UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERISATION OF BACTERIOCINOGENIC *LACTOBACILLUS PLANTARUM* ISOLATED FROM MALAYSIAN FERMENTED FOOD

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By

MORTEZA SHOJAEI MOGHADAM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,

In Fulfilment of Requirements for the Degree of Master of Science

DEDICATIONS

I wish to dedicate this work to my grandmother who passed away during my study and I missed the chance to participate in her funeral. God bless her soul.

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October 2009

Chairman: Dr. Foo Hooi Ling, Ph.D

Faculty: Biotechnology and Biomolecular Sciences

Molecular approaches were used in this study to characterize six bacteriocinogenic *Lactobacillus plantarum* strains isolated from Malaysian foods since biochemical approaches could not differentiate them distinctively. The *Lb. plantarum* strains were initially identified as *Lb. plantarum* I with 99.9% similarity by the analysis of carbohydrate fermentation pattern using API CHL50 identification kit. The biochemical identification result was further confirmed by analyzing partial sequence of 16S rDNA that showed 99-100 % similarity to *Lb. plantarum*. Identification up to genus level was also achieved when Amplified Ribosomal DNA Restriction Analaysis (ARDRA) was applied with *Lactococcus lactis* MG1363, *Lb. plantarum* ATCC 11305, *Lb. johnsonii*, *Streptococcus thermophilus* BAA 250 and *Pediococcus acidilactici* 446 as reference strains. Furthermore, the studied *Lb. plantarum* strains were characterized using genotypic methods: plasmid profiling, randomly amplified polymorphic DNA (RAPD), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), repetitive extragenic

palindromes (Rep)-PCR as well as 16S-23S rDNA (ITS1) and 23S-5S rDNA (ITS2) spacer regions analyses. The strain RG14 was successfully differentiated from others by plasmid profiling. Results from RAPD study in which 6 arbitrary primers were tested, revealed slight differences in the genome of six *Lb. plantarum* strains. Moreover, sequence analysis of ITS1 revealed a four base pair variable region from which the strains could be divided into four groups. Comparative analysis of ITS1 with 17 *Lb. plantarum* strains available in GenBank confirmed the variability of this region and showed that the genotype of the studied strains are not present in the strains used for comparative analysis. As for PCR-RFLP study, the studied strains were initially screened for the presence of structural bacteriocin genes. It was found that all studied strains harboured the novel combination of *plantaricin EF* (*Pln EF*) and *plantaricin W* (*Pln W*), which had not been reported elsewhere. However, the PCR-RFLP technique was not discriminative when the *Pln EF* genes were digested with restriction enzymes *Hind*III, *Mbo*I and *Pst*I. Although rep-PCR showed strong typing ability, the banding pattern was not discriminative. The ITS2 region showed an extra 5S rDNA sequence downstream of the ribosomal DNA region. The ITS2 region, however, was highly conserved among the strains and encodes rRNA that form secondary structure with the predicted free energy of -11.5 Kcal/mol. In conclusion, the studied strains are novel bacteriocinogenic *Lb. plantarum*, which were successfully discriminated in a polyphasic approaches using plasmid profiling, RAPD and ITS1 analysis with the RAPD technique showing the highest discriminatory power.

PENCIRIAN MOLEKULAR *LACTOBACILLUS PLANTARUM* BAKTERIOSINOGENIK YANG DIPENCILKAN DARIPADA MAKANAN TERTAPAI MALAYSIA

Oleh

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Teknik molekul digunakan dalam kajian ini untuk mencirikan enam strain bakteriosinogenik *Lactobacillus palantarum* yang dipencilkan daripada makanan Malaysia memandangkan teknik biokimia tidak dapat membezakan enam strain tersebut secara sepsifik. Strain *Lb. plantarum* yang dikenalpasti pada awalnya sebagai *Lb. plantarum* I dengan 99.9% persamaan melalui analisis fermentasi karbohidrat API CHL50. Keputusan biokimia ini dikenalpasti dengan analisis separa jujukan bagi 16S rDNA yang telah menunjukkan 99-100% persamaan dengan *Lb. plantarum*. Identifikasi pada paras genera dicapai dengan teknik "Amplified Ribosomal DNA Restriction Analaysis (ARDRA)" yang mengunakan *Lactococcus lactis* MG1363, *Lb. plantarum* ATCC 11305, *Lb. johnsenii*, *Streptococcus thermophilus* BAA 250 dan *Pediococcus acidilactici* 446 sebagai strain rujukan.

