UNIVERSITI PUTRA MALAYSIA

INHIBITORY EFFECT OF BIOFILM-FORMING Lactobacillus plantarum *PA21 ISOLATED FROM TROPICAL PLANT PANDANUS ON FOODBORNE PATHOGENS*

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By

TANNAZ JALILSOOD

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

April 2017

DEDICATION

Dedicated to my father and my Aunt and my dear friend Media who have been a source of inspiration which contributed imensly to the success of this thesis.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

INHIBITORY EFFECT OF BIOFILM-FORMING *Lactobacillus plantarum* PA21 ISOLATED FROM TROPICAL PLANT PANDANUS ON FOODBORNE PATHOGENS

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April 2017

Chairman : Professor Raha Abdul Rahim, PhD
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Bacterial biofilms are a preferred mode of growth for many types of microorganisms in their natural environments. The ability of pathogens to integrate within a biofilm is pivotal to their survival. Alternatively, new opportunities are now arising with the rapidly expanding potential of lactic acid bacteria (LAB) biofilms as biocontrol agents against foodborne pathogens. The present study was carried out to evaluate the effectiveness of a new *Lactobacillus plantarum* PA21 against several pathogenic and food-spoilage bacteria in the biofilm and planktonic phases. In addition, the attention was focused on the use of this isolate as a new host to investigate *Lactobacillus* key regulatory proteins in biofilm formation for further biotechnological applications.

Towards this objective, LAB was isolated from tropical plant *Pandanus amaryllifolius*. A new isolate was identified as *Lactobacillus plantarum* PA21 which showed biofilm formation in either pure culture and or in combination with several pathogenic and food-spoilage bacteria, such as *Salmonella enterica*, *Bacillus cereus*, *Pseudomonas fluorescens*, and *Aeromonas hydrophila*. Exposure to *Lb. plantarum* PA21 has significantly reduced the number of *P. fluorescens, A. hydrophila* and *B. cereus* cells in the planktonic and biofilm forms over 2-, 4- and 6-day time periods. However, despite the reduction in *S. enterica* cells, this pathogen showed the most resistance when co-cultured with *Lb. plantarum* PA21 and could not be eliminated entirely, either in the planktonic or biofilm phase.

Lb. plantarum PA21 was also found to be able to constitutively express *gfp* (green fluorescent protein) gene when transformed with the expression vector pMG36e, suggesting its capability of being a host for heterologous protein production.

Moreover, the gene expression ability of PA21 has allowed the identification the EAL containing protein for the first time in *Lactobacillus* spp, which inversely regulates biofilm formation and acts as a key regulatory protein in biofilm dispersal. By reading the optical density and viable cell count results, EAL_{21} overexpression in PA21 showed decreased adhesion compared to the wild type strain and significantly lowered the mean of cell counting results by 4.7 log.

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

KESAN PERENCATAN OLEH *Lactobacillus plantarum* PA21 DARI TUMBUHAN TROPIKA PADA PATOGEN BAWAAN MAKANAN

Oleh

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Biofilm bakteria adalah mod pertumbuhan yang digemari untuk pelbagai jenis mikroorganisma dalam persekitaran semula jadi mereka. Keupayaan patogen untuk mengintegrasi ke dalam biofilm adalahpenting untuk kelangsungan hidup mereka. Selain itu,bakteria laktik asid adalah jenis bakteria yang mempunyai banyak potensi dan peluang baru yang sedang berkembang pesat sebagai agen kawalan biologi terhadap patogen bawaan makanan. Kajian ini telah dijalankan untuk menilai keberkesanan *Lactobacillus* pencilan baru terhadap beberapa bakteria patogenik dan bakteria perosak makanan dalam biofilm dan fasa plankton. Di samping itu, kajian ini memberi tumpuan kepada penggunaan isolat ini sebagaiperumah baru untuk mengkaji protein pengawalaturan utama *Lactobacillus* dalam pembentukan biofilm untuk aplikasi bioteknologi selanjutnya.

Bagi mencapai matlamat ini, pemencilan strain LAB daripada tumbuhan tropika *Pandanus amaryllifolius* (Pokok Pandan) telah dilakukan. Pencilan baru telah dikenal pasti sebagai *Lactobacillus plantarum* PA21 yang menunjukkan pembentukan biofilm sama ada dalamkultur tulen dan atau dalam kombinasi dengan beberapa bakteria patogenik dan bakteria perosakmakanan seperti *Salmonella enterica, Bacillus cereus, Pseudomonas fluorescens* dan *Aeromonas hydrophila*. Bilangan sel *P. fluorescens, A. hydrophila* dan *B. cereus* dalam bentuk planktonic dan biofilm berkurangandengan ketara apabila didedahkan kepada *Lb. plantarum* PA21 dalam jangka waktu 2, 4, dan 6 hari. Walaubagaimanapun, di sebalik penurunan bilangan sel *S. enterica*, patogen ini menunjukkan rintangan yang paling tinggi apabila dikultur bersama *LB. plantarum* PA21 dan tidak boleh dihapuskan sama sekali, sama ada dalam fasa plankton atau biofilm.

