UNIVERSITI PUTRA MALAYSIA

INFLUENCE OF BIOFILM-FORMING LACTIC ACID BACTERIA AGAINST SELECTED PATHOGENIC BACTERIA

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By

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Chairman: Wan Zuhainis Saad, PhD
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The threat of pathogenic bacteria is a major health concern in the food and healthcare industry. Accordingly, numerous studies have shown that certain diseases are largely caused by the biofilms formed by the persistent pathogenic bacteria. By omitting the options of chemical preservatives or antibiotics, lactic acid bacteria (LAB) have shown great potential in inhibiting many of these pathogens. However, majority studies on antimicrobial properties of LAB use only the conventional planktonic forms of LAB. This study evaluates the potential application of LAB biofilms to control or inhibit the formation of biofilms by pathogenic bacteria. It was thus proposed that the biofilms formed by LAB could be a promising tool for the control of pathogen biofilm formation. In this study, biofilm-forming LAB was isolated from local fruits and dairy products. From the total of 21 distinguished LAB isolates, two isolates (Isolate Y1 and Isolate KF) were selected based on their prominent inhibition against test pathogens (using spot-on-agar method and agar-well-diffusion assay) and efficient biofilm production [using tissue culture plate (TCP) method] compared to other isolates. They were then identified as *Lactobacillus casei* Y1 and *Lactobacillus plantarum* KF, respectively using 16S rDNA gene sequencing. The influence of incubation time, temperature and aeration on the biofilm production of *Lb. casei* Y1 and *Lb. plantarum* KF was also investigated using TCP method. For both the isolates, maximum biofilm production was found to be at the 48th h of incubation time, at the temperature of 35°C and under anaerobic condition. The inhibitory activity of both the selected LAB biofilms was evaluated against Methicillin-Resistant *Staphylococcus aureus*, MRSA (IMR code: S547), *Listeria monocytogenes* (IMR code: L10) and *Escherichia coli* O157:H7 (IMR code: E187), using *Lb. plantarum* ATCC 8014 as the reference strain, preliminarily at the 48th hour. However, from the initial finding, only biofilms of MRSA (S547) were found to be susceptible by all the selected LAB biofilms. As such only MRSA (S547) was selected for the detailed study of their biofilm inhibition using biofilms of LAB. When LAB biofilms and MRSA (S547) were co-cultured, both LAB biofilms showed significant potential in reducing the adherent and planktonic population of MRSA

(S547). *Lactobacillus casei* Y1 showed the highest reduction of MRSA biofilms, by 3.53 log at 48 hours while *Lb. plantarum* KF records the highest reduction of 2.64 log at 36 hours. In inhibiting planktonic population of MRSA (S547), both *Lb. casei* Y1 and *Lb. plantarum* KF biofilms recorded their maximum reduction of 4.13 log and 3.41 log at 24 hours, respectively. Despite their inhibitory effect being time-dependent, both LAB biofilms exhibited good potential in controlling the biofilm and planktonic population of MRSA (S547). The results from this study could highlight the importance of analysing biofilms of LAB to enhance their antimicrobial efficacy. Preferably, these protective biofilms of LAB could also be a better alternative to control the formation of biofilms by pathogens such as MRSA.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGARUH BAKTERIA ASID LAKTIK PEMBENTUK BIOFILM TERHADAP BAKTERIA PATOGEN YANG TERPILIH

