

The Pennsylvania State University

The Graduate School

Department of Food Science

**EFFECT OF STARCH SPHERULITES ON SURVIVAL OF BIFIDOBACTERIA
IN THE PRESENCE OF ACID OR BILE**

A Thesis in

Food Science

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

May 2009

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ABSTRACT

Probiotics are live microorganisms which have been shown to confer a health benefit on the host, when administered in sufficient amounts (FAO/WHO, 2001). Many of the probiotic bacteria currently used in foods and dietary supplements are members of Lactic Acid Bacteria. Strains of the genus *Bifidobacterium* are widely used as probiotics for human consumption. Bifidobacteria are often sensitive to stresses encountered during production and storage of food and during passage through the gastrointestinal tract. Thus, the overall purpose of this research was to determine if adhesion of bifidobacteria to starch-based spherulites can improve their survival when exposed to acid or bile.

The first objective of this work was to develop a procedure to prepare spherulites in adequate quantities for use in adhesion and survival studies. Spherulites were prepared successfully in a Parr reactor (pressure vessel) using potato starch, high amylose maize starch (Hylon VII) and purified potato amylose. Conditions were developed to dry spherulites using vacuum filtration and solvent dehydration. Spherulites were characterized with respect to size (particle size analysis) and shape (optical microscopy). Similar morphology and size were observed for both washed and dried spherulites.

The second objective was to determine if a variety of bifidobacterial strains could adhere to potato amylose spherulites and high amylose maize starch granules. The strains differed in their ability to adhere to spherulites and Hylon VII with adhesion to spherulites significantly higher ($p = 0.001$) than Hylon VII for the majority of the strains.

The final objective was to investigate the protective effect of adherence of bifidobacteria to spherulites or Hylon VII on their survival when exposed to acid or bile.

Adhesion to spherulites or Hylon VII did not improve the survival of bifidobacteria. The number of bifidobacteria surviving after 3 h exposure to acid (pH 1.8, 2.9 and 7.2) or bile (0, 0.5 and 1% oxgall) was significantly affected by type of strain used, pH and bile concentration. The strains analyzed were not tolerant to pH 1.8 whereas survived well at pH 2.9 and 7.2 and in bile solutions containing 0, 0.5 and 1% oxgall.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Bb-12	<i>Bifidobacterium animalis</i> ssp. <i>lactis</i> Bb-12
bp	Base pair
BSH	Bile salt hydrolase
CFU	Colony-forming unit
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
dNTPs	Deoxyribonucleotide triphosphates (mixture of four nucleotides)
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EDTA	Ethylene diaminetetraacetic acid
FAO	Food and Agriculture Organization of the United Nations
g	Gram
GG	<i>Lactobacillus rhamnosus</i> GG
GI	Gastrointestinal
GLM	General linear model
HMP	Human microbiome project
h	Hour
HVII	Granular high amylose maize starch (Hylon VII)
IBD	Inflammatory bowel disease

kb	Kilobase pair
min	Minute
MRS	deMan Rogosa and Sharpe (medium)
NAD	Nicotinamide adenine dinucleotide
NIH	National Institutes of Health
OD	Optical density
ORS	Oral rehydration solution
PAS	Potato amylose spherulites
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RB	Roberts-Briczinski culture collection
rDNA	Ribosomal DNA
rpm	Revolutions per minute
RS	Resistant starch
SCFA	Short chain fatty acid
s	Second
TBE	Tris/borate/EDTA
TOS	Trans-galactooligosaccharides
U.S.A.	United States of America
UV	Ultraviolet
WAXS	Wide-angle X-ray scattering
WHO	World Health Organization of the United Nations

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Dr. Robert F. Roberts for his guidance and support throughout this research. I greatly appreciate his encouragement and patience which was invaluable for the thesis completion. I would also like to thank Dr. Gregory R. Ziegler and Dr. Stephen J. Knabel for serving on my thesis committee. They were always interested and willing to help with the project. I would like to express my gratitude towards Dr. Nancy Ostiguy from the Department of Entomology for advising me with the statistical analysis. I would like to acknowledge Center for Food Manufacturing for providing financial support for this work.

I am thankful to my lab mates and fellow graduate students in the department for their help and friendship and providing me necessary training with various equipment. Particularly, I appreciate Lyubov Tmanova for being such a wonderful friend and supporting me in difficult times.

Finally, I would like to thank my parents and family for their continuous faith, love and support which made this thesis possible. I deeply appreciate the moral support, help and enthusiasm from my close friends Ravi, Swetha and Soujanya and for patiently listening to me during all stressful situations.

DEDICATION

I would like to dedicate this thesis to my father Ramakoti Chittiprolu who has been a great inspiration to me throughout my life and to the memory of my mother Sathyavathi Chittiprolu. My dad motivated me every step of the way through his constant encouragement, guidance, care, love and faith in me. I am extremely grateful to him for instilling confidence and optimism in all my endeavors. I owe my father for the person I am and for what I achieved today.

Chapter 1

Review of the literature

1.1 Overview

The intestinal microflora plays an important role in maintaining health in humans and animals, and disturbances in the composition and activity of these microbes can negatively influence health. One way to improve the intestinal microbiota is through the ingestion of live beneficial organisms often termed ‘probiotics’. Probiotic organisms have been studied for over a century for their potential health benefits in humans. In order to understand how probiotics may influence the intestinal microbial population, it is important to know about the human microbiome. Thus, this review begins with an introduction to human microbiota followed by definitions of probiotics and their reported health benefits. Although many genera of microorganisms have been considered as probiotics, this review focuses on *Bifidobacterium*. Viability of bifidobacteria is affected by several factors including exposure to acid, bile, oxygen and when incorporated into food products. One of the several approaches suggested to improve survival of bifidobacteria in adverse conditions is through adhesion to resistant starches. Thus, this paper highlights the factors affecting survival of bifidobacteria and methods for improving survival as well as the potential of starch spherulites (a type of resistant starch) as a means to protect bifidobacteria.

1.2 The human microbiome

The microbial ecosystem of humans is extremely complex and harbors about 100 trillion bacteria which constitute 1-2% of the body's mass. A healthy human body contains 10 times more bacterial than human cells and approximately 100 times more microbial genes than human genes (Ley *et al.*, 2007, Phillips, 2008). The genomes of all these microbial cells are collectively defined as the 'Microbiome' (Turrioni *et al.*, 2008). The gastrointestinal (GI) tract of humans is colonized by more than 500 different species of bacteria with the numbers varying between stomach, small intestine and colon (Fooks *et al.*, 1999, Leahy *et al.*, 2005). The average population of microflora in the stomach and duodenum is usually below 10^3 /g due to the low pH and consists of gram positive bacteria such as lactobacilli, streptococci and yeasts. The jejunum and ileum have higher numbers ranging from 10^4 to 10^8 /g which include gram negative facultatively anaerobic *Enterobacteriaceae* and anaerobic groups (*Bacteroides*, *Bifidobacterium*, *Fusobacterium* and lactobacilli). The human large intestine contains a highly complex microbial community with the population ranging from 10^{11} to 10^{12} /g and is dominated by strict anaerobes such as *Bacteroides*, *Bifidobacterium*, *Eubacterium* and *Peptostreptococcus*, with *Enterobacteriaceae*, streptococci, lactobacilli and clostridia in lower amounts (Fooks *et al.*, 1999, Holzappel *et al.*, 1998).

A large portion of the colonic microflora has not been cultured by traditional culturing techniques and still remains uncharacterized. Novel culture-independent approaches have been developed to analyze this complex ecosystem. Metagenomics is a culture-independent analysis of the microbiome in a particular environment through

sequence-based, compositional and/or functional analyses. Metagenomic analysis involves isolation of DNA from a defined environment followed by cloning of DNA into a suitable host and further analyzing the resultant DNA for sequences and functions of interest (Handelsman, 2004). A most recent initiative in metagenomics known as the Human Microbiome Project (HMP) was launched by National Institutes of Health (NIH) in 2008 with the aim of characterizing various human microbiomes and their role in human health and disease (<http://nihroadmap.nih.gov/hmp/>). HMP is described as a ‘logical conceptual and experimental extension of the Human Genome Project’ completed in 2003 which helped in understanding the complete human genome. Many outcomes can be expected from HMP including identification of new biomarkers of health, new enzymes produced by the human microflora with applications in biotechnology and most importantly, HMP will result in a deeper understanding of the nutritional requirement of humans (Turnbaugh *et al.*, 2007). Another potential outcome of HMP will be to aid in understanding the impact of “probiotics” delivered in food or dietary supplements on human health.

1.3 Definitions: Probiotics, Prebiotics and Synbiotics

Probiotics and prebiotics or their combination (synbiotics) have been receiving great attention in the past few years’ because of their potential for promoting human health. The number of “health promoting” organisms in the gastrointestinal tract can be increased in two ways (Suskovic *et al.*, 2001). One approach is by oral administration of live beneficial microorganisms (use of probiotics) and the second is to provide those

beneficial bacteria already existing in the intestine with selective carbon and energy sources that give them a competitive advantage over other bacteria in the system i.e., through the use of prebiotics.

The word **probiotic** is derived from two Greek words which together mean ‘for life’ (Fooks *et al.*, 1999). There are several published definitions of the term ‘probiotic’. These definitions vary with respect to route of administration, site and mode of action, and in an absolute requirement for viability. The most widely accepted definition was put forth by a group of experts working on the evaluation of probiotics in food convened by the Food and the Agricultural Organization (FAO) of the World Health Organization and is “live microorganisms which when administered in adequate amounts confer a health effect on the host” (FAO/WHO, 2001).

The term **prebiotic** was originally defined as “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health” (Gibson and Roberfroid, 1995). Some of the effects of the non digestible carbohydrates in the GI system include delayed gastric emptying, improved glucose tolerance, bulking effect, binding and sequestration of bile acids resulting indirectly in decreased fat and cholesterol absorption, and modulation of carbohydrate fermentation by anaerobic bacteria leading to production of short chain carboxylic acids and lowering of pH in the colon (Roberfroid, 1996). Taking into account the research published since its introduction, the original prebiotic concept was reviewed recently in terms of three criteria: (i) resistance to acidity, enzyme hydrolysis and gastrointestinal absorption, (ii) fermentation by members of the intestinal microbiota, and (iii) selective stimulation of

the intestinal bacteria associated with health promoting effects (Gibson *et al.*, 2004).

Based on these criteria, the term prebiotic has been re-defined as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health” (Gibson *et al.*, 2004). Currently, only two food ingredients satisfy these criteria: inulin and trans-galactooligosaccharides (TOS). There are other promising dietary carbohydrates that may serve as prebiotic compounds such as isomaltooligosaccharides, soybean oligosaccharides, lactosucrose, xylooligosaccharides and glucooligosaccharides, but these still require additional studies (Roberfroid, 2007).

The combination of probiotic microorganisms with appropriate prebiotic ingredients is termed synbiotics (Gibson and Roberfroid, 1995). A **synbiotic** is defined as “a mixture of probiotic and prebiotic that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare” (Gibson and Roberfroid, 1995). An example of a synbiotic would be a fructo-oligosaccharide in conjunction with a bifidobacterial strain. This combination could lead to advantages to the host offered by each component of the mixture i.e., probiotic and prebiotic (because of the availability of specific substrate for probiotic fermentation (Fooks *et al.*, 1999).

1.4 Role of probiotics in human health

In the early 20th century, Elie Metchnikoff, a Nobel laureate from the Pasteur Institute in Paris, proposed consumption of fermented milk products containing *Lactobacilli* reduced the number of toxin producing bacteria in the gut and contributed to the longevity of Bulgarian peasants (Metchnikoff, 1908). Metchnikoff suggested that ‘the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes’ (Metchnikoff, 1908). This observation led to great scientific interest in the role of specific probiotic bacteria in promoting health.

1.4.1 Mechanism of action

The exact manner in which probiotics act to confer health benefits is still uncertain, and many mechanisms have been hypothesized including (Fooks *et al.*, 1999, Gill and Guarner, 2004, Suskovic *et al.*, 2001, Vijaya *et al.*, 2005) :

- Competition for nutrients and adhesion sites
- Modulation of the immune system by increasing production of immunoglobulins or stimulating specific and non-specific immune responses
- Adhesion to the intestinal mucosa
- Antagonistic activity towards enteric pathogens by lowering the gut pH through production of short chain fatty acids (SCFA) and antimicrobial substances (e.g. lactic acid, acetic acid, bacteriocins or hydrogen peroxide), and prevention of pathogen adhesion or activation

- Inhibition of action of microbial toxins, suppression of toxin production and blocking of toxin receptor sites
- Alteration of microbial metabolism in the intestinal tract
- Restoration of the normal intestinal flora during antibiotic therapy
- Neutralization of dietary carcinogens.

Understanding the possible mechanisms by which probiotic bacteria exert their effect still remains an active area of research.

1.4.2 Health effects of probiotics

The putative health effects of specific probiotics vary with the genera and species of microorganisms, and are considered to be strain specific i.e., not all members of a specific species may provide the putative health benefits. There are a number of health benefits associated with probiotics, with some of them well documented and established, while others are proven only in animal models and still need to be substantiated in human studies. The potential role of probiotic microorganisms have been reported in various areas including prevention and treatment of GI diseases, modulation of immune system, protection against cancer, alleviation of lactose intolerance and allergies, hypocholesterolemic actions, and some nutritional benefits. A few of the reported benefits are cited below.

Probiotics may play a role in the treatment of GI disorders such as inflammatory bowel disease (IBD e.g., Crohn's disease and ulcerative colitis) by modulation of immune response, competitive exclusion, production of antimicrobials, modification of

intestinal permeability and decomposition of pathogenic antigens. Some probiotics also aid in prevention of infections caused by *Helicobacter pylori* through competitive exclusion, production of antimicrobials and lactic acid, and antipathogen activity (Gill and Guarner, 2004, Mercenier *et al.*, 2003, Ouwehand and Vesterlund, 2003, Sullivan and Nord, 2002, Vasijevic and Shah, 2008). Consumption of probiotic cultures have been shown to prevent or reduce the occurrence and duration of diarrhea (rotavirus, traveler's, *Clostridium difficile*, or antibiotic-associated diarrhea), through competitive exclusion, and/or improved immune response (Bengmark, 2001, Ouwehand *et al.*, 2002, Sanders, 2003, Sullivan and Nord, 2002). There have also been some studies on the efficacy of probiotics in urinary tract infections and vaginosis (Ouwehand and Vesterlund, 2003, Sanders, 1999, Senok *et al.*, 2005, Vijaya *et al.*, 2005), and on anti-colon cancer effects (Bengmark, 2001, Kailasapathy and Chin, 2000, Mercenier *et al.*, 2003, Ouwehand *et al.*, 2002).

Probiotic strains have the ability to modulate the immune system by enhancing non-specific defense against infection as well as increasing the phagocytic activity of white blood cells and increasing secretory IgA production (Mercenier *et al.*, 2003, Ouwehand *et al.*, 2002, Sanders, 1999, Senok *et al.*, 2005). Specific probiotics may have anticarcinogenic activity, through stimulation of the immune system, inhibition of carcinogen/pre-carcinogen formation, suppression of carcinogen producing bacteria, and/or mutagen absorption (Fooks *et al.*, 1999, Gill and Guarner, 2004, Kailasapathy and Chin, 2000, Sanders, 2003). The potential for probiotics to alleviate allergic reactions (atopic eczema or food allergies) through prevention of antigen translocation into the blood stream and through restoration of the homeostasis of the immune system, has also

been studied extensively (Bengmark, 2001, Isolauri *et al.*, 2000, Kalliomaki *et al.*, 2001, Vasijevic and Shah, 2008). It has been shown that specific probiotic strains play a role in deconjugation of bile acids and assimilation of cholesterol, leading to reduction in serum cholesterol and reducing the risk of cardiovascular diseases (Fooks *et al.*, 1999, Gill and Guarner, 2004, Gomes and Malcata, 1999, Kailasapathy and Chin, 2000, Mercenier *et al.*, 2003).

In addition to the above benefits, some probiotic bacteria can also improve the nutritional value of product when incorporated (through synthesis of higher levels of B vitamins and certain free amino acids, viz. methionine, lysine and tryptophan) and also improve the digestibility of foods by aiding the breakdown of proteins, fats and carbohydrates (Gomes and Malcata, 1999). Probiotic cultures have also been shown to alleviate lactose intolerance by delivering bacterial β -galactosidase into the human GI tract that hydrolyses lactose, and thus prevents the symptoms associated with this condition (Fooks *et al.*, 1999, Kailasapathy and Chin, 2000, Ouwehand and Vesterlund, 2003, Vasijevic and Shah, 2008).

1.4.3 Commercial probiotic strains

The most commonly used probiotic organisms are from the genera *Bifidobacterium* and *Lactobacillus* (Donohue, 2006). However, strains from other genera such as *Enterococcus*, *Pediococcus*, *Leuconostoc*, *Streptococcus*, *Lactococcus* and non lactic acid bacteria (Propionibacteria and *Saccharomyces* (yeast)) have also been proposed as probiotics (Holzapfel *et al.*, 2001). Numerous strains have been evaluated for

their potential health benefits. Table 1-1 lists some commercial probiotic strains currently marketed in different products in U.S.A. which have been studied in controlled human trials for the listed benefits. Specific probiotic strains that are consumed regularly are discussed below.

Lactobacillus rhamnosus GG (GG, ATCC 53103; Valio, Finland) is one of the most widely studied probiotic strains. In the U.S.A., GG is being sold as a dietary supplement in a product called Culturelle®. *Lactobacillus* GG was isolated from a healthy human intestinal tract in 1985 by Sherwood Gorbach and Barry Goldin and is stable to acid and bile, able to attach to mucosal cells of the human intestinal tract and produces lactic acid (US patent 4839281). Health benefits associated with the consumption of GG include prevention and treatment of diarrhea (rotavirus, *C. difficile* and antibiotic-associated diarrhea), alleviation of atopic eczema and modulation of immune response (Saarela *et al.*, 2000, Salminen *et al.*, 1996).

Lactobacillus casei Shirota was discovered by Minoru Shirota in 1930 and has been marketed in Japan since 1935 in a fermented dairy product known as Yakult® (www.yakultusa.com). *L. casei* Shirota has been reported to lower fecal enzyme activity, modulate intestinal bacteria, reduce recurrence of superficial bladder cancer, prevent rotavirus diarrhea, enhance immunity and have positive effects in the treatment of cervical cancer (Ouweland *et al.*, 1999, Saarela *et al.*, 2000, Salminen *et al.*, 1996).

Bifidobacterium animalis ssp. *lactis* is a widely used probiotic culture. Some of the commercial *B. animalis* ssp. *lactis* strains that have been extensively studied are Bb-

12, HN019 and DN-173 010. *Bifidobacterium animalis* ssp. *lactis* Bb-12 (Chr. Hansen, Denmark) is the most widely studied probiotic strain of bifidobacteria. Bb-12 transiently colonizes the gastrointestinal tract and has been shown to reduce incidence of traveler's diarrhea, treat rotavirus diarrhea, modulate immune response, and alleviate atopic eczema and constipation (Ouwehand *et al.*, 2002, Saarela *et al.*, 2000). *Bifidobacterium animalis* ssp. *lactis* HN019 was isolated from commercial yogurt and is marketed as DR10 by Fonterra, New Zealand and HOWARU Bifido by Danisco, U.S.A. (Sanders, 2006). It has been reported to have antagonistic activity against pathogens including *Escherichia coli*, *Salmonella*, *Clostridium* and *Bacteroides*, and to enhance immunity in children and adults (Chiang *et al.*, 2000, Gill *et al.*, 2001, Gopal *et al.*, 2003, Ouwehand and Philipp, 2004). *Bifidobacterium animalis* ssp. *lactis* DN-173 010 (Danone, France), used in the product Activia™, has been associated with reducing the colonic transit time and providing relief in constipated individuals (Bouvier *et al.*, 2001, Marteau *et al.*, 2002, Meance *et al.*, 2001, Yang *et al.*, 2008).

Table 1-1: Some commercially available probiotic products in the U.S.A. that have been evaluated in controlled human studies to provide support for the listed indications.

(http://www.usprobiotics.org/docs/Probiotic_Products.pdf; Sanders M.E. 2008).

Commercial strain designation	Product (Format)	Indication	References
<i>L. rhamnosus</i> GG (LGG)	Culturelle (capsule)	Infant diarrhea	(Szajewska <i>et al.</i> , 2007)
VSL#3 (8-strain combination of 3 <i>Bifidobacterium</i> strains, 4 <i>Lactobacillus</i> strains and <i>S. thermophilus</i>)	VSL#3 (powder)	Inflammatory bowel conditions (primary evidence in pouchitis)	(Chapman <i>et al.</i> , 2006)
<i>S. cerevisiae</i> (<i>S. boulardii</i>)	Florastar (powder) Lalflor (capsule)	Antibiotic-associated and <i>C. difficile</i> diarrhea	(Johnston <i>et al.</i> , 2006, McFarland, 2006, Szajewska and Mrukowicz, 2005)
<i>L. rhamnosus</i> GG (LGG)	Culturelle (capsule)		(Katz, 2006)
<i>L. casei</i> DN-114 001	DanActive (fermented milk)		(Hickson <i>et al.</i> , 2007)
<i>L. acidophilus</i> CL 1285 plus <i>L. casei</i> Lbc80r	BioK+CL1285 (fermented milk, soy milk, capsule)		(Beausoleil <i>et al.</i> , 2007)
<i>B. animalis</i> ssp. <i>lactis</i> DN-173 010 (<i>Bifidus regularis</i>)	Activia (yogurt)	Gut transit time/ bowel function	(Marteau <i>et al.</i> , 2002)
<i>L. casei</i> Shirota	Yakult (daily dose drink)		(Koebnick <i>et al.</i> , 2003)
<i>L. reuteri</i> ATCC 55730 (Protectis)	BioGaia chewable gut health tablets; BioGaia gut health probiotic straws	Keeping healthy	(Tubelius <i>et al.</i> , 2005, Weizman and Alsheikh, 2006)
<i>L. rhamnosus</i> GG (LGG)	Culturelle (capsule)		(Hatakka <i>et al.</i> , 2001)
<i>L. casei</i> DN-114 001	DanActive (fermented milk)		(Pedone <i>et al.</i> , 2000)

Table 1-1 (continued)

Commercial strain designation	Product (Format)	Indication	References
Most strains of <i>L. bulgaricus</i> and/or <i>S. thermophilus</i>	All yogurts with live, active cultures	Lactose maldigestion	(de Vrese <i>et al.</i> , 2001)
<i>L. reuteri</i> ATCC 55730 (Protectis)	Reuteri drops	Colic in infants	(Savino <i>et al.</i> , 2007)
<i>B. animalis</i> ssp. <i>lactis</i> HN019 (HOWARU Bifido or DR10)	Strain sold as an ingredient for dairy and supplement products	Immune support	(Sanders, 2006)
<i>L. reuteri</i> ATCC 55730	BioGala chewable gut health tablets; BioGala gut health probiotic straws		(Valeur <i>et al.</i> , 2004)
<i>L. rhamnosus</i> GG (LGG)	Culturelle (capsule) Danimals (drinkable yogurt)		(Kekkonen <i>et al.</i> , 2008, Piirainen <i>et al.</i> , 2008)
<i>L. casei</i> Shirota	Yakult (daily dose drink)		(Takeda and Okumura, 2007)
<i>B. animalis</i> ssp. <i>lactis</i> Bb-12	Good start natural cultures (Infant formula); Live active cheese; Yo-Plus yogurt		(Kekkonen <i>et al.</i> , 2008)
<i>L. casei</i> DN-114 001	DanActive (fermented milk)		(Parra <i>et al.</i> , 2004)
<i>L. rhamnosus</i> GR-1 plus <i>L. reuteri</i> RC-14	Fem-Dophilus (capsules)		Bacterial vaginosis
<i>B. infantis</i> 35624 (Bifantis™)	Align (capsules)	Mild to moderate irritable bowel syndrome symptoms	(Whorwell <i>et al.</i> , 2006)
<i>L. plantarum</i> 299v (Lp299v)	Very Good Belly (juice)		(Niedzielin <i>et al.</i> , 2001)

1.5 Criteria for consideration as a probiotic bacterium

There are several desirable properties expected of a potential probiotic strain in order for it to be able to exert beneficial effects. It is important to recognize that not every potential probiotic strain will possess every property.

Criteria for selection of probiotic microorganisms can be divided into three major categories: safety, functional and technological (Saarela *et al.*, 2000). In terms of safety, the strain should have an origin similar to its intended host, be accurately identified and characterized to the strain level, be non-pathogenic and non-toxic, and should not carry transmissible antibiotic resistance genes (Mattila-Sandholm *et al.*, 2002, Mercenier *et al.*, 2003, Ouwehand *et al.*, 1999).

Several aspects of functionality have to be considered when evaluating potential probiotic bacteria. Some of these factors are related to survival in the host including acid tolerance, bile tolerance, tolerance to human gastric juice, adherence to the intestinal mucosa and colonization potential in the human GI tract. Other factors are related to specific health promoting activities in the host including production of antimicrobial substances, immunostimulation, antagonistic activity towards pathogenic bacteria as well as antimutagenic and anticarcinogenic properties (Kailasapathy and Chin, 2000, Klaenhammer and Kullen, 1999, Saarela *et al.*, 2000).

Technologically, the strain to be considered as a probiotic should remain viable during large-scale production and processing, should be stable in the product during storage and delivery, should be genetically stable and possess phage resistance. In

addition, they should have good sensory properties and should not impart any undesirable characteristics when included in foods (Klaenhammer and Kullen, 1999, Saarela *et al.*, 2000, Vasijevic and Shah, 2008).

1.6 Taxonomy of *Bifidobacterium*

Bifidobacterium spp. have been reported to be beneficial probiotic organisms that provide excellent therapeutic benefits (Gomes and Malcata, 1999). Bifidobacteria are gram-positive, non-spore forming, non-motile, non-gas producing, catalase-negative, anaerobic bacteria with a variety of shapes ranging from short, curvy rods to bifurcated, Y-shaped or clubbed rods (Biavati *et al.*, 2000, Gomes and Malcata, 1999) and are a major type of saccharolytic bacteria in the colon (Gibson and Roberfroid, 1995).

Henry Tissier, a French pediatrician discovered this genus, originally termed *Bacillus bifidus*, in 1899 from the feces of breast-fed infants while studying infantile diarrhea (Leahy *et al.*, 2005). He suggested these bacteria could be used to treat patients with diarrhea to help in restoration of healthy gut microflora. There has been extensive research on the properties of these organisms since that time.

Bifidobacterium spp. are normal inhabitants of the human intestine and are found at high concentrations in the feces of breast-fed infants. Bifidobacteria constitute up to 99% of the infant intestinal microflora, but account for less than 15% of the microorganisms present in the adults and are considered the fourth most prominent type of bacteria among the GI flora (Rao *et al.*, 1989). The optimum growth temperature for bifidobacteria is 37 – 41°C (minimum growth temperature is 25 – 28°C and maximum is

43 – 45°C) (Gomes and Malcata, 1999, Roy, 2001). There is no growth of bifidobacteria below 20°C and above 46°C, except *B. thermoacidophilum* which can grow at 49.5°C (Dong *et al.*, 2000). Sensitivity to oxygen varies accordingly to the species and the strains used (Biavati *et al.*, 2000).

