

Storage Stability and Folate Requirements of a Commercial Probiotic
Bifidobacteria and *Bifidobacterium longum* DJO10A

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OMER F CELIK

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Daniel J. O'Sullivan, Adviser

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Dedication

This thesis is dedicated to my parents and siblings for their unconditional love and support.

Abstract

Using trehalose as the cryoprotectant, a freeze-drying protocol with almost no loss of viability was developed for stress-sensitive strains of bifidobacteria. The resilience of *B. animalis* subsp. *lactis* Bb-12, a common probiotic used in food products, to a wide range of storage conditions was demonstrated while a stress-sensitive strain, *B. longum* DJO10A, required optimum conditions of frozen storage, water activities controlled between 0.11 and 0.22 and replacement of oxygen with nitrogen to maintain viability.

The predicted models based on genome sequences for the folate biosynthetic abilities of several bifidobacteria were examined and the accuracy of these models for the tested strains was functionally evaluated. This study indicated that some bifidobacteria have potential to supplement folate while some can act as folate scavengers, including strain Bb-12. To maintain adequate dietary folate intake it may be conceivable to include additional folic acid in foods containing high levels of folate depleting bifidobacteria.

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Chapter I - Bifidobacteria: The Journey Back To the Intestine

1. Human Gastrointestinal Microflora

1.1. Development of the Intestinal Microbiota in Neonates and Factors Influencing its Composition

The human body consists of internal components not exposed to the environment and external components that are. The skin, lungs, mouth, throat, nose, stomach, vagina/urethra, and intestines are the primary constituent parts exposed to the environment. The outer parts are permanently exposed to the surrounding environment and this enables microorganisms to come in contact with the body through contact with surfaces, foods consumed and air inhaled. The gastrointestinal tract (GIT), which is generally > 8 meters long in adults, but varies from person to person, largely depending on weight, constitutes the major part of the outside of the body and refers to all the structures from the mouth to the anus including the esophagus, stomach, small and large intestines (Hounnou et al., 2002). The GIT is defined as a very large and dynamic ecosystem hosting either resident members or transient microorganisms introduced from the environment primarily through ingested foods and can be commensal, pathogens or beneficial. Based on culturing and microscopic analysis it was originally estimated that the human GIT contains 400-500 bacterial species, which constitute only a small portion of the whole microbiome (Moore and Holdeman, 1974). The human intestinal tract harbors 10^{13} to 10^{14} microorganisms whose genomes contain at least 100-fold more genes than the complete human genome (Gill et al., 2006). However, the knowledge of this complex ecosystem is still limited and its microbial composition varies greatly between individuals due to factors such as genetics, age, health conditions and diet.

There are a few reports on the presence of bacteria in amniotic fluid and meconium (DiGiulio et al., 2008, Jiménez et al., 2008) as well as the presence of bacterial

DNA in placenta (Satokari et al., 2009) that may suggest an internal route that enables bacterial transition from maternal to fetal GIT, but this concept requires further studies to be proven. The fetal GIT is generally acknowledged to be sterile and its colonization begins with its exposure to bacteria from the mother during birth and the surrounding environment. Although the GIT is usually first colonized by facultative anaerobes such as *Streptococcus* and coliforms, it is suggested that strict anaerobes such as *Bifidobacterium*, *Clostridium*, and *Bacteroides* dominate gradually with the consumption of oxygen and formation of a reduced environment in the GIT (Favier et al., 2002). However there are several factors influencing the postnatal colonization of the GIT: mode of delivery, type of infant feeding, gestational age, infant hospitalization and antibiotic use (Penders et al., 2006).

The initial colonizers of the GIT originate from the vaginal and fecal microbiota of mothers for vaginally delivered babies, while the hospital environment is a larger determinant of the microbial composition for caesarian delivered babies. Studies indicated that infants born through cesarean section had lower numbers of *Bifidobacterium* and *Bacteroides*, and they were more often colonized with *Clostridium difficile* (Penders et al., 2006). Colonization of the GIT in preterm infants was found to be affected by gestational age, mainly due to antibiotic usage in most preterm infants. However, a study on 52 premature infants ranging between 30 - 35 weeks, showed bifidobacterial colonization is significantly delayed regardless of delivery type and antibiotic usage (Butel et al., 2007). In general colonization of beneficial bacteria such as lactobacilli and bifidobacteria was found to be delayed in preterm infants whereas the

number of potentially pathogenic bacteria such as *E.coli* and enterococci was found to be high (Westerbeek et al., 2006).

The development and composition of microbiota is highly influenced by the diet. Higher abundance of bifidobacteria and lower abundance of facultative anaerobes were detected in the GI microbiota of breast-fed infants compared to formula-fed infants in different studies (Favier et al., 2002, Harmsen et al., 2000). While Penders et al. (2005) found no such difference, that study used real time PCR with primer pairs that were not sufficiently validated for specificity and may have amplified DNA for some of the thousands of species present in the GIT. The intestinal flora of formula-fed infants is more diverse and often contains more *Bacteroides*, *Clostridium*, and *Enterobacteriaceae* while the intestinal flora of breast fed infants is generally dominated by *Bifidobacterium* and other lactic acid producing bacteria. Although formula-fed infants were shown to have less bifidobacteria, supplementation of formulas with fructooligosaccharides (FOS) and galactooligosaccharides (GOS) was shown to increase the number of bifidobacteria in fecal samples (Haarman and Knol, 2005, Klaassens et al., 2009)

Hospitalization after birth resulted in higher *Clostridium difficile* colonization and its prevalence was found to be increased ~ 13% per day of hospitalization (Penders et al., 2006). Antibiotic use alters the GIT microbiota depending on the type of antibiotic and dosage applied. A significant decrease in the counts of *Bifidobacterium* and *Bacteroides* species in infants receiving oral antibiotic therapy was reported by Penders et al. (2006).

1.2. Microbial Diversity in the Human Gastrointestinal Tract (GIT)

The mouth is the starting point of the GIT and the oral environment is a suitable environment for bacterial growth and according to the online human oral microbiome database (HOMD), the human oral environment currently contains 179 characterized genera, comprising nearly 700 species (Chen et al., 2010). However, metagenomic analyses have suggested the real number of species is several thousands (Keijsers et al., 2008). Besides being very individualistic, the oral microbiota is mainly composed of the four phyla, *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* (Bik et al., 2010, Zaura et al., 2009). The esophagus and the stomach were thought to be unsuitable for microbial growth by microbiologists early on. Any bacteria detected were considered as transients rather than residents, mainly due to the extremely low pH in the stomach and very short transition time in the esophagus (Franklin and Skoryna, 1966, Lau et al., 1981). However, the esophagus is considered as a potential environment for bacterial colonization due to its large mucosal surface downstream of the oropharynx. According to a study by Pei et al. (2004) ~ 100 species were found to be residents of the normal esophagus with more than 80% of them characterized and cultivable. However, dominant phylotypes in the esophagus were found to be similar to upstream members in the oral cavity and the throat suggesting the bacteria that are present are usually those that have been swallowed with the food.

The stomach typically hosts ~ 10^3 cfu per gram of gastric content (Franklin and Skoryna, 1966, Giannella et al., 1972). Besides *Helicobacter pylori*, a stomach pathogen, and bacteria that can tolerate the gastric environment, such as lactobacilli and streptococci, some other proteobacteria specifically associated with the stomach environment were found in a study by Andersson et al. (2008). The stomach microbiota is

mainly dominated by the four phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*, similar to the oral cavity (Bik et al., 2006, Maldonado-Contreras et al., 2011). The effect of *H. pylori* status on the diversity of gastric microbiota is not clear. While Bik et al. (2006) found no difference in abundance of *H. pylori* from gastric biopsy samples of 23 patients, Andersson et al. (2008) detected a more diverse microbiota in the 6 patients of negative *H. pylori* status

The small intestine is the place where food degradation and absorption of nutrients mainly occurs. It consists of three distinct parts, the duodenum, the jejunum and the ileum and the pH increases throughout the small intestine, reaching 6.5-7.5 in the distal part (Evans et al., 1988). There are many nutrients absorbed through the small intestine, especially in the duodenum. These nutrients are also available for utilization by the microbiota, but there are several challenges they have to face concurrently. One challenge is the short transit time which is estimated to be ~ 2.5 hours (Hung et al., 2006). Secreted compounds such as bile salts also play an important role in shaping the diversification of the microbiota in the small intestine due to their strong bactericidal activity while facilitating the digestion of nutrients. Another challenge for bacterial colonization is immunoglobulin A (IgA) which limits microbial penetration into the mucosa.

In general, microbial numbers and diversity increase further down in the jejunum and ileum however, studies on the microbial composition of the small intestine are limited due to its poor accessibility and sampling difficulties. Up to recently, samplings were based on biopsies that were obtained either from the mouth or rectum using catheters which provide limited amounts of material and are open to contamination while

passing through the other sections of the GIT. Another problem with this type of sampling is the prior use of a GI evacuation process which may influence the makeup of the natural microbiota. In recent studies, subjects were chosen among ileostomists (patients whose large intestine is removed) mainly because of the ease of access, providing the use of non-invasive methods and sufficient amount of material. Among the groups defined from ileal effluents, *Streptococcus* sp. and *Clostridium* sp. were found to be dominant based on 16S rRNA and metagenomic approaches (Booijink et al., 2010, Zoetendal et al., 2012). However it should be considered that samples from ileostomy subjects may not be good representatives of natural small intestinal microbiota.

The colon has a large impact on health with its large microbial population, which is estimated to be $\sim 10^{11}$ - 10^{12} microbial cells per gram of material. Inflammatory bowel disease has been linked to the loss of bacterial diversity in the colon (Ott et al., 2004). Microbial colonization in the colon is facilitated by relatively favorable conditions of colon, such as a higher pH, larger volume, lower bile salt concentration, and longer retention time due to slower peristalsis (Walter and Ley, 2011). Although the colon is rich in undigested polysaccharides, the main energy sources for the microbes in the colon, the nutrient composition is unsteady and it is dependent on the individual dietary habits (Alles et al., 1996). The colon has the highest microbial population in the body, but its microbial composition is primarily dominated by the four bacterial phyla: *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Proteobacteria* (Eckburg et al., 2005). This is low compared to soil which typically has nine dominant phyla (Janssen, 2006). Two of these, *Bacteroidetes* and *Firmicutes*, were found to represent more than 90% of the total microbial population based on a 16S rRNA gene sequencing analysis (Eckburg

et al., 2005), but this may be an over representation because on the inherent bias of universal primers. In a recent metagenomic study as part of the EU funded Metagenomics of Human Intestinal Tract (MetaHit) project, it was estimated from a study of 124 individuals that each individual person harbored more than 1,000 species, of which 160 were largely shared among the population (Qin et al., 2010).

2. Probiotics

2.1. History of Probiotics

“Probiotic”, a term derived from Greek, meaning prolife or for life, was likely first used by Kollath in 1953 to define plant based components to treat patients malnourished due to a diet of highly refined foods (Kollath, 1953). As research in the field developed, it was redefined as “organisms and substances that contribute to the intestinal microbial balance” by Parker (1974), and subsequently as “a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance” by Fuller (1992). As can be seen the definition has been modified in a way highlighting the importance of live cells and its health beneficial effects on the host as essential features of a probiotic. The most commonly accepted definition proposed in 2001 by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), described probiotics as “live microorganisms that confer a health benefit on the host when administered in adequate amounts” (FAO/WHO, 2001).

Although the current definition of the term probiotics is relatively new, as a concept, it dates back to the early 1900s with the contribution of two important pioneer figures. The first one was Tissier (1900), who cultured and fed bifidobacteria to infants suffering from diarrhea, given that these ‘bifid’ shaped bacteria dominate the feces of

breast fed infants, but are absent from infants with diarrhea. The second one was Eli Metchnikoff (Metchnikoff et al., 1908) who attributed the health and longevity in Bulgarian rural populations to their consumption of lactic acid producing bacteria present in fermented dairy products. Specifically, he suggested that detrimental microorganisms in the intestine can be replaced by lactic acid bacteria by consuming appropriate fermented foods.

Scientists mostly agree that probiotics are live microorganisms but there is still an ongoing argument for the inclusion of nonviable bacteria in the definition. At the core of this argument is the belief that dead cells, metabolites, and fermentation products derived from bacteria may also provide specific health benefits such as improved digestion of lactose and some immune modulation activities. However, they are not considered to be probiotics because they don't meet the current definition for probiotics. For example, Naidu et al. (1999) used the concept of "Probiotic - Active Substance" to define "cellular complex of lactic acid bacteria that may beneficially modulate the immune system independent from viability by interacting with the host mucosa". In the same light, Taverniti and Guglielmetti (2011) argued that dead microbial cells or cell fractions can still act as probiotics in promoting health and suggested another term "paraprobiotic" to define those non-viable bacteria or crude cell-extracts.

2.2. Marketing of Probiotics

The global market size of probiotics is forecasted to be \$31.1 billion by 2014 and over \$40 billion by 2018 (Frost & Sullivan, 2012). Although the probiotic market is not as well established in North America compared to Japan and Europe, it is currently rapidly growing. The probiotic market is expected to grow ~ 7% globally whereas the

annual growth rate for North America is projected to be 13.6% between 2011 and 2015 (Frost & Sullivan, 2012). Dairy products, especially yogurts, are the main food products used as carriers for probiotic microorganisms. *Lactobacillus* species such as *L. acidophilus* and *L. casei* and *Bifidobacterium animalis* subsp. *lactis* are the most common probiotic cultures used commercially. Some of the strains that are used commonly in commercial food products are given in Table I-1. Some other lactic acid bacteria such as *Enterococcus*, non-lactic acid bacteria such as *E. coli* strain Nissle and *Bacillus coagulans*, and a yeast, *Saccharomyces boulardii*, are sometimes claimed as probiotics due to studies suggesting beneficial effects. However, as they are not normally part of the GI resident flora these strains require more studies in order to be considered true probiotic organisms.

Table I-1. *Lactobacillus* and *Bifidobacterium* containing food products in grocery stores in the Twin Cities Metro Area

Manufacturer (State)	Product	Cultures¹ listed on the food product²
Brown Cow Farm (CA)	Brown Cow Yogurt	Bifidus, LA
Cascade Fresh, Inc. (WA)	Cascade Fresh Yogurt	<i>B. longum</i> , <i>B. bifidum</i> , <i>B. infantis</i> , LA, LC, LR
Chobani, Inc. (NY)	Chobani Greek Yogurt	Bifidus, LA, LC
Dannon Co, Inc. (NY)	DanActive Drinkable Yogurt	<i>L. casei</i> DN-114 001
	Activia Yogurt	<i>B. lactis</i> DN 173-010
FAGE USA Dairy Industry Inc. (NY)	Fage Greek Yogurt	Bifidus, LA, LC
Green Valley Organics (CA)	Green Valley Kefir	<i>B. bifidum</i> , LA, LC, LR
Kalona SuperNatural (IA)	Kalona Yogurt	Bifidus, LA
Liberty Brand Products, Inc. (MN)	Liberté Méditerranée Yogurt	Bifidobacteria sp., LA, <i>L. paracasei</i>
Lifeway Foods, Inc. (IL)	Lifeway Kefir	<i>B. breve</i> , <i>B. lactis</i> , <i>B. longum</i> , LA, LC, <i>L. lactis</i> , <i>L. plantarum</i> , <i>L. reuteri</i> , LR
	Lifeway Probugs Smoothie	<i>B. breve</i> , <i>B. longum</i> , LA, LC, <i>L. lactis</i> , <i>L. plantarum</i> , LR
NextFoods (CO)	GoodBelly + Juice Drink/ Straight Shot Oat Milk	<i>L. plantarum</i> 299V
Noosa Yoghurts (CO)	Noosa Yoghurt	Bifidus, LA, LC
Old Chatham Shepherding Company (NY)	Old Chatham Sheep Milk Yogurt	Bifidus, LA
Redwood Hill Farm & Creamery (CA)	Redwood Hill Farm Goat Milk Kefir	<i>B. bifidum</i> , LA, LC, LR
	Redwood Hill Farm Goat Milk Yogurt	Bifidus, LA
Seven Stars Farm (PA)	Seven Stars Farm Yogurt	Bifidus, LA
Springfield Creamery (OR)	Nancy's Organic Yogurt/Probiotic Greek Yogurt	<i>B. animalis</i> subsp. <i>lactis</i> BB-12, <i>L. acidophilus</i> LA-5, LC, <i>L. rhamnosus</i> LB3
Stonyfield Farm (NH)	Stonyfield Super Smoothie/YoKids-	Bifidus, LA, LC, LR

	YoBaby-YoToddler Yogurt	
	Stonyfield Blends Creamy Yogurt/O'Soy Organic Soy Yogurt/Greek Yogurt	Bifidus, LA, LC
The Greek Gods, LLC (WA)	Greek Gods Yogurt	<i>Bifidobacterium</i> , LA, LC
<i>The Icelandic Milk and Skyr Corporation (NY)</i>	Siggi's Yogurt	<i>B. lactis</i> , LA
	Siggi's Drinkable Yogurt	<i>Bifidobacterium</i> sp., LA, LC, <i>L. delbrueckii</i> subsp. <i>lactis</i> , LR
Turtle Mountain, LLC (OR)	So Delicious Cultured Almond/Coconut Milk	<i>B. animalis</i> , <i>B. bifidum</i> , LA, LR
<i>Wallaby Yogurt Company (CA)</i>	Wallaby Yogurt/Greek Yogurt	Bifidus, LA
WholeSoy & Co. (CA)	WholeSoy & Co Yogurt	<i>B. bifidum</i> , LA
Yakult U.S.A. Inc. (CA)	Yakult Cultured Milk	<i>L. casei</i> Shirota
YoFarm Yogurt Co. (CT)	Yopa Greek Yogurt	Bifidus, LA, LC
	YoCrunch Yogurt	Bifidus, LA
Yoplait USA, Inc. (MN)	Yoplait Simplait Yogurt	LA

¹Only cultures representing *Bifidobacterium* and *Lactobacillus* are listed and *Lactobacillus bulgaricus*, a yogurt starter culture is excluded.

²LA: *Lactobacillus acidophilus*, LC: *Lactobacillus casei*, LR: *Lactobacillus rhamnosus*

3. Bifidobacteria

3.1. History, Taxonomy, Ecology and Morphology of Bifidobacteria

Bifidobacteria were first isolated by Henri Tissier from the feces of breast-fed infants. These bacteria were initially called *Bacillus bifidus* due to their distinctive bifid morphology (Tissier, 1900) and are currently included in the genus, *Bifidobacterium*. Although this genus was first proposed by Orla Jensen in 1924, they were initially classified as members of *Bacteroides* and subsequently *Lactobacillus* due to their obligate fermentative features as well as their morphologies. They were reclassified as a separate genus in the 8th edition of Bergey's Manual in 1974 and denominated as *Bifidobacterium*. According to current taxonomy, *Bifidobacterium* is a member of the *Actinobacteria* phylum.

Bifidobacteria are gram-positive, non-motile, non-spore-forming, anaerobic, non-gas producing bacteria with high G+C content (55-67%) (Gomes and Malcata, 1999). Currently, there are 41 species included in the genus *Bifidobacterium* and there are 29 complete *Bifidobacterium* genome sequences available (Table I-2). Besides being normal inhabitants of the GIT of humans, bifidobacteria have been isolated from a variety of ecological niches in the environment, such as human vagina, blood, oral cavity, intestines of animals and insects, food and sewage (Ventura et al., 2007). Although some species are thought to be strictly of human origin, such as *B. dentium* and *B. adolescentis*, an ecological survey of bifidobacteria has shown that they can also be found in non-human hosts (Lamendella et al., 2008). *B. adolescentis*, *B. catenulatum* and *B. longum* subsp. *longum* are representatives of adult bifidobacteria populations, whereas *B. breve*, *B. bifidum*, and *B. longum* subsp. *infantis* are more common in infants (Roger et al., 2010). *B. animalis*, *B. pseudolongum* and *B. thermophilum* are known as animal derived species.

