UNIVERSITI PUTRA MALAYSIA

THE BINDING OF *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 TOMUTAGENIC/CARCINOGENIC HETEROCYCLIC AROMATIC AMINESIN AN *IN VITRO* STUDY

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FSTM 2010 1

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By

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THE BINDING OF *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 TO MUTAGENIC/CARCINOGENIC HETEROCYCLIC AROMATIC AMINES IN AN *IN VITRO* STUDY

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January 2010

Chairman : Professor Mohd Yazid Abdul Manap, PhD

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Consumption of probiotic microorganisms has been associated with decreased risk of colon cancer and reported to have antimutagenic/anticarcinogenic properties. Lactic acid bacteria (LAB) existing in the colon may exert an anticarcinogenic action, but the mechanism is still poorly understood. One possible mechanism for this effect involves physical binding of the mutagenic compounds, such as heterocyclic amines, to the bacteria. Therefore, the purpose of this study was to study the binding assay of mutagenic/carcinogenic heterocyclic aromatic amines (HCAs) to *Bifidobacterium pseudocatenulatum* G4, a species which has not been explored yet as a commercial probiotic, *in vitro*. The effect of two gram positive bacteria: *Bifidobacterium pseudocatenulatum* G4 and *Bifidobacterium longum* BB536 (a commercial probiotic used as a reference strain), and a gram negative bacterium: human intestinal strain *Escherichia coli* ATCC 25922 at the colon

environmental pH and temperature were studied. *In vitro* binding of five common mutagens namely; IQ, MeIQx, PhIP, Trp-p-2 and 7, 8-DiMeIQx to the bacterial strains, in two media of different pH levels was analyzed using HPLC. Moreover, the tolerance of *B. pseudocatenulatum* G4 to simulated colonic pH and the survival of this strain in the presence of carcinogens were evaluated. The results showed that HCAs were able to bind all bacterial strains tested *in vitro.* A significant decrease ($P < 0.05$) in the sum of polar and apolar HCAs compared to untreated samples was observed. *B. pseudocatenulatum* G4 showed the highest decrease in the total HCAs content, followed by *B. longum*, and *E. coli*. The binding of freeze-dried cells to HCAs was pH dependant; the highest binding occured in the descending region pH of the colon and rectum at pH 6.8. In this study, the binding capacities of gram-positive and gram-negative bacteria to pyrolyzates were compared, the gram positive strains were found consistently more effective than gram negative strain. Although we observed a significant decrease in the amount of HCAs in the presence of three different cell concentrations (10^6,10^8 and 10^{10} cfu/g) of *B. pseudocatenulatum* G4, the highest decrease was detected at the concentration of 10^{10} cfu/g. The average binding (%) of IQ, MeIQx, 7,8DiMeIQx, Trp-p-2, and PhIP were 86.77, 92.58, 80.45, 85.68, 79.32, respectively, and differed significantly ($P < 0.05$) depending on the mutagen. The resistance of G4 to simulated colonic pH showed a high survival rate with little or no decrease in cell count. Also, the survival of *B. pseudocatenulatum* G4 in the presence of selected carcinogens showed no reduction in the number of living bacterial cells as compared with the control. These results revealed that *B. pseudocatenulatum* G4 potentially can reduce the concentration of mutagenic/carcinogenic HCAs markedly in human intestinal tracts and colons.

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

PERLEKATAN *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 TERHADAP MUTAGENIK/KARSINOGENIK HETEROSIKLIK AROMATIK AMINA DI DALAM KAJIAN *IN VITRO*

Oleh

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Penggunaan mikroorganisma probiotik seringkali dikaitkan dengan pengurangan risiko kanser kolon dan juga telah dilaporkan mempunyai ciri-ciri antimutagenik/antikarsinogenik. Kehadiran laktik asid bakteria pada kolon manusia mungkin mempengaruhi tindakan antikarsinogenik tetapi mekanismanya masih kurang difahami. Salah satu mekanisme yang munasabah untuk tindakan ini ialah penglibatan pelekatan fizikal sebatian mutagenik seperti heterosiklik amina kepada bakteria. Oleh itu, tujuan penyelidikan ini adalah untuk mengkaji pencerakinan ikatan mutagenik/karsinogenik daripada heterosiklik aromatik amina terhadap *B.pseudocatenulatum* G4, iaitu satu spesis yang tidak pernah diteliti lagi sebagai probiotik komersial, secara *in vitro*. Kesan dua bakteria Gram Positif: *B.pseudocatenulatum* G4 dan *B. longum* BB536 (sejenis probiotik komersial yang digunakan sebagai strain rujukan) dan satu bakteria Gram Negatif: strain dari usus manusia *E. coli* ATCC 25922 pada pH dan suhu persekitaran kolon telah dikaji.

