

**EVALUATION OF PROBIOTICS ON
CONSTIPATION**

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UNIVERSITI SAINS MALAYSIA

2019

EVALUATION OF PROBIOTICS ON CONSTIPATION

by

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**Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science**

May 2020

ACKNOWLEDGEMENT

In the name of Allah, The Most Gracious, The Most Merciful, Shalawat and Salam
for the Holy Prophet Muhammad P.B.U.H.

First and foremost, I would like to express my gratitude to my supervisor, Professor Dr. Liong Min Tze for her guidance, patience, understanding and most importantly, she has provided me with positive encouragement as well as warm spirit to complete this thesis. It has been a great pleasure and honor for me to have her as my supervisor. I would like to extend my heartfelt gratitude to my dear lab mates; namely Lee Ching, Yan Yan, Amy Lau, Chuah, Jia Sin, Cheng Chung, Abegal and Fatin for their guidance, help and all the supports given during the ups and downs we have had throughout my study. Above all, I would like to thank my beloved family for their endless support, love as well as encouragement at the time when I need the most.

So, I thank you to all those who were kind to help and provide me with guidance during all this time. Whom I may not mention their name, but I would never forget how happy and grateful I am. Truly I am thankful.

Thank you.

MOHAMAD HAFIS BIN JAAFAR

2020

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$$\text{Fecal moisture content} = \frac{(\text{fecal wet weight} - \text{fecal dry weight})}{\text{fecal wet weight}} \times 100$$

LIST OF ABBREVIATIONS

· OH	Hydroxyl radical
A/G	Albumin/globulin ratio
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMDI	Advanced Medical and Dental Institute
ANOVA	Analysis of variance
API	Analytic profile index
AQP3	Aquaporin 3
ARF	Animal Research Facilities
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Bax	BCL2 Associated X
Bcl-2	B-cell lymphoma 2
BMI	Body mass index
BSF	Bristol Stool Form
CASP3	Caspase-3
cDNA	Complementary DNA
CFS	Cell-free supernatant
CFU	Colony-forming unit
COX-2	Cyclooxygenase-2
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPPH	Diphenylpicrylhydrazyl
DPX	Dibutyl phthalate-polystyrene-xylene
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organisation of the United Nations
FeCl ₃	Ferric chloride
FISH	Fluorescence in situ hybridization
FOS	Fructo-oligosaccharide
FRAP	Ferric reducing antioxidant power
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Guanine-cytosine
GC-MS	Gas chromatography-mass spectrometry
GOS	Galacto-oligosaccharides
H & E	Hematoxylin and Eosin
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
HAPCs	High-amplitude propagated contractions
HCl	Hydrochloric acid

hTERT	Telomerase consists of protein subunit
IACUC	Institutional Animal Care and Use Committee
IFCC	International Federation for Clinical Chemistry
IL-1 β	Interleukin 1 beta
IL-8	Interleukin 8
Inc.	Incorporated
INU	Inulin
K ₂ EDTA	Dipotassium EDTA
LAB	Lactic acid bacteria
MAP	Mitogen-activated protein
MgCl ₂	Magnesium chloride
MMSE	Mini-Mental State Examination
mRNA	Messenger RNA
MRS	de Man-Rogosa-Sharpe
mtDNA	Mitochondrial DNA
MUC3	Mucin 3
NB	Nutrient broth
NCBI	National Center for Biotechnology Information
NF κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHI	National Health Insurance
NIH	National Institutes of Health
O ₂ ⁻	Superoxide anion
OD	Optical density
OTUs	Operational taxonomic units
p53	Tumor protein p53
PCR	Polymerase chain reaction
PGE ₂ .EP ₂	Prostaglandin E2 receptor 2
PKC	Protein kinase C
PTGER	Prostaglandin E Receptor
QIIME	Quantitative insights into microbiota ecology
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
rRNA	Ribosomal RNA
SCFA	Short-chain fatty acids
SCFA	Short-chain fatty acid
scg	Single copy gene
Sdn. Bhd.	“Sendirian Berhad”
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
sPLS-DA	Sparse partial least square discrimination analysis

T/S	Telomere to single-copy gene ratio
TE	Tris-EDTA buffer
TGF- β	Transforming growth factor beta
Th	T helper
TNF- α	Tumor necrosis factor alpha
TPTZ	Tripyridyltriazine
Treg	Regulatory T
TRFs	Terminal restriction fragments
Tris-HCl	Tris hydrochloride
TSB	Tryptic soy broth
UK	United Kingdom
USA	United States of America
USD	United States dollar
USM	Universiti Sains Malaysia
UV	Ultraviolet
VFA	Visceral fat area
WHO	World Health Organisation

LIST OF SYMBOLS

<	less than
>	more than
%	percent
α	alpha
β	beta
$^{\circ}\text{C}$	degree Celsius
γ	gamma
g	gravitational force
h	hour
L	liter
min	minute
mg	milligram
M	molar
nm	nanometer
®	registered trademark
s	second
U	enzyme unit
v/v	volume over volume
w/v	weight over volume

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PENILAIAN PROBIOTIK TERHADAP SEMBELIT

ABSTRAK

Sembelit merujuk kepada kesukaran dalam proses penyahtinjaan. Keadaan berkaitan dengan pergerakan usus yang jarang berlaku untuk mengosongkan sistem gastrousus yang pada akhirnya menghasilkan tinja yang keras. Walau bagaimanapun, sembelit bukanlah akibat fisiologi penuaan yang normal: kurang pergerakan, ubat-ubatan, dan kesan penyakit lain mungkin menyebabkan peningkatan kekerapan berlaku pada orang tua. Oleh itu, kajian ini bertujuan untuk lebih memahami dan menilai kesan potensi *Lactobacillus* sp. sebagai probiotik dan mekanismanya untuk mengurangkan sembelit sebagai terapeutik halangan alternatif kepada *lactulose* untuk rawatan sembelit kepada model haiwan tua. Sifat probiotik *L. fermentum* DR9, *L. plantarum* DR7, *L. sakei* Probio65 dan *L. casei* Y telah dicirikan dalam kajian ini. Pencirian fisiologi menggunakan sistem *API 50 CHL* telah menunjukkan profil pengasidan yang berbeza antara *strain* bakteria asid laktik (*LAB*) yang lain. *Strain LAB* telah dinilai untuk keupayaan mereka untuk menggunakan prebiotik, dimana *GOS* telah digunakan oleh *L. plantarum* DR7, yang mana ia telah menunjukkan pertumbuhan yang ketara berbanding *strain LAB* yang lain ($p < 0.005$). Supernatant bebas sel (*CFS*) tidak-netral dari *L. fermentum* DR9, *L. plantarum* DR7, *L. sakei* Probio65 dan *L. casei* Y telah menunjukkan penahanan pertumbuhan yang ketara terhadap kedua-dua antagonis *E. coli* dan *Salmonella* sp. ($p < 0.005$). Kuasa pengurangan ferik *CFS* dari *L. fermentum* DR9 dan *L. plantarum* DR7 adalah tinggi yang ketara berbanding *strain LAB* yang lain ($p < 0.005$). Penilaian kesan laksatif *LAB*

menggunakan in-vivo telah dilaksanakan pada tikus *Sprague Dawley* jantan penuaan pramatang yang dirawat dengan *L. fermentum* DR9 dan *L. plantarum* DR7 (10 log CFU / kg) melalui pemberian oral dan pengurangan sembelit pengaruh-*loperamide* telah diperhatikan. Peningkatan saiz pukal dan sedikit melembutkan tinja apabila pemberian *L. fermentum* DR9 dan *L. plantarum* DR7, digambarkan menggunakan *Bristol Stool Chart*. Tambahan pula, kandungan kelembapan tinja yang lebih tinggi didapati selepas pemberian *L. fermentum* DR9 dan *L. plantarum* DR7 apabila dibandingkan dengan kumpulan kawalan *loperamide* ($p < 0.005$). Terdapat bukti bahawa kepelbagaian mikrobiota dalam usus telah berubah dengan pemberian *L. fermentum* DR9 dan *L. plantarum* DR7. Klustering skor sPLS-DA yang kontras dalam kelimpahan mikrobiota di peringkat keluarga dan genus telah digambarkan selepas pemberian *L. fermentum* DR9 dan *L. plantarum* DR7. Analisis metabolit larut air dari tinja tikus telah menunjukkan pengurangan ketara kepekatan *threonine* selepas pemberian *L. fermentum* DR9 dan *L. plantarum* DR7 ($p < 0.005$). Manakala, tiada perubahan dalam kepekatan *SCFA*. Penyiasatan lanjut menunjukkan bahawa tiada kesan pada gerakan gastrousus yang diuji melalui masa perjalanan *carmine* dan nisbah perjalanan arang di antara kumpulan ujikaji. Walau bagaimanapun, analisis *histopathology* telah menunjukkan jumlah sel goblet yang ketara tingginya dalam sampel colon dari kumpulan rawatan *L. fermentum* DR9 dan *L. plantarum* DR7 ($p < 0.005$). Oleh itu, sebagai kesimpulan kajian ini menggambarkan potensi *Lactobacillus* sp. sebagai probiotik untuk memperbaiki kesan buruk sembelit pengaruh-*loperamide* yang mempamerkan laksatif osmotik tanpa menyebabkankan cirit-birit bergantung kepada jenis strain yang digunakan.

EVALUATION OF PROBIOTICS ON CONSTIPATION

ABSTRACT

Constipation refers to difficulties in the defecation process. A condition associated with infrequent bowel movement in emptying the gastrointestinal system that ultimately produces a hardened fecal matter. However, constipation is not the physiologic aftermath of normal aging: decrease mobility, medication, and other comorbid medical conditions may all lead to its increased prevalence in older adults. Thus, the present study aimed to better understand and evaluate the potential effects of *Lactobacillus* sp. as probiotic and its mechanism to alleviate constipation as the alternative therapeutic intervention for constipation treatment to lactulose in the aging animal model. The probiotic properties of *L. fermentum* DR9, *L. plantarum* DR7, *L. sakei* Probio65 and *L. casei* Y were characterized in the present study. The physiological characterization using the analytic profile index (API) 50 CHL system had shown a distinct acidification profile among lactic acid bacteria (LAB) strains. The LAB strains were assessed for their ability to utilize prebiotic, where galactooligosaccharides (GOS) were utilized by *L. plantarum* DR7, as it showed significant growth compared to other LAB strains ($p < 0.005$). Non-neutralize cell-free supernatant (CFS) from *L. fermentum* DR9, *L. plantarum* DR7, *L. sakei* Probio65 and *L. casei* Y showed to significantly suppresses the growth of both antagonists *E. coli* and *Salmonella* sp. ($p < 0.005$). The ferric reducing power of CFS from *L. fermentum* DR9 and *L. plantarum* DR7 was significantly higher than other LAB strains ($p < 0.005$). In vivo laxative effect evaluation of LAB was performed in premature

