UNIVERSITI PUTRA MALAYSIA

ASSESSMENT OF *Bifidobacterium pseudocatenulatum* G4 AS A PROBIOTIC

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DOCTOR OF PHILOSOPHY

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By

BARKA MOHAMMED KABEIR

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

March 2009

DEDICATION

The work is dedicated to my parents Mohd Kabeir and Hawa' Nurain; to all members of my big family. It also goes to teachers, scientists, researchers, and all seekers for knowledge.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

ASSESSMENT OF *Bifidobacterium pseudocatenulatum* G4 AS A PROBIOTIC

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BARKA MOHAMMED KABEIR

March 2009

Chairman: Professor Mohd Yazid Abdul Manap, PhD

Faculty : Food Science and Technology

This study has demonstrated the probiotic criteria of *Bifidobacterium pseudocatenulatum* G4 related to safety profile; effects on intestinal microbiota; interactions with prebiotics; and the growth in peanut milk (PM) and survivability during the storage. The safety profile of the strain G4 was evaluated in groups of BALB/c mice, fed different concentrations of *Bifidobacterium* strains for a period of 28 days. At the end of the experimental period, the highest dose of 11 log CFU *B. pseudocatenulatum* G4 /day did not affect the general health of mice nor cause any toxicity to blood based on biochemistry and haematology measurements. Pathogenicity symptoms were not detected in the internal organs; serum enzymes of liver and kidney; and histology of ceacum, ileum and colon of the treated mice; thus, the strain G4 could be a safe probiotic for food application. The effects of feeding strain G4 on selected microbiota community and metabolic activity in ceacum and colon of rats were also examined in 30

days feeding trail. The rat groups received skim milk contained either viable or metabolic product of *B. pseudocatenulatum* G4. The viable supplement of strain G4 increased profile of bifidobacteria, Lactobacillus and total anaerobes in ceacum and colon of the fed rats; while it decreased total aerobes and potential pathogens (staphylococcus, enterococcus and enterobacteriacea) of the same intestinal regions. However, salmonella and coliform were maintained mostly unchanged. In addition, the viable supplement of strain G4 has significantly ($p< 0.05$) increased short chain fatty acids (acetic, propionic, and butyric) of ceacum and colon. Commercial prebiotics [inulin and fructo-oligosaccharide (FOS)], together with sorbitol, arabinan and inoculum concentration, were tested by fractional factorial design (FFD) to determine their impact on growth of *B. pseudocatenulatum* G4 in skim milk (SM) medium. At 48h incubation, bacterial growth was mainly influenced by FOS and inoculum concentration. Central composite design (CCD) was adopted using FOS and inoculum concentration at 48h incubation to develop the statistical model for optimization. The model predicted that 2.46 log CFU/ml produced the optimum growth increase of *B. pseudocatenulatum* G4. The combination that produced the optimum point was 2.86% FOS (g/v) and 0.67% inoculum concentration (v/v). At this point of optimum combination, validation experiment recorded 2.40 ± l0.02 log CFU/ml. Further application in 1-L bioreactor for 24h showed higher growth of 2.95 log CFU/ml as compared to 2.46 CFU/ml of the optimum model prediction. The growth of strain G4 was evaluated in peanut milk (PM), and growth amounting 7.12 log CFU/ml was attained at 24h incubation. Supplementation with FOS produced growth of 8.35 log CFU/ml, which comparable to the 8.25 log CFU/ml

of Trypticase phytone yeast extract (TPY) obtained at 21h incubation. During the storage of fermented PM at 25 ºC for a period of two weeks, the viable cells of strain G4 decreased to a level < 7 log CFU/ml, which did not fulfill the requirement of probiotics food. However, at two weeks refrigeration (4 ºC) storage, the viability of strain G4 was maintained high at acceptable level of > 7 log CFU CFU/ml fermented PM products. Therefore, *B. pseudocatenulatum* G4 assessed to be a safe probiotic microorganism for synbiotics formulation with FOS and incorporation into peanut milk (PM) for delivery purposes.

Abstrak tesis yang dikemukakan Senat Universiti Putra Malaysia sebagi memenuhi keperluan untuk ijazah Doktor Falsafah

Penilaian *Bifidobacterium pseudocatenulatum* G4 sebagai bakteria probiotik

Oleh

BARKA MOHAMMED KABEIR

Mac 2009

Pengerusi : Profesor Mohd Yazid Abdul Manap, PhD

Fakulti : Fakulti Sains dan Teknologi Makanan

Kajian ini telah mengutarakan ciri-ciri probiotik strain *Bifidobacterium pseudocatenulatum* G4 dari segi profil keselamatan, kesan ke atas mikrobiota usus, interaksi dengan prebiotik, dan kebolehan untuk tumbuh serta hidup dalam media berasaskan susu skim kacang tanah. Profil keselamatan strain G4 dinilai dalam beberapa kumpulan tikus BALB/c yang di beri makan strain *Bifidobacterium* pada kepekatan yang berbeza selama 28 hari. Pada akhir eksperimen, di dapati pemberian B. *pseudocatenulatum* G4/hari tidak mempengaruhi kesihatan tikus mahupun memberi kesan toksik dalam darah secara biokimia atau ukuran haematologi. Tiada sebarang patogenik simptom di kesan dalam organ dalaman, enzim-enzim serum hati dan buah pinggang, dan histologi cecum, usus kecil dan kolon. Oleh itu, strain *B. pseudocatenulatum* G4 adalah mikroorganisma probiotik yang selamat dan mempunyai potensi untuk di guna dalam aplikasi makanan. Kesan memakan strain G4 keatas komuniti

