UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION AND EXPLOITATION OF LACTOBACILLUS STRAINS AS POTENTIAL PROBIOTICS FOR HUMANS AND CHICKENS

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IB 2013 41

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By

PARISA SHOKRYAZDAN

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

August 2013

DEDICATION

This thesis is dedicated to my husband and my parents for their love, endless support and encouragement

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

CHARACTERIZATION AND EXPLOITATION OF *LACTOBACILLUS* STRAINS AS POTENTIAL PROBIOTICS FOR HUMANS AND CHICKENS

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Chairman: Professor Ho Yin Wan, PhD

Institute: Bioscience

Among the lactic acid bacteria, lactobacilli are the most common microorganisms used as probiotics. Many different strains of *Lactobacillus* have been reported to be suitable probiotics for different hosts and new strains are being discovered as potential candidates. It has been suggested that there are beneficial lactic acid bacterial strains in human milk and feces, fermented foods and chicken intestines which can be used as probiotics. Thus, this study was conducted to isolate, identify and characterize some new lactic acid bacterial strains from the above-mentioned sources with a view to exploit them as potential probiotics for humans and chickens.

One hundred and eighty-two lactic acid bacterial strains were isolated and 12 strains which showed bile and acid tolerance (growth measured as cell turbidity) were selected and identified using carbohydrate fermentation patterns and 16S rRNA gene sequences. All 12 strains belonged to the genus *Lactobacillus*, of which three strains were *L. buchneri*, three were *L. casei*, one was *L. acidophilus*, two were *L. fermentum* and three were *L. salivarius*.

The 12 *Lactobacillus* strains and two commercial reference strains, *L. casei* Shirota and *L. reuteri* C10, were tested for their ability to survive in the gastrointestinal tract using the following characteristics: tolerance to acid, bile salt and pancreatic enzyme (growth measured as viable counts of colony forming units [CFU]/ml) and adherence to intestinal cell line. The results showed that all 12 *Lactobacillus* strains were able to tolerate acid, bile and pancreatic enzyme. All 12 *Lactobacillus* strains also exhibited good adherence to the intestinal cell line (10.3 to 37.7 *Lactobacillus* cells per Caco-2 cell).

In vitro assays on their bioactivities showed that all 12 *Lactobacillus* strains exhibited good antagonistic activity against a wide range of pathogens and most of the strains had significantly higher inhibitory effect against the pathogens than the two commercial reference strains. The antagonistic activity was due to production of organic acids. All strains also showed cholesterol-reducing activity, good antioxidant activity and production of useful enzymes. The cholesterol-reducing and antioxidant activities of many of the strains were significantly higher than those of the two commercial reference strains. Only three *L. salivarius* strains which were isolated from chicken intestines, and *L. acidophilus* HM1 isolated from human milk showed moderate to high BSH activity. None of the *Lactobacillus* strains produced harmful enzymes or biogenic amines. Three *Lactobacillus* strains (*L. acidophilus* HM1, *L. fermentum* HM3 and *L. buchneri* FD2) selected for their good adherence ability (33.5, 37.7 and 35.9 *Lactobacillus* cells per Caco-2 cell, respectively), and the commercial reference strain *L. casei* Shirota (19.7 *Lactobacillus* cells per Caco-2 cell) were investigated for their cytotoxic activity against three cancer cell lines and a normal cell line. The results showed that the three *Lactobacillus* strains showed selectivity in killing cancer cells when compared to the normal cells. However, the commercial reference strain, *L. casei* Shirota, did not show selectivity toward the cancer or normal cells.

The efficacy of a mixture of three *L. salivarius* strains isolated from chicken intestines, which showed good probiotic traits, was evaluated in broiler chickens. The results revealed that the mixture of *Lactobacillus* strains significantly improved the body weight, body weight gain and feed conversion ratio, increased beneficial cecal bacteria such as lactobacilli and bifidobacteria, decreased harmful cecal bacteria such as *E. coli* and total aerobes, decreased serum total cholesterol, low density lipoprotein-cholesterol and triglycerides, reduced harmful cecal bacterial enzymes, β-glucosidase and β-glucuronidase, and improved the histomorphology of the gut by increasing villus heights and villus height:crypt depth ratio of broiler chickens.

Two *Lactobacillus* strains (*L. fermentum* HM3 and *L. buchneri* FD2 isolated from human milk and fermented dates, respectively), with very good probiotic characteristics were selected as potential probiotic for humans and a safety assessment of them was carried out using acute and subacute oral toxicity tests in Sprague-Dawley rats. Results showed that both strains were safe and even at a high concentration of 10^{10} CFU/kg BW/day there were no observed adverse effects on growth, feed consumption, cellular blood components, serum biochemistry and vital organs of the animals fed *Lactobacillus* strains. The two *Lactobacillus* strains also increased beneficial cecal bacterial populations, and decreased pathogenic bacterial populations and harmful intestinal bacterial enzymes.

In conclusion, all 12 *Lactobacillus* strains isolated in the present study showed good probiotic characteristics and bioactivities *in vitro*. *In vivo* study of three *L. salivarius* strains in broiler chickens indicated that they improved performance and well-being of chickens, and could be good probiotics for chickens, while toxicity tests on *L. fermentum* HM3 and *L. buchneri* FD2 in rats showed that they are safe, and could be further investigated and exploited as probiotics for humans.

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

PENCIRIAN DAN EKSPLOITASI STRAIN *LACTOBACILLUS* SEBAGAI PROBIOTIK BERPOTENSI UNTUK MANUSIA DAN AYAM PEDAGING

Oleh

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Diantara laktik asid bakteria, lactobacilli adalah mikroorganisma yang biasa digunakan sebagai probiotik. Pelbagai strain *Lactobacillus* telah dilaporkan sebagai probiotik yang sesuai untuk perumah yang berbeza dan juga strain-strain baru sedang diterokai sebagai calon-calon yang berpotensi. Ada beberapa cadangan bahawa terdapat strain laktik asid bakteria yang bermanfaat dari sumber susu manusia dan najis, makanan ditapai dan usus haiwan yang boleh digunakan sebagai probiotik. Oleh itu, kajian ini dijalankan untuk mengasingkan, mengenal pasti dan mencirikan beberapa laktik asid bakteria dari sumber yang disebut di atas dengan tujuan untuk mengeksploitasi potensi makanan tambahan probiotik untuk manusia dan ayam.

Seratus lapan puluh dua isolat laktik asid bakteria telah diisolat dan 12 strain yang menunjukkan toleransi asid dan hempedu (pertumbuhan diukur sebagai kekeruhan sel) telah dipilih dan dikenal pasti menggunakan corak penapaian karbohidrat dan 16S rRNA urutan gen. Kesemua 12 jenis strain telah dikenalpasti sebagai genus *Lactobacillus*, di mana tiga adalah jenis strain *L. buchneri*,

tiga lagi strain *L. casei*, satu strain *L. Acidophilus*, dua strain *L. fermentum* dan tiga strain *L. Salivarius*.

12 *Lactobacillus* strain dan dua strain rujukan komersial, *L. casei* Shirota dan *L. reuteri* C10, telah diuji keupayaanya untuk terus hidup dalam saluran gastrousus dengan menggunakan ciri-ciri ujian berikut: toleransi kepada asid, garam hempedu dan enzim pankreas (pertumbuhan diukur dari segi kiraan koloni hidup/ml (CFU/ml) dan daya lekatan pada sel usus. Hasil kajian menunjukkan bahawa kesemua 12 jenis *Lactobacillus* strain dapat bertolak ansur dengan asid, hempedu dan pankreas enzim. Semua 12 *Lactobacillus* jenis juga menunjukkan daya lekatan yang baik pada sel usus (10.3 to 37.7 *Lactobacillus* sel per Caco-2 sel).

Hasil kajian bioaktiviti menunjukkan bahawa kesemua 12 *Lactobacillus* strain menunjukkan aktiviti antagonistik yang berkesan terhadap pelbagai patogen dan kebanyakannya mempunyai kesan bersignifikan lebih tinggi terhadap patogen daripada dua jenis rujukan komersial. Aktiviti antagonistik disebabkan oleh pengeluaran asid organik. Semua jenis strain juga menunjukkan aktiviti penurunan paras kolesterol, aktiviti antioksida yang baik dan pengeluaran enzim yang berguna. Penurunan paras kolesterol dan antioksida aktiviti kebanyakan jenis strain adalah bersignifikan berbanding dengan kedua-dua jenis strain rujukan komersial. Hanya tiga jenis *L. salivarius* yang telah diisolat daripada usus ayam, dan *L. acidophilus* HM1 diisolat daripada susu manusia menunjukkan aktiviti BSH dari sederhana hingga tinggi. Tiada strain *Lactobacillus* yang menghasilkan enzim berbahaya atau amina biogenik. Tiga jenis *Lactobacillus* (*L. acidophilus* HM1, *L. fermentum* HM3 dan *L. buchneri* FD2) yang dipilih berdasarkan keupayaan daya lekatan yang baik (33.5, 37.7 and 35.9 *Lactobacillus* sel per Caco-2 sel), dan strain rujukan komersial *L. casei* Shirota telah dikaji untuk aktiviti sitotoksik mereka terhadap tiga sel kanser dan sel normal. Hasil kajian menunjukkan bahawa tiga jenis strain *Lactobacillus* menunjukkan pemilihan dalam membunuh sel-sel kanser berbanding dengan sel-sel normal. Walau bagaimanapun, strain rujukan komersial, *L. casei* Shirota, tidak menunjukkan pemilihan ke arah kanser atau sel-sel normal.

Keberkesanan campuran tiga jenis strain *L. salivarius* yang di asingkan daripada usus ayam, yang menunjukkan sifat-sifat probiotik yang baik, telah dinilai dalam ayam daging. Keputusan menunjukkan bahawa campuran strain *Lactobacillus* meningkatkan berat badan (BW), kadar pertumbuhan berat badan dan nisbah penukaran makanan, peningkatan bakteria sekum berfaedah seperti lactobacilli dan bifidobakteria, menurun bakteria sekum berbahaya seperti *E. coli* dan jumlah bakteria aerob, penurunan jumlah kolesterol serum, ketumpatan rendah lipoprotein kolesterol dan trigliserida, mengurangkan enzim berbahaya bakteria sekum, β-glucosidase dan β-glucuronidase, dan meningkatkan kualiti histomorphologi usus oleh tahap villus yang semakin meningkat dan ketinggian villus:nisbah crypt depth ayam daging.

Dua jenis strain *Lactobacillus* (*L. fermentum* HM3 dan *L. buchneri* FD2 diisolat daripada susu manusia dan kurma ditapai masing-masing) dengan ciri-ciri probiotik yang sangat baik telah dipilih sebagai potensi probiotik untuk manusia dan penilaian keselamatan mereka telah dijalankan dengan menggunakan akut dan subakut ujian ketoksikan oral pada tikus Sprague-Dawley. Keputusan ujian menunjukkan bahawa kedua-dua jenis strain adalah selamat diguna walaupun pada kepekatan yang tinggi 10^{10} CFU / kg BW/hari dimana tiada kesan buruk diperhatikan kepada pertumbuhan, pengambilan makanan, komponen darah selular, serum biokimia dan organ-organ penting dalam haiwan yang diberi makanan tambahan yang mengandungi jenis strain *Lactobacillus*. Kedua-dua jenis strain *Lactobacillus* juga meningkatkan populasi bakteria sekum yang bermanfaat, dan menurunkan populasi bakteria berpatogenik dan enzim berbahaya bakteria usus.

Kesimpulannya, kesemua 12 jenis strain *Lactobacillus* diasingkan dalam kajian ini menunjukkan ciri-ciri dan bioaktiviti yang baik dalam kajian secara *in vitro*. Dalam kajian secara *in vivo*, tiga jenis strain *L. salivarius* dalam ayam daging menunjukkan bahawa mereka meningkatkan prestasi dan kesihatan ayam, dan berpotensi untuk dijadikan sebagai probiotik ayam, manakala ujian ketoksikan *L. fermentum* HM3 dan *L. buchneri* FD2 pada tikus menunjukkan bahawa kedua-dua strain bukan toksik,boleh dikaji lagi dan dieksploitasi sebagai probiotik untuk manusia.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my utmost gratitude to the chairman of the supervisory committee, Professor Dr. Ho Yin Wan, for being so approachable, and for her invaluable advice, guidance, support, encouragement, tolerance and patience throughout the course of my study. I am really grateful to have been given this opportunity to pursue my study under her supervision.

I would also like to express my deepest thanks to Associate Professor Dr. Kalavathy Ramasamy, Associate Professor Dr. Liang Juan Boo and Associate Professor Dr. Sieo Chin Chin who are members of the supervisory committee for their advice, invaluable guidance, hospitality, support and encouragement throughout the duration of my study.

I would like to thank Associate Professor Dr. Noorjahan Banu Mohamed Alitheen for her help, support and guidance in the cell culture, and Profesor Dato' Dr. Tengku Azmi Tengku Ibrahim for his help, guidance and assistance in the histopathology of rats.

My appreciations are extended to Mr. Khairul Kamar Bakri, staff of the Probiotic and Prebiotic Programme, Laboratory of Vaccine and Immunotherapeutics, Institute of Bioscience, for his technical support and kind assistance.

Thanks are also due to all my labmates, who made me feel welcome in Malaysia, and revealed to me a side of South East Asia that I could never have experienced on my own.

Last but not least, very special thanks to my loving husband, Mohammad, for his endless love, strong support, constant encouragement, infinite patience and understanding, and kind assistance without which this study would be much harder to pursue and accomplish.

I certify that a Thesis Examination Committee has met on 29 August 2013 to conduct the final examination of Parisa Shokryazdan on her thesis entitled "Characterization and Exploitation of *Lactobacillus* Strains as Potential Probiotics for Humans and Chickens" 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 submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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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 or concurrently submitted for any other degree at Universiti Putra Malaysia or other institutions.

PARISA SHOKRYAZDAN

Date: 29 August 2013

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ABTS	2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
AACC	American Association for Clinical Chemistry
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BSH	Bile Salt Hydrolase
Ca	Calcium
CFU	Colony Forming Units
Cl	Chloride
Cq	Quantification cycle
Creat	Creatinine
CVD	Cardiovascular diseases
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPPH	2,2-Diphenyl-1-picrylhydrazyl
E	Amplification efficiency
EDTA	Ethylene Diamine Tetraacetic Acid
Eosin.	Eosinophils
FAO	Food and Agriculture Organization
FBS	Fetal Bovine Serum
FID	Flame Ionization Detector
fL	Femtoliters

FRAP	Ferric Reducing Ability of Plasma
GC	Gas Chromatograph
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GDCA	Glycodeoxycholic acid
GIA	Global Industry Analysts
GIT	Gastrointestinal Tract
Glu.	Glucose
GRAS	Generally Recognized As Safe
HB	Hemoglobin
HDL	High Density Lipoprotein
HPLC	High Performance Liquid Chromatography
ISAPP	International Scientific Association for Probiotics and Prebiotics
K	Potassium
LB	Luria-bertani
LDL	Low Density Lipoprotein
Lymp.	Lymphocytes
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
Mono.	Monocytes
MRS	De Man, Rogosa and Sharpe
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na	Sodium
NCBI	National Center for Biotechnology Information
Neut.	Neutrophils
NRC	National Research Council
NTC	No-template control
OCDE	Organisation de Coopération et de Développement Economiques
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development
OPA	O-phthalaldehyde

P	Phosphorus
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
QPS	Qualified Presumption of Safety
RBC	Red Blood Cell Count
RFU	Relative Fluorescence Units
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal ribonucleic acid
SAS	Statistical Analysis System
T. bil.	Total bilirubin
T. chol.	Total cholesterol
T. prot.	Total protein
TBA	Thiobarbituric acid
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TDCA	Taurodeoxycholic acid
TEA	Tris-acetate EDTA
TEAC	Trolox Equivalent Antioxidant Capacity
Thrombo.	Thrombocytes
TLCA	Taurolithocholic acid
TPTZ	2,4,6-Tri (2-pyridyl)-1,3,5-triazine
Trig.	Triglycerides
URI	Genomics and Sequencing Center of University of Rhode Island
VFA	Volatile Fatty Acids
WBC	White Blood Cell Count
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

More than two thousand years ago, Hippocrates said: "Let food be thy medicine and medicine be thy food". This quote is a tenet for many people today. As nowadays people are more conscious and aware about the role of diet in their health, interest and demand for "healthy" foods have increased considerably (Suvarna and Boby, 2005). Hence, the food industry is expected to provide a healthcare market by offering functional foods, or foods that promote health beyond providing basic nutrition. A rapidly expanding portion of this market belongs to probiotics and prebiotics. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001), and prebiotics are "non-digestible food ingredients that beneficially affect the host by stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve host health" (Gibson and Roberfroid, 1995). According to the Global Industrial Analysis (GIA, 2012), the probiotic market alone is predicted to be worth about US $ 29 billion in 2015.

In addition to the food industry for human, feed industry for livestock, especially poultry, is another sector for the probiotic market. For over 50 years, antibiotic growth promoters were commonly used at subtherapeutical levels to promote growth and feed efficiency in poultry (Kabir, 2009). However, in recent years, increasing concern on the rampant use of antibiotics, which can lead to development of antibiotic-resistant bacteria and antibiotic residues in animal products has led to restriction or total ban of antibiotics as growth promoters in animal production (Patterson and Burkholder, 2003). As a result, the concept of using probiotics as a possible alternative to antibiotic growth promoters has attracted considerable interest (Kabir, 2009). That is because probiotics have the potential to reduce enteric diseases and enhance productivity of poultry. Moreover, with rising levels of healthcare awareness, the demand for drug-free animal meat and products is set to increase.

Bifidobacteria and lactic acid bacteria, especially *Lactobacillus*, are the most common types of microorganisms used as probiotics in the food and feed industries (Conway, 1996; Vankerckhoven *et al.*, 2008). That is because lactic acid bacteria and bifidobacteria have good functional effects as probiotics and they are regarded as friendly bacteria and "Generally Recognized As Safe (GRAS)" (Collins *et al.*, 1998). Although many strains of lactic acid bacteria have been found to have probiotic characteristics and have been developed as suitable probiotic supplements for different hosts, there is still a continued search for more new probiotic strains with better health promoting properties than existing ones.

Human milk is an important factor in the colonization and development of beneficial bacteria particularly bifidobacteria in the intestine of breast-fed newborns, and it has been suggested that human milk contains bacterial strains with potential to be used as probiotic agents (Martin *et al.*, 2004). Feces of healthy breast-fed infants have also been reported to contain high numbers of lactic acid bacteria (Hopkins *et al.*, 2005). In addition, many studies have shown that fermented foods contain potential probiotic strains. (Heller, 2001; Muyanja *et al.*, 2003; Leroy and De-Vuyst, 2004; Mathara *et al.*, 2008). It has been recommended that bacterial strain to be used as probiotic for animals should be isolated from the gastrointestinal tract (GIT) of the same type of animal in order to have more specific application for the animal (Kizerwetter-Swida and Binek, 2005). For example, the GIT of chickens will be a suitable source for potential probiotic strains for chicken and poultry.

As a result of the growing interest in probiotics, many purported probiotic products have been marketed without proper studies on the probiotic properties of the strains, giving rise to problems of inconsistent efficacy of the products. Several studies have reported misidentification or mislabeling of probiotic species or presence of unspecified species in many commercial probiotic products (Hamilton-Miller and Shah, 1996; Canganella *et al.*, 1997; Klein *et al.*, 1998; Hamilton-Miller *et al.*, 1999; Schillinger, 1999). Since the properties of probiotic are strain specific, the quality of products is closely linked to the individual strains in the products, thus, they should be correctly identified, and their probiotic properties properly studied. In 2001, FAO/WHO produced a set of guidelines for the evaluation of probiotics in food in which they recommended that every potential probiotic strain must be correctly identified, followed by various *in vitro* assays to investigate its functional properties and *in vivo* trials for its safety. This is because probiotic properties are strain specific and cannot be extrapolated to the whole genus or species.

Thus, the present study was initiated to isolate, identify and characterize some new lactic acid bacterial strains from human milk, infant feces, fermented grapes and dates and chicken intestine with a view to exploit them as potential probiotic supplements for humans and chickens. *In vitro* studies on the bioactivities and good probiotic properties of the isolated bacterial strains were first carried out for selection of potential probiotic candidates, followed by *in vivo* studies using animal models to evaluate their safety and efficacy.

The specific objectives of this study were:

i) To isolate some new lactic acid bacterial strains from human milk, infant feces, fermented grapes and dates and chicken intestines
ii) To identify the isolated bacterial strains using morphological, biochemical and molecular characteristics
iii) To assess (*in vitro*) the characteristics associated with survival of the isolated bacterial strains in the GIT
iv) To investigate (*in vitro*) the bioactivities of the isolated bacterial strains
v) To evaluate the efficacy of selected probiotic strains intended for chickens using a broiler chicken trial
vi) To study the safety of selected probiotic strains intended for humans using a rat model

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LIST OF PUBLICATIONS

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1. Shokryazdan, P., R. Kalavathy, C.C. Sieo, N. B. Alitheen, J. B. Liang, M. F. Jahromi and Y. W. Ho. Isolation and characterization of *Lactobacillus* strains as potential probiotics for chickens. *Pertanika Journal of Tropical Agricultural Science*. In press.

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2. Shokryazdan, P., C.C. Sieo, R. Kalavathy, J. B. Liang, M.F. Jahromi and Y. W. Ho. Antimicrobial activity of *Lactobacillus salivarius* isolated from chicken. The 3rd International Conference on Sustainable Animal Agriculture for Developing Countries (SAADC 2011), July 26-29, 2011. Nakhon Ratchasima, Thailand. P:258.

3. Shokryazdan, P., C.C. Sieo, R. Kalavathy, J.B. Liang, M.F. Jahromi, and Y. W. Ho. Bile Salt Hydrolase Activity of *Lactobacillus* Strains Isolated From Chicken Intestine. The 14th Animal Science Congress of the Asian-Australasian Association of Animal Production Societies (AAAP 2010), August 23-27, 2010. Pingtung, Taiwan. Vol:2, P:309.