aging male Sprague Dawley rat treated with *L. fermentum* DR9 and *L. plantarum* DR7 (10 log CFU/kg) via oral administration and the alleviation of loperamide-induced constipation was observed. Increased fecal bulk and slight softening of fecal upon administration of *L. fermentum* DR9, and *L. plantarum* DR7 in loperamide-induced constipation group was described using the Bristol Stool Chart. Coupled with a higher fecal moisture content was observed following the administration of *L. fermentum* DR9 and *L. plantarum* DR7 ($p < 0.005$). There is evidence that the gut microbiota diversity has changed with the administration of *L. fermentum* DR9 and *L. plantarum* DR7. Illustrated by contrast clustering of sparse partial least square discrimination analysis (sPLS-DA) score in the microbiota abundance at the family and genes level after administration of *L. fermentum* DR9 and *L. plantarum* DR7. The water-soluble metabolite analysis from rat's fecal showed a significant reduction of threonine concentration after administration of *L. fermentum* DR9 and *L. plantarum* DR7 ($p < 0.005$). Whereas, there are no changes in short-chain fatty acid (SCFA) concentration. Further investigation showed that there was no effect on gastrointestinal motility tested via carmine travel time and charcoal travel ratio among the experimental group. However, the histopathological analysis showed a significant high goblet cell count in the colon sample from *L. fermentum* DR9 and *L. plantarum* DR7 treatment group ($p < 0.005$). Thus, to conclude the present study illustrated the potential of *Lactobacillus* sp. as probiotics to ameliorate the adverse effect of loperamide-induced constipation, which exhibits osmotic laxatives without causing diarrhea in a strain-dependent manner.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Constipation is a common gastrointestinal symptom in which refer to difficulties in the defecation process. A condition commonly associated with infrequent movement in emptying the gastrointestinal system that ultimately produce a harden fecal matter. The symptom typically synonymous with abdominal pain, bloating, and the sensation of unsatisfactory expulsion of fecal matter from the rectum. A healthy digestive system, according to physicians, is range between three bowel movement per day and/or three per week for healthy adults. Often, a higher frequency in babies with around four bowel movements whereas usually around three per day in younger human. Although the occasional constipation is very common in general population due to its multifactorial causes, the prolong of constipation episode however can interfere with the ability to go about daily tasks and reduces the quality of life (Forootan *et al.*, 2018).

The occurrence of constipation has been reported to be more than 30 % of the general population with the elderly adult being mostly affected. The prevalence of constipation episodes increases with age, where 3 out of 100 adults ages 60 and older in the United State population have suffered from constipation (Andromanakos *et al.*, 2006). Thus, prolonged medical attention as well as long-term care in the infirmary or nursing homes due to the frequency of constipation episodes among these elderly patients (Bouras and Tangalos, 2009). The difficulty in fecal passage along the

gastrointestinal tract, the expulsion of hard or bulging fecal matter, and the need for excessive force or manipulation during fecal excretion, however, are not physiologic aftermath of normal aging. The pathogenesis of this common gastrointestinal problem is multifactorial – centered on anomalies anatomy of the body, disturbance in the hormone balance, genetic predisposition, inadequate fluid intake, lack of mobility, poor fiber consumption, side effects of medications or socioeconomic status, *et cetera* (Benninga *et al.*, 2005).

For one, it is impossible to put a stopper on the decaying process of our delicate body, but the episodes of constipation can surely be prevented in some cases. The conventional treatment for constipation that primarily addresses dietary advice and toilet habits education, which has been reported to not always bring about the desired improvement and satisfactory relief (Pohl *et al.*, 2008). Usually, simple changes in lifestyle and diet as the non-pharmacological treatment does not always improve constipation. Thus, the recommendation to use the laxative substance to alleviate the constipation episodes come to play. The constipation treatment and management come with varying degrees of efficacy as well as cost, which bring about a substantial economic impact on the patients (Rao and Go, 2010). The laxative, a heterogeneous group of drugs or substances which includes a wide spectrum of products that a difference in pharmacological characteristics and the mechanism of actions. Yet most laxatives have a common ground of stimulating the excretion process or softening the density of fecal matter in order to facilitate evacuation. Nevertheless, the application of laxatives must be tailor-made for each patient with meticulous attention to comorbid medical conditions, medication interaction and its adverse, effects especially in the older adults (De Giorgio *et al.*, 2015).

Over the last two decade, there has been growing attention on both basic and clinical science in probiotic due to its ability to give benefits to human. Probiotic according to World Health Organization refers as “live-microorganisms which, when administered in adequate amounts, confer health benefits on the host. Probiotic such as lactic acid bacteria and *Bifidobacteria* have been proven to give diverse therapeutic benefits to human. Evident suggests that probiotics may reduce constipation related condition as an alternative treatment. Previous studies have shown that this viable microorganism found to be useful in alleviating certain constipation condition among adults (Agrawal *et al.*, 2009; Higashikawa *et al.*, 2010) with limited data. Thus, probiotic as an acute treatment for constipation is yet to be proven while the clinical application is still considered investigational. The present study aims to investigate the potential of selected *Lactobacillus* sp. in the therapeutic intervention for constipation treatment to milk sugar lactose (lactulose) in an aging model.

1.2 Research Objective

The main objective of the present study was to better understand and analyze the potential benefits of *Lactobacillus* sp. as well as its mechanism to alleviate constipation in an aging model. The specific objectives were as follows:

1. To characterize the probiotic properties of *L. fermentum* DR9 and *L. plantarum* DR7 as a putative probiotic strain along with commercial probiotic; *L. sakei* Probio65 and *L. casei* Y.
2. To analyze the laxative effect of *L. fermentum* DR9 and *L. plantarum* DR7 on D-galactose induced aging rat model, as well as to evaluate the gut microbiota and metabolite changes associated with constipation conditions.
3. To determine the modulatory effect on the laxative attribute of *L. fermentum* DR9 and *L. plantarum* DR7 administration on gastrointestinal motility and intestinal morphology in loperamide-induced constipation.

CHAPTER 2

LITERATURE REVIEW

2.1 Lactic Acid Bacteria

Lactic acid bacteria are a taxonomical order of *Lactobacillales* which delineate as acid tolerance, gram-positive with either bacilli (rod) or cocci (spherical) shaped bacteria with a DNA base arrangement of low GC (less than 53 mol % guanine-cytosine content). These bacteria generally are non-respiratory but aerotolerant, non-sporulating and lack catalase which undergone carbohydrate homolactic fermentation to produce metabolic end product primarily lactic acid. “Lactic acid bacteria” (LAB), the term formerly used constantly allude to “milk-souring organism” at the dawn of the 20th century. The similitudes between milk-souring organisms and bacteria producing lactic acid were soon discovered. A detailed volume wrote by Orla-Jansen in 1919 which outline the modern fundamental of LAB classification. The genus *Enterococcus*, *Lactobacillus*, *Lactococcus*, and *Streptococcus* constitute a representation of the order, are the commoner in the food manufacturing industry which also as probiotic candidates (Klaenhammer and Kullen, 1999; Tamime, 2003). However, interest in the study of probiotic dated back to Henri Tissier, a French pediatrician. Early in his work had isolated a bacterium characterized by a peculiar, Y-shaped morphology in the intestinal microflora of breast-fed baby. Subsequently, in 1906, he discovered that junior with diarrhea had in their fecal a low number of these “bifid” bacteria which conversely, plenteous in healthy children. Henry later recommended that the administration of *Bifidobacteria* to subject suffer from diarrhea to encourage rehabilitate a healthy gastrointestinal microflora (Tissier, 1906). At this

time, the Russian born Nobel laureate, Élie Metchnikoff discovered that the consumption of a fermented dairy product – yogurt containing lactic acid bacteria at regular manner was incidental with enhanced health in Bulgarian farmer populations and had lived a longer life. In 1907, Metchnikoff wrote “The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes” in his volume based on his discovery- *The Prolongation of Life* (Metchnikoff, 1907). The book contains the first scientific description of the enormous potential to improve human health through consuming substances, which favorably alter the gastrointestinal microflora – a concept now widely known as the probiotic principle (Gogineni *et al.*, 2013).

Hence, the work of Henri and Metchnikoff were the earliest to make scientific postulation regarding the probiotic used of the bacteria ahead of the time even before the terminology that we used and know today was coined. It was later the term “probiotic” was devised, in 1960 to name substances produced by the microorganisms which promoted the growth of another microorganism (Lilly and Stillwell, 1965). Since then, the increasing interest in probiotic had mainly pivoted on the microbial nature of probiotics on improving gastrointestinal health. The term was derived from Latin preposition *pro* and Greek noun βίος (*bios*), exactly means “for life” (Hamilton-Miller *et al.*, 2003) and the definition has been redefined with time. Until recently, The Food and Agriculture Organisation of the United Nations (FAO) and the World Health Organisation (WHO) has defined probiotics as “live microorganisms, which when administered in adequate amounts, confer a health benefit on the host” (FAO/WHO, 2006).

Over the centuries ago today, substantial research and development had opened up doors for the utilization of probiotics, also emphasize its remedial benefits such as prevention and cure for certain diseases. Probiotics was so enticing that the commercial and industrialization exploitation instantly followed their scientific work, which recently gained tremendous demand in the global market (Ringel *et al.*, 2012). In 2015, the global retail market value for probiotics products worth USD 41 billion large after growing an impressive 64 percent from 2017, worth USD 14.9 billion. In fact, the market size on probiotics products is expected to exceed USD 64 billion large by the year 2023 (Dover, 2016; Feldman, 2016). A broad member of the genera *Bifidobacterium* and *Lactobacillus* are commonly used, but not exclusively, as probiotic microorganisms which currently available to the customer worldwide (de Simone, 2018). The ecological interactions on gastrointestinal microflora of the probiotic supplement concept, are imperative to better understand the relevance for human health.

Probiotics have been studied for decades, mainly accentuating on promoting general gastrointestinal health like alleviating intestinal disorder and preserving a healthy microflora inhabitant (Verna and Lucak, 2010). Lately, with technological advances, numerous studies had shown that probiotics exert health-promoting effect stretch over gastrointestinal wellbeing. A complex interaction between limbic system located in the brain and enteric microbiota in deep gastrointestinal was found exist, denominate as “gut-brain axis” (Rhee *et al.*, 2009). Understanding into gut-brain crosstalk have uncovered an intricate bidirectional communication network that ensures an appropriate manner to preserve gastrointestinal homeostasis and multitude likelihood effects in higher cerebral abilities (Carabotti *et al.*, 2015).

Although there have been a great number of scientific evidences on remedial benefits of probiotics, it is imperative to note that the effect is strain-specific in action and neither positive nor negative reaction of one probiotic strain should not be extrapolated to another strain (McFarland *et al.*, 2018). Therefore, accurate taxonomical characterization and identification of the interest bacterial strain are paramount for the refinement in experimental design to predict the probiotic effect on a specific medical condition

2.1.1 Probiotics; Health and Remedial Benefits

Probiotic represent a heterogeneous group of microorganisms from genera *Bifidobacterium* and *Lactobacillus* that possess a wide range of health and remedial benefits. Initially, probiotics are known to exert health benefits toward the host exclusively via altering gastrointestinal microflora as well as preserving gastrointestinal homeostasis. The gastrointestinal modulation action of probiotics in alleviating intestinal disorder includes antibiotic-associated diarrhea, chronic inflammation, irritable bowel disease, infectious diarrhea, and lactose intolerance (Hibberd *et al.*, 2015; Khalesi *et al.*, 2019). The mechanistic probiotic function lies with its ability to exert antimicrobial factor, enhance intestinal barrier function, and immunomodulatory effects.

The capacity of probiotic strain to suppress growth or/and eliminate pathogens is via the production of antimicrobial compounds, including bacteriocins or microcins and short-chain fatty acids (Lebeer *et al.*, 2008; Collins *et al.*, 2017). In particular, the present of *Lactobacillus reuteri* (*L. reuteri*) via twice-daily administration found to

protect colonization and ameliorate disease from enterohemorrhagic *Escherichia coli* (*E. coli*) in germfree murine study (Eaton *et al.*, 2011). Toxin production by antagonist *E. coli* and toxin transfer from the intestinal cavity to the blood circulatory was found to be vulnerable with oral administration with *Bifidobacterium longum* (Yoshimura *et al.*, 2010). However, there are no significant in *E. coli* cell count in the fecal sample among *Bifidobacterium*-associated experimental groups. Bacterial cell-cell communication (Kaper and Sperandio, 2005; Oleskin and Shenderov, 2016), as well as competition and cooperation for nutrients (Sonnenburg *et al.*, 2006; Desai *et al.*, 2016) apart from the materialization of antimicrobial compounds by probiotics strain demonstrate a multitude likelihood mechanism of microbe-microbe interaction to exert antimicrobial factor.

Probiotic bacteria have been proven to enhance intestinal barrier function, which includes metabolic interaction, induction of mucins, and tight junction preservation. Metabolic phenotype interaction between probiotic bacteria and the host could regulate the essential nutritive capacity of the gastrointestinal tissue. A holistic system investigation demonstrates the metabolic effects of daily administration to either *L. paracasei* NCC2461 or *L. rhamnosus* NCC4007 in germfree murine colonized with human infant microflora model. An integrated top-down systems biology approach used to observe changes in a broad spectrum of pathways outcomes such as amino acid metabolism, organic methylamines, and short-chain fatty acids (SCFAs) like butanoate of the samples; taken from the cecum, fecal, ileum, liver, plasma, and urine. Changes in microbiota profile after probiotic administration has shown to alter hepatic lipid metabolism, reduced plasma lipoprotein, increased triglyceride levels, and seemingly excite glycolysis (Martin *et al.*, 2008; Heinken and

Thiele, 2015). Probiotics have shown to strengthen the gastrointestinal mucosa via increasing extracellular MUC3 mucin expression or secretion by goblet cells. The adherent of *Lactobacillus* spp. had shown to stimulate the MUC3 mucin expression in human intestinal epithelial cells (Mack *et al.*, 2003; Bron *et al.*, 2017). Furthermore, probiotic administration could enhance tight junction integrity which recent study demonstrated that probiotic-secretory proteins compound from *L. rhamnosus* protects the intestinal epithelial tight junctions from disruption induced by hydrogen peroxide, via a PKC- and MAP kinase-dependent mechanism (Seth *et al.*, 2008). Thereby, decreasing gastrointestinal epithelial paracellular permeability to intraluminal pathogens and toxins. A significant decreased in epithelial membrane permeability after *L. plantarum* and *L. rhamnosus* treatment of the cell indicates a “strengthening” of the gastrointestinal barrier (Blackwood *et al.*, 2017).

Immunomodulatory effect of probiotic bacteria in the gastrointestinal system, which includes promoting tolerogenic dendritic cell as well as regulate T cell phenotypes, suppressing inflammatory cytokine production, and intensify natural killer cell activity (Ng *et al.*, 2008). Treatment with *L. acidophilus* A4 extracts had shown to induce a significant upregulation of the mRNA levels of IL-1 β , IL-8, and TNF- α which paramount regulatory factor in gastrointestinal immune system responds (Kim *et al.*, 2008). Regular consumption of probiotic bacteria has shown to modulate innate immune cells such as B cells, T helper 1 (Th1), Th2, Th17 and regulatory T (Treg) cells which draws parallel to general health and the pathogenesis of immune disorders. Increased in the abundance of probiotic bacteria within the gastrointestinal lumen revealed itself to ameliorate immune dysfunction also associated conditions like

allergies, atopic dermatitis, and multiple sclerosis. (Kiseleva and Novik, 2013; Dargahi *et al.*, 2018).

The expensive technological advancement in research tools has not only to broaden the knowledge of health and remedial benefits of probiotics also germinate new interest for a more comprehensive study related to anti-cancer properties, metabolic and neurodegenerative diseases. The genetic potential of gastrointestinal microbiota analyses is imperative toward understanding its impact on human health and wellbeing. The deoxyribonucleic acid (DNA) sequencing coupled with metagenomic sequencing and proteomics techniques unveiled that the number of microbes in the human gastrointestinal system is 10 times more than our mammalian cells. The genes set carried in these microorganisms are approximately 150 times larger than the entire human genome which vastly accepted as the ‘second genome’ in the human body, recently (Qin *et al.*, 2010).

The gravity of gastrointestinal microbiota on host’s general health and wellbeing has been demonstrated using germfree murine model (Cénié *et al.*, 2014). Considering its significant part in maintaining the balance of gastrointestinal microflora, probiotics contribution on general host health and wellbeing is of great interest. Probiotics have been demonstrated in a countless study through intricate interrelated mechanisms to promote health and remedial benefits, are summarized in the table 2.1.

Table 2.1 Health and remedial claimed benefits of probiotics.

Application	Probiotic strains	Health and remedial claimed	Reference
Allergic and recurrent	<i>Bacillus clausii</i> , <i>L. rhamnosus</i>	Modulates cytokine profiles (by increases IL-10 and TGF- β) which induces Treg cells; prevents recurrent respiratory infections and shortens duration; Accelerates oral tolerance acquisition in cow's milk allergic; limit T-helper (Th)/Th2 bias	Ciprandi <i>et al.</i> , 2005; Cosenza <i>et al.</i> , 2015
Anti-cancer	<i>L. acidophilus</i> , <i>L. rhamnosus</i> ,	Reduce risk of colon cancer; reduces the expression of β -catenin and the inflammatory proteins COX-2, NF κ B-p65, and TNF α ; the anti-apoptotic protein Bcl-2, but increased the expression of the pro-apoptotic proteins Bax, casp3 and p53	Gamallat <i>et al.</i> , 2016; Banna <i>et al.</i> , 2017
Eczema and skin health	<i>L. bulgaris</i> , <i>L. casei</i> , <i>L. johnsonii</i>	Enhancing the skin natural defence barriers; produce antimicrobial peptides that benefit immune responses and eliminate pathogens; improve acne symptoms, atopic dermatitis; protect skin against ultraviolet radiation	Al-Ghazzewi and Tester, 2014; Roudsari <i>et al.</i> , 2015
Genitourinary health	<i>L. acidophilus</i> , <i>L. fermentum</i> , <i>L. rhamnosus</i>	Reduce the risk of bladder and vaginal infections; relieve pain and complications associated with infection; restore healthy microflora of the vagina; prevent urogenital infections	Reid <i>et al.</i> , 2001; Williams, 2010

Application	Probiotic strains	Health and remedial claimed	Reference
Neurodegenerative impairment	<i>Bifidobacterium bifidum</i> (<i>B. bifidum</i>), <i>B. longum</i> , <i>L. helveticus</i>	Improve mood and psychological distress; reduced anxiety-like behaviour; significant improvement in the Mini-Mental State Examination (MMSE) score in Alzheimer's patients	Messaoudi <i>et al.</i> , 2011; Akbari <i>et al.</i> , 2016
Obesity and weight loss	<i>L. rhamnosus</i> , <i>L. gasseri</i>	Significant reductions in fat mass deposit and circulating leptin concentrations; induces weight loss; reduction in body mass index (BMI), abdominal VFA, waist and hip circumferences	Sanchez <i>et al.</i> , 2014; Sáez-Lara <i>et al.</i> , 2016
Oral health	<i>L. reuteri</i> , <i>L. casei</i> , <i>Streptococcus salivarius</i> , <i>Weissella cibaria</i>	Reduce oral volatile sulfur compounds levels; improve halitosis, chronic periodontitis and maintain oral microflora ecology	Burton <i>et al.</i> , 2005; Meurman and Stamatova, 2007; Allaker and Stephen, 2017

2.1.2 Properties of Probiotic Strains

The collaborative effort between the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) Consultation for the refinement of the probiotic's scientific evaluation. To be classified as a potent probiotic, not only the bacteria strain must exert therapeutic benefits on the host, but selection criteria-origin and functional aspects are an absolute fundamental. The bacteria strain must be characterized and identified by using phenotypic strategy first, upon observation as potentially beneficial in which could be conducted using analytic profile index (API), molecular techniques-16s rRNA or specific-specific PCR method (Fijan, 2014; Ceapa *et al.*, 2015). Then, the genotypic strategy could be performed using the most robust phylogenetic identification analysis – 16s rRNA sequencing to unveil accurate taxonomical details up to the species level. The use of 16S rRNA gene sequences as the housekeeping genetic marker due to its presence in nearly all bacteria, sizeable for bioinformatics purposes, combine with its sequences over time has not changed which gives this method an edge of great accuracy and feasibility, particularly for mundane isolation work (Janda and Abbott, 2007; Bayili *et al.*, 2019). The species-specific identification of unidentified probiotic bacteria employs species-specific primer/probes alignment derived from 16S or 23S rRNA sequences. This assay offers a rapid, sensitive, and highly specific alternative to conventional method which paramount in the identification of bacteria in clinical specimens since many therapeutic benefits of probiotics are strain-specific dependence (Dickson *et al.*, 2005; Archer and Halami, 2015).

The biochemical-based analysis for bacteria strains physiological characterization using analytic profile index (API) system from BioMerieux, France. A homogenize bacterial culture was subjected to the commercial kit, API 50 CHL system to reveal the carbohydrate-fermentation and esculin hydrolysis fingerprint. The API 50 CHL system acidification which indicated by color changes is one of the most preferred phenotypic procedures used, it gives between 78.2 – 99.9 % fermentation profile accuracy (Nigatu, 2000; Moraes *et al.*, 2013).

The term prebiotics are defined as, “a 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 that have the potential to improve host health” (Gibson and Roberfroid, 1995; Gibson *et al.*, 2017). It has been observed that the availability of carbohydrate compounds that escape metabolic digestion as well as absorption in the small bowel segment of the gastrointestinal system, in which become a great influence for microflora establishment in the colon (Kaplan and Hutkins, 2000). The fermentable carbohydrate mainly used as prebiotics include inulin, oligosaccharide, resistant starch or wheat bran (De Vrese and Schrezenmeir, 2008). A group of naturally occurring compounds, inulin is made of polysaccharides found in plants which often extracted from chicory for the industrial purpose (Roberfroid, 2005). It consists of a repetitive fructosyl moiety and chain-terminating glucosyl moieties which are linked by $\beta(2,1)$ bonds, a heterogeneous collection of fructose polymers (Barclay *et al.*, 2016). Oligosaccharide in other hand, is a short saccharide polymer compound made up by small number simple sugar (monosaccharides). Naturally occurring oligosaccharides consist of glycosidic residues such as fructose in

fructo-oligosaccharide (FOS) and galacto-oligosaccharides (GOS) (Grimoud *et al.*, 2010).

FOS occurs naturally, in which sometimes called oligofructose are commonly used as prebiotic as well as an alternative sweetener with high commercial demand for healthier and calorie-reduced foods. It is made of a mixture oligosaccharides polymer consisting a glucose monomer and varying number of fructose moieties linked by $\beta(2,1)$ glycosidic bond (Spiegel *et al.*, 1994). GOS are produced through the enzymatic transgalactosylation of lactose with a terminal glucose unit, originate from bovine milk. The glycosidic bonds configuration make them resilient against α -amylase hydrolysis by salivary and gastrointestinal digestive as well as pancreatic enzymes (Jeurink *et al.*, 2013). These soluble, non-digestible and fermentable fibers have to be present as a part of the food's ingredients in amounts of 30 – 60 mg/g for solid food and 15 – 30 mg/g for liquid food, to exert its prebiotic effect (Oliveira *et al.*, 2009). The prebiotic beneficial effect depends on the actual number of the beneficial bacteria count in the host's gastrointestinal system, which has led to the functional development of the product that integrates both beneficial bacteria and prebiotic (Scholz-Ahrens *et al.*, 2016). Additionally, prebiotic by themselves combine with their symbiotic interaction with probiotic bacteria have been proven to inhibit the growth of pathogenic bacteria in human, *in vitro* as well as the experimental animal model. The additional of prebiotics component in food product not only proven to increase the probiotic bacteria in the human gastrointestinal system but also protect and stimulate their growth during the product's shelf life (Özer *et al.*, 2005; Scholz-Ahrens *et al.*, 2016; de Almeida *et al.*, 2018).

The escalating episode of antibiotic resistance in healthcare management has catalyzed the scientific venture on new antibacterial compounds and alternative strategies, such as the application of probiotic bacteria. The gastrointestinal microflora is an intricate balance ecosystem populated over 400 bacterial species where anaerobes bacteria outnumber facultative anaerobes. The microflora is sparse in the stomach and upper segment of the gastrointestinal system, whereas thriving in the lower bowel. The infection with opportunistic pathogen however from contaminated food substance as well as the side effect of antibiotics treatment that potentially disturb the harmonious balance of the normal microflora, in which can favor both infections by exogenous pathogens and overgrowth by endogenous pathogens (Gorbach, 1996; Modi *et al.*, 2014; Bäumlner and Sperandio, 2016). Probiotic bacteria, namely *L. rhamnosus* GG has proven to inhibit the biofilm formation of antagonist *E. coli* and *Salmonella* sp. which preventing the production of toxin and pathogen-associated diseases. Both *E. coli* and *Salmonella* sp. are common pathogens that inhabit the gastrointestinal system (Petrova *et al.*, 2016).

In addition, probiotics bacteria have shown to exhibit antioxidant activity and reduced damages caused by oxidative. The oxidation reactions are an absolute essential in living organism for energy production. The abnormal formation of reactive chemical species and the accumulation of free radical materialize *in vivo* can lead to the damage of carbohydrates, lipids, nucleic acids, and proteins in living cells and tissues. Thus, the antioxidant additives strategies, in which utilize naturally occurring compound to prevent the abnormal oxidation of cellular substrates have proven its capacity to protect against oxidative damages (Sies, 1997; Halliwell and Gutteridge, 2015). The administration with *L. fermentum* ME-3 shown to not only able to eradicate

Salmonella Typhimurium-infected murine model as well as exhibit high total antioxidative activity (Mikelsaar, & Zilmer, 2009).

Thus, according to expert consultation from FAO/WHO, the selected bacterial strains must fulfill criteria as follow: 1) alive when administered; 2) accurate taxonomical characterization and identification defined microbe and/or cocktail of microbes, at genus, species, and strain level; 3) undergone a standard controlled scientific evaluation to document specific benefits on specific medical condition; 4) safe for its intended application to be denominated as probiotic (Sanders, 2000).

2.2 Aging

A natural event, aging is the process of becoming older in which is inevitable in all living organism. Aging is defined as progressive physiological capability degeneration over time. It represents an intricate process caused by the deterioration in age-specific fitness components of an organism like age-related capacity and efficiency (Flatt, 2012; Lipsky and King, 2015). The term refers to grey hair affairs in humans, becoming older in most animals and fungi. However, for some like single-celled organisms, perennial plants, and elementary animals are potentially biologically immortal. At the biological level, damages accumulation overtime in an organism caused by aging results in more than just decline in the mechanic physical performance but also deterioration vital cellular process and mental health which eventually entail morbidity and mortality (Ferrucci *et al.*, 2008). The human chronological age of 65 years old, is generally accepted in most of the developed countries as an elderly or old

(WHO, 2002). In 2012 the global population reached 7 billion where 562 million large, 8 percent of them were aged 65 and over. As the world population grows 3 years later in 2015, the aged population reached 8.5 percent with an additional 55 million aged people. The aged population is estimated to be projected to near double, 1.6 billion globally from 2025 to 2050 (NHI, 2016).

With time as we aged, one may experience certain age-related conditions such as decline in coordination and strength, hearing ability, impaired vision, metabolic syndrome like cardiovascular and diabetes problem, as well as neurological disorder like dementia (Ferrucci *et al.*, 2008; Belikov, 2018). A global phenomenon, population aging contributes to cultural, economic, and social challenges to individuals, families, societies and the global community with escalating healthcare management, lesser labor force participation, pension and poverty right (UNFPA, 2012; NHI, 2016). Therefore, it is an absolute essential to recognize the fundamental biological mechanism of aging to develop new interventions for the diagnosis, early detection, prevention, and treatment that detain aging and/or foster healthier aging.

2.2.1 Mechanism of Aging

The process of growing old in an organism is a multifactorial process that revolves around two main theories namely, genetically programmed and damage-related accumulation modulation. The measure of aging is varying substantially across different organism as well as among species. The genetically programmed factors obey the biological timetable which regulates the growth and development of the newborn until its final stage of life. This regulation depends on the intricate changes in

instrumental gene expression that affect the biological system responsible for defense, maintenance, and repair responses. In other hands, damaged-related factor includes environmental and internal assaults to living organism that generate accumulative damage at various levels. Damage-related elements include environmental and internal assaults to living organisms that caused cumulative damage at various degree. It is either because of the naturally occurring toxic by-products of metabolism and/or inefficient defensive or repair system at the cellular level, accumulates throughout the entire organism lifespan and eventually foster aging (Holliday, 2004; Kirkwood, 2005; LA,2006). The main theory of aging process in various organisms, especially human are oxidative stress and telomere erosion which will be discussed in this literature review.

As we age, the episode of cancer and neurodegenerative diseases arises, likely because of the increased in the potentially harmful molecule accumulation in the cellular system such as reactive oxygen species (ROS). This phenomenon occurs at the biochemical level, also known as free radical theory, resultant damage due to redox imbalances by which there is an elevation in the destructive free radical molecule and a reduction in antioxidant protection (Bokov *et al.*, 2004; Birch-Machin and Bowman, 2016). Highly active molecules, ROS consist of a diverse number of chemical species including hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and superoxide anion (O_2^-) which commonly produced as by-product during cellular metabolic reaction especially in mitochondria (Harman, 2002). The powerhouse of the cell, mitochondria is responsible to generate energy in the form of adenosine triphosphate (ATP) while at the same time, releasing oxygen through a transport chain reaction. Then, the oxygen is consumed during mitochondrial respiration which later reduced to hydrogen

peroxide and superoxide radical (Cui *et al.*, 2012; Sergiev *et al.*, 2015). ROS can also be generated due to other environmental factors such as chemical oxidation, inflammation cytokines, radiation, stress, and toxin.

Once they are produced, accumulated ROS molecules react with lipids, nucleic acids, and protein to effectuate oxidative damage, which attributes in a variety of age-related condition such as cancer and neurodegenerative diseases (Evans, *et al.*, 2004). The damaging effect of accumulated ROS and the link to the aging process is ascribed by its deleterious DNA lesion, mutation on the mitochondrial DNA (mtDNA), as well as rapid oxidative reaction with lipids and protein that commonly observed in the aged cellular organism (Cui *et al.*, 2012). Under the normal cellular condition, ROS molecules are sustained at the physiological levels by several endogenous antioxidant systems, such as catalase, glutathione peroxidases, glutathione reductase, and superoxide dismutase. Located in a different cellular compartment, these endogenous antioxidant systems are complex and they often complementary and/or redundant in various conditions. The physiological levels of ROS play an important role in mediating cell signaling by interacting with the redox state. In other hands, oxidative damage to cellular components and activate several cell death pathways caused by pathological levels of ROS (Sohal and Orr, 2012; Labunskyy and Gladyshev, 2013; Dai *et al.*, 2014).

Telomeres, a specific DNA-protein complex composed of the highly conserved nucleotides sequence of 'TTAGGG' located at each end of a chromosome. Together the DNA and protein complex configure a loop structure, which protect the genome from end-to-end interchromosomal fusion, nucleolytic degeneration, unnecessary recombination, and repair (Houben *et al.*, 2008). The telomeres length shortens at each cycle of cell division, because of the inability of DNA polymerase to completely replicate all the way to the ends of chromosomal DNA, denominated as "end-replication-problem" (Olovnikov, 1973). Telomere renders cell to undergo a definite replicative process, which every cell experience replicative senescence. A naturally occurring selection process where the numbers of cell division are limited and predetermined as the region in DNA at the most end of the chromosome dematerialize. Thereby, when telomere length reaches its critical limit overtime, the cell exits the cell-division cycle and encounter apoptosis and/or senescence. Telomere length has therefore been observed to be negatively associated with the actual chronological age, it determines the lifespan of an organism down to its (organism) cell also play a vital part in preserving the genetic information of our genome (Heidinger *et al.*, 2012; Broer *et al.*, 2014).

The rate of telomere length shortening can be affected by a multitude of additional factors, including oxidative stress. Certain lifestyle factor, however, may accelerate telomere shortening via inducing damage to DNA in general or worst, directly at telomere complex and therefore affecting general health and lifespan of an organism or individual (Sanders and Newman, 2013). Telomere shortening can be restored by the action of telomerase which extends the telomeric region in DNA. This enzyme, telomerase consists of protein subunit (hTERT) and RNA subunit (hTR), but

presence exclusively in a certain type of cell with an indefinite proliferating ability like germline and stem cells, as well as cancerous cells. Telomerase preserves the telomere length by adding 'TTAGGG' sequence repeats at the end of the chromosome in DNA (Shammas *et al.*, 2004). Therefore, telomere length does not shorten in stem cells where telomerase enzyme is active, on the contrary, inactive in normal somatic cells. Thus, the measurement of telomere length often used as a biological clock to extrapolate the lifespan of an organism or individual (Weinert and Timiras, 2003; Heidinger *et al.*, 2012).

Telomere shortening has proven to cause a series of cascading age-related conditions, for example, cell death, genomic instability, and eventually senescence which is driven by the deleterious action of chronic inflammation and oxidative stress (Hou *et al.*, 2015). These observations marked the importance of telomere as one of the institutional factors for age-associated pathologies, therefore underline its role as an aging biomarker. The measurement of telomere length can be estimated using various techniques including fluorescence *in situ* hybridization (FISH), the traditional Southern blot analysis of the terminal restriction fragments (TRFs) length, and quantitative polymerase chain reaction (qPCR) amplification (Bhattacharyya *et al.*, 2017). However, in the present study, we utilize the quantitative PCR amplification technique to estimate the telomere length with a primer pair which measures the telomeric repeat copy number and its ratio to the copy number of a single-copy gene. The ratio value generated should be proportional to the average telomere length of DNA sample, expressed as telomere to single-copy gene ratio (T/S) (Cawthon, 2002).

2.2.2 In-Vivo Aging Model

The experimental animal model is a valuable tool to study the biological development and progression of the disease or certain condition, investigate hypotheses generated from clinical finding, and evaluate the efficacy of the possible interventions. This comparative medicine concept, in which the laboratory animal model shares behavioral, physiological, or other characteristics with us – humans. Recently, the animal model is employed in nearly all fields of biomedical study including, but not exclusively to, basic biology, behavior, immunology, infectious disease, and oncology (Ericsson *et al.*, 2013). Premature aging in laboratory animal such as murine can be chemically induced by using D-galactose (D-gal), commonly through subcutaneous injection. The chronic low dose administration of D-gal in laboratory murine has shown previously to undergone accelerated aging via increase oxidative stress accumulation and glycation end-product. The D-gal induce animals were reported to exhibit condition parallel to the naturally aged animals (Song *et al.*, 1999; Chen *et al.*, 2018). The D-gal induction has been observed to causes caspase-dependent apoptosis, hampered immune system, mitochondrial dysfunction as well as neurobehavioral changes including cognition and motor impairment; neurodegeneration, and reduced neurogenesis (Zhou *et al.*, 2013).