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Ancaman bakteria patogenik dalam industri makanan dan persekitaran rawatan adalah isu yang membimbangkan dari aspek keselamatan dan kesihatan. Banyak kajian telah membuktikan bahawa sebahagian besar daripada penyakit-penyakit tertentu berpunca dari pembentukan biofilm tegar oleh bakteria patogenik. Dengan mengabaikan pilihan bahan pengawet kimia atau antibiotik, bakteriosin yang dihasilkan bakteria asid laktik (BAL) telah menunjukkan potensi yang besar dalam membendung banyak jenis patogen. Walau bagaimanapun, majoriti kajian mengenai ciri-ciri antimikrob bakteriosin hanya menggunakan BAL dalam fasa planktonik. Kajian ini menilai potensi biofilm BAL untuk mencegah atau mengurangkan pembentukan biofilm oleh bakteria patogenik. Oleh itu, idea kepentingan biofilm BAL sebagai ejen pengawal pembentukan biofilm patogen telah dicadangkan. Dalam kajian ini, BAL pembentuk biofilm yang mempamerkan ciri-ciri antimikrob telah diasingkan daripada buah-buahan dan produk tenusu tempatan. Daripada sejumlah 21 BAL, dua isolat (Isolat Y1 dan Isolat KF) telah dipilih berdasarkan sifat antimikrob (menggunakan kaedah titisan atas agar dan teknik penyerapan lubang) dan pembentukan biofilm (melalui kaedah pengujian penempelan plat mikrotiter) yang cekap berbanding BAL lain. Melalui penjujukan gen 16S rDNA, isolat tersebut telah dikenalpasti sebagai *Lactobacillus casei* Y1 dan *Lactobacillus plantarum* KF. Pengaruh faktor seperti masa pengeraman, suhu dan pengudaraan terhadap pengeluaran biofilm oleh *Lb. casei* Y1 dan *Lb. plantarum* KF turut disiasat menggunakan kaedah pengujian penempelan plat mikrotiter. Kedua-dua BAL terbabit telah menghasilkan biofilm maksimum pada jam ke-48, pada suhu 35°C dan di bawah keadaan anaerobik. Aktiviti pencegahan biofilm oleh *Lb. casei* Y1 dan *Lb. plantarum* KF telah dinilai (pada peringkat saringan, jam ke-48) terhadap Methicillin-Rentan *Staphylococcus aureus*, MRSA (Kod IMR: S547), *Listeria monocytogenes* (Kod IMR: L10) dan *Escherichia coli* O157: H7 (Kod IMR: E187), dengan menggunakan *Lb. plantarum* ATCC 8014 sebagai bakteria rujukan. Walau bagaimanapun, kajian awal menunjukkan bahawa hanya biofilm MRSA (S547) dapat dibendung oleh kesemua biofilm BAL yang terpilih. Oleh itu hanya MRSA (S547) telah dipilih untuk kajian yang lebih terperinci mengenai tahap perencatan biofilmnya menggunakan biofilm

BAL. Apabila biofilm BAL dikulturkan dengan MRSA (S547), kedua-dua biofilm BAL menunjukkan potensi yang besar dalam mengurangkan populasi planktonik dan biofilm MRSA (S547). *Lb. casei* Y1 menunjukkan penurunan tertinggi biofilm MRSA (S547) pada jam yang ke-48 (kadar pengurangan: 3.53 log) manakala *Lb. plantarum* KF merekodkan penurunan tertinggisebanyak 2.64 log pada jam ke-36. Dalam mencegah MRSA planktonik, biofilm *Lb. casei* Y1 dan *Lb. plantarum* KF merekodkan pengurangan maksimum 4.13 log dan 3.41 log pada jam ke-24. Biofilm *Lb. casei* Y1 terbukti lebih baik (lebih baik daripada bakteria rujukan) berbanding dengan *Lb. plantarum* KF dalam membendung planktonik dan biofilm MRSA. Walaupun secara keseluruhannya, aktiviti pencegahan kesemua biofilm BAL adalah tertakluk kepada masa, potensi yang baik telah dipamerkan dalam mengawal biofilm dan planktonik MRSA (S547). Hasil kajian ini menekankan kepentingan menganalisis biofilm BAL untuk peningkatan keberkesanan antimikrobial. Penggunaan biofilm perlindungan BAL juga boleh menjadi alternatif yang lebih baik untuk mengawal pembentukan biofilm patogen seperti MRSA.