Lb. plantarum PA21 juga didapati boleh mengekspresi gen GFP (protein pendarfluor hijau) secara berterusan apabiladitransformasi dengan vector pengekspresan pMG36e, ini membuktikan keupayaannya untuk menjadi bakteria perumah bagi penghasilan protein heterolog Selain itu, keupayaan pengekspresan gen oleh PA21 telah membolehkan pengenalpastian protein yang mengandungi EAL buat pertama kali dalam *Lactobacillus* spp., yang mana telah mengawal pembentukan biofilm secara songsang dan bertindak sebagai protein pengawalaturan utama dalam penyebaran biofilm Berdasarkan keputusan ketumpatan optik dan kiraan sel, ekspresi berlebihan EAL_{21}di dalam PA21 menunjukkan penurunan pada lekatan berbanding strain jenis liar dankeputusan min kiraan sel turun dengan ketara sebanyak 4.7 log.

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I certify that a Thesis Examination Committee has met on 20 April 2017 to conduct the final examination of Tannaz Jalilsood on her thesis entitled "Inhibitory Effect of Biofilm-Forming *Lactobacillus plantarum* PA21 Isolated from Tropical Plant Pandanus on Foodborne Pathogens" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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

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LIST OF ABBREVIATIONS

A	Aeromonas
AFM	Atomic Force Microscopy
AI	Autoinducer
AHL	N-acylhomoserine Lactones
API	Analytical Profile Index
ATP	Adenosine Triphosphate
ATR	Acid Tolerance Response
B	Bacillus
BCIP/NBT	3-Bromo-4-Chloro-5-Indolyl Phosphate and Nitro Blue Tetrazolium
CaCl2	Calcium chloride
CBD	Calgary Biofilm Device
c-di-GMP	Bis- (3′, 5′)-Cyclic-dimeric-Guanosine Monophoshate (c-di-GMP)
CE	Competitive Exclusion
CFU	Colony Forming Unit
CRD	Completely Randomized Design
CSLM	Confocal Scanning Laser Microscopy
CVC	Central Venous Catheter
DGC	Diguanylate Cyclases
DMRT	Duncan's Multiple Range Test
EAL E[A/E]L	amino acid motif
EDTA	Ethylenediaminetetraacetic Acid
EIIA	EIIA(Glc) β-glucosides-specific IIA component

E	Escherichia
EPS	Extracellular Polymeric Substance
ESEM	Environmental Scanning Electron Microscopy
EU	European Union
g	Gram
g	Relative centrifugation force
G1	c-di-GMP guanine base-1
GFP	Green Fluorescent Protein
GGDEF	GG[D/E]EF amino acid motif
GIT	Gastrointestinal Tract
GM17	M17 medium with 0.5 % (w/v) glucose
g/mol	Gram per mol
GRAS	Generally-regarded-as-safe
GTP	Guanosine Triphosphate
h	Hour
HMM	Hidden Markov Model
IBD	Inflammatory Bowel Disease
kDa	Kilo dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
L	Litre
LAB	Lactic Acid Bacteria
Lactobacillus	Lb.
Lactococcus	Lc.
Leuconostoc	Leuc.
LB	Luria Bertani

L	Listeria
LM	Light Microscopy
LPS	Lipopolysaccharides
MCS	Multiple Cloning Site
Me1	One metal-ion
Me2	Two-metal-ion
mm	Millimeter
min	Minute
mg	Milligram
MgCl2	Magnesium Chloride
µg	Microgram
µL	Microlitre
µm	Micrometer
mM	Millimolar
MRS	de Man, Rogosa and Sharpe
MTP	Microtiter Plate Assay
MW	Molecular weights
NaCl	Sodium chloride
ND	Not detected
OD	Optical density
OMPs	Outer-membrane Proteins
%	Percentage
P	Pseudomonas
P1/P2	phosphate groups of cyclic di-GMP
PCR	Polymerase Chain Reaction

