UNIVERSITI PUTRA MALAYSIA

SYNERGISTIC EFFECT OF BIFIDOBACTERIUM PSEUDOCATENULATUM AND FRUCTOOLIGOSACCHARIDES AGAINST ESCHERICHIA COLI

LIM LONG CHANG.

FSMB 2004 12

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By

LIM LONG CHANG

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

April 2004

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

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Chairman: Associate Professor Mohd Yazid Manap, Ph.D.

Faculty : Food Science and Biotechnology

8 wild type strains of *Bifidobacterium pseudocatenulatum* and 4 commercial strains (*B. pseudocatenulatum* JCM 1200, *B. infantis* ATCC 15698, *B. breve* ATCC 26720 *and B. longum* BB536) were screened for inulinase activity on modified PY-O and PY-I agar. Wild type *B. pseudocatenulatum* F117 and reference *B. pseudocatenulatum* JCM 1200 were determined to have the highest inulinase activity. This activity was apparently higher on PY-O agar in comparison to PY-I agar. In batch cultivation, growth of *B. pseudocatenulatum* F117 was enhanced in PY-O medium (0.38 h^{-1} and 2.64 x 10^8 cfu/mL), compared to PY-G (0.48 h^{-1} and 5.45 x 10^8 cfu/mL) and PY-I medium (0.20 h^{-1} and 1.70 x 10^8 cfu/mL) from the respective, initial specific growth rate and maximum growth. Acetic, lactic and formic acid production was also found to be relatively higher in PY-O medium (38.60 mM; 37.48 mM; 7.37 mM) compared to PY-G medium (11.95 mM; 22.77 mM; 5.91 mM) or PY-I medium (12.34 mM; 19.73 mM; 0 mM). In therapeutic

study, the antagonistic effect against *E. coli* V157 by *B. pseudocatenulatum* F117 was contributed by pH lowering of the growth medium. This effect was especially intensified at pH below 5.0. Antagonistic effect found in PY-O medium was greater compared to that of PY-G medium. The kinetics of antagonistic effect could be divided into two apparent phases. It was the condition before (first phase) and after (second phase) *B. pseudocatenulatum* F117 to achieve the maximum growth (10^9 cfu/mL), with relatively intense antagonistic effect at second phase. Organic acid was highly produced at second phase (PY-G medium: 45.57±8.99 mM; PY-O medium: 61.54±11.92 mM) rather than first phase (PY-G medium: 25.59±3.16; PY-O medium: 30.46±7.21 mM) implicative of the importance of probiotic concentration for effective antagonism. Oligofructose was found to be able to stimulate the growth of *B. pseudocatenulatum* F117 and consequently shorten the time for the maximum growth to achieve, from 18 hr in PY-G medium to 12 hr in PY-O medium. Besides, lactic acid production was initiated 6 hr earlier in PY-O medium than to PY-G medium, which could be an added inhibitory advantage. With low bifidobacteria dose (10^5 cfu/mL), the antagonistic effect displayed was quite identical to a higher dose (10^8 cfu/mL) employed. *B. pseudocatenulatum* F117 was found to obtain the maximum growth in 18 hr for the both trial doses in either growth medium. In prophylactic study, PY-O medium could not be observed to further enhance the antagonistic effect in PY-G medium. *E. coli* V157 (10^8 cfu/mL) was unable to sustain and multiply to a higher population but decreased in numbers in either growth mediums. Finally, a higher oligofructose concentration (1.0 %) was shown to dramatically improve the antagonistic effect as compared to the lower

concentration (0.5 %) used, in both therapeutic and prophylactic study. This effect was again due to an even higher amount of organic acids produced.

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

KESAN SINERGISTIK DI ANTARA *BIFIDOBACTERIUM PSEUDOCATENULATUM* DAN OLIGOFRUKTOSA TERHADAP *ESCHERICHIA COLI*

Oleh

LIM LONG CHANG

April 2004

Pengerusi: Profesor Madya Mohd Yazid Manap, Ph.D.

