2015

Development and Evaluation of Fiber and Pectin Based Delivery Systems for Delivering *Lactobacillus plantarum*

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DEVELOPMENT AND EVALUATION OF FIBER AND PECTIN BASED DELIVERY SYSTEMS FOR DELIVERING *LACTOBACILLUS PLANTARUM*

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Nutrition and Food Sciences

by
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December 2015

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest sincere gratitude and appreciation to Prof. Subramaniam Sathivel, my major advisor, for his valuable advice, continuous guidance, encouragement, constructive criticism, and kindness throughout my study, which enable me to carry out this dissertation successfully. My special thanks will be extended to all committee members, Dr. Joan King, Dr. Charles Boeneke, Dr. Joseph Bankston, and Dr. Ioan Negulescu, for giving valuable suggestions. I would like to thank Dr. Ted Gauthier, a director of LSU AgCenter Biotechnology Laboratory, and his staff, Ms. Tamara Chouljenko and Dr. Dong Liu, for providing me with laboratory facilities, helpful suggestions and kindness. My appreciation also goes to Thai Royal Government for their financial support during studying. Many thanks are also expressed to all colleagues in the food engineering and bioprocessing group for their distinguishable cheerfulness, encouragement, support and friendship. Finally, I wish to express my greatly appreciation to my family, for their love, understanding, patience, support, encouragement and everything throughout from the beginning.

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ABSTRACT

Probiotics may improve gut microbial composition and immune function. Introduction of probiotics via foods or supplements may result in the probiotics not surviving during processing and passage through the stomach to the large intestine. The overall objective of this study was to develop and investigate three delivery systems for delivering live probiotic cells (*Lactobacillus plantarum* NRRL B-4496) (LP). The three delivery systems were: (1) immobilized LP (~10^8-10^9 CFU/mL LP Free Cells) on purple rice bran fiber (PRF) (Delivery system 1), (2) encapsulated LP with combined pectin-rice bran extract (Delivery system 2), and (3) double encapsulated LP with protective agents (Delivery system 3). All three delivery systems were frozen prior to freeze drying and they were tested for viability of LP during processing and under gastrointestinal fluid conditions and compared with free LP cells. PRF protected cells in Delivery system 1 had less than 1 log reduction of viable cells, while the control (free LP cells) had reductions greater than 6 logs after freeze drying. The log reductions of viable LP cells protected with PRF after freeze drying and 12 weeks storage at 4 °C were between 0.7 and 1.3 log cycles. Delivery system 2 had significantly higher viability under gut conditions than free LP cells prior to freeze drying. However, the encapsulated LP did not survive during freeze drying. The third delivery system was developed by mixing LP cells with a protective agent including maltodextrin, wheat dextrin soluble fiber, or hi-maize starch. They were double encapsulated, first with pectin-rice bran extract then with whey protein isolate. Delivery system 3 had greater numbers of viable cells than delivery system 2 after freeze drying. The whey protein isolate coating significantly improved cell viability of the encapsulated cells during freeze drying. Hi-maize starch provided better protection to the encapsulated cells during freeze drying and in simulated gastrointestinal conditions than maltrodextrin and wheat dextrin soluble fiber. Encapsulation of *L. plantarum*

with hi-maize starch in freeze dried pectin-rice bran capsules would be a novel synbiotic supplement that may potentially be incorporated into food products such as nutrition bars, cereal products or dairy products.

CHAPTER 1 INTRODUCTION

Probiotics are well known for beneficial health effects on the host, such as improvement of gut microbial composition, protection against pathogenic bacteria, and modulation of immune systems. Probiotics have been incorporated into various food products, including both dairy products, such as yogurt, cheese, and ice cream, and non-dairy products i.e. cereals and juices. The global market of probiotic products has a tendency to increase at a compound annual growth rate of 6.8% and is expected to reach $37.9 billion in 2018 (Sharma, Tomar, Goswami, Sangwan, & Singh, 2014). For health benefits, viability of probiotic cells is a paramount factor that needs to be considered. Typically, probiotic concentration levels of 10^6 to 10^7 CFU/g, or greater, are desirable for their application as food supplements. In addition, probiotics need to have good survivability during the digestion process so that they reach the site of action, the large intestine, in sufficient numbers and viability for functionality.

However, introduction of probiotics via foods may result in the probiotics not surviving during processing and passage through the gastrointestinal tracts. Losses of probiotic cells could result from not only unfavorable conditions of food processing, such as heating, freezing, dehydration, and acidification, but also digestive system environments which contain gastric fluids (high acid level), bile salts, and bile enzymes. Poor probiotic viability was found in products containing free probiotic cells. The number of viable *Lactobacillus casei* added to yogurt dramatically decreased from 10^7 to less than 10 CFU/g within 30 min after exposure to pH 2, while the viable cells in low-fat cheese was reduced to 10^5 and 10^4 CFU/g after 30 and 120 min incubation at pH 2, respectively (Sharp, McMahon, & Broadbent, 2008). Providing probiotics with a physical barrier can be an approach to resist harmful environments, improve probiotic viability, and delivery through the stomach to the large intestine.

Immobilization and encapsulation are techniques that can be used to develop delivery systems for probiotics, which provide protection of bacteria cells. Dietary fibers may be utilized to immobilize bacterial cells throughout the fiber matrix. They provide surfaces for bacteria to attach onto, and they function as a protective agent against physiochemical changes due to adverse pH, temperature, and biles. For example, *L. casei* immobilized on wheat grains had a large number of viable cells after freeze drying and during storage for 12 months at -18°C (Bosnea et al., 2009). Oat bran fibers improved the survival of *L. casei* during dehydration and storage at room temperature. The cells adhered to oat bran fibers had better survival in simulated gastric acid (at pH 1.5) and bile salt media than did free cells after incubating for 2 h (Guergoletto, Magnani, Martin, Andrade, & Garcia, 2010). Pectin is a soluble fiber that can form three dimensional rigid and water insoluble hydrogels with a continuous layer. These hydrogels encapsulate bacterial cells within their core matrix. Pectin hydrogels/capsules stay intact in the stomach due to resistance to gastric acid and intestinal enzymes but can be degraded by colonic bacteria in the large intestine (Sriamornsak, 2003). The degradation by colonic bacteria releases the encapsulated cells. Viability of *Lactobacillus rhamnosus* in gastric conditions at pH 2 was improved when the cell was encapsulated with pectin (Gerez, Font de Valdez, Gigante, & Grosso, 2012). Encapsulated *Lactobacillus acidophilus* in pectin capsules had 1.51 log cycle reductions after incubating in simulated gastric (pH 3) and intestinal (pH 7) juices, while a reduction of 3.54 log cycles was observed in non-encapsulated cells (Gebara et al., 2013).

The overall objective of this dissertation was to develop and evaluate fiber and/or pectin based delivery systems for delivering *Lactobacillus plantarum*. To accomplish this, three constituent studies were conducted: (1) immobilization of *L. plantarum* on purple rice bran fiber, (2) development of a combined pectin-rice bran extract delivery system to improve *L. platarum*

viability under acid and bile conditions, and (3) double encapsulation of *L. plantarum* with protective agents to improve cell viability after freeze drying and during simulated gastrointestinal conditions.

CHAPTER 2 LITERATURE REVIEW

2.1. Probiotics

2.1.1. Definition and classification

The term "probiotic" was first used in 1965 by Lilly and Stillwell to describe "substances secreted by one microorganism which stimulates the growth of another" (Schrezenmeir & de Vrese, 2001). The term has been redefined by several researchers. In 1989, probiotic was redefined as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). This definition had increased focus on viability of probiotics and beneficial effects on the host. The definition of probiotic was improved by Food and Agriculture Organization of the United Nations and World Health Organization as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). Most probiotics are lactic acid bacteria (LAB). LAB are gram-negative, non-spore forming, and non-aerobic, and are aero-tolerant and acid tolerant bacteria (Agrawal, 2005). They are cocci or rods, which produce lactic acid as a major end product after carbohydrate fermentation (Wee, Kim, & Ryu, 2006). The important genera of LAB are *Lactobacilli*, *Bifidobacteria* and *Enterococci* (Agrawal, 2005); these genera are listed in Table 2.1. However, some non-lactic acid bacteria such as *Bacillus cereus* var. toyoi and *Escherichia coli* strain Nissle and some yeast are also considered as probiotics (Holzapfel, Haberer, Snel, Schillinger, & Huis in't Veld, 1998).

2.2.2. Probiotic products and their viability

Digestive health products are one of the most successful categories of functional foods. These include probiotic, prebiotic and dietary fiber products. Valls et al. (2013) mentioned that digestive health had been the most used assertion made on new functional food launches between

2005 and 2009. Between 2004 and 2009 the global market for pre- and probiotic yogurt grew to 128%, from $3.3 billion to $7.6 billion, while the drinking yogurt market increased 44% to $11.2 billion (Valls et al., 2013).

Table 2.1. Microorganisms used as probiotics

Lactobacillus sp.	*Bifidobacterium* sp.	Other LAB	*Bacillus* sp.	Non LAB
L. acidophilus	*B. adolescentis*	*Enterococcus*	*B. cereus*	*Clostridium*
L. casei	*B. animalis*	*faecalis*	*B. clausii*	*butyricum*
L. delbrueckii	*B. animalis* subsp.	*E. faecium*	*B. coagulans*	*Escherichia*
subsp.	*lactis*	*Lactococcus*	*B. licheniformis*	*coli*
delbrueckii	*B. bifidum*	*lactis*	*B. mesentericus*	*Propionibacterium*
L. delbrueckii	*B. breve*	*Leuconostoc*	*B. subtillis*	*freudenreichii*
subsp. *bulgaricus*	*B. longum* subsp.	*mesenteroides*		*Saccharomyces*
L. delbrueckii	*infantis*	*Pediococcus*		*cerevisiae*
subsp. *lactis*	*B. longum* subsp.	*acidilactici*		subsp.
L. helveticus	*longum*	*P. pentosaceus*		*cerevisiae*
L. fermentum		*Streptococcus*		*Saccharomyces*
L. johnsonii		*salivarius*		*cerevisiae*
L. leichmanii		*S. macedonicus*		subsp.
= delbrueckii		*S. mitis*		*boulardii*
subsp. *lactis*		*S. sanguis*		
L. paracasei		*S. thermophilus*		
L. plantarum				
L. reuteri				
L. rhamnosus				
L. sakei				

Source: modified from Foligne, Daniel, and Pot (2013)

Recently, it was reported that global markets of probiotic products have a tendency to grow at a CAGR (compound annual growth rate) of 6.8% and were expected to reach $37.9 billion in 2018 (Sharma et al., 2014). In the United State, 19% of American adults in 2008 had purchased a pre/probiotic yogurt in the previous 3 months. 24% of women had consumed those products which was two-fold higher than men. The majority of purchasers (30%) were in the 45 to 54 age range (Granato, Branco, Cruz, Faria, & Shah, 2010). Similarly, in Western Europe probiotic foods are a huge consumer market. In 2008 the probiotic market earned more than 1.4 billion euros. Yogurt and desserts were the biggest sector, which was about 1 billion euros

(Saxelin, 2008). Japan is one of the biggest world-wild functional food markets. Sixty-five probiotic products with 16 different strains had been listed as foods for specialized health use (FOSHU) in 2005. This increased to 73 products in 2008 (Amagase, 2008; Fukushima & Hurt, 2011).

Fermented dairy products such as yogurts, kefir, and cultured drinks are the major category of probiotic products. Milk and dairy products have been used as the main vehicle to deliver probiotics through human GI tracts for decades. The traditional yogurts are prepared by allowing yogurt cultures containing *L. bulgaricus* and *Streptococcus thermophiles* to ferment milk (Ranadheera, Baines, & Adams, 2010). However, some studies found that the conventional yogurt starter bacteria failed to survive through the intestinal gut or that their viability was lower than the minimum requirement ($< 10^6$ cfu/g) (Plessas, Bosnea, Alexopoulos, & Bezirtzoglou, 2012). Incorporating extra probiotics to yogurts such as *L. acidophilus* or *B. bifidum* has been suggested method to add more nutritional-physiological value (Lourens-Hattingh & Viljoen, 2001; Mortazavian et al., 2006). Ataie-Jafari, Larijani, Alavi Majd, and Tahbaz (2009) reported that compared with traditional yogurt consumption of yogurt fortified with *L. acidophilus* and *B. lactis* significantly decreased serum total cholesterol in mildly to moderately hypercholesterolemic subjects,. Cheese is one of the food products appealing to many palates. Cheese consumption has been growing in various countries in past decades (Gomes da Cruz, Alonso Buriti, Batista de Souza, Fonseca Faria, & Isay Saad, 2009). Several studies have mentioned that cheese functioned as a better probiotic carrier than yogurts. The high pH, fat content, buffering capacity, and dense protein matrix of cheese could help improve probiotic viability during storage and during passage through the gastrointestinal tract (Boylston, Vinderola, Ghoddusi, & Reinheimer, 2004; Phillips, Kailasapathy, & Tran, 2006). Sharp et al.

(2008) found that the number of viable *L. casei* added in yogurt dramatically decreased from 10^7 to less than 10 CFU/g within 30 min exposure to pH 2, while the strain in low-fat cheese was reduced to 10^5 CFU/g after 30-min incubation and had 10^4 CFU/g after 120 min. Ice cream is likely to be a good probiotic carrier due to its composition including milk proteins, fat and lactose, as well as other compounds. Fortification of ice cream by probiotics could increase the value of ice cream since it would become a functional food (Cruz, Antunes, Sousa, Faria, & Saad, 2009). *Lactobacillus acidophilus*, *L. agilis*, and *L. rhamnosus* were added into ice cream containing either sucrose or aspartame. Survivability of the cells was determined monthly during storage at -20 °C. The results showed that their stable viability and properties, including resistance to bile salts, antibiotics, and acidic conditions. Addition of the probiotic did not affect the ice cream characteristics (Basyigit, Kuleasan, & Karahan, 2006). Similarly, probiotic ice cream containing *L. casei* and *L. rhamnosus* had large lactic acid bacteria counts after frozen stage, ranging from 6.5 to 6.9 log cfu/g, while the control (without cells added) had less than 3 log cfu/g. No cell loss was observed during storage at -20 °C for 16 weeks. For the sensory assessment, probiotic vanilla ice cream had slightly lower taste intensity than the control ice cream (Di Criscio et al., 2010). Overrun levels of ice cream were reported to negatively affect probiotic viability due to probiotics lack of an oxygen-scavenging system. Microaerophilic and anaerobic probiotics are unable to reduce hydrogen peroxide, a toxic oxygen metabolite, which causes cell death (Vasiljevic & Shah, 2008). Ferraz et al. (2012) suggested that to maintain probiotic viability through the ice cream shelf-life, lower overrun levels should be obtained during manufacture. The authors found that a 90% overrun negatively affected viability of *L. acidophilus*, decreasing viable cell count by 2 log CFU/g after 60 days of frozen storage.

Besides dairy products, fruit and vegetable based probiotic products have been launched in order to satisfy vegetarians, consumers suffering from lactose intolerance, and consumers with cholesterol-restricted diets. The probiotics are incorporated into fruit pieces, fruit and vegetable juices and cereal bars. In one study, *L. rhamnosus* attached to apple wedges by dipping the apple into a probiotic solution. The apple contained high cell viability (~10^8cfu/g) during storage for 10 days at 4 °C in modified atmosphere packaging. Moreover, the sensory evaluation showed that average overall acceptability of the fresh prepared probiotic apple wedges had no significant difference with the control apple wedges (Roßle, Auty, Brunton, Gormley, & Butler, 2010). Dried apple cubes containing *L. plantarum* and *L. kefir* were analyzed for cell viability. It was found that the number of viable cells decreased by 2 log cycles after drying at 40 °C for 27 h. After storage at 4 °C for 3 months, the dried apples had only a 1 log reduction of cells. Cells in apples stored at room temperature died after 1 month of storage (Rego et al., 2013). Similarly, probiotic pomegranate juice containing *L. plantarum*, *L. delbruekii*, *L. paracasei*, and *L. acidophilus* had high cell survivability (~10^8 cfu/mL) after fermentation for 72 h. The number of viable cells was gradually decreased after storage for 2 weeks at 4 °C and no cells were detected after 4 weeks of storage (Mousavi, Mousavi, Razavi, Emam-Djomeh, & Kiani, 2011). Apple juices fortified with the addition of oligofructose or sucralose as sugar substitutes and *L. paracasei* were evaluated for sensory acceptability. Although the probiotics increased the turbidity of the juice, it had no effects on acceptance (appearance, aroma, flavor, texture and overall impression). The acceptance was positive on sweet taste, sweet aroma and bitter aftertaste. On the contrary, it was negative on apple flavor, apple aroma, darker color and sour taste (Pimentel, Madrona, & Prudencio, 2015). *L. rhamnosus* was inoculated into six cooked grains including buckwheat, dark buckwheat, barley, oat, soya, and chickpea, which were then

molded and fermented at 37 °C for 10 h. It was found that the cells multiplied in the final product of all grain types, yielding cell densities of 6.68–7.58 log CFU/g. The freshly prepared probiotic grain was acceptable to consumers but sensory scores decreased when the stored probiotic grain was evaluated. The researchers stated that lower acceptability scores were possibly due to the probiotic metabolites produced during storage (Kockova & Valik, 2014).

2.1.3. Probiotic functions and resulting health claims

Probiotics have many health benefits including reduction of infections, prevention of certain types of acute diarrhea, reduction of the risk of antibiotic-associated symptoms and improvement of lactose tolerance. Modulation of gut microbiota and immunomodulation by probiotics are also well documented health benefits. Recently probiotics have been reported to decrease cholesterol as well as prevent some cancers. A more detailed discussion follows.

1) Prevention of infectious diarrhea

It has been well established that probiotics help relieve acute infectious diarrhea especially in infants and children. Competitive exclusion is a major mechanism of probiotics and is responsible for the healing effect of infectious diarrhea (Saxelin, Tynkkynen, Mattila-Sandholm, & de Vos, 2005). Some probiotics have an ability to adhere to the epithelial wall, resulting in competition between probiotics and intestinal pathogens for the same adhesive receptors (Oelschlaeger, 2010). Allen, Martinez, Gregorio, and Dans (2010) revealed effects of probiotics on acute infectious diarrhea from 63 studies which included a total of 8,014 participants-mainly infants and young children. Overall probiotics shortened the duration of diarrhea and reduce its severity. The study supported the use of probiotics in acute, infectious diarrhoea. *L. rhamnosus* GG was reported to have the most consistent effect on prevention of acute infectious diarrhea in infants and children, when compared with other effective probiotics

(Szajewska & Mrukowicz, 2001). The use of probiotics helps prevent diarrhea caused by antibiotics particularly in children. Antibiotics negatively affected colonic microflora leading to changes of carbohydrate metabolism and antimicrobial activity in the colon. As the results, osmotic diarrhea and diarrhea caused by pathogens could occur (Salvini, 2013). Sixteen clinical studies (3,432 children) related to the prevention of antibiotic-associated diarrhea (AAD) by probiotics (*Lactobacilus* sp., *Bifidobacterium* sp., *Streptococcus* sp., or *Saccharomyces boulardii* alone or in combination) were reviewed and analyzed by Johnston, Goldenberg Joshua, Vandvik Per, Sun, and Guyatt Gordon (2011). The analyses stated that 15 out of 16 trials reported the benefits of probiotics against AAD compared to active, placebo or treatment controls. The incidence of AAD in the probiotic group was 9%, while 18% was found in the control group. The data indicated that a high dosage of *L. rhamnosus* and *S. boulardii* (5-40x10^9 CFU/day) was likely to prevent the onset of AAD with no serious side effects. *Clostridium difficile*-associated diseases (CDAD) are also reported to be relieved by probiotics. CDAD occur by transmission of *C. difficile* via the fecal-oral route, for example from the contamination of the hands of healthcare workers. A meta-analysis of 471 people across CDAD studies showed that the treatments of probiotics reduced a 71% of CDAC risk (Avadhani & Miley, 2011).

2) Improvement of lactose tolerance

Lactose intolerance, the gastrointestinal symptoms such as abdominal pain, flatulence, bloating, nausea, or diarrheas, results from lactose maldigestion, the inability to completely digest lactose (Hertzler & Clancy, 2003). Probiotics alleviate lactose intolerance by reducing lactose contents or by releasing lactase (Rolfe, 2000). Effects of kefir, a fermented milk beverage that contains different cultures than yogurt, on fifteen lactose maldigesters were evaluated and compared with the effect of yogurt and milk. The kinetics of hydrogen production

after ingestion was used to determine lactose maldigestion. The results showed that the hydrogen was not significantly different in yogurt and kefir treatments while milk had significantly increased hydrogen. Flatulence, the most common symptom in lactose intolerance, was severe in the subjects consuming milk. On the other hand, no differences in flatus severity were reported between yogurts and kefir treatments. The highest β-galactosidase activity was found in the subjects taking kefir (Hertzler & Clancy, 2003). Consuming of yogurt enriched with *Bifidobacterium animalis* and probiotic *B. longum* was reported to reduce lactose intolerance symptons in eleven adult lactose maldigesters. The study found that a number of total bacteria and fecal β-galactosidase activity were increased after 2 week supplementation. The symptom scores after lactose challenge was decreased (He et al., 2008). Almeida, Lorena, Pavan, Akasaka, and Mesquita (2012) also mentioned that the symptoms of lactose intolerance were reduced after lactose-intolerant patients consumed *L. casei* Shirota and *B. breve* Yakult. Hydrogen production of subject consuming probiotic treatments was lower than the baseline group, and was the same as that of the patients who received lactase.as same as the patients received lactase.

3) Modulation of gut microbiota

Probiotics have an influence on the ecosystem balance and/or metabolism characteristics of intestinal microbiota (Rabot, Rafter, Rijkers, Watzl, & Antoine, 2010). Probiotics stimulate the growth of indigenous bacteria resulting in increasing not only in the number of the bacteria but also the density (Ohashi & Ushida, 2009). This plays an important role in antagonism against pathogenic bacteria by reducing luminal pH, inhibiting bacterial adherence and translocation, or producing antibacterial substances and defenses. The production of a physiologically restrictive environment (pH, redox potential, and hydrogen sulfide production) helps resists colonization of pathogens (Ng, Hart, Kamm, Stagg, & Knight, 2009).

Effects of probiotics on the composition of the intestinal microbiota in humans were shown in several studies. Tuohy et al. (2007) mentioned that the number of fecal lactobacilli recovered from volunteers consuming a fermented milk drink (*L. casei*) was significantly increased after ingestion and that there was no change in the level of *L. casei* in the recovered fecal samples during the study period (21 days). The impact of yogurt consumption supplemented with *B. animalis* subsp. *lactis* (BB12) and *L. acidophilus* (LA-5) on fecal bacterial counts of healthy adults was studied. The results demonstrated that the healthy subjects consuming the yogurt had higher fecal numbers of BB12, LA5, and total lactobacilli, but lower enterococci than the placebo group (Savard et al., 2011). Similarly, it was evident that elderly volunteers who consumed cheese containing *L. rhamnosus* HN001 and *L. acidophilus* NCFM had increased number of the bacteria in fecal samples and lower counts of *Clostridium difficile* compared to the plain cheese group (Lahtinen et al., 2012).

4) Immunomodulation

The immunomodulatoty effect is one of the crucial benefits of probiotics. As well-known, segregation of immune systems is related to intestinal epithelial cells where microbiota in the gut lumen attached. The bacteria recognize receptors on the epithelial cell surface and bind to the receptors, immunological defense mechanisms such as the production of pro- and anti-inflammatory cytokines are triggered (Saxelin et al., 2005). Cytokines such as antigen presenting cells (APCs) and T lymphocytes have a positive impact on inflammatory bowel disease (IBD), irritable bowel syndromes, and allergies (McGovern & Powrie, 2007). Transforming growth factor beta (TFG-β) and interleukins such as IL-4, IL-5, IL-6 and IL-10 are multiple cytokines required for IgA promotion and maturation (Corthesy, 2007). IgA or immunoglobulin plays key roles in immune protection (Woof & Kerr, 2006). It has been reported that probiotics could

modulate cytokine profiles and activate monocytes and macrophages. This plays a pivotal role in activation of antigen-specific immunity and stimulation of IgA immunity. The result is related to promoting of IgE induction and allergic responses (Drago, Nicola, Iemoli, Banfi, & De Vecchi, 2010).

In clinical human studies, consumption of probiotic products or probiotic supplements helps enhance and/or modulate immune systems. According to the study of Parra, Martínez de Morentin, Cobo, Mateos, and Martínez (2004), forty-five healthy volunteers receiving fermented milk containing *L. casei* DN114001 increased of oxidative burst capacity of monocytes and natural killer cells tumoricidal activity, indicating that the bacterial strain could modulate the innate immune defense. Olivares et al. (2006) found that yogurt and a new fermented product containing *L. gasseri* CECT 5714 and *L. coryniformis* CECT 5711 increased the proportion of monocytes and neutrophils of healthy adult subjects as well as their phagocytic activity. *L. gasseri* CECT 5714 and *L. coryniformis* CECT 5711 induced an increase in natural killer cells proportion and IgA concentrations. It was reported that the use of probiotics in patients with ulcerative colitis reduced the colonic concentration of IL-6, colonic myeloperoxidase activity, and the level of fecal calprotectin, resulting in amelioration of colonic inflammation (Hegazy & El-Bedewy, 2010).

5) Lowering levels of cholesterol

Recently cholesterol lowering probiotics have been studied by many researchers. Several *in vitro* and *in vivo* studies reveled probiotics' the ability to assimilate cholesterol and/or deconjugate cholesterol to bile acids through bile salt hydrolase. These result in reduction of cholesterol and beneficial changes of lipid profile (Homayouni, Payahoo, & Azizi, 2012; Ooi & Liong, 2010). Guo et al. (2011) evaluated the effects of probiotic consumption on blood lipid in

13 trials that included a total of 485 participants with high, borderline high or normal cholesterol levels by a meta-analysis. They compared the treated probiotic groups to the control, and found that the pooled mean net change in total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were lower by 6.40 mg/dl, 4.90 mg/dl, 0.11 mg/dl, and 3.95 mg/dl, respectively. This indicated that a rich probiotic diet decreased total cholesterol and LDL cholesterol concentration in plasma of subjects with high, borderline high and normal cholesterol levels. The result was also confirmed by other studies in later years. Asemi et al. (2011) mentioned that consumption of enriched probiotic yogurt for 9 weeks reduced concentrations of total cholesterol (53.7 mg/dl), HDL-cholesterol (9.8 mg/dl), and triglyceride (42.8 mg/dl) in pregnant women. However, no difference in the reduction of total cholesterol and HDL-cholesterol concentrations was observed when compared to traditional yogurt. A yogurt containing microencapsulated *L. reuteri* NCIMB 30242 was found to significantly reduce LDL-cholesterol (8·92 %), total cholesterol (4·81 %) and non-HDL-cholesterol (6·01 %) in hypercholesterolaemic adults over placebo (Jones, Martoni, Parent, & Prakash, 2012). Type 2 diabetes mellitus patients taking probiotic yogurt containing *L. acidophilus* La-5 and *B. lactis* Bb-12 for 8 weeks had a significant reduction in the ratio between LDL-cholesterol and HDL-cholesterol compared to type 2 patients consuming conventional yogurt, while there was no difference in the concentration of total cholesterol, triglyceride, LDL-cholesterol and HDL-cholesterol.

6) Prevention of colorectal cancer

A number of *in vitro* and animal studies have demonstrated that probiotics could contribute to colorectal cancer (CRC) prevention. Anti-CRC mechanisms of probiotics are complex. According to Chong (2014), Intraluminal, systemic, and direct effects of probiotics on

intestinal mucosa are related to CRC prevention. Intraluminal effects include competitive exclusion, modulation of gut microbiota, reduction of carcinogenic secondary bile acids, binding of carcinogens and mutagens, and increasing short chain fatty acids production. Multifaceted immunomodulatories, in particular its ability to modulate intestinal inflammation, play an important role in decreasing the risk of CRC and reduction of DNA damages. Suppression of aberrant crypt foci formation on intestinal mucosa has also been mentioned as direct anti-CRC effects of probiotics. Anticancer activity of probiotics is strain dependent. *L. reuteri* ATCC PTA 6475, *L. rhamnosus* GG, *L. paracasei* IMPC2.1, and *L. acidophilus* could inhibit the growth of colon cancer cell lines and induce their apoptosis. Iyer et al. (2008) revealed that *L. reuteri* inactivated a tumor necrosis factor induced nuclear factor-κB in a dose and time-dependent manner by regulating cell proliferation, resulting in promoting apoptosis of activated immune cells. *L. rhamnosus* GG and *L. paracasei* IMPC2.1 were found to cause significant reduction in proliferation activity of DLD-1 cells, colon cell lines, after 24 and 48 h of attachment (Orlando et al., 2012). An *in vivo* studie revealed that rats fed with *L. rhamnosus* GG or *L. acidophilus* NCDC #15 and injected with 1,2 dimethylhydrazine dihydrochloride (DMH) to induce chemical colon carcinogenesis had reduction in Aberrant crypts, and recognizable mucosal alterations. In histopathological studies, it was found that *L. acidophilus* + DMH-treated rats had moderate infiltration of lymphocytes with edema in submucosa and mucosa, whereas *L. rhamnosus* + DMH had normal morphology of the colon (closely packed glands with few lymphocytes) (Verma & Shukla, 2013). *L. plantarum* AS1 was reported to reduce colon tumor volume diameter and total number of tumors induced by DMH in probiotic pre- and post-treated rats. The number of tumors was reduced from 2.16 tumors per rat to 1.8 tumors per rat in the

pretreated rats and to 1.6 tumors per rat in the post treated rats (Kumar et al., 2012). To confirm the potential role of probiotics in CRC prevention, human studies are required.

2.2. Human gastrointestinal tract and physiology

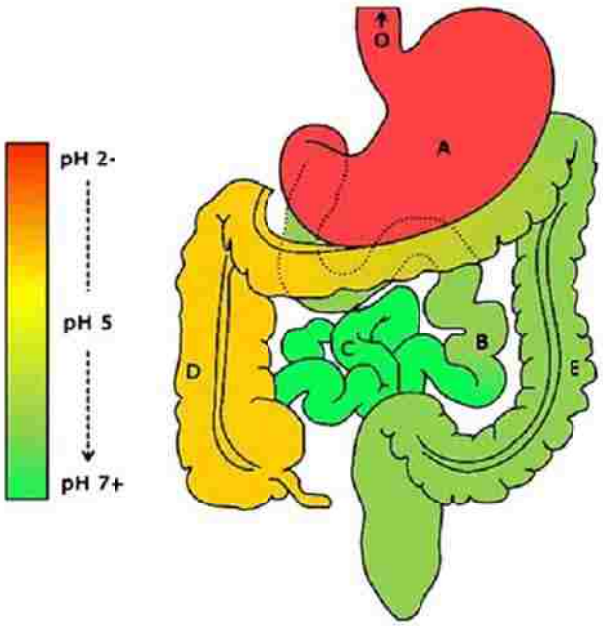
The gastrointestinal (GI) tract is a hollow muscular tube which functions for digestion, absorption, excretion, and protection (Cheng et al., 2010). The GI tract includes the mouth, pharynx, esophagus, stomach, small intestine, and large intestine. Each section has its own physiology (Figure 2.1), which needs to be considered to design delivery systems for controlled release. According to Cook, Tzortzis, Charalampopoulos, and Khutoryanskiy (2012), delivery systems convey bioactive components pass esophagus quickly. Only 10 to 14 seconds are needed. The system then reaches to the stomach which is a crucial section for pH-sensitive components such as probiotic cells. High acidity levels in the stomach could cause greatest loss of bacteria viability. The pH of the stomach is in the range of pH 1 to 2.5 but it can be as high as 5 in fed patients. The transit time is often reported between 0.5 and 2 hours. The pH and transit time of the stomach are highly variable and are dependent on many factors, such as time since eating and age. The stomach's fluid capacity ranges from 50 mL in a fasted state to as much as 1500 mL. The stomach has epithelium cells that can secrete a proteolytic enzyme (pepsin), the hormone gastrin, and hydrochloric acid (Daniels & Allum, 2005). After passage through the stomach, a delivery system enters into the small intestine. The small intestine is divided into the duodenum, the first short sessile, jejunum, a long coiled part constituting about two-fifths of the small intestine, and the ileum, the distal part of the small intestine which constitutes about three-fifths of the small intestine. (Sinha & Kumria, 2003). The small intestine has a pH ranging between 6.15 and 7.35 in the proximal region and a pH of 6.80 to 7.88 in the distal part of the small intestine. The transit time is approximately 3 to 4 h; however it can be varied depending on

individual subjects and formulation and dietary factors (Yu & Amidon, 1998). The duodenum contains some aerobic and gram positive bacteria (10^3 to 10^4 CFU/g), while a few microorganisms such as *Lactobacillus* sp. and *Enterococcus* sp. are found in the jejunum and the upper ileum. In the distal ileum, the bacterial concentration become greater and gram-negative bacteria start to out complete the gram-positive organisms (Sinha & Kumria, 2003). The large intestine is the large section into which the delivery system conveys a compound. It is divided into ascending, transverse, descending, and sigmoid regions. The pH of the colon varies depending on the section and the transit time, typically reported at 6 to 32 h (Cook et al., 2012). The colon is home to a large concentration of microbiota (which is 10^{10} to 10^{12} CFU/g) (Schrezenmeir & de Vrese, 2001). These bacteria are responsible for fermentation of protein and polysaccharides and can release the compounds that are carried by a delivery system. The fermentation contributes to formation of short chain fatty acids and fecal bulking, as well as increase transit time of colonic contents (Topping & Clifton, 2001a)

2.3. Dietary fibers and their physiological effects

The American Association of Cereal Chemists defined dietary fibers as "the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine" (AACC, 2000). The dietary fibers can be derived from plants, vegetables, cereal grains, woody plants, fruits, legumes, leguminous plants, etc. They are classified into soluble and insoluble fibers. Soluble fibers are pectins, gums, inulin-type fructans and some hemicelluloses, while insoluble fibers consist of lignin, cellulose and some hemicelluloses. Most fibers include approximately one-third soluble and two-thirds insoluble fibers (Wong & Jenkins, 2007). Dietary fibers are composed of non-digestible poly- and oligosaccharides and compounds in plants. Table 2.2 displays

components of dietary fibers as listed by AACC. Dietary fibers have physiological effects on human health such as attenuating blood glucose and cholesterol levels as well as improving colonic fermentation and bowel functions (Raninen, Lappi, Mykkanen, & Poutanen, 2011). These effects can contribute to reducing blood pressure, improving serum lipid concentration, promoting regularity, inducing weight loss, and enhancing immune functions. These result in reduction of risk of several diseases, such as coronary heart diseases, diabetes, obesity, and certain gastrointestinal disorders (Anderson et al., 2009). According to Tungland and Meyer (2002), the GI tract is the primary area where dietary fibers function. Intake of dietary fiber affects absorption of nutrients, carbohydrate and fat metabolism, and sterol metabolism. Dietary fiber also influences colonic fermentation and stool production.



Letter	Region	pH	Transit Time
O	Oesophagus	~7.0	10-14 seconds
A	Stomach	1-2.5 (up to 5 fed)	Half emptying: ~80.5 mins
B	Proximal Small Intestine	6.15-7.35	3.2 ±1.6 hrs (combined)
C	Distal Small Intestine	6.80-7.88	
D	Ascending Colon	5.26-6.72	Highly variable, dependent on bowel evacuations
E	Descending Colon	5.20-7.02	

Figure 2.1. Characteristics of human gastrointestinal tracts

Source: Cook et al. (2012)

Table 2.2. Constituents of dietary fibers

Non starch polysaccharides and oligosaccharides	Analagous carbohydrates	Lignin substances
Cellulose	Indigestible dextrins	Waxes
Hemicellulose	Resistant maltodextrins	Phytate
Arabinoxylans	Resistant potato dextrins	Cutin
Arabinogalactans	Synthesized carbohydrates	Saponins
Polyfructoses	compounds	Suberin
Inulin	Polydextrose	Tannin
Oligofructans	Methyl cellulose	
Galacto-oligosaccharides	Hydroxypropylmethyl	
Gums	cellulose	
Mucilages	Resistant starches	
Pectins		

Source: AACC (2000)

2.3.1. Physiological effects of dietary fibers in the small intestine

Dietary fibers are reported to have an influence on rheology of the small intestine. Soluble fibers such as pectin or guar gum can increase the gut viscosity by forming gels, causing delay of gastric emptying and increase small intestinal transit time. Fibers with a high water holding capacity play an important role in the volume and bulk of the intestinal content. The change in gut rheology slows diffusion of glucose and increases the unstirred water layer at the surface of the small intestine, resulting in delay of glucose absorption (Guillon & Champ, 2000). Effects of dietary fibers in small intestinal simulation were investigated. The researchers reported that viscosity of small intestinal simulation was increased after incubating between 3 and 9 h with guar gum, oat bran and rice bran. Guar gum and oat bran had viscous characteristics, indicating the ability to attenuate blood glucose and lipid, while viscous characteristics were not found in wheat bran, rice bran and wood cellulose (Dikeman, Murphy, & Fahey, 2006). Insoluble fibers derived from peel of *Citrus sinensis* L. cv. Liucheng (Liucheng sweet orange) could adsorb glucose, retard glucose diffusion, and inhibit the activity of α-amylase. The result

might delay the rate of glucose absorption and further lower the concentration of postprandial serum glucose (Ahmed, Sairam, & Urooj, 2011).

Dietary fibers can bind to bile acids and metabolize cholesterols, which can affect the digestion and absorption of lipids in the small intestine (Rodriguez, Jimenez, Fernandez-Bolanos, Guillen, & Heredia, 2006). The binding capacity of soluble dietary fiber from apple peel to bile acids and to cholesterol was compared to insoluble dietary fiber from wheat bran and soybean seed hulls, and to a mixture of soluble and insoluble dietary fiber. Soluble dietary fiber had the greatest binding capacity to bile acids and cholesterol, followed by the fiber mixture, and insoluble dietary fiber (Zhang, Huang, & Ou, 2011). In contrast, Kahlon and Woodruff (2003) reported that dehulled barley had the highest relative binding to bile acids (57%), followed by rice bran (49%), β-glucan enriched barley (40%), and oat bran (30%), The relative binding to bile acids was calculated based on bile acid binding to cholestyramine as 100%. The author suggested that bile acid binding to rice bran, oat bran, and β-glucan enriched barley may be related to their insoluble fiber contents. Soluble fibers more effectively lower low-density lipoprotein (LDL) cholesterol than insoluble fibers. Babio, Balanza, Basulto, Bullo, and Salas-Salvado (2010) mentioned that soluble fibers affected metabolic pathways of hepatic cholesterol and lipoprotein metabolism, as the result of modification in volume, bulk, and viscosity in the intestinal lumen by fibers. The alteration of metabolic pathways leads to lowering of LDL cholesterol. Soluble fibers can also reduce plasma cholesterol by lowering absorption of intestinal bile acid. The interruption of the enterohepatic bile acid circulation elevated fecal bile acid loss and its de novo synthesis in liver. In clinical studies, it was suggested that intake of 5–15 g per day of soluble fiber yielded a 5–13% reduction in LDL-cholesterol levels in both men and women (Anderson et al., 2009). A meta-analysis of 126 studies involving 5,590 subjects

indicated that consumption of 3 g per day of oat or barley β-glucan or more could decrease blood cholesterol. The results showed that consumption of oat and barley β-glucan reduced total cholesterol by 0.60 mmol/L, LDL cholesterol by 0.66 mmol/L, triglyceride by 0.04 mmol/L and increased high density lipoprotein (HDL) cholesterol by 0.03 mmol/L (Tiwari & Cummins, 2011). Insoluble fiber enriched polyphenol was found to have an effect on serum blood cholesterol in hypercholesterolemic patients. Consumption of insoluble fiber rich in polyphenols lowered the total cholesterol, LDL cholesterol, LDL: HDL cholesterol ratio, and triglycerides by 17.8%, 22.59%, 26.2% and 16.3% after 4 weeks consumption compared with the baseline (Ruiz-Roso, Quintela, de la Fuente, Haya, & Perez-Olleros, 2010).

2.3.2. Physiological effects of dietary fibers in the large intestine

The large intestine contains a numerically large and diverse range of bacteria. More than 500 bacterial species with up to 10^{10} CFU/g of intestinal contents are found in the large intestine (Hold, Pryde, Russell, Furrie, & Flint, 2002). Dietary fibers play an important role in colonic fermentation. They provide the colonic bacteria with energy sources and are then fermented to short-chain fatty acids (SCFAs) and bacterial mass (Tungland & Meyer, 2002). Increase of SCFAs levels contributes to prevention of colon cancers and gastrointestinal disorders (Wong, de Souza, Kendall, Emam, & Jenkins, 2006). Fermentability of fibers is dependent on physiochemical properties of fibers such as solubility, type of linkages, degree of polymerization, and transit time (Raninen et al., 2011). Production of SCFAs from seven different dietary fibers by intestinal microflora was compared by Pylkas, Juneja, and Slavin (2005). The results showed that SCFAs production was dependent on fiber sources. After 24 h incubation, the highest total SCFAs concentration was found in hydrolyzed guar gum and galactomannan, followed by indigestible dextrin, arabinogalctan, polydextrose, psyllium husk,

and methylcellulose, respectively. Butyrate production was greater in the fiber treatments compared to the glucose control. Rice bran fibers were reported as a novel prebiotic. The fiber was utilized by lactobacilli to produce some SCFAs, while butyrate was produced when the fiber was fermented by *Eubacterium limosum* (Osamu et al., 2010). Oat bran could be degraded by human fecal bacteria and altered to SCFAs including acetate, lactate, propionate, and butyrate. Concentration of butyrate from oat bran fermentation was not different compared to prebiotic fructo-oligasaccharide fermentation but higher than in glucose fermentation. Oat bran could also increase the number of bifidobacterium and lactobacilli and had the same pattern as fermentation of prebiotic fructo-oligosaccharide (Kedia, Vazquez, Charalampopoulos, & Pandiella, 2009). Mice fed with rice bran had higher colonization of native lactobacilli in fecal samples, compared to a control. The author suggested that induction of lactobacilli was possibly related to an increase of mucosal IgA response (Henderson, Kumar, Barnett, Dow, & Ryan, 2012).

Insoluble fibers play an important role in bowel functions by increasing fecal volume and weight (bulking effect) and decreasing transit time. Raninen et al. (2011) mentioned that intake of fibers derived from bran or whole grains of 11 to 30 g/d helped decrease transit time, increase stool weight and frequency, and improved stool consistency. Consumption of low fiber diets could lead to the formation of very compact feces which might promote oncogenesis, resulting from large exposure time of the intestinal mucosa, to cancer-risk agents (Rodriguez et al., 2006). Compared to a low fiber diet, high fiber cereal breakfasts namely All- Bran, Bran Buds with Corn and Bran Buds with Psyllium significantly increased fecal bulk and bowel movement as well as decrease intestinal transit time in healthy persons. The largest fecal wet weight was found in the subjects consuming Bran Buds with Psyllium (Vuksan et al., 2008). Fecal bulk and moisture were increased when hamsters were fed with insoluble fibers derived from passion fruit

seeds. The levels of triglyceride and total cholesterol in serum as well as liver cholesterol were reported to be increased, in contrast the levels of total lipids, cholesterol, and bile acids in feces were decreased. The authors noted that cholesterol and lipid lowering effects might be partly due to the ability of the insoluble fibers in promoting the excretion of lipids and bile acids via feces (Chau & Huang, 2005).

2.4. Immobilization and encapsulation

Cell immobilization refers to trapping of microorganisms within or throughout a matrix (Mitropoulou, Nedovic, Goyal, & Kourkoutas, 2013). It has been applied to enzymes, proteins, and alcohol beverages. This technology provides several advantages such as enhancing fermentation productivity, improving continuous processes, increasing cell stability, and lowering costs of recovery, recycling, and downstream processing (Kosseva, 2011). One of the well-known applications of cell immobilization is production of high fructose corn syrup (HFCS). According to Kosseva (2011), in 1969, Tekasaki and his colleagues discovered that *Streptomyces albus,* glucose isomerase production bacteria, was able to grow on crude xylans such as cereal bran or straw. During growth on xylans, the cells could retain their enzyme activity in a prolonged process and cell lysis was prevented at operating temperature (60 °C). This could lay a foundation on the reuse of whole-cell biocatalyst, and continuous processing in a column reactor. Use of cell immobilized-biocatalyst technology was used to produce HFCS on a commercial scale in 1975 and has been continuously since then. In addition to enzyme production purposes, immobilization techniques have been used to protect probiotics during processing and improve their stability during storage. Immobilization of lactobacilli on wheat dextrin, oat bran fiber, bacterial cellulose, and mungbean fibers were reported to help enhance viability of the cell during dehydrating, freeze-drying and storage (Guergoletto et al., 2010;

Hongpattarakere, Rattanaubon, & Buntin, 2013; Jagannath, Raju, & Bawa, 2010; Saarela, Virkajarvi, Nohynek, Vaari, & Matto, 2006). Cell immobilization is conducted by replicating the conditions under which cells grow on surfaces since microorganism themselves have ability to naturally adhere to different types of support materials (Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004). Immobilization techniques can be divided into three main categories based on the physical mechanism employed, namely attachment or adsorption on solid carrier surfaces, entrapment within a porous matrix, and self-aggregation by flocculation or with crosslinking agents as shown in Figure 2.2 (Pilkington, Margaritis, Mensour, & Russell, 1998). Some papers include cell containment behind barriers or encapsulation as one of immobilization methods.

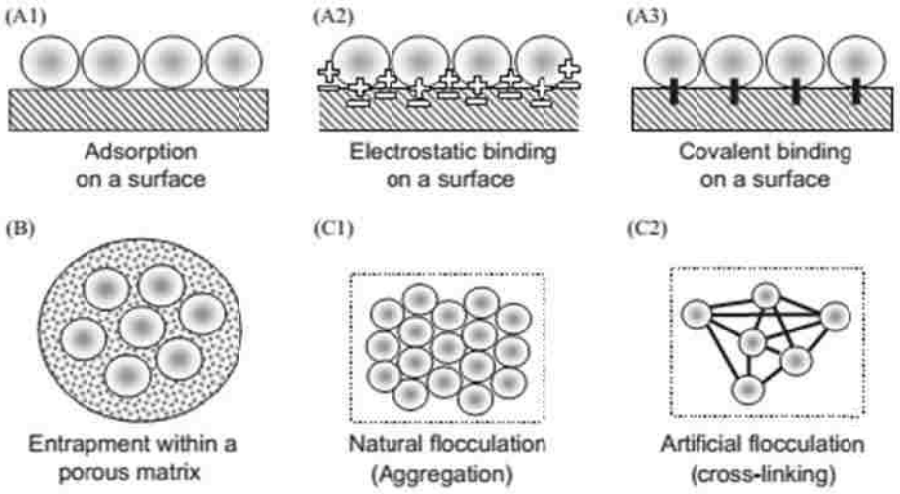


Figure 2.2. Basis methods of cell immobilization (A=immobilization on solid surface, B=entrapment within porous matrix, and C=cell flocculation)

Source: Kourkoutas et al. (2004)

Encapsulation refers to a process of forming a continuous layer entrapping a whole compound within a matrix core. The technique can produce particles or capsules with diameters of a few nanometers to a few millimeters. The entrapped substance, usually an active compound, is normally called the core material or internal phase, while the substance encapsulating the core

material can be termed the carrier material, wall material, matrix, or external phase (Zuidam & Shimoni, 2010). Cell encapsulation was first applied in the biotechnology area. The encapsulated cells helped improve efficiency of processes by allowing rapid and efficient separation of cells and metabolites. The technique was then introduced to the pharmaceutical sector for drug and vaccine delivery and to the food industry (Nedovic, Kalusevic, Manojlovic, Levic, & Bugarski, 2011).

In the food industry, encapsulation techniques have been applied to bioactive molecules such as antioxidants, vitamins, essential oils, or flavors and living microorganisms (probiotics) for several purposes. Encapsulation helps slow down degradation/inactivation of active compounds and protect them from adverse effects during processing and storage (de Vos, Faas, Spasojevic, & Sikkema, 2010). The technology serves as a barrier between the encapsulated compounds and surrounding environments, which helps stabilize food ingredients, allow aroma or flavor differentiation or mask bad odors (Champagne & Fustier, 2007). For probiotics, encapsulation provides protection to the cells and their functionalities against unfavorable factors (i.e. heat, moisture, light, or oxygen) not only during production, but also when passing through the gastrointestinal system (Anal & Singh, 2007). Encapsulation can also modify physical characteristics of original materials to allow easy handling, separation of components that would react to others, and uniform dispersion (Desai & Jin, 2005). Additionally, delivery of active compounds to the right place at a right time or controlled release is one of the important roles of encapsulation. Controlled release could improve the effectiveness of active ingredients, increase the application range of food ingredients and ensure optimal dosage (Desai & Jin, 2005). A specific barrier formed by encapsulation can serve to manipulate the release of active compounds to provide functional benefits and unique sensory experiences (Lakkis, 2007). For example,

encapsulation of probiotics contributes to cell stability and viability in a food matrix and during passage through the gut and allows progressive liberation of the cells in the intestine (Nazzaro, Fratianni, Orlando, & Coppola, 2012).

2.4.1. Immobilization of probiotics on a dietary fiber based matrix

As mentioned earlier, probiotics provide a variety of health benefits, if they survive until they reach the intestine and colonize the host. The use of plant-based matrixes to enhance probiotic survival during processing, formulation, and passage through the gastrointestinal tract was demonstrated in several studies. According to Saarela et al. (2006), immobilization of *L. rhamnosus* on wheat dextrin and polydextrose had good cell viability after freeze-drying and during storage. Only 0.7 and 1.3 log reductions were reported in wheat dextrin and polydextrose-freeze dried cells, respectively, after storage at 37 °C for 4 weeks. The freeze-dried cells with the fibers were incorporated into chocolate-coated breakfast cereals and stored for 7 months. This showed that freeze dried cells with polydextrose was more stable than those with wheat dextrin. *L. casei* immobilized on wheat grains had a large number of viable cells after freeze drying. The cell morphology was retained and no shrinkage was observed. Freeze dried *L. casei* on wheat grains was highly stable during storage for 12 months at -18 °C (Bosnea et al., 2009). Oat bran fiber was found to improve the survival of *L. casei* during dehydration and storage at room temperature. The cells adhered to oat bran fiber had better survival in gastric (at pH 1.5) and in bile salt media than did free cells after incubating for 2 h (Guergoletto, Magnani, Martin, Andrade, & Garcia, 2010). Jagannath et al. (2010) found that nata or bacteria cellulose could function as a cryoprotectant and an immobilized support for freeze dried lactobacilli. After freeze drying, the viability of lactobacilli attaching on nata had approximately a 3 log cycle reduction. The freeze-dried cells with nata had ~10^5 CFU/g viable cells after storage for 60 days at 4 °C.

The presence of mungbean fiber helped maintain viability of *L. plantarum* after freeze drying and during storage. It was observed that in freezed-dried cells, a thin layer of mungbean fiber coated the cells, which could indicate that the fiber served as a physical barrier, protecting the cells from freeze-drying process (Hongpattarakere et al., 2013).

2.4.2. Encapsulation of bioactive components using a hydrogel-base matrix

1) Hydrogels

Hydrogels or hydrocolloid gels are cross-linked polymers with the ability to swell in an aqueous medium (Kim, Bae, & Okano, 1992). Hydrogels can be applied in several areas including the pharmaceutical, medical, cosmetic, and food industries. A major applications is to use hydrogels to encapsulate drug, probiotics, and bioactive molecules and to control their release (Burey, Bhandari, Howes, & Gidley, 2008). Hydrogels can be either physically or chemically formed (called physical and chemical gels, respectively) by using natural or synthetic polymers. Physical gels are achieved via physical processes including association, aggregation, crystallization, complexation, and hydrogen bonding, while chemical gels are prepared by chemical processes (Omidian & Park, 2012). Figure 2.3 illustrates the difference of physical and chemical gel preparations. Hydrogels can also be classified according to their responses to the environmental conditions such as pH, temperature, and the composition of the surrounding liquid (Figure 2.4). Hydrogels react to environmental changes by changing their size or shape. Ionic hydrogels are sensitive to and respond to pH changes. Hydrogels containing hydrophobic groups swell and shrink in response to temperature changes. Non-ionic hydrogels are more stable than ionic hydrogels in a salt swelling media and in a nonsolvent media (Omidian & Park, 2012). Hydrogels are versatile. Their characteristics are dependent on the types of polymers, the network formation mechanism and the processing method used for gel formation (Burey et al.,

2008). They also have many different physical forms, including solid molded forms, pressed powder matrices, microparticles, coatings, membrane or sheet, encapsulated solids, and liquid (Hoffman, 2002).

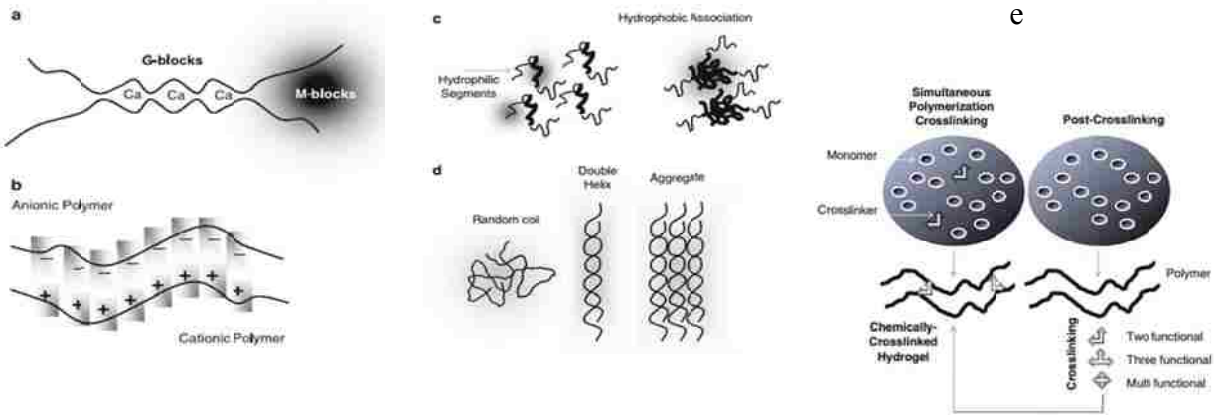


Figure 2.3. Examples of (a) physical hydrogels crosslinked by ion–polymer complexation, (b) polymer–polymer complexation, (c) hydrophobic association, (d) chain aggregation, and (e) chemical hydrogels

Source: Omidian and Park (2012)

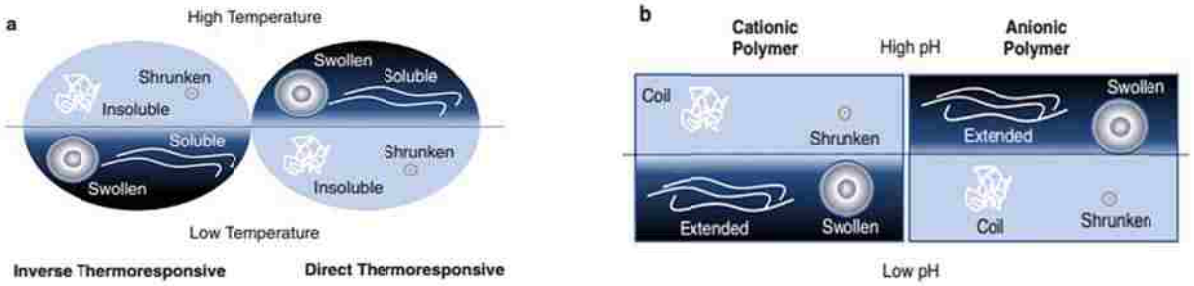


Figure 2.4. Hydrogel responses dependence on (a) temperature and (b) pH

Source: Omidian and Park (2012)

2) Ionotropic gelation

Ionotropic gelation is a mechanism of physical gel formation. It is based on the ability of polyelectrolytes to cross link in the presence of counter ions. Typically, negatively charged polymers are cross-linked by cations from an aqueous solution. Bioactive compounds can be

loaded into the hydrogel by combining the gelation with emulsification, coacervation, or extrusion dripping. Ionotropic gelation is carried out by two diffusion setting techniques, external gelation and internal gelation. For external gelation, a polymer solution is introduced into an ionic solution. The ions diffuse into the polymer solution to form a three dimensional lattice of ionically crosslinked moieties during gelation occurring from the surface to the core. This technique can cause non-homogenous hydrogels and a firm outer surface but soft core gel. For Internal gelation, an inactive form of the ion is sufficiently dispersed in the polymer solution. The ion is then activated and released by pH adjustment to form hydrogels. Although this technique has been developed to overcome non-homogeneity of the hydrogels in external gelation, the problem could still occur if gelation happens prior to adequate ion dispersion. In addition, it is evident that hydrogels from internal gelation have less dense matrices than externally cross-linked hydrogels with large pore size leading to low loading efficiency and fast release rates (Chan, Lee, & Heng, 2006).

There are a wide and diverse range of polymers available with which to fabricate hydrogels. Alginate is the most common natural polymer used to form the hydrogels. This polymer is reported to be suitable for bioactive food components and living cell encapsulation as well as for drug or protein delivery (Matricardi, Meo, Coviello, & Alhaique, 2008). Alginate-based hydrogels are used to facilitate controlled release of bioactive molecules in the colon (Shah, Shah, & Amin, 2011). Pectin is generally used for targeted drug delivery. It remains intact in the stomach and the small intestine. It is degraded by enzymes secreted by the host microbiota in the large intestine (Liu, Fishman, & Hicks, 2007). Carboxymethly cellulose is applied in enzyme immobilization, dry removal, and drug and probiotic delivery (Chitprasert, Sudsai, & Rodklongtan, 2012). It has gastric acid resistance and intestinal solubility characteristics (Kamel,

Ali, Jahangir, Shah, & El-Gendy, 2008). Gelatin, carrageenan, and gellan gum are also widely used for encapsulation of active compounds by ionotropic gelation method (Patil, Chavanke, & Wagh, 2012).

3) Production of hydrogels by ionotropic gelation/extrusion dripping

The extrusion dripping technique has been extensively applied for encapsulation of living cells and bioactive ingredients. This method can be done by forcing a mixed solution of biopolymers (mainly alginate, carboxymethy cellulose, or pectin) with active ingredients through nozzles or small openings in droplet-generating devices into a bath containing a cross-linking solution (de Vos et al., 2010). In the laboratory, the solution is forced through a syringe needle to form droplets. For large scale production, multiple-nozzle systems, spinning disc atomizers, or jet-cutter techniques are employed to produce droplets (Figure 2.5).

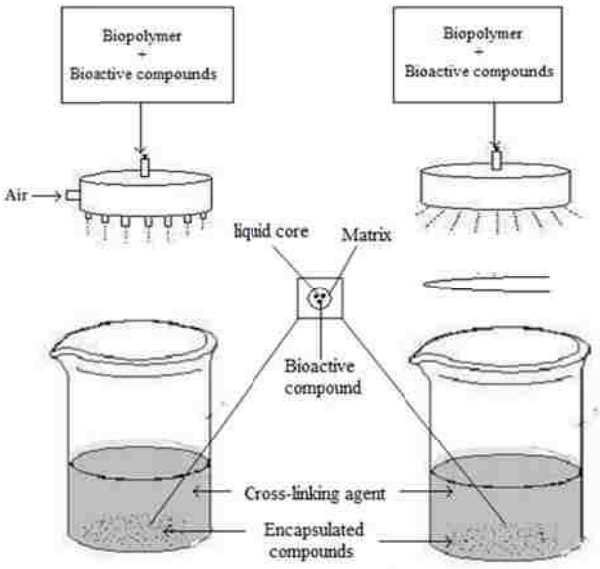


Figure 2.5. Extrusion dripping technologies by simple needle droplet generator using air driven (left) and pinning disk (right)

Source: Solanki et al. (2013)

The bead size and shape depend on the liquid formulation and parameter setup such as solution viscosity, surface tension, tip size, collecting distance etc. Some trial-and-error attempts

are often required (Lee, Ravindra, & Chan, 2013). The technique has many advantages particularly for living cells and sensitive bioactive compounds. It is relatively simple, gentle and non-toxic. The process can be operated under both aerobic and anaerobic conditions (de Vos et al., 2010). However, a major drawback of this method is that the matrix has high porosity allowing fast and easy diffusion of water and other fluids in and out of the matrix (Rathore, Desai, Liew, Chan, & Heng, 2013). The encapsulates are less stable due to rapid cross-linking and hardening at the surfaces of the beads delaying the movement of cross-linking ions into the inner core (Liu et al., 2002). To overcome these problems coating the beads with chitosan or whey protein isolate and/or adding filler agents such as starch or rice bran have been suggested (Chitprasert et al., 2012; Gerez et al., 2012; Kanmani et al., 2011; Martin, Lara-Villoslada, Ruiz, & Morales, 2013). *Lactobacillus plantarum* encapsulated by alginate covered with a whey protein isolate layer had better survival than uncoated treatments after exposure to simulated gastric fluid (pH 1.8) and to simulated intestinal fluid (pH 6.5) (Gbassi, Vandamme, Ennahar, & Marchioni, 2009). Whey protein isolate and starch could function as barriers to solvent flow and delay release of active compounds. Table 2.3 lists some studies of probiotic and bioactive compound encapsulation by the ionotropic gelation/extrusion dripping process.

Table 2.3. Studies of probiotic and bioactive compound encapsulation prepared by iontropic gelation/extrusion dripping process

Probiotics	Materials	Outcomes	Reference
L. plantarum	Alginate coated with whey protein isolate	The beads had higher cell viability than uncoated beads when they were exposed to simulated gastric acid fluid and only coated beads had cell survive in simulated intestinal fluid	Gbassi et al. (2009)

Table 2.3. continued

Probiotics	Materials	Outcomes	Reference
L. acidophilus	Pectin coated with why protein isolate	The beads had a high encapsulation yield and positively affected cell viability when cells were exposed to simulated gastrointestinal tract conditions. The beads released the cells in simulated intestinal fluid.	Gebara et al. (2013)
L. bulgaricus	Carageenan-locus bean gum coated with milk	The microsphere provided protection to the cells. More than 8 log CFU/g were recovered when they were incubated in simulated gastric fluid and in bile salt conditions, only 1.5 log reduction of cell viability was found. The cells were completely released in 45 min under simulated intestinal fluid.	Shi et al. (2013)
Lactobacillus rhamnosus	Alginate-locus bean gum coated with chitosan	The encapsulated cells could encounter stress upon freeze drying, heat and acid exposure. A majority of the cells released in simulated intestinal fluid rather than simulated gastric fluid.	Cheow, Kiew, and Hadinoto (2014)

2.6. Pectin based hydrogel beads

2.6.1. Pectin

Pectin is a complex mixture of polysaccharides, present in the middle lamella and primary cell wall of higher order plants. Commercial pectin is mainly derived from citrus peel and apple pomace, by-products of juice manufacturing. Figure 2.6 shows chemical structure of pectin consisting of three main building blocks including homogalacturonan (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Fraeye, Duvetter, Doungla, Van Loey, & Hendrickx, 2010b) HG, RG-I, and RG-II form a continuous backbone by covalent bonding (Coenen, Bakx, Verhoef, Schols, & Voragen, 2007; Vincken et al., 2003). HG are unbranched molecules composed of 1,4-linked α-D-galacturonic acid units, which are called smooth regions (Fraeye et al., 2010b). The GalA units have carboxyl groups, some of which are naturally methyl esterified. RG-I is referred as hairy regions. It has a backbone of the repeating

disaccharide [-4)-α-D-GalA-(1,2)-α-L-Rha-(1-]$_n$ composed of GalA and rhamnose (Rha) residues. The Rha residues are substituted with side chains of a single sugar unit (mainly arabinose and galactose) or complex polymers (arabinans, galactans and arabinogalactans). RG-II is composed of approximately nine 1,4-linked α-D-GalA residues with four heteropolymeric side chains of 11 different monosaccharides. Some of the monosaccharides are uncommon sugars, such as apiose, aceric acid and 2-keto-3-deoxy-D-manno-octulosonic acid.

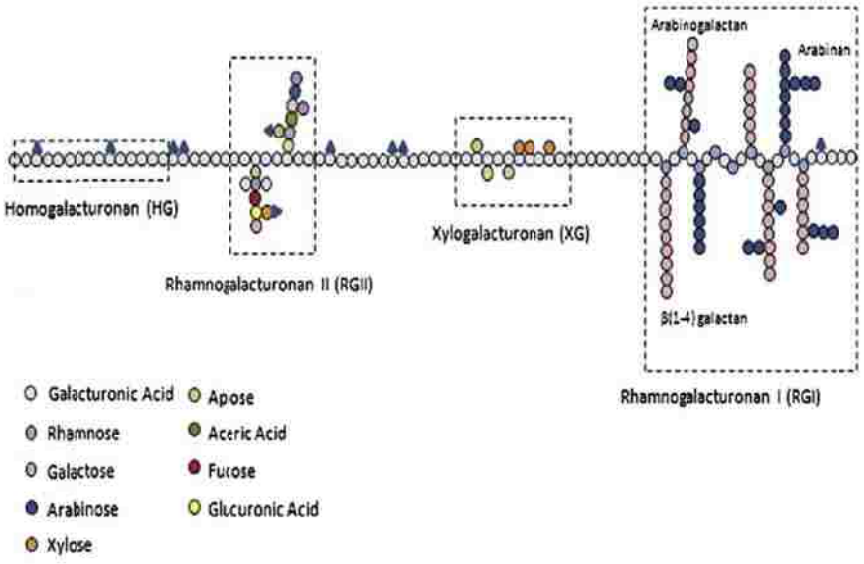


Figure 2.6. Schematic diagrams of pectin structure

Source: Maxwell, Belshaw, Waldron, and Morris (2012)

Pectin is classified into two groups based on degree of esterification (DE). As mentioned above, HG is partially esterified by methyl groups. The ratio of esterified GalA groups to total GalA groups is referred to the DE. The DE is dependent on species, tissue, and maturity of the plant (Sriamornsak, 2003). Pectin in which the DE is higher than 50% is known as high methoxyl pectin. With less than 50% DE, pectin is referred to low methoxyl pectin (Ridley, O'Neill, & Mohnen, 2001). The DE plays an important role in pectin properties particularly in pectin gelation. High methoxyl pectin forms a gel in acidic mediums (pH < 3.5) with minimal

amounts of soluble solid. Low methoxyl pectin requires the presence of calcium or other divalent cations to form a gel (Fraeye et al., 2010a). Pectin gelation is discussed in the next section.

2.6.2. Pectin as a bioactive polysaccharide

Pectin is well-known as a rich source of soluble dietary fiber (SDF), which is associated with gastrointestinal health, cholesterol reduction, and weight management (Olivares et al., 2006; Wicker et al., 2014; Willats, Knox, & Mikkelsen, 2006). Pectin may prevent and reduce carcinogenesis (Maxwell et al., 2012). Pectin is considered a highly fermentable substance. When it reaches to the large intestine, it will be fermented by gut microflora and degraded to oligosaccharides and smaller metabolites. Fermentation of pectin increased the fecal bulk and exhibited bifidogenic and prebiotic properties in a recent study (Nazzaro et al., 2012). Pectin modulated gut metabolism by improving the growth of *Bifidobacterium* and *Lactobacillus* sp., leading to an increase of digestion and decrease of inflammation. A number of *Bifidobacterium* sp. and *Lactobacillus* sp. derived from fecal bacteria of ulcerative colitis patients and fermented in pectin fraction media were higher than in the control; acetate levels were also higher (Vigsnæs, Holck, Meyer, & Licht, 2011). Pectin oligosaccharides (POS) from bergamot peel increased the number of bifidobacteria and lactobacilli while decreasing clostridial populations. It was also observed that POS had higher prebiotic index (PI) than fructo-oligosaccharide (Mandalari et al., 2007). The stimulation of *Bifidobacterium* sp. and *Lactobacillus* sp. also contributes to their action as immunomodulaters, inhibition of pathogens, reduction of ammonia formation, lowering of blood cholesterol levels and restoration of normal flora during antibiotic therapy (Blaut, 2002). POS could increase short chain fatty acid (SCFA) concentrations, particularly that of acetate, propionate and butyrate. Fermentation of POS from apple pomace by fecal inoculum increased SCFA concentrations, resulting in pH lowering (Gullon, Gullon, Sanz,

Alonso, & Parajo, 2011). High generation of acids with low pH upon fermentation was desired as it helped inhibit the overgrowth of pathogens such as *E. coli* and *Salmonella* sp. (Topping & Clifton, 2001b).

Pectin has an ability to lower blood cholesterol levels. Highly viscous fibers such as pectin restrict the formation of micelles, the absorbable form of cholesterol, or decrease its diffusion rate (Gunness & Gidley, 2010). An early study revealed that both low and high methoxyl pectin reduced total cholesterol (Judd & Truswell, 1982), however their efficiencies are dependent on physico-chemical properties including viscosity, molecular weight (MW) and degree of esterification (DE) (Brouns et al., 2012). That study showed that high MW and high DE pectin such as apple or citrus pectin (DE-70) had the highest reduction of low-density lipoprotein (LDL) cholesterol in hypercholesterolemic patients, followed by apple or citrus pectin (DE-35), orange pulp fiber (DE-70), low-MW pectin (DE-70), and citrus pectin (DE-0). Highly viscous pectin (13 mPa.s viscosity) provided significantly lower plasma cholesterol concentrations in hamsters than a pectin with low viscosity (7 mPa.s) (Terpstra, Lapre, de Vries, & Beynen, 1998). Cholesterol lowering is also related to the interaction between bile acids and pectin in the small intestine. Bile acids function as an emulsifier, facilitating the formation of micelles which promote digestion and absorption of dietary fat. Bile acids are synthesized in the human liver and reabsorbed the intestinal tract (Einarsson et al., 1991). The interaction of pectin and bile acids reduces reabsorption of bile acids in the small intestine resulting in an increase of bile acids, which are transported to the colon and deconjugated or partly dehydroxylated by enzymes of the microflora. This probably had an effect on reduction of serum cholesterol levels due to increased hepatic synthesis of bile acids and liver depletion of cholesterol (Dongowski & Lorenz, 2004). They studied the effects of pectin (with different degrees of methylation) on

cholesterol and bile acids concentrations in conventional rats and found that LDL-cholesterol in the rats fed the pectin-containing diets was lowered significantly after 21 days and bile acid concentrations were also reduced in all groups.

In addition, pectin possibly reduces the risk of some cancers. Although the mechanism has been unclear, it is evident that pectin fragments with small molecular weight (modified pectin) can bind to cancer galectin-3 recognition domain, inhibiting cell adhesion and migration and preventing apoptosis (Maxwell et al., 2012). Modified citrus pectin (MCP) was reported to inhibit cell proliferation and apoptosis of prostate cancer cell lines (Jun & Katz, 2010) and colon carcinoma and erythroleukemia cell lines (Bergman, Djaldetti, Salman, & Bessler, 2010). MCP was fed in animal studies employing mice as colon cancer models. The result showed that liver metastasis, the main cause impacting the therapeutic effect and postoperative prognosis of colorectal cancer, was significant lower in the MCP-diet group (Liu, Huang, Yang, Lu, & Yu, 2008). The effects of modified apple pectin (MAP) on a mouse model of colitis-associated colon cancer were also studied. The result showed that MAP prevented tumor formation and decreased inflammation (Li et al., 2012).

2.6.3. Low-methoxyl pectin hydrogels

1) Low-methoxyl pectin gelation

As mentioned earlier, pectin is classified into low methoxyl (LM) and high methoxyl (HM) pectin. They have different gelation mechanisms. HM pectin requires high concentrations of sucrose or other sugars (typically ~60-65%wt) under an acidic condition, while LM pectin can form gels in the presence of divalent ions. In this research we focused on LM pectin only. As well-known, LM pectin requires the presence of divalent ions, generally calcium (Ca) ion, to

form gels. The gelation occurs by forming of junction zones, as illustrated by well known “egg box” model (Figure 2.7).

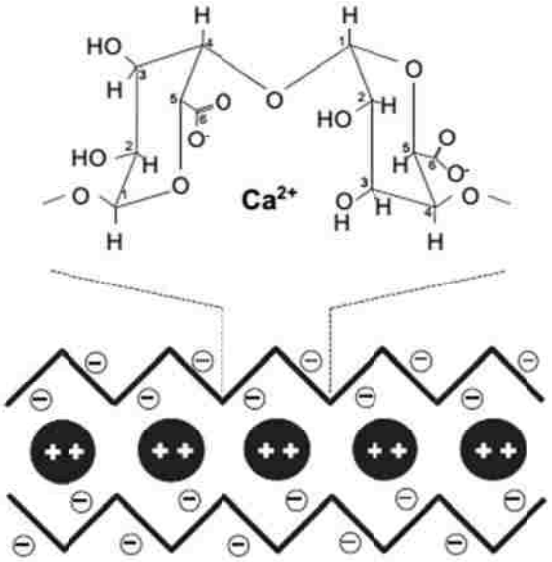


Figure 2.7. Schematic representation of the ‘‘egg-box’’ model for junction zone formation in pectin calcium gels

Source: Fraeye et al. (2010b)

The junction zone is created by binding of calcium ions on two-fold symmetrical, antiparallel polygalacturonant chains, which are packed in the interstices of the twisted chains (analogy with a corrugated egg-box). As one of the chains is slightly shifted with respect to the other, the association is better described as a shifted egg box. The egg-box formed between two neighbouring chains is linked intermolecularly through electrostatic interactions, hydrogen bonds, and van der Waals interactions.