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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 Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
A_{595}	Absorbance at 595 nm
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
AU	Arbitary unit
AWDA	Agar well diffusion assay
BLAST	Basic Local Alignment Search Tool
CFS	Cell-free supernatant
CFU	Colony-forming unit
CO_2	Carbon dioxide
et al.	and friends
LAB	Lactic acid bacteria
log	logarithm
MRS	de Man, Rogosa and Sharpe
MRSA	Methicillin resistant *Staphylococcus aureus*
NCBI	National Center of Biotechnology Information
O_2	Oxygen
QS	Quorum sensing
SD	Standard deviation
SEM	Scanning electron microscopy
TCP	Tissue-culture plate
TSA	Tryptic soy agar
TSB	Tryptic soy broth
v/v	Volume over volume ratio
w/v	Weight over volume ratio

CHAPTER 1

INTRODUCTION

Through many advances in related studies, microbiologists worldwide are now accepting the universality of biofilms as an important phase in microbial studies. Microbial research across various fields and disciplines has embraced the biofilm-based approach instead of the conventional planktonic phase alone (Prakash *et al*., 2003). While this approach could be the key for tackling biofilms of pathogens, biofilms of beneficial bacteria, like lactic acid bacteria (LAB) could also be studied for their advantage as protective biofilms.

A typical lactic acid bacterium can be characterized as gram-positive, non- sporing, non-respiring fermentative rod or coccus which produces lactic acid as a major end product (Axelsson, 2004). The antimicrobial effects of LAB can be attributed to their production of organic acid, hydrogen peroxide, diacetyl, reuterin, bacteriocin and many others (Reis *et al*., 2012). Bacteriocins differ from antibiotics due to their proteinaceous nature and their commonly narrow spectrum of inhibition (Saeed *et al*., 2014). Most antibacterial studies of LAB were focused on their planktonic phase and this approach is equally crucial for an early indication of potential antibacterial LAB. However, using the biofilm-based approach, biofilm- forming LAB could be used as protective biofilms against the persistent biofilm of pathogens. Essentially, studying the biofilm phenotype of LAB could enhance their antibacterial activity from a fresh perspective.

A fine example of biofilm-forming pathogen that is particularly difficult to eradicate is methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA is actually formed through the acquisition of *mecA* gene by S. *aureus* strains, which results in their resistance against β-lactam antibiotics (Fuda *et al*., 2004). The fact that *S. aureus* thrives as the normal flora of human body would definitely impact MRSA eradication. Furthermore, the genetic makeup of MRSA is highly flexible as evidenced by the vancomycin minimum inhibitory concentration (MIC) creep in its treatment (Dhand and Sakoulas, 2012). This means the efficacy of vancomycin as the gold standard treatment against MRSA has been regressing (Ho *et al*., 2010). This blooming pattern of MRSA infections is further exacerbated by its ability to form biofilms (Smith *et al*., 2008).

The adherence ability of MRSA to form biofilms is considered as one of its virulence mechanism (Watkins *et al*., 2012). In the healthcare settings, the threat of MRSA biofilms persists in chronic wound infections and indwelling medical device infections (Mermel *et al*., 2009). Biofilms are generally defined as surface-adhering communities of microorganisms embedded in an extracellular polymeric substance (EPS) (Hoiby *et al*., 2010; Maric and Vranes, 2007). The microbial cells within biofilms show exceptional durability against various biocides as well as the human

immune response that makes their eradication much tougher (Bryers, 2008). Overall, the expanding rate of MRSA's resistance and their tendency to form biofilms indicate that their eradication measures should not depend solely on antibiotic discoveries (Schellenberg, 2006). Alternatively, the use of probiotic strains like LAB could be a safer eradication option as they are less likely to elevate the multi-drug resistance of MRSA (Roghmann and McGrail, 2006). In this study, the inhibitory action of LAB biofilms against the biofilm and planktonic population of MRSA (S547) was investigated.

The objectives of this study were as follows:

1. To isolate lactic acid bacteria (LAB) with antibacterial properties.
2. To evaluate the biofilm production of selected LAB isolates, identify them using molecular identification and determine the influence of incubation time, temperature and aeration on their biofilm production.
3. To investigate the antibacterial potential of selected LAB biofilms to inhibit the planktonic and biofilm population of selected pathogens

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