Currently there are 29 species in the genus *Bifidobacterium* (Ventura *et al.*, 2007). The species which were isolated from the humans (feces of infants and adults, dental caries, adult human blood) are: *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. gallicum*, *B. longum* biotype *infantis*, *B. longum* biotype *longum*, *B. pseudocatenulatum* and *B. scardovii*. Species isolated from swine feces include *B. choerinum*, *B. psychroaerophilum*, *B. longum* biotype *suis*, *B. pseudolongum* ssp. *pseudolongum*, *B. thermoacidophilum* ssp. *porcinum* and *B. thermophilum*. Those species found in feces and rumen of calf, cow, rabbit and chicken are *B. animalis* ssp. *animalis*, *B. boum*, *B. magnum*, *B. pseudolongum* ssp. *globosum*, *B. cuniculi*, *B. merycicum*, *B. saeculare*, *B. gallinarum*, *B. pullorum* and *B. ruminatum*. There are three species isolated from the hindgut of honeybees (*B. asteroides*, *B. indicum* and *B. coryneforme*), two species from sewage (*B. subtile* and *B. minimum*), and *B. thermoacidophilum* ssp. *thermoacidophilum* from waste water. *B. animalis* ssp. *lactis* was isolated from yogurt (Meile *et al.*, 1997).

Species of *Bifidobacterium* commonly used as probiotics in humans include *B. adolescentis*, *B. animalis* ssp. *lactis*, *B. bifidum*, *B. breve*, *B. infantis* and *B. longum* (Anal and Singh, 2007, Roy, 2005).

1.7 Survival of bifidobacteria

The health benefits attributed to *Bifidobacterium* spp. can be expected only when the organisms are able to survive and be metabolically active under adverse environmental conditions. Thus, understanding the survival of bifidobacterial strains in GI tract and in probiotic containing products is essential in the strain selection process.

1.7.1 Survival in the presence of acid

Bifidobacteria are considered acid tolerant but not acidophilic (Biavati and Mattarelli, 2006). Optimal pH for growth of bifidobacteria is between 6.5 and 7.0 with little or no growth below pH 5 or above 8 (Biavati *et al.*, 2000, Doleyres and Lacroix, 2005, Roy, 2001), with the exception of *B. thermoacidophilum* which can grow at pH 4 (Dong *et al.*, 2000). The pH may go as low as 1.5 in the stomach and the surroundings of the GI tract and bifidobacteria must be able to survive these conditions and reach the intestines (Kailasapathy and Chin, 2000).

Previous studies have shown survival of bifidobacteria was low under simulated gastric conditions. The viability of bifidobacteria depends on pH of the system, the length of exposure to acid, and the species and strains used (Bezkorovainy, 2001, Charteris *et al.*, 1998). Some strains are rapidly killed in the stomach while others can survive passage through the gut in high numbers. It has been shown that *B. longum* survived better in acidic conditions (pH 1.0, 2.0 and 3.0 for 3 h) than *B. infantis*, *B. adolescentis* or *B. bifidum* (Clark *et al.*, 1993). Similar to these results, in another study where survival of 9 strains of *Bifidobacterium* spp. in acidic conditions was studied, *B. longum* 1941 and

B. pseudolongum 20099 showed the greatest survival, and *B. adolescentis*, *B. bifidum* and *B. breve* survived poorly at all pH levels (1.5, 2.0, 2.5 and 3.0 for 3 h). Survival of *B. infantis* 1912 was reduced at pH 1.5, 2 and 2.5 (Lankaputhra and Shah, 1995). However, Liong and Shah (2005) reported that *B. longum* BB536 and *B. infantis* ATCC 17930 were more acid tolerant than *B. breve* ATCC 15698, *B. longum* 1941 and *B. infantis* 1912 when exposed to pH 2.0 for 120 min (Liong and Shah, 2005). Thus, it can be noted that acid tolerance varies greatly with strain type.

1.7.2 Survival in the presence of bile

Bile is a yellow-green aqueous solution constituting majorly of bile acids, cholesterol, phospholipids, and the pigment biliverdin and acts as a biological detergent which emulsifies and solubilizes fats. It is synthesized in the pericentral hepatocytes of the liver from cholesterol and stored in the gall bladder. During digestion, bile is secreted into the duodenum as conjugates of glycine or taurine and facilitates fat absorption (see review by Begley *et al.* (2005)). During this process, bile salts can be transformed by the intestinal microflora. The primary bile salts produced in the liver can be deconjugated by bile salt hydrolases (BSH) resulting in the formation of free/unconjugated bile acids (mainly cholic and chenodeoxycholic) and amino acid residues. Subsequently, 7 α -dehydroxylation can take place and convert these primary bile acids to secondary bile acids (deoxycholic and lithocholic) (Noriega *et al.*, 2004).

Survival of bifidobacteria in the presence of bile varies with both concentration and strain studied. Bile concentration can reach up to 2% during the first hour of

digestion and may drop to 0.5% in the second hour (Clark and Martin, 1994). Ibrahim and Bezkorvainy (1993) studied the tolerance of *B. longum*, *B. infantis*, *B. bifidum* and *B. breve* to 0, 0.06, 0.15 and 0.3% sodium glycolate in tryptone-peptone-yeast extract (TPY) medium by measuring optical density (OD) for up to 48 h. *Bifidobacterium infantis* showed the best survival, followed by *B. bifidum* and *B. breve*, whereas *B. longum* showed the least resistance. In contrast to this, Clark and Martin (1994) showed that *B. longum* exhibited better tolerance to bile than *B. infantis*, *B. bifidum* and *B. adolescentis* in 2% and 4% oxgall after 12 h incubation at 37°C. Similarly, Lankaputhra and Shah (1995) reported that among 9 strains of *Bifidobacterium* spp., *B. longum* 1941, *B. pseudolongum* 20099 and *B. infantis* 1912 showed the best tolerance during three hours in bile (0, 1 and 1.5%). It was also shown that though *B. longum* 1941 was bile tolerant, the other *B. longum* strain (20097) was not so tolerant. In another study, it was reported that *B. longum* BB536 was the most efficient among the eight bifidobacterial strains tested in the presence of six different conjugated bile salts (Grill *et al.*, 1995). Consistent with the above studies, Liong and Shah (2005) demonstrated that *B. longum* 1941, *B. infantis* 1912, *B. infantis* ATCC 17930, *B. longum* BB536 and *B. breve* ATCC 15698 were able to grow in 0.3% (w/v) of oxgall, cholic acid or taurocholic acid in MRS-LC broth.

1.7.3 Acid and bile tolerance of *B. animalis* ssp. *lactis*

Previous literature indicates that *B. animalis* ssp. *lactis* strains are more resistant to acid and bile *in vitro* than other bifidobacteria (Hansen *et al.*, 2002, Matsumoto *et al.*,

2004, Matto *et al.*, 2004, Saarela *et al.*, 2005) and also survive transit in the human GI tract (Alander *et al.*, 2001). Hansen *et al.* (2002) demonstrated that among the nine bifidobacterial strains screened for resistance to simulated gastric juice and bile salts, Bb-12 showed the greatest survival in the presence of acid (pH 2, 3 and 6 for 2 h) and oxgall (0, 0.5 and 1 % for 24 h). In another study by Saarela *et al.* (2005), Bb-12 cells grown in fermenters in general edible medium (GEM) for 15 or 22 h were able to survive well in PBS adjusted to pH 3, but poorly at pH 2.5 (2 h exposure). The cells also tolerated 1% bile acids in PBS (pH 7.2) after 3 h incubation. The sensitivity of Bb-12 to very low pH (close to 1) was reported by Favaro-Trindade *et al.* (2000). Bb-12 showed a reduction of 1 log at pH 2 after 2 h and was completely eliminated at pH 1 after 1 h. However, the strain was tolerant to 2 and 4% oxgall (which are considered to be higher than the normal bile concentrations in the human intestine) even after 12 h incubation demonstrating the extreme bile resistance of Bb-12. Matto *et al.* (2004) studied the acid (pH 3 for 1 h), bile (1.5% bile extract for 3 h) and oxygen tolerance (ambient) of 34 intestinal bifidobacterial isolates and 8 reference strains. Besides the *B. animalis* ssp. *lactis* isolates which were highly similar to the reference (DSMZ 10140), none of the other isolates exhibited good tolerance to acid, bile and oxygen (Matto *et al.*, 2004). Similarly, *B. animalis* ssp. *lactis* strains displayed higher survival than strains from other *Bifidobacterium* spp. during simulated gastrointestinal transit (Masco *et al.*, 2007). The acid and bile tolerance of strains used in commercial products was also demonstrated earlier. *Bifidobacterium animalis* ssp. *lactis* HN019 has exhibited good survival at pH 3 for 3 h and also in MRS medium containing oxgall (0, 0.4, 0.8 and 1%) at 37°C (Prasad *et al.*, 1998). Similarly,

another strain *B. animalis* ssp. *lactis* DN-173 010 used in Activia®, has been shown to survive human intestinal transit (Duez *et al.*, 2000).

The mechanism for acid tolerance in *B. animalis* ssp. *lactis* may be dependent on the membrane H⁺-ATPase activity induced at low pH (Matsumoto *et al.*, 2004, Ventura *et al.*, 2004a). When bacteria are exposed to low pH, the intracellular pH decreases followed by an increase in the amount of F₁F₀-ATPase. This enzyme catalyzes the ATP-driven translocation of protons from the cytoplasm (Ventura *et al.*, 2004b). Such enzyme activity has been observed in *B. animalis* ssp. *lactis* (Matto *et al.*, 2006). The F₁F₀-ATPase is encoded by the *atp* operon and this operon has been sequenced from *B. animalis* ssp. *lactis* DSMZ 10140 (Ventura *et al.*, 2004b). It was shown that H⁺-ATPase activity increased at low pH (4) for acid tolerant strains (*B. lactis* and *B. animalis*) but was lower for non-acid tolerant strains (*B. adolescentis*, *B. bifidum*, *B. breve*, *B. cantenulatum*, *B. infantis*, *B. longum* and *B. pseudocantenulatum*). The increased acid tolerance for *B. lactis* and *B. animalis* strains was related to the rapid increase in H⁺-ATPase activity under acid conditions. These researchers emphasized that when strains are exposed to low pH, it is important that there is an increase in H⁺-ATPase activity quickly in order to release H⁺ to maintain a constant intracellular pH (Matsumoto *et al.*, 2004).

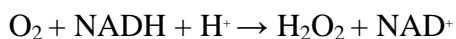
The mechanisms underlying bile tolerance of *Bifidobacterium* and *Lactobacillus* are still poorly understood (Begley *et al.*, 2005). Some researchers suggest that bile salt hydrolases may be involved in the resistance mechanism. Deconjugation by these enzymes may decrease the toxicity of the conjugated bile salts against the bacteria producing it and thereby increase bile resistance (Desmet *et al.*, 1995, Grill *et al.*, 1995).

Recently, it was shown that the F_1F_0 -ATPase was involved in bile tolerance of *B. animalis* ssp. *lactis* (Sanchez *et al.*, 2006). In this study, it was suggested that the bile-adapted *B. animalis* ssp. *lactis* strains (IPLA 4549 and its derivative 4549Ox) tolerated bile by increasing the intracellular ATP and inducing proton pumping by the F_1F_0 -ATPase and thus maintaining the internal pH. The mechanisms involved in the adaptation and resistance of these strains to bile was further studied, wherein the proteomes of both strains were compared in the presence and absence of bile (Sanchez *et al.*, 2007). The results indicated that several physiological mechanisms including changes associated with nitrogen metabolism, changes in fatty acid biosynthesis, increase in amount of molecular chaperones, changes in redox potential of the cell and changes in manner of energy production can jointly impact the toxicity of bile to the cells.

1.7.4 Oxygen toxicity

Bifidobacterium spp. which are strictly anaerobic are susceptible to the toxic effects of oxygen. Exposure to oxygen results in the accumulation of toxic oxygenic metabolites such as superoxide anion (O_2^-), hydroxyl radical (OH \cdot), and hydrogen peroxide (H_2O_2) in the cell, eventually leading to cell death (Talwalkar and Kailasapathy, 2004). Sensitivity to oxygen in bifidobacteria had been reported to be strain dependent (Beerens *et al.*, 2000, Bolduc *et al.*, 2006, Shimamura *et al.*, 1992). Beerens *et al.* (2000) evaluated the effect of exposure to air on 84 strains of bifidobacteria. It was shown that strains belonging to species of animal origin (*B. thermophilum*, *B. pseudolongum* ssp. *pseudolongum*, *B. pseudolongum* ssp. *globosum*) were more resistant than those of human

origin. Exposure to air for 4 days at 20°C in brain heart infusion broth reduced the levels of 84 strains of bifidobacteria by less than 1 log. When a similar experiment was conducted on *B. pseudolongum* ssp. *pseudolongum* and *B. thermophilum* strains at 4°C, the majority of the strains survived without any decline. The authors indicated that oxygenation during processing is unlikely to affect bifidobacterial survival but other factors such as product formulation (presence of nitrites or other ingredients), water activity and relative humidity may be significant (Beerens *et al.*, 2000). In another study, to determine the relationship between oxygen sensitivity and oxygen metabolism, *B. infantis* ATCC 15697, *B. longum* ATCC 15707 and *B. breve* ATCC 15700 showed growth under conditions of partial aeration (occasional shaking for 10 s every 30 min), while growth of *B. adolescentis* ATCC 15703 was inhibited by low concentrations of oxygen. All the strains exhibited significant growth in an anaerobic environment but were suppressed in an aerobic environment (constant shaking at 45 rpm) (Shimamura *et al.*, 1992). The researchers suggested the activities of NADH-oxidase and NADH-peroxidase play a role in antioxidative defense, while the contribution of superoxide dismutase enzyme was probably lower. NADH-oxidase and NADH-peroxidase catalyze the following reactions in *Bifidobacterium* species, respectively:



In these reactions, oxygen is being converted to water and thus, these enzymes may play role in reducing oxygen toxicity in *Bifidobacterium* species. Consistent with these findings, it was demonstrated that NADH-oxidase and NADH-peroxidase activities increased with increasing oxygen concentration from 0 to 21% and no conclusive trend

was observed for superoxide dismutase with the oxygen concentration (Talwalkar and Kailasapathy, 2003b). Thus, oxygen toxicity is another important factor to be considered that can influence survival in a probiotic containing product.

1.7.5 Survival in dairy products

Bifidobacteria have been incorporated in various products such as yogurt, frozen desserts, mayonnaise, sour cream, buttermilk, cheese, powdered milk, spreads and fruit juices (Khalil and Mansour, 1998). Products such as infant formula, cereal, granola and chocolate bars formulated to contain bifidobacteria are also available in the market.

It is not precisely clear how many probiotic organisms are required to achieve beneficial health effects. However, in general, minimum numbers of 10^6 CFU/g and even higher have been recommended at the time of consumption (Roy, 2005). Further, the product should be consumed regularly in adequate quantities so the gut receives an adequate dose of organisms, taking into account the losses encountered by the gastric stresses (Ross *et al.*, 2005). However, the dose for the probiotic to be effective may depend on the strain used, the form of food in which it is ingested (Roy, 2005, Stanton *et al.*, 2001) and also varies with the desired health effects. Thus, manufacturers generally add higher numbers of bacteria initially to ensure the final product at the end of its shelf-life contains 10^6 to 10^8 CFU/g (Champagne *et al.*, 2005). In order to help consumers differentiate between yogurts containing live active cultures and which do not, the National Yogurt Association introduced a 'Live Active Culture' seal. This seal helps the consumers identify yogurt containing 10^8 viable lactic acid bacteria per g or frozen yogurt

containing 10^7 per g at the time of manufacture. However, these numbers do not distinguish probiotic bacteria from starter culture bacteria (*L. bulgaricus* and *S. thermophilus*). These counts represent the total number of live cultures and do not indicate the quantity of each species or strain used in the product (Sanders, 2003, Senok *et al.*, 2005).

Several studies have shown that probiotic bacteria may not survive in high levels when incorporated into highly acidic products like yogurt (Dave and Shah, 1997, Micanel *et al.*, 1997, Schillinger, 1999, Shah *et al.*, 1995, Vinderola *et al.*, 2000), fermented frozen dairy desserts (Ravula and Shah, 1998, Shah and Ravula, 2000, Sheu *et al.*, 1993), and fermented milk (Berrada *et al.*, 1991, Samona and Robinson, 1994). Additionally, there are studies on the survival of probiotics when incorporated in ice cream (Alamprese *et al.*, 2005, Godward and Kailasapathy, 2003), cheese (Gardiner *et al.*, 1999, Kailasapathy and Masondole, 2005, Phillips *et al.*, 2006) and mayonnaise (Khalil and Mansour, 1998).

It has been reported the viability of probiotic cultures in dairy products is affected by pH, titrable acidity, hydrogen peroxide, oxygen content, storage temperature, species and the strains used, and concentrations of acids and buffers present (Dave and Shah, 1997, Godward and Kailasapathy, 2003, Lankaputhra *et al.*, 1996, Micanel *et al.*, 1997, Samona and Robinson, 1994, Shin *et al.*, 2000, Talwalkar and Kailasapathy, 2003a). Survival of some *Bifidobacterium* spp. in yogurts has been shown to be low, due to their intolerance of acid conditions and the presence of other cultures, such as *L. delbrueckii* ssp. *bulgaricus* (Grosso and Trindade, 2004). Shah *et al.* (1995) demonstrated *B. bifidum* from 5 commercial yogurts showed a decline in viable numbers during storage for 5

weeks and suggested the reason might be the decrease in pH values during the storage period. In another study, commercial yogurts were obtained from four manufacturers one day post manufacture and analyzed for the microflora present for a period of 5-6 weeks (Micanel *et al.*, 1997). Out of the three products containing bifidobacteria, one maintained high levels ($>10^6$ CFU/g), the second showed a sharp decline within two weeks (1.5×10^5 to $< 10^3$ CFU/g) and no viable cultures were detected in the third ($<10^3$ CFU/g). Storage temperature (4°C and 10°C) had very little effect on viability and there was no noticeable effect of fat on survival. Dave and Shah (1997) studied the viability of probiotic bacteria during manufacture and 35 days storage in yogurt made from four commercial starter cultures. The increase in number of viable bifidobacteria during manufacture and storage was related to the species and strains of the yogurt organisms. Viability of bifidobacteria increased with a decrease in the dissolved oxygen concentration and was also affected by storage temperature of 10°C.

Factors such as acidity of the product, freeze injury, sugar concentration and oxygen toxicity may influence the viability of probiotics in frozen yogurt or fermented dairy desserts (Ravula and Shah, 1998). In their study, a total of 41 strains (yogurt bacteria, *Lactobacillus acidophilus* and *Bifidobacterium* spp.) were screened for the ability to survive frozen conditions, presence of sugar (8 and 16%) and acidic conditions. All the strains survived freezing and sugar concentration and the majority of the strains survived at pH greater than 4. The importance of choosing a strain resistant to acidity in commercial fermented milks was demonstrated by Berrada *et al.* (1991). In this study, it was shown that not all commercial fermented milks contain bifidobacteria which survive through the GI tract and during the product storage. Previous literature indicates that ice

cream could be a suitable vehicle for delivering probiotic bacteria. In a study by Hekmat and McMahon (1992), high counts of *B. bifidum* (10^7 CFU/ml) have been reported in probiotic ice cream even after 17 weeks of frozen storage (Hekmat and McMahon, 1992). Similarly, it was shown that *B. infantis* 1912 survived well in ice cream at -20°C over a period of 24 weeks. The high survival might be due to the protection provided by the total solids in the mix and high fat content of the ice cream (Godward and Kailasapathy, 2003). Thus, the survival of bifidobacteria in dairy products is influenced by several factors including strain, interactions between the species in the product, acidity, oxygen content and storage temperature.

Since the success of food products containing bifidobacteria depends on their viability during conditions existing in the upper GI tract and during the production and shelf life of the product (Sun and Griffiths, 2000), various methods have been used to improve the resistance of probiotic bacteria when exposed to adverse conditions. Different approaches to maintain viability have been evaluated, such as micro-encapsulation, appropriate selection of acid and bile resistant strains, use of oxygen-impermeable containers, two-step fermentation, stress adaptation, incorporation of micro-nutrients such as peptides and amino acids (Picot and Lacroix, 2004, Shah, 2000), addition of prebiotics (Bruno *et al.*, 2002, Capela *et al.*, 2006, Chen *et al.*, 2005, Donkor *et al.*, 2007, Iyer and Kailasapathy, 2005) and adhesion to granular starches (Crittenden *et al.*, 2001a, Wang *et al.*, 1999a).

1.8 Methods for improving survival

1.8.1 Micro-encapsulation

Micro-encapsulation is a process in which viable cells are retained within an encapsulating material that provides a physical barrier against the external environment and reduces cell injury or loss (Krasaekoopt *et al.*, 2003). Micro-encapsulation has also been defined as a chemical or mechanical process in which a primary ingredient is covered by a layer of another material, providing protection and facilitating controlled release of the primary ingredients (Chen *et al.*, 2005). If the appropriate barrier materials are chosen, encapsulation can help to protect the bacteria from the adverse conditions of the GI tract, releasing them in the intestine where the chances of survival and colonization are higher (Godward and Kailasapathy, 2003).

Encapsulated probiotic bacteria could have applications in many fermented dairy products such as yogurt, cheese, cultured cream and frozen dairy desserts, and for biomass production (Kailasapathy, 2002, Krasaekoopt *et al.*, 2003). Various techniques used for encapsulating probiotic microorganisms are extrusion, emulsion, spray drying, spray-congealing, fluidized-bed coating/air-suspension, and coacervation/phase separation (Anal and Singh, 2007). Previous reports suggest microencapsulation can provide protection to probiotic cultures from high oxygen levels (Talwalkar and Kailasapathy, 2003a), during manufacture and storage of yogurt (Adhikari *et al.*, 2003, Adhikari *et al.*, 2000, Iyer and Kailasapathy, 2005), freezing (Shah and Ravula, 2000), and during simulated GI conditions (Chandramouli *et al.*, 2004, Favaro-Trindade and

Grosso, 2002, Liserre *et al.*, 2007, Picot and Lacroix, 2004, Sridar *et al.*, 2003, Wen-Chian Lian *et al.*, 2003).

Probiotic cultures are generally supplied in the form of concentrated powders (spray dried, freeze dried or fluidized bed dried) for their ease of use in commercial food applications (Krasaekoopt *et al.*, 2003). However, it has been reported that cultures encapsulated by these techniques do not survive well due to stresses during the manufacturing process and are not completely protected from the adverse conditions in the product or in the stomach/intestinal tract. Encapsulation in biodegradable hydrocolloid polymers entraps the cells within the matrix and provides protection in such adverse conditions (Anal and Singh, 2007, Krasaekoopt *et al.*, 2003). Different materials have been used for encapsulation of bifidobacteria, including starch (Iyer and Kailasapathy, 2005, Lahtinen *et al.*, 2007, Lian *et al.*, 2003, Mattila-Sandholm *et al.*, 2002, Myllarinen *et al.*, 2000, O'Riordan *et al.*, 2001a), alginate (Favaro-Trindade and Grosso, 2002, Hansen *et al.*, 2002, Khalil and Mansour, 1998), alginate-starch (Khalida Sultana *et al.*, 2000), cellulose acetate phthalate (Favaro-Trindade and Grosso, 2002, Rao *et al.*, 1989), k-carrageenan (Adhikari *et al.*, 2003, Adhikari *et al.*, 2000), whey protein (Picot and Lacroix, 2004) and gellan- xanthan gum (McMaster *et al.*, 2005b, Sun and Griffiths, 2000). Encapsulation in polymer systems have some advantages including ease in: handling and preparation on a lab scale, quantification of the number of cells in the beads allowing dosage control, incorporation of cryo- and osmo-protective components into the matrix and application of an additional surface coating. However, scaling up of the process is difficult and processing costs are also high. In addition, most of these microcapsules are porous and allow water and other fluids in and out of the matrix.

Another point to be considered is the size of the capsule which can affect the sensory and textural properties of the product when added. Thus, the major challenge is to select the appropriate encapsulation technique and encapsulating material (Anal and Singh, 2007).

1.8.2 Adhesion to starch granules

Attachment of bacterial cells to a substrate might be ‘non-specific and reversible’ or ‘specific and irreversible’. When cells become associated with a surface, initial attachment is based on physicochemical attractive forces which may be reversible or irreversible. This can be followed by firm attachment mediated by production of exopolysaccharides or specific ligands that may interact with the surface (Hood and Zottola, 1995, Palmer *et al.*, 2007). Definitions for the terms ‘attachment’ and ‘adhesion’ are not always clear or consistent. However, in previous literature evaluating the interaction of bifidobacteria with starch, the term ‘adhesion’ was used and this term is used throughout the present work and should be understood to include both specific and non-specific bacterial starch interactions.

Bacterial adhesion to starch may provide advantages in new probiotic technologies to enhance the delivery of viable and metabolically active probiotics to the intestinal tract (Crittenden *et al.*, 2001a). The adhesion of bifidobacteria to starch granules has been studied previously (Crittenden *et al.*, 2001a, O’Riordan *et al.*, 2001b, Wang *et al.*, 1999a). In a study evaluating the protective effects of high amylose maize starch granules on the survival of *Bifidobacterium* spp. in the mouse intestinal tract, an adhesion assay was performed to determine the adhesion of bifidobacteria to starch

granules (Wang *et al.*, 1999a). In this method, the bacterial cells (*Bifidobacterium* spp. LaftiTM 8B and LaftiTM 13B) were harvested by centrifugation at 10000 rpm for 5 min. The harvested cells were washed with PBS buffer and re-suspended pellets were mixed with uncooked and precooked starch solution (heated at 90°C for 30 min). The mixture was incubated in a 37°C water bath for 30 min. The supernatant was removed and the precipitate was washed with PBS buffer to remove the loosely bound bacteria. Phase contrast microscopy was used to verify whether or not bacteria adhered to starch. Survival of strains (in the presence and absence of high amylose starch granules) when exposed to pH 2.3, 3.5 and 6.5 as well as 0.03 and 0.5 % w/v bile acids was determined. Growth in the presence of high amylose starch granules led to enhanced survival of the strains. This assay could be used as a preliminary test to examine the adhesion of bifidobacteria to starch. The percentage adhesion was not determined.

In another study, adhesion of bifidobacteria to starch granules was studied based on the principle of starch granule sedimentation (Crittenden *et al.*, 2001a). The cells of the strain being studied were washed with 0.1M phosphate buffer and re-suspended in the same buffer. The bacterial suspension was mixed with an equal volume of a suspension of starch granules and the suspension was allowed to stand at room temperature for 1 h to allow the starch to sediment. Optical density (OD) values of the tubes containing bacteria plus starch and control tubes with bacteria but no starch and starch but no bacteria were measured. These values were used to calculate percentage adhesion. A limitation of this study was that no microscopic technique was used to verify adhesion of the bacteria to starch.

Adhesion of *Vibrio cholerae* to granular starches was determined based on the principle of centrifugal sedimentation (Gancz *et al.*, 2005). In this work, an overnight culture of bacteria was harvested by centrifugation and then washed three times with Oral Rehydration Solution (ORS). Starch was added to the culture and then mixed for 2 min. The starch granules were removed by centrifugal sedimentation (320 g for 30 s) and then the supernatant was serially diluted to determine the viable count. Light microscopy was used to verify removal of starch from the supernatant. The optimum combination of centrifugation time and speed facilitating maximum starch removal with minimum bacterial precipitation is an important factor to be considered in this study. Viable counts before and after centrifugal sedimentation with these optimal parameters could be used to calculate % adhesion.

