UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION AND IMMUNOMODULATION OF BIFIDOBACTERIUM PSEUDOCATENULATUM G4 AND BIFIDOBACTERIUM LONGUM BB536 IN VITRO

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FSTM 2015 3

CHARACTERIZATION AND IMMUNOMODULATION OF *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 AND *BIFIDOBACTERIUM LONGUM* BB536 *IN VITRO*

By

FATEMEH AHMADI

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

February 2015

DEDICATION

I dedicate this thesis to my mother Marziyeh and My father Mohsen who have raised me to be the person who I am today. Thank you for all the unconditional love, guidance and support that you always given me.

To my a dearest brothers Ali who has given lots of love and support to continuance my education

To my beloved husbands, Behnam Shafiei Astani, for his immense support, patience, and encourgment

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

CHARACTERIZATION AND IMMUNOMODULATION OF *BIFIDOBACTERIUM PSEUDOCATENULATUM* G4 AND *BIFIDOBACTERIUM LONGUM* BB536 *IN VITRO*

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February 2015

Chairman: Professor Dato' Mohd Yazid Abdul Manap, PhD
Faculty: Food Science and Technology

Probiotics bacteria have fundamental and important roles in the human immune system; hence they are also called ''good bacteria''. Probiotic bacteria such as *Bifidobacterium pseudocatenulatum* G4 and *Bifidobacterium longum* BB536 are natural members of the human intestinal tract that have immonodulatory activity. They stimulate dendritic cells, peyer's patches, lymphocytes such as B- lymphocytes, NK cells and T- cells. Thus, they inhibit the growth of pathogenic bacteria. In the first part of this study, the effect of colonic pH (5.7, 6.4 and 6.9) and the different concentration of hydrogen peroxide (H_2O_2) (50, and 100 µg/l) on bifidobacteria growth were evaluated.

Modulation of host immunity is one of the proposed benefits of the consumption of probiotics such as *Bifidobacterium pseudocatenulatum* G4 and *Bifidobacterium longum* BB536. On the other hand, activity of the bacteria can be changed using different pH and high concentration of H_2O_2 which will have an inhibitory effect on bacteria growth. Also, Preparation of bifidobacteria with different method such as heat treatment and sonication can enable the active substances to be accessible to relevant lymphoid cells. In another word, exposure of the immunopotentiators may have occurred as the result of leakage of intracellular components or disruption of huge complex of cell wall materials.

Two strains of *B. pseudocatenulatum* G4 and *B. longum* BB536 were examined for their survival in colonic pH. The survival rate of bifidobacteria in colonic pH did not vary among isolates. *B. pseudocatenulatum* G4 and *B. longum* BB536 maintain high viability of > 10^8 cfu/ml during 3 hours of incubation in the different pH solution. Hence, bifidobacteria can grow in different colonic pH (5.7, 6.4 and 6.9) with no reduction in the number of living bacterial cells as compared to the control group. The survival rate of bifidobacteria at H_2O_2 concentrations varied during 36 hours. The growth of two strains of bifidobacteria decreased significantly ($P < 0.05$) at 100 µg/ml H_2O_2 during 36 hours. On the other hand, these results revealed that bifidobacteria growth can be reduced by different concentration of H_2O_2 (50 and 100 µg/l) during 36 hours. High concentration of H_2O_2 causes the reduction of immune responses and the mediation of host defence. One of the most important criteria of the bifidobacteria is the ability to increase the immune system in order to increase the interleukins and

cytokines. Live and heat-treated bifidobacteria were used in this study to measure cytokine production such as IL-12, IL-6, IL-4, IFN-γ and IgA after isolation of mice Peyer's patches. Mice that were orally administered with heat-treated bacteria (*B. pseudocatenulatum* G4 and *B. longum* BB536) for seven consecutive days showed an increase in immunomodulatory responses because of the production of heat shock protein during heating process. So the bacteria can induce inflammatory cytokine by activation bacterial mechanisms by pattern-recognition receptors such as toll like receptors (TLRs) Therefore, the bifidobacteria induce more inflammatory cytokines but there are no any significant differences between these two strains of bifidobacteria. The level of IFN-γ significant increased more than other cytokines therefore, it has a stronger immunomodulatory effect ($p < 0.05$).

This study also showed that live and heat-treated bifidobacteria might induce different immune responses by increasing the level of cytokines and immunomodulatory responses through the Peyer's patches (PPs) activation. It is hypothesised that heat-treated bacteria increase the production of cytokines and immunomodulatory effect; nonetheless, comparative studies on the immunological properties that support the selection of strains of the same species for specific health benefits are needed in future.

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

PENCIRIAN DAN IMMUNOMODULATION OF BIFIDOBACTERIUM PSEUDOCATENULATUM G4 DAN BIFIDOBACTERIUM LONGUM BB536 IN VITRO

Oleh
FATEMEH AHMADY

Februari 2015

Pengerusi: Professor Dato' Mohd Yazid Abdul Manap, PhD
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Bakteria probiotik mempunyai peranan asas dan penting dalam sistem imun manusia; oleh itu bakteria itu juga dikenali sebagai "bakteria baik". Bakteria probiotik seperti *Bifidobakterium pseudocatenulatum* G4 dan *Bifidobakterium longum* BB536 terdapat secara semula jadi dalam saluran usus manusia yang mempunyai aktiviti penyesuaian imunologi. Bakteria tersebut merangsang sel-sel dendrit, tompok Peyer, limfosit seperti limfosit B-sel-sel NK dan T. Oleh itu, bakteria tersebut menghalang pertumbuhan bakteria patogen. Dalam bahagian pertama kajian ini, kesan pH kolon (5.7, 6.4 dan 6.9) dan kepekatan hidrogen peroksida (H_2O_2) yang berbeza (50, dan 100 μg/l) pada pertumbuhan bifidobakteria yang telah dinilai.

Modulasi imuniti perumah adalah salah satu manfaat cadangan penggunaan probiotik seperti *Bifidobakterium pseudocatenulatum* G4 dan *Bifidobakterium longum* BB536. Sebaliknya, aktiviti bakteria boleh ditukar dengan menggunakan pH yang berbeza dan H_2O_2 berkepekatan tinggi yang akan mempunyai kesan yang merencat pertumbuhan bakteria. Selain itu, penyediaan bifidobakteria dengan kaedah yang berbeza seperti rawatan haba dan sonikasi membolehkan sel-sel limfoid yang berkaitan untuk mengakses bahan-bahan aktif . Dalam erti kata lain, pendedahan imunopotensiator mungkin telah berlaku akibat kebocoran komponen intrasel atau gangguan kompleks besar bagi bahan dinding sel.

Dua strain *B. pseudocatenulatum* G4 dan *B. longum* BB536 telah diperiksa untuk kelangsungan hidup bakteria tersebut dalam pH kolon. Kadar kelangsungan hidup bifidobakteria dalam pH kolon tidak berbeza antara asingan. *B. pseudocatenulatum* G4 dan *B. longum* BB536 mengekalkan kebolehhidupan yang tinggi > 10^8 cfu/ml selama 3 jam pengeraman dalam larutan pH yang berbeza. Oleh itu, *bifidobakteria* boleh tumbuh dalam pH kolon yang berbeza (5.7, 6.4 dan 6.9) tanpa pengurangan dalam bilangan sel-sel hidup bakteria berbanding dengan kumpulan kawalan. Kadar kelangsungan hidup *bifidobakteria* pada kepekatan H_2O_2 berubah dalam masa 36 jam. Pertumbuhan dua strain *bifidobakteria* menurun dengan ketara ($P < 0.05$) pada 100 μg/ml H_2O_2 semasa tempoh 36 jam. Sebaliknya, keputusan ini menunjukkan bahawa pertumbuhan *bifidobakteria* boleh dikurangkan dengan kepekatan yang berbeza H_2O_2 (50 dan 100 μg/l) semasa tempoh 36 jam. Kepekatan tinggi H_2O_2 menyebabkan pengurangan tindak balas imun dan pengantaraan pertahanan perumah. Salah satu kriteria yang paling

penting bagi *bifidobakteria* adalah keupayaan untuk meningkatkan sistem imun untuk meningkatkan interleukin dan sitokin. *Bifidobakteria* hidup dan terawat haba telah digunakan dalam kajian ini untuk mengukur pengeluaran sitokin seperti IL-12, IL-6, IL-4, IFN-γ dan IgA selepas pengasingan tikus tompok Peyer. Tikus yang diberi makan dengan bakteria terawat haba (*B. pseudocatenulatum* G4 dan *B. longum* BB536) selama tujuh hari berturut-turut menunjukkan peningkatan dalam tindak balas penyesuaian imunologi disebabkan oleh penghasilan protein kejutan haba semasa proses pemanasan. Oleh itu, bakteria boleh menyebabkan radang sitokin dengan mekanisme pengaktifan bakteria oleh reseptor pengiktirafan corak iaitu tol seperti reseptor (TLR). Oleh itu, bifidobakteria mendorong lebih radang sitokin tetapi tidak terdapat apa-apa perbezaan yang ketara antara kedua-dua strain *bifidobakteria.* Tahap IFN-γ meningkat dengan ketara lebih daripada sitokin lain. Oleh itu, ia mempunyai kesan penyesuaian imunologi yang lebih kukuh ($p < 0.05$).

Kajian ini juga menunjukkan bahawa bifidobakteria hidup dan terawat haba mungkin menyebabkan tindak balas imun yang berbeza dengan meningkatkan tahap sitokin dan tindak balas penyesuaian imunologi melalui tompok pengaktifan Peyer (PP) ini. Ia dihipotesiskan bahawa bakteria terawat haba meningkatkan pengeluaran sitokin dan kesan penyesuaian imunologi; namun begitu, kajian perbandingan ke atas sifat imunologi yang menyokong pemilihan strain daripada spesies yang sama untuk manfaat kesihatan tertentu diperlukan pada masa akan datang.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank almighty ALLAH for all He has done in my life, and I praise him for everything.

I especially want to extend my truly kind and devoted supervisor Professor Dato' Dr. Mohd Yazid Abdul Manap. I would never have conducted this research without his continuing guidance and effective suggestions. His ever-ethical devotion to science became the inspiration to my research style.

I wish to express my heartfelt thanks and deepest gratitude to my dear co-supervisor Associate Professor Dr. Son Radu who provided this project for me and giving valuable time to discuss and for suggesting valuable information during the study. My grateful appreciation also goes to my co-supervisors Associate Professor Dr. Intan Hakimah Ismail and Dr. Mehrnoush Amid who was always supportive and never withheld providing whatever I needed.

I am certainly grateful and indebted to my amazing and dearest friends Dr. Kajal Sarsaifi and Dr Ali Etemad who constantly encouraged and helps me during my study.

My profound and warmest thanks and love to my mother for her love, patience, and encouragement and to my father for his constant support, love and prayers throughout my study. I am forever indebted to my parents. My heartfelt thanks are extended to my wife, for her care, endless emotional support, love and assistance throughout my course of study. Last but certainly not least, I wish to express my sincere appreciation to all those who not mentioned here that helped me to ensure the completion of my research.

I certify that a Thesis Examination Committee has met on ---- to conduct the final examination of Fatemeh Ahmadi on his thesis entitled "Characterization and Immunomodulation of *Bifidobacterium Pseudocatenulatum* G4 and *Bifidobacterium Longum* Bb536 *In Vitro*" 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 degree of Master of Science.

Members of the Thesis Examination Committee were 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 Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

%	Percentage
/	Per
<	Less than
µg	Microgram
µl	Microlitr
GI	Gastrointestinal
cfu	Colony Forming Unit
g	Gram
IgA	Immunoglobulin A
PPs	Peyer's Patches
H_2O_2	Hydrogen Peroxide
NK	Natural killer
IL	Interleukin
HCA	Heterocyclic Aromatic Amine
CpG	Cystosin Phosphtate Guanosin
DC	Dendritic Cell
APCs	Antigen Presenting Cells
MHC	Major Histocompatibility Complex
TCR	T-cell Receptor
Th	T-Helper
Treg	T-Regulatory
HLA	Human Leukocyte Antigen
TNF	Tumor Necrosis Factor
IFN	Interferon
TGF	Transforming Growth Factor
sIgA	Secretory Immunoglobulin A
UC	Ulcerative Colities
CD	Crohn's Disease
IBD	Inflammatory Bowel Disease
MC	Mast cell
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
TPY	Trypticase Phytone Yeast
MRS	Man Ragosa
v/v	Volume Per Volume
w/v	Weight Per Volume
PBS	Phosphate Buffer Salin
EDTA	Ethylenediaminetetraacetic Acid
Mm	Milimolar
rpm	Revolutions Per Minute
HCL	Hydrochloric Acid
h	Hour
min	Minute
OD	Optical Density
B.	Bifidobacteria
TLRs	Toll-Like Receptors
PAMPS	Pathogen Associated Molecular Pattern
ELISA	Enzyme-Linked Immunosorbent Assay

CHAPTER 1

INTRODUCTION

1.1 Study Background

Based on a definition approved by FAO/WHO, probiotics are ''Live microorganisms, which control human health'' (Parvez *et al.*, 2006). These good bacteria are accessible to consumers in various forms of food and dietary supplements (Holzapfel and Schillinger, 2002). They have important roles in antimicrobial activity, enhancement of barrier function, and immunomodulation, so probiotics have significant role in the immune system. For example, antimicrobial activity affects luminal pH and decreases it; and it also secretes antimicrobial peptides, which inhibit bacterial invasion (Mai and Draganov, 2009).

Intestinal flora and the associated immune response are the targets of ingested probiotics, but consequent researches and scientific studies on non-intestinal infections are limited (de Verse & Schrenzenmeir, 2002). Probiotic therapeutic bacteria compete with pathogens for nutrients and mucosal adherence and modulation of mucosal immune functions (O'sullivan *et al.,* 2005). A large number of reports have established the effect of different microorganisms for preventing and curing disorders and strengthening the human immune system. Bifidobacteria have various immunological functions such as mutagenic activity, anti tumor effects and increasing the number of macrophages (Cao *et al.*, 2011).

Immunomodulation is a process that alters the immune system of organisms by interfering with its function as in immunostimulation or immunosuppression (Corthésy *et al.*, 2007). Immune regulation is a multipart section between effectors cells and regulatory cells and there are disparities in their immunological mechanisms (Sforcin, 2007). There is some evidence to improve gastrointestinal (GI) health through valuable food including probiotics and prebiotics. They can prevent colon cancer in humans by strengthening the human immune system. Probiotics bacteria such as bifidobacteria have an immunomodulatory effect on the human immune system, so they can prevent the risk of colon cancer (Delcenserie *et al.*, 2008).

Immunotherapy is a medical term defined as the treatment of human diseases by enhancing, inducing or suppressing an immune response (Khan *et al.*, 2011). Immunotherapies that increase an immune response are classified as activation immunotherapies, and immunotherapies that decrease the immune response are classified as suppression immunotherapies (Khan *et al.*, 2011).

The human intestinal microbiota is the main cause of microbial stimulation that can both benefit and also damages human health. It is a major mediator that contributes to the increase and improvement of the immune system, oral tolerance and immunity (Arrieta *et al.*, 2010). Therefore, the microbiota may be responsible for identifying inflammatory reaction in allergy and in bowel diseases. Probotics such aas bifidobacteria can induce an inflammatory process by stabilizing the intestinal

microbiota environment. Immunomodulation and immunostimulation are the major mechanisms of probiotics that work against many harmful bacteria (Yan and Polk, 2010).

Bifidobacteria are natural members of the human intestinal tract and they occur in concentrations of 10^9 to 10^{11} CFU g^{-1}. Bifidobacteria also have an important role in product fermentation. They are referred as probiotics bacteria because of their beneficial role in the human intestine such as raising protein digestion, improving intestinal microflora, human immune system activation and prevention of colon cancer (Gourbeyre *et al.*, 2011).

Bifidobacterium pseudocatenulatum G4 is one of the common bacterial species which are isolated from infant faeces in Malaysia (Kabeir *et al.*, 2005; Yazid *et al.*, 1998). It is well adapted to the colonic environment as reflected by its ability to adapt to low pH and high concentration of bile salts. Studies have demonstrated that the probiotic strain G4 has been identified as a safe probiotic that can be used in functional food for human consumption (Gourbeyre *et al.*, 2011). *Bifidobacterium longum* is an anaerobic species that can be found in infant's intestine. They prevent the growth of gram negative bacteria such as lactic acid bacteria by producing lactic acid and absorbing the sugar complex compound in human breast milk (Fooks and Gibson, 2002).

1.2 Problem Statements

Modulation of host immunity is one of the proposed benefits of the consumption of probiotics such as *Bifidobacterium pseudocatenulatum* G4 and *Bifidobacterium longum* BB536. On the other hand, activity of the bacteria can be changed using different pH and H_2O_2 concentration which will have an inhibitory effect on bacteria growth.

Also, Preparation of bifidobacteria with different method such as heat treatment and sonication can enable the active substances to be accessible to relevant lymphoid cells. In another word, exposure of the immunopotentiators may have occurred as the result of leakage of intracellular components or disruption of huge complex of cell wall materials. On the other hand, preparation of heat-treated bifidobacteria might induce the thermal denaturation of proteins on the cell surface of bacteria. Although the chemical changes in proteins cannot be detected, heat-treated bifidobacteria can induce immunomodulatory effect. It is hypothesised that heat-treated bacteria increase the production of cytokines and immunomodulatory effect; nonetheless, comparative studies on the immunological properties that support the selection of strains of the same species for specific health benefits are needed in future.

1.3 Significance of the Present Study

Bifidobacterium pseudocatenulatum G4 and *Bifidobacterium longum* BB536 are becoming increasingly important as source of probiotic in treatment of infection and diseases. This study will provide information on immunomodulatory activity of probiotic bacteria such as *Bifidobacterium pseudocatenulatum* G4 and *Bifidobacterium longum* BB536. Findings of study will provide some insight to the treatment of immune-related disease such as infectious gastroenteritis, allergic disease and inflammatory bowel disease.

1.4 General Objective

The main purpose of this study was to determine the characteristics and immunomodulation function of *Bifidobacterium pseudocatenulatum* (G4) and *Bifidobacterium longum* BB536 *in vitro*.

1.5 Specific Objectives

1. To evaluate the survival of *B. pseudocatenulatum* G4 and *B. longum* BB536 characteristics in different colonic pH and H_2O_2 concentrations.

2. To compare the immunomodutory effect of live and heat- treated *B.pseudocatenulatum* G4 and *B. longum* BB536 using animal feeding study.

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