Seterusnya, strain yang dikaji dibezakan dengan menggunakan kaedah genotaip: profil plasmid, "randomly amplified polymorphic DNA (RAPD)", "PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)", "Repetitive extragenic palindromes (Rep)-PCR" dan "16S-23S rDNA (ITS1)" dan "23S-5S rDNA (ITS2) intergenic spacer region analisis". Strain RG14 berjaya dibezakan daripada strain lain melalui teknik profil plasmid. Keputusan kajian RAPD yang dijalankan melalui enam primer arbitari menunjukan sedikit pembezaan dalam kandungan gen enam *Lb. plantarum* yang dikaji. Secara tambahan, analisis jujukan ITS1 menunjukkan empat pasang bes daripada bahagian berubah membezakan strain yang dikaji kepada empat kumpulan. Analisis perbandingan ITS1 dengan 17 strain *Lb. plantarum* dalam GenBank membuktikan bahagian berubah dan keputusan menunjukkan genotaip strain yang dikaji tidak terdapat dalam strain lain yang digunakan untuk analisis perbandingan. Bagi kajian PCR-RFLP, kehadiran gen struktur bakteriosin dalam strain yang dikaji telah dikenalpasti dan didapati bahawa semua strain mengandungi kombinasi *plantaricin EF* (*Pln EF*) and *plantaricin W* (*Pln W*) yang unik yang tidak pernah dilaporkan dalam kajian lain. Walaubagaimanapun, teknik PCR-RFLP tidak dapat menunjukkan perbezaan antara strain yang dikaji apabila gen *Pln EF* dihadam dengan *Hind*III, *Mbo*I and *Pst*I. Walaupun rep-PCR mempunyai kebolehan penyisihan yang kuat, corak jalur yang diperolehi dalam kajian ini tidak menunjukkan sebarang perbezaan. Bahagian ITS2 menunjukkan satu jujukan 5S rDNA tambahan di bawah bahagian ribosom DNA. Bahagian ITS2 amat dipulihara antara strain yang dikaji dan ia juga mengekodkan rRNA yang membentuk struktur sekunder dengan tenaga yang dijangka -11.5 Kcal/mol. Secara kesimpulan, semua strain yang dikaji dibuktikan adalah bakteriosinogenik *Lb. plantarum* yang novel, di mana strain ini telah berjaya dibezakan melalui kaedah profil plasmid, RAPD and

ITS1 analisis dengan teknik RAPD menunjukkan keupayaan pembezaan yang tertinggi berbanding dengan teknik lain.

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APPROVAL SHEET 1

I certify that an Examination Committee has met on **14/10/2009** to conduct the final examination of **Morteza Shojaei Moghadam** on his degree of **Master of Science** thesis entitled "**Molecular Characterization of Bacteriocinogenic *Lactobacillus plantarum* Strains Isolated from Malaysian Food**" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the degree of Master of Science.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institutions.

____

MORTEZA SHOJAEI MOGHADAM

Date: ____________

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LIST OF ABBREVIATIONS

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~	Approximately
°C	Degree Celsius
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
ATCC	America Type Culture Collection
ARDRA	Amplified Ribosomal DNA Restriction Analysis
BLAST	Basic Local Alignment Search Tool
bp	~~base~~ Base pair
DNA	~~deoxyribonucleic~~ Deoxyribonucleic acid
dNTP	~~deoxyribonucleotide~~ Deoxyribonucleotide triphosphate
dsDNA	~~double~~double-stranded DNA
EDTA	~~ethylene~~ Ethylene diamine tetraacetic acid
EMBL	European Molecular Biology Laboratory
EMP	Embden-meyerhof-Parnas
G+C	~~guanine~~ Guanine plus cytosine
g	~~gravity~~ Gravity force
g	Gram
GRAS	~~generally~~ Generally regarded as safe
GTE	Glucose-Tris-EDTA
ITS-PCR	Intergenic spacer region-PCR
ITS1	16S-23S rDNA intergenic spacer region
ITS1-S	Short form of ITS1
ITS1-L	Long form of ITS1
ITS2	23S-5S rDNA intergenic spacer region
ITS2-S	Short form of ITS2
ITS2-L	Long form of ITS2
kb	~~kilo~~ Kilo base pair
kDa	~~kilo~~ Kilo Dalton
LAB	~~lactic~~ Lactic acid bacteria
Lb	*Lactobacillus*
Lc	*Lactococcus*
M	Molar
$MgCl_2$	Magnesium chloride
min	Minute
mm	Milimeter
mL	Mililiter
MRS	Man Rogosa and Sharpe
N	Normality
Na	Sodium
NaOH	~~sodium~~ Sodium hydroxide
NCBI	National Center for Biotechnology Information
nm	Nanometer
nt	~~nucleotide~~Nucleotide
ORF	Open Reading Frame
PCI	~~phenol~~Phenol-chloroform-isoamyl alcohol
PCR	~~polymerase~~ Polymerase chain reaction
P	*Pediococcus*

