UNIVERSITI PUTRA MALAYSIA

EFFECTS OF LACTOBACILLUS STRAINS AS A PROBIOTIC AND A HYPOLIPIDAEMIC AGENT FOR CHICKENS

KALAVATHY RAMASAMY

EFFECTS OF *LACTOBACILLUS* STRAINS AS A PROBIOTIC AND A HYPOLIPIDAEMIC AGENT FOR CHICKENS

By

KALAVATHY RAMASAMY

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

August 2003

Abstract of the thesis submitted to the senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

EFFECTS OF *LACTOBACILLUS* STRAINS AS A PROBIOTIC AND A HYPOLIPIDAEMIC AGENT FOR CHICKENS

By

KALAVATHY RAMASAMY

August 2003

Chairman : Professor Dr. Ho Yin Wan
Institute : Bioscience

In recent years, there has been considerable interest in the beneficial effects of probiotics (direct-fed microbials, which include *Lactobacillus*) to modulate the lipid metabolism. However, the mechanism(s) involved remains unclear. A series of experiments was carried out to investigate the ability of 12 *Lactobacillus* strains to deconjugate bile salts and to remove cholesterol *in vitro*, and to assess their potential as a probiotic and as a hypolipidaemic agent for broilers and laying hens. Bile salt hydrolase (BSH) activity (resulting in bile salt deconjugation) of intestinal bacteria is closely linked to the lowering of cholesterol. The results of the *in vitro* studies showed that all the 12 *Lactobacillus* strains could deconjugate sodium glychocholate (GCA) and sodium taurocholate (TCA) bile salts, and all the strains, except *L. fermentum* I 24, had a higher affinity for GCA. However, only eight strains could deconjugate sodium taurodeoxycholate (TDCA). This indicates that the BSH of the *Lactobacillus* strains is substrate specific. The 12 *Lactobacillus* strains showed significant differences in their ability to reduce cholesterol from the growth medium (27 to 85 %) with or without bile salt, indicating that bile salt is not a prerequisite for the removal of cholesterol. *Lactobacillus acidophilus* I 16, *L. crispatus* I 12, *L.*

brevis C 17 and I 211, and *L. fermentum* I 24 and I 25 removed cholesterol from the growth medium mainly through assimilation of cholesterol into the cells. On the other hand, *L. brevis* C 1, C 10, I 23 and I 218, and *L. fermentum* C 16 removed cholesterol through both assimilation and co-precipitation of deconjugated bile salt with cholesterol at low pH. The *Lactobacillus* strains assimilated more esterified than non-esterified cholesterol and the assimilated cholesterol was tightly bound to the cells. Cells grown in the presence of cholesterol were more resistant to lysis by sonication than when grown in its absence, suggesting a possible alteration of the cell wall or membrane by the assimilated cholesterol. Cholesterol removal by the *Lactobacillus* strains was also affected by Tween 80.

The feeding trials showed that the supplementation of a mixture of the 12 *Lactobacillus* cultures (LC), as a probiotic for broilers, significantly improved growth equivalent to that provided by the antibiotic, oxytetracycline, but the feed conversion ratio was better in LC-fed broilers. The supplementation of LC also significantly lowered the total cholesterol, low density lipoprotein cholesterol and triglycerides of the serum; the cholesterol of the carcass and liver; abdominal fat deposition; and fat contents of the liver, muscle and carcass of broilers; but there was little effect on the fatty acid compositions of the liver, muscle and carcass.

In laying hens, the supplementation of LC improved the feed efficiency and hen-day egg production during the early stage of the laying cycle, and increased egg weight and influenced a shift from small and medium to large and extra large eggs throughout the laying cycle. However, LC had very little effect on improving the fatty acid composition, and the cholesterol and total fat contents of eggs.

