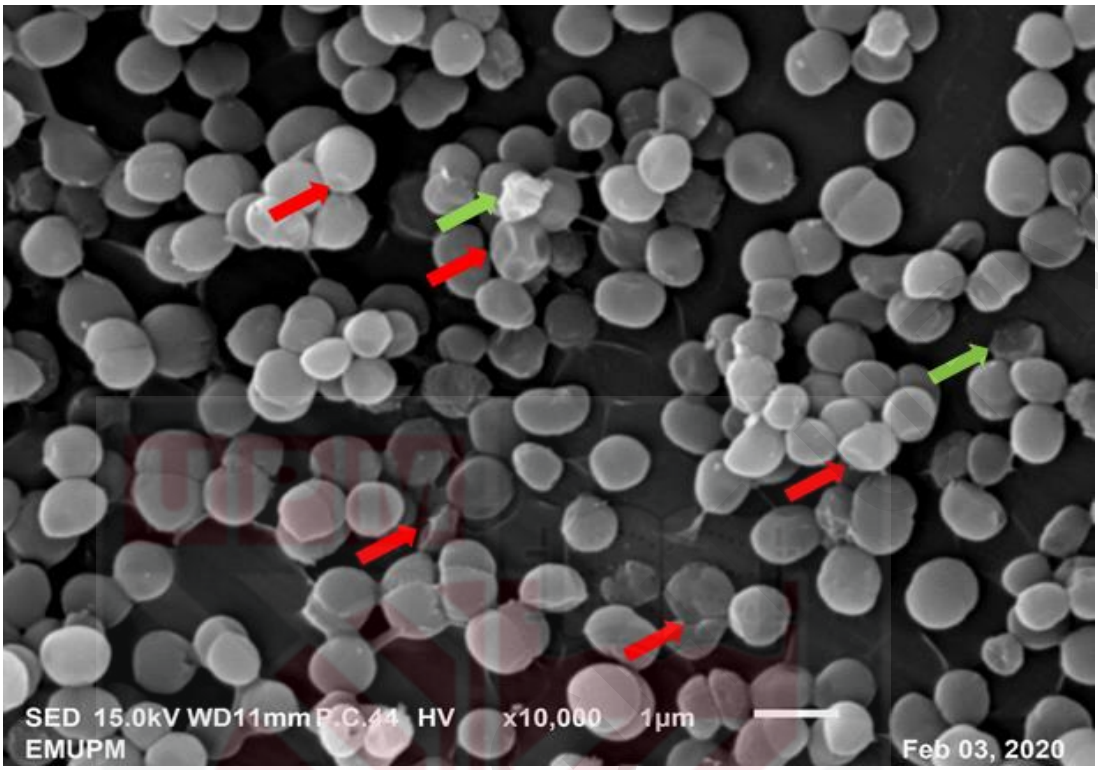

Figure 4.3.3: MRSA treated with AMPs from *L.lactis* gh1

Figure 4.3.3 shows the SEM image demonstrating the antibacterial effect of AMPs derived from *L.lactis* gh1 against MRSA after 12 h incubation. The treatment caused pitting of membranes (red arrow) which was attributed to the same mechanism explained in Figure 2.3. Additionally, lysis of some cells (green arrows) can also be observed which might be associated with the loss of structural integrity and stability of the membrane that eventually result in cell death. Scale bars correspond to 1µm.

4.3.4 TEM image of untreated MRSA

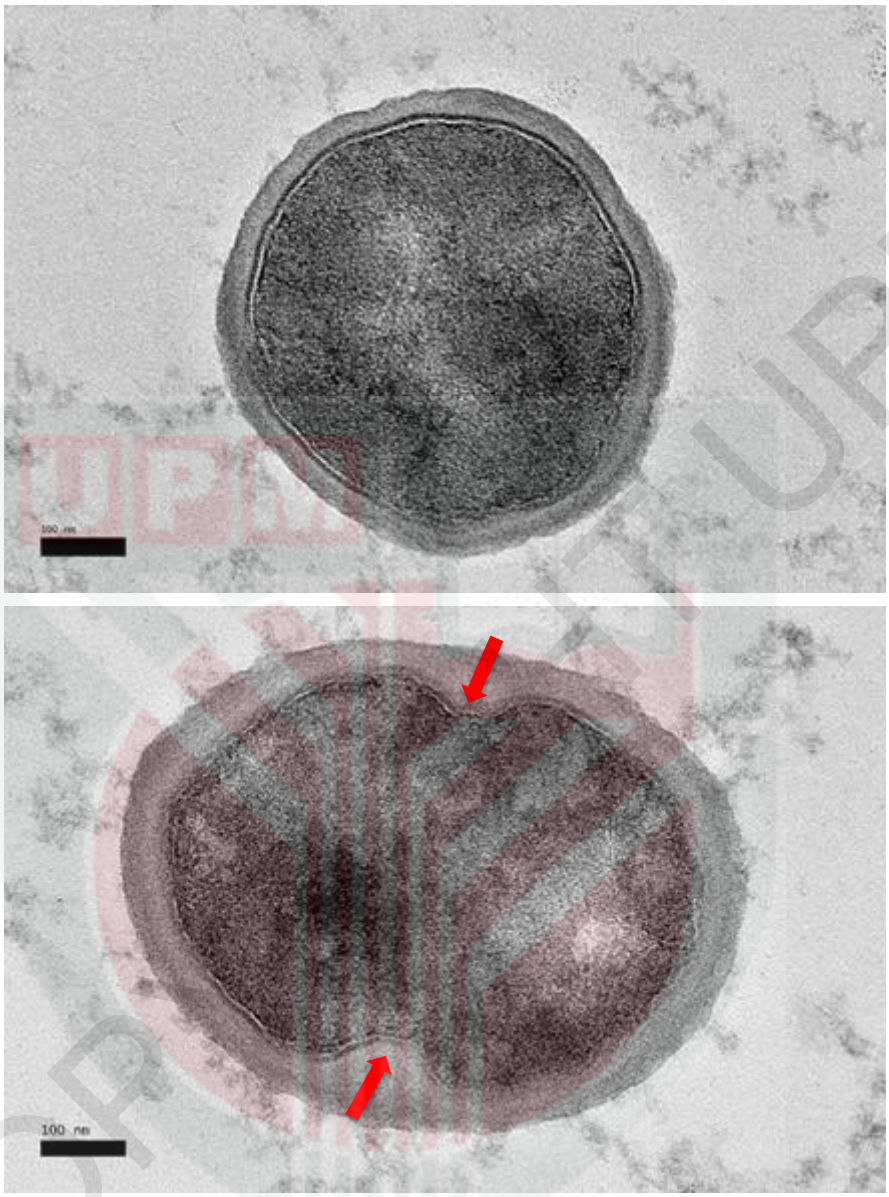

Figure 4.3.4 TEM image of untreated MRSA

Figure 4.3.4 shows the untreated bacterial cells displaying well defined spherical shape morphology and smooth surfaces with obvious boundaries between them where the inner and outer membranes are visible as continuous structures. There is also indication of regular growth of binary fission. The periplasmic space had a uniform appearance and thin. Scale bar corresponds to 200 μm.

