



UNIVERSITI PUTRA MALAYSIA

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

**LEE CHIN MEI
IB 2009 16**



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

By

LEE CHIN MEI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfillment of the Requirements for the Degree of Master of Science**

December 2009



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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

By

LEE CHIN MEI

December 2009

Chairperson: Dr. Siew 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 *Lactobacillus* 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 the 16S 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

Pengerusi: Dr. Sieo Chin Chin, PhD

Institut: Biosains

Penggunaan probiotik sebagai alternatif untuk penggalak pertumbuhan antibiotik telah memperolehi populariti 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 spesifik 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 pengenpastian, 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.

ACKNOWLEDGEMENTS

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

~William Shakespeare

This study would not have been possible without the help and support of many people. Firstly, I would like to convey my sincere gratitude to the chairperson of the supervisory committee, Dr. Sieo Chin Chin, for her invaluable assistance, support and guidance. Her boundless energy and enthusiasm in research has inspired and motivated me in my endeavors as a student and as a researcher. In addition, she is always approachable, accessible and helpful which made my work so much easier and enjoyable and the research more rewarding.

I am deeply grateful to the members of the supervisory committee, especially Professor Dr. Ho Yin Wan, for her advice, endless support and encouragement, and constructive suggestions which have nourished my intellectual maturity. I would also like to thank Professor Dr. Norhani Abdullah for her kind assistance, guidance, encouragement and support.

I am indebted to Associate Professor Dr. Michael Wong Vui Ling who has ignited my passion for research especially in the field of molecular biology. I also wish to thank Dr. Cheach Yoke Kqueen and Dr. Jong Bor Chyan for their kind assistance.

Special appreciation goes to Madam Haw Ah Kam, Mr. Khairul Kamal Bakri and Mr. Nagayah Muniandy, staff of the Microbial Biotechnology Unit, Laboratory of



Industrial Biotechnology, Institute of Bioscience, for their technical assistance throughout the course of my study.

I would like to extend my gratitude to the Ministry of Science, Technology and Innovation for providing the financial support under the National Science Fellowship for the course of my study.

I am very grateful to all my labmates, Chiun Yee, Sor Sing, Tiam Poh, Ida, Hiao Ling and Mus for their friendship, help and encouragement. Thanks for making the long hours in the lab a pleasant and memorable one.

Words fail me to express my appreciation to my family. I would like to express my deepest gratitude to my late mother. Although she is no longer with us, she is forever loved and remembered. I am indebted to my father for his understanding, patience, support and endless love throughout the course of my study. Thanks are also due to my dear brothers for being supportive and caring siblings.



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.

Members of the Examination Committee are as follows:

Jana Ong Abdullah, PhD

Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Raha Abdul Rahim, PhD

Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Suhaimi Mustaffa, PhD

Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Thong Kwai Lin, PhD

Professor
Institute of Biological Sciences
University of Malaya
(External Examiner)

BUJANG KIM HUAT, PhD.

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:



This thesis was submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirements for the degree of Master of Science. The members of the Supervisory Committee are as follows:

Sieo Chin Chin, Ph.D.

Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Ho Yin Wan, Ph.D.

Professor
Insitute of Bioscience
Universiti Putra Malaysia
(Member)

Norhani Abdullah, Ph.D.

Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

HASANAH MOHD GHAZALI, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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.

LEE CHIN MEI

Date: 18 February 2010



TABLE OF CONTENTS

	Page
ABSTRACT	ii
ABSTRAK	v
ACKNOWLEDGEMENTS	viii
APPROVAL	x
DECLARATION	xii
LIST OF TABLES	xvi
LIST OF FIGURES	xvii
LIST OF ABBREVIATIONS	xxi
CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	
2.1 The gastrointestinal microbiota of chickens	5
2.2 The <i>Lactobacillus</i> genus	7
2.3 <i>Lactobacillus</i> as probiotic	9
2.4 Identification and discrimination of <i>Lactobacillus</i>	12
2.4.1 Conventional methods	12
2.4.2 Molecular methods	13
2.5 Quantification of <i>Lactobacillus</i>	27
2.5.1 Real-time quantitative PCR	27
2.5.2 Fluorescence <i>in situ</i> hybridization (FISH)	32
2.5.3 Flow cytometry (FCM)	34
3 IDENTIFICATION OF PROBIOTIC <i>LACTOBACILLUS</i> STRAINS BY 16S RIBOSOMAL RNA GENE AND 16S-23S RIBOSOMAL RNA GENE ISR SEQUENCE ANALYSES	
3.1 Introduction	36
3.2 Materials and methods	38
3.2.1 Strains, media and cultivation conditions	38
3.2.2 Genomic DNA extraction	38
3.2.3 PCR amplification of 16S rRNA gene	40
3.2.4 PCR amplification of 16S-23S rRNA ISR	40
3.2.5 Agarose gel electrophoresis	42
3.2.6 Cloning and sequencing of the PCR amplicons	42
3.2.7 Sequence alignments and phylogenetic inference	43
3.2.8 Deposition of the nucleotide sequences in the public database	44
3.3 Results	44
3.3.1 Nucleotide analysis and identification of probiotic <i>Lactobacillus</i> strains based on the 16S rRNA gene sequences	44

3.3.2	Phylogenetic analysis based on the 16S rRNA gene	54
3.3.3	Nucleotide analysis and identification of probiotic <i>Lactobacillus</i> strains based on the 16S-23S rRNA gene ISR sequences	54
3.3.4	Phylogenetic analysis based on 16S-23S rRNA gene ISR	60
3.4	Discussion	62
4	CHARACTERISATIONS OF <i>LACTOBACILLUS</i> STRAINS BY REPETITIVE ELEMENT SEQUENCE-BASED PCR (REP-PCR) AND AMPLIFIED RIBOSOMAL DNA RESTRICTION ANALYSIS (ARDRA)	
4.1	Introduction	69
4.2	Materials and methods	71
4.2.1	Strains, medium and cultivation condition	71
4.2.2	Rep-PCR	71
4.2.3	ARDRA	74
4.2.4	Numerical analyses of rep-PCR and ARDRA fingerprints	75
4.3	Results	76
4.3.1	Rep-PCR fingerprints	76
4.3.2	Numerical analysis of Rep-PCR fingerprints	82
4.3.3	ARDRA fingerprint patterns	90
4.3.4	Numerical analysis of ADRDA fingerprints	97
4.4	Discussion	105
5	QUANTIFICATION OF <i>LACTOBACILLUS</i> STRAINS BY SYBR GREEN I REAL-TIME QUANTITATIVE PCR TARGETING THE 16S RIBOSOMAL RNA GENE	
5.1	Introduction	114
5.2	Materials and methods	115
5.2.1	Strains, medium and cultivation condition	115
5.2.2	Isolation of genomic DNA	116
5.2.3	Design of PCR primers	116
5.2.4	Validation of the specificity of designed primers	117
5.2.5	Estimation of the 16S rRNA gene copy numbers in <i>Lactobacillus</i> strains	117
5.2.6	Real-time PCR	120
5.2.7	Standard curves construction	121
5.2.8	Quantification of <i>Lactobacillus</i> species	122
5.3	Results	123
5.3.1	Design of 16S rRNA gene-targeted primers	123
5.3.2	Specificity of the 16S rRNA gene-targeted primers	127
5.3.3	Estimation of the 16S rRNA gene copy numbers in <i>Lactobacillus</i> strains	131
5.3.4	Real-time PCR assays	140
5.3.5	Validation of real-time PCR assays	145

5.3.6	Standard curves generation	145
5.3.7	Quantification of <i>Lactobacillus</i> species	149
5.4	Discussion	156
6	GENERAL DISCUSSION AND RECOMMENDATIONS FOR FUTURE RESEARCH	164
7	CONCLUSION	171
	REFERENCES	173
	APPENDICE	195
	BIODATA OF STUDENT	196
	LIST OF PUBLICATIONS	197



LIST OF TABLES

Table		Page
3.1	<i>Lactobacillus</i> strains and site of isolation from the gastrointestinal tract of chickens.	39
3.2	Sequences of the oligonucleotide primers used for PCR amplification.	41
3.3	<i>Lactobacillus</i> strains identified by the 16S rRNA gene and 16S-23S rRNA gene ISR sequences.	46
3.4	Similarity level of the 16S rRNA gene sequences of the 12 <i>Lactobacillus</i> strains.	53
3.5	Similarity matrix of the 16S-23S rRNA gene ISR sequences of the 12 <i>Lactobacillus</i> strains.	59
4.1	Twelve <i>Lactobacillus</i> strains and type strains of five <i>Lactobacillus</i> species used in this study.	72
4.2	Sequences of the primers used for PCR amplification.	73
4.3	Numbers and sizes of DNA fragments obtained from the fingerprint patterns of ERIC-, REP-, BOX- and (GTG) ₅ -PCR.	81
4.4	Numbers and sizes of DNA fragments derived from the fingerprint patterns of ARDRA using restriction enzymes AluI, HaeIII, Sau3AI and TaqI.	97
4.5	Theoretical and experimental ARDRA profiles of some representative <i>Lactobacillus</i> strains generated with AluI, HaeIII, Sau3AI and TaqI restriction enzymes.	98
5.1	PCR primers for real-time PCR assay.	124



