UNIVERSITI PUTRA MALAYSIA

ADHESION PROPERTIES OF *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 TO HT-29 EPITHELIUM CELL LINE

ALI KAHTAN SULIMAN

FSTM 2009 9

ADHESION PROPERTIES OF *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 TO HT-29 EPITHELIUM CELL LINE

BY

ALI KAHTAN SULIMAN

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

July 2009

Abstract of thesis presented to the Senate of University Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

ADHESION AND ADHESION INHIBITION PROPERTIES OF *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 TO HT-29 EPITHELIUM CELL LINE

BY

ALI KAHTAN SULAIMAN

July 2009

Chairman : Professor Mohd Yazid Abdul Manap, PhD

Faculty : Food Science and Technology

Bifidobacterium pseudocatenulatum G4 has been recently identified as a safe probiotic for incorporation into functional food for human consumption. Preliminary investigations showed that the probiotic candidate *B. pseudocatenulatum* G4 strain possesses the required criteria for a being successful probiotic microorganism. Further enhancement of these criteria was undertaken by studying the adherence and inhibition properties potential of this probiotic candidate. Human colon carcinoma epithelium cell line HT-29 was used to evaluate the adherence of *B. pseudocatenulatum* G4, in simulated environmental factors of the colon, namely pH, calcium ions, and cholic acid. The effect of this strain on enhancing intestinal tract resistance to pathogenic *Escherichia coli* and *Clostridium* infections was examined.

Three different assays were used in order to differentiate between the competition, exclusion, and displacement of the pathogens by *B. pseudocatenulatum* G4.

The adherence ability of *B. pseudocatenulatum* G4 to HT-29 cell line was investigated as *in vitro* model. In addition the morphology observation of the organism was done by using scanning electron microscopy (SEM). The effect of human colon environmental factors on the adhesion quality was studied. The results showed that the highest adhesion was in the ascending and transverse acidic regions of the colon. Calcium was shown to increase, while cholic acid was shown to decrease the adhesion of *B. pseudocatenulatum* G4 to HT-29. The inhibitory effect of *B. pseudocatenulatum* G4 on the adherence of *Escherichia coli* O157:H7, *Clostridium scindens* and *Clostridium hiranonis* was demonstrated. A decrease in the number of adhering pathogens was observed.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah master sains

PERLEKATAN DAN KEBOLEHAN MERENCAT PERLEKATAN *BIFIDOBACTERIUM PSEUDOCATANULATUM* G4 PADA PERMUKAAN SEL EPITHELIUM HT-29

Oleh

ALI KAHTAN SULAIMAN

Juli 2009

Pengerusi : Profesor Mohd Yazid Abdul Manap, PhD

Fakulti : Sains dan Teknologi Makanan

Bifidobacterium pseudocatanulatum G4 telah dikenalpasti sebagai probiotik yang selamat untuk tambah ke dalam makanan berfungsi untuk kegunaan manusia. Kajian awal menunjukkkan strain *B. pseudocatanulatum* G4 mempunyai kriteria yang diperlukan untuk bertindak sebagai mikroorganisma probiotik yang berjaya. Kriteria selanjutnya telah diselidik melalui kajian potensi kebolehan melekat and merencat perlekatan. Lapisan sel epithelium karsinoma kolon manusia HT-29 telah digunakan untuk menilai kebolehan melekat strain *B. pseudocatanulatum* G4 di dalam persekitaran kolon simulasi. Faktor-faktor persekitaran kolon yang disimulasi adalah pH, ion kalsium and asid kolik. Kesan strain ini dalam meningkatkan kerentangan saluran usus terhadap jangkitan *Esherichia coli* and *Clostridum* telah kaji. Tiga

analisis yang berlainan telah digunakan untuk mengenalpasti samada terdapat persaingan, pengusiran dan pengambilalihan antara pathogen dan *B. pseudocatanulatum* G4. Kebolehan melekat *B. pseudocatanulatum* G4 kepada lapisan sel HT-29 telah diperhatikan menggunakan model *in vitro*. Kesan factor-faktor persekitaran dalam kolon manusia terhadap kualiti kebolehan melekat juga telah dikaji. Selain itu, morfologi organisma telah diperhatikan menggunakan mikroskop pengimbas elektron. Keputusan kajian menunjukkan kebolehan melekat paling tinggi *B. pseudocatanulatum* G4 adalah pada bahagian kolon menaik dan melintang. Kehadiran kalsium dapat meningkatkan kebolehan melekat *B. pseudocatanulatum* G4 pada HT-29 tetapi asid kolik mempuyai kesan yang sebaliknya. Kesan kerencatan oleh *B. pseudocatanulatum* G4 terhadap kebolehan melekat *E. coli* O157:H7, *Clostridium scindens* and *Clostridium hiranonis* telah dipamerkan. Pengurangan bilangan pathogen yang melekat telah diperhatikan.

ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my supervisor, Dr. Mohd Yazid Abdul Manap. His wide knowledge and his logical way of thinking have provided me with great values. Without his outstanding leadership, invaluable suggestions, and constrictive criticisms, this work would not be made possible. My sincere appreciation also goes to the members of my supervisory committee: Dr. Shuhaimi Mustafa, Dr. Hasanah Mohd Ghazali, and Dr. Zamberi sekawi for their concrete advice, understanding, and constant encouragement throughout this study.

Special thanks are extended to Mr. Farid Azizi Jalilian for his help while I was struggling with my tissue culture work. My deep thanks also go to my sister Maha for her patience, encouragement, and great help. And not forgetting my homemates for their kind assistance through my study.

Last but not least, my deepest appreciation is expressed to my family members: my father, my mother, my sisters, my brothers, and my aunt for their support through good and bad times.

This thesis submitted to the Senate of University Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory are as follow:

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

Hasana Mohd Ghazali, PhD
Professor
Faculty of Food Science and Technology
University Putra Malaysia
(Member)

Shuhaimi Mustaf, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
University Putra Malaysia
(Member)

Zamberi Sekawi, PhD
Associate Professor
Faculty of Medicine and Community Health
University Putra Malaysia
(Member)

_____
HASANAH MOHD. GHAZALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 16 October 2009

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 been previously or concurrently submitted for any other degree at UPM or other institution.

_____

Ali Kahtan Suleiman

Date:

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LEST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CFU	Colony Forming Unit
CO_2	Carbon dioxide
DMEM	Dulbecco's modified Eagle's minimal essential medium
DNA	Deoxyribonucleic acid
e.g.	Example gratia (for example)
et at.	Et cetera (and company)
FBS	Fetal Calf Serum
GIT	Gastrointestinal tract
H	Hour
H^+	Hydrogen ion
HCL	Hydrochloric acid
JCM	Japan Collocation of Microbiology
L	Liter
LAB	Lactic Acid Bacteria
Log	Logarithm
M	Molar
mL	Milliliter
mM	Millimolar
MRS	De Man Rogosa Sharpe Media
NaOH	Sodium Hydroxide
PBS	Phosphor Buffer Saline

RAPD	Randomly amplified polymorphic DNA
rpm	Revolution per minute
SEM	Scanning electron microscopy
S.D.	Stander Deviation
spp.	Species
v/v	Volume per volume
WHO	World Health Organization
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

The normal microflora of the human body is extensive and diverse. The largest portion is found in the gastrointestinal tract (GIT), specifically the colon. Bifidobacteria represents one of the predominant groups of the GIT microflora in breast-fed children (Caglar *et al*., 2005), and it has been the focus of researches on probiotic in recent years.

There are more than 10^{13} – 10^{14} total bacteria in the human GIT, most of them are anaerobes. Bifidobacteria was discovered in 1899 as naturally present in the colon microflora, including *Bifidobacterium bifidum* and *Bifidobacterium longum*. Tissier has isolated the genus *Bifidobacterium* from the stool of human beings and observed that it represents up to 25% of the cultivable faecal bacteria in adults and 80% of it in infants (Salminen *et al*., 2004; Picard *et al*., 2005).

In 1989, Fuller defined probiotic as "a live microbial feed supplement which beneficially affects on the host animal by improving its microbial balance." Probiotic survive gastric acidity, bile, and pancreatic secretions. They attach to the epithelial cells and colonize in the intestinal tract, where they inhibit the pathogenic bacteria

and stimulate the immune system (Saito, 2004; Del Piano *et al*., 2006). It is probable that adhesion bacteria interact to a greater degree with the intestinal mucosa and therefore, with the host as in comparison to flora bacteria, many of which have little or no influence on the host (Del Piano *et al*, 2006).

Many of *Bifidobacterium* human isolation species are probiotic as *B. longum, B. breve, B. lactis, B. bifidum,* and *B. pseudocatenulatum* (Ouwehand *et al*., 2002; Shuhaimi *et al*., 2004). Since few studies have been carried out on *Bifidobacterium pseudocatenulatum* G4, this research is a comprehensive study on this species. A special emphasis has been made on the species isolation by PCR- based and 16S rDNA partial sequences analysis methods (Shuhaimi *et al*., 2002), a generation of genomic DNA fingerprints of *B. pseudocatenulatum* G4 is isolated by RAPD (Shuhaimi *et al*., 2001).

The theoretical benefits of probiotic bifidobacteria are mediated by modulating the functionality of the intestinal microbial flora, gut barrier, and host immune system. Their therapeutic and prophylactic roles have been proposed and trailed in animals and human beings. Probiotic bifidobacteria adhesion properties, resistance to infectious diseases, and prevention of colon cancer have been assessed in current years (Salminen *et al*, 2004). A number of bifidobacteria have now a long history of safe use in dairy products; *B. adolescentis, B. animalis, B. lactis, B. bifidum, B. breve, and B. longum* are generally regarded as safe status (Salminen *et al*., 2004).

Probiotic strains adhesion to the intestinal surface and their following colonization in the human GIT have been suggested as an important requirement for probiotic action. Adhesive strains of probiotic bacteria probably persist longer in the intestinal tract and thus have better potential of showing metabolic and immunomodulatory effects than non adhesive strains. Adhesion provides an interaction with the mucosal surface, facilitating the contact with gut associated lymphoid tissue and mediating local and systematic immune effects. Adhesion may also provide means of competitive exclusion of pathogenic bacteria from the intestinal epithelium (Saarela *et al*., 2000).

The bacterial surface properties determine their ability to adhere to intestinal mucus, enterocyte cells, and gut epithelial tissue (Saarela *et al*., 2000). These surface properties also determine the microorganism's resistance to pH and bile, and its production of antimicrobial substances. Resistance to bile, however, is one of the criteria used to select probiotic strains that would potentially be capable of performing effectively in the GIT (Begley *et al*., 2005). As the calcium receptor is expressed along the entire GIT, the present of the calcium ion in human colons may enhance the adhesion of probiotic (Larsen *et al*., 2007).

Clearly avoiding pathogenic colonization and reducing the risk of potential pathogenic bacteria is beneficial to the host. The idea is that probiotic change the host's normal microflora from a potential harmful composition towards a beneficial one. Generally, this means reducing the growth of pathogens and increasing lactobacilli and/ or bifidobacteria (Ouwehand *et al*., 2002).

Further assessment of probiotic qualities, this study is focused on the adhesion and adhesion inhibition properties of *B. pseudocatenulatum* G4 with the following objectives:

1. Evaluating the effect of time, pH, calcium, and cholic acid on *B. pseudocatenulatum* G4 adhesion quality.

2. Determines the ability of *B. pseudocatenulatum* G4 to impair the adhesion of several pathogens.

CHAPTER 2

LITRETURE REVIEW

2.1 Definition of Probiotic

Recent researches regarding probiotics as beneficial bacteria, concentrate basically on Lactobacillus spp. and *Bifidobacterium spp*. Bifidobacteria represents one of the predominant groups in the gastrointestinal tract (GIT). However; the presence of bifidobacteria has decreased after vertebrate weaning and potentially pathogenic bacteria begin to predominate. Some bifidobacteria was documented today as probiotic, that is to say, bacteria which improve the properties of the intestinal flora and contribute to better health (Haschke *et al*., 1998).

In the last century, microbiologists differentiated between microbiota in the GIT of healthy individuals from those found in diseased individual. The beneficial microorganisms found in the GIT are termed as probiotic. Probiotic, meaning "for life," are microorganisms that have proven to extract healthy promoting influences in human beings and animals (Parvez *et al*., 2006). Probiotic by the general accepted definition is a live microbial feed supplement which beneficially affects the host animal by improving intestinal microbial balance (Ferna´ndez *et al*., 2003). The term 'probiotic' was first used by Lilly and Stillwed in (1965) to describe substances secreted by one microorganism which stimulate the growth of another. A powerful evolution of this definition was coined by Parker in (1974), who proposed that

probiotic are "organisms and substances which contribute to intestinal microbial balance". Fuller; then modified the definition in (1989) to "a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance". Kalantzopoulos (1997), defined probiotic as "fermented food containing specific live microorganisms or live microbial food or feed supplement, which beneficial affects the human or the host animal by improving its intestinal microbial balance". Salminen *et al*., in (1998), defined probiotic as "food which contains live bacteria which are beneficial to health". While Marteautal, (2002) defined them as preparations or components of microbial cells that have beneficial effects on the health.

The first probiotic species been introduced into research were *Lactobacillus acidophilus* by Hull *et al.,* (1984) and *Bifidobacteruim bifidum* by Holcombh *et al*., (1991). The idea was that bacteria in fermented products compete with microorganisms that were harmful to health. The bacterial population of the human GIT constitutes an enormously complex ecosystem. Most of the organisms are beneficial e.g. *Bifidobacterium* and *Lactobacillus* but some are harmful e.g. *Salamonella spp.*, *Helicobacter pylor*, *Escherichia coli*, and *Clostridium spp.* Most of the beneficial bacteria fall into the group of organisms known as lactic acid bacteria. These organisms are usually produced and consumed in the form of yoghurt, fermented milk or other fermented foods. Some of the positive impacts of lactic acid bacteria include consumption, improving intestinal tract health, strengthening the immune system, enhancing bioavailability of nutrients, and reducing the risk of certain cancers (Parvez *et al.,* 2006).