UNIVERSITI PUTRA MALAYSIA

DISCOVERY AND CHARACTERIZATION OF ANTILISTERIAL PROTEINS FROM Paenibacillus polymyxa Kp10 USING GENOME MINING AND MASS SPECTROMETRY FOR FOOD APPLICATION

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By

NUR FADHILAH KHAIRIL MOKHTAR

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DISCOVERY AND CHARACTERIZATION OF ANTILISTERIAL PROTEINS FROM *Paenibacillus polymyxa* Kp10 USING GENOME MINING AND MASS SPECTROMETRY FOR FOOD APPLICATION

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Chairman : Professor Datuk Wira Raha binti Hj Abdul Rahim, PhD
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The prevalence of food poisoning cases causing listeriosis is worrisome, requiring advancement in food preservation strategies. The use of chemical preservatives raises health concern, therefore prompted the search of new natural preservatives such as bioactive proteins. A variety of bacteria isolated from dairy and fermented products have been known to produce various antimicrobial proteins beneficial for food and pharmaceutical industries. Previously, unidentified pure bacterial isolate originated from milk curd has been found to exert antimicrobial activity against *Listeria monocytogenes* ATCC 15313. Despite promising property exerted by the bacteria, the protein(s) responsible for the antilisterial activity was not yet identified and characterized, which mask its true potential for industrial application. The bacterium is hypothesized to encode antimicrobial genes in its genome and expresses one or more antilisterial proteins with a varying degree of stability against various conditions. Hence, this study aimed to identify the bacterium, followed by identification and characterization of potential genes and proteins contributing to the bacterial antilisterial property. Genome mining strategies in combination with mass spectrometric analysis were conducted to predict and identify the antilisterial proteins. The antilisterial activity of these proteins was verified through heterologous protein expression in *Escherichia coli*, followed by physicochemical stability assays against different pH, temperature and proteases. 16S rDNA sequence analysis of the pure isolate confirmed its identity as *Paenibacillus polymyxa* and designated as *P. polymyxa* Kp10. Genome mining of the bacterial draft genome sequence successfully determined eight putative ribosomally-synthesized antimicrobial proteins and six non-ribosomally synthesised antimicrobial peptide/polyketides. Out of the fourteen predicted proteins and peptides, two histone-like DNA binding proteins HU precedingly detected *in silico* were identified by the mass spectrometric analysis of the partially purified antilisterial proteins. Additionally, the active fraction also contains

translation initiation factor IF-1 and a 50S ribosomal protein L29. All of the antilisterial proteins produced by *P. polymyxa* Kp10 exhibited a various degree of physicochemical stability against different pH, temperature, proteases, and in the meat homogenates. Overall, the proteins are stable at high temperature, but sensitive to proteases and high alkaline pH. Proteins' stability in chicken, salmon and Ultra High Temperature (UHT) processed-milk was significantly compromised but excellent stability was observed in beef and simulated meat gravy food model. Bactericidal action of the proteins was confirmed by their ability to reduce and totally inhibit the growth of *L. monocytogenes* in simulated meat gravy food model. This study highlighted the potential of antimicrobial proteins and peptides from *P. polymyxa* Kp10 for manipulation in food industry, which warrants further investigations.

Keywords: *Paenibacillus polymyxa*, antilisterial proteins, genome mining, mass spectrometry, structural regulatory proteins

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENEROKAAN DAN PENCIRIAN PROTEIN ANTILISTERIA DARI *Paenibacillus polymyxa* Kp10 MENGGUNAKAN TEKNIK PERLOMBONGAN GENOM DAN ANALISIS SPEKTROMETRI JISIM UNTUK DIGUNAKAN DALAM MAKANAN

Oleh

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Kes keracunan makanan yang menyebabkan jangkitan listeriosis adalah sangat membimbangkan. Ia memerlukan penambahbaikan dalam strategi pengawetan makanan. Penggunaan bahan pengawet kimia pula berpotensi menimbulkan masalah kesihatan. Keadaan ini mendorong penyelidikan bagi mencari bahan pengawet semula jadi baru seperti protein bioaktif. Pelbagai bakteria yang diasingkan dari produk tenusu dan produk fermentasi telah diketahui menghasilkan pelbagai protein antimikrob yang bermanfaat untuk industri makanan dan farmaseutikal. Penyelidikan sebelum ini telah berjaya mengasingkan dan menulenkan bakteria yang tidak dikenali yang berasal dari gumpalan susu. Ia didapati mempunyai aktiviti antimikrob terhadap *Listeria monocytogenes* ATCC 15313. Walaupun sifat yang dimiliki oleh bakteria ini sangat baik, protein yang bertanggungjawab terhadap aktiviti antilisteria ini masih belum dapat dikenal pasti dan dicirikan. Ini menyebabkan, potensi sebenar protein ini dalam industri tidak dapat dinilai. Hipotesis kajian ini adalah bakteria ini mengekodkan gen antimikrob di dalam genomnya dan mengungkapkan satu atau lebih protein antilisteria dengan ciri-ciri kestabilan yang berbeza pada pelbagai keadaan. Oleh itu, projek ini bertujuan untuk mengenal pasti identiti bakteria, diikuti dengan pengenalpastian dan pencirian gen dan protein yang berpotensi menyumbang kepada sifat antilisteria. Kombinasi antara strategi perlombongan genom dan analisis spektrometri jisim dijalankan untuk meramal dan mengenal pasti protein antilisteria. Aktiviti protein antilisteria ini pula telah ditentu-sahkan melalui pengungkapan protein secara heterolog di dalam *Escherichia coli*, diikuti dengan ujian kestabilan fisikokimia terhadap pH, suhu dan enzim protease. Analisis jujukan 16S rDNA mengesahkan identiti pencilan bakteria ini sebagai *Paenibacillus polymyxa*, yang kemudian digelar sebagai *P. polymyxa* Kp10. Perlombongan draf genom bakteria ini berjaya menemukan lapan protein