Some animal species are host specific and are only present in certain animals, such as *B. magnum* and *B. cuniculi*, which have only been found in rabbits, *B. pullorum* and *B. gallinarum* only found in the chickens, *B. suis* only in pigs, and *B. asteroides* and *B. indicum* in honeybees. *B. minimum* and *B. subtile* have only been found in sewage.

Bifidobacteria have a characteristic morphology with V- or Y- shaped rods, usually referred to as bifid shape. However, culture conditions can affect the morphology of bifidobacteria and lead to the formation of different cellular forms. According to a study by Rasić and Kurmann (1983) bifidobacteria are mostly rod-shaped in their natural habitat and branching occurs under unfavorable conditions. Lack of certain aminoacids in the growth medium such as alanine, aspartic acid, glutamic acid, and serine, or the aminosugar N-acetylglucosamine, which is involved in the synthesis of peptidoglycan (Glick et al., 1960), can result in an increase in branching and induces the bifid shape of bifidobacteria. Inadequate concentrations of sodium or calcium ions are other factors that influence the morphology of bifidobacteria (Kojima et al., 1968). Tissier also implied that this branched shape is a response to acidity, temperature, and nitrogen sources. Some bifidobacteria have shown elongated cell morphology when exposed to oxygen due to incomplete cell division (Simpson et al., 2004). The morphology of bifidobacteria also varies among species and depends on the age of culture.

Table I-2. Available complete genome sequences of *Bifidobacterium*¹

Bacterium	Strain	Genome Size (Mb)	Center	Date Released
<i>B. longum</i>	NCC2705	2.26	Nestle Research Center, Switzerland	10/17/02
<i>B. adolescentis</i>	ATCC 15703	2.09	Gifu University, Life Science Research Center, Japan	12/05/06
<i>B. longum</i>	DJO10A	2.3	University of Minnesota, MN, USA	06/05/08
<i>B. longum</i> subsp. <i>infantis</i>	ATCC 15697	2.83	DOE Joint Genome Institute, CA, USA	11/20/08
<i>B. animalis</i> subsp. <i>lactis</i>	AD011	1.93	Korea Research Institute of Bioscience and Biotechnology, Korea	01/06/09
<i>B. animalis</i> subsp. <i>lactis</i>	BL-04	1.93	Danisco USA Inc.	06/19/09
<i>B. animalis</i> subsp. <i>lactis</i>	DSM 10140	1.93	Danisco USA Inc.	06/19/09
<i>B. dentium</i>	Bd1	2.6	University of Parma, Italy	01/05/10
<i>B. animalis</i> subsp. <i>lactis</i>	BB-12	1.94	Chr. Hansen, Denmark	02/19/10
<i>B. longum</i> subsp. <i>longum</i>	F8	2.38	Wellcome Trust Sanger Institute, UK	03/25/10
<i>B. animalis</i> subsp. <i>lactis</i>	V9	1.94	Southwest Agricultural University, China	05/05/10
<i>B. longum</i> subsp. <i>longum</i>	JDM301	2.48	Genome Sequencing Center (GSC) at Washington University, St. Louis, MO, USA	05/18/10
<i>B. bifidum</i>	S17	2.2	Institute of Microbiology and Biotechnology, University of Ulm, Germany	10/19/10
<i>B. bifidum</i>	PRL2010	2.2	University of Parma, Italy	11/02/10
<i>B. longum</i> subsp. <i>longum</i>	BBMN68	2.27	Southwest Agricultural University, China	11/10/10
<i>B. longum</i> subsp. <i>infantis</i>	157F	2.41	University of Tokyo, Japan	01/28/11
<i>B. longum</i> subsp. <i>longum</i>	JCM 1217	2.39	University of Tokyo, Japan	01/28/11
<i>B. longum</i> subsp. <i>infantis</i>	ATCC 15697	2.83	University of Tokyo, Japan	01/28/11
<i>B. breve</i>	ACS-071-V-	2.3	J. Craig Venter Institute,	05/17/11

	Sch8b		USA	
<i>B. longum</i> subsp. <i>longum</i>	KACC91563	2.4	National Livestock Research Institute, Suwon, Korea	06/28/11
<i>B. breve</i>	UCC2003	2.4	National University of Ireland, Cork, Ireland	07/06/11
<i>B. animalis</i> subsp. <i>lactis</i>	CNCM I-2494	1.94	Genome Sequencing Center (GSC) at Washington University, St. Louis, MO, USA	07/18/11
<i>B. animalis</i> subsp. <i>lactis</i>	BLC1	1.94	University of Parma, Italy	09/01/11
<i>B. animalis</i> subsp. <i>lactis</i>	ATCC 25527	1.93	Pennsylvania State University, PA, USA	04/30/12
<i>B. animalis</i> subsp. <i>lactis</i>	Bi-07	1.94	DuPont Nutrition and Health, WI, USA	05/07/12
<i>B. animalis</i> subsp. <i>lactis</i>	B420	1.94	DuPont Nutrition and Health, WI, USA	05/07/12
<i>B. bifidum</i>	BGN4	2.22	Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Korea	06/05/12
<i>B. asteroides</i>	PRL2011	2.17	National University of Ireland, Cork, Ireland.	10/09/12
<i>B. thermophilum</i>	RBL67	2.29	Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland	03/20/13

¹Sequences those are present in GenBank as of 05/15/2013.

3.2. Tolerance of Bifidobacteria to Technological and Gastrointestinal Stress Factors

3.2.1. Temperature Tolerance

Bifidobacteria are exposed to temperature fluctuations starting from preparation to their consumption as probiotic. Tolerance to temperature stresses is an effective factor on the viability and stability hence crucial for bifidobacteria. The optimum temperature for growth of human isolated strains of bifidobacteria is between 36 and 38°C. Gavini et al. (1991) found out growth at 45°C can be used as a discriminative property for classification of human and animal originated bifidobacterial strains since, animal originated strains are able to grow at this temperature whereas the majority of the human strains are not. However, an isolate of *B. thermacidophilum* from baby feces was found to be able to grow at 47°C (von Ah et al., 2007). Another isolate from waste water of a bean-curd farm can grow at 49.5°C (Dong et al., 2000) which is the highest growth temperature known for bifidobacteria. Although generally bifidobacteria cannot grow below 20°C, *B. psychraerophilum*, an isolate from a pig, has been shown to grow at temperatures as low as 4°C (Simpson et al., 2004). Growth temperature is found to be effective not only on morphology but also on the adhesion properties of bifidobacteria (Mattarelli et al., 1999).

3.2.2. Oxygen Sensitivity

Bifidobacteria are classified as strict anaerobes, but their tolerance to oxygen differs between species and strains (Simpson et al., 2004, Talwalkar and Kailasapathy, 2004). *B. psychraerophilum*, an isolate from pig caecum, is aerotolerant and it is the first bifidobacteria reported which is able to grow on agar media under aerobic conditions (Simpson et al., 2004). Kawasaki et al. (2006) reported that growth of *B. thermophilum*

and *B. boum* was stimulated in the presence of up to 10% and 20% oxygen, respectively whereas *B. bifidum* and *B. longum* were inhibited under high oxygen concentrations (10 and 20%) due to the accumulation of H₂O₂ in the medium. In the subsequent study it was shown that growth of *B. thermophilum* and *B. boum* was stimulated in the presence of 1% CO₂, under an atmosphere containing 5% O₂ (Kawasaki et al., 2007). It was also reported that some *Bifidobacterium* species, such as *B. globosum*, and *B. thermophilum*, form colonies under air when enriched with 10% CO₂ (Li et al., 2010). Sensitivity of four different *Bifidobacterium* species, *B. breve*, *B. infantis*, *B. adolescentis* and *B. longum*, to oxygen was tested in a study by Shimamura et al. (1992) and only *B. adolescentis* was found to be significantly suppressed by low concentrations of oxygen (obtained via constant shaking at 45 rpm) whereas the others reached an optical density (660 nm) of 60 to 70% of that observed with the anaerobic environment. Among bifidobacteria *B. scardovii* was described as a facultative anaerobe (Hoyles et al., 2002) and *B. animalis* subsp. *lactis* is known as oxygen tolerant mainly because of its adaptation to the fermented milk environment..

3.2.3. Acid and bile tolerance

According to the current definition, probiotics have to be alive when they are consumed in order to provide beneficial health effects although there are many studies suggesting that probiotics confer health benefits regardless of viability (reviewed, Kataria et al., 2009). Acid and bile tolerance are important characteristics of probiotic bifidobacteria since they are exposed to bile and harsh acidic conditions throughout the GIT.

Bifidobacteria generate acetic acid in addition to lactic acid (in the molar ratio of 3:2) through fermentation. Therefore, many strains are fairly acid-tolerant and the optimum pH for growth is determined to be between 6.5 and 7.0. No growth is recorded at pH lower than 4.5 or higher than 8.5 with the exception of *B. thermacidophilum*, which is able to grow at pH 4.0 (Dong et al., 2000). *B. dentium*, which is isolated from the oral cavity, and *B. longum* were shown to be able to survive under prolonged exposure to pH 4.0, which is even more resistant than *Streptococcus mutans*, the main contributor to tooth decay in humans (Nakajo et al., 2010). In addition, *B. animalis* subsp. *lactis* was shown to be able to survive in strong acidic conditions (Masco et al., 2007, Vernazza et al., 2006), including synthetic human gastric fluid at a pH of 3.5 (Maus and Ingham, 2003). Even though bifidobacteria are known as acid-tolerant, they are not acidophilic microorganisms. The viability for bifidobacteria under acidic conditions (pH < 3) was found to be growth phase dependent and stationary phase cells retained viability better than actively growing cells (Waddington et al., 2010).

The most common method to test the resistance to high acidity is based on the ability to survive under exposure to low pHs, usually pH 3 or lower, for certain time periods. These conditions are used to demonstrate the stomach conditions during digestion. Although many probiotics do not have sufficient resistance, this can be improved by exposure to acidic environments. Exposure to moderate or increasing acidity allows an adaptation to acidic conditions, which is known as adaptive acid tolerance response (ATR) (O'Sullivan and Condon, 1997). In general viability of lactobacilli was shown to be more pronounced in comparison to bifidobacteria after heat and acid treatments (Saarela et al., 2004).

Acid tolerance is also important for the incorporation of probiotics into acidic foods such as yogurts and fruit juices. Microencapsulation of probiotic cells is gaining attention to increase their viability in yogurt (Kailasapathy, 2006). UV mutagenesis followed by passage in acidic mediums has been shown to enable having more acid-resistant Bb-12 mutants. Furthermore, it was shown that resilience to acidic conditions should be tested using acidic food matrixes rather than hydrochloric acid since stability predictions are not correlated for these two conditions (Saarela et al., 2011).

Bile is produced by the liver and secreted from the gall bladder into the duodenum where it acts as a surfactant and aids in the digestion of lipids by emulsifying the fats contained in foods. On the other hand it is an antimicrobial compound since it disrupts the cell structure not only via changing the fatty acid and phospholipid composition but also modulating the expression of membrane proteins (Ruiz et al., 2007). Therefore, resistance to bile is an important selection criterion for probiotic bifidobacteria. Among the probiotic strains tested in the study by Vinderola and Reinheimer (2003), *Lactobacillus acidophilus* showed the highest bile tolerance followed by the other probiotic lactobacilli and bifidobacteria tested. Among *Bifidobacterium* species tested, *B. bifidum* was shown to be more resistant than *B. longum*. However, *B. longum* strain BL was found to be as resistant as *B. bifidum* strains suggesting strain dependency of bile resistance. In another study *B. animalis* subsp. *lactis* and *B. bifidum* were found to be more resistant to bile on agar compared to broth based on growth tests.

3.2.4 Adhesion

Adhesion to the intestinal cells is an important characteristic for survival and activity of probiotics, especially in the small intestine which has relatively lower retention time and constant efflux. Adherent strains potentially persist longer in the GIT and have more opportunity to show beneficial effects (Saarela et al., 2000). Furthermore, adhesion is suggested to be correlated with immunomodulation effect of probiotics. Different in vitro model systems have been developed for the selection of potentially adherent probiotic strains. Among these Caco-2 cells, a cell line used as an in vitro model for intestinal epithelium, are mostly used for testing the adherence ability of probiotics to intestinal cells. KATO III (Del Re et al., 2000), HT-29 (Ali et al., 2008), and HT-29 MTX (Gopal et al., 2001), a mucus secreting cell line, are other cell lines that are used to demonstrate adherence properties of bifidobacteria. HT-29 MTX was thought to be a better representative for the mucus secreting enterocytes and adhesion of different probiotics displayed 2-3 times greater adhesion with this cell line comparatively (Gopal et al., 2001). Although attachment to these cell lines can be used as an indicator for potential adherence properties, failures should not be evaluated as a precise inability to adhere due to limitations of the assay (O'Sullivan, 2001).

Adhesion properties of bifidobacteria were found to be strain dependent and correlated with bile resistance (Gueimonde et al., 2005). In different studies it was found that different strains of bifidobacteria had better adhesion properties when applied in combination with *L. rhamnosus* GG suggesting a combination of certain strains of probiotics may increase the adhesion level of particular individual strains (Collado et al., 2007, Juntunen et al., 2001, Ouwehand et al., 2000). Bifidobacteria were previously

reported to inhibit the adhesion of gram-negative pathogens such as enterotoxigenic *E. coli* H10407, enteropathogenic *E. coli* JPN15, *E. coli* O157:H7 and *Salmonella typhimurium* SL 1344 (Bernet et al., 1993, Gagnon et al., 2004, Gopal et al., 2001, Lievin et al., 2000). In a study by Collado et al. (2005) bifidobacteria of animal origin were shown to have greater adherence and pathogen displacement ability than human strains. In another study it was shown that greater adhesion and pathogen displacement ability occurred with bifidobacteria which were incubated at pH = 2.0 for 16 h to induce acid resistance (Collado et al., 2006).

It was demonstrated that adhesion properties of bifidobacteria were influenced by the presence of specific sugars, bile salts, pH and incubation time. Bile is known to affect hydrophobic components of the bacterial cell and thus may alter the adhesion properties. Ouwehand et al. (2001) reported that pretreatment with 1% bile causes a reduction in the adhesive capacity of *B. animalis* subsp. *lactis* Bb12. Adhesion levels of all *Bifidobacterium* strains tested were reduced between 7% and 74% in the presence of 0.3% bile (Gueimonde et al., 2005). Adhesion was increased in the presence of fucose and mannose (Guglielmetti et al., 2009) and higher adhesion levels were obtained as incubation time with the cell line increased from 15 min to 120 min (Ali et al., 2008). Although a higher pH was generally thought to give better adhesion due to alterations on the cell lines at lower pH, these studies suggest that the effect of pH depends on the cell lines used and strains tested .

3.2.5. Antimicrobial Activity

A huge competition takes place in the intestinal environment. Resident and transient microorganisms compete for the nutrients. Antimicrobial activity also provides a superior advantage for probiotics against pathogenic and the other intestinal residents. There are metabolites associated with the lactic acid bacteria that can ensure antimicrobial activity such as organic acids and hydrogen peroxide. All lactic acid bacteria produce organic acids but only some produce bacteriocins, which are peptide based antimicrobials. Bacteriocins provide advantages to probiotics through their antimicrobial feature against other colonizers of the intestinal microflora. They may also serve as signaling peptides, signaling other bacteria or the immune system (Dobson et al., 2012).

Bacteriocins differ from traditional antibiotics in several aspects. Unlike antibiotics which are synthesized by enzymes, bacteriocins are ribosomally synthesized and they are thought to be able to inhibit similar or closely related bacteria. Nisin is produced by specific strains of *Lactococcus lactis* and has been used in many food products for preservation purposes. It is a member of the lantibiotic family of bacteriocins, which exhibits broad antimicrobial spectrum. Although they have a broader antimicrobial spectrum, they are primarily effective only on gram positive bacteria.

Until recently only *B. bifidum* (Yildirim et al., 1999) and *B. infantis* (Cheikhoussef et al., 2010) were shown to produce bacteriocins among *Bifidobacterium* species suggesting bifidobacteria may have different competition mechanisms involved for competition in the large intestine. However, recently bisin, a lantibiotic produced by *B. longum* subsp. *longum* DJO10A, was shown to have natural potential inhibitory effects

against members of the *Enterobacteriaceae* (O'Sullivan and Lee, 2011), a gram-negative bacteria family embracing familiar pathogens such as *Salmonella* and *E. coli*.

3.2.6. Safety Aspects

Safety is the first thing that has to be considered for a new probiotic strain. All strains have unique properties and probiotic activities of different strains may differ within the same species (Soccol et al., 2010). Therefore, detailed identification of the genus, species, and strain is required. There are a number of studies reporting that the identity of microorganisms isolated from probiotic products often do not correspond to the information stated on the product label (Gueimonde et al., 2004, Temmerman et al., 2003). A study by Huys et al. (2006) was done to evaluate the accuracy of identities of commercial probiotic cultures to address the problem of mislabeled probiotic products. Results of this study show that 28% of the commercial probiotic cultures were misidentified at the genus or species level by their manufacturers.

Potential virulence factors and invasion potential for a new strain also need to be studied in terms of safety (Sanders et al., 2010) although no known virulence factors have been identified for both *Lactobacillus* (Vesterlund et al., 2007) and *Bifidobacterium* (Ouwehand et al., 2004). *Lactobacillus* and *Bifidobacterium* are considered safe due to not only their association with fermented food and the normal microbiota of the human body, but are also very rarely involved in infections in humans. *L. casei* and *L. rhamnosus* are the species most frequently associated with bacteremia and endocarditis. Among these there are only 2 cases of *Lactobacillus* infection which may be linked with probiotic consumption (Land et al., 2005). There are very few cases of bifidobacteremia reported and non-probiotic *B. dentium* was implicated in most cases (Meile et al., 2008).

Although bifidobacteria has been consumed in infant formulas worldwide for almost 20 years, there are no reported cases associated with any pathologic or adverse effects (Saavedra, 2007). On the other hand, some microorganisms used as probiotics such as *Bacillus*, *Streptococcus*, *Enterococcus* and *Escherichia* pose a greater threat than others. Therefore, the accuracy of identification to the strain level is a critical step in the safety evaluation of these particular genera.

Antibiotic resistance and transferability is another major problem that needs to be addressed for the safety of probiotics. Antibiotic resistance can be either intrinsic, or can be acquired by mutation or by added genes. The primary threat of acquired resistance is the ability of that resistance to be horizontally transferred from probiotic strains to residents of gut microbiota, particularly pathogens. Functional investigation of antibiotic resistance transferability, using animal studies or clinical trials, provides a more solid argument for safety issues. Nowadays, it is also feasible to obtain a genome sequence and its analysis for possible gene transfer mechanisms. Also, it is important to examine the sequence for known drug resistance markers in order to determine if the genes are present.

Chapter II - Factors influencing the stability of freeze-dried stress-resilient and stress-sensitive strains of bifidobacteria

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Freeze-drying is a common method for preservation of probiotics including bifidobacteria for further industrial applications. However, the stability of freeze-dried bifidobacteria varies depending on the freeze-drying method and following storage conditions. The primary goals of this study were to develop an optimized freeze-drying procedure and to determine the effects of temperature, water activity and atmosphere on survival of freeze-dried bifidobacteria. To address these goals, a commercially used bifidobacteria strain that is quite resilient to stress, *Bifidobacterium animalis* subsp. *lactis* Bb-12, and a characterized intestinal strain that is more sensitive to stress conditions, *B. longum* DJO10A, were used. A freeze-drying protocol was developed using trehalose as the cryoprotectant which resulted in almost no loss of viability during freeze-drying. Resuscitation medium, temperature and time did not significantly influence recovery rates when this cryoprotectant was used. The effects of temperature (- 80 to 45°C), water activity (0.02 to 0.92) and atmosphere (air, vacuum and nitrogen) were evaluated for the stability of the freeze-dried powders during storage. Freeze-dried *B. animalis* subsp. *lactis* Bb-12 was found to survive under all conditions tested with optimum survival at temperatures up to room temperature, water activities up to 0.44 and all 3 atmospheres tested. The intestinal adapted strain *B. longum* DJO10A was much more sensitive to the different storage conditions, but using optimum conditions could be adequately maintained. These optimum storage conditions included frozen storage, replacement of oxygen with nitrogen and water activities controlled between 0.11 and 0.22. These results indicated that an optimized storage environment is required to maintain viability of stress sensitive bifidobacteria strains while stress resilient bifidobacteria strains can maintain

viability over a wide range of storage conditions, which is practical for countries where controlled cold storage conditions may not be readily available.