2) Factors affecting LM pectin gelation

Factors affecting LM pectin gelation are divided into two main categories, namely intrinsic and extrinsic factors. Intrinsic factors are primarily related to pectin structural characteristics such as amount and distribution of methyl esters, chain length, pectin side chains, amidation and acetylation. Extrinsic factors are related to gel compositions and environmental

conditions, such as calcium ion and pectin content, pH, sugar content and temperature. It has been found that a single factor has an influence on several other factors. For example, the impact of gel properties with increasing calcium ion concentration is strongly affected the amount of methyl esters in pectin, pectin concentration, or pH of the gel.

(a) Intrinsic factors

The ability to form egg box junction zones is depended on degree methylation (DM), the amount of methoxylated galacturonic acid (GalA) residues, and its pattern. The lower the DM is, the higher the egg box that is formed. This contributes to an increase of Ca ion binding capacity, resulting in higher gel strength (Fraeye et al., 2010b). It was found that the modulus of elasticity of pectin gel was increased with decreased DM and that the gel became brittle when pectin had very low DM (Fraeye et al., 2010a). With decreasing DM, the chain length of the interjunction zones was shortened because a larger proportion of the pectin chains was bound in junction zones. This caused reduction of network flexibility (MacDougall, Needs, Rigby, & Ring, 1996). The pattern or distribution of non-methoxylated GalA residues also affects the gel properties. Pectin with blockwise distribution of free carboxyl groups bound to Ca ion more tightly (Ralet, Dronnet, Buchholt, & Thibault, 2001) and exhibited stronger gels (Willats, McCartney, Mackie, & Knox, 2001). Pectin with blockwise distribution was also reported to be able to associate by egg-box formation at higher DM, compared to pectin with random distribution (Liners, Thibault, & Van Cutsem, 1992).

Molecular mass of pectin plays an important role in gel properties. Gel strength was reduced with decrease of pectin molecular mass. When pectin was depolymerized (>1% GalA bonds cleaved), the gel network broke down, resulting in decrease of gel elasticity (Fraeye et al., 2010b). As formation of a continuous network needs at least two binding sites per chain,

strong depolymerization can overly shorten the chains and reduce the gelation efficiency (Capel, Nicolai, Durand, Boulenguer, & Langendorff, 2006). However when the depolymerization is limited, loss of network strength could be resolved by increasing ionic crosslinks. Addition of excessive amount of Ca ions is recommended (Fraeye et al., 2009).

Pectin can be modified by demethoxylating the polymer in the presence of ammonia. The modified pectin is then called as amidated pectin. The added amide groups change the viscoelastic properties of pectin gels. Amidation can increase pectin gel strength. In the absence of calcium, amidated pectin was able to form strong gels at pHs below 3, while non-amidated pectin yielded much weaker gels (Lootens et al., 2003). Reduction of pH lowered charge density of pectin molecules, resulting in decrease of electrostatic repulsion, and inducing aggregation of pectin chains to form gels (Cardoso, Coimbra, & Lopes da Silva, 2003). With non-amidated pectin, coarse gel was formed, while the amide groups in amidated pectin helped reinforce the gel via hydrogen bonding and inhibited coarsening. Moreover, in the presence of Ca ions, decreasing the pH reduced the elasticity of non-amidated pectin gels. On the contrary, the gels from amidated pectin were reinforced. This obviously indicated that amidation contributed to acid induced gelation (Lootens et al., 2003).

(b) Extrinsic factors

Calcium and pectin contents have major impacts on pectin gelation. According to the egg box model, two-fold symmetrical helices of pectin are bound by Ca ions. The molar ratio, $R = 2[Ca^{2+}]/[COO^{-}]$, is an important parameter. Gel strength increased with R due to more amount of egg boxes. All calcium ions are theoretically bound to the egg boxes when R is at least 0.5. However, when R is above a certain value, phase separation could occur. The type of phase separation is different, dependent on pectin concentrations. At a low concentration, pectin

separates because of a salting out effect. Alternatively, gel syneresis is likely to occur at a high pectin concentration. In addition to divalent ions (Ca^{2+}), monovalent ions such as Na^{+} or K^{+} could help enhance gel formation. The larger monovalent cation yielded higher gel strength (Yoo et al., 2009). When R is constant, increase of pectin concentration yields higher gel strength (Fraeye et al., 2010a). At a lower concentration, the gel is formed by intramolecular ionic bonding, which is not effective for gel elasticity (Capel et al., 2006). On the contrary, increase of pectin concentrations decreases the fraction of the intramolecular ionic bonding, promoting effective junction zones (Cardoso et al., 2003).

The pH of the pectin solution is also one of the important factors affecting pectin gel formation. Decrease of pH reduces the pectin charge density, resulting in lower sensitivity of pectin to calcium ions. Pectin can form weak gels in the absence of Ca at pH below 2.0. The carboxyl groups in a pectin molecule are almost fully protonated while the electrostatic interactions are neglected. Moreover, it was found that the carboxyl groups functioned as hydrogen-bond donors when the pH was below 3.5 (pK_a of pectin), inducing the gel formation by association of three-fold helices through cooperative hydrogen bonding (Gilsenan, Richardson, & Morris, 2000). On the contrary, the pectin is almost fully charged when the pH is above 4.5. It has remarkably electrostatic interactions that can form gels easily in the presence of calcium ions. At greater pH, the gel properties are independent of pH. The microstructure of pectin revealed that pectin gels at pH 7 were denser than at pH 3. Pore sizes were in a range of 100 nm and 300-400 nm for the gel at pH 7 and pH 3, respectively (Lofgren, Guillotin, & Hermansson, 2005).

Sol-gel transition occurs at a temperature, depending on pectin structure and gel compositions (Lootens et al., 2003). Mixing pectin and calcium ions at a high temperature

decreased the gel strength due to formation of short junction zones, on the other hand, highly cooperative helix junctions followed by aggregation was formed at lower temperature (Cardenas, Goycoolea, & Rinaudo, 2008). Increasing of temperature reduces the concentration of crosslinks and has a tendency to break the gel more than form it (Lootens et al., 2003). High temperatures also degrad the pectin chain, negatively affecting texture properties of the gels (Fraeye et al., 2007).

Properly amounts of sugar added to pectin solution increases pectin gel rigidity. Gel strength and firmness were improved when sucrose (10-20%) was added to a pectin solution. However, the gels were weaker, featured, and started syneresis when sugar was added more than 20% (El-Nawawi & Heikal, 1995). The effects of sugar on gel strength are varied, depending on sugar concentration, types of sugar, its structural characteristics, and pH (Fraeye et al., 2010b). Pectin gels with fructose and sorbitol had lower gel rigidity than those with sucrose and glucose (Grosso, Bobbio, & Airoldi, 2000). The researcher stated that reduction of gel rigidity could result from the capacity of sugar to form complex cations in competition with pectin. Fructose and sorbitol were able to form a complex with Ca^{2+}. This decreased the availability of the cation to associate with pectin molecules, causing lower gel rigidity. On the contrary, sucrose and glucose formed no complexes with Ca^{2+}. Sucrose was reported to improve pectin-pectin interactions by reducing the water content and stabilizing the crosslink junctions through its specific spacing of the hydroxyl groups (Lofgren et al., 2005).

2.6.4. Applications of pectin based hydrogels in microorganisms

Pectin based hydrogels are mainly developed for drug delivery systems. A few studies were reported the use of pectin based hydrogels for microorganisms. Pectin hydrogel beads were initially developed to immobilize yeast and bacteria cells for a continuous fermentation process

as an alternative to alginate gels. The beads are expected to be reused as many times as possible. Calcium alginate gels were reported to be unstable in the presence of calcium chelators such as phosphate, lactate or citrate and cations such as sodium, magnesium. This could cause alginate beads to disintegrate during fermentations. In contrast, calcium pectate gels were found to be less sensitive to ions and chemical reagents. Bacterial cells, *Nocardia tartaricans*, immobilized in calcium pectate gels had notable high stability during storage and during semi-continuous and continuous processing in both stirred batch and packed-bed reactors. The gel could resist the destructive effects of tartaric acid, a product from fermentation, for 21 h. On the contrary, calcium alginate gels were destroyed within 30 min. To increase the gel stability, the calcium pectate gels loaded with *N. tartaricans* were hardened by glutaraldehyde. It was determined that the hardened pectate gels would be last long more than 360 days in the presence of high concentration of tartaric acid, while hardened calcium alginate could remain in the acid condition for only 3 h (Kurillova et al., 2000). Comparison of alginate and pectin based hydrogels for production of poultry probiotics was studied. The researcher revealed that the pectin gels were more stable than alginate gels and that their stability was enhanced by coating with chitosan. Coating the pectin gels with chitosan effectively limited cell release during fermentation compared to the uncoated pectin gels, while there was no significant difference between cell release for coated or uncoated alginate gels. Limiting cell release helps the gel to be reusable for three or four fermentation cycles (Voo, Ravindra, Tey, & Chan, 2011).

Pectin was mixed with alginate in order to improve the mechanical and chemical stability of alginate gels and their encapsulation effectiveness. The pectin-alginate hydrogels were then used to encapsulate *Lactobacillus casei*. The addition of pectin to alginate increased the encapsulation efficiency from 54.3% to 79.2%. It was also found that, compared to alginate

hydrogels, pectin-alginate hydrogels increased decaying time of the encapsulated cells during storage in yogurt and had higher cell viability after exposure to simulated gastrointestinal conditions (Sandoval-Castilla, Lobato-Calleros, García-Galindo, Alvarez-Ramírez, & Vernon-Carter, 2010). *Lactobacillus rhamnosus* encapsulated by pectin and coated with whey protein isolate showed high viability after freeze drying. The freeze dried microcapsules had no loss and 75% loss of viability after 120-h incubation in simulated gastric fluid at pH 2.0 and 1.2, respectively, while no viable cells were detected in non-encapsulated cells (Gerez et al., 2012). Pectin was also used to encapsulate *Lactobacillus acidophilus*. The researcher reported that the pectin microparticles remained intact in simulated gastric juice at pH 1.2 and 3.0 for 120 min and in simulated intestinal juice at pH 7.0 for 300 min. Viability reduction of the encapsulated cells was lower than the non-encapsulated cells after exposure to simulated gastric juice at pH 3.0 and simulated intestinal juice at pH 7.0. However, the encapsulation could not protect the cells when they were exposed at pH 1.2 (Gebara et al., 2013).

2.7. References

AACC. (2000). The definition of dietary fibre. *Cereal Food World, 46*(3), 112-126.

Agrawal, R. (2005). Probiotics: An emerging food supplement with health benefits. [Article]. *Food Biotechnology, 19*(3), 227-246. doi: 10.1080/08905430500316474

Ahmed, F., Sairam, S., & Urooj, A. (2011). In vitro hypoglycemic effects of selected dietary fiber sources. *Journal of food science and technology, 48*(3), 285-289. doi: 10.1007/s13197-010-0153-7

Allen, S. J., Martinez, E. G., Gregorio, G. V., & Dans, L. F. (2010). Probiotics for treating acute infectious diarrhoea. *Cochrane Database of Systematic Reviews,* (11). Retrieved from http://onlinelibrary.wiley.com/doi/10.1002/14651858.CD003048.pub3/abstract doi:10.1002/14651858.CD003048.pub3

Almeida, C. C., Lorena, S. L. S., Pavan, C. R., Akasaka, H. M. I., & Mesquita, M. A. (2012). Beneficial effects of long-term consumption of a probiotic combination of *Lactobacillus casei shirota* and *Bifidobacterium breve* yakult may persist after suspension of therapy in lactose-intolerant patients. *Nutrition in Clinical Practice, 27*(2), 247-251. doi: 10.1177/0884533612440289

Amagase, H. (2008). Current marketplace for probiotics: a Japanese perspective. *Clinical Infectious Diseases, 46*(Supplement 2), S73-S75. doi: 10.1086/523338

Anal, A. K., & Singh, H. (2007). Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. *Trends in Food Science & Technology, 18*(5), 240-251. doi: http://dx.doi.org/10.1016/j.tifs.2007.01.004

Anderson, J. W., Baird, P., Davis, R. H., Ferreri, S., Knudtson, M., Koraym, A., . . . Williams, C. L. (2009). Health benefits of dietary fiber. [Journal Article]. *Nutrition Reviews 67*(4), 188-205. doi: 10.1111/j.1753-4887.2009.00189.x

Asemi, Z., Samimi, M., Tabasi, Z., Talebian, P., Azarbad, Z., Hydarzadeh, Z., & Esmaillzadeh, A. (2011). Effect of daily consumption of probiotic yoghurt on lipid profiles in pregnant women: a randomized controlled clinical trial. *The Journal of Maternal-Fetal & Neonatal Medicine, 25*(9), 1552-1556. doi: 10.3109/14767058.2011.640372

Ataie-Jafari, A., Larijani, B., Alavi Majd, H., & Tahbaz, F. (2009). Cholesterol-lowering effect of probiotic yogurt in comparison with ordinary yogurt in mildly to moderately hypercholesterolemic subjects. *Annals of Nutrition and Metabolism, 54*(1), 22-27.

Avadhani, A., & Miley, H. (2011). Probiotics for prevention of antibiotic-associated diarrhea and Clostridium difficile-associated disease in hospitalized adults-A meta-analysis. [Article]. *Journal of the American Academy of Nurse Practitioners, 23*(6), 269-274. doi: 10.1111/j.1745-7599.2011.00617.x

Babio, N., Balanza, R., Basulto, J., Bullo, M., & Salas-Salvado, J. (2010). Dietary fibre: influence on body weight, glycemic control and plasma cholesterol profile. [Article]. *Nutricion Hospitalaria, 25*(3), 327-340.

Basyigit, G., Kuleasan, H., & Karahan, A. (2006). Viability of human-derived probiotic lactobacilli in ice cream produced with sucrose and aspartame. *Journal of Industrial Microbiology and Biotechnology, 33*(9), 796-800. doi: 10.1007/s10295-006-0128-x

Bergman, M., Djaldetti, M., Salman, H., & Bessler, H. (2010). Effect of citrus pectin on malignant cell proliferation. *Biomedicine & Pharmacotherapy, 64*(1), 44-47. doi: http://dx.doi.org/10.1016/j.biopha.2009.03.004

Blaut, M. (2002). Relationship of prebiotics and food to intestinal microflora. *European Journal of Nutrition, 41*(1), i11-i16. doi: 10.1007/s00394-002-1102-7

Bosnea, L. A., Kourkoutas, Y., Albantaki, N., Tzia, C., Koutinas, A. A., & Kanellaki, M. (2009). Functionality of freeze-dried *L. casei* cells immobilized on wheat grains. *LWT - Food Science and Technology, 42*(10), 1696-1702. doi: http://dx.doi.org/10.1016/j.lwt.2009.05.011

Boylston, T. D., Vinderola, C. G., Ghoddusi, H. B., & Reinheimer, J. A. (2004). Incorporation of bifidobacteria into cheeses: challenges and rewards. *International Dairy Journal, 14*(5), 375-387. doi: http://dx.doi.org/10.1016/j.idairyj.2003.08.008

Brouns, F., Theuwissen, E., Adam, A., Bell, M., Berger, A., & Mensink, R. P. (2012). Cholesterol-lowering properties of different pectin types in mildly hyper-cholesterolemic men and women. *Eur J Clin Nutr, 66*(5), 591-599.

Burey, P., Bhandari, B. R., Howes, T., & Gidley, M. J. (2008). Hydrocolloid Gel Particles: Formation, Characterization, and Application. *Critical Reviews in Food Science and Nutrition, 48*(5), 361-377. doi: 10.1080/10408390701347801

Capel, F., Nicolai, T., Durand, D., Boulenguer, P., & Langendorff, V. (2006). Calcium and acid induced gelation of (amidated) low methoxyl pectin. *Food Hydrocolloids, 20*(6), 901-907. doi: http://dx.doi.org/10.1016/j.foodhyd.2005.09.004

Cardenas, A., Goycoolea, F. M., & Rinaudo, M. (2008). On the gelling behaviour of 'nopal' (Opuntia ficus indica) low methoxyl pectin. *Carbohydrate Polymers, 73*(2), 212-222. doi: http://dx.doi.org/10.1016/j.carbpol.2007.11.017

Cardoso, S. M., Coimbra, M. A., & Lopes da Silva, J. A. (2003). Temperature dependence of the formation and melting of pectin–Ca^{2+} networks: a rheological study. *Food Hydrocolloids, 17*(6), 801-807. doi: http://dx.doi.org/10.1016/S0268-005X(03)00101-2

Champagne, C. P., & Fustier, P. (2007). Microencapsulation for the improved delivery of bioactive compounds into foods. *Current Opinion in Biotechnology, 18*(2), 184-190. doi: http://dx.doi.org/10.1016/j.copbio.2007.03.001

Chan, L. W., Lee, H. Y., & Heng, P. W. S. (2006). Mechanisms of external and internal gelation and their impact on the functions of alginate as a coat and delivery system. *Carbohydrate Polymers, 63*(2), 176-187. doi: http://dx.doi.org/10.1016/j.carbpol.2005.07.033

Chau, C.-F., & Huang, Y.-L. (2005). Effects of the insoluble fiber derived from *Passiflora edulis* seed on plasma and hepatic lipids and fecal output. *Molecular Nutrition & Food Research, 49*(8), 786-790. doi: 10.1002/mnfr.200500060

Cheng, L. K., O'Grady, G., Du, P., Egbuji, J. U., Windsor, J. A., & Pullan, A. J. (2010). Gastrointestinal system. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine, 2*(1), 65-79. doi: 10.1002/wsbm.19

Cheow, W. S., Kiew, T. Y., & Hadinoto, K. (2014). Controlled release of *Lactobacillus rhamnosus* biofilm probiotics from alginate-locust bean gum microcapsules. *Carbohydrate Polymers, 103*(0), 587-595. doi: http://dx.doi.org/10.1016/j.carbpol.2014.01.036

Chitprasert, P., Sudsai, P., & Rodklongtan, A. (2012). Aluminum carboxymethyl cellulose–rice bran microcapsules: Enhancing survival of *Lactobacillus reuteri* KUB-AC5. *Carbohydrate Polymers, 90*(1), 78-86. doi: http://dx.doi.org/10.1016/j.carbpol.2012.04.065

Chong, E. S. L. (2014). A potential role of probiotics in colorectal cancer prevention: review of possible mechanisms of action. *World Journal of Microbiology and Biotechnology, 30*(2), 351-374. doi: 10.1007/s11274-013-1499-6

Coenen, G. J., Bakx, E. J., Verhoef, R. P., Schols, H. A., & Voragen, A. G. J. (2007). Identification of the connecting linkage between homo- or xylogalacturonan and rhamnogalacturonan type I. *Carbohydrate Polymers, 70*(2), 224-235. doi: http://dx.doi.org/10.1016/j.carbpol.2007.04.007

Cook, M. T., Tzortzis, G., Charalampopoulos, D., & Khutoryanskiy, V. V. (2012). Microencapsulation of probiotics for gastrointestinal delivery. *Journal of Controlled Release, 162*(1), 56-67. doi: http://dx.doi.org/10.1016/j.jconrel.2012.06.003

Corthesy, B. (2007). Roundtrip ticket for secretory IgA: Role in mucosal homeostasis? *The Journal of Immunology, 178*(1), 27-32. doi: 10.4049/jimmunol.178.1.27

Cruz, A. G., Antunes, A. E. C., Sousa, A. L. O. P., Faria, J. A. F., & Saad, S. M. I. (2009). Ice-cream as a probiotic food carrier. *Food Research International, 42*(9), 1233-1239. doi: http://dx.doi.org/10.1016/j.foodres.2009.03.020

Daniels, I., & Allum, W. (2005). The Anatomy and physiology of the stomach *Upper Gastrointestinal Surgery* (pp. 17-37): Springer London.

de Vos, P., Faas, M. M., Spasojevic, M., & Sikkema, J. (2010). Encapsulation for preservation of functionality and targeted delivery of bioactive food components. *International Dairy Journal, 20*(4), 292-302. doi: http://dx.doi.org/10.1016/j.idairyj.2009.11.008

Desai, K. G. H., & Jin, P. H. (2005). Recent developments in microencapsulation of food ingredients. *Drying Technology, 23*(7), 1361-1394. doi: 10.1081/DRT-200063478

Di Criscio, T., Fratianni, A., Mignogna, R., Cinquanta, L., Coppola, R., Sorrentino, E., & Panfili, G. (2010). Production of functional probiotic, prebiotic, and synbiotic ice creams. *Journal of Dairy Science, 93*(10), 4555-4564. doi: 10.3168/jds.2010-3355

Dikeman, C. L., Murphy, M. R., & Fahey, G. C. (2006). Dietary fibers affect viscosity of solutions and simulated human gastric and small intestinal digesta. *The Journal of Nutrition, 136*(4), 913-919.

Dongowski, G., & Lorenz, A. (2004). Intestinal steroids in rats are influenced by the structural parameters of pectin. *The Journal of Nutritional Biochemistry, 15*(4), 196-205. doi: http://dx.doi.org/10.1016/S0955-2863(03)00080-9

Drago, L., Nicola, L., Iemoli, E., Banfi, G., & De Vecchi, E. (2010). Strain-dependent release of cytokines modulatedby Lactobacillus salivarius human isolates in anin vitro model. [Article]. *BMC Research Notes, 3*, 44-48. doi: 10.1186/1756-0500-3-44

Einarsson, K., Ericsson, S., Ewerth, S., Reihner, E., Rudling, M., Stahlberg, D., & Angelin, B. (1991). Bile acid sequestrants: mechanisms of action on bile acid and cholesterol

metabolism. *European Journal of Clinical Pharmacology, 40*(1), S53-S58. doi: 10.1007/bf03216291

El-Nawawi, S. A., & Heikal, Y. A. (1995). Factors affecting the production of low-ester pectin gels. *Carbohydrate Polymers, 26*(3), 189-193. doi: http://dx.doi.org/10.1016/0144-8617(94)00100-8

FAO/WHO. (2001). Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Córdoba, Argentina.

Ferraz, J. L., Cruz, A. G., Cadena, R. S., Freitas, M. Q., Pinto, U. M., Carvalho, C. C., . . . Bolini, H. M. A. (2012). Sensory acceptance and survival of probiotic bacteria in ice cream produced with different overrun levels. *Journal of Food Science, 77*(1), S24-S28. doi: 10.1111/j.1750-3841.2011.02508.x

Foligne, B., Daniel, C., & Pot, B. (2013). Probiotics from research to market: the possibilities, risks and challenges. *Current Opinion in Microbiology, 16*(3), 284-292. doi: http://dx.doi.org/10.1016/j.mib.2013.06.008

Fraeye, I., Colle, I., Vandevenne, E., Duvetter, T., Van Buggenhout, S., Moldenaers, P., . . . Hendrickx, M. (2010a). Influence of pectin structure on texture of pectin–calcium gels. *Innovative Food Science & Emerging Technologies, 11*(2), 401-409. doi: http://dx.doi.org/10.1016/j.ifset.2009.08.015

Fraeye, I., De Roeck, A., Duvetter, T., Verlent, I., Hendrickx, M., & Van Loey, A. (2007). Influence of pectin properties and processing conditions on thermal pectin degradation. *Food Chemistry, 105*(2), 555-563. doi: http://dx.doi.org/10.1016/j.foodchem.2007.04.009

Fraeye, I., Doungla, E., Duvetter, T., Moldenaers, P., Van Loey, A., & Hendrickx, M. (2009). Influence of intrinsic and extrinsic factors on rheology of pectin–calcium gels. *Food Hydrocolloids, 23*(8), 2069-2077. doi: http://dx.doi.org/10.1016/j.foodhyd.2009.03.022

Fraeye, I., Duvetter, T., Doungla, E., Van Loey, A., & Hendrickx, M. (2010b). Fine-tuning the properties of pectin–calcium gels by control of pectin fine structure, gel composition and environmental conditions. *Trends in Food Science & Technology, 21*(5), 219-228. doi: http://dx.doi.org/10.1016/j.tifs.2010.02.001

Fukushima, Y., & Hurt, E. (2011). Probiotic health claims in Japan and Europe In K. Sonomoto & A. Yokota (Eds.), Lactic acid bacteria and bifidobacteria: current progress in advanced research. Norfolk, UK: Caister Academic Press.

Fuller, R. (1989). Probiotics in man and animals. *Journal of Applied Microbiology, 66*(5), 365-378. doi: 10.1111/j.1365-2672.1989.tb05105.x

Gbassi, G. K., Vandamme, T., Ennahar, S., & Marchioni, E. (2009). Microencapsulation of *Lactobacillus plantarum* spp in an alginate matrix coated with whey proteins.

International Journal of Food Microbiology, 129(1), 103-105. doi: http://dx.doi.org/10.1016/j.ijfoodmicro.2008.11.012

Gebara, C., Chaves, K. S., Ribeiro, M. C. E., Souza, F. N., Grosso, C. R. F., & Gigante, M. L. (2013). Viability of *Lactobacillus acidophilus* La5 in pectin–whey protein microparticles during exposure to simulated gastrointestinal conditions. *Food Research International, 51*(2), 872-878. doi: http://dx.doi.org/10.1016/j.foodres.2013.02.008

Gerez, C. L., Font de Valdez, G., Gigante, M. L., & Grosso, C. R. F. (2012). Whey protein coating bead improves the survival of the probiotic *Lactobacillus rhamnosus* CRL 1505 to low pH. *Letters in Applied Microbiology, 54*(6), 552-556. doi: 10.1111/j.1472-765X.2012.03247.x

Gilsenan, P. M., Richardson, R. K., & Morris, E. R. (2000). Thermally reversible acid-induced gelation of low-methoxy pectin. *Carbohydrate Polymers, 41*(4), 339-349. doi: http://dx.doi.org/10.1016/S0144-8617(99)00119-8

Gomes da Cruz, A., Alonso Buriti, F. C., Batista de Souza, C. H., Fonseca Faria, J. A., & Isay Saad, S. M. (2009). Probiotic cheese: Health benefits, technological and stability aspects. *Trends in Food Science & Technology, 20*(8), 344-354. doi: http://dx.doi.org/10.1016/j.tifs.2009.05.001

Granato, D., Branco, G. F., Cruz, A. G., Faria, J. d. A. F., & Shah, N. P. (2010). Probiotic Dairy Products as Functional Foods. *Comprehensive Reviews in Food Science and Food Safety, 9*(5), 455-470. doi: 10.1111/j.1541-4337.2010.00120.x

Grosso, C. R. F., Bobbio, P. A., & Airoldi, C. (2000). Effect of sugar and sorbitol on the formation of low methoxyl pectin gels. *Carbohydrate Polymers, 41*(4), 421-424. doi: http://dx.doi.org/10.1016/S0144-8617(99)00099-5

Guergoletto, K. B., Magnani, M., Martin, J. S., Andrade, C. G. T. d. J., & Garcia, S. (2010). Survival of *Lactobacillus casei* (LC-1) adhered to prebiotic vegetal fibers. *Innovative Food Science & Emerging Technologies, 11*(2), 415-421. doi: http://dx.doi.org/10.1016/j.ifset.2009.11.003

Guillon, F., & Champ, M. (2000). Structural and physical properties of dietary fibres, and consequences of processing on human physiology. *Food Research International, 33*(3–4), 233-245. doi: http://dx.doi.org/10.1016/S0963-9969(00)00038-7

Gullon, B., Gullon, P., Sanz, Y., Alonso, J. L., & Parajo, J. C. (2011). Prebiotic potential of a refined product containing pectic oligosaccharides. *LWT - Food Science and Technology, 44*(8), 1687-1696. doi: http://dx.doi.org/10.1016/j.lwt.2011.03.006

Gunness, P., & Gidley, M. J. (2010). Mechanisms underlying the cholesterol-lowering properties of soluble dietary fibre polysaccharides. [10.1039/C0FO00080A]. *Food & Function, 1*(2), 149-155. doi: 10.1039/c0fo00080a

Guo, Z., Liu, X. M., Zhang, Q. X., Shen, Z., Tian, F. W., Zhang, H., . . . Chen, W. (2011). Influence of consumption of probiotics on the plasma lipid profile: A meta-analysis of randomised controlled trials. *Nutrition, Metabolism and Cardiovascular Diseases, 21*(11), 844-850. doi: http://dx.doi.org/10.1016/j.numecd.2011.04.008

He, T., Priebe, M. G., Zhong, Y., Huang, C., Harmsen, H. J. M., Raangs, G. C., . . . Vonk, R. J. (2008). Effects of yogurt and bifidobacteria supplementation on the colonic microbiota in lactose-intolerant subjects. *Journal of Applied Microbiology, 104*(2), 595-604. doi: 10.1111/j.1365-2672.2007.03579.x

Hegazy, S. K., & El-Bedewy, M. M. (2010). Effect of probiotics on pro-inflammatory cytokines and NF-κB activation in ulcerative colitis. *World Journal of Gastroenterology : WJG, 16*(33), 4145-4151. doi: 10.3748/wjg.v16.i33.4145

Henderson, A. J., Kumar, A., Barnett, B., Dow, S. W., & Ryan, E. P. (2012). Consumption of rice bran increases mucosal immunoglobulin A concentrations and numbers of intestinal *Lactobacillus* spp. *Journal of Medicinal Food, 15*(5), 469-475. doi: 10.1089/jmf.2011.0213

Hertzler, S. R., & Clancy, S. M. (2003). Kefir improves lactose digestion and tolerance in adults with lactose maldigestion. *Journal of the American Dietetic Association, 103*(5), 582-587. doi: http://dx.doi.org/10.1053/jada.2003.50111

Hoffman, A. S. (2002). Hydrogels for biomedical applications. *Advanced Drug Delivery Reviews, 54*(1), 3-12. doi: http://dx.doi.org/10.1016/S0169-409X(01)00239-3

Hold, G. L., Pryde, S. E., Russell, V. J., Furrie, E., & Flint, H. J. (2002). Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. [10.1111/j.1574-6941.2002.tb00904.x]. *FEMS Microbiology Ecology, 39*(1), 33-39.

Holzapfel, W. H., Haberer, P., Snel, J., Schillinger, U., & Huis in't Veld, J. H. J. (1998). Overview of gut flora and probiotics. *International Journal of Food Microbiology, 41*(2), 85-101. doi: http://dx.doi.org/10.1016/S0168-1605(98)00044-0

Homayouni, A., Payahoo, L., & Azizi, A. (2012). Effects of probiotics on lipid profile: A review. *American Journal of Food Technology, 7*, 251-265.

Hongpattarakere, T., Rattanaubon, P., & Buntin, N. (2013). Improvement of freeze-dried *Lactobacillus Plantarum* survival using water extracts and crude fibers from food crops. *Food and Bioprocess Technology, 6*(8), 1885-1896. doi: 10.1007/s11947-012-1018-z

Iyer, C., Kosters, A., Sethi, G., Kunnumakkara, A. B., Aggarwal, B. B., & Versalovic, J. (2008). Probiotic *Lactobacillus reuteri* promotes TNF-induced apoptosis in human myeloid leukemia-derived cells by modulation of NF-κB and MAPK signalling. *Cellular Microbiology, 10*(7), 1442-1452. doi: 10.1111/j.1462-5822.2008.01137.x

Jagannath, A., Raju, P. S., & Bawa, A. S. (2010). Comparative evaluation of bacterial cellulose (nata) as a cryoprotectant and carrier support during the freeze drying process of

probiotic lactic acid bacteria. *LWT - Food Science and Technology, 43*(8), 1197-1203. doi: http://dx.doi.org/10.1016/j.lwt.2010.03.009

Johnston, B. C., Goldenberg Joshua, Z., Vandvik Per, O., Sun, X., & Guyatt Gordon, H. (2011). Probiotics for the prevention of pediatric antibiotic-associated diarrhea. *Cochrane Database of Systematic Reviews,* (11). Retrieved from http://onlinelibrary.wiley.com/doi/10.1002/14651858.CD004827.pub3/abstract doi:10.1002/14651858.CD004827.pub3

Jones, M. L., Martoni, C. J., Parent, M., & Prakash, S. (2012). Cholesterol-lowering efficacy of a microencapsulated bile salt hydrolase-active Lactobacillus reuteri NCIMB 30242 yoghurt formulation in hypercholesterolaemic adults. [10.1017/S0007114511004703]. *British Journal of Nutrition, 107*(10), 1505-1513.

Judd, P. A., & Truswell, A. S. (1982). Comparison of the effects of high- and low-methoxyl pectins on blood and faecal lipids in man. *British Journal of Nutrition, 48*(03), 451-458. doi: doi:10.1079/BJN19820130

Jun, Y., & Katz, A. (2010). PectaSol-C modified citrus pectin induces apoptosis and inhibition of proliferation in human and mouse androgen-dependent and -independent prostate cancer cells. *Integrative Cancer Therapies, 9*(2), 197-203. doi: 10.1177/1534735410369672

Kahlon, T. S., & Woodruff, C. L. (2003). In vitro binding of bile acids by rice bran, oat bran, barley and β-glucan enriched barley. *Cereal Chemistry Journal, 80*(3), 260-263. doi: 10.1094/cchem.2003.80.3.260

Kamel, S., Ali, N., Jahangir, K., Shah, S. M., & El-Gendy, A. A. (2008). Pharmaceutical significance of cellulose: a review. *eXPRESS Polymer Letters, 2*, 758–778.

Kanmani, P., Kumar, R. S., Yuvaraj, N., Paari, K. A., Pattukumar, V., & Arul, V. (2011). Cryopreservation and microencapsulation of a probiotic in alginate-chitosan capsules improves survival in simulated gastrointestinal conditions. *Biotechnology and Bioprocess Engineering, 16*(6), 1106-1114. doi: 10.1007/s12257-011-0068-9

Kedia, G., Vazquez, J., Charalampopoulos, D., & Pandiella, S. (2009). In vitro fermentation of oat bran obtained by debranning with a mixed Cclture of human fecal bacteria. *Current Microbiology, 58*(4), 338-342. doi: 10.1007/s00284-008-9335-1

Kim, S., Bae, Y., & Okano, T. (1992). Hydrogels: swelling, drug loading, and release. *Pharmaceutical Research, 9*(3), 283-290. doi: 10.1023/a:1015887213431

Kockova, M., & Valik, L. (2014). Development of new cereal-, pseudocereal-, and cereal-leguminous-based probiotic foods. *Czech Journal of Food Sciences, 32*, 391-397.

Kosseva, M. R. (2011). Immobilization of microbial cells in food fermentation processes. *Food and Bioprocess Technology, 4*(6), 1089-1118. doi: 10.1007/s11947-010-0435-0

Kourkoutas, Y., Bekatorou, A., Banat, I. M., Marchant, R., & Koutinas, A. A. (2004). Immobilization technologies and support materials suitable in alcohol beverages production: a review. *Food Microbiology, 21*(4), 377-397. doi: http://dx.doi.org/10.1016/j.fm.2003.10.005

Kumar, R. S., Kanmani, P., Yuvaraj, N., Paari, K. A., Pattukumar, V., Thirunavukkarasu, C., & Arul, V. (2012). *Lactobacillus plantarum* AS1 isolated from south indian fermented food kallappam suppress 1,2-dimethyl hydrazine (DMH)-induced colorectal cancer in male wistar rats. *Applied Biochemistry and Biotechnology, 166*(3), 620-631. doi: 10.1007/s12010-011-9453-2

Kurillova, L., Gemeiner, P., Vikartovska A., Mikova, H., Rosenberg, M., & Ilavsky, M. (2000). Calcium pectate gel beads for cell entrapment. 6. Morphology of stabilized and hardened calcium pectate gel beads with cells for immobilized biotechnology. *Journal of Microencapsulation, 17*(3), 279-296. doi: doi:10.1080/026520400288265

Lahtinen, S., Forssten, S., Aakko, J., Granlund, L., Rautonen, N., Salminen, S., . . . Ouwehand, A. (2012). Probiotic cheese containing *Lactobacillus rhamnosus* HN001 and *Lactobacillus acidophilus* NCFM® modifies subpopulations of fecal lactobacilli and *Clostridium difficile* in the elderly. *AGE, 34*(1), 133-143. doi: 10.1007/s11357-011-9208-6

Lakkis, J. M. (2007). Introduction *Encapsulation and controlled release technologies in food systems* (pp. 1-11): Blackwell Publishing.