4.3.5 TEM image of MRSA treated with AMPs from *P.acidilactici* KP10

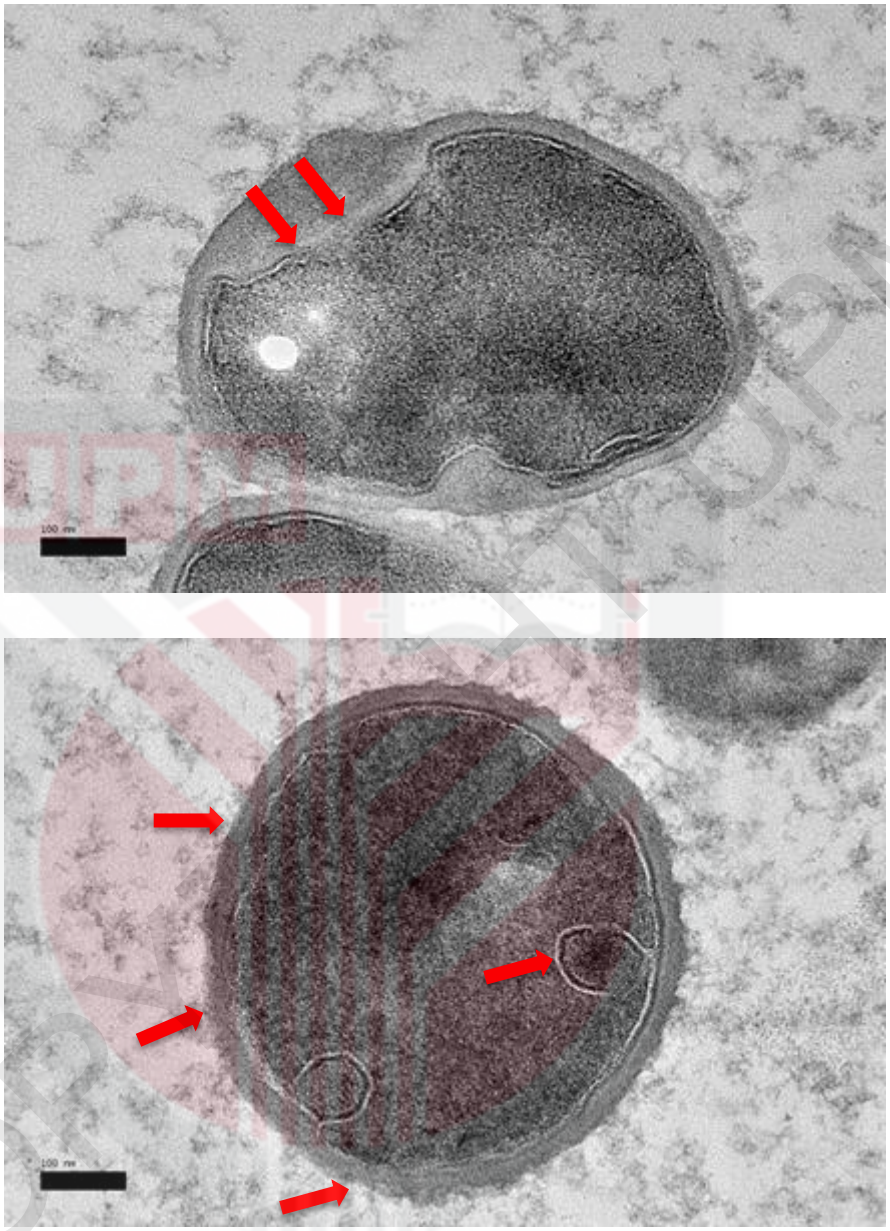

Figure 4.3.5 TEM image of MRSA treated with AMPs from *P.acidilactici* KP10

In Figure 4.3.5, following 12 h treatment with AMPs from *P.acidilactici* KP10, the coccoid shape of MRSA was not preserved. The outer membrane is weakened but the inner membrane remained intact although slightly waved. There is also swelling in the periplasmic space and some mesosome-like structures (red arrow) can be observed. Scale bar corresponds to 200 μm

4.3.6 TEM image of MRSA treated with AMPs from *L.lactis* gh1

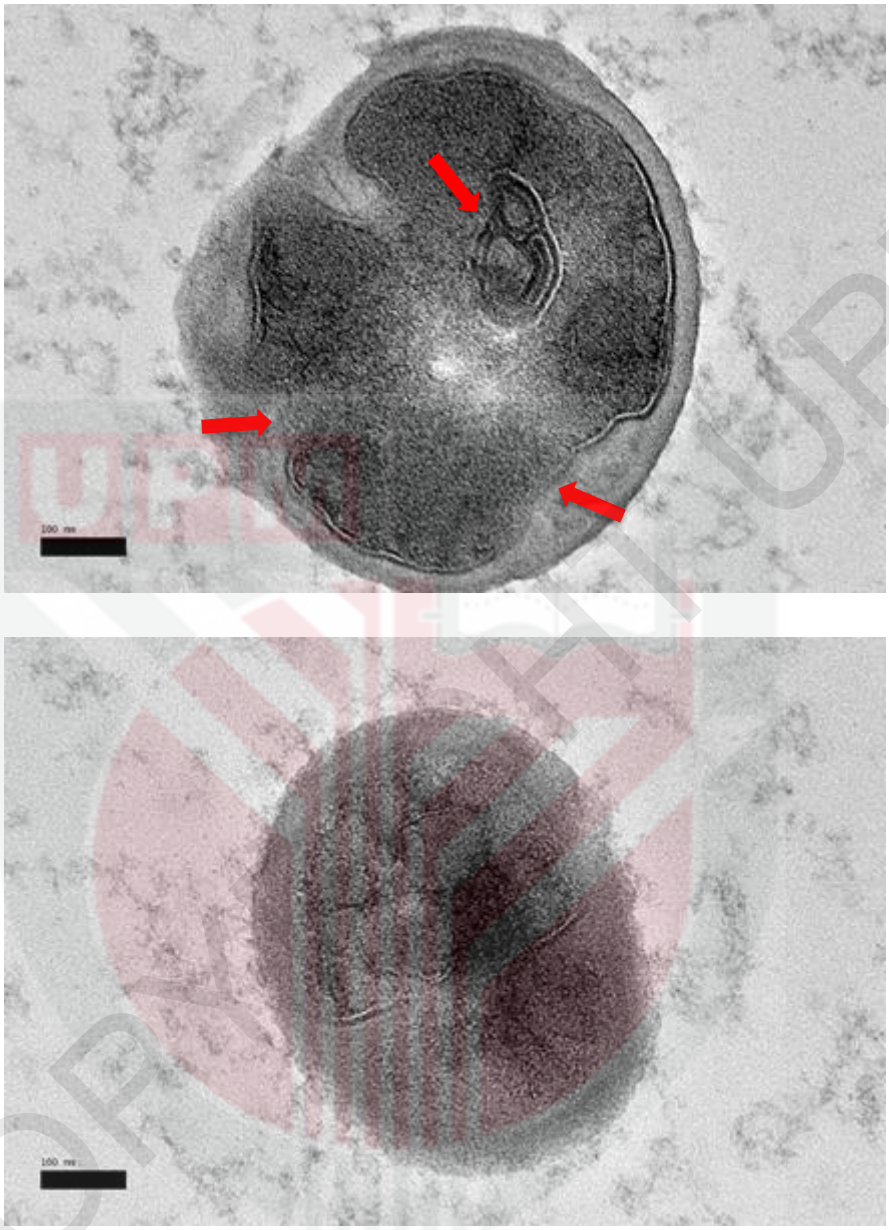

Figure 4.3.6 TEM images of MRSA treated with AMPs from *L.lactis* gh1

The images in Figure 4.3.6 observed using TEM confirmed the weakening of both the outer and inner cell membrane. Both membranes were compromised with interrupted stretches which causes the boundaries between the treated cells to be significantly blurred. This condition was indicative of damaged cell walls. Subsequently, the presence of double-layered mesosome-like structures can be

observed in the cytoplasm. In addition, the periplasmic space is also swollen. Scale bar corresponds to 200 μm.

4.4 Ultrastructure of untreated VRE and VRE treated with AMPs derived from *P.acidilactici* KP10 and *L.lactis* gh1

4.4.1 SEM image of untreated VRE

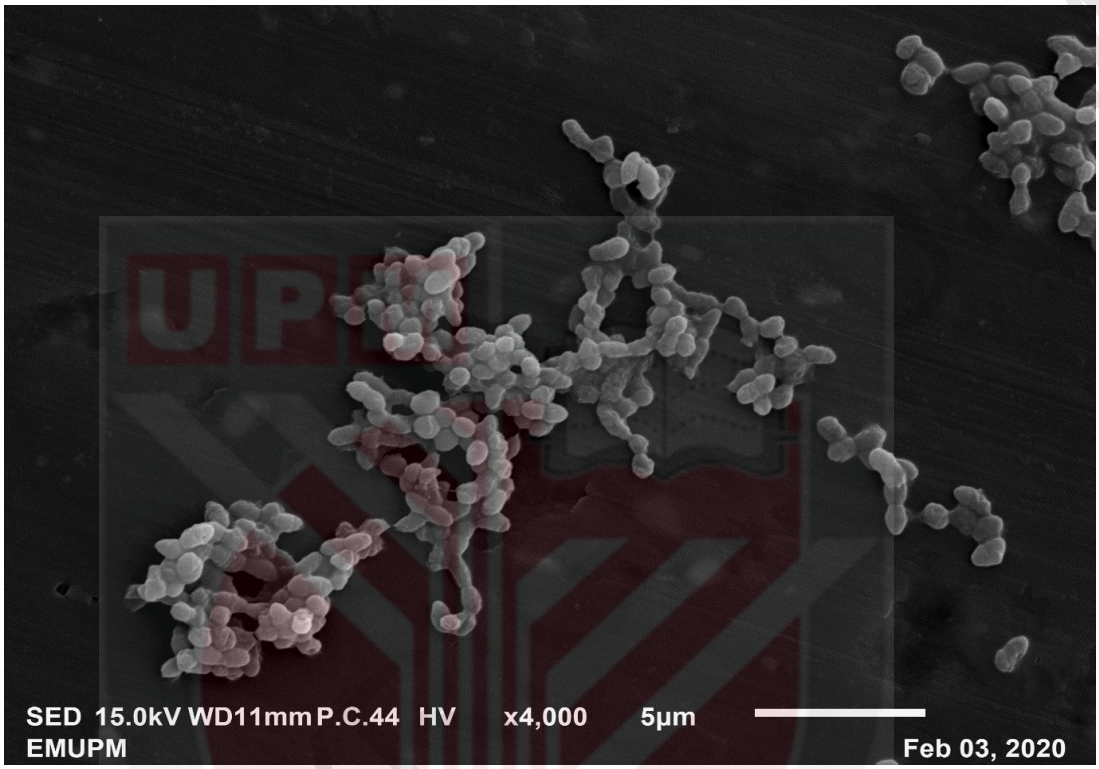

Figure 4.4.1: untreated MRSA

In Figure 4.4.1, the untreated bacteria exhibited smooth and intact surfaces with structure integrity.

4.4.2 SEM image of VRE treated with AMPs from *P.acidilactici* KP10

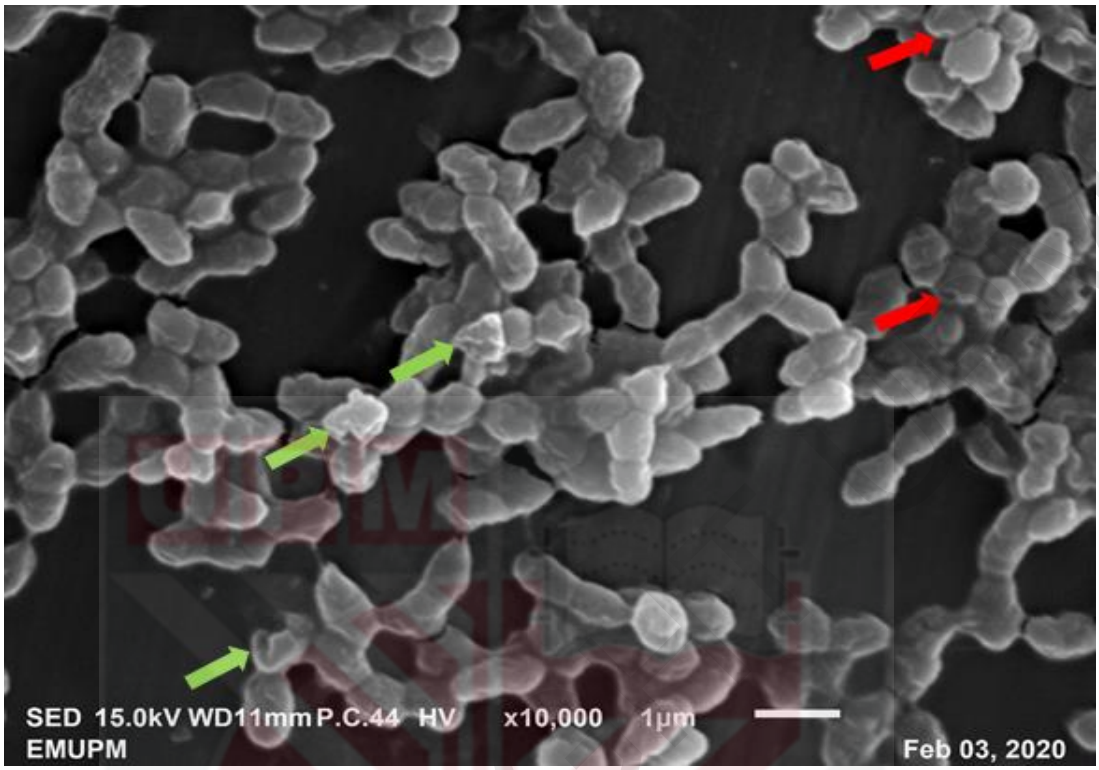

Figure 4.4.2: VRE treated with AMPs from *P.acidilactici* KP10

Figure 4.4.2 shows the SEM image demonstrating the antibacterial effect of AMPs derived from *P.acidilactici* KP10 against VRE after 12 h incubation. The diplococci shape of VRE was not preserved. The bacterial cells also displayed surface irregularity and indentations (red arrow). Some completely burst cells were also observed (green arrow). Scale bars corresponds to 1µm

4.4.3 SEM image of VRE treated with AMPs from *L.lactis* gh1

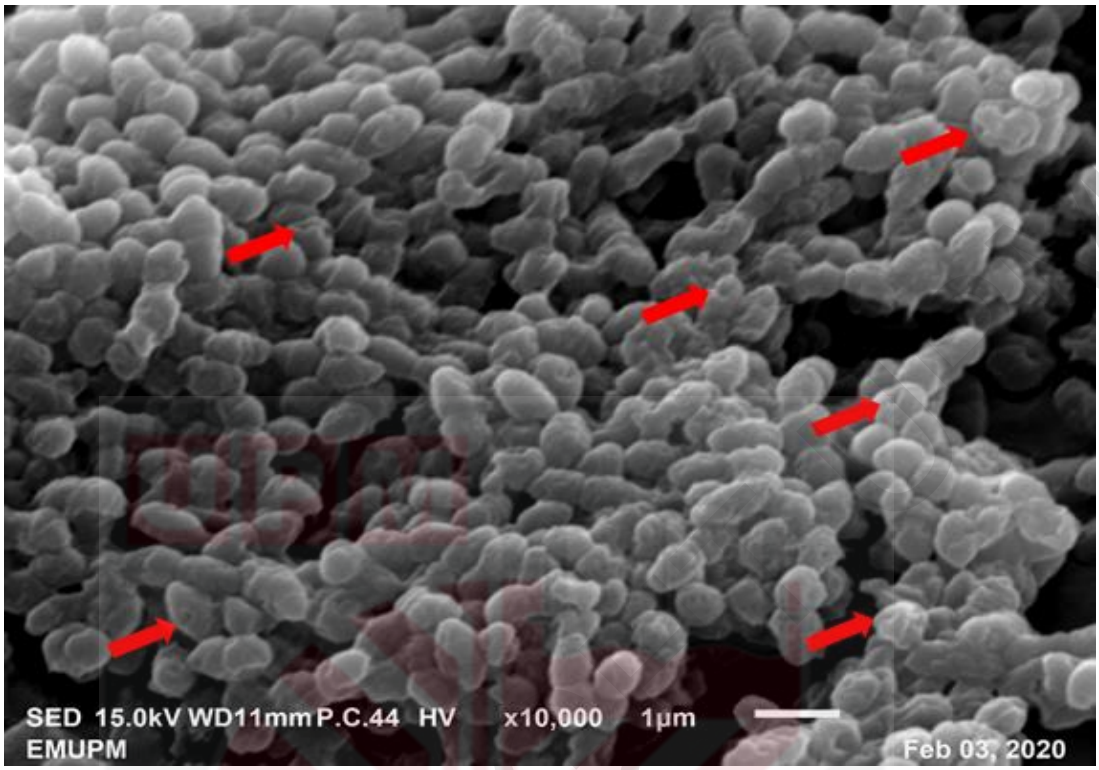

Figure 4.4.3: VRE treated with AMPs from *L.lactis* gh1

The SEM image in Figure 4.4.3 demonstrated the antibacterial effect of AMPs derived from *L.lactis* gh1 against VRE after 12 h of incubation. The surface of bacterial cells appeared to be aggregated with dimples and blisters. The treatment also caused abundant pitting of membranes (red arrow). Scale bars corresponds to 1µm.