LIST OF FIGURES

Figure		Page
2.1	Secondary structure model for prokaryotic 16S rRNA gene in which V1-V9 indicate the variable regions.	15
2.2	Organisation of a typical <i>rrn</i> operon which contains the 16S, 23S and 5S rRNA genes.	18
2.3	Amplification curve of a specific gene illustrating increase in fluorescent reporter signal with each PCR cycle.	29
2.4	Measurement of fluorescence emitted during the DNA amplification can be accomplished by using dsDNA intercalating dye like (a) SYBR Green I, or FRET-based probes such as (b) taqman and (c) molecular beacons.	30
3.1	PCR amplification of the 16S rRNA gene from 12 <i>Lactobacillus</i> strains.	45
3.2	Multiple alignment of the 16S rRNA gene sequences of <i>Lactobacillus</i> strains C 1, C 10, C 16, C 17, I 12, I 16, I 23, I 24, I 25, I 26, I 211 and I 218.	47
3.3	Phylogenetic tree of <i>Lactobacillus</i> strains based on the neighbour-joining method of the 16S rRNA gene sequences.	55
3.4	Agarose gel electrophoresis of the 16S-23S rRNA gene intergenic spacer regions amplified from 12 <i>Lactobacillus</i> strains.	56
3.5	ISR sequences comparison of <i>Lactobacillus</i> strains C 1, C 10, C 16, C 17, I 12, I 16, I 23, I 24, I 25, I 26, I 211 and I 218.	58
3.6	Phylogenetic tree based on the 16S-23S ISR sequences constructed by neighbour-joining method showing relationships of the 12 <i>Lactobacillus</i> strains and other selected member of the <i>Lactobacillus</i> genus.	61
4.1	(a) Banding profiles and (b) digitised gel image of <i>Lactobacillus</i> strains generated by ERIC-PCR.	77
4.2	(a) REP-PCR and (b) digitised banding patterns of <i>Lactobacillus</i> strains.	78
4.3	(a) BOX-PCR and (b) digitised banding profiles of <i>Lactobacillus</i> strains.	79



4.4	(a) DNA fingerprints and (b) digitised fingerprints of <i>Lactobacillus</i> strains generated by primer (GTG) ₅ .	80
4.5	Dendrogram generated from cluster analysis of the BOX-PCR fingerprints of <i>Lactobacillus</i> strains.	83
4.6	Dendrogram derived from numerical analysis of (GTG) ₅ -PCR profiles of <i>Lactobacillus</i> strains.	84
4.7	Dendrogram representing genetic relationships among <i>Lactobacillus</i> strains based on REP-PCR fingerprints.	85
4.8	ERIC-PCR generated dendrogram of 17 <i>Lactobacillus</i> strains.	86
4.9	Dendrogram based on the numerical analysis of composite data of rep-PCR.	87
4.10	The 16S rRNA gene of the 17 <i>Lactobacillus</i> strains amplified using primer LG (F) and LR (R).	91
4.11	(a) Agarose gel electrophoresis of ARDRA profiles and (b) digitised gel image of the 17 <i>Lactobacillus</i> strains generated using AluI restriction enzyme.	93
4.12	(a) ARDRA and (b) digitised patterns of the 17 <i>Lactobacillus</i> strains generated using HaeIII restriction enzyme.	94
4.13	(a) Restriction profiles of 16S rRNA gene and (b) digitised restriction profiles of the 17 <i>Lactobacillus</i> strains generated by Sau3AI restriction enzyme.	95
4.14	(a) ARDRA patterns and (b) digitised gel image of the 17 <i>Lactobacillus</i> strains generated by TaqI restriction enzyme.	96
4.15	Dendrogram constructed based on the UPGMA clustering with Dice coefficient (expressed as a percentage value) of ARDRA profiles generated with TaqI restriction enzyme.	100
4.16	Dendrogram derived from Sau3AI ARDRA profiles of the 17 <i>Lactobacillus</i> strains.	101
4.17	Dendrogram based on the UPGMA clustering of the Dice similarity coefficients of ARDRA patterns generated with AluI for the 17 <i>Lactobacillus</i> strains.	103
4.18	Dendrogram showing similarity among the 17 <i>Lactobacillus</i> strains based on the numerical analysis of ARDRA patterns generated with HaeIII restriction enzyme.	104



