UNIVERSITI PUTRA MALAYSIA

EFFECTS OF *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 ON *CLOSTRIDIUM SCINDENS* AND *CLOSTRIDIUM HIRANONIS*

BABAK RASTI

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By

BABAK RASTI

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

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Chairman : Professor Mohd Yazid Abdul Manap, PhD

Faculty : Food Science and Technology

The purpose of this study was to investigate the deoxycholic acid (DCA) reduction by *Bifidobacterium pseudocatenulatum* G4 at *in vitro* conditions with the emphasis of its bile salt deconjugation ability and antagonistic activity against 7α-dehydroxylating bacteria. *B. pseudocatenulatum* G4 showed antagonistic activity against *Clostridium scindens* and *C. hiranonis* at colonic pH (5.7, 6.2 and 6.8), with highest activity (4mm inhibition zone) at pH 5.7 against *C. hiranonis*. Growth rates of *B. pseudocatenulatum* G4 (time required for the initial absorbance at zero time to increase by 0.3 units), decreased in the presence of 2.0% oxgall compared to absence of oxgall. Effect of *B. pseudocatenulatum* G4 on pH reduction was measured; *B. pseudocatenulatum* G4 reduced pH from 6.8 to 3.9 in TPY broth supplemented with 0.1% and pH 3.74 in TPY broth without oxgall (control), compared to pH 5.28 in TPY broth supplemented with

2.0% oxgall. This results showed oxgall exert inhibitory effect on pH reduction by *B. pseudocatenulatum* G4.

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. pseudocatenulatum* G4 gave deconjugation rate (disappearance of conjugated bile acid) in TPY broth supplemented with 0.25 mM (the highest concentration in colon) and 5.0 mM (the highest concentration in small intestine) of all 6 different conjugated bile acids (TCA, GCA, TCDCA, GCDCA, TDCA, and GDCA). Generally *B. pseudocatenulatum* G4 deconjugated glycoconjugated bile acids in higher amount compare to tauroconjugates. The percentage of deconjugation activity was higher in TPY medium supplemented with 0.25mM bile acids compared to TPY broth with 5.0mM bile acids. On other hand, pH 6.2 was observed as the optimum pH for BSH activity. Full factorial statistical design was used to evaluate the effects of three factors (pH, *B. pseudocatenulatum* G4 concentrations and *Clostridiums*) with different levels and interactions between them, on DCA production. Statistical analysis for results of the *in vitro* experiment showed that total DCA concentration produced by *C. scindens* and *C. hiranonis* in the presence of *B. pseudocatenulatum* G4 and low pH, were lower than the control group with highest pH and in the absence of *B. pseudocatenulatum* G4.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia Untuk memenuhi keperluan untuk ijazah Master Sains

EFFECTS OF *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 ON *CLOSTRIDIUM SCINDENS* AND *CLOSTRIDIUM HIRANONIS*

Oleh

BABAK RASTI

June 2009

Pengerusi : Profesor Mohd Yazid Abdul Manap, PhD

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Tujuan kajian ini adalah untuk menyiasat penurunan asid deoksikolik (DCA) oleh *Bifidobacterium pseudocatenulatum* G4 pada keadaan *in-vitro* dengan penekanan terhadap kebolehan mengdekonjugat garam hempedu dan aktiviti antagonistiknya terhadap bakteria 7α-dehidroksilasi. *B. pseudocatenulatum* G4 menunjukkan aktiviti antagonistik terhadap *Clostridium scindens* dan *C. hiranonis* pada pH kolon (5.7, 6.2 dan 6.8), dengan aktiviti tertinggi (zon perencatan 4mm) pada pH 5.7 terhadap *C. hiranonis*. Kadar pertumbuhan *B. pseudocatenulatum* G4 (masa yang diperlukan untuk penyerapan awal pada masa kosong untuk meningkat sebanyak 0.3 unit), menurun dengan kehadiran 2.0% oxgall berbanding dengan ketidakhadiran oxgall. *B. pseudocatenulatum* G4 menurunkan pH daripada 6.8 kepada 3.9 dalam broth TPY yang ditambahkan 0.1% oxgall dan 3.74 dalam broth TPY tanpa oxgall (kawalan). Berbanding dengan pH 5.28

dalam broth TPY yang ditambahkan 2.0% oxgall, keputusan ini menunjukkan oxgall mengeluarkan efek perencatan terhadap penurunan pH oleh *B. pseudocatenulatum* G4.

Aktiviti hidrolase garam hempedu, yang mana pengukuran aktiviti enzim adalah bertanggungjawab kepada dekonjugasi garam hempedu, dikuantitikan dengan asai kromatografi cecair tekanan tinggi (HPLC). *B. pseudocatenulatum* G4 adalah isolat dengan kadar dekonjugasi (asid hempedu konjugat menghilang) yang tinggi dalam broth TPY yang ditambahkan dengan 0.25 mM (kepekatan tertinggi dalam kolon) dan 5.0 mM (kepekatan tertinggi dalam usus kecil) semua jenis asid hempedu. Secara umum, strain ini mengdekonjugasikan asid-asid hempedu glikokonjugat dalam kuantiti yang lebih tinggi berbanding taurokonjugat. Begitu juga dalam peratusan keseluruhan aktiviti dekonjugasi adalah lebih tinggi dalam medium TPY yang ditambahkan dengan 0.25mM asid hempedu berbanding dengan broth TPY dengan 5.0mM asid hempedu. Sementara itu, pH 6.2 diperhatikan sebagai pH optimum untuk aktiviti BSH. Rekabentuk statistic factorial penuh telah digunkan untuk menilai kesan factor-faktor (pH, kepekatan *B. pseudocatenulatum* G4 dan *Clostridium*s) dengan peringkat yang berbeza dan hubungan di antara mereka, atas penghasilan DCA. Keputusan eksperimen ini menunjukkan bahawa jumlah kepekatan DCA yang dihasilkan oleh *C. scindens* dan *C. hiranonis* dalam kehadiran *B. pseudocatenulatum* G4 pada pH rendah adalah lebih rendah daripada kumpulan kawalan pada pH tinggi dengan ketidakhadiran *B. pseudocatenulatum* G4.

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This thesis is dedicated to my loving parents, whose love and support allowed me to complete this education and for their continuing encouragement and support all these years. It is also dedicated to the rest of my family who provided the support that helped me to make it through the tough times.

I certify that a Thesis Examination Committee has met on 19 June 2009 to conduct the final examination of Babak Rasti on his thesis entitled "Effects of *Bifidobacterium pseudocatenulatum* G4 on *Clostridium scindens* and *Clostridium hiranonis*" 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 Master of Science.

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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, and is not concurrently, submitted for any other degree at University Putra Malaysia or at any other institution.

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BABAK RASTI

Date: 15 June 2009

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LIST OF ABBREVIATIONS

%	Percentage
/	Per
<	Less than
µg	Microgram
µL	Microliter
BA	Bile acid
Bai	Bile acid inducible
BCS	Biopharmaceutical classification system
BSEP	Bile salt export pump
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CE	Cholesterol ester
cfu	Colony forming units
CoA	Coenzyme A
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
d	Day
DCA	Deoxycholic acid
DG	Diglyceride

DNA	Deoxyribonucleic acid
e.g.	For example
EC	Enzyme Commission number
ERIC	Enterobacterial repetitive intergenic consensus
et al.	And others
etc.	Et cetera
FBA	Free bile acid
FFA	Free fatty acids
g	Gram
G+C	Guanine-cytosine content
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GDCA	Glycodeoxycholic acid
GI	Gastrointestinal
GIT	Gastrointestinal tract
h	Hour
HCL	Hydrochloric acid
HPLC	High performance liquid chromatography
HSDH	Hydroxysteroid dehydrogenase
i.e.	That is
iNOS	Inducible nitric oxide synthase
K_2HPO_4	Potassium phosphate (dibasic)
kg	Kilogram

KH_2PO_4	Potassium phosphate (monobasic)
L	Liter
LAB	Lactic acid bacteria
LCA	Lithocholic acid
L-NAME	N-nitro-L-arginine methyl ester
$\log_{10}$	Logarithm to the base 10
LOX	Lipoxygenase
mg	Milligram
MG	Monoglycerides
min	Minute
mL	Milliliter
MMC	Migrating motor complex
mRNA	Messenger ribonucleic acid
N	Nitrogen
N	Normal
NaDOC	Sodium deoxycholate
NMR	Nuclear magnetic resonance
NOS	Nitric oxide synthase
NTCP	Na+-taurocholate co-transporting polypeptide
OFN	Oxygen free nitrogen
P	Probability
PBA	Primary bile acid
PBS	Phosphate buffer solution

PCR	Polymerase chain reaction
PDTC	Pyrrolidine dithiocarbamate
pKa	Acid dissociation constant
PLA2	Phospholipase A2
ppm	Parts per million
RAPD	Random amplification of polymorphic DNA
rDNA	Ribosomal DNA
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
s	Second
SBA	Secondary bile acid
SE	Standard error
SEM	Standard error of the mean
sp.	Specie
spp.	Species
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TDCA	Taurodeoxycholic acid
TTFA	Thenotrifluoro acetone
UC	Unesterified cholesterol
UDCA	Ursodeoxycholic acid
vs.	Versus
w/v	Weight per volume