antimikrob yang disintesis oleh ribosom dan enam protein antimikrob/poliketida yang disintensis tanpa ribosom. Dari empat belas protein dan peptida dua protein pengikat DNA seperti histon HU yang sebelum ini dikesan secara *in silico* telah dikenalpasti melalui analisis spektrometri jisim protein separa diekstrak. Selain itu, pecahan protein aktif itu juga mengandungi protein faktor inisiasi translasi IF-1 dan ribosom 50S L29. Semua protein antilisteria yang dihasilkan oleh *P. polymyxa* Kp10 memperlihatkan pelbagai tahap kestabilan fisikokimia terhadap pH, suhu, protease, dan homogenat daging yang berbeza. Secara keseluruhannya, protein adalah stabil pada suhu tinggi, tetapi, keadaan pH beralkali dan kehadiran protease mengganggu kestabilan protein. Kestabilian protein juga terganggu di dalam daging ayam, salmon dan susu lembu diproses pada suhu ultra tinggi (UHT). Walaubagaimanpun, kestabilan yang sangat baik didapati apabila protein berada di dalam daging dan simulasi model makanan daging berkuah. Tindakan bakterisidal protein ini juga telah disahkan melalui kemampuannya untuk mengurangkan dan merencat pertumbuhan *L. monocytogenes* dalam simulasi model daging berkuah. Kajian ini menonjolkan potensi protein dan peptida antimikrob dari *P. polymyxa* Kp10 untuk dimanipulasikan penggunaannya dalam industri makanan, yang memerlukan penyelidikan lanjut.

Kata kunci: *Paenibacillus polymyxa*, protein antilisteria, perlombongan genom, spektrometri jisim, protein pengawalan berstruktur

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CHAPTER 1

INTRODUCTION

Food safety has been a worldwide concern as approximately 600 million people were reported to be affected by consumption of contaminated food yearly, which results in the death of almost 420,000 people (Mehlhorn, 2015). *Escherichia coli (E. coli)*, *Listeria monocytogenes (L. monocytogenes)*, *Staphylococcus aureus (S. aureus)*, and *Salmonella* sp., are among major food borne pathogens commonly found in contaminated food products and food manufacturing facilities (Newell et al., 2010). *L. monocytogenes*, for an instance, cause listeriosis with a high fatality rate among neonates, pregnant women, elderly and immunocompromised individuals (Buchanan, Gorris, Hayman, Jackson, & Whiting, 2017). Invasive listeriosis often causes diarrhea and fever in healthy adults. In contrast, significantly serious clinical symptoms such as sepsis, bacteremia, and pneumonia are often seen in highly risk people post infection, while non-invasive listeriosis causes febrile gastroenteritis in healthy people (Dhama et al., 2015; Doganay, 2003). The ability of *L. monocytogenes* to retain the viability in harsh conditions (eg; the presence or absence of oxygen, extreme pH, salt concentration and temperature) has contributed to their cross-contamination in food during processing (Radoshevich & Cossart, 2018). Recently, the cases of *L. monocytogenes* contamination in countries across the world have been postulated to be due to globalization of food supply and increased consumption of raw and ready-to-eat raw products (Gandhi and Chikindas 2007; Swaminathan and Gerner-Smidt 2007). Utilization of artificial and chemical food preservatives has caused great health concerns, therefore natural preservative is greatly sought by consumers (Carocho et al. 2014; Pongsavee 2015). However, there is limited number of natural preservatives available since ancient times such as salt, sugar, and vinegar (Amit, Uddin, Rahman, Islam, & Khan, 2017; Carocho et al., 2014; Zhou, Xu, & Liu, 2010). These natural preservatives have slight limitations. For an instance, high salt concentration is needed to reduce the water activity thus simultaneously interrupt microbial growth in food. However, high salt content in food products has been linked to cardiovascular diseases such as hypertension among consumers (Albarracín, Sánchez, Grau, & Barat, 2011). Nutritional value of high salt-containing food is also compromized due to elimination of water-soluble vitamins and minerals. Besides, the use of salting as a sole food preservation method is inadequate to fully combat microbial spoilage in food (Albarracín et al., 2011).

