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ENCAPSULATION OF PROBIOTIC MICROORGANISMS IN FOOD-GRADE HYDROGEL MICROBEADS FOR IMPROVING LONG-TERM STORAGE AND ORAL DELIVERY

A Thesis Presented

by

TIMOTHY W. YEUNG

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

September 2016

Department of Food Science

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ENCAPSULATION OF PROBIOTIC MICROORGANISMS IN FOOD-GRADE HYDROGEL MICROBEADS FOR IMPROVING LONG-TERM STORAGE AND ORAL DELIVERY

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by

TIMOTHY W. YEUNG

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DEDICATION

This thesis is dedicated to my grandfather, Joe Hing Lowe (1933-2015), who painted the world as a marvelous place to explore.

ACKNOWLEDGMENTS

I would like to first thank Izlia Arroyo-Maya, Elif Üçok, and Kendra Tiani for their contributions to this work. Without them, I would not have become a published scientist this early in my career.

Next, I thank the members of my committee. I thank David A. Sela for all his support and guidance. His passion for doing good science makes him a vital part of the department. Thanks to D. Julian McClements and Lynne McLandsborough for their thoughtful advice and support in and out of the classroom.

I also thank the lab coordinators of the Department of Food Science, Cynthia Kane, Jean Alamed, and Ruth Witkowsky, for their assistance in acquiring supplies and training me to operate equipment properly. Their efforts made all experiments go smoothly and safely.

I thank all my colleagues in and out of the Sela Lab who directly or indirectly contributed to the projects presented herein. This includes my professors, TAs, and classmates at Penn State; my undergraduate experience prepared me to undertake graduate work with less hardship.

Lastly, and most importantly, I thank my parents for their love and support verbally, spiritually, and financially.

-Timothy Yeung

ABSTRACT

ENCAPSULATION OF PROBIOTIC MICROORGANISMS IN FOOD-GRADE HYDROGEL MICROBEADS FOR IMPROVING LONG-TERM STORAGE AND ORAL DELIVERY

SEPTEMBER 2016

TIMOTHY W. YEUNG, B.S., PENNSYLVANIA STATE UNIVERSITY

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Probiotics die over time during processing, storage and digestion, resulting in reduced health benefits to the consumer. Microencapsulation of microorganisms is an effective way to improve probiotic viability by restricting cell exposure to extreme conditions through the gastrointestinal tract until release in the colon. In this work, appearance and survival of encapsulated probiotic species from two genera was explored. *Lactococcus lactis* and *Bifidobacterium longum* were suspended in calcium alginate microbeads by spraying droplets of alginate-probiotic mixture into calcium chloride solution. This produced uniformly shaped transparent microbeads with high encapsulation yield. Encapsulating *Lactococcus lactis* extended viability during dry room temperature storage. Encapsulating *Bifidobacterium longum* revealed high variation between eight different strains from subspecies *longum* and *infantis*. Coating alginate particles with chitosan did not improve viability and, viability of free and encapsulated bifidobacteria decreased when exposed to simulated gastric and intestinal conditions. Data from these studies suggest microencapsulating probiotic cells is an invaluable process to extending cell viability. Future research should optimize current formulations to improve encapsulation yield and cell survival during processing, storage, and gastrointestinal transit.

TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS v

ABSTRACT vi

LIST OF TABLES ix

LIST OF FIGURES x

CHAPTER

1. LITERATURE REVIEW 1

    1.1 Introduction 1

    1.2 Probiotics 2

    1.3 Microencapsulation 3

    1.4 OBJECTIVES 10

2. MICROENCAPSULATION OF PROBIOTICS IN HYDROGEL PARTICLES: ENHANCING *LACTOCOCCUS LACTIS* SUBSP. *CREMORIS* LM0230 VIABILITY USING CALCIUM ALGINATE BEADS 11

    2.1 Abstract 12

    2.2 Introduction 13

    2.3 Materials and methods 16

    2.4 Results 19

    2.5 Discussion 25

    2.6 Acknowledgements 29

3. MICROENCAPSULATION IN ALGINATE AND CHITOSAN MICROGELS TO ENHANCE VIABILITY OF *BIFIDOBACTERIUM LONGUM* FOR ORAL DELIVERY 30

    3.1 Abstract 31

    3.2 Introduction 32

Page

3.3 Materials and methods 34

3.4 Results 42

3.5 Discussion 52

3.6 Acknowledgements 55

4. CONCLUSION 56

APPENDICES

A: SUPPLEMENTAL DATA 57

B: EXTENDING VIABILITY OF *LACTOBACILLUS PLANTARUM* AND *LACTOBACILLUS JOHNSONII* BY MICROENCAPSULATION IN ALGINATE MICROGELS 61

BIBLIOGRAPHY 75

LIST OF TABLES

Table Page

2.1 Mean particle diameters of alginate beads prepared with and without *Lactococcus lactis* LM0230 cells for two gelation time periods 20

3.1 *Bifidobacterium longum* strains selected for encapsulation..35

3.2 Mean particle diameters of alginate and chitosan-coated alginate beads containing strains of *Bifidobacterium longum*..42

3.3 Zeta potential of alginate and chitosan-coated alginate beads containing strains of *Bifidobacterium longum*...46

3.4 Survival of free and encapsulated *Bifidobacterium longum* subsp. *infantis* UMA 299 in three separate simulated digestion phases 52

A.1 Survival of *Lactococcus lactis* subjected to drying at room temperature (22-25°C) 57

A.2 Viability of free *Bifidobacterium longum* cells during refrigerated storage 58

A.3 Viability of *Bifidobacterium longum cells* encapsulated in calcium alginate during refrigerated storage...59

A.4 Viability of *Bifidobacterium longum cells* encapsulated in chitosan-coated alginate during refrigerated storage 60

B.1 Mean particle diameters of alginate beads containing *Lactobacillus plantarum* and *Lactobacillus johnsonii* 68

B.2 Viability of free and encapsulated *Lactobacillus plantarum* and *Lactobacillus johnsonii* during refrigerated storage 72

B.3 Simulated digestion of free and encapsulated *Lactobacillus plantarum* and *Lactobacillus johnsonii* cells...73

LIST OF FIGURES

Figure Page

1.1 Experimental design for immobilizing probiotics in alginate microbeads 8

2.1 Particle size distribution of alginate beads prepared with and without *Lactococcus lactis* LM0230 cells for two gelation time periods 21

2.2 Optical microscope images of (A) *Lactococcus lactis* LM0230 (40×), (B) unfilled alginate microbead (40×), and filled alginate beads gelled for (C) 1 hour (40×) and (D) 24 hours (40×) 22

2.3 Scanning electron microscope images of (A) *Lactococcus lactis* LM0230 (5000×), (B) unfilled alginate microbead (500×), and filled alginate beads gelled for (C) 1 hour (400×) and (D) 24 hours (1100×) 23

2.4 Confocal microscope images of (A) *Lactococcus lactis* LM0230, (B) unfilled alginate microbead, and (C) filled alginate beads...24

2.5 Survival of free and encapsulated *Lactococcus lactis* LM0230 in calcium alginate beads during dry room temperature storage..25

3.1 Experimental design for simulated digestion of free and encapsulated *Bifidobacterium longum* subsp. *infantis* UMA 299 41

3.2 Optical microscope images of (A) *Bifidobacterium longum* subsp. *infantis* UMA 300 (20×), (B) unfilled alginate bead (20×), (C) filled alginate bead with *B. longum* UMA 306 (20×), (D) *B. longum* subsp. *longum* UMA 318 (20×), (E) unfilled chitosan-coated alginate bead (20×), and (F) filled chitosan-coated alginate bead with *B. infantis* UMA 299 (20×) 44

3.3 Scanning electron microscope images of (A) *Bifidobacterium longum* subsp. *infantis* UMA299, (B) unfilled alginate bead, (C) unfilled chitosan-coated alginate bead, (D) *B. longum* subsp. *longum* UMA 306, (E) alginate bead containing *B. longum* subsp. *longum* UMA 401, and (F) chitosan-coated alginate bead containing *B. longum* subsp. *infantis* UMA 300 45

3.4 Survival of free *Bifidobacterium longum* cells in wet refrigerated storage over time 47

3.5 Survival of *Bifidobacterium longum* cells in calcium alginate microbeads in wet refrigerated storage over time 49

3.6 Survival of *Bifidobacterium longum* cells in chitosan-coated alginate microbeads in wet refrigerated storage over time 50

B.1 Particle size distribution of alginate beads containing *Lactobacillus plantarum* and *Lactobacillus johnsonii* 68

B.2 Optical microscope images of (A) alginate microbead containing *Lactobacillus plantarum* (20×), (B) alginate microbead containing *Lactobacillus johnsonii* (20×).......... 69

B.3 Scanning electron microscope images of (A) *Lactobacillus plantarum* ATCC BAA-793 (4000×), (B) alginate bead containing *L. plantarum* (300×), (C) *Lactobacillus johnsonii* ATCC 33200 (2000×), (D) alginate bead containing *L. johnsonii* (500×) 70

B.4 Survival of free and encapsulated *Lactobacillus plantarum* and *Lactobacillus johnsonii* during refrigerated storage 71

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Scientific understanding of probiotics has been developing ever since the term was introduced by Lilly and Stillwell (1965). Put simply, probiotics are microorganisms that confer health benefits on the consumer host, particularly associated with the gastrointestinal (GI) tract. Current technical definitions of the term probiotic emphasize the *viability* and *concentration* of microorganisms needed for the consumer to gain health benefits (Schrezenmeir and de Vrese, 2001). Benefits from probiotics include modulating inflammatory response in the colon, strengthening the immune system, preventing growth of pathogens, and improving nutrient absorption (Lian et al., 2003; Parvez et al., 2006; Anal and Singh, 2007). While probiotic consumption in function foods and supplements have become increasingly popular, studies on their efficacy need to be established.

Major criteria for determining efficacy include proper identification of strains used by the consumer, viability of those strains at the time of consumption, and consistent quality of the product (Lewis et al., 2015). A minimum of 10^6 - 10^8 CFU/mL present in the consumed product is the recommended concentration to be effective (Krasaekoopt et al., 2003; Amine et al., 2014b). This concentration is initially added as free cells into fermented products, but overall viability degrades during storage. While conventional methods add free cells several log CFU higher than the suggested dose, intrinsic factors of the food product such as low pH, low water activity, and presence of antibiotic substances can accelerate decline of probiotic viability below the recommended dose (Rokka and Rantamäki, 2010). Free cells are also exposed to extreme conditions during

digestion. Low pH in the gastric phase and potent enzymes throughout the GIT can cause significant loss of cell viability. As few as 20-40% log CFU probiotic cells can survive gastrointestinal transit, depending on the species and strain (Bezkorovainy, 2001). Thus, processing methods to mitigate degradation of cell viability are necessary to impart health benefits despite long storage periods as well as gastrointestinal transit.

1.2 Probiotic bacteria

Several diverse groups of microorganisms are considered probiotic with a wide range of health benefits. The two major groups are lactic acid bacteria and bifidobacteria. Lactic acid bacteria (LAB) are a large group of Gram positive microorganisms found in a diverse range of environments. Several genera within this group include *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc, Oenococcus*, *Pediococcus*, and *Streptococcus* and are associated with the surfaces of vegetation and dairy products. LAB all have the ability to produce lactic acid as an end product, and are often used for fermentative processes (Price et al., 2012). As a result, many LAB have high acid tolerance, allowing them to survive gastrointestinal transit, colonize the host colon and, outcompete growth of pathogens. This group is highly diverse, producing many different end-products other than lactic acid, which affect flavor profiles of the food products they are added into. While many species within this group are considered spoilage organisms, others are generally recognized as safe (GRAS) and are used to ferment food products such as cheeses, yogurt, and sauerkraut.

Bifidobacteria are a smaller group of anaerobic Gram positive bacteria closely associated with the mammalian gut. Most species within this group are considered probiotic, breaking down a wide variety of indigestible carbohydrates (Chaplin et al.,

2015). Bifidobacteria may even have a role in establishing host gut microbiome shortly after birth (Sela and Mills, 2010). Like LAB, bifidobacteria produce lactic acid. Presence of oxygen can cause irreparable oxidative damage to bifidobacteria, depending on their aerotolerance. Some species like *B. animalis* are faculatative, while others like *B. longum* are strictly anaerobic. However, *B. longum* has been found to colonize the infant gut more effectively than *B. animalis* (Underwood et al., 2013). Preparation techniques need to be adapted to minimize oxygen permeability during storage as supplements or within food products (O'Riordan et al., 2001). Acid resistance of bifidobacteria is strain dependent. *B. longum* and *B. bifidum* strains was shown to be more sensitive to highly acidic (pH 2) conditions than *B. lactis*, *B. breve*, and *B. adolescentis* strains (Hansen et al., 2002). However, *B. longum* and *B. breve* strains were less sensitive than *B. bifidum* and *B. adolescentis* strains when exposed long term to more alkaline (pH 4) conditions (Sun and Griffiths, 2000).

Other probiotics include certain bacterial strains of *E. coli* and *Bacillus coagulans*. Most fungi are not probiotic, with possible exceptions of a few *Saccharomyces* species which are facultative anaerobes. Novel probiotic strains and combinations of these strains continue to be discovered and evaluated for human consumption and other agricultural applications (Fernández-Murga and Sanz, 2016; Neau et al., 2016; Salvetti et al., 2016).

1.3 Microencapsulation

Encapsulation is a way to protect bacteria against severe environmental factors by suspending cells within hydrogel particles bacteria will survive during processing and storage (Anal and Singh, 2007; Champagne and Fustier, 2007). Providing live probiotic

cells with a physical barrier is an increasingly popular approach to protecting them from adverse conditions within the product and during digestion (Kailasapathy, 2009). This process can be physicochemical and/or mechanical in order to entrap an agent in a material resulting in the production of particles ranging from 1-1000 μm, which isolate them and delay their release.

Several reviews have thoroughly catalogued studies examining the effects of encapsulating probiotics, classifying them based on probiotic species, encapsulation materials, and processing methods utilized (Krasaekoopt et al., 2003; Rokka and Rantamäki, 2010; Chávarri et al., 2012; Solanki et al., 2013). Studies have commonly reported variability in responses to growth or stability between strains (Godward, 2000; Krasaekoopt, 2004; Capela et al., 2006; Ranadheera et al., 2010). Therefore, in order to describe the effect of encapsulation techniques on probiotic bacteria viability, it is desirable to select cultures that are sensitive to various environmental stresses. In addition, one problem associated with microencapsulated cultures for foods is their effect on sensory properties (Karimi, 2011). Particle size and cellular stress are important factors of encapsulation technologies which can impact texture and taste perception (Hansen et al., 2002; Burgain et al., 2011). There is interest in comparing different encapsulation technologies in order to improve the utilization of beneficial probiotics microorganisms in the food industry.

The most important factors influencing the efficiency and acceptability of encapsulation include starting materials used to encapsulate probiotic cells, the encapsulation processing method employed, and particle size resulting from processing.

1.3.1 Materials used for microencapsulation

A wide range of food-grade polymers have been used for encapsulating probiotic cells: starch, pectin, cellulose, gelatin, alginate, chitosan, gums like guar, gellan, and carrageenan, and composites of these (Kosaraju, 2005; Prakash et al., 2011; Sarao and M, 2015). Probiotics can be encapsulated by simply suspending cells within hydrogel particles made of one or more polymers or suspending cells within complex multi-layered hydrogel particles. The latter method typically requires gelling the internal encapsulation followed by coating the particles with a secondary (and even tertiary) layer (Sarao et al., 2015). Multi-layered encapsulation is favorable, because each polymer has its limitations in terms of stability. Gums like carrageenan require increased temperature to induce gelation (Anal and Singh, 2007). Starch cannot form beads alone, and requires chemical modification (Rokka and Rantamäki, 2010). Chitosan dissolves in low pH, releasing its content before reaching the colon (Kosaraju, 2005). Alginate, the most commonly used to encapsulate probiotics, requires calcium ions to form a gel matrix around core material via cross-linking (Kailasapathy, 2002; Kosaraju, 2005). Several studies suggested that encapsulating probiotics with alginate coated with chitosan allowed better viability than encapsulating with alginate alone (Lee et al., 2004; Iyer and Kailasapathy, 2005; Kamalian et al., 2014). Chemical modification of polymers can also improve encapsulation functionality. Encapsulating *Bifidobacterium longum* with N-palmitoylated alginate has previously shown to increase encapsulation yield compared to encapsulating with native alginate (Amine et al., 2014b). Encapsulation with modified starch may slow coating solvation in the upper GI tract and additionally give prebiotic effect to gut microflora (Kosaraju, 2005).

A synbiotic preparation, that is co-encapsulation of probiotics with prebiotics, is thought to enhance cell viability by strengthening the hydrogel structure and/or improving the fitness of the microbial cells. An *in vitro* study found that *Lactobacillus acidophilus* strains encapsulated with high amylose corn starch significantly increase viability counts during acidification (Sultana et al., 2000). Another *in vitro* study supported previous results of encapsulation with starch. *L. acidophilus* with prebiotics inulin and oligofructose also enhanced viability, but not as much as the corn starch. The authors suggest that the 1.0% (w/v) starch granules blocked pores in the polymer matrix, restricting cell exposure to low pH. However, interaction of the encapsulation material with prebiotics may also hinder capsule integrity, depending on prebiotic concentration (Iyer and Kailasapathy, 2005). Fritzen-Freire et al. (2012) did a similar study encapsulating *Bifidobacterium BB-12* in reconstituted skim milk mixed with inulin or oligofructose. Microbeads with oligofructose-enriched inulin and microbeads with oligofructose protected bifidobacteria better than other samples during storage.

Several studies suggested that chitosan with alginate allowed better viability than alginate alone (Krasaekoopt, 2004; Lee et al., 2004; Iyer and Kailasapathy, 2005). Incorporating modified polymers can affect encapsulation functionality. Beads containing *B. longum* sp. made with N-palmitoylated alginate have higher encapsulation yield than that made with native alginate (Amine et al., 2014b). Encapsulation with modified starch may slow coating solvation in the upper GI tract and give prebiotic effect to gut microflora (Kosaraju, 2005).

1.3.2 Processing methods of encapsulation

Several methods exist for microencapsulation production, but the most common techniques are spray drying, extrusion, and emulsion (Rokka and Rantamäki, 2010). Spray drying involves atomizing solution containing core material (*i.e.* probiotic cells) and polymer into a heated chamber, leaving dried beads of encapsulated material (Chávarri et al., 2012; Solanki et al., 2013). Spray-drying is an inexpensive process widely used on the industrial scale. However, high temperature and desiccation cause initial cell population to decrease (Kailasapathy, 2002; Rokka and Rantamäki, 2010). Encapsulation materials used in this process are also limited to prevent sticking to the collection vessel. Nonetheless this technology can be applied to heat-tolerant probiotic strains. O'Riordan et al. (2001) studied the heat effect of spray drying *Bifidobacterium* PL-1 in starch. More cells survived processing in comparison to free cells. This suggests that starch had a protective effect on the encapsulated cells. It was suggested that air temperature of 100°C was best to minimize cell death to less than 1 log, while producing dry microbeads. However, less than one-third of the cells could be recovered using such conditions (O'Riordan et al., 2001).

The oldest method of microencapsulation, extrusion technique, involves pumping a core material-polymer solution (typically alginate) through a syringe or microfluidics system into a hardening solution (calcium ions), resulting on hydrated hydrogel microparticles (Figure 1.1). Technology employing this method has been further developed to optimize consistency and speed at which wet particles can be produced (Brandenberger et al., 1999; Whelehan and Marison, 2011; Seiffert, 2013). Extrusion encapsulation is also inexpensive, but typically used to produce hydrogel particles larger

particle sizes above 1 mm, which can affect sensory perception if added into food products (Rokka and Rantamäki, 2010; Solanki et al., 2013). This is generally controlled by nozzle size and concentration of polymer solution, but viscosity limits these adjustments.

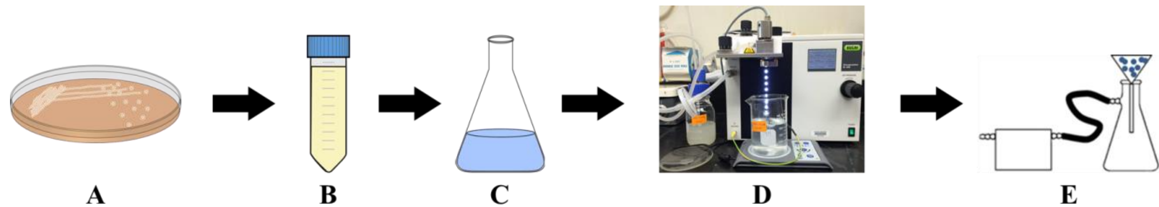

Figure 1.1: Experimental design for immobilizing probiotics in alginate microbeads. (A) An isolated colony of probiotic cell culture is obtained. (B) The isolate is inoculated into growth media and incubated at set temperature. The growth is then centrifuged, and re-suspended to appropriate concentration. (C) The cell suspension is dispersed in sodium alginate solution prior to extrusion. (D) Droplets of cell-polymer solution are sprayed into cross-linking solution, forming hydrogel microbeads. (E) The microbeads are filtered and rinsed before storage.

As its name suggests, emulsion-based encapsulation involves emulsifying core material – polymer solution in a lipid base followed by breaking the emulsion by adding a hydrophilic hardening solution. The beads are subsequently filtered out and washed to remove residual oils (Sheu and Marshall, 1993; Kailasapathy, 2002). This method produces smaller sized particles and can be scaled up, but overall size distribution tends to be high and processing is more costly due to the use of oils and surfactants (Chávarri et al., 2012; Solanki et al., 2013). Residual oil on the microencapsulation particles may also affect nutritional composition and oxidative stability of food products (Kailasapathy, 2002).

1.3.3 Encapsulation particle size

Particle size distribution of microencapsulated beads can affect physical and biochemical acceptability of product. Hansen et al. (2002) encapsulated various *Bifidobacterium* sp. in alginate and exposed them to GI conditions. They observed that alginate beads 1 mm in diameter were unsuitable for incorporation in milk, adversely affecting mouth feel. The ideal size for particles containg probiotics was suggested be around <100 µm in diameter, small enough to minimize texture change and large enough to effectively protect immobilized cells (McMaster and Kokott, 2005; Martín et al., 2015).

Cell metabolism may be altered by particle size which influences diffusion of external factors into the beads. Because of this, off-flavors or other compounds which are atypical of products which use free cells. Hansen et al. (2002) found that yogurt containing encapsulated cells tasted more bitter than yogurt prepared with free cells; they thought that peptide production by the probiotics was the cause. Although this observation has been noted in sensory tests of microbeads in foods, little research has critically analyzed the metabolic change of encapsulated bifidobacteria which results in altered sensory perception (Karimi, 2011). Particle size and cellular stress are important factors of encapsulation technologies which can impact texture and taste perception (Hansen et al., 2002; Burgain et al., 2011).

1.4 Objectives

The purpose of this research was to establish a standardized encapsulation method to compare the survival of free and encapsulated probiotic bacteria during storage and simulated digestion.

The **objectives** were as follows:

1. Track viability of free and encapsulated *Lactococcus lactis* during storage in room temperature conditions.
2. Evaluate variation in survival between several strains of free and encapsulated *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium longum* subsp. *longum* during refrigerated storage and in simulated digestion phases.

CHAPTER 2

MICROENCAPSULATION OF PROBIOTICS IN HYDROGEL PARTICLES: ENHANCING *LACTOCOCCUS LACTIS* SUBSP. *CREMORIS* LM0230 VIABILITY USING CALCIUM ALGINATE BEADS

Published in:

Yeung, T.W., Arroyo-Maya, I.J., McClements, D.J., and Sela, D.A. (2016). Microencapsulation of probiotics in hydrogel particles: enhancing *Lactococcus lactis* subsp. *cremoris* LM0230 viability using calcium alginate beads. Food & Function 7(4), 1797-1804. doi: 10.1039/C5FO00801H.

2.1 Abstract

Probiotics are beneficial microbes often added to food products to enhance the health and wellness of consumers. A major limitation to producing efficacious functional foods containing probiotic cells is their tendency to lose viability during storage and gastrointestinal transit. In this study, the impact of encapsulating probiotics within food-grade hydrogel particles to mitigate sensitivity to environmental stresses was examined. Confocal fluorescence microscopy confirmed that *Lactococcus lactis* were trapped within calcium alginate beads formed by dripping a probiotic-alginate mixture into a calcium solution. Encapsulation improved the viability of the probiotics during aerobic storage: After seven days, less than a two-log reduction was observed in encapsulated cells stored at room temperature, demonstrating that a high concentration of cells survived relative to non-encapsulated bacteria. These hydrogel beads may have applications for improving the stability and efficacy of probiotics in functional foods.

2.2 Introduction

Lactococcus lactis are members of the polyphyletic clade of microorganisms referred collectively as the lactic acid bacteria. *L. lactis* are Gram-positive, low G+C, acid tolerant, non-spore-forming cocci that are often employed in dairy fermentations and are naturally associated with plants (Stark and Sherman, 1935; Price et al., 2012). In addition to food fermentations, *L. lactis* has been employed as a delivery vehicle to secrete interleukin-10 *in situ* in order to reduce colitis in murine (Steidler et al., 2000), porcine (Steidler et al., 2003), and human subjects (Braat et al., 2006). In addition, *L. lactis* has been demonstrated as a versatile platform to deliver vaccines to mucosal tissues (Ramirez et al., 2010). Despite their utility as a genetically malleable delivery vehicle, *L. lactis* is not a typical resident of the human gut microbiome and is confronted with a variety of physicochemical, enzymatic, and biological hurdles while transiting the gastrointestinal tract (GIT). As a consequence, there is typically a major reduction in the number of viable cells reaching the colon after oral ingestion.

In the food industry, probiotic bacteria are often incorporated into functional food or beverage products. To exert their beneficial health effects, the probiotics must remain sufficiently viable throughout the manufacture, transport and storage of a product, as well as during passage through the relatively harsh environment of the GIT. Several studies have previously shown that probiotic preparations containing free cells are highly vulnerable to degradation within food products and during passage through the GIT (Sheu and Marshall, 1993; Sultana et al., 2000; Sun and Griffiths, 2000; de Vos et al., 2010). The required dose of probiotics depends on the bacterial strain and the food application (Sanders, 2008), but relatively high concentrations are sought (e.g. 10^6-10^8

CFU/g) (Ouwehand and Salminen, 1998; Krasaekoopt et al., 2003; Talwalkar and Kailasapathy, 2003; Roy, 2005). The high susceptibility of probiotics to degradation means that effective encapsulation strategies are required to protect them within foods and the upper GIT, but then release them within the colon where they can exert their beneficial effects.

Numerous approaches can be used to encapsulate probiotics based on different physicochemical and mechanical methods (Kailasapathy, 2002; Anal and Singh, 2007; Rokka and Rantamäki, 2010). Extrusion encapsulation is one of the most simple and effective methods of encapsulation probiotics (Krasaekoopt et al., 2003). This approach usually involves encapsulating bacterial cells within hydrogel particles fabricated from food-grade biopolymers (Kosaraju, 2005). The probiotic-loaded hydrogel particles are formed using a number of steps: (*i*) the probiotic bacteria are dispersed within an aqueous biopolymer solution that is capable of being gelled: (*ii*) this mixture is extruded into a gelling environment through a small nozzle to form small biopolymer droplets with bacteria inside; (*iii*) the biopolymer droplets are then stabilized against dissociation or aggregation using gelation and/or coating methods. The resulting hydrogel particles can then be collected, washed, and dried. This process produces a powdered form of the probiotic cells that can be conveniently incorporated into functional foods (Conway et al., 2001; Lian et al., 2003; Oliveira et al., 2007), while maintaining the viability of the bacterial cells (Trindade, 2000; Favaro-Trindade and Grosso, 2002; Baur and Sinclair, 2006; Liserre et al., 2007; Oliveira et al., 2007; Shima et al., 2009; Thantsha et al., 2009). If the biopolymers used are indigestible within the upper GIT (*i.e.,* dietary fibers) and maintain their physical integrity, then the hydrogel particles may retain and protect the

bacteria until they reach the colon where they are released when the biopolymer matrix is digested by colonic bacteria. Few encapsulation studies have specifically investigated *L. lactis* as its probiotic function may best lie in its ability to deliver heterologous bioactive molecules *in situ* rather than direct interactions with microbiota or their host (Champagne et al., 1992; Morin et al., 1992; Groboillot et al., 1993; Hyndman et al., 1993; Klinkenberg et al., 2001; Divya and Nampoothiri, 2015). Thus the lactococcal cell represents the primary vehicle with the single-layer biopolymer encapsulant the secondary shield.

Alginate is one of the most widely used food-grade biopolymers for encapsulation purposes as it is a naturally-occurring polysaccharide that is non-toxic to both probiotic cells and humans (Gudmund, 2006; Quignard et al., 2008)). Moreover, alginate forms hydrogel matrices around bacterial cells using mild processing conditions that promote cellular integrity (Morin et al., 1992; Zhou et al., 1998; Divya and Nampoothiri, 2015). Typically, the bacteria are mixed with an alginate solution that is then injected into an aqueous calcium solution, which leads to the formation of probiotic-loaded calcium alginate beads. In this case, the alginate molecules were physically cross-linked by electrostatic bridging between anionic carboxyl groups on the alginate molecules and the cationic divalent calcium ions. Previous studies have shown that hydrogel particles can preserve probiotic cells over time when stored under wet conditions or when exposed to simulated GIT fluids (Morin et al., 1992; Groboillot et al., 1993; Divya and Nampoothiri, 2015). However, there has been little research on the preservation of probiotics under dry conditions, which may be important for future commercial application of lactococcal-

based probiotics in a variety of food matrices. The aim of this study was therefore to investigate the protection of encapsulated *L. lactis* cells under dry aerobic conditions.

2.3 Materials and methods

2.3.1 Bacteria propagation and general growth conditions

Lactococcus lactis subsp. cremoris LM0230 were routinely stored at -80 °C in deMan, Rogosa, Sharpe (MRS) broth (Oxoid, Basingstoke, Hampshire, UK) enriched with 50% glycerol (Sigma, St. Louis, MO, USA). The bacteria were grown in MRS broth at 37 °C for 22 h, and maintained on MRS agar (Oxoid). Anaerobic conditions were maintained using a double chamber anaerobic hood with airlock (82% N_2, 10% CO_2 and 7% H_2; Coy Laboratory products, Grass Lake, MI, USA). Cells were harvested by centrifugation at 4000 *g* for 15 min followed by separation from the supernatant.

2.3.2 Probiotic microencapsulation

L. lactis cells were encapsulated by means of ionotropic gelation using an automated encapsulation instrument with a vibrating extrusion nozzle. 96 mL of 1% (w/v) alginate solution (HG400, TIC Gums, Belcamp, MD, USA) was prepared and autoclaved. The sterile alginate solution was mixed with 4 mL of ~10^9 CFU/mL probiotic bacteria that had been concentrated in sterile 0.85% sodium chloride solution (Fisher, Fair Lawn, NJ, USA). The polymeric matrix was agitated to uniformly distribute cells throughout the mixture. The mixture was left to stand for 5 min to allow dissolved air to leave the solution prior to bead preparation using an automated encapsulation device (Büchi B-390 Encapsulator, Flawil, Switzerland) with a nozzle size of 120 µm, as per the manufacturer’s standard operating conditions (frequency 800 Hz, electrode 800 V, pressure 300 mbar). The beads were collected into 300 mL of 0.1 M calcium chloride

solution (Sigma, St. Louis, MO, USA). After 1 or 24 hours under continuous agitation to allow cross-linking, the beads were vacuum-filtered, rinsed with sterile deionized water (200 mL), and then filtered again. The resulting samples were stored aerobically in sterile Petri dishes at 24-26 °C for up to 4 weeks to model long-term storage conditions.

Unfilled alginate beads were prepared in parallel, with the exception of not adding *L. lactis* to the alginate solution. 100 mL of 1% alginate solution was extruded into 300 mL of 0.1 M $CaCl_2$ solution under continuous agitation. The working parameters (nozzle diameter, frequency, charge and pressure), filtering steps and storage conditions used were the same as those for the preparation of filled alginate beads.

2.3.3 Determination of calcium alginate bead size distribution

The particle size distribution was determined by static light scattering (Mastersizer S, Malvern Instruments, Worcestershire, UK). Samples were diluted and re-suspended in water (1 mL beads in 10 mL) to avoid multiple scattering effects and to ensure they were homogeneous prior to analysis. Volume-based (D [4,3]) and surface-based (D [3,2]) mean diameter measurements were obtained for all samples.

2.3.4 Optical microscopy

The overall appearance of filled and unfilled calcium alginate beads was inspected and characterized by optical microscopy. A microscope (C1 Digital Eclipse, Nikon, Tokyo, Japan) with 20, 40 and 60× objective lenses was used to capture images of the beads. Alginate suspensions were vortexed to separate individual beads. All optical images were taken using a digital camera and then characterized using the instrument software (EZ CSI version 3.8, Nikon).

2.3.5 Scanning electronic microscopy (SEM)

The microstructure of the beads was also characterized using a scanning electron microscope (JCM-6000 NeoScope Benchtop SEM, Jeol, Tokyo, Japan). To prepare the samples prior to analysis, calcium alginate beads were air-dried at room temperature for at least 24 hours on Petri dishes. Subsequently, the beads were spattered with 10 nm gold and mounted on an aluminum stud, which was loaded into the microscope. Images of the calcium alginate beads were recorded in randomly selected fields.

2.3.6 Confocal scanning laser microscopy (CLSM)

To locate and visualize *L. lactis* cells within the alginate capsules it was necessary to label the cells with a dye (acridine orange). Briefly, an aliquot of the sample containing alginate beads was suspended in a solution of 0.1% acridine orange hemi (zinc chloride) salt (Sigma, St. Louis, MO, USA) for 30 min to stain the microorganisms (i.e. DNA). Excess fluorochrome was removed by washing (3×). The samples were drop-casted onto glass slides and were observed using a confocal laser-scanning microscope (C1 Digital Eclipse, Nikon, Tokyo, Japan) with an excitation and emission wavelength of 455 and 490 nm respectively.

2.3.7 Enumeration of viable cells

To determine viable counts of encapsulated bacteria, 0.1 g of alginate beads was re-suspended in 9.9 mL of 10% sodium citrate dihydrate solution (pH 8.2; Fisher, Fairlawn, NJ, USA) followed by vortexing for 10 minutes at room temperature. The number of released cells was determined by plate count on solid agar, serial dilutions of dissolved beads (10^2-10^7) were plated in duplicate and incubated at 37 °C anaerobically for 40 h. Samples were taken once per week over a four-week period. The initial cell

count (before bead formation) was determined by plating serial dilutions (10^5 -10^8) in duplicate of the starting alginate-bacteria mixture before gelation on MRS agar. These were incubated at 37° C under anaerobic conditions for 24 h followed by cell enumeration.

Free cells (non-encapsulated bacteria) were also maintained under aerobic conditions at 24-26°C to compare with encapsulated cells. Survival of free cells was determined using a spread plate count on MRS agar. Dried cell pellets were re-suspended in 50 mL sterile distilled water and vortexed. Serial dilutions (10^0-10^8) of the cell suspension were sampled in triplicate and incubated at 37°C anaerobically for 40 h followed by enumeration. Samples were taken every other day over a two-week period.

2.3.8 Statistical analysis

The mean of two or three individual determinations was used to calculate cell counts and particle size. Cell counts and particle size distributions were analyzed using 2-sample t-test (α=0.05). Significant differences among individual means were determined using Tukey's HSD test.

2.4 Results

Mean particle diameters and particle size distributions of alginate beads prepared with and without *L. lactis* cells using 1 or 24-hour gelation periods were compared with each other (Table 2.1, Figure 2.1). After 1 hour incubation in the calcium bath, the probiotic-loaded alginate beads had a relatively wide particle size range (diameter from 7 to 480 µm) with an average diameter of 161 ± 107 µm. After 24 hour incubation, the particle size increased slightly but not significantly, with a range from 7 to 650 µm and an average diameter of 188 ± 132 µm in diameter. These values were not significantly

different from the unloaded alginate beads prepared in the absence of probiotics, which had average particle diameters of 130± 94 and 155 ±116 μm for 1 and 24 hour gelation respectively. Thus, the presence of the bacterial cells in the initial alginate solution did not significantly increase average bead size.

Table 2.1: Mean particle diameters of alginate beads prepared with and without *Lactococcus lactis* cells for two gelation time periods. Averages were determined by static light scattering. Values are shown as volume-based (D [4,3]) or surface-based (D [3,2]) mean ± standard deviation.

Beads	**D [4,3]**	**D [3,2]**
	μm	
No cells, 1 h gelation	198 ± 94[a]	130 ± 94[b]
No cells, 24 h gelation	241 ± 94[a]	155 ±116[b]
Cells, 1 h gelation	232 ± 89[a]	161 ±107[b]
Cells, 24 h gelation	281 ± 119[a]	188 ±132[b]

Optical micrograph images of free cells, immobilized cells, and unfilled alginate beads were captured for comparison (Figure 2.2). The free cells were coccoid shaped and densely clustered. Similar cell morphology was observed for the probiotics trapped within the filled alginate beads. Unfilled beads did not contain any visible microorganisms. Fresh beads appeared spherical, but flattened into irregularly shaped granules as they dried as seen in SEM analysis. Beads gelled for 1 and 24 hours did not differ morphologically regardless of hydration status.

