MECHANISTICS OF SECRETOME PROTEINS FROM *Paenibacillus polymyxa* Kp10 AND *Lactococcus lactis* Gh1 AGAINST METHICILLIN-RESISTANT *Staphylococcus aureus* AND VANCOMYCIN-RESISTANT *Enterococcus*

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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January 2023

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Resistance in pathogenic bacteria has emerged as a major global public health concern. Antibiotic-resistant bacterial infections are a major cause of patient mortality and morbidity, and rising antibiotic resistance is seriously compromising the vast medical advances made possible by antibiotics over the last decade. Hence, alternative approaches in controlling the bacterial infections are urgently needed. *Paenibacillus polymyxa* Kp10 (Kp10) and *Lactococcus lactis* Gh1 (Gh1) both are bacterial isolates that were believed to exhibit antimicrobial activity. Therefore, the effectiveness of secretome protein extracts isolated from Kp10 and Gh1 as the therapeutic agent in the treatment against antibiotic-resistant bacterial strains, namely, vancomycin-resistant *Enterococcus* (VRE*)* and methicillin-resistant *Staphylococcus aureus* (MRSA) were investigated. The main objective of this study is to determine the inhibition mechanisms of secretome protein extracted from Kp10 and Gh1 against MRSA and VRE bacteria. Minimal Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and time-to-kill assays were used to determine the sensitivity and viability of MRSA and VRE bacterial cells following treatment with the secretome proteins of Kp10 and Gh1. Next, to determine the morphological changes of MRSA and VRE after treated with Kp10 and Gh1, the microscopic analysis using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were observed. Then, to elucidate the antimicrobial mechanism of secretome protein of Kp10 and Gh1 against MRSA and VRE, 2D gel and sonication proteomic analysis based on time dependent manner by using liquid chromatography-mass spectrometry (LCMS) were run by comparing upregulated and downregulated proteins. Subsequently, the proton motive force study included the efflux of ATP; the pH gradient and the membrane potential study in treated MRSA and VRE were conducted. The differential proteins expression in MRSA and VRE treated with secretome proteins Kp10 and Gh1 in time dependent manner were analyzed. Protein extracts were obtained from treated MRSA and VRE cells by sonication and the protein profiling were identified by using liquid chromatography-mass spectrometry (LCMS). The safety of both secretome proteins in human cell also were

evaluated by the characterization of serum stability towards secretome proteins and their potential toxicity towards Medical Research Council cell strain 5 cell (MRC5), a kind of human lung cells. MRSA and VRE bacteria that were found to be sensitive to secretome proteins of Kp10 and Gh1 were treated with the respective secretome protein extract and were found to display several distinguished and apparent signs of morphological and internal composition changes, based on the microscopic analysis. Several proteins that were found to be important in cell wall functions and cell division, cell wall biosynthesis/ protein synthesis and the stress response were identified to be down-regulated or up-regulated in both treated cells without changing the membrane potential gradient. Next, the cytotoxicity test suggested that there were no cytotoxic effects have been observed on both secretome proteins when treated on MRC5 cells. Hence, there is no IC50 was determined. Finally, the preliminary test on the effect of secretome proteins in human serum was done using the agar well diffusion method. From this study, there are no significant changes in the inhibition zone of secretome proteins in serum when treated on the bacterial strain, thus giving the initial impression that peptide is safe to use in the human body. In conclusion, secretome proteins of Kp10 and Gh1 were demonstrated to reduce the growth number of VRE and MRSA by damaging the cell membrane, suggesting that both secretome proteins may serve as a potential therapeutic agent for antibiotic-resistant pathogen.

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MEKANISTIK PROTEIN REMBESAN DARI *Paenibacillus polymyxa* Kp10 DAN *Lactococcus lactis* Gh1 TERHADAP *Staphylococcus aureus* TAHAN METHICILLIN DAN *Enterococcus* TAHAN VANCOMYCIN

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Kemunculan rintangan dalam bakteria patogen telah menjadi ancaman awam yang ketara terhadap kesihatan global. Jangkitan bakteria tahan antibiotik telah menyebabkan kematian dan morbiditi terhadap pesakit yang besar, dan peningkatan rintangan antibiotik secara serius mengancam kemajuan antibiotik dalam perubatan sepanjang dekad yang lalu. Oleh itu, pendekatan alternatif untuk mengawal jangkitan bakteria amat diperlukan. *Paenibacillus polymyxa* Kp10 (Kp10) dan *Lactococcus lactis* Gh1 (Gh1) kedua-duanya adalah pengasingan bakteria yang dipercayai mempamerkan aktiviti antimikrob. Oleh itu, keberkesanan ekstrak protein rembesan yang diasingkan dari Kp10 dan Gh1 sebagai agen terapeutik dalam rawatan terhadap jangkitan strain bakteria tahan antibiotik, iaitu, *Enterococcus* tahan vancomycin (VRE) dan *Staphylococcus aureus* tahan methicillin (MRSA) telah dikaji. Objektif utama kajian ini adalah untuk menentukan mekanisme perencatan protein rembesan yang diekstrak dari Kp10 dan Gh1 terhadap bakteria MRSA dan VRE. Kepekatan Minimal Inhibitory (MIC), Kepekatan Bakteria Minimum (MBC) dan ujian masa untuk membunuh bacteria digunakan untuk menentukan sensitiviti dan daya maju sel bakteria MRSA dan VRE berikutan rawatan dengan protein rembesan dari Kp10 dan Gh1. Kemudian, untuk menentukan perubahan morfologi MRSA dan VRE selepas dirawat dengan Kp10 dan Gh1, analisis mikroskopik menggunakan mikroskopi elektron pengimbasan (SEM) dan mikroskopi elektron penghantaran (TEM) telah diperhatikan. Seterusnya, ntuk menjelaskan mekanisme antimikrob protein rembesan Kp10 dan Gh1 terhadap MRSA dan VRE, analisis proteomik 2D gel dan protein sonikasi yang bergantung pada masa rawatan yang berbeza telah dijalankan dengan menggunakan spektrometri jisim kromatografi cecair (LCMS) dengan membandingkan protein yang dikawal selia dan kajian daya motif proton termasuk efflux ATP; kecerunan pH dan kajian potensi membran dalam MRSA dan VRE yang dirawat juga turut dijalankan dijalankan. Keselamatan kedua-dua protein rembesan dalam sel manusia juga dinilai oleh pencirian kestabilan serum terhadap protein rembesan dan potensi ketoksikan mereka terhadap sel Majlis Penyelidikan Perubatan strain 5 sel (MRC5*)*, sejenis sel paru-paru manusia. Bakteria MRSA dan VRE yang

didapati sensitif terhadap Kp10 dan Gh1 dirawat dengan ekstrak protein rembesan masing-masing dan didapati menunjukkan beberapa tanda-tanda perubahan morfologi dan komposisi dalaman yang jelas berdasarkan analisis mikroskopik. Beberapa rembesan protein yang didapati penting dalam fungsi dinding sel dan pembahagian sel, biosintesis dinding sel / sintesis protein dan tindak balas tekanan telah dikenal pasti untuk semakin meningkat atau semakin menurun dalam kedua-dua sel yang dirawat tanpa mengubah kecerunan potensi membran. Seterusnya, ujian sitotoksik menunjukkan bahawa tiada kesan sitotoksik terhadap kedua-dua rembesan protein telah diperhatikan apabila dirawat pada sel MRC5 sehingga kepekatan yang dikenal pasti. Oleh itu, tiada IC50 ditentukan. Akhirnya, ujian awal mengenai kesan rembesan protein dalam serum manusia yang dilakukan secara ringkas menggunakan kaedah penyebaran agar telah menunjukkan bahawa tiada perubahan ketara dalam zon perencatan rembesan protein apabila dibandingkan dengan rembesan protein dalam serum setelah dirawat pada strain bakteria, dengan itu memberikan kesan awal bahawa rembesan protein dari kedua-dua sumber adalah selamat untuk digunakan dalam tubuh manusia. Dari kajian ini, dapat disimpulkan bahawa protein rembesan yang berasal dari protein rembesan Kp10 dan Gh1 mempunyai aktiviti antibakteria dan terbukti dapat mengurangkan jumlah pertumbuhan VRE dan MRSA dengan merosakkan membran sel, menunjukkan bahawa kedua-dua protein rembesan tersebut sebagai agen terapeutik yang berpotensi untuk patogen tahan antibiotik.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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$(NH_4)_2SO_4$	Ammonium sulfate
$\Delta\psi$	membrane potential
ΔpH	pH gradient
&	And
%	Percentage
$	Dollar
ºC	degree Celsius
2-DE	2 dimensional
ABC	ATP-binding cassete
ACN	Acetonitrile
AMPs	Antimicrobial peptides
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCECF	20-70-bis(carboxyethyl)-56-carboxyfluorescein
BHI	Brain Heart Infusion
BLIS	Bacteriocin-like-inhibitory substance
Boc	ert-butyloxycarbonyl
BR	Binding region
BSA	Bovine serum albumin
CAMA	Cecropin A-Magainin 2
Cbz	Carbobenzoxy
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
COG	Clusters of Orthologous