The use of bacterial-derived antimicrobial proteins has been a better alternative due to its efficiency in microbial growth control, stability in vast food matrices, and does not cause health-related problems (Choyam, Srivastava, Shin, & Kammara, 2019). The large bacterial diversity has contributed to the varieties of discovered antimicrobial proteins to date. Antimicrobial proteins include ribosomally synthesized antimicrobial protein (such as bacteriocin, ribosomal proteins and histone-like DNA binding protein) and non-ribosomally synthesized antimicrobial proteins such as polyketides and lipopeptides (V. Ahmad et al., 2017; Carvalho et al., 2018; Caulier et al., 2019; Gharsallaoui, Oulahal, Joly, & Degraeve, 2016;

Malheiros, Cuccovia, & Franco, 2016). Besides extensive researches carried out to determine novel antimicrobial proteins produced by lactic acid bacteria, *Paenibacillus*; a gram-positive bacterium that is commonly associated with plant-growth promoting activities, has been recently found to produce novel antimicrobial proteins such as paenibacillin and paenilan (Huang & Yousef, 2012; Park, Kim, Park, Choi, & Park, 2017). Therefore, in this study, we aim to discover and characterize the antimicrobial proteins from a pure culture of an unidentified bacterium to explore its potential as antimicrobial agent for food preservation. This unidentified bacterium has been previously isolated from milk curd and exhibited high antilisterial activity. This study has successfully identified the bacterial strain and its associated antilisterial proteins, followed by purification and characterization of the antilisterial proteins using molecular, *in silico* and proteomic approaches to investigate its bio-preservation potential in food

1.1 Problem statement

The existing food preservatives suffer several drawbacks such as safety and nutritional issues, therefore the needs of natural and effective food preservative are rising. Previously, unidentified pure bacterial isolate originated from milk curd has been found to exert antimicrobial activity against *L. monocytogenes* ATCC 15313. However, despite promising antilisterial property exerted by the bacteria, the identity and characteristics of the protein(s) responsible for the antilisterial activity remains elusive, which mask its true potential for industrial application. While sufficiently pure proteins are needed for protein identification, unavailability of existing knowledge on the properties of the antilisterial protein(s) produced by this isolate demands for development of suitable purification methodologies. Furthermore, the absence of information on their stability prohibits further manipulation and optimization of the proteins for future use in the food industry. Therefore, assessment on their physicochemical stability in different environmental conditions and food models is necessary.

1.2 Research Questions

1) What is the identity of species and biochemical characteristics of the bacterium producing the antilisterial proteins?
2) Assisted by genome mining approach, what are the antimicrobial proteins-related genes encoded in the bacterial genome?
3) How could the antilisterial proteins of *P. polymyxa* Kp10 be purified and identified?
4) Can the antilisterial activity of the partially purified proteins be reproduced using the recombinant protein to validate its activity?
5) Are the antilisterial proteins physicochemically stable in different environmental conditions and food models?

1.3 Hypothesis

1) The bacterial isolate displaying antilisterial activity is a specific bacterium with particular biochemical characteristics
2) The bacterium harbors multiple antimicrobial proteins-related genes encoded in its genome predicted through in silico genome mining approach
3) Antilisterial proteins of *P. polymyxa* Kp10 could be purified using consecutive purification based on molecular weight and isoelectric point differences and further identified using mass spectrometry
4) The recombinant proteins will show antilisterial activity similar to that of the partially purified protein counterparts, hence validating their antilisterial activity
5) The antilisterial proteins display a varying degree of physicochemical stability upon exposure to different environmental conditions and food models

1.4 Aim and specific objectives

This study aims to discover antilisterial proteins of milk-curd originated bacteria through genomic and proteomic approaches and characterize their suitability for food application. The specific objectives in this study are:

1) To identify and characterize a milk curd-originated bacterium (designated as Kp10) displaying antilisterial activity using 16S rRNA gene sequencing and biochemical assays
2) To identify antimicrobial protein-encoded genes and the biosynthetic gene clusters of *P. polymyxa* Kp10 using genome mining approach
3) To purify and identify the antilisterial proteins produced by *Paenibacillus polymyxa* Kp10 using consecutive purification techniques and nanoLC-MS/MS.
4) To verify the antilisterial activity of the identified proteins through heterologous protein expression in *E. coli* and biological assays
5) To examine physicochemical stability of the recombinant antilisterial proteins at different environmental conditions and food models

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