Lee, B. B., Ravindra, P., & Chan, E. S. (2013). Size and shape of calcium alginate beads produced by extrusion dripping. *Chemical Engineering & Technology, 36*(10), 1627-1642. doi: 10.1002/ceat.201300230

Li, Y., Liu, L., Niu, Y., Feng, J., Sun, Y., Kong, X., . . . Mei, Q. (2012). Modified apple polysaccharide prevents against tumorigenesis in a mouse model of colitis-associated colon cancer: role of galectin-3 and apoptosis in cancer prevention. *European Journal of Nutrition, 51*(1), 107-117. doi: 10.1007/s00394-011-0194-3

Liners, F., Thibault, J.-F., & Van Cutsem, P. (1992). Influence of the degree of polymerization of oligogalacturonates and of esterification pattern of pectin on their recognition by monoclonal antibodies. *Plant Physiology, 99*(3), 1099-1104.

Liu, H. Y., Huang, Z.-L., Yang, G.-H., Lu, W.-Q., & Yu, N.-R. (2008). Inhibitory effect of modified citrus pectin on liver metastases in a mouse colon cancer model. *World Journal of Gastroenterology : WJG, 14*(48), 7386-7391. doi: 10.3748/wjg.14.7386

Liu, L. S., Fishman, M. L., & Hicks, K. B. (2007). Pectin in controlled drug delivery - a review. *Cellulose, 14*(1), 15-24. doi: DOI 10.1007/s10570-006-9095-7

Liu, X. D., Yu, W. Y., Zhang, Y., Xue, W. M., Yu, W. T., Xiong, Y., . . . Yuan, Q. (2002). Characterization of structure and diffusion behaviour of Ca-alginate beads prepared with

external or internal calcium sources. *Journal of Microencapsulation, 19*(6), 775-782. doi: doi:10.1080/0265204021000022743

Lofgren, C., Guillotin, S., & Hermansson, A.-M. (2005). Microstructure and kinetic rheological behavior of amidated and nonamidated LM pectin gels. *Biomacromolecules, 7*(1), 114-121. doi: 10.1021/bm050459r

Lootens, D., Capel, F., Durand, D., Nicolai, T., Boulenguer, P., & Langendorff, V. (2003). Influence of pH, Ca concentration, temperature and amidation on the gelation of low methoxyl pectin. *Food Hydrocolloids, 17*(3), 237-244. doi: http://dx.doi.org/10.1016/S0268-005X(02)00056-5

Lourens-Hattingh, A., & Viljoen, B. C. (2001). Yogurt as probiotic carrier food. *International Dairy Journal, 11*(1–2), 1-17. doi: http://dx.doi.org/10.1016/S0958-6946(01)00036-X

MacDougall, A. J., Needs, P. W., Rigby, N. M., & Ring, S. G. (1996). Calcium gelation of pectic polysaccharides isolated from unripe tomato fruit. *Carbohydrate Research, 293*(2), 235-249. doi: http://dx.doi.org/10.1016/0008-6215(96)00197-8

Mandalari, G., Nueno Palop, C., Tuohy, K., Gibson, G. R., Bennett, R. N., Waldron, K. W., . . . Faulds, C. B. (2007). In vitro evaluation of the prebiotic activity of a pectic oligosaccharide-rich extract enzymatically derived from bergamot peel. *Applied Microbiology and Biotechnology, 73*(5), 1173-1179. doi: 10.1007/s00253-006-0561-9

Martin, M. J., Lara-Villoslada, F., Ruiz, M. A., & Morales, M. E. (2013). Effect of unmodified starch on viability of alginate-encapsulated *Lactobacillus fermentum* CECT5716. *LWT - Food Science and Technology, 53*(2), 480-486. doi: http://dx.doi.org/10.1016/j.lwt.2013.03.019

Matricardi, P., Meo, C. D., Coviello, T., & Alhaique, F. (2008). Recent advances and perspectives on coated alginate microspheres for modified drug delivery. *Expert Opinion on Drug Delivery, 5*(4), 417-425. doi: doi:10.1517/17425247.5.4.417

Maxwell, E. G., Belshaw, N. J., Waldron, K. W., & Morris, V. J. (2012). Pectin – An emerging new bioactive food polysaccharide. *Trends in Food Science & Technology, 24*(2), 64-73. doi: http://dx.doi.org/10.1016/j.tifs.2011.11.002

McGovern, D., & Powrie, F. (2007). The IL23 axis plays a key role in the pathogenesis of IBD. *Gut, 56*(10), 1333-1336. doi: 10.1136/gut.2006.115402

Mitropoulou, G., Nedovic, V., Goyal, A., & Kourkoutas, Y. (2013). Immobilization technologies in probiotic food production. *Journal of Nutrition and Metabolism, 2013*, 15. doi: 10.1155/2013/716861

Mortazavian, A. M., Ehsani, M. R., Mousavi, S. M., Reinheimer, J. A., Emamdjomeh, Z., Sohrabvandi, S., & Rezaei, K. (2006). Preliminary investigation of the combined effect of heat treatment and incubation temperature on the viability of the probiotic micro-

organisms in freshly made yogurt. *International Journal of Dairy Technology, 59*(1), 8-11. doi: 10.1111/j.1471-0307.2006.00216.x

Mousavi, Z. E., Mousavi, S. M., Razavi, S. H., Emam-Djomeh, Z., & Kiani, H. (2011). Fermentation of pomegranate juice by probiotic lactic acid bacteria. *World Journal of Microbiology and Biotechnology, 27*(1), 123-128. doi: 10.1007/s11274-010-0436-1

Nazzaro, F., Fratianni, F., Orlando, P., & Coppola, R. (2012). Biochemical traits, survival and biological properties of the probiotic *Lactobacillus plantarum* grown in the presence of prebiotic inulin and pectin as energy source. *Pharmaceuticals, 5*(5), 481-492.

Nedovic, V., Kalusevic, A., Manojlovic, V., Levic, S., & Bugarski, B. (2011). An overview of encapsulation technologies for food applications. *Procedia Food Science, 1*, 1806-1815. doi: http://dx.doi.org/10.1016/j.profoo.2011.09.265

Ng, S. C., Hart, A. L., Kamm, M. A., Stagg, A. J., & Knight, S. C. (2009). Mechanisms of action of probiotics: Recent advances. *Inflammatory Bowel Diseases, 15*(2), 300-310. doi: 10.1002/ibd.20602

Oelschlaeger, T. A. (2010). Mechanisms of probiotic actions – A review. *International Journal of Medical Microbiology, 300*(1), 57-62. doi: http://dx.doi.org/10.1016/j.ijmm.2009.08.005

Ohashi, Y., & Ushida, K. (2009). Health-beneficial effects of probiotics: Its mode of action. *Animal Science Journal, 80*(4), 361-371. doi: 10.1111/j.1740-0929.2009.00645.x

Olivares, M., Diaz-Ropero, M. P., Gomez, N., Lara-Villoslada, F., Sierra, S., Maldonado, J. A., . . . Xau, J. (2006). The consumption of two new probiotic strains, *Lactobacillus gasseri* CECT 5714 and *Lactobacillus coryniformis* CECT 5711, boosts the immune system of healthy humans. *International Microbiology, 9*(1), 47-52.

Omidian, H., & Park, K. (2012). Hydrogels. In J. Siepmann, R. A. Siegel & M. J. Rathbone (Eds.), *Fundamentals and applications of controlled release drug delivery* (pp. 75-105): Springer US.

Ooi, L.-G., & Liong, M.-T. (2010). Cholesterol-lowering effects of probiotics and prebiotics: A review of in *vivo* and in *vitro* findings. *International Journal of Molecular Sciences, 11*(6), 2499-2522. doi: 10.3390/ijms11062499

Orlando, A., Refolo, M. G., Messa, C., Amati, L., Lavermicocca, P., Guerra, V., & Russo, F. (2012). Antiproliferative and proapoptotic effects of viable or heat-killed *Lactobacillus paracasei* IMPC2.1 and *Lactobacillus rhamnosus* GG in HGC-27 gastric and DLD-1 colon cell lines. *Nutrition and Cancer, 64*(7), 1103-1111. doi: 10.1080/01635581.2012.717676

Osamu, K., Keiichi, M., Yutaka, K., Minoru, Y., Akira, A., & Michio, S. (2010). Preventive effects of enzyme-treated rice fiber in a restraint stress-induced irritable bowel syndrome model. *Int J Mol Med, 25*(4), 547-555.

Parra, M. D., Martínez de Morentin, B. E., Cobo, J. M., Mateos, A., & Martínez, J. A. (2004). Daily ingestion of fermented milk containing Lactobacillus casei DN114001 improves innate defense capacity in healthy middle-aged people. *Journal of Physiology and Biochemistry, 60*(2), 85-91. doi: 10.1007/bf03168444

Patil, P., Chavanke, D., & Wagh, M. (2012). A review on ionotropic gelation method: novel approach for controlled gastroretentive gelispheres. *International Journal of Pharmacy & Pharmaceutical Sciences, 24*, 27-32.

Phillips, M., Kailasapathy, K., & Tran, L. (2006). Viability of commercial probiotic cultures (*L. acidophilus*, *Bifidobacterium* sp., *L. casei, L. paracasei* and *L. rhamnosus*) in cheddar cheese. *International Journal of Food Microbiology, 108*(2), 276-280. doi: http://dx.doi.org/10.1016/j.ijfoodmicro.2005.12.009

Pilkington, P. H., Margaritis, A., Mensour, N. A., & Russell, I. (1998). Fundametals of immobilised yeast cells for continuous beer fermentation:A review. *Journal of the Institute of Brewing, 104*(1), 19-31. doi: 10.1002/j.2050-0416.1998.tb00970.x

Pimentel, T. C., Madrona, G. S., & Prudencio, S. H. (2015). Probiotic clarified apple juice with oligofructose or sucralose as sugar substitutes: sensory profile and acceptability. *LWT - Food Science and Technology, 62*(1), 838–846. doi: http://dx.doi.org/10.1016/j.lwt.2014.08.001

Plessas, S., Bosnea, L., Alexopoulos, A., & Bezirtzoglou, E. (2012). Potential effects of probiotics in cheese and yogurt production: A review. *Engineering in Life Sciences, 12*(4), 433-440. doi: 10.1002/elsc.201100122

Pylkas, A. M., Juneja, L. R., & Slavin, J. L. (2005). Comparison of different fibers for *in vitro* production of short chain fatty acids by intestinal microflora. *Journal of Medicinal Food, 8*(1), 113-116. doi: 10.1089/jmf.2005.8.113

Rabot, S., Rafter, J., Rijkers, G. T., Watzl, B., & Antoine, J.-M. (2010). Guidance for substantiating the evidence for beneficial effects of probiotics: Impact of probiotics on digestive system metabolism. *The Journal of Nutrition, 140*(3), 677S-689S. doi: 10.3945/jn.109.113738

Ralet, M.-C., Dronnet, V., Buchholt, H. C., & Thibault, J.-F. (2001). Enzymatically and chemically de-esterified lime pectins: characterisation, polyelectrolyte behaviour and calcium binding properties. *Carbohydrate Research, 336*(2), 117-125. doi: http://dx.doi.org/10.1016/S0008-6215(01)00248-8

Ranadheera, R. D. C. S., Baines, S. K., & Adams, M. C. (2010). Importance of food in probiotic efficacy. *Food Research International, 43*(1), 1-7. doi: http://dx.doi.org/10.1016/j.foodres.2009.09.009

Raninen, K., Lappi, J., Mykkanen, H., & Poutanen, K. (2011). Dietary fiber type reflects physiological functionality: comparison of grain fiber, inulin, and polydextrose. *Nutrition Reviews, 69*(1), 9-21. doi: 10.1111/j.1753-4887.2010.00358.x

Rathore, S., Desai, P. M., Liew, C. V., Chan, L. W., & Heng, P. W. S. (2013). Microencapsulation of microbial cells. *Journal of Food Engineering, 116*(2), 369-381. doi: http://dx.doi.org/10.1016/j.jfoodeng.2012.12.022

Rego, A., Freixo, R., Silva, J., Gibbs, P., Morais, A. M. M. B., & Teixeira, P. (2013). A functional dried fruit matrix incorporated with probiotic strains: *Lactobacillus Plantarum* and *Lactobacillus Kefir*. *Focusing on Modern Food Industry, 2*(3), 138-143.

Ridley, B. L., O'Neill, M. A., & Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry, 57*(6), 929-967. doi: http://dx.doi.org/10.1016/S0031-9422(01)00113-3

Rodriguez, R., Jimenez, A., Fernandez-Bolanos, J., Guillen, R., & Heredia, A. (2006). Dietary fibre from vegetable products as source of functional ingredients. *Trends in Food Science & Technology, 17*(1), 3-15. doi: http://dx.doi.org/10.1016/j.tifs.2005.10.002

Rolfe, R. D. (2000). The role of probiotic cultures in the control of gastrointestinal health. *The Journal of Nutrition, 130*(2), 396.

Roßle, C., Auty, M. A. E., Brunton, N., Gormley, R. T., & Butler, F. (2010). Evaluation of fresh-cut apple slices enriched with probiotic bacteria. *Innovative Food Science & Emerging Technologies, 11*(1), 203-209. doi: http://dx.doi.org/10.1016/j.ifset.2009.08.016

Ruiz-Roso, B., Quintela, J., de la Fuente, E., Haya, J., & Perez-Olleros, L. (2010). Insoluble carob fiber rich in polyphenols lowers total and LDL cholesterol in hypercholesterolemic sujects. *Plant Foods for Human Nutrition, 65*(1), 50-56. doi: 10.1007/s11130-009-0153-9

Saarela, M., Virkajarvi, I., Nohynek, L., Vaari, A., & Matto, J. (2006). Fibres as carriers for *Lactobacillus rhamnosus* during freeze-drying and storage in apple juice and chocolate-coated breakfast cereals. *International Journal of Food Microbiology, 112*(2), 171-178. doi: http://dx.doi.org/10.1016/j.ijfoodmicro.2006.05.019

Salvini, F. (2013). Probiotics and prevention of antibiotic associated diarrhoea in children *International Journal of Probiotics and Prebiotics 8*(1), 29-32.

Sandoval-Castilla, O., Lobato-Calleros, C., García-Galindo, H. S., Alvarez-Ramírez, J., & Vernon-Carter, E. J. (2010). Textural properties of alginate–pectin beads and survivability of entrapped Lb. casei in simulated gastrointestinal conditions and in yoghurt. *Food Research International, 43*(1), 111-117. doi: http://dx.doi.org/10.1016/j.foodres.2009.09.010

Savard, P., Lamarche, B., Paradis, M.-E., Thiboutot, H., Laurin, É., & Roy, D. (2011). Impact of *Bifidobacterium animalis* subsp. lactis BB-12 and, *Lactobacillus acidophilus* LA-5-containing yoghurt, on fecal bacterial counts of healthy adults. *International Journal of Food Microbiology, 149*(1), 50-57. doi: http://dx.doi.org/10.1016/j.ijfoodmicro.2010.12.026

Saxelin, M. (2008). Probiotic formulations and applications, the current probiotics market, and changes in the marketplace: A European perspective. *Clinical Infectious Diseases, 46*(Supplement 2), S76-S79. doi: 10.1086/523337

Saxelin, M., Tynkkynen, S., Mattila-Sandholm, T., & de Vos, W. M. (2005). Probiotic and other functional microbes: from markets to mechanisms. *Current Opinion in Biotechnology, 16*(2), 204-211. doi: http://dx.doi.org/10.1016/j.copbio.2005.02.003

Schrezenmeir, J., & de Vrese, M. (2001). Probiotics, prebiotics, and synbiotics—approaching a definition. *The American Journal of Clinical Nutrition, 73*(2), 361s-364s.

Shah, N., Shah, T., & Amin, A. (2011). Polysaccharides: a targeting strategy for colonic drug delivery. *Expert Opinion on Drug Delivery, 8*(6), 779-796. doi: doi:10.1517/17425247.2011.574121

Sharma, P., Tomar, S. K., Goswami, P., Sangwan, V., & Singh, R. (2014). Antibiotic resistance among commercially available probiotics. *Food Research International, 57*(0), 176-195. doi: http://dx.doi.org/10.1016/j.foodres.2014.01.025

Sharp, M. D., McMahon, D. J., & Broadbent, J. R. (2008). Comparative evaluation of yogurt and low-fat cheddar cheese as delivery media for probiotic *Lactobacillus casei*. *Journal of Food Science, 73*(7), M375-M377. doi: 10.1111/j.1750-3841.2008.00882.x

Shi, L.-E., Li, Z.-H., Zhang, Z.-L., Zhang, T.-T., Yu, W.-M., Zhou, M.-L., & Tang, Z.-X. (2013). Encapsulation of *Lactobacillus bulgaricus* in carrageenan-locust bean gum coated milk microspheres with double layer structure. *LWT - Food Science and Technology, 54*(1), 147-151. doi: http://dx.doi.org/10.1016/j.lwt.2013.05.027

Sinha, V. R., & Kumria, R. (2003). Microbially triggered drug delivery to the colon. *European Journal of Pharmaceutical Sciences, 18*(1), 3-18. doi: http://dx.doi.org/10.1016/S0928-0987(02)00221-X

Solanki, H. K., Pawar, D. D., Shah, D. A., Prajapati, V. D., Jani, G. K., Mulla, A. M., & Thakar, P. M. (2013). Development of microencapsulation delivery system for long-term preservation of probiotics as biotherapeutics agent. *BioMed Research International, 2013*, 21. doi: 10.1155/2013/620719

Sriamornsak, P. (2003). Chemistry of pectin and its pharmaceutical uses: A review. *Silpakorn University International Journal 1*(3), 206-228.

Szajewska, H., & Mrukowicz, J. Z. (2001). Probiotics in the treatment and prevention of acute infectious diarrhea in infants and children: A systematic review of published randomized, double-blind, placebo-controlled trials. *Journal of Pediatric Gastroenterology and Nutrition, 33*, S17-S25.

Terpstra, A. H. M., Lapre, J. A., de Vries, H. T., & Beynen, A. C. (1998). Dietary pectin with high viscosity lowers plasma and liver cholesterol concentration and plasma cholesteryl ester transfer protein activity in hamsters. *The Journal of Nutrition, 128*(11), 1944-1949.

Tiwari, U., & Cummins, E. (2011). Meta-analysis of the effect of β-glucan intake on blood cholesterol and glucose levels. *Nutrition, 27*(10), 1008-1016. doi: http://dx.doi.org/10.1016/j.nut.2010.11.006

Topping, D. L., & Clifton, P. M. (2001a). Short-chain atty acids and human colonic function: roles of resistant starch and nonnstarch polysaccharides. *Physiological Reviews, 81*(3), 1031-1064.

Topping, D. L., & Clifton, P. M. (2001b). *Short-chain fatty acids and human colonic function: roles of resistant rtarch and nonstarch polysaccharides* (Vol. 81).

Tungland, B. C., & Meyer, D. (2002). Nondigestible oligo- and polysaccharides (dietary fiber): their physiology and role in human health and food. *Comprehensive Reviews in Food Science and Food Safety, 1*(3), 90-109. doi: 10.1111/j.1541-4337.2002.tb00009.x

Tuohy, K. M., Pinart-Gilberga, M., Jones, M., Hoyles, L., McCartney, A. L., & Gibson, G. R. (2007). Survivability of a probiotic *Lactobacillus casei* in the gastrointestinal tract of healthy human volunteers and its impact on the faecal microflora. *Journal of Applied Microbiology, 102*(4), 1026-1032. doi: 10.1111/j.1365-2672.2006.03154.x

Valls, J., Pasamontes, N., Pantaleon, A., Vinaixa, S., Vaque, M., Soler, A., . . . Gomez, X. (2013). Prospects of functional foods/nutraceuticals and markets. In K. G. Ramawat & J.-M. Mérillon (Eds.), *Natural Products* (pp. 2491-2525): Springer Berlin Heidelberg.

Vasiljevic, T., & Shah, N. P. (2008). Probiotics—from metchnikoff to bioactives. *International Dairy Journal, 18*(7), 714-728. doi: http://dx.doi.org/10.1016/j.idairyj.2008.03.004

Verma, A., & Shukla, G. (2013). Probiotics *Lactobacillus rhamnosus* GG , *Lactobacillus acidophilus* suppresses DMH-induced procarcinogenic fecal enzymes and preneoplastic aberrant crypt foci in early colon carcinogenesis in sprague dawley rats. [Article]. *Nutrition & Cancer, 65*(1), 84-91. doi: 10.1080/01635581.2013.741746

Vigsnæs, L. K., Holck, J., Meyer, A. S., & Licht, T. R. (2011). *In vitro* fermentation of sugar beet arabino-oligosaccharides by fecal microbiota obtained from patients with ulcerative colitis to selectively stimulate the growth of *Bifidobacterium* spp. and *Lactobacillus* spp. *Applied and Environmental Microbiology, 77*(23), 8336-8344. doi: 10.1128/aem.05895-11

Vincken, J., Schols, H. A., Oomen, R. J., McCann, M. C., Ulvskov, P., Voragen, A. G., & Visser, R. G. (2003). If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell Wall architecture. *Plant Physiology, 132*(4), 1781-1789.

Voo, W.-P., Ravindra, P., Tey, B.-T., & Chan, E.-S. (2011). Comparison of alginate and pectin based beads for production of poultry probiotic cells. *Journal of Bioscience and Bioengineering, 111*(3), 294-299. doi: http://dx.doi.org/10.1016/j.jbiosc.2010.11.010

Vuksan, V., Jenkins, A. L., Jenkins, D. J., Rogovik, A. L., Sievenpiper, J. L., & Jovanovski, E. (2008). Using cereal to increase dietary fiber intake to the recommended level and the

effect of fiber on bowel function in healthy persons consuming North American diets. *The American Journal of Clinical Nutrition, 88*(5), 1256-1262. doi: 10.3945/ajcn.2008.25956

Wee, Y.-J., Kim, J.-N., & Ryu, H.-W. (2006). Biotechnological production of lactic acid and its recent applications. *Food Technology and Biotechnology, 44*(2), 163-172.

Wicker, L., Kim, Y., Kim, M.-J., Thirkield, B., Lin, Z., & Jung, J. (2014). Pectin as a bioactive polysaccharide – Extracting tailored function from less. *Food Hydrocolloids, 42, Part 2*(0), 251-259. doi: http://dx.doi.org/10.1016/j.foodhyd.2014.01.002

Willats, W. G. T., Knox, J. P., & Mikkelsen, J. D. (2006). Pectin: new insights into an old polymer are starting to gel. *Trends in Food Science & Technology, 17*(3), 97-104. doi: http://dx.doi.org/10.1016/j.tifs.2005.10.008

Willats, W. G. T., McCartney, L., Mackie, W., & Knox, J. P. (2001). Pectin: cell biology and prospects for functional analysis. *Plant Molecular Biology, 47*(1-2), 9-27. doi: 10.1023/a:1010662911148

Wong, J. M. W., de Souza, R., Kendall, C. W. C., Emam, A., & Jenkins, D. J. A. (2006). Colonic Health: fermentation and short chain fatty acids. *Journal of Clinical Gastroenterology, 40*(3), 235-243.

Wong, J. M. W., & Jenkins, D. J. A. (2007). Carbohydrate digestibility and metabolic effects. *The Journal of Nutrition, 137*(11), 2539S-2546S.

Woof, J. M., & Kerr, M. A. (2006). The function of immunoglobulin A in immunity. *The Journal of Pathology, 208*(2), 270-282. doi: 10.1002/path.1877

Yoo, S. H., Lee, B. H., Savary, B. J., Lee, S., Lee, H. G., & Hotchkiss, A. T. (2009). Characteristics of enzymatically-deesterified pectin gels produced in the presence of monovalent ionic salts. *Food Hydrocolloids, 23*(7), 1926-1929. doi: http://dx.doi.org/10.1016/j.foodhyd.2009.02.006

Yu, L. X., & Amidon, G. L. (1998). Characterization of small intestinal transit time distribution in humans. *International Journal of Pharmaceutics, 171*(2), 157-163. doi: http://dx.doi.org/10.1016/S0378-5173(98)00174-4

Zhang, N., Huang, C., & Ou, S. (2011). *In vitro* binding capacities of three dietary fibers and their mixture for four toxic elements, cholesterol, and bile acid. *Journal of Hazardous Materials, 186*(1), 236-239. doi: http://dx.doi.org/10.1016/j.jhazmat.2010.10.120

Zuidam, N., & Shimoni, E. (2010). Overview of microencapsulates for use in food products or processes and methods to make them. In N. J. Zuidam & V. Nedovic (Eds.), *Encapsulation technologies for active food ingredients and food processing* (pp. 3-29): Springer New York.

CHAPTER 3 EFFECTS OF ENZYMATICALLY EXTRACTED PURPLE RICE BRAN FIBERS AS A PROTECTANT OF *L. PLANTARUM* NRRL B-4496 DURING FREEZING, FREEZE DRYING, AND STORAGE

3.1. Abstract

This study investigated purple rice bran-fiber (PRF) as a protectant for *Lactobacillus plantarum* NRRL B-4496 (LP) during freezing, freeze drying, and storage. PRF was enzymatically extracted from purple rice bran. *L. plantarum* NRRL B-4496 was grown in MRS broth, centrifuged, and immobilized on PRF suspension. LP cells immobilized on PRF (LP-PRF) and free LP cell (control) samples were frozen in either air blast (AF) or cryogenic freezers (CF) before freeze drying. Freeze-dried (FLP) samples were stored either at room temperature or at refrigerated temperatures. For either freezing method, PRF protected cells had less than one log reduction of viable cells while the control had reductions greater than six logs after freeze drying. The counts of viable LP cells protected with PRF after freeze drying and 12 weeks storage at 4^{o} C for AF and CF treatments were 7.55±0.07 and 7.49±0.06 log CFU/g, respectively. The viable LP-PRF cell count for CF was significantly lower than for AF after 12 weeks at room temperature. PRF improved LP survival in both AF and CF samples in bile. This study demonstrated that freezing methods affected LP viability during storage and that PRF could protect at both refrigerated and room temperatures.

Keywords: Purple rice bran fiber, *Lactobacillus plantarum*, Freeze drying

This chapter previously appeared as Arranee Chotiko and Subramaniam Sathivel, Effect of enzymatically extracted purple rice bran fiber as a protectant of *L. plantarum* NRRL-B4496 during freezing, freeze drying, and storage, June 9 2014. It is reprinted by permission of Elsevier—see the permission letter for proper acknowledgment phrase in Appendix B (see page 139).

3.2. Introduction

The American Association of Cereal Chemists defined dietary fiber as "the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine." (AACC, 2001). Dietary fibers are composed of non-starch polysaccharides, such as cellulose, hemicelluloses, or pectin, oligosaccharides such as resistant starches, resistant maltodextrins, or indigestible dextrin, and some lignin substances including phytate, cutin and tannin. (Lattimer & Haub, 2010). Common food sources for these fibers are whole grains, legumes, fruits, and vegetables (Slavin, 2008). Besides their well-known benefits of reducing the risk of chronic diseases, some dietary fibers could exhibit prebiotic effects, enhancing the growth of colonic bacteria or probiotics resulting in improving the host's health (Manning & Gibson, 2004) Some of the fibers can also be used as probiotic protectants, protecting the probiotic cells during down-stream processing, formulation and storage (Saarela, Virkajärvi, Nohynek, Vaari, & Mättö, 2006). Thus, these positive interactions between prebiotics (dietary fibers) and probiotics would contribute to functional foods and nutraceutical products.

Survival of the bacteria during processing and storage is a necessity for effective probiotic products. Most marketed probiotics markets have been preserved by lyophilization or freeze drying. Although it is a gentle method, losses of cell viability occur, particularly during freezing (Meng, Stanton, Fitzgerald, Daly, & Ross, 2008). For this reason, protective agents such as skim milk, sucrose, dextran or polyethylene glycol are commonly required to maintain high levels of cell viability during freeze drying and storage (Li et al., 2011). Several dietary fibers have been reported to have potential as protectants for probiotics. Saarela et al. (2006) found that survival of *Lactobacillus rhamnosus* E800 was increased after freeze drying when the cell was

adhered to wheat dextrin. Oat bran fiber also improved the survival of *Lactobacillus casei* during dehydration and storage at room temperature (Guergoletto, Magnani, Martin, Andrade, & Garcia, 2010). Similarly, *Nata* or bacteria cellulose could function as a cryoprotectant and an immobilized support for freeze dried lactobacilli (Jagannath, Raju, & Bawa, 2010). Recently, Hongpattarakere, Rattanaubon, and Buntin (2013) revealed that the presence of mungbean fiber helped maintain viability of *L. plantarum* after freeze drying and during storage.

Rice bran, a by-product from rice milling, is a rich source of protein, fat, dietary fiber and phytochemicals helpful in promoting human health. In particular, purple rice bran, an excellent source of natural antioxidants including tocopherol, tocotrienol, and oryzanol, is a better source of antioxidants than the regular rice bran (Jang & Xu, 2009). Kanauchi et al. (2010) demonstrated that enzyme-treated rice bran fiber, which was a novel prebiotic, could decrease major symptoms of irritable bowel syndrome. Moreover, it could reduce inflammation in the colon by modulating the colonic environment and stimulating immune cell differentiation (Komiyama et al., 2011).

Freeze drying consists of two main processes: freezing and drying by sublimation. Freezing, especially freezing rates, has been reported as a crucial factor affecting viability of lactic acid bacteria after drying (Morgan, Herman, White, & Vesey, 2006). Therefore, it is important to determine the effect of combined freezing and freeze drying processes on the viability and stability of probiotic cells in addition to evaluating purple rice bran fiber as a probiotic protectant. In the present study, dietary fiber enzymatically extracted from purple rice (*Blanca Isabel*) bran was selected to be used as a cryoprotectant for *L. plantarum*. The aim of this study was to examine effects of purple rice bran fiber and freezing methods (air blast freezing and cryogenic freezing) on the viability of LP after freezing, freeze drying, and storage.

3.3. Material and methods

3.3.1. Extraction of purple rice bran fiber (PRF)

PRF was prepared by the enzymatic extraction described by Kanauchi et al. (2010) with some modifications. Three hundred grams of defatted purple rice bran was suspended in 1200 mL of distilled water. Nine-tenths mL of heat-stable amylase (Sigma-Aldrich, St. Louis, MO) was subsequently added. The mixture was incubated at 80 °C for 1 h with continuous stirring at 2400 rpm (IKA RW 20 digital, IKA Works Inc., NC). The mixture was filtered through a 75-µm sieve (Fisherbrand Test Sieve, Fisher Scientific Co., PA.). The insoluble fraction was recovered and re-suspended in distilled water. The pH was then adjusted to 8 by NaOH (Sigma-Aldrich, St. Louis, MO), followed by addition of 0.45 mL of Alcalase (Sigma-Aldrich, St. Louis, MO). The re-suspended mixture was continuously stirred at 650 rpm and maintained in a water bath (Microprocessor Controlled 280 Series Water bath, Thermo Scientific Inc., MA.) at 63 °C for 5 h. After hydrolysis, the insoluble residue was isolated using a 75-µm sieve and then was suspended in distilled water. The pH of mixture was adjusted to 4.5 by HCl. A 2.4 g of hemicellulase (Sigma-Aldrich, St. Louis, MO) was added to the suspension and incubated at 40 °C for 12 h with 650 rpm stirring. The insoluble fraction was collected by filtering through a 75-µm sieve and washed with distilled water 4 times prior to drying in a dehydrator (Excalibur 2900ECB food dehydrator, Excalibur®, FL) at 60°C for 8 h to obtain the PRF. The proximate analysis of the PRF was determined. The antioxidant activity, total phenolic contents, moisture contents, and colors were also obtained.

3.3.2. Microorganism

A lyophilized *L. plantarum* NRRL B-4496 isolated from sauerkraut was kindly provided by ARS Culture Collection (Washington DC, US). The culture was activated in deMan Rogosa

Sharpe (MRS) broth (Neogen Corporation, Lansing, MI). Twenty five mL of the strain was subsequently inoculated in MRS broth (500 mL) and incubated at 37 °C for 16 h to reach stationary phase. Cell pellets were harvested by centrifugation at 12,000 x g for 10 min at 4 °C. The pellets were washed three times and suspended in sterile distilled water.

3.3.3. Preparation of freeze-dried L. plantarum adhered on PRF

A 5.5 g of PRF was mixed with 55 mL of cell pellet suspension in a sterile weighting boat. The bacterium-PRF suspension was kept at room temperature for 1 h (Guergoletto et al., 2010). Then, the suspension was divided into two groups; one was frozen using an air blast freezer (Master-Bilt Products, New Albany, MS) at -20 °C for 24 h, while the other group was frozen in a cabinet cryogenic freezer (Air Liquide Co., Houston, TX) with liquid nitrogen (Air Liquide, Houston, TX, USA). After reaching an internal temperature of -20 °C, the samples were stored at -20 °C for 24 h in a regular freezer. All samples were subsequently dried in a freeze dryer (Virtis Genesis 35 xl, SP Scientific, PA). After freeze drying, the freeze dried cells adhered on PRF (FLP-PRF) were measured for moisture content and color. Two gram samples of FLP-PRF were loaded in glass bottles and stored either at ambient temperature or at refrigerated temperature to examine cell stability during storage.

3.3.4. Proximate analysis of PRF

PRF was analyzed for moisture content, fat, protein, ash, total dietary fiber. The moisture content was determined according to AOAC standard methods 930.15 (AOAC, 2005) and then the fat was extracted with Soxhlet extraction from the dehydrated PRF according to AOAC standard methods 920.39 (AOAC, 2005). The protein content was determined according to AOAC procedure 992.15 (AOAC, 2005) using a Perkin Elmer Model 2410 Nitrogen Analyzer (Perkin Elmer Instruments, Norwalk, CT). The ash content was determined with a Thermolyne

Type 6000 muffle furnace (Thermo Scientific, Lawrence, KS) at 549 ºC as described in AOAC method 920.153 (AOAC, 2005). The total dietary fiber was determined according to AOAC method 985.29 (AOAC, 2005) using an enzymatic-gravimetric method.

3.3.5. Determination of antioxidant activity and total phenolic content of PRF

Antioxidant activity and total phenolic content of the PRF were determined. Antioxidant activity of PRF was measured using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) method described by Jun, Song, Yang, Youn, and Kim (2012). Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard. Antioxidant activity was expressed as %Antioxidant activity = [(absorbance 515 nm of control – absorbance 515 nm of sample)/absorbance 515 nm of control] × 100. The result was reported as Trolox equivalents. Total phenolic content was determined according to the method of Jun et al. (2012). Gallic acid was used as a standard, and total phenolic contents were expressed as gallic acid equivalents. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

3.3.6. Colors of PRF and FLP-PRF

Colors of PRF and FLP-PRE were determined using the chroma meter LABSCAN XE (Hunterlab, VA, USA). The data was reported in CIELAB color scales (L*, a*, and b*). Chroma and hue angle value were calculated with Eq. 3.1 and 3.2, respectively.

$$\text{Chroma} = [a^{*2} + b^{*2}]\ ½ \qquad (3.1)$$

$$\text{Hue angle} = \tan^{-1}(b^*/a^*) \qquad (3.2)$$

3.3.7. Scanning electron microscopy (SEM)

FLP-PRF samples were mounted on aluminum SEM stubs and then coated with gold: palladium (60:40) in an Edwards S150 sputter coater. The morphology of FLP-PRF was observed under a scanning electron microscope (JSM-6610LV, JEOL Ltd. Japan).

3.3.8. Cell viability of *L. plantarum* after freezing and freeze drying

Cell viability was determined according to the method described by Jagannath et al. (2010) with some modification. After freezing, the LP-PRF was thawed at room temperature. One mL of thawed sample was added to 9 mL of 0.85 g/100 mL sterile saline solution. Regarding FLP-PRF, one g of sample was mixed with 9 mL of the saline solution in a stomacher (AES Carboratoire easy MIX, AEC Chemunex, NJ) for 2 min. Serial dilutions were performed in the saline solution. The pour plating method using MRS agar (Neogen Corporation, Lansing, MI) with 0.75 g/100 mL $CaCO_3$ (Sigma-Aldrich, St. Louis, MO) as the media was used in enumeration of the cells. The plates were incubated at 37 °C and were enumerated for colony forming units per gram (CFU/g) after 48 h.

3.3.9. Acid and bile tolerances

Acid and bile tolerances were determined according to the method described by Cebeci and Gürakan (2003) with some modifications. Both free cells and FLP-PRF were considered. Free cells were prepared by growing the strain in MRS broth for 16 h. Determination of acid tolerance was accomplished by adding 1 g of FLP-PRF or 1 mL of free cells to 30 mL of sterile acidified MRS broth adjusted to a pH of 2.0 or 3.0. The cells were incubated at 37 °C and collected after 1 and 2 h incubation. In the bile tolerance test, FLP-PRF samples and free cells were separately exposed to 30 mL of MRS broth containing either 0.3g/100mL, 0.5g/100mL, or 1g/100mL oxgall (Sigma-Aldrich, St. Louis, MO) and incubated at 37 °C. Samples were tested after incubation for 12 and 24 h. The viable cells of both acid and bile tolerance studies were enumerated on MRS agar containing 0.75 g/100 mL $CaCO_3$ after incubation at 37 °C for 48 h.