1.8.3 Resistant starch

Starch is generally digested to glucose and absorbed in the small intestine (Thompson, 2000). However, some starches are not completely digested during passage through the stomach and small intestine and are fermented by the colonic bacteria to short chain fatty acids (SCFA) that are readily absorbed (Cummings and Macfarlane, 1997a, b). Such starch has been termed “Resistant starch”. Resistant starch (RS) was defined by EURESTA (European Food-Linked Agro-Industrial Research - Concerted Action on Resistant Starch 1991) as the “sum of starch and products of starch degradation products not absorbed in the small intestine of healthy individuals”. Resistant starch was originally divided into 3 types based on their resistance to digestion: RS₁ – Physically inaccessible,

e.g., partly milled grains or seeds, RS₂ – Resistant granules, e.g., raw potato, green bananas, some legumes and high amylose maize starch, RS₃ – Retrograded starches, e.g., cooked and cooled potato, bread and corn flakes (Englyst *et al.*, 1992). An additional type of RS is obtained by chemical modification – RS₄, e.g., etherized, esterified or cross-bonded starches used in processed foods (Brown, 1996).

Resistant starch can help assure viability of probiotic organisms in the product and during transit through the intestinal tract (Wang *et al.*, 1999a) and might act as a potential prebiotic (Brown *et al.*, 1997, Crittenden *et al.*, 2001b). Use of RS has some advantages including protection of probiotic bacteria during transit through GI tract, provision of fermentable substrate for some intestinal microflora, including *Bifidobacterium*, acting as a functional prebiotic for some probiotic microbes, improving viability of probiotics in processed foods and enhancing survival/proliferation of some probiotics in the large bowel (Brown *et al.*, 1997, Brown *et al.*, 1998).

Bifidobacterium spp. may play a role in the utilization of starch granules, particularly high amylose maize starch granules (Wang *et al.*, 1999b). The amylose content in high amylose maize varieties has a direct relationship to the level of resistant starch and dietary fiber content in the material (Brown *et al.*, 1995). Wang *et al.* (1999a) reported that the addition of high amylose maize starch had a protective effect for *Bifidobacterium* strains when exposed to low pH and bile acids. As the granules pass through the GI tract to the colon relatively undigested due to their crystalline structure and granular conformation, bacteria adhering to them may reach the colon in higher numbers (Brown *et al.*, 1998, Wang *et al.*, 1999a). It was shown that *Bifidobacterium* Lafti™ 8B and *Bifidobacterium* Lafti™ 13B grown in the presence of high amylose

maize starch decreased by 2.5 log units after 6 h at pH 3.5, in contrast to the cells of the same strains grown in glucose which were not culturable following acid treatment. It was also reported that *Bifidobacterium* Lafti™ 8B cells grown in glucose declined by 2 log units at 0.05% bile acids for 6 h, while there was no loss in the cells grown in high amylose maize starch. Glucose grown cells of *Bifidobacterium* Lafti™ 13B decreased by 3 logs but high amylose maize starch grown cells did not reduce at all. However, Lafti™ 13B cells grown in glucose at 0% bile acids also showed a decline of 2.5 logs. Thus it is not clear if there is any protection offered by high amylose maize starch in the presence of bile. The authors suggest the bulking capacity of RS may modify the pH of the stomach and bile concentration level in the small intestine resulting in enhanced survival of probiotics in GI tract. The authors also attributed the increase in survival of the strains to the fact that cells adhered to the starch granules. In agreement with this reasoning, it has been observed in natural environments that attached bacteria demonstrate enhanced resistance to environmental stresses and degraded the unabsorbed substrates (Kirchman and Mitchell, 1982).

Previously it has been reported that the inclusion of RS in the diet increased the numbers of bifidobacteria in the intestinal tract in animal models (Brown *et al.*, 1997, Brown *et al.*, 1998, Wang *et al.*, 1999a, Wang *et al.*, 2002b). Addition of RS resulted in a six-fold better recovery of the strain *Bifidobacterium* Lafti™ 13B from the feces of mice after oral administration of bacteria in a medium containing amylose (Wang *et al.*, 1999a). Similarly, it has been shown that there was an increased starch concentration in the proximal colon of pigs fed a diet containing high amylose starch. This was consistent with enhanced bacterial fermentation in the large bowel (Topping *et al.*, 1997). In

concordance with these findings, when pigs were fed with high amylose starch, the fecal numbers of *B. longum* were higher (by 0.79 log CFU/g wet wt) than with a low amylose starch (Brown *et al.*, 1997). The beneficial bacteria utilize some dietary carbohydrates or prebiotics that are able to survive passage through the GI tract and grow in the bowel (Brown *et al.*, 1998).

RS could protect probiotic bacteria during processing, storage and GI transit by offering an ideal surface for probiotics to adhere and acting as a matrix to support the adhered bacteria (Brown, 2004, Crittenden *et al.*, 2001a). Further, adhesion of the bacterial strains to the resistant starch core may facilitate encapsulation of the probiotics which would ensure continued interaction between the probiotic and prebiotic (Crittenden *et al.*, 2001a, Mattila-Sandholm *et al.*, 2002).

1.9 Spherulitic starches

Spherulites or spherocrystals are ‘spherically symmetric arrays of chain-folded lamellae’ (Khoury and Passaglia, 1976). Crystallization of synthetic polymers from melts or from concentrated solutions commonly leads to the formation of radially symmetric polycrystalline aggregates called spherulites. Spherulites are birefringent, and display a ‘Maltese cross’ extinction pattern when viewed between crossed polarizers (Nordmark and Ziegler, 2002a, b). The birefringence is caused by the preferred orientation of anisotropic crystallites (Ring *et al.*, 1987).

Guenet (1996) described the general conditions that favor the formation of spherulites or gels on cooling synthetic polymer solutions. Gelatinization temperature is

the temperature below which only a gel is formed and above which only spherulitic structures will be observed. The ease of formation of spherulites was linked to the rigidity of the chain structure. Spherulite formation is inhibited by factors which favor chain stiffening, such as helix formation which hinders chain folding and instead leads to gel formation. Solutions of crystallizable synthetic polymers can form either spherulites or gels when cooled, whereas biopolymers usually produce gels (Guenet, 1996). However, such crystalline morphologies have been observed among biopolymers such as starches. It has been shown recently that native starch granules can form spherulites, when heated to $> 170^{\circ}\text{C}$ in the presence of excess moisture (> 70 wt %) and rapidly quenched (Nordmark and Ziegler, 2002a, b, Ziegler *et al.*, 2003). Above 170°C , there is an increase in chain flexibility due to helix \rightarrow coil conformational changes that might lead to the formation of spherulites in long chain linear or lightly branched starch (Ziegler *et al.*, 2003). Starch spherulites are semi-crystalline and are similar but not identical to granular starches (Nordmark and Ziegler, 2002a). Spherulitic starches are a type of resistant starches.

The final morphology of starch spherulites will be affected by factors such as maximum heating temperature, cooling rate, quench temperature, and amylose content (Creek *et al.*, 2006). Spherulite formation potential will also vary with the botanical origin of the starch used. Native starches normally show two types of crystallinity as observed by wide-angle X-ray scattering (WAXS): A-type and B-type (Ziegler *et al.*, 2005). A-type crystallinity is seen in normal cereal starches, while B-type is most often found in tuber starches. A third type of crystallinity, referred to as C-type that consists of both crystalline types is observed in legume starches. Spherulite formation was favored in

starches having a greater proportion of linear fractions (amylose) and B-type starches (Ring *et al.*, 1987, Ziegler *et al.*, 2003). It was shown earlier that linear polymers form spherulites more rapidly than their branched fractions (Creek *et al.*, 2006, Philips, 1994) and highly branched molecules inhibit spherulitic crystallization (Chowdhury *et al.*, 1998, Ziegler *et al.*, 2003).

When starches are heated above the gelatinization temperature, amylose and amylopectin disperse in water and upon cooling can spontaneously reassociate and form crystallites that resist enzymatic hydrolysis (Brown, 1996). There have been no reports on the protective effects of starch spherulites on survival of bifidobacteria. This raised the question whether bifidobacteria can adhere to starch spherulites and whether bifidobacteria adhering to spherulites show enhanced survival.

1.10 Goal and hypothesis of the work

The hypothesis for this work was adhesion of bifidobacteria to starch spherulites will improve their survival when exposed to acid or bile. The objective was to evaluate the effect of adhesion to spherulitic starch on survival of bifidobacteria in the presence of acid or bile salts. With the above in mind, the specific goals of this project were to:

- Prepare starch spherulites in large quantities and further characterize them.
- Evaluate adhesion of a collection of bifidobacterial strains to starch spherulites.
- Determine the effect of adhesion of bifidobacteria to starch spherulites on their survival in the presence of acid or bile.

Chapter 2

Production and characterization of spherulites

2.1 Introduction

Spherulites are semi crystalline entities that display a ‘Maltese Cross’ extinction pattern when viewed between crossed polarizers and are more or less radially symmetric (Creek *et al.*, 2006, Khoury and Passaglia, 1976). Synthetic polymers most commonly crystallize from melts or concentrated solutions in the form of spherulites but such spherulites are rarely found in biopolymers (Guenet, 1996). However, spherulites have been prepared from native starch granules when aqueous suspensions were heated to $>170^{\circ}\text{C}$ and then rapidly quenched (Nordmark and Ziegler, 2002a, b, Ziegler *et al.*, 2003).

Native starch granules have been considered natural spherulites because they exhibit the maltese cross pattern when viewed with crossed polarizers (Buleon *et al.*, 1998, Ring *et al.*, 1987) and it has been shown that spherulitic crystallization *in vivo* results in starch granule initiation (Chatterjee, 2007, Ziegler *et al.*, 2005). The inner structure of spherulites can be determined by examining birefringence. Starch granules are generally positively birefringent, but starch spherulites exhibited primarily negative birefringence with some displaying positive birefringence (Nordmark and Ziegler, 2002a). Factors such as maximum heating temperature, cooling rate, quench temperature, and amylose content have been shown to affect the morphology of spherulites (Creek *et*

al., 2006). In starch, it has been demonstrated that a higher proportion of linear fraction (amylose) favors spherulite formation (Nordmark and Ziegler, 2002a, b, Ziegler *et al.*, 2003). In agreement with this observation, it has been shown in synthetic polymers that spherulitic crystallization was hindered by heavy branching, whereas favored in molecules with less branching (Chowdhury *et al.*, 1998, Ring *et al.*, 1987).

Aqueous samples of native starch can be heated and cooled in DSC pans to prepare very small amounts (mg) of spherulites (Nordmark and Ziegler, 2002a, b, Ziegler *et al.*, 2003). The present study required production of spherulites in relatively large quantities (grams), as well as development of a suitable method to obtain dried spherulites that could be stored for use in microbial adhesion and survival experiments. The morphology, size and thermal properties of the spherulites prepared were also determined.

2.2 Materials and methods

2.2.1 Materials

Potato amylose (> 99% pure, A0512-25G) and potato starch were purchased from Sigma Aldrich Corp. (St. Louis, MO). Granular high amylose (*ae* 70) maize starch was obtained from National Starch and Chemical Company (Bridgewater, NJ). Ethyl alcohol (USP/ACS reagent grade) was supplied by Pharmaco (Brookfield, CT) and acetone (ACS grade) was purchased from EMD chemicals (Gibbstown, NJ). Distilled water was used to make all solutions.

2.2.2 Making spherulites

2.2.2.1 Production of washed spherulites

Conditions employed for production of spherulites were adapted from the work of Nordmark and Ziegler (2002a, b). In initial attempts to produce spherulites in a reactor (Parr instrument company, Moline, IL), a 10% (w/w) suspension of potato starch in distilled water (10 ml) was used as the base material. After mixing, the sample was placed in the reactor and heated at temperatures ranging from 180 to 205°C in a still air oven for different time periods (20 – 45 min) followed by quenching in ice-water slush for varying times (20 – 60 min). The sample was then held at room temperature for 24 h. The reactor was opened and the sample was observed for the presence of spherulites using optical microscopy as described in section **2.2.3.1**. Since spherulites were not produced in the still air oven, probably due to inadequate heating, a mechanical convection oven (Precision Scientific, Inc., Chicago, IL) was used to produce spherulites. In these experiments, samples were heated at 185°C for different times (25 - 60 min) and quenched for varying times (30 - 60 min). Based upon the results of these preliminary experiments, the following method was employed for production of spherulites for further experiments. Initially, high amylose maize starch (HVII) was used for method development as it was cheaper. However, to prevent potential confounding by artifacts from the presence of granular starch, spherulites for use in adhesion and survival experiments were produced from commercially available purified potato amylose.

A 10 % (w/w) suspension (3 ml) of sample was made with distilled water and heated in the Parr reactor. Sample was heated to 185°C in the convection oven and maintained at that temperature for 30 min followed by quenching in a 10°C water bath (Fisher Scientific, Pittsburgh, PA) for 30 min. The reactor was then held at room temperature for 24 h. The spherulites formed were transferred into a 50 ml sterile centrifuge tube under sterile conditions. The reactor was rinsed with 1 ml sterile distilled water and the contents were emptied into the centrifuge tube. Sample was then vortexed for 1 minute followed by centrifugation at 5000 rpm for 5 min (Beckman GPR centrifuge, Beckman Instruments, Inc., Fullerton, CA) to harvest the spherulites. After removal of the supernatant, the spherulites were re-suspended in 1 ml of sterile distilled water, and are referred to as 'washed' spherulites.

2.2.2.2 Drying of spherulites

Two methods were evaluated for drying spherulites. Initially, washed spherulites were dried in a dessicator containing calcium sulfate (Drierite) for 24-72 h. This method proved unsatisfactory. As an alternative, washed spherulites were subjected to vacuum filtration using a 0.45 µ filter (Millipore Corporation, Bedford, MA) followed by solvent dehydration using distilled water first, followed by 50 % ethanol, 95 % ethanol and finally by acetone. The final pellet obtained was scraped into an aluminium pan and dried in a dessicator overnight. Dried spherulites were then transferred into a sterile tube and stored in a dessicator for further use.

2.2.3 Characterization of spherulites

2.2.3.1 Optical microscopy

Spherulites prepared as described in section **2.2.2.1** from potato starch were observed under bright field and polarized light using an Olympus BX-41 microscope (Hitech Instruments, Edgemont, PA), and photographed with a SPOT insight QE camera (Hitech Instruments, Edgemont, PA) using SPOT analytical and controlling software (SPOT Diagnostic Instruments, Sterling Heights, MI).

Washed and dried potato amylose spherulites (PAS), native HVII granules and HVII spherulites (sections **2.2.2.1** and **2.2.2.2**), were viewed using bright field and polarized light microscopy on an Olympus BX-40 microscope (Hitech Instruments, Edgemont, PA) equipped with a DP71 camera and PAX-it image management and image analysis software (Midwest Information Systems, Inc., Villa Park, IL).

2.2.3.2 Particle size analysis

The particle size distribution of washed PAS, dried PAS, Hylon VII and HVII spherulites was measured using a Mastersizer particle size analyzer (Malvern Instruments Ltd., England). Spherulites were dispersed in distilled water, vortexed for 1 min and treated in a sonicator at full power (Branson Ultrasonic Cleaner, Branson Cleaning Equipment Company, Shelton, CT) for different times (0, 10, 20 and 30 min) and the particle size was determined after each treatment. A magnetic stirrer mixed the dispersion constantly during analysis in the Mastersizer cell. The particle size is reported as the

mean diameter over the volume distribution D [4, 3]. Particle size analysis was done in triplicate.

The number average diameter of spherulites and starch granules was measured by analyzing the light microscopic images using PAX-it image management and image analysis software (Midwest Information Systems, Inc., Villa Park, IL). The procedure for measuring the number average diameter was obtained from previous work (Chatterjee, 2007). The diameter of the spherulites and starch granules was measured manually by using 'line measurement' along the longest axis and 'circle measurement' along the circumference of the sample. Three microscopic images (50-60 particles per image) were analyzed for each sample and the number average diameter was the average of the lengths obtained by line measurement and diameters by circle measurement.

2.2.3.3 Differential scanning calorimetry (DSC)

Aqueous suspensions of 10% (w/w) dried PAS and HVII in distilled water were prepared in 60 μ l stainless steel DSC pans (Perkin-Elmer Instruments, Norwalk, CT). The pans were hermetically sealed and stored overnight at 20°C to facilitate moisture equilibration. Samples were heated to 180°C at a rate of 10°C/min in a DSC-7 (Perkin-Elmer Instruments, Norwalk, CT), and the melting curves were analyzed using the Pyris software (Perkin-Elmer Instruments, Norwalk, CT). The peak of the endothermic reaction was taken as dissolution temperature. Analyses were done in duplicate.

2.3 Results and discussion

2.3.1 Production of spherulites in large quantities

In previous work, spherulites were prepared in DSC pans from various starch suspensions in the presence of excess water when heated to temperatures $>170^{\circ}\text{C}$ and rapidly quenched (Creek *et al.*, 2007, Nordmark and Ziegler, 2002a, b, Ziegler *et al.*, 2003). Initial conditions for spherulite formation in a Parr reactor were attempted based on these results. It was a challenge to produce spherulites in the reactor because the level of control of heating and cooling rates was much less defined when compared to production in a DSC pan. Potato starch was used as the test material for development of working conditions for spherulite production, since it had been reported potato starch formed spherulites easily and in high number (Ziegler *et al.*, 2003).

When aqueous samples (10% w/w) of potato starch were heated in a still air oven to temperatures in the range of $180 - 205^{\circ}\text{C}$ for varying times (20 – 45 min) and rapidly quenched in ice-water slush (20 – 60 min), the resulting samples were viscous and transparent and formed gel like structures. Microscopic examination revealed that no spherulites were formed. The reason for this observation could be the samples in the reactor were not reaching the temperature required to facilitate spherulite formation. Ziegler *et al.* (2003) indicated the maximum heating temperature plays a role in spherulite development. To facilitate heating, all further experiments were conducted using a hot air convection oven to produce spherulites. In these experiments, samples were heated at 185°C for different times (25 - 60 min) and quenched for varying times (30 - 60 min). Samples from these experiments were observed to be pasty and turbid and

revealed spherulitic morphology when observed using polarized light microscopy. However, some grainy, non-spherulitic material was also visible in the bright field images (Figure 2-1). Since the conditions described in section 2.2.2.1 were successful in producing spherulites consistently, they were employed for further research. Using these conditions, potato starch formed numerous spherulites easily although they varied widely in size (Figure 2-1). The results are in agreement with the available data (Ziegler *et al.*, 2003).

2.3.2 Production of dry spherulites from Hylon VII

Native Hylon VII granules demonstrated positive birefringence under polarized light and the shape varied from circular to longitudinal [Figure 2-2 (C)]. The number average size diameter was $10.84 \pm 0.34 \mu\text{m}$. The size ranged from 5 to 20 μm which is consistent with the particle size analysis data (Figure 2-3). Particle size analysis showed a D [4, 3] of $9.7 \pm 0.41 \mu\text{m}$. Particle size range was consistent with previous literature (Blaszczak *et al.*, 2005, Buleon *et al.*, 1998).

Spherulites from Hylon VII were used to develop the drying method. Spherulites were prepared from Hylon VII and dried as described in section 2.2.2.2. Hylon VII could readily form round, well dispersed spherulites that displayed the ‘maltese cross’ extinction pattern (Figures 2-4 and 2-6). This was not surprising as high amylose maize starch granules have previously shown good spherulite forming ability (Nordmark and Ziegler, 2002a, b, Ziegler *et al.*, 2003). Spherulites from HVII exhibited negative birefringence in agreement with the work of Nordmark and Ziegler (2002a, b). However,

in contrast to this, positive birefringence was also observed in previous literature (Creek, 2007). Negative birefringence indicates that main axes of polymer chains were tangential to the radial direction of spherulites while positive birefringence implies that orientation of main chain axes were radial (Barham *et al.*, 1974, Wang *et al.*, 2002a). The particle size of HVII spherulites varied from 5 to 50 μm (Figures 2-5 and 2-7) and showed a D [4, 3] of $16.81 \pm 0.57 \mu\text{m}$.

Two methods were evaluated for preparing dry spherulites. When the spherulites were dried in a dessicator (for 24-72 h), the sample was flaky and the dessicator method required a long time to dry because of the high moisture content of the initial spherulite preparation. After grinding the spherulites with a mortar and pestle, they were observed under polarized light. As shown in Figure 2-4 (B), microscopic evaluation revealed clumps of spherulitic material sticking to each other and some crushed material was also evident. This was reflected in the particle size analysis data (Figure 2-5). Spherulites were dispersed in distilled water and sonicated for different times in an effort to ensure the distribution represented individual particle and not the clumps. Sonication resulted in a narrower size distribution of the spherulites when compared to the unsonicated samples. However, even after sonication for 30 min, a broad particle size distribution was observed suggesting the clumps were not completely distributed.

In an effort to alleviate the issue of clumping, samples were dried using vacuum filtration followed by solvent dehydration in ethanol and acetone. This technique removed most of the water from the sample and thus, dried spherulites could be prepared in less than a day. After vacuum filtration and dehydration, samples were ground with a mortar and pestle resulting in formation of fine powder. Spherulites with less clumps and

no flakes that were well dispersed and similar to washed spherulites were observed under polarized light microscopy [Figure 2-6 (A, B)]. Particle size analysis also revealed that spherulites dried in this manner were similar to the washed PAS (Figure 2-7). On sonication, the particle size range narrowed and after 10 min, further sonication had very little effect on the distribution. The size distribution of the washed spherulites was similar to the dried spherulites. Thus, vacuum filtration followed by solvent dehydration technique was used to prepare the dried spherulites used in adhesion and survival experiments.

2.3.3 Size and shape of potato amylose spherulites

Potato amylose spherulites were prepared and dried by vacuum filtration (section 2.2.2). Potato amylose produced numerous, well-developed, spherical, positively birefringent entities that displayed the maltese cross pattern (i.e., spherulites) [Figure 2-2 (A, B)]. Such positive birefringence was observed previously in A-type crystalline amylose spherulites (Helbert *et al.*, 1993). As mentioned previously, spherulite formation has been observed most often in polymers with higher content of linear fractions and highly branched polymers like amylopectin hinder spherulitic crystallization (Nordmark and Ziegler, 2002a, b, Ziegler *et al.*, 2003). The branched polymers form structures that do not exhibit the maltese cross. These are referred to as ‘non-spherulitic material’. Such non-spherulitic material was absent in the images of spherulites under bright field microscopy (data not shown). Thus, potato amylose which had excellent spherulite

forming ability and was commercially available was used to produce spherulites for adhesion and survival experiments.

Particle size analysis of spherulites produced from potato amylose using the conditions described in section 2.2.2 exhibited a wide size range (Figures 2-3 and 2-8) consistent with previous observations (Creek *et al.*, 2007). There was very little change in the particle size distribution of PAS after sonication beyond 10 min. Figure 2-3 represents the size distribution of spherulites used for adhesion and survival experiments described in Chapter 3. The size of dried spherulites varied between 5- 60 μm and had a D [4, 3] of $34.58 \pm 4.23 \mu\text{m}$. The number average diameter of washed spherulites was $11.72 \pm 0.92 \mu\text{m}$ and for dried spherulites was $9.99 \pm 1.05 \mu\text{m}$. Similar morphology was observed for both washed and dried spherulites [Figure 2-2 (A, B)].

2.3.4 Thermal properties

The thermal properties of dried potato amylose spherulites and native Hylon VII granules were determined by Differential Scanning Calorimetry (DSC). Aqueous samples of dry PAS (10%) heated from 20 to 180°C at 10°C/min exhibited a broad endotherm ranging from 90 to 135°C, with a peak dissociation temperature of $108.75 \pm 0.36^\circ\text{C}$ and an average enthalpy of $4.42 \pm 0.35 \text{ J/g}$ [Figure 2-9 (A)]. The broad thermogram could be due to the presence of crystallites of different size distribution or varying order (Creek *et al.*, 2007, Ziegler *et al.*, 2003).

Native Hylon VII granules also showed a broad endotherm from 65 to 110 °C with a peak temperature of $90.75 \pm 1.77^\circ\text{C}$ and an enthalpy of $14.3 \pm 0.77 \text{ J/g}$ [Figure 2-9

(B)]. Such broad peaks have been reported previously for Hylon VII with temperature ranging from 69-110 °C and enthalpy of 16.5 ± 1.6 J/g (Augustin *et al.*, 2008). Similarly, an enthalpy of 15.6 J/g was also reported earlier for Hylon VII (Xie and Liu, 2004). Therefore it can be said that these endotherms are consistent with available literature.

2.4 Conclusions

Spherulites could be produced successfully in large quantities on a lab scale by heating in a Parr reactor to 185°C for 30 min followed by quenching in a 10°C water bath for 30 min. Vacuum filtration followed by solvent dehydration was found to be suitable for drying spherulites. Potato amylose, Hylon VII and potato starch with higher percentage of linear fraction formed spherulites easily in accordance with previous research. Spherulites demonstrated consistent size using microscopy and particle size analysis. Using these conditions, about 100 reactions (batches) were performed to produce ~19 g of spherulites.

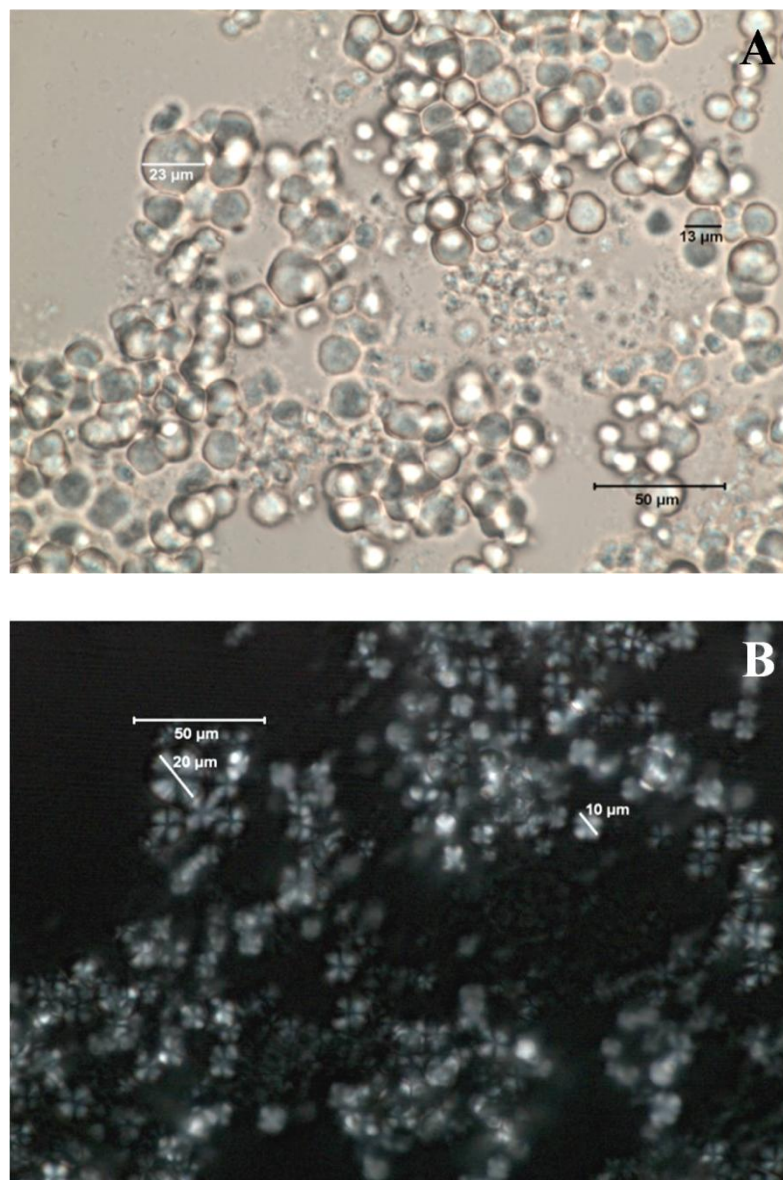


Figure 2-1: Spherulites produced from potato starch by heating at 185°C for 30 min followed by quenching in ice-water slush for 30 min: A) Bright field; B) Polarized light. Bar Scale: 50 μm.

