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MOLECULAR APPROACHES FOR IDENTIFICATION, CHARACTERISATION AND QUANTIFICATION OF PROBIOTIC *LACTOBACILLUS* STRAINS FOR POULTRY

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By

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Chairperson: Dr. Sieo Chin Chin, PhD

Institute: Bioscience

The use of probiotics as an alternative to antibiotic growth promoter has gained popularity in the commercial poultry industry in view of the hazards posed by antibiotics to human and animal health. However, the science behind the probiotic microorganisms has been poor with respect to their identity at the species and strain level, their interaction with the host animal, and their efficacy in poultry practices. Thus, a reliable and efficient method is essential to monitor the probiotic microorganisms and to perform quality control of commercial probiotic products. In the present study, molecular methods were applied for reidentification, characterisation and enumeration of 12 probiotic *Lactobacillus* strains which were previously identified with classical biochemical tests.

Based on comparative sequence analyses of the 16S ribosomal RNA (rRNA) gene and 16S-23S rRNA gene intergenic spacer region (ISR), discrepancies were found in the identification of nine out of the 12 *Lactobacillus* strains, namely, *L. brevis* C 1, *L. brevis* C 10, *L. fermentum* C 16, *L. brevis* C 17, *L. crispatus* I 12, *L. acidophilus* I 16,

L. fermentum I 24, *L. fermentum* I 25 and *L. acidophilus* I 26. These strains were reidentified as *L. reuteri* C 1, *L. reuteri* C 10, *L. reuteri* C 16, *L. panis* C 17, *L. brevis* I 12, *L. gallinarum* I 16, *L. salivarius* I 24, *L. brevis* I 25 and *L. gallinarum* I 26. The rate of misidentification is high when conventional identification methods are used.

To further characterise the 12 *Lactobacillu*s strains, repetitive element sequence-based PCR (rep-PCR) and amplified ribosomal DNA restriction analysis (ARDRA) were employed. Rep-PCR was able to discriminate *L. reuteri* C 10, *L. panis* C 17 and *L. salivarius* I 24 up to strain level. However, *L. brevis* I 12, I 23, I 25, I 211 and I 218, *L. reuteri* C 1 and C 16, and *L. gallinarum* I 16 and I 26 could only be differentiated up to species level. A lower discriminatory power was demonstrated by ARDRA as it could only distinguished *L. reuteri* C 10 and *L. panis* C 17 into single strains. The 16S rRNA gene restriction patterns were able to further distinguished *L. gallinarum* I 16 and I 26 into single strains. *Lactobacillus brevis* I 12, I 23, I 25, I 211 and I 218 seem to be multiple clones of the same bacterial strain as are *L. reuteri* C 1 and C 16.

SYBR Green I real-time quantitative PCR was employed for the quantification of five representative species of the *Lactobacillus* strains. The primers designed from the variable regions of the 16S rRNA gene were found to be target-specific except for the primers targeting *L. gallinarum* which were group-specific. The *Lactobacillus* strains were estimated to have four to seven copies of the16S rRNA gene. The copy numbers of *L. gallinarum* and *L. panis* reported in the present study are the first record. The real-time PCR quantification protocol developed in this study was compared with the conventional culture quantification method. It was found that the

quantification results produced by real-time PCR for *L. reuteri* and *L. panis* were highly similar with the conventional method. Higher values of bacterial number were determined for *L. gallinarum* and *L. salivarius*, and lower values of bacterial number were obtained from *L. brevis* when real-time PCR was compared with the conventional culture method.

The results of this study demonstrated that molecular techniques offer reliable, efficient and accurate identification, characterisation as well as quantification of *Lactobacillus* strains. Application of molecular-based techniques provides significant advantages over the traditional method in this respect. The results of the present study will be potentially useful in the strategic formulation and development of a more effective probiotic.

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

PENDEKATAN-PENDEKATAN MOLEKUL UNTUK PENGENALPASTIAN, PENCIRIAN DAN PENGKUANTITIAN STRAIN PROBIOTIK *LACTOBACILLUS* UNTUK AYAM

By

LEE CHIN MEI

Disember 2009

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Penggunaan probiotik sebagai alternatif untuk penggalak pertumbuhan antibiotik telah memperolehi popularity dalam industri penternakan ayam memandangkan risiko yang ditimbulkan oleh antibiotik ke atas kesihatan manusia dan haiwan. Walau bagaimanapun, sains di sebalik probiotik mikroorganisma masih kurang difahami terutamanya dalam aspek pengenalpastian identiti ke tahap spesies dan strain, interaksi probiotik mikroorganisma dengan haiwan perumah dan keberkesanannya dalam industri penternakan ayam. Oleh itu, suatu kaedah yang tepat dan cekap adalah penting dalam usaha pemantauan probiotik mikroorganisma dan pengawalan mutu produk probiotik. Justeru itu, dalam kajian ini, kaedah-kaedah biologi molekul telah digunakan dalam pengenalpastian semula, pencirian dan pengkuantitian 12 strain probiotik *Lactobacillus* yang pernah dikenalpasti sebelum ini dengan ujian biokimia klasik.