4.4.4 TEM image of untreated VRE

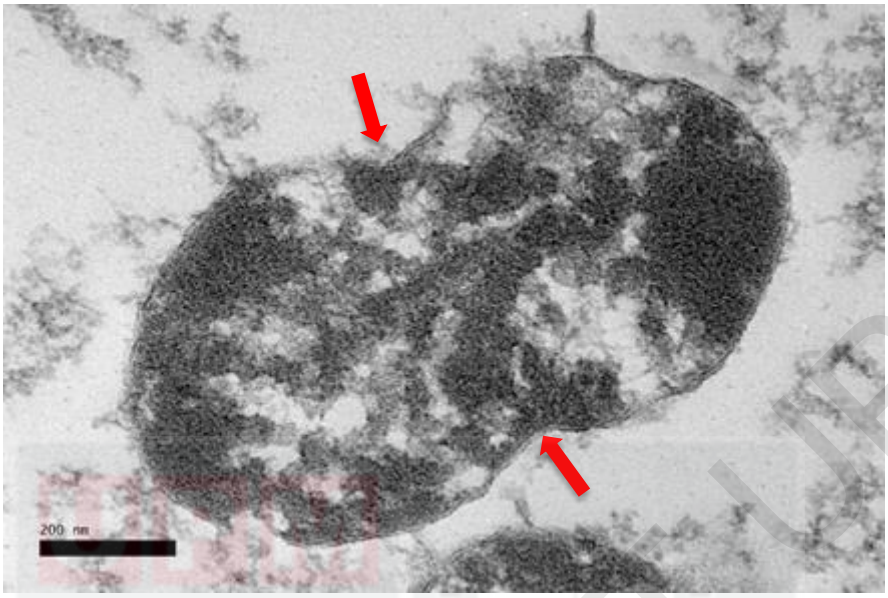

Figure 4.4.4 TEM images of untreated VRE

In the control group demonstrated in Figure 4.4.4, the bacterial cells show normal morphology and homogenous cytoplasm. The cell wall and membrane are intact and there is no noticeable damage. There is also indication of regular growth of binary fission Scale bar corresponds to 200 µm.

4.4.5 TEM image of VRE treated with AMPs from *P.acidilactici* KP10

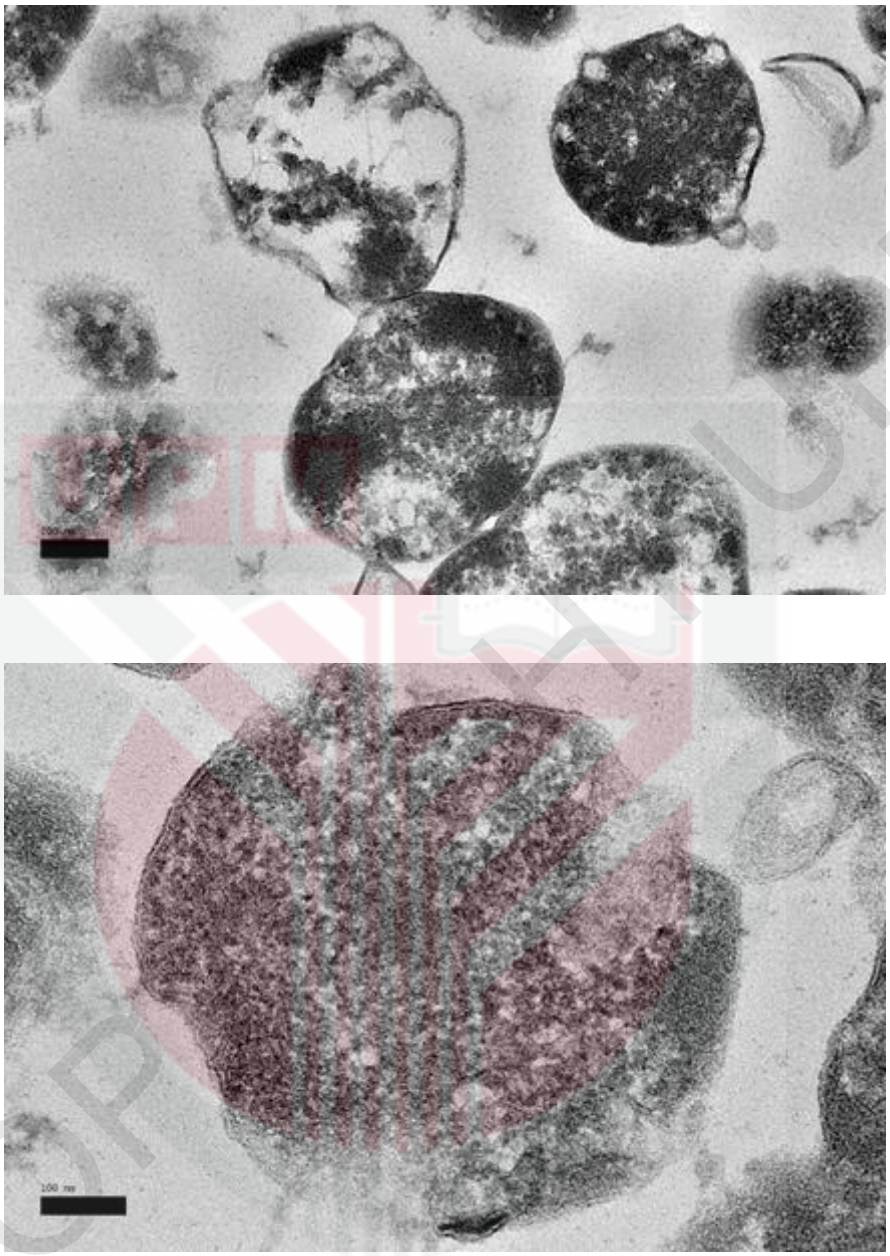

Figure 4.4.5 TEM image of VRE treated with AMPs from *P.acidilactici* KP10

In Figure 4.4.5, TEM images demonstrated the antibacterial effect of AMPs derived from *P.acidilactici* KP10 on VRE after 12 h incubation. The micrographs confirmed remarkable changes in the shape and size of the cells. The membranes displayed partial disruption and permeabilization which leads to leakage of cell contents. The treated bacterial cells also showed lytic effects.