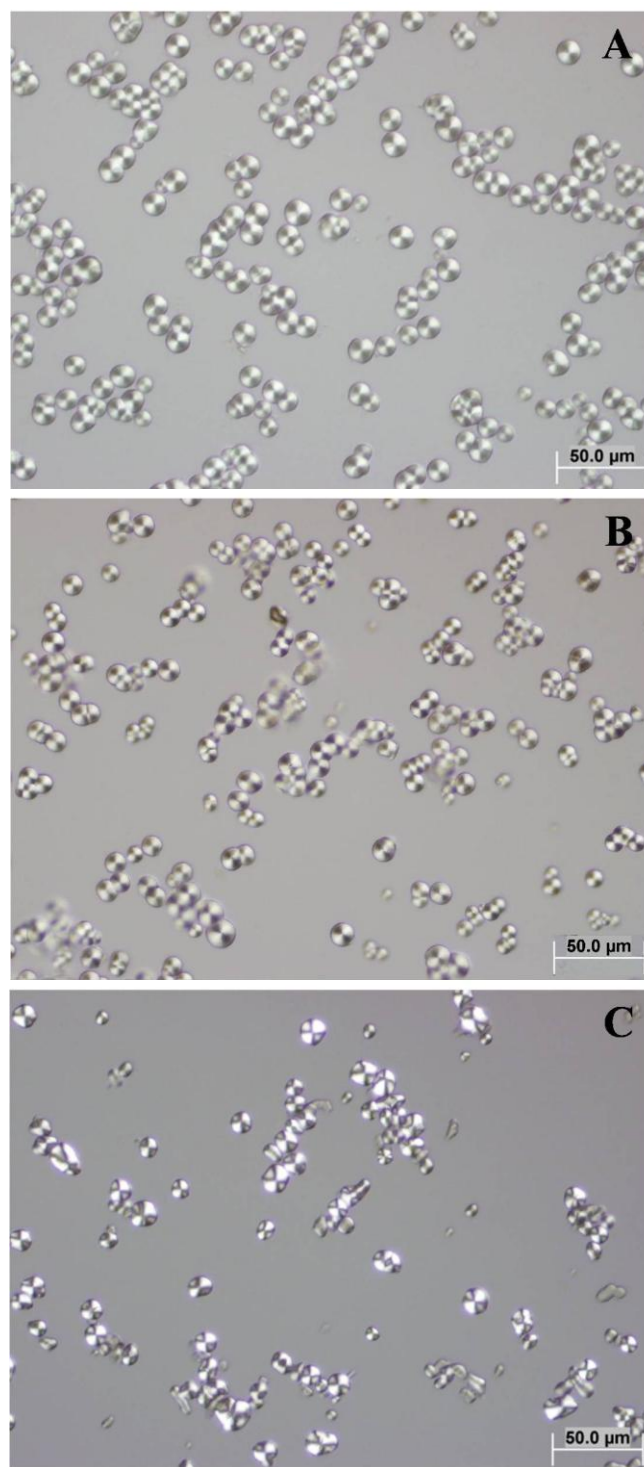


Figure 2-2: Polarized light images of: A) Washed potato amylose spherulites; B) Dried potato amylose spherulites; C) Native Hylon VII granules. Bar Scale: 50 μm.

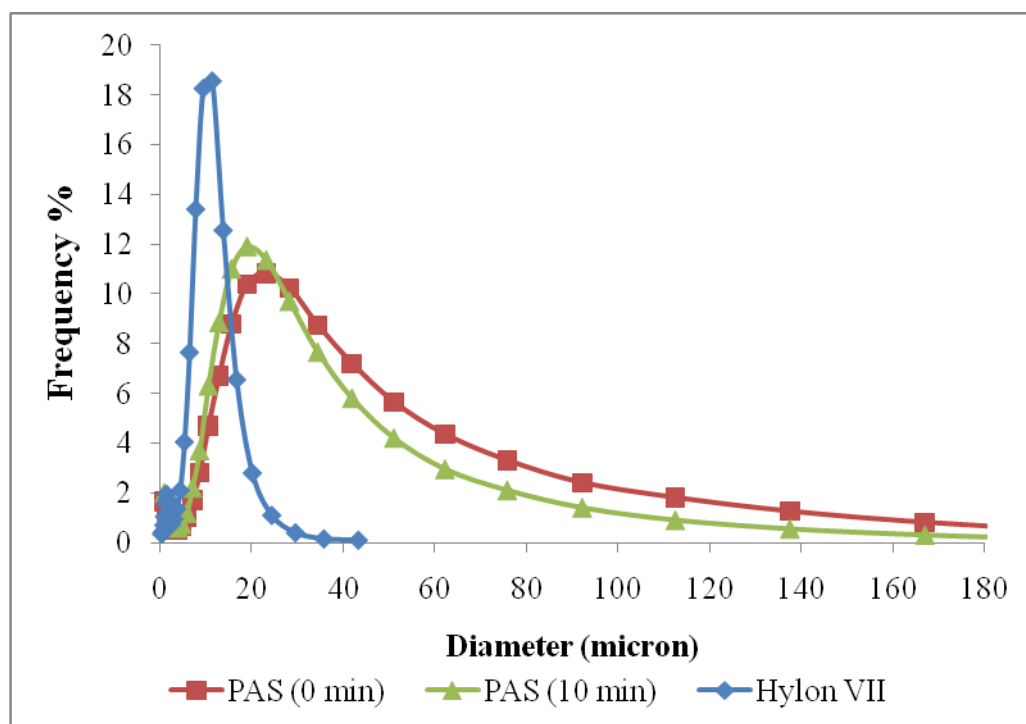


Figure 2-3: Particle size distribution of native Hylon VII starch granules and potato amylose spherulites (PAS) before and after sonication in an ultrasonic bath for 10 min. Analysis was done in triplicate.

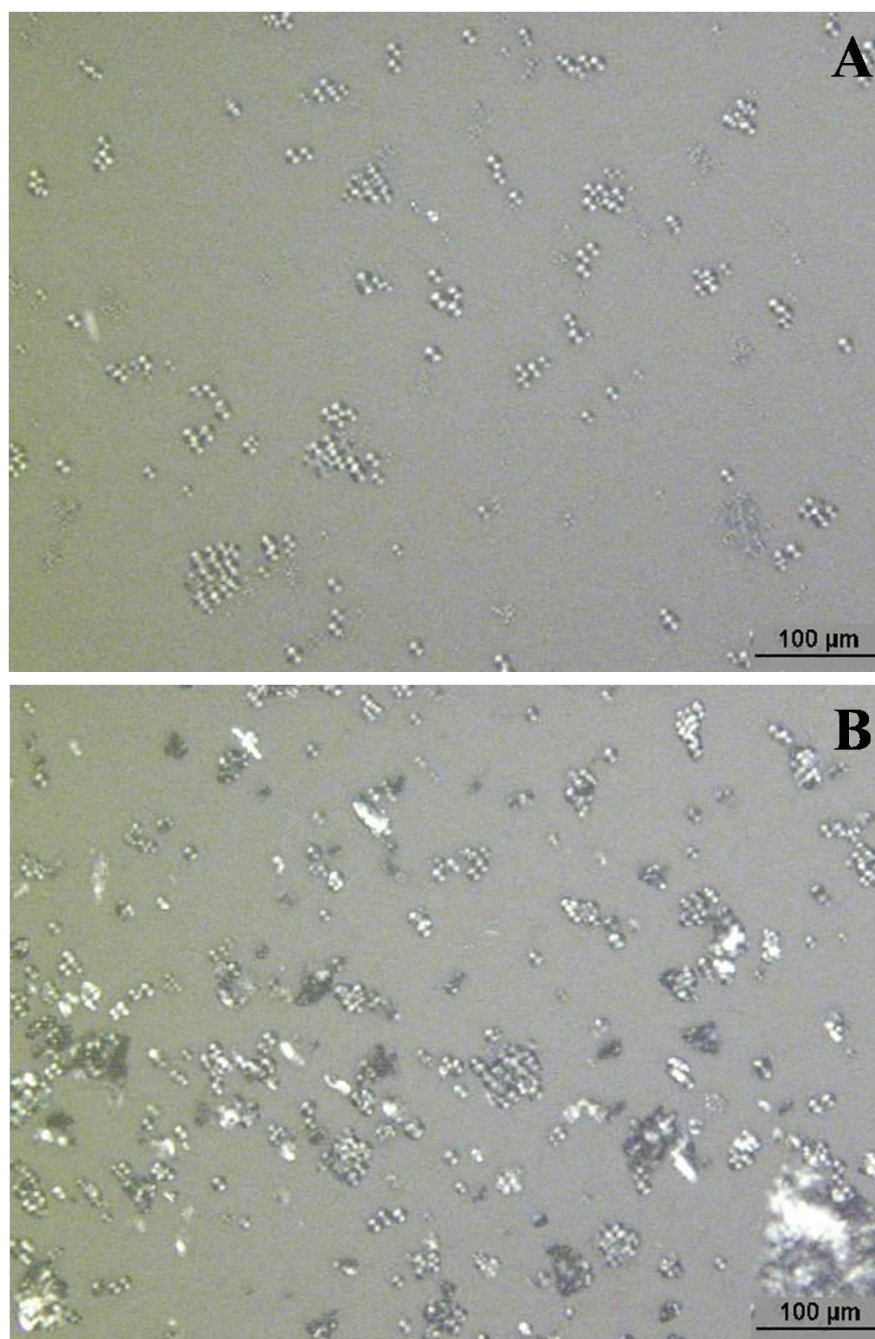


Figure 2-4: Polarized light images of spherulites produced from Hylon VII: A) Washed spherulites; B) Spherulites dried in a desiccator. Bar scale: 100 μm.

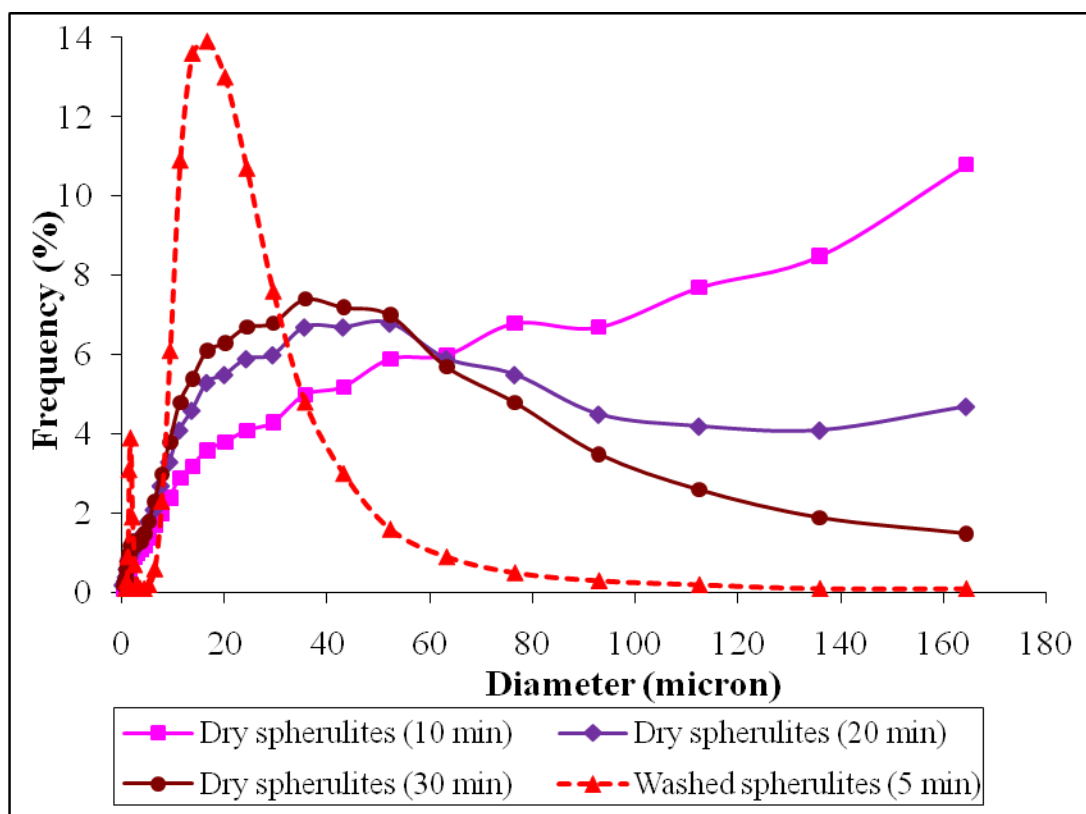


Figure 2-5: Particle size distribution of washed Hylon VII spherulites and spherulites dried in a dessicator after sonication in an ultrasonic bath for different times. This is representative data from a single experiment.

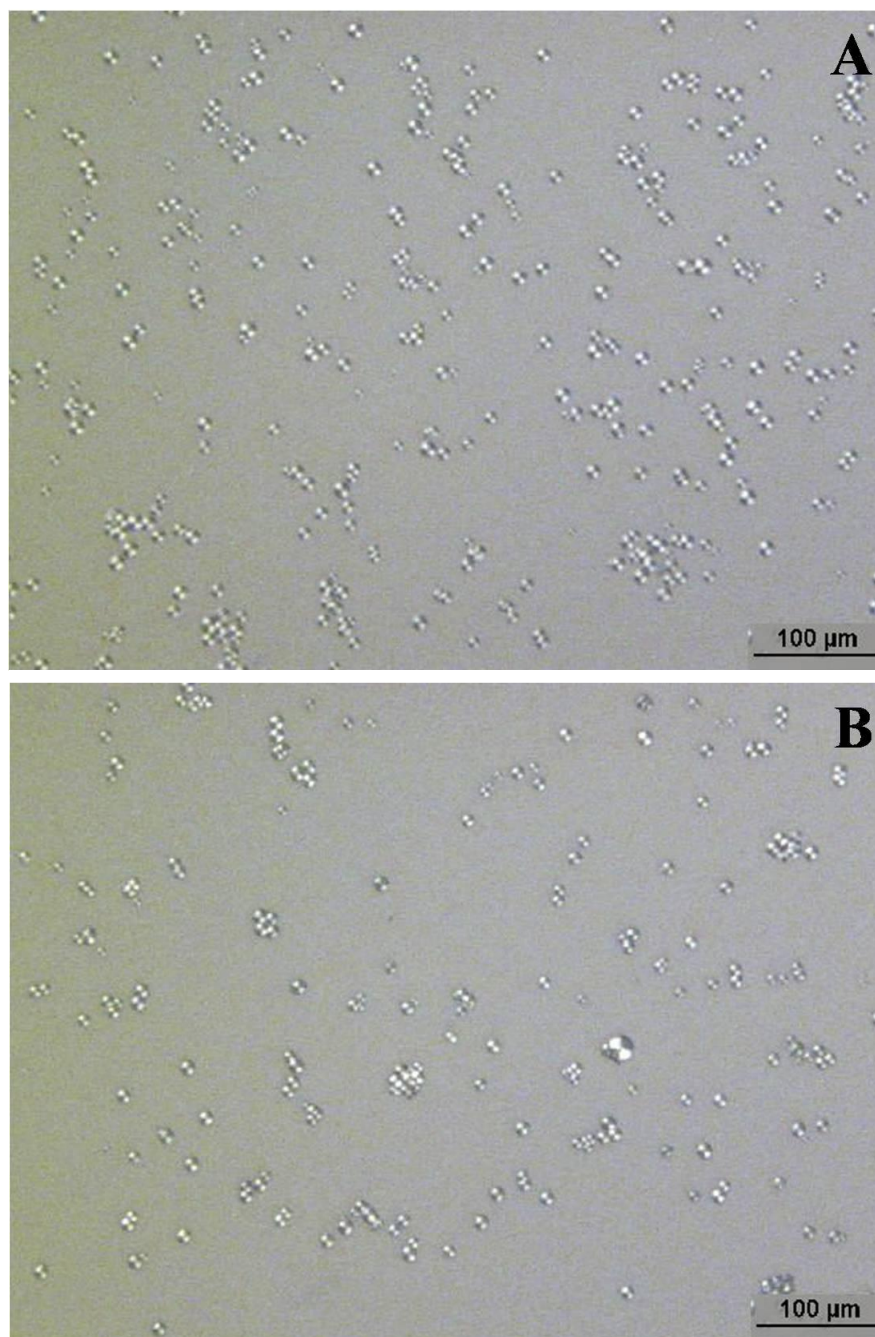


Figure 2-6: Polarized light images of spherulites produced from Hylon VII: A) Washed spherulites; B) Spherulites dried by vacuum filtration. Bar scale: 100 μm .

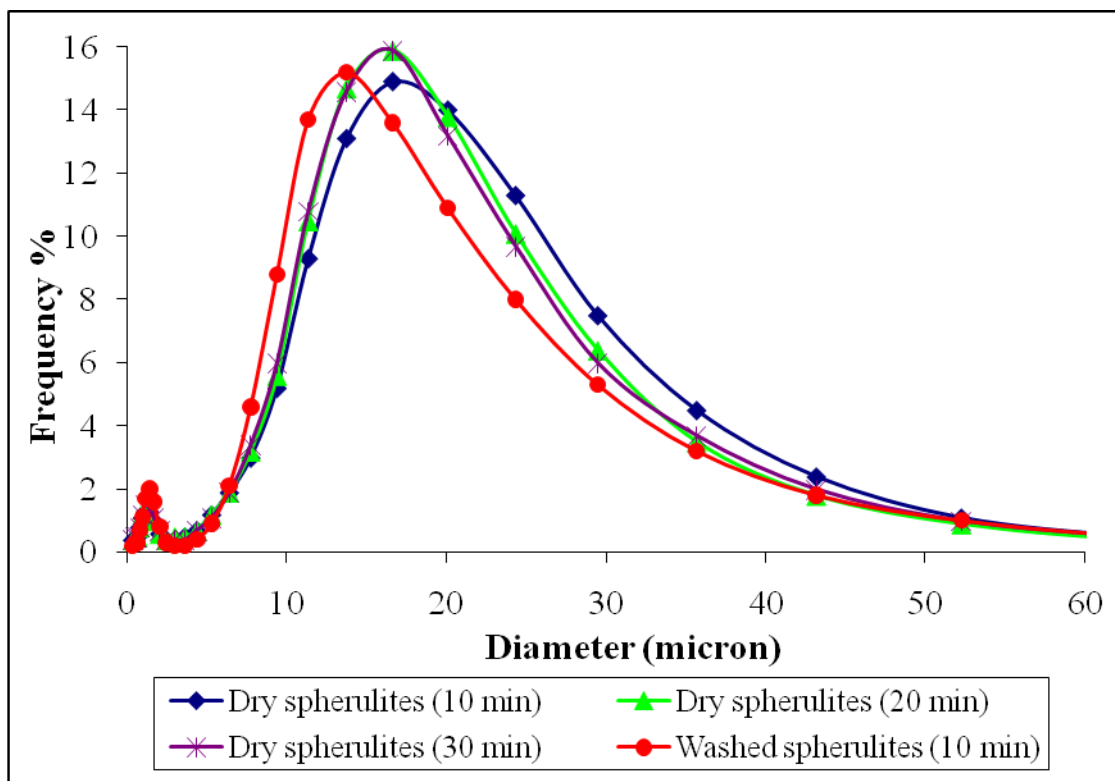


Figure 2-7: Particle size distribution of washed Hylon VII spherulites and spherulites dried by vacuum filtration and solvent dehydration after sonication in an ultrasonic bath for different times. This is representative data from a single experiment.

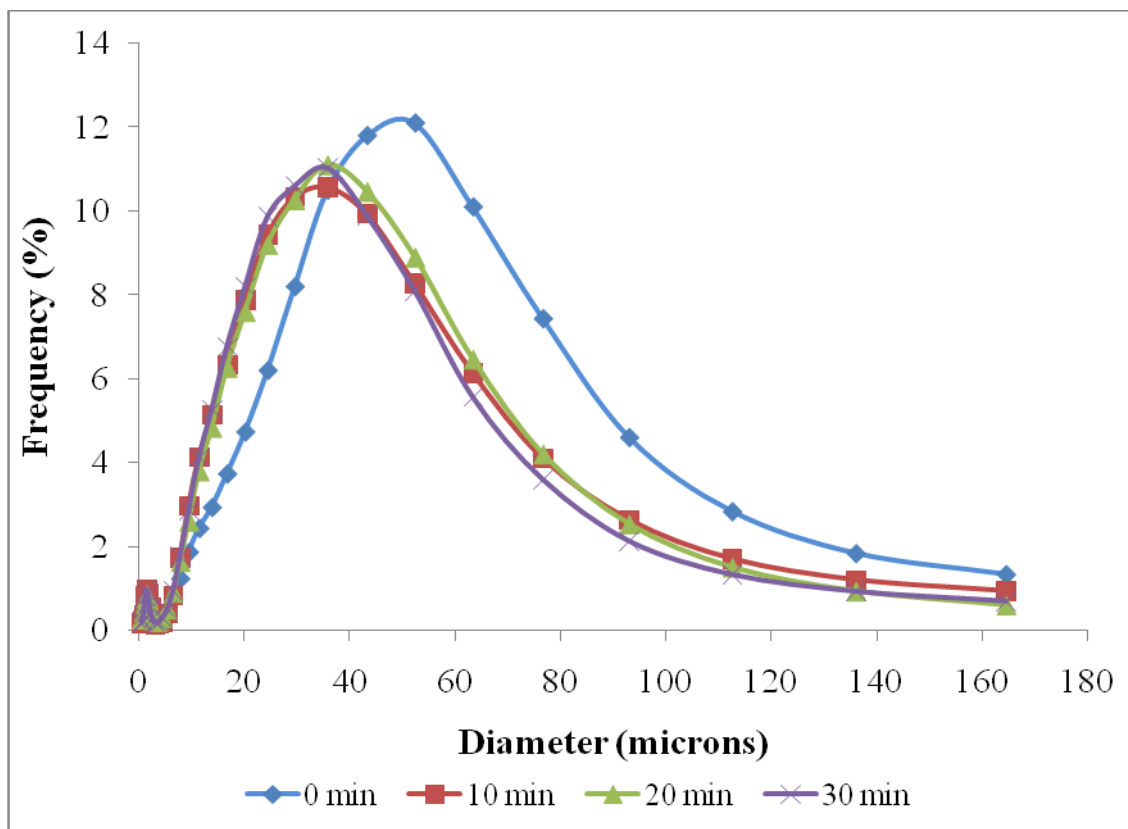


Figure 2-8: Particle size distribution of spherulites produced from potato amylose dried by vacuum filtration and solvent dehydration after sonication in an ultrasonic bath for different times. Analysis was done in triplicate.

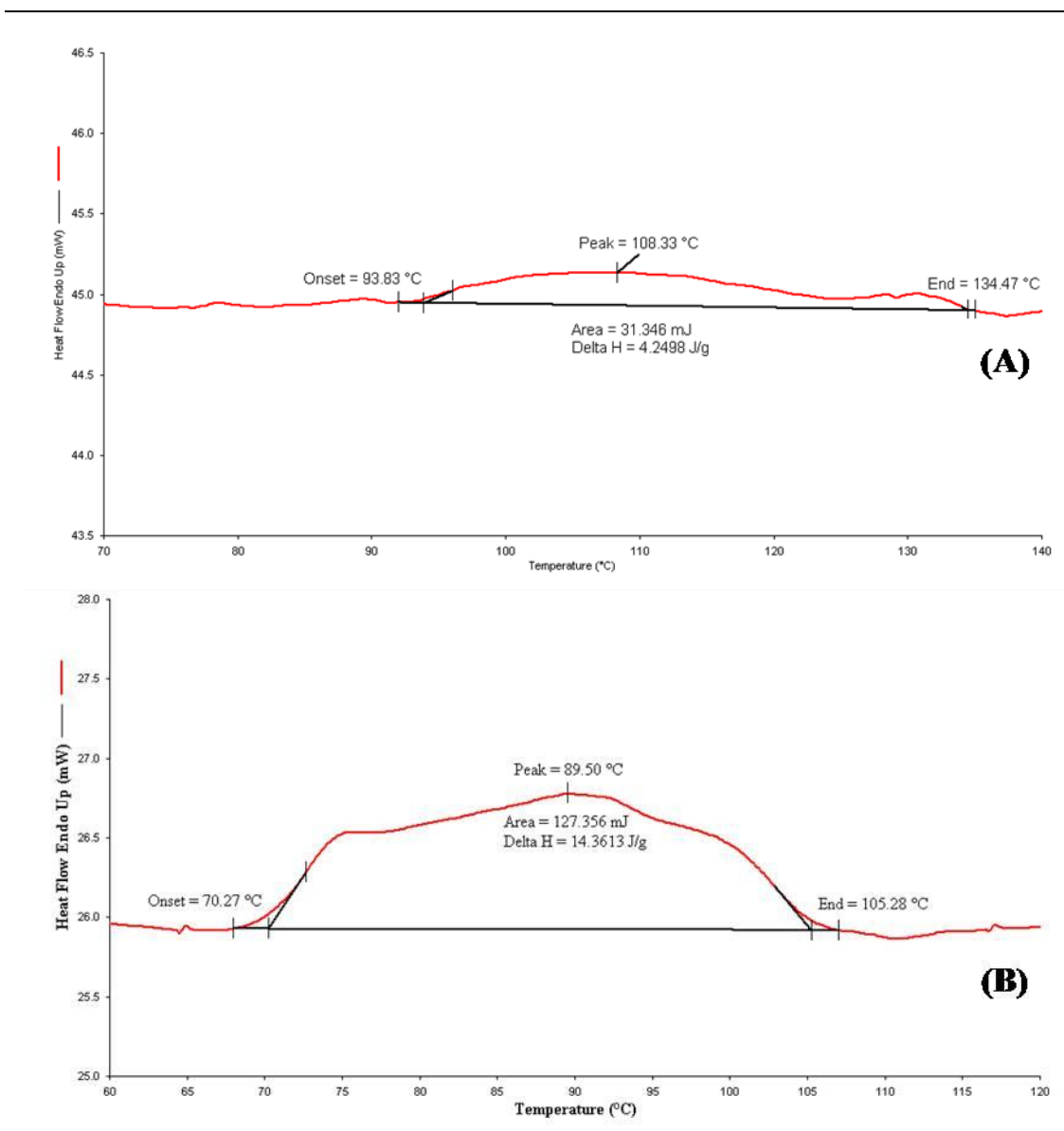


Figure 2-9: DSC endotherms of 10% (w/w) of: A) Dried potato amylose spherulites and B) Native Hylon VII granules when heated from 20°C to 180°C at 10°C/min. This is representative data from a single experiment.