Fakulti : Sains Makanan dan Bioteknologi

8 stren liar *Bifidobacterium pseudocatenulatum* dan 4 stren *komersial (B. pseudocatenulatum* JCM 1200, *B. infantis* ATCC 15698, *B. breve* ATCC 26720 dan *B. longum* BB536) disaring untuk aktiviti inulinase ke atas agar gubahan PY-O dan PY-I. Stren liar *B. pseudocatenulatum* F117 dan kawalan *B. pseudocatenulatum* JCM 1200 didapati memperoleh aktiviti inulinase yang tertinggi. Aktiviti tersebut adalah lebih ketara tinggi pada agar PY-O dibanding dengan agar PY-I. Dalam pengkulturan tertutup, pertumbuhan *B. pseudocatenulatum* F117 dipertingkatkan dalam media PY-O (0.38 h^{-1} and 2.64 x 10^8 cfu/mL) berbanding dengan media PY-G (0.48 h^{-1} and 5.45 x 10^8 cfu/mL) dan PY-I (0.20 h^{-1} and 1.70 x 10^8 cfu/mL) dari segi kadar pertumbuhan spesifik awal dan pertumbuhan maksimum masing-masing. Penghasilan asid asitik, laktik dan formik juga didapati lebih tinggi dalam media PY-O (38.60 mM; 37.48 mM; 7.37 mM) dibanding dengan media PY-G (11.95 mM; 22.77 mM; 5.91 mM) mahupun media PY-I (12.34 mM; 19.73 mM; 0 mM). Dalam

kajian pemulihan, kesan antagonistik terhadap *E. coli* V157 daripada *B. pseudocatenulatum* F117 adalah disebabkan oleh penurunan pH media pertumbuhan. Kesan ini menjadi lebih hebat di bawah pH 5.0. Kesan antagonistik di dalam media PY-O adalah lebih hebat dibandingkan di dalam media PY-G. Kinetics dalam kesan antagonistik dapat dibahagikan kepada dua fasa yang jelas. Ini adalah keadaan sebelum (fasa pertama) dan selepas (fasa kedua) *B. pseudocatenulatum* F117 mencapai pertumbuhan maksimumnya (10^9 cfu/mL), di mana kesan antagonistik yang lebih hebat berlaku pada fasa yang kedua. Asid organik banyak dihasilkan pada fasa kedua (media PY-G: 45.57±8.99 mM; media PY-O: 61.54±11.92 mM) berbanding dengan fasa pertama (media PY-G: 25.59±3.16; media PY-O: 30.46±7.21 mM), justerunya mengimplikasikan kepentingan kandungan probiotik untuk antagonisma yang berkesan. Oligofruktosa didapati merangsangkan pertumbuhan *B. pseudocatenulatum* F117 dan seterusnya mengurangkan tempoh untuk mencapai pertumbuhan maksimumnya (10^9 cfu/mL), daripada 18 j dalam media PY-G kepada 12 j dalam media PY-O. Di samping itu, penghasilan asid laktik bermula 6 j lebih awal dalam media PY-O daripada dalam media PY-G, kemungkinan besar menjadi kebaikan tambahan faktor perencatan. Pada dos bifidobakteria yang rendah (10^5 cfu/mL), kesan antagonistik yang dihasilkan adalah agak sama dengan dos yang tinggi (10^8 cfu/mL) digunakan. *B. pseudocatenulatum* F117 didapati mencapai pertumbuhan maksimum dalam 18 j untuk kedua-dua dos dalam mana-mana media pertumbuhan. Dalam kajian pencegahan, media PY-O didapati tidak meningkatkan kesan antagonistik lagi sepertimana yang diperhatikan dalam media PY-G. *E. coli* (10^8 cfu/mL) tidak dapat mengekal mahupun

membangun kepada populasi yang lebih tinggi tetapi berkurangan dalam bilangannya. Akhirnya, kepekatan oligofruktosa yang tinggi (1 %) didapati menghasilkan kesan antagonistik yang lebih hebat daripda kepekatan rendahnya (0.5 %) dalam kedua-duanya kajian pemulihan dan pencegahan. Kesan ini disebabkan oleh penghasilan asid organik yang lebih tinggi lagi.