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

KESAN PELBAGAI STRAIN *LACTOBACILLUS* SEBAGAI PROBIOTIK DAN AGEN HIPOLIPIDIMIK UNTUK AYAM

Oleh

KALAVATHY RAMASAMY

Ogos 2003

Pengerusi : Profesor Dr. Ho Yin Wan
Institut : Biosains

Sejak kebelakangan ini, kecenderungan untuk menggunakan probiotik (mikrob makanan, termasuk *Lactobacillus*) dalam mengawal atur metabolisma lipid semakin berkembang. Namun demikian, mekanisma yang terlibat masih tidak jelas. Satu siri eksperimen telah dijalankan untuk mengkaji keupayaan 12 strain *Lactobacillus* untuk melakukan dikonjugasi garam hempedu (garam konjugat) dan mengurangkan kolesterol secara *in vitro*, serta kesannya sebagai probiotik dan agen hypolipidimik terhadap ayam pedaging dan ayam penelur. Aktiviti enzim "bile salt hydrolase (BSH)" (yang menyebabkan dikonjugasi garam hempedu) usus berkait rapat dengan pengurangan kolesterol. Hasil kajian *in vitro* menunjukkan bahawa kesemua 12 strain *Lactobacillus* berupaya melakukan dikonjugasi garam "glychocholate" (GCA) dan garam "taurocholate" (TCA), dan kesemua strain, kecuali *L. fermentum* I 24, menunjukkan afiniti yang lebih tinggi terhadap GCA. Tetapi hanya lapan strain berupaya melakukan dikonjugasi garam "taurodeoxycholate". Ini menunjukkan bahawa aktiviti BSH *Lactobacillus* adalah spesifik substrat. Duabelas strain *Lactobacillus* ini juga menunjukkan keupayaan untuk mengurangkan kolesterol dari media kultur (25 hingga 85 %) yang ada atau tiada garam hempedu. Pengurangan kolesterol dari media kultur oleh *L. acidophilus*

I 16, *L. crispatus* I 12, *L. brevis* C 17 dan I 211, dan *L. fermentum* I 24 dan I 25 adalah terutamanya melalui asimilasi kolesterol oleh sel. Pengurangan kolesterol oleh *L. brevis* C 1, C 10, I 23 dan I 218, dan *L. fermentum* C 16 pula, adalah melalui asimilasi dan juga ko-mendakan garam hempedu tak berkonjugat bersama kolesterol pada pH yang rendah. Strain *Lactobacillus* mengasimilasi lebih banyak kolesterol ester berbanding dengan kolesterol bebas dan kolesterol yang diasimilasi didapati terikat dengan kuat pada sel. Sel yang ditumbuhkan bersama kolesterol juga lebih resistan kepada sonikasi, mencadangkan bahawa pengubahsuaian pada dinding atau membran sel berlaku setelah mengasimilasi kolesterol. Pengurangan kolesterol oleh strain *Lactobacillus* juga bergantung pada Tween 80.

Hasil kajian *in vivo* menunjukkan bahawa campuran 12 strain *Lactobacillus* (LC), sebagai probiotik pada ayam pedaging dapat meningkatkan berat badan sama seperti antibiotik "oxytetracycline", tetapi kadar penukaran makanan ayam adalah lebih baik pada ayam yang di beri LC. Penambahan LC pada ayam juga dapat menurunkan paras "total" kolesterol, "low density lipoprotein" kolesterol dan trigliserida di serum; kandungan kolesterol pada karkas dan hati; lemak berlebihan pada bahagian abdomen; dan kandungan lemak pada hati, otot dan karkas; tetapi tidak berupaya mengubah profil asid lemak pada hati, otot dan karkas.

Ayam penelur yang di beri LC dapat meningkatkan kadar penukaran makanan dan produksi telur pada peringkat awal peneluran serta dapat menghasilkan telur yang lebih berat dan saiz yang lebih besar sepanjang proses peneluran. Namun demikian, LC kurang berkesan untuk mengubah profil asid lemak, atau menurunkan paras kolesterol dan lemak di telur.

ACKNOWLEDGEMENTS

I wish to express my deep appreciation and most sincere gratitude to the chairman of the supervisory committee, Professor Dr. Ho Yin Wan, for her invaluable guidance and advice, endless support, patience, and encouragement throughout the duration of this study and for her critical analysis, constructive criticism and helpful suggestions during the preparation of my thesis.