Chapter 3

Effect of spherulites on survival of bifidobacteria in the presence of acid or bile

3.1 Introduction

Probiotic bacteria are live microorganisms that when ingested in adequate numbers will have beneficial effects on health and well being (FAO/WHO, 2001). Probiotic bacteria are widely delivered in fermented dairy products, which benefit the health of consumers by exerting a positive effect on the intestinal microbial balance (Fuller, 1989). Bacteria commonly used as probiotics for human consumption belong to the genera *Bifidobacterium* and *Lactobacillus* (Kailasapathy and Chin, 2000, Sanders, 1999). Bifidobacteria are one of the most promising organisms used to promote the intestinal health of humans (Champagne *et al.*, 2005). Bifidobacteria are gram-positive, non-spore forming, non-gas producing, homo-fermentative, anaerobic bacteria which constitute 5 – 10% of the total flora in the human intestine (Roy, 2001, Vasijevic and Shah, 2008). There are several putative health benefits associated with bifidobacteria, including regulating intestinal flora by preventing colonization of pathogens, activating the immune system, increasing protein digestion, amelioration of diarrhea or constipation, and production of antimicrobials and vitamins (Biavati and Mattarelli, 2006, Ishibashi and Shimamura, 1993).

Maintaining the viability and stability of probiotics in food products and during passage through the human gastrointestinal tract has been a challenge for the industry.

The ability of probiotics to survive through the upper GI tract depends on stomach acidity, the length of exposure to acid, the concentration of and length of exposure to bile salts, the level of bile salt hydrolase activity, as well as the species and strains used (Bezkorovainy, 2001). Bifidobacteria have exhibited wide variation in their resistance to low pH and bile salts (Charteris *et al.*, 1998, Clark *et al.*, 1993, Clark and Martin, 1994, Ibrahim and Bezkorovainy, 1993, Lankaputhra and Shah, 1995). Different approaches have been used to improve the survival of bifidobacteria under adverse conditions including micro-encapsulation, appropriate selection of acid and bile resistant strains, use of oxygen-impermeable containers, two-step fermentation, stress adaptation, incorporation of micro-nutrients such as peptides and amino acids and addition of prebiotics (Capela *et al.*, 2006, Donkor *et al.*, 2007, Picot and Lacroix, 2004). One strategy proposed to improve delivery of viable bifidobacteria to the intestinal tract is via adhesion of these organisms to granular starches (Crittenden *et al.*, 2001a, Wang *et al.*, 1999a).

Some starches, termed Resistant starches (RS) escape digestion in the small intestine and arrive in the colon where they are digested by intestinal bacteria (Cummings and Macfarlane, 1997a, b). It has also been shown that RS can stimulate proliferation of bifidobacteria in the intestinal tract both *in vitro* and *in vivo* (Brown *et al.*, 1997, Brown *et al.*, 1998, Wang *et al.*, 2002b). There have been reports demonstrating that bifidobacteria can adhere to such starches (Crittenden *et al.*, 2001a, O'Riordan *et al.*, 2001b, Wang *et al.*, 1999a). Wang *et al.* (1999a) observed that *Bifidobacterium* spp. showed improved survival in the mouse intestinal tract in the presence of high amylose maize starch (a type of RS). The authors proposed the improved survival in the presence

of high amylose maize starch was probably due to adhesion of the bacteria to starch. This explanation was in agreement with work done by Kirchman and Mitchell (1982), where adhering bacteria were considered more resistant to environmental stresses than unattached bacteria.

Recently, spherulites have been developed from certain kind of native starches and the crystalline materials are similar to, but not identical to granular starches (Nordmark and Ziegler, 2002a, b, Ziegler *et al.*, 2003). It has been hypothesized that spherulites have resistance properties similar to native starches. There have been no reports on the protective effects of starch spherulites on survival of bifidobacteria. Thus, the aim of this research was to determine the ability of bifidobacterial strains to adhere to starch spherulites and further to investigate survival of bifidobacteria adhering to spherulites in the presence of acid or bile salts. Since spherulite formation is favored in starches with higher amylose content (Nordmark and Ziegler, 2002a, b), potato amylose was chosen as raw material for spherulite formation.

3.2 Materials and methods

3.2.1 Bifidobacterial strains

Strains of bifidobacteria were obtained from the ATCC (American Type Culture Collection, Manassas, VA) and the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) as well as from commercial suppliers. Commercial strains were

obtained previously from six commercial starter culture suppliers and were designated as “RB” followed by a random four digit number for identification purposes and maintained as frozen stocks at -70°C (Briczinski, 2007). Prior to use in the current experiments, frozen stock cultures were revived in 10 ml fresh MRS broth (de Man - rogosa Sharpe; Difco, Becton, Dickson and Company, Sparks, MD) at 37°C for 24 h in an anaerobic chamber (Anaerobe Systems, Inc., San Jose, CA) with an atmosphere consisting of 10% carbon dioxide, 5% hydrogen and 85% nitrogen. Turbid cultures were then streaked for isolation on MRS agar and incubated at 37°C for 72 h in the anaerobic chamber. A single isolated colony from the agar plate was transferred to 10 ml MRS broth and incubated for 24 h at 37°C. Turbid broth was then mixed in equal amounts with 20% glycerol (w/v) (Fisher Scientific, Fairlawn, NJ). This suspension was transferred into sterile cryovials (Nalgene, Rochester, NY) and frozen at -70°C. The strains were activated from stock cultures by transferring 200 µl of frozen stock into 10 ml fresh MRS broth or transferring an isolated colony from a MRS agar plate streaked with stock culture to 10 ml broth and incubating at 37°C for 24 h in the anaerobic chamber.

3.2.2 Identification of *Bifidobacterium* strains by PCR

Bifidobacterial strains used in survival studies were verified as being members of *Bifidobacterium* genus and *B. animalis* ssp. *lactis* using the protocols described in the work of Briczinski (2007). The protocol for genus identification was based on that of Kaufmann *et al.* (1997) and sub-species specific identification on Ventura *et al.* (2001).

3.2.2.1 Genus identification of *Bifidobacterium* using genus-specific primers

Frozen cultures were streaked onto MRS agar plates and incubated at 37°C for 48-72 h under anaerobic conditions. A single or partial colony was transferred from a plate into the bottom of a 0.2 ml thin walled PCR tube (Corning, Inc., Corning, NY) using a sterile inoculating needle. Cell lysis was performed by microwaving the PCR tubes for 7 min (700 W; (Kullen *et al.*, 1997)). The genus-specific primers Im26 and Im3 (Integrated DNA Technologies, Coralville, IA; Table 3-1) target a 1.35 kb region of the 16S rDNA in *Bifidobacterium* (Kaufmann *et al.*, 1997). The PCR amplification mixture (50 µl) contained 1 µM of each primer, 10 µL of *Taq* DNA Polymerase 5X reaction buffer with MgCl₂, 300 µM of PCR Nucleotide Mix, 2 U of *Taq* DNA Polymerase (Promega Corporation, Madison, WI). The negative control consisted of the complete reaction mixture without template DNA. PCR was performed in an Eppendorf Mastercycler (Brinkmann Instruments, Westbury, NY). The PCR conditions consisted of denaturation at 94°C for 60 s, annealing at 57°C for 3 min and elongation at 72°C for 4 min. After 35 cycles, the samples were cooled down to 4°C.

Ampified DNA was fractionated by submerged horizontal gel electrophoresis on a 1% agarose gel (Promega Corporation, Madison, WI) using 0.5X TBE buffer (EMD Chemicals, Gibbstown, NJ) at 100 volts. A 100-bp DNA ladder (Promega) was used as a molecular weight marker. The gel was stained with ethidium bromide solution (Promega, 0.4 mg/L) and visualized on a UV transilluminator (302 nm). Images were obtained with an AlphaImager 3300 Gel Documentation System (Alpha Innotech Corp., San Leandro, CA).

3.2.2.2 Sub-species specific identification of *Bifidobacterium animalis ssp. lactis*

Cells were lysed using the microwave method described above. *Bifidobacterium animalis ssp. lactis* was confirmed using the sub-species specific primers Bflac2 and Bflac5 (Ventura *et al.*, 2001). The Bflac2 primer targets a region of the 16S rRNA gene, whereas the oligonucleotide Bflac5 targets the 16S-23S rRNA spacer region. The PCR reaction mixture (50 µl) consisted of 1 µM of each primer (Integrated DNA technologies), 10 µL of *Taq* DNA Polymerase 5X reaction buffer with MgCl₂, 300 µM of PCR Nucleotide Mix, 2.5 U of *Taq* DNA Polymerase (Promega Corporation, Madison, WI). The PCR amplification parameters were: initial denaturation at 95°C for 5 min; 30 cycles of: denaturation at 95°C for 30 s, annealing at 58°C for 60 s, extension at 72°C for 2 min; and final elongation at 72°C for 7 min. Samples were cooled down to 4°C. Electrophoresis (~ 70 V) and gel visualization were performed as described above.

3.2.3 Starch samples

Potato amylose (> 99% pure, A0512-25G) was purchased from Sigma Aldrich Corp. (St. Louis, MO). Granular high amylose (*ae* 70) maize starch (Hylon VII) was obtained from National Starch and Chemical Company (Bridgewater, NJ). Dried potato amylose spherulites (PAS) were prepared as described in sections 2.2.2.1 and 2.2.2.2. A composite sample of PAS (~19 g prepared in 100 batches) was used as the test material for adhesion and survival studies.

3.2.4 Adhesion of bifidobacteria to starches

Adhesion of bifidobacteria to intact high amylose maize starch granules (HVII) was determined using the co-sedimentation assay previously described by Crittenden *et al.* (2001a). The cells were transferred twice into fresh media before being used in the adhesion experiments. A 24 h activated culture (10 ml) (see section 3.2.1) was harvested by centrifugation (5000 rpm, 5 min) and then washed twice with 10 ml of 0.1 M phosphate buffer (pH 7). Washed cells were re-suspended in 10 ml of phosphate buffer. Washed bacterial suspension and a previously prepared suspension of starch (10 g l⁻¹ in 0.1 M phosphate buffer) were mixed in equal volumes (2 ml each) in a 1 cm diameter test tube (VWR International LLC, Westchester, PA), vortexed and allowed to stand at room temperature for 1 h to allow sedimentation of starch. Two 150 µl samples were taken from 0.5 cm below the surface of liquid, and the absorbance was measured at 540 nm (A₅₄₀) with a Microplate Autoreader (Bio-tek Instruments Inc., VT). Phosphate buffer was used as a blank for microplate readings. The percent adhesion of each strain was calculated by comparing the A₅₄₀ of tubes with adhered cells to controls containing bacteria without starch (free cells) and starch without bacteria. The percentage of cells that adhered to the starch and then co-sedimented to the bottom of the test tube was calculated as follows:

$$\% \text{ of adhering cells by absorbance method} = 100 * \left[1 - \frac{(a - b)}{c} \right] \%$$

where *a* is the A₅₄₀ of sample containing starch and bacteria (adhered cells), *b* is the A₅₄₀ of control containing starch without bacteria, and *c* is the A₅₄₀ of control containing

bacteria without starch (free cells). The sediment obtained after 1 h of sedimentation was observed under phase contrast microscope to see if bacteria adhered to the starch.

In preliminary experiments, the supernatant or the top 100 µl of the control and treatment suspensions, before and after sedimentation, were serially diluted and spread plated on MRS agar to obtain the viable counts. The plates were incubated at 37°C under anaerobic conditions for 72 h. Percent adhesion was calculated by:

$$\% \text{ of adhering cells by viable count method} = 100 * \left[1 - \frac{A}{B} \right] \%$$

where A is the CFU of treatment containing starch and bacteria (adhered cells) and B is the CFU of bacteria control without starch (free cells). Since in preliminary experiments, absorbance and viable count methods yielded similar results, the absorbance method was used for further screening of *Bifidobacterium* strains for their adhesion to Hylon VII and potato amylose spherulites. Among the tested strains, two adherent strains (*B. pseudolongum* ATCC 25526, *B. choerinum* ATCC 27686) and two non-adherent strains (*B. animalis* ssp. *lactis* DSMZ 10140, *B. animalis* ssp. *lactis* Bb-12) were employed in further investigations.

3.2.5 Optical microscopy

The adhesion experiment was performed as described in the above section for the two adhering and two non-adhering strains chosen for use in survival studies. After 1 h of sedimentation, the buffers containing free cells, cells adhered to PAS and HVII were vortexed to form uniform suspensions. For each treatment, a drop of the suspension was

mounted on a clean microscope slide and covered with a cover slip. The samples were viewed using polarized light and phase contrast microscopy on an Olympus BX-40 microscope (Hitech Instruments, Edgemont, PA) equipped with a DP71 camera and PAX-it image management and image analysis software (Midwest Information Systems, Inc., Villa Park, IL).

3.2.6 Survival studies

The strains were activated from stocks stored at -70°C in 20% glycerol and inoculated into fresh MRS broth and incubated for 24 h at 37°C as described previously (section 3.2.1). After activation, the cells were transferred twice into fresh media before being used in the survival experiments. Two adhering and two non-adhering strains obtained from the adhesion experiments were chosen for use in the survival studies.

3.2.6.1 Survival in the presence of acid or bile

The acid sensitivity of free and adhered cells was studied using a method developed from the work of Lankaputhra and Shah (1995) and Wang *et al.* (1999a). Briefly, a suspension containing cells and starch obtained after 1 h sedimentation from the adhesion experiments was vortexed to form a uniform mixture. One ml of this suspension was added to 9 ml of glycine - HCl buffer adjusted to pH 1.8, 2.9 or to sterile 0.1% (w/v) peptone water (pH 7.2) (Bacto, Becton, Dickson and Company, Sparks, MD) and incubated at 37°C for 3 h. Glycine - HCl buffer was prepared as follows: 25 ml of a

0.2 mol l⁻¹ solution of glycine (MP Biomedicals, Inc., Solon, OH) added to x ml of 0.2 mol l⁻¹ HCl (ACS grade, Fisher Scientific, Fairlawn, NJ) and made up to 100 ml with distilled water (x= 30.55 for pH 1.8 and x= 6.1 for pH 2.9) (Vernazza *et al.*, 2006).

Aliquots of 100 µl of the acid treated cells or controls were taken after 0, 60, 120 and 180 min and spread onto MRS agar plates with 0.5% g/L L-cysteine-HCl (Sigma-aldrich, Inc., St. Louis, MO). The plates were incubated under anaerobic conditions at 37°C for 72 h.

Similarly, survival in the presence of bile salts was tested based on a method developed from the work of Wang *et al.* (1999a) and Hansen *et al.* (2002). Aliquots (1 ml) of the mixture containing adhered cells were added to 9 ml of PBS, pH 7.4 with different concentrations (0, 0.5 and 1%) of oxgall (Difco, Becton, Dickson and Company, Sparks, MD). The samples were incubated at 37°C for 3 h. Aliquots of 100 µl were taken at 0, 90 and 180 min and the viable counts were determined. The plates were incubated under anaerobic conditions at 37°C for 72 h prior to enumeration.

3.2.7 Statistical analysis

Statistical analysis of data was carried out using the statistical software package SPSS version 17.0 (SPSS Inc., Chicago, IL). Adhesion and survival in acid experiments were performed in triplicate and survival in bile experiments were repeated twice. Data are presented as mean ± standard error of means. Significant differences in the adhesion values obtained by viable count and absorbance method was tested by one way analysis of variance (ANOVA). The differences in the mean adhesion values of different

Bifidobacterium strains to Hylon VII and potato amylose spherulites was statistically analyzed using Nested two way ANOVA. Statistical analysis was performed by grouping the strains by species with the factor 'strain' nested within 'species'. Further, differences in the mean adhesion values of *B. animalis* ssp. *lactis* strains to HVII and PAS were analyzed using two factor ANOVA. ANOVA using General Linear Model (GLM) for a Univariate design was used to test for significant differences between the mean values of log reduction. Multiple comparisons of means for all the tests were done by Tukey's post hoc test. Differences were considered significant at $p \leq 0.05$. Summaries of the statistical outputs appear in **Appendix A**.

The effect of species and starch on adhesion values was determined statistically by: Intercept + Species + Starch + Strain(Species) + (Species*Starch).

The interaction of *B. animalis* ssp. *lactis* strains and starch on adhesion values was analyzed by:

Intercept + Strain + Starch + (Strain *Starch).

The model for testing effect of strain, starch and pH on log reduction values in the presence of acid was:

Intercept + Strain + Starch + pH + (Strain*Starch) + (Starch*pH) + (pH*Strain) + (Strain*Starch*pH).

Similarly, the interactions between strain, starch and oxgall and their effect on log reduction values in the presence of bile were tested by:

Intercept + Strain + Starch + Oxgall + (Strain*Starch) + (Starch* Oxgall) + (Oxgall *Strain) + (Strain*Starch* Oxgall).

3.3 Results and Discussion

3.3.1 Genus-specific PCR

Genus-specific PCR using primers lm26 and lm3 was performed for the four strains (*B. pseudolongum* ATCC 25526, *B. animalis* ssp. *lactis* DSMZ 10140, *B. choerinum* ATCC 27686 and *B. animalis* ssp. *lactis* Bb-12) used in the survival studies to confirm that the organisms belong to the genus *Bifidobacterium*. Results of the PCR analysis are shown in Figure 3-1. A 1.35 kb amplicon was observed in all the four strains confirming that the strains belong to the genus *Bifidobacterium*.

3.3.2 Sub-species specific PCR

PCR analysis was performed for all strains of *Bifidobacterium* used in survival studies to identify *Bifidobacterium animalis* ssp. *lactis* using sub-species specific primers Bflac2 and Bflac5. The results of the PCR reactions appear in Figure 3-2. *B. animalis* ssp. *lactis* DSMZ 10140 and *B. animalis* ssp. *lactis* Bb-12 were confirmed as members of the sub-species based on the presence of 680 bp amplicon. The 680 bp amplicon was absent in *B. pseudolongum* ATCC 25526 and *B. choerinum* ATCC 27686 indicating that they do not classify as *B. animalis* ssp. *lactis*. The primary purpose of this analysis was to confirm the non-*B. animalis* ssp. *lactis* strains were not contaminated (as often is the case with commercial preparations (Briczinski, 2007)).

3.3.3 Adhesion of bifidobacteria to starch

In initial experiments, two methods were evaluated for determining adhesion of 4 bifidobacterial strains to Hylon VII. Table 3-2 shows the comparison of adhesion values obtained by absorbance assay and viable count method. Results indicated adhesion values obtained by both the methods were not significantly different for a particular strain and therefore the less expensive absorbance method was chosen for use in further studies.

The ability of 38 *Bifidobacterium* strains to adhere to potato amylose spherulites and granular Hylon VII was studied using the absorbance assay (Table 3-3). Some of the strains had negative adhesion values and these were considered non-adherent (showing 0% or no adhesion) for discussion purpose. However, statistical analysis was performed using the actual numerical values obtained during the experiments. Bacterial strains differed in their ability to adhere to starch with adhesion values ranging from 0-80%. For statistical analysis, the strains were grouped by species and the main effects of 'species' and 'starch' and the nested main effect of strain(species) along with interactions on the adhesion values was evaluated. Statistical analysis revealed that 'species', 'starch' and the nested strain(species) factor had a significant effect on the adhesion values observed ($p = 0.000$, 0.001 and 0.000 respectively) and also revealed a significant interaction between the terms species and starch ($p = 0.000$). *Bifidobacterium pseudolongum* was shown to be highly adherent (~80%) followed by *B. choerinum* with moderate adhesion (~50%). The two adherent species were significantly different from each other and all the other organisms examined. *Bifidobacterium animalis* ssp. *lactis* displayed low adhesion with the mean adhesion values differing significantly from all other species except for *B.*

adolescentis ($p = 0.089$) and *B. bifidum* ($p = 0.108$). All the other species (*B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. infantis* and *B. longum*) were non-adherent and did not differ significantly in their adhesion from each other. Adhesion of all the species was significantly higher to PAS than HVII except for *B. infantis*. However, since *B. infantis* demonstrated negative adhesion values to both HVII and PAS, this does not have any practical relevance (non-adherent to HVII and PAS).

The effect of factor strain nested within a particular species was significant. Since most of the strains used for the screening belonged to *B. animalis* ssp. *lactis*, the effect of strain type and starch on the adhesion percentages within the species *B. animalis* ssp. *lactis* was determined using a two factor ANOVA. Strain and starch and their interaction had a significant effect on adhesion within the species ($p = 0.000$ for all the terms). There was a significant difference in the ability of *B. animalis* ssp. *lactis* strains to adhere to HVII and PAS with values ranging from 0 to 40%. All the strains adhered to a significantly higher degree to PAS than HVII.

Not all bifidobacterial strains adhered to starch which is consistent with the literature (Crittenden *et al.*, 2001a). Among the strains tested, two adherent (*B. pseudolongum* ATCC 25526 and *B. choerinum* ATCC 27686) and two non-adherent strains (*B. animalis* ssp. *lactis* DSMZ 10140 and *B. animalis* ssp. *lactis* Bb-12) were chosen for further experiments. *B. pseudolongum* ATCC 25526 exhibited strong adhesion to both PAS and HVII (~80%) and *B. animalis* ssp. *lactis* DSMZ 10140 was poorly adherent. The findings for these strains with HVII are in agreement with the available literature (Crittenden *et al.*, 2001a). *B. choerinum* ATCC 27686 (~50%) was moderately adherent to both the starches. *B. animalis* ssp. *lactis* Bb-12 adhered poorly to both PAS

and HVII. However, this strain (Bb-12) has been reported to adhere to a higher level with potato starch ($64.1 \pm 10.4\%$) (Lahtinen *et al.*, 2007). The type and size of starch granule and the method used to prepare bacterial cells might lead to the differences observed. For the majority of the strains evaluated, adhesion to PAS was higher than to HVII.

Crittenden *et al.* (2001a) suggested the specific binding capacity of the starch (number of bacteria per gram) was correlated to granule surface area. In contrast to this, our results indicate that adhesion of bifidobacteria to PAS, which had a lower specific surface area (surface area of a unit volume of particles; $0.95 \pm 0.1 \text{ m}^2/\text{cc}$), was higher than HVII granules ($\text{SSA} = 1.153 \pm 0.11 \text{ m}^2/\text{cc}$). It could be that the surface properties of potato amylose spherulites allow higher adhesion.

There have been previous reports on the adhesion of strains of *Bifidobacterium* to granular starch (Crittenden *et al.*, 2001a, O'Riordan *et al.*, 2001b, Wang *et al.*, 1999a). The mechanism involved in adhesion of bifidobacteria to starch was examined earlier (Crittenden *et al.*, 2001a) and it was observed that specific cell surface proteins that bind to α -1, 4-linked glucose saccharides were involved in adhesion. Similarly in another study, it was suggested attachment of two human-derived *Bifidobacterium* strains, PL1 and PL2, to amylo maize starch granules was mediated by a cell wall-associated proteinaceous factor and the binding occurred only when the cells were grown in media containing starch or maltose, but not glucose (O'Riordan *et al.*, 2001b). Consistent with these findings, it has been suggested adhesion of *Lactobacillus amylovorus* to corn starch granules may be mediated by cell-surface proteins or glycoproteins (Imam and Harryokuru, 1991). Binding of starch to the cell surface facilitated through specific outer membrane proteins was also observed in *Bacteroides thetaiotaomicron* (Anderson and

Salysers, 1989a, b, Reeves *et al.*, 1997, Tancula *et al.*, 1992). In this organism, the amylolytic enzymes were not extracellular but cell-associated and starch binding was necessary for starch utilization. However, this was not the case in bifidobacteria as no relation was observed between starch utilization and starch adhesion for the *Bifidobacterium* strains examined (Crittenden *et al.*, 2001a). In their study, all the amylolytic strains were not adherent but all the highly adherent strains displayed amylolytic activity. In agreement with this, the highly adherent strain *B. pseudolongum* ATCC 25526 was reported to hydrolyze starch granules in previous literature (Wang *et al.*, 1999b). It has been proposed that adhesion of bifidobacteria to starch may depend on the amount of cell bound starch-degrading enzymes in the strains (Crittenden *et al.*, 2001a, Wang *et al.*, 1999b). However, further research is needed to understand the nature of the proteins and the mechanism involved in starch adhesion by bifidobacteria.

3.3.4 Microscopy

Cells adhered to PAS and HVII for two adhering and two non-adhering strains (*B. pseudolongum* ATCC 25526, *B. choerinum* ATCC 27686, *B. animalis* ssp. *lactis* DSMZ 10140 and *B. animalis* ssp. *lactis* Bb-12) were examined under polarized light and phase contrast microscopy (see section 3.2.5) to confirm the adhesion of bifidobacteria to the starch (Figures 3-3, 3-4, 3-5 and 3-6). The controls containing free cells without starch and starch without bacteria exhibited no aggregates under the microscope.

Bifidobacterium pseudolongum ATCC 25526 and *B. choerinum* ATCC 27686 adhered to PAS and HVII in accordance with the adhesion data (Figures 3-3 and 3-4). It

can be seen in the images that the number of free cells of *B. pseudolongum* ATCC 25526 in Figure 3-3 (A) is higher compared to the number in Figure 3-3 (B, C, D, E). In the presence of starch, cells of the highly adherent strain *B. pseudolongum* ATCC 25526 (~80% adhesion; Table 3-3) adhered to the surface of starch and formed clumps. Most of the cells appeared to adhere to starch particles resulting in lower numbers of free bacteria. Percent adhesion was calculated by measuring the percentage of cells that adhered to the starch and co-sedimented to the bottom of test tube. This means that for the strains with higher adhesion values, a lower number of free cells are present in the supernatant as majority of the cells adhere to the starch and settle down. Figure 3-3 is supportive of the above explanation.

Similar behavior was observed for *B. choerinum* ATCC 27686 which showed moderate adhesion (~50%; Table 3-3). *B. choerinum* ATCC 27686 also formed clumps with bacteria adhering to the starch particles, but more free bacteria were observed in the mixture than when compared to *B. pseudolongum* ATCC 25526 (Figure 3-4). Some of the bacteria adhered to the starch while some appeared free in the buffer consistent with the adhesion values.

For the two non-adherent strains, there were no cells adhering to Hylon VII and limited cells attaching to spherulites (Figures 3-5 and 3-6). A large number of free cells were observed for both the strains in the presence of HVII and PAS. The proportions of free cells are consistent with their respective adhesion percentages (Table 3-3).

3.3.5 Survival studies

Figures 3-7, 3-8, 3-9 and 3-10 represent survival of the two adhering strains (*B. pseudolongum* ATCC 25526 and *B. choerinum* ATCC 27686) and two non-adherent strains (*B. animalis* ssp. *lactis* DSMZ 10140 and *B. animalis* ssp. *lactis* Bb-12) in the presence of acid (pH 7.2, 2.9 and 1.8) for different time periods (0, 1, 2 and 3 h) at 37°C. Free and adhered cells of all the strains could survive well in pH 7.2 and 2.9 but could not tolerate pH 1.8. Free and adhered cells of *B. pseudolongum* ATCC 25526 (Figure 3-7) were reduced by about 4-5 logs after 1 h in pH 1.8 and could not be detected after 2 h. *B. choerinum* ATCC 27686 was slightly more sensitive than *B. pseudolongum* ATCC 25526 (Figure 3-9). For this strain, no viable cells could be enumerated after 1 h in pH 1.8 buffer. The two *B. animalis* ssp. *lactis* strains reduced by only 1 or 2 logs in the presence of pH 1.8 after 1 h (Figures 3-8 and 3-10). However, both the strains were undetectable after 2 h of incubation. Thus, we can say that all the strains were sensitive to pH 1.8 although the rate at which they were killed was different.

