UNIVERSITI PUTRA MALAYSIA

THE ROLE OF BIFIDOBACTERIUM SPP. ON CHOLESTROL ASSIMILATION IN THE IN VITRO AND IN VIVO STUDIES

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By

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Chairman: **Mohd Yazid Abdul Manap, Ph.D**

Faculty: **Food Science and Biotechnology**

The purpose of this study was to investigate the cholesterol removal by some bifidobacterium spp. at *in vitro* and *in vivo* conditions with the emphasis of their bile salt deconjugation ability. Bile salt hydrolase (BSH) activity, which is the measurement of enzyme activity responsible for bile salt deconjugation, was quantified by high pressure liquid chromatography (HPLC) assay. *B. infantis* G001204 was the isolate with the highest deconjugation rate in TPY broth supplemented with 5mM GCDC. Generally all the isolates deconjugated glycoconjugated bile acid in higher amount ($P<0.05$) compared to tauroconjugates. Likewise in overall the percentage of deconjugation activity was higher in TPY medium supplemented with 5mM bile acids ($P<0.05$) compared to the TPY broth with 10mM bile acids. Cholesterol removal from media was strain-dependent. The percentage of cholesterol assimilated in TPY containing 0.52mM cholesterol plus bile acids ranged from 4.0% for *B. infantis* F41134 to 47.0% for *B. infantis* G001204. The presence of bile salt was prerequisite for cholesterol removal.

Results of the *in vivo* experiment showed that total cholesterol concentration in rats fed on the high-cholesterol diet plus either *B. infantis* G001204 or *B. animalis* ATCC 27672 in a 2-week period were significantly ($P<0.05$) lower than the control group. Total fecal bile acid excretion increased in animal groups throughout the high-cholesterol diet feeding and probiotic-treated groups had higher excretion rate of fecal bile acids compared to the control significantly ($P<0.05$).

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

PERANAN *BIFIDOBACTERIUM* SPP. DALAM ASSIMILASI KOLESTROL MELAUI KAJIAN *IN VITRO* AND *IN VIVO*

Oleh

MARYAM REZAEI SABET

Julai 2001

Pengerusi: **Mohd Yazid Abdul Manap, Ph.D**

Fakulti: **Sains Makanan dan Bioteknologi**

Objektif eksperimen ini ialah untuk mengkaji aktiviti menyah-kolesterol oleh spesis Bifidobakteria tertentu pada keadaan *in vitro* dan *in vivo* dengan keutamaan diberi kepada kebolehan mendekonjugasi garam hempedu.. Aktiviti enzim bile salt hydrolase (BSH) yang bertanggungjawab didalam mendekonjugasi garam hempedu telah diukur dengan menggunakan HPLC. *B. infantis* G001204 menunjukkan aktiviti dekonjugasi tertinggi didalam kaldu TPY yang ditambah dengan 5mM GCDC. Secara umumnya, semua isolat mendekonjugasi garam hempedu terkonjugasi pada amaun yang tertinggi ($P<0.05$) jika dibandingkan dengan tauroconjugat dan, peratusan aktiviti dekonjugasi adalah lebih tinggi di dalam medium TPY yang ditambah dengan 5mM asid hempedu ($P<0.05$) jika dibandingkan dengan kaldu TPY yang mengandungi 10mM asid hempedu. Strain yang berlainan mempunyai kapasiti menyah-kolesterol yang berlainan dimana peratusan kolesterol yang diasimilasi di dalam TPY dengan 0.52mM kolesterol dan asid hempedu adalah berjulat antara 4.0% untuk *B. infantis* F41134 dan 47.0% untuk *B.*

infantis G001204. Kehadiran garam hempedu adalah sebagai petunjuk awal kolesterol telah disingkirkan. Keputusan *in vivo* menunjukkan jumlah kepekatan kolesterol pada tikus yang telah diberi makan diet kolesterol yang tinggi bersama-sama dengan *B. infantis* G001204 atau *B. animalis* ATCC 27672 dalam tempoh 2 minggu adalah lebih rendah dari kumpulan kawalan ($P<0.05$). Jumlah penyingkiran asid hempedu melalui najis secara umumnya meningkat bagi semua kumpulan haiwan yang diberi diet berkolesterol tinggi. Walaubagaimanapun, kumpulan haiwan yang diberi diet mengandungi proboitik mempunyai kadar penyingkiran asid hempedu yang ketara jika dibandingkan dengan kawalan ($P<0.05$).

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Finally my deepest regard to my parents, sisters and brother for their continued love and support.

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.

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(MARYAM REZAEI SABET)

Date:

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Plate **Page**

LIST OF ABBREVIATIONS

A	absorbance
ANOVA	analysis of variance
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSH	bile salt hydrolase
C	carbon
Ca	calcium
cfu	colony forming unit
cfu/ml	colony forming unit / milliliter
CHD	coronary heart disease
CV	coefficient of variation
CVD	cardiovascular disease
EHC	entrohepatic circulation
FH	familial hypercholesterolemia
FM	fermented milk
g	gram
g	gravity
g/l	gram/liter
GC	glycocholic acid
GCDC	glycochenodeoxycholic acid
GDC	glycodeoxycholic acid
GIT	gastrointestinal tract
GLM	general linear models
h	hour
H	hydrogen
H_2O_2	hydrogen peroxide
HC + B.a	high-cholesterol diet plus *B. animalis* ATCC 27672
HC + G4	high-cholesterol diet plus *B. infantis* G001204
HC	high-cholesterol diet
HCl	hydrochloric acid
HDL	high density lipoproteins
HMG-CoA	hydroxy methyl glutaryl Coenzyme A
HPLC	high pressure liquid chromatography
Kg	kilogram
LC	*Lactobacillus casei*
LDL	low density lipoproteins
log	logarithm
LP	LC protease-treated preparation