4.4.6 TEM image of VRE treated with AMPs from *L.lactis* gh1

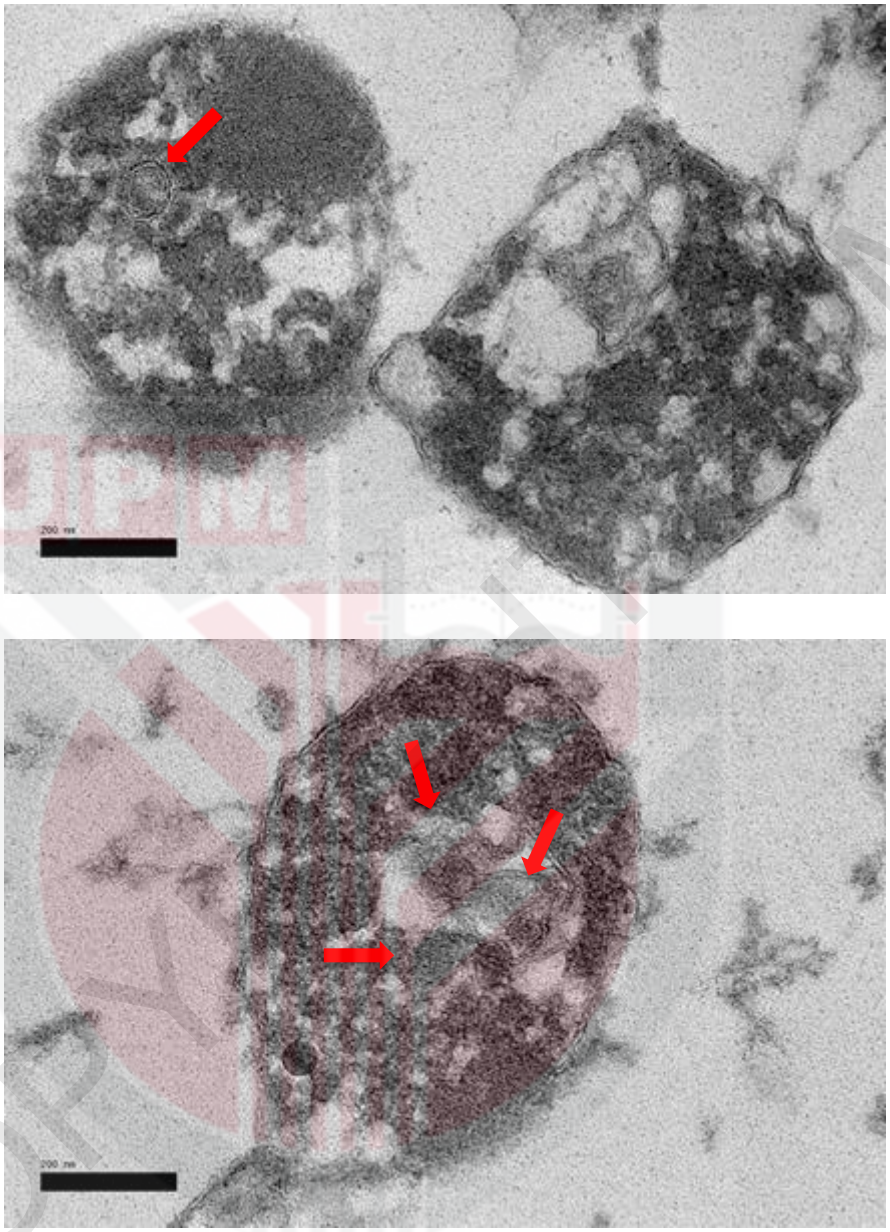

Figure 4.4.6 TEM image of VRE treated with AMPs from *L.lactis* gh1

In Figure 4.4.6, it can be observed that the enterococcoidal shape of VRE was severely compromised and there was clear deformation of the cell surface. *L.lactis* gh1 treatment for 12 h also reported the perforation of bacteria membrane at polar ends which subsequently leads to cytoplasm leakage. Double-layered mesosome-like structures could also be seen in the cytoplasm. Scale bar corresponds to 200 μm.

CHAPTER 5

DISCUSSION

5.1 MIC and MBC

Minimal inhibitory concentrations (MIC) of AMPs from *P.acidilactici* KP10 and *L.lactis* gh1 are listed in Table 4.1 and Table 4.2 respectively. Both MRSA and VRE strains used in the study were susceptible to AMPs isolated from *P.acidilactici* KP10 and *L.lactis* gh1, with MICs ranging 0.34688 - 11.10006 µg/mL.

Microdilution method employed to calculate the respective MIC and MBC of AMPs derived from *P.acidilactici* KP10 and *L.lactis* gh1 allowed the estimation of the lowest concentration of AMPs that either totally inhibit or suppress growth of bacteria in culture. Both the MIC and MBC tests complement each other. In this study, the MIC is the lowest concentration of AMPs that inhibit the growth of VRE and MRSA. At MIC, there is no visual turbidity in the liquid culture medium. However, plating the bacteria treated with MIC of AMPs onto agar still results in bacterial growth because the AMP is not strong enough to cause death of bacteria cells. In contrast, the MBC is recorded as the lowest concentration of AMPs which lowers the viability of the initial bacterial inoculum by >99.9% reduction. It was determined by subculturing the broth dilution of concentration higher than the MIC to fresh agar plates. The lowest concentration that totally prevented the growth of bacteria on plate implies the MBC. This method is also useful to determine whether the concentration of AMP or time plays a bigger role in bacterial killing.

The MIC of *P.acidilactici* KP10 and *L.lactis* gh1 against MRSA were 11.10006 µg/mL and 2.95325 µg/mL respectively. As for VRE, the MIC of AMPs

from *P.acidilactici* KP10 was recorded to be 0.34688 μg/mL and the MIC of AMPs from *L.lactis* gh1 was 1.47662 μg/mL. In MBC testing plate, the reaction of VRE against AMPs from *P.acidilactici* KP10 was recorded to be 0.69375 μg/mL while the reaction against *L.lactis* gh1 obtained the values of 2.95325 μg/mL. As for the reaction of MRSA against *P.acidilactici* KP10 and *L.lactis* gh1, MBC value was recorded to be 11.10006 μg/mL and 5.09649 μg/mL respectively.

In respect to the MBC and MIC value, AMPs derived from both *P.acidilactici* KP10 and *L.lactis* gh1 were found to be more effective against VRE compared to MRSA where lower concentration of both AMPs were needed to inhibit the growth of VRE compared to MRSA. This might be attributed to the differences in cell wall compositions and growth rates (Radziq et al., 2013).

5.2 SEM and TEM of MRSA and VRE

To study the morphological alteration of MRSA and VRE, we employed the powerful scanning and transmission electron microscopy, both of which are predominant for providing information on the structure of cells and their enclosures of singular organelles (Erlandsen, Kristich, Dunny and Wells, 2004). The observation from electron microscopy serves to support the inhibitory activity of the AMPs that were suggested by *in vitro* microbiological techniques which in this study was the MIC and MBC testing. SEM and TEM were highly advantageous in terms of portraying the bacteriostatic/bactericidal activity of a wide variety of antibacterial agents at certain doses. Through these combined techniques, we were able to compare the structure of cell membrane, the roughness and irregularity of outer surface and subcellular structure of treated bacteria that are otherwise not visible

under traditional light microscopes (Khalifa, Friberg, Illing, & Rask-Andersen, 2003).

Essentially, there is still insufficient evidence on how specific bacterial strains respond to particular antimicrobial agents and to date, resistance still remains an ongoing problem. Although published reports still do not agree on definite antibacterial mechanisms of AMPs, majority of them suggest that AMPs exhibit their bacteria-killing action through cell membrane disruption and penetration since the target of most antimicrobial agents is intracellular organelles. Accordingly, electron microscopies were employed to study the *in vitro* effect of AMPs against VRE and MRSA due to their ability to examine thin specimens and display the interior image of cells following AMPs penetration. In addition, SEM and TEM allowed the identification of structural alteration associated with the presence or absence of peptides at specific doses hence, revealing the potential antimicrobial effect of different agents. In this study, the independent morphological parameters of VRE and MRSA and their respective organelles represent their sensitivity and susceptibility status towards AMPs from *P.acidilactici* KP10 and *L.lactis* gh1.

Small bacteria like VRE and MRSA that have a diameter of less than 1 mm enables electron microscopy to execute single-cell profiling instead of the conventional single-cell imaging. To support the observed differences in treated bacteria, untreated bacteria were studied as negative growth control. Representative images of MRSA by SEM illustrated how the bacteria exist as normal and rounded appearing cells within clusters in the control culture. The SEM images obtained from treated VRE and MRSA revealed surface roughness and several distinguished and apparent signs of membrane alteration which includes pitted membranes and

formation of deep craters and blisters. Some of the bacteria cells were also completely lysed, as can be seen in Figure 4.4.2. It was also revealed that the cells experienced shredding and rupturing of cell walls which subsequently resulted in shape distortion and severe destruction.

The changes observed on the bacteria cell membrane suggested the possibility of cytoplasm leakage and loss of functionality associated with the incorporation of AMPs. From the micrographs obtained from treated bacterial cells, almost all of them showed severe membrane damages. The breaks observed in cell membrane were hypothesized from previous study to cause potassium ions to exit the cell which then leads to respiration inhibition and promote propidium iodide uptake (Price-Whelan et al., 2013). It was also suggested that destabilization of the outer membrane was brought about by the action of AMPs that act on the lipopolysaccharide layer and substitute for the Mg^{2+} ions (Da Silva & Teschke, 2003). The destabilization of the outer membrane eventually enables AMPs to fill the periplasmic space through promotion of AMPs penetration. Whereas, the creation of holes was attributed to alteration in the cytoskeleton matrix.