The log reduction values of all the strains when exposed to different pH conditions for 3 h at 37°C is shown in Table 3-4. Statistical analysis was performed to determine the effect of strain, starch and pH and their interactions on the log reduction values of bifidobacteria using GLM Univariate analysis followed by Tukey's pairwise comparison test. Both strain and pH and the interaction term (strain*pH) had a significant effect ($p = 0.001$, 0.000 , 0.000 respectively) on the response. Starch had no significant effect ($p = 0.744$) on the log reduction values implying no protective effect of adherence to HVII or PAS on survival of bifidobacterial strains analyzed. Exposure to various levels

of acid (pH 7.2, 2.9 and 1.8) resulted in significantly different levels of cell death (see Table 3-4). Exposure of the cells to pH 1.8 (the most extreme condition evaluated) significantly reduced the population of bifidobacterial strains when compared to the other two pH conditions. The two non-adherent *B. animalis* ssp. *lactis* strains showed similar survival in the different pH conditions. *B. animalis* ssp. *lactis* DSMZ 10140 was not significantly different from any other strain. The two adherent strains (*B. pseudolongum* ATCC 25526 and *B. choerinum* ATCC 27686) significantly differed from *B. animalis* ssp. *lactis* Bb-12 in survival. The adherent strains displayed slightly lower log reduction than the non adherent strains. While these numbers are statistically significant, they are probably not practically significant.

Similarly, the survival of the four strains in the presence of oxgall was studied (Figures 3-11, 3-12, 3-13 and 3-14). Initially, cells of *B. pseudolongum* ATCC 25526 and *B. animalis* ssp. *lactis* DSMZ 10140 were exposed to 0, 0.5 and 1% oxgall in PBS for 3 h at 37°C. Both strains showed good survival (< 1.5 log reduction) at all concentrations of oxgall. Since 1% oxgall (~equivalent to 10% bile) was very high when compared to normal concentrations found in the GI tract, the survival of other two strains (*B. choerinum* ATCC 27686 and *B. animalis* ssp. *lactis* Bb-12) was determined only in presence of 0 and 0.5% oxgall. These strains could also tolerate the bile conditions studied. Thus, all the strains examined exhibited resistance to bile under the conditions of the assay.

The effect of strain, oxgall, starch and their interactions on the survival was also analyzed statistically. Strain and oxgall showed a significant effect ($p = 0.00$ and 0.01 respectively) on the survival and there were no other significant interactions observed.

The presence of starch did not significantly affect ($p = 0.93$) the log reduction values. Exposure to 0.5% oxgall resulted in relatively higher reduction of population when compared to control (no oxgall) (mean difference = 0.34 logs). Statistically, all the strains demonstrated similar survival in the presence of oxgall, the only exception being *B. choerinum* ATCC 27686. This strain differed significantly in log reduction from the other strains except *B. pseudolongum* ATCC 25526 which had near significance ($p = 0.058$).

In this study, *B. pseudolongum* ATCC 25526 could survive well at pH 2.9 and 7.2 and in the presence of oxgall (0, 0.5 and 1%) but was sensitive to exposure to pH 1.8. Tolerance of this strain to acid and bile has been reported previously. It was shown that *B. pseudolongum* ATCC 25526 can grow in MRS broth with pH adjusted to as low as 4 and in the presence of 0.3% (w/v) bovine bile salts when incubated for 18 h at 37°C (Gagnon *et al.*, 2004). In agreement with these results, the strain was also reported to grow in TPY broth adjusted to a pH of 4.5 and up to bile concentration of 0.4% (w/v) after 24 h incubation (Wilkie, 2006). From these studies, it can be said that growth of *B. pseudolongum* ATCC 25526 is sensitive to lower pH (< 4) and higher bile concentration.

B. animalis ssp. *lactis* strains are generally more resistant to low pH and bile than the other bifidobacterial strains. In this work, both strains of *B. animalis* ssp. *lactis* were shown to survive exposure to pH 7.2 and 2.9. Cell death occurred after 2 h incubation in pH 1.8 buffer indicating their sensitivity to very low pH. Consistent with these findings, it was shown that free cells of Bb-12 were extremely sensitive to pH 1.5 and decreased from 10^9 CFU/g to < 100 CFU/g (detection limit) after 15 min exposure (Liserre *et al.*, 2007). Similarly, it was reported that Bb-12 grown in a fermenter survived well in PBS buffer adjusted to pH 3 but poorly in pH 2.5 (~4 log reduction) after 2 h exposure at 37°C

(Saarela *et al.*, 2005). However, Hansen *et al.* (2002) reported survival of Bb-12 in simulated gastric juice with pH adjusted to 2, 3 and 6 for 2 h at 37°C. At pH 2, Bb-12 showed only 1.3 log reduction compared to the control (pH 6). Vernazza *et al.* (2006) also demonstrated tolerance of Bb-12 to pH 1.8, 2.9 and 3.7. In this study, the acid tolerance was studied after exposure to only 20 min and cannot be compared directly to our work. In another study, Bb-12 showed only 1 log reduction at pH 2 after 2 h exposure 37°C and was completely killed at pH 1 after 1 h (Favaro-Trindade and Grosso, 2000). The authors indicate the therapeutic benefits of the strain might be limited if the pH of the stomach drops close to 1.

Our work also indicates good survival of *B. animalis* ssp. *lactis* strains in the presence of oxgall (0, 0.5 and 1%) in PBS (3 h exposure). These results are in agreement with Hansen *et al.* (2002) where Bb-12 showed resistance to oxgall in milk yeast medium (0, 0.5 and 1%) for 24 h at 37°C. Similarly, the strain was extremely tolerant to 2 and 4% bile solutions after exposure for 12 h at 37°C (Favaro-Trindade and Grosso, 2000). Bile tolerance of Bb-12 was also demonstrated by Vernazza *et al.* (2006) where growth was observed in WC broth containing 1% oxgall.

The acid and bile resistance of *B. animalis* ssp. *lactis* DSMZ 10140 has also been previously reported. McMaster *et al.* (2005a) demonstrated populations of *B. animalis* ssp. *lactis* DSMZ 10140 decreased by about 1.5 logs approximately after 2 h exposure to gastric solution at pH 1.5 and by 3 logs after 4 h. In contrast to this, our study showed that DSMZ 10140 was killed after 2 h exposure to pH 1.8. In another study, it was shown that DSMZ 10140 survived well (< 1 log reduction) in pancreatic and bile solution (0.75% and 0.25% (w/v) respectively) for 4 h (McMaster *et al.*, 2005a). Similarly, Matto

et al. (2004) reported the acid (pH 3 for 1 h) and bile (1.5% bile extract for 3 h) tolerance of *B. animalis* ssp. *lactis* DSMZ 10140. These results are consistent with our work.

It is difficult to directly compare studies evaluating survival in the presence of acid or bile since different concentrations, exposure periods, acid and bile sources, and experimental designs are commonly employed. However, these studies indicate the sensitivity of the strains when exposed to extreme acid or bile conditions. It should be noted that studying survival in buffers of defined pH values or bile acids may lead to underestimation of the probiotics potential to survive as these conditions are generally harsher than those encountered in the GI tract.

Adhesion to HVII and PAS demonstrated no protection to bifidobacteria when exposed to acid or bile. There have been no reports on protection offered by PAS on bifidobacteria. However, survival of bifidobacteria when grown in media containing HVII or glucose and when exposed to low pH and bile acids was reported earlier (Wang *et al.*, 1999a). In this study, both the strains *Bifidobacterium* Lafti™ 8B and *Bifidobacterium* Lafti™ 13B grown in media containing HVII or glucose died after 10 min exposure to pH 2.3. The effect of HVII was seen at pH 3.5 where the counts decreased by 2.5 log units after 6 h, in comparison to the cells grown in glucose which were not culturable. These results are in accordance with our findings where free and adhered cells could not be detected at pH 1.8 after 2 h but survived well at higher pH. However, the protective of HVII at higher pH was not observed in our work as all the strains reported excellent survival. It is possible that HVII may show some protection at higher pH but none at lower pH. Wang *et al.* (1999a) also state that HVII had a protective effect when the strains were exposed to bile. *Bifidobacterium* Lafti™ 8B cells grown in

glucose declined by 2 log units at 0.05% bile acids for 6 h, while there was no loss in the cells grown in HVII. Glucose grown cells of Lafti™ 13B decreased by 3 logs whereas HVII grown cells showed no reduction. However, the cells of Lafti™ 13B grown in glucose with 0% bile also showed a reduction of 2.5 logs. Thus, it is not clear if the reduction observed for glucose grown cells was due to the presence of bile and it could be that HVII is not providing any protection in the presence of bile. In that case, it would be in agreement with our results, where no effect of starch on survival in presence of oxgall was observed. In the study by Wang *et al.* (1999a), adhesion was considered as a possible mechanism for improved survival in presence of acid and bile. However, work by Crittenden *et al.* (2001a), showed that adhesion of bifidobacteria to starch was inhibited in environment containing acid and protease but was unaffected by the presence of bile. This may explain the inability of starch to show any protection at lower pH in the present study. It may be that pH decreases adhesion to starch or spherulites. It may also be that cells gradually detach from the surface of starch or spherulites and are then exposed to the adverse conditions resulting in reduced survival.

Thus, it is important to study the mechanism involved in the adhesion of bifidobacteria to starch. Knowledge of factors influencing adhesion may help in successfully delivering viable cells to the GI tract. It would be interesting to examine the ability of bifidobacteria to utilize spherulites and understand the relation between adhesion and spherulite utilization. Bifidobacteria adhering to spherulites may offer a selective advantage in the host intestine by utilizing the substrate. Further, adhesion of the bacterial strains to the spherulites may facilitate encapsulation of the probiotics using

this starch which ensures interaction between probiotics and spherulites and protects the bacteria during passage through the GI tract.

3.4 Conclusions

The bifidobacterial strains evaluated adhered to varying degrees to potato amylose spherulites and high amylose maize starch. Adhesion of bifidobacteria was significantly higher to PAS than native granules of Hylon VII. There was no protective effect of adhesion to PAS or HVII on survival of adhering or non-adhering *Bifidobacterium* strains tested when exposed to acid or bile. Survival of bifidobacteria varied with the type of the strain, pH and concentration of bile used. All the strains tested were sensitive to pH 1.8 and survived well at higher pH (2.9 and 7.2) and in bile solutions (0, 0.5 and 1% oxgall).

Table 3-1: PCR primers used in this work

Primer	Sequence (5' → 3')	Product size	Reference
<i>Bifidobacterium</i> genus			
lm26 (F ²)	GATTCTGGCTCAGGATGAACG	1.35 kb	(Kaufmann <i>et al.</i> , 1997)
lm3 (R)	CGGGTGCTI ¹ CCCACCTTTCATG		
<i>Bifidobacterium animalis ssp. lactis</i>			
Bflac2 (F)	GTGGAGACACGGTTTCCC	680 bp	(Ventura <i>et al.</i> , 2001)
Bflac5 (R)	CACACCACACAATCCAATAC		

¹Inosine, matches A, T, G, and C.

²F - Forward, R - Reverse.

Table 3-2: Comparison of adhesion of bifidobacteria to high amylose maize starch granules measured using absorbance and viable count methods.

Values are the means of duplicate samples \pm standard error of means. Adhesion values within the same strain with the same superscript are not significantly different at $p \leq 0.05$.

Strain	Adhesion%	
	Absorbance ¹	Viable Count ²
<i>B. pseudolongum</i> ATCC 25526	84.77 \pm 9.21 ^a	89.78 \pm 11.64 ^a
<i>B. animalis</i> ssp. <i>lactis</i> DSMZ 10140	3.84 \pm 2.66 ^a	2.80 \pm 1.69 ^a
<i>B. animalis</i> ssp. <i>lactis</i> Bb-12	-5.56 \pm 6.92 ^a	0.13 \pm 4.90 ^a
<i>B. longum</i> ATCC 15708	1.38 \pm 0.93 ^a	1.33 \pm 3.82 ^a

$$^1 \text{ \% adhesion} = 100 * \left[1 - \frac{(a-b)}{c} \right] \%$$

where a is the absorbance (540 nm, A_{540}) of sample containing starch and bacteria (adhered cells), b is the A_{540} of control containing starch without bacteria, and c is the A_{540} of control containing bacteria without starch (free cells).

$$^2 \text{ \% adhesion} = 100 * \left[1 - \frac{A}{B} \right] \%$$

where A is the CFU of treatment containing starch and bacteria (adhered cells) and B is the CFU of bacteria control without starch (free cells).

Table 3-3: Adhesion of bifidobacteria to high amylose maize starch granules (HVII) and potato amylose spherulites (PAS). Values are the means of triplicate samples \pm standard error of means. Negative values are considered as '0' or non-adherent.

Strain	Adhesion%	
	HVII	PAS
<i>B. pseudolongum</i> ATCC 25526	77.17 \pm 6.90	80.51 \pm 4.27
<i>B. animalis</i> ssp. <i>lactis</i> DSMZ 10140	0.38 \pm 2.30	30.24 \pm 9.39
<i>B. choerinum</i> ATCC 27686	49.55 \pm 13.05	50.64 \pm 5.96
<i>B. animalis</i> ssp. <i>lactis</i> RB 1573	-3.88 \pm 5.26	41.78 \pm 7.14
<i>B. animalis</i> ssp. <i>lactis</i> RB 9321	1.71 \pm 1.67	25.56 \pm 9.87
<i>B. animalis</i> ssp. <i>lactis</i> RB 5859	-0.39 \pm 5.24	25.01 \pm 10.67
<i>B. animalis</i> ssp. <i>lactis</i> RB 1791	0.07 \pm 3.34	23.17 \pm 4.34
<i>B. animalis</i> ssp. <i>lactis</i> LAFTI B94	5.78 \pm 4.77	21.48 \pm 8.72
<i>B. animalis</i> ssp. <i>lactis</i> Bb-12	-3.32 \pm 4.90	21.35 \pm 7.43
<i>B. animalis</i> ssp. <i>lactis</i> RB 1280	-2.41 \pm 0.71	20.87 \pm 10.75
<i>B. animalis</i> ssp. <i>lactis</i> RB 4753	-4.74 \pm 3.91	19.85 \pm 8.41
<i>B. animalis</i> ssp. <i>lactis</i> RB 9632	0.50 \pm 2.67	19.73 \pm 6.19
<i>B. animalis</i> ssp. <i>lactis</i> RB 5251	1.02 \pm 3.84	19.71 \pm 1.15
<i>B. animalis</i> ssp. <i>lactis</i> RB 4052	-0.28 \pm 5.70	19.46 \pm 9.81
<i>B. animalis</i> ssp. <i>lactis</i> RB 4536	4.14 \pm 7.83	18.99 \pm 6.44
<i>B. animalis</i> ssp. <i>lactis</i> RB 7339	2.14 \pm 0.46	18.36 \pm 7.49
<i>B. animalis</i> ssp. <i>lactis</i> RB 4825	-0.94 \pm 3.20	14.83 \pm 7.59
<i>B. animalis</i> ssp. <i>lactis</i> RB 5851	-4.08 \pm 5.17	14.62 \pm 2.59
<i>B. animalis</i> ssp. <i>lactis</i> RB 1281	0.41 \pm 2.06	12.86 \pm 5.34
<i>B. bifidum</i> ATCC 29521	2.11 \pm 0.77	12.80 \pm 5.66
<i>B. animalis</i> ssp. <i>lactis</i> RB 0171	-1.84 \pm 5.88	12.58 \pm 7.20
<i>B. animalis</i> ssp. <i>lactis</i> RB 3046	0.45 \pm 1.60	12.51 \pm 3.97
<i>B. animalis</i> ssp. <i>lactis</i> ATCC 27536	-2.82 \pm 2.45	11.21 \pm 5.05
<i>B. animalis</i> ssp. <i>lactis</i> RB 8613	-2.70 \pm 3.53	10.66 \pm 7.86
<i>B. longum</i> RB 3982	-2.38 \pm 3.98	8.46 \pm 4.72
<i>B. animalis</i> ssp. <i>lactis</i> RB 5422	-0.26 \pm 4.71	8.21 \pm 5.48
<i>B. animalis</i> ATCC 27672	-8.79 \pm 4.15	4.52 \pm 2.65
<i>B. breve</i> RB 5333	0.57 \pm 2.07	3.70 \pm 3.89
<i>B. animalis</i> ssp. <i>lactis</i> RB 5733	-1.78 \pm 1.89	3.62 \pm 1.43
<i>B. breve</i> ATCC 15698	-2.66 \pm 5.40	3.43 \pm 4.36
<i>B. bifidum</i> ATCC 15696	-5.97 \pm 2.55	3.04 \pm 3.81
<i>B. animalis</i> ATCC 25527	2.98 \pm 3.24	2.69 \pm 4.59
<i>B. adolescentis</i> ATCC 15703	-0.36 \pm 5.57	1.62 \pm 2.52
<i>B. animalis</i> ssp. <i>lactis</i> RB 7239	-2.81 \pm 5.91	1.43 \pm 4.60
<i>B. longum</i> ATCC 15707	-2.27 \pm 6.22	-1.19 \pm 4.01
<i>B. breve</i> ATCC 15700	-2.27 \pm 3.59	-4.87 \pm 6.02
<i>B. infantis</i> ATCC 15697	-0.03 \pm 8.11	-4.92 \pm 6.26
<i>B. longum</i> ATCC 15708	-1.55 \pm 2.36	-6.01 \pm 4.24

Table 3-4: Survival of bifidobacteria after exposure for 3 h in the presence of acid (control (7.2), pH 1.8 and pH 2.9) at 37°C. Values are means of triplicate samples \pm standard error of the means.

Strain	Starch	Log reduction		
		Control	pH 1.8	pH 2.9
<i>B. pseudolongum</i> ATCC 25526	Free cells	0.03 \pm 0.12	8.02 \pm 0.18	0.17 \pm 0.10
	Cells adhered to HVII	0.10 \pm 0.02	8.12 \pm 0.25	-0.01 \pm 0.14
	Cells adhered to PAS	0.06 \pm 0.25	8.01 \pm 0.22	0.12 \pm 0.16
<i>B. choerinum</i> ATCC 27686	Free cells	-0.37 \pm 0.68	7.87 \pm 0.36	0.67 \pm 0.16
	Cells adhered to HVII	-0.34 \pm 0.82	7.61 \pm 0.58	0.44 \pm 0.22
	Cells adhered to PAS	-0.21 \pm 0.34	7.61 \pm 0.59	0.43 \pm 0.22
<i>B. animalis</i> ssp. <i>lactis</i> DSMZ 10140	Free cells	0.12 \pm 0.09	8.34 \pm 0.32	0.08 \pm 0.26
	Cells adhered to HVII	0.02 \pm 0.08	8.38 \pm 0.30	-0.06 \pm 0.21
	Cells adhered to PAS	0.00 \pm 0.10	8.42 \pm 0.24	-0.01 \pm 0.11
<i>B. animalis</i> ssp. <i>lactis</i> Bb-12	Free cells	-0.02 \pm 0.11	8.72 \pm 0.17	0.13 \pm 0.12
	Cells adhered to HVII	0.05 \pm 0.02	8.70 \pm 0.19	0.12 \pm 0.15
	Cells adhered to PAS	0.09 \pm 0.08	8.73 \pm 0.08	0.08 \pm 0.08

Table 3-5: Survival of bifidobacteria after exposure for 3 h in the presence of oxgall in PBS (0 and 0.5%) at 37°C. Values are means of duplicate samples \pm standard error of the means.

Strain	Starch	Log reduction	
		0%	0.5%
<i>B. pseudolongum</i> ATCC 25526	Free cells	0.54 \pm 0.06	0.66 \pm 0.05
	Cells adhered to HVII	-0.05 \pm 0.04	1.04 \pm 0.62
	Cells adhered to PAS	0.45 \pm 0.02	1.21 \pm 0.54
<i>B. choerinum</i> ATCC 27686	Free cells	0.09 \pm 0.20	0.22 \pm 0.10
	Cells adhered to HVII	0.38 \pm 0.55	0.15 \pm 0.05
	Cells adhered to PAS	0.18 \pm 1.00	0.04 \pm 0.15
<i>B. animalis</i> ssp. <i>lactis</i> DSMZ 10140	Free cells	1.13 \pm 0.40	0.97 \pm 0.67
	Cells adhered to HVII	0.64 \pm 0.27	1.40 \pm 0.83
	Cells adhered to PAS	1.01 \pm 0.16	0.99 \pm 0.59
<i>B. animalis</i> ssp. <i>lactis</i> Bb-12	Free cells	0.88 \pm 0.34	1.29 \pm 0.20
	Cells adhered to HVII	0.60 \pm 0.13	1.46 \pm 0.29
	Cells adhered to PAS	0.84 \pm 0.11	1.37 \pm 0.55

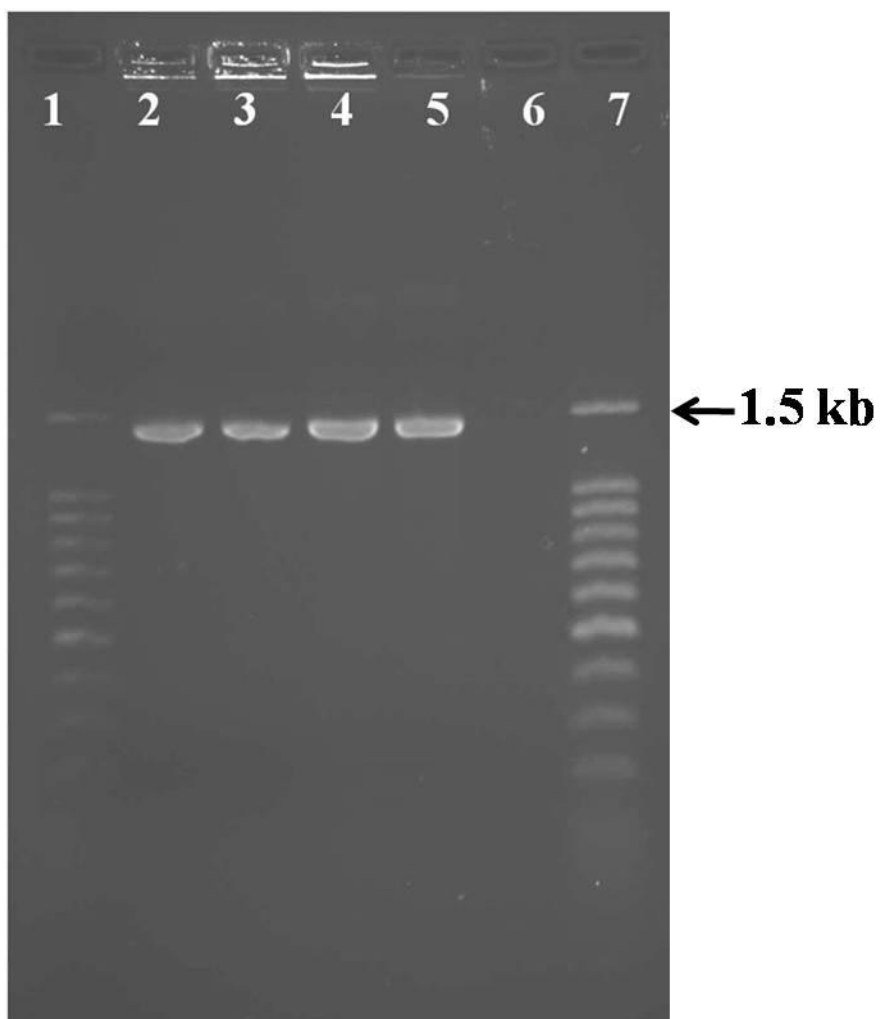


Figure 3-1: PCR analysis of 16S rDNA of *Bifidobacterium* strains chosen for survival assays with genus-specific primers lm26 and lm3.

Presence of 1.35 kb fragment indicates the genus *Bifidobacterium*. Lanes 1 and 7: 100-bp ladder, molecular weight marker, lane 2: *B. pseudolongum* ATCC 25526, lane 3: *B. choerinum* ATCC 27686, lane 4: *B. animalis* ssp. *lactis* DSMZ 10140, lane 5: *B. animalis* ssp. *lactis* Bb-12, lane 6: negative control.

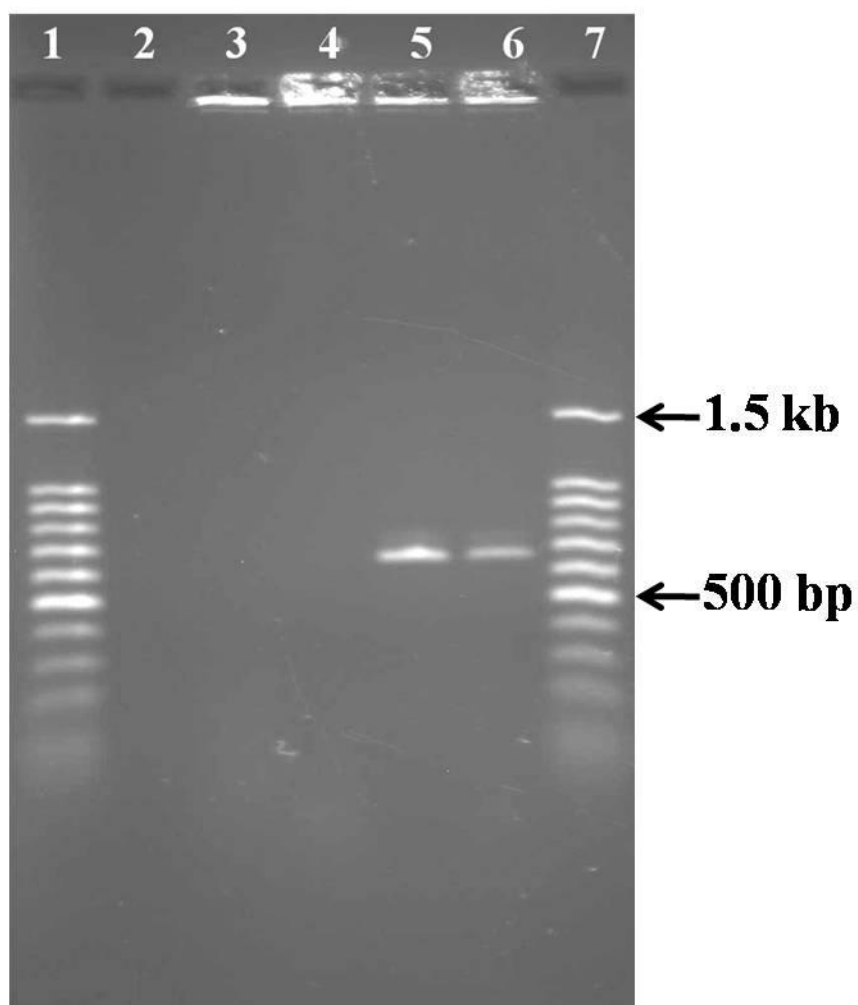


Figure 3-2: PCR analysis of *Bifidobacterium* strains used in survival experiments with *B. animalis* ssp. *lactis* sub species-specific primers Bflac2 and Bflac5.