3.3.10. Stability of FLP-PRF during storage

Cell viability of FLP-PRF samples stored at either ambient or refrigerated temperatures was investigated after storage for up to 12 weeks. A new bottle was opened at every time interval. One gram of FLP-PRF was mixed with 9 mL of 0.85g/100mL sterile saline solution in a stomacher (AES Carboratoire easy MIX, AEC Chemunex, NJ) for 2 min prior to performing decimal dilution. The viable cells on MRS agar containing 0.75g/100mL $CaCO_3$ were counted after 48 h incubation at 37 °C. The specific rate of degradation (k) of FLP-PRF was calculated according to Eq 3.3 (Korakoch et al., 2005)

$$\log N = \log N_0 - kt \qquad (3.3)$$

where N_0 is the initial (time $t = t_o$) number of viable cells (CFU/g of solids), N is the number of viable cells (CFU/g of solids) at time t (week), k is the specific rate of degradation ($week^{-1}$) and t is the storage time.

3.3.11. Statistical analysis

All values were means and standard deviations of three determinations. Means values from statistical analysis was condcuted with the SAS (Statistical Analysis System) software (version 9.2) (SAS Institute Inc., Cary, NC, USA) to test the significance of the differences among the different treatments.

3.4. Results and Discussions

3.4.1. Evaluation of PRF and FLP-PRF

As shown in Table 3.1, the major dry components of PRF were dietary fiber (66.34 g/100g), protein (20.7 g/100g), fat (9.05 g/100g), and ash (2.25 g/100g). Digestible carbohydrate was 1.56 g/100g in the PRF. The composition of PRF was within the range reported by other researches (Abdul-Hamid & Luan, 2000; Choi et al., 2011; Kanauchi et al., 2010). PRF had

antioxidant activity and contained phenolic compounds. Vitaglione, Napolitano, and Fogliano (2008) suggested that most of phenolic compounds in bran covalently bound to cell wall polysaccharides via ester bonds, called as dietary-fiber phenolic compounds including ferulic acid, diferulic acids ,ρ-coumaric acid, caffeic acid, and benzoic acid derivatives.

Table 3.1. Proximate analysis, antioxidant activity, and total phenolic contents of PRF

Compositions	g/100 g (dry basis)
Proteins	20.7±0.14
Fats	9.05±0.10
Carbohydrates*	67.9±0.22
Total dietary fibers	66.3±1.21
Ash	2.25±0.15
Antioxidant activity (μmol TE/kg)	1350.38±12.84
Total phenolic contents (mg GA/kg)	260.3±1.45

*****Carbohydrate (g/100 g) was calculated by the formula, 100 - protein - lipids - ash. Values are means ± standard deviation of triplicate measurements.

The moisture content of PRF and FLP-PRF is listed in Table 3.2. The moisture of PRF was 5.68 g/100g which was not significantly different than FLP-PRF frozen by air blast freezing, 5.23 g/100g ($P \leq 0.05$). The moisture content of FLP-PRF cryogenically frozen (7.22 g/100g) was significantly higher than both PRF and air blast frozen FLP-PRF ($P \leq 0.05$). All color values (L*, a*, b*, chroma, and hue angle) of FLP-PRF for both AF and CF treatments were significantly different than those of PRF ($P \leq 0.05$). The color of FLP-PRF was darker than PRF.

3.4.2. Viability of L. plantarum adhered on PRF after freezing and after freeze-drying

Viability of *L. plantarum,* both as free cells and as cells adhered on PRF and frozen by different freezing methods, is shown in Figure 3.1. Free cells, the cells without any protectants, were used as a control.

Table 3.2. Moisture contents and colors of PRF and FLP-PRF

Physicochemical properties	PRF	FLP-PRF	
		Air blast freezing (AF)	Cryogenic freezing (CF)
Moisture (g/100g)	5.68±0.27[b]	5.23±0.31[b]	7.22±0.12[a]
L*	32.0±1.61[a]	25.53±0.88[b]	27.72±0.32[b]
a*	7.39±0.03[b]	8.77±0.14[a]	8.61±0.09[a]
b*	4.65±0.06[b]	6.05±0.14[a]	6.14±0.33[a]
Chroma	38.11±0.44[b]	56.82±2.03[a]	55.95±2.87[a]
Hue angle	32.20±0.31[b]	34.60±0.27[a]	35.05±1.17[a]

PRF= purple rice bran fiber, FLP-PRF = freeze dried *L. plantarum* adhered on purple rice bran fiber.[a,b]Means ± standard deviation with different letters within the same row were significantly different ($P \leq 0.05$).

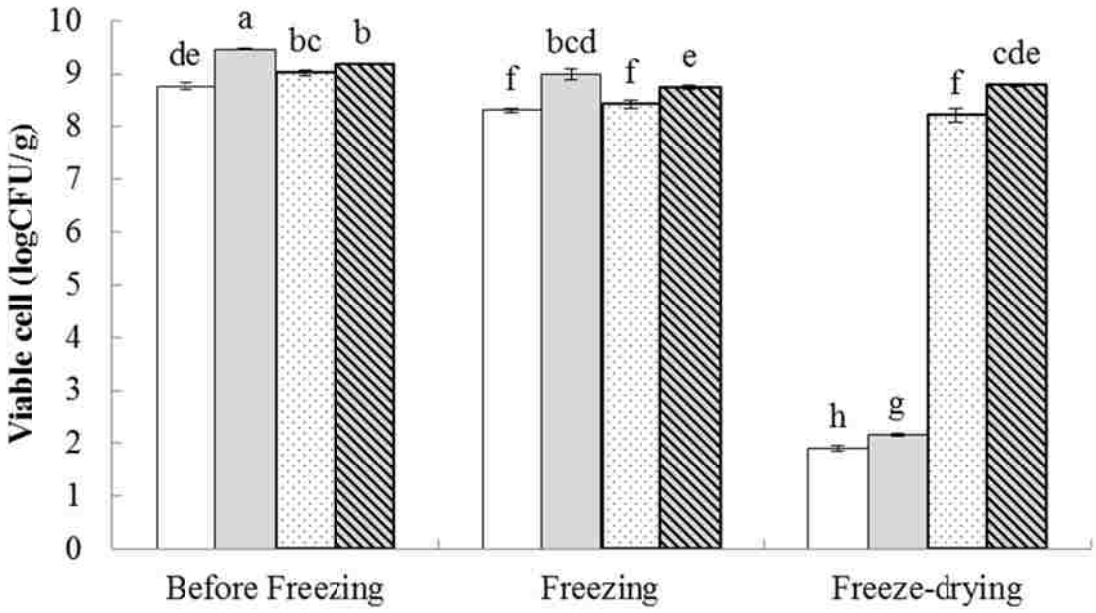


Figure 3.1. Viability of *L. plantarum* adhered on PRF before and after freezing and freeze drying. □ = Free cells/AF (cells without any protectant, grown in MRS broth for 16 h and frozen by air blast freezer), □ = Free cells/CF (cells without any protectant, grown in MRS broth for 16 h and cryogenically frozen), ⊡ = LP-PRF/AF (*L. plantarum* adhered on purple rice bran fiber frozen by air blast freezer), and ▨ = LP-PRF/CF (*L. plantarum* adhered on purple rice bran fiber cryogenically frozen). [a-h]means with different letters in different treatments before and after freezing and freeze drying are significantly different ($P \leq 0.05$).

All treatments had high survival rates with less than 1 log reduction after freezing. After freeze drying, the numbers of viable cells for the free cell control were dramatically decreased to 1.91±0.04 log CFU/g for the AF treatment and 2.17±0.03 log CUF/g for the CF treatment. On the other hand, the cells with PRF in both the AF and CF treatments (LP-PRF/AF and LP-PRF/CF) maintained much greater viability. LP-PRF/CF had 8.78±0.10 log CFU/g viable cells, while LP-PRF/AF had 8.22±0.13 log CFU/g viable cells. This indicated that PRF successfully helped protect the viability of the cells during freeze drying. PRF may have functioned as a physical barrier protecting the cells from unfavorable environments. As Figure 3.2 shown, the cells attached to the fiber matrix after freeze drying.

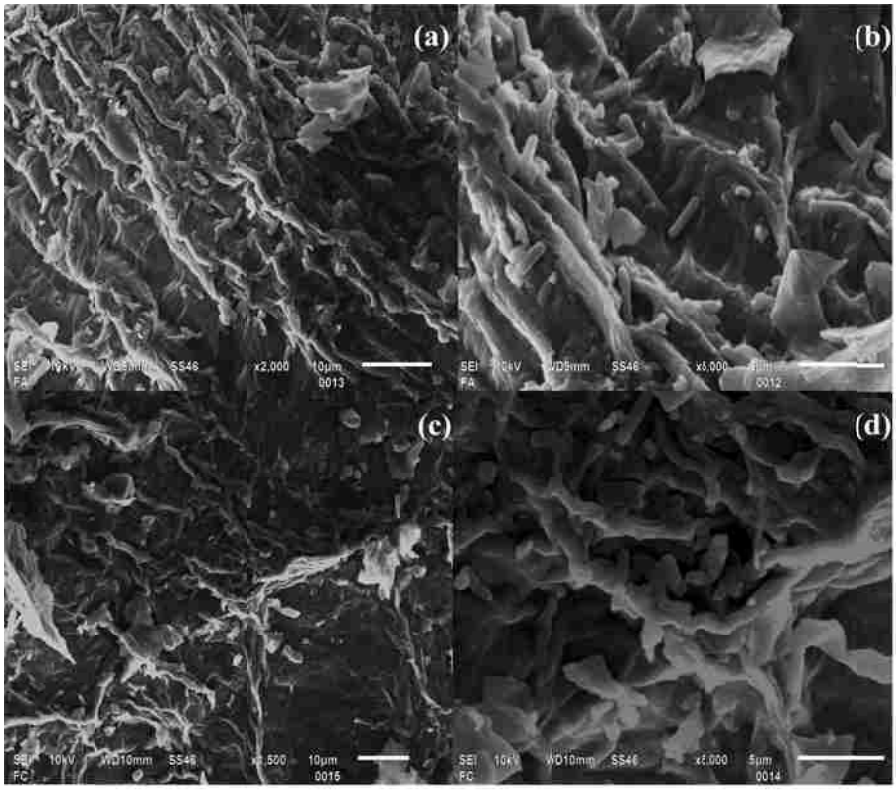


Figure 3.2. Scanning electron micrographs of freeze-dried *L. plantarum* adhered on PRF and frozen by air blast freezing (a and b) or by cryogenic freezing (c and d).

Jagannath et al. (2010) suggested that a disorganized arrangement of overlapping and twisting fibrous strands could have the potential to hold bacteria in the spaces and on the surface. It has been reported that insoluble fibers have potential as probiotic protectants by their reducing bacteria inactivation during freezing, drying, and storage (Charalampopoulos, Wang, Pandiella, & Webb, 2002; Guergoletto et al., 2010; Hongpattarakere et al., 2013; Saarela et al., 2006).

3.4.3. Acid and bile tolerance of freeze dried *L. plantarum* adhered on PRF

This strain of *L. plantarum* has been reported as acid tolerant (Reddy, Raghavendra, Kumar, Misra, & Prapulla, 2007) and capable of surviving in acidic mediums of both pH 2 and pH 3. In our study, the number of viable free cells was decreased less than 1 log after incubation for either 1 or 2 h in either pH condition (Figure 3.3). The number of viable cells for FLP-PRF after incubation at pH 3 for 2 h was 7.75±0.10 log CFU/g and 7.64±0.07 log CFU/g for AF and CF treatments, respectively. These values were not significantly different ($P \leq 0.05$). After 2 h incubation at pH 2, the viable cells of FLP-PRF/CF (6.06±0.01 log CFU/g) was significantly lower than FLP-PRF/AF (6.99±0.12 log CFU/g) ($P \leq 0.05$). This might indicate that cryogenic freezing, a rapid freezing method, could cause some cell damage resulting in the reduction of acid tolerance. Bâati, Fabre-Gea, Auriol, and Blanc (2000) reported that high freezing rates failed to improve cell viability and also might have detrimental effects on cells. When a high freezing rate was applied, membranes could be ruptured due to osmotic fluxes.(Volkert, Ananta, Luscher, & Knorr, 2008). This phenomenon could probably affect the proton permeability of plasma membrane, contributing to the regulation of intracellular pH which directly relates to the acid-stress response of microorganisms.

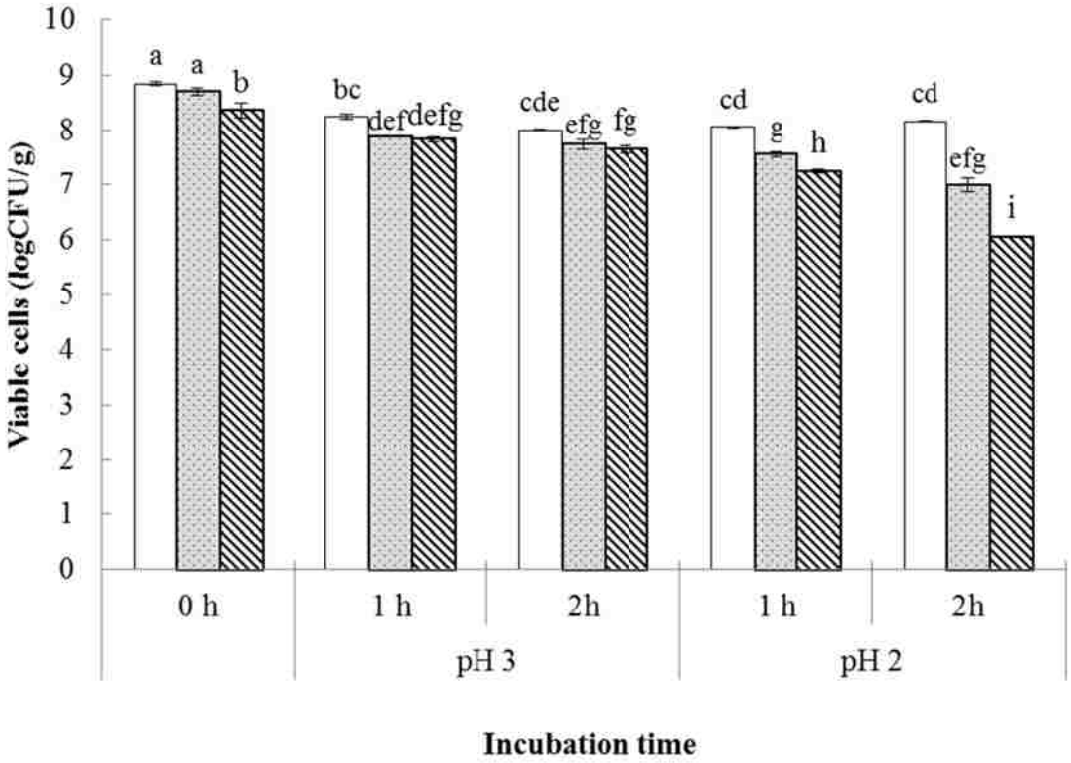


Figure 3.3. Viability of freeze dried *L. plantarum* adhered to PRF and frozen by air blast freezing (FLP-PRF/AF) and by cryogenic freezing (FLP-PRF/CF) and viability of the control (under acidic conditions. □ = Free cells (cells without any protectant and grown in MRS broth for 16 h), ▨ = FLP-PRF/AF (freeze dried *L. plantarum* adhered on purple rice bran fiber frozen by air blast freezer), and ▩ = FLP-PRF/CF (freeze dried *L. plantarum* adhered on purple rice bran fiber cryogenically frozen). $^{a\text{-}i}$means with different letters in different treatments before and after incubation are significantly different ($P \leq 0.05$).

In the bile tolerance study, the number of viable cells of all treatments was decreased by increasing oxgall concentration (Figure 3.4). Oxgall is bile that can emulsify and solubilize fats. It damages cells by lysing plasma membranes mainly composed of phospholipids (Begley, Gahan, & Hill, 2005). The number of viable cells of FLP-PRF in both AF and CF treatments was significantly higher than the number of viable free cells (control) ($P \leq 0.05$). After incubation for 24 h in 1% oxgall media, no free cells survived. Regardless of freezing method, FLP-PRF had cells survive in all concentrations of oxgall. Thus, it seemed that PRF provided the cells with protection from the bile.

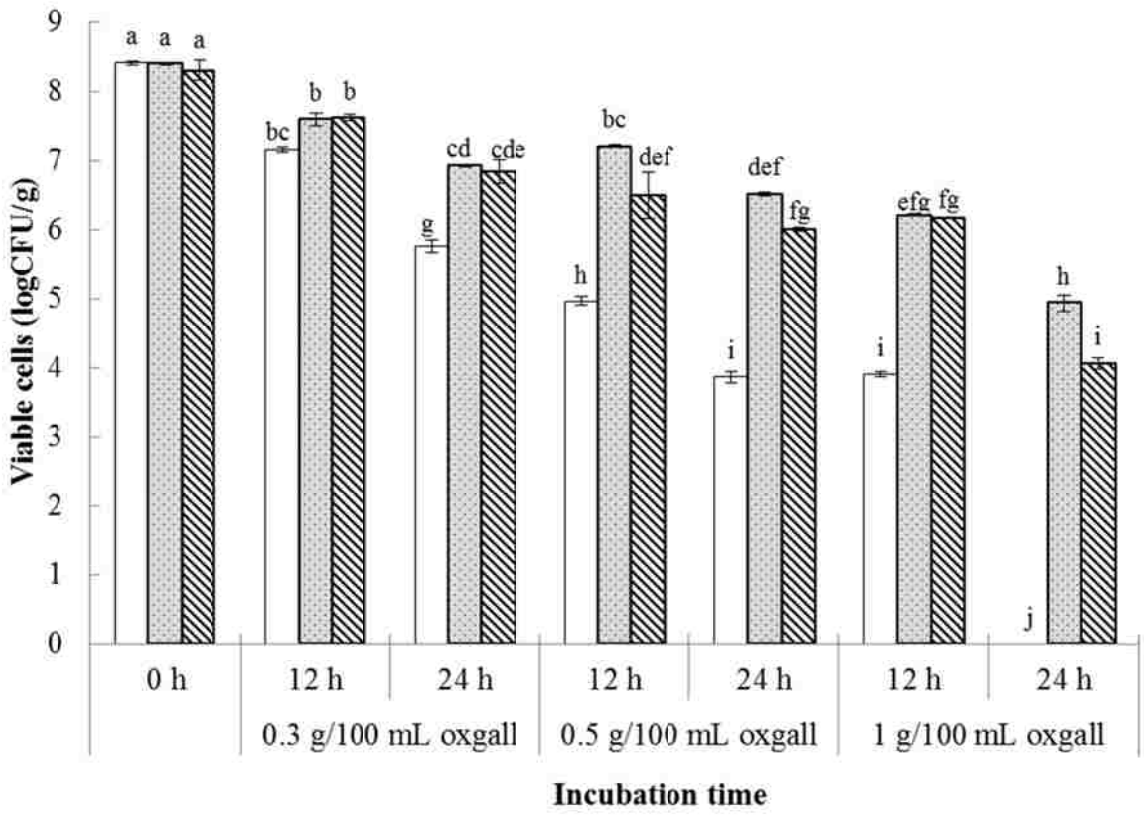


Figure 3.4. Viability of freeze dried *L. plantarum* adhered to PRF and frozen by air blast freezing (FLP-PRF/AF) and by cryogenic freezing (FLP-PRF/CF) and viability of the control (free cells) under bile salt conditions. □ = Free cells (cells without any protectant and grown in MRS broth for 16 h), ▨ = FLP-PRF/AF (freeze dried *L. plantarum* adhered on purple rice bran fiber frozen by air blast freezer), and ▧ = FLP-PRF/CF (freeze dried *L. plantarum* adhered on purple rice bran fiber cryogenically frozen). $^{a-j}$means with different letters in different treatment before and after incubation are significantly different ($P \leq 0.05$).

According to Dongowski (2007), dietary fiber was able to interact with bile acids; however, the mechanism has not been clearly understood. Kahlon and Chow (2000) mentioned that rice bran had bile acid binding ability higher than wheat bran, oat bran, and corn bran. It was also reported that bile acid binding appeared to be related to the content of insoluble dietary fiber (Kahlon & Woodruff, 2003). Moreover, similar to the acid tolerance results, FLP-PRF/AF had a significantly higher number of viable cells than FLP-PRF/CF ($P \leq 0.05$). Previous research has indicated that bile sensitivity could be used to predict the relative level of freezing injury to lactic

acid bacteria. Bile sensitive cells were likely to become non-viable during storage more rapidly than bile resistant cells (Heenan, Adams, Hosken, & Fleet, 2004). Thus, the result could confirm that cryogenic freezing negatively affected cell activities.

3.4.4. Cell stability during storage

After 12-week storage at 4°C, less than 1 log cycle reduction was observed in both FLP-PRF/AF and FLP-PRF/CF (Figure 3.5). The viable cell counts were 7.55±0.07 and 7.49±0.06 log CFU/g for FLP-PRF/AF and FLP-PRF/CF, respectively. The specific rate of degradation (*k*) of FLP-PRF/AF (0.053±0.001 $week^{-1}$) was significantly lower than FLP-PRF/CF (0.115±0.002 $week^{-1}$) ($P \leq 0.05$). Regarding the room temperature storage condition, the viability of the freeze dried cells in both AF and CF were reduced to about 1 log cycle after storage for 4 weeks. By the end of the 12-week period, the number of viable cells had been gradually decreased to 5.02±0.09 log CFU/g for FLP-PRF/AF and to 4.32±0.03 log CFU/g for FLP-PRF/CF. Similar to the 4°C storage condition results, the degradation rate of the cryogenically frozen FLP-PRF (0.362±0.001 $week^{1}$) was significantly higher than the air blast frozen FLP-PRF (0.283±0.001 $week^{-1}$). These results were in agreement with those of Péter and Reichart (2001) who noted that slow freezing positively affected the survival of *L. plantarum,* compared to fast freezing, At a high freezing rate, cell injury would ensue due to mechanical forces originated from intracellular ice formation (Volkert et al., 2008). Our study indicates that PRF may become a preferred protectant for freeze dried *L. plantarum* cells during storage. For example, in contrast with our PRF results, viable counts of freeze dried *L. plantarum* incorporated with inulin and gum acacia was considerably reduced after storage at 25°C for 1 month (Dhewa, Pant, & Mishra, 2011). Likewise, the number of freeze dried *L. plantarum* immobilized on mungbean crude fiber had 3 log reductions after storage for 4 weeks at 30°C (Hongpattarakere et al., 2013).

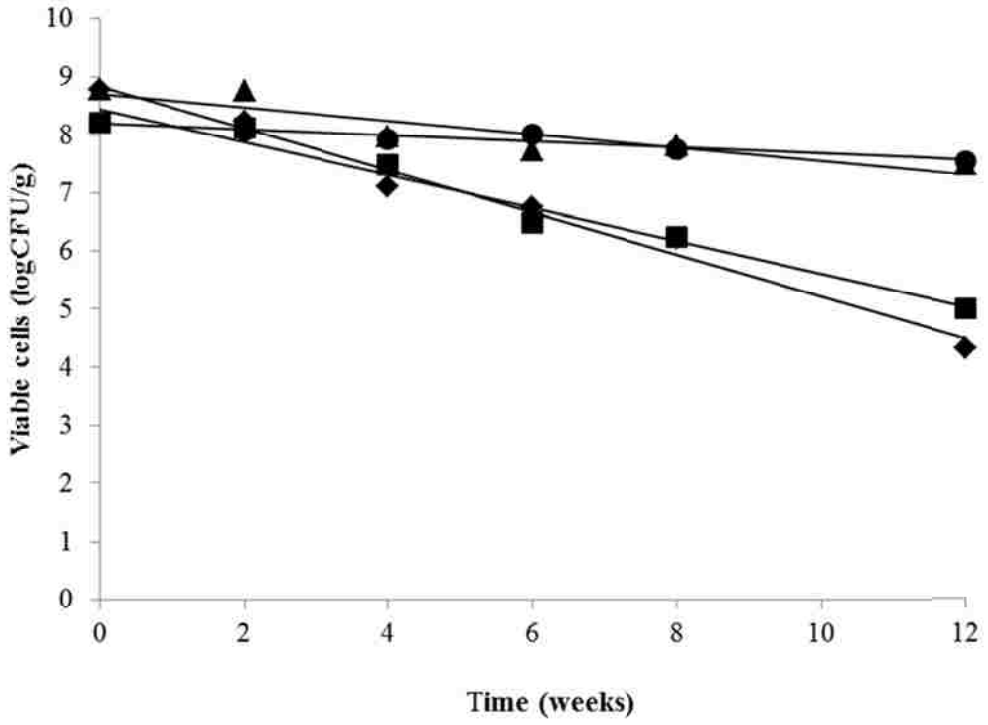


Figure 3.5. Viability of freeze dried *L. plantarum* adhered to PRF and frozen by air blast freezing (FLP-PRF/AF) and by cryogenic freezing (FLP-PRF/CF) during storage at 4°C (4T) and at room temperature (RT). ●— = FLP-PRF/AF-4T, ■— = FLP-PRF/AF-RT, ▲— = FLP-PRF/CF-RT, and ◆— = FLP-PRF/CF-4T.

3.6. Conclusions

This study demonstrated that purple rice bran fiber functioned as a fiber matrix, supporting and protecting the viability of the cells during freeze drying and storage. The fiber also helped protect the cells from bile. Freezing methods had an influence on the viability of the cells. Cryogenically frozen cells had lower survival, compared with air blast frozen cells. The result indicated that cryogenic freezing caused cell damage resulting in loss of acid and bile tolerance and loss of cell stability during storage. In summary, the study successfully developed a new probiotic-fiber supplement that would be incorporated into food products (e.g. nutrition

bars, cereal products or dairy products). The application of the probiotic-purple rice bran fiber supplement in food systems are recommended to study.

3.7. References

AACC. (2001). The definition of dietary fibre. *Cereal food world, 46*(3), 112-126.

Abdul-Hamid, A., & Luan, Y. S. (2000). Functional properties of dietary fibre prepared from defatted rice bran. *Food Chemistry, 68*(1), 15-19.

AOAC. (2005). *Official Methods of Analysis of AOAC Internation* (18 ed.). Gaithersburg, MD: Association of Official Analytical Chemists International.

Bâati, L. l., Fabre-Gea, C., Auriol, D., & Blanc, P. J. (2000). Study of the cryotolerance of *Lactobacillus acidophilus*: effect of culture and freezing conditions on the viability and cellular protein levels. *International journal of food microbiology, 59*(3), 241-247.

Begley, M., Gahan, C. G. M., & Hill, C. (2005). The interaction between bacteria and bile. *FEMS microbiology reviews, 29*(4), 625-651.

Cebeci, A., & Gürakan, C. (2003). Properties of potential probiotic *Lactobacillus plantarum* strains. *Food microbiology, 20*(5), 511-518.

Charalampopoulos, D., Wang, R., Pandiella, S. S., & Webb, C. (2002). Application of cereals and cereal components in functional foods: a review. *International journal of food microbiology, 79*(1–2), 131-141.

Choi, Y.-S., Choi, J.-H., Han, D.-J., Kim, H.-Y., Lee, M.-A., Kim, H.-W., . . . Kim, C.-J. (2011). Effects of rice bran fiber on heat-induced gel prepared with pork salt-soluble meat proteins in model system. *Meat Science, 88*(1), 59-66.

Dhewa, T., Pant, S., & Mishra, V. (2011). Development of freeze dried synbiotic formulation using a probiotic strain of *Lactobacillus plantarum*. *Journal of food science and technology*, 1-7. doi: 10.1007/s13197-011-0457-2

Dongowski, G. (2007). Interactions between dietary fibre-rich preparations and glycoconjugated bile acids in vitro. *Food Chemistry, 104*(1), 390-397.

Fernando, W. M. A. D. B., Brennan, C. S., Flint, S., Ranaweera, K. K. D. S., Bamunuarachchi, A., & Morton, H. R. (2010). Enhancement of short chain fatty acid formation by pure cultures of probiotics on rice fibre. *International journal of food science and technology, 45*(4), 690-696.

Goffman, F. D., & Bergman, C. J. (2004). Rice kernel phenolic content and its relationship with antiradical efficiency. *Journal of the science of food and agriculture, 84*(10), 1235-1240.

Guergoletto, K. B., Magnani, M., Martin, J. S., Andrade, C. G. T. d. J., & Garcia, S. (2010). Survival of *Lactobacillus casei* (LC-1) adhered to prebiotic vegetal fibers. *Innovative food Science & emerging technologies, 11*(2), 415-421.

Heenan, C. N., Adams, M. C., Hosken, R. W., & Fleet, G. H. (2004). Survival and sensory acceptability of probiotic microorganisms in a nonfermented frozen vegetarian dessert. *LWT - food science and technology, 37*(4), 461-466.

Hongpattarakere, T., Rattanaubon, P., & Buntin, N. (2013). Improvement of freeze-dried *Lactobacillus Plantarum* survival using water extracts and crude fibers from food crops. *Food and bioprocess technology, 6*(8), 1885-1896.

Jagannath, A., Raju, P. S., & Bawa, A. S. (2010). Comparative evaluation of bacterial cellulose (nata) as a cryoprotectant and carrier support during the freeze drying process of probiotic lactic acid bacteria. *LWT - food science and technology, 43*(8), 1197-1203.

Jang, S., & Xu, Z. (2009). Lipophilic and hydrophilic antioxidants and their antioxidant activities in purple rice bran. *Journal of agricultural and food chemistry, 57*(3), 858-862.

Jun, H.-I., Song, G.-S., Yang, E.-I., Youn, Y., & Kim, Y.-S. (2012). Antioxidant activities and phenolic compounds of pigmented rice bran extracts. *Journal of food science, 77*(7), C759-C764.

Kahlon, T. S., & Chow, F. I. (2000). In Vitro binding of bile acids by rice bran, oat bran, wheat bran, and corn bran. *Cereal Chemistry, 77*(4), 518-521.

Kahlon, T. S., & Woodruff, C. L. (2003). In vitro binding of bile acids by rice bran, oat bran, barley and β-glucan enriched barley. *Cereal Chemistry, 80*(3), 260-263.

Kanauchi, O., Mitsuyama, K., Komiyama, Y., Yagi, M., Andoh, A., & Sata, M. (2010). Preventive effects of enzyme-treated rice fiber in a restraint stress-induced irritable bowel syndrome model. *International journal of molecular medicine 25*(4), 547-555.

Komiyama, Y., Andoh, A., Fujiwara, D., Ohmae, H., Araki, Y., Fujiyama, Y., . . . Kanauchi, O. (2011). New prebiotics from rice bran ameliorate inflammation in murine colitis models through the modulation of intestinal homeostasis and the mucosal immune system. *Scandinavian journal of gastroenterology, 46*(1), 40-52.

Korakoch, H., Sukyai, P., Loiseau, G., Nitisinprasert, S., Montet, D., & Wanchaitanawong, P. (2005). Prediction on the stability of spray-dried *Lactobacillus reuteri* KUB-AC5 by arrhenius equation for long-term storage. *Journal of microbiology and biotechnology, 15*(6), 1178-1182.

Lattimer, J. M., & Haub, M. D. (2010). Effects of dietary fiber and its components on metabolic health. *Nutrients, 2*(12), 1266-1289.

Li, B., Tian, F., Liu, X., Zhao, J., Zhang, H., & Chen, W. (2011). Effects of cryoprotectants on viability of *Lactobacillus reuteri* CICC6226. *Applied microbiology and biotechnology, 92*(3), 609-616.

Manning, T. S., & Gibson, G. R. (2004). Prebiotics. *Best practice & research clinical gastroenterology 18*(2), 287-298.

McKee, L. H., & Latner, T. A. (2000). Underutilized sources of dietary fiber: A review. *Plant foods for human nutrition 55*(4), 285-304.

Meng, X. C., Stanton, C., Fitzgerald, G. F., Daly, C., & Ross, R. P. (2008). Anhydrobiotics: The challenges of drying probiotic cultures. *Food Chemistry, 106*(4), 1406-1416.

Morgan, C. A., Herman, N., White, P. A., & Vesey, G. (2006). Preservation of micro-organisms by drying; A review. *Journal of microbiological methods, 66*(2), 183-193.

Péter, G., & Reichart, O. (2001). The effect of growth phase, cryoprotectants and freezing rate on the survival of selected microorganisms during freezing and thawing. *Acta alimentaria academiae scientiarium hungaricae, 30*(1), 89-97.

Reddy, K. B. P. K., Raghavendra, P., Kumar, B. G., Misra, M. C., & Prapulla, S. G. (2007). Screening of probiotic properties of lactic acid bacteria isolated from *Kanjika*, an ayruvedic lactic acid fermented product: An in-vitro evaluation. *Journal of general and applied microbiology, 53*(3), 207-213.

Saarela, M., Virkajärvi, I., Nohynek, L., Vaari, A., & Mättö, J. (2006). Fibres as carriers for *Lactobacillus rhamnosus* during freeze-drying and storage in apple juice and chocolate-coated breakfast cereals. *International journal of food microbiology, 112*(2), 171-178.

Slavin, J. (2008). Position of the American Dietetic Association: health implications of dietary fiber. *Journal of the american dietetic association, 108*(10), 1716-1731.

Vitaglione, P., Napolitano, A., & Fogliano, V. (2008). Cereal dietary fibre: a natural functional ingredient to deliver phenolic compounds into the gut. *Trends in Food Science & Technology, 19*(9), 451-463.

Volkert, M., Ananta, E., Luscher, C., & Knorr, D. (2008). Effect of air freezing, spray freezing, and pressure shift freezing on membrane integrity and viability of *Lactobacillus rhamnosus* GG. *Journal of food engineering, 87*(4), 532-540.

CHAPTER 4 DEVELOPMENT OF A COMBINED LOW METHOXYL PECTIN AND RICE BRAN EXTRACT DELIVERY SYSTEM TO IMPROVE THE VIABILITY OF *L. PLANTARUM* UNDER ACID AND BILE CONDITIONS

4.1. Abstract

A combined pectin-rice bran delivery system for *Lactobacillus plantarum* NRRL-B4496 (LP) was developed. Four pectin (PE)-rice bran extract (RB) gel solutions were prepared: (1) 2.0 g/100 mL PE with 0.5 g/100 mL RB, (2) 2.0 g/100 mL PE with 1.0 g/100 mL RB, (3) 2.0 g/100 mL PE with 2.0 g/100 mL RB, and (4) 2.0 g/100 mL PE (control). *L. plantarum* was grown in MRS broth, centrifuged, and mixed with the gel solutions. The capsules loaded with *L. plantarum* (LP/PE-RB capsules) were then prepared by ionotropic gelation. PE-RB gel solutions exhibited pseudoplastic behavior. The gel solution containing 2.0 g/100 mL RB had the highest consistency and viscosity. All LP/PE-RB capsules had similar diameter. Both the sphericity and the encapsulation efficiency of the capsules were increased with higher RB content, while the hardness and springiness were decreased. When exposed to acidic and bile salt conditions, the viability of encapsulated cells was higher than free cells. The study demonstrated that PE-RB capsules could have potential as a delivery system for *L. plantarum*.

Keywords: Pectin-rice bran delivery system, *Lactobacillus plantarum,* capsules

4.2. Introduction

Probiotics are well-known for their health promoting effects. They can relieve diarrhea, reduce colonization of pathogenic bacteria and intestinal inflammation, alleviate lactose

This chapter previously appeared as Arranee Chotiko and Subramaniam Sathivel, Development of a combined low-methoxyl-pectin and rice-bran-extract delivery system to improve the viability of *Lactobacillus plantarum* under acid and bile conditions, October 28, 2015. It is reprinted by permission of Elsevier—see the permission letter for proper acknowledgment phrase in Appendix B (see page 144).

intolerance, reduce blood cholesterol, and possess anti-colon cancer effects (Ratna, Chauhan, Dixit, Babu, & Jamil, 2009; Saxelin, Tynkkynen, Mattila-Sandholm, & de Vos, 2005). The probiotic activity depends on the dose levels and the probiotic's viability in products and in gut environments (Kailasapathy & Chin, 2000). The recommended levels of live probiotic bacteria for addition to food products is at least 10^6-10^7 cfu/g (Floch et al., 2008). Therefore, it is necessary to maintain high levels of probiotics in products before consumption and to ensure good survival during the digestion processes to reach the sites of action with sufficient numbers and viability to provide health benefits.

Encapsulation is the packaging technology of active ingredients in small capsules that release their content at controlled rates over prolonged periods of time (Corbo, Bevilacqua, Gallo, Speranza, & Sinigaglia, 2013). Different encapsulation techniques are used for probiotics to enhance their viability and for target delivery, generally, including spray drying, freeze drying, emulsion, and extrusion (Huq, Khan, Khan, Riedl, & Lacroix, 2012). According to Krasaekoopt, Bhandari, and Deeth (2003), probiotic powder encapsulated by drying processes is released in food products. This results in loss of protection and greater deterioration in unfavorable environments, such as during the passage through the gastrointestinal (GI) tract. (de Castro-Cislaghi, Silva, Fritzen-Freire, Lorenz, & Sant'Anna, 2012) found that spray dried *Bifidobacterium animalis* subsp. *lactis* Bb-12 powder with whey had greater decrease in viability at low pH and bile conditions than free cells. On the other hand, encapsulation of probiotics in a hydrocolloid gel matrix provides protection against GI conditions. Encapsulated *B. animalis* Bb-12 in milk protein or alginate-chitosan capsules formed by gelation methods had a larger number of viable cells than free cells at GI conditions (Heidebach, Forst, & Kulozik, 2009; Liserre, Re, & Franco, 2007).

Encapsulation of probiotics in gel/bead matrix, such as sodium alginate (Sathyabama, Ranjith, Bruntha, Vijayabharathi, & Brindha, 2014), carrageenan (Hernandez-Rodriguez, Lobato-Calleros, Pimentel-Gonzalez, & Vernon-Carter, 2014), carboxymethyl cellulose (Chitprasert, Sudsai, & Rodklongtan, 2012), and gelatin (Annan, Borza, & Hansen, 2008) has been successfully studied. However, there are few reports on encapsulation of probiotics using pectin matrix, although it has been widely used in the pharmaceutical industry as a delivery vehicle for colon-specific oral drugs. Pectin is a complexly structured polysaccharide predominantly composed of homogalacturonan, a homopolymer of partially methyl-esterified (1-4)-linked α-D-galacturonic acid, and a range of neutral sugars such as rhamnose, galactose, or arabinose (Maxwell, Belshaw, Waldron, & Morris, 2012). It can form three dimensional rigid and water insoluble hydrogels by calcium-induced ionotropic gelation (Lee, Kim, Chung, & Lee, 2009 & Lee, 2009). Pectin is a soluble fiber that is resistant to GI conditions and degradable by colonic bacteria (Cabrera, Cambier, & Cutsem, 2011). It also has prebiotic properties, which can enhance the growth of *Bifidobacterium* and *Lactobacillus* sp. (Wicker et al., 2014).

Pectin has been reported to be less sensitive to chemical agents and more resistant to GI environments than alginate. Voo, Ravindra, Tey, and Chan (2011) found that pectin based beads containing poultry probiotic cells had higher mechanical strength than alginate beads. As a result, cell release from pectin beads was less than that from alginate beads during fermentation. Viability of *Lactobacillus rhamnosus* in gastric conditions at pH 2 was improved when the cell was encapsulated with pectin (Gerez, Font de Valdez, Gigante, & Grosso, 2012). In a recent study, Gebara et al. (2013) encapsulated *Lactobacillus acidophilus* in pectin beads by ionotropic gelation. The result showed that the reduction of encapsulated cells was 1.51 log cycles after

incubating in simulated gastric (pH 3) and intestinal (pH 7) juices, while a reduction of 3.54 log cycles was observed in non-encapsulated cells.

Pectin's high porosity and weak binding behavior to a crosslink agent (Ca^{2+}) are disadvantages, limiting the use of pectin in encapsulation technology (Chan et al., 2011; Fang et al., 2008). Adding a filler agent, such as starch or rice bran was suggested to overcome the problem and to increase the protective effect for probiotic delivery. Chan et al. (2011) revealed that starch filler could improve sphericity, flowability, and mechanical strength and reduce porosity of the calcium alginate beads. Viability of *Lactobacillus casei* encapsulated in the beads with starch filler was higher than the control after lyophilization and during storage. Chitprasert et al. (2012) determined that encapsulation of *Lactobacillus reuteri* in aluminum carboxymethyl cellulose–rice bran capsules helped increase microencapsulation yield and contributed to cell survival during heat exposure.

A pectin (PE) and rice bran extract (RB) delivery system may protect viable *L. plantarum* cells under acidic and bile conditions. There are either no data or only limited information available on effects of PE and RB delivery systems containing probiotics at acidic and bile conditions. Therefore, the intention of this study was to develop and analyze PE-RB loaded with *L. plantarum* capsules. The effects of RB on physical properties of the capsules were investigated as well as the viability of the encapsulated cells after encapsulation and exposure to acidic and bile conditions. The *L. plantarum* NRRL-B4496 strain used is a probiotic strain used in fermented food products (Fijan, 2014; Pedreschi, Campos, Noratto, Chirinos, & Cisneros-Zevallos, 2003; Upadhyay, 2014). It has the ability to inhibit the growth of some bacteria that cause digestive illness, such as *Helicobacter pylori* (Apostolidis, Kwon, Shinde, Ghaedian, & Shetty, 2011) and *Listeria monocytogenes* (Upadhyay, 2014). The *L. plantarum* also reduces

hypolipidemic activity (Haroun, Refaat, El-Waseif, El-Menoufy, & Amin, 2013). Additionally, it shows antioxidant (Das & Goyal, 2015) and antifungal activities (Cortes-Zavaleta, Lopez-Malo, Hernandez-Mendoza, & Garcia, 2014).

4.3. Materials and methods

4.3.1. Cell culture preparation

Lactobacillus plantarum NRRL B-4496 (LP) isolated from pickled cabbage was kindly provided by ARS Culture Collection (Washington DC, US). The frozen stock culture was reactivated twice in de Man Rogosa Sharpe (MRS) broth (Neogen Corporation, Lansing, MI). The culture (25 mL) was subsequently inoculated in MRS broth (500 mL) and incubated at 37 °C for 16 h to reach stationary phase. Cell pellets were harvested by centrifuging at 10,000 x g for 10 min at 4 °C (Beckman J2-HC, Beckman Coulter, Inc., Brea, CA). The pellets were washed three times and suspended in sterile distilled water (LP suspension).

4.3.2. Preparation of pectin-rice bran loaded with *L. plantarum* capsules

The preparation was performed as described by Lee et al. (2009) with some modifications. First, pectin-rice bran gel solutions loaded (PE-RB gel solution) with LP were prepared by mixing a LP suspension (~10^9cfu/mL) with 2 g/100 mL of low-methoxyl pectin (PE) (TIC PRETESTED® pectin LM 32 powder), provided by TIC Gums Inc (Belcamp, MD) and rice bran extract (RB) (Ribus Inc. (St. Louis, MO). The proximate composition of RB is 17 g/100 g protein, 41 g/100 g carbohydrate, 22 g/100 g fat, 13 g/100 g ash, and 7 g/100 g water. The required concentrations of RB were 0.5 g/100 mL, 1 g/100 mL, and 2 g/100 mL. PE gel solution (without RB) containing LP was used to produce control (LP/PE) capsules. All gel solutions with LP were then stirred for 1 h, extruded through a 23G needle by a syringe pump at a flow rate of 1.2 mL/min, and dropped into calcium chloride ($CaCl_2$) solution (4 g/100 mL) (Fisher Scientific

Inc., Pittsburgh, PA). The pectin-rice bran loaded with LP (LP/PE-RB) capsules were immediately formed and continuously hardened in $CaCl_2$ solution for 30 min. The capsules were collected, washed twice, and kept at 4 °C for further analysis.

4.3.3. Rheological properties of PE-RB gel solutions

Flow behavior of the PE-RB gel solutions without LP was measured using an AR 2000 Ex Rheometer (TA Instruments, New Castle, DE) fitted with a plate geometry (a steel plate with a 40-mm diameter, having a 200 μm gap between the two plates). Each sample was placed on the temperature-controlled parallel plate at 25 °C. The shear stress was measured at shear rates from 1 to100 s^{-1}. The flow properties of the gel sample were characterized by the power law, shown in Eq. 4.1.

$$\sigma = K\gamma^n \qquad (4.1)$$

where σ = shear stress (Pa), γ = shear rate (s^{-1}), K = consistency index (Pa s^n), and n = flow behavior index. A plot of log σ against log γ was constructed, and the magnitudes of K and n were determined from the resulting straight line intercept and slope, respectively. The mean values of n, K, and apparent viscosity for triplicate gel solution samples were reported.

4.3.4. Determination of LP/PE-RB capsule size and shape

Thirty capsule diameters were measured with a stereomicroscope (Zeiss SteREO Lumar.V12, Thornwood, NY), using an image analyzer software. The capsule shape was quantified by the sphericity factor (SF), shown in Eq. 4.2 (Chan et al., 2011).

$$SF = \frac{d_{max} - d_{min}}{d_{max} + d_{min}} \qquad (4.2)$$

where d_{max} is the largest diameter and d_{min} is the smallest diameter perpendicular to d_{max}.

4.3.5. Determination of encapsulation efficiency

The encapsulation efficiency (EE) represented a combined measure of the efficacy of entrapment and survival of viable cells during the microencapsulation procedure, calculated according to Eq. 4.3 (Gebara et al., 2013).

$$EE\ (\%) = \frac{N}{N_0} \times 100 \qquad (4.3)$$

where EE is the encapsulation efficiency, expressed in percentage, N is the number of cells released from the beads (log CFU/g of capsules), and N_0 is the number of cells in the gel solution (log CFU/g of LP suspension).

To determine the number of cells in the capsules, the encapsulated LP was released according to the method described by Sathyabama et al. (2014) with some modifications. One gram of the capsules was added to 99 mL of 0.1 M phosphate buffer (pH 7.2) and stirred at 250 rpm by a shaker (Lab line incubator shaker model 3525, Fisher Scientific Inc., Pittsburgh, PA) for 30 min. The colony forming units (CFU/g) was examined by pour-plate method on MRS agar containing $CaCO_3$ (0.6g/100mL) (Fisher Scientific Inc., Pittsburgh, PA). The plate was incubated at 37 °C for 24-48 h.

4.3.6. Textural properties of LP/PE-RB capsules

The LP/PE-RB capsules were analyzed for texture profile as described by Sandoval-Castilla, Lobato-Calleros, Garcia-Galindo, Alvarez-Ramírez, and Vernon-Carter (2010) with some modification. An Instron Universal Testing Device (Model 5544, Norwood, MA) equipped with a 5 Kg-load cell was used for determination. The capsule samples (5 g) were placed on a fixed bottom plate under the probe. The contact force was controlled at 0.005 N. The samples were compressed 30%, using two compression cycles at a constant crosshead velocity of 30 mm min^{-1}. Hardness, cohesiveness, and springiness (ratio between the areas under the compression

and decompression curves) were analyzed using the software Bluehill Materials Testing Software (Bluehill 3, version 3.13, 2010, Instron).

4.3.7. Scanning electron micrographs of LP/PE-RB capsules

The LP/PE-RB capsules were incubated overnight in a mixture of ethanol, acetic acid, and formaldehyde. After incubation, they were rinsed and dehydrated with ethanol. Then they were dried with liquid CO_2 using a critical point-dryer. The dried capsules were cut, mounted on aluminum SEM stubs, and coated with gold:palladium (60:40) in an Edwards S150 sputter coater. The capsule morphology was observed under a scanning electron microscope (JSM-6610LV, JEOL Ltd. Japan).

4.3.8. Survival of the cells in LP/PE-RB capsules under acidic and bile conditions

Acid and bile tolerance of the encapsulated cells and of free cells was determined according to the method described by Ding and Shah (2007). One gram of capsules and 1 mL of free cells (~10^8 CFU/g) were inoculated into acidified MRS broth (pH 3.0) to measure acid tolerance and separately into MRS broth containing 1 g/100 mL oxgall (Sigma Aldrich, St. Louis, MO) to measure bile tolerance. They were then incubated at 37 °C for 2 h (acid) and 24 h (bile) to determine acid and bile tolerances. The encapsulated LP was released from the capsules and enumerated according to the method described in section 4.3.5.

4.3.9. Statistical analysis

All values were means and standard deviations of three determinations. Statistical analysis on the mean values was conducted with the SAS (Statistical Analysis System) software (version 9.4) (SAS Institute Inc., Cary, NC, USA) to test for differences among the treatments ($P \leq 0.05$).

4.4. Results and discussions

4.4.1. Rheological properties of PE-RB gel solutions

Flow behavior and apparent viscosity of the gel solutions are shown in Table 4.1. The results indicated that the rheological properties of gel solutions were affected by RB concentrations. All gel solutions exhibited non-Newtonian or pseudoplastic behaviors, as evidenced by the flow index (*n*) which was less than 1.0 (Paredes, Rao, & Bourne, 1989).

Table 4.1. Flow behavior properties of PE-RB gel solutions at 25°C

Gel solutions	RB concentration (g/100 mL)	*n*	*K* (Pa s^n)	Viscosity (Pa s)
PE (control)	0	0.968±0.014[a]	0.064±0.005[b]	0.056±0.007[b]
PE-0.5RB	0.5	0.961±0.013[a]	0.021±0.001[c]	0.018±0.001[c]
PE-1.0RB	1	0.865±0.024[b]	0.092±0.009[b]	0.050±0.001[b]
PE-2.0RB	2	0.275±0.035[c]	5.711±0.865[a]	0.213±0.007[a]

Gel solutions of PE, PE-0.5RB, PE-1.0RB, and PE-2.0RB are gel solution containing 2.0 g/100 mL pectin without rice bran extract, 2.0 g/100 mL pectin with 0.5 g/100 mL rice bran extract, 2.0 g/100 mL pectin with 1.0 g/100 mL rice bran extract, and 2.0 g/100 mL pectin with 2.0 g/100 mL rice bran extract, respectively. [a-c]Means ± standard deviation with different letters within the same column are significantly different ($P \leq 0.05$).

The *n* obtained in the current study was 0.968±0.014, 0.961±0.013, 0.865±0.024, and 0.275±0.035 for PE, PE-0.5RB, PE-1.0RB, and PE-2.0RB gel solutions, respectively. PE and PE-0.5RB gel solutions demonstrated a nearly Newtonian like fluid behavior. Pseudoplasticity of the gel solutions increased significantly with higher RB concentrations. The PE-2.0RB gel solution had significantly highest consistency (5.711±0.865 Pa s^n) ($P \leq 0.05$). Moreover, RB concentration greatly influenced gel solution viscosity. The PE-0.5RB gel solution had significantly lower viscosity than the PE gel solution (0.018±0.001 and 0.056±0.007 Pa s, respectively) ($P \leq 0.05$). RB functions as a processing aid for extrusion processes, helping to reduce a product's surface irregularity and increase production rates (Hammond (2000). In

general, viscosities of polymers decrease when the proper amount of processing aid is used (Achilleos, Georgiou, & Hatzikiriakos, 2002). This could possibly explain the reduction of viscosity at low levels of RB (PE-0.5RB gel solution), followed by increased viscosity at higher RB concentrations. The PE-2.0RB gel solution had a viscosity value of 0.213±0.007 Pa s, which was significantly higher than PE-1.0RB gel solution (0.050±0.001 Pa s) and the PE gel solution. Some interactions between RB and PE may have occurred, causing changes to their functional properties. Our lab examined structural aspects of PE-RB powder by Fourier Transform Infrared Spectroscopy (FTIR) and found certain changes in spectra profiles of PE-RB powder, compared to PE or RB alone (see Appendix A). RB contains high protein and fat content. Certain functional groups of RB protein and fatty acids are mainly involved in the interaction, as reported by Chitprasert et al. (2012)

4.4.2. LP/PE-RB capsule size, shape, and encapsulation efficiency

As shown in Table 4.2, all capsules had similar sizes ($P \leq 0.05$). The sphericity factor (SF) was used to determine the shape of the capsule samples. Capsules with SF less than 0.05 are considered to be spherical beads (Lee, Ravindra, & Chan, 2013). LP/PE-2.0RB capsules had the lowest SF (0.03±0.02) ($P \leq 0.05$). The highest SF was in LP/PE-0.5RB (0.07±0.05) capsules, which were not significantly different compared to LP/PE samples (0.06±0.04) ($P \leq 0.05$). The appearance of the LP/PE-RB capsules is shown in Figure 4.1. The LP/PE-2.0RB capsules had spherical shape, and were light brown in color due to the natural color of RB. LP/PE, LP/PE-0.5RB, and LP/PE-1.0RB samples were unable to form spherical particles which may be due to insufficient RB and pectin concentration. These results are compatible with those of Chitprasert et al. (2012) who determined that the capsules produced from carboxymethyl cellulose became more spherical when rice bran was added. This effect may be viscosity dependent, as noted by

Chan et al. (2011). Lee et al. (2013) reported that spherical beads or capsules could not be formed if the gel solutions had a viscosity between 60-150 cp. Although all gel solutions in our study had viscosities out of this critical range, only PE-2.0RB gel solution had a higher viscosity than the critical value (0.213 Pa.s. or 213 cp).

Table 4.2. Diameter, sphericity factor, and encapsulation yield of LP/PE-RB capsules

Capsules	RB concentration (g/100 mL)	Diameter of beads (Hammond)	Sphericity factor (SF)	Encapsulation Efficiency (EE) (%)
LP/PE	0	6.54±0.53[a]	0.06±0.04[a]	83.23±3.41[c]
LP/PE-0.5RB	0.5	6.52±0.57[a]	0.07±0.05[a]	90.94±1.98[b]
LP/PE-1.0RB	1	6.23±0.52[a]	0.05±0.03[b]	93.40±1.64[ab]
LP/PE-2.0RB	2	6.37±0.28[a]	0.03±0.02[b]	95.44±1.22[a]

LP/PE = *L. plantarum* loaded in pectin capsules without rice bran extract (control). LP/PE-0.5RB, LP/PE-1.0RB, and LP/PE-2.0RB = *L. plantarum* loaded in pectin capsules containing 0.5 g/100 mL, 1.0 g/100 mL, and 2.0 g/100 mL rice bran extract, respectively. [a-b]Means ± standard deviation with different letters within the same column are significantly different ($P \leq 0.05$).

Table 4.2 demonstrated that LP/PE-RB capsules showed high encapsulation efficiency (EE) in all samples (less than one log cycle of viable cells reduction). LP/PE-RB capsules contained more than 10^7 CFU/g of viable cells. Mattila-Sandholm et al. (2002) suggested that for colonization, viable population of probiotics should be in a range of 10^7-10^9 CFU/g. High EE would be attributed to the pectin shell. White, Budarin, and Clark (2010) have reported that pectin is categorized as a nanoporous polymer, having pore sizes between 2 to 50 nm. It is well known that bacteria sizes are about 0.2 μm in diameter and 2-8 μm in length. Thus, pectin has considerable capacity for cell entrapment. It was obvious that RB helped improve the EE of LP/PE-RB capsules. The EE monotonically increased with RB. LP/PE-2.0RB samples had the highest EE (95.44±1.22%), followed by LP/PE-1.0RB (93.40±1.64%), LP/PE-0.5RB (90.94±1.98%), and LP/PE capsules (83.23±3.41%). The increase of EE probably resulted from

higher sphericity of the LP capsules containing RB, leading to a reduction of cell losses during the gelation process. Woo et al. (2007) mentioned that irregular and tear shape beads could cause the release of encapsulants.

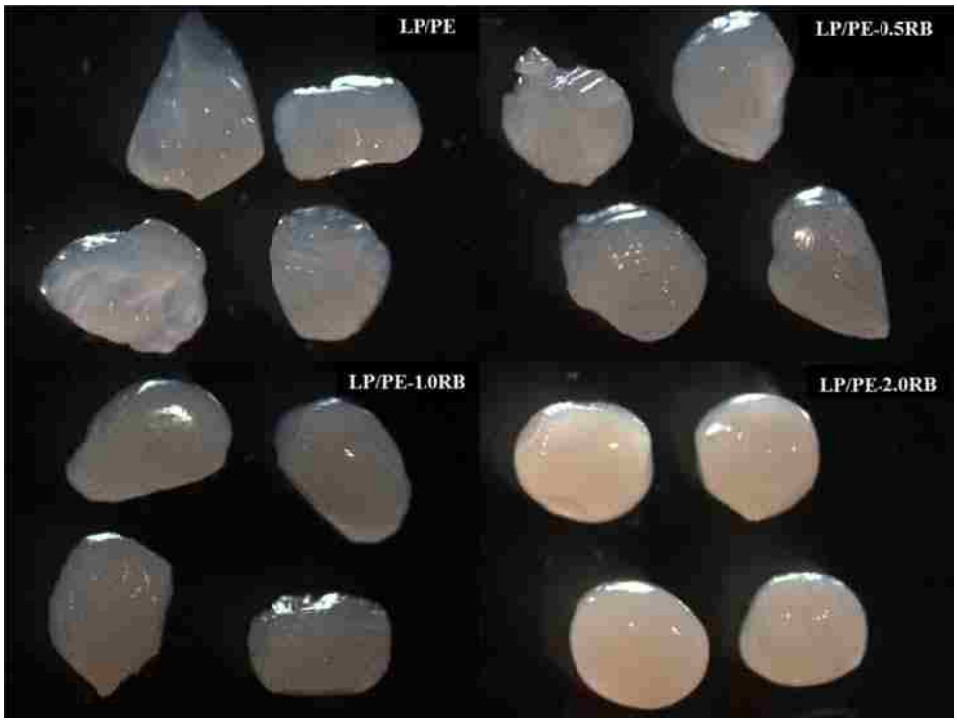


Figure 4.1. *L. plantarum*-loaded pectin-rice bran extract capsules. LP/PE = *L. plantarum* loaded in pectin capsules without rice bran extract (control). LP/PE-0.5RB, LP/PE-1.0RB, and LP/PE-2.0RB = *L. plantarum* loaded in pectin capsules containing 0.5 g/100 mL, 1.0 g/100 mL, and 2.0 g/100 mL rice bran extract, respectively.

4.4.3. Textural properties of LP/PE-RB capsules

The textural properties of the LP/PE-RB capsules were affected by RB concentration (Table 4.3). Hardness and springiness decreased with increasing RB content. The lowest hardness and springiness were in LP/PE-2.0RB capsules (8.61±1.74 N and 0.97±0.01 mm, respectively) ($P \leq 0.05$). These values were not significantly different from LP/PE-1.0RB samples (11.49±1.18 N and 0.99±0.02 mm, respectively) ($P \leq 0.05$). Similarly, no significant differences were observed in hardness and springiness between LP/PE-0.5RB and LP/PE samples ($P \leq 0.05$). All treatment samples had similar cohesiveness ($P \leq 0.05$). The softer texture

of LP/PE-1.0RB and LP/PE-2.0RB capsules possibly occurred because of protein and fat in RB. Liu, Xu, and Guo (2008) observed that protein is broken up by water and functions as a lubricant, providing smoothness and softness. Fat can also act as a lubricant by changing compression properties of the gel matrix and decreasing its breakdown forces (Pereira, Matia-Merino, Jones, and Singh (2006). Moreover, lipids increase the apparent viscosity of the gel matrix, resulting in a lower friction coefficient and consequently changing the gel's lubricant properties (Chojnicka, Sala, de Kruif, and van de Velde (2009). Costas, Pera, Lopez, Mechetti, and Castro (2012) noted that an increase of viscosity leads to reduction of gel deformation which affects textural properties of the gels.

Table 4.3. Textural properties of LP/PE-RB capsules

Capsules	RB concentration (g/100mL)	Hardness (N)	Springiness (mm)	Cohesiveness (ratio)
LP/PE	0	14.59 ± 0.75^{a}	1.43 ± 0.11^{a}	0.63 ± 0.01^{a}
LP/PE-0.5RB	0.5	14.90 ± 0.89^{a}	1.29 ± 0.19^{a}	0.63 ± 0.05^{a}
LP/PE-1.0RB	1	11.49 ± 1.18^{ab}	0.99 ± 0.02^{b}	0.63 ± 0.05^{a}
LP/PE-2.0RB	2	8.61 ± 1.74^{b}	0.97 ± 0.01^{b}	0.58 ± 0.01^{a}

LP/PE = *L. plantarum* loaded in pectin capsules without rice bran extract (control); LP/PE-0.5RB, LP/PE-1.0RB, and LP/PE-2.0RB = *L. plantarum* loaded in pectin capsules containing 0.5 g/100 mL, 1.0 g/100 mL, and 2.0 g/100 mL of the rice bran extract, respectively. $^{a-c}$Means ± standard deviation with different letters within the same column are significantly different ($P \leq 0.05$).

4.4.4. Morphology of LP/PE-RB capsules

Scanning electron micrographs of the external and internal structures of LP/PE-RB capsules are shown in Figure 4.2. Cracks were observed on the surface of all LP-bead samples (Figure 4.2a). In accordance with literature, this was presumably due to loss of water and collapse of the gel matrix during sample drying (Badve, Sher, Korde, & Pawar, 2007; Jung, Arnold, & Wicker, 2013). Further, it was reported that crosslinking fixatives and the base buffer

using used in sample preparation could weaken the hydrated capsules integrity (Allan-Wojtas, Truelstrup Hansen, & Paulson, 2008). In the present study, cells were covered with a thin matrix and distributed under the surface (Figure 4.2b); there were no cells observed on the surface. The results were similar to the microstructures recently revealed by Jimenez-Pranteda et al. (2012) and Martin, Lara-Villoslada, Ruiz, and Morales (2013). In our study, it was evident that the bacteria cells were randomly entrapped in the mesh-like network (indicated by white arrows), as illustrated in Figure 4.2c. At higher concentrations of RB, a greater network was observed. This could suggest that there were some interactions or overlaps between RB and the PE matrix. In this regard, our results were in agreement with the study of Chitprasert et al. (2012). They found that it was more difficult to find *Lactobacillus reuteri* entrapped in aluminum carboxymethy cellulose capsules with added RB (AlCMC-RB), than in capsules without RB. They suggested that AlCMC-RB consisted of a dense matrix of RB sheets and AlCMC-entrapped RB interstices.

4.4.5. Survival of the cells in LP/PE-RB beads under acidic and bile conditions

The viability of LP under acidified media (pH 3.0) is shown in Figure 4.3. The results demonstrated that the cells in all samples exhibited good acid survivability after 2 h incubation, which is supported by the work of Chotiko and Sathivel (2014). Regarding reduction of the viable cells, viability of LP in all capsules, except LP/PE-0.5RB samples, had lower log reduction than free cells ($P \leq 0.05$). After incubation, the free cells had 0.97±0.01 log CFU/g reduction. The PE/PE-1.0RB sample had the least viable cell reduction (0.41±0.07 log CFU/g), followed by LP/PE-2.0RB (0.48±0.05 log CFU/g), LP/PE (0.63±0.10 log CFU/g), and LP/PE-0.5RB (1.05±0.09 log CFU/g). The greatest number of viable cells was found in LP/PE-2.0RB

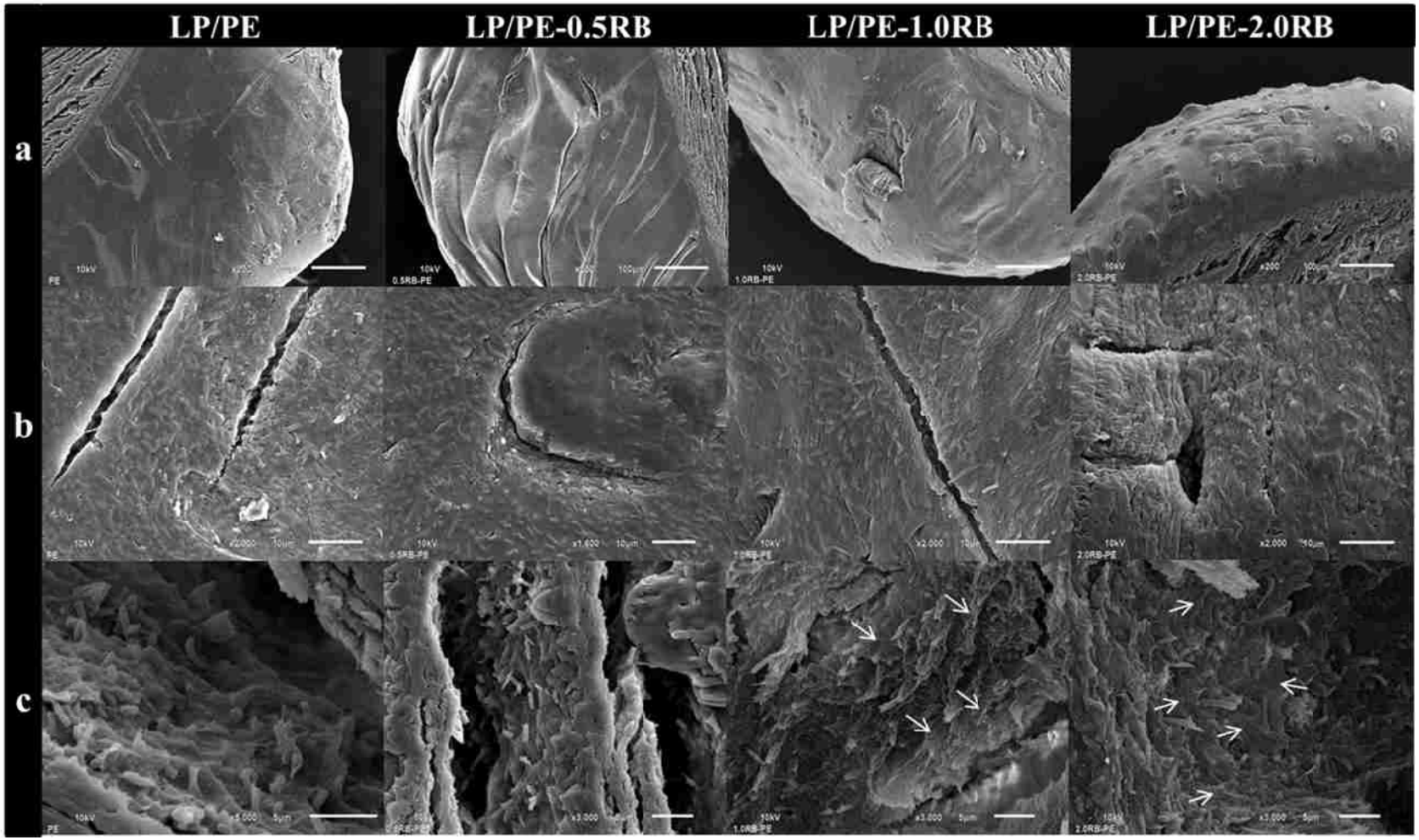


Figure 4.2. Scanning electron micrographs of LP/PE-RB capsules illustrating surface area of the capsules at low magnification (a), at high magnification (b), and capsule cross sections (c). LP/PE = *L. plantarum* loaded in pectin capsules without rice bran extract (control). LP/PE-0.5RB, LP/PE-1.0RB, and LP/PE-2.0RB = *L. plantarum* loaded in pectin capsules containing 0.5 g/100 mL, 1.0 g/100 mL, and 2.0g/100 mL of the rice bran extract, respectively.

capsules (8.30±0.01 log CFU/g), which was not significantly different from the number of viable cells in LP/PE-1.0RB (8.16±0.04 log CFU/g) ($P \leq 0.05$). This indicated that pectin could protect the cell from acidic conditions and that sufficient concentrations of RB were able to enhance the protection of the encapsulated cells.

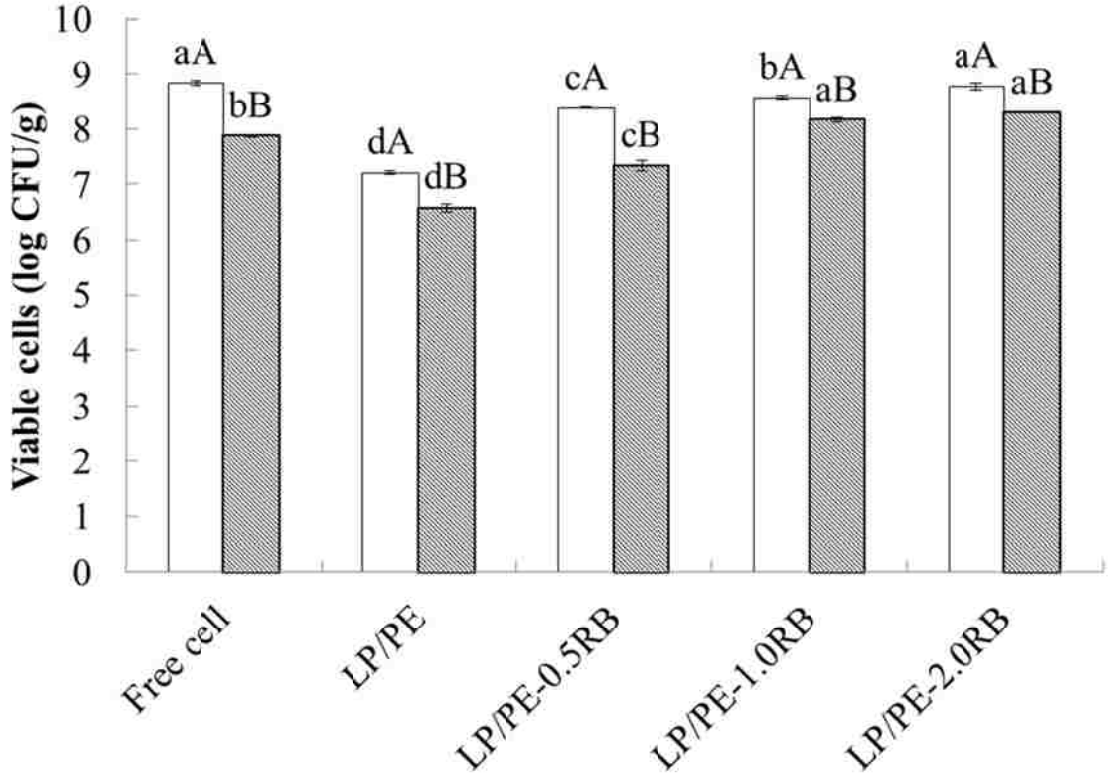


Figure 4.3. Viability of *L. plantarum*-loaded calcium pectinate-rice bran capsules before (□) and after (▨) incubating in acidified MRS at pH 3.0. Free cells = *L. plantarum* grown in MRS broth for 16 h, LP/PE = *L. plantarum* loaded in pectin capsules without rice bran extract (control). LP/PE-0.5RB, LP/PE-1.0RB, and LP/PE-2.0RB = *L. plantarum* loaded in pectin capsules containing 0.5g/100mL, 1.0g/100mL, and 2.0g/100mL of the rice bran extract, respectively. ^{A,B}Means ± standard deviation with different letters within the same treatment are significantly different ($P \leq 0.05$). $^{a\text{-}d}$Means ± standard deviation with different letters within different treatments (before and after incubation) are significantly different ($P \leq 0.05$).

Protective effects of biopolymer encapsulation on probiotic survivability have been studied by several researchers. Their results indicated that encapsulated bacteria exhibited a significantly greater number of viable cells than free or non-encapsulated cells when were

exposed to low pH conditions (Mokarram, Mortazavi, Najafi, & Shahidi, 2009; Nazzaro, Fratianni, Coppola, Sada, & Orlando, 2009; Sabikhi, Babu, Thompkinson, & Kapila, 2010; Shi et al., 2013). Recently, Gebara et al. (2013) reported that viability of *L. acidophilus* LA 5 after exposure to simulated gastric juice (pH 3.0) was increased when the cell was encapsulated with 2 g/100 mL amidated pectin. de Vos, Faas, Spasojevic, and Sikkema (2010) suggested that the formation of hydrogels acted as a physical barrier, delaying penetration of fluids into the cells. Sandoval-Castilla et al. (2010) found that addition of pectin to alginate beads loaded with *L. casei* slows the diffusion rate of growth inhibition compounds, such as acids and hydrogen peroxide into the cells. In addition, our results suggest that at low pH viability of LP in the capsules was enhanced by the presence of RB. This is possibly due to their structural stability.

Regarding the effect of mechanical strength (hardness) of the gel matrix on cell viability under acidic conditions, our results contradict those reported by Zhao et al. (2015). They found that viability of encapsulated cells in simulated gastric juice was positively correlated with mechanical strength of the capsules, and that greater mechanical strength led to a more integrated structure and a smaller mesh size of the network. In our study, the mechanical strength of PE capsules containing RB was lower than the capsules without RB. However, they had more mesh-like networks, as shown in Figure 4.2c. This could effectively help protect the cells from acid penetration. This is in agreement with the results reported by Chitprasert et al. (2012) that the addition of rice bran to carboxyl methyl cellulose beads provided a high density structure to the encapsulating matrix, which contributed to high survival rates of probiotic bacteria. Lactobacilli survive under acidic conditions when they can maintain a pH gradient between the medium and their cytoplasm (Charalampopoulos, Pandiella, & Webb, 2003). The mesh matrix formed by RB

could increase the diffusion path length (Chitprasert et al., 2012), which possibly reduced the diffusion rate of acid into the encapsulated cells.

To obtain a measure of bile tolerance both encapsulated LP and free cells were exposed to media containing high oxgall concentration (1 g/100 mL). Oxgall functions as an emulsifier and fat solubilizer, hydrolyzing plasma membranes of bacteria cells, resulting in cell damage (Begley, Gahan, & Hill, 2005). In our study, encapsulated LP of all samples had higher cell viability than the free cells (Figure 4.4). After 24-h incubation, the number of free cells was reduced to 3.51±0.01 log CFU/g (5.35±0.01 log CFU/g reduction). A greater number of viable cells was observed for LP/PE capsules (5.80±0.15 log CFU/g) ($P \leq 0.05$). These cells had undergone a reduction of 2.35±0.06 log CFU/g, indicating that a pectin matrix could protect LP from bile effects. According to Cheewatanakornkool et al. (2012), pectins have the ability to bind with bile salts, however their binding efficiency depends upon pectin sources. Many studies have reported that encapsulation of probiotic bacteria in polysaccharide matrices, such as alginate, pectin, or carrageenan successfully enhanced the survival of the bacteria during exposure to 1-3% bile salt solutions (Rokka & Rantamäki, 2010; Sandoval-Castilla et al., 2010; Shi et al., 2013).

RB also helped protect the survival of LP from bile salts. After incubation under the bile condition, the number of viable cells in LP/PE capsules with RB was greater than either that of capsules without RB or free cells ($P \leq 0.05$). LP/PE-1.0RB capsules had the most viable cells (6.87±0.18 log CFU/g) after incubation, which was not significantly different from LP/PE-2.0RB (6.80±0.01 log CFU/g) and LP/PR-0.5RB (6.71±0.09 log CFU/g). As viable cells in the tested acid condition were reduced less than one log, approximately 10^6 CFU of LP/g of PE-RB capsules could survive for colonization. Compared to LP/PE-RB capsules, the greater

mechanical strength of LP/PE capsules did not contribute to increase of cell viability. According to Zhao et al. (2015), survivability of encapsuleated cells in bile conditions were not increased when mechanical strength of matrix was higher. Similar to the acid tolerant study, RB possibly provided the capsules with higher networks and diffusion path lengths, reducing penetration of bile solution and consequently decreasing cell losses.

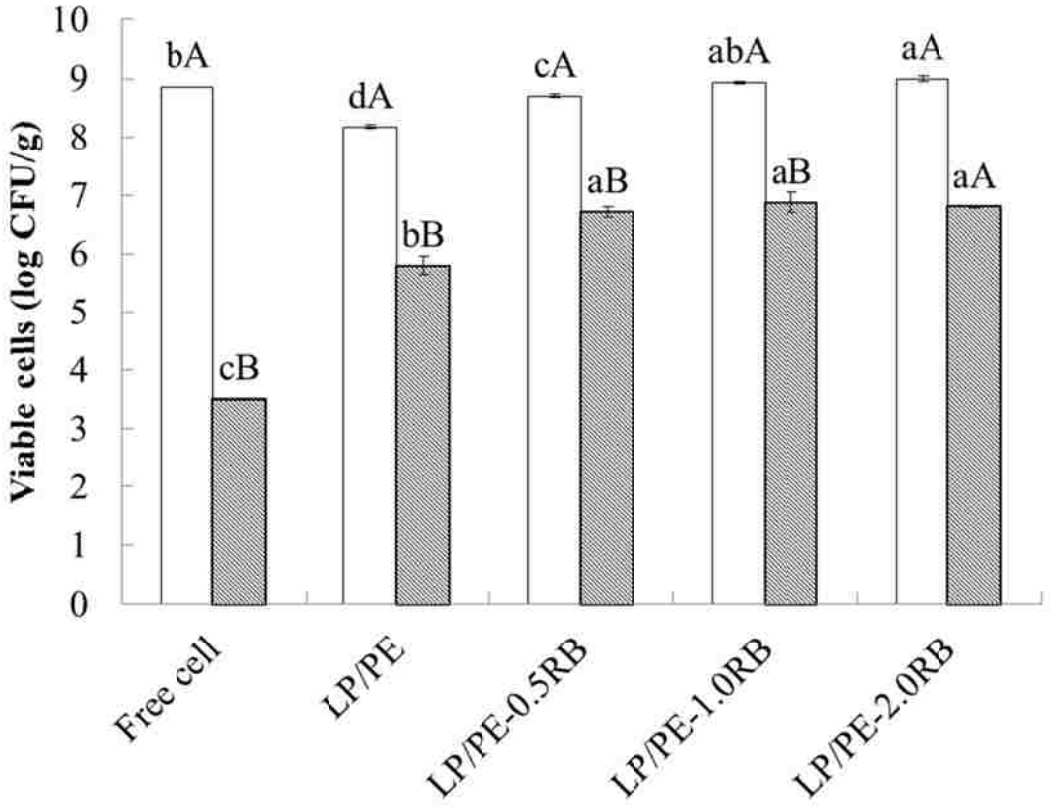


Figure 4.4. Viability of *L. plantarum*-loaded calcium pectinate-rice bran capsules before (□) and after (▨) incubating in MRS containing 1 g/100 mL oxgall. Free cells = *L. plantarum* grown in MRS broth for 16 h, LP/PE = *L. plantarum* loaded in pectin capsules without rice bran (control). LP/PE-0.5RB, LP/PE-1.0RB, and LP/PE-2.0RB = *L. plantarum* loaded in pectin capsules containing 0.5g/100mL, 1.0g/100mL, and 2.0g/100mL rice bran extract, respectively. ^{A,B}Means ± standard deviation with different letters within the same treatment are significantly different ($P \leq 0.05$). $^{a-d}$Means ± standard deviation with different letters within different treatments (before and after incubation) are significantly different ($P \leq 0.05$).

4.5. Conclusion

The use of pectin and rice bran extract to obtain capsules loaded with *L. plantarum* NRRL-B4496 could improve encapsulation efficiency and sphericity of the capsules. Addition of 2 g/100 mL RB yielded the highest encapsulation efficiency and highest sphericity, however it reduced hardness and springiness of the capsules. The rice bran extract helped create a mesh-like network in the calcium pectinate-based capsules, contributing to enhancement of cell viability after exposure to acid and bile conditions. In summary, the pectin-rice bran extract capsules could be used as a new vehicle for probiotic bacteria and incorporated into some food products such as yogurt, cereal bars, or fruit juices.

4.6. References

Achilleos, E. C., Georgiou, G., & Hatzikiriakos, S. G. (2002). Role of processing aids in the extrusion of molten polymers. *Journal of Vinyl and Additive Technology, 8*(1), 7-24. doi: 10.1002/vnl.10340

Allan-Wojtas, P., Truelstrup Hansen, L., & Paulson, A. T. (2008). Microstructural studies of probiotic bacteria-loaded alginate microcapsules using standard electron microscopy techniques and anhydrous fixation. *LWT - Food Science and Technology, 41*(1), 101-108. doi: http://dx.doi.org/10.1016/j.lwt.2007.02.003

Annan, N. T., Borza, A. D., & Hansen, L. T. (2008). Encapsulation in alginate-coated gelatin microspheres improves survival of the probiotic *Bifidobacterium adolescentis* 15703T during exposure to simulated gastro-intestinal conditions. *Food Research International, 41*(2), 184-193. doi: http://dx.doi.org/10.1016/j.foodres.2007.11.001

Apostolidis, E., Kwon, Y. I., Shinde, R., Ghaedian, R., & Shetty, K. (2011). Inhibition of Helicobacter pylori by Fermented Milk and Soymilk Using Select Lactic Acid Bacteria and Link to Enrichment of Lactic Acid and Phenolic Content. *Food Biotechnology, 25*(1), 58-76. doi: 10.1080/08905436.2011.547118

Badve, S. S., Sher, P., Korde, A., & Pawar, A. P. (2007). Development of hollow/porous calcium pectinate beads for floating-pulsatile drug delivery. *European Journal of Pharmaceutics and Biopharmaceutics, 65*(1), 85-93. doi: http://dx.doi.org/10.1016/j.ejpb.2006.07.010

Cabrera, J. C., Cambier, P., & Cutsem, P. V. (2011). Drug encapsulation in pectin hydrogel beads-A systematic study of stimulated digestion media. *International Journal of Pharmacy and Pharmaceutical Sciences, 3*(5), 292-299.

Chan, E.-S., Wong, S.-L., Lee, P.-P., Lee, J.-S., Ti, T. B., Zhang, Z., . . . Yim, Z.-H. (2011). Effects of starch filler on the physical properties of lyophilized calcium–alginate beads and the viability of encapsulated cells. *Carbohydrate Polymers, 83*(1), 225-232. doi: http://dx.doi.org/10.1016/j.carbpol.2010.07.044

Charalampopoulos, D., Pandiella, S. S., & Webb, C. (2003). Evaluation of the effect of malt, wheat and barley extracts on the viability of potentially probiotic lactic acid bacteria under acidic conditions. *International Journal of Food Microbiology, 82*(2), 133-141. doi: 10.1016/s0168-1605(02)00248-9

Chitprasert, P., Sudsai, P., & Rodklongtan, A. (2012). Aluminum carboxymethyl cellulose–rice bran microcapsules: Enhancing survival of *Lactobacillus reuteri* KUB-AC5. *Carbohydrate Polymers, 90*(1), 78-86. doi: http://dx.doi.org/10.1016/j.carbpol.2012.04.065

Chojnicka, A., Sala, G., de Kruif, C. G., & van de Velde, F. (2009). The interactions between oil droplets and gel matrix affect the lubrication properties of sheared emulsion-filled gels. *Food Hydrocolloids, 23*(3), 1038-1046. doi: http://dx.doi.org/10.1016/j.foodhyd.2008.08.008

Chotiko, A., & Sathivel, S. (2014). Effects of enzymatically-extracted purple rice bran fiber as a protectant of *L. plantarum* NRRL B-4496 during freezing, freeze drying, and storage. *LWT - Food Science and Technology, 59*(1), 59-64. doi: http://dx.doi.org/10.1016/j.lwt.2014.05.056

Corbo, M. R., Bevilacqua, A., Gallo, M., Speranza, B., & Sinigaglia, M. (2013). Immobilization and microencapsulation of *Lactobacillus plantarum*: Performances and in vivo applications. *Innovative Food Science & Emerging Technologies, 18*(0), 196-201. doi: http://dx.doi.org/10.1016/j.ifset.2012.12.004

Cortes-Zavaleta, O., Lopez-Malo, A., Hernandez-Mendoza, A., & Garcia, H. S. (2014). Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *International Journal of Food Microbiology, 173*, 30-35. doi: http://dx.doi.org/10.1016/j.ijfoodmicro.2013.12.016

Costas, L., Pera, L., Lopez, A., Mechetti, M., & Castro, G. (2012). Controlled Release of Sulfasalazine Release from "Smart" Pectin Gel Microspheres under Physiological Simulated Fluids. *Applied Biochemistry and Biotechnology, 167*(5), 1396-1407. doi: 10.1007/s12010-012-9615-x

Das, D., & Goyal, A. (2015). Antioxidant activity and γ-aminobutyric acid (GABA) producing ability of probiotic Lactobacillus plantarum DM5 isolated from Marcha of Sikkim. *LWT - Food Science and Technology, 61*(1), 263-268. doi: http://dx.doi.org/10.1016/j.lwt.2014.11.013

de Castro-Cislaghi, F. P., Silva, C. D. R. E., Fritzen-Freire, C. B., Lorenz, J. G., & Sant'Anna, E. S. (2012). Bifidobacterium Bb-12 microencapsulated by spray drying with whey: Survival under simulated gastrointestinal conditions, tolerance to NaCl, and viability

during storage. *Journal of Food Engineering, 113*(2), 186-193. doi: http://dx.doi.org/10.1016/j.jfoodeng.2012.06.006

de Vos, P., Faas, M. M., Spasojevic, M., & Sikkema, J. (2010). Encapsulation for preservation of functionality and targeted delivery of bioactive food components. *International Dairy Journal, 20*(4), 292-302. doi: http://dx.doi.org/10.1016/j.idairyj.2009.11.008

Ding, W. K., & Shah, N. P. (2007). Acid, bile, and heat tolerance of free and microencapsulated probiotic bacteria. *Journal of Food Science, 72*(9), M446-M450. doi: 10.1111/j.1750-3841.2007.00565.x

Fang, Y., Al-Assaf, S., Phillips, G. O., Nishinari, K., Funami, T., & Williams, P. A. (2008). Binding behavior of calcium to polyuronates: Comparison of pectin with alginate. *Carbohydrate Polymers, 72*(2), 334-341. doi: http://dx.doi.org/10.1016/j.carbpol.2007.08.021

Fijan, S. (2014). Microorganisms with Claimed Probiotic Properties: An Overview of Recent Literature. *International Journal of Environmental Research and Public Health, 11*(5), 4745-4767. doi: 10.3390/ijerph110504745

Floch, M. H., Walker, W. A., Guandalini, S., Hibberd, P., Gorbach, S., Surawicz, C., . . . Dieleman, L. A. (2008). Recommendations for Probiotic Use—2008. *Journal of Clinical Gastroenterology, 42*, S104-S108 110.1097/MCG.1090b1013e31816b31903f.

Gebara, C., Chaves, K. S., Ribeiro, M. C. E., Souza, F. N., Grosso, C. R. F., & Gigante, M. L. (2013). Viability of *Lactobacillus acidophilus* La5 in pectin–whey protein microparticles during exposure to simulated gastrointestinal conditions. *Food Research International, 51*(2), 872-878. doi: http://dx.doi.org/10.1016/j.foodres.2013.02.008

Gerez, C. L., Font de Valdez, G., Gigante, M. L., & Grosso, C. R. F. (2012). Whey protein coating bead improves the survival of the probiotic *Lactobacillus rhamnosus* CRL 1505 to low pH. *Letters in Applied Microbiology, 54*(6), 552-556. doi: 10.1111/j.1472-765X.2012.03247.x

Hammond, N. A. (2000). US Patent No. 6,054,149. Woodland, CA: US.

Haroun, B. M., Refaat, B. M., El-Waseif, A. A., El-Menoufy, H. A., & Amin, H. A. (2013). Hypolipidemic activity of the probiotic *Lactobacillus plantarum* NRRL B-4496 and their prebiotic exopolysaccharide in *vitro* and in *vivo*. *Journal of Applied Sciences Research, 9*(1), 1015-1020.

Heidebach, T., Forst, P., & Kulozik, U. (2009). Microencapsulation of probiotic cells by means of rennet-gelation of milk proteins. *Food Hydrocolloids, 23*(7), 1670-1677. doi: http://dx.doi.org/10.1016/j.foodhyd.2009.01.006

Hernandez-Rodriguez, L., Lobato-Calleros, C., Pimentel-Gonzalez, D. J., & Vernon-Carter, E. J. (2014). *Lactobacillus plantarum* protection by entrapment in whey protein isolate: κ-

carrageenan complex coacervates. *Food Hydrocolloids, 36*(0), 181-188. doi: http://dx.doi.org/10.1016/j.foodhyd.2013.09.018

Huq, T., Khan, A., Khan, R. A., Riedl, B., & Lacroix, M. (2012). Encapsulation of probiotic bacteria in biopolymeric system. *Critical Reviews in Food Science and Nutrition, 53*(9), 909-916. doi: 10.1080/10408398.2011.573152

Jimenez-Pranteda, M. L., Poncelet, D., Nader-Macias, M. E., Arcos, A., Aguilera, M., Monteoliva-Sanchez, M., & Ramos-Cormenzana, A. (2012). Stability of lactobacilli encapsulated in various microbial polymers. *Journal of Bioscience and Bioengineering, 113*(2), 179-184. doi: http://dx.doi.org/10.1016/j.jbiosc.2011.10.010

Jung, J., Arnold, R. D., & Wicker, L. (2013). Pectin and charge modified pectin hydrogel beads as a colon-targeted drug delivery carrier. *Colloids and Surfaces B: Biointerfaces, 104*(0), 116-121. doi: http://dx.doi.org/10.1016/j.colsurfb.2012.11.042

Kailasapathy, K., & Chin, J. (2000). Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. *Immunol Cell Biol, 78*(1), 80-88.

Krasaekoopt, W., Bhandari, B., & Deeth, H. (2003). Evaluation of encapsulation techniques of probiotics for yoghurt. *International Dairy Journal, 13*(1), 3-13. doi: http://dx.doi.org/10.1016/S0958-6946(02)00155-3

Lee, B. B., Ravindra, P., & Chan, E. S. (2013). Size and shape of calcium aginate beads produced by extrusion dripping. *Chemical Engineering & Technology, 36*(10), 1627-1642. doi: 10.1002/ceat.201300230

Lee, J.-S., Kim, E.-J., Chung, D., & Lee, H. G. (2009). Characteristics and antioxidant activity of catechin-loaded calcium pectinate gel beads prepared by internal gelation. *Colloids and Surfaces B: Biointerfaces, 74*(1), 17-22. doi: http://dx.doi.org/10.1016/j.colsurfb.2009.06.018

Liserre, A. M., Re, M. I., & Franco, B. D. G. M. (2007). Microencapsulation of *Bifidobacterium animalis* subsp. lactis in modified alginate-chitosan beads and evaluation of survival in simulated gastrointestinal conditions. *Food Biotechnology, 21*(1), 1-16. doi: 10.1080/08905430701191064

Liu, H., Xu, X. M., & Guo, S. D. (2008). Comparison of full-fat and low-fat cheese analogues with or without pectin gel through microstructure, texture, rheology, thermal and sensory analysis. *International Journal of Food Science & Technology, 43*(9), 1581-1592. doi: 10.1111/j.1365-2621.2007.01616.x

Martin, M. J., Lara-Villoslada, F., Ruiz, M. A., & Morales, M. E. (2013). Effect of unmodified starch on viability of alginate-encapsulated *Lactobacillus fermentum* CECT5716. *LWT - Food Science and Technology, 53*(2), 480-486. doi: http://dx.doi.org/10.1016/j.lwt.2013.03.019

Maxwell, E. G., Belshaw, N. J., Waldron, K. W., & Morris, V. J. (2012). Pectin – An emerging new bioactive food polysaccharide. *Trends in Food Science & Technology, 24*(2), 64-73. doi: http://dx.doi.org/10.1016/j.tifs.2011.11.002

Mokarram, R. R., Mortazavi, S. A., Najafi, M. B. H., & Shahidi, F. (2009). The influence of multi stage alginate coating on survivability of potential probiotic bacteria in simulated gastric and intestinal juice. *Food Research International, 42*(8), 1040-1045. doi: http://dx.doi.org/10.1016/j.foodres.2009.04.023

Nazzaro, F., Fratianni, F., Coppola, R., Sada, A., & Orlando, P. (2009). Fermentative ability of alginate-prebiotic encapsulated *Lactobacillus acidophilus* and survival under simulated gastrointestinal conditions. *Journal of Functional Foods, 1*(3), 319-323. doi: http://dx.doi.org/10.1016/j.jff.2009.02.001

Paredes, M. D. C., Rao, M. A., & Bourne, M. C. (1989). Rheological characterization of salad dressings 2: effect of storage. *Journal of Texture Studies, 20*(2), 235-250. doi: 10.1111/j.1745-4603.1989.tb00436.x

Pedreschi, R., Campos, D., Noratto, G., Chirinos, R., & Cisneros-Zevallos, L. (2003). Andean yacon root (*Smallanthus sonchifolius* Poepp. Endl) fructooligosaccharides as a potential novel source of prebiotics. *Journal of Agricultural and Food Chemistry, 51*(18), 5278-5284. doi: 10.1021/jf0344744

Pereira, R., Matia-Merino, L., Jones, V., & Singh, H. (2006). Influence of fat on the perceived texture of set acid milk gels: a sensory perspective. *Food Hydrocolloids, 20*(2–3), 305-313. doi: http://dx.doi.org/10.1016/j.foodhyd.2005.01.009

Ratna, S. M., Chauhan, P., Dixit, K., Babu, S., & Jamil, K. (2009). Probiotics as complementary therapy for hypercholesterolemia. *Biology & Medicine, 1*(4), 1-13.

Sabikhi, L., Babu, R., Thompkinson, D. K., & Kapila, S. (2010). Resistance of microencapsulated *Lactobacillus acidophilus* LA1 to processing treatments and simulated gut conditions. *Food and Bioprocess Technology, 3*(4), 586-593. doi: 10.1007/s11947-008-0135-1

Sandoval-Castilla, O., Lobato-Calleros, C., Garcia-Galindo, H. S., Alvarez-Ramírez, J., & Vernon-Carter, E. J. (2010). Textural properties of alginate–pectin beads and survivability of entrapped *Lb. casei* in simulated gastrointestinal conditions and in yoghurt. *Food Research International, 43*(1), 111-117. doi: http://dx.doi.org/10.1016/j.foodres.2009.09.010

Sathyabama, S., Ranjith, K. M., Bruntha, D. P., Vijayabharathi, R., & Brindha, P. V. (2014). Co-encapsulation of probiotics with prebiotics on alginate matrix and its effect on viability in simulated gastric environment. *LWT - Food Science and Technology, 57*(1), 419-425. doi: http://dx.doi.org/10.1016/j.lwt.2013.12.024

Saxelin, M., Tynkkynen, S., Mattila-Sandholm, T., & de Vos, W. M. (2005). Probiotic and other functional microbes: from markets to mechanisms. *Current Opinion in Biotechnology, 16*(2), 204-211. doi: http://dx.doi.org/10.1016/j.copbio.2005.02.003

Shi, L. E., Li, Z. H., Li, D. T., Xu, M., Chen, H. Y., Zhang, Z. L., & Tang, Z. X. (2013). Encapsulation of probiotic *Lactobacillus bulgaricus* in alginate–milk microspheres and evaluation of the survival in simulated gastrointestinal conditions. *Journal of Food Engineering, 117*(1), 99-104. doi: http://dx.doi.org/10.1016/j.jfoodeng.2013.02.012

Upadhyay, A. (2014). *Investigating the potential of plant-derived antimicrobials and probiotic bacteria for controlling Listeria monocytogenes.* Doctoral dissertation, University of Connecticut - Storrs.

Voo, W.-P., Ravindra, P., Tey, B.-T., & Chan, E.-S. (2011). Comparison of alginate and pectin based beads for production of poultry probiotic cells. *Journal of Bioscience and Bioengineering, 111*(3), 294-299. doi: http://dx.doi.org/10.1016/j.jbiosc.2010.11.010

White, R. J., Budarin, V. L., & Clark, J. H. (2010). Pectin-derived porous materials. *Chemistry – A European Journal, 16*(4), 1326-1335. doi: 10.1002/chem.200901879

Wicker, L., Kim, Y., Kim, M.-J., Thirkield, B., Lin, Z., & Jung, J. (2014). Pectin as a bioactive polysaccharide – Extracting tailored function from less. *Food Hydrocolloids, 42, Part 2*(0), 251-259. doi: http://dx.doi.org/10.1016/j.foodhyd.2014.01.002

Woo, J. W., Roh, H. J., Park, H. D., Ji, C. I., Lee, Y. B., & Kim, S. B. (2007). Sphericity optimization of calcium alginate gel beads and the effects of processing conditions on their physical properties *Food Sci. Biotechnol., 16*, 715–721.

Zhao, M., Qu, F., Cai, S., Fang, Y., Nishinari, K., Phillips, G., & Jiang, F. (2015). Microencapsulation of *Lactobacillus acidophilus* CGMCC1.2686: correlation between bacteria survivability and physical properties of microcapsules. *Food Biophysics, 10*(3), 292-299. doi: 10.1007/s11483-014-9389-5

CHAPTER 5 DOUBLE ENCAPSULATION OF *L. PLANTARUM* WITH PROTECTIVE AGENTS IN PECTIN-RICE BRAN EXTRACT CAPSULES WITH WHEY PROTEIN ISOLATE COATING TO IMPROVE CELL VIABLITY AFTER FREEZE DRYING AND DURING SIMULATED GASTROINTESTINAL CONDITIONS

5.1. Abstract

Three protective agents, maltrodextrin, wheat dextrin soluble fiber, and hi-maize starch were incorporated into pectin-rice bran capsules loaded with *Lactobacillus plantarum* prepared by ionotropic gelation. The capsules were coated with and without whey protein isolate and then freeze dried. The viability of the encapsulated cells in the freeze dried capsules was evaluated after freeze drying and at simulated gastrointestinal conditions. All uncoated and coated pectin-rice bran capsules yielded high encapsulation efficiency (> 95%). The whey protein isolate coating significantly enhanced viability of the encapsulated cells after the freeze drying. The freeze dried capsules with hi-maize starch (FHMC) had the highest cell viability, 8.63±0.01 and 5.63±0.02 log CFU/g for the coated and uncoated capsules, respectively. In simulated gastrointestinal conditions, only 0.89 and 2.12 log cycles was reduced when the encapsulated cells of FHMC were exposed to fed state with a copious meal (at pH 3.0, followed by pH 7.0) and with a standard meal (at pH 2.5, followed by pH 6.5) condition, respectively. Due to the effect of pH 1.8, low number of viable cells was recovered from FHMC (3.27±0.13 log CFU/g) after incubating in a fasted state without meal, while there was no survival cells found in other treatments. The study demonstrated that the freeze dried pectin-rice bran capsules containing hi-maize starch with whey protein isolate coating effectively improved viability of *L. plantarum*.

Keywords: probiotics, encapsulation, freeze drying, pectin, whey protein isolate

This chapter previously submitted to a peer reviewed publication as Arranee Chotiko and Subramaniam Sathivel, Double encapsulation of *L. plantarum* with protective agents in pectin-rice bran extract capsules with whey protein isolate coating to improve cell viability after freeze drying and during simulated gastrointestinal conditions.

5.2. Introduction

Probiotics have been well known for their health benefits such as reducing pathogenic bacteria colonization, alleviating diarrhea, reducing intestinal inflammation, lowering blood cholesterol, and for potentially having anti-colon cancer activity (Ratna Sudha, Chauhan, Dixit, Babu, & Jamil, 2009; Saxelin, Tynkkynen, Mattila-Sandholm, & de Vos, 2005). They have been incorporated into many foods such as yogurt, cheese, and fruit and vegetable juices (Ranadheera, Baines, & Adams, 2010; Rivera-Espinoza & Gallardo-Navarro, 2010). For beneficial health effects, the number of live probiotic bacteria in food products is recommended to be at least 10^6-10^7 cfu/g (Floch et al., 2008). However, that an effective number of live probiotic bacterial food products is dependent upon the number of probiotics decreased during formulation, down-stream processing, and storage as well as during passage through the gastrointestinal tract (Saarela, Virkajarvi, Nohynek, Vaari, & Matto, 2006).

Encapsulation technology can help protect probiotics from the undesired conditions and function as a vehicle, to deliver them to the intestine with sufficient number and viability to exert their benefits. Encapsulation is a process of forming a continuous layer entrapping a whole compound within a matrix core (Zuidam & Shimoni, 2010). According to Anal and Singh (2007), probiotics can be encapsulated by various techniques such as spray drying, freeze drying, emulsion, and extrusion. These various techniques may not have the same efficacy, for example, it has been reported that the probiotics encapsulated by drying processes were released when applied to food products, resulting in loss of protection in unfavorable environments such as passage through gastrointestinal tracts. On the other hand, the bead matrix in which probiotics were entrapped or immobilized by emulsion or extrusion techniques provided protection against the conditions (Krasaekoopt, Bhandari, and Deeth (2003).

Pectin is a soluble fiber, which is resistant to gastrointestinal conditions and can be degraded by microorganisms. It is considered as highly fermentable substances for gut microflora. Fermentation of pectin increased in the fecal bulk and exhibited bifidogenic and prebiotic properties (Nazzaro, Fratianni, Orlando, & Coppola, 2012). A number of *Bifidobacterium* sp. and *Lactobacillus* sp. derived from fecal bacteria of ulcerative colitis patients and fermented in pectin fraction media was higher than control as well as acetate levels (Vigsnæs, Holck, Meyer, & Licht, 2011). Use of pectin hydrogels as a matrix for probiotic delivery has been reported to improve cell viability at gastrointestinal conditions. It forms three dimensional rigid and water insoluble hydrogels by calcium-induced ionotropic gelation (Lee, Kim, Chung, & Lee, 2009). Pectin microparticles loaded with *Lactobacillus acidophilus* remained intact in simulated gastric juice at pH 1.2 and 3.0 for 120 min and in simulated intestinal juice at pH 7.0 for 300 min. After exposure to simulated gastric juice at pH 3.0 and simulated intestinal juice at pH 7.0, viability reduction of pectin encapsulated cells was lower than the non-encapsulated (Gebara et al., 2013). Similarly, viability of *Lactobacillus rhamnosus* under a gastric condition at pH 2 was improved when the cell was encapsulated with pectin coated with whey protein isolate (Gerez, Font de Valdez, Gigante, & Grosso, 2012). In our previous study, we found that pectin with rice bran extract capsules could enhance the viability of *Lactobacillus plantarum* when they were incubated under acid (pH 3.0) and bile (1.0% oxgall) conditions for 2 h and 24 h, respectively.

For long-term storage, probiotics are usually preserved by freeze drying. Although it is a gentle method, losses of cell viability occur due to freeze damage. Protective agents are required to reduce cell damage during freeze drying and to avoid intracellular ice formation by raising the glass-phase transition temperature (Meng, Stanton, Fitzgerald, Daly, & Ross, 2008). Sugars such

as sucrose, lactose, trehalose, and maltodextrin and some prebiotics were used to increase cell viability of probiotic bacteria after freeze drying (Reddy, Awasthi, Madhu, & Prapulla, 2009; Semyonov et al., 2010). Wheat dextrin was reported to protect viability of *L. rhamnosus* during freeze drying and maintain their viability during storage for 4 weeks at 37 °C (Saarela et al., 2006). Encapsulation of *Lactobacillus casei* and *Bifidobacterium lactis* in alginate-resistant starch (high amylose corn starch) beads had a high number of cells survive during freezing. The viability of the encapsulated cells was higher by 30%, compared to non-encapsulated cells during storage for 180 days in ice-cream (-20 °C) (Homayouni, Azizi, Ehsani, Yarmand, & Razavi, 2008).

Whey protein isolate can be used as a coating material for various hydrocolloid matrices and as a wall material for spray drying or freeze drying of probiotics to improve probiotic viability. Gbassi, Vandamme, Ennahar, and Marchioni (2009) reported that WPI coated alginate beads of *L. plantarum* had better cell survival than uncoated beads after exposure to simulated gastric and intestinal fluids. *L. casei* encapsulated in alginate beads coated with WPI had high cell viability after incubating in simulated gastrointestinal conditions for 24 h (Smilkov et al., 2014). Encapsulation of spray-freeze dried and freeze dried *L. plantarum* in WPI was reported to protect the cells in simulated gastrointestinal fluids up to 4 h (Dolly, Anishaparvin, Joseph, & Anandharamakrishnan, 2011). Viability of *L. plantarum* mixed with a mixture of sodium alginate and WPI after freeze drying was 9-12% higher than that obtained from the spray drying process (Rajam, Karthik, Parthasarathi, Joseph, & Anandharamakrishnan, 2012).

The objectives of this study were to develop freeze-dried pectin-rice bran capsules as a delivery vehicle for *L. plantarum* and evaluate the effects of protective agents and whey protein

isolate coating on the cell viability after freeze drying and during exposure to simulated gastrointestinal conditions.

5.3. Materials and methods

5.3.1. Cell culture preparation

L. plantarum NRRL B-4496 (LP) isolated from sauerkraut, was kindly provided by ARS Culture Collection (Washington DC, US). The frozen stock culture was reactivated twice in deMan Rogosa Sharpe (MRS) broth (Neogen Corporation, Lansing, MI). Twenty five mL of the strain was subsequently inoculated in MRS broth (500 mL) and incubated at 37 °C for 16 h to reach stationary phase. Cell pellets were harvested by centrifugation at 10,000 x g for 10 min at 4 °C (Beckman J2-HC, Beckman Coulter, Inc., Brea, CA). The pellets were washed three times and suspended in sterile distilled water to obtain concentrated LP (~10^9 CFU/ml). The concentrated LP was then mixed with protective agents (20 g/100 mL) including maltodextrin (MD) (Dextrose Equivalent of 9–13, NOW Foods Company, Bloomingdale, IL), wheat dextrin soluble fiber (WF) (Nutriose®FM06, Ingredion Incorporated, Westchester, IL), and hi-maize starch (HM) (Ingredion Incorporated, Westchester, IL) to obtain concentrated LP with protective agents, LP+MD, LP+WF, and LP+HM, respectively. In order to obtain LP+HM, HM was dissolved in distilled water, autoclaved at 121 °C for 15 min and cooled in a refrigerator overnight. The concentrated LP was then centrifuged and re-suspended in the HM solution.

5.3.2. Encapsulation of *L. plantarum* in pectin-rice bran capsules

Capsule preparation was performed as described by Lee et al. (2009) with some modifications. First, pectin-rice bran (PE-RB) gel solutions were prepared by mixing low-methoxyl pectin (TIC PRETESTED® pectin LM 32 powder), that was provided by TIC Gums Inc. (Belcamp, MD), and rice bran extract, provided by Ribus Inc. (St. Louis, MO), for 1 hour at

room temperature. Concentrated LP with a protective agent, (LP+MD, LP+WF, or LP+HM) was added separately into gel solutions at the ratio of 1:4 and stirred at room temperature for 30 min. Concentrated LP with no protective agents (LP-NP) was used to prepare control capsules. Each gel solution containing LP had a final concentration of 2 g/100 mL PE and 2 g/100 mL RB. These solutions were extruded through a 21G needle by a syringe pump (Pump 11, Harvard Apparatus, Holliston, MA) at 1.2 mL/min and dropped into a crosslink solution, 4 g/100 mL of calcium chloride (Fisher Scientific Inc., Pittsburgh, PA) with and without whey protein isolate (WPI) (BiPRO®, Davisco Food International, Inc., Eden Prairie, MN) at a concentration of 8 g/100 mL. The crosslink solution with WPI was adjusted to pH 4.0 and stirred for 1 h before use. The PE-RB loaded with LP plus protective agent capsules were immediately formed and continuously hardened in the crosslink solution for 30 min. The wet capsules, PE-RB capsules loaded LP with MD (MDC), WF (WFC), HM (HMC), and no protective agents (NPC) were collected, washed, and cryogenically frozen by liquid nitrogen (Air Liquide Co., Houston, TX) until the temperature of capsules reached -85 °C. They were subsequently placed into a freeze dryer (Heto PowerDry LL3000, Thermo Scientific, Laurel, MD) for 40 h. The freeze-dried MDC (FMDC), freeze dried WFC (FWFC), freeze dried HMC (FHMC), and freeze-dried NPC (FNPC) were then stored in glass bottles in a refrigerator.

5.3.3. Enumeration of *L. plantarum* viable cells in pectin-rice bran beads

To determine the number of the cells in the wet and in the freeze dried capsules, the encapsulated LP was released as the method described by Sathyabama, Ranjith kumar, Bruntha devi, Vijayabharathi, and Brindha priyadharisini (2014) with some modifications. One gram of the wet and/or lyophilized capsules was added to 99 mL of 0.1 M phosphate buffer (pH 7.2) and stirred at 250 rpm in a shaker (Lab line incubator shaker model 3525, Fisher Scientific Inc.,

Pittsburgh, PA) for 30 min. The colony forming units (CFU/g) was examined by the pour-plate method on MRS agar containing 0.6 g/100 mL of $CaCO_3$ (Fisher Scientific Inc., Pittsburgh, PA). The plate was incubated at 37 °C for 24-48 h.

5.3.4. Encapsulation efficiency

The encapsulation efficiency represented a combined measurement of the efficacy of entrapment and survival of viable cells during the microencapsulation procedure, calculated according to Eq. 5.1 (Gebara et al., 2013)

$$EE\ (\%) = \frac{N}{N_0} \times 100 \qquad (5.1)$$

where EE is the encapsulation efficiency, expressed in percentage; N is the number of the cells released from the wet beads (log CFU/g); and N_0 is the number of the cells added to the gel solution (log CFU/g).

5.3.5. Sizes, shapes, and bulk density of freeze-dried pectin-rice bran loaded with LP capsules

The diameter of thirty capsules was measured under a stereomicroscope (Zeiss SteREO Lumar.V12, Thornwood, NY) using image analyzer software. The capsule shape was quantified by the sphericity factor (SF), shown in Eq. 5.2 (Chan et al., 2011)

$$SF = \frac{d_{max} - d_{min}}{d_{max} + d_{min}} \qquad (5.2)$$

where d_{max} is the largest diameter and d_{min} is the smallest diameter perpendicular to d_{max}.