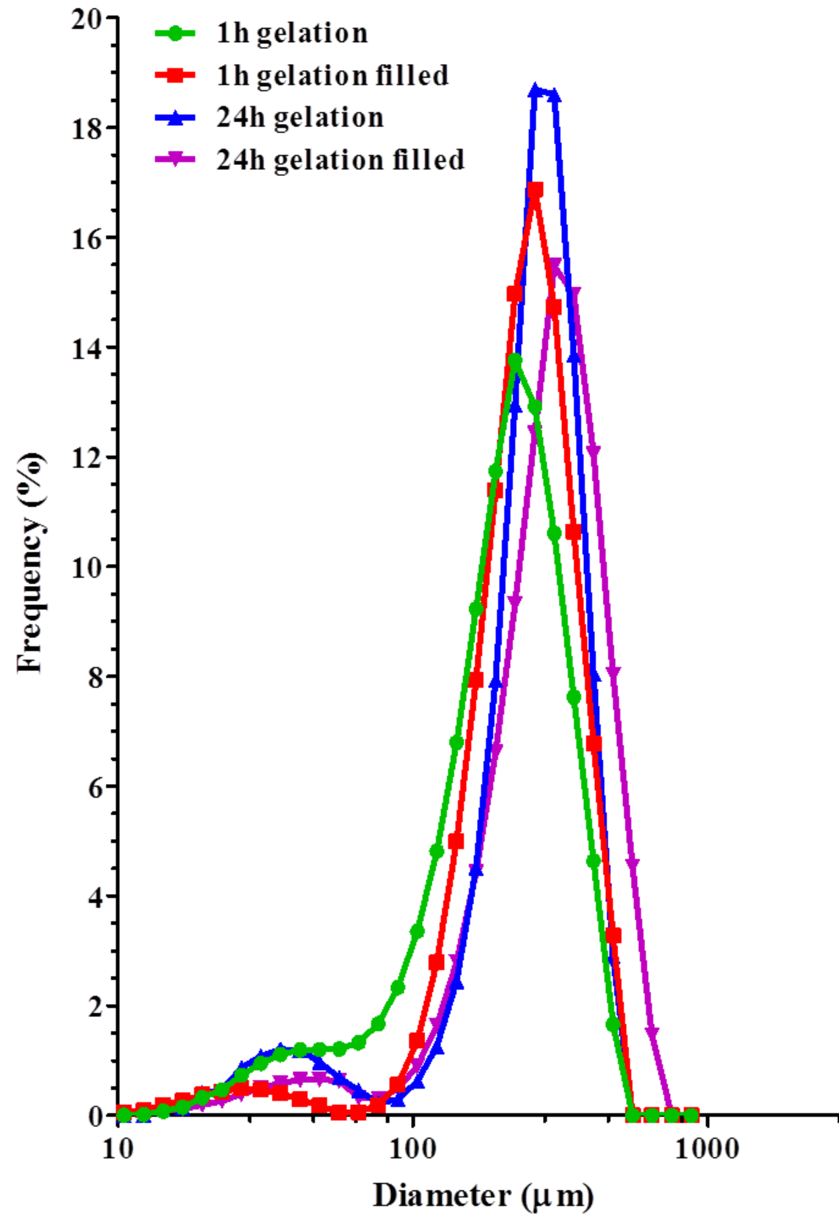

Figure 2.1: Particle size distribution of alginate beads prepared with and without *Lactococcus lactis* cells for two gelation time periods. Fresh samples were analyzed by static light scattering. Samples include beads that lacked or contained *L. lactis* and gelled for 1 or 24 hours.

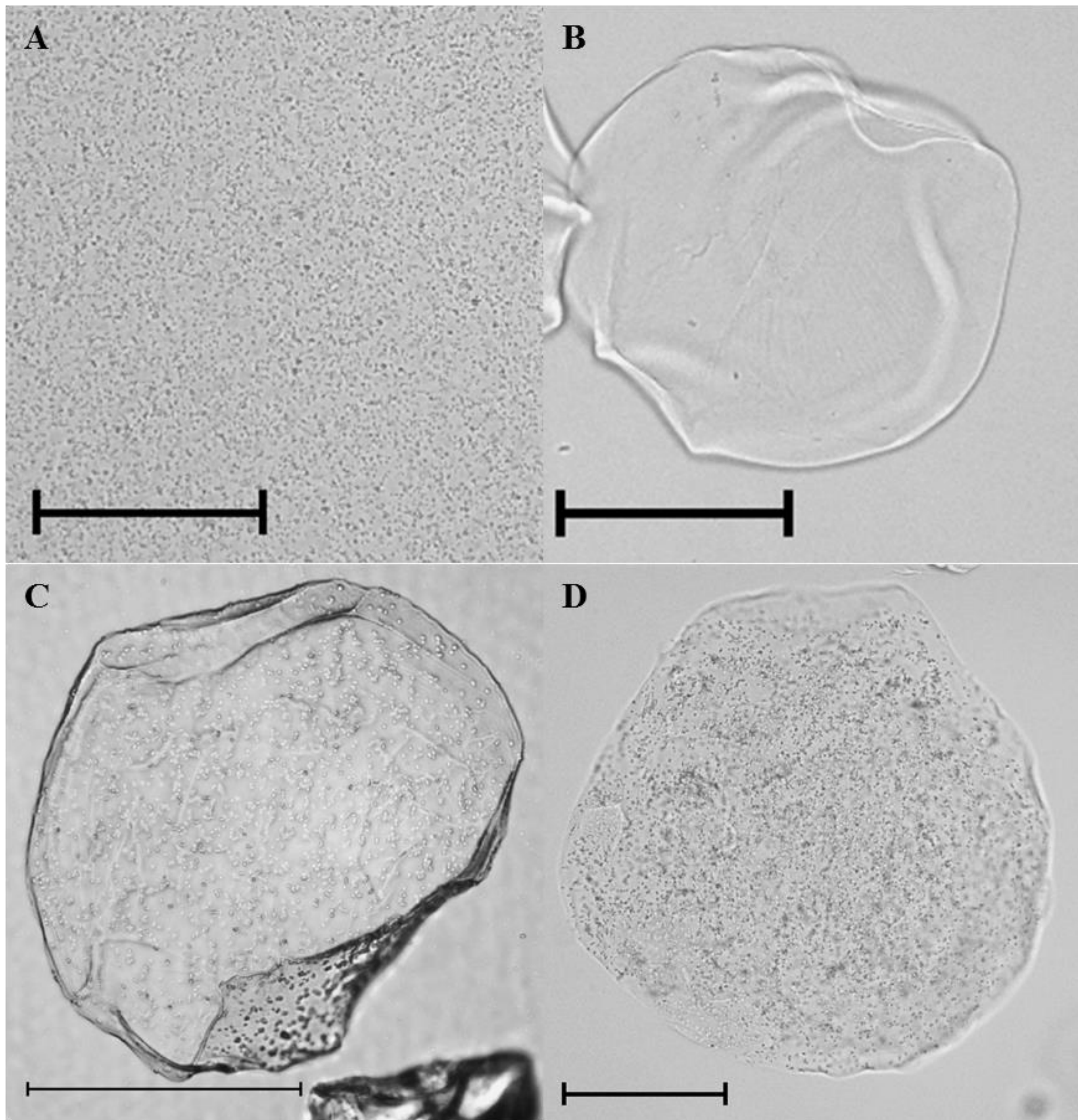

Figure 2.2: Optical microscope images of (A) *Lactococcus lactis* LM0230 (40×), (B) unfilled alginate microbead (40×), and filled alginate beads gelled for (C) 1 hour (40×) and (D) 24 hours (40×). All scale bars represent 100 µm.

Scanning electron micrographs also showed the presence of coccoid cells that were around 1 to 2 µm in length protruding from filled alginate beads (Figure 2.3). The unfilled alginate beads lacked these protrusions, consistent with the lack of bacterial cells incorporated in the preparation. Alginate beads gelled for 1 and 24 hours lost their

spherical structure during desiccation and exhibited irregular shapes with pronounced wrinkles and folds.

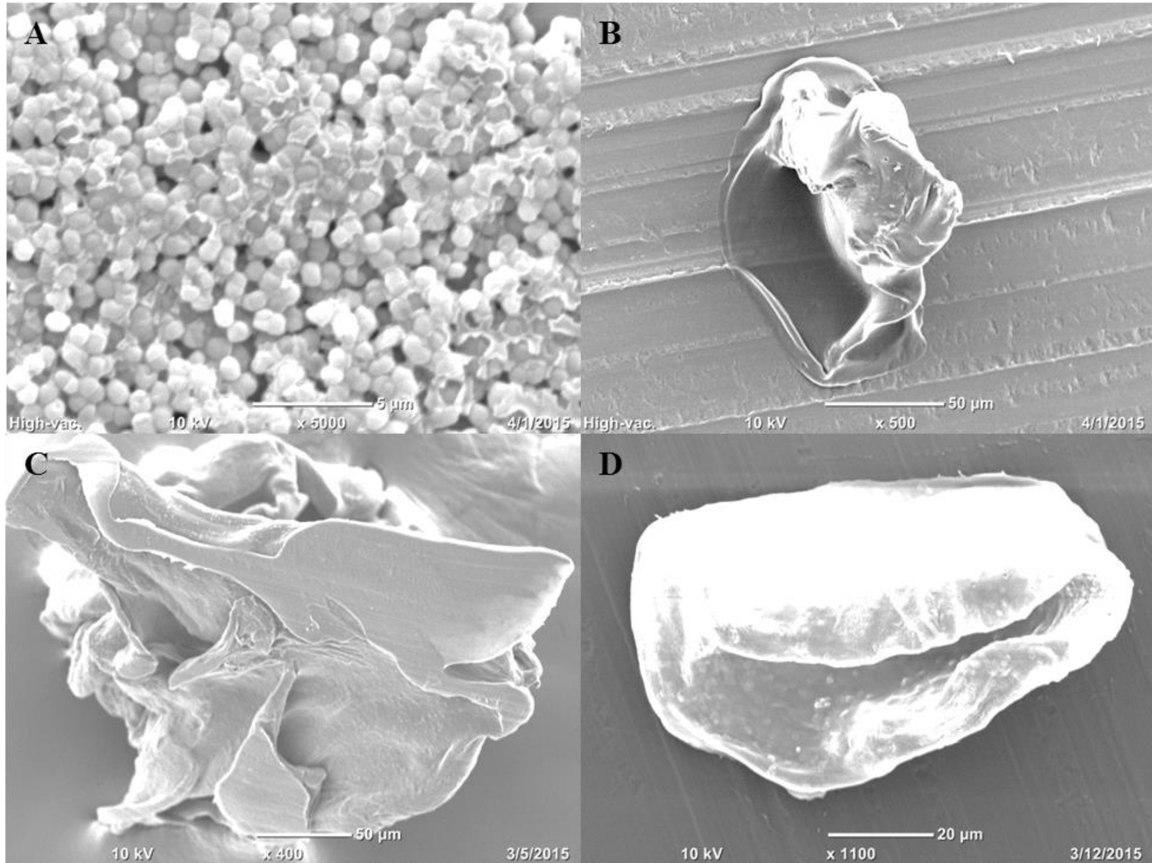

Figure 2.3: Scanning electron microscope images of (A) *Lactococcus lactis* LM0230 (5000×), (B) unfilled alginate microbead (500×), and filled alginate beads gelled for (C) 1 hour (400×) and (D) 24 hours (1100×). Samples were dried before sputter-coating with gold. SEM was set at high-vacuum, 10 kV.

Confocal microscopy was also employed to detect the location of the bacteria within the hydrogel beads, by using acridine orange, which intercalates into bacterial DNA (Figure 2.4). The free cells interacting with the stain produced green fluorescence. As expected, empty microbeads did not fluoresce brightly due to the absence of bacteria. In contrast, bacteria encapsulated in alginate beads provided strong fluorescence concomitant with the presence of cells.

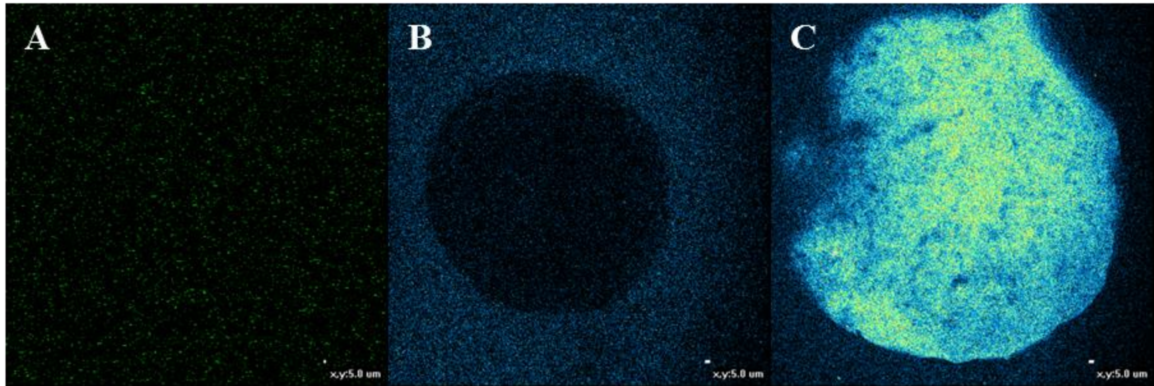

Figure 2.4: Confocal microscope images of (A) *Lactococcus lactis* LM0230, (B) unfilled alginate microbead, and (C) filled alginate beads. Samples were stained with acridine orange fluorescent dye to detect DNA. All scale bars represent 5 µm.

The total cell population decreased significantly over several weeks for all samples (Figure 2.5, Table A.1). After 1 week, the average cell population showed a 1.5 log reduction from beads gelled for 24 hours and a 1.7 log reduction for those gelled for 1 hour. In contrast, a 5.4 log reduction occurred over the same time for free cells. After about two weeks, a 3.1 and 4.1 log reduction was shown for 24 and 1 hour gelled beads respectively. The decrease in cell viability over time was not significantly different between beads gelled for 1 hour and 24 hours.

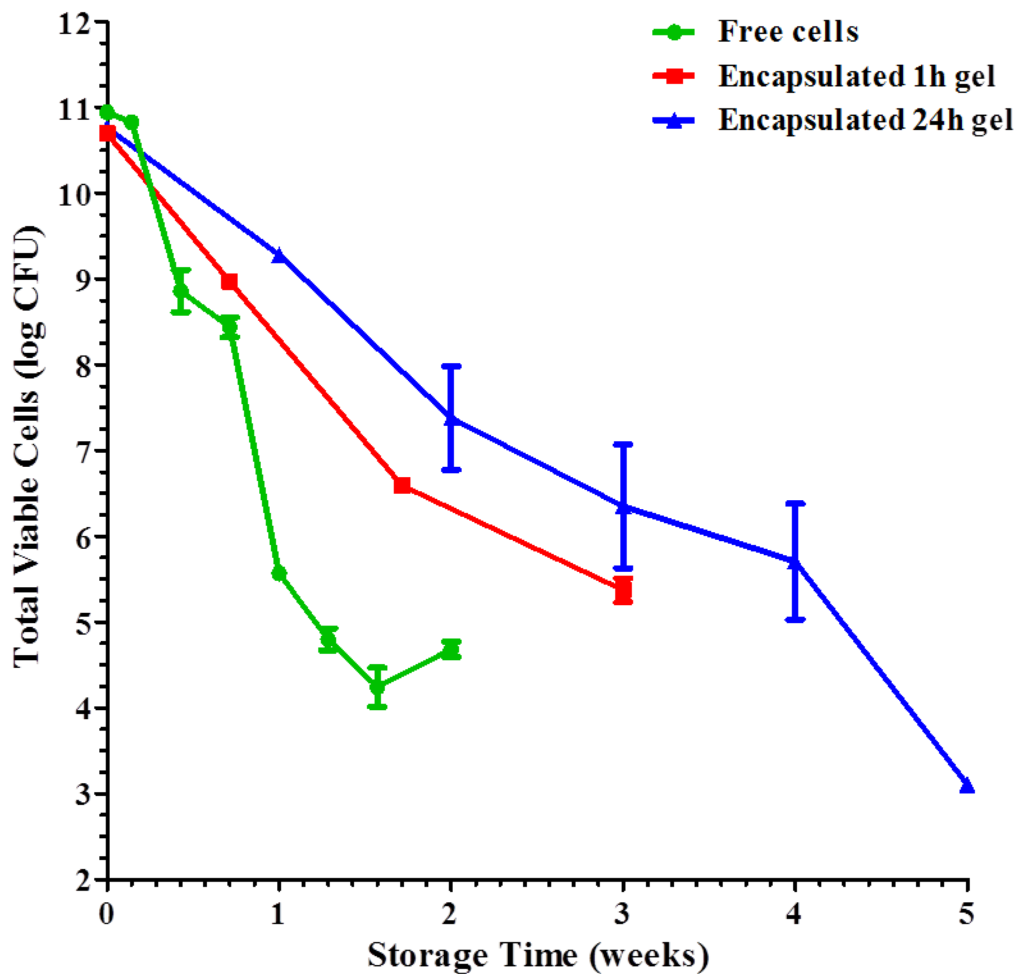

Figure 2.5: Survival of free and encapsulated *Lactococcus lactis* LM0230 in calcium alginate beads during dry room temperature storage. Counts are based on samples spread-plated on MRS and incubated at 37°C anaerobically. Error bars represent standard error of replicate samples.

2.5 Discussion

The encapsulation method used produced alginate beads that were able to protect live *L. lactis* cells against loss of viability over several weeks. Previous studies have not reported particle size distributions of *L. lactis* encapsulated solely within calcium alginate beads. Nevertheless, they have been reported for other types of hydrogel particles. For example, Groboillot et al. (1993) reported that *L. lactis* encapsulated within beads

prepared using 2% chitosan using an emulsion-templating method yielded beads with mean diameter of 0 µm (Groboillot et al., 1993). Whereas Hyndman et al. (1993) encapsulated bacteria within beads prepared using 24% gelatin in a similar method that had mean diameters around 124 µm (Hyndman et al., 1993). The dimensions of the beads produced may be important for a number of reasons. First, it may influence the effectiveness of the hydrogel beads to maintain the viability of the probiotics. Second, it may influence the physical stability of the beads within food products, *e.g.,* to sedimentation, flocculation, and coalescence. Third, it may influence the physicochemical properties of food products that the beads are incorporated into, such as rheology and appearance. Finally, it may influence the sensory perception of a functional food product, as large beads may lead to a grainy mouthfeel. Future studies in our laboratory will therefore examine the influence of particle dimensions on the functional performance of hydrogel beads loaded with *L. lactis* cells.

The optical and electron microscopy images confirmed that the probiotic bacteria were trapped inside of the calcium alginate beads, and that the bacteria had dimensions of about 1 to 2 µm in length. These results are consistent with previous microstructure studies of encapsulated bacteria (Hansen et al., 2002; Fareez et al., 2015). Alginate alone is able to encapsulate *L. lactis* by cross-linking via calcium ions although the beads formed typically have a relatively low mechanical stability and are sensitive to chelating compounds such as phosphate, citrate, EDTA and lactate, or anti-gelling cations such as Na^+ and Mg^{2+} (Willaert, 1996). Such factors may be found in bioprocessing environments or within the gastrointestinal tract (Wyss et al., 2004). For this reason, it may be necessary to improve the encapsulation process by blending the alginate with other

polymers or by adding additional coats to the beads (Krasaekoopt et al., 2003). There are a number of important criteria for any successful delivery system for *L. lactis:* the probiotics should initially be located within the interior of the hydrogel beads; the beads should provide an internal environment that protects the bacteria from degradation; the beads should maintain their physical integrity throughout manufacture, transport, and storage, and well as during passage through the GIT; the bacterial cells should be released after arrival in the colon.

A medium viscosity sodium alginate was used for these experiments to prepare the calcium alginate beads. We also investigated the use of a low viscosity sodium alginate, although we observed poor performance, *i.e.* the beads were too fragile to handle properly (data not shown). Beads stored in dry aerobic (room temperature) conditions contained viable cells for over 4 weeks representing a significant improvement over the non-encapsulated (free) cells. The total cell count fell below 10^6 CFU/g after 3 weeks indicating a considerable stability relative to the control conditions and typical of handling lactococcal strains in the laboratory. Ultimately the diffusion of oxygen as well as slow drying may have caused the eventual decrease in viable counts as expected. The cell count model used assumes a uniform concentration of cells throughout the samples. However, heterogeneous exposure to the atmosphere in combination to irregular bead shape could cause variation of cell viability within beads, which will be explored in future studies. Previous studies have stored microbeads as freeze-dried samples (Lee et al., 2004; Chávarri et al., 2010; Kanmani et al., 2011), as hydrated samples at refrigeration temperatures (Sheu and Marshall, 1993; Sultana et al., 2000), or as frozen samples (Amine et al., 2014b), before incorporation into a food product or performing

microbial count analysis. These storage methods maintained the spherical shape of the original alginate beads, but have the disadvantage that greater energy needs were required to prepare or store the samples.