An amplicon of 680 bp indicates the presence of *B. animalis* ssp. *lactis*. Lanes 1 and 7: 100-bp ladder, molecular weight marker, lane 2: negative control, lane 3: *B. pseudolongum* ATCC 25526, lane 4: *B. choerinum* ATCC 27686, lane 5: *B. animalis* ssp. *lactis* DSMZ 10140, lane 6: *B. animalis* ssp. *lactis* Bb-12.

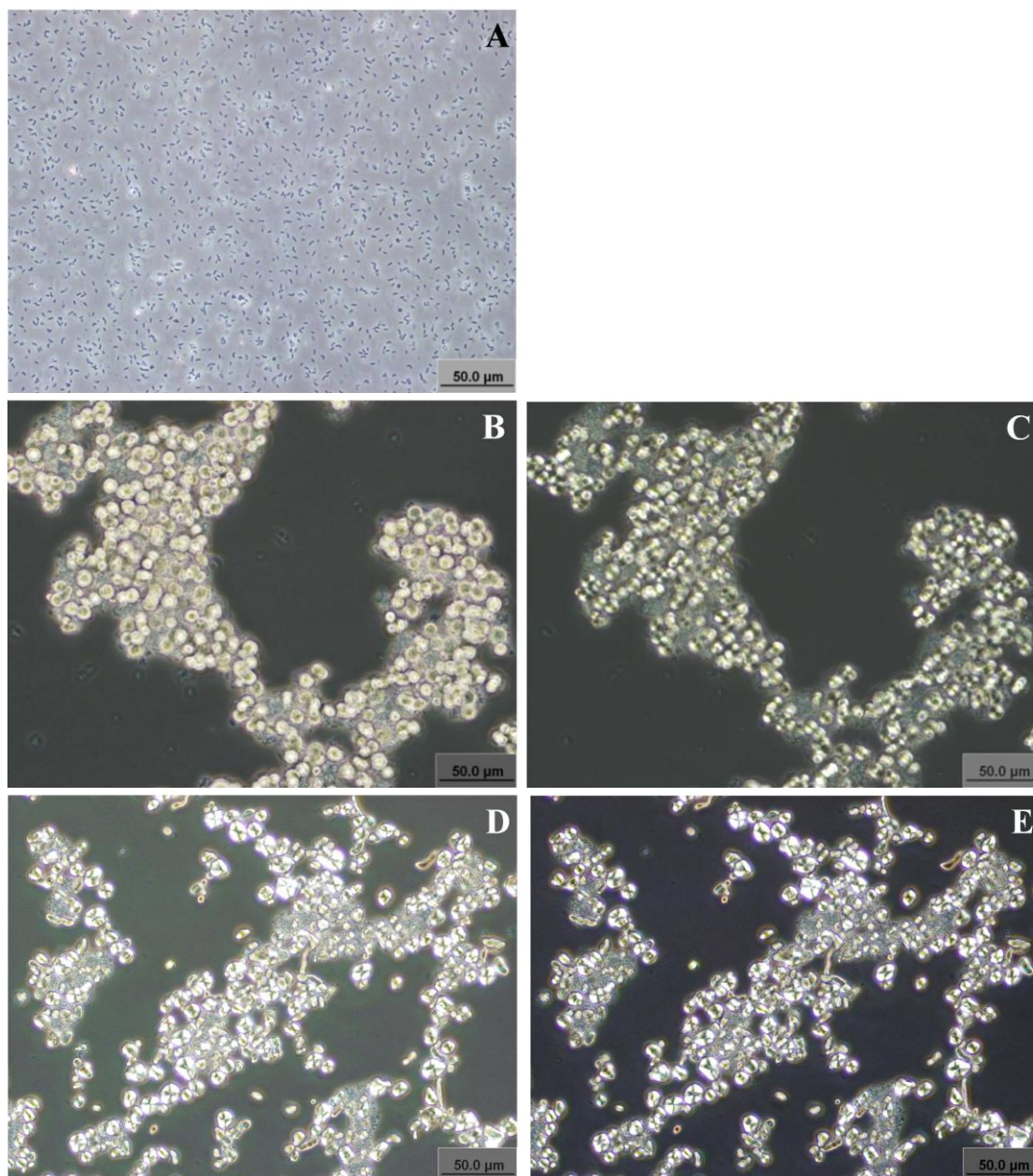


Figure 3-3: Micrographs of *B. pseudolongum* ATCC 25526: A) in buffer; (B, C) in the presence of potato amylose spherulites; (D, E) in the presence of native Hylon VII granules. (A, B, D) – phase contrast; (C, E) – polarized light and phase contrast. Bar scale: 50 μm.

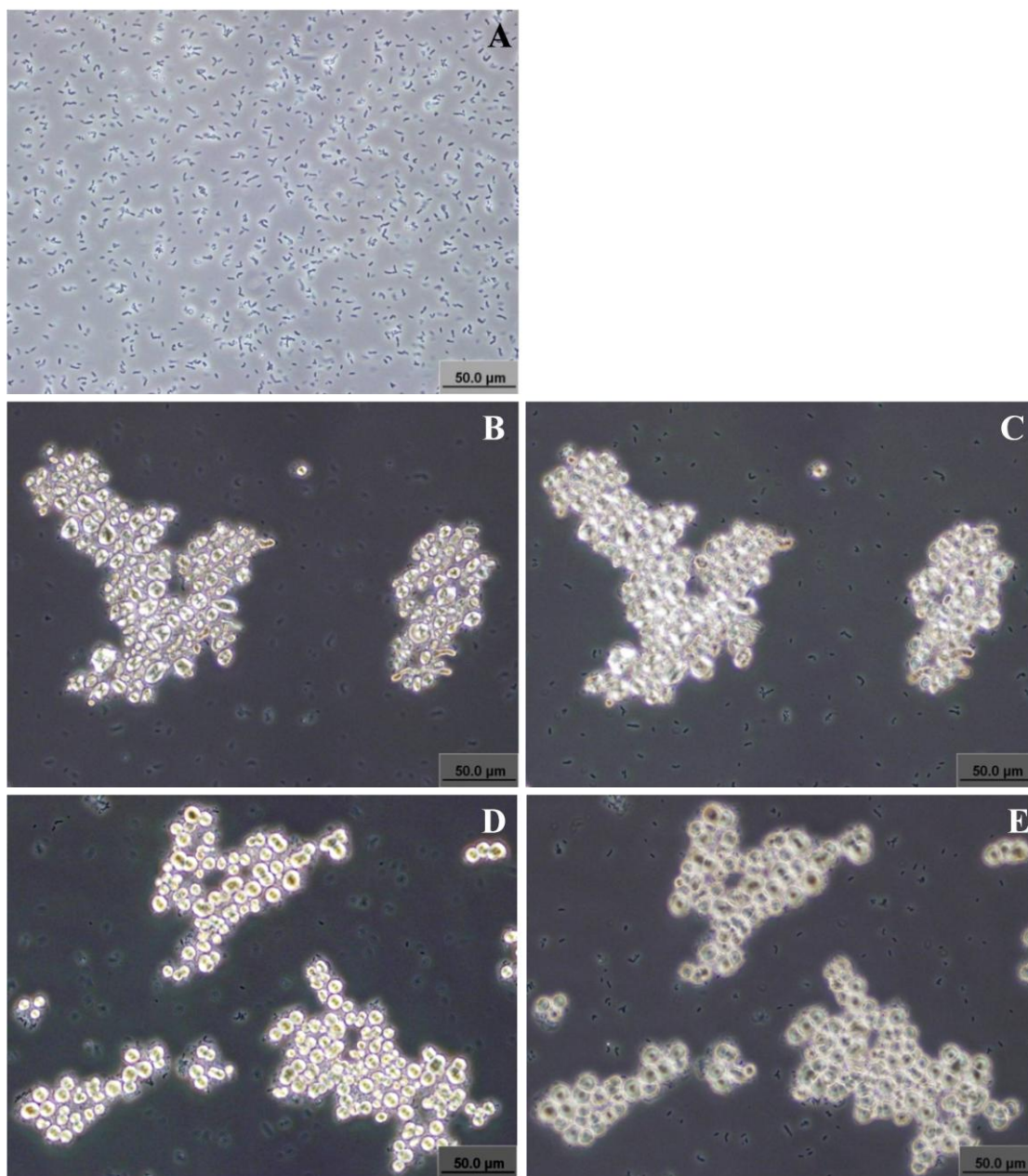


Figure 3-4: Polarized light and phase contrast micrographs of *B. choerinum* ATCC 27686: A) in buffer; (B, C) in the presence of native Hylon VII granules; (D, E) in the presence of potato amylose spherulites. Bar scale: 50 μm.

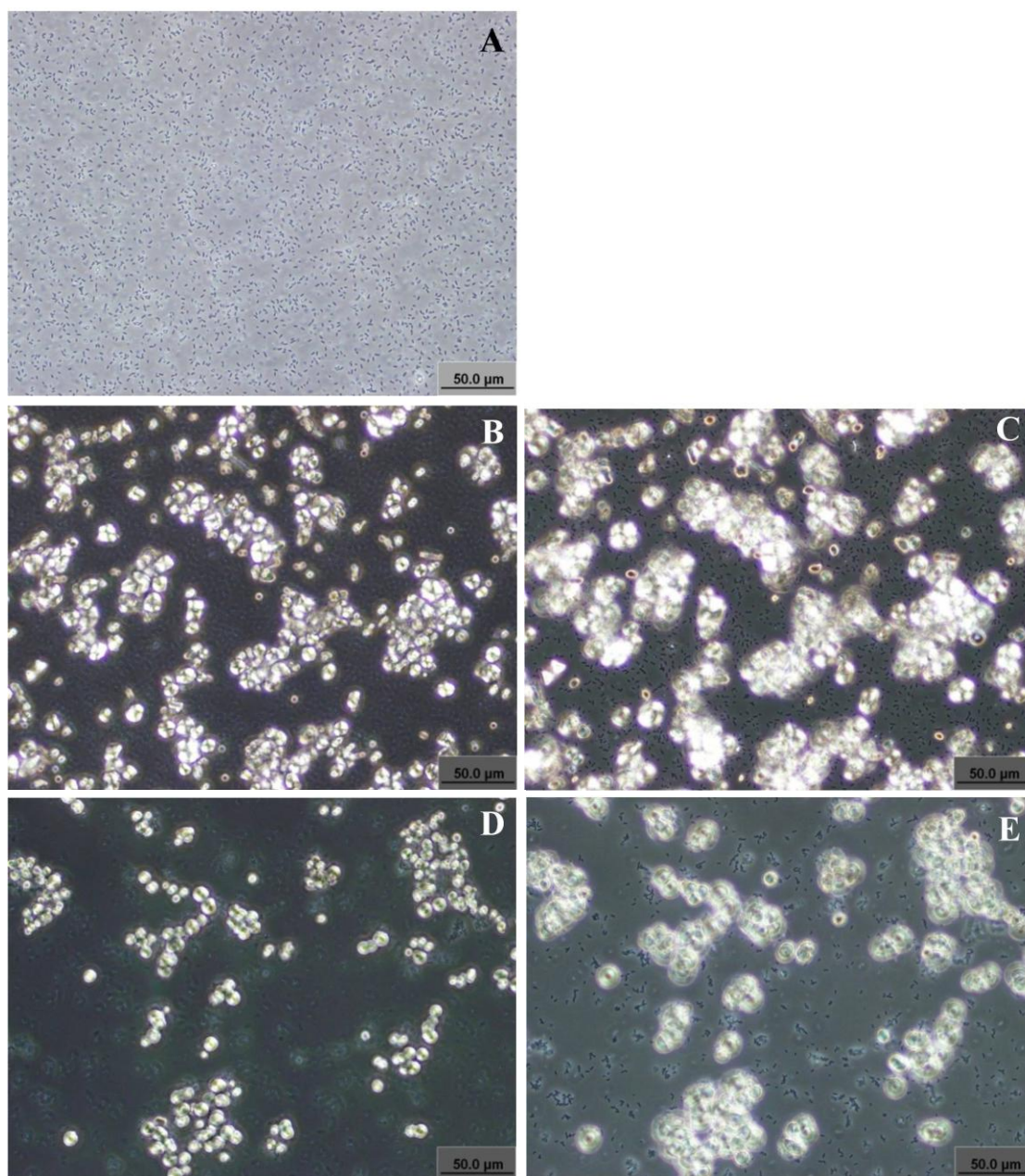


Figure 3-5: Polarized light and phase contrast micrographs of *B. animalis* ssp. *lactis* DSMZ 10140: A) in buffer; (B, C) in the presence of native Hylon VII granules; (D, E) in the presence of potato amylose spherulites. Bar scale: 50 μm.

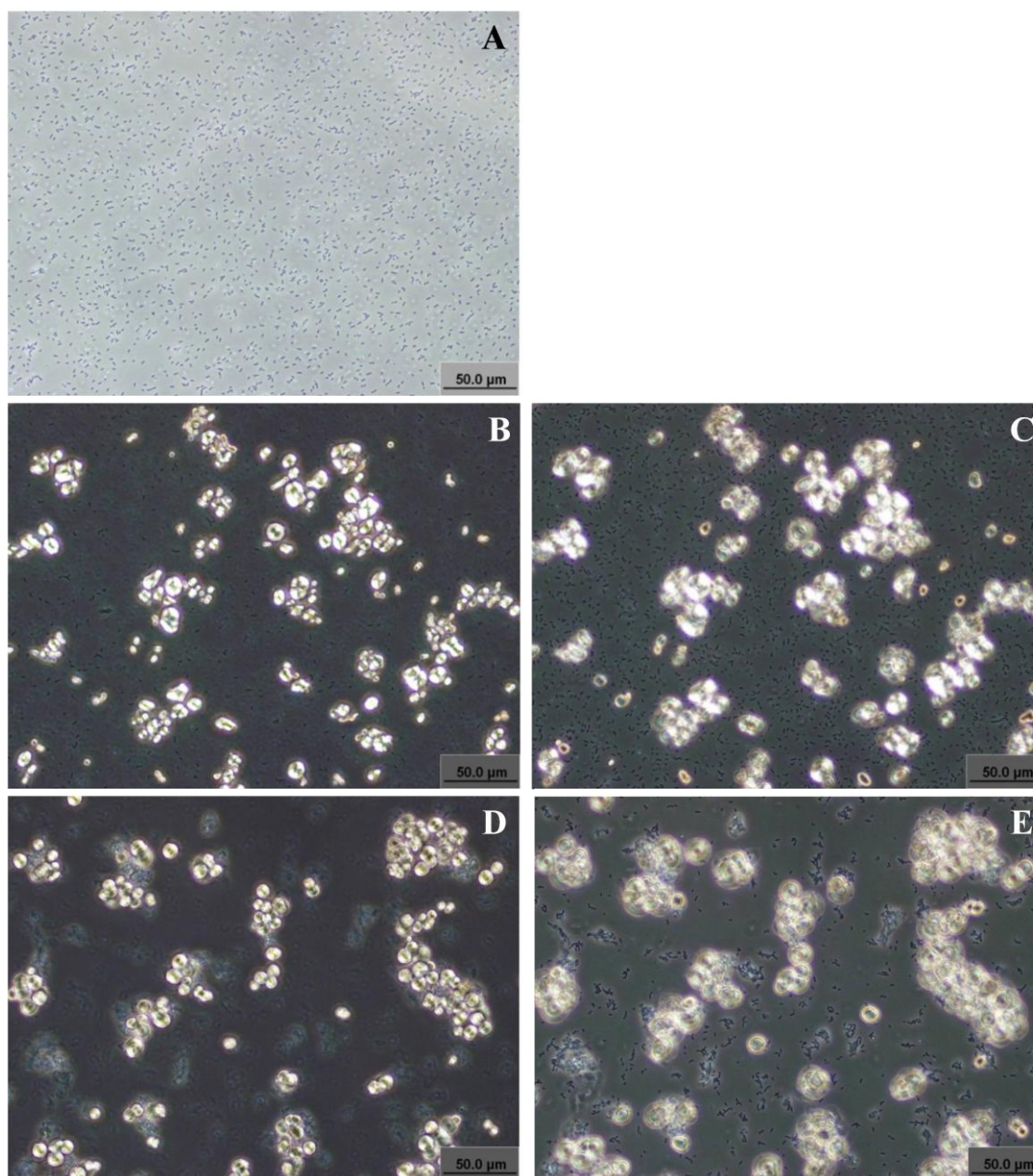


Figure 3-6: Polarized light and phase contrast micrographs of *B. animalis* ssp. *lactis* Bb-12: A) in buffer; (B, C) in the presence of native Hylon VII granules; (D, E) in the presence of potato amylose spherulites. Bar scale: 50 μm.

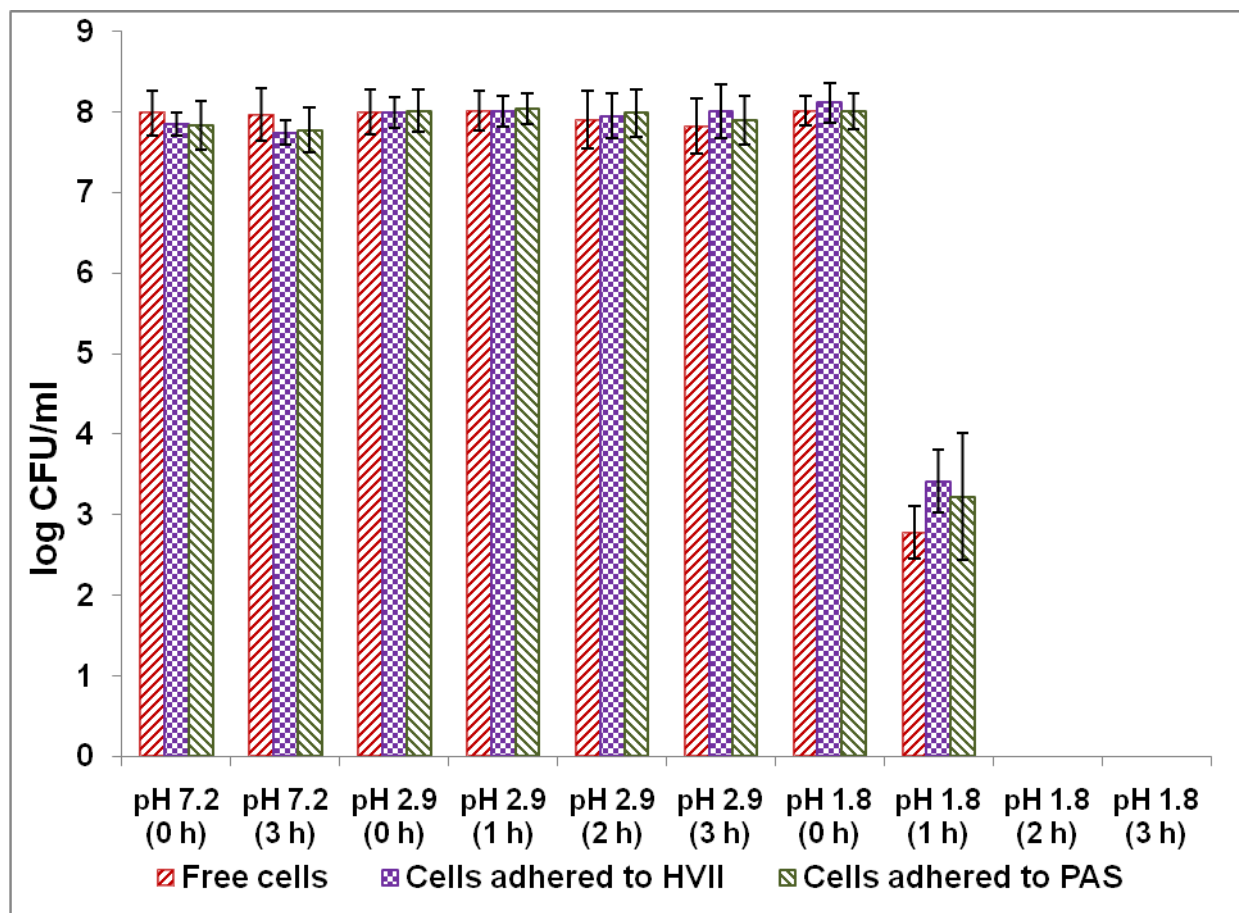


Figure 3-7: Survival of *B. pseudolongum* ATCC 25526 when exposed to different pH conditions for 0, 1, 2 and 3 h at 37°C. The numbers in the parentheses on the x-axis represents exposure time (h). The error bars represent standard error of means (n=3).

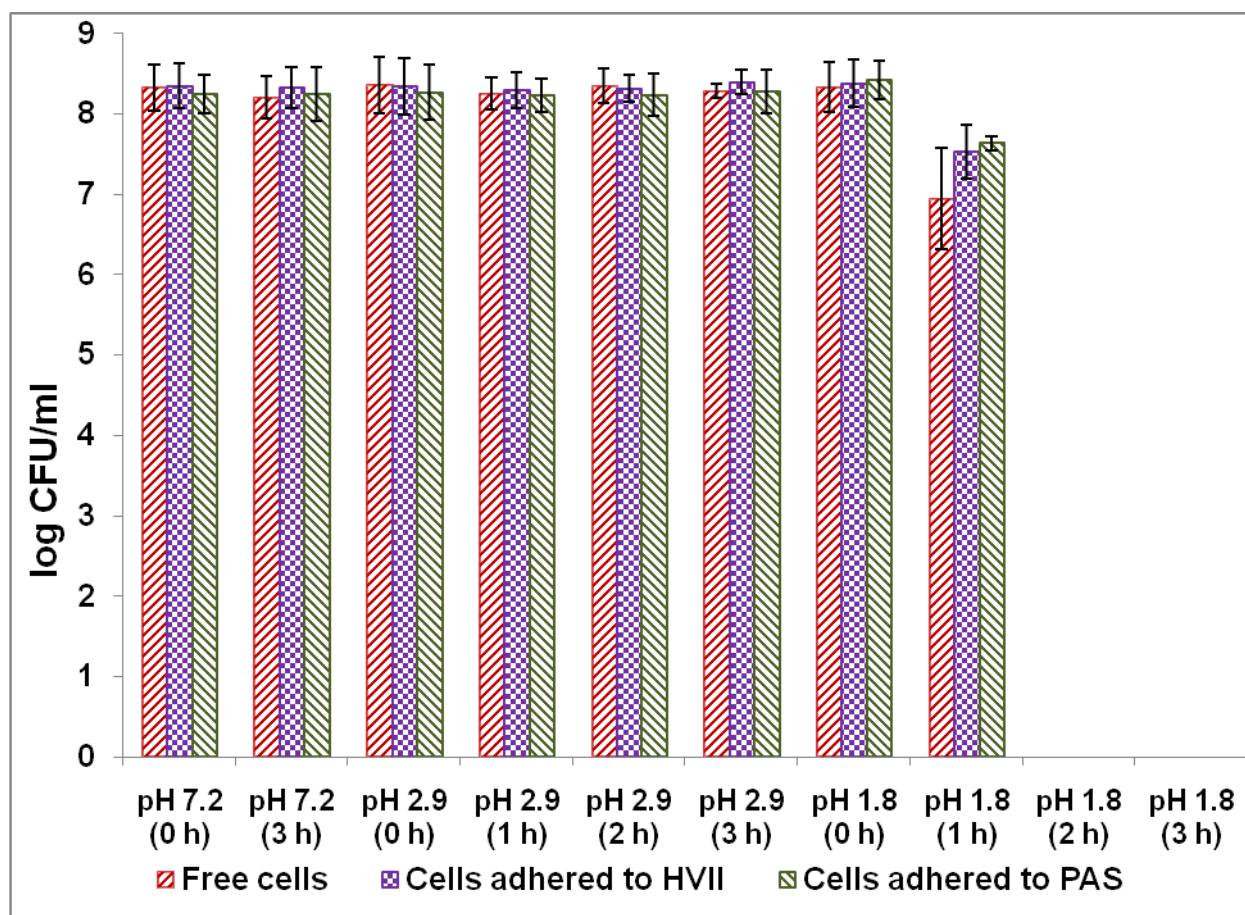


Figure 3-8: Survival of *B. animalis* ssp. *lactis* DSMZ 10140 when exposed to different pH conditions for 0, 1, 2 and 3 h at 37°C. The numbers in the parantheses on the x-axis represents exposure time (h). The error bars represent standard error of means (n=3).

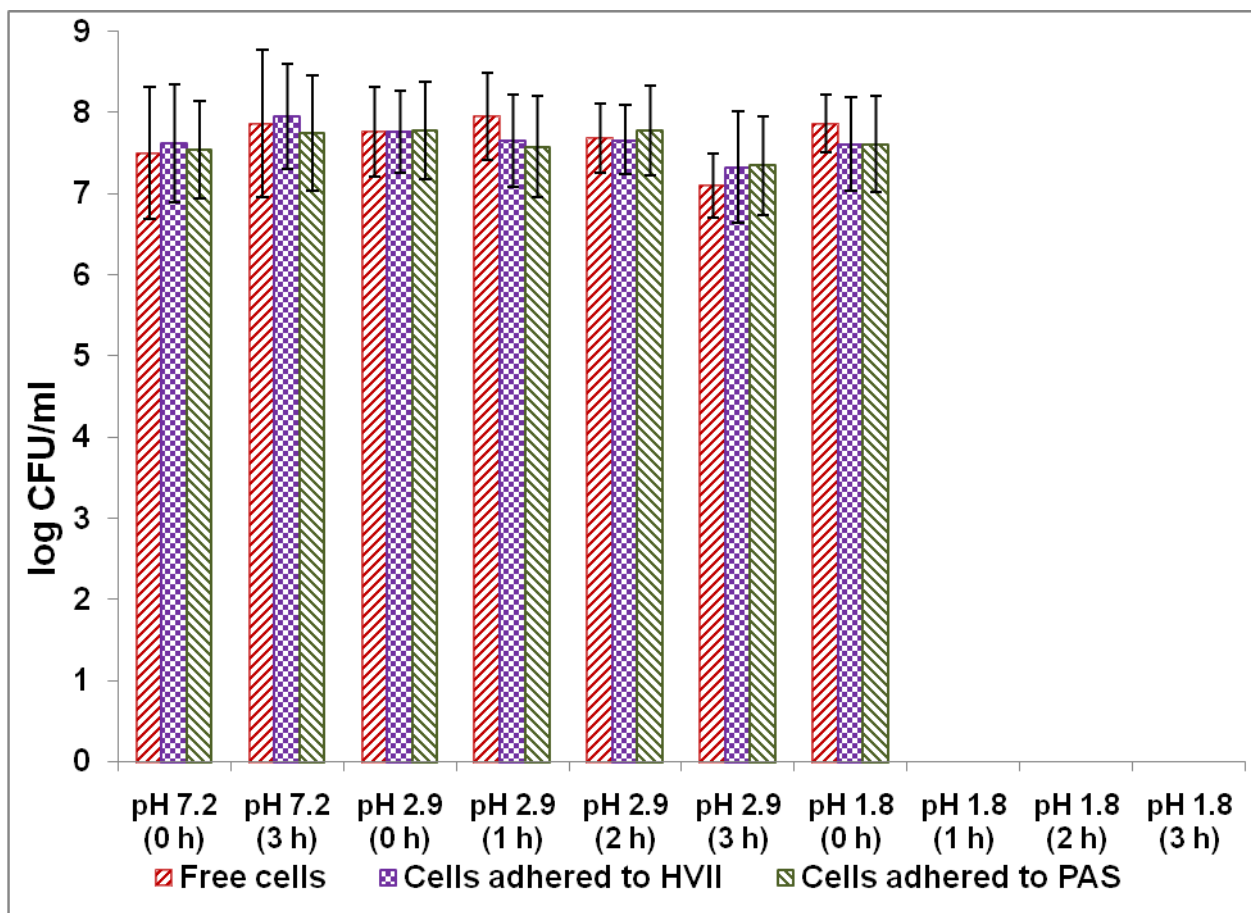


Figure 3-9: Survival of *B. choerinum* ATCC 27686 when exposed to different pH conditions for 0, 1, 2 and 3 h at 37°C. The numbers in the parentheses on the x-axis represents exposure time (h). The error bars represent standard error of means (n=3).