LP80	*Lactobacillus plantarum* 80
LSD	least of significant difference
M	molar
mg/dl	milligram/ deciliter
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
mM/g	millimole/gram
MRS	de Man Rogosa Sharpe medium
Na_2HPO_4	sodium phosphate dibasic
NaCl	sodium chloride
NAD	nicotine amide adenine
NaH_2PO_4	sodium phosphate mono basic
NaOH	sodium hydroxide
nm	nanometer
OD	optical density
OH	hydroxyl group
P	probability
PCBH1	overproducing LP80
pH	power of hydrogen
pKa	ionization constant
SAS	Statistical Analysis System
SD	standard deviation
Spp	species
St + G4	standard commercial diet plus *B. infantis* G001204
St	standard commercial diet
TC	taurocholic acid
TCA	trichloroacetic acid
TCDC	taurochenodeoxycholic acid
TDC	taurodeoxycholic acid
TPY	Trypticase Phytone Yeast extract
TSA	Tryptone Soy Agar
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight
WT	wild type
%	per cent
°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometer
<	less than

CHAPTER I

INTRODUCTION

During the last few decades, numerous epidemiological, laboratory and clinical studies have demonstrated the positive correlation between elevated serum cholesterol levels and increased risk for atherosclerosis and coronary heart disease (CHD), the latter being a major cause of death in Western countries (Jacobson, 2000; Leiter, 2000; Smith, 1997).

Potential hypocholesterolemic food products are continuously being developed in order to control serum cholesterol levels in persons with abnormally high levels. Food is the major source of microorganisms that can improve health or cause illnesses to the human or animal host. The intestinal tract has great importance since most of the nutrients are absorbed through it. The physiological parameters include pH, concentrations of gastric and small intestine enzymes, concentration of bile salts, the kinetics of passage of chyme through the stomach and intestine and bowel movement are perhaps the most important factors for determining the selection of the microorganisms which can remain in the gastrointestinal tract (Binder *et al.* 1975). Among those microorganisms, which can survive conditions of the gastrointestinal tract, is bifidobacteria.

Bifidobacteria is a gram positive bacteria that can be isolated from feces of human at any age. One of the most interesting characteristic of bifidobacteria is its bile salt deconjugation activity that may provide health benefits to human. Whereas bile salt metabolism and cholesterol metabolism are closely linked, any change in intestinal bile salt availability might influence cholesterol absorption. The ingestion of bifidobacteria might effect cholesterol levels through interference with bile salt metabolism via interruption of the enterohepatic circulation. They have ability to hydrolyse bile salts and produced free (deconjugated) bile salts. Free bile salts are more easily precipitated at low pH or with Ca^{2+} (Van der Meer & De Vries, 1985), hence they are excreted more rapidly in the feces. This amount then requires to be newly synthesized from cholesterol to maintain bile salt homeostasis.

To obtain strains of bifidobacteria that may improve the health of humans or animals, it is necessary to isolate microorganisms that can survive the acidic conditions of the stomach, and the high concentration of bile acids in the upper part of digestive tract. Also, bifidobacteria should be able to compete with the other microorganisms of the intestinal microflora.

The specific objectives of this study were: 1) to determine if bifidobacteria can deconjugate both tauro- and glyco-conjugated bile acids, 2) determining if bifidobacteria can remove cholesterol from laboratory media, and 3) if bifidobacteria can reduce serum cholesterol level in animal model fed high-cholesterol diet.

CHAPTER II

LITERATURE REVIEW

Cholesterol

The compound now known as cholesterol was described for the first time in the latter half of the 18th century. Poulletier de la Salle (1769) obtained the cholesterol from the alcohol-soluble part of human gallstones (Dam, 1958). In 1816, Chevreul introduced the designation *cholesterine* from Greek: chole, bile; and steros, solid (Dam, 1958). Cholesterol is doubtless the most publicized lipid in nature, because of the strong correlation between high levels of cholesterol in the blood and the incidence of diseases of the cardiovascular system in human (Lehninger *et al.* 1993). It is a major component of animal plasma membranes and occurs in lesser amounts in the membranes of their subcellular organelles. Its polar OH group gives it a weak amphiphilic character, whereas its fused ring system provides it with greater rigidity than other membrane lipids. Cholesterol is therefore an important determinant of membrane properties. Its also abundant in blood plasma lipoproteins where ~70% of it is esterified to long-chain fatty acids to form cholesteryl esters (Voet & Voet, 1990).

Cholesterol Biosynthesis

Although the cholesterol structure suggests complexity in its biosynthesis, all of its carbon atoms are provided by a single precursor- acetate. The cholesterol biosynthesis occurs in four stages. In stage 1, the three acetate units condense to form a six-carbon intermediate, mevalonate. Stage 2, involves the conversion of mevalonate into activated isoprene units, and stage 3, the polymerization of six 5-carbon isoprene units to form the 30-carbon linear structure of squalene. Finally (stage 4), the cyclization of squalene forms the four rings of the steroid nucleus, and a further series of changes (oxidation, removal or migration of methyl groups) leads to final product, cholesterol (Zubay, 1998).

Cholesterol Metabolism

Cholesterol is the precursor of steroid hormones and bile acids. Quantitatively the most important pathway for the excretion of cholesterol in mammals is the formation of bile acids (also called bile salts). The major bile acids, cholic acid and chenodeoxycholic acid, are synthesized in the liver and secreted as glycine or taurine conjugates into the gallbladder. From there, they are secreted into the small intestine where they act as emulsifying agents in the digestion and absorption of fats and fat-soluble vitamins. An efficient recycling system allows the bile acids to reenter the blood stream and return to the liver for reuse several times each day. Less