The micrographs obtained from TEM of bacterial cells treated with MICs of both AMPs confirmed their interaction with inner and outer membrane, its disturbance at polar ends and cellular content leakage. Our TEM images of VRE bacterial cells that were incubated with AMPs from *P.acidilactici* KP10 (Figure 4.4.5) revealed damaged membrane and lysis of some cells as also noted in SEM images. On the other hand, our TEM images of VRE cells incubated with AMPs from *L.lactis* gh1 (Figure 4.4.6) revealed obviously compromised cell shape and membrane disruption particularly at the polar ends which cause leakage of

cytoplasm. The location might be attributed to the preferential interaction of positively charged AMPs with cardiolipin-rich domains that are negatively charged (Mileykovskaya & Dowhan, 2000). Another significant impact of both peptides on VRE and MRSA was evident from the presence of swollen periplasmic space caused by disintegration of membrane and increased penetration of AMPs (Hartmann, 2010). The mesosome-like structures present in the cytoplasm of treated bacterial cells are a simple physicochemical process. They are artefacts that were known to form due to chemical deterioration of membrane during chemical fixation for electron microscopy (Balkwill & Stevens, 1980).

The results obtained from coupled SEM and TEM analysis gave valuable information on the susceptibility and sensitivity of these resistant bacteria pathogens towards alternative therapeutic at subcellular resolution. Apart from making electron microscopy a potential platform for ultrastructure investigation of single cells, it will also eventually widen the microbiology field to new potential, especially in the field of developing new antimicrobials compounds in treating diseases.

CHAPTER 6

CONCLUSION

6.1 Conclusion

The emergence of drug-resistant bacteria and the serious problems they carry requires urgent development of alternative therapeutics. In this respect, AMPs is among the most heavily researched of the alternatives to antibiotics and are studied as promising antimicrobial agents. From present study, it can be concluded that AMPs derived from *P.acidilactici* KP10 and *L.lactis* gh1 have antibacterial activity and were proven to reduce the growth number of VRE and MRSA by damaging the cell membrane. Bacterial cells that were treated with these peptides expressed various phases of cell death ranging from cell membrane disruption to lysis.

6.2 Recommendation

The research on the potential of AMPs as alternatives to antibiotics are still steadily on-going. Although there are already AMPs that were approved by the Food and Drug Administration (FDA), there are still several obstacles to be overcome before they can finally be applied clinically. In the future, researchers can employ different analytical tools to distinguish the multiple distinct mechanisms of the AMPs isolated from *P.acidilactici* kp10 and *L.lactis* gh1 against VRE and MRSA. From the results of current study, additional studies using *in vivo* models would be necessary and might give valuable information to confirm the effectiveness of these AMP as well as providing appropriate scientific justification.

REFERENCES

Abbasiliasi, S., Tan, J. S., Bashokouh, F., Ibrahim, T. A., Mustafa, S., Vakhshiteh, F., … Ariff, A. B. (2017). In vitro assessment of Pediococcus acidilactici Kp10 for its potential use in the food industry. *BMC Microbiology*, *17*(1). doi:10.1186/s12866-017-1000-z

Almeida, P. F., & Pokorny, A. (2009). Mechanisms of Antimicrobial, Cytolytic, and Cell-Penetrating Peptides: From Kinetics to Thermodynamics. *Biochemistry*, *48*(34), 8083-8093. doi:10.1021/bi900914g

Anunthawan T, de la Fuente-Núñez C, Hancock RE, Klaynongsruang S. (2015). Cationic amphipathic peptides KT2 and RT2 are taken up into bacterial cells and kill planktonic and biofilm bacteria. *Biochim Biophys Acta*. 1848(6):1352–1358.

Anne Pereira, H. (2006). Novel therapies based on cationic antimicrobial peptides. *Current Pharmaceutical Biotechnology*, *7*(4), 229-234. https://doi.org/10.2174/138920106777950771

Arthur, M., & Courvalin, P. (1993). Genetics and mechanisms of glycopeptide resistance in enterococci. *Antimicrobial Agents and Chemotherapy*, *37*(8), 1563-1571. doi:10.1128/aac.37.8.1563

Aslam, B., Wang, W., Arshad, M. I., Khurshid, M., Muzammil, S., Rasool, M. H., … Baloch, Z. (2018). Antibiotic resistance: a rundown of a global crisis. *Infection and drug resistance, 11*, 1645–1658. doi:10.2147/IDR.S173867

B. Som, A., Vemparala, S., Ivanov, I., & Tew, G. N. (2008). Synthetic mimics of antimicrobial peptides. *Biopolymers*, *90*(2), 83-93. doi:10.1002/bip.20970

Bæk, K. T., Gründling, A., Mogensen, R. G., Thøgersen, L., Petersen, A., Paulander, W., & Frees, D. (2014). β-Lactam resistance in methicillin-resistant Staphylococcus aureus USA300 is increased by inactivation of the ClpXP protease. *Antimicrobial agents and chemotherapy*, *58*(8), 4593–4603. doi:10.1128/AAC.02802-14

Bahar, A., & Ren, D. (2013). Antimicrobial Peptides. *Pharmaceuticals*, *6*(12), 1543-1575. doi:10.3390/ph6121543

Balaban, N. Q. (2004). Bacterial Persistence as a Phenotypic Switch. *Science*, *305*(5690), 1622-1625. doi:10.1126/science.1099390

Balkwill, D. L., & Stevens, S. E. (1980). Effects of penicillin G on mesosome-like structures in Agmenellum quadruplicatum. *Antimicrobial Agents and Chemotherapy*, *17*(3), 506-509. https://doi.org/10.1128/aac.17.3.506

Bartlett, J. G., Gilbert, D. N., & Spellberg, B. (2013). Seven ways to preserve the miracle of antibiotics. *Clinical Infectious Diseases*, *56*(10), 1445-1450. https://doi.org/10.1093/cid/cit070

Beniac, D. R., Hiebert, S. L., Siemens, C. G., Corbett, C. R., & Booth, T. F. (2015). A mobile biosafety microanalysis system for infectious agents. *Scientific Reports*, *5*(1). doi:10.1038/srep09505

Beniac, D., Siemens, C., Wright, C., & Booth, T. (2014). A Filtration Based Technique for Simultaneous SEM and TEM Sample Preparation for the

Rapid Detection of Pathogens. *Viruses*, *6*(9), 3458-3471. doi:10.3390/v6093458

Bergmans, L., Moisiadis, P., Van Meerbeek, B., Quirynen, M., & Lambrechts, P. (2005). Microscopic observation of bacteria: review highlighting the use of environmental SEM. *International Endodontic Journal*, *38*(11), 775-788. doi:10.1111/j.1365-2591.2005.00999.x

Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol*. 3, 238–250. doi: 10.1038/nrmicro1098

Bugg, T. D., Wright, G. D., Dutka-Malen, S., Arthur, M., Courvalin, P., & Walsh, C. T. (1991). Molecular basis for vancomycin resistance in Enterococcus faecium BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. *Biochemistry*, *30*(43), 10408-10415. doi:10.1021/bi00107a007

Butaye, P., Devriese, L. A., & Haesebrouck, F. (2003). Antimicrobial growth promoters used in animal feed: Effects of less well known antibiotics on Gram-positive bacteria. *Clinical Microbiology Reviews*, *16*(2), 175-188. https://doi.org/10.1128/cmr.16.2.175-188.2003

Bush, K. (2013). Proliferation and significance of clinically relevant β-lactamases. *Annals of the New York Academy of Sciences*, *1277*(1), 84-90. https://doi.org/10.1111/nyas.12023

Centers for Disease Control and Prevention, Office of Infectious Disease Antibiotic resistance threats in the United States, 2013. Apr, 2013. Available at:

http://www.cdc.gov/drugresistance/threat-report-2013. Accessed January 28, 2015.