I am deeply grateful and indebted to Associate Professor Dr. Norhani Abdullah and Dr. Clemente Michael Wong, who are members of the supervisory committee, for their kind assistance, advice and guidance throughout the course of my work and in the preparation of the thesis.

Special appreciation goes to Tan Sri Dato Dr. Syed Jalaludin Syed Salim (who was a member of the supervisory committee till his retirement in 2001) for his wise counsel, support and constant encouragement.

My heartfelt appreciations are extended to Madam Haw Ah Kam, Mr. Khairul Kamar Bakri, Mr. Nagayah Muniandy, Mr. Jivanathan Arumugam and Mr. Paimon Lugiman, staff of the Enzyme and Microbial Technology Laboratory, and Mr. Saparin Denim and Mr. Ibrahim Mohsin, staff of Animal Nutrition Laboratory, for their technical support and kind assistance. Thanks are also due to Dr. Goh Yong Meng for his assistance on the preparation of samples for the fatty acid determination using Gas Chromatography.

I wish to extend my sincere thanks to my post graduate friends Chin Chin, Latiffah, Lan, Darlis, Vicky, Wan, Thongsuk, Lee, Pit Kang and Sidieg for their friendship, support, encouragement and their sense of humor that made the many hours in the laboratory very pleasant, which contributed to the successful completion of this work.

Finally, very special thanks are due to my family for their unconditional love, untiring patience, support and encouragement, which inspired and motivated me throughout the course of this study.

I certify that an Examination Committee met on 6th August 2003 to conduct the final examination of Kalavathy Ramasamy on her Doctor of Philosophy thesis entitled "Effects of *Lactobacillus* Strains as a Probiotic and a Hypolipidaemic Agent for Chickens" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

ABDUL RAZAK ALIMON, Ph.D.
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

HO YIN WAN, Ph.D.
Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

NORHANI ABDULLAH, Ph.D.
Associate Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

CLEMENTE MICHAEL WONG, Ph.D.
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

HYUNG TAI SHIN, Ph.D.
Professor
Department of Food and Bioresources
Faculty of Life Science and Technology
Sung Kyun Kwan University
300 Chunchun-Dong, Jangan-Ku
Suwon 440-746, Republic of Korea
(Independent Examiner)

GULAM RUSUL RAHMAT ALI, Ph.D.
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 4 SEP 2003

This thesis submitted to the Senate of 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 are as follows:

HO YIN WAN, Ph.D.
Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Chairman)

NORHANI ABDULLAH, Ph.D.
Associate Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

CLEMENTE MICHAEL WONG, Ph.D.
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

AINI IDERIS, Ph.D.
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 16 SEP 2003

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 UPM or other institutions.

KALAVATHY RAMASAMY

Date: 22/9/03

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAP	Aminoantipyrine
ADP	Adenosine diphosphate
AFTA	Asean Free Trade Centre
AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
BSH	Bile salt hydrolase
CFU	Colony forming unit
cm	centimetre
CP	Cell pellet
d	Day
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organisation
FDA	Food and Drug Administrations
g	gram
GC	Gas Chromatography
GCA	Sodium glychocholate
GRAS	Generally Recognized as Safe
h	hour
H_2O_2	Hydrogen peroxide
HACCP	Hazzard Analysis Critical Control Points
HBA	Hydroxybenzoic acid
HDL	High density lipoprotein
HMG CoA	Hydroxymethylglutaryl coenzyme A
HPLC	High Performance Liquid Chromatography
HU	Haugh unit
IDL	Intermediate density lipoprotein
IU	International Unit
kg	kilogram
KIC	α-ketoisocaproic acid
KOH	Potassium hydroxide
l	litre
LABIP	International Platform for Lactic Acid Bacteria
LC	A mixture of 12 *Lactobacillus* cultures
LDL	Low density lipoprotein
M	Molar
m	metre
mg	milligram
min	minute
MJ	megajoules
mRNA	Messenger Ribonucleic Acid
MRS	Man Rogoso Sharpe
MRSB	MRS containing bile salt
MRSC	MRS containing cholesterol
MRSBC	MRS containing bile salt and cholesterol
MRS-TDCA	MRS agar supplemented with 0.5 % sodium taurodeoxycholate
MUFA	Monounsaturated fatty acids
NaCl	Sodium chloride
NaOH	Sodium hydroxide

