# 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**

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**MASTER OF SCIENCE**
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**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**

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**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**

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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.

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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.

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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:

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## 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.

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**MUHAMMAD ANAS BIN OTHAMAN**

Date: 13 November 2009

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| | |
|---|---|
| $^{o}C$ | 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 20$^{th}$ 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: