A RANDOMIZED, DOUBLE BLIND, PLACEBO-CONTROLLED STUDY ON THE EFFICACY OF *BIFIDOBACTERIUM LONGUM* BB536 IN REDUCING SYMPTOMS OF RESPIRATORY AND GASTROINTESTINAL DISEASES AMONG YOUNG CHILDREN

by

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LIST OF ABBREVIATIONS

AAD	Antibiotic-associated diarrhea
ANOVA	Analysis of variance
AOM	Acute otitis media
APS	Adenosine 5'-phosphosulfate
ARI	Acute respiratory infections
ATP	Adenosine triphosphate
BALT	Bronchus-associated lymphoid tissue
BILQ	Basic Information and Lifestyle Questionnaire
BMI	Body mass index
bp	base pair
CDD	*Clostridium difficile*-associated diarrhea
CFU	Colony forming unit
CI	Confidence intervals
CVI	Content validity index
dATP	Deoxyadenosine triphosphate
DCs	Dendritic cells
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dNMP	Deoxynucleoside monophosphate
dNTPs	Deoxynucleotide triphosphates
dTTP	Deoxythymidine triphosphate

ENT	Ear, nose and throat
FDA	Food and Drug Administration
FOSHU	Food for Specified Health Uses
FVI	Face validity index
GALT	Gut-associated lymphoid tissue
GEE	Generalized estimating equations
GIT	Gastrointestinal tract
GRAS	Generally Recognised as Safe
HACCP	Hazard analysis and critical control points
HCQ	Health Condition Questionnaire
IBD	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
I-CVI	Item-level content validity index
IFN-γ	Interferon-γ
IgA	Immunoglobulin A
IL	Interleukin
LRTI	Lower respiratory tract infections
MANOVA	Multivariate analysis of variance
MLN	Mesenteric lymph nodes
NEC	Necrotizing enterocolitis
NGS	Next generation sequencing
OTUs	Operational taxonomic units
PCR	Polymerase chain reaction
PCoA	Principal coordinate analysis
PEG	Polyethylene glycol

Pi	Inorganic phosphate
PPi	Pyrophosphate
QIIME	Quantitative Insights into Microbial Ecology
RNA	Ribonucleic acid
RSVs	Rhinoviruses
RTI	Respiratory tract infections
SBS	Sequencing-by-synthesis
SCFAs	Short chain fatty acids
S-CVI	Scale-level content validity index
S-CVI/Ave	Averaging calculation method
S-CVI/UA	Universal agreement calculation method
SDS	Sodium dodecyl sulfate
TE	Tris-EDTA
Th	T helper cell
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
Treg	T regulatory cell
URTI	Upper respiratory tract infections
WHO	World Health Organization

KAJIAN RAWAK, DUBEL-BUTA DAN TERKAWALKAN PLASEBO KE ATAS KEBERKESANAN *BIFIDOBACTERIUM LONGUM* BB536 DALAM MENGURANGKAN GEJALA PENYAKIT PERNAFASAN DAN GASTROUSUS DALAM KALANGAN KANAK-KANAK

ABSTRAK

Probiotik telah digunakan secara meluas dan mungkin berkesan dalam meringankan gejala dan mengurangkan kejadian penyakit pernafasan dan gastrousus, tetapi keberkesanannya masih kontroversi disebabkan kebergantungan kepada strain dan perumah. Kanak-kanak yang menghadiri pusat jagaan harian adalah berisiko 1.5-3.0 kali lebih tinggi dijangkiti penyakit pernafasan dan gastrousus dibandingkan mereka yang tidak menghadiri pusat jagaan harian. Kajian percubaan rawak, dubel-buta dan terkawalkan plasebo yang telah dilakukan bagi tempoh 10 bulan ini bertujuan untuk mengkaji kesan pengambilan *Bifidobacterium longum* BB536 dalam mencegah kejadian cirit-birit akut dan / atau penyakit yang berkaitan dengan sistem pernafasan dalam kalangan kanak-kanak muda di Malaysia. Seramai 520 kanak-kanak muda yang sihat berumur dari 2 hingga 6 tahun telah direkrut dan diagihkan secara rawak kepada dua kumpulan untuk menerima sama ada serbuk BB536 dalam dos tetap sebanyak $5x10^9$ CFU/hari atau serbuk plasebo, selama lima hari seminggu bagi tempoh 10 bulan. Serbuk BB536 dan plasebo tersebut yang diimport daripada Morinaga Milk Industry Co. Ltd, Japan, telah dihasilkan oleh loji perkilangan yang telah mendapat pensijilan sistem analisis hazard dan titik kawalan kritikal (HACCP). Serbuk BB536 mengandungi probiotik *Bifidobacterium longum* BB536 dan dekstrin sebagai penghantar manakala plasebo mengandungi 100% dekstrin. Soal selidik dasar (Soalselidik Maklumat Asas dan Gayahidup) diagihkan dan dikumpulkan sebelum

intervensi manakala soal selidik bulanan (Soalselidik Kesihatan Bulanan) diagihkan dan dikumpulkan setiap bulan. Sampel najis telah dikutip pada bulan ke-0, 5 dan 10 kajian untuk analisis profil mikrobiota usus dan kepekatan immunoglobulin A (IgA) fekal. Keputusan menunjukkan tiada perbezaan yang signifikan dalam insiden dan gejala cirit-birit antara kumpulan BB536 dan kumpulan plasebo. Perbandingan gejala jangkitan pernafasan menunjukkan pengurangan yang signifikan ($P<0.05$) dalam gejala sakit tekak ($P = 0.018$), tetapi tiada pengurangan signifikan untuk demam ($P = 0.084$), batuk ($P = 0.087$) dan hidung berair ($P = 0.087$) dalam kumpulan BB536 berbanding dengan kumpulan plasebo pada tahap keyakinan 95%. Sampel najis dianalisis melalui amplikon penjujukan pemprosesan tinggi yang diperolehi dari rantau V3-V4 gen 16S rRNA. Perbandingan kelimpahan relatif bagi spesies menunjukkan peningkatan signifikan dalam kelimpahan genus *Bacteroides* dan *Prevotella* selepas pengambilan BB536, yang diandaikan bertanggungjawab dalam pengawalan tindak balas imun dalam paru-paru melalui penghasilan asid lemak berantai pendek. Analisis koordinat prinsipal menunjukkan corak pengelompokan mikrobiota usus yang signifikan di peringkat genus dalam kumpulan BB536 di mana mikrobiota berkelompok bersama-sama manakala kumpulan plasebo menunjukkan corak yang lebih tersebar, menunjukkan bahawa komposisi mikrobiota lebih serupa dalam kumpulan rawatan. Kepekatan immunoglobulin A dalam sampel najis tidak berbeza secara signifikan antara kumpulan plasebo dan kumpulan BB536. Kedua-dua kumpulan menunjukkan peningkatan dalam kepekatan IgA pada musim hujan apabila kecenderungan untuk jangkitan adalah lebih tinggi. Kesimpulannya, keputusan ini menunjukkan bahawa pengambilan BB536 boleh mengurangkan sakit tekak semasa jangkitan pernafasan, tetapi tiada kesan dalam mengubah komposisi mikrobiota usus dalam kalangan kanak-kanak di Malaysia.

A RANDOMIZED, DOUBLE BLIND, PLACEBO-CONTROLLED STUDY ON THE EFFICACY OF *BIFIDOBACTERIUM LONGUM* BB536 IN REDUCING SYMPTOMS OF RESPIRATORY AND GASTROINTESTINAL DISEASES AMONG YOUNG CHILDREN

ABSTRACT

Probiotics have been extensively used and may be effective in alleviating symptoms and reducing the occurrences of gastrointestinal and respiratory diseases, but their efficacy remains controversial due to strain and host dependency. Children who are attending day care centres have 1.5 to 3.0 times higher risk of getting respiratory and gastrointestinal diseases than those who are not attending day care centres. This randomized, double-blind and placebo-controlled trial which was carried out for a period of 10 months was aimed to evaluate the effects of oral administration of *Bifidobacterium longum* BB536 in preventing acute diarrhea and/or respiratory-related diseases among young children in Malaysia. A total of 520 healthy young children aged from 2 to 6 years were recruited and randomized into two groups to receive either BB536 powder at a fixed dosage of $5x10^9$ CFU/day or placebo powder for five days per week for 10 months. The BB536 powder and placebo powder, which were imported from Morinaga Milk Industry Co., Ltd., Japan, have been manufactured under a HACCP certified manufacturing plant. The BB536 powder contains probiotic BB536 and dextrin as carrier while placebo contains 100% dextrin. Baseline questionnaires (Basic Lifestyle and Demographic Information Questionnaire) were distributed and collected before the intervention was started while monthly questionnaires (Health Condition Questionnaire) were distributed and collected every month. Fecal samples were collected at 0^{th}, 5^{th} and 10^{th} month of the study for gut

microbiota profile and fecal immunoglobulin A (IgA) concentration analysis. Results showed no significant difference in diarrhea incidences and symptoms between the BB536 group and placebo group. Comparison of the symptoms of respiratory diseases indicated a significant reduction ($P<0.05$) in sore throat ($P=0.018$), but no significant reduction in fever ($P=0.084$), cough ($P=0.087$) and runny nose ($P=0.087$) in the BB536 group compared with the placebo group at 95% confidence level. Fecal samples were analyzed by high-throughput sequencing amplicons derived from the V3-V4 region of the 16S rRNA gene. Comparison of relative abundance of species showed significant increment in the abundance of genera *Bacteroides* and *Prevotella* on BB536 intake which was postulated to regulate immune responses in the lung through short-chain fatty acids production. Principal coordinate analysis showed significant clustering pattern of the gut microbiota at genus level in BB536 group where the microbiota clustered together while placebo group showed a more dispersed pattern, indicating a more similar microbiota composition within the treatment group. The concentrations of the fecal immunoglobulin A were not significantly different between placebo and BB536 groups. Both groups showed increment in IgA concentrations during wet season when infections were prone to occur. In conclusion, these results suggest that as well as changing the gut microbiota population, administration of BB536 may alleviate sore throat during respiratory infections but showed no significant reduction in other symptoms such as fever, cough and runny nose among young children in Malaysia.

CHAPTER 1

INTRODUCTION

1.1 Background

In the human body, abundant of microbial cells colonize on every surface, such as the skin, oral cavity, urogenital tract, gastrointestinal tract and respiratory tract (Gerritsen *et al.*, 2011; Mikami *et al.*, 2012; Picard *et al.*, 2005; Putignani *et al.*, 2014). Microbial colonization is much more preferred in gastrointestinal tract which harbours a huge and complex diversity of microorganisms including both pathogenic and beneficial bacteria as well as viruses (Glendinning and Free, 2014; Holzapfel, 2006; Sekirov *et al.*, 2010). These diverse species of bacteria are pivotal in regulating the balance of the bacteria ecosystem in the gastrointestinal tract and maintain health of the human host (Schell *et al.*, 2002). Imbalance of the intestinal microbiota composition could cause diarrhea and respiratory diseases, thus negatively influencing the host (Hawrelak and Myers, 2004).

Acute diarrhea, gastroenteritis, inflammatory bowel disease (IBD), irritable bowel syndrome (Nicholson *et al.*, 2012) and others are categorized as gastrointestinal diseases. Acute gastroenteritis is one of the major causes of morbidity among young children, resulting in significant health costs. Statistics showed that among infants and young children or preschool children, severe gastroenteritis is a worldwide concern, with two million hospitalizations of children under five years of age for diarrhea (Jungersen *et al.*, 2014; Walker *et al.*, 2012). In Malaysia, the reported cases of acute gastrointestinal diseases including acute diarrhea have been observed throughout the

year (Cheah *et al.*, 2011; Hsu *et al.*, 2005; Hung *et al.*, 2006). Diarrhea is commonly defined as passage of three or more loose or watery stools in the last 24 hours (WHO, 2013).

Respiratory diseases, comprising of upper and lower respiratory tract infections, are the most common acute diseases among children. This had contributed to the major cause of the morbidity and mortality globally (Lehtoranta *et al.*, 2014; Vouloumanou *et al.*, 2009). The occurrence of respiratory tract infections is much dependent on the weather of the region (Rahman *et al.*, 2014; Sam *et al.*, 2010; Simonsen *et al.*, 2011). For example, in Malaysia, most occurrences of respiratory tract infections among young children or preschool children peak in midyear which is during dry season (April to June) and during wet season in the year end (October to January) (Rahman *et al.*, 2014; Sam *et al.*, 2010).

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2006). Probiotic bacteria, which beneficially affect the host by improving the intestinal microbial balance, may affect the immune response, thus boosting the body system to combat against diseases (Hatakka *et al.*, 2001).

Several studies on probiotic prevention of acute diarrhea in daycare centres worldwide with the use of *Bifidobacterium* as probiotics (alone or in combination with *Streptococcus thermophiles, Lactobacillus rhamnosus, Lactobacillus acidophilus*), showed clear evidence of efficacy to reduce diarrhea-related symptoms and duration in a strain-dependent and dose-dependent manner (Allen *et al.*, 2009; Guandalini, 2011; Kloster Smerud *et al.*, 2008).

Probiotics on the other hand have also gained increasing evidence as an effective prophylaxis in preventing respiratory tract infections by reducing the

incidence, duration, and/or severity of respiratory diseases, particularly upper respiratory tract infections (Gill *et al.*, 2012; Lehtoranta *et al.*, 2014). Among clinical trials conducted with *Bifidobacteria* strains, duration of respiratory infections and symptoms of respiratory infection such as fever, cough and rhinorrhea were significantly reduced, relative to placebo (Cazzola *et al.*, 2010; Gerasimov *et al.*, 2016; Hatakka *et al.*, 2007; Leyer *et al.*, 2009; Rerksuppaphol and Rerksuppaphol, 2012; Taipale *et al.*, 2011; Taipale *et al.*, 2016).

Among all the previous studies done on probiotic effects on acute diarrhea and respiratory diseases, there are many different probiotic strains which showed their efficacy in reducing the occurrences and symptoms. Probiotic strain *Bifidobacterium longum* BB536 is recognised as one of the best-known probiotic strains in the world which is widely applied. It has been extensively studied in both clinical research and technical development for improving intestinal environment, prevention of diarrhea, alleviation of constipation as well as supporting immune system (Akatsu *et al.*, 2013; Kondo *et al.*, 2013; Namba *et al.*, 2010; Yaeshima *et al.*, 1997). However, to our knowledge, the efficacy of *Bifidobacterium longum* BB536 in respiratory and gastrointestinal diseases has not yet been clinically studied among children in Malaysia. Considering that past studies have reported positive outcomes of bifidobacteria in alleviating the symptoms of the diseases, we hypothesized that administration of *Bifidobacterium longum* BB536 could reduce the symptoms during the occurrence of acute diarrhea and respiratory diseases. As these infections are common and are major causes of children's morbidity and mortality, in this project, we hope to resolve these global issues via bifido approach.

More than 70% of all the microorganisms found in the human body can be found in the gastrointestinal tract (GIT). The composition of the gastrointestinal tract

microbiota varies between individuals (Claesson *et al.*, 2012). Fecal microbiota profiles can be determined through pyrosequencing, to provide an insight into the association of the gastrointestinal microbiota with certain diseases and probiotic ingestion. Through the pyrosequencing, method of DNA sequencing, microbial diversity, mainly in the gastrointestinal tract and other different environments such as skin, vagina, nasopharynx and oropharynx can be better understood (Siqueira *et al.*, 2012). Previous studies have indicated that probiotic administrations are able to modify the composition of the fecal microbiota (Kristensen *et al.*, 2016). Hence, in this study, the putative changes of the fecal microbiota profile between probiotic group and placebo group will be further elucidated.

Immunoglobulin (Ig) A concentration is affected by the presence of probiotics (Lopéz *et al.*, 2011). It is responsible in protecting host against pathogens through immunomodulatory mechanism. It is also involved in regulating the balance of the intestinal microbiota by preventing the penetration of the enteric pathogens into the epithelial surface and clearing all the pathogens. IgA is the most abundant immunoglobulin class found in the body, primarily at the mucosal surfaces (Isolauri *et al.*, 2001). It is mainly produced in the inductive sites known as Peyer's patches (PPs) in the small intestines (Rios *et al.*, 2015). The production of IgA is promoted by the presence of cytokine interleukin (IL) 17 which is activated by T-helper (Th) 17 (Blutt and Conner, 2013). Studies demonstrated that bifidobacteria enhanced the production of cytokines which promoted the secretion of IgA. Therefore, it was aimed to compare the difference of the IgA secretion between probiotic group and placebo group.

1.2 Aim and objectives of research

The aim of this study was to evaluate the effects of *Bifidobacterium longum* BB536 administered orally for a period of 10 months in reducing acute diarrhea and/or respiratory diseases among children in Malaysia. The specific objectives of this study were:

1) To compare the severity of acute diarrhea between the subjects in *Bifidobacterium longum* BB536 group and placebo group for 10 months in terms of total days, total occurrences and number of symptoms occurred during the event of diarrhea.
2) To compare the severity of upper respiratory diseases between the subjects in *Bifidobacterium longum* BB536 group and placebo group for 10 months in terms of total days, total occurrences as well as number and types of symptoms occurred during the event of respiratory diseases.
3) To compare the changes of fecal microbiota profiles of the subjects in *Bifidobacterium longum* BB536 group and placebo group across 10 months.
4) To compare Immunoglobulin (Ig) A concentration from fecal samples of the subjects in *Bifidobacterium longum* BB536 group and placebo group across 10 months.

CHAPTER 2

LITERATURE REVIEW

2.1 Gastrointestinal microbiota

Gastrointestinal tract of a human is densely populated with over 500-1000 different species of bacteria, contributing to a total weight of several hundred grams (Gerritsen *et al.*, 2011; Mikami *et al.*, 2012; Makino *et al.*, 2013; Picard *et al.*, 2005; Sjogren *et al.*, 2009). With its large surface area, equivalent to the size of a tennis court (200 m^2), and richness in nutrients, human gastrointestinal tract contains over 70% of all the microorganisms in the human body (Holzapfel, 2006; Sekirov *et al.*, 2010). Most of the bacteria present are obligate anaerobes and are found on epithelial surface, mucus layer in the intestine or intestinal lumen (Picard *et al.*, 2005; Sekirov *et al.*, 2010). Four predominant bacterial phyla found in the human gastrointestinal tract are *Firmicutes, Bacteroidetes, Proteobacteria,* and *Actinobacteria* (Glendinning and Free, 2014; Lee and O'Sullivan, 2010; Sartor and Mazmanlan, 2012).

Homeostasis of the commensal microbiota in gastrointestinal ecosystem is pivotal in maintaining host's health (Hawrelak and Myers, 2004; Miyazaki, 2015; Ohno, 2015). The gut microbiota are responsible for a multitude of important functions such as metabolizing exfoliated epithelial cells, complex carbohydrates to short chain fatty acids (SCFAs) (Rauch and Lynch, 2012; Sartor and Mazmanlan, 2012); production of essential vitamins (Glendinning and Free, 2014); drug metabolism and toxicity (Aw and Fukuda, 2015); fortification of intestinal epithelial barrier (Rauch

and Lynch, 2012); as well as modulation of immune responses in host (Hemarajata and Versalovic, 2013; Rauch and Lynch, 2012).

Microbiota perturbation can lead to gastrointestinal disorders such as inflammatory bowel diseases (IBD), irritable bowel syndrome (Nicholson *et al.*, 2012), diarrhea, constipation and necrotizing enterocolitis (NEC) (Hawrelak and Myers, 2004; Sartor and Mazmanlan, 2012; Schulz *et al.*, 2014; Turroni *et al.*, 2012); atopic diseases such as atopic dermatitis, allergic rhinitis, asthma and food allergy (Toh *et al.*, 2012; Yesilova *et al.*, 2012); and respiratory tract infections, namely URTIs and LRTIs (Vouloumanou *et al.*, 2009).

2.2 Emergence of probiotics

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2006). The word probiotic means "for life" which was derived from Greek "pro bios" (Soccol *et al.*, 2010). They were only known as beneficial bacteria at the beginning of the 20th century by a Russian Nobel Prize winner, Elie Metchnikoff who linked the health and longevity with the consumption of yogurt containing lactic-acid-producing bacteria (LAB) such as lactobacilli (Anukam and Reid, 2007; Gogineni *et al.*, 2013; Rauch and Lynch, 2012; Soccol *et al.*, 2010). Later in 1899, a French pediatrician, Henry Tissier, isolated *Bifidobacteria* from the fecal samples of breast-fed infants to treat diarrhea through their oral administration (Anukam and Reid, 2007; Gogineni *et al.*, 2013; Lee and O'Sullivan, 2010; Soccol *et al.*, 2010). Since last century, bifidobacteria were highly recommended due to their potential health benefits (Lee and O'Sullivan, 2010).

Progressing through the years, both community and health care providers are increasingly aware of the relation of nutrition, diet and health. Therefore, probiotics

have recently gained tremendous popularity and interest (Ringel *et al.*, 2012). Statistics showed that the global market of the probiotics was worth $14.9 billion in 2007, nearly $16 billion in 2008, and $36.6 billion in 2015. The market value is expected to exceed $64 billion by 2023 (Dover, 2016). Probiotics are marketed for different applications, such as functional foods and beverage, dietary supplements in infant formula, food and nutritional supplements and also in animal feed (Soccol *et al.*, 2010). Geographically, the global probiotic market is divided as the North America (United States), Europe (United Kingdom, France and Germany), Asia Pacific (China, Japan, India), Latin America (Brazil), Middle East and Africa. United Kingdom and Germany account for about 45% of the total probiotic market while Japan is the second largest market (BioMedTrends, 2017).

2.3 Classification and beneficial effects of probiotics

2.3.1 *In-vitro* studies

To qualify as a probiotic, a viable bacterial strain must be safe for human use. Strains such as lactobacilli and bifidobacteria are commonly isolated from healthy human (Boyle *et al.*, 2006; Isolauri *et al.*, 2001; Kailasapathy and Chin, 2000; Soccol *et al.*, 2010). The strain must be stable against bile, acid, enzyme and oxygen; able to adhere to intestinal mucosa; able to colonize in the human gastrointestinal tract; able to reduce pathogen adhesion to mucosal surfaces and able to produce antimicrobial substances (Boyle *et al.*, 2006; FAO/WHO, 2002; Isolauri *et al.*, 2001; Kailasapathy and Chin, 2000). In addition, the strain must be viable and metabolically active within gastrointestinal tract (Kailasapathy and Chin, 2000). General findings of the *in-vitro* studies of bifidobacteria are summarized in Table 2.1.

Table 2.1 Summary of the *in-vitro* studies involving bifidobacteria.

Properties	Findings
Resistance to bile	• *B. longum, B. pseudocatenulatum* and *B. bifidum* were able to grow in the presence of 2% of bovine bile (Delgado *et al.*, 2008). • Bifidobacteria contain several genes and proteins, eg bile salt hydrolase and bile efflux transporters which confer in bile resistance (Grimm *et al.*, 2014)
Resistance to acid	• *B. longum* and *B. pseudocatenulatum* were still viable at low pH of 3.5 (Delgado *et al.*, 2008)
Adherence to intestinal mucosa	• Strains of *B. adolescentis, B. lactis, B. longum, B. bifidum, B. infantis* and *B. breve* were reported to adhere to Caco-2, T84 and HT-29 cell lines in strain specific manner (Preising *et al.*, 2010)
Antimicrobial activity	• Pathogenic *C. difficile* is a toxin-producing bacterium which normally causes diarrhea and colitis. *In vitro* analysis to test the ability of probiotic strains in inhibiting pathogen's growth showed about 60.4% of *C. difficile* were killed after 3 hours while 99.7% were killed after 24-hour incubation with *B. breve* and *B. lactis* (Lee *et al.*, 2013)
Anti-inflammatory properties	• Strains of bifidobacteria acted in a strain-dependent manner in inhibiting lipopolysaccharide-induced IL-8 secretion in both Caco-2 and T84 cell lines (Preising *et al.*, 2010)

IL: interleukin

2.3.2 *In-vivo* studies and clinical trials

Lactobacilli and bifidobacteria are common in exerting therapeutic and health benefits to the host by altering or modifying the host's gastrointestinal microflora to restore microbial balance (Kailasapathy and Chin, 2000; Rauch and Lynch, 2012; Ritchie and Romanuk, 2012). They confer health benefits through enhancement of immune system, prevention of intestinal infections, alleviation of diarrhea, prevention of food allergy, anti-carcinogenic effect, prevention of upper gastrointestinal tract

diseases, improvement of lactose intolerance, prevention of hypercholesterolemia, prevention of osteoporosis and stabilization of the gastrointestinal mucosal barrier (Boyle *et al.*, 2006; Hao *et al.*, 2015; Kailasapathy and Chin, 2000; Rauch and Lynch, 2012; Ringel *et al.*, 2012; Soccol *et al.*, 2010). A summary of the *in-vivo* findings is shown in Table 2.2.

Probiotics have been widely studied in various clinical trials worldwide, applied in healthy or diseased conditions, involving subjects ranging from infants to the elderly. Clinical trials are referred to as research studies which are controlled scientifically with adherence to good clinical practices (GCP) for the evaluation of the safety and effectiveness of a therapeutic agent, involving human volunteers with their consent (Mahan, 2014; Thorat *et al.*, 2010). Clinical trials can be conducted as randomized controlled trial or non-randomized controlled trial. Ideally, a clinical study should be randomized to ensure the allocation of subjects to the different treatment groups in a balanced manner to maximize the homogeneity between two groups (Meinert, 2012; Rohrig *et al.*, 2009). Randomization can be stratified according to gender or age to produce a parallel ratio of subjects in both groups. A trial should also involve masking or blinding, either single-blind or double-blind. Single-blind is where only the subject enrolled is unaware which treatment is received while double-blind refers to both subject and investigator being unaware of which treatment is planned (Rohrig *et al.*, 2009). Double-blind design is able to exclude possible factors such as sex, age or social differences, which will eventually affect the evaluation of the therapy and the measures of outcomes. A masked design of trial is more reliable than without blinding, therefore, ensure the quality of the study (Meinert, 2012). Besides, placebo-controlled studies are also widely applied as well as in probiotic studies, where the group receiving desired probiotic strain is compared to the control group receiving

preparation containing no probiotic. This is widely applied to compare the effectiveness of the probiotic in alleviating or preventing certain medical conditions.

Table 2.2 Summary of the *in-vivo* studies involving bifidobacteria.

Properties	Findings
Resistance to bile	• Resistance to bile is critical for the colonization of the GIT with microorganism for host health. • Recombinant strain of *B. breve* increased GIT persistence and protected the mice against infection of *L. monocytogenes* (Grimm *et al.*, 2014)
Adherence to intestinal mucosa	• Bifidobacteria have been studied in healthy volunteers and patients at different dosage ranging from 10^6-10^{12} CFU/mL/day. Results showed that *B. longum* at dosage of 10^{11} CFU/mL/day for 40 days showed survival in the gut and lowered the population of pathogenic bacteria such as enterobacteriaceae and clostridia (Fujiwara *et al.*, 2001) • *B. lactis* strain BB12 at dosage of 10^6 CFU/mL/day showed prophylactic action against acute diarrhea in infants younger than 8 months (Chouragui *et al.*, 2004)
Antimicrobial activity	• Strains of bifidobacteria showed protective effects against infections caused by *C. rodentium* in mice model through the production of exopolysaccharides (EPS) and short chain fatty acids (SCFAs) (Grimm *et al.*, 2014)
Anti-inflammatory properties	• *B. bifidum* reduced the inflammatory scores of trinitribenzene sulfonic acid (TNBS) colitis from 4.7 to 3.2 (P=0.023) in murine model. • Appearance of the mice's colon wall was normal and solid fecal was observed upon feeding with bifidobacteria (Preising *et al.*, 2010)

2.4 Bifidobacteria

2.4.1 General features of bifidobacteria

Bifidobacteria are known as one of the main potentially health-enhancing probiotic bacteria as they are natural predominant inhabitants in human gastrointestinal tract (Picard *et al.*, 2005; Tan *et al.*, 2015). Bifidobacteria show no health risk for they have a long history of consumption in the form of fermented milk (Picard *et al.*, 2005). Bifidobacteria are generally characterized as high-G+C Gram-positive prokaryotes, non-spore-forming, non-motile, catalase-negative and obligate anaerobic bacteria (Lee and O'Sullivan, 2010; Martinez *et al.*, 2013; Schell *et al.*, 2002). They were described as pleomorphic rods with different shapes, including curved, short and bifurcated Y shapes (Martinez *et al.*, 2013; Schell *et al.*, 2002). They produce acetic and lactic acids through the bifid shunt pathway by metabolizing lactose, glucose, galactose and fructose (Lee and O'Sullivan, 2010; Wasilewska *et al.*, 2003).

Genus *Bifidobacterium,* belongs to the phylum *Actinobacteria,* which is one of the main phylum in the *Bacteria* domain (Lee and O'Sullivan, 2010). To-date, there are 32 species in the genus *Bifidobacterium* (Baffoni *et al.*, 2013; Schell *et al.*, 2002). They were isolated from different ecological niches, which are human gastrointestinal tract (GIT), oral cavity, food, animal gastrointestinal tract, insect intestine, and sewage (Bottacini *et al.*, 2014; Ventura *et al.*, 2012). Among these niches, human GIT contains the vast majority of bifidobacteria where they are naturally present as the dominant commensal colonic microbiota (Martinez *et al.*, 2013; Picard *et al.*, 2005; Schell *et al.*, 2002). In the human GIT, *Bifidobacterium longum* subsp. *longum, B. longum* subsp. *infantis, B. pseudolongum, B. bifidum, B. animalis* subsp. *lactis, B. adolescentis, B. pseudocatenulatum, B. catenulatum, B. angulatum* and *B. breve* are the most abundant bifidobacterial species (Grimm *et al.*, 2014; Turroni *et al.*, 2014; Ventura *et al.*, 2012).

The population of bifidobacterial species and their compositions in humans vary across age. Martinez *et al.* (2013) and Grimm *et al.* (2014) reviewed that approximately 91% of the bifidobacteria were found in a newborn's GIT while the composition was reduced to 3-7% in an adult's GIT (Leke *et al.*, 2007; Turroni *et al.*, 2014). In addition, the composition of the bifidobacteria in infants is also different to than those in adults' GIT, and the bifidobacteria are known as infant-specific and adult-specific bifidobacterial species. Infant-specific bifidobacterial species are *B. breve, B. bifidum,* and *B. longum* subsp. *infantis* while adult-specific bifidobacterial species are *B. adolescentis, B. catenulatum/pseudocatenulatum,* and *B. longum* subsp. *longum* species (Ventura *et al.*, 2012).

2.4.2 Health benefits

Bifidobacteria colonization of the gastrointestinal tract during infancy is critical as it was reviewed that insufficient colonization resulted in abuse of antibiotics, causing inflammation, autoimmune and atopic diseases (Dong *et al.*, 2010; Gueimonde *et al.*, 2007; Gill *et al.*, 2012). Establishment of bifidobacteria is also vital in modulation of immune system and anti-inflammatory role on the mucosal surface (Mikami *et al.*, 2009). Also, studies showed that bifidobacteria are able to break down indigestible polysaccharides into readily absorbed short chain fatty acids (SCFAs), leading to an increase of bifidobacterial populations in the gastrointestinal tract, as well as promoting enrichment of the other beneficial bacteria in the lower gastrointestinal tract where they can utilize the SCFAs (Fushinobu, 2010; Kitaoka, 2012; Lee and O'Sullivan, 2010; Pokusaeva *et al.*, 2011). The high number of bifidobacteria in infants as compared to that in adults may correlate with the low levels of putrefactive products such as ammonia, indole, *p*-cresol and phenol (Lee and

O'Sullivan, 2010). This suggests that supplementation of bifidobacteria can restore the homeostasis of intestinal microflora.

Members of the genus bifidobacterium have been extensively studied for their health benefits on the host. Bifidobacteria have been reported in reducing or treating numerous diseases such as acute diarrhea, necrotizing entercolitis, constipation, atopic diseases and respiratory tract infections as well as preventing infections through competitive exclusion (Lau *et al.*, 2015; O'Callaghan and van Sinderen, 2016). Several studies, including human studies have been carried out to understand the possible mechanisms of the bifidobacteria in reducing or treating the diseases (Table 2.3).

2.4.3 *Bifidobacterium longum* BB536

Bifidobacterium longum BB536 is one of the best-known probiotic strains in the world. It was originally isolated from a healthy infant back in 1969 in Japan. It has been widely applied in milk, yogurt, infant formula the dairy industry, and as health supplements, which are commercially available in more than 30 countries, including Malaysia and Japan. *B. longum* BB536 is manufactured by Morinaga Milk Industry Co., Ltd., Japan.

B. longum BB536 has been extensively studied in both clinical research and technical development with more than 100 scientific publications. Based on *in-vitro, in-vivo* and clinical studies, BB536's long consumption history of more than 30 years, supports the safety and health benefits of the strain. These include improving intestinal environment, prevention of diarrhea, alleviation of constipation as well as supporting immune system (Akatsu *et al.*, 2013; Kondo *et al.*, 2013; Namba *et al.*, 2010; Yaeshima *et al.*, 1997).

Table 2.3 Health benefits of bifidobacteria in different diseases

No.	Diseases/Conditions	Possible mechanisms of bifidobacteria	Outcomes from clinical studies
1.	Acute diarrhea	• Modulation of the host immune system • Restoration of the composition of GIT microbiota • Production of bacteriocins and SCFAs to alter pH of GIT • Inhibit colonization of enteropathogens	• Reduced in mean duration of diarrhea • Lower concentrations of fecal rotavirus and *E. coli* in rotavirus diarrhea • Downregulation of pro-inflammatory cytokine such as TNF-α • Upregulation of anti-inflammatory cytokines such as IL-10 and IFN-γ (Allen *et al.*, 2009; Shu *et al.*, 2001; Salari *et al.*, 2012)
2.	Necrotizing enterocolitis (NEC)	• Improve development of cellular junctional proteins in the intestinal epithelium • Promote production of IgA • Enhance production of anti-inflammatory cytokines	• Incidence of NEC was reduced from 57% to 17% • Suppressed TLR4-triggered inflammatory responses (Khailova *et al.*, 2009)
3.	Constipation	• Promote reduction in pH through production of SCFAs and prevents colonization with pathogenic bacteria • Stimulation of water and electrolyte secretion • Decrease colonic transit time	• Increased defecation frequency and mean stool consistency score • Decreased pain during defecation, episodes of fecal incontinence and abdominal pain (Tabbers *et al.*, 2011)
4.	Atopic disease	• Modulation of immune responses • Activation of dendritic cells and skewing of T-helper (Th1/Th2) • Upregulation on anti-inflammatory cytokines such as interferon (IFN)-γ	• Reduced allergic symptoms • Decreased the atopic dermatitis scores (SCORAD) from 16 to 0 after 2 months of intervention (Michail, 2009; Taniuchi *et al.*, 2005; Xiao *et al.*, 2006; Yesilova *et al.*, 2012)
5.	Respiratory tract infections	• Increasing immune cell activity • Increasing local and systemic antibody production • Inducing phenotypic changes in dendritic cells	• Sig. reduction in the incidence and duration of fever, coughing and rhinorrhea • Lower no. of subjects experienced episodes of respiratory infections compared to control group. • Fewer episodes of fever (Taipale *et al.*, 2011; Weizman *et al.*, 2005)

2.5 Respiratory diseases

Acute respiratory infections (ARI) and chronic respiratory infections are the common examples of respiratory diseases. They represent one of the major health issues among children. Statistically, acute respiratory infections are responsible for pediatric morbidity and mortality among children under five years old (Araujo *et al.*, 2015; Madhi and Klugman, 2006; Simoes *et al.*, 2006; Wang *et al.*, 2016). It is estimated that 1.9 million to 2.2 million of children died of acute respiratory infection worldwide annually (Madhi and Klugman, 2006). Acute respiratory infections also accounted for approximately 60% of all pediatric illnesses, 22-26.7% of all hospitalizations and 33.5-59% of all general practitioner consultations (Gerasimov *et al.*, 2016; Simoes *et al.*, 2006; Tregoning and Schwarze, 2010). Economically, acute respiratory infections have resulted in significant impact on the health care infrastructure among countries (Gerasimov *et al.*, 2016; Tregoning and Schwarze, 2010; Wang *et al.*, 2016). As the acute respiratory infections among children are still a great global challenge for public health, effective treatments and prophylaxes for the acute respiratory infections are of utmost importance.

Acute respiratory infections can be classified as upper respiratory tract infections (URTI) and lower respiratory tract infections (LRTI). The URTI is referred to the infection that occurs in the airways from the nostrils to the vocal cords in the larynx. This includes conditions such as common cold, otitis media, pharyngitis, laryngitis and sinusitis. URTIs are known as the most common infectious diseases (Simoes *et al.*, 2006). The LRTI refers to the infection occurring in the airways from the trachea and the bronchi to the bronchioles and the alveoli, for example bronchitis and pneumonia (Araujo *et al.*, 2015; Simoes *et al.*, 2006; Tregoning and Schwarze, 2010; Thompson *et al.*, 2013; Wang *et al.*, 2016). Such acute respiratory infections are

mostly caused by rhinoviruses (RSVs), parainfluenza and influenza viruses, human metapneumovirus, and adenoviruses (Simoes *et al.*, 2006). Common symptoms found in acute respiratory infections are fever, runny nose, nasal congestion, sore throat, cough, sneezing, increased mucus production or wheezing (Agustina *et al.*, 2012; Cazzola *et al.*, 2010; Eccles, 2007; Gerasimov *et al.*, 2016; Kumpu *et al.*, 2012; Rerksuppaphol and Rerksuppaphol, 2012; Thompson *et al.*, 2013; Wang *et al.*, 2016). However, symptoms developed in LRTI are generally more severe than that in URTI. These include severe cough, breathlessness, respiratory distress or reduced breath sounds due to trapping of air and peripheral hyperinflation of the lung. Therefore, LRTI might be a great threat to the children and hospitalization is usually required (Tregoning and Schwarze, 2010; Wang *et al.*, 2016).

In Malaysia, it was reviewed (Sam *et al.*, 2010) that the occurrence of acute respiratory infections is seasonal, where the occurrences peak in midyear (during the month of May) and year end (months of November and December). The dry season reported during midyear while wet season at year end in Malaysia potentially resulted in the occurrence of ARIs (Rahman *et al.*, 2014; Saat *et al.*, 2010; Sam, 2015). During the dry season, the number of particulates such as smoke, dust, pollen, soot and liquid droplets suspended in the air increased. In industrialized area, haze or air pollution as a result from deforestation and land-clearing also increased the number of particle pollutions in the air. The particle pollutions may trigger the inflammation process in the respiratory tract and promote the access of pathogenic microorganisms into the airways and develop inflammation (Rosa *et al.*, 2008; Tekverk *et al.*, 2015). On the other hand, elevated humidity due to excess rainfall encouraged the development of fungi and certain viruses with seasonal behaviour, resulting in conditions such as cough, allergy and respiratory tract infections among children (Rosa *et al.*, 2008).

2.5.1 Effects of probiotics on respiratory diseases: *in-vivo* evidences

To-date, there are accumulating evidences through clinical trials showing that probiotics exhibit positive effects in the alleviation of acute respiratory infections in both children and adults (Araujo *et al.*, 2015; Cazzola *et al.*, 2010; Gerasimov *et al.*, 2016; Rerksuppaphol and Rerksuppaphol, 2012; Taipale *et al.*, 2016). Children who attend day care centres or preschools are especially exposed to higher risk of acquiring acute respiratory infections. This is due to their close physical contact among children that favours the transmission of infectious diseases. In a clinical study done in Finland of children receiving either probiotic *L. rhamnosus* GG in milk, the days of day care absenteeism due to illness was significantly lesser when compared with control group. In addition, children in the *L. rhamnosus* GG group had fewer respiratory tract infections with complications (otitis media, sinusitis, bronchitis and pneumonia) and less prescribed antibiotic treatments (Lehtoranta, 2012). *Lactobacillus* group showed significantly longer duration without respiratory symptoms as compared to the control group (Hatakka *et al.*, 2001). Another study done in Croatia where children attending day care were randomly allocated to either receiving *L. rhamnosus* GG in milk or placebo for three months, the children in *L. rhamnosus* GG group were observed to have significantly reduced risk of upper respiratory tract infection that lasted more than three days (Hojsak *et al.*, 2010). The effects of a probiotic mixture on alleviation of respiratory illnesses were also investigated by Hatakka and his colleagues (2007) in Finland among the otitis-prone children from 10 months to six years of age. This placebo-controlled study involved 24 weeks of intervention with capsule containing mixture of *Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Bifidobacterium breve* 99 and *Propionibacterium freudenreichii* JS showed reduction in the occurrence of recurrent respiratory illness. When the effects of probiotics were studied on the

respiratory pathogens in the nasopharyngeal airway, the number of human bocavirus was reduced significantly in the nasopharynx of these children, indicating that probiotics may be more effective against respiratory tract infections of viral origin (Lehtoranta, 2012). Also, administration of *Bifidobacterium longum* BB536 was shown to alleviate symptoms of the respiratory infections (Xiao *et al.*, 2006; Xiao *et al.*, 2007). Namba *et al.* (2010) also reported that the incidence and occurrence of influenza were significantly lower in *Bifidobacterium longum* BB536 group, relative to the placebo group.

2.5.2 Clinical studies on the effects of bifidobacteria on respiratory diseases

Bifidobacteria had been extensively evaluated for their efficacies in preventing or reducing incidences of respiratory diseases, particularly in upper respiratory tract infections (URTI) (Gill *et al.*, 2012; Lehtoranta *et al.*, 2014). Collectively, a number of randomized controlled trials showed positive outcomes of the administration of bifidobacteria in reducing the incidences as well as symptoms of respiratory diseases among subjects aged from one month up to 12 years old (Cazzola *et al.*, 2010; Gerasimov *et al.*, 2016; Hatakka *et al.*, 2007; Leyer *et al.*, 2009; Rerksuppaphol and Rerksuppaphol, 2012; Taipale *et al.*, 2011) but a few showed no effect on the incidences (Hojsak *et al.*, 2015; Hojsak *et al.*, 2016; Kloster Smerud *et al.*, 2008) (Table 2.4).

Table 2.4 Randomized, placebo-controlled trials involving bifidobacteria strains in respiratory diseases.

i) **Trials with positive outcomes**

Treatment	Probiotic strains	Dosage (Duration of study)	Age range	Subjects (Randomized); (included in the analysis)	Outcomes	Country	Reference
Common winter diseases	*L. helveticus* R0052, *B. infantis* R0033, *B. bifidum* R0071	3 x 10^9 CFU/day (3 months)	3 – 7 years old	135 (62T/73P); (62T/73P)	• No. of children suffered at least one ENT and respiratory tract disorder symptoms reduced	France	Cazzola *et al.*, 2010
Acute respiratory infections (ARI)	*L. acidophilus* DDS-1 (NCIMB 30333), *B. lactis* UABLA-12 (NCIMB 30334)	5 x 10^{10} CFU/day (14 days or till resolution of secondary ARI)	3 – 12 years old	210 (113T/112P); (113T/112P)	• Time to resolution of the secondary ARI was shorter • Median severity of ARI was lower	Ukraine	Gerasimov *et al.*, 2016
Acute otitis media	*L. rhamnosus* GG (ATCC53103), *L. rhamnosus* LC705, *B. breve* 99, *P. freudenreichii* spp. *shermanii* JS	8-9 x 10^9 CFU/capsule of each strain, 1 capsule/day (6 months)	10 months – 6 years old	309 (155T/154P); (135T/134P)	• Tendency in reducing recurrent of respiratory infections	Finland	Hatakka *et al.*, 2007
Common cold	*L. acidophilus*, *B. bifidum*	10^9 bacteria each/capsule; 2 capsules/day (3 months)	8 – 13 years old	80 (40T/40P); (40T/40P)	• Symptoms of common cold (fever, cough, rhinorrhea) reduced	Thailand	Rerksuppaphol and Rerksuppaphol, 2012

*****Intervention 1: 110/110; Intervention 2: 112/112

AOM= Acute otitis media; ENT=ear, nose and throat; P=placebo; T=intervention; CFU=colony forming unit

Continued Table 2.4

ii) **Trials with insignificant outcomes**

Treatment	Probiotic strains	Dosage (Duration of study)	Age range	Subjects (Randomized); (included in the analysis)	Outcomes	Country	Reference
Respiratory tract infections	*B. animalis* subsp. *lactis*	10^9 CFU/day (entire duration of hospitalization)	1 – 18 years old	727 (362T/365P); (362T/365P)	• No significant in incidence, duration and severity of respiratory tract infections • No significant in duration of hospitalization • No significant in antibiotic use	Croatia	Hojsak *et al.*, 2015
Respiratory tract infections	*L. rhamnosus* GG and *L. acidophilus* LA-5, *B. lactis* BB-12	10^9 CFU/day of *Lactobacillus* 10^7 CFU/day of BB-12 (7 months)	12 – 36 months	240 (117T/123P); (97T/102P)	• No significant in days of respiratory symptoms	Norway	Kloster Smerud *et al.*, 2008
Common infections	*B. animalis* subsp. *lactis*	10^9 CFU/day (3 months)	1 – 8 years old	210 (104T/106P); (104T/106P)	• No significant in number of children with infections • No significant in the duration of respiratory infections	Croatia	Hojsak *et al.*, 2016
Infectious diseases	*B. animalis* subsp. *lactis* BB-12 (DSM15954)	1 x 10^{11} CFU/day (7 months)	1 – 2 months	109 (55T/54P); (34T/35P)	• Reduced incidence of respiratory infection • No significant on fever episodes, antibiotic treatments, AOM	Finland	Taipale *et al.*, 2011

*****Intervention 1: 110/110; Intervention 2: 112/112

AOM= Acute otitis media; ENT=ear, nose and throat; P=placebo; T=intervention; CFU=colony forming unit

2.6 Gastrointestinal diseases

Gastrointestinal diseases include irritable bowel syndrome (Nicholson *et al.*, 2012), inflammatory bowel diseases (IBD), *Helicobacter pylori* infection, necrotizing enterocolitis (NEC), pouchitis, antibiotic associated diarrhea (AAD), *Clostridium difficile*-associated diarrhea (CDD), traveler's diarrhea, infectious diarrhea and acute diarrhea (Ringel *et al.*, 2012; Ritchie and Romanuk, 2012). Among these gastrointestinal diseases, acute diarrhea is the second leading cause of morbidity and mortality among children under five years of age globally, such that nearly 1.7 billion cases of diarrhea and 760 thousand cases of diarrhea-related death are reported every year (Ahmadi *et al.*, 2015; Kolader *et al.*, 2013; WHO, 2013). While in the year 2015, WHO claimed that as many as 5.9 million children under five years of age died due to preventable diseases including diarrhea (WHO, 2016). Among the children in this age group, those attending day care centres or preschool are 2-3 times higher risk in acquiring acute gastrointestinal infections like diarrhea (Hatakka *et al.*, 2001; Hojsak *et al.*, 2016; Kloster Smerud *et al.*, 2008). Diarrhea is commonly defined as three or more loose or watery stools in the last 24 hours (WHO, 2013).

In Malaysia, reported cases of acute diarrhea and gastroenteritis have been observed throughout the year, with rotavirus-associated diarrhea being the most identified gastrointestinal disorder (Cheah *et al.*, 2011; Hsu *et al.*, 2005; Hung *et al.*, 2006). Although data on the occurrence of gastrointestinal diseases, specifically for children are rare, general data from the Malaysian population revealed that acute diarrhea occurrence happened throughout the year with several peaks in the early of the year (month of January), midyear (month of May till July) and year end (months of November and December) (Cheah *et al.*, 2011; Hsu *et al.*, 2005).

Evidences suggested that gastrointestinal diseases have been linked to dysbiosis or imbalance of the gastrointestinal microbial community (Lau *et al.*, 2015; Rauch and Lynch, 2012; Ringel *et al.*, 2012; Ritchie and Romanuk, 2012). Dysbiosis is referred to as "qualitative and quantitative changes in the intestinal flora, their metabolic activity and their local distribution" (Hawrelak and Myers, 2004). Perturbations in the microbial communities may contribute to disease susceptibility (Hemarajata and Versalovic, 2013).

2.6.1 Effects of probiotics on gastrointestinal diseases: *in-vivo* evidences

Emerging evidences showed that probiotic supplementation is able to ameliorate diseases by restructuring the microbiota community (Forbes *et al.*, 2016; Hemarajata and Versalovic, 2013; Hojsak *et al.*, 2016; Kloster Smerud *et al.*, 2008; Rauch and Lynch, 2012; Ringel *et al.*, 2012). In several randomized, double-blind and placebo-controlled studies on probiotic prevention of acute diarrhea in daycare centres worldwide, probiotics tested such as *Lactobacillus* GG, *Bifidobacterium lactis* (alone or in combination with *Streptococcus thermophiles*), *L. reuteri*, *L. casei* and *L. acidophilus* showed a clear evidence of efficacy to reduce diarrhea-related symptoms in a strain-dependent and dose-dependent manner (Guandalini, 2011; Kloster Smerud *et al.*, 2008). Out of the 15 clinical studies reviewed, participants who were administered probiotics were less likely to have diarrhea lasting more than three days, indicating the efficacy of probiotics not only in preventing occurrence of diarrhea, but also in treating episodes of diarrhea. Additionally, it was found that probiotics intake, as was reported in 12 studies performed on infants and children, reduced the mean duration of diarrhea by 29.20 hours (Allen *et al.*, 2009). In the study by Grandy *et al.* (2010), the effect of probiotics *Lactobacillus acidophilus*, *L. rhamnosus*, *B. longum*

and *Saccharomyces boulardii* also showed that intervention groups had a shorter time of vomiting periods as compared with controls, during the probiotics treatment of acute rotavirus diarrhea. Also, a clinical study carried out by Colombel *et al.* (1987) showed that with the administration of yogurt containing *B. longum* BB536, there was no diarrhea case reported among 10 adult volunteers. They also found a great decrease in the numbers of fecal *Clostridium* in the *B. longum* BB536 administered group compared to that of the placebo group.

2.6.2 Clinical studies on the effects of bifidobacteria on gastrointestinal diseases

There are increasing evidence reported that bifidobacteria demonstrated positive effects on various gastrointestinal diseases. However, the evidences are still limited (Table 2.5). In a study done by Weizman and colleagues (2005), two interventions using two probiotics strains: *Bifidobacterium lactis* (BB-12) and *Lactobacillus reuteri* (55730) which were supplemented in cow's milk formula showed significant reduction in days with diarrhea and episodes of diarrhea in both probiotics groups. Besides, another study which involved children attending day-care exhibited a significant (P=0.020) reduction in the treatment group receiving probiotic milk drink containing a combination of three lactic acid bacteria: LGG, *L. acidophilus* LA-5 and *Bifidobacteria* Bb-12 (Kloster Smerud *et al.*, 2008).

Bifidobacteria are also effective in ameliorating antibiotic-associated diarrhea (Picard *et al.*, 2005). A study done by Corrêa *et al.* (2005) which involved the administration of commercial probiotic formula containing *Bifidobacterium lactis* and *Streptococcus thermophiles*, showed a significant lower in the number of antibiotic-associated diarrhea (AAD), 16.3% in treatment group as compared to 31.2% in placebo group.