Pln	Plantaricin
Pg	Picogram
RAPD	~~randomly~~ Randomly amplified polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
RE	~~restriction~~ Restriction enzyme
Rep-PCR	Repetitive Extragenic Palindromes-PCR
RFLP	~~restriction~~ Restriction fragment length polymorphism
RNA	~~ribonucleic~~ Ribonucleic acid
rRNA	~~ribosomal~~ Ribosomal RNA
rrn	~~ribosomal~~ Ribosomal DNA operon
sdH_2O	~~sterile~~ Sterile distilled water
SDS	~~sodium~~ Sodium dodecyl sulphate
S	*Streptococcus*
TAE	Tris-acetate-EDTA
Taq	Thermus aquaticus
TE	Tris-EDTA
T_a	Anealing Temperature
T_m	~~melting~~ Melting temperature
tRNA	Transfer RNA
U	~~unit~~Unit
UV	Ultra Violet
V	Volt
v/v	~~volume~~ Volume per volume
w/v	~~weight~~ Weight per volume
µg	Microgram
µL	Microliter
µg/mL	Microgram per milliliter
µM	Micromolar

CHAPTER 1

INTRODUCTION

1.1 Background

The biosphere was formed by and is completely dependent on the metabolism of microorganisms and on their interactions with each other. Currently, it is estimated that there are about 4-6 $\times 10^{30}$ different prokaryotic cells, exceeding, by various orders of magnitude, all plant and animal diversity. This enormous genetic variability is the result of rare mutations and recombination events (Pontes *et al.*, 2007). These reasons together with expectations about specialized species with novel enzymatic functions, new products and beneficial characteristics, have made bacterial identification as a growing field of interest within microbiology and led researchers to examine the enormous unknown diversity of prokaryotes present in different ecosystems (Temmerman *et al.*, 2004; Pontes *et al.*, 2007).

It is noteworthy that in some fields such as diagnostic and food industries, it is critically important to identify the isolates even up to the strain level. In such areas, where lactic acid bacteria (LAB) are extensively being used, strain-level identification is of great importance as some of the health beneficial effects of these bacteria have been reported to be strain specific (Ouwehand *et al.*, 2002; Temmerman *et al.*, 2004). Initially, phenotypic methods was widely used for identification and discrimination of bacteria, but it was soon revealed that these methods are not effective, as similar phenotypes displayed by strains do not always correspond to similar or even closely related genotypes. Consequently, there has been a shift towards the use of genotypic

characterization methods in order to provide a more distinctive classification and differentiation of bacteria (Temmerman *et al.*, 2004).

In contrast to phenotypic methods, the genotypic methods directly analyze the DNA and tend to have higher resolution. In general, many genotypic methods are based on the principle of polymerase chain reaction (PCR) and electrophoretic separation of DNA fragments of different molecular size (Olive and Bean, 1999; Temmerman *et al.*, 2004).

Various genotypic techniques have been developed and successfully applied to differentiate and characterize bacteria, such as amplified ribosomal DNA restriction DNA analysis (ARDRA) (Carmen and Hernández, 2007), species-specific primers based on other parts of ribosomal DNA (*rrn*) operon (Chenguad *et al*., 2001; Bringel and Hubert, 2003; Felis and Dellaglio, 2007) as well as *recA* (Torriani *et al*., 2001), randomly amplified polymorphic DNA (RAPD), plasmid profiling, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) (Olive and Bean, 1999; Temmerman *et al.*, 2004) and repetitive extragenic palindromes (rep)-PCR (Gevers *et al*., 2001). However, the discriminatory power of the above-mentioned methods has been at genus to strain level depending on the bacterial type. Since each method has its own advantages and disadvantages, distinctive discriminatory molecular characterization studies have to be conducted in a combination of various molecular methods.

Bacteriocinogenic *Lb. plantarum* species is of high value particularly in food industry. They have been isolated and characterized from various sources. However, negligible

reports are available from those isolated from Malaysian foods. Surveys and comparison among available data showed that members of bacteriocinogenic *Lb. plantarum* species produce various plantaricins. Moreover, in almost all similar studies, bacteria from ecologically different sources were subjected to molecular characterization and those strains isolated from ecologically similar sources have been always assumed to be the same.

