UNIVERSITI PUTRA MALAYSIA

IN-VITRO SYNERGESTIC EFFECTS BETWEEN *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 AND INULIN ON HUMAN GASTROINTESTINAL TRACT MICROBIAL COMPOSITION

MUHAMMAD ANAS BIN OTHAMAN

FSTM 2009 24

IN-VITRO SYNERGESTIC EFFECTS BETWEEN *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 AND INULIN ON HUMAN GASTROINTESTINAL TRACT MICROBIAL COMPOSITION

MUHAMMAD ANAS BIN OTHAMAN

MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA

2009

IN-VITRO SYNERGESTIC EFFECTS BETWEEN *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 AND INULIN ON HUMAN GASTROINTESTINAL TRACT MICROBIAL COMPOSITION

By

MUHAMMAD ANAS BIN OTHAMAN

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

September 2009

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

IN-VITRO SYNERGESTIC EFFECTS BETWEEN _BIFIDOBACTERIUM PSEUDOCATENULATUM_ G4 AND INULIN ON HUMAN GASTROINTESTINAL TRACT MICROBIAL COMPOSITION

By

MUHAMMAD ANAS BIN OTHAMAN

September 2009

Chairman : Professor Mohd Yazid Abdul Manap, PhD

Faculty : Food Science and Technology

The eagerness in finding the most effective probiotic strain has attracted many investigations. *Bifidobacterium pseudocatenulatum* G4, strain isolated from free-living infant was reported to have characteristics as probiotic candidate. Meanwhile, inulin is a known natural source of carbon that can act as a prebiotic substance. The consumption of probiotic, prebiotic, and its combination (synbiotic) was reported to have the ability to alter microbial composition in human gastrointestinal tract (GIT). In this study, the effects of *B. pseudocatenulatum* G4 (probiotic), inulin (prebiotic) and its combination (synbiotic) towards the human GIT microbial composition were evaluated *in vitro*. The effects of inulin incorporated in chocolate products as one of its ingredients were also tested. Real-time PCR assay with selected genus- and species-specific primers were used as a tool in identification and enumeration of selected bacterial strain in fermentation of mixture of bacteria from human faecal sample while dilution and plate count technique was used to enumerate the bacterial cell in fermentation of pure culture bacteria. The morphology of the tested *Bifidobacterium* strains was observed and the species was

confirmed by molecular method targeting 16S rRNA gene. In pure culture batch fermentation of tryptone peptone yeast (TPY) medium supplemented with 0.5% inulin, *B. pseudocatenulatum* G4 grew at the growth rate of 0.53 ± 0.06 $\log_{10}$ h^{-1} as compared to other *Bifidobacterium* strains namely *B. breve* ATCC 15700, *B. longum* BB536, and *B. infantis* ATCC 15697 which grew at 0.45 ± 0.04 $\log_{10}$ h^{-1}, 0.31 ± 0.08 $\log_{10}$ h^{-1}, and 0.72 ± 0.03 $\log_{10}$ h^{-1}, respectively. The same amount of inulin was then introduced into dark- and milk chocolate and caused *B. pseudocatenulatum* G4, *B. breve* ATCC 15700, *B. longum* BB536, and *B. infantis* ATCC 15697 to grow at 0.54 ± 0.06, 0.44 ± 0.04, 0.36 ± 0.05, 0.73 ± 0.02 $\log_{10}$ h^{-1} for dark chocolate and 0.57 ± 0.05, 0.46 ± 0.03, 0.41 ± 0.04, 0.75 ± 0.01 $\log_{10}$ h^{-1} for milk chocolate respectively. Some of the chocolate ingredients had also influenced the growth of *B. pseudocatenulatum* G4. The addition of 0.5% of cocoa liquor in TPY medium caused *B. pseudocatenulatum* G4 to grow at 0.29 ± 0.03 $\log_{10}$ h^{-1}, and isomalt at 0.59 ± 0.05 $\log_{10}$ h^{-1} compared to TPY medium without any additional carbon source which grew at 0.19 ± 0.02 $\log_{10}$ h^{-1}, while the addition of cocoa butter did not support the growth of *B. pseudocatenulatum* G4. In 24 hours batch fermentation of human faecal bacteria, *B. pseudocatenulatum* G4 (Probiotic) showed its probiotic effects by inhibiting the growth of *Salmonella* and *Enterococcus faecalis*. The addition of inulin (Prebiotic) selectively supported the growth of *Bifidobacterium* and *Lactobacillus* as well as inhibits the growth of *Bacteroides*, *Salmonella*, and *E. faecalis.* The synbiotic combination of *B. pseudocatenulatum* G4 and inulin (Synbiotic) showed a synergestic effect as they reduced the number of *Bacteroides, Salmonella,* and *E. faecalis* better than Probiotic or Prebiotic alone. Synbiotic chocolate preparations (DCsynbiotic and MCsynbiotic) showed better synergestic effect with *B. pseudocatenulatum* G4