Cetinkaya, Y., Falk, P., & Mayhall, C. G., (2000). Vancomycin-resistant enterococci. *Clinical microbiology reviews*, *13*(4), 686–707. doi:10.1128/cmr.13.4.686-707.2000

Chambers, H. F., & DeLeo, F. R. (2009). Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nature Reviews Microbiology*, *7*(9), 629-641. https://doi.org/10.1038/nrmicro2200

Coates, A. R., Halls, G., & Hu, Y. (2011). Novel classes of antibiotics or more of the same?. *British journal of pharmacology*, *163*(1), 184–194. doi:10.1111/j.1476-5381.2011.01250.x

Cosgrove, S. E., Sakoulas, G., Perencevich, E. N., Schwaber, M. J., Karchmer, A. W., & Carmeli, Y. (2003). Comparison of mortality associated with methicillin-resistant and methicillin-susceptiblestaphylococcus aureusBacteremia: A meta-analysis. *Clinical Infectious Diseases*, *36*(1), 53-59. https://doi.org/10.1086/345476

Da Silva, A., & Teschke, O. (2003). Effects of the antimicrobial peptide PGLa on live Escherichia coli. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1643*(1-3), 95-103. https://doi.org/10.1016/j.bbamcr.2003.10.001

Duong-Ly, K. C., & Gabelli, S. B. (2014). Salting out of Proteins Using Ammonium Sulfate Precipitation. *Methods in Enzymology*, 85-94. doi:10.1016/b978-0-12-420119-4.00007-0

Easton, D. M., Nijnik, A., Mayer, M. L., & Hancock, R. E. (2009). Potential of immunomodulatory host defense peptides as novel anti-infectives. *Trends in Biotechnology*, *27*(10), 582-590. doi:10.1016/j.tibtech.2009.07.00

Erlandsen, S. L., Kristich, C. J., Dunny, G. M., & Wells, C. L. (2004). High-resolution visualization of the microbial Glycocalyx with low-voltage scanning electron microscopy: Dependence on cationic dyes. *Journal of Histochemistry & Cytochemistry*, *52*(11), 1427-1435. https://doi.org/10.1369/jhc.4a6428.2004

Evers, S., & Courvalin, P. (1996). Regulation of VanB-type vancomycin resistance gene expression by the VanS(B)-VanR (B) two-component regulatory system in Enterococcus faecalis V583. *Journal of bacteriology*, *178*(5), 1302-1309. doi:10.1128/jb.178.5.1302-1309.1996

Fines, M., Perichon, B., Reynolds, P., Sahm, D. F., & Courvalin, P. (1999). VanE, a New Type of Acquired Glycopeptide Resistance in Enterococcus faecalis BM4405. *Antimicrobial Agents and Chemotherapy*, *43*(9), 2161-2164. doi:10.1128/aac.43.9.2161

Gaspar, D., Veiga, A. S., & Castanho, M. A. (2013). From antimicrobial to anticancer peptides. A review. *Frontiers in Microbiology*, *4*. https://doi.org/10.3389/fmicb.2013.00294

Golding, C. G., Lamboo, L. L., Beniac, D. R., & Booth, T. F. (2016). The scanning electron microscope in microbiology and diagnosis of infectious disease. *Scientific reports*, *6*, 26516. doi:10.1038/srep26516

Golkar, Z., Bagasra, O., & Pace, D. G. (2014). Bacteriophage therapy: A potential solution for the antibiotic resistance crisis. *The Journal of Infection in Developing Countries*, *8*(02), 129-136. https://doi.org/10.3855/jidc.3573

Goossens, H., Ferech, M., Vanderstichele, R., & Elseviers, M. (2005). Outpatient antibiotic use in Europe and association with resistance: A cross-national database study. *The Lancet*, *365*(9459), 579-587. https://doi.org/10.1016/s0140-6736(05)70799-6

Gordon, Y. J., Romanowski, E. G., & McDermott, A. M. (2005). A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Current Eye Research*, *30*(7), 505-515. https://doi.org/10.1080/02713680590968637

Gross, M. (2013). Antibiotics in crisis. *Current Biology*, *23*(24), R1063-R1065. https://doi.org/10.1016/j.cub.2013.11.057

Hartmann, M., Berditsch, M., Hawecker, J., Ardakani, M. F., Gerthsen, D., & Ulrich, A. S. (2010). Damage of the bacterial cell envelope by antimicrobial peptides gramicidin S and PGLa as revealed by transmission and scanning electron microscopy. *Antimicrobial Agents and Chemotherapy*, *54*(8), 3132-3142. https://doi.org/10.1128/aac.00124-10

Hancock, R. E., and Rozek, A. (2002). Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiol. Lett*. 206, 143–149. doi: 10.1111/j.1574-6968.2002.tb11000.x

Hawker, J. I., Smith, S., Smith, G. E., Morbey, R., Johnson, A. P., Fleming, D. M., Shallcross, L., & Hayward, A. C. (2014). Trends in antibiotic prescribing in primary care for clinical syndromes subject to national recommendations to reduce antibiotic resistance, UK 1995-2011: Analysis of a large database of primary care consultations. *Journal of Antimicrobial Chemotherapy*, *69*(12), 3423-3430. https://doi.org/10.1093/jac/dku291

Hayles, M. F., Stokes, D. J., Phifer, D., & Findlay, K. C. (2007). A technique for improved focused ion beam milling of cryo-prepared life science specimens. *Journal of Microscopy*, *226*(3), 263-269. doi:10.1111/j.1365-2818.2007.01775.x

Herathge, N., George, J., & Rowley, D. (2011). Differential antimicrobial activities of Human Beta-Defensins against Methicillin Resistant (MRSA) and Methicillin sensitive (MSSA) Staphylococcus aureus. *Science and Technology Against Microbial Pathogens*. doi:10.1142/9789814354868_0003

Karthikeya, V., & Santhosh, S. (2009). Study of Bacteriocin as a food preservative and the L. acidophilus strain as probiotic. *Pakistan Journal of Nutrition*, *8*(4), 335-340. https://doi.org/10.3923/pjn.2009.335.340

Kim, H., Jang, J. H., Kim, S. C., & Cho, J. H. (2013). De Novo generation of short antimicrobial peptides with enhanced stability and cell specificity. *Journal of Antimicrobial Chemotherapy*, *69*(1), 121-132. https://doi.org/10.1093/jac/dkt322

Jakel CE, Meschenmoser K, Kim Y, Weiher H, Schmidt-Wolf IG. (2012) Efficacy of a proapoptotic peptide towards cancer cells. *In Vivo*. 2012;26:419–26

Jawan, R., Abbasiliasi, S., Mustafa, S., Halim, M., & B. Ariff, A. (2018). In Vitro Properties Analysis of Potential Probiotic of Isolated Lactic Acid Bacteria from Milk By-Product. *Borneo Journal of Medical Sciences (BJMS)*, 61. Retrieved from https://jurcon.ums.edu.my/ojums/index.php/bjms/article/view/1402

Khalifa, S. A., Friberg, U., Illing, R., & Rask-Andersen, H. (2003). Synaptophysin immunohistochemistry in the human cochlea. *Hearing Research, 185*(1-2), 35-42. doi:10.1016/s0378-5955(03)00228-4

Koch, A. L. (2003). Bacterial wall as target for attack. *Clinical Microbiology Reviews*, *16*(4), 673-687. https://doi.org/10.1128/cmr.16.4.673-687.2003

Koprivnjak, T., & Peschel, A. (2011). Bacterial resistance mechanisms against host defense peptides. *Cellular and Molecular Life Sciences*, *68*(13), 2243-2254. https://doi.org/10.1007/s00018-011-0716-4

Leclercq, R., & Courvalin, P. (1997). Resistance to Glycopeptides in Enterococci. *Clinical Infectious Diseases*, *24*(4), 545-556. doi:10.1093/clind/24.4.545

Lei, J., Sun, L., Huang, S., Zhu, C., Li, P., He, J., … He, Q. (2019). The antimicrobial peptides and their potential clinical applications. *American journal of translational research*, *11*(7), 3919–3931.