Six *Lb. plantarum* strains studied in this project were previously isolated from steamed fish, fermented tapioca and tempeh (Lim, 2003; Woo, 2001). Although the studied strains were isolated from different foods, phenotypic methods failed to differentiate the strains (Lim, 2003). Therefore, this study was conducted to identify and characterize six bacteriocinogenic *Lb. plantarum* strains isolated from Malaysian foods using molecular approaches.

1.2 Objectives

a) To identify the bacteriocinogenic *Lb. plantarum* strains isolated from Malaysian food.

b) To differentiate the strains using amplified ribosomal DNA analysis.

c) To differentiate the strains using analysis of ribosomal DNA intergenic spacer regions.

d) To differentiate the strains using locus specific restriction fragment length polymorphism.

e) To differentiate the strains using plasmid profiling.

f) To differentiate the strains using randomly amplified polymorphic DNA.

g) To differentiate the strains using repetitive extragenic palindromes-PCR.

CHAPTER 2

LITERATURE REVIEW

2.1 Lactic acid bacteria (LAB)

The lactic acid bacteria (LAB) emerged around 3 billion years ago, probably before the photosynthetic cyanobacteria. Their expansion has really begun with the apparition of milk-producing mammals, over 65 million years ago. However, the first registered usage comes from the discovery of small vases punched by small holes, near the Neufchatel Lake, over 3000 years BC. Since those days, humans are able to control milk curdling (Champomier-Vergès *et al.*, 2002).

Lactic acid bacteria are now constituted of a heterogeneous group of Gram-positive bacteria with a strictly fermentative metabolism from which lactic acid is the key metabolite (Carr *et al.*, 2002). LAB are catalase-negative, non-spore forming, micro-aerobe to strictly anaerobe, and appear either in rod or coccid shape. LAB consist of number of genera: *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Oenococcus*, *Enterococcus* and *Streptococcus, Weisella, Carnobacterium, Tetragenococcus* and *Bifidobacterium* (Klein *et al.*, 1998; Carr *et al.*, 2002; Temmerman *et al.*, 2004). They are widespread in nature and commonly found in milk and dairy products, plant materials, silage, and intestinal tract and mucous membranes of humans and animals.

Lactic acid bacteria fall into two major groups based on their glucose fermentation: The homofermenters which produce lactic acid as the major product of fermentation of

glucose and the heterofermenters which produce a number of products besides lactic acid, including carbon dioxide, acetic acid, and ethanol from the fermentation of glucose (Carr *et al.*, 2002). All of the above mentioned genera are extensively being used in food industry due to their GRAS "Generally Regarded As Safe" status (Carr *et al.*, 2002).

In spite of the GRAS status of LAB, there have been reports on the involvement of these bacteria on human clinical infections. In a report by Aguirre and Collins (1993), a number of cases in which LAB have been implicated in human disease were reviewed and cited.

The classification of LAB has been largely based on the morphology, mode of glucose fermentation, growth at different temperatures, configuration of lactic acid produced, ability to grow at high salt concentrations, and acid/alkaline tolerance (Axelsson, 2004). By developing molecular techniques, however, classification of LAB have been dramatically changed and their identification was facilitated (Olive and Bean, 1999; Busch and Nitschko, 1999; Axelsson, 2004). For example, Streptococci were divided into three genetically distinct genera: *Streptococcus*, *Enterococcus* and *Lactococcus* (Stiles and Holzapfel, 1997; Champomier-Vergès *et al.*, 2002). Members of LAB have small genome with low G+C (guanine plus cytosine) content (<50%) (Klein *et al.*, 1998).

2.2 The genus *Lactobacillus*

2.2.1 Classical classification

The Lactobacilli were originally classified by Orla and Jensen (1919) into the homofermenters and heteroferementers based on the amount of lactic acid formed during glucose fermentation. Orla and Jensen (1919) also divided the Lactobacilli into the three groups (the Thermobacteria, Streptobacteria, and the Betabacteria) based on the growth temperature and biochemical reactions (Table 2.1). Although those three groups have been replaced for the most part, the three names are still commonly used and are defined according to growth temperature, ability to ferment pentoses, ability to produce carbon dioxide from glucose or gluconate, requirement for thiamine, formation of lactic acid as a major product of fermentation, homofermentative or heterofermentative type of fermentation, reduction of fructose to mannitol and hydrolysis of arginine (As cited in Carr *et al.*, 2002).