compared to Synbiotic when *Bifidobacterium* increased at 1.64 $\log_{10}$ (DCsynbiotic) and 1.67 $\log_{10}$ cells/ml (MCsynbiotic) from the initial counts. *Lactobacillus* also increased its cell number higher than Synbiotic treatment. Nevertheless, synbiotic chocolate preparations also gave a positive result towards the growth of potential pathogenic bacteria when compared to Synbiotic. However, the inhibition pattern still can be observed on *Salmonella* and *E. faecalis* when compared to glucose (control). The antimicrobial action was largely due to the pattern of lactic and acetic acid production in fermentation. Here, the synbiotic approach was more efficient than prebiotic or probiotic alone to modulate the human GIT microbial composition and *B. pseudocatenulatum* G4 with inulin is a compatible synbiotic pair to perform the function.

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

KESAN SINERGISTIK *IN-VITRO* DI ANTARA *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 DAN INULIN TERHADAP KOMPOSISI MIKROB DALAM SALURAN GASTRO-USUS MANUSIA

Oleh

MUHAMMAD ANAS BIN OTHAMAN

September 2009

Pengerusi : Professor Mohd Yazid Abdul Manap, Ph D

Fakulti : Sains dan Teknologi Makanan

Keinginan untuk mencari strain probiotik yang paling berkesan telah menarik minat banyak penyelidikan. *Bifidobacterium pseudocatenulatum* G4, strain yang dipencilkan dari najis bayi telah dilaporkan mempunyai ciri-ciri sebagai calon probiotik. Manakala inulin sedia kala diketahui sebagai sumber karbon yang boleh bertindak sebagai bahan prebiotik. Pengambilan produk probiotik, prebiotik dan kombinasinya (sinbiotik) telah dilaporkan mempunyai kebolehan untuk merubah komposisi mikroorganisma di dalam saluran usus manusia. Oleh itu, dalam kajian ini, kesan *B. pseudocatenulatum* G4 (probiotik), inulin (prebiotik) dan kombinasinya (sinbiotik) terhadap komposisi mikroorganisma di dalam saluran usus manusia telah dinilai secara *in vitro*. Kesan inulin yang telah dicampurkan ke dalam produk coklat sebagai salah satu bahan ramuan dalam pembuatannya juga telah diuji. Reaksi rantaian polimerase-masa nyata (real-time PCR) bersama pemula (primer) spesifik kepada genus dan spesis bakteria terpilih telah digunakan sebagai alat untuk mengenal pasti dan mengira jumlah bakteria terpilih dalam fermentasi bakteria campuran dari sampel najis manusia manakala teknik pencairan dan