1. Introduction

The observations of Metchnikoff et al. (1908) on the health benefits of ingesting lactic acid producing bacteria laid the groundwork for the current probiotic era, which is a rapidly growing field worldwide. Dairy foods are particularly good vehicles for the delivery of probiotics to humans, as lactic acid producing cultures are suited to this environment. The first commercial probiotic drink Yakult, containing *Lactobacillus casei* Shirota, was introduced in 1935 in Japan and is still sold today throughout the world (Fukushima and Hurt, 2011). Bifidobacteria were subsequently introduced due to their association with healthy intestines in breast fed infants and also their reduced numbers in the elderly with a concomitant reduction in gut health. In the last 20 years, the global probiotic market has been growing due to considerable progress in probiotic research and sales are estimated to reach \$19.6 billion in 2013 (Granato et al., 2010). Currently, *Lactobacillus* and *Bifidobacterium* are the most common probiotic genera used in food products.

There are many challenges in the development of probiotic-containing food products such as selection of effective strains and their survival during processing and storage. An important component of current selection practices for bifidobacteria for use as probiotics is their ability to survive food processing and storage. Their viability has been a technological issue for food manufacturers, particularly fermented foods such as yogurt. As bifidobacteria are largely obligate anaerobic bacteria and not very acid tolerant, they are less stable in yogurts compared to lactobacilli (El-Dieb et al., 2012). Hence viability of bifidobacteria becomes an important issue and until technological

advancements are made in protecting their viability in foods, strains are selected primarily for this feature rather than the myriad of other characteristics that pertain to their probiotic functioning in the gut.

Drying cultures can enable long term storage and transportation without the need for refrigeration, if conditions are optimized. However, conditions are not the same for all cultures, with some cultures, particularly many bifidobacteria cultures, being particularly sensitive (Meng et al., 2008). While dried cultures provides advantages through shipping and storage, there are critical parameters that affect the viability following their incorporation into foods, such as the nature of the food matrix, rehydration temperature, powder-to-liquid ratios and rehydration time (Champagne, 2009). Freeze-drying and spray drying are the two methods for drying of probiotics that are currently used, with spray drying the preferred choice because of cost effectiveness. In terms of viability, freeze-drying is the best process known to date but its cost has hindered its use in large-scale processes. Spray drying can be used for some cultures, but the conditions require optimization since probiotics are sensitive to heat (Chávez and Ledebøer, 2007). Studies have substantiated that bifidobacteria in general are very sensitive to spray drying, but gave superior survival rates for freeze-drying in different media (Chávez and Ledebøer, 2007, Wang et al., 2004, Wong et al., 2010). Based on these findings, freeze-drying became a popular method of stabilizing bifidobacteria before incorporation into food products.

Maintaining viability not only during the freeze-drying process but also during storage is a critical challenge for commercial production of bifidobacteria for probiotic applications (Saarela et al., 2005). Cryoprotectants such as polymers and sugars have

been involved in the freeze-drying process to improve the survival rate of bifidobacteria (Kiviharju et al., 2005, Saarela et al., 2005). However, the ability to survive during freeze-drying and subsequent storage is not linked. Therefore, factors that affect survival during storage need to be elaborated (Champagne et al., 2005). Storage temperatures, exposure to oxygen and water activity are several critical factors that affect viability of dried probiotics (Chávez and Ledebøer, 2007) only a few studies showing the detrimental effects of storage temperature and water activity on freeze-dried bifidobacteria (Abe et al., 2009a, Bruno and Shah, 2003, Champagne et al., 1996).

While bifidobacteria with high stress tolerance are commonly used in food products, there is an interest in using other less tolerant strains that may be more suited to competing in the gut, provided that suitable conditions were developed to maintain their viability in foods. The objectives of this study were to develop a freeze-drying protocol for bifidobacteria, including stress sensitive strains, and to evaluate the effects of temperature, water activity and atmosphere during subsequent storage of the freeze-dried powders. Two potential probiotic bifidobacteria with different stress tolerances were used to achieve these objectives. One strain represented the most stress adapted group of bifidobacteria, *B. animalis* subsp. *lactis* (Simpson et al., 2005), and the other strain, *B. longum* DJO10A, a characterized intestinal strain with minimum pure culture adaptation, represented a highly stress sensitive group of bifidobacteria.

2. Materials and Methods

2.1. Bacterial Strains, Growth Media, and Culture Growth Conditions

Two strains of bifidobacteria were used for this study; *Bifidobacterium animalis* subsp. *lactis* Bb-12 and *B. longum* subsp. *longum* DJO10A. Strain Bb-12 which is a common commercial probiotic used in many food products was obtained from Chr. Hansen Inc., Milwaukee, WI. Strain DJO10A is an intestinal strain which was isolated and characterized in our laboratory (Islam, 2006). Cultures were used from frozen stocks stored at -80°C stocked in Man, Rogosa, and Sharpe (MRS; BD Biosciences, San Jose, CA) containing 15% glycerol. Cultures were cultivated in MRS and incubated at 37°C anaerobically. A selective medium for bifidobacteria, bifidobacteria iodoacetate selective medium (BIM-25; (Munoa and Pares, 1988), was used consisting of 3.8 % reinforced clostridial agar (RCM; Oxoid, Hampshire, England), 0.005% L-cysteine·HCl, 0.002% nalidixic acid, 0.00085% polymyxin B sulfate, 0.005% kanamycin sulfate, 0.0035% iodoacetic acid, 0.0025% 2,3,5 triphenyl tetrazolium chloride and 1.8 % agar.

2.2. Preparation of Cultures for Freeze-Drying

Strains of both Bb-12 and DJO10A were inoculated from stock cultures into tubes of MRS containing 0.05% L-cysteine·HCl and incubated anaerobically at 37°C for one day. Each culture was sub-inoculated at 2% into 4 L MRS+0.05% L-cysteine·HCl and incubated anaerobically at 37°C. Optical density at 600 nm (OD₆₀₀) was checked for determination of growth kinetics and when an OD₆₀₀ of 1.0 was reached, and the following freeze-drying procedure was applied.

2.3. Preparation of Freeze-Drying Buffers

Sodium phosphate buffer (pH=6.8) was used as the base buffer after autoclaving at 121°C for 15 minutes. Base buffer was supplemented with dried skim milk (DSM; Difco), trehalose and/or sucrose as cryoprotectants. Four different buffers; sodium phosphate buffer, sodium phosphate buffer + 5% DSM, sodium phosphate buffer + 5% DSM + 4% trehalose and sodium phosphate buffer + 5% DSM + 4% trehalose + 5% sucrose were tested to observe their effects on the survival of bifidobacteria after freeze-drying.

2.4. Freeze-Drying Procedure

Previous procedures (Bruno and Shah, 2003, Saarela et al., 2006) were used to develop a suitable freeze-drying procedure for this study. Cultures were grown in MRS+0.05% L-cysteine HCl as described above and OD₆₀₀ was checked periodically. When the OD₆₀₀ was 1.0 the following additional steps were applied to both cultures.

Two 1 ml aliquots of culture were taken for subsequent TAP-PCR analysis and enumeration of viable cell counts after serial dilution. The remaining culture was divided into two centrifuge tubes and centrifuged at 4,500 X g for 15 min at 4°C and the supernatant was discarded. The pellets in each tube were resuspended with 20 ml of 0.1 M sodium phosphate buffer and transferred to 50 ml falcon tubes. Tubes were centrifuged at 2,792 X g for 15 min at 4°C and the supernatant was discarded. The pellets in each tube were re-suspended with 10 ml of one of the four freeze-drying buffers and transferred to new 50 ml falcon tubes. The tubes containing resuspended cells were sealed with parafilm and aluminum foil and were frozen at - 80°C overnight. The cultures

were then dried in a freeze-dryer (Labconco FreeZone 6 L) that was set to dry at -55°C and pressure at 0.018 mbar for 45 hours.

2.5. Preparation of Resuscitation Media

Three types of media were tested for the selection of optimum resuscitation medium; molecular water, MRS+0.05% L-cysteine·HCl and MRS+0.05% L-cysteine·HCl supplemented with 5% DSM.

2.6. Viable Plate Counts

2.6.1. Viable Cell Counts before Freeze-Drying

Cultures were serially diluted (1 in 9 ml) in MRS+0.05% L-cysteine·HCl medium up to 10^{-8} and followed by spread-plating on fresh agar plates of the same medium. Selected dilutions were also plated on BIM-25 agar for verification. All plates were incubated anaerobically at 37°C for 2 days.

2.6.2. Viable Cell Counts after Freeze-Drying

The equivalent quantity of freeze-dried powders required for viable cell counts were determined based on the total volume of culture that had been used for freeze-drying and the amount of powder gained after freeze-drying. Freeze-dried powder was resuspended in 10 ml of one of three resuscitation media by vortexing for ~ 1min. This culture was then serially diluted (1 in 9 ml) in fresh MRS+0.05% L-cysteine·HCl media and followed by spread-plating on fresh agar plates of the same medium. All plates were incubated anaerobically at 37°C for 2 days.

2.7. DNA Fingerprinting of Cultures

To ensure the purity of the cultures before and after freeze-drying a DNA fingerprint was obtained before and after culture preparations. A fingerprinting procedure

that had this capability was triplicate arbitrarily primed-PCR (TAP-PCR) and this was carried out essentially as described in Cusick and O'Sullivan (2000). Specifically, 1.5 ml of overnight broth cultures were pelleted and resuspended in 200 μ l of molecular grade water. Cells were disrupted by agitation with 0.5 volume glass beads ($< 106 \mu\text{m}$ diameter; Sigma, St. Louis, MO) in a Minibeadbeater-8 (Biospec Products, Bartlesville, OK) for 30 s. After a 10-fold dilution, 2 μ l was used as the DNA template. The reaction was performed using 100 pMol of the primer P32 (5'-CAGCAGCCGCGGTAATWC), which has homology to a universally conserved region of the 16S rRNA gene, 1 μ l Taq polymerase (New England Biolabs, Ipswich, MA) along with 5 μ l of the reaction buffer (New England Biolabs) and 0.2 mM dNTP (Promega, Madison, WI) in a 50 μ l final volume. PCR mixtures were overlaid with 25 μ l of mineral oil. Amplification was carried out using a Robocycler Gradient Thermocycler (Agilent Technologies, Santa Clara, Ca) with the following conditions: one cycle of 94°C for 5 min, followed by 40 cycles of 94°C for 1 min and 38, 40, and 42°C for 1 min, followed by 72°C for 2 min, followed by a final extension step at 72°C for 10 minutes.

2.8. Storage Conditions

Three different storage conditions were evaluated. The first one evaluated the effect of different temperatures. Freeze-dried Bb-12 and DJO10A powders were stored at different temperatures (-80, -20, 4, 21, 37 and 45°C) and the viabilities were monitored periodically using viable cell counts.

The second parameter examined was water activity (a_w). Freeze-dried cultures of bifidobacteria were stored at nine different water activity conditions ranging from 0.02 to 0.92. Saturated salt solutions were utilized to provide the indicated water activities as

follows: Calcium chloride ($a_w = 0.02$), lithium chloride ($a_w = 0.11$), potassium acetate ($a_w = 0.22$), magnesium chloride ($a_w = 0.32$), potassium carbonate ($a_w = 0.44$), magnesium nitrate ($a_w = 0.50$), sodium acetate ($a_w = 0.67$), ammonium sulphate ($a_w = 0.79$), sodium phosphate dibasic ($a_w = 0.92$) (Gopalakrishna and Prabhakar, 1983). Freeze-dried powders were spread over shallow containers and placed in mason jars filled with saturated salt solutions. All jars were stored at room temperature (21°C).

The third parameter examined was atmospheric conditions. Normal air, nitrogen based anaerobic atmosphere and vacuum to represent the absence of an atmosphere were applied. Freeze-dried powders were placed in anaerobic jars and jars were immediately closed tightly. Air was evacuated by vacuum. A nitrogen anaerobic atmosphere was provided by pumping nitrogen inside the jar after all the air was vacuumed. All jars were stored at room temperature (21°C). Samples were taken at indicated intervals for analysis of culture viability. All samples were analyzed in triplicate and data were statistically analyzed using the Microsoft Excel software program (Redmond, WA).

3. Results

3.1. Selection of Optimum Cryoprotectant and Resuscitation Medium

Four different cryoprotectants and three resuscitation media were tested in combination for determination of the optimum cryoprotectant and resuscitation medium. *B. longum* DJO10A was used as the model culture for selection of the optimum cryoprotectant and resuscitation medium as it has previously been shown to be more sensitive to processing conditions compared to *B. animalis* subsp. *lactis* Bb-12 (Scheller and O'Sullivan, 2011). The highest recovery rate was obtained when sodium phosphate buffer + 5% DSM + 4% trehalose was used as the cryoprotectant regardless of the resuscitation media (Figure II-1). Although MRS+0.05% L-cysteine·HCl+5% DSM had a slightly higher recovery rate comparatively, resuscitation media did not significantly influence recovery rates when this cryoprotectant was used. However, MRS+0.05% L-cysteine·HCl gave better recovery rates in general. Therefore, sodium phosphate buffer + 5% DSM + 4% trehalose was selected as the optimum cryoprotectant and MRS+0.05% L-cysteine·HCl was selected as the resuscitation medium for further freeze-drying applications.

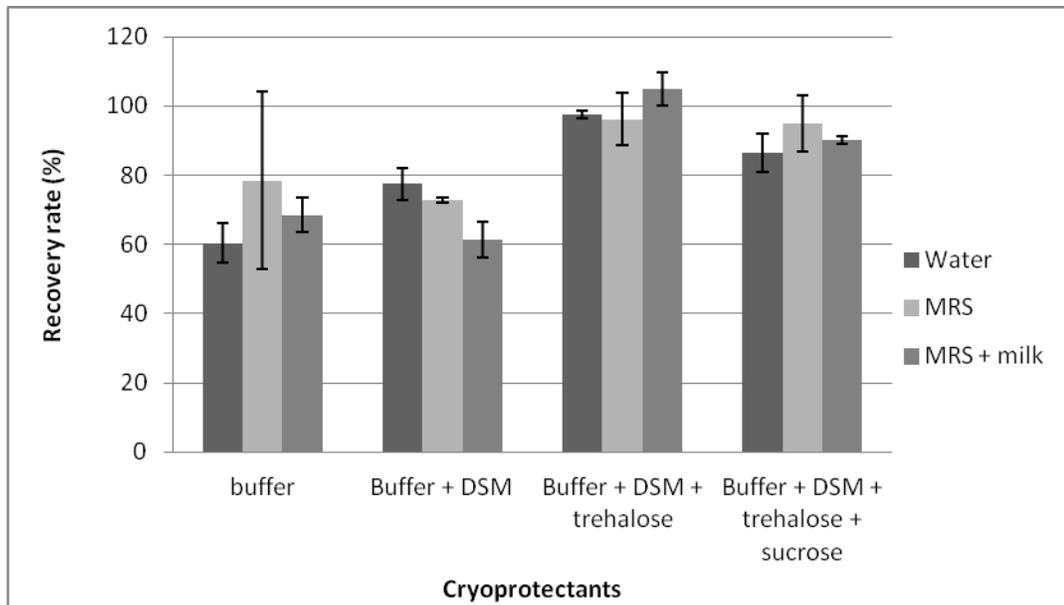


Figure II-1. Recovery rates (%) of *B. longum* DJO10A in different resuscitation media after freeze-drying with different cryoprotectants. Each bar represents the average of three replicas and the error bars show standard deviations.

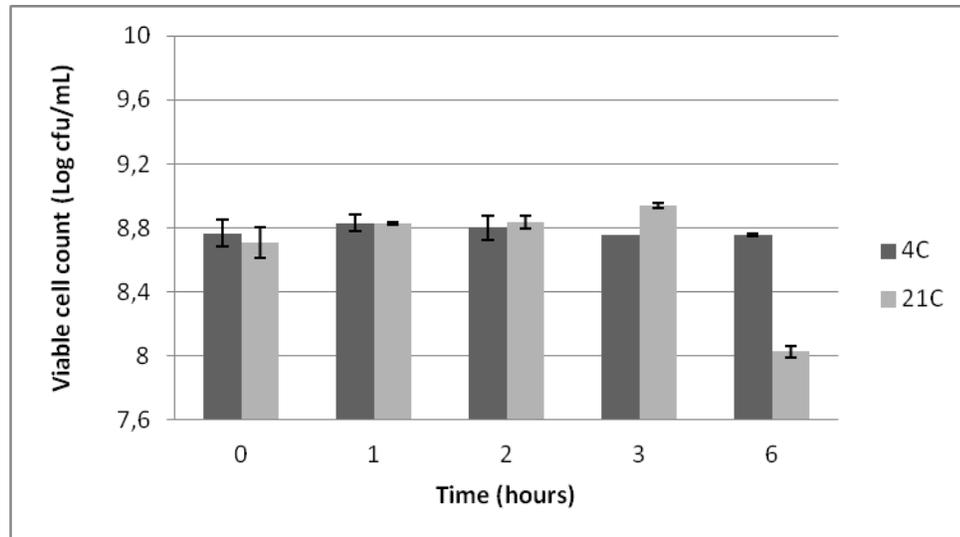
3.2. Evaluation of Resuscitation Time and Temperature for Freeze-Dried Bifidobacteria in Milk

Freshly freeze-dried powders of strain DJO10A and Bb-12 were resuscitated in sterilized skim milk that had initial temperatures of 4 and 21°C. Viable cell counts were carried out and recorded as “0 hours”. The tubes were then stored at 4°C and 21°C and viable cell counts were enumerated at 1, 2, 3 and 6 hours to observe the effect of different temperatures on the resuscitation times of freeze-dried cultures of bifidobacteria in milk. Resuscitation time and temperature did not increase the viability of either strain DJO10A or Bb-12 (Figure II-2). However, extended resuscitation for 6 hours at 21°C reduced viability of strain DJO10A, while strain Bb-12 resumed growing and increased in numbers.

3.3. DNA Fingerprints of Freeze-Dried Cells

A TAP-PCR DNA fingerprint demonstrated that both strains have distinctive fingerprints and the before and after fingerprints are identical (Figure II-3). Therefore, no detectable contamination or chromosomal damage occurred during the freeze-drying procedure based on this fingerprinting analysis.

a)



b)

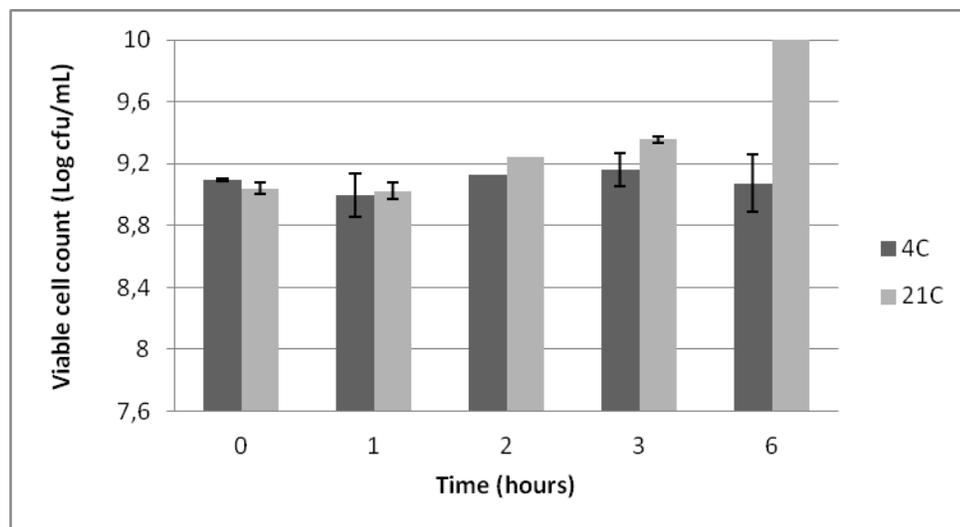


Figure II-2. Resuscitation time in milk at 4 or 21°C for a) *B. longum* DJO10A; and b) *B. animalis* subsp. *lactis* Bb-12. Each bar represents the average of three replicates, and the error bars show standard deviations.