Determination of bulk density was conducted according to the method described by Sandoval-Castilla, Lobato-Calleros, García-Galindo, Alvarez-Ramírez, and Vernon-Carter (2010). One gram of freeze-dried capsules was weighed and poured into a 25-mL graduate cylinder, from which the bulk volume was determined. The bulk density was calculated by dividing the mass by the bulk volume.

5.3.6. Scanning electron micrographs of lyophilized pectin-rice bran loaded with *L. plantarum* capsules

The freeze-dried capsules were cross sectioned and mounted on aluminum SEM stubs. The samples were coated with gold:palladium (60:40) in an Edwards S150 sputter coater. The capsule morphology was observed under a scanning electron microscope (JSM-6610LV, JEOL Ltd. Japan).

5.3.7. Viability of *L. plantarum* in freeze-dried pectin-rice bran capsules during exposure to simulated gastrointestinal fluids

The assay was carried out as described by Gbassi, Vandamme, Yolou, and Marchioni (2011) and Sathyabama et al. (2014). Phosphate buffer saline solution used as simulated gastrointestinal fluids (SGIF) consisted of 8g/L of NaCl (Fisher Scientific Inc., Pittsburgh, PA), 0.2g/L of Na_2HPO_4 (Fisher Scientific Inc., Pittsburgh, PA), and 1.44 g/L of KH_2PO_4 (Sigma-Aldrich, St. Louis, MO). The pH of the buffer was adjusted according to the gastrointestinal tract conditions shown in Table 5.1.

Table 5.1. *In vitro* experimental conditions of gastrointestinal tract

Parameter	Stomach	Intestine
Incubation time	2 h	4 h
pH		
• copious meal	3.0	7.0
• standard meal	2.5	6.5
• without meal	1.8	6.0

The freeze dried capsules (0.5 g) were added into SGIFs (50 mL) at pHs of 1.8, 2.5, and 3.0 for 2 hours (stomach incubation time) at 37 °C in an orbital shaker with 100-rpm agitation then removed from the acid conditions and sequentially placed into SGIFs (50mL) at pHs of 6.0, 6.5, and 7.0, respectively. The samples were incubated at 37 °C for additional 4 h (intestinal incubation time) with 100-rpm agitation. At the end of incubation period, the capsules were collected, disintegrated, and enumerated for viable cells.

5.3.8. Statistical analysis

All values are means and standard deviations of two determinations. Mean values from statistical analysis were determined with the SAS (Statistical Analysis System) software (version 9.4) (SAS Institute Inc., Cary, NC, USA). ANOVA and Tukey's studentized range test were carried out to determine differences among treatments at the significant level of $P \leq 0.05$.

5.4. Results and discussion

5.4.1. Encapsulation efficiency

As shown in Figure 5.1, all treatments had excellent encapsulation efficiency (EE), greater than 95%. EEs of the samples were not significantly different. The large EEs were attributed to the ability of pectin to entrap cells. This ability is due to the minuscule pore sizes of pectin, which are in a range from 2 to 50 nm (Gbassi et al., 2011; White, Budarin, & Clark, 2010). During PE-RB capsule formation, only water molecules and particles smaller than the pectin pore sizes were able to diffuse from the capsules, while the bacteria cells which are considerable larger than pore sizes of pectin (0.2 μm in diameter and 2-8 μm in length) were entrapped inside of the capsules. Addition of rice bran extract possibly helped reduce cell losses during hardening in the crosslink solution. In our previous study, it was shown that addition of rice bran extract improved EE of pectin capsules loaded with LP (Chotiko & Sathivel, 2016). The result also indicated that adding of protectants and coating with WPI had no effect on EE. The EE depends on encapsulation techniques, wall materials used, and microorganisms. High EE (99.9%) was observed in encapsulation of Lactobacilli and *Bifidobacterium bifidum* in alginate beads prepared by extrusion-ionotropic gelation. Coating the beads with chitosan, alginate, and combined poly-L-lysine with alginate was reported to have no significant difference in EE, compared with the uncoated beads (Krasaekoopt, Bhandari, & Deeth, 2004).

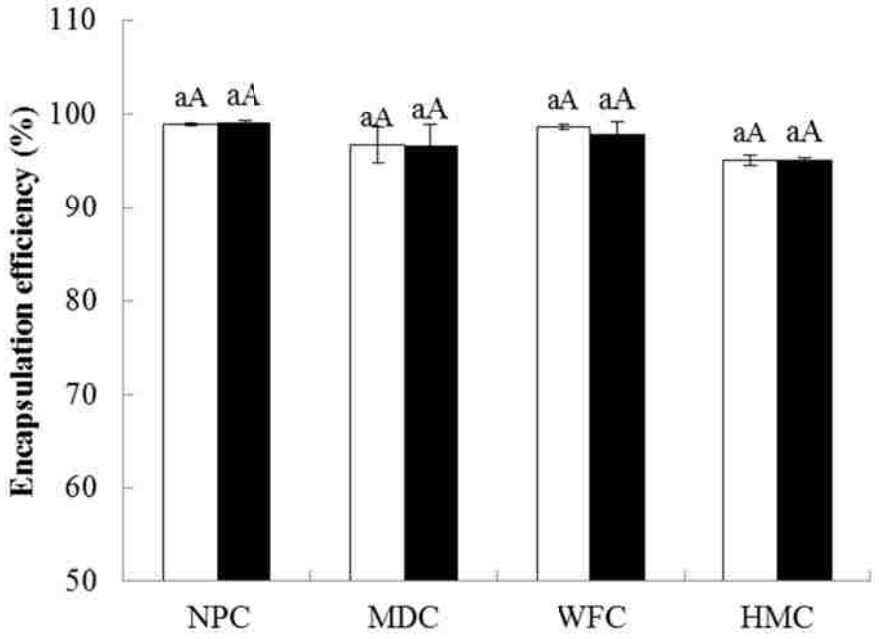


Figure 5.1. Encapsulation efficiency of pectin-rice bran capsules loaded with *L. plantarum* uncoated (□) and coated (■) with whey protein isolate; NPC, MDC, WFC, and HMC were pectin-rice bran capsules loaded with *L. plantarum* with: no protectants, maltodextrin, wheat dextrin soluble fiber, and hi-maize resistance starch, respectively. [A]Means ± standard deviation with same letters within the same treatment are not significantly different ($P \leq 0.05$). [a]Means ± standard deviation with same letters within a different treatment between uncoated and coated capsules with the same protective agents are not significantly different ($P \leq 0.05$).

The EEs obtained in our study were higher than those reported in other studies. For example, Gebara et al. (2013) found that microencapsulation of *L. acidophilus* by emulsion-ionotropic gelation using pectin as a wall material and coated with WPI had 84.35% EE. Similar to our results, the researcher reported that EE of the coated and uncoated samples were not significantly different. Trabelsi et al. (2013) mentioned that EE of alginate beads coated with chitosan containing *L. plantarum*, which were prepared by extrusion-ionotropic gelation was 80.98%. The EE of *L. casei* in alginate-pectin beads varied between 54.3% and 79.2% (Corbo, Bevilacqua, Gallo, Speranza, & Sinigaglia, 2013).

5.4.2. Viability of *L. plantarum* in pectin-rice bran capsules after freeze drying

Viability of freeze-dried LP was improved by use of protective agents and a coating material, such as WPI. Before freeze drying, the number of viable cells in all samples was similar, approximately 10 log CUF/g (dry basis) (Figure 5.2a). After freeze drying (Figure 5.2b), the results demonstrated that the freeze dried capsules coated with WPI had significantly greater cells survive than the uncoated samples ($P \leq 0.05$). WPI could cover the capsules and protect the cells from freeze-drying injuries. WPI has isoelectric point of approximately 5.2 and at pH 4 (the pH value of our $CaCl_2$ crosslinking solution with WPI), the compound contains positive charges. As pectin is an anionic polymer, electrostatic association between pectin and WPI is created by interaction of the positive charges of protein patches (mainly $–NH^{+3}$ groups) and the negative charges carried by carboxyl groups of pectin polymer (Gentès, St-Gelais, & Turgeon, 2010). Souza et al. (2012) mentioned that electrostatic interaction between pectin and whey proteins can occur at pH below 4.5. In addition, whey protein can be used as a protectant for microorganisms during freeze drying. Protein can accumulate within the cells resulting in reduction of the osmotic difference between the internal and external environments (Meng et al., 2008). The different polysaccharides also had different protective effects on the viability of encapsulated LP during freeze drying. Without a protective agent, uncoated FNPC had the lowest number of viable cells compared with other treatments, which was 2.30±0.01 log CFU/g with decrease of 8.19 log reductions. Reddy et al. (2009) found that viability of lactobacilli was reduced up to 50% in the absence of protective agents. Powder of *L. plantarum* NRRL-B4495 had severe decreases in the cell viability when the pure culture was freeze-dried without any protective agents (Chotiko & Sathivel, 2014). Meng et al. (2008) stated that the loss of cell viability noted

after freeze drying is mainly caused by extracellular ice formation during freezing. Ice formation leads to increase of extracellular osmolality, resulting in dehydration of the cell.

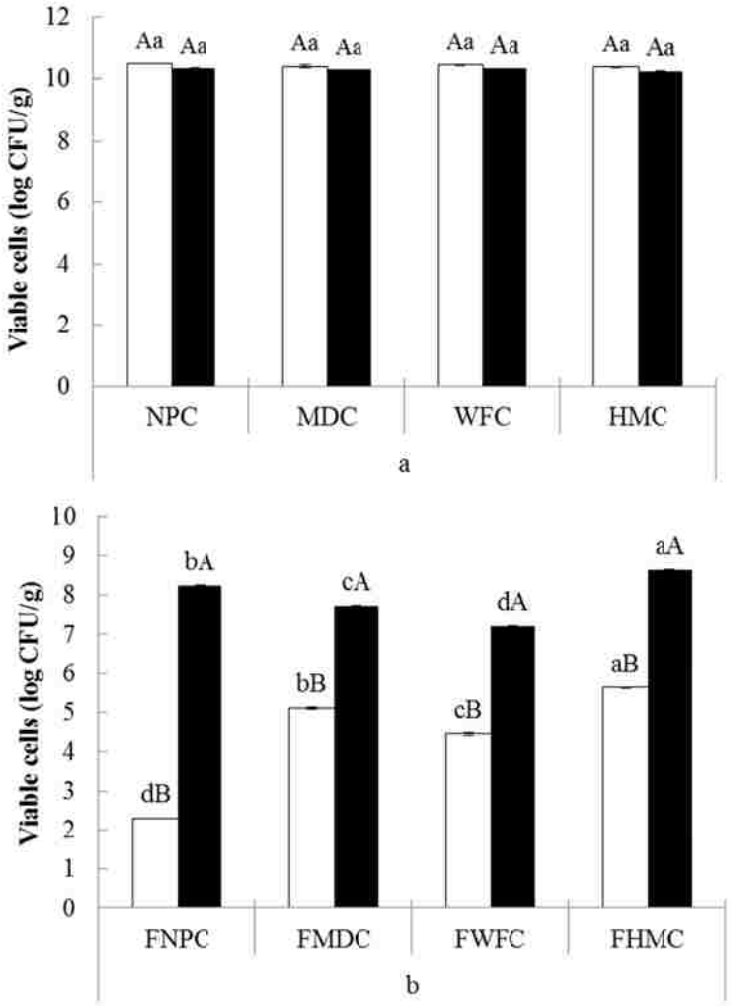


Figure 5.2. Viability of *L. plantarum* in pectin-rice bran capsules containing different protectants uncoated (□) and coated (■) with whey protein isolate before (a) and after (b) freeze drying; NPC, MDC, WDC, and HMC were pectin-rice bran capsules loaded with *L. plantarum* with: no protectants, maltodextrin, wheat dextrin soluble fiber, and hi-maize starch, respectively. FNPC, FMDC, FWFC, and FHMC were freeze-dried NPC, WDC, WFC, and HMC respectively. [a-d]Means with different letters within the same color indicate significant differences ($P \leq 0.05$). [A,B]Means with different letters between uncoated and coated treatments with the same protective agents indicate significant differences ($P \leq 0.05$).

The greatest number of viable cells after freeze drying was found in FHMC, which was 8.63±0.01 log CFU/g and 5.63±0.02 log CFU/g for coated and uncoated treatments ($P \leq 0.05$).

The coated FHMC had a 1.59 log CFU/g reduction, while uncoated FHMC showed a 4.75 log reduction. Martin, Lara-Villoslada, Ruiz, and Morales (2013) reported that addition of starch into probiotic encapsulations improved polymeric networks and partially isolated the cells from environmental conditions. No losses of *Lactobacillus fermentum* was found after freeze drying when the cells were encapsulated in alginate mixed with corn starch. Crittenden et al. (2001) mentioned that resistant starch offered a surface for probiotic adherence, providing robustness and resilience to environmental stresses. This possibly indicated that the hi-maize resistant starch (HM) functioned as an attachment matrix, helping the cells avoid effects of ice crystallization and extracellular osmolality.

Coated FMDC had significantly more viable cells (7.72±0.03 log CFU/g) than FWFC (7.21±0.02 log CUF/g) ($P \leq 0.05$). The number of viable cells of uncoated FMDC was also significantly greater than the viable cell counts in uncoated FWFC, 5.10±0.03 and 4.44±0.04 log CFU/g, respectively ($P \leq 0.05$). Viable cell reductions were 2.56, 3.11, 5.30, and 5.99 log CFU/g for coated FMDC, coated FWFC, uncoated FMDC, and uncoated FWFC, respectively. The result indicated that maltodextrin (MD) protected the cells from freeze drying damages more effectively than wheat dextrin soluble fiber (WF). This was possibly because MD has a smaller molecular weight than WF. The lower molecular weight of dextrins yields higher bacterial survival during freezing and after drying (Semyonov et al., 2010). In dehydration processes, sugars and some polysaccharides can function as a water replacer (Santivarangkna, Higl, & Foerst, 2008). It is noted that removal of water from bacteria cells interrupts the structure of phospholipids in cell membranes, causing large lateral compressive stresses in the plane of the membrane and consequently leading to cell membrane transition and leakage (Santivarangkna, Kulozik, & Foerst, 2008). Hydroxyl groups of the sugars bind to the phosphate group of

phospholipids though hydrogen bonds at the surface of the cell membrane lipid bilayer (Pereira & Hünenberger, 2006). This helps stabilize the cell membrane during dehydration. It has been revealed that the interaction of polysaccharides and phospholipids mainly depend on the flexibility of the structure. Vereyken, van Kuik, Evers, Rijken, and de Kruijff (2003) reported branched polysaccharide dextran rarely interacts with phospholipids. Unexpectedly, FNPC coated with WPI had significantly greater cell viability (8.24±0.01 log CFU/g) than the coated capsules of FMDC and FWFC. Viable cell reduction of coated FNPC was 2.08 log cycles. The reason might be because addition of MD or WF hindered the interactions between pectin and WPI.

5.4.3. Sizes, shapes, and bulk density of freeze-dried pectin-rice bran loaded with *L. plantarum* capsules

The diameters of freeze-dried PE-RB loaded with LP capsules were shown in Table 5.2. Uncoated FNPC had the largest diameter (3.05±0.50 mm) and were more irregular than other uncoated treatments. There was no significant difference in diameters of uncoated FMDC (2.81±0.43 mm), uncoated FWFC (2.68±0.28 mm), and uncoated FHMC (2.64±0.28 mm). The results also showed that FHMC, FMDDC, and FWFC coated with WPI had significantly larger diameters than uncoated samples, which were 3.18±0.38, 2.94±0.63, and 3.21±0.41 mm, respectively. FHMC, FMDDC, and FWFC coated with WPI had similar diameter compared to the coated FNPC (3.20±0.51 mm). The results were in agreement with Mokarram, Mortazavi, Najafi, and Shahidi (2009), who reported that diameter of alginate gels were increased by multi stage alginate coating. Coatings of chitosan, combined poly-L-lysine with alginate, or alginate was also reported to increase the diameter of alginate beads of encapsulated probiotics (Krasaekoopt et al., 2004).

Table 5.2. Sizes of freeze dried pectin-rice bran loaded with LP capsules

Capsules	Diameter (mm)	
	Uncoated	Coated
FNPC	3.05 ± 0.50^{aA}	3.20 ± 0.51^{aA}
FMDC	2.81 ± 0.43^{abB}	3.03 ± 0.63^{aA}
FWFC	2.68 ± 0.28^{bB}	3.21 ± 0.41^{aA}
FHMC	2.64 ± 0.28^{bB}	3.18 ± 0.38^{aA}

FNPC, FMDC, FWFC, and FHMC were freeze-dried pectin-rice bran capsules loaded with *L. plantarum* with: no protectants, maltodextrin, wheat dextrin soluble fiber, and hi-maize starch, respectively. ^{a,b}Means ± standard deviation with different letters within the same column indicate significant differences ($P \leq 0.05$). ^{A,B}Means ± standard deviation with different letters between uncoated and coated treatments with the same protective agents indicate significant differences ($P \leq 0.05$).

Capsules' shape was characterized by their sphericity factor (SF) (Table 5.3). The lower the number are, the more spherical the capsule is (Lee, Ravindra, & Chan, 2013). The lowest SF was observed in uncoated FHMC (0.07±0.03) ($P \leq 0.05$). The largest SF was found in uncoated FNPC (0.21±0.13), which was not significantly different than uncoated FMDC (0.19±0.10) and uncoated FWFC (0.12±0.07). The result indicated that HM could enhance sphericity of freeze dried capsules. According to Chan et al. (2011), removal of water from hydrogel capsules caused the hydrogels to collapse, resulting in reduction of sphericity and shape changes from spherical to irregular shapes. Addition of a filler agent such as starch into gel capsules helped maintain the capsules shape during drying. Starch acted as a structure support to protect the gel capsules from collapse and shrinkage. Starch in alginate gels could create a new matrix or a co-matrix with the polymer by touching of starch granules themselves or binding of starch granules within or between alginate matrixes (Rassis, Saguy, & Nussinovitch, 2002). WPI molecules trended to randomly layer on the gel capsules, and SF of the freeze dried capsules seemed to be independent of WPI coating. Significantly increase of SF was found in coated FHMC (0.11±0.09) when compared to uncoated FHMC. In contrast, coated FMDC had significantly lower SF (0.14±0.07) than uncoated FMDC (0.19±0.10). No significantly difference of SF was

shown between uncoated and coated FNPC (0.15±0.10) as well as FWFC (0.12±0.07 for uncoated FWFC and 0.15±0.09 for coated FWFC).

Table 5.3. Shapes of freeze dried pectin-rice bran loaded with LP capsules

Capsules	Sphericity factor	
	Uncoated	Coated
FNPC	0.21 ± 0.13^{aA}	0.15 ± 0.10^{aA}
FMDC	0.19 ± 0.10^{aA}	0.14 ± 0.07^{aB}
FWFC	0.12 ± 0.07^{bA}	0.15 ± 0.09^{aA}
FHMC	0.07 ± 0.04^{bB}	0.11 ± 0.09^{aA}

FNPC, FMDC, FWFC, and FHMC are freeze-dried pectin-rice bran capsules loaded with *L. plantarum* with: no protectants, maltodextrin, wheat dextrin soluble fiber, and hi-maize starch, respectively. ^{a,b}Means ± standard deviation with different letters within the same column indicate significant differences ($P \leq 0.05$). ^{A,B}Means ± standard deviation with different letters between uncoated and coated treatments with the same protective agents indicate significant differences ($P \leq 0.05$).

In the present work, bulk density refers to the mass of capsules divided by the total volume including the capsule volume, the inter-particle void volume, and the internal pore volume. As shown in Table 5.4, uncoated FHMC had significantly higher bulk density (0.105±0.003 g/mL) than uncoated FMDC (0.090±0.001 g/mL), uncoated FWFD (0.090±0.001 g/mL), and uncoated FNPC (0.089±0.002 g/mL) ($P \leq 0.05$). For coated capsules, the highest bulk density was obtained from coated FHMC (0.109±0.002 g/mL), which was not significantly different than coated FWFD (0.105±0.003 g/mL) but significantly greater than coated FMDC (0.101±0.002 g/mL) and coated FNPC (0.090±0.001 g/mL) ($P \leq 0.05$). Coating with WPI significantly increased the bulk density of FMDC and FWFC. Bulk density of capsules is mainly dependent upon size, shape, and surface characteristics of the capsules (Rajam & Anandharamakrishnan, 2015). High bulk density in FHMC treatments was probably due to greater sphericity than FNPC, FMDC, and FWFC. Irregular shapes increases external voids, leading to higher bulk volume which in turn causes lower loose bulk density (Caparino et al., 2012).

Table 5.4. Bulk density of freeze dried pectin-rice bran loaded with LP capsules

Capsules	Bulk density (g/mL)	
	Uncoated	Coated
FNPC	0.089 ± 0.002^{bA}	0.090 ± 0.001^{cA}
FMDC	0.090 ± 0.001^{bB}	0.101 ± 0.002^{bA}
FWFC	0.090 ± 0.001^{bB}	0.105 ± 0.003^{abA}
FHMC	0.105 ± 0.003^{aA}	0.109 ± 0.002^{aA}

FNPC, FMDC, FWFC, and FHMC are freeze-dried pectin-rice bran capsules loaded with *L. plantarum* with: no protectants, maltodextrin, wheat dextrin soluble fiber, and hi-maize starch, respectively. $^{a-c}$Means ± standard deviation with different letters within the same column indicate significant differences ($P \leq 0.05$). ^{A,B}Means ± standard deviation with different letters between uncoated and coated treatments with the same protective agents indicate significant differences ($P \leq 0.05$).

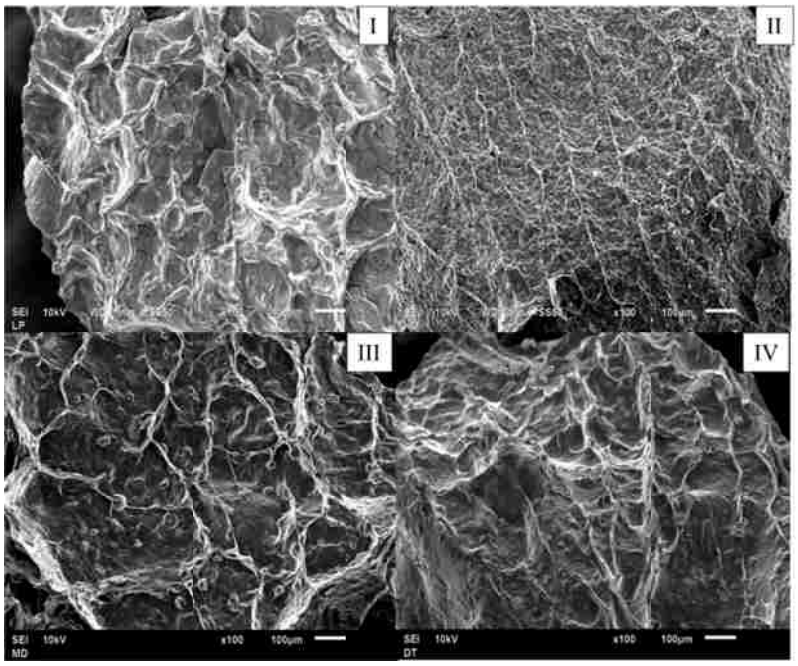
5.4.4. Morphology of freeze-dried pectin-rice bran capsules from scanning electron microscope

The exterior surface of uncoated and coated freeze-dried PE-RB capsules loaded with LP is displayed in Figure 5.3. Due to dehydration effects, all capsules were collapsed. Pereira and Hünenberger (2006) mentioned that freeze drying collapsed the wall of calcium pectinate gel beads causing fragile structure. In our results, cracks were obviously seen on the surface of uncoated FNPC (Figure 5.3a I). Uncoated FHMC had rough surfaces containing a number of attached starch particles (Figure 5.3a II). All coated capsules were randomly covered with WPI particles and no visible cracks were found as illustrated in Figure 5.3b. Freeze dried particles of WPI resembled broken glass or a flake-like structure, similar to the result reported by Ezhilarasi, Indrani, Jena, and Anandharamakrishnan (2013). The interior of the fractured freeze dried capsules are shown in Figure 5.4, indicating a large number of bacteria cells randomly distributed and attached on the capsule matrix of all treatments. Cracks were obvious in uncoated FNPC and FWFC. Bacteria cells in uncoated FHMC aggregated on starch particles (Figure 5.4a II), confirming our hypothesis that starch could provide LP with an adherence matrix. In Figure 5.4b, the cells attached on the matrix of coated capsules were less visible than that which was on

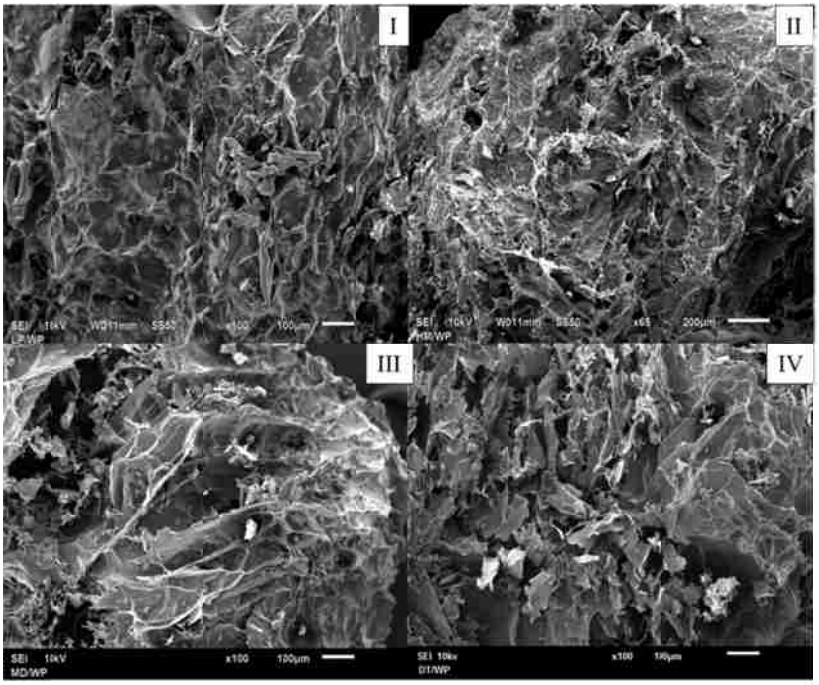
the uncoated capsule, a result of thin layering of WPI on the capsule. Some cracks were observed on the coated FNPC.

5.4.5. Viability of *L. plantarum* in freeze-dried pectin-rice bran capsules coated with WPI during exposure to simulated conditions of a gastrointestinal tract

Because of low cell viability of the uncoated capsules after freeze drying process, only the PE-RB capsules with WPI coating were only selected to test the viability of the encapsulated LP during exposure to simulated gastrointestinal tract. Conditions in a gastrointestinal tract are different as a result of contents and locations. The stomach is a crucial section for pH-sensitive components such as probiotic cells. In the fasted state, the stomach of healthy subjects has a range of pH from 1.3 to 2.5, while eating can increase the pH to a 4.5 to 5.8 range (Kong & Singh, 2008). In the small intestine, the pH changed to pH 6 and gradually increases to about pH 7.4 in the terminal ileum (Fallingborg, 1999). In this study, three main conditions were simulated, including a fed state with a copious meal (pH 3.0 for 2 h, followed by pH 7.0 for 4 h), a fed state with a standard meal (pH 2.5 for 2 h, followed by pH 6.5 for 4 h), and a fasted state without meal (pH 1.8 for 2 h, followed by pH 6.0 for 4 h). Viability of the encapsulated LP in freeze dried capsules during a fed state with a copious meal condition is illustrated in Figure 5.5. Following exposure to the simulated conditions, the viable cells in the capsules were enumerated after incubation for 6 h. The results showed that the number of viable cells of all treatments was significantly reduced ($P \leq 0.05$). The highest viability of encapsulated LP was found in coated FHMC (7.38±0.01 log CFU/g), only a 0.89 log CFU/g reduction. The number of viable cells in coated FWFC (6.76±0.02 log CFU/g) was significantly higher than coated FNPC (6.50±0.01 log CFU/g) and FMDC (6.07±0.02 log CFU/g) ($P \leq 0.05$). Their viable cell log reductions were 1.00, 1.68, and 1.86 log CFU/g for coated FWFC, FNPC, and FMDC respectively.

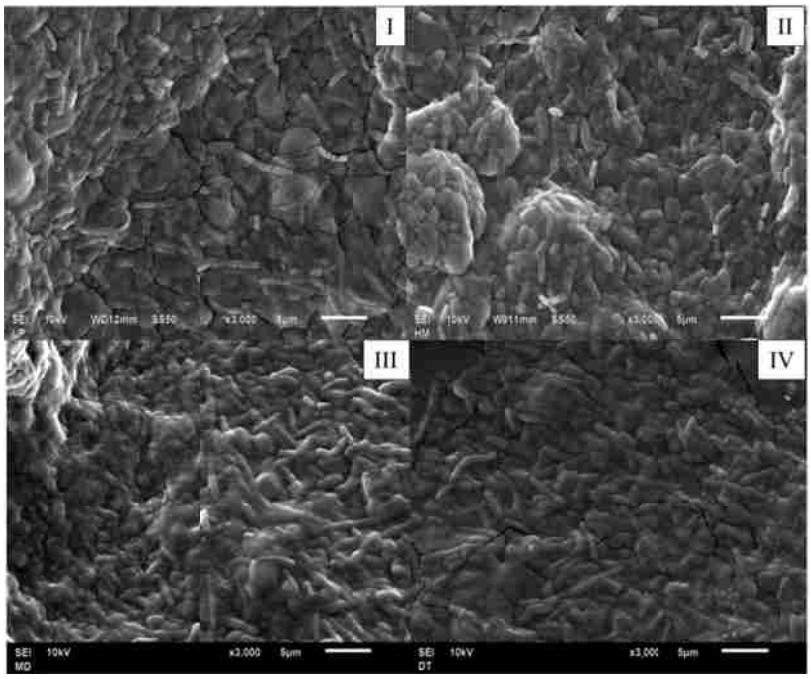


a



b

Figure 5.3. Scanning electron micrographs of surface area of freeze dried pectin-rice bran capsules loaded with *L.plantarum* and protective agents (a = uncoated capsules and b = WPI coated capsules). I = FNPC, II = FHMC, III = FMDC, and IV = FWFC. FNPC, FMDC, FWFC, and FHMC are freeze-dried pectin-rice bran capsules loaded with *L. plantarum* with: no protectants, maltodextrin, wheat dextrin soluble fiber, and hi-maize starch, respectively.



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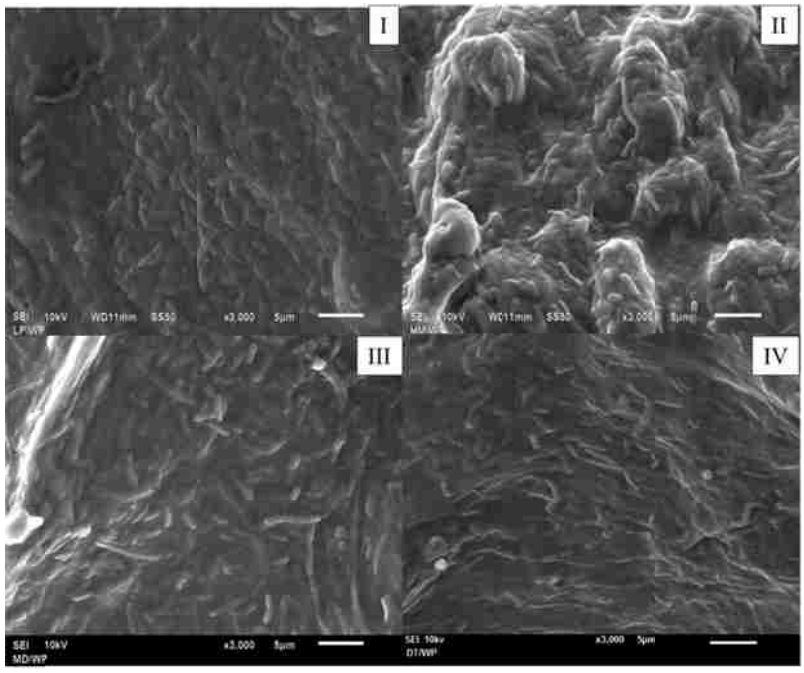

b

Figure 5.4. Scanning electron micrographs of cross-scetions of freeze dried pectin-rice bran capsules loaded with *L.plantarum* and protective agents (a = uncoated capsuels and b = WPI coated capsules). I = FLPC, II = FHMC, III = FMDC, and IV = FWFC. FNPC, FMDC, FWFC, and FHMC are freeze-dried pectin-rice bran capsules loaded with *L. plantarum* with: no protectants, maltodextrin, wheat dextrin soluble fiber, and hi-maize starch, respectively.

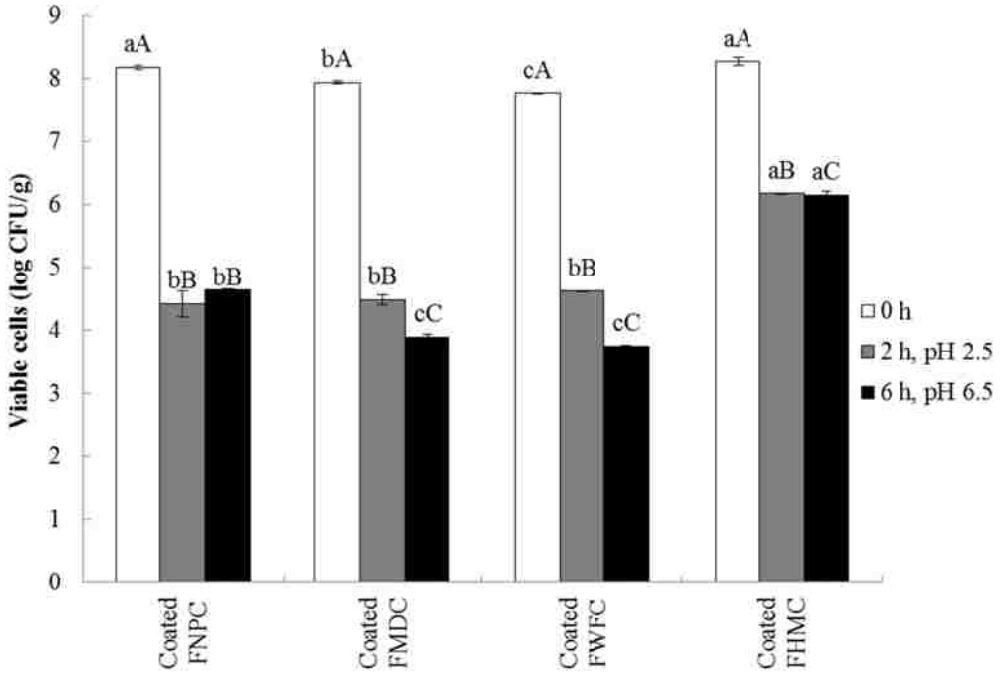

Figure 5.5. Viability of *L. plantarum* loaded in freeze-dried pectin-rice bran extract capsules in a simulated gastrointestinal condition of fed state with a copious meal (□ at 0 h, ▨ at stomach (pH 3.0 and duration time = 2 h), and ■ at intestine (pH 7.0 and duration time =4 h). FNPC, FMDC, FWFC, and FHMC are freeze-dried pectin-rice bran capsules loaded with *L. plantarum* with: no protectants, maltodextrin, wheat dextrin soluble fiber, and hi-maize starch, respectively; $^{a-d}$Means with different letters within the same color indicate significant differences ($P \leq 0.05$). $^{A-C}$Means with different letters of the same protective agents before and after incubation indicate significant differences ($P \leq 0.05$).

Compared to a fed state with a copious meal, a fed state with a standard meal resulted in more cell losses which may be attributable to the lower pH in the mimicked stomach condition (Figure 5.6). Coated FHMC had the greatest number of viable cells (6.14±0.03 log CFU/g), having undergone only a 2.12 log reduction. There was no significant difference in the number of recovered viable cells in coated FWFC (3.75±0.05 log CFU/g), FMDC (3.89±0.01 log CFU/g), and FNPC (4.64±0.04 log CFU/g). Their log reductions were 4.05, 4.01, and 3.53 log CFU/g, respectively. The lowest number of recovered viable cells from encapsulated LP was obtained from the fasted state condition and is attributed to the effect of pH 1.8 (the most acidic condition). The number of viable cells in coated FNPC, FMDC, and FWFC were below the

detection limit (< 2 log CFU/g). Only a few numbers of viable cells in coated FHMC were recovered (3.27±0.13 log CFU/g (5.00 log reduction)). Because of low cell counts, study of cell viability in the simulated intestinal fluid for the fasted condition was not conducted.