Perlekatan *in vitro* lima jenis mutagen iaitu; IQ, MeIQx, PhIP, Trp-p-2 dan 7,8-DiMeIQx terhadap strain-strain bakteria, pada dua jenis medium yang mempunyai pH yang berbeza telah dianalisis menggunakan Kromatografi Cecair Berkeupayaan Tinggi. Tambahan lagi, ketahanan *B. pseudocatenulatum* G4 terhadap simulasi pH kolon dan kemandirian strain ini terhadap kehadiran bahan karsinogenik juga telah dinilai. Keputusan kajian menunjukkan bahawa HCA berkebolehan untuk melekat dengan kesemua strain bakteria secara *in vitro*. Pengurangan nyata ($P < 0.05$) dalam jumlah polar dan apolar HCA dalam perbandingan dengan sampel yang tidak dirawat telah diperhatikan. *B. pseudocatenulatum* G4 telah menunjukkan pengurangan paling tinggi dalam jumlah keseluruhan kandungan HCA, diikuti oleh *B.longum* dan *E. coli*. Keputusan menunjukkan bahawa perlekatan sel-sel bakteria sejuk beku-kering terhadap HCA adalah pH tanggungan; perlekatan paling tinggi telah dicapai di kawasan pH kolon yang menurun dan rektum pada pH 6.8. Dalam kajian ini juga, kapasiti perlekatan bakteria Gram-positif dan Gram-negatif dengan pyrolizat telah dibandingkan. Strain Gram-positif didapati lebih berkesan secara konsisten berbanding strain Gram-negatif.Walaupun pemerhatian kajian mendapati terdapat pengurangan yang nyata pada kuantiti HCA terhadap tiga kepekatan sel *B. pseudocatenulatum* G4 yang berbeza iaitu 10^6, 10^8, dan 10^{10} cfu g^{-1}, tetapi penurunan yang paling tinggi dikesan adalah pada kepekatan 10^{10} cfu g^{-1}. Nilai peratus purata perlekatan IQ, MeIQx, 7,8- DiMeIQx, Trp-p-2 dan PhIP masing-masing adalah 86.77, 92.58, 80.45, 85.68 dan 79.32 serta berbeza secara nyata ($P < 0.05$) bergantung kepada mutagen. Daya tahan rintangan G4 terhadap simulasi pH kolon menunjukkan kadar kemandirian yang tinggi dengan sedikit atau tiada penurunan dalam kiraan bilangan sel. Juga, kadar kemandirian *B. pseudocatenulatum* G4 dalam kehadiran karsinogen terpilih menunjukkan tiada

penurunan di dalam bilangan sel bakteria hidup jika dibandingkan dengan kawalan. Keputusan ini memperlihatkan bahawa *B*. *pseudocatenulatum* G4 berpotensi untuk megurangkan kadar kepekatan mutagenik/karsinogenik HCA dengan ketara pada saluran usus dan kolon manusia.

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I certify that an Examination Committee has met on **……** to conduct the final examination of **Farnaz Faridnia** on her Master thesis entitled "**The Binding of *Bifidobacterium Pseudocatenulatum* G4 to Mutagenic/ Carcinogenic Heterocyclic Aromatic Amines in an *in vitro* Study**" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the (Name of relevant degree).

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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 Universiti Putra Malaysia or at any other institutions.

FARNAZ FARIDNIA

Date: 1.03.2010

TABLE OF CONTENTS

		Page
ABSTRACT		ii
ABSTRAK		iv
ACKNOWLEDGEMENTS		vii
APPROVAL		ix
DECLARATION		xi
LIST OF TABLES		xv
LIST OF FIGURES		xvii
LIST OF ABBREVIATIONS		xx
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	7
	2.1 Epidemiology	7
	2.1.1 History of Heterocyclic Amines discovery	9
	2.1.2 Human Exposure and Cancer Risks	13
	2.1.3 Classification of Heterocyclic Amines	14
	2.1.4 Carcinogenesis	15
	2.2 Heterocyclic Amines and DNA Adduct Formation	18
	2.2.1 Mutagenic activity and Ames test	20
	2.3 Formation of dietary Heterocyclic Amines	23
	2.3.1 Factors Affecting the Formation of Heterocyclic Amines	25
	2.3.2 Metabolism of HCAs in Experimental Laboratory Animals and Humans	26
	2.3.3 Reduction or Inhibition of Heterocyclic Amines	30
	2.3.4 Binding and Degrading Potential Carcinogens/ Mutagens	30
	2.4 Gastrointestinal Physiology and Functions	33
	2.4.1 Gut Microflora	36
	2.4.2 Modulation of the Intestinal Microflora and Its Metabolism	37
	2.4.3 Role of the Gut Microflora in Cancer	38
	2.5 Etiology of Colon Cancer	38
	2.6 Food Born Genotoxic Substances and Cancer	40
	2.7 Functional Foods	40
	2.8 Probiotics	41
	2.8.1 Proposed Mechanism of Action and Properties of Probiotic	42
	2.8.2 Pathogen Prohibition Activity of Probiotics	44

2.8.3 Mechanisms of Anti-mutagenicity and Anti-carcinogenicity of Probiotics 44
2.8.4 Mechanisms by which Probiotic Bacteria may Inhibit Colon Cancer 46
2.8.5 The Anti-Genotoxic Activity of Probiotics 47
2.9 Bifidobacteria and their Beneficial Effects 48
2.9.1 Bifidobacterium Pseudocatenulatum G4 50
2.9.2 Bifidobacterium longum BB536 51
2.9.3 The Safety of Probiotics 52

3 MATERIALS AND METHODS 53
3.1 Bacterial Strains and Growth Conditions 53
3.2 Growth Phases of Bifidobacterial strains 55
3.3 Survival of Bacterial Strains to Simulated Colonic pH Environment 56
3.3.1 Preparation of Solutions to simulate pH of Human Colon 56
3.3.2 Survival of *B. pseudocatenulatum* G4 at Different pH Conditions 56
3.3.3 Microbiological Analysis 57
3.4 Survival of *B. pseudocatenulatum* G4 in the Presence of HCAs 58
3.4.1 Maintenance of Viable Cells 58
3.4.2 Preparation of Methanolic Solutions of HCAs 58
3.4.3 Growth of Bifidobacteria in the Presence of HCAs 58
3.4.4 Statistical Analysis 59
3.5 Binding of Mutagenic Heterocyclic Amines by Bacterial Cells 59
3.5.1 Preparation of bacterial cells 59
3.5.2 Sample preparation 60
3.5.3 Preparation of Lyophilized Cells 61
3.5.4 Mutagenic Chemicals used in the study 62
3.5.5 *In vitro* Binding assay 63
3.5.6 Chromatography System and Operating Conditions 64
3.5.7 Statistical Analysis 66

4 RESULTS AND DISCUSSION 67
4.1 Morphological Observation Using Gram Staining and Microscopic Method 67
4.2 Growth Phases of Bifidobacterial Strains 70
4.3 Survival of Bacterial Strains to Simulated Colonic pH Environment 73
4.4 *In vitro* Binding of HCAs to bacterial cells 79
4.5 Effects of pH on binding of heterocyclic amines to the bacterial cells 89
4.6 Effect of Cell Concentration on the Binding of HCAs to freeze-dried Cells 99

4.7 Survival of *B. pseudocatenulatum* G4 in the presence of Heterocyclic Amines 102

5 GENERAL CONCLUSION 105

REFERENCES 108
APPENDICES 126
BIODATA OF STUDENT 139
LIST OF PUBLICATIONS 139

LIST OF TABLES

Table		Page
2.1	Chemical names, abbreviations and years of discovery of the different heterocyclic amines	12
2.2	Abbreviations of HCAs and their mutagenicities in *S. typhimurium* TA98	23
2.3	Antigenotoxicity of Probiotics and Prebiotics *in vitro* and *in vivo*	35
3.1	List of Bacterial Strains used in the present study	54
3.2	Identification and analytical information of HCAs	62
4.1	Definition of terms used for the description of bifidobacteria	67
4.2	Effect of simulated colonic pH (5.6, 6.6, and 6.8) on cell viability	74
4.3	Viability of bacterial strains according to their resistance to colonic pH	75
4.4	Summary of probiotic screening studies done on *B. pseudocatenulatum* G4 obtained from previous researchers.	78
4.5	Percentage of *in vitro* binding of mutagens by cells of *B. pseudocatenulatum* G4, *B. longum* BB536 and *E. coli* (10^8 cfu/ ml)	81
4.6	Significance probability (*P*-value and *F*-value), R^2 of the independent variables effects in the full factorial design with bound HCAs with *B. pseudocatenulatum* G4 (10^{10}cfu/ml) as the response	93