Berdasarkan analisis perbandingan jujukan 16S ribosomal RNA (rRNA) gen dan 16S-23S rRNA gen 'intergenic spacer region' (ISR), percanggahan telah ditemui dalam pengenalpastian sembilan daripada 12 strain *Lactobacillus*, iaitu *L. brevis* C 1, *L. brevis* C 10, *L. fermentum* C 16, *L. brevis* C 17, *L. crispatus* I 12, *L. acidophilus* I 16, *L. fermentum* I 24, *L. fermentum* I 25 dan *L. acidophilus* I 26. Melalui kaedah biologi molekul, strain-strain ini dikenalpasti semula sebagai *L. reuteri* C 1, *L. reuteri* C 10, *L. reuteri* C 16, *L. panis* C 17, *L. brevis* I 12, *L. gallinarum* I 16, *L. salivarius* I 24, *L. brevis* I 25 dan *L. gallinarum* I 26. Kesalahan dalam pengenalpastian identiti probiotik *Lactobacillus* dikesan pada kadar yang tinggi apabila ujian biokimia klasik digunakan.

Dalam usaha untuk melanjutkan pencirian 12 strain *Lactobacillus*, kaedah 'repetitive element sequence-based PCR' (rep-PCR) dan 'amplified ribosomal DNA restriction analysis' (ARDRA) telah digunakan. Rep-PCR berupaya mendiskriminasi *L. reuteri* C 10, *L. panis* C 17 dan *L. salivarius* I 24 ke tahap strain. Akan tetapi, *L. brevis* I 12, I 23, I 25, I 211 dan I 218, *L. reuteri* C 1 dan C 16, dan *L. gallinarum* I 16 dan I 26 hanya boleh dibezakan sehingga tahap spesies. Kuasa diskriminasi yang lebih rendah telah ditunjukkan oleh ARDRA di mana ia hanya boleh membezakan *L. reuteri* C 10 dan *L. panis* C 17 ke strain tunggal. '16S rRNA gene restriction patterns' berupaya melanjutkan perbezaan *L. gallinarum* I 16 and I 26 sehingga ke tahap strain. *Lactobacillus brevis* I 12, I 23, I 25, I 211 and I 218 berkemungkinan adalah klon-klon daripada strain bakteria yang sama, begitu juga dengan *L reuteri* C 1 dan C 16.

'SYBR Green I real-time quantitative PCR' telah digunakan dalam kuantifikasi lima spesies *Lactobacillus* yang terpilih. Primer yang diterbitkan daripada tapak

perubahan 16S rRNA gen didapati spesifik dengan sasaran probiotik *Lactobacillus* kecuali primer yang mensasarkan *L. gallinarum*. Primer ini adalah specifik dengan sekumpulan spesies *Lactobacillus*. Strain-strain *Lactobacillus* telah dianggarkan mempunyai empat hingga tujuh salinan 16S rRNA gen. Nombor salinan untuk *L. gallinarum* dan *L. panis* yang dilaporkan dalam kajian ini adalah yang pertama dalam rekod. Protokol pengkuantitian 'real-time PCR' yang dibangunkan dalam kajian ini telah dibandingkan dengan kaedah pengkuantitian konvensional. Keputusan pengkuantitian yang dihasilkan oleh kaedah 'real-time PCR' untuk *L. reuteri* dan *L. panis* adalah amat sama dengan kaedah konvensional. Pengkuantitian jumlah bakteria secara berlebihan untuk *L. gallinarum* dan *L. salivarius*, dan kekurangan dalam pengaggaran jumlah bakteria untuk *L brevis* telah dikesan melalui 'real-time PCR'.

Hasil kajian ini menunjukkan bahawa teknik-teknik biologi molekul menawarkan pengenalpastian, pencirian dan pengkuantitian strain *Lactobacillus* yang boleh dipercayai, cekap dan tepat. Aplikasi teknik-teknik biologi molekul memberikan lebih manfaat berbanding kaedah tradisional dalam aspek-aspek tersebut. Hasil kajian ini berpotensi untuk digunakan dalam usaha formulasi strategik dan pembangunan probiotik yang berkesan.

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I can no other answer make, but, thanks, and thanks.

~William Shakespeare

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I certify that an Examination Committee met on 15th December 2009 to conduct the final examination of Lee Chin Mei on her Master of Science thesis entitled "Molecular Approaches for Identification, Characterisation and Quantification of Probiotic *Lactobacillus* Strains for Poultry" 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 Master of Science.