PDEs	Phosphodiesterases
PMSF	Phenylmethane-Sulfonyl Fluoride
PTS	Phosphoenol-pyruvate phosphotransferase System
QS	Quorum Sensing
RCR	Rolling Cycle Replication
RE	Restriction Enzyme
S	Salmonella
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SGM17	Sucrose Glucose and M17
SGM17MC	SGM17 containing 0.2 M MgCl2 and 0.02 M CaCl2
spp	Subspecies
Staph	Staphylococcus
T3SS	Type III Secretion System
TA	Thymidine-Adenine
TAE	Tris base, acetic acid and EDTA
TBST	Tris-Buffered Saline Tween-20
TE	10mM Tris-HCl [pH 7.5], 1 mM EDTA
TEM	Transmission Electron Microscopy
V	Volume
w/v	Weight per volume
WHO	World Health Organization
w.t	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

CHAPTER 1

INTRODUCTION

Bacterial biofilms are a natural complex of microorganisms embedded in a protective slimy matrix composed of various types of polysaccharides, proteins, nucleic acids and lipids (Flemming & Wingender, 2010). The ability to form a biofilm is an important property for both pathogenic bacteria and useful bacteria used in diverse processes, such as fermentation and/or the preservation of food and feed. The food industry faces serious challenges due to equipment impairment caused by metal corrosion in pipelines resulting from chemical and biological reactions by resident biofilms (Bremer, Fillery, & McQuillan, 2006; Gram, Bagge-Ravn, Ng, Gymoese, & Vogel, 2007; Marc W, 1998; Vieira, Melo, & Pinheiro, 1993). Biofilms are resistant to antimicrobial agents and present major challenges in the application of disinfectant treatments (Manuel Simoes, Bennett, & Rosa, 2009). The adhesion capacity of food and water-borne pathogens, such as *Salmonella* spp., *Bacillus cereus*, *Pseudomonas fluorescens* and *Aeromonas hydrophila,* which develop biofilms in food-processing plants, lead to the transmission of diseases and decreased product shelf-life (Dogan & Boor, 2003; Elhariry, 2011; Kreske, Ryu, Pettigrew, & Beuchat, 2006; Lindsay, Brozel, Mostert, & Von Holy, 2002; Sharma & Anand, 2002). Biofilm constitute an attachment of microorganism in complex 3-dimensional structures that adhere on surfaces and eventually forms the biofilm matrix (Sutherland, 2001). Biofilms development as a part of bacterial life cycle can alter phenotypic and genotypic functions of different microorganisms, including foodborne pathogens (Donlan, 2002). After the discovery of biofilms, much research has focused on monospecies or pure cultures; however, most biofilm in the environment consist of multiple bacterial species having metabolic cooperation. Although various studies have shed light on the importance of biofilm communities and interspecies interactions in mixed-species biofilms, research is still in its infancy.

Majority of the pathogenic bacteria related to food-borne diseases are able to form biofilm on different materials and under various types of environmental conditions encountered in food processing plants. *Pseudomonas* spp. produces lipolytic and proteolytic enzymes that survive pasteurization and decrease the shelf life of dairy products (Dogan & Boor, 2003). In commercial dairy plants, *B. cereus* easily spreads in food production systems via sporulation, where the hydrophobicity and stress resistance of spores allow bacteria to easily attach to food processing plant (Lindsay, Brözel, & von Holy, 2006; Paidhungat, 2002). The genus *Salmonella,* with multiple tolerances known as a cross-protection phenomenon, is an important enteropathogenic pathogen that causes salmonellosis, which results in thousands of death every year (Høiby, Bjarnsholt, Givskov, Molin, & Ciofu, 2010; Leyer & Johnson, 1993; Xu, Lee, & Ahn, 2008). In addition to all of the pathogens mentioned above, *Aeromoans* spp. is also considered an opportunistic aquatic pathogen with the ability to form a biofilm on the surface of green leafy vegetables, such as cabbage and lettuce. It can also be found on minimally processed salad and juice prepared from fresh vegetables in low numbers (Janda & Abbott, 2010). The diverse pathogens present in specific food niches and the natural mixed-species biofilms express cooperative behavior instead of expected competitive selection between different microorganisms. In this regard, some Lactic Acid Bacteria (LAB) biofilms were discovered to have positive properties that could prevent these critical activities (Guerrieri et al., 2009; Speranza, Sinigaglia, & Corbo, 2009).

Lactic acid bacteria are well known as beneficial bacteria and include probiotic bacteria that positively affect the prevention of gastrointestinal related diseases, improve digestion by alleviating lactose intolerance (Levri, Ketvertis, Deramo, Merenstein, & D Amico, 2005), prevent intestinal tract infections (Reid et al., 2005), reduce the chances of inflammatory bowel disease or reactions to alergens (Bongaerts & Severijnen, 2005; Viljanen et al., 2005), and ease the absorption of nutrients (Amdekar, Dwivedi, Roy, Kushwah, & Singh, 2010; Delcenserie et al., 2008).

The LAB species are frequently detected as dominant bacteria in the gut of both mature animals and foods. These organisms use their ability to outnumber pathogens by creating an environment that is unfavorable for colonization rather than conducting physical attack and defeat missions. The lactic acid bacteria and their metabolites frequently used as a popular method of natural protection. Lactic acid bacteria provide another protective shield when forming biofilm. The use of biofilm by probiotic bacteria, such as *Lactobacillus* spp., and adherence to the mucosa of the host is consider to be a beneficial property, because it ensures colonization and stability in the mucosal surface, and also has antagonistic properties and contributes efficient defense against pathogenic bacteria by avoiding colonization (Terraf, Tomás, Nader-Macías, & Silva, 2012). Another effective approach to eradicate biofilm formation by unwanted bacteria is the adhesion of LAB biofilms to grow on hard surfaces that cause reduction of proliferation of other microbes, based on the CE (Competitive Exclusion) principle (Salas-Jara, Ilabaca, Vega, & García, 2016). Due to their health-promoting properties, LAB, particularly lactobacilli, are valued as candidates for cancer therapy, vaccine delivery, and immune-modulators (Bernardeau, Guguen, & Vernoux, 2006). Among lactobacilli, *Lactobacillus plantarum* is an excellent candidate for genetic engineering and shows great potential as a live vector for the improvement of therapeutic peptides that target bacterial pathogens (Diep, Mathiesen, Eijsink, & Nes, 2009). Current biofilm preventive strategies by *Lactobacillus* against pathogenic bacteria are essentially aimed at production of antimicrobial metabolites or inhibitory extracellular polymeric substance (EPS) surrounding the pathogenic bacteria. It has been investigated that production of extracellular polymeric substance (EPS) has been carried out by some biofilm forming strains, which successfully were shown to suppress biofilm formation by certain pathogens (Fracchia, Cavallo, Allegrone, & Martinotti, 2010; Walencka, Różalska, Sadowska, & Różalska, 2008). During the last years, some strains of *Lactobacillus* have been reported to have unique capacity to form biofilms on abiotic surfaces (glass or polystyrene) (Aoudia et al., 2016; Bujňáková & Kmeť, 2012; Ramírez, Smid, Abee, & Groot, 2015; Terraf et al., 2012). However, recent studies suggested that competition for adhesion sites and nutrients could also interfere with biofilm formation in pathogenic organisms, modulating *Lactobacillus*-

pathogen interfaces (Simões, Simões, & Vieira, 2010). To date, few studies have addressed this issue in multispecies biofilm context. In this regard, new information on *Lactobacillus* interactions with mixed biofilm communities is therefore needed.

Previously, it has been shown that biofilm formation and dispersal are regulated by several key regulatory proteins (Flemming & Wingender, 2010). These core proteins involved in the synthesis of adhesions and biofilm matrix components are evidently known, providing a tool for biofilm formation control. Engineering of even more efficient biofilm producers may be achieved by manipulating metabolic pathways via over-expression or down-regulation/knock-out of specific target proteins, which can mediate cell-to-cell interconnections or promote early biofilm formation and thereby bacterial survival.

To determine the feasibility of using *Lactobacillus* as an alternative host for a biological control strategy against different food-borne pathogens, it must be able to express the genes of interest under an inducible or constitutive expression systems (Chassy & Flickinger, 1987). The use of several constitutive promoters for heterologous expression in lactobacilli, including the P_{ldhL}, P_{slpA}, P_{144}, and lactococcal P_{23} promoters (Stephenson, Moore, & Allison, 2011), has become practical after successful electroporation in *Lactobacillus casei* (Chassy & Flickinger, 1987). As such, in the present study, apart from evaluating the effectiveness of the new *Lb plantarum* PA21 with adhesive properties to inhibit several pathogenic and food-spoilage bacteria, we also verified the ability of this strain to function as a host for future genetic engineering work which would improve biofilm production in this strain and provide insights regarding different aspects of the adhesion process. The specific objectives were as follows:

1. To isolate and identify LAB with high probability for biofilm formation
2. To examine the influence of the LAB isolate on pathogen attachment and vice versa.
3. To investigate the ability of the LAB isolates to be used as host for genetic engineering.
4. To over-express and study the effect of proteins consisting of the EAL domain, the products of which are involved in the signalling system and biofilm format

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