4.19	Dendrogram representing the genetic relationships among the 17 <i>Lactobacillus</i> strains based on the composite analysis of ARDRA patterns from restriction enzymes AluI, HaeIII, Sau3AI and TaqI.	106
5.1	An alignment of the sequences of 16S rRNA gene in <i>Lactobacillus</i> strains.	125
5.2	The sequences of the 16S rRNA gene region of the 12 <i>Lactobacillus</i> strains and reference strains were aligned.	126
5.3	The 16S rRNA gene sequences comparison of <i>Lactobacillus</i> strains.	128
5.4	Multiple sequence alignment of the 16S rRNA gene region of <i>Lactobacillus</i> strains.	129
5.5	The sequences of the 16S rRNA gene of the 12 <i>Lactobacillus</i> strains and the selected reference strains were aligned.	130
5.6	Validation of the specificity of primers Reu (F) and Reu (R) using DNA from 17 <i>Lactobacillus</i> strains.	132
5.7	The specificity of primers Bre (F) and Bre (R) were verified by conventional PCR using DNA of 12 probiotic <i>Lactobacillus</i> strains and 5 type strains.	133
5.8	PCR amplification for the specificity validation of primers Pan (F) and Pan (R) using genomic DNA from <i>Lactobacillus</i> strains.	134
5.9	Validation of the specificity of primers Sal (F) and Sal (R) using DNA of 12 probiotic <i>Lactobacillus</i> strains and 5 type strains.	135
5.10	Validation of the specificity of primers Gal (F) and Gal (R) were carried out using DNA of 12 probiotic <i>Lactobacillus</i> strains, five <i>Lactobacillus</i> type strains representing the five species of the 12 <i>Lactobacillus</i> strains and two closely related <i>Lactobacillus</i> strains.	136
5.11	16S rRNA gene copy number of <i>L. salivarius</i> I 24.	137
5.12	Southern hybridisation of <i>L. panis</i> C 17 genomic DNA with biotinylated 16S rRNA gene probe.	138
5.13	Southern hybridisation with genomic DNA of <i>L. gallinarum</i> (a) I 16 and (b) I 26.	139
5.14	Representative Southern hybridisation blot of <i>L. brevis</i> strain.	141
5.15	Southern blot analysis of the 16S rRNA gene of (a) <i>L. reuteri</i> C 1, (b) C 16 and (c) C 10.	142

5.16	Amplification plots obtained from real-time PCR assay of <i>Lactobacillus</i> strains with primers targeted to (a) <i>L. brevis</i> and (b) <i>L. reuteri</i> .	143
5.17	Amplification plots generated from the amplification of <i>Lactobacillus</i> strains with primers specific to (a) <i>L. panis</i> and (b) <i>L. salivarius</i> .	143
5.18	Amplification plot of <i>Lactobacillus</i> strains generated with primers specific to <i>L. gallinarum</i> .	144
5.19	Dissociation curves for the 12 <i>Lactobacillus</i> strains using primers targeted to (a) <i>L. brevis</i> and (b) <i>L. reuteri</i> .	146
5.20	Dissociation curve analysis of <i>Lactobacillus</i> strains using primers specific to (a) <i>L. gallinarum</i> and (b) <i>L. salivarius</i> .	147
5.21	Dissociation curve generated from amplification of <i>Lactobacillus</i> strains using primers Pan (F) and Pan (R).	148
5.22	Standard curves generated from serial dilutions of plasmids containing the 16S rRNA genes of (a) <i>L. brevis</i> and (b) <i>L. reuteri</i> ranging from 10^3 to 10^8 copies.	150
5.23	Standard curves of real-time PCR obtained from serial dilutions of plasmids containing the 16S rRNA genes of (a) <i>L. gallinarum</i> and (b) <i>L. salivarius</i> ranging from 10^3 to 10^8 copies.	151
5.24	Standard curve of real-time PCR for <i>L. panis</i> generated from serial dilutions of plasmid containing the 16S rRNA gene ranging from 10^3 to 10^8 copies.	152
5.25	Quantification of (a) <i>L. reuteri</i> and (b) <i>L. panis</i> using real-time quantitative PCR and the conventional culture method.	153
5.26	Enumeration of (a) <i>L. salivarius</i> and (b) <i>L. gallinarum</i> with real-time quantitative PCR and culture method.	154
5.27	Quantitative analysis of <i>L. brevis</i> with real-time quantitative PCR and culture method.	155

LIST OF ABBREVIATIONS

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
<i>D</i>	- 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 <i>in situ</i> hybridisation
FRET	- fluorescence resonance energy transfer
G	- guanine
<i>g</i>	- gravity
g	- gram
mg	- milligram
μ g	- microgram
HCl	- hydrochloric acid
ISR	- Intergenic spacer region
LB	- Luria-Bertani
M	- molar / molarity
mM	- millimolar
μ M	- micromolar
ml	- millilitre
MEGA	- Molecular Evolutionary Genetic Analysis
MRS	- de Man, Rogosa and Sharpe
NTC	- no-template control
μ l	- 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
<i>rrn</i>	- ribosomal ribonucleic acid operons
<i>rrnDB</i>	- 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
ΔR_n	- 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 microorganism 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