ACKNOWLEDGEMENTS

First of all, please let me ask for forgiveness upon all the mistakes done inadvertently in thoughts, words or actions to everyone along my way in carrying out these experiments. Sorry if I have not fulfilled the expectation that you all wish for. I sincerely repent myself, Namo Amituofo!

Next, may I extend my heartfelt gratitude to my chairperson of supervisory committee, Assoc. Prof. Dr. Mohd Yazid Abd Manap, for his invaluable advises and guidance throughout the course of my study. My thanks also to Assoc. Prof. Dr. Arbakariya Ariff and Assoc. Prof. Dr. Sharifah Kharidah, being my co-supervisors for their patience and endurance with my progress.

Also not forget to forward a special thank to Mr. Halim for helping me out in the HPLC analysis. Thanks to my senior, Dr. Shuhaimi for his voluntarily helps in familiarizing me the fundamental laboratory skills before I can help myself very well later. My deepest thanks must go equally to the rest of all the faculty staffs who accompanied me like a happy family that help to put away my homesickness here.

Finally, but not least, I wish to call upon my grateful appreciation to these peoples who cheered me up always when I encountered failure and disappointments: Lovely Miss Levina, Anis, Leng Choo, Siew Eim, Sek Yee, Hooi Ling, Wendy, Santhy, brother Sohail and both my parents. Thanks a lot! Amituofo.

I certify that an Examination Committee met on 22nd April 2004 to conduct the final examination of Lim Long Chang on his Master of Science thesis entitled "Synergistic Effect of *Bifidobacterium pseudocatenulatum* and Fructooligosaccharides against *Escherichia coli*" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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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 Supervisory Committee are as follows:

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Date: 08 SEP 2004

DECLARATION

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

LIM LONG CHANG

Date: 27/06/2004

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LISTS OF ABBREVIATIONS

ATP	:	adenosine triphosphate
ATCC	:	American Type Culture Collection
ca.	:	calculated
CFC	:	continuous flow culture
cfu	:	colony forming unit
conc.	:	concentration
d	:	day
D	:	dilution rate
DP	:	degree of polymerization
E. coli	:	*Escherichia coli*
ENDO	:	a European Commission-funded project on NDOs
e.g.	:	*example gratia* (for example)
et al.	:	et cetera (and company)
FOS	:	fructooligosaccharides
Fruc	:	fructose
g	:	gram
GALT	:	gut-associated lymphoid tissues
GF2	:	1-kestose
GIT	:	gastrointestinal tract
Glu	:	glucose
h	:	hour

h^{-1}	:	per hour
H+	:	hydrogen ion
HCl	:	hydrochloric acid
HCO_3^-	:	hydrogen carbonate ion
H_2SO_4	:	sulphuric acid
i.e.	:	*id est* (that is)
JCM	:	Japan Collection of Microorganisms
L	:	liter
LAB	:	lactic acid bacteria
log	:	logarithm
M	:	molar
MCS	:	multiple chamber operation systems
mL	:	milliliter
mM	:	millimolar
n	:	number
N	:	normality
NaOH	:	sodium hydroxide
NDOs	:	non-digestible oligosaccharides
O_2	:	oxygen
PY-G	:	peptone yeast glucose
PY-O	:	peptone yeast oligofructose

PY-I	:	peptone yeast inulin
R	:	resident time
rDNA	:	ribosomal deoxyribonucleic acid
rpm	:	revolution per minute
SCFA	:	short chain fatty acids
SCFAH	:	protonated short chain fatty acids
SOS	:	soybean oligosaccharides
spp	:	species
Th	:	T helper cells
TOS	:	transgalactosyl-oligosaccharides
TPY	:	trypticase-phytone-yeast extract
v	:	volume
XOS	:	xylo-oligosaccharides
μL	:	microliter
△	:	transmembrane pH gradient
α	:	alpha
β	:	beta
%	:	percentage
^{0}C	:	degree Celsius
/	:	per
μ	:	specific growth rate
μ_m	:	maximum specific growth rate

