PURIFICATION, CHARACTERIZATION, AND MICROEMULSION SPRAY FORMULATION OF ENTEROCIN SECRETED BY *Enterococcus faecium* CC2 AGAINST ORAL PATHOGEN *Streptococcus mutans* UKMCC 1019

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2023

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

July 2023

ACKNOWLEDGEMENT

I would like to express my gratitude to Dr. Tan Joo Shun, my supervisor, who was the driving factor behind my PhD's research. His important guidance and the degree of flexibility he granted me were critical to the success of this project. I am particularly grateful to Associate Prof. Dr. Lee Chee Keong, my co-supervisor, for his unfailing support and encouragement during my candidature. This thesis would not have been completed without their guidance, patience and encouragement.

I would like to thank the rest of my postgraduate colleagues in Bioprocess Technology division, School of Technology Industry, who work hard together and support each other while our research. We had spent a lot of meaningful time together, sharing problems and thoughts for finishing our own projects. Besides that, I would like to credit to all lab assistants especially in Bioprocess Technology division, Mr. Azmaizan and Mrs. Najmah.

Last but not least, this study was funded by USM Fellowship. Not forgetting, I would like to convey my sincere thanks to my beloved parents for their love, support, tolerance, and understanding. Apart from my efforts, the completion of this PhD's research could not have been possible without the efforts and cooperation of everyone.

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LIST OF SYMBOLS

℃	Degree Celsius
µ	Micro
%	Percentage
±	Plus-minus Sign
g	Gram
h	Hour
min	Minute

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ANOVA	Analysis Of Variance
CCD	Central Composite Design
CFU	Colony Form Unit
DMSO	Dimethyl Sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DNA	Deoxyribonucleic Acid
Da	Dalton
DNS	3, 5-dinitrosalicylic acid
HPLC	High Performance Liquid Chromatography
HCl	Hydrochloride acid
LAB	Lactic Acid Bacteria
LC-MS	Liquid Chromatography-Mass Spectrometry
MS	Mass spectrometry
MBC	Minimal Bacteriological Concentration
MIC	Minimal Inhibition Concentration
mL	Millilitre
MRS	De Man, Rogosa and Sharpe
m/z	Mass Divided by Charge Number
NaCl	Sodium Chloride
nm	Nanometre

pH	Potential of Hydrogen
PCR	Principal Component Regression
PEG	Polyethylene glycol
RNA	Ribonucleic Acid
rpm	Revolution Per Minute
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscope
% w/w	Weight percentage

LIST OF APPENDICES

PENULENAN, PENCIRIAN, DAN FORMULASI SEMBURAN MIKROEMULSI UNTUK ENTEROSIN YANG DIREMBESKAN OLEH *Enterococcus faecium* CC2 TERHADAP PATOGEN MULUT *Streptococcus mutans* UKMCC 1019

ABSTRAK

Karies gigi adalah penyakit tidak berjangkit yang paling biasa di seluruh dunia dan *Streptococcus mutans* adalah patogen oral utama yang menyumbang kepada karies gigi. Penggunaan ubat kumur chlorhexidine dalam penghambatan *S. mutans* boleh menyebabkan beberapa kesan sampingan kepada manusia dan juga berbahaya bagi kehidupan akuatik. Bahan perencatan menyerupai bacteriocin (BLIS) daripada bakteria asid laktik (LAB) boleh menjadi pengganti chlorhexidine dalam penghambatan *S. mutans*. Dalam kajian ini, BLIS yang dihasilkan oleh *Enterococcus faecium* CC2 menunjukkan aktiviti penghambatan tertinggi terhadap *S. mutans* UKMCC 1019 antara 61 LAB. BLIS yang dihasilkan oleh *E. faecium* CC2 mempunyai toleransi tinggi terhadap persekitaran berasid, alkali dan suhu tinggi. Ia adalah bahan semula jadi proteinaceous dan mempunyai 4406.655 AU / mL dan 268.592 AU / mL aktiviti penghambatan terhadap *S. mutans* UKMCC 1019 dan *Candida albicans* masing-masing. Lisis sel *S. mutans* UKMCC 1019 dikesankan bawah TEM. Pemurnian separa BLIS dilakukan dengan pemendakan amonium sulfat, diikuti oleh sistem dua fasa berair berasaskan cecair ionik (ILATPS) dan polietilena glikol (PEG) sistem dua fasa berair (ATPS), masing-masing. Faktor pemurnian tertinggi, hasil pemulihan dan pekali partisi yang dicapai dari ATPS berasaskan PEG adalah tinggi berbanding dengan ILATPS.

Jisim molekul BLIS yang disucikan sebahagiannya dijumpai sekitar 6 kDa oleh natrium dodecyl sulfate-polyacrylamide gel elektroforesis (SDS-PAGE). Dari kromatografi cecair berprestasi tinggi (HPLC), pecahan yang memberikan aktiviti perencatan dikumpulkan dan dianalisis sebagai enterocin-HF dengan jisim molekul 5.949 kDa dengan kromatografi cecair – spektrometri jisim (LC – MS). MIC, MBC dan masa untuk membunuh *S. mutans* UKMCC 1019 bagi BLIS yang disucikan sebahagian dari ATPS berasaskan PEG adalah rendah berbanding dengan ILATPS. Ujian sitotoksisitas menunjukkan bahawa 1 mg / mL BLIS yang disucikan sebahagiannya dijumpai dalam julat yang boleh diterima bagi dua sistem ini. Formulasi yang dipilih adalah 0.5% w/w minyak, 0.5% w/w campuran antara nisbah Tween 80 dan PEG 400 (1: 1) dan 1 mg / mL BLIS yang disucikan sebahagian berdasarkan aktiviti penghambatan dan penampilan visual selepas dua minggu. Formulasi yang dipilih menunjukkan kestabilan termodinamik yang tinggi terhadap sentrifugasi, kitaran pemanasan / penyejukan dan tekanan terma. Ia dibuktikan tidak beracun dan mempunyai aktiviti antioksidan. Semburan oral BLIS yang diformulasikan stabil dalam 6 minggu pertama untuk semua suhu penyimpanan dari segi pencemaran mikroba, aktiviti antimikroba, penampilan visual, pH, kelikatan, ukuran zarah, taburan ukuran dan potensi zeta. Masa yang diperlukan oleh formulasi yang dipilih untuk membunuh *S. mutans* UKMCC 1019 ditingkatkan dari 8h ke 5h.