Li, B., & Webster, T. J. (2018). Bacteria antibiotic resistance: New challenges and opportunities for implant-associated orthopedic infections. *Journal of*

orthopaedic research : official publication of the Orthopaedic Research Society, *36*(1), 22–32. doi:10.1002/jor.23656

Li, J., Koh, J., Liu, S., Lakshminarayanan, R., Verma, C. S., & Beuerman, R. W. (2017). Membrane active antimicrobial peptides: Translating mechanistic insights to design. *Frontiers in Neuroscience*, *11*. https://doi.org/10.3389/fnins.2017.00073

Liu, C., Bayer, A., Cosgrove, S. E., Daum, R. S., Fridkin, S. K., Gorwitz, R. J., Kaplan, S. L., Karchmer, A. W., Levine, D. P., Murray, B. E., Rybak, M. J., Talan, D. A., & Chambers, H. F. (2011). Clinical practice guidelines by the Infectious Diseases Society of America for the treatment of methicillin-resistant staphylococcus aureus infections in adults and children. *Clinical Infectious Diseases*, *52*(3), e18-e55. https://doi.org/10.1093/cid/ciq146

Malanovic, N., & Lohner, K. (2016). Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1858*(5), 936-946. https://doi.org/10.1016/j.bbamem.2015.11.004

McMurry, L., Petrucci, R. E., & Levy, S. B. (1980). Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in escherichia coli. *Proceedings of the National Academy of Sciences*, *77*(7), 3974-3977. https://doi.org/10.1073/pnas.77.7.3974

Michael, C. A., Dominey-Howes, D., & Labbate, M. (2014). The antimicrobial resistance crisis: Causes, consequences, and management. *Frontiers in Public Health*, *2*. https://doi.org/10.3389/fpubh.2014.00145

Mileykovskaya, E., & Dowhan, W. (2000). Visualization of Phospholipid Domains inEscherichia coli by Using the Cardiolipin-Specific Fluorescent Dye 10-N-Nonyl Acridine Orange. *Journal of Bacteriology*, *182*(4), 1172-1175. https://doi.org/10.1128/jb.182.4.1172-1175.2000

Moellering, R. C. (1992). Emergence of Enterococcus as a Significant Pathogen. *Clinical Infectious Diseases*, *14*(6), 1173-1178. doi:10.1093/clinids/14.6.1173

Moropoulou, A., Zendri, E., Ortiz, P., Delegou, E. T., Ntoutsi, I., Balliana, E., … Ortiz, R. (2019). Scanning Microscopy Techniques as an Assessment Tool of Materials and Interventions for the Protection of Built Cultural Heritage. *Scanning*, *2019*, 5376214. doi:10.1155/2019/5376214

Munita, J. M., & Arias, C. A. (2016). Mechanisms of antibiotic resistance. *Microbiology Spectrum*, *4*(2). https://doi.org/10.1128/microbiolspec.vmbf-0016-2015

Murray, B. E. (1990). The life and times of the Enterococcus. *Clinical Microbiology Reviews*, *3*(1), 46-65. doi:10.1128/cmr.3.1.46

Nguyen, L. T., Haney, E. F., and Vogel, H. J. (2011). The expanding scope of antimicrobial peptide structures and their modes of action. *Trends Biotechnol*. 29, 464–472. doi: 10.1016/j.tibtech.2011.05.001

Park, Y., & Hahm, K. (2005). Antimicrobial Peptides (AMPs): Peptide Structure and Mode of Action. *BMB Reports*, *38*(5), 507-516. doi:10.5483/bmbrep.2005.38.5.507

Piddock, L. J. (2006). Clinically relevant Chromosomally encoded Multidrug resistance efflux pumps in bacteria. *Clinical Microbiology Reviews*, *19*(2), 382-402. https://doi.org/10.1128/cmr.19.2.382-402.2006

Piddock, L. J. (2012). The crisis of no new antibiotics—what is the way forward? *The Lancet Infectious Diseases*, *12*(3), 249-253. https://doi.org/10.1016/s1473-3099(11)70316-4

Price-Whelan, A., Poon, C. K., Benson, M. A., Eidem, T. T., Roux, C. M., Boyd, J. M., … Krulwich, T. A. (2013). Transcriptional Profiling of Staphylococcus aureus During Growth in 2 M NaCl Leads to Clarification of Physiological Roles for Kdp and Ktr K+ Uptake Systems. *mBio*, *4*(4). doi:10.1128/mbio.00407-13

Radzig, M., Nadtochenko, V., Koksharova, O., Kiwi, J., Lipasova, V., & Khmel, I. (2013). Antibacterial effects of silver nanoparticles on gram-negative bacteria: Influence on the growth and biofilms formation, mechanisms of action. *Colloids and Surfaces B: Biointerfaces*, *102*, 300-306. doi:10.1016/j.colsurfb.2012.07.039

Rammelkamp, C. H., & Maxon, T. (1942). Resistance of staphylococcus aureus to the action of penicillin. *Experimental Biology and Medicine*, *51*(3), 386-389. https://doi.org/10.3181/00379727-51-13986

Read, A. F., & Woods, R. J. (2014). Antibiotic resistance management. *Evolution, Medicine, and Public Health*, *2014*(1), 147-147. doi:10.1093/emph/eou024

Reddy, K.V.R.,Yedery,R.D.,andAranha,C.(2004).Antimicrobial peptides:premisesandpromises. *Int. J.Antimicrob.Agents* 24, 536–547.doi: 10.1016/j.ijantimicag.2004.09.005

Salgado C, Farr B. (2002). The cost of vancomycin-resistance: a meta-analysis. In: Program and abstracts of the 12th Annual Scientific Meeting of the Society for Healthcare Epidemiology of America; April 6-9, 2002; Salt Lake City. Abstract 113.

Schleifer, K. H., & Kilpper-Balz, R. (1984). Transfer of Streptococcus faecalis and Streptococcus faecium to the Genus Enterococcus nom. rev. as Enterococcus faecalis comb. nov. and Enterococcus faecium comb. nov. *International Journal of Systematic Bacteriology*, *34*(1), 31-34. doi:10.1099/00207713-34-1-31

Seo, M.-D.,Won,H.-S.,Kim,J.-H.,Mishig-Ochir,T.,andLee,B.-J.(2012). Antimicrobial peptidesfortherapeuticapplications:areview. *Molecules* 17, 12276–12286. doi:10.3390/molecules171012276

Skinner, D. (1941). Significance of bacteremia caused by staphylococcus aureus. *Archives of Internal Medicine*, *68*(5), 851. https://doi.org/10.1001/archinte.1941.00200110003001

Stapleton, P. D., & Taylor, P. W. (2002). Methicillin resistance in staphylococcus aureus: Mechanisms and modulation. *Science Progress*, *85*(1), 57-72. https://doi.org/10.3184/003685002783238870

Teixeira, V., Feio, M. J., and Bastos, M. (2012). Role of lipids in the interaction of antimicrobial peptides with membranes. *Prog. Lipid Res.* 51, 149–177. doi: 10.1016/j.plipres.2011.12.005

Thiberge, S., Nechushtan, A., Sprinzak, D., Gileadi, O., Behar, V., Zik, O., … Moses, E. (2004). Scanning electron microscopy of cells and tissues under fully hydrated conditions. *Proceedings of the National Academy of Sciences*, *101*(10), 3346-3351. doi:10.1073/pnas.0400088101

Williams, D. B., & Carter, C. B. (1996). The transmission electron microscope. *Transmission Electron Microscopy*, 3-17. https://doi.org/10.1007/978-1-4757-2519-3_1

Wilson, D. N. (2013). Ribosome-targeting antibiotics and mechanisms of bacterial resistance. *Nature Reviews Microbiology*, *12*(1), 35-48. https://doi.org/10.1038/nrmicro3155

Winey, M., Meehl, J. B., O'Toole, E. T., & Giddings, T. H. (2014). Conventional transmission electron microscopy. *Molecular Biology of the Cell*, *25*(3), 319-323. doi:10.1091/mbc.e12-12-0863

Woodford, N., Johnson, A. P., Morrison, D., & Speller, D. C. (1995). Current perspectives on glycopeptide resistance. *Clinical Microbiology Reviews*, *8*(4), 585-615. https://doi.org/10.1128/cmr.8.4.585

Yeaman, M. R., & Yount, N. Y. (2003). Mechanisms of antimicrobial peptide action and resistance. *Pharmacological Reviews*, *55*(1), 27-55. https://doi.org/10.1124/pr.55.1.2

Zanetti, M. (2003). Cathelicidins, multifunctional peptides of the innate immunity. *Journal of Leukocyte Biology*, *75*(1), 39-48. doi:10.1189/jlb.0403147

Zhang, L., & Gallo, R. L. (2016). Antimicrobial peptides. *Current Biology*, *26*(1), R14-R19. doi:10.1016/j.cub.2015.11.017