DiSC3(5)	3,3-Dipropylthiadicarbocyanine iodide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dpi	Dots per inch
DRAMP	Data repository of antimicrobial peptides
DTT	Dithiothreitol
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
ELISA	enzyme-linked immunoassay
EPL	Expressed protein ligation
ER	Endoplasmin reticulum
et. al.,	and others
FA	Formic acid
FBA	Fructose-bisphosphate aldolase
Fn	Fibronectin
G	Gram
GarQ	Garvicin Q
Gh1	*Lactococcus lactis* Gh1
GRE	Gentamicin-resistance enterococcus
GTP	guanosine triphosphate
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HGT	horizontal gene transfer
hr	hour
HT29	human colorectal adenocarcinoma cell line
IC50	concentration that reduces the effect by 50%
IPG	immobilized pH gradient
IU	International unit

K	Potassium
$K_3Fe(CN)_6$	Potassium ferrocyanide
KCl	Potassium chloride
kDA	kilodalton
Kp10	*Paenibacillus polymyxa* Kp10
kV	kilovolt
L	Liter
LC-MS	Liquid chromatography–mass spectrometry
LL-37	37 amino acid cationic peptide
m/z	mass to charge ratio
MBC	Minimum Bactericidal Concentration
mg	milligram
mL	millilitre
MH	Muller Hilton
MIC	Minimum Inhibitory Concentration
min	Minutes
MK0	MRSA treated with Kp10 at 0 hr
MK12	MRSA treated with Kp10 at 12 hr
MK18	MRSA treated with Kp10 at 18 hr
MK6	MRSA treated with Kp10 at 6 hr
ML0	MRSA treated with Gh1 at 0 hr
ML12	MRSA treated with Gh1 at 12 hr
ML18	MRSA treated with Gh1 at 18 hr
ML6	MRSA treated with Gh1 at 6 hr
MR-CoNS	Methicillin-resistant coagulase-negative *staphylococci*
MRC5	Medical Research Council cell strain 5
mRNA	messenger RNA

MRS	De Man, Rogosa and Sharpe
MRSA	Methicillin-resistant *Staphylococcus aureus*
ms	Mass spectra
MSA	Mannitol Salt Agar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
$Na_2S_2O_3$	sodium thiosulfate
NCL	native chemical ligation
NH_4HCO_3	Ammonium bicarbonate
NRR	Non repeat region
OD	Optical density
PDB	Protein data bank
PDR	Pan-drug-resistant
pH	Potential of hydrogen
PsDef1	Pine defensin 1
R	Resistance
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
S	Second
S. aureus	*Staphylococcus aureus*
SEM	Scanning electron microscopy
sp.	Species
SPSS	Solid phase peptide synthesis
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
tRNA	Transfer RNA

U87 MG	Uppsala 87 malignant glioma
UniProt	Universal protein
US	United states
USA	United States of America
V	Volt
VK0	VRE treated with KP10 at 0 hr
VK12	VRE treated with KP10 at 12 hr
VK18	VRE treated with KP10 at 18 hr
VK6	VRE treated with KP10 at 6 hr
VL0	VRE treated with Gh1 at 0 hr
VL12	VRE treated with Gh1 at 12 hr
VL18	VRE treated with Gh1 at 18 hr
VL6	VRE treated with Gh1 at 6 hr
VRE	Vancomycin resistant *enterococci*
X.laevis	Xenopus laevis
XDR	Extensively drug resistant

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Antibiotic resistance in pathogenic bacteria has become a major global public health concern. Vancomycin-resistant *Enterococcus* (VRE) and Methicillin-resistant *Staphylococcus aureus* (MRSA) are among the known prominent antibiotic-resistant bacteria. They have resulted in a significant number of patient deaths and financial burdens on health-care systems (Ventola, 2015; Dadgostar, 2019). Moreover, antibiotic resistance poses a significant risk to current medical advances, which is heading toward a post-antibiotic era in which common infections and minor injuries can once again lead to death. (Golkar, Bagasra & Pace, 2014; Ventola, 2015). The effectiveness of traditional antibiotics has deteriorated dramatically over time, and more effective therapeutic agents against infections caused by antibiotic-resistant bacteria are desperately needed (Golkar, Bagasra, & Pace, 2014; Sengupta, Chattopadhyay & Grossart, 2013; Wright, 2014). Various studies are currently looking for alternative substances that could potentially replace existing antibiotics.

Secretome protein has been shown to have antimicrobial activity due to the presence of antimicrobial elements that can inhibit bacterial growth and could potentially replace antibiotics (Damayanti, 2021). As the number of antibiotic-resistance cases rises, the use of secretome protein may be one of the options to tackle drug resistance. Secretome protein has been shown in some studies to release antimicrobial peptides (AMPs) such as cathelicidin, RNase3, human -defensins, and calprotectin (Kasiri et al., 2016). For example, secretome protein that was found in adipose tissue was observed to suppress the growth of *Staphylococcus aureus* via the increased activity of cathelicidin (Yagi et al., 2020; Harman et al., 2017). Based on these intriguing findings, the understanding of antimicrobial properties found in secretome proteins should be expanded.

AMPs in secretome protein act as host defenses, where most of them have been isolated from eukaryotes, such as animals, plants, and fungi (Zainal Baharin et al., 2021; Kumar, Kizhakkedathu & Straus, 2018). Some bacteria are also found to produce secretome proteins that can inhibit food-borne pathogens and other pathogenic bacteria (Vieco-Saiz et al., 2019). For example, the secretome protein on bacteria found in skin wounds inhibited the growth of Gram-positive bacteria *Staphylococcus aureus* and Gram-negative *Escherichia coli* by producing secreted factors that can affect bacterial membranes (Harman et al., 2017). For that reason, more potential bacteria should be searched and explored.

In this study, *Paenibacillus polymyxa* Kp10 (Kp10) and *Lactococcus lactis* Gh1 (Gh1) were chosen because of their ability to kill some bacteria based on previous studies (Mokhtar et al., 2020; Suzuki, 2021). For example, Gh1 has been shown to have antimicrobial activity against pathogenic *Staphylococcus aureus, Listeria monocytogens*, *Salmonella* and *Bacillus cereus* (Jawan et al., 2020), and Kp10 has been shown to have antimicrobial activity against *Escherichia coli* (Mokhtar et al., 2020). Because of their distinct characteristics, a focus on their secretome extract with abilities as therapeutic agents against antibiotic-resistant pathogens should be further documented for inhibition mechanisms. This is a common drug discovery strategy that can provide insight into the mechanism of protein activity of the bacterial extract.