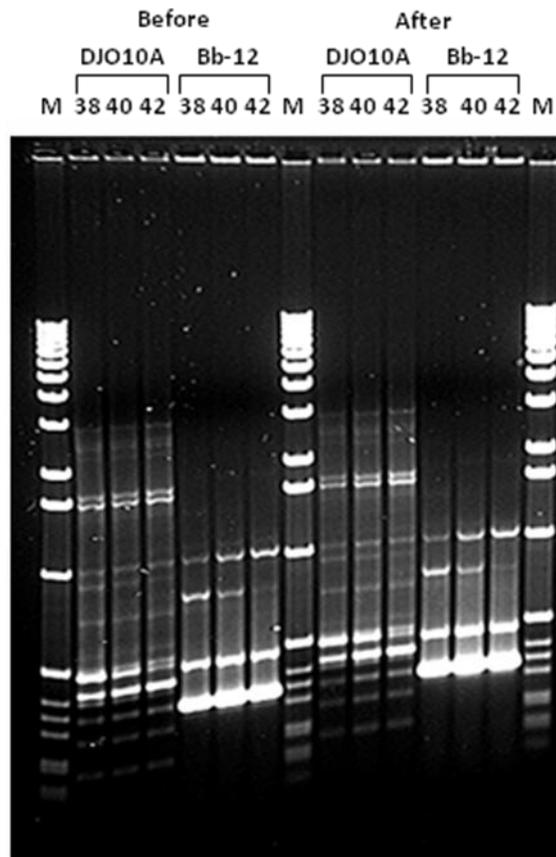


Figure II-3. TAP-PCR DNA fingerprints of both *B. longum* DJO10A and *B. animalis* subsp. *lactis* Bb-12 cell batches before and after freeze-drying. Numbers above the lanes represent annealing temperatures (°C). “M” indicates 1-kb DNA ladder (Invitrogen).

3.4. Effect of Temperature on Survival of Freeze-Dried Bifidobacteria during Storage

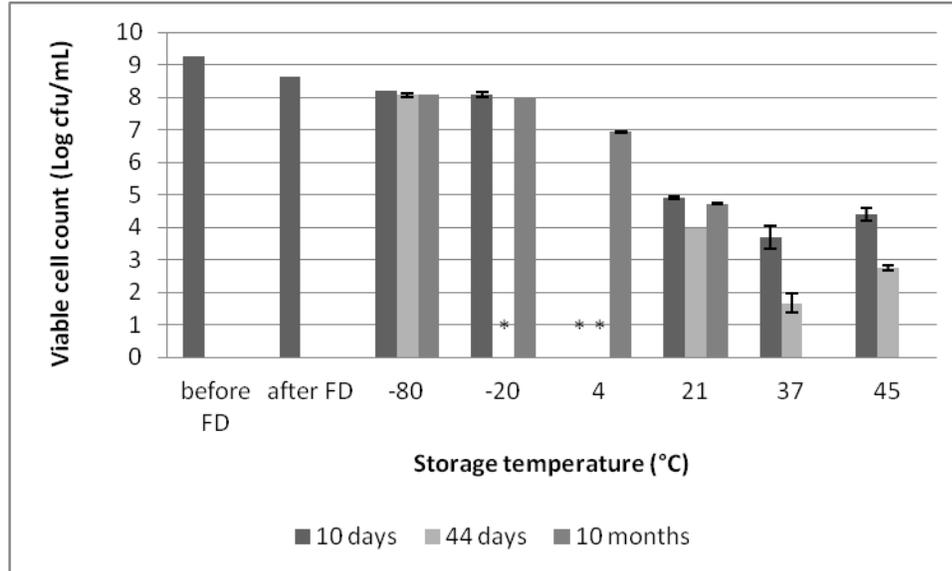
The recovery rate for strain DJO10A was significantly affected by storage temperature. In general, the recovery rate of the DJO10A powders decreased as the storage temperature increased above freezing (Figure II-4a). The highest viability was obtained at frozen storage conditions, in which no considerable loss of viability was observed even after 10 months. Freeze-dried powders of strain Bb-12 did not show a significant reduction in viability during 10 months of storage up to 21°C (Figure II-4b). The highest reduction in viability occurred after 10 months at 37 and 45°C which is about 3 logs.

3.5. Effect of Water Activity (a_w) on Survival of Freeze-Dried Bifidobacteria during Storage

Freeze-dried powders that were stored at different water activities had markedly different physical properties. Powders represented from dusty to sticky properties as the water activity increases and powders that were stored at $a_w = 0.92$ became a mixture of powder and water. The colors of the powders also changed ranging from cream and yellow to orange and brown (Figure II-5). Freeze-dried powders of DJO10A that were stored at higher water activities (0.67 - 0.79 and 0.92) could not be dissolved completely in the resuscitation medium. This prevented accurate viable cell counts for these storage conditions. While similar effects were observed for Bb-12 freeze-dried powders viable plate counts could be performed for all conditions except $a_w = 0.92$. After 45 days of

storage visible molds started to appear on the freeze-dried powders stored at $a_w = 0.92$. Therefore, no more viable cell counts were performed after 45 days for both cultures at this water activity.

a)



b)

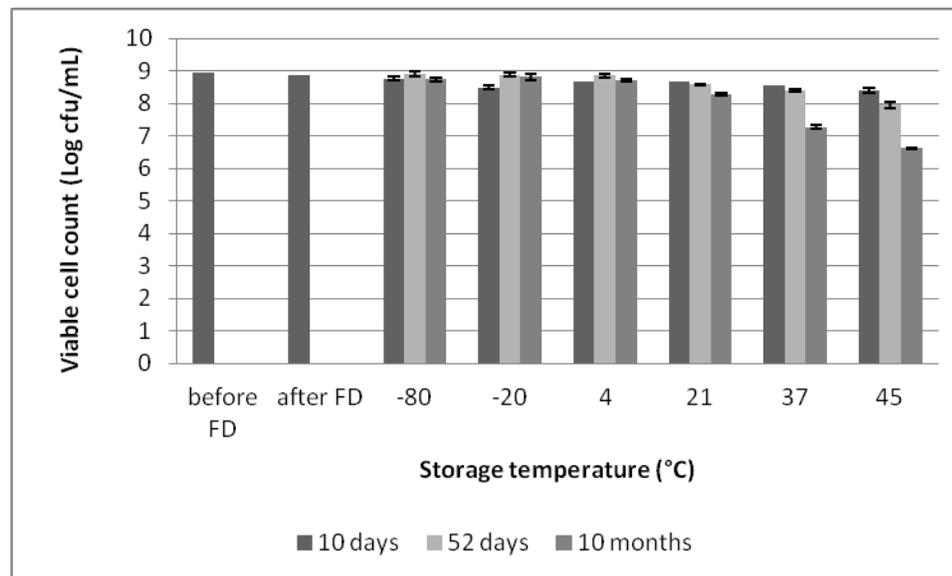


Figure II-4. Viable cell counts for a) *B. longum* DJO10A; and b) *B. animalis* subsp. *lactis* Bb-12 stored at different temperatures. Each bar represents the average of three replicates, and the error bars show standard deviations. Asterisks (*) indicate undetermined viable cell counts due to technical problems.

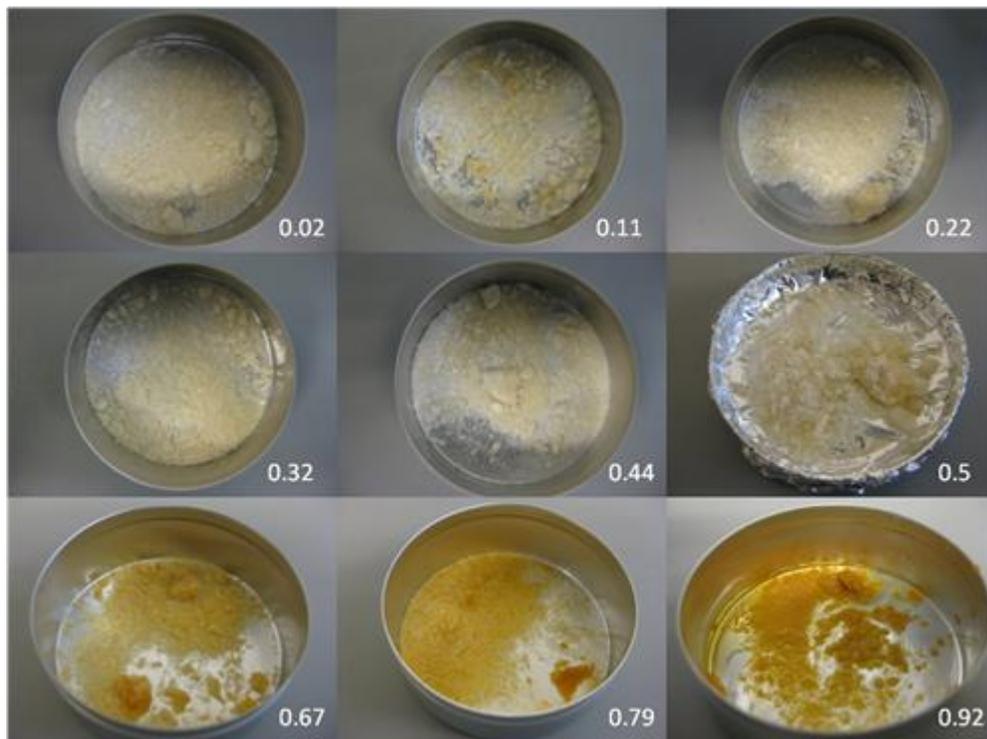


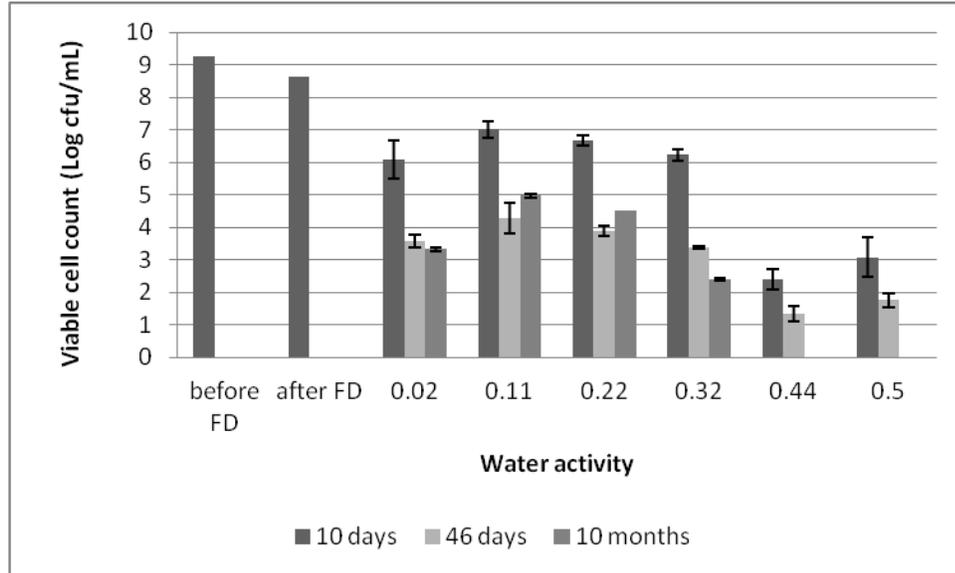
Figure II-5. Physical appearance of freeze-dried cultures of *B. longum* DJO10A under different water activities after 10 days. Color version is available in the online PDF version.

The viability of freeze-dried DJO10A decreased at different rates during storage at different water activities. After 46 days of storage, powders that were stored at $a_w = 0.11$ and $a_w = 0.22$ had the highest viability, respectively whereas $a_w = 0.44$ and $a_w = 0.5$ had the lowest viability (Figure II-6a). The viability of freeze-dried BB-12 was much higher than DJO10A over storage time. Its viability did not change significantly over 10 months of storage at conditions of $a_w = 0.44$ or less. Although viability was still remarkable up to 50 days at higher water activities, no viable cells were detected after 10 months of storage for $a_w = 0.67$ or higher. After 10 months the viability of freeze-dried Bb-12 stored at $a_w = 0.5$ dropped significantly, almost 3 logs and no accurate viable cell counts could be obtained for higher water activities due to solubility problems (Figure II-6b).

3.6. Effect of Atmospheric Conditions on Survival of Freeze-Dried Bifidobacteria during Storage

After 10 months of storage no viable cells could be detected for DJO10A under both vacuum and air at room temperature, as determined by no growth of the resuscitated powders on MRS agar plates (Figure II-7a). Temperature is the main factor affecting viability but it's obvious that exposure to either air or vacuum has a detrimental effect on DJO10A. No considerable difference was found between air and nitrogen after short time storage. However, nitrogen was found to be the most suitable atmosphere for the extended storage of freeze-dried DJO10A. The nitrogen atmosphere caused about a 5 log reduction in viability during the first period but viability was kept constant after that up to 10 months. The affect of different atmospheric conditions on the viability of freeze-dried Bb-12 did not differ from each other and almost no loss of viability was observed (Figure II-7b).

a)



b)

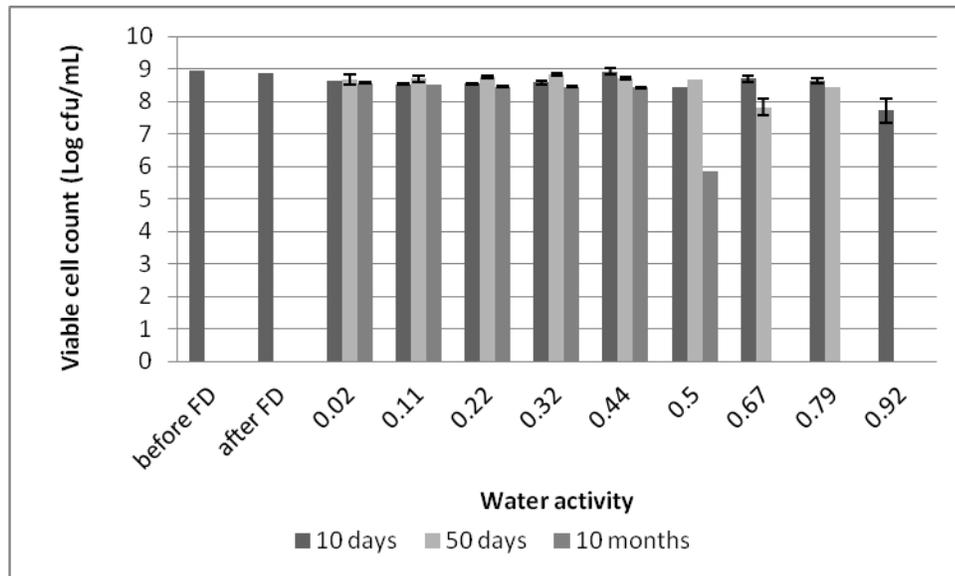
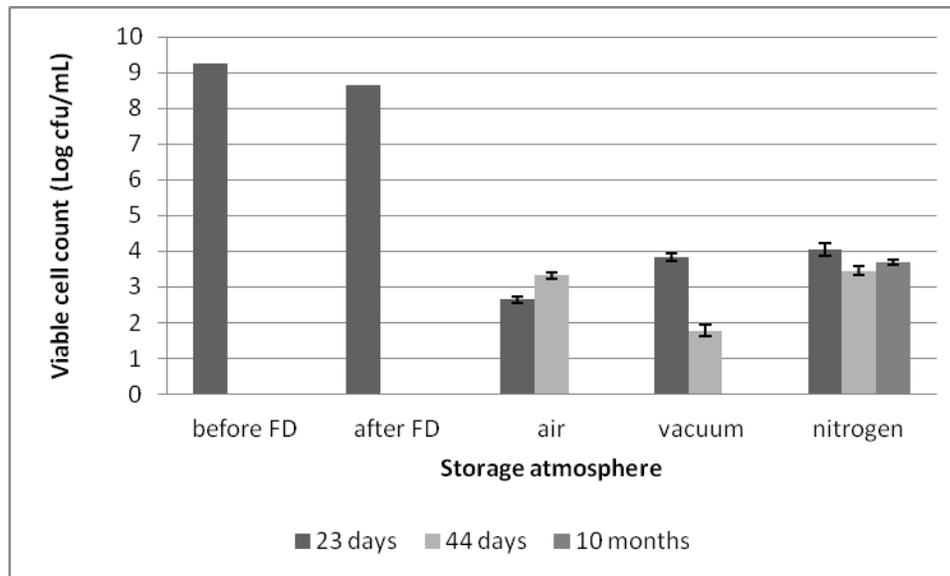


Figure II-6. Viable cell counts for a) *B. longum* DJO10A; and b) *B. animalis* subsp. *lactis* Bb-12 stored at different water activities (a_w). Each bar represents the average of three replicates, and the error bars show standard deviations.

a)



b)

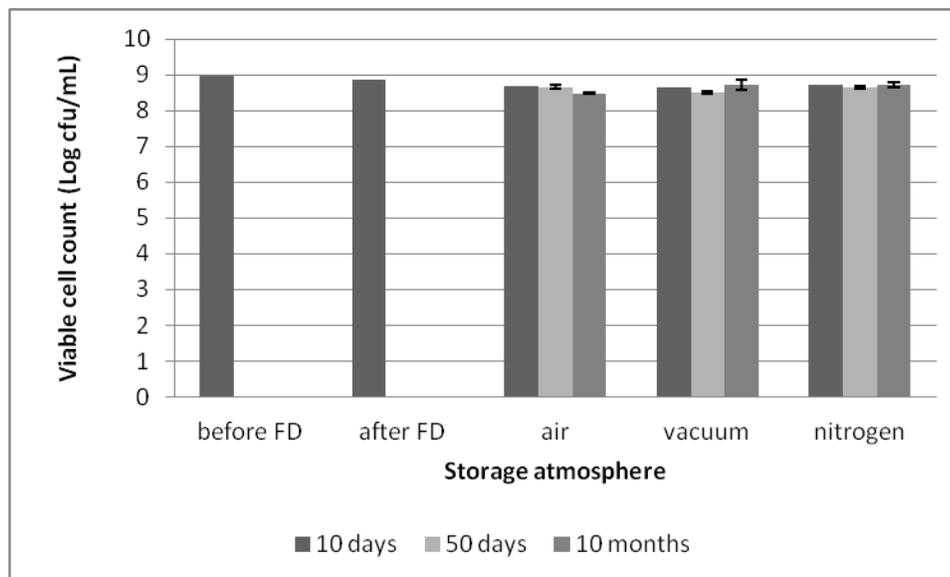


Figure II-7. Viable cell counts for a) *B. longum* DJO10A; and b) *B. animalis* subsp. *lactis* Bb-12 stored at different atmospheres (at room temperature, 21°C). Each bar represents the average of three replicates, and the error bars show standard deviations.