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ABSTRACT

Dental caries is the most common noncommunicable disease worldwide and *Streptococcus mutans* is the main oral pathogen which contributes to it. Using of chlorhexidine mouthwash in the inhibition of *S. mutans* may cause some side effects to human beings and also harmful to aquatic life. Bacteriocin-like inhibitory substance (BLIS) from lactic acid bacteria can be the replacement for chlorhexidine in the inhibition of *S. mutans*. In this study, out of the 61 LAB strains, the BLIS produced by *Enterococcus faecium* CC2 showed highest inhibition activity against *S. mutans* UKMCC 1019. BLIS produced by *E. faecium* CC2 have high tolerance to acidic, alkaline and high temperature environment. It is a proteinaceous nature substance that possess of 4406.655 AU/mL and 268.592 AU/mL inhibition activity against *S. mutans* UKMCC 1019 and *Candida albicans*, respectively. Under TEM, cell lysis of *S. mutans* UKMCC 1019 was observed. Partial purification of BLIS was done by ammonium sulphate precipitation, following by ionic liquid based aqueous two-phase system (ILATPS) and polyethylene glycol (PEG) based aqueous two-phase system (ATPS), respectively. The highest purification factor, recovery yield and partition coefficient achieved from PEG based ATPS were higher than ILATPS. The molecular mass of partially purified BLIS was found around 6 kDa by sodium dodecyl sulphate-

polyacrylamide gel electrophoresis (SDS-PAGE). From high-performance liquid chromatography (HPLC), the fraction that gives inhibition activity were collected and analyzed as enterocin-HF with molecular mass of 5.949 kDa by liquid chromatography–mass spectrometry (LC–MS). The MIC, MBC and time needed to kill *S. mutans* UKMCC 1019 of partially purified BLIS from PEG based ATPS were lower than ILATPS. The cytotoxicity test showed that concentration of 1 mg/mL of partially purified BLIS was found within the acceptable range for both systems. The selected formulation was 0.5% w/w of oils, 0.5% w/w of mixture of Tween 80 and PEG 400 (ratio of 1:1) and 1 mg/mL of partially purified BLIS based on inhibition activity and visual appearances after two weeks. The selected formulation showed high thermodynamic stability towards centrifugation, heating/cooling cycle and thermal stress. It was non-toxic and possess antioxidant activity. The formulated BLIS oral spray was stable in the first 6 weeks for all storage temperature in terms of microbial contamination, antimicrobial activities, visual appearances, pH, viscosity, particle size, size distribution and zeta potential. The time needed for partially purified BLIS to kill *S. mutans* UKMCC 1019 was improved to 5h from 8h after formulating.

CHAPTER 1

INTRODUCTION

1.1 Research background

One of the most common and widespread oral health problems is dental caries. Dental caries is an obliteration of human's dental hard tissues, which is majorly caused by dental biofilm or plaque forming by cariogenic microorganism. Acid is released after the breakdown process of food debris on tooth by the cariogenic microorganism, leading to dissolving of enamel or dentin of the tooth (Ozdemir, 2013). According to World Health Organization (WHO), there are around 2 billion people suffering from dental caries of permanent teeth1 and 520 million children suffering from dental caries of primary teeth. Over 400 microbial species that can be found in normal adolescent human mouth, *Streptococcus mutans* is found to be the most virulent species that acts as a dental caries initiator as it can be transmitted both horizontally and vertically (Joel & Ramteke, 2017; Ozdemir, 2013).

S. mutans is known as a facultative anaerobe, Gram-positive coccus which commonly adheres to human oral cavity. Facultative anaerobe is described as an organism that can synthesis ATP aerobically in the present of oxygen respiration, but fermentation in the absent of oxygen. It is a homofermentative bacteria and it has higher acidic property as compared to other oral streptococci (Daboor, Masood, Al Azab, & Nori, 2015). Dental caries normally will be initiated by the transforming of sucrose to extracellular polysaccharides by *S. mutans* which involves the enzyme glucosyltransferase. The polysaccharides possess α (1–3) glucose linkage, creating an

environment for the attachment of bacterium (Bowen & Koo, 2011). Lipo teichoic acid is produced by *S. mutans* for it to adhere to the external enamel, thus enhances the colonization process. By breaking down sugar for energy, the pH of the oral environment will be lowered and hence creating acidic environment which causes demineralization of enamel dentine, which is also called as dental caries (Klein, Hwang, Santos, Campanella, & Koo, 2015). From previous studies, *Streptococcus sanguinis* was also found as a commensal bacterium in oral biofilm formation and it had antagonistic effect against *S. mutans*. *S. sanguinis* was proved that it can repress the growth of *S. mutans* by producing H2O2. Conversely, *S. sanguinis* was suppressed by *S. mutans* by secreting mutacin I and IX. (Zhu, Macleod, Kitten, & Xu, 2018). Besides that, *S. mutans* was found to have a synbiotic relationship *Candida albicans* synergizes in plaque forming in human oral environment, which making them more resistant to the human oral environment. (Bachtiar & Bachtiar, 2018).

The global demand for chemical free, less harmful and easier solutions to health problems has increased in past few years. Recently, the prevention of dental caries using various bacteriocin produced by lactic acid bacteria (LAB) has been attempted.

1.2 Problem statement

Chlorhexidine has been used in mouth wash or mouth spray to reduce the number of *S. mutans* for caries prevention. Normally, 0.2% of chlorhexidine was used in oral products to reduce the salivary *S. mutans*. However, using of chlorhexidine mouthwash may cause some sides effects, including taste changes, staining teeth, sore throat, tongue-tip irritation, shortness of breath, nasal congestion, swelling, skin rash and

mouth irritation. One of the studies showed that about 31.4% of people had these adverse events after using chlorhexidine mouthwash for 4 months (McCoy et al., 2008). It was also proved to have high level of toxicity to the algae and crustacean. This raises concerns about its potential effects in aquatic food webs, since these organisms are in the base of trophic chains. (Jesus et al., 2013). The prevention of dental caries using a natural active ingredient that is less harmful and environment friendly has been attempted. Bacteriocin or BLIS will be a good candidate to replace chlorhexidine in oral products.Bacteriocins are natural antimicrobial peptides with antimicrobial action against other bacteria and have no toxicity. To date only few bacteriocins have been used commercially. From previous studies, *Lactobacillus plantarum* and *L. acidophilus* are probiotic candidates with respect to biofilm inhibitory activity of *S. mutans* (Keller, Hasslöf, Stecksén-Blicks, & Twetman, 2011). This may be partially due to the fact that newly discovered bacteriocins have not yet to be fully characterized.

The difficulty of purification of targeted protein from crude extracted is the major cost for protein manufacturers with around 80% of the total cost (P. T. Wingfield, 2015). One of the papers demonstrates that purification of enterocin B from *E. faecium* can be done through cation-exchange chromatography and reverse-phase high-performance liquid chromatography (Dündar et al., 2015). Furthermore, one of the studies shows that the crude extracted bacteriocin from *Enterococcus thailandicus* can be purified using cation exchange and size exclusion chromatography (Al-Madboly et al., 2020). However, there are few major drawbacks of these purification methods, which includes expensive, low productivity, time consuming and not easy to be scaled up due to professional skills needed in the operation (Nfha Aziz et al., 2017). Recently, PEG-

based and ionic liquid based aqueous two-phase systems were found to have many benefits as compared to other purification methods such as it can be scaled up easily, fast in mass transfer and balance, effective, non-viscous, less emulsion to form, elimination of volatile organic usage and gentle biocompatible environment (Alvarez-Guerra et al., 2016).

The storage stability of BLIS will be the challenge due to the proteinaceous nature of BLIS. Improper storage temperature or formulation may cause aggregation and denaturation of BLIS, leading to decrease in its inhibition activity (Freire et al., 2013). Previous study showed that the inhibition activity of Propionicin PLG-I, a bacteriocin produced by *Propionibacterium thoenii* strain P127 was reduced slowly after store at 25°C (Lyon & Glatz, 1993). Besides that, one of the papers proved that the inhibition activity of BLIS from *Pediococcus acidilactici* kp10 decreased more than 80% after storing at 4°C, -20°C, and -80°C for three months (Md Sidek et al., 2018). Hence, formulation of BLIS is very important to improve the stability and extend the shelf life. Microemulsion formulation has been much focused by many researchers in the formulation of proteins mainly due to its high thermodynamic stability (Lopes, 2014). Microemulsions (MEs) are defined as isotropic mixtures that make up of oil, water and surfactant with or without cosurfactants (Coneac et al., 2015). They are clear transparent liquid with small particle size, ranged from 10 to 200 nm (Subongkot & Ngawhirunpat, 2017). They are used largely as drug carrier systems for topical, oral, and parenteral administration of drugs as they can be prepared and scaled up easily, they are stable thermodynamically and they improve the drug solubility and bioavailability. Besides that, they improve the therapeutic efficacy of drugs significantly which can reduce the

drug dosage to be used and drug toxicity (Suhail et al., 2021). The investigation on oral spray that containing purified BLIS, which can be used as a novel oral product in future will be done in this research.

1.3 Research scope and objectives

This study focused on characterization, purification and formulation of BLIS produced by lactic acid bacteria against oral pathogen *S. mutans* UKMCC 1019. The lactic acid bacteria were isolated from different sources such as kimchi, chicken, coffee wastes, vegetables waste water and others. The BLIS produced by LAB that gives the highest antimicrobial activity against *S. mutans* UKMCC 1019 was selected to be further characterized, purified and formulated. The two purification methods were used to purify the BLIS selected, including PEG-based ATPS and ionic liquid based ATPS. The responses of the two systems were compared. Then, the partially purified BLIS was proceeded with microemulsion-based BLIS oral spray formulation.

The objectives of this study were:

i. To isolate and characterize of lactic acid bacteria with ability to secrete bacteriocin-like inhibitory substance (BLIS) for the inhibition of oral pathogen *Streptococcus mutans* UKMCC 1019

ii. To optimize the effect of parameters on partial purification of BLIS from selected lactic acid bacteria by ionic liquid based aqueous two-phase system (ILATPS) using response surface methodology (RSM)

iii. To optimize the effect of parameters on partial purification of BLIS from selected lactic acid bacteria by polyethylene glycol based aqueous two phase system (PEG based ATPS) using response surface methodology (RSM)

iv. To develop, characterize and evaluate the microemulsion-based BLIS oral spray with high stability and inhibition activity against *Streptococcus mutans* UKMCC 1019

CHAPTER 2

LITERATURE REVIEW

2.1 *Streptococcus mutans*

James Kilian Clarke (1886–1955) originally identified *S. mutans* after isolating it from a carious lesion and researches about dental caries were started by scientist around 1960s. Dental caries in humans have been linked to *S. mutans* as the main pathogenic agent. *S. mutans* is a Gram-positive pathogen that can be found abundantly in oral. It can grow in the environment with temperature around 18-40°C (Banas & Drake, 2018). Their capacity to create the biofilm known as dental plaque on tooth surfaces is a crucial aspect of its virulence. This organism also makes surface proteins that work together to form dental plaque and lead to dental caries, including glucosyltransferases, several glucan-binding proteins, protein antigen c, and collagen-binding protein. Quorum-sensing mechanisms are used by *S. mutans* to control their reactions to environmental stress. The so-called two-component signal transduction system, which increase their ability to control their gene expression and coordinate activities in handling environmental stress, is a key signalling mechanism. Regarding *S. mutans*, it has been discovered that the comCDE-encoded signal peptide-mediated quorum-sensing system is a control mechanism deal with cell density and some environmental changes by producing a peptide signal molecule known as competence-stimulating peptide. Mutacins-producing ability of *S. mutans* is one of their main virulence factors. Two-component signal transduction systems are frequently used by them to control the expression of the bacteriocin gene and are also connected to their ability to produce biofilms. *S. mutans* produces lipo teichoic acid to help it stick to the exterior enamel, which speeds up the colonisation process. The pH of the oral

environment will be decreased by the breakdown of sugar for energy, creating an acidic environment that leads to demineralization of enamel and dentine (Harper et al., 2021).

2.2 *Enterococcus faecium*

Enterococci were first isolated from human fecal flora in 1899. However, until 1984, they were still categorized as Streptococci. The earliest *Streptococcus faecium* was identified in 1919. *E. faecium* is a Gram-positive, facultative anaerobe in coccus shape that exist singly or in groups. It is lactic acid bacteria that usually can be found in gastrointestinal tract, oral cavity, and vaginal tract of many animals. The life shelf of *E. faecium* can be very long as it can grow in the alkaline, acidic, isotonic, or hypertonic environments at temperature with the range of 10-45°C (Zhou et al., 2020). There were many researches had been done on the investigation of antibacterial activities *E. faecium* against targeted pathogens. *E. faecium* SF68 was proved to be effective agent in the reduction intestinal inflammation. It was used to treat diarrhea, especially in cases of antibiotic-associated diarrhea (Torres-Henderson et al., 2017). Besides that, *E. faecium* Smr18 had inhibition activity against *Salmonella enterica* and it could be a candidate for the treatment of typhoid. The antibacterial ability of bacteriocin extracted from *E. faecium* was also investigated in previous studies. Enterocins L50A and L50B produced by *E. faecium* LCW 44 was revealed to have inhibition activities against *L. monocytogenes* (Vimont et al., 2017). Furthermore, enterocin 12a extracted from *E. faecium* 12a was claimed as a novel bacteriocin that has anticancer properties against human cell lines (P. Sharma et al., 2021) .

2.3 Bacteriocin

Pathogens raise concerns from us due to their impacts on human health by infection via foods, livestock or animals. Previously, antimicrobial drugs or antibiotics have been chosen in the treatment of infectious diseases caused by pathogens. However, the increase of the usage of antibiotics in killing pathogens has led to the emergence of antibiotic-resistant bacteria, which will be the next challenges for humankind. Recently, studies on bacteriocin or bacteriocin-like inhibitory substances (BLIS) have been focused on finding out an alternative therapeutic option for bacterial infections. Bacteriocin has been chosen as a potential drug candidate to replace chemicals and antibiotics in future due to its lower toxicity and proteinaceous nature. Bacteriocin is a natural antimicrobial peptide produced by bacteria to protect themselves from other bacteria or pathogens by inhibiting or killing it without harming themselves. Bacteriocin is usually produced by lactic acid bacteria (LAB) or other bacteria, such as *Bacillus* strains, *Staphylococcus* strains and *Escherichia coli* strains. LAB is a group of Gram-positive bacteria with high tolerance to acidic environment, non-spore forming, either rod or spherical in shape, which shares similar characteristics in terms of metabolism and physiology (Bintsis, 2018). They play a vital role in fermentation by using carbohydrates as their main source, producing lactic acids as the main product (Bintsis, 2018). Basically, LAB can be divided into a few genera, which comprise *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Oenococcus, Tetragenococcus, Vagococcus* and *Weissella* (Mokoena, 2017). They are mainly grouped into two categories, which are homofermentative and heterofermentative LAB. During the process of fermentation, lactic acid is mainly

produced by homofermentative LAB, whereas lactic acid, acetic acid or alcohol and carbon dioxide are produced by heterofermentative LAB (Endo & Dicks, 2014).

There are some differences between antibiotics and bacteriocin. First, the antibacterial activity of antibiotics is broad-spectrum, while the antibacterial activity of bacteriocin is narrow-spectrum. Furthermore, antibiotics is produced from metabolic pathway, whereas the production of bacteriocin takes place in ribosomes. Usually, the molecular weight of bacteriocin is larger than antibiotics. Furthermore, the usage of antibiotics in killing pathogens may cause a disruption of the gut microbiota, as it is not only killing the targeted microbial community, but also the surrounding microbial community, as shown in Figure 2.1 (a) (S. Kim et al., 2017). Consequently, the disruption of the gut microbiota leads to immunological, metabolic and neurological disorders. In contrast, bacteriocin re-shape the microbiota by killing the targeted pathogens without killing the other surrounding microbial community, as shown in Figure 2.1 (b) (Hemarajata & Versalovic, 2013).

The mechanism of action of bacteriocin and antibiotic against targeted microbiota was different. Normally, bacteriocin will bind to cell walls of sensitive microbes, motive ionic imbalances and produce spores. Bacteriocins such as nisin, salivaricin and lacticin work by causing a pore in the membranes of their target bacteria or by interfering with the manufacture of their cell walls. Notably, it has been demonstrated that they bind to lipid II, obstruct the movement of peptidoglycan subunits from the cytoplasm into the cell wall, and subsequently prevent the development of cell walls. They can also use the docking molecule lipid II to start the creation of pores and cause fast cell death. In a series of processes, commencing with the contact of the bacteriocin with the membrane and ending with its inclusion in the phospholipidic bilayer, low molecular weight intracellular substances including amino

acids, ions, and ATP are released through the formation of pores in the membranes of the target cells. These cationic peptides engage in interaction with the cytoplasmic membrane's anionic surface. By electrostatic interactions, the C-terminal region, which contains the majority of positively charged residues, attaches to the head group of anionic phospholipids in the cytoplasmic membrane. The hydrophobic residues of bacteriocin N-terminal region typically act as a conduit for their entry into the cytoplasmic membrane. The "barrel-stave" and "wedge" models are two alternative theories that have been put out to explain how bacteriocins generate pores. The bacteriocin monomers bind to the outer leaflet in the barrel-stave model by electrostatic contact with the leaflet oriented parallel to the membrane surface. As a result of the interaction between the hydrophobic lipid core of the membrane and the non-polar side chains of the bacteriocin, a water-filled pore is created. The quantity of peptides involved affects the size and stability of the barrel-stave pore. However, the anionic head groups of the phospholipids interact with the cationic bacteriocins in the wedge model, causing a localised distortion of the membrane that allows the bacteriocin's hydrophobic residues to be inserted into the membrane. For antibiotic, they kill bacteria by destroying their cell walls and cell membranes, inhibits protein, RNA and DNA synthesis in the cell. Different types of antibiotics have different mechanism actions against targeted pathogens. For beta-lactam antibiotics, the primary targets of the β-lactam agents are the penicillin binding proteins (PBPs). It has been proposed that the D-alanyl D-alanine section of the peptide chain, which is typically bound by PBP, is mimicked by the β -lactam ring. PBP interacts with the β-lactam ring and isn't used in the production of fresh peptidoglycan. The lysis of the bacterium is caused by the rupture of the peptidoglycan layer. Tetracyclines, including tetracycline, chlortetracycline, doxycycline, and minocycline, act on the conserved

sequences of the 30S ribosomal subunit's 16S r-RNA to block t-RNA binding to the A site. Chloramphenicol interacts with the conserved regions of the 50S subunit's 23S r-RNA's peptidyl transferase cavity. Therefore, it prevents t-RNA from binding to the ribosome's A site, thereby inhibiting protein production. By concentrating on the conserved sequences of the peptidyl transferase centre of the 23S r-RNA of the 50S ribosomal subunit, macrolides it affects the first stage of protein synthesis, known as translocation. For Oxazolidinones, it inhibits protein synthesis by binding to 23Sr RNA of the 50S subunit, following by suppress 70S inhibition and interact with peptidyl-t-RNA. The fluoroquinolone (FQ) inhibits the enzyme bacterial DNA gyrase, which nicks the double-stranded DNA, introducing negative supercoils and then reseals the nicked ends. This is very vital to prevent excessive positive supercoiling of the strands when they separate to permit replication or transcription.

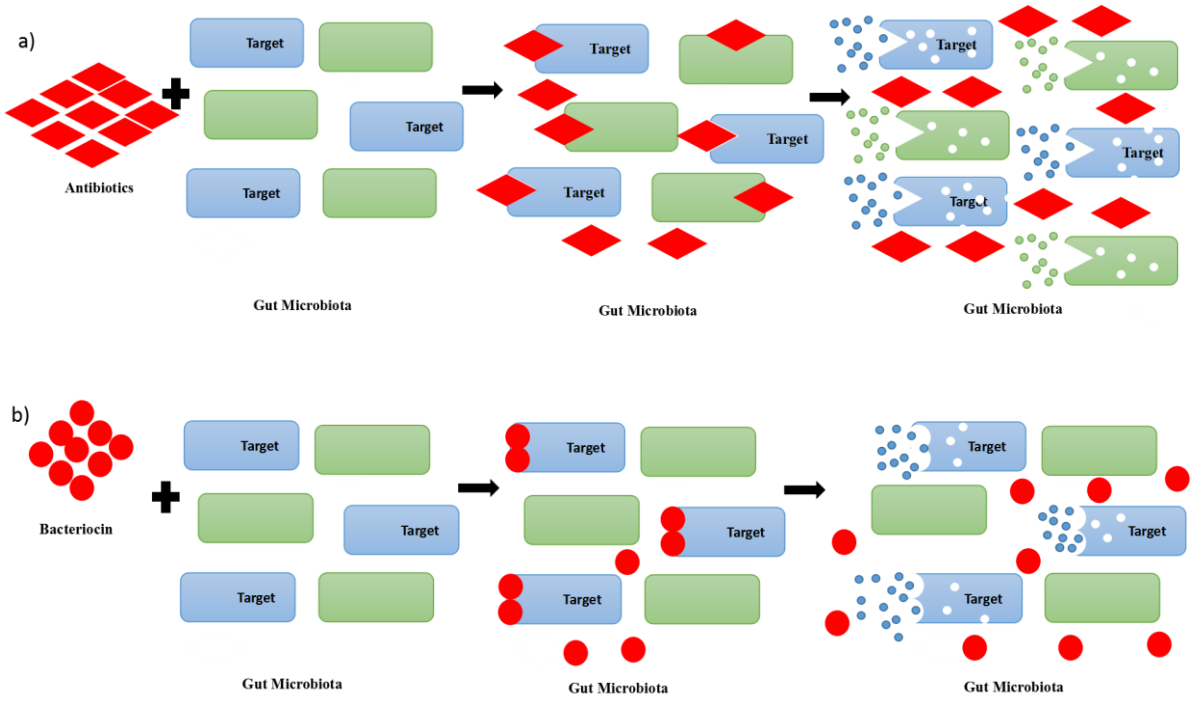

Figure 2.1 Killing or inhibition actions of (a) antibiotics and (b) bacteriocin on gut microbiota

From previous studies, some LAB was found to have an ability to produce antimicrobial substances, especially bacteriocin, to protect themself from other spoilage bacteria and pathogen.Bacteriocins can be categorized into mainly three classes. Class I bacteriocins are made of peptides that are small in size, which is less than 5 kDa. They contain specific post-translationally modified residues, which include lanthionine and β-methyllanthionine. The representative bacteriocin from class I are nisin Z, A and Q, enterocin W and nukacin ISK-1 (Perez et al., 2014). Class II bacteriocins are bacteriocin with sizes of less than 10 kDa, resistant to heat, non-modified and hydrophobic peptides. Usually, they can be grouped into two sub-classes, which are class IIa and class IIb. Class IIa bacteriocin, such as leucocin A and pediocin PA1, are widely used in food preservation due to its pediocin-like *Listeria*. Class IIb bacteriocins exert or improve the antimicrobial effects *via* synergistic activity of two complementary peptides, such as plantaricin A and enterocin X. They contain amphiphilic and hydrophobic regions, and are mostly cationic and active in the range of nanomolar to picomolar concentrations (Endo & Dicks, 2014). Class III bacteriocins are made of large proteins with sizes of more than 30 kDa, and are likely to be altered or degraded when subjected to heat. The representative bacteriocins from class III are lysostaphin, enterolysin A, and helveticin J (Nilsen et al., 2003). Nisin Z is a bacteriocin used to reduce the adhesion of *Candida albicans* to human oral cells (Shin et al., 2016). It is a class I bacteriocin with a size of 3.5 kDa that can be extracted from *Lactococcus lactis* NZ22186 or *L. lactis* NZ9800 (Shin et al., 2016). Besides that, the combination of nisin, pediocin 34 and enterocin FH99 was found to have high antimicrobial activity against *Listeria monocytogenes* ATCC 53135 (Kaur et al., 2013).

From previous studies, several bacteriocins were proved to have antimicrobial activities against *S. mutans*. Lacticin 3147 was found to be effective bacteriocin in the prevention of dental carries. It was proved to exert inhibitory effect against *S. mutans* which was the common pathogen cause oral plaque formation in humans. Previous study reported that the concentration of Lacticin 3147 needed to inhibit 50% growth of *S. mutans* was at 1280-5120 AU/mL. The study claimed that the *S. mutans* strain in human saliva could be eliminated if 40,000 AU/mL was used. Besides that, fermencin SD11 was proved to be a useful antimicrobial agent to against *S. mutans* ATCC 25175. Fermencin SD11 was proved to exhibit more than 125, 000 AU/mL of antimicrobial activity against *S. mutans* ATCC 25175. Furthermore, thermophilin 110 extracted from *S. thermophilus* B59671showed inhibition activity against *S. mutans* UA159. The batch cell culture of *S. mutans* UA159 was inhibited by using thermophilin 110 with concentration of or more than 80 AU/mL, whereas the biofilm growth of it could be inhibited by using thermophilin 110 with concentration of or more than 160 AU/mL.

2.2 Types of bacteriocin

The antimicrobial effects of penicillin against a wide range of pathogens were considered as a big contribution in the medical field to cure many infections caused by bacteria or virus. During the 1950s and 1960s, there were a lot of new antibiotics had been discovered and used in the treatment of infectious disease. However, the findings of new antibiotics were decreasing after 1985. Meanwhile, the discoveries of bacteria that resistant to antibiotics were increasing significantly and this had threatened human beings. Pathogens had been proved to develop mechanisms for resisting to drugs, in which the drug binding sites were altered and the access of the antimicrobial

agents to its intracellular site had been reduced or inhibited. One of the examples of drug-resistant bacteria is MRSA which is the general pathogen that may cause skin infection. MRSA was first discovered in hospital during 1960s after methicillin was used wisely in the treatment of skin infection. This problem alerted humans to find out alternative antimicrobial agents that can be used in killing or inhibit pathogens. Bacteriocin, with proteinaceous nature, was recommended by many researches to replace antibiotics for the treatment of infectious disease due to its low toxicity. Many researches have been done on investigation of bacteriocin which can be used to solve human health's problems such as urinary tract infection, skin infection, diarrhoea, dental carries, lung infection, bloodstream infection, mastitis, respiratory tract infection and cancer. Table 2.1 shows different types of bacteriocin, their producer, characteristics and applications.

2.2.1 Nisin

Nisin, a bacteriocin produced by *Lactococcus lactis* spp, is found to be used not only in the food preservation, but also can be used in the treatment of infectious disease caused by bacteria. Skin infection is usually caused by *S. aureus* and the usage of methicillin in the treatment of the infection has emerged the MRSA (Shin et al., 2016). Recently, there are many researches have been done on the investigation of bacteriocin to replace methicillin in the treatment of skin infection as the failure to treat the infection caused by MRSA may lead to death. Electrospun nanofibre wound dressing that comprises of nisin has been proved can be used in wound healing to prevent skin infection by MRSA. In the treatment of skin infection, wound dressing that contains nisin was found to be effective in reduction of the colonization of *S. aureus* and it fastened the wound healing process. Besides that, nisin was demonstrated to have antimicrobial effect against *Clostridium difficile*. *C. difficile* is a

bacterium that may causes diarrhoea and inflammation of colon (H. T. Yang et al., 2017). Previous studies proved that older people in hospital or for those who is taking anitibiotics for long term treatment may be infected by *C.difficle* as compared to others. Around a half million of citizens from United States was reported to be infected by *C. difficle* every year and the infection cases was getting severe and more difficult to be treated. Nisin A and nisin Z were claimed to be effective bacteriocin that can be utilized in the inhibition of the growth of *C. difficle.* The minimum inhibitory concentration (MIC) of nisin A and nisin Z were 0.8 μg/mL and 6.2 μg/mL, respectively. Furthermore, dental carries are a common health problem that affects all ages of human being. Nisin was also proved to have inhibitory abilities against pathogens that cause dental carries, which were *Streptococcus mutans*, *Streptococcus sanguinis* and *Streptococcus sobrinus*. The MIC of nisin needed to inhibit the growth of *S. mutans, S. sanguinis* and *S. sobrinus* are 625-1250 IU/mL, 156.25-312.5 IU/mL and 1250-2500 IU/mL, respectively.

In addition, nisin Z is also demonstrated to be used for the treatment of mucosal and bloodstream infections caused by *C. albicans*. Bloodstream infection which is also called as candidemia can lead to infection of other parts of body such as eyes, kidney, liver, and brain by the spreading of the *C. albicans* from your bloodstream. Candidemia may cause illness such as fever, skin rash, low blood pressure and abdominal pain as well as death if without treatment. Previous studies proved that nisin Z was able to inhibit the growth of *C. albicans* at 500 μg/mL by disturbing the cell membrane structure of *C. albicans* and increasing the granulation of the cytoplasm. Besides that, nisin F was also proved to have inhibition activity against *S. aureus* that may cause infection of respiratory tract. 8192 AU/mL of nisin F was proved to has significant antimicrobial effect against *S. aureus*. Furthermore,

nisin were demonstrated to be used in the treatment of lung infections and stomach ulcers to inhibit the growth of *P. aeruginosa* and *Helicobacter pylori*, respectively. In addition, nisin A was also proved to be alternative agent used in the treatment of cancer. Nisin A was found can be used to reduce tumorigenesis by inducing preferential apoptosis and the combination of nisin with doxorubicin was claimed to be effective agent used in the reduction of tumour severity in skin carcinogenesis (Joo et al., 2012).

2.2.2 Lacticin

Lacticin is a broad-spectrum bacteriocin produced by *L. lactis subsp* that belongs to lantibiotics groups. Lacticin 3147 produced by *L. lactis* ssp. *lactis* DPC3147 has been demonstrated to exert antimicrobial effect against a range of pathogenic bacteria (Ryan et al., 2022). Lacticin 3147 was proved to be effective antimicrobial agent that can be used in the treatment of skin and surgical site or prosthetic joint infection. The most common bacterium that causes skin infection is *S. aureus.* Lacticin 3147 was proved to have inhibitory ability on the growth of MRSA ST291. Lacticin 3147 showed the lower MIC against MRSA ST291 as compared to nisin Z and penicillin G, which is 3.85 μg/mL. Besides that, Lacticin 3147 was claimed to have inhibition activity against the growth of *Cutibacterium acnes*. *C. acnes* is grouped as a lipophilic anaerobic, Gram-positive bacterium that can be found commonly on the normal skin, oral environment, nose, urogenital tract and large intestine. *C. acnes* may cause infections during orthopedic surgery especially which is related to implants and prosthetic joint infections. The MIC needed for the inhibition of the growth of *C. acnes* LMG 16711 was 2.50 μg/mL.

In addition, lacticins A164 and BH5 produced by *L. lactis* subsp. lactis A164 and *L. lactis* subsp. lactis BH5, respectively, were demonstrated as the strongest bacteriocins among lacticins that could be used in the treatment of stomach ulcers caused by *H. pylori. H. pylori* is a pathogen that can be found in the gastric mucosa. It is the main root cause of gastritis and peptic ulcer. The antimicrobial activities of lacticin A164 and lacticin BH5 against *H. pylori* ATCC 43504 were 1,310,000 AU/mL and 655,000 AU/mL, respectively. MIC of lacticin A164 and lacticin BH5 needed to inhibit the growth of *H. pylori* ATCC 43504 was 12.5 mg/l (McAuliffe et al., 1998).

2.2.3 Salivaricin

Salivaricin is a bacteriocin that produced by *Streptococcus salivarius* and belongs to class II lantibiotics. Many reasearches proved that salivaricin has contributed to the health sustainability in humans. Salivaricin was proved not only can be used to maintain oral health, but also can be used in the treatment or prevention of skin or lung infections. *Streptococcus pneumoniae* is a pathogen that causes pneumonia, sinus infection, ear infection, bacteremia and meningtis. Salivaricin A2 produced by *S. salivarius* K12 was demonstrated to exert antimicrobial effects against *S. pneumoniae* ATCC 27336, AI8, AI11, AI14, AI6 and AI7. The MIC of salivaricin A2 to be required in the inhibition of the growth of *S. pneumoniae* ATCC 27336, AI8, AI11, AI14, AI6 and AI7 were 32, 32, 16, 32, 128 and 128 μg/mL, respectively. Besides that, *Streptococcus pyogenes* is a pathogen that causes many infectious diseases in humans such as scarlet fever, rheumatic fever, pharyngitis, tonsillitis, cellulitis, erysipelas and necrotizing fasciitis. Salivaricin B from *S. salivarius* K12 was found to exert inhibition activities against *S. pyogenes* with MIC of 2.16 μM. The growth of *S. pyogenes* was proved to be inhibted by 50% by using 1.0 μM of

salivaricin B. The inhibition activity can be increased to 90% by using 2.0 μM of salivaricin B and the cell will be totally inhibited if it was treated with 2.5 μM.

Furthermore, salivaricin B was also indicated to have inhibition activity against *Corynebacterium spp GH17*. *Corynebacterium spp* was found in triggering the diseases such as pharyngitis, endocarditis, gastrointestinal tract infection and skin infection. The MIC for the inhibition of growth of *Corynebacterium spp GH17* by salivaricin B was 2690 nM. Besides that, Salivaricin D from *S. salivarius* 5M6c was proved to exert inhibitory ability against *Clostridium bifermentans*. *C. bifermentans* is a gram-positive, spore-forming, anaerobic bacterium that always cause infectious diseases such as empyema and pneumonia to humans. The MIC of salivaricin D for the inhibition of the growth of *C. bifermentans* was found at around 0.01 nM. *Leuconostoc lactis* is a gram-positive, catalase-negative and facultatively anaerobic which may cause infections in immunocompromised hosts. Up to now, there is still limited of treatment and antibiotics that available for the infections caused by *Ln. Lactis*. To solve this problem, salivaricin D was claimed to have antimicrobial effect on *Ln. lactis.* The MIC of the salivaricin D to inhibit the growth of *Ln. lactis* was proved at around 0.1 nM. Salivaricin D was also demonstrated as antimicrobial agent in the inhibition of the growth of *S. pneumoniae* D39, TIGR4 and R6 for the prevention of pneumonia. The MIC of salivaricin D to inhibit the growth of *S. pneumoniae* D39, TIGR4 and R6 were proved at 0.03, 0.06 and 12, respectively. Besides that, salivaricin D was also exerting inhibitory effects against *S. pyogenes* 08198 with MIC value of 7.2 nM.

2.2.4 Subtilosin

Subtilosin is a bacteriocin that can be produced by Bacillus strains. *Bacillus* spp is aerobic, Gram-positive bacteria with rod shape which can be usually found in soil, water or natural flora in the intestines. Subtilosin A is a bacteriocin with a molecular weight of 3398.9 which consists of 32 usual amino acid and some non-amino acid residues. Subtilosin A, produced by *B. subtilis* 168, was claimed to exert antimicrobial effects against many Gram-positive and Gram-negative pathogens. Subtilosin A was proved to have inhibitory effect against *E. faecalis*. *E. faecalis* is a Gram-positive bacterium that may causes infections in humans especially infection of urinary tract. The MIC at 3.125 mg/L of subtilosin A was demonstrated to have significant inhibition activity against *E. faecalis* OGX-1. Besides that, subtilosin A was proved that it could be used to inhibit the growth of *Porphyromonas gingivalis*. *P. gingivalis* is a Gram-negative, anaerobic, non-motile bacterium with rod shape which binds to human oral cavity and always cause periodontitis and tooth loss. The MIC of subtilosin A needed for the inhibition of the growth of *P. gingivalis* ATCC 33277, *P. gingivalis* ATCC 33277 KDP129, *P. gingivalis* ATCC 33277 KDP133 and *P. gingivalis* W83 were 3.125, 3.125, 6.25 and >100 mg/L, respectively (Martin-Visscher et al., 2011).

Furthermore, subtilosin A was proved to exert inhibitory effect against *Klebsiella pneumoniae. K. pneumoniae* is a Gram-negative, non-motile and rod-shaped facultative anaerobe which may causes pneumonia, urinary tract infection, skin infections and meningitis. The MIC of subtilosin A for the inhibition of the growth of *K. pneumoniae ATCC 4352*, *K. pneumoniae UMN1*, *K. pneumoniae UMN5*, *K. pneumoniae UMN3* and *K. pneumoniae UMN4* were >200, >200, 1.25, 5.0 and 25.0 mg/L, respectively. Subtilosin A was also found to have inhibitory effect against *K.*

rhizophila. *K. rhizophila* is a Gram-positive bacterium that can be normally found on normal skin and mucous membrane of humans, but it may cause infections such as methylmalonic aciduria in immunocompromised patients. 1.25 mg/L of subtilosin A was proved to be the MIC needed to inhibit *K. rhizophila* ATCC 9341. In addition, the growth of *Enterobacter aerogenes* was demonstrated to be inhibited by subtilosin A. *E. aerogenes*, known as *Klebsiella aerogenes* now, is a Gram-negative bacterium with rod shape which may cause gastrointestinal infection, meningitis, urinary tract infection, skin infection and respiratory infection. The growth of *E. aerogenes* ATCC 13408 was proved to be inhibited by the MIC of subtilosin A at 1.25 mg/L. Subtilosin A was also claimed to have inhibition activity against *S. pyogenes*, a pathogen that may cause infections in human. The MIC of Subtilosin A to inhibit S. pyogenes ATCC 19615 was proved at 1.25 mg/L. Besides that, subtilosin A was proved to be used as an antimicrobial agent in the treatment of shigellosis. Shigellosis is the infection that may be caused by *Shigella sonnei*, leading to diarrhea, fever and stomach cramps. 1.25 mg/L of subtilosin A was prove to be the MIC to inhibit the growth of *S. sonnei* ATCC 25931. Moreover, the inhibition of the growth of *P. aeruginosa* was proved can be done by using subtilosin A. *P. aeruginosa* is a Gram-negative, encapsulated, rod-shaped bacterium that may cause infectious diseases such as pneumonia, urinary tract infections and bacteremia in humans. The MIC of subtilosin A needed to inhibit the growth of *P. aeruginosa* ATCC 9027 was 50 mg/L. In addition, subtilosin A was prove to exert inhibitory effect against *S. gordonii* Challis which was the pathogen found in oral cavity to cause dental plaques. The MIC of 83.25 of subtilosin A was claimed to exert significant inhibitory effect on *S. gordonii* Challis ATCC 49818 (Vading et al., 2018).