WHO	World Health Organisation
x	Time

CHAPTER 1

INTRODUCTION

Bile acids are saturated, hydroxylated C-24 cyclopentanephenanthrene sterols synthesized from cholesterol in hepatocytes. The two primary bile acids synthesized in the human liver are cholic acid (CA; 3α, 7α, 12α-trihydroxy-5bcholan-24-oic acid) and chenodeoxycholic acid (CDCA; 3α, 7α-dihydroxy-5b-cholan-24-oic acid). Bile acids are further metabolized by the liver via conjugation (N-acyl amidation) to glycine or taurine. Thus, at physiological pH, conjugated bile acids are almost fully ionized and may be termed bile salts (McGarr *et al*., 2005). Bile salts are secreted actively across the canalicular membrane and are carried in bile to the gallbladder, where they are concentrated during the inter-digestive period. After a meal, the release of cholecystokinin from the duodenum stimulates the gallbladder to contract, causing bile to flow into the duodenum.

Bile salts are highly effective detergents that promote solubilization, digestion, and absorption of dietary lipids and lipid-soluble vitamins throughout the small intestine. High concentrations of bile salts are maintained in the duodenum, jejunum, and proximal ileum, where fat digestion and absorption take place. Bile salts are then absorbed through high-affinity active transport in the distal ileum (Vlahcevic *et al*., 1996). Upon entering the bloodstream, bile salts are complexes to plasma proteins and returned to the liver. Upon reaching the liver, they are cleared efficiently from the circulation by active transporters on the sinusoidal membrane of hepatocytes and rapidly

secreted into bile. This process is known as the enterohepatic circulation (Ridlon *et al*., 2006).

However, roughly 5% (approximately 400–800 mg) of the bile acid pool escapes ileal absorption and enters the large intestines each day and becomes substrate for significant microbial biotransforming reactions in the large bowel (Doerner *et al*., 1997). The secondary bile acids deoxycholic acid (DCA; 3α, 12α-dihydroxy- 5b-cholan-24-oic acid) and lithocholic acid (LCA; 3α-hydroxy-5b-cholan-24-oic acid) are produced solely by microbial biotransforming reactions (7α-dehydroxylation by colonic bacteria) in the human large intestine. Of the three hydroxyl groups (3α, 7α and 12α) of naturally occurring bile acids, the 7α-hydroxyl-group is the most readily degraded by human intestinal flora (Macdonald *et al*., 1982). 7α-dehydroxylation is the most quantitatively important bacterial bile salt biotransformation in the human colon. Unlike rodents, the human liver cannot 7α-hydroxylate DCA, forming CA. Hence, under normal physiological conditions, there is no metabolic pathway for removing DCA from the bile acid pool in humans. The amount of DCA in the bile acid pool is a function of at least three variables: 1) the rate of formation and absorption of DCA through the colon (input); 2) colonic transit time; and 3) colonic pH. High levels of DCA in blood, bile, and feces have been correlated with an increased risk of cholesterol gallstone disease and colon cancer (Ridlon *et al*., 2006).

Current research however, has demonstrated the important role that the normal gastrointestinal microflora plays in animal health and nutrition, as well as highlighting

the ecological significance of intestinal pathogens. Members of the genera *Clostridium* (*C. scindens*, *C. hiranonis*) and *Eubacterium* are the predominant intestinal species exhibiting bile acid 7α-dehydroxylating activity. *Eubacterium* sp. strain VPI 12708 has been shown to have a multistep bile acid 7α-dehydroxylation pathway with most of the required enzymes (Doerner *et al*., 1997). High levels of CA 7α-dehydroxylating fecal bacteria have been correlated with increased amounts of DCA in bile of a subset of cholesterol gallstone patients. Secondary bile acids, may contribute to the pathogenesis of colon cancer, gallstones, and other gastrointestinal (GI) diseases (Ridlon *et al*., 2006). However, base on speculation, the ways to preventing the occurrence of these mechanisms are: 1) eliminating primary bile acids in colon (input); 2) decrease in the number of bile acid 7α-dehydroxylating bacteria; and 3) eliminating deoxycholic acid (secondary bile acid; output).

As conjugated bile salts possess antimicrobial activity, bacteria seem to have evolved to produce bile salt hydrolase (BSH) to neutralize this adverse activity. A number of bacterial strains possessing deconjugating activity such as anaerobic *Lactobacillaceae* and *Bifidobacteria* have been isolated and these have been shown to be present in ileal and fecal content (Yazid *et al*., 1999; Mariam *et al*., 2004; Suresh Kumar *et al*., 2006).

Bifidobacteria are natural members of the human intestinal microbiota, in which they occur at concentrations of 10^9 to 10^{11} cfu/ g of feces, and represent up to 91% of the total gut population during the early stages of life. The mechanisms allowing intestinal bacteria to resist physiological bile salts concentrations remain poorly understood and