4. Discussion

Keeping probiotics alive is an important task for food manufacturers since viability has been defined as important for probiotics to provide health benefits (FAO/WHO, 2001). Thus, the freeze-drying process is commonly used for the preservation and storage of probiotics for industrial applications. In this study, a freeze-drying procedure using trehalose as the cryoprotectant was developed and effects of various temperatures, water activities and atmospheres on the stability of a freeze-dried commercial strain and a minimally cultured intestinal strain of bifidobacteria during storage were investigated.

The freeze-drying process can cause cellular damage due to the aqueous nature of the contents. Hence, sugars are widely used as cryoprotectants, with non-reducing disaccharides with high glass transition temperatures (T_g) shown to provide higher viability for freeze-dried probiotics (Miao et al., 2008, Siaterlis et al., 2009, Zayed and Roos, 2004). Our study established the cryoprotective effect of trehalose, a non-reducing disaccharide, as an effective cryoprotectant for freeze-drying of bifidobacteria.

Injured cells during freeze-drying can be recovered by applying suitable resuscitation conditions such as resuscitation medium, time and temperature (Carvalho et al., 2004). Variable recovery rates were found depending on the cryoprotective agents used in this study. Appreciable values were obtained even when distilled water was used as the resuscitation medium. These results suggest that the effect of selected resuscitation media on the recovery rate of freeze-dried *B. longum* DJO10A is not significant and more dependent on the cryoprotective agent used.

Confirmation of strain identity is another key point to assure that no contamination is occurred during the freeze-drying process. TAP-PCR DNA

fingerprinting has previously been known to be a convenient method for differentiation of bifidobacteria profiles (Cusick and O'Sullivan, 2000) and in this study was used to ensure the purity of cultures before and after freeze-drying.

Higher storage temperatures yielded lower recovery rates for strain DJO10A, which is consistent with the findings for other bifidobacteria freeze-dried powders (Abe et al., 2009b, Bruno and Shah, 2003, Champagne et al., 1996). Freeze-dried powders of the commercial probiotic Bb-12 were much more resilient to high storage temperatures where viability was kept constant even at room temperature after 10 months of storage period. The 3 log reduction in viability of Bb-12 at 45°C is promising for storage in tropical countries that are not conducive to controlled storage temperatures.

After 10 months of storage at room temperature viability was protected only at water activities lower than 0.44, with an optimum of 0.11, for freeze-dried DJO10A powder. Viability decreased as a_w increased or decreased from 0.11. These results correlate with the findings of other studies where higher stability of strains of *B. longum* is associated with lower water activities (Abe et al., 2009a, Nagawa et al., 1988). However, strain Bb-12 was found to be more tolerant to different water activity conditions. Although Chávez and Ledebøer (2007) suggested water activities below 0.25 for better survival of freeze-dried Bb-12, based on this study, Bb-12 can be stored at water activities up to 0.44 without any loss of viability. This difference may be ascribed to different freeze-drying procedures applied and cryoprotectants used in this study. Despite the fact that the commercial isolate Bb-12 can tolerate a wider range of water activity conditions without any loss in viability, this study demonstrates that the viability

of sensitive cultures, like strain DJO10A, can be kept at reasonable levels when they are stored under controlled water activity conditions.

The water activity was found to be an important factor not only on the stability but also on the physical properties of freeze-dried bifidobacteria such as solubility and color. Solubility problems and color change at higher water activities are maybe associated with various types of nonenzymatic browning reactions occurring, including Maillard reaction (Kurtmann et al., 2009, Stapelfeldt et al., 1997). Trehalose has no role in these reactions as it is a non-reducing sugar; but, lactose from the skim milk powder may have contributed to these reactions. Although loss of solubility at high water activities was a problem for both cultures, it was much more pronounced for DJO10A cells, which may be due to tendency of this strain to form clumps in broth media.

Nitrogen was the only atmosphere that protected the viability of freeze-dried DJO10A up to 10 months of storage. In order to prevent detrimental effects of oxygen Chávez and Ledebøer (2007) suggested vacuum or replacement oxygen with nitrogen for the storage of freeze-dried bifidobacteria. All storage conditions were comparable for the commercial strain Bb-12, consistent with its superior tolerance to stress conditions.

Bifidobacteria are anaerobic bacteria but their oxygen tolerance varies from very low (e.g., *B. longum*) to quite high (e.g., *B. animalis* subsp. *lactis*) (Simpson et al., 2005). When testing the effect of temperature, freeze-dried cultures were simultaneously exposed to air. No loss of viability was observed for Bb-12 in both setups. However, results for DJO10A were found to be distinctly different where 4 logs recovery detected at room temperature test but no recovery obtained in the atmosphere setup at the same temperature. This may be explained by a greater exposure to air when testing the effect of

atmosphere, where the caps were removed from the storage tubes inside the anaerobic jars, whereas for the temperature experiment the storage tubes were capped. In this study, the survival rate of freeze-dried DJO10A was highly dependent on the amount of air, but it had no influence on the survival of freeze-dried Bb-12.

5. Conclusion

This study demonstrated that trehalose provided a superior level of protection during freeze-drying of bifidobacteria. It also verified the resilience of freeze-dried Bb-12 powders to a wide range of storage conditions which makes it an attractive and common probiotic used in the food industry, while also determining suitable storage conditions for sensitive strains such as DJO10A. Frozen storage, replacement of oxygen with nitrogen and water activities controlled between 0.11 and 0.22 were the optimum conditions for the storage of freeze-dried bifidobacteria powders.

6. Acknowledgments

This research was funded in part by the Dairy Management Incorporated (DMI). Ju-Hoon Lee is acknowledged for discussions on optimizing freeze-drying conditions for bifidobacteria.

Chapter III - Functional Analysis of Folate Production by Different Bifidobacteria

Five strains of *Bifidobacterium*, including the widely used commercial probiotic *B. animalis* subsp. *lactis* Bb-12, were screened to investigate their *de novo* folate production ability in the light of predicted models based on available genome sequences. As previously developed folate free media could not support the growth of these strains, even with added folate, a new medium (YNB+) was developed. *B. breve* ATCC 15701 and *B. longum* DJO10A grew independent from pABA and folate whereas *B. longum* subsp. *infantis* RECb4 required pABA for growth. Consistent with its genome prediction, strain Bb-12 required folate for its growth even in the presence of pABA. An LC-MS methodology was used to quantify different forms of folate in MRS + L-cysteine medium. Strains RECb4 and Bb-12 were potential scavengers while strain DJO10A and *B. bifidum* ATCC 15696 were potential producers based on these quantification results. Although *B. breve* ATCC 15701 was able to grow regardless of folate, it depleted folate when it was readily available. Folate production was found to be a strain dependent feature and was influenced by the growth stage of the cultures. This study suggests that although some bifidobacteria have the capability to produce folate, some can act as depleters when folate is readily available in the environment. Given that strains of *B. animalis* subsp. *lactis* are extensively used as probiotics and are folate scavengers, it may be advisable to include additional folic acid in foods in which these and other folate depleting cultures are used in high concentrations.

1. Introduction

Vitamins are organic compounds that are essential for metabolic functions within organisms. They have a variety of functions such as acting as cofactors for enzymes and providing antioxidant properties. However, humans are not able to synthesize all vitamins that are required. Some vitamins require precursors to be synthesized in the human body such as retinol which can be synthesized from beta carotene. Another example is vitamin D₃ which is synthesized in the human skin from 7-dehydrocholesterol with exposure to adequate sunlight. Most of the vitamins, particularly vitamins B and K, are not synthesized by the human body and they have to be supplemented from exogenous sources such as diet and vitamin supplements. The human gut microbiota is another important source of vitamins (Hill, 1997).

The human gut microbiota is a large community composed of different types of microorganisms. Certain members of this population have been known to produce vitamin K and some of the B vitamins, including biotin and folic acid. *Bacteroides* species were found to be one group of microorganisms capable of producing vitamin K (Fernandez and Collins, 1987). In addition, a metagenomic analysis of the human distal gut microbiome was found to be enriched with COGs (Clusters of Orthologous Groups) that are predicted to be involved in the synthesis of B vitamins including folate, thiamine, vitamin B₆ and vitamin B₁₂ (Gill et al., 2006).

Folate, also commonly known as vitamin B₉ and pteroyl glutamic acid, is very sparsely found in nature. Folic acid represents the chemically synthesized form of the vitamin which is used in fortified foods and dietary supplements (Quinlivan et al., 2006). The chemical structure of folic acid consists of para-aminobenzoic acid (pABA) in the center, linked to a pteridine ring and an L-glutamic acid (Figure III-1). Natural sources of

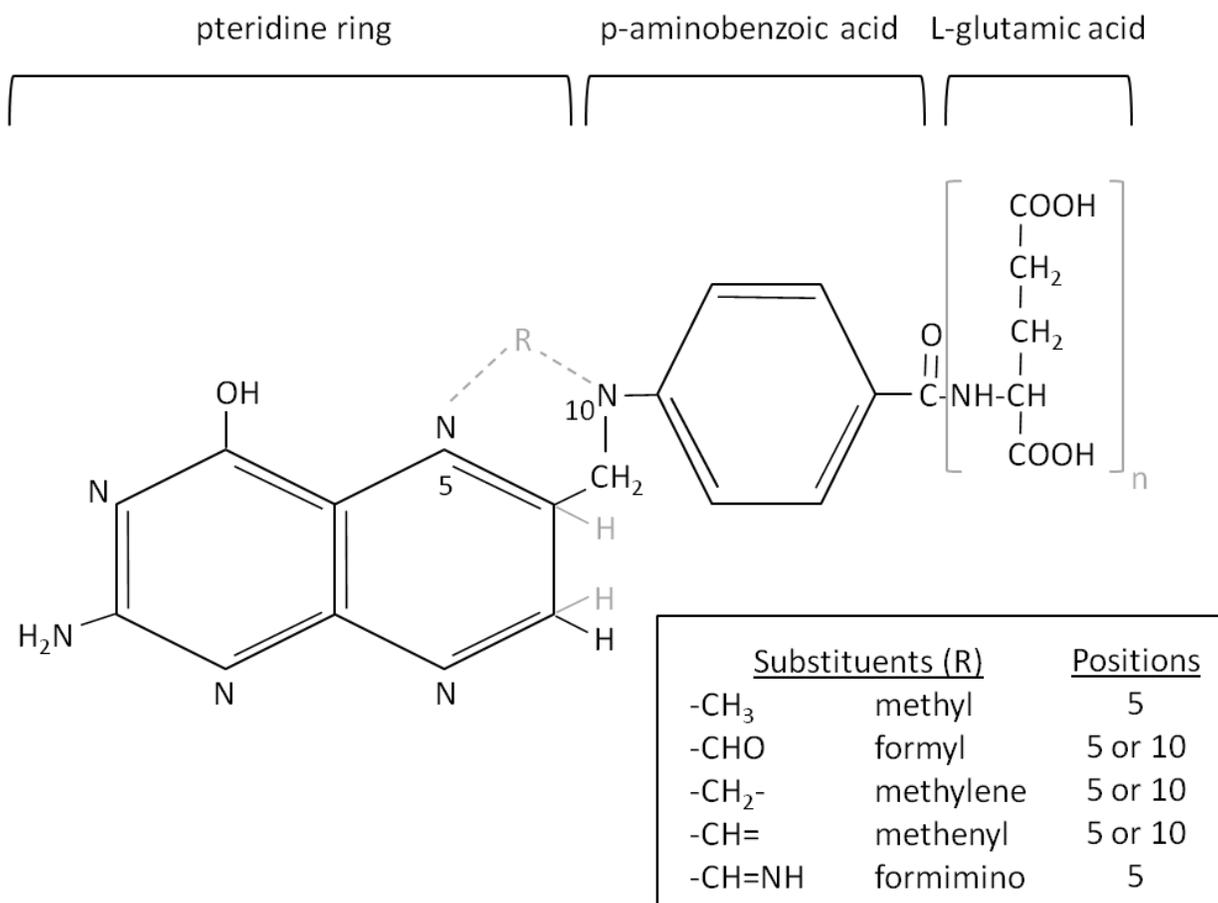


Figure III-1. Structure of folic acid and native food folate. Black color represents the structure of folic acid and grey color represents additional groups present in the structure of folate (n; indicates the number of L-glutamic acid residues).

folate contain different structures, depending on the substitute group on the pteridine ring, the reduction state of the pteroyl group and the number of glutamyl residues attached to the pteroyl group. Although pteroylpolyglutamates exist in nature with up to 11 glutamic acid residues, the most commonly occurring pteroylpolyglutamates contain up to 7 glutamic acid residues. The five different substitute groups that can attach to the pteridine ring at either the N5 or N10 position are: methyl, formyl, formimino, methylene and methenyl (Quinlivan et al., 2006), (LeBlanc et al., 2007). Their stabilities differ with 5-formyl-tetrahydrofolate (folinic acid) being the most stable, followed by 5-methyl-tetrahydrofolate (5-methyl-THF), 10-formyl-THF and THF (Forssen et al., 2000). Folates have a crucial role in metabolic pathways such as DNA and RNA biosynthesis and they are essential in DNA replication and methylation (Iyer and Tomar, 2009). They also support the synthesis of purines and some amino acids, such as methionine, by acting as one-carbon unit donors (Quinlivan et al., 2006).

While all *Eubacteria* and *Eukarya* either require or synthesize folate, some *Archaea* do not and likely evolved alternative pathways for purine biosynthesis (White, 1997). Humans have an absolute requirement for folate and its deficiency has been associated with a variety of health problems such as coronary heart fatalities (Morrison et al., 1996), cancer (Duthie et al., 2002) and neural tube defects in developing fetuses (FAO, 2001). Therefore, folate has to be supplemented from exogenous sources such as diet and nutraceuticals. While folate is the most common form naturally found in foods, it is very unstable compared to the unreduced folic acid and loses its activity due to oxidation causing considerable loss of available folate in foods (Scott, 1999). The bio-availability of natural folate is highly dependent on the cleavage of the polyglutamate

chain by the intestinal conjugase and this reduces its bioavailability several fold compared to folic acid since this reaction is not complete (Scott, 1999). In general, diet itself is not sufficient to provide adequate amounts of folate. Therefore folate fortification programs of certain foods have been implemented by several countries, such as USA, Canada and Australia (Lawrence et al., 2009). In the US, fortification of enriched white flours and other grain foods such as pasta became mandatory since 1998 (Sherwood et al., 2006). The Center for Disease Control (CDC) reported a ~ 30% decline in neural tube defects in the 24 months post fortification period (CDC, 2005). When the FDA initiated the fortification program, a 100 µg/day increase in folate intake was predicted. According to follow up studies the real increase in folate intake from dietary folate was more than double of the projected amount (Choumenkovitch et al., 2002, Quinlivan and Gregory III, 2003) but still under the upper intake level. While the recommended daily intake (RDI) for adults is 400 µg, pregnant women require more and supplementation is needed to achieve this (Sherwood et al., 2006).

Intestinal bifidobacteria have been reported to synthesize a number of B vitamins such as thiamine, nicotinic acid, pyridoxine and folic acid in different concentrations (Deguchi et al., 1985) but are believed to be non-producers for vitamin K (Fernandez and Collins, 1987). Ingesting folate producing *Bifidobacterium* strains and fructooligosaccharide (FOS) was found to result in a significant increase in serum folate levels in Wistar rats (Pompei et al., 2007b). This suggests that ingesting folate producing bifidobacteria may contribute to an increase in folate absorption into the blood stream. However, the contribution of microbial folate for enhancing folate status may not be as

great in humans, as rats consume their own feces and this could cause more folate absorption through the small intestine (Krause et al., 1996).

Selection of proper strains of *Bifidobacterium* was shown to enhance the natural folate content of fermented milk suggesting the potential use of *Bifidobacterium* strains in fermented dairy products (Crittenden et al., 2003, Lin and Young, 2000). Some strains of *Bifidobacterium* were found to be depleting 5-methyl-THF in milk indicating the importance of strain selection (Holasoava et al., 2004). In vitro studies for folate production by 76 strains of different species of *Bifidobacterium* from both human and animal sources found just 17 could grow in a folate-free medium and produce folate (D'Aimmo et al., 2012, Pompei et al., 2007a). All of these 17 strains were from a human origin suggesting a higher incidence of folate producing bifidobacteria in humans compared to animals, a finding substantiated by D'Aimmo et al. (2012). These studies also confirm that folate production is a characteristic of strains rather than species. Screening of *Bifidobacterium* strains for folate producing ability is still an issue due to the lack of a general synthetic medium where most *Bifidobacterium* spp. can grow (Rossi et al., 2011).

Based on the availability of genome sequences for several *Bifidobacterium* species, predicted metabolic models based on gene content can be derived. The objective of this study is to examine the predicted models for the folate biosynthetic abilities of several bifidobacteria and to functionally evaluate the accuracy of these models for the different strains tested.

2. Materials and Methods

2.1. Bacterial Strains and Folate-free Media

Five strains of *Bifidobacterium* representing four different species were used for this study: *B. animalis* subsp. *lactis* Bb-12 (Chr. Hansen Inc, Milwaukee, WI), *B. longum* subsp. *infantis* RECb4 (Kullen et al., 1997), *B. bifidum* ATCC 15696 and *B. breve* ATCC 15701 (American Type Culture Collection, Manassas, VA) and *B. longum* DJO10A which was isolated and characterized in our laboratory (Islam, 2006).

Three different folate-free media were prepared for testing the folate dependency of selected *Bifidobacterium* strains: (i) Folate-free medium SM7 (Pompei et al., 2007a); (ii) minimum culture medium no. 7 (Mogna and Strozzi, 2007); and (iii) a folate-free medium (YNB⁺) developed containing vitamin assay casamino acids (15 g l⁻¹) and proteose peptone No:3 (2 g l⁻¹), both from BD Biosciences, with urea (2 g l⁻¹), sodium acetate (10 g l⁻¹), ammonium sulfate (10 g l⁻¹), Tween 80 (1 g l⁻¹), manganese sulfate (10 mg l⁻¹), FeSO₄ (0.01 g l⁻¹), boric acid (0.5 mg l⁻¹), zinc sulfate (0.4 mg l⁻¹) from Thermo Fisher Scientific Inc., and sodium chloride (0.2 g l⁻¹), monopotassium phosphate (1 g l⁻¹) (both from Mallinckrodt), sodium molybdate (0.2 mg l⁻¹) (Merck & Co., Inc.), copper sulfate (0.04 mg l⁻¹) (Curtin Matheson Scientific Inc.), ascorbic acid (10 g l⁻¹), calcium chloride (0.1 g l⁻¹), L-cysteine (0.5 g l⁻¹), L-asparagine (0.2 g l⁻¹), L-cystine (0.1 g l⁻¹), DL-methionine (0.02 g l⁻¹), DL-tryptophane (0.2 g l⁻¹), DL-histidine (0.01 g l⁻¹), xanthine (0.01 g l⁻¹), FeCl₃·6H₂O (0.8 mg l⁻¹), potassium iodide (0.1 mg l⁻¹), adenine, guanine, cytosine, uracil (16 mg l⁻¹ each), calcium pantothenate (400 µg l⁻¹), myo-inositol (2 mg l⁻¹), niacin (400 µg l⁻¹), pyridoxine hydrochloride (400 µg l⁻¹), biotin (2 µg l⁻¹), riboflavin (200 µg l⁻¹), thiamine hydrochloride (400 µg l⁻¹), vitamin B12 (cyanocobalamin) (500 µg l⁻¹). The pH was adjusted to 7.0 using 10 M NaOH, and the medium was autoclaved at

110°C for 30 min. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g l^{-1}) and lactose (20 g l^{-1}) were autoclaved separately and added to the medium afterwards. The media were supplemented with para-aminobenzoic acid (pABA) ($200 \text{ } \mu\text{g l}^{-1}$) and folic acid ($10 \text{ } \mu\text{g l}^{-1}$) depending on the objective of the experiments. The medium was prepared fresh each time. All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

2.2. Culture Conditions

Bifidobacterium strains were inoculated from frozen stocks stored at -80°C into Lactobacilli MRS broth (BD Biosciences) containing 0.5 g l^{-1} L-cysteine·HCl and were incubated at 37°C for 24 h. Bifidobacteria were routinely grown at 37°C under anaerobic conditions by filling growth tubes to maximum capacity and using screw caps to conceal the tops. Cells from the MRS cultures were subinoculated (5%, vol/vol) into 16 ml of YNB^+ folate-free medium and incubated at 37°C for 24 hours to remove any residual folate carried over from the prior medium. To address the folate dependency, the cultures were subinoculated (5%, vol/vol) one more time into 16 ml tubes of the same medium. The cultures were then dispensed ($270 \text{ } \mu\text{l}$) into a 100 well honeycomb microplate and overlaid with mineral oil ($30 \text{ } \mu\text{l}$) and incubated at 37°C for 48 h using a Bioscreen CTM growth chamber (Growth Curves). Readings were obtained with 1 h intervals after 15 seconds of shaking at medium speed. The 16 ml tubes that contained the remaining cultures were also incubated at 37°C and optical densities at 600 nm (OD_{600}) were simultaneously checked using a Spectronic 20D (Milton Roy).