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Date: 11 Febuary 2010

DECLARATION

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

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LEE CHIN MEI

Date: 18 February 2010

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ARDRA	- amplified ribosomal DNA restriction analysis
BLAST	- Basic Local Alignment Search Tool
bp	- basepair
kb	- kilobasepair
BSA	- bovine serum albumin
C	- cytosine
cfu	- colony forming unit
Ct	- threshold cycle
D	- discriminatory index
DGGE	- denaturing gel electrophoresis
DMSO	- dimethyl sulphoxide
DNA	- deoxyribonucleic acid
dNTP	- deoxyribonucleotide triphosphate
ds	- double stranded
ERIC	- enterobacterial repetitive intergenic consensus
EDTA	- ethylene diamine tetracetate
E_s	- amplification efficiency
FCM	- flow cytometry
FISH	- Fluorescence *in situ* hybridisation
FRET	- fluorescence resonance energy transfer
G	- guanine
g	- gravity
g	- gram
mg	- milligram
µg	- microgram
HCl	- hydrochloric acid
ISR	- Intergenic spacer region
LB	- Luria-Bertani
M	- molar / molarity
mM	- millimolar
µM	- micromolar
ml	- millilitre
MEGA	- Molecular Evolutionary Genetic Analysis
MRS	- de Man, Rogosa and Sharpe
NTC	- no-template control
µl	- microliter
ng	- nanogram
OD	- optical density
PCR	- polymerase chain reaction
PCR-ELISA	- PCR-enzyme linked immunosorbent assay
PFGE	- pulse field gel electrophoresis
pmole	- picomole
R^2	- correlation coefficient
RAPD	- randomly amplified polymorphic DNA
REP	- repetitive extragenic palindromic
rep-PCR	- repetitive element sequence-based polymerase chain reaction

RDPII	- Ribosomal Database II
RFLP	- restriction fragment length polymorphism
rrn	- ribosomal ribonucleic acid operons
*rrn*DB	- Ribosomal RNA Operon Copy Number Database
rRNA	- ribosomal ribonucleic acid
tRNA	- transfer ribonucleic acid
SB	- sodium boric acid
SDS	- sodium dodecyl sulfate / sodium lauryl sulfate
SSC	- standard saline citrate
TAE	- Tris-acetate EDTA
TBE	- Tris-borate EDTA
TE	- Tris-EDTA
U	- unit
UPGMA	- unweighted pair group method using arithmetic averages
UV	- ultraviolet
V	- volt
V1	- variable region one
v/v	- volume per volume
w/v	- weight per volume
×	- times
X-Gal	- 5-bromo-4-chloro-3-indoyl-b-D-galactoside
ΔRn	- normalised reporter signal

CHAPTER 1

INTRODUCTION

The gastrointestinal tract of animal is colonised by a large and complex collection of intestinal microflora. The intestinal microbiota, which are attached to the intestinal epithelial cells, play an important role in maintaining the health of the host animal. Thus, the exploitation of intestinal microflora as probiotic has become an area of great interest. A probiotic is "a preparation consisting of live microorganisms or microbial stimulants which affects the indigenous microflora of the recipient animal, plant or food in a beneficial way" (Fuller, 1995). In the livestock industry, application of probiotic has been adopted over the past three decades and continues to gain momentum owing to the phasing out or severe restriction of antibiotic applications for non-medicinal purpose in many countries.

Lactobacillus is the principal microrganism used as probiotic to improve livestock nutrition and health in animal production (Timmerman *et al.*, 2006). It is the most predominant species in the avian alimentary tract (Lu *et al.*, 2003; Bjerrum *et al.*, 2006) and has been credited with an impressive list of therapeutic and prophylactic properties. The supplementation of probiotics has been reported to improve the growth performance of chickens through increased feed digestibility and feed utilisation (Nahashon *et al.*, 1994; Vicente *et al.*, 2007). Bacterial antagonism towards intestinal pathogen has also been demonstrated by probiotic *Lactobacillus* strains (Patterson and Burkholder, 2003). In addition, probiotics have been found to stimulate the gastrointestinal tract immune system (Huang *et al.*, 2004) and reduce fat and cholesterol levels in chickens (Kalavathy *et al.*, 2006).

Although the benefits of incorporating probiotics in animal feeds are well substantiated, studies have shown that the efficacy of probiotics in poultry practice has been inconsistent (Simmering and Blaut, 2001). The interactions of the microbes and the host animals still remain obscure and the science behind the function of probiotic requires stringent interpretation (Reid *et al.*, 2003). Furthermore, numerous studies have elucidated deficiencies in the microbiological quality and labelling of commercial probiotic products (Coeuret *et al.*, 2004). Some probiotic products have been mislabelled with respect to the bacterial species or the number of microorganisms present in the products. These problems arise because of lack of accurate and sensitive identification methods to monitor and keep track of the probiotic microorganisms during production as well as upon consumption by the hosts.

Traditional culture-dependant methods, which include morphological and biochemical characterisations (phenotypic traits), are still the routine procedures to identify bacteria despite being labour-intensive and time-consuming (Charteris *et al.*, 1997). Misidentifications of bacterial species using biochemical methods are common as phenotypic characterisation is unreliable and is affected by changes of the environmental conditions (Randazzo *et al.*, 2004). In the case of multi-strain probiotic, which has been reported to be more effective than mono-strain probiotic (Timmerman *et al.*, 2004), enumeration of a particular species by conventional method cannot be achieved since the phenotypic traits of the different species are not clearly distinguishable. This leads to difficulty in monitoring the quality of the final products. These factors have spurred the development of molecular-based identification and detection methods as an alternative to the phenotypic identification