CHAPTER 1

INTRODUCTION

Gut health is currently getting much attention among researchers and public. This is mainly because many of the physical health problems were found closely related to the improper functionality of gut. Over the years, study of the microbiological system in the gut has slowly uncovered the general effects brought to the host. Evolved from birth, this group of microorganism has adapted and changed according to the maturity of gut, which make them present as one entity in the so-called "symbiosis" relationship. This is the most important fact that many researchers have been neglecting so far, by putting less attention to the biochemistry of these microorganisms. These organisms and their metabolic activities are not inert to the human host and have both positive and negative impacts on health. The balance of this ecosystem is dynamic and may be altered by several factors such as aging, medication, stress, diet, and some other environmental factors (Conway, 2001). The maintenance of a community of bacteria, which contains a predominance of beneficial species and minimal putrefactive processes, is believed to be important for maintaining intestinal health.

The concept of probiotic appears when effort is made to modify or alter the composition of the microfloras in the gut to a remedial one by directly introducing the biologically important beneficial microorganism (Gibson and Roberfroid, 1995).

Most of the attempt microorganisms are of human origin and largely represented by *Bifidobacterium* and *Lactobacilli* (Silvi *et al.*, 2003). These microorganisms are found to be the predominant gut floras in the breastfed infants and also in healthy adults. The beneficial influences of probiotic on human gut floras include factors such as antagonistic effects, competition and immune effects. Improved resistance to pathogen offers the most promise for development of efficacious probiotics. The use of probiotic bacterial cultures stimulates the growth of preferred microorganism, crowds out potentially harmful bacteria and reinforces the body's natural defense mechanisms (Conway, 2001).

Currently, the prebiotic strategy is proposed as an alternative mode to improve the well being of gut. This is the application of indigestible food ingredient that has impact on the metabolism of intestinal microorganisms (Holzapfel and Schillinger, 2002). In the scientific literature, most of the data on prebiotic effect deals with inulin and fructooligosaccharides, and a range of commercial products have been available for many years. First the majority of data were produced on the ability of these carbohydrates to increase the amount of bifidobacteria in faeces. During recent years, more emphasis has been put on physiological functions, such as gut function, colon cancer biomarkers, calcium absorption and lipid metabolism (Puupponen-Pimia *et al.*, 2002).

Due to the potential synergy between probiotic and prebiotic, foods containing a combination of these ingredients are often referred to as synbiotic

(Gibson and Roberfroid, 1995). Prebiotic might influence the growth and survival of the probiotic and starter in fermented dairy products. One may also expect the prebiotic to serve as a preliminary growth substance while introducing a biologically important probiotic into the gut. This is especially important in view of the weakness of probiotic surviving through gastrointestinal tract to reach an allocated site such as in the small and large bowel. Nonetheless, endeavors have been made to improve the competency of the probiotic consumed. Some researchers suggested having pre-adaptation of the probiotic to prebiotic prior to consumption. This might post competitive advantage for the probiotic if it is consumed concurrently with the prebiotic (Conway, 2001). On the other hand, the correct pair of probiotic and prebiotic is designed to ensure a successful establishment of probiotic in the gut among the presence of the large microbial assemblage (Fooks and Gibson, 2002). This is true when the substrate affinity of probiotic, for example *Bifidobacterium*, is varied among species and strains. Therefore, in applying the principle of synbiotic, besides proper strains selection for a characteristic probiotic, one has to take into consideration the substrate affinity of these probiotic as well. Therefore, current research is trying to focus on several topics regarding the application of synbiotic in vitro as below:

1. To compare substrate affinity of 8 locally isolated wild type *Bifidobacterium pseudocatenulatum* and 4 commercial *Bifidobacterium* spp. towards oligofructose and inulin,