2.3. Folate Extraction

Folate was extracted from cultures of bifidobacteria following the protocol by Lin and Young (2000) with some modifications. Cultures were grown in MRS + 0.5% L-

cysteine twice before they were transferred at 2% to 100 ml of the same medium to initiate folate extraction. Immediately, 6 ml of the culture, representing the “0 h” sample, was pipetted into a 50 ml polypropylene centrifuge tube (Corning Inc.) and the pH was adjusted to 4.5 ± 0.05 with lactic acid (85%) followed by adding 10 ml of extraction buffer (0.1 M sodium phosphate buffer containing 0.5% Na-ascorbate, pH = 4.5). The mixture was placed in boiling water for 15 min and subsequently centrifuged at 5,000 X *g* for 10 min (4°C) in an Allegra 25R centrifuge with a TA-14-50 rotor (Beckman Coulter). Three ml of the supernatant was transferred to a 15 ml polypropylene centrifuge tube (Corning Inc.) followed by adding 400 µl of human plasma (Sigma-Aldrich) and incubation at 37°C and 225 rpm in an Innova 2300 platform shaker (New Brunswick Scientific Co.) for 1 h to initiate deconjugation of folate. The tube was then placed in boiling water for 5 min to stop the reaction and centrifuged at 15,000 X *g* for 15 min (4°C) in an Allegra 25R centrifuge with a TA-14-50 rotor (Beckman Coulter). The supernatant was filtered through a 0.22 µm filter and stored at - 20°C.

2.4. Genome Sequence Analysis

The available genome sequences for bifidobacteria were analyzed to predict folate biosynthesis pathways using the KEGG database (Kanehisa et al., 2012). The availability of genes were evaluated via BLASTp (Altschul et al., 1990) using the reference proteins sequences from *B. dentium* Bd1 and *B. adolescentis* ATCC 15703 since they carry all the genes involved in folate biosynthesis, except for the gene encoding dihydroneopterin triphosphate pyrophosphatase (EC 3.6.1.-). This protein sequence was obtained from *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC BAA-365, as it was the closest phylogenetic relative for bifidobacteria available in GenBank.

2.5. Quantification of Folate by Liquid Chromatography – Mass Spectrometry (LC-MS)

Folic acid, 5-Methyltetrahydrofolic acid disodium salt (5-MTHF), tetrahydrofolic acid (THF), folinic acid calcium salt hydrate (all from Sigma-Aldrich) were used as standards. Stock solutions were prepared in 2% acetonitrile/ 98% water/ 0.1% formic acid with concentrations of 5mM for folic acid and 5-MTHF, 2.8 mM for THF and 20 mM for folinic acid for construction of standard curves. The Applied Biosystem 4000 iontrap (Applied Biosystems) fitted with a turbo V electrospray source was optimized for each reagent using direct injection. For each sample, 2 μ l was diluted into 10 μ l of 2% acetonitrile/ 98% water/ 0.1% formic acid. The samples were then injected using an Agilent autosampler (Agilent Technologies). An analytical Agilent Eclipse XDB-C8 column (4.8 x 150 mm, 5 μ M; Agilent Technologies) was connected to the Applied Biosystem 4000 iontrap. The samples were subjected to a linear gradient beginning from 2% acetonitrile/ 0.1% formic acid to a final concentration of 98% acetonitrile and 0.1% formic acid for 15 min at a column flow rate of 500 μ l/min. Transitions monitored were the m/z 442 \rightarrow m/z 295 for folic acid; m/z 460 \rightarrow m/z 313 for 5-MTHF; m/z 446 \rightarrow m/z 299 for THF and m/z 474 \rightarrow m/z 327 for folinic acid. Standard curves were constructed using a mix of varying concentrations of folinic acid, folic acid and 5-MTHF. The purity of THF acid didn't allow mixing and the standard curve for THF was constructed independently. The data was analyzed using MultiQuant™ Software 2.0 (AB Sciex) providing the peak area for the transitions. LC retention times varied when analytes were run in the load buffer in comparison to retention time of analytes in the presence of sample buffer. Therefore retention times were determined for each analyte by introducing

the analyte into the sample buffer. Peaks at these retention times were used for quantitation.

3. Results

3.1. Predicted Folate Biosynthesis Pathways for Selected Bifidobacteria

Based on the analysis of available genome sequences, it was found that only strains of *B. bifidum* (all three) and *B. longum* subsp. *infantis* (one of two) carry all the known genes required for folate biosynthesis. Strains of *B. breve* and *B. longum* DJO10A lack only one gene whereas *B. animalis* subsp. *lactis* Bb-12 lacks several genes required for biosynthesis of folate (Figure III-2). The two precursors of folate, pABA and 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP), and the enzyme dihydropteroate synthase (EC 2.5.1.15) are required for *de novo* folate biosynthesis. All strains were predicted to possess the two enzymes, aminodeoxychorismate synthase (EC 2.6.1.85) and aminodeoxychorismate lyase (EC 4.1.3.38), required for the synthesis of pABA from chorismate. Excluding *B. bifidum* and *B. longum* subsp. *infantis*, all strains lack the genes encoding either of the phosphatases catalyzing the reaction for the formation of 7,8-dihydroneopterin suggesting that they require DHPPP supplementation. However, *B. animalis* subsp. *lactis* Bb-12 lacks the gene encoding the enzyme (EC 2.5.1.15) catalyzing the reaction between pABA and DHPPP so, it should be auxotrophic for *de novo* folate biosynthesis even when both precursors are present.

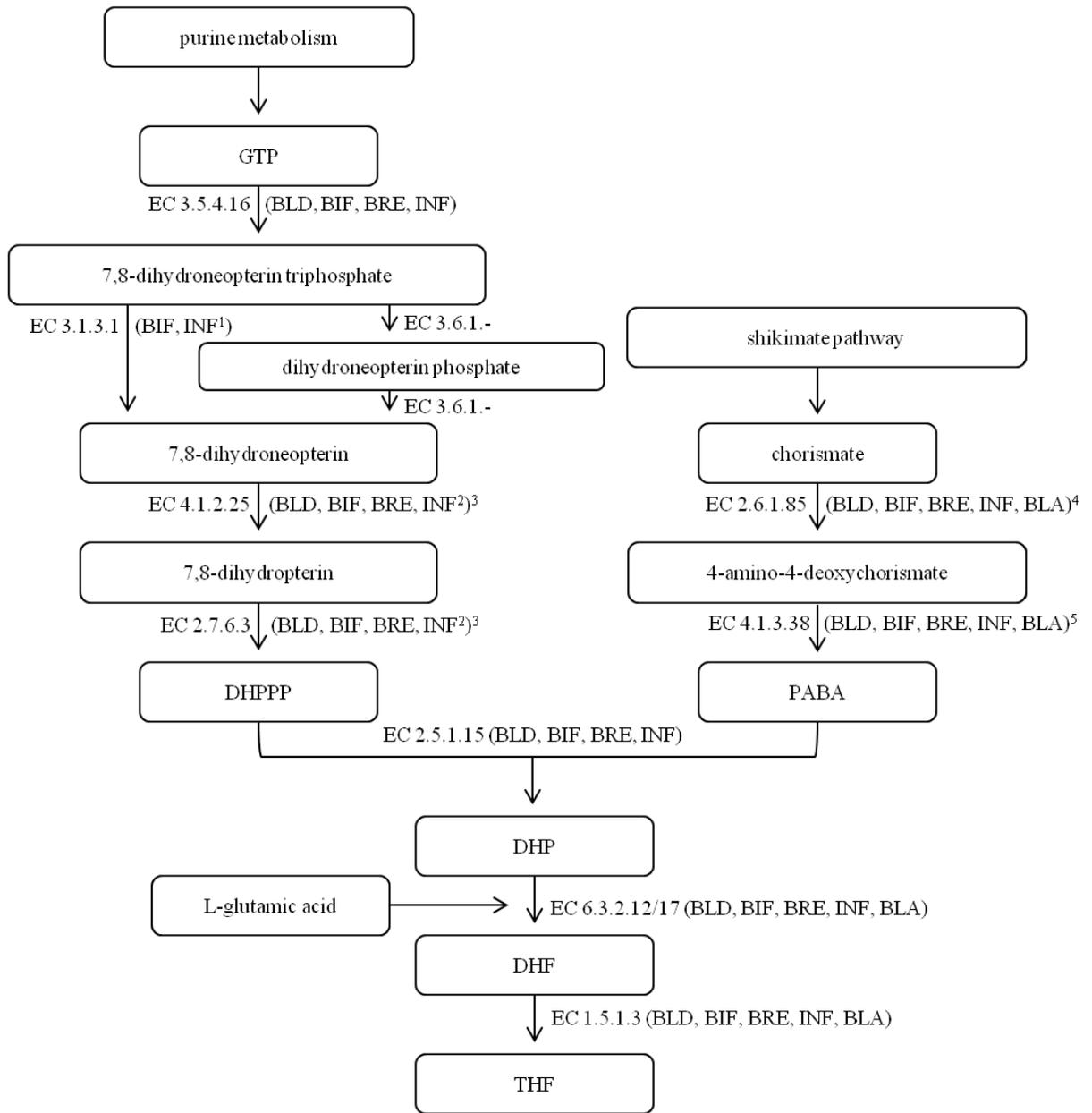


Figure III-2. Predictable folate biosynthesis pathway for bifidobacteria based on available genome sequences. Abbreviations: GTP, guanosine triphosphate; DHPPP, 6-hydroxymethyl-7,8-dihydropterin pyrophosphate; pABA, para-aminobenzoic acid; DHP, 7,8-dihydropterate; DHF, dihydrofolate; THF, tetrahydrofolate. Letters near the enzyme codes indicate the bifidobacteria that carry the encoding genes with the following annotations: BLD: *B. longum* DJO10A; BLA: *B. animalis* subsp. *lactis* Bb-12, BIF: *B.*

bifidum BGN4, PRL 2010 and S17; BRE: *B. breve* UCC2003 and ACS-071-V-Sch8b; INF: *B. longum* subsp. *infantis* 157F and ATCC 15697. EC 1.5.1.3: dihydrofolate reductase; EC 2.5.1.15: dihydropteroate synthase; EC 2.6.1.85: aminodeoxychorismate synthase; EC 2.7.6.3: 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase; EC 3.1.3.1: alkaline phosphatase; EC 3.5.4.16: GTP cyclohydrolase I; EC 3.6.1.-: dihydroneopterin triphosphate pyrophosphatase; EC 4.1.2.25: dihydroneopterin aldolase, EC 4.1.3.38: aminodeoxychorismate lyase; EC 6.3.2.12/17: dihydrofolate/tetrahydrofolate synthase. EC 4.1.2.25 and EC 2.7.6.3 represent a bifunctional protein, encoded by the same gene (Garçon et al., 2006) .

¹: This gene is absent in one of the sequenced strains of *B. longum* subsp. *infantis* 157F whereas it is present in the other sequenced strain, ATCC 15697.

²: This gene is present in sequenced strains *B. longum* subsp. *infantis* 157F and ATCC 15697. However, it is gene BLIF_1645 in strain 157F that currently is annotated as a hypothetical protein in GenBank.

³: The C-terminal of a hypothetical protein BIF_00485 in *B. animalis* subsp. *lactis* Bb-12, exhibits significant similarity (48% identity/69% similarity) to an internal portion of this bifunctional enzyme in *B. dentium*. In *B. animalis* subsp. *lactis* DSM 10140 it is annotated as this bifunctional enzyme, presumably based on this C-terminal similarity.

⁴: This gene is present in all strains but with a variety of annotations such as glutamine amidotransferase, glutamine amidotransferase of anthranilate synthase, anthranilate/para-aminobenzoate synthases component II and para-aminobenzoate synthase glutamine amidotransferase.

⁵: This gene is present in all strains with different annotations such as aminodeoxychorismate lyase, conserved hypothetical protein with aminodeoxychorismate lyase domain, hypothetical protein or YceG protein family. It is annotated as membrane associated protein in strain Bb-12.

3.2. Development of a Folate-free Medium for Bifidobacteria

A complete folate-free medium for Bifidobacteria is expected to provide adequate growth when folic acid is present. Two model organisms, a commercially used probiotic strain, *B. animalis* subsp. *lactis* Bb-12 and a minimally cultured intestinal strain, *B. longum* DJO10A, were selected to evaluate the suitability of the previously developed folate-free media, SM7 and MM7, for growth. After 48 hours of incubation, strain DJO10A did not grow in either media containing added folate, whereas strain Bb-12 grew only in MM7 up to a maximum OD₆₀₀ of 0.2 which is not satisfactory. Hence, there was a need for a suitable folate-free medium suitable for adequate growth of bifidobacteria.

Although both folate-free media mentioned were based on the composition of yeast nitrogen base (BD Biosciences) with additional compounds, they lack several compounds such as inositol, calcium chloride and the aminoacids: methionine, histidine and tryptophan. MM7 contained some extra compounds that are present in yeast nitrogen base and allowed some growth of strain Bb-12. Therefore, a new medium was constructed based on the composition of MM7 with additional components (Figure III-3). Glucose was replaced with lactose as strain DJO10A tended to form clumps when glucose had been used. Vitamin B12 was added based on the medium developed for the growth of *B. animalis* by Kongo et al. (2003). Nitrogen bases were added based on the composition of Folic AOAC Medium (BD Biosciences) that was developed for the microbiological assay of folate using *Enterococcus hirae* ATCC 8043 as the test bacteria. The aminoacids, L-cystine and L-asparagine were supplemented due to their low concentrations in the vitamin assay casaminoacids. Proteose peptone No:3 (BD Biosciences) is the major component for the Bifidobacteria Low-Iron Medium with

added Iron (BLIM + Fe) (Islam, 2006) which is used for cultivation of bifidobacteria, and supplemented with a minimum concentration to support the growth of bifidobacteria in YNB⁺. When folic acid was included, the OD₆₀₀ for strains Bb-12 and DJO10A were 0.57 and 0.74 using a Bioscreen CTM growth monitor; 0.58 and 1.0 using the manual readings, respectively. The corresponding maximum OD₆₀₀ for *B. longum* subsp. *infantis* RECb4 was 0.55 (manual reading = 0.57) and 0.84 for *B. breve* ATCC 15701 (manual reading = 1.27). However, *B. bifidum* ATCC 15696 was not able to grow in YNB⁺ even in the presence of folic acid (data not shown).

3.3. Growth Kinetics of Bifidobacteria in YNB⁺

Cultures of *Bifidobacterium* were grown in the absence of pABA and/or folic acid to functionally test their predicted ability of folate biosynthesis. Although the manual OD₆₀₀ readings were higher, similar growth curves were observed based on the OD₆₀₀ readings procured from the Bioscreen CTM (Figure III-4 and Figure III-5). *B. longum* DJO10A and *B. breve* ATCC 15701 were able to grow having no need for either folic acid or pABA supplementation. *B. animalis* subsp. *lactis* Bb-12 was not able to grow in the presence of pABA and required folic acid supplementation in order to grow in YNB⁺. *B. longum* subsp. *infantis* RECb4 required either pABA or folic acid for its growth.

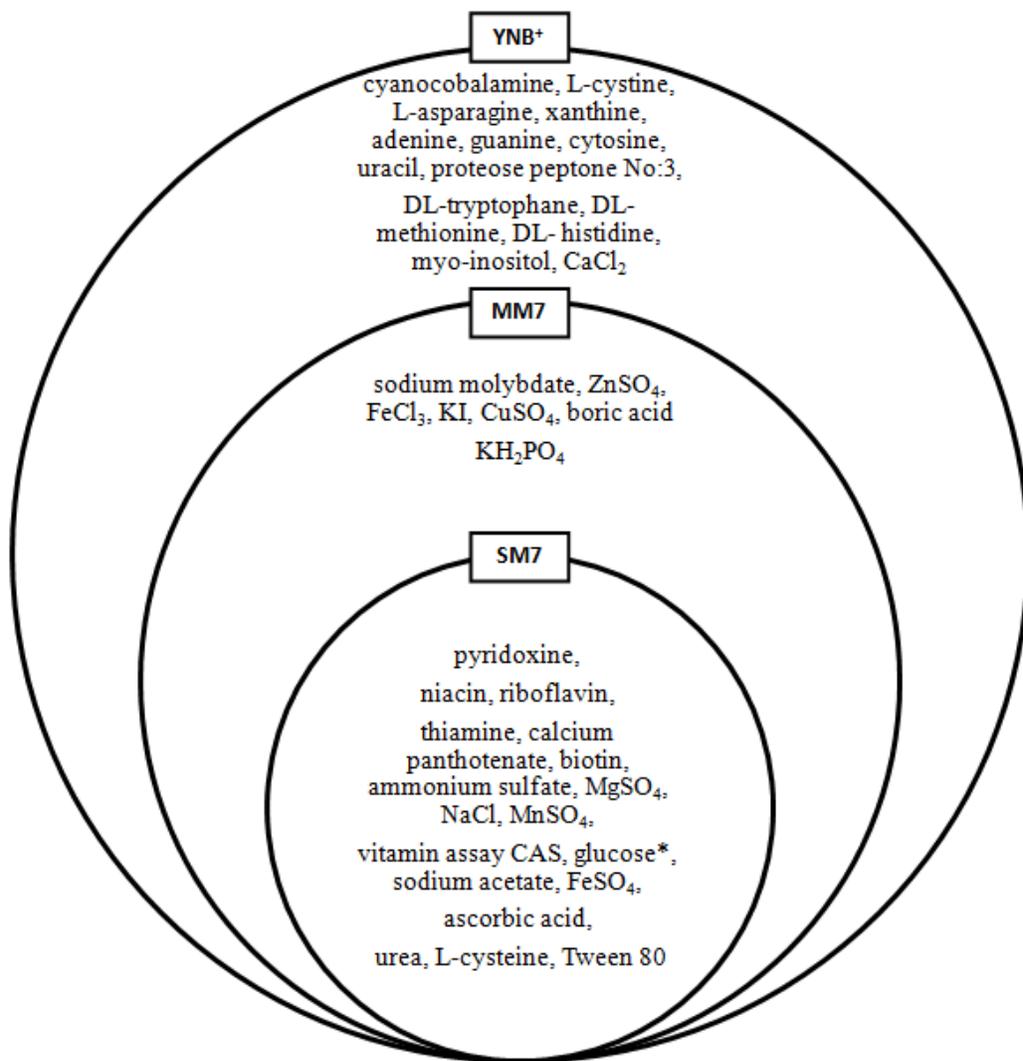


Figure III-3. Composition of the folate-free media: YNB⁺, MM7 and SM7. *, indicates glucose was replaced with lactose in YNB⁺.