ND	No data
OD	Optical density
OTC	Oxytetracycline
PTA	Phototungstic Acid
PPLO	Pleuropneumonia-like organism
PUFA	Polyunsaturated fatty acids
SAS	Stastical Analysis Software
SCFA	Short chain fatty acids
SFA	Saturated fatty acids
ST	Supernatant
TCA	Sodium taurocholate
TDCA	Sodium taurodeoxycholate
tRNA	Transfer Ribonucleic Acid
UFA	Unsaturated fatty acids
μg	microgram
μl	microlitre
VLDL	Very low density lipoprotein
W	Watt
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

The worldwide poultry industry provides a substantial proportion of the nutritional requirement of the human population. Poultry meat is perceived to be lean and low in cholesterol, so it may come as a surprise to learn that poultry scientists and producers are increasingly concerned about the amount of fat present in chicken meat. Chambers *et al.* (1981), Lin (1981) and Havenstein *et al.* (1994) reported that, as a result of selection strategy for body weight gain or growth rate, modern fast-growing broilers have been found to contain about four times higher amounts of abdominal fat than those in the 1960s. Eggs have also been viewed with suspicion today because of their high cholesterol content (Stadelman, 1999). In the US, egg consumption has declined from 256 eggs per capita per year in 1985 to 235 in 1995 (USDA, 1997). The lipid composition of animal products is a primary consumer concern as high fat and cholesterol intakes have been implicated to contribute to coronary heart disease, the most common chronic illness in developed countries. To the poultry producers, on the other hand, excess fat is an economic burden, as fat is lost during processing of the carcass or of the meat, resulting in lower meat yields and, furthermore, the discarded abdominal fat and visceral fat increases waste management problems. This has put the poultry production system under pressure and, therefore, much attention is now directed towards producing healthier meat and eggs such that the lipid fraction is improved (reduced cholesterol and fat and improvement of the fatty acid make-up). Animal feed strategies, genetic selections, and gene manipulation are some of the techniques that have been developed to alter the lipid composition in broilers (Jiménez-Colmenero, 2000) and egg yolk (Hargis, 1988). However, very often these techniques are cost prohibitive or may impair performances and, therefore, not economically feasible to be applied at commercial scale. Animal welfare and environmental issues may also be linked in the application of these techniques.

Performance and economic returns are one of the main concerns of the commercial poultry industry. To achieve these goals, very often, intensive farming systems are adopted, subjecting broilers and laying hens to various stressful situations. Stress may lower the body's defense mechanism and create an imbalance in the intestinal microflora (Fuller, 1999), which in turn increases susceptibility to infectious diseases, resulting in poor performance. Efforts to prevent or reduce avian diseases include improved management practices, but inevitably at a cost, because this requires high quality feed manufacturing and feeding systems where the environment and the feed are relatively pathogen-free (Zhang-Barber *et al*., 1999). The benefits of incorporating antibiotic growth promoters in animal feeds are well substantiated (Bedford, 2000). These products have been used for many years by the poultry industry and have proved to be an effective way of enhancing animal status, uniformity and production efficiency. The Union of Concerned Scientists recently estimated that, each year, 11.2 million kg of antimicrobials are given to animals for non-therapeutic purposes, and 900,000 kg are given for theraphy, thus, it is fair to state that substantial amounts of antimicrobials are administered to food animals for growth promotion and feed efficiency in the absence of known disease (Gorbach, 2001). However, the use of antibiotics as growth promoters is severely restricted or totally banned in poultry production in many countries, largely because of concern on the development of resistant bacterial strains and residual toxicity in