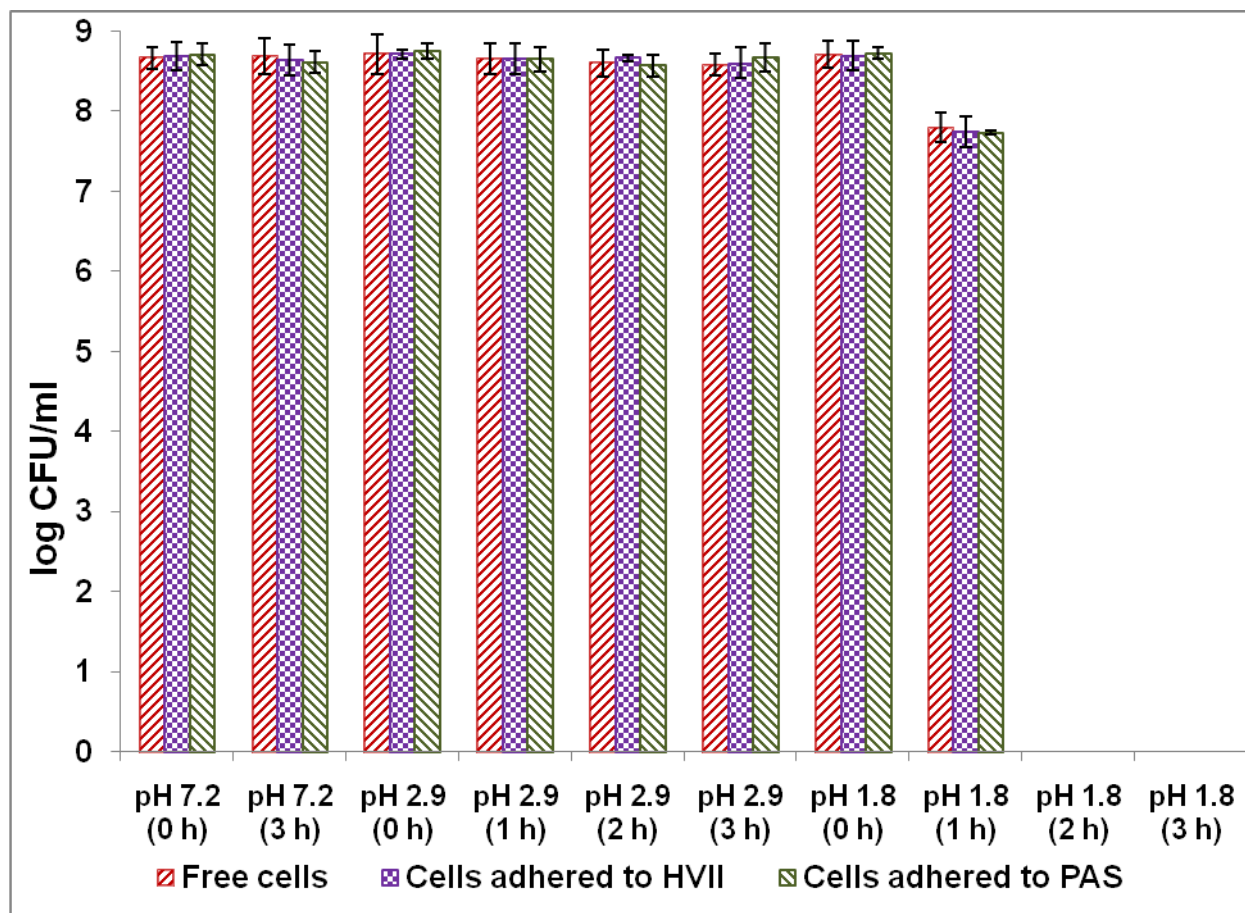


Figure 3-10: Survival of *B. animalis* ssp. *lactis* Bb-12 when exposed to different different pH conditions for 0, 1, 2 and 3 h at 37°C. The numbers in the parantheses on the x-axis represents exposure time (h). The error bars represent standard error of means (n=3).

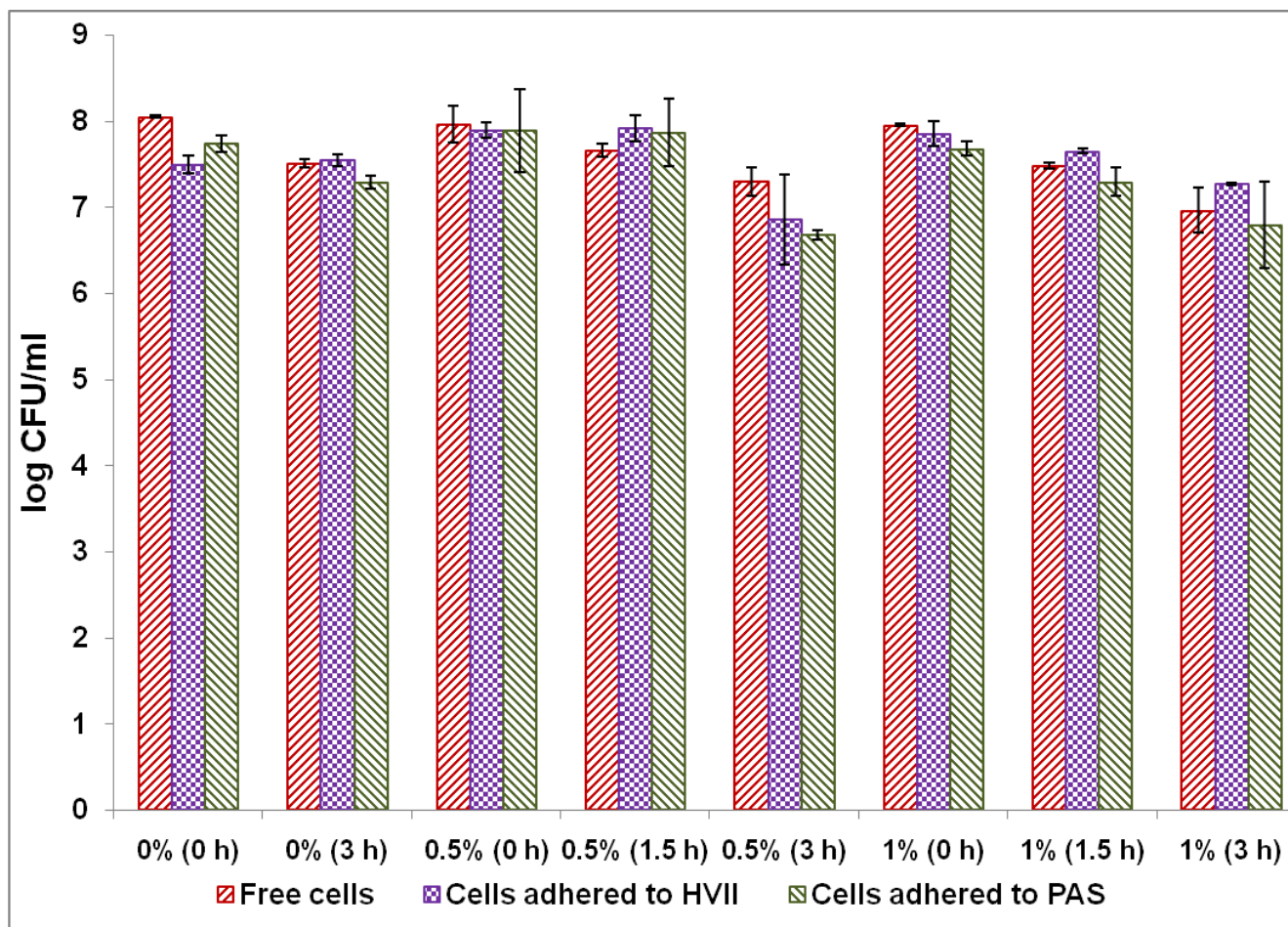


Figure 3-11: Survival of *B. pseudolongum* ATCC 25526 when exposed to different concentrations of oxgall in PBS (0, 0.5 and 1%) for 0, 1.5 and 3 h at 37°C. The numbers in the parentheses on the x-axis represents exposure time (h). The error bars represent standard error of means (n=2).

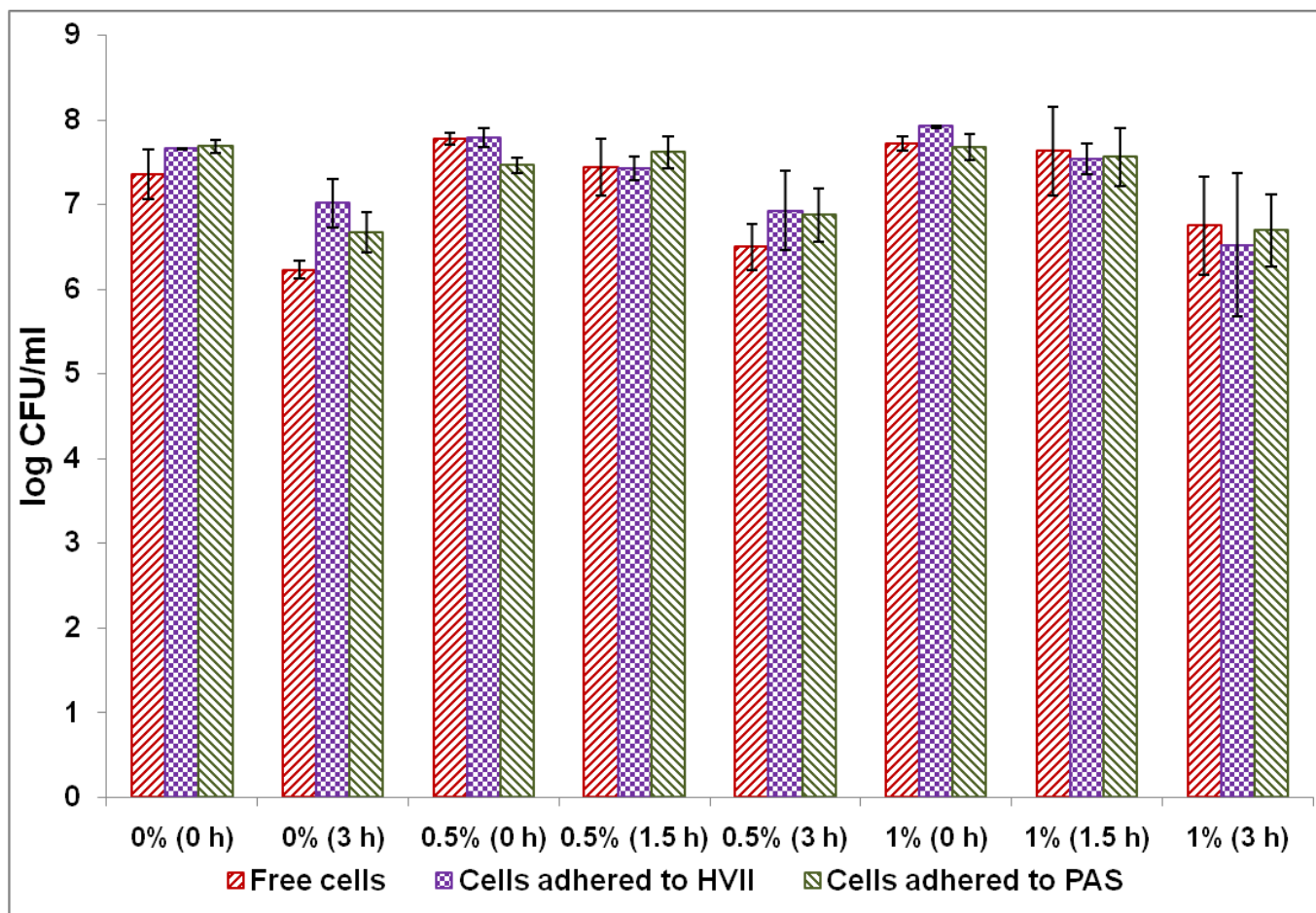


Figure 3-12: Survival of *B. animalis* ssp. *lactis* DSMZ 10140 when exposed to different concentrations of oxgall in PBS (0, 0.5 and 1%) for 0, 1.5 and 3 h at 37°C. The numbers in the parentheses on the x-axis represents exposure time (h). The error bars represent standard error of means (n=2).

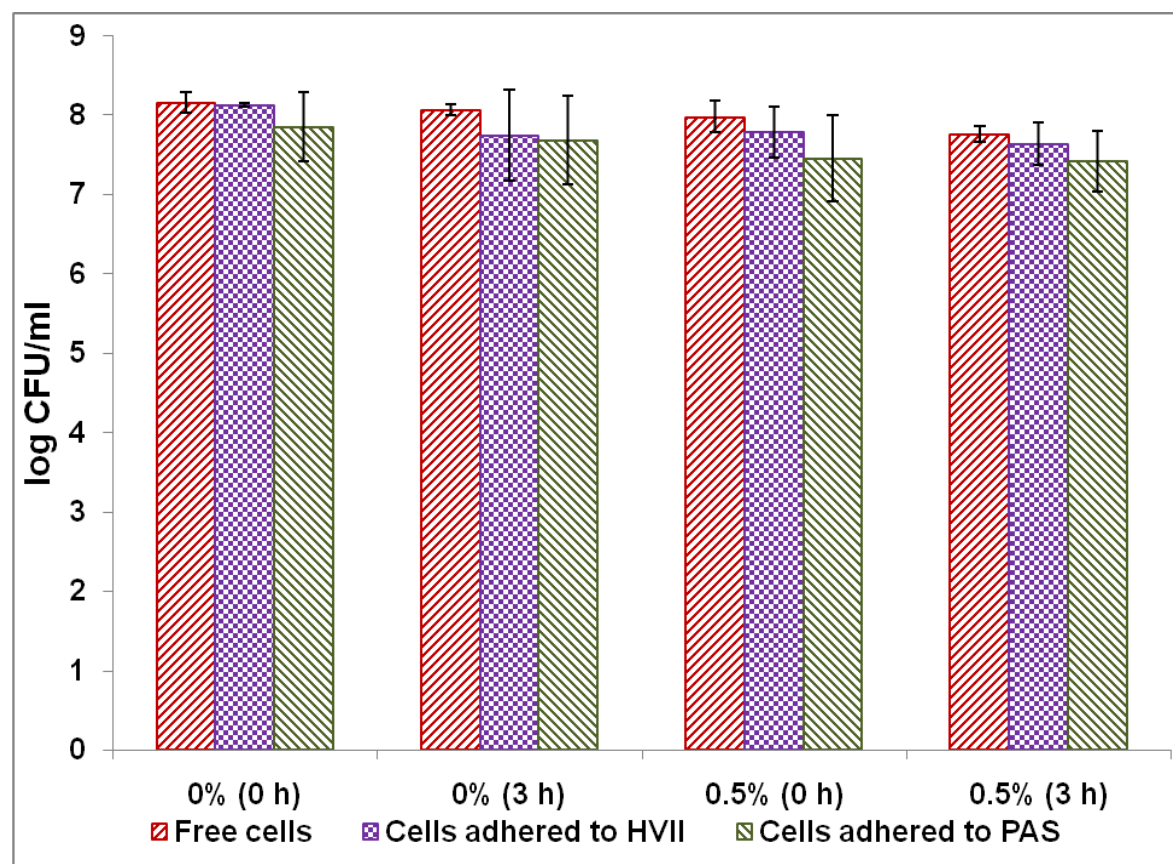


Figure 3-13: Survival of *B. choerinum* ATCC 27686 when exposed to different concentrations of oxgall in PBS (0 and 0.5%) for 0 and 3 h at 37°C. The numbers in the parantheses on the x-axis represents exposure time (h). The error bars represent standard error of means (n=2).

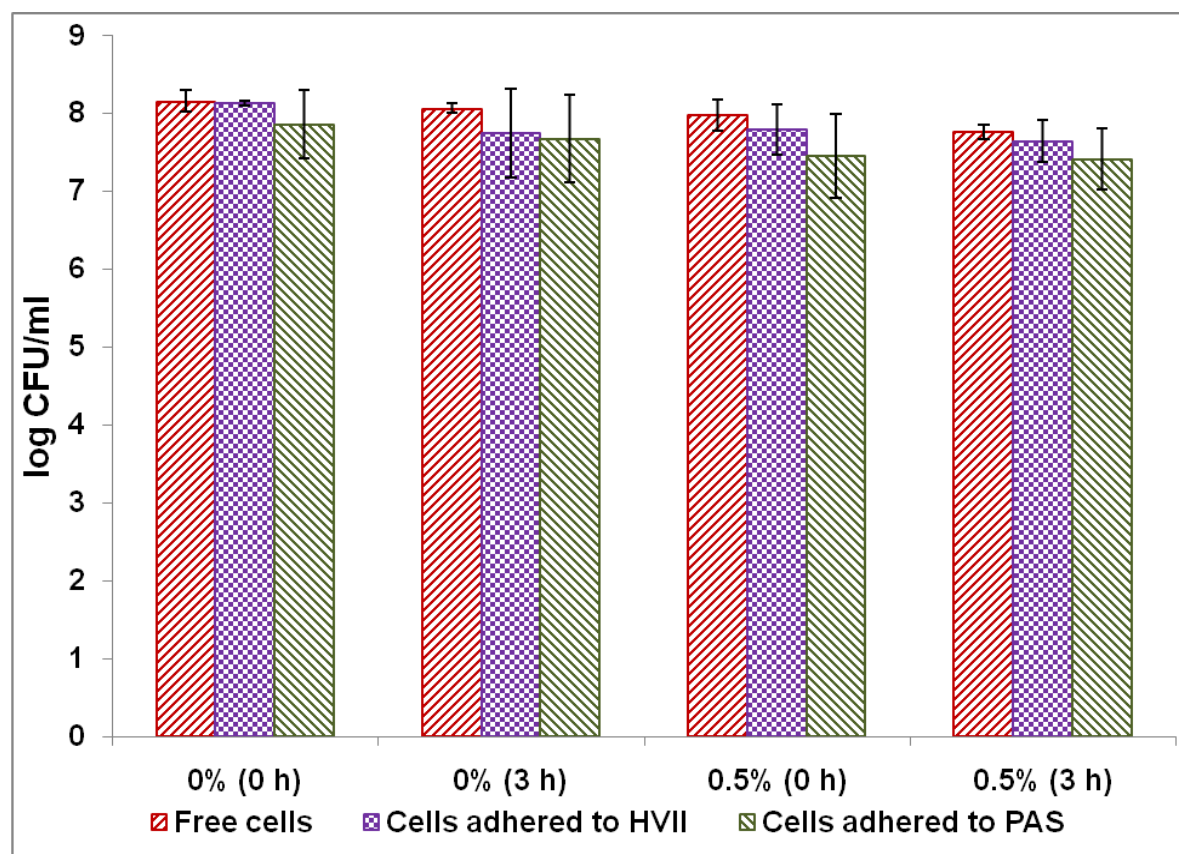


Figure 3-14: Survival of *B. animalis* ssp. *lactis* Bb-12 when exposed to different concentrations of oxgall in PBS (0 and 0.5%) for 0 and 3 h at 37°C. The numbers in the parantheses on the x-axis represents exposure time (h). The error bars represent standard error of means (n=2)

Chapter 4

Summary and future research

4.1 Summary of the work

The goal of this project was to study the survival of bifidobacteria in the presence of spherulites when exposed to acid or bile. Spherulites could be produced in a reactor when starches with high amylose content (potato, HVII and potato amylose) were heated to 185°C and rapidly quenched. Drying of the spherulites using vacuum filtration followed by solvent dehydration resulted in spherulites similar in morphology and size to the washed spherulites.

Bifidobacterial strains exhibited different degrees of adhesion to PAS and HVII, with adhesion of majority of the strains being higher to PAS than HVII. All the four strains used in the survival studies were identified correctly at the genus level and at the sub-species level with *B. animalis* ssp. *lactis* specific primers. Microscopic images of the four strains chosen for survival studies in the presence of PAS and HVII were supportive of the adhesion results. Survival of bifidobacterial strains was dependent on the strain type, pH and bile concentration used. All the strains were sensitive to pH 1.8 but extremely tolerant to higher pH and bile (0, 0.5 and 1% oxgall). Adhesion to PAS or HVII could not enhance the survival of bifidobacterial strains analyzed in acid or bile.

Several methods have been used to improve the survival of bifidobacteria in adverse conditions. However, this is the first study on protective effects of spherulites on

survival of bifidobacterial strains. Although spherulites could not improve survival, future research involving, studying the mechanism for adhesion, utilization of spherulites by bifidobacteria and further encapsulating the strains within the spherulitic starch may have potential applications in successfully delivering viable bifidobacteria to the human intestine.

4.2 Suggestions for future research

The present research indicates that presence of PAS or HVII had limited influence on survival of bifidobacteria. Understanding the mechanism involved in bifidobacteria adhering to spherulites or starch may help in prolonging viability in adverse environmental conditions. The strains used in this study survived well in bile and at pH 2.9 and 7.2. It would be interesting to look at the effect of spherulites on adhering bacteria that were sensitive to acid and bile. It would also be useful to determine spherulite utilization by bifidobacteria and the impact of adhesion on spherulite utilization. Bacteria adhering to the spherulites could utilize the substrate and offer selective advantages in the host.

In the current work, spherulites could be produced successfully in a reactor but several batches of spherulites were made to obtain sufficient quantity for the microbial experiments. It would be interesting to obtain a method to prepare spherulites in large scale from one single batch. This could minimize the variation in heating and cooling rate that might occur during production in a reactor for several days. It would also be useful to encapsulate bifidobacteria within spherulitic starches during their production by

introducing bacteria during the cooling cycle before the starch crystallizes and forms spherulites. This could ensure association between bifidobacteria and spherulites and protect the bacteria during product processing and storage and during passage through the human GI tract. Survival of encapsulated and free cells in acid and bile salts and when incorporated in yogurt could also be determined. It would also be interesting to encapsulate bifidobacteria in spherulites that also contain buffer or to prepare time-release capsules that are broken down by enzymes in the intestines and not by enzymes in the stomach.

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Appendix A

Statistical outputs

Table A-1: ANOVA table comparing the adhesion values obtained by absorbance and viable count method for a particular strain. Differences were considered significant at $p \leq 0.05$.

		Sum of Squares	df	Mean Square	F	Sig.
10140 ¹	Between Groups	1.092	1	1.092	.220	.685
	Within Groups	9.925	2	4.962		
	Total	11.017	3			
15708 ²	Between Groups	0.002	1	.002	.000	.987
	Within Groups	15.451	2	7.726		
	Total	15.454	3			
Bb-12 ³	Between Groups	32.262	1	32.262	.897	.444
	Within Groups	71.934	2	35.967		
	Total	104.197	3			
25526 ⁴	Between Groups	25.150	1	25.150	.228	.680
	Within Groups	220.356	2	110.178		
	Total	245.506	3			

¹ *B. animalis* ssp. *lactis* DSMZ 10140

² *B. longum* ATCC 15708

³ *B. animalis* ssp. *lactis* Bb-12

⁴ *B. pseudolongum* ATCC 25526

Table A-2: Results of nested two way ANOVA to determine the effect of starch and species on the mean adhesion values of different bifidobacterial strains. Factor strain is nested within factor species. Differences were considered significant at $p \leq 0.05$.

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	Hypothesis	17901.948	1	17901.948	149.489	.000	.838
	Error	3472.882	29	119.755 ^a			
Species	Hypothesis	45267.347	8	5658.418	47.250	.000	.929
	Error	3472.882	29	119.755 ^a			
Starch	Hypothesis	443.418	1	443.418	10.398	.001	.054
	Error	7718.609	181	42.644 ^b			
Strain(Species)	Hypothesis	3472.882	29	119.755	2.808	.000	.310
	Error	7718.609	181	42.644 ^b			
Species * Starch	Hypothesis	3275.912	8	409.489	9.602	.000	.298
	Error	7718.609	181	42.644 ^b			

^a MS(Strain(Species))

^b MS(Error)

Design: Intercept + Species + Starch + Strain(Species) + Species * Starch

Table A-3: Results of two way ANOVA to determine the effect of starch and strain on the mean adhesion values of *B. animalis* ssp. *lactis* strains. Differences were considered significant at $p \leq 0.05$.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	17785.621 ^a	47	378.417	11.160	.000	.845
Intercept	10635.109	1	10635.109	313.640	.000	.766
Strain	2910.550	23	126.546	3.732	.000	.472
Starch	12303.616	1	12303.616	362.845	.000	.791
Strain * Starch	2571.455	23	111.802	3.297	.000	.441
Error	3255.235	96	33.909			
Total	31675.966	144				
Corrected Total	21040.856	143				

^a R Squared = .845 (Adjusted R Squared = .770)

Design: Intercept + Strain + Starch + Strain * Starch

Table A-4: Results of GLM univariate analysis to determine the effect of strain, starch and pH on the mean log reduction values of *Bifidobacterium* strains chosen for survival studies. Differences were considered significant at $p \leq 0.05$.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	1599.026 ^a	35	45.686	548.985	.000	.996
Intercept	837.020	1	837.020	10057.946	.000	.993
Starch	0.049	2	0.025	0.296	.744	.008
pH	1591.168	2	795.584	9560.029	.000	.996
Strain	1.475	3	0.492	5.908	.001	.198
Starch * pH	0.106	4	0.027	0.319	.865	.017
Starch * Strain	0.086	6	0.014	0.172	.984	.014
pH * Strain	5.926	6	0.988	11.867	.000	.497
Starch * pH * Strain	0.216	12	0.018	0.217	.997	.035
Error	5.992	72	0.083			
Total	2442.038	108				
Corrected Total	1605.018	107				

^a R Squared = .996 (Adjusted R Squared = .994)

Design: Intercept + Starch + pH + Strain + Starch * pH + Starch * Strain + pH * Strain + Starch * pH * Strain

Table A-5: Results of GLM univariate analysis to determine the effect of strain, starch and oxgall on the mean log reduction values of *Bifidobacterium* strains chosen for survival studies. Differences were considered significant at $p \leq 0.05$.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	10.182 ^a	23	0.443	2.442	.017	.701
Intercept	25.459	1	25.459	140.469	.000	.854
Strain	6.208	3	2.069	11.417	.000	.588
Oxgall	1.406	1	1.406	7.757	.010	.244
Starch	0.027	2	0.013	0.073	.930	.006
Strain * Oxgall	1.109	3	0.370	2.039	.135	.203
Strain * Starch	0.276	6	0.046	0.254	.953	.060
Oxgall * Starch	0.510	2	0.255	1.407	.264	.105
Strain * Oxgall * Starch	0.647	6	0.108	0.595	.731	.129
Error	4.350	24	.181			
Total	39.990	48				
Corrected Total	14.531	47				

^aR Squared = .701 (Adjusted R Squared = .414)

Design: Intercept + Strain + Oxgall + Starch + Strain * Oxgall + Strain * Starch + Oxgall * Starch + Strain * Oxgall * Starch