mikrobiota terpilih dan aktiviti metabolik pada cecum dan kolon tikus telah diuji selama 30 hari. Kumpulan tikus tersebut menerima diet susu skim yang mengandungi B. *pseudocatenulatum* G4 hidup atau produk metabolik B. *pseudocatenulatum* G4. Kesan pemberian strain G4 yang hidup telah meningkatkan populasi Bifidobacteria, Lactobacillus dan keseluruhan bakteria anearob dalam cecum dan kolon tikus yang di uji, manakala bilangan keseluruhan bakteria aerob dan bakteria yang berpotensi sebagai patogen seperti staphylococcus, enterocuccus dan enterobacteriacea telah menurun. Bagaimanapun, hampir sedikit atau tiada langsung perubahan pada bilangan Salmonella and coliform. Pemberian strain G4 yang hidup juga telah meningkatkan kandungan asid lemak berantai pendek (acetic, propionic, dan butyric) secara signifikan ($p>0.05$) dalam cecum dan kolon tikus yang di uji. Komersial prebiotik terpilih [inulin dan fructo-oligosaccharide (FOS)] serta sorbitol, arabinan dan kepekatan inokulum telah diuji menggunakan fractional factorial design (FFD) untuk mengetahui kesan interaksi terhadap pertumbuhan B. *pseudocatenulatum* G4 dalam media berasaskan susu skim. Setelah 48 jam inkubasi, didapati sebahagian besar pertumbuhan bakteria dipengaruhi oleh fructo-oligosaccharide (FOS) dan kadar kepekatan inokulum. Penurunan pertumbuhan telah diperhatikan dalam semua sampel selepas 72 jam inkubasi hasil dari pembentukan asid laktik. Central composite design (CCD) telah digunakan ke atas FOS dan kadar inokulum pada 48 jam inkubasi pengeraman untuk menghasilkan model statistik bagi tujuan pengoptimaan. Model tersebut meramal 2.461 log CFU/ml sebagai peningkatan pertumbuhan optimum *Bifidobacterium pseudocatenulatum* G4. Kombinasi yang menghasilkan titik

optimum tersebut adalah 2.86 % FOS (g/v) and 0.67 % kadar inokulum (v/v). Model tersebut seterusnya disahkan dalam bioreaktor 1L untuk 24 jam. Keputusannya menunjukkan peningkatan pertumbuhan lebih tinggi iaitu 2.95 log CFU/ml jika dibandingkan dengan ramalan model optima iaitu 2.46 CFU/ml. Pertumbuhan strain G4 telah dinilai dalam produk berasaskan susu kacang tanah dan kadar pertumbuhan selepas 24 jam inkubasi adalah 7.12 log CFU/ml. Pertumbuhan ini telah dirangsang oleh kesan pemberian frocto-oligosacharide (FOS). Pertumbuhan yang tinggi ialah 8.35 log CFU/ml di dalam susu kacang tanah + FOS dan ianya setanding dengan 8.25 log CFU/ml dalam TPY yang di perolehi selepas 21 jam inkubasi. Strain G4 hidup dalam sampel terfermentasi yang disimpan pada 25^{o}C selama 2 minggu telah menurun kepada paras < 7 log CFU/ml, yang mana ianya tidak menepati keperluan sebagai probiotik makanan. Bagaimanapun, selepas penyimpanan selama 2 minggu pada 4^{o}C, pertumbuhan strain G4 kekal tinggi melangkaui paras yang disyorkan iaitu sepuluh juta sel per ml produk. Kesimpulannya, *B. pseudocatenulatum* G4 mempunyai potensi sebagai bakteria probiotik. Ianya adalah mikroorganisma probiotik yang selamat untuk tujuan formulasi synbiotik bersama FOS dan dicampurkan ke dalam produk berasaskan susu kacang tanah selain susu skim.

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I certify that an Examination Committee has met on 30 March 2009 to conduct the final examination of Barka Mohammed Kabeir on his Doctor of Philosophy thesis entitled "Assessment of *Bifidobacterium pseudocatenulatum* G4 as a probiotic" in accordance with Universiti Putra Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Member of the Examination Committee are as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Date: 9 July 2009

DECLARATION

I declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that is has not been previously and is not concurrently, submitted for any other degree at UPM or at any other institution.

BARKA MOHAMMED KABEIR

Date: 24 May 2009

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LIST OF ABBREVIATIONS

G	gram
ºC	degree Celsius
%	Percentage
et al.	et cetera (and company)
WHO	World Health Organization
PCR	Polymerase chain reaction
16S rRNA	Ribosomal ribonucleic acid
Kb	kilo base pair
bp	base pair
JCM	Japan Collection of Microorganism
MRS	de Mann-Rogosa-Sharpe
nm	nanometer
OD	Optical density
PBS	phosphate buffer saline
CFU	colony Forming Unit
mmol	milimolar
ml	milliliter
x g	gravity
EDTA	Ethylenediaminetetraacetic acid
H&E	Haematoxylin and Eosin
mg	milligram
ANOVA	Analysis of variance