2.2.5 Mersacidin

Mersacidin is a bacteriocin that belongs to lantibiotic group, which is consisting of 20 amino acids and lanthionine group in its structure. It is produced by Bacillus spp and proved to have inhibition activities against Gram-positive pathogens. *S. aureus* is a Gram-positive bacterium with rod shape which may cause infectious diseases in human such as abscesses, furuncle, bloodstream infection and pneumonia. Mersacidin produced by Bacillus sp. strain HIL Y-85,54728 was claimed to exert antimicrobial effects on few *S. aureus* strains, which including *S. aureus* SH1000, R33 MRSA, SG511, SA137/93A and SA137/93G. The MIC of mersacidin needed to inhibit the growth of *S. aureus* SH1000, R33 MRSA, SG511, SA137/93A and SA137/93G was proved at 32, 32, 1, 35 and 30 μg/mL, respectively. Besides that, mersacidin was also proved to have inhibition activities against *S. pneumonia*e BAA-255, a pathogen that causes pneumonia in humans. The MIC of mersacidin needed to inhibit the growth of *S. pneumonia*e BAA-255 was 2 μg/mL. Furthermore, mersacidin was demonstrated to have inhibition activity against *Micrococcus luteus. M. luteus* is a Gram-positive and non-motile bacterium that has been reported as a opportunistic pathogen that may cause pneumonia and meningitis. The MIC of mersacidin needed to inhibit *M. luteus* ATCC4698 was 1.2 μg/mL. In addition, mersacidin was also proved to have inhibitory effect on the *E. faecium and E. faecalis* strains which might cause infections in abdomen, skin, bloodstream and urinary tract. The MIC of mersacidin needed for the inhibition of the growth of *E. faecium* ATCC19579, *E. faecalis* ATCC29212 and *E. faecium* 7131121 VRE were proved at 32, 64, 64 μg/mL, respectively.

2.2.6 Enterocin

Enterocin is bacteriocin produced by *Enterococcus spp* that active against Gram-positive pathogents. Enterocin A was claimed to exert inhibitory effect against *L. monocytogenes*. *L. monocytogenes* is a facultative anaerobic that may cause listeriosis. The groups of people that can be easily infected by *L. monocytogenes* are newborns, older adults, pregnant women and people with immunodeficiency disorders. The MIC of enterocin A needed to inhibit the growth of *L. monocytogenes* EGDe was around 4.57 μg/mL. The inhibition activity of enterocin A against *L. monocytogenes* EGDe was found can be improved by the combination of both enterocin A and thyme essential oil, in which only 0.9 and 1.2 μg/mL of MIC of enterocin and thyme essential oil, respectively, needed for the inhibition of the *L. monocytogenes EGDe*. Besides that, enterocin LD3 from *Enterococcus hirae* LD3 was proved to have antimicrobial effects against *M. luteus* MTCC 106 with MIC at 80 μg/mL and MBC at 128 μg/mL. Enterocin A was also found to have inhibition activity against *E. coli* NCDC 135 with MIC at 112 μg/mL and MBC at 180 μg/mL. In addition, enterocin AS-48 was proved to have inhibition against *B. cereus* ATCC 14579. *B. cereus* is a Gram-positive, motile and facultative anaerobe with rod shape which can be usually found in soil environment and cause diarrhoea and infections in respiration tract and wounds. The MIC of enterocin AS-48 needed for the inhibition of the growth of *B. cereus* ATCC 14579 was 2.5 μg/mL. Enterocin E20C produced by *E. hirae* 20C was also demonstrated to exert antimicrobial effect against *Salmonella enterica. S. enterica* is a Gram-negative and facultative aerobe with rod in shape that may cause gastroenteritis, bacteremia and enteric fever. The MIC of enterocin E20C needed for the inhibition of the growth of *S. enterica was* 0.5 μg/mL.

2.2.7 Epidermin

Epidermin is a tetracyclic bacteriocin that belongs to type A lantibiotic. It is comprising of 21 amino acids with meso-lanthionine, 3-methyllanthionine, and *S*-(2-aminovinyl)-D-cysteine in structure. Epidermin that produced by *Staphylococcus epidermidis* exhibits antimicrobial activities against a wide range of Gram-positive bacteria. Epidermin was claimed to exhibit inhibition activities against pathogens *S. aureus* SG 511 and E 88 which might cause respiratory tract, skin and surgical site infections. The MIC of epidermin needed to inhibit the growth of *S. aureus* SG 511 and E 88 were same at 8 μg/mL. Besides that, epidermin was also applied in the treatment of infection caused by *S. epidermidis. S. epidermidis* is Gram-positive and facultative anaerobe that can be usually found in normal human flora. The 6.25 μg/mL of MIC of epidermin was needed for the inhibition of *S. epidermidis* 12228. Futhermore, epidermin was also claimed to exhibit inhibitory effect against *S. pneumoniae* ATCC 6302, a pathogen that may cause pneumonia. The MIC of 4 μg/mL of epidermin was required to inhibit the growth of *S. pneumoniae* ATCC 6302.

Moreover, *S. pyogenes* ATCC 8668, a pathogen that might cause respiratory tract infection in human was found to be inhibited by epidermin with MIC of 1 μg/mL. Epidermin was also proved to against *S. faecalis* ATCC 29212 that may cause infections in urinary tract, wound and soft tissue. The MIC of epidermin needed to inhibit the growth of *S. faecalis* ATCC 29212 was proved at 64 μg/mL. In addition, epidermin was proved to have inhibition activity against *Corynebacteriurn xerosis. C. xerosis* is a Gram-positive aerobe that can be found in normal flora of the nasopharynx and skin and may cause endocarditis, cerebrospinal fluid shunt infection in an infant, mediastinitis and spontaneous bacterial peritonitis. The MIC of epidermin needed to exert inhibition activity against *C. xerosis* was 1 μg/mL. Besides that, *M.*