Drying has been previously shown to have a deleterious effect on probiotic viability (Efiuvwevwere et al., 1999). *L. lactis* was previously shown to undergo a 3-4 log reduction after 3 days when dried at 20 and 30 °C (Efiuvwevwere et al., 1999). In contrast, our encapsulated samples underwent a 3-4 log reduction after 2 weeks. It should be noted that their study used *L. lactis subsp. lactis*, started with lower concentration of cells and may have had a few variations in methodological parameters.

Increasing the gelation time from 1 hour to 24 hours did not add statistically significant protection to immobilized cells, although the general trend was increased viability. The maturation kinetics of calcium alginate beads (unfilled) was previously characterized, and it was noted that the density of the beads decreased over time, then levelled off after about 6 hours (Puguan et al., 2014). This suggests that syneresis occurred, producing drier beads with increased gelation time. This in turn may influence drying rate, and alter cell viability. The effect of gelation time on cell viability has not been previously explored and remains a target parameter to optimize in future studies.

In summary, we demonstrated that encapsulation of *L. lactis* in calcium alginate microbeads shielded the microbes from stresses encountered during drying conditions. Dehydration of the samples caused the alginate beads to adopt irregular-shaped structures, but the viability of the microbial cells was greatly increased compared to non-encapsulated cells. In the future, preserving spherical morphology of the beads may be

achieved by incorporating other biopolymers into the coating material to potentially increased strength and decrease porosity.

2.6 Acknowledgements

We thank Jean Alamed and various individuals at the University of Massachusetts for their technical assistance and helpful conversations concerning this project. IA thanks the Consejo Nacional de Ciencia y Tecnología (CONACYT) in Mexico for funding support (registration number 208139).

CHAPTER 3

MICROENCAPSULATION IN ALGINATE AND CHITOSAN MICROGELS TO ENHANCE VIABILITY OF *BIFIDOBACTERIUM LONGUM* FOR ORAL DELIVERY

Published in:

Yeung, T.W., Üçok, E.F., Tiani, K.A., McClements, D.J., and Sela, D.A. (2016). Microencapsulation in Alginate and Chitosan Microgels to Enhance Viability of *Bifidobacterium longum* for Oral Delivery. Frontiers in Microbiology 7. doi: 10.3389/fmicb.2016.00494.

3.1 Abstract

Probiotic microorganisms are incorporated into a wide variety of foods, supplements, and pharmaceuticals to promote human health and wellness. However, maintaining bacterial cell viability during storage and gastrointestinal transit remains a challenge. Encapsulation of bifidobacteria within food-grade hydrogel particles potentially mitigates their sensitivity to environmental stresses. In this study, phylogenetically related *Bifidobacterium longum* subspecies and strains were encapsulated in core-shell microgels consisting of an alginate core and a microgel shell. Encapsulating obligate anaerobes *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium longum* subsp. *longum* strains showed little difference in viability over time, suggesting minimal divergence in stress response programs. This includes viability under aerobic storage conditions and modeled gastrointestinal tract conditions. Coating alginate microgels with chitosan did not improve viability compared to cells encapsulated in alginate microgels alone; this suggests that modifying the surface charge alone does not enhance viability. Thus hydrogel beads have great potential for improving the stability and efficacy of bifidobacterial probiotics in various nutritional interventions.

3.2 Introduction

Beneficial bacteria are often incorporated into functional foods and nutritional interventions to be ingested orally as probiotics. This includes humans and livestock animals that receive direct-fed microbials to enhance health outcomes and reduce pathogen load (Braat et al., 2006; Puccio et al., 2007; Neal-McKinney et al., 2012; Watson and Preedy, 2015). *Bifidobacterium longum* colonizes the human gastrointestinal tract (GIT) and is one of the 48 recognized taxa that are encompassed within the genus *Bifidobacterium* (Garrido et al., 2013; Sun et al., 2015). This obligate anaerobe is one of the earliest colonizers of the infant GIT, and is present in lower concentrations in the adult gut (Schell et al., 2002; Sela et al., 2008). The bifidobacterial taxa *longum, infantis,* and *suis* were previously classified as discrete species, but more recently they were reclassified as subspecies of *Bifidobacterium longum* (Sakata et al., 2002). Their unification as a single species is based primarily on genomic and phenotypic similarities shared between these groups.

B. longum is deployed in several probiotic applications using a variety of delivery formats (Adhikari et al., 2000; Fortin et al., 2011; Amine et al., 2014a; Lewis et al., 2015). A relatively large dose of probiotics is recommended to impart health benefits, typically 10^6-10^7 CFU/g per day (Krasaekoopt et al., 2003; Roy, 2005). However, the direct incorporation of free probiotic cells into food products and supplements results in a significant decrease in cell viability throughout storage and gastrointestinal transit (Sultana et al., 2000; de Vos et al., 2010). Therefore, prolonged storage and the process of ingesting these probiotics may reduce their viability below recommended levels to achieve health benefits. Microencapsulating probiotic cells within hydrogel matrices

protects them against extrinsic environmental factors thereby enhancing bacterial survival during processing, storage, and digestion (de Vos et al., 2010; Fareez et al., 2015; Yeung et al., 2016a). Encapsulation may also dictate the controlled release of the probiotic at the precise anatomical site of activity within the gastrointestinal tract, thereby enhancing the efficacy of the probiotic through specific targeting after oral delivery (de Barros et al., 2015; Zhang et al., 2015b).

Several biopolymer materials are available to encapsulate microbes in hydrogel matrices, depending on the desired physicochemical properties of the delivery vehicle. The most commonly used food-grade biopolymers are proteins (*e.g.,* whey proteins and caseins) and carbohydrates (*e.g.,* starch and gums) (Bagchi et al., 2010; Gaonkar et al., 2014; Etchepare et al., 2015). For many food applications, it is advantageous to encapsulate probiotics within hydrogel beads that trap bacteria within small particles containing cross-linked biopolymer molecules. These microgels must be engineered to encapsulate high concentrations of probiotics and protect them from environmental stresses, such as acidic pH, bile salts, and digestive enzymes (Zhang et al., 2015a). Alginate has been widely used as a biopolymer suitable for food applications as it is relatively inexpensive, easy to gel, biodegradable, and compatible with many food systems (Gombotz and Wee, 2012; Lee and Mooney, 2012). Indeed, recently studies have shown that lactococcal-based probiotics can be encapsulated within alginate microgels to improve their stability (Yeung et al., 2016a).

There are appreciable differences between probiotic strain tolerance towards environmental and gastrointestinal stresses. Consequently, it is possible to identify particular strains that are more resistant to these stresses than others, which are therefore

more suitable for commercial application (Godward, 2000; Krasaekoopt, 2004; Capela et al., 2006). As an anaerobe, bifidobacteria are sensitive to oxygen exposure and to other environmental stresses during the preparative phase prior to probiotic deployment. Therefore, bifidobacterial probiotics may be encapsulated to restrict oxidative damage during preparation and storage and to limit exposure to degradative processes within the gastrointestinal tract.

The aim of this study was to design, fabricate, and characterize a food-grade encapsulation system to protect *B. longum* cells during simulated storage and gastrointestinal passage. Previously, we demonstrated that encapsulation of probiotics within alginate microgels could improve their viability during storage (Yeung et al., 2016a). In the current study, we encapsulated *Bifidobacterium longum* cells within alginate beads to determine if their viability could be enhanced in storage and gastrointestinal transit. Moreover, the impact of coating these alginate beads with a layer of chitosan was investigated as well. Chitosan coated alginate beads have previously been used to enhance the mucoadhesive properties of probiotic bacteria (Chen et al., 2013).

3.3 Materials and methods

3.3.1 Preparation of bacterial cultures

Four strains of both *Bifidobacterium longum* subsp. *longum* (*B. longum*) and *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) were studied (Table 3.1). Stock solutions were maintained by storing bacteria at -80 °C in deMann, Regosa, Sharpe media with 0.05% L-cysteine in 25% glycerol. Bacteria were propagated in MRS with L-cysteine (MRSC) at 37°C for 24 h, checked for purity, and maintained on MRSC agar anaerobically. Anaerobic conditions were maintained in a double chamber anaerobic

hood with an airlock (88% N_2, 10% CO_2 and 2% H_2) from Coy Laboratory Products (Grass Lake, Mississippi, USA).

Table 3.1: *Bifidobacterium longum* strains selected for encapsulation. Four strains are classified under subspecies *infantis*, and the other four strains are classified under subspecies *longum*.

Subspecies	Strain designation
infantis	UMA 298
	UMA 299
	UMA 300
	UMA 305
longum	UMA 306
	UMA 318
	UMA 401
	UMA 402

Isolated colonies were routinely propagated in MRSC broth (50 mL) for 40 hours at 37° C. Cells were harvested by centrifugation at 4000 *g* for 10 min, washed twice with 0.85% NaCl (physiological saline) solution (25 mL), and suspended in 0.85% NaCl (2 mL). The resulting cell suspensions were used either directly for assessing survival of free cells (*i.e.* no encapsulation) or subjected to encapsulation as described in section 2.3. Free cell suspensions (2 mL) were stored in 0.85% NaCl solution (50 mL) at 2-5 °C for up to five weeks to model long-term storage conditions.

3.3.2 General chemicals used in encapsulation and modeled digestion

For bacterial culture preparation, MRS broth was obtained from Becton Dickinson and Company (Sparks, Maryland, USA). Agar, L-cysteine hydrochloric acid, and sodium chloride (NaCl) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Glycerol and sodium citrate dihydrate was purchased from Fisher Scientific (Fair Lawn, New Jersey, USA).

For encapsulation experiments, sodium alginate (TICA-algin® HG 400 powder) was donated by TIC Gums (White Marsh, Maryland, USA). Calcium chloride hexahydrate, chitosan (medium molecular weight) was obtained from Sigma Aldrich. Glacial acetic acid was purchased from Fisher Scientific.

For simulated digestion, ammonium nitrate, bile extract porcine, lipase from porcine pancreas type II, pepsin from porcine gastric mucosa, porcine gastric mucin type II, potassium chloride, potassium citrate, potassium phosphate, sodium DL-lactate, sodium hydroxide (NaOH), and uric acid sodium salt were also purchased from Sigma Aldrich. Hydrochloric acid (HCl), phosphate buffer saline (PBS), and urea were purchased from Fisher Scientific.

3.3.3 Microencapsulation of bifidobacterial cells

Bifidobacteria were encapsulated within alginate microgels using an injection-gelation method (Whelehan and Marison, 2011; Seiffert, 2013). Briefly, 1% (w/v) sodium alginate solution was prepared, autoclaved, and then cooled to ambient temperature. The sterile alginate solution (198 mL) was mixed with 2 mL of ~10^9 CFU/mL probiotic organisms suspended in physiological saline. The polymeric solution was agitated to uniformly distribute cells throughout the mixture. The alginate beads were prepared aseptically using an encapsulator (Büchi B-390®, Büchi Labortechnik AG, Flawil, Switzerland) with a nozzle size of 120 µm, using the manufacturer's standard operating conditions (amplitude 3, frequency 800 Hz, electrode 800 V, pressure 250-300 mbar). Aliquots of probiotic/alginate solution were injected into 0.1 M calcium chloride solution (350 mL). After 1-hour gelation under agitation, the resulting calcium alginate beads were collected by filtration, rinsed with sterile deionized water (200 mL), and re-

filtered. Microbeads (~30 mL) were stored in physiological saline solution (50 mL) at 4° C for up to 4 weeks to model long-term storage conditions. This process was repeated for all eight strains of bifidobacteria.

Unfilled alginate beads were prepared identically but without the addition of bacterial strains to the alginate solution. 1% alginate solution (200 mL) was extruded into of 0.1 M $CaCl_2$ (350 mL) solution under continuous agitation. The working parameters (nozzle diameter, frequency, charge and pressure), filtering steps and storage conditions used were the same as those for the preparation of filled alginate beads.

An aqueous chitosan solution (0.4% w/v) was prepared as described previously by Zhou et al. (1998). Briefly, chitosan (0.4 g) was dissolved in distilled water (90 mL) and glacial acetic acid (0.8 mL). The pH was adjusted to 5.0-5.1 with NaOH, and the total volume was adjusted to 100 mL. The solution was autoclaved and filtered to remove undissolved solids. Subsequently, the alginate beads were submerged in the chitosan solution to provide a secondary coating by electrostatic attraction of the cationic chitosan molecules to the surfaces of the anionic alginate beads. The mixture was agitated for one hour before filtering and rinsing beads with sterile distilled water. Chitosan-coated alginate beads were then stored and analyzed.

3.3.4 Particle size distribution

The particle size distribution was determined by static light scattering (Mastersizer S, Malvern Instruments, Worcestershire, UK). Each sample (1-2 mL) was suspended in distilled water (10 mL) and vortexed to avoid multiple scattering effects and to ensure homogeneity prior to analysis. Volume-weighted (D [4,3]) and surface-weighted (D [3,2]) mean particle diameters were obtained for all samples.

3.3.5 Optical microscopy

The overall appearance of alginate and chitosan-coated alginate beads was characterized with an optical microscope (C1 Digital Eclipse, Nikon, Tokyo, Japan). Microgel suspensions (1-2 mL) were immersed in physiological saline (10 mL) and vortexed to separate individual beads. Optical images were obtained using a digital camera and further analyzed using the instrument software (EZ CSI version 3.8, Nikon).

3.3.6 Scanning electronic microscopy (SEM)

The bead microstructure was characterized using a bench-top scanning electron microscope (JCM-6000 NeoScope, JEOL, Tokyo, Japan). To prepare the samples prior to analysis, alginate and chitosan-coated alginate beads were freeze-dried and sputter-coated with gold (10 nm) before loading onto the microscope. Images of the microgels were documented in representative fields.

3.3.7 Electrical properties

The surface potential (ζ-potential) of alginate and chitosan-coated alginate microgels was evaluated by electrophoretic light scattering (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK). For each sample, refrigerated microgels (1-2 mL) were suspended in distilled water (10 mL) and vortexed to separate the beads. Samples were then loaded into the measurement cells and analyzed.

3.3.8 Modeled long-term storage conditions of encapsulated bifidobacteria

Total cell counts of free and encapsulated bifidobacteria were determined by a modified drop plate method as previously described (Herigstad et al., 2001). Briefly, 10 drops (10 μL) of a dilution within a series (10^0 – 10^7) were deposited on MRSC agar plates and counted after incubation under anaerobic conditions at 37°C.

To determine viable counts of the encapsulated bacteria, beads (1 mL) were re-suspended in 10% sodium citrate dihydrate solution (9 mL) followed by vortexing. The number of released cells was determined by plate count using MRSC agar, dilutions of dissolved beads (10^{-1}-10^{-7}) were plated in duplicate and incubated at 37°C anaerobically for 40 h. For lower viability samples later, beads (2 mL) were re-suspended in 10% sodium citrate dihydrate solution (2 mL) instead, and dilutions (10^{0}-10^{-3}) were plated as before. Samples were taken over a four-week period on days 0 (initial), 1, 3, 5, 7, 10, 14, 21, and 28. Days 17 and 24 were also plated for free cell samples.

3.3.9 In vitro simulated digestion of free and encapsulated bifidobacteria in chitosan-coated alginate microbeads

Free and encapsulated bifidobacteria were exposed to simulated digestion phases (Figure 3.1). Fluids used in *in vitro* modeling of digestion were prepared based on the method described by Li et al. (2011). One liter of modeled saliva stock solution was prepared with ammonium nitrate (0.328 g), potassium chloride (0.202 g), potassium citrate (0.308 g), potassium phosphate (0.636 g), sodium chloride (1.594 g), sodium DL-lactate (0.146 g), urea (0.198 g), and uric acid sodium salt (0.021 g) in distilled water. The stock solution was then filter-sterilized. The day before digestion experiments were carried out, the salivary phase was prepared by adding porcine gastric mucin type II (2.4 g) to saliva stock solution (80 mL). The solution was stirred overnight at room temperature to completely dissolve the powder.

One liter of simulated gastric stock solution was prepared by adding sodium chloride (2 g) and hydrochloric acid (7 mL) to distilled water and filter sterilizing. The simulated intestinal stock solution (500 ml) was prepared by adding calcium chloride

hexahydrate (27.38 g) and sodium chloride (109.685g) to distilled water and autoclaved. Pepsin extracted from porcine gastric mucosa (0.32 g) was then added to gastric stock solution (100 mL).

The day before digestion experiments were carried out, porcine bile extract (0.75 g) was added to PBS solution (14 mL) for the modeled intestinal phase. The solution was stirred overnight at room temperature to completely dissolve the powder. Lipase from porcine pancreas type II (0.24 g) was dissolved in PBS solution (10 mL); the solution (5 mL) was then added with bile salt solution (7 mL) and intestinal stock solution (33 mL).

Free and encapsulated bifidobacteria cells were separately added to simulated saliva fluids (22 mL, pH adjusted to 6.7-6.8), simulated gastric fluids (45 mL, pH adjusted to 2.5-2.6) or simulated intestinal fluids (45 mL, pH adjusted to 7.0-7.2). Dilutions (10^0-10^5) were plated on MRSC agar for initial, 5, 10, 15, 20, or 30 minutes exposure and incubated anaerobically for at least 48 hours.

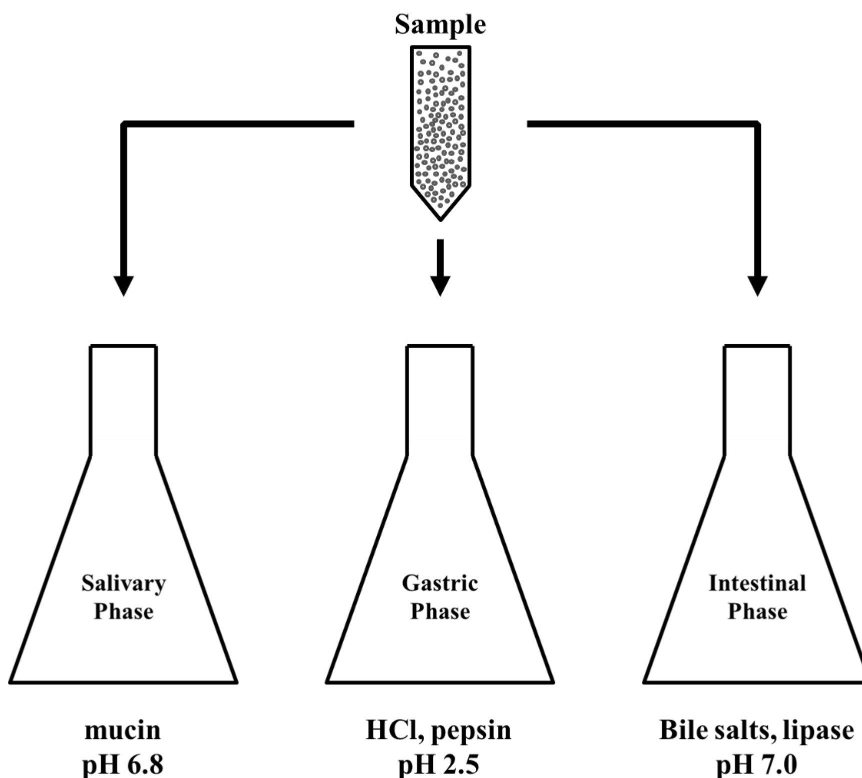

Figure 3.1: Experimental design for simulated digestion of free and encapsulated *Bifidobacterium longum* subsp. *infantis* UMA 299. Samples were exposed to salivary (containing mucin, pH 6.7-6.8), gastric (containing hydrochloric acid and pepsin, pH 2.5-2.6), and intestinal (containing bile salts and lipase, pH 7.0-7.2) phases separately. One milliliter samples were obtained at times 0 (before exposure), 5, 10, 15, and 30 minutes exposure.