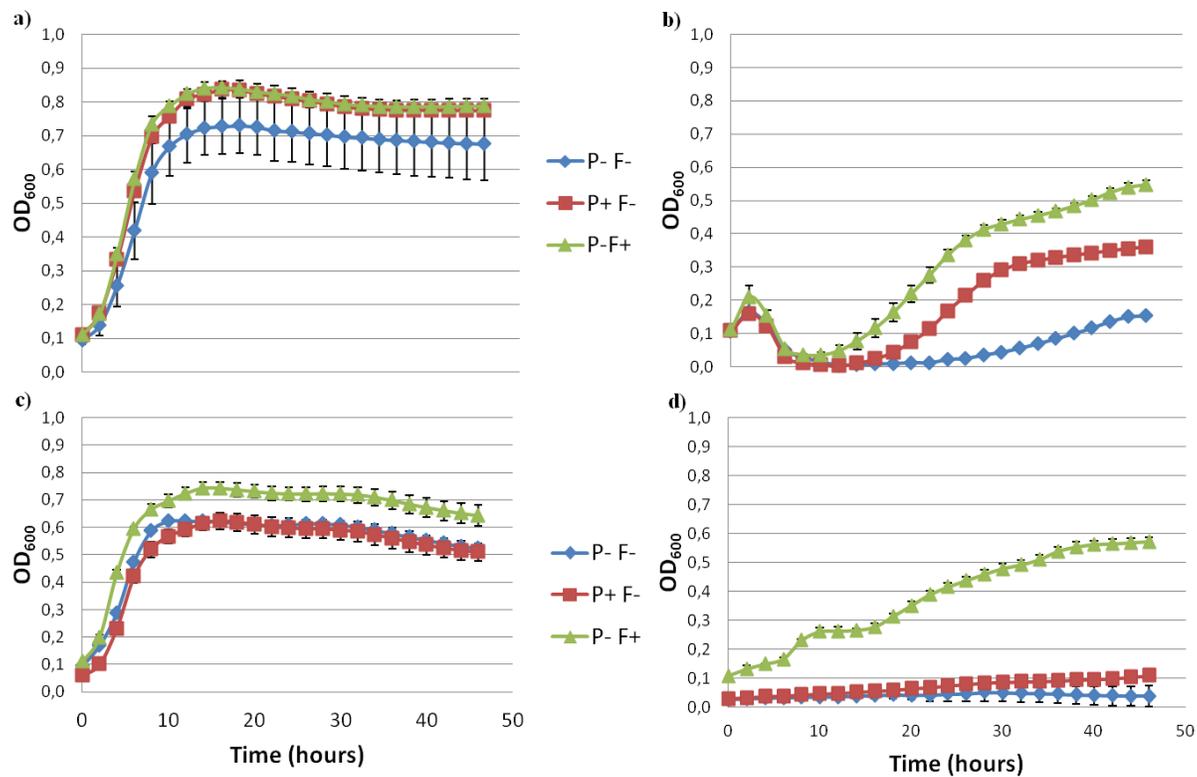


Figure III-4. Growth curves from Bioscreen CTM readings for a) *B. breve* ATCC 15701, b) *B. longum* subsp. *infantis* RECb4, c) *B. longum* DJO10A and d) *B. animalis* subsp. *lactis* Bb-12 in the YNB⁺ without pABA and folic acid (P⁻ F⁻); only with pABA (P⁺ F⁻) and only with folic acid (P⁻ F⁺). All values represent the average of five replicates, and the error bars show standard deviations.

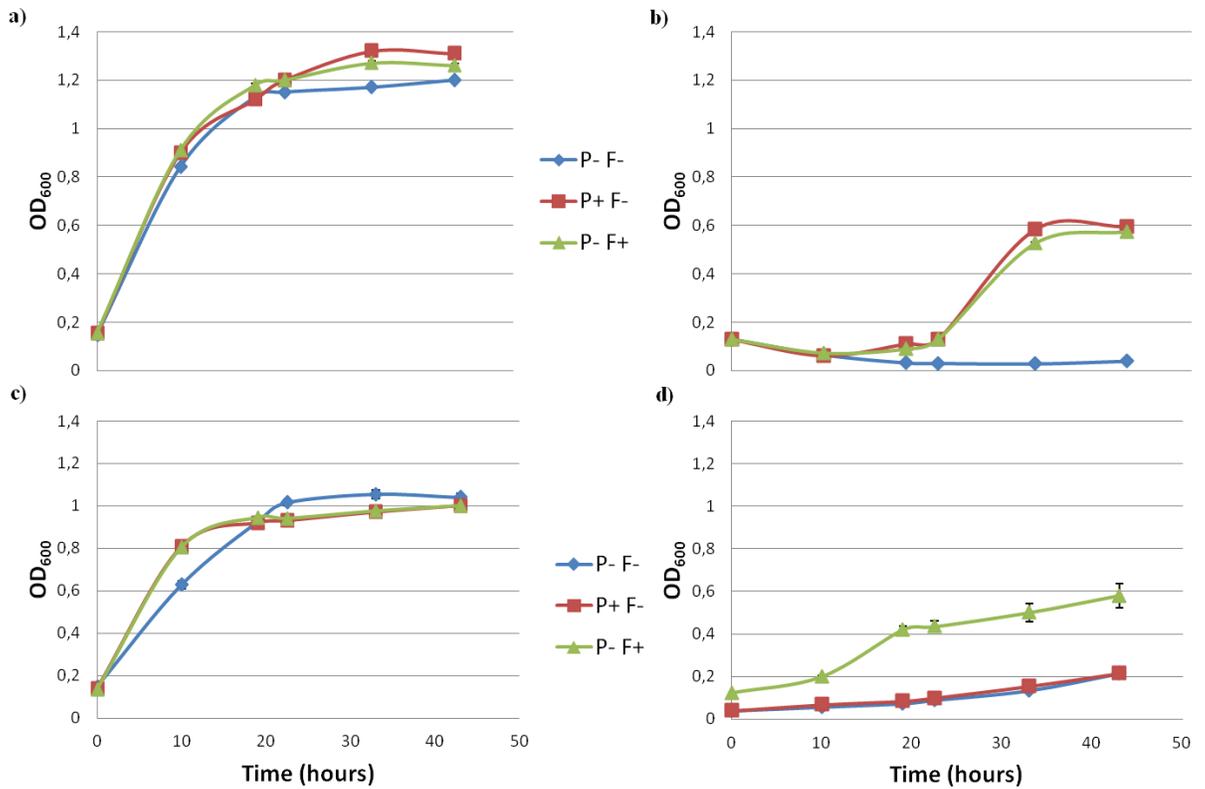


Figure III-5. Growth curves from manual readings for a) *B. breve* ATCC 15701, b) *B. longum* subsp. *infantis* RECb4, c) *B. longum* DJO10A and d) *B. animalis* subsp. *lactis* Bb-12 in the YNB⁺ without pABA and folic acid (P⁻ F⁻); only with pABA (P⁺ F⁻) and only with folic acid (P⁻ F⁺). All values represent the average of 5 replicates, and the error bars show standard deviations.

3.4. Growth Kinetics of Bifidobacteria in MRS

The quantification of folate produced by the bifidobacteria cultures was performed in MRS + 0.5% L-cysteine due to the limited growth of some strains in YNB⁺ even when folic acid was supplemented. Growth kinetics of selected strains was needed to determine the time points for folate extraction and quantification. All strains had similar pH change patterns during their growth. Although *B. longum* subsp. *infantis* RECb4 and *B. bifidum* ATCC 15696 had a slightly longer lag phase, all strains reached an OD₆₀₀ of 1.0 or higher after 14 hours of incubation (Figure III-6). To address the affect of growth stage on folate concentration 0, 6, 12 and 24 h time points were selected representing the lag phase, exponential phase, early stationary phase and late stationary phases.

3.5. Quantification of Folate Derivatives in MRS Cultured with Bifidobacteria

The concentrations for all folate derivatives were represented as net concentration and obtained by subtracting the initial concentration in MRS from the actual concentrations. Standard curves were constructed for each folate derivative and standard deviations from folic acid, folinic acid and 5-MTHF standards were evaluated by replicating measurements in triplicate (Figure III-7). Measurements for the four individual folate compounds revealed that folic acid, folinic acid and THF showed consistent patterns (Figure III-8). However, measurements for 5-MTHF were not consistent and the standard error for this compound within the low concentration levels found here was measured at 101% compared to 32% and lower for the other three derivatives at this concentration range. Therefore, the 5-MTHF measurements were deemed unreliable and were excluded from the folate analysis. Folinic acid was

determined to be the derivative utilized most followed by folic acid and THF, respectively. *B. longum* subsp. *infantis* RECb4, *B. breve* ATCC 15701 and *B. animalis* subsp. *lactis* Bb-12 were constant depleters for the three folate derivatives. They started depletion after 6 h and reached the greatest depletion at 24 h. Although both *B. longum* DJO10A and *B. bifidum* ATCC 15696 produced some of the derivatives only *B. longum* DJO10A was a producer at the end of the incubation period. The concentrations for folate derivatives for each strain are given in Table III-1.

Total folate concentrations were calculated from folinic acid, folic acid and THF, given the error for 5-MTHF was too large in the low concentration range of these samples. After 24 h incubation it was evident that *B. animalis* subsp. *lactis* Bb-12, *B. breve* ATCC 15701 and *B. longum* subsp. *infantis* RECb4 were significant depleters of folate in the medium (Figure III-9). *B. bifidum* ATCC 15696 depleted folate to a lesser extent, while *B. longum* DJO10A did not deplete folate. Strain DJO10A exhibited a tendency to balance the folate concentration in the environment during the incubation period. Sample chromatograms of all folate derivatives are displayed in Figure III-10 for strain DJO10A at 12 hours of incubation.

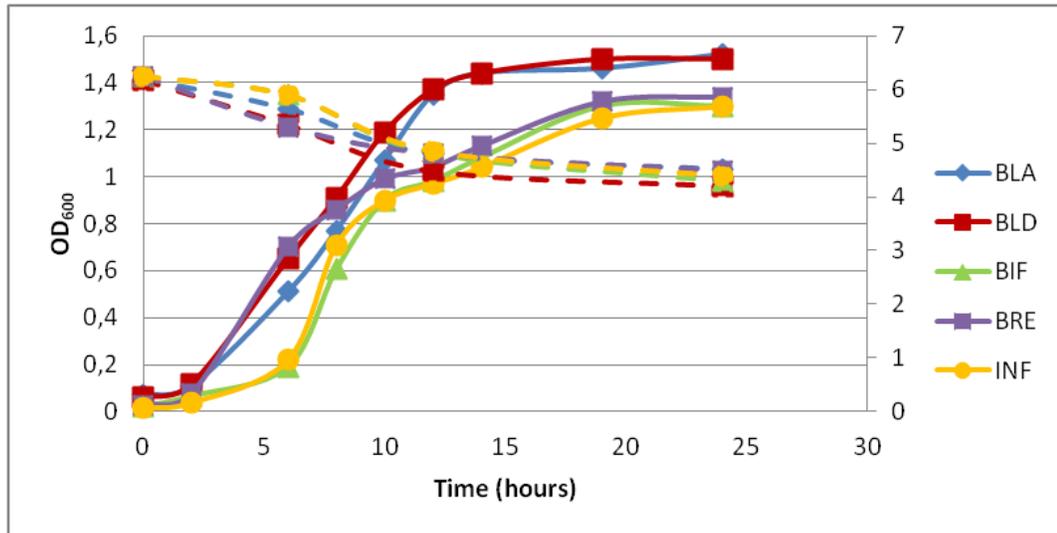


Figure III-6. Changes in pH (dashed lines) and OD₆₀₀ (solid lines) for cultures of bifidobacteria during growth in MRS + L-cysteine. BLD: *B. longum* DJO10A; BLA: *B. animalis* subsp. *lactis* Bb-12, BIF: *B. bifidum* ATCC 15696; BRE: *B. breve* ATCC 15701; INF: *B. longum* subsp. *infantis* RECb4.

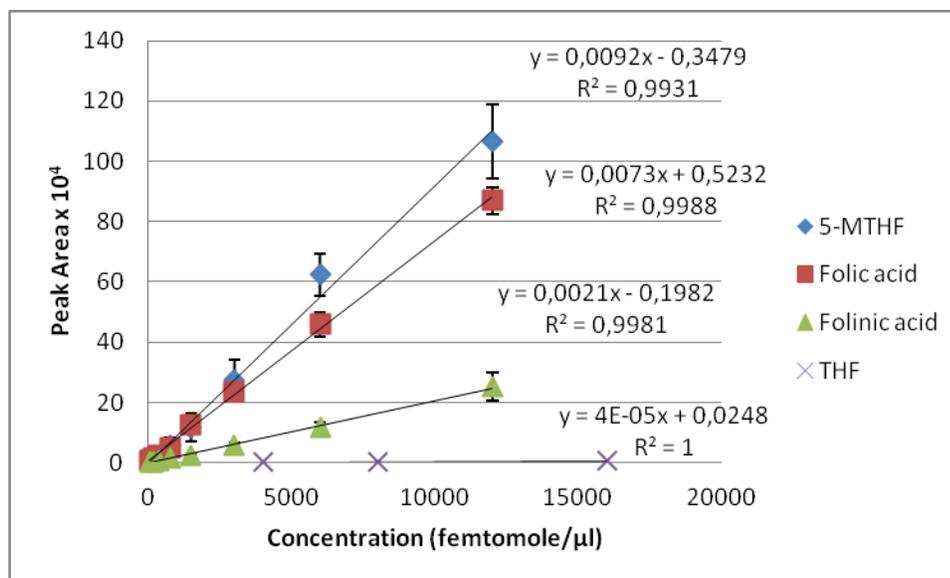


Figure III-7. Standard curves for folate derivatives. The equations and the square regression coefficients (R^2) for the standard curves are given. The peak areas of the compounds were measured based on LC-MS results as a function of known initial concentrations. Values are the means of three measurements \pm standard errors of the mean (except THF).

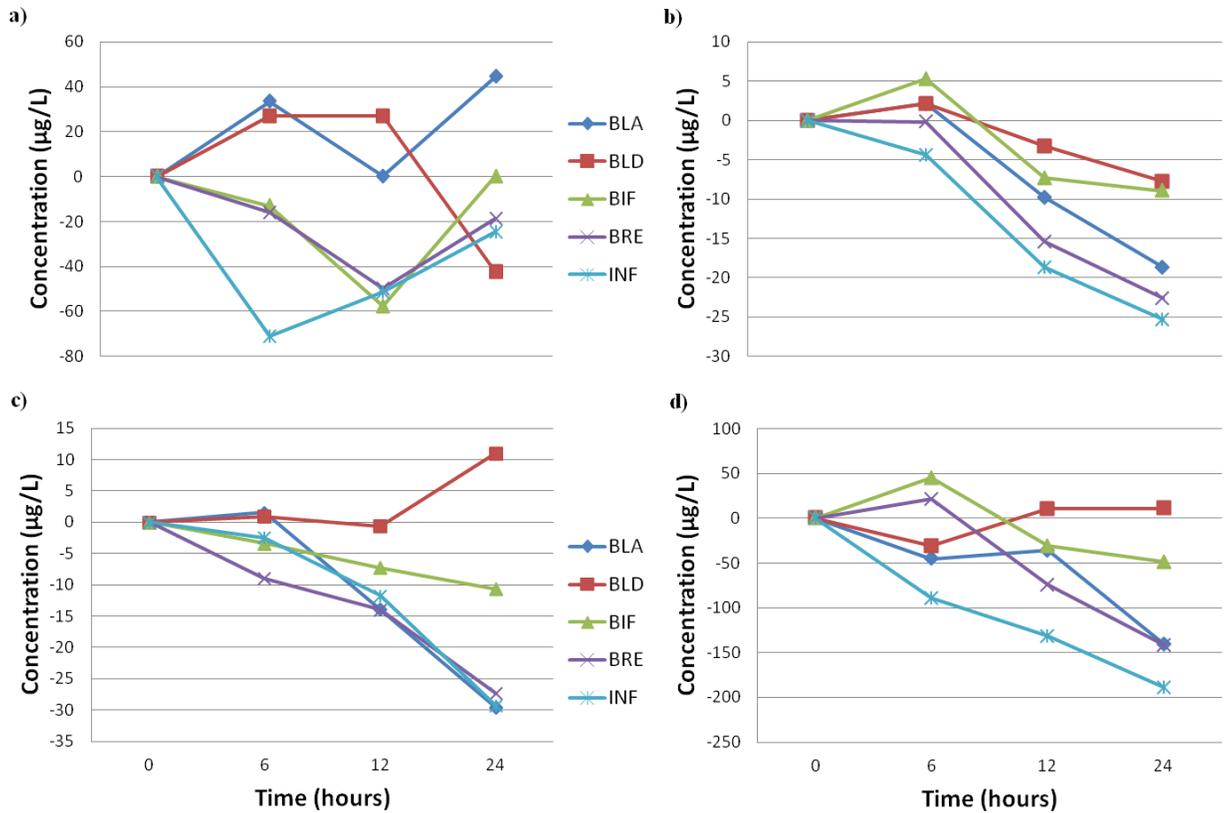


Figure III-8. Effect of incubation time on the concentrations of folate derivatives. a) 5-MTHF, b) THF, c) Folic acid, d) Folinic acid. Negative values show the reduction from initial concentrations in MRS + L-cysteine. BLD: *B. longum* DJO10A; BLA: *B. animalis* subsp. *lactis* Bb-12, BIF: *B. bifidum* ATCC 15696; BRE: *B. breve* ATCC 15701; INF: *B. longum* subsp. *infantis* RECb4.

Table III-1. Net concentrations ($\mu\text{g/L}$) of folate derivatives during incubation¹.

Hours	Folic acid			5-MTHF			Folinic acid			THF		
	6	12	24	6	12	24	6	12	24	6	12	24
<i>B. bifidum</i> ATCC 15696	-7.0	-14.9	-21.9	-13.0	-57.7	+0.3	+45.1	-31.1	-48.9	+5.3	-7.3	-8.9
<i>B. breve</i> ATCC 15701	-18.5	-28.8	-56.2	-15.9	-49.9	-18.6	+21.7	-74.1	-141.3	-0.2	-15.5	-22.6
<i>B. longum</i> subsp. <i>infantis</i> RECb4	-5.1	-24.1	-60.3	-71.0	-51.5	-24.6	-89.0	-131.4	-188.7	-4.4	-18.7	-25.4
<i>B. longum</i> DJO10A	+1.9	-1.4	+22.6	+27.1	+27.1	-42.5	-31.1	+10.3	+10.9	+2.1	-3.3	-7.8
<i>B. animalis</i> subsp. <i>lactis</i> Bb-12	+3.2	-28.6	-60.8	+33.5	+0.1	+44.5	-45.4	-36.3	-140.4	+2.1	-9.8	-18.7

¹: Represent the changes in folate concentration in MRS + L-cysteine medium.