pengiraan kultur dalam piring digunakan untuk mengira sel bakteria dalam fermentasi kultur bakteria tulen. Morfologi kesemua strain bifidobacteria yang dikaji telah diperhatikan dan pengesahan di peringkat spesis pula dilakukan menggunakan teknik molekular yang mensasarkan gen 16S rRNA. Dalam fermentasi sesekelompok kultur tulen media TPY yang dibekalkan dengan 0.5% inulin, kadar pertumbuhan *B. psuedocatenulatum* G4 adalah pada 0.53 ± 0.06 $\log_{10}$ jam^{-1}. Dalam perbandingan bersama strain bifidobacteria yang lain, *B. breve* ATCC 15700, *B. longum* BB536, dan *B. infantis* ATCC 15697, masing-masing tumbuh pada 0.45 ± 0.04 $\log_{10}$ jam^{-1}, 0.31 ± 0.08 $\log_{10}$ jam^{-1}, and 0.72 ± 0.03 $\log_{10}$ jam^{-1}. Kemudian, jumlah inulin yang sama dimasukkan ke dalam coklat hitam dan coklat susu dan menyebabkan *B. pseudocatenulatum* G4, *B. breve* ATCC 15700, *B. longum* BB536, dan *B. infantis* ATCC 15697 masing-masing tumbuh pada kadar 0.54 ± 0.06, 0.44 ± 0.04, 0.36 ± 0.05, 0.73 ± 0.02 $\log_{10}$ jam^{-1} untuk coklat hitam dan 0.57 ± 0.05, 0.46 ± 0.03, 0.41 ± 0.04, 0.75 ± 0.01 $\log_{10}$ jam^{-1} untuk coklat susu. Sebahagian dari bahan-bahan dalam coklat juga mempengaruhi pertumbuhan *B. pseudocatenulatum* G4. Penambahan likur koko dan isomalt dalam media TPY (0.5%) menjadikan *B. pseudocatenulatum* G4 membiak, masing-masing pada kadar 0.29 ± 0.03 $\log_{10}$ jam^{-1}, dan 0.59 ± 0.05 $\log_{10}$ jam^{-1} berbandingkan dengan media TPY tanpa apa-apa sumber karbon yang hanya menunjukkan *B. pseudocatenulatum* G4 membiak pada kadar 0.19 ± 0.02 $\log_{10}$ jam^{-1}, manakala penambahan mentega koko pula tidak menyokong pertumbuhan *B. pseudocatenulatum* G4. Dalam 24 jam fermentasi sesekelompok statik kultur campuran dari najis manusia, *B. pseudocatenulatum* G4 (Probiotic) telah menunjukkan kesan probiotiknya apabila merencat pertumbuhan *Salmonella* dan *Enterococcus faecalis*. Inulin juga menunjukkan kesan prebiotiknya apabila menyokong

pertumbuhan *Bifidobacterium* dan *Lactobacillus* secara selektif dan merencat pertumbuhan *Bacteroides*, *Salmonella*, and *E. faecalis*. Kombinasi sinbiotik oleh *B. pseudocatenulatum* G4 dan inulin menunjukkan kesan sinergistik apabila menurunkan jumlah bilangan *Bacteroiodes*, *Salmonella* and *E. faecalis* lebih baik dari apa yang dilakukan oleh sediaan Probiotic dan sediaan Prebiotic sahaja. Sediaan sinbiotik coklat (DCsynbiotik dan MCsynbiotic) juga menunjukkan kesan sinergistik yang lebih baik bersama *B. pseudocatenulatum* G4 apabila masing-masing meningkatkan bilangan *Bifidobacterium* sebanyak 1.64 $\log_{10}$ and 1.67 $\log_{10}$ sel/ml daripada kiraan permulaan. Peningkatan sel *Lactobacillus* juga lebih tinggi jika dibandingkan dengan sediaan Synbiotic. Selain daripada itu, persediaan sinbiotik coklat juga memberi keputusan yang positif terhadap pertumbuhan bakteria patogen apabila dibandingkan dengan sediaan Synbiotic. Walaupun begitu, corak perencatan masih lagi boleh diperhatikan ke atas *Salmonella* dan *E. faecalis* apabila dibandingkan dengan glukos (kawalan). Kesan antimikrob yang ditunjukkan dipengaruhi besar oleh corak penghasilan asid laktik dan asid asetik dalam fermentasi. Disini, pendekatan sinbiotik adalah lebih berkesan daripada prebiotik dan probiotik bersendirian dalam mengubahsuai komposisi mikroorganisma di dalam saluran usus manusia dan *B. pseudocatenulatum* G4 bersama inulin adalah pasangan sinbiotik yang sesuai untuk melakukan tugas ini.

ACKNOWLEDGEMENTS

First of all, I want to give my warmest appreciation to my Chairperson of Supervisory Committee, Prof. Dr. Mohd Yazid bin Abd Manap who had lead and guide me in doing research. A bunch of appreciation also to my co-supervisors, Dr Shuhaimi bin Mustafa and Prof. Madya Dr. Loong Yik Yee for their invaluable advices and guidance throughout my research studies.

I also want to extent my gratitude to all the science officers and laboratory assistances in Food Biotechnology laboratory, Basic Biotechnology laboratory (202), HPLC room, and Food Biochemistry Laboratory. Without their help, none of this work would have been possible.

Also special thanks to all my labmates in Food Biotechnology laboratory, Barka, Arizou, Stephnie, Farrah, Nassim, Amir, Paghty, Babak, Farnaz, Ali and also in my Basic Biotechnology Laboratory, Hamim, Huda, Raba'atun, Zarizal, Yamin, Faizah, Khalilah, and Salma, who had always cheered me up and lend their hands whenever I was in trouble, thanks a lot!

Last but not least, I would like to thank my beloved parents who encourage me to further study in this field. The moral support from my lovely wife, Wan Malliati bte Wan Mustafa which kept me focus and work in high spirit. This work is also dedicated to both of my cute and amazing daughter and son, Aina Malliani and Muhammad Aidif.

I certify that an Examination Committee has met on **16th September 2009** to conduct the final examination of Muhammad Anas bin Othaman on his Master of Science thesis entitled **"The in-vitro study of synergistic effects between *Bifidobacterium pseudocatenulatum* G4 and inulin on human gastrointestinal tract microbial composition"** 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 Science degree.

Azizah Abdul Hamid, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Nazamid Saari, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Internal Examiner)

Fatimah Abu Bakar, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Internal Examiner)

Ainon Hamzah, PhD
Associate Professor
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
(External Examiner)

_____

BUJANG KIM HUAT, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Mohd Yazid Abd Manap, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Shuhaimi Mustafa, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Loong Yik Yee, MD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

_____

HASANAH MOHD GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 14 January 2010

DECLARATION

I hereby declare that the thesis is based on my original work except for quotation 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.

_____

MUHAMMAD ANAS BIN OTHAMAN

Date: 13 November 2009

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

^{o}C	Degree Celsius
$k(h^{-1})$	growth rate constant
µL	Micro liter
µM	Micro molar
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cfu	Colony forming unit
cn	Copy number
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DP	Degree of polimerization
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
e.g	*Example gratia* (for example)
et al.	Et cetera (and company)
FOSHU	Foods for Special Health Use
FOS	Fructooligosaccharides
g	gram
GET	Glucose-EDTA-Tris
GIT	Gastrointestinal tract

h	Hour
H_2SO_4	Sulphuric acid
HCl	Hydrocloric acid
HPLC	High performance liquid chromatography
i.e.	id est (that is)
IPTG	Isopropyl-ß-D-thiogalactopyranoside
JCM	Japan Collection of Microorganism
kb	Kilo base pair
kV	Kilo volt
L	Liter
LB	Lysogeny broth
LAB	Lactic acid bacteria
Log	Logarithm
M	Molar
min	Minute
Mg	Magnesium
$MgCl_2$	Magnesium Chloride
mL	Mililiter
mM	Milimolar
N	Normality
NaOH	Sodium Hydroxide
NCBI	National Centre of Biotechnology
OFN	Oxigen free nitrogen

OS	Oligosaccharides
PCR	Polymerase chain reaction
PBS	Phosphate-buffered Saline
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
rpm	Revolution per minute
s	Second
S.D.	Standard deviation
SOC	Super Optimal Broth (with catabolite)
spp.	Species
TPY	Trypticase-Phytone- Yeast Extract
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

CHAPTER 1

INTRODUCTION

The awareness on keeping good gastrointestinal tract (GIT) health among the public has risen nowadays. Inflammatory bowel disease (IBS), irritable bowel syndrome (IBS), Crohn's disease, ulcerative colitis and Celiac disease are the common problem in gut due to unbalance of bacteria in GIT (Bradesi et al. 2003; Asakura et al. 2008; Collado et al. 2008). Many factors affect the population of intestinal bacteria including diet, age, sex, use of drugs, surgery, and some diseases. Researches showed that maintaining a proper balance of bacteria in GIT is the key to good gut health (Gorbach 2000) and one of the ways to achieve it is by consuming probiotics and prebiotics.

Probiotics and prebiotics are already a reputable sector in the health food market of developed countries. Probiotic dairy products were reported as one of the most developed and well-liked functional food products in European market (Shortt et al. 2004). The market is currently estimated to be worth more than USD 2 billion per annum (Saxelin 2008). In the USA, the annual market value of functional foods is estimated to be around USD80 billion in 2000. An estimated USD 1.86 billion is contributed by probiotics, and probiotic related products. The value is expected to grow to USD3.5 billion by 2007 (Sanders 1998). In Japan, the market size for probiotics and other dietary supplement segments, dominated (80%) the Foods for Special Health Use (FOSHU) category. The FOSHU market is estimated to worth more than USD 10 billion, annually (Amagase 2008). Meanwhile, in Malaysia,

probiotics and prebiotics are an emerging health food concept. Probiotic and prebiotic food products have a huge potential in the functional food market in Malaysia. Hence, special attention is required to develop the sector to be market driven and most important is to use locally manufactured products and expertise in order to participate and compete in the main stream of the field.