4.7	Mean values of bound HCAs with *B. pseudocatenulatum* G4 (10^{10}cfu/ml) in two different pH medium.	93
A.1	Regression Equation for HCAs	127
A.2	HCAs retention times according to HPLC chromatogram	131
A.3	Significance probability of the independent variables effects in the full factorial design with bound HCAs as the response	132
A.4	Interaction between pH, HCAs and bacterial cells (10^{8}cfu/ml)	133
A.5	Mean values of bound mutagen in two pH mediums obtained from the interaction between pH and HCAs	133
A.6	TPY medium composition	136
A.7	Carcinogenicity of Heterocyclic Amines in Rats and Mice	138

LIST OF FIGURES

Figure		Page
2.1	Structures of heterocyclic amines	11
2.2	Chemical structures of precursors for IQ type HCAs	25
2.3	Species differences in regioselective of CYP1A2 oxidation (bioactivation and detoxication) of 8-MeIQx and PhIP	27
2.4	Major pathways of metabolism of PhIP in experimental animals and humans	28
2.5	Schematic representation of the interactions between intestinal bacteria and HCAs	29
2.6	Electron Micrograph of *B. pseudocatenulatum* G4	51
4.1	Compound light microscope image of *B. pseudocatenulatum* G4 after gram staining	68
4.2	Microscopic Image of *B. pseudocatenulatum* G4 Grown on Trypticase Phytone Yeast (TPY) Broth Medium after Gram Staining	69
4.3	Compound light microscope image of *B. longum* BB536 after Gram staining	69
4.4	Log, Stationary, and Death Phases for *B. pseudocatenulatum* G4	71
4.5	Log, Stationary, and Death Phases for *B. longum* BB536	71

4.6	pH Curve during *B. pseudocatenulatum* G4 and *B. longum* BB536 Growth in Broth Culture Media for 24 h	72
4.7	Interaction between HCAs and *B. pseudocatenulatum* G4, *B. longum* BB536 and *E. coli* (10^8cfu/ml) at pH: (A) pH= 5.6; and (B) pH= 6.8 after incubation. Means with the same superscript letters were not significantly different (Duncan's Multiple Range Test; $P < 0.05$)	82
4.8	Different pH in human small and large intestine	90
4.9	Effects of pH on *in vitro* HCAs Binding to freeze-dried cells of *B. pseudocatenulatum* G4 at 10^{10}cfu/ml in two different pH medium	92
4.10	*In vitro* binding of potent mutagenic pyrolysates to cells of *B. pseudocatenulatum* G4 at 10^{10} cfu/ml in two different pH medium	95
4.11	Interaction between HCAs and *B. pseudocatenulatum* G4 different concentrations (cfu/ml). Means with the same superscript letter are not significantly different at $P < 0.05$	100
4.12	Growth of *B. pseudocatenulatum* G4 at 10^6 cfu/ ml in the presence of HCAs	103
4.13	Growth of *B. pseudocatenulatum* G4 at 10^8 cfu/ ml in the presence of HCAs	103
4.14	Growth of *B. pseudocatenulatum* G4 at 10^{10} cfu/ ml in the presence of HCAs	104
A.1	HPLC chromatograms (λ =265nm) of heterocyclic aromatic amines: Standard mixture	127
A.2	Standard curve for IQ obtained by UV detection	128

A.3	Standard curve for MeIQx obtained by UV detection	128
A.4	Standard curve for 7,8 DiMeIQx obtained by UV detection	129
A.5	Standard curve for Trp-p-2 obtained by UV detection	129
A.6	Standard curve for PhIP obtained by UV detection	130
A.7	Anatomy, physiology, and microbiology of the GIT	134
A.8	Shimadzu LC-20AT HPLC with Shimadzu SPD-M20A Detector	137
A.9	Anaerobic jars containing Anaerocult A	137

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
A_{620}	Absorbency under 620 nm
BHI	Brain Heart Infusion Medium
cfu	Colony forming units
conc.	Concentration
CO_2	Carbon dioxide
CRC	Colorectal Cancer
d	Day
7, 8DiMeIQx	3, 7, 8-trimethyl-3H-imidazo [4, 5-f] quinoxalin-2-amine
DNA	Deoxyribonucleic acid
DNMRT	Duncan's New Multiple Range Test
g	unit for measuring centrifugation force
GI	Gastrointestinal
GIT	Gastrointestinal tract
Glu	glucose
GRAS	Generally regarded as safe
H^+	Hydrogen ion
HCA	Heterocyclic Aromatic Amine
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography

i.e.	*id est* (that is)
IQ	2-amino-3-methyl-3H-imidazo [4, 5-F] quinoline
kg	Kilogram
L	Liter
LAB	Lactic acid-producing bacteria
Log_{10}	Logarithm to the base 10
M	Molar
MeIQx	2-amino-3, 8-dimethylimidazo [4, 5-*f*] quinoxaline
mg	Milligram
min	Minute
mL	Milliliter
MRS	de Man Rogosa Sharpe Medium
N	Nitrogen
N	Normality
NDMA	N-nitrosodimethylamine
NaOH	Sodium hydroxide
P	Probability
PBS	Phosphate buffered Saline (Buffer Solution)
PhIP	2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine
ppm	Parts per million
RCBD	Randomized Complete Block Design
RNA	Ribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid

RNA	Ribonucleic acid
rpm	Revolution per minute
SCFA	Short Chain Fatty Acids
S.D.	Standard Deviation
SE	Standard Error
SEM	Standard Error of the Mean
Sp.	Specie
spp.	Species
TPY	Trypticase -Phytone-Yeast Extract
Trp-p-2	3-amino-1-methyl-5H-pyrido [4, 3-b] indole
UV	Ultra Violet ray
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization
α	alpha
β	beta

CHAPTER 1

INTRODUCTION

Colorectal cancer (CRC) is the second major cause of death from cancer in Europe and in the USA. It has been estimated that about one third of human cancers are related to diet (Yun, 2004). Dietary factors and colonic microbiota seem to play a crucial role in colorectal carcinogenesis (Gooderham *et al*., 2007; Capurso *et al*., 2006). The molecular genetic mechanisms of CRC are well established, but environmental factors such as diet also have a major role in development of sporadic colon cancer. Regular consumption of dietary fat and red meat, especially processed meat, has been associated with high risk of colon cancer. In contrast, a high intake of fruits and vegetables, whole grain cereals, fish, and calcium have been associated with reduced risk (Bingham, 1999; Rafter and Glinghammar, 1998).

A group of carcinogens of dietary origin are the heterocyclic aromatic amines, formed in protein-rich muscle foods during high temperature of cooking or grilling (Melo *et al*., 2008; Cheng *et al*., 2006). Heterocyclic aromatic amines (HCAs) were first discovered in cooked foods by Sugimura and his collaborators more than 25 years ago (Sugimura *et al*., 2004). More than 20 HCAs have since been isolated and identified in cooked foods. Among these amines, Trp-p-1 (3-amino-1, 4-dimethyl-5-H-pyrido [4, 3-b] indole); Trp-p-2 (3-amino-1-methyl-5-H-pyrido [4, 3-b] indole); IQ (2-amino-3-methylimidazo[4,5,-f]quinoline); MeIQx (2-amino-3,8-imethylimidazo [4,5-f]quinoxaline), and PhIP (2-amino-methyl-6-phenylimidazo[4,5-b]pyridine) are carcinogenic in rats and mice, and IQ was also

found to be carcinogenic in monkeys (Ohgaki *et al*.,1991; Sugimura, 1997). Their formation is dependent upon the type of meat, cooking method, temperature and cooking time. Several HCAs have been classified as possible/probable carcinogens by the International Agency for Research on Cancer (IARC) (Melo *et al*., 2008). They have been tested for their mutagenic activity in assays *in vitro* and *in vivo* with positive results of toxicity for most of them (Schut and Snyderwine, 1999). The most abundant of these heterocyclic amines, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) has been shown to specifically induce tumors of the colon, breast, and prostate in rats which, co-incidentally are the three commonest sites of diet-associated cancer in Western society (Gooderham *et al*., 2007; Zhu *et al*., 2006). Recently a correlation between pancreatic cancer and meat intake, especially those cooked at high temperatures, suggests the involvement of HCAs consumption (Smith *et al*., 2008). Therefore, reducing human exposure to these compounds is highly recommended.

However, intestinal bacteria could also play a part in initiation of colon cancer through production of carcinogens, co-carcinogens, or pro-carcinogens. Some intestinal microorganisms strongly increase damage to DNA in colon cells induced by heterocyclic amines, whereas other intestinal bacteria can take up and detoxify such compounds (Wollowski *et al*., 2001). Bacteria of the *Bacteroides* and *Clostridium* genera increase the incidence and growth rate of colonic tumors induced in animals, whereas other genera such as *Lactobacillus* and *Bifidobacteria* prevent tumorigenesis (O'Mahony *et al*., 2001; Onoue *et al*., 1997). The harmful bacteria possess several enzymes (e.g. *β*-glucuronidase, nitroreductase) responsible for conversion of procarcinogens into carcinogenic substances, such as