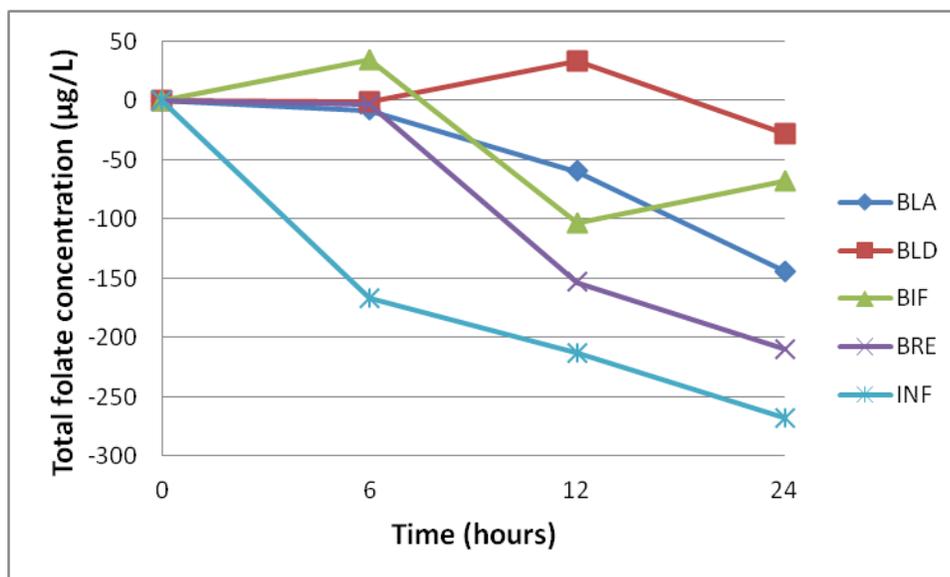


Figure III-9. Effect of incubation time on the total net folate concentration (except 5-MTHF) for cultures of bifidobacteria. Negative values show the reduction from initial folate concentration in MRS + L-cysteine. BLD: *B. longum* DJO10A; BLA: *B. animalis* subsp. *lactis* Bb-12; BIF: *B. bifidum* ATCC 15696; BRE: *B. breve* ATCC 15701; and INF: *B. longum* subsp. *infantis* RECb4.

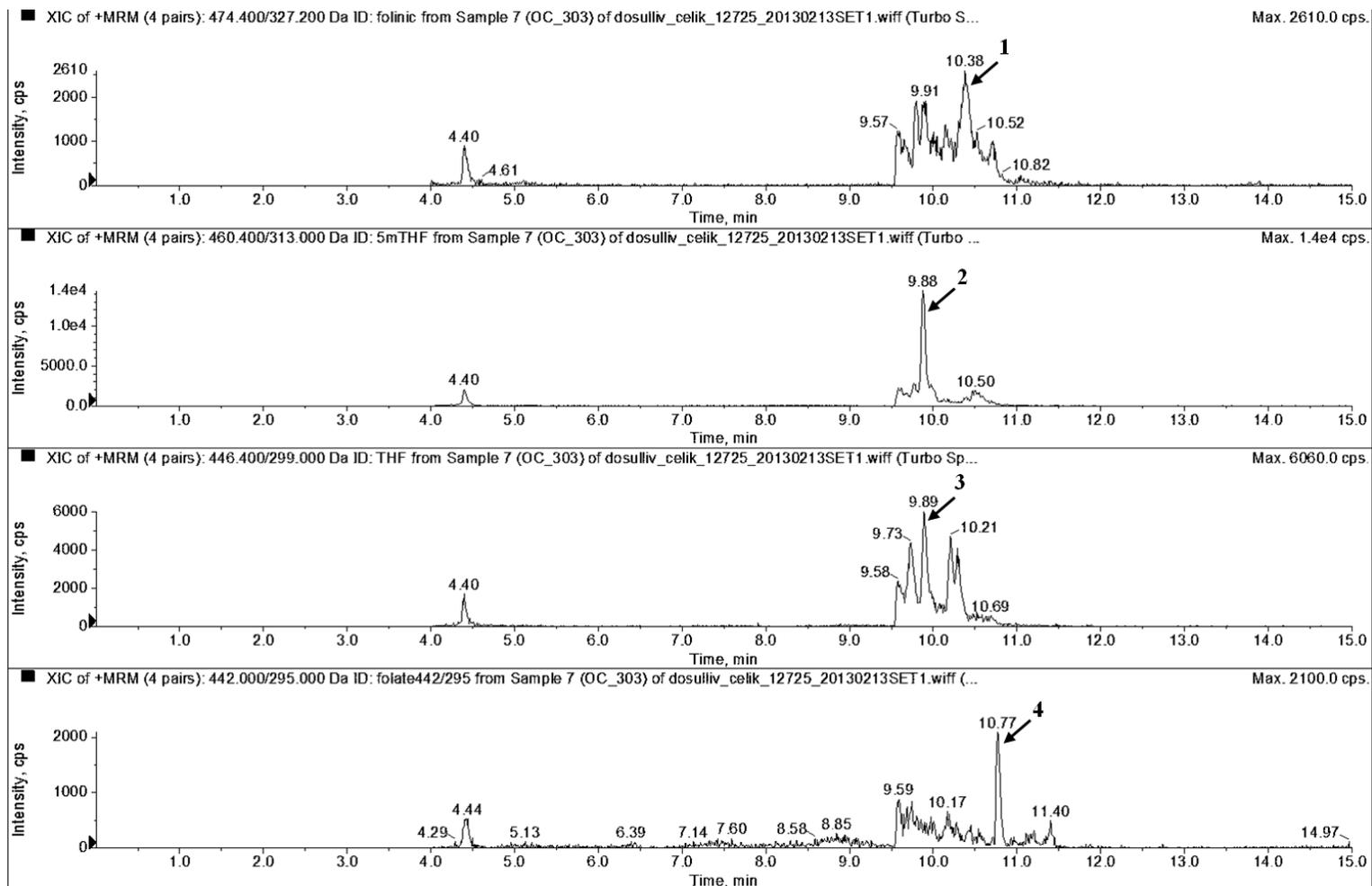


Figure III-10. LC-MS chromatogram for *B. longum* DJO10A at 12 hours incubation. 1) Folinic acid, 2) 5-MTHF, 3) THF, 4) Folic acid.

4. Discussion

Species of *Bifidobacterium* and *Lactobacillus* are the most common probiotic bacteria used in food products. Lactobacilli generally are depleters of folic acid, with *Lactobacillus casei* being traditionally used for microbiological assays for folic acid (Landy and Dicken, 1942). Genome analysis substantiates that all the sequenced lactobacilli lack the genes for the biosynthesis of pABA and are predicted to be auxotrophic for folate (reviewed, Rossi et al., 2011). Therefore, studies mostly focus on bifidobacteria to investigate their potential to be used as folate suppliers in fermented dairy products (Holasova et al., 2004, Lin and Young, 2000). Among the sequenced strains of *Bifidobacterium*, *B. bifidum*, *B. longum* subsp. *infantis*, *B. dentium* and *B. adolescentis* carry the full set of genes, whereas strains of *B. animalis* subsp. *lactis* lack several genes and are predicted to be auxotrophic for *de novo* folate biosynthesis. In light of the predicted folate biosynthesis pathways from available genomes, this study functionally investigated the folate scavenging or supplying potential of several bifidobacteria including a common commercial probiotic strain, *B. animalis* subsp. *lactis* Bb-12, and a characterized intestinal strain, *B. longum* DJO10A.

Although there are several folate-free media developed for bifidobacteria, such as SM7 (Pompei et al., 2007a) and MM7 (Mogna and Strozzi, 2007), they did not support the growth of cultures in this study even when folic acid was included in the media. Therefore, a new folate-free medium, YNB⁺, containing additional compounds was developed as part of this study. Except for *B. bifidum* ATCC 15696, all strains grew sufficiently in the presence of folate.

The folate dependency of *B. animalis* subsp. *lactis* Bb-12 was evaluated using growth tests in the folate-free medium and it substantiated the genome prediction for this

strain. The growth results for strain Bb-12 were also consistent with the studies of D'Aimmo et al. (2012) and Pompei et al. (2007a) where they found that strain DSMZ10140, another sequenced strain of *B. animalis* subsp. *lactis* which lacks folate genes, was not able to grow in folate-free media that were developed. However, the latter study used SM7 medium, which was found in this study to be incapable of supporting the growth of *B. animalis* subsp. *lactis* even with added folate.

Contrary to their genome predictions, *B. longum* DJO10A and *B. breve* ATCC 15701 were found to be able to grow independently from both pABA and folate whereas *B. longum* subsp. *infantis* RECb4 only grew when either pABA or folic acid was included in the medium. Given, both *B. longum* DJO10A and *B. breve* ATCC 15701 lack the specific genes encoding alkaline phosphatase (EC 3.1.3.1) and dihydroneopterin triphosphate pyrophosphatase (EC 3.6.1.-), they likely use different types of pyrophosphatases and alkaline phosphatases that are encoded in their genomes. The genome of strain DJO10A contains a cluster of orthologous groups (COGs) corresponding to an alkaline phosphatase family (COG1368), which could replace the functions of the missing genes in the predicted pathway. It can therefore be assumed, that the lack of specific genes encoding these two phosphatases cannot be used as a prediction of folate dependency.

Given that *B. longum* subsp. *infantis* RECb4 grew when pABA was supplied, this indicates that the genes encoding the enzymes on the left side of the folate biosynthesis pathway (Figure III-2) must be functioning and one or both of the genes encoding aminodeoxychorismate synthase (EC 2.6.1.85) and aminodeoxychorismate lyase (EC 4.1.3.38) are either missing or not functional. This was surprising as both sequenced

strains of *B. longum* subsp. *infantis* contain both these genes. This substantiates strain differences in folate dependency in this subspecies, similar to the gene encoding EC 3.1.3.1: alkaline phosphatase where one of the sequenced strains, 157F, lacked the gene while the other, strain ATCC 15697, contained it.

Corresponding to its genome prediction and growth tests, quantification results designated *B. animalis* supsp. *lactis* Bb-12 as a depleter as total folate and individual folate derivatives, except for 5-MTHF were depleted (Figure III-8 and Figure III-9). Given the standard error for quantification of this particular derivative at low concentrations was more than 100%, it cannot be concluded that the strain produced this compound. However, given this strain has enzymes that convert different folate derivatives into THF, it is feasible that it could convert folate derivatives in the medium into 5-MTHF, thus increasing the levels of this derivative. A study that quantified folate production by strain Bb-12 using a microbiological assay found that it produced folate (0.6 and 0.1 $\mu\text{g/g}$ in the cell biomass and supernatant, respectively) when it was grown in the presence of folate (Herranen et al., 2010). However, this study did not present any statistical data regarding the standard deviations and therefore the folate concentrations may not be reliable. Another study by D'Aimmo et al. (2012) found that all strains of *B. animalis* produced folate when they were grown in the presence of folate. This study only quantified two forms of folate, THF and 5-MTHF, inside the cells using an HPLC method. The folate produced by strains of *B. animalis* is only in 5-MTHF form and could be the folate taken up from the readily available folate contained in the medium. Their inability to grow in the folate-free medium that was developed also suggest that they

cannot synthesize folate *de novo* however no information was given about their growth ability when folate was included in the medium.

B. breve ATCC 15701 and *B. longum* subsp. *infantis* RECb4 were also found to be depleters when folate was readily available in the growth medium. *B. breve* ATCC 15701 was expected to produce folate based on its predicted genome and the growth test results obtained. Therefore, this particular strain can sense the folate in the environment and down regulate the expression of the genes responsible for folate production inside the cell. Although the genome prediction suggests that strain RECb4 likely possesses all the genes required for folate biosynthesis it was not expected to produce folate as it cannot grow in the folate-free medium. Based on the quantification results, strain RECb4 was found to be a major depleter for all folate derivatives, substantiating the growth data. Supporting the strain difference, D'Aimmo et al. (2012) and Pompei et al. (2007a) found that only some strains of *B. longum* subsp. *infantis* tested were able to grow and make folate in folate-free media containing pABA suggesting some were capable of producing folate from pABA, while others could not further substantiating the strain differences for folate production in this subspecies. However, neither of these studies investigated the growth and folate production when pABA was excluded from the medium.

Unlike *B. breve* ATCC 15701, *B. longum* DJO10A was found to be a potential folate producer based on all experiments carried out. It can contribute to both individual and total folate concentrations when it was grown in MRS. The folate biosynthesis ability of *B. longum* is consistent with several other studies which determined strains of *B. longum* as folate producers in either a folate containing medium, or milk (D'Aimmo et al., 2012, Holasova et al., 2004, Lin and Young, 2000).

B. bifidum ATCC 15696 was determined to be another potential folate producer based on the quantification analysis. Given that it can synthesize folate but cannot grow in YNB⁺ with added folate, it is obvious that it requires extra growth factors that are missing in YNB⁺ medium. In a study by Holasova et al. (2004) two strains of *B. bifidum* were found to produce 5-MTHF in milk, substantiating the potential of this species to produce folate.

5. Conclusion

This study shows that *B. longum* DJO10A and *B. bifidum* ATCC15696 are potential folate suppliers whereas the other tested strains may act as potential folate scavengers in an environment supplied with folate. We found that the commercial probiotic *B. animalis* subsp. *lactis* Bb-12 with a concentration of $\sim 10^{11}$ cfu/L utilized up to ~ 200 μ g of folate in a liter of MRS, which is more than the folate content of milk. Considering that the RDI for folate is 400 μ g, it may be advisable to include additional folic acid in food products in which this and other folate depleting cultures are used at high concentrations. Selection of folate producing bifidobacteria for use as probiotics may nutritionally enhance the folate status of the host.

Chapter IV - Overall Conclusions

1. Introduction

Probiotics are selected primarily for their ability to withstand stress conditions and maintain viability given that probiotics should be alive when they are consumed to fully elicit potential health benefits. However, many studies have shown that commercial products often did not contain the indicated or required number of probiotic microorganisms (Gueimonde et al., 2004, Temmerman et al., 2003). This is more pronounced for bifidobacteria, which are known as technologically sensitive strains. Although there has been a significant interest in incorporating probiotic bifidobacteria into food products mainly due to their perceived potential health benefits, their low survival under stress conditions limits their use. The majority of probiotic foods contain strains of *Bifidobacterium animalis* subsp. *lactis*, as they are generally more tolerant to acid and oxygen. However there is also interest in using other less stress tolerant bifidobacteria, especially human isolates which may be more suited to the human gut environment, provided that their viability is maintained in foods.

One way of maintaining viability at required levels is incorporation of bifidobacteria into foods using suitable preservation methods. Although spray drying is of significant commercial interest due to its relatively low cost, the high temperature applied can cause structural and physiological injury to the bifidobacteria cells and result in substantial loss of viability. Therefore, freeze-drying comes out as an alternative process for drying of temperature sensitive cultures. Freeze drying was found to give better survival rates compared to spray drying (Chávez and Ledebøer, 2007) and viability was increased using sugars and polymers as cryoprotectants (Miao et al., 2008). The storage and resuscitation conditions for freeze-dried cultures are also crucial to keep their viability high until their incorporation to food products. However, little documented

information is available on the relative effects of storage parameters on freeze dried bifidobacteria. By developing a freeze drying method yielding high viability and understanding how storage conditions affect viability will allow better survival rates for bifidobacteria and may remove the current obstacles against using of sensitive strains as probiotics.

Although it is not a requirement, the ability to synthesize vitamins, particularly essential vitamins, is a plus when selecting probiotics. One example is folic acid which is of significant interest since its deficiency is associated with a variety of serious health problems such as colon cancer, anemia and neural tube defects. Folate can be found in foods naturally. However its concentration is not very high and it is unstable compared to folic acid, the chemical form. Therefore, folic acid fortification programs were initiated in many countries, including the USA. In the US, fortification of enriched white flours and other grain foods such as pasta became mandatory since 1998. This resulted in a significant decline in infant neural tube defects in the following two years. On the other hand, it was also found that the folic acid intake from fortified foods was more than the projected amount possibly due to over-fortification of folate and underestimated amounts of food intake levels (Quinlivan and Gregory III, 2003). Considering that excess folic acid can mask vitamin B12 deficiency, this enhanced the importance of natural folate and use of starter cultures and probiotics for natural folate supplementation.

Functional analysis has demonstrated that strains of *Lactobacillus* species, including commercial probiotic lactobacilli, are generally depleters of folate which is consistent with their genome predictions (Rossi et al., 2011). On the contrary, bifidobacteria are generally known as potential folate producers. Based on the analysis of

genome sequences for predictable metabolic pathways, *B. animalis* subsp. *lactis* strains commonly used in foods, lack several genes required for de novo folate synthesis.

Although there are studies targeting incorporating folate producing bifidobacteria into foods, no studies have looked at the folate scavenging potential of commercial probiotic strains. Therefore, it would be of interest to functionally evaluate the accuracy of predicted models for the folate biosynthetic abilities of selected bifidobacteria and their folate producing or scavenging capacities.

2. Contributions of this research to the field

Improving viability of potential probiotic bifidobacteria in order to be used in foods is an important technological challenge for probiotics. In this thesis, high survival rates were obtained by freeze-drying *B. longum* DJO10A, representing a stress-sensitive group of bifidobacteria, using trehalose as the cryoprotectant. This study not only verified the resilience of freeze-dried *B. animalis* subsp. *lactis* Bb-12 to a wide range of storage conditions, but also determined suitable storage conditions for sensitive strains. Frozen storage with water activities controlled between 0.11 and 0.22 and replacement of oxygen with nitrogen were the optimal conditions found to maintain satisfactory viability levels for sensitive strains of bifidobacteria. This information will enhance the use of freeze-dried bifidobacteria for food applications, particularly in countries where cold storage is not readily available.

As part of this research, a new folate-free medium was developed which can be used for screening the folate synthesizing abilities of not all but some species of bifidobacteria. It was shown that folate production is growth stage dependent and can be regulated based on availability of folate in the environment. Furthermore, this study confirmed the folate scavenging potential of *B. animalis* subsp. *lactis* Bb-12 which is

consistent with the genome prediction of available strains of this subspecies. Therefore, inclusion of extra folic acid or addition of folic acid producing strains should be considered for foods that contain high levels of folate scavenging probiotic strains. These findings will highlight the importance of selection of probiotic strains for food applications and may open up a new horizon for selection criteria for probiotics.

3. Future directions of this research field

In parallel to the probiotic market, our understanding of characteristics of probiotics is growing with emerging technologies and the increasing number of sequenced genomes. Freeze-drying stands out with its superior viability for sensitive strains of bifidobacteria. However, further research is needed to determine how different factors affect their viability during storage. In particular, understanding what mechanisms lead to loss of viability under high water activities will allow taking steps for improved survival and longer shelf-life for freeze-dried bifidobacteria, especially under humid climates. These reactions are likely related with lipid oxidation and Maillard reactions. Addition of antioxidants into cryoprotectants could be tested to prevent possible lipid oxidation reactions and maintain viability during storage. Similarly, addition of sulphur containing aminoacids reduces the possible Maillard reaction rate and help increasing the survival rate during storage. Also, testing viability under combinations of a variety of water activities and atmosphere conditions should provide more details about possible reactions that cause loss of viability. Another aspect that needs to be investigated is how the probiotic functionality of freeze-dried cells is affected during the drying process and storage, and if it can be recovered using optimal resuscitation conditions.

Food companies like to claim as many health benefits as they can. Claiming supplementation of folic acid for consumers, particularly for pregnant women, may

enhance the market share for probiotics. However, this will bring along the need for verification of this health claim which is currently an ongoing problem in the probiotic market. Although *Bifidobacterium* spp. are predicted to be good candidates for folate production, functional investigation of this characteristic is required in target food environments using standardized quantification methods. Development of a general defined medium for cultivation of *Bifidobacterium* spp. would allow an extensive screening of bifidobacteria for their ability to synthesize not only folate but also other vitamins. A better understanding of folate production by bifidobacteria *in vivo* and its absorption through the colon will be needed to propose folic acid supplementation after ingestion of probiotics. Above all, a deeper investigation of the folate scavenging risk of probiotic strains that are currently in use is required. Another future research area of interest could be using gene transfer methods such as transformation (Wegkamp et al., 2004) or conjugation (Dominguez and O'Sullivan, 2013) to transfer genes required for *de novo* synthesis of folate to probiotic strains that lack required genes. In order to supply adequate folate, folate production can be increased using metabolic engineering (Santos et al., 2008, Sybesma et al., 2003) for folate producer strains of probiotic bifidobacteria. In the future, this line of research will highlight the importance of judicious selection of probiotics for folate supplementation and potentially allow the use of stress sensitive bifidobacteria strains as probiotics in food applications benefiting both manufacturers and consumers.

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