3.3.10 Statistical analysis

The mean of 2 or 3 individual determinations was used to calculate particle size, ζ-potential, and cell counts. Analysis of variance (ANOVA) followed by Tukey honest significant difference test was use to analyze all data and compare individual means. This was performed using statistical software (GraphPad Prism 6, GraphPad Software, La Jolla, California, USA).

3.4 Results

Light scattering was used to determine the mean particle diameter of the different microgel samples (Table 3.2). The mean particle sizes of alginate beads containing similar strains were similar, ranging from 135 to 185 µm (D [3,2]) for encapsulated *B. infantis* strains and 149 to 216 µm (D [3,2]) for encapsulated *B. longum* strains. The chitosan-coated alginate beads were significantly larger compared than the alginate beads, ranging from 191 to 292 µm (D [3,2]). This increase in particle size may have been because of the additional coating formed by the alginate molecules, or because of some aggregation of the microgels. Microgel aggregation may have occurred due to bridging flocculation, which is the ability of the chitosan cation to adsorb to the surfaces of two or more anionic alginate beads. Additional information about the structural configuration of the microgels was therefore obtained through microscopy.

Table 3.2: Mean particle diameters of alginate and chitosan-coated alginate beads containing strains of *Bifidobacterium longum*. Averages were determined by static light scattering. Values are shown as volume-based (D [4,3]) and surface-based (D [3,2]) mean ± standard deviation. Values followed by the same letters in the same column are not significantly different ($p > 0.05$) from each other.

Beads			D [4,3]	D [3,2]
			µm	
Alginate	Subsp. *infantis*	UMA 298	233 ± 4^{ab}	167 ± 6^{abc}
		UMA 299	230 ± 3^{ab}	162 ± 3^{b}
		UMA 300	251 ± 6^{a}	185 ± 12^{cd}
		UMA 305	211 ± 4^{b}	135 ± 2^{e}
	Subsp. *longum*	UMA 306	247 ± 13^{ac}	164 ± 4^{ab}
		UMA 318	228 ± 11^{ab}	149 ± 13^{abe}
		UMA 401	277 ± 4^{cd}	216 ± 3^{f}
		UMA 402	287 ± 13^{de}	195 ± 3^{dfg}
Chit.-alg.	Subsp. *infantis*	UMA 299	327 ± 2^{fg}	292 ± 3^{h}
		UMA 300	344 ± 14^{f}	237 ± 3^{i}
	Subsp. *longum*	UMA 401	310 ± 20^{eg}	213 ± 4^{f}
		UMA 402	315 ± 26^{efg}	191 ± 11^{g}

The structures of samples containing free cells or bacterial-loaded microgels were determined using optical microscopy immediately after encapsulation (Figure 3.2). Free cells appeared rod-shaped as expected for bifidobacteria (Figures 3.2A and 3.2D). The unfilled alginate and chitosan-coated alginate microgels were similar in morphology, although the individual coated alginate beads did appear larger than the uncoated ones, which is consistent with the particle size analysis (Figures 3.2B and 3.2E). Encapsulated bifidobacteria were clearly visualized within the microgels for both alginate and chitosan-coated alginate microgels (Figures 3.2C and 3.2F). The bifidobacterial-loaded alginate and chitosan-coated alginate beads had a similar external appearance as the equivalent unloaded beads. The microgels were generally spherical with diameters around 100 to 300 μm for all samples.

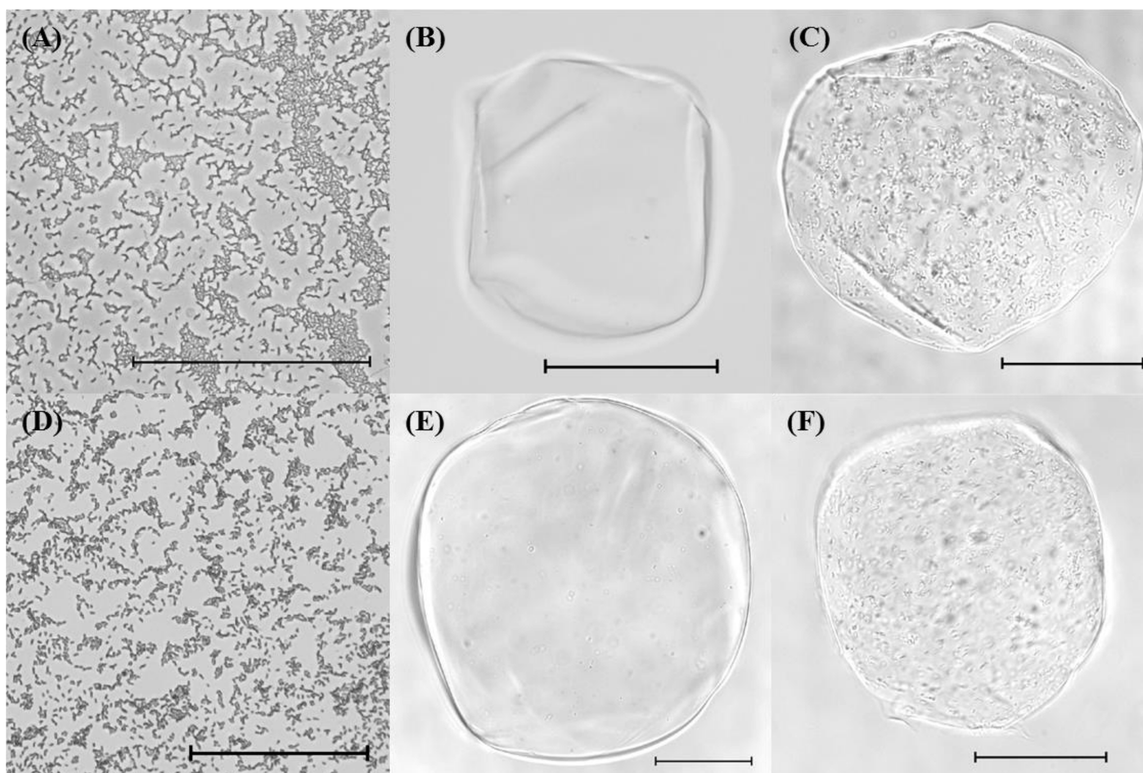

Figure 3.2: Optical microscope images of (A) *Bifidobacterium longum* subsp. *infantis* UMA 300 (20×), (B) unfilled alginate bead (20×), (C) filled alginate bead with *B. longum* subsp. *longum* UMA 306 (20×), (D) *B. longum* subsp. *longum* UMA 318 (20×), (E) unfilled chitosan-coated alginate bead (20×), and (F) filled chitosan-coated alginate bead with *B. longum* subsp. *infantis* UMA 299 (20×). All scale bars represent 100 μm.

Scanning electron microscopy (SEM) was used to inspect the structure of the alginate and chitosan-coated alginate beads (Figure 3.3). Freeze-dried microgels were uniform in size and shape. However, the surfaces of the microgels observed by SEM appeared wrinkled, whereas they presented as smooth when observed by optical microscopy. This is likely due to sublimation of water originally trapped within the hydrogel matrix, as has been described previously (Yeung et al., 2016a). The chitosan-coated alginate beads appeared to be more irregular in shape compared to alginate beads. Qualitatively, the alginate beads had smoother wrinkles and microstructures, whereas the chitosan-coated beads exhibited sharp jagged edges. This observation suggests that the

chitosan layer has been successfully deposited onto the external surfaces of the alginate microgels.

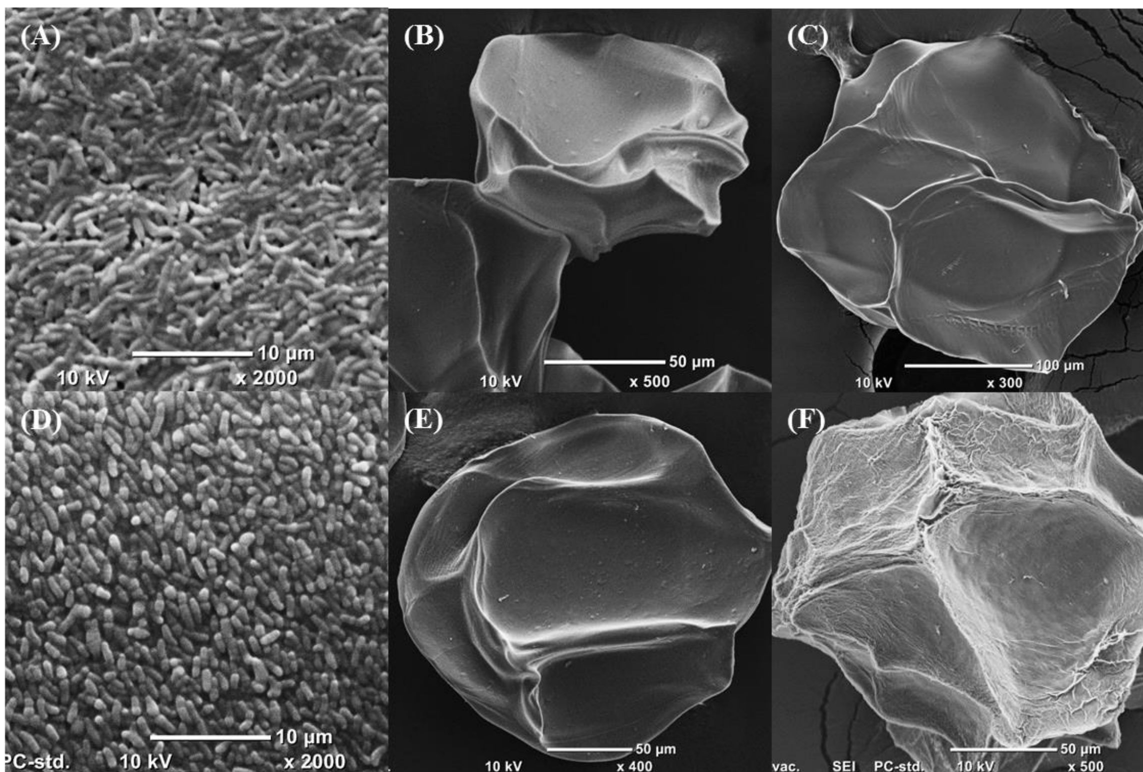

Figure 3.3: Scanning electron microscope images of (A) *Bifidobacterium longum* subsp. *infantis* UMA299, (B) unfilled alginate bead, (C) unfilled chitosan-coated alginate bead, (D) *B. longum* subsp. *longum* UMA 306, (E) alginate bead containing *B. longum* subsp. *longum* UMA 401, and (F) chitosan-coated alginate bead containing *B. longum* subsp. *infantis* UMA 300. Samples were dried before sputter-coating with gold. SEM was set at high-vacuum, 10 kV.

Electrophoretic light scattering was used to evaluate the electrical characteristics of the microgels (Table 3.3). The ζ-potentials of all the alginate beads were negative, ranging from -4.2 to -9.4 mV for *B. infantis* and -2.6 to -4.4 mV for *B. longum* as predicted with this coating. In contrast, all chitosan-coated alginate bead samples had positive surface potentials ranging from +9.9 to +14.9 mV for *B. infantis* and +0.8 to +9.0 mV for *B. longum*. These results indicate that the cationic chitosan molecules formed a secondary shell around the anionic calcium alginate beads.

Table 3.3: Zeta potential of alginate and chitosan-coated alginate beads containing strains of *Bifidobacterium longum*. Values are shown as mean ± standard deviation. Values followed by the same letters are not significantly different ($p > 0.05$) from each other.

Beads			mV
Alginate	Subsp. *infantis*	UMA 298	-5.23 ± 2.06^{ab}
		UMA 299	-9.42 ± 2.54^{a}
		UMA 300	-8.73 ± 4.88^{a}
		UMA 305	-4.15 ± 1.17^{ab}
	Subsp. *longum*	UMA 306	-3.14 ± 2.24^{ab}
		UMA 318	-2.60 ± 0.04^{ab}
		UMA 401	-4.38 ± 0.64^{ab}
		UMA 402	-4.28 ± 1.12^{ab}
Chit.-alg.	Subsp. *infantis*	UMA 299	9.92 ± 3.92^{c}
		UMA 300	14.87 ± 4.26^{c}
	Subsp. *longum*	UMA 401	0.79 ± 2.53^{bd}
		UMA 402	9.03 ± 4.90^{cd}

The viability of four *B. longum* and four *B. infantis* strains that were not encapsulated was determined during five weeks of storage (Figure 3.4, Table A.2). As expected, there was a decrease in the viability of the bifidobacteria evaluated, but the rate of the decrease was strain dependent. A sharp decrease in viability was observed for *B. infantis* UMA318 and *B. longum* UMA401, diminishing by 9-10 log CFU over the course of a week under aerobic conditions. *B. infantis* UMA 300 and *B. infantis* UMA 305 remained viable for slightly longer, with a 10-log reduction observed within 10 days. Whereas, *B. infantis* UMA 298 and *B. infantis* 306 exhibited a 9-10 log decrease over 2 weeks of storage. Interestingly, *B. infantis* UMA 299 and *B. longum* UMA 402 survived the longest, as viable cell counts diminished by 7-8 logs over 3 weeks before decreasing to undetectable levels.

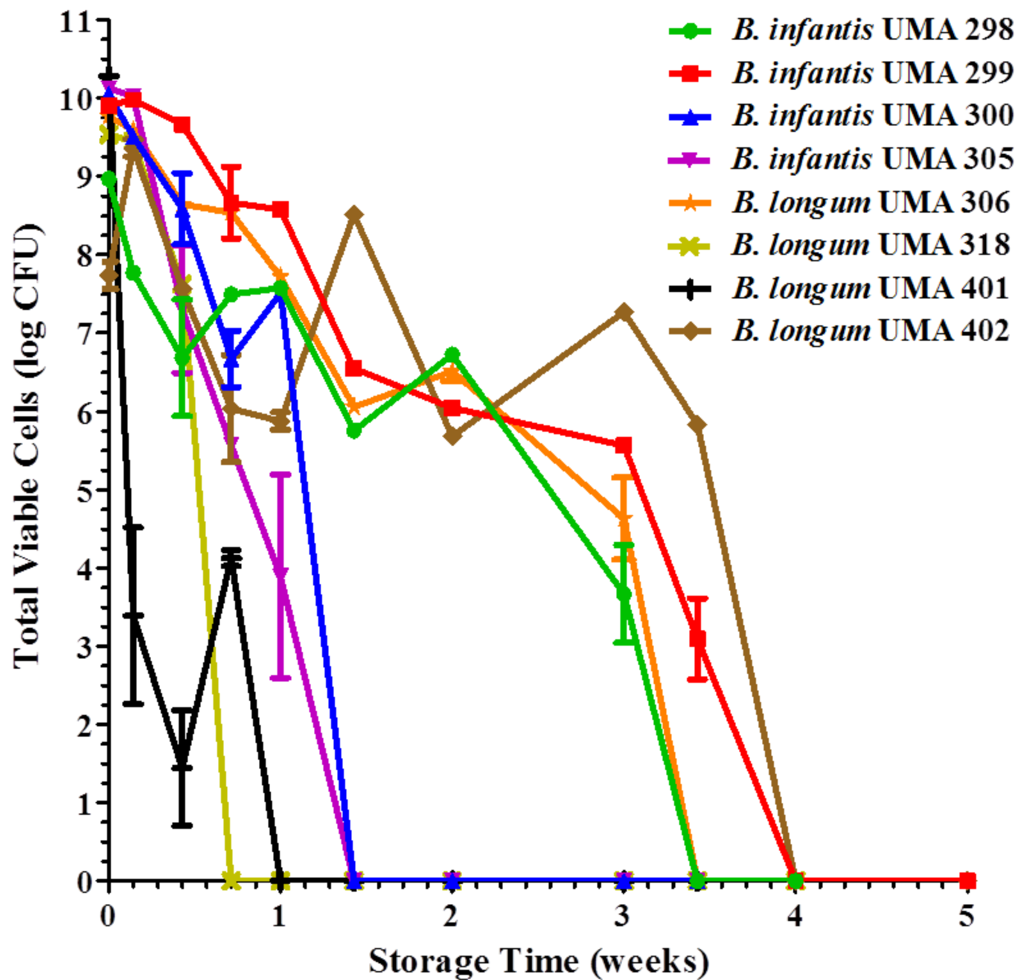

Figure 3.4: Survival of free *Bifidobacterium longum* cells in wet refrigerated storage over time. Counts based on samples drop-plated on MRSC agar incubated at 37°C anaerobically. Error bars indicate the standard error of replicate counts.

Viability following encapsulation was determined for all eight bifidobacterial strains (Figure 3.5, Table A.3). There were distinct differences between the effects of encapsulation depending on strain type. The cell viability of *B. infantis* UMA 298, *B. infantis* UMA 305, and *B. longum* UMA 318 stains rapidly decreased and were undetectable after 3 days. Unexpectedly, *B. infantis* UMA 298 and *B. infantis* UMA 305 in alginate were inactivated faster than the corresponding free cells, being undetectable after 24 and 10 days respectively. Viability of the encapsulated *B. longum* UMA 318 was

identical to that of free cells. *B. infantis* UMA 300, *B. longum* UMA 306, *B. longum* 401, and *B. longum* 402 maintained viable populations that decreased by 3-4 log CFU after 10 days of storage before rapidly decreasing to zero. Encapsulated *B. infantis* UMA 300 survived three days longer than corresponding free cells; encapsulated *B. longum* UMA 401 lasted a week longer than free cells. Encapsulated *B. longum* UMA 306 survived similarly to free cells, and encapsulated *B. longum* UMA 402 survived over a week shorter than corresponding free cells. Interestingly, *B. infantis* UMA 299 viability was enhanced as it experienced a 5 log CFU reduction in 3 weeks compared to an 8 log CFU reduction during this time for the non-encapsulated cells. Thus, encapsulating with alginate extended cell viability of *B. infantis* UMA 299 and 300 by a few days, and extended viability of *B. longum* UMA 401 cells by a week. Encapsulating other *B. infantis* and *B. longum* strains did not appear to extend viability over the storage conditions used.

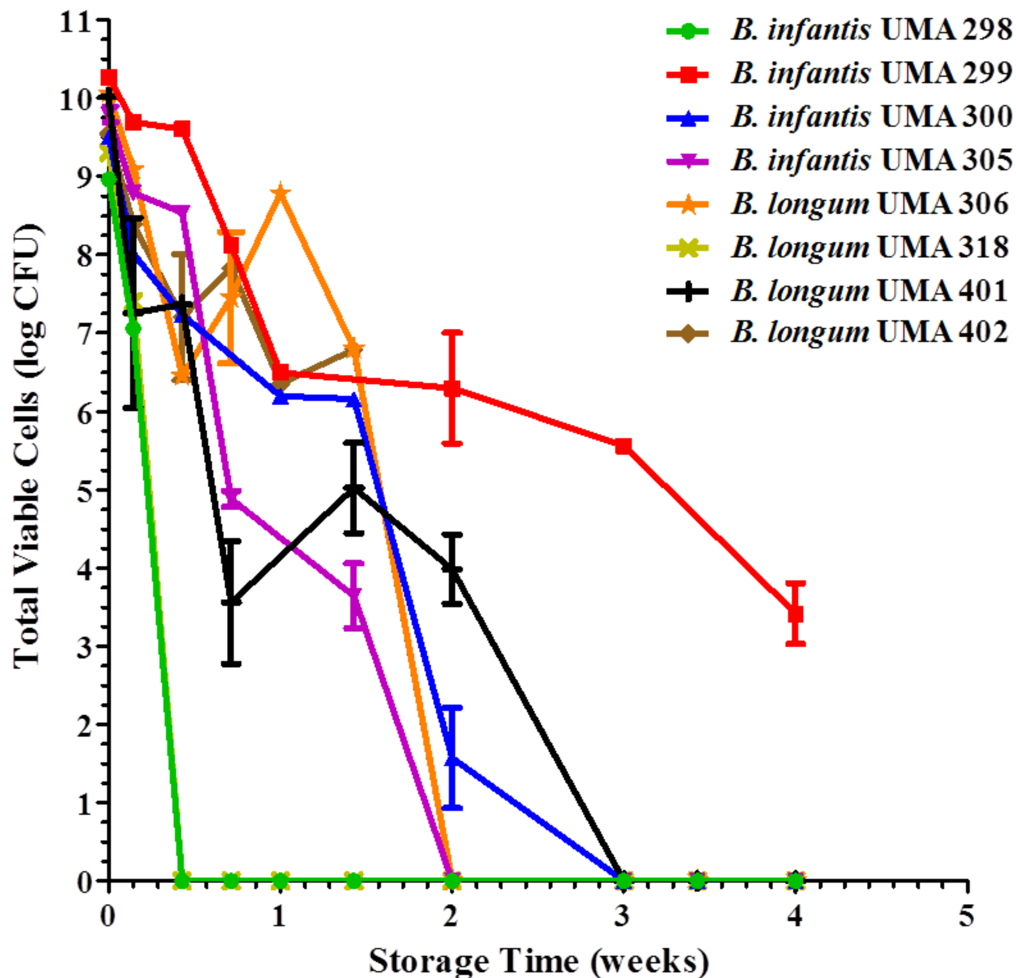

Figure 3.5: Survival of *Bifidobacterium longum* cells in calcium alginate microbeads in wet refrigerated storage over time. Counts based on samples drop-plated on MRSC agar and incubated at 37°C anaerobically. Error bars indicate the standard error of replicate counts.

Two strains each of *B. infantis* (UMA 299 and 300) and *B. longum* (UMA 401 and 402) were encapsulated in a secondary coating of chitosan applied to the alginate bead core and submitted to testing over time (Figure 3.6, Table A.4). *B. longum* UMA 401 and 402 both decreased 3-4 log within three days, and fell to undetectable levels by two weeks. Viability of *B. infantis* UMA 299 and *B. infantis* UMA 300 decreased only 2 logs in five days, before falling to undetectable levels after two weeks. Encapsulating

bifidobacterial cells in chitosan-coated alginate beads did not appear to extend viability compared with uncoated alginate microbeads.

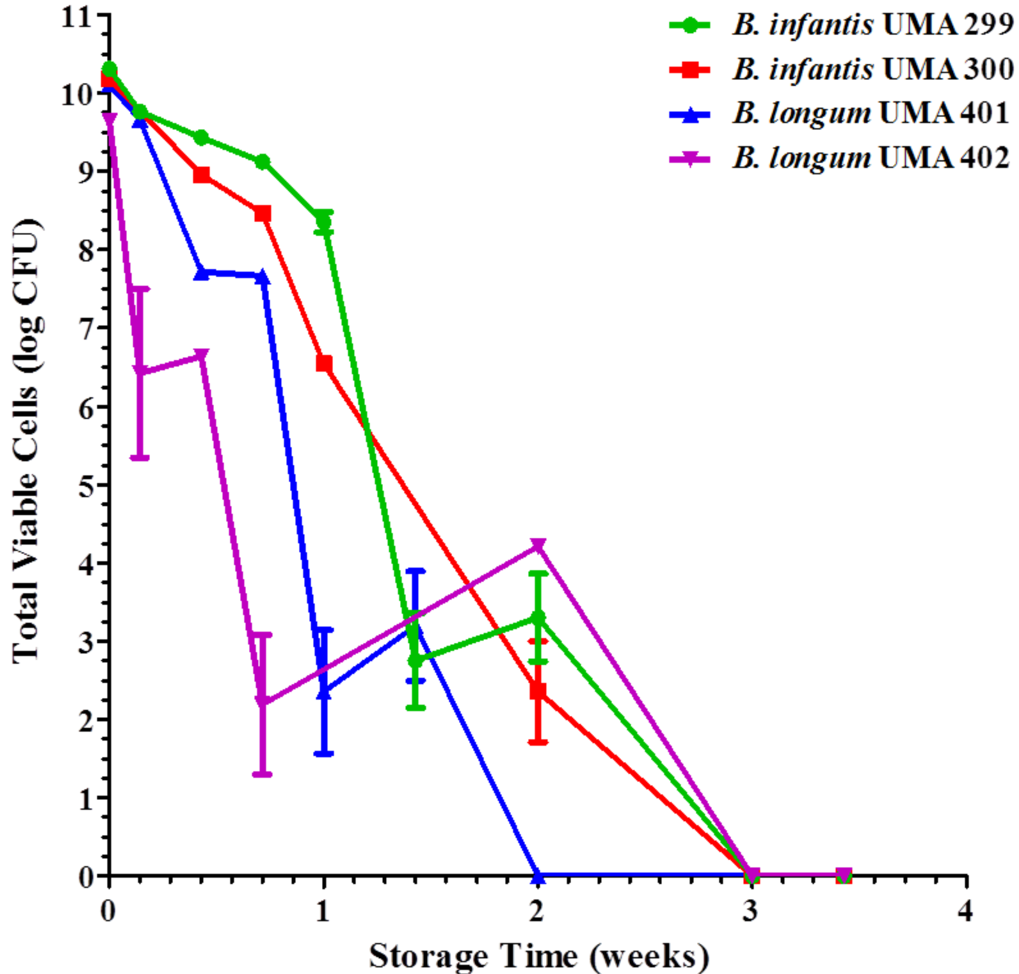

Figure 3.6: Survival of *Bifidobacterium longum* cells in chitosan-coated alginate microbeads in wet refrigerated storage over time. Counts based on samples drop-plated on MRSC agar and incubated at 37°C anaerobically. Error bars indicate the standard error of replicate counts.

Free and bifidobacterial cells encapsulated in chitosan-coated alginate beads were subjected to simulated digestion in a GIT model as previously described (Li et al., 2011). Free *B. infantis* UMA 299 and cells encapsulated chitosan-coated alginate were immersed separately in simulated salivary, gastric, and intestinal phases and assessed over time for

cell viability (Table 3.4). The bacteria appeared to be relatively stable within simulated saliva fluids, as less than a one log CFU reduction was experienced in 30 minutes of exposure regardless of encapsulation. The model salivary juice did not greatly inhibit cell viability in general, as less than one log CFU reduction was experienced in 30 minutes of exposure regardless of encapsulation. However, microencapsulation provided enhanced protection for UMA299 by shielding the strain from the low pH of the gastric phase. Encapsulated cells decreased by 1.4 logs CFU, whereas untreated cells decreased by 2.7 logs following exposure to pH 2.5 conditions (5 minutes). This indicates a significant, albeit fleeting protection afforded to the encapsulated cells as viability was abrogated after 10 minutes of exposure to the gastric phase. Similarly, UMA299 cell viability was not detectible after 5 minutes of exposure to the intestinal phase. *B. longum* UMA 402 encapsulated in chitosan-coated alginate was also subjected to simulated digestion. As with *B. infantis*, cell viability remained stable in the modeled salivary phase, but underwent a 6-log reduction after only a few minutes exposure to gastric phase (pH 2.5).

Table 3.4: Survival of free and encapsulated *Bifidobacterium longum* subsp. *infantis* UMA 299 in three separate simulated digestion phases. Counts based on samples drop-plated on MRSC agar and incubated at 37°C anaerobically. Values are shown as mean ± standard error of replicate counts. Means within each column followed by the same lowercase letters are not significantly different (p > 0.05) from each other. Means within each row followed by the same uppercase letters are not significantly different (p > 0.05) from each other.

	Free			**Encapsulated**		
	Saliva	**Gastric**	**Intestinal**	**Saliva**	**Gastric**	**Intestinal**
pH	6.74	2.53	7.04	6.78	2.57	7.12
Time (minutes)	**log CFU**					
0	9.63 ±0.07^{aA}	9.63 ±0.07^{aA}	9.63 ±0.07^{aA}	8.40 ±0.84^{abB}	8.40 ±0.84^{aB}	8.40 ±0.84^{aB}
5	9.17 ±0.06^{a}	6.99 ±0.03^{b}	ND	8.10 ±0.05^{a}	6.90 ±0.04^{a}	ND
10	9.28 ±0.09^{a}	ND	ND	7.89 ±0.07^{a}	ND	ND
15	9.14 ±0.06^{a}	ND	ND	6.42 ±1.07^{b}	ND	ND
30	9.01 ±0.06^{a}	ND	ND	8.14 ±0.040^{a}	ND	ND

3.5 Discussion

Initially, chitosan-coating of alginate beads was postulated to enhance the viability of encapsulated probiotics by reducing their exposure to environmental stresses during storage and within the gastrointestinal tract. Accordingly, the influence of encapsulation on a panel of *B. longum* strains to assess differential viability was systematically studied. The calcium alginate beads formed using an injection-gelation method were roughly spherical in shape, negatively charged, and had dimensions around 130 to 220 µm. Coating the alginate beads with chitosan caused a small increase in their size and changed their charge from negative to positive. Optical microscopy confirmed that the bifidobacteria were immobilized within the hydrogel beads, which is consistent

with previous encapsulation studies (Hansen et al., 2002; Fareez et al., 2015; Yeung et al., 2016a).

Interestingly, encapsulation of bifidobacteria in chitosan-coated alginate beads led to an appreciable improvement in their storage or gastrointestinal stability. One possible explanation for this observation is that the alginate hydrogel used had relatively large pores, and so small molecules, such as oxygen, acids, bile salts, or digestive enzymes, could easily diffuse into the microgels and inactivate the encapsulated bacteria (McClements, 2015). These results suggest that a simple secondary layer of chitosan alone will not fully protect encapsulated bifidobacteria, and that further optimization is required to engineer more effective delivery systems. Previous studies have shown that alginate has a prebiotic effect on bifidobacteria, which might account for its ability to enhanced viability, potentially through a non-encapsulation mechanism (Wang et al., 2006; Ramnani et al., 2012). In future studies, it may be useful to examine the influence of different biopolymer materials and methods on the ability of microgels to enhance probiotic viability. As an example, the hydrogel pore size may be decreased to limit molecular diffusion, with the addition of anti-oxidants to limit oxidation reactions and prebiotics to stimulate probiotic growth in the colon. Since bifidobacteria ferment oligosaccharides within the gut, a synbiotic approach that integrates prebiotic substrates including plant or milk oligosaccharides may advance bifidobacterial-based delivery (Sela, 2011). Alternatively, judicious selection of strain selection that are resistant to acids, bile salts, or digestive enzymes may enhance the delivery scheme. However, previous studies indicate that most bifidobacteria strains typically exhibit a significant

decrease in survival around pH 4 which would necessitate shielding from gastric conditions (Sun and Griffiths, 2000).

Bifidobacteria have been exposed to simulated digestive fluids in previously conducted studies (O'Riordan et al., 2001; Hansen et al., 2002; Kamalian et al., 2014). Although specific strains tested and experimental schemes vary between studies. Hansen et al. (2002) encapsulated several bifidobacterial strains in microgels formed by an emulsion-templating method, and then exposed them to simulated gastric and small intestinal fluids. In this study, *B. infantis* and *B. longum* strains showed a 4-6 log CFU/mL decrease between exposure to gastric fluids set at pH 6.0 and pH 2.0 for two hours, and 3-5 log CFU/mL reduction between exposure to intestinal fluid containing 0% and 1% bile for 24 hours. In a separate experiment, encapsulated *B. longum* experienced a 5-log CFU/mL reduction after 30 minutes exposure to gastric juice (pH 2.0). In the study herein, *B. infantis* UMA 299 encapsulated in chitosan-coated alginate underwent an 8-log reduction in a 10-minute exposure to gastric fluid (pH 2.6), and an 8-log reduction in 5 minutes exposure to intestinal fluid. This study included 0.75% bile extract, pepsin, and lipases were included in the gastric and intestinal fluids, for the purpose of simulating the harsh conditions of the human GIT. In an additional study, an emulsion encapsulation method was performed on *Bifidobacterium pseudocatenulatum* G4 in chitosan-coated alginate and exposed to gastric conditions (pH 1.5) for two hours followed by intestinal phase for five hours (Kamalian et al., 2014). The encapsulated *B. pseudocatenulatum* experienced a 4-log reduction when encapsulated in alginate and a 2-log reduction in chitosan-coated alginate, relative to the 5-log reduction in the control. However, this was accomplished in the absence of digestive enzymes or bile salts in simulated gastric and

intestinal fluids that would present additional hurdles to the bifidobacterial cells. O'Riordan et al. (2001) studied spray-dried *Bifidobacterium* spp. PL1 in starch and subjected the resultant granules to simulated digestion. After 3 hours of exposure to buffer with pH 2.8, they were unable to detect viable cells as well as other sampling points in between 0 and 3 hours. This is consistent with the results presented in this study.

In summary, bifidobacterial viability following encapsulation varied between subspecies as well as strains. This suggests that there is a range of genotypic and phenotypic factors contributing to stress responses that promote enhanced viability. Further functional genomic analysis of encapsulated probiotic organisms can aid in matching strains with the particular encapsulation process to optimize cell integrity during storage. Moreover, similar approaches may be used in selecting ideal delivery vehicles to shield bifidobacteria during GIT transit to arrive intact and metabolically poised to exert beneficial activities in the distal colon.

3.6 Acknowledgements

We thank David Prodanas for assistance with freeze-drying samples prior to SEM and Ezgi Özcan for assistance with statistical analysis. We also thank Jean Alamed and various individuals at the University of Massachusetts for helpful advice. EFÜ thanks the Scientific and Technological Research Council of Turkey (TUBITAK) for financial support under Grant BIDEB 2214/A.

CHAPTER 4

CONCLUSION

The studies presented herein showed that encapsulating *Lactococcus lactis* LM0230 improved viability during room-temperature (24-26°C) storage, while encapsulating *Bifidobacterium longum* strains improved viability of some strains during refrigerated (4°C) storage. This suggests that variations in oxygen tolerance as well as other stress-inducing factors are species and strain-dependent. Hence, future studies should optimize encapsulation formulation based on the genetic properties of the probiotic strain, continue observing the effects of encapsulating other novel probiotic strains (see Appendix B), and expand knowledge of prebiotic-probiotic interactions. Incorporating antioxidants and buffering agents with encapsulated probiotics may improve survival of bacterial strains sensitive to oxygen and high acidity. *In vitro* encapsulation experiments of newly discovered or potential probiotics can characterize their survival traits and evaluate their safety for use in foods, supplements, or medical applications. Lastly, co-encapsulating beneficial microorganisms with indigestible or bioactive food components may yield additive or synergistic health benefits for the consumer. These areas of research will improve overall survival of probiotic delivery to the colon, and therefore improve host health.

APPENDIX A

SUPPLEMENTAL DATA

Corresponding data for graphs shown in Chapters 2 and 3 are included here.

Table A.1: Survival of *Lactococcus lactis* subjected to drying at room temperature (22-25°C). Values are based on duplicate (with †) and triplicate counts of samples spread-plated on MRS and incubated at 37°C anaerobically and are shown as mean ± standard error of the mean. Corresponding letters indicate values are not significantly different ($p > 0.05$).

Time	Free Cells	1 h gelation	24 h gelation
	log CFU		
0 d	10.94 ± 0.03^{a}	10.69 ± 0.03^{ab}	10.77 ± 0.03^{a}
1 d	10.83 ± 0.02^{a}	-	-
3 d	8.86 ± 0.25^{cd}	-	-
5 d	$8.44 \pm 0.12^{\dagger cde}$	8.97 ± 0.01^{bcd}	-
7 d	$5.57 \pm 0.02^{\dagger gh}$	-	$9.28 \pm 0.05^{\dagger abc}$
9 d	4.80 ± 0.13^{hi}	-	-
11 d	$4.24 \pm 0.23^{\dagger hi}$	6.60 ± 0.05^{efg}	-
2 wk	4.68 ± 0.09^{hi}	-	7.38 ± 0.60^{def}
3 wk	-	5.37 ± 0.14^{gh}	6.35 ± 0.72^{fg}
4 wk	-	-	5.71 ± 0.68^{gh}
5 wk	-	-	$3.11 \pm 0.08^{\dagger i}$

Table A.2: Viability of free *Bifidobacterium longum* cells during refrigerated storage. Values based on samples drop-plated on MRSC agar and incubated at 37°C anaerobically and are shown as mean ± standard error of mean. Means within each column followed by the same lowercase letters are not significantly different ($p > 0.05$) from each other. Means within each row followed by the same uppercase letters are not significantly different ($p > 0.05$) from each other.

Time (days)	*B. longum* subsp. *infantis*				*B. longum* subsp. *longum*			
	UMA 298	UMA 299	UMA 300	UMA 305	UMA 306	UMA 318	UMA 401	UMA 402
	log CFU							
0	8.967 ±0.044^{aA}	9.908 ±0.043^{aAB}	10.05 ±0.018^{aBC}	10.13 ±0.084^{aBD}	9.766 ±0.060^{aACDE}	9.536 ±0.056^{aACDF}	10.28 ±0.047^{aBEF}	7.739 ±0.175^{aG}
1	7.770 ±0.057^{abA}	9.980 ±0.017^{aB}	9.509 ±0.072^{aB}	10.025 ±0.033^{aB}	9.592 ±0.050^{abB}	9.451 ±0.060^{aB}	3.388 ±1.130^{bC}	9.365 ±0.107^{bB}
3	6.683 ±0.745^{bcA}	9.667 ±0.053^{abB}	8.588 ±0.454^{aB}	7.303 ±0.813^{bA}	8.648 ±0.072^{abcBC}	7.623 ±0.027^{bAC}	1.445 ±0.736^{cD}	7.566 ±0.071^{aAC}
5	7.493 ±0.080^{bAB}	8.666 ±0.459^{abC}	6.672 ±0.357^{bB}	5.572 ±0.073^{cD}	8.539 ±0.074^{bcAC}	ND	4.126 ±0.101^{bF}	6.036 ±0.685^{cBD}
7	7.574 ±0.072^{bA}	8.579 ±0.072^{bA}	7.510 ±0.056^{bA}	3.895 ±1.298^{dB}	7.719 ±0.058^{cA}	ND	ND	5.875 ±0.113^{cD}
10	5.754 ±0.061^{cA}	6.549 ±0.071^{cA}	ND	ND	6.057 ±0.077^{dA}	ND	ND	8.516 ±0.065^{abC}
14	6.722 ±0.060^{bcA}	6.045 ±0.027^{cA}	ND	ND	6.511 ±0.120^{dA}	ND	ND	5.682 ±0.057^{cA}
21	3.666 ±0.624^{dA}	5.563 ±0.085^{cB}	ND	ND	4.628 ±0.523^{eAB}	ND	ND	7.270 ±0.022^{aD}
24	ND	3.093 ±0.517^{dB}	ND	ND	ND	ND	ND	5.833 ±0.018^{cC}
28	ND	ND	ND	ND	ND	ND	ND	ND
35	ND	ND	ND	ND	ND	ND	ND	ND

Table A.3: Viability of *Bifidobacterium longum cells* encapsulated in calcium alginate during refrigerated storage. Values based on samples drop-plated on MRSC agar and incubated at 37°C anaerobically and are shown as mean ± standard error of mean. Means within each column followed by the same lowercase letters are not significantly different ($p > 0.05$) from each other. Means within each row followed by the same uppercase letters are not significantly different ($p > 0.05$) from each other.

Time (days)	***B. longum* subsp. *infantis***				***B. longum* subsp. *longum***			
	UMA 298	**UMA 299**	**UMA 300**	**UMA 305**	**UMA 306**	**UMA 318**	**UMA 401**	**UMA 402**
	log CFU							
0	8.959 $\pm 0.049^{aA}$	10.27 $\pm 0.054^{aB}$	9.508 $\pm 0.029^{aAB}$	9.793 $\pm 0.100^{aAB}$	10.07 $\pm 0.052^{aBC}$	9.312 $\pm 0.026^{aACD}$	10.01 $\pm 0.069^{aBD}$	9.545 $\pm 0.027^{aAB}$
1	7.058 $\pm 0.085^{bA}$	9.694 $\pm 0.033^{aB}$	8.018 $\pm 0.059^{bCD}$	8.791 $\pm 0.051^{bCE}$	9.088 $\pm 0.087^{bBEF}$	7.406 $\pm 0.047^{bAD}$	7.258 $\pm 1.214^{bCF}$	8.394 $\pm 0.059^{bCF}$
3	ND	9.603 $\pm 0.020^{aB}$	7.233 $\pm 0.075^{bCD}$	8.536 $\pm 0.030^{bE}$	6.457 $\pm 0.063^{cdC}$	ND	7.364 $\pm 0.056^{cDF}$	7.202 $\pm 0.804^{cdCF}$
5	ND	8.121 $\pm 0.052^{bB}$	-	4.883 $\pm 0.103^{cC}$	7.453 $\pm 0.839^{bB}$	ND	3.559 $\pm 0.782^{dD}$	7.831 $\pm 0.021^{cB}$
7	ND	6.497 $\pm 0.030^{cB}$	6.196 $\pm 0.075^{cB}$	-	8.785 $\pm 0.037^{bC}$	ND	N/A	6.355 $\pm 0.089^{dB}$
10	ND	-	6.159 $\pm 0.041^{cB}$	3.641 $\pm 0.413^{dC}$	6.802 $\pm 0.070^{cdB}$	ND	5.020 $\pm 0.578^{eD}$	6.790 $\pm 0.025^{dB}$
14	ND	6.298 $\pm 0.705^{cB}$	1.571 $\pm 0.642^{dC}$	ND	ND	ND	3.984 $\pm 0.444^{dD}$	ND
21	ND	5.561 $\pm 0.031^{cB}$	ND	ND	ND	ND	ND	ND
24	ND	N/A	ND	ND	ND	ND	ND	ND
28	ND	3.415 $\pm 0.392^{dB}$	ND	ND	ND	ND	ND	ND
35	ND	ND	ND	ND	ND	ND	ND	ND

Table A.4: Viability of *Bifidobacterium longum cells* encapsulated in chitosan-coated alginate during refrigerated storage. Values based on samples drop-plated on MRSC agar and incubated at 37°C anaerobically and are shown as mean ± standard error of mean. Means within each column followed by the same lowercase letters are not significantly different ($p > 0.05$) from each other. Means within each row followed by the same uppercase letters are not significantly different ($p > 0.05$) from each other.

Time (days)	***B. longum* subsp. *infantis***		***B. longum* subsp. *longum***	
	UMA 299	**UMA 300**	**UMA 401**	**UMA 402**
	log CFU			
0	10.31 $\pm 0.037^{aA}$	10.19 $\pm 0.052^{aA}$	10.11 $\pm 0.042^{aA}$	9.637 $\pm 0.023^{aA}$
1	9.764 $\pm 0.035^{aA}$	-	9.660 $\pm 0.028^{aA}$	6.424 $\pm 1.075^{bC}$
3	9.435 $\pm 0.032^{abA}$	8.960 $\pm 0.061^{abA}$	7.717 $\pm 0.024^{bB}$	6.638 $\pm 0.046^{bB}$
5	9.122 $\pm 0.080^{abA}$	8.469 $\pm 0.046^{bAB}$	7.665 $\pm 0.028^{bB}$	2.191 $\pm 0.894^{cC}$
7	8.356 $\pm 0.128^{bA}$	6.544 $\pm 0.063^{cB}$	2.356 $\pm 0.788^{cC}$	-
10	2.753 $\pm 0.603^{cA}$	-	3.194 $\pm 0.698^{cA}$	-
14	3.304 $\pm 0.558^{cAB}$	2.357 $\pm 0.643^{dA}$	ND	4.212 $\pm 0.076^{dB}$
21	ND	ND	ND	ND
24	ND	ND	ND	ND
28	ND	ND	ND	ND
35	ND	ND	ND	ND

APPENDIX B

EXTENDING VIABILITY *OF LACTOBACILLUS PLANTARUM* AND *LACTOBACILLUS JOHNSONII* BY MICROENCAPSULATION IN ALGINATE MICROGELS

Introduction

Lactobacillus plantarum is a facultative Gram-positive rod-shaped bacterial species commonly found on the surfaces of vegetation and is widely used for plant-based fermentations (Boekhorst et al., 2004; Ferreira dos Santos et al., 2016). *L. plantarum* is known to be relatively tolerant to acid and bile salts and can also secrete antimicrobial compounds and adhere to the gut epithelial surface, discouraging growth and colonization by pathogenic microorganisms (Cebeci and Gürakan, 2003; Ingham et al., 2008). *Lactobacillus johnsonii* is a more fastidious related bacterial species that also resides in the human gastrointestinal tract and has documented benefits to human health similar to *Lactobacillus plantarum*, such as adhesion to gut epithelial cells to compete with pathogens and immunomodulation (Boekhorst et al., 2004; Pridmore et al., 2004; Garrido et al., 2005; Hertzberger et al., 2013).

Depending on the specific strain of *Lactobacilli,* the survival rate during gastrointestinal transit is twenty to forty percent due to the challenges of gastric acidity and bile salts present in the intestine (Bezkorovainy, 2001). Microencapsulating *L. plantarum* and *L. johnsonii* in food-grade alginate shows promise to increase survival of bacteria through the harsh conditions of the gastrointestinal tract. Although several studies have previously observed the effects of encapsulating *L. plantarum* (Ding and Shah, 2007; Gbassi et al., 2009; Brinques and Ayub, 2011), to the authors' knowledge, *L.*

johnsonii has never been encapsulated. In this study, we compare the survival of free and encapsulated *L. plantarum* and *L. johnsonii* cells during refrigerated storage as well as during exposure to simulated digestion phases.

Materials and methods

General chemicals used in encapsulation and modeled digestion

For bacterial culture preparation, Lactobacilli MRS broth was obtained from Becton Dickinson and Company (Sparks, Maryland, USA). Agar and L-cysteine hydrochloric acid were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Glycerol, sodium chloride (NaCl), and sodium citrate dihydrate was purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). For encapsulation experiments, sodium alginate (TICA-algin HG 400 powder) was donated by TIC Gums (White Marsh, Maryland, USA). Calcium chloride hexahydrate was obtained from Sigma Aldrich. Glacial acetic acid was purchased from Fisher Scientific. For simulated digestion, ammonium nitrate, bile extract porcine, lipase from porcine pancreas type II, pepsin from porcine gastric mucosa, porcine gastric mucin type II, potassium chloride, potassium citrate, potassium phosphate, sodium DL-lactate, sodium hydroxide (NaOH), and uric acid sodium salt were also purchased from Sigma Aldrich. Hydrochloric acid (HCl), phosphate buffer saline (PBS), and urea were purchased from Fisher Scientific.

Bacterial culture conditions

Lactobacillus johnsonii ATCC 33200 and *Lactobacillus plantarum* ATCC BAA-793 cultures were obtained from the American Type Culture Collection and propagated in deMan, Rogosa, Sharpe broth supplemented with 0.05% L-cysteine (MRSC). Stock cultures were stored in MRSC with 25% glycerol at -80°C.

Isolated colonies of *L. johnsonii* and *L. plantarum* were inoculated into MRSC broth (50 mL) and incubated aerobically for 24 h at 37°C. The bacteria were then harvested by centrifugation at 4000 *g* for 10 min, and re-suspended in physiological saline. This was done in duplicate. These cell suspensions were used for encapsulation or survival of free cells during long-term storage.

Microencapsulation of probiotics

1% (w/v) sodium alginate solution (HG400, TIC Gums, White Marsh, MD, USA) was sterilized by autoclaving. Next, ~10^9 CFU mL^{-1} probiotic bacteria in physiological saline (2 mL) were added to the 1% alginate solution (148 mL) and dispersed evenly by gentle agitation. The hydrogel microbeads were formed using an encapsulator machine (Büchi B-390 Encapsulator, Flawil, Switzerland) using a vibrating extrusion nozzle of 120 µm with standard settings (frequency 800 Hz, electrode 800 V, air pressure 300-330 mbar, amplitude 3). The liquid polymer-cell droplets were sprayed into sterile 0.10 M calcium chloride solution (225 mL) which continuously stirred at 300 rpm. After the mixture was exhausted, the beads were stirred continuously in calcium chloride solution for 1 hour to complete cross-linking. Finally, the alginate microbeads (~30 mL) were rinsed with sterile distilled water, filtered, and stored in physiological saline (50 mL) at 4°C for 10 weeks to monitor shelf-life. A sample of beads (5 mL) was reserved for optical microscopy, scanning electron microscopy, and particle size analysis.

Particle size analysis of alginate microbeads

The particle size distribution of the calcium alginate beads was evaluated immediately following encapsulation using a laser diffraction particle size analysis system (Mastersizer S, Malvern Instruments, Worcestershire, UK). Samples for particle

size analysis (2-3 mL) were suspended in 10 mL of distilled water. Volume-based (D [4,3]) and surface-based (D [3,2]) mean particle diameters were collected for all samples. This was done in duplicate for all samples.

Optical Microscopy

The morphology of probiotic-filled calcium alginate microbeads was examined immediately following encapsulation using optical microscopy to ensure beads were of the correct size and shape. Images were captured using a microscope (C1 Digital Eclipse, Nikon, Tokyo, Japan) with 20× and 40× objective lenses and analyzed using instrument software (EZ CSI version 3.8, Nikon).

Scanning electron microscopy (SEM)

A bench-top scanning electron microscope (JCM-6000 NeoScope, JEOL, Tokyo, Japan) was used to capture images of the alginate microbeads. Alginate beads were dried for several days on aluminum foil and sputter-coated with 10 nm of gold prior to being loaded into the SEM. Free cells were also viewed by SEM to periodically check for contamination.

Cell enumeration of *Lactobacillus* cells during storage and simulated digestion

Cell viability was assessed over time using a modified drop plate method as previously described (Herigstad et al., 2001). Briefly, 10 drops (10 µL) of each dilution within a series (10^{0}-10^{-7} for free cells and 10^{-1}-10^{-7} for encapsulated cells) were dropped onto MRSC agar plates and incubated aerobically for at least 24 hours at 37°C. Dilutions having 3-30 visible CFU per droplet were used to estimate total cell viability for each

time point. This method was used to evaluate cell viability during refrigerated storage and exposure to simulated digestion fluids.

To assess survival of free cells during long-term storage, cell suspension (2 mL, prepared as described in section 2.2) were transferred to physiological saline (50 mL) and stored at 4°C for 10 weeks. To assess survival of encapsulated cells during long-term storage, beads (~30 mL) were stored in physiological saline (50 mL) at 4°C for 10 weeks. Cell counts of the encapsulated cells were measured at days 0, 1, 3, 5, 7, 10, 14, 17, 28, and then once weekly until day 80. At each of these time points, cell-containing alginate microbeads (1 mL) was suspended in 10% (w/v) sodium citrate dihydrate solution (pH 8.2, 9 mL) and vortexed to dissolve the beads and release the cells. Serial dilutions were prepared from this 10^{-1} sample. Cell counts of the free cells were taken at days 0, 1, 3, 5, 7, 10, 14, 17, 28, and then every 7 days after until day 80. Serial dilutions were prepared from the initial sample (10^{0}).

Chemicals used in the simulated digestion were ammonium nitrate, porcine bile extract, lipase from porcine pancreas type II, pepsin from porcine gastric mucosa, porcine gastric mucin type II, potassium chloride, potassium citrate, potassium phosphate, sodium DL-lactate, sodium hydroxide, uric acid sodium salt, hydrochloric acid, phosphate buffer saline (PBS), and urea.

The artificial saliva stock solution (1 L) was prepared by stirring the following in distilled water until dissolved: sodium chloride (1.594 g), ammonium nitrate (0.328 g), potassium phosphate (0.636 g), potassium chloride (0.202 g), potassium citrate (0.308 g), uric acid sodium salt (0.021 g), sodium DL-lactate (0.146 g), and urea (0.198 g). The day prior to the simulated digestion of *L. plantarum*, the artificial saliva work solution was

prepared by adding porcine gastric mucin type II (3 g) to artificial saliva stock solution (100 mL) and stirring the solution overnight at room temperature.

The simulated gastric fluid (1 L) was prepared by stirring sodium chloride (2 g) and 6 M hydrochloric acid (7 mL) into distilled water. On the day of the simulated digestion experiments, the gastric fluid work solution was prepared by adding pepsin (0.32 g) to simulated gastric fluid stock solution (100 mL) and stirred at room temperature.

The intestinal phase stock solution (500 mL) was prepared by stirring calcium chloride (18.375 g) and sodium chloride (109.685 g) with distilled water. The bile salt solution was prepared 24 h before experimentation by adding bile salts (0.5357 g) to phosphate buffer (10 mL) and stirring overnight. Approximately 45 min prior to the intestinal phase, lipase (0.168 g) was added to phosphate buffer (7 mL) and stirred for 30 min. The simulated intestinal work solution was prepared by adding phosphate buffered saline solution (35 mL) intestinal phase stock solution (2 mL), bile salt solution (4.7 mL), and lipase solution (3.3 mL).

During the simulated digestion experiments, free (5 mL) and encapsulated (5 g) *Lactobacillus* cells were each added to artificial saliva work solution (45 mL, pH adjusted to 6.74-8.80), simulated gastric fluid (45 mL, pH adjusted to 2.44-2.52), and simulated intestinal fluid (45 mL, pH adjusted to 6.85-6.95) and kept at 37°C in a shaking incubator (MaxQ 6000, Thermo Scientific, Waltham, MA, USA) at 110 rpm. Samples (1 mL) were taken from each of the solutions at 5, 10, 15, and 30 minutes for the salivary phase and at 5, 15, 30, 60, 90, and 120 minutes for the gastric and intestinal phases. Dilutions (10^0-

10^{-7}) were plated onto MRSC agar plates and incubated at 37°C overnight for cell enumeration.

Statistical analysis

Particle size analysis results are shown as the mean of triplicate values. For cell enumeration counts, the mean value of 10 replicates (drops) was used to calculate cell counts for each free and encapsulated sample. Duplicate encapsulations were performed for *L. plantarum* and *L. johnsonii*. Analysis of variance (ANOVA) followed by Tukey honest significant difference test was determined using statistical analysis software package (GraphPad Prism 7, GraphPad Software, La Jolla, CA, USA).

Results

Laser diffraction particle size analysis was used to determine the mean diameter and particle size distribution of the *Lactobacilli*-containing calcium alginate microbeads (Table B.1, Figure B.1). The diameters of the alginate beads containing encapsulated *L. johnsonii* ranged from 126-188 µm (D [3,2]), while the diameters of the *L. plantarum*-containing beads ranged from 208-226 µm (D [3,2]). There was no statistical difference in the average diameters (D [3,2]) of the two replicates of beads containing *L. plantarum*, as well as the beads containing *L. plantarum* and replicate B of the beads containing *L. johnsonii.* The diameter (D [3,2]) of replicate A of the beads containing *L. johnsonii* was statistically different in size. However, there was no statistically significant effect on the storage times of the encapsulated *L. plantarum* and *L. johnsonii* at day 70 (Figure B.4).

Table B.1: Mean particle diameters of alginate beads containing *Lactobacillus plantarum* and *Lactobacillus johnsonii*. Averages were determined by static light scattering. Values are shown as volume-based (D[4,3]) and surface-based (D[3,2]) mean ± standard deviation.

Alginate Beads	Replicate	D [4,3]	D [3,2]
		µm	
L. plantarum	1	334 ± 27[a]	208 ± 13[a]
	2	337 ± 10[a]	226 ± 9[a]
L. johnsonii	1	184 ± 6[b]	126 ± 3[b]
	2	254 ± 6[c]	188 ± 12[a]

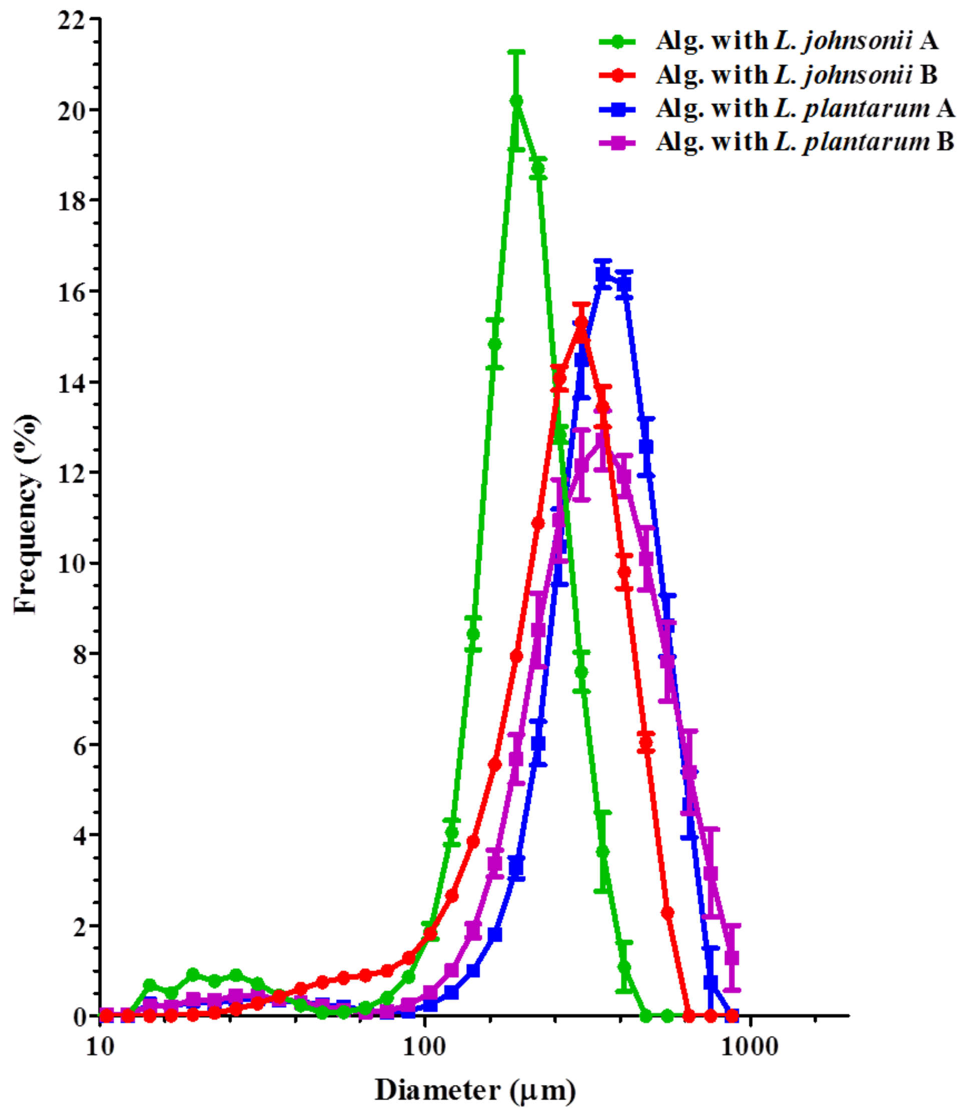

Figure B.1: Particle size distribution of alginate beads containing *Lactobacillus plantarum* and *Lactobacillus johnsonii*. Fresh samples were analyzed by static light scattering. Error bars represent the standard error of the mean.

Optical microscopy was used to examine the structure of the calcium alginate microbeads containing *L. plantarum* and *L. johnsonii*. The hydrogel microbeads were spherical with small indentations and were ~150-350 µm in diameter for both the *L. plantarum* and *L. johnsonii* containing beads (Figure B.2). This was consistent with the particle size analysis data (Table B.1). The *Lactobacillus* cells were visibly immobilized within the transparent hydrogel microbeads, confirming that the encapsulation was achieved. Within the microbeads, *L. plantarum* cells appeared to be spread uniformly throughout the hydrogel, while *L. johnsonii* cells appeared to be more aggregated.

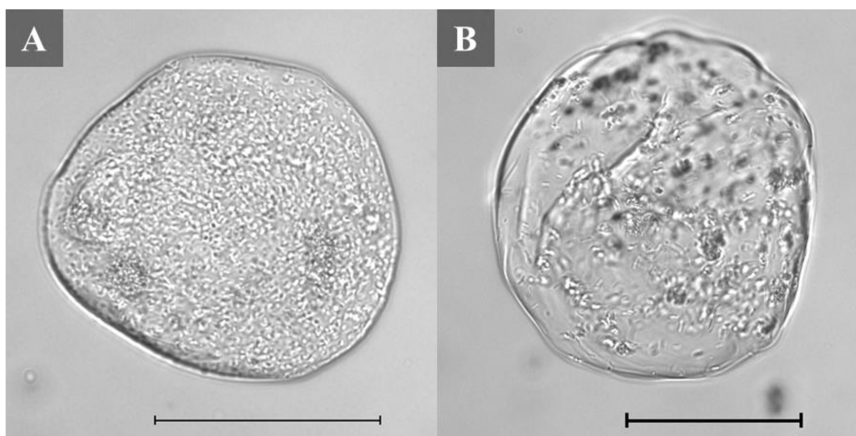

Figure B.2: Optical microscope images of (A) alginate microbead containing *Lactobacillus plantarum* (20×), (B) alginate microbead containing *Lactobacillus johnsonii* (20×). Scale bars represent 100 µm.

Scanning electron microscopy (SEM) was used to visualize the morphology of the calcium alginate beads. SEM was also used to visualize the free *L. plantarum* and *L. johnsonii* and to check for contamination. The free *L. plantarum* and *L. johnsonii* cells appeared consistent to their known morphologies, suggesting no contamination occurred (Figure B.3A, B.3C). The dried beads appeared more irregular than the wet beads visualized using optical microscopy (Figure B.2). The beads appeared to be collapsed from their previous spherical shape due to the loss of water formerly trapped inside the

hydrogel. The microbeads all exhibited diameters of ~150-350 µm. The surface of the beads containing *L. plantarum* and *L. johnsonii* were similar in appearance (Figure B.3B, B.3D).

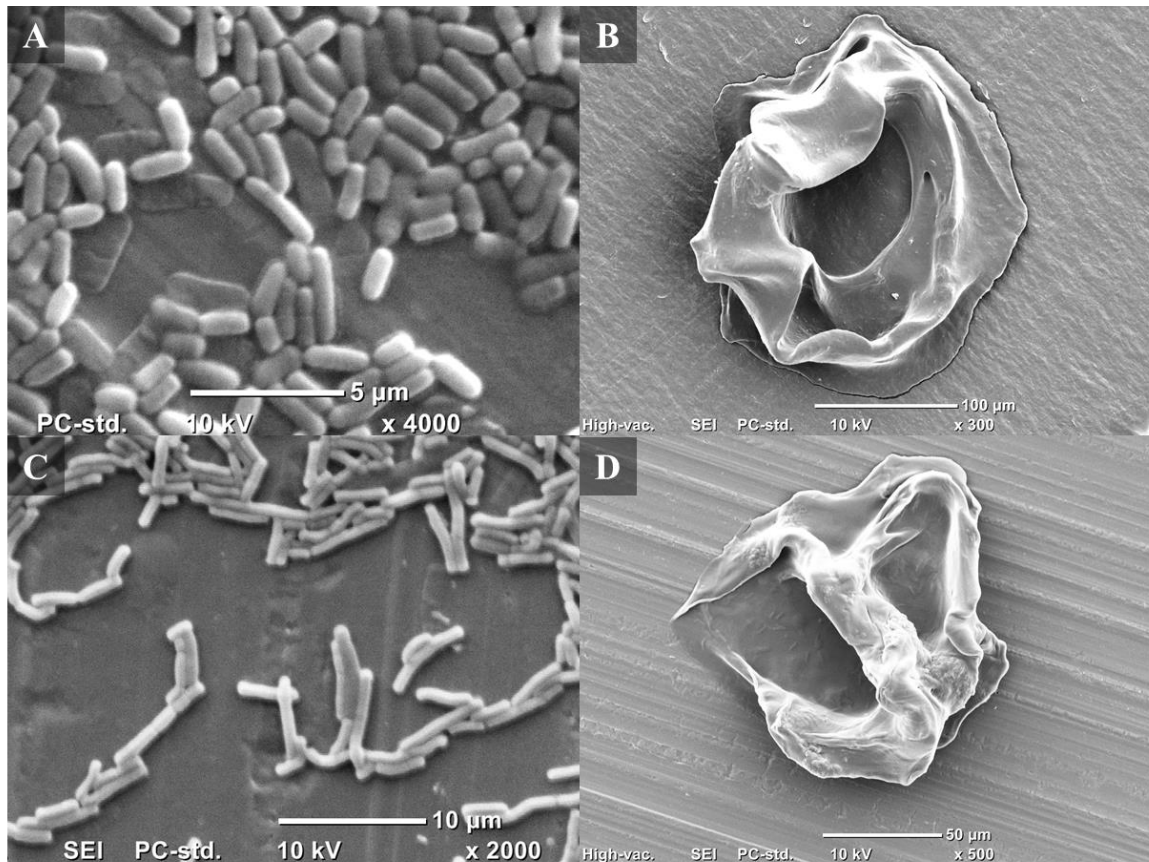

Figure B.3: Scanning electron microscope images of (A) *Lactobacillus plantarum* ATCC BAA-793 (4000×), (B) alginate bead containing *L. plantarum* (300×), (C) *Lactobacillus johnsonii* ATCC 33200 (2000×), (D) alginate bead containing *L. johnsonii* (500×). Samples were dried before sputter-coating with gold. SEM was set at high-vacuum, 10 kV. Scale bars for (A), (B), (C), and (D) represent 5, 100, 10, and 50 µm respectively.

Cell viabilities of free and encapsulated *L. plantarum* and *L. johnsonii* were compared over time during refrigerated storage (Figure B.4, Table B.2). Free *L. johnsonii* cells showed the shortest period of viability remaining consistently around 10 logs CFU until day 10 and then sharply dropped after day 17, reaching undetectable levels by day 31. Encapsulated *L. johnsonii* cells maintained high viability, having 9.09 logs CFU after

56 days of storage. This was only a 1 log decrease from the initial encapsulation of *L. johnsonii* compared to a 10 log reduction over 31 days for the free *L. johnsonii* cells. Thus, encapsulation of *L. johnsonii* significantly extended cell viability during long-term storage.

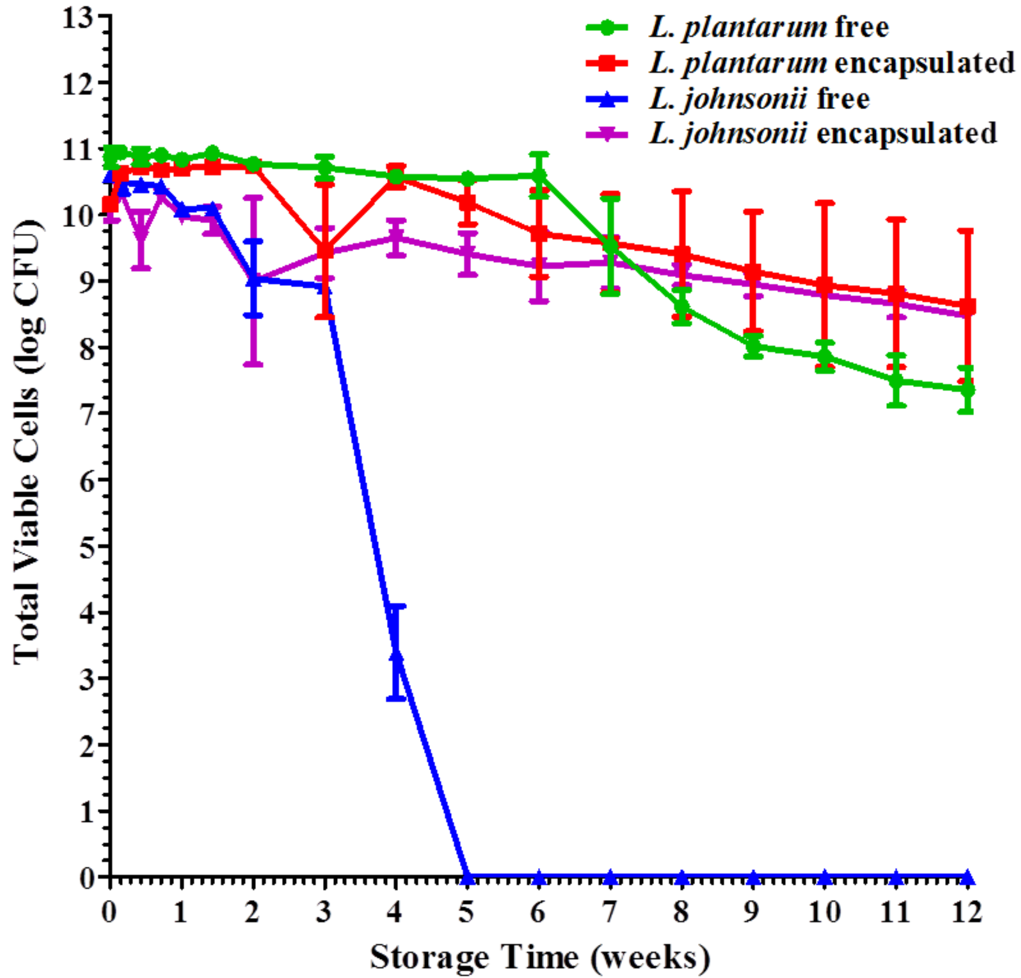

Figure B.4: Survival of free and encapsulated *Lactobacillus plantarum* and *Lactobacillus johnsonii* during refrigerated storage. Cell counts are based on samples drop-plated on MRSC agar and incubating at 37°C aerobically. Error bars indicate the standard error of the mean.

Table B.2: Viability of free and encapsulated *Lactobacillus plantarum* and *Lactobacillus johnsonii* during refrigerated storage. Cell counts are based on samples drop-plated on MRSC agar and incubating at 37°C aerobically. Values are shown as mean ± standard error of replicate counts. Means within each column followed by the same lowercase letters are not significantly different ($p > 0.05$) from each other. Means within each row followed by the same uppercase letters are not significantly different ($p > 0.05$) from each other.

Time	*Lactobacillus plantarum*		*Lactobacillus johnsonii*	
	Free	Encapsulated	Free	Encapsulated
	log CFU			
Day 0	$10.9{\pm}0.148^{aA}$	$10.2{\pm}0.0757^{abA}$	$10.6{\pm}0.0239^{aA}$	$10.1{\pm}0.145^{abA}$
Day 1	$10.9{\pm}0.0965^{aA}$	$10.6{\pm}0.0578^{aA}$	$10.5{\pm}0.186^{aA}$	$10.4{\pm}0.0540^{aA}$
Day 3	$10.9{\pm}0.118^{aA}$	$10.7{\pm}0.0949^{aA}$	$10.5{\pm}0.0658^{aA}$	$9.62{\pm}0.432^{abA}$
Day 5	$10.9{\pm}0.0982^{aA}$	$10.7{\pm}0.0481^{abA}$	$10.4{\pm}0.0406^{aA}$	$10.3{\pm}0.0408^{abA}$
Week 1	$10.8{\pm}0.0822^{aA}$	$10.7{\pm}0.0576^{aA}$	$10.1{\pm}0.0513^{aA}$	$9.98{\pm}0.106^{abA}$
Day 10	$10.9{\pm}0.0390^{aA}$	$10.7{\pm}0.0489^{aA}$	$10.1{\pm}0.0292^{aA}$	$9.92{\pm}0.205^{abA}$
Week 2	$10.8{\pm}0.0811^{aA}$	$10.7{\pm}0.0781^{aA}$	$9.04{\pm}0.557^{aB}$	$8.99{\pm}1.26^{abA}$
Week 3	$10.7{\pm}0.164^{aA}$	$10.5{\pm}0.0871^{abAB}$	$8.91{\pm}0.0630^{aB}$	$10.0{\pm}0.204^{abA}$
Week 4	$10.6{\pm}0.0833^{aA}$	$10.6{\pm}0.164^{abA}$	$3.39{\pm}0.702^{bB}$	$9.65{\pm}0.267^{abA}$
Week 5	$10.5{\pm}0.0892^{aA}$	$10.2{\pm}0.333^{abA}$	ND^{cB}	$9.41{\pm}0.318^{abA}$
Week 6	$10.6{\pm}0.317^{aA}$	$9.71{\pm}0.650^{abA}$	-	$9.22{\pm}0.530^{abA}$
Week 7	$9.52{\pm}0.719^{abA}$	$9.57{\pm}0.742^{abA}$	-	$9.27{\pm}0.390^{abA}$
Week 8	$8.61{\pm}0.252^{bcA}$	$9.40{\pm}0.947^{abA}$	-	$9.09{\pm}0.158^{abA}$
Week 9	$8.01{\pm}0.159^{bcA}$	$9.14{\pm}0.905^{abA}$	-	$8.95{\pm}0.178^{abA}$
Week 10	$7.86{\pm}0.210^{bcA}$	$8.93{\pm}1.23^{abA}$	-	$8.78{\pm}0.0587^{abA}$
Week 11	$7.50{\pm}0.379^{cA}$	$8.81{\pm}1.12^{bA}$	-	$8.65{\pm}0.206^{abA}$
Week 12	$7.36{\pm}0.336^{cA}$	$8.62{\pm}1.14^{bA}$	-	$8.47{\pm}0.0194^{bA}$

Free and encapsulated *L. plantarum* both maintained high viability over the 12 week storage period; there was no statistical difference in cell viability between free and encapsulated *L. plantarum* during long-term storage. By day 70, average log CFU of the encapsulated cells was 2-log higher than free cells (not statistically significant).

While encapsulating *L. plantarum* did not significantly extend shelf life during storage, survivability in simulated digestion fluids differed between free and encapsulated cells (Table B.3). Encapsulated *L. plantarum* was 4 log CFU higher than free *L. plantarum* after 60 minutes exposure to gastric phase. When exposed to intestinal phase,

Table B.3: Simulated digestion of free and encapsulated *Lactobacillus plantarum* and *Lactobacillus johnsonii* cells. Counts based on samples drop-plated on MRSC agar and incubated at 37°C aerobically. Values are shown as mean cell number ± standard error of duplicate results. Within each treatment, means within each column followed by the same letters are not significantly different ($p > 0.05$) from each other.

L. plantarum	**Free cells**			**Encapsulated cells**		
	Salivary	**Gastric**	**Intestinal**	**Salivary**	**Gastric**	**Intestinal**
pH	6.74-6.80	2.44-2.52	6.96-6.98	6.72-6.89	2.44-2.48	6.85-6.95
Time (min)	**log CFU**					
0	9.86±0.0241[a]	9.86±0.0241[a]	9.86±0.0241[a]	10.1±0.138[a]	10.1±0.138[a]	10.1±0.138[a]
5	9.74±0.125[a]	9.09±1.68[a]	8.83±0.471[a]	9.98±0.413[a]	9.97±0.159[a]	9.35±0.336[a]
10	9.77±0.0541[a]	-	-	10.3±0.0266[a]	-	-
15	9.26±0.479[a]	6.88±2.83[ab]	8.64±0.220[a]	9.19±1.07[a]	9.42±0.497[a]	9.39±0.470[a]
30	9.82±0.0372[a]	5.00±4.33[bc]	8.64±0.283[a]	9.71±0.543[a]	9.26±0.156[a]	8.69±1.16[a]
60	-	2.96±2.96[cd]	8.42±0.664[a]	-	7.01±1.05[a]	9.20±0.438[a]
90	-	ND[d]	8.51±0.551[a]	-	0.570±0.570[b]	9.27±0.541[a]
120	-	ND[d]	8.35±0.473[a]	-	0.855±0.855[b]	9.26±0.594[a]
L. johnsonii	**Free cells**			**Encapsulated cells**		
	Salivary	**Gastric**	**Intestinal**	**Salivary**	**Gastric**	**Intestinal**
pH	6.83-6.97	2.55-2.66	7.02-7.08	6.85-6.89	2.48-2.59	7.08-7.14
Time (min)	**log CFU**					
0	8.15±1.53[a]	8.15±1.53[a]	8.15±1.53[a]	7.96±1.61[a]	7.96±1.61[a]	7.96±1.61[a]
5	7.94±1.60[a]	8.10±1.70[a]	ND[b]	8.09±1.75[a]	8.03±1.48[a]	<5.33±1.63[ab]
10	7.96±1.56[a]	-	-	8.09±1.74[a]	-	-
15	7.52±1.06[a]	8.06±1.73[a]	ND[b]	8.01±1.58[a]	8.30±1.82[a]	<5.79±2.09[ab]
30	8.00±1.60[a]	<7.07±2.37[a]	ND[b]	8.17±1.64[a]	7.98±1.57[a]	<3.87±0.175[b]
60	-	>6.28±1.90[a]	ND[b]	-	>7.76±1.41[a]	<5.75±2.05[ab]
90	-	>7.08±1.09[a]	ND[b]	-	>7.06±1.12[a]	<5.81±2.11[ab]
120	-	>6.47±0.709[a]	ND[b]	-	>7.06±1.12[a]	<5.85±2.15[ab]

free *L. plantarum* only decreased by 1.5 log CFU, and encapsulated *L. plantarum* decreased by 0.84 log CFU after 120 minutes.

Encapsulating *L. johnsonii* in alginate did not significantly improve cell viability when exposed to the three simulated digestion phases. Indeed, effectively no change in viability was seen for both free and encapsulated cell in salivary phase, similar to that of *L. plantarum*. Free and encapsulated *L. johnsonii* cell viability decreased when exposed to gastric phase, but not as drastically as *L. plantarum*. Free *L. johnsonii* decreased 1.1 log CFU in 30 minutes and maintained high viability over the 120 minutes time period. Similarly, viability of encapsulated *L. johnsonii* decreased 0.9 log over 90 minutes. Unlike *L. plantarum* samples, both free and encapsulated L. johnsonii rapidly decreased in viability under exposure to simulated intestinal phase, suggesting high sensitivity to bile salts and lipase.

Acknowledgements

The authors thank Jean Alamed and Cynthia Kane for technical assistance on the project. Ezgi Ozcan is thanked for her assistance with statistical analysis. TY thanks the Center for Produce Safety (CPS) for financial support under Grant SCB14056.

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