Although the mechanism of secretome protein derived from Kp10 and GH1 action in pathogen inhibition remains unknown, bacteriocins with a conserved amino acid sequence and low molecular weight are generally hypothesized to interact with bacterial membranes, particularly negatively charged bacterial cell membranes (Negash & Tsehai, 2020), causing cell membrane damage and thus impairing the transport of large molecules (e.g. proteins), resulting in cell death and a disrupted cell. Furthermore, these bacteriocins have the ability to cross bacterial membranes and act on intracellular targets (Lei et al., 2019).

Several studies have shown that different bacterial strains can inhibit the growth of pathogenic microorganisms and degrade mycotoxins. Furthermore, studies have been conducted to describe the probiotic properties and antimicrobial activity of bacterial strain's extracts isolated from various sources (Ndiaye et al., 2022; Vieco-Saiz et al., 2019; Azat et al. 2016). Although bacterial strains are commonly used as culture starters and source for bacteriocins as food preservatives, more research on their inhibitory potential against antibiotic-resistant pathogens is required. As a result, the use of various current technologies to identify and characterize new bacterial strains carrying high potential of antimicrobial secretome proteins are warranted to allow better elucidation of the mechanisms.

Therefore, the purpose of this study is to investigate the properties of bacterial isolates; Kp10 and Gh1, as well as their ability to inhibit antibiotic-resistant pathogens. The inhibition mechanisms of secretome protein extracted from Kp10 and Gh1 against VRE and MRSA bacteria were studied through the determination of the sensitivity and viability of the cells and the morphological changes by microscopic studies by using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The antimicrobial mechanism of secretome protein of Kp10 and Gh1 against MRSA and VRE were analyzed by by using 2-DE gel and protein sonication proteomic analysis based on time dependent manner with liquid chromatography-mass spectrometry (LCMS). The safety of secretome proteins derived from Kp10 and Gh1 in human cell were also evaluated by the characterization of serum stability towards both secretome proteins and their potential toxicity towards MRC5 cells, a type of human lung cells. The findings of this study may provide detailed biological observations on the potential of the

secrotome proteins of Kp10 and Gh1 to be used as the therapeutic agents and eventually to reduce reliance on antibiotics.

1.2 Proplem Statement

The emergence of antibiotic-resistant pathogens worldwide is partly associated with the use of some available antibiotics that impose some identical mechanisms of inhibition. This issue has been identified as a major public health threat affecting human health globally. Multidrug-resistant pathogens have emerged not only in hospital settings but are now frequently identified in community settings, implying that an antibiotic-resistant bacteria reservoir exists outside of the hospital.

VRE and MRSA are two of the most common pathogens, causing a significant number of deaths and a financial burden on healthcare systems (Ventola, 2015; Dadgostar, 2019). Controlling bacterial infections is an ongoing process, and as antibiotic resistance spreads, an alternative antimicrobial agent is required to address the issues associated with MRSA and VRE infections.

Kp10 and Gh1 are well-known probiotic bacterial strains with antimicrobial activity, which could be used to develop antitherapeutic candidates. As a result, the secretome proteins Kp10 and Gh1 containing AMPs with a novel inhibition mechanism are being studied. Understanding the inhibition mechanism of resistance is critical for developing strategies to suppress resistance emergence and spread, as well as novel therapeutic approaches against multidrug-resistant organisms.

1.3 Objectives

1.3.1 General Objective

The general objective of this study is to determine the inhibition mechanisms of secretome protein extracted from Kp10 and Gh1 against MRSA and VRE bacteria.

1.3.2 Specific Objectives

a. To determine the sensitivity and viability of MRSA and VRE bacterial cells following treatment with the secretome proteins of Kp10 and Gh1.

b. To determine the morphological changes of MRSA and VRE after treatment with Kp10 and Gh1 based on microscopic analysis.

c. To elucidate the antimicrobial mechanism of secretome protein of Kp10 and Gh1 against MRSA and VRE based on 2D gel from 2D-electrophoresis and LC MS-gel image analyses.

d. To compare differential proteins expression in response to secretome proteins of Kp10 and Gh1 in MRSA and VRE in time-dependent manner.

e. To evaluate the safety of secretome proteins derived from Kp10 and Gh1 based on serum stability and potential toxicity in MRC5 cells.

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