The concept of ingesting live microorganisms for therapeutic and prophylactic purposes can be traced back to the beginning of the 20th century (Metchnikoff 1910). Ever since such discovery had been made, there has been rapid growth in research regarding the use of live bacterial cells to benefit humankind. Probiotic organisms influence the physiological and pathological process of the host by modifying the intestinal microbiota, thereby affecting human health (Erickson and Hubbard 2000). They have been used in the prevention and treatment of many GIT disorders, such as inflammatory bowel disease, antibiotic-related diarrhea, and post-resection disorders such as pouchitis (Gorbach 2000). Most of the applied probiotic microorganisms are of human origin and are largely represented by *Bifidobacterium* and *Lactobacillus* species in the commercial. For the past several years, research in our laboratory has focused on *Bifidobacterium* strain that shows the most probiotic characteristics. *Bifidobacterium pseudocatenulatum* G4 was isolated from free-living breast-fed infant by Yazid *et al*. (1999a) and has shown some characteristics as a probiotic candidate.

Prebiotics is defined as non digestible food ingredient that beneficially affects the host by selectively 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). It is

a recent novel food concept that includes food ingredients that are not digested in the human upper intestinal tract and commonly used as a food additive (fiber), fat replacer, sugar substitute, or emulsifier. Dietary modulation of the gut microflora by prebiotics is designed to improve human health by reducing disease risk through the large intestinal surface with various physiological process and stimulating the numbers and/or activities of the bifidobacteria and lactobacilli (Manning and Gibson 2004). Prebiotics for which sufficient data are available for their classification as functional food ingredients are the inulin-type fructans, which include native inulin, enzymatically hydrolysed inulin or oligofructose, and synthetic fructo-oligosaccharides (FOSs) (Roberfroid and Delzenne 1998; Roberfroid et al. 1998).

Inulin is a plant-derived carbohydrate with the benefits of soluble dietary fiber (Schneeman 1999). It was reported to be fermented by resident bacterial groups such as bifidobacteria once reaches the colon intact (Roberfroid et al. 1998). In addition, the bifidogenic effect of inulin has been well proven (Gibson and Roberfroid 1995; Kolida and Gibson 2007).

Prebiotics are consumed by human generally in the form of ingredients in food products. Many food companies especially companies that produce dairy products take a prospect in developing a new product that contains prebiotic substances for the health claim. Inulin and fructo-oligosaccharides (FOS) have been studied in terms of their prebiotic activity in many studies. However, when it comes to its role as a food ingredient, only few tests took place. Most studies of prebiotics have involved the consumption of inulin- or oligosaccharide-containing powders, which may not be

relevant to everyday life because the substrates, for commercial use, would be incorporated into a food product (Tannock et al. 2004).

Probiotics, when applied in conjugation with prebiotics give rise to another possibility in microflora management technique known as synbiotics (Gibson and Roberfroid 1995). This combination could confer greater advantages to the host owing to greater survivality of probiotic candidate due to availability of substrate for its fermentation. Probiotics, prebiotics and synbiotics can be classified as health-enhancing and health-promoting functional food concepts. However, only a few combinations of pre/probiotics have been evaluated as synbiotics, with only a limited number determining effects on the human faecal microbiota using reliable molecular techniques (Saulnier 2007) and to date, finding the novel probiotic strain and the best synbiotic preparation still remains a challenge (Liong and Shah 2008). Thus, this study was conducted to elucidate the effect of synbiotic preparation of our own probiotic candidate, *B. pseudocatenulatum* G4 with inulin towards the bacterial composition in human GIT using real-time PCR assays. Here, we describe the first stages in the development of a synbiotic, by first determine the ability of probiotic strain of interest to metabolize prebiotic substance of interest, then by evaluating their effect on the faecal microbiota *in vitro*, to assess whether the synbiotic can have a superior functionality as compared with its probiotic/prebiotic components. Synbiotic function was also tested in food products to see whether the functionality of synbiotic was affected or not by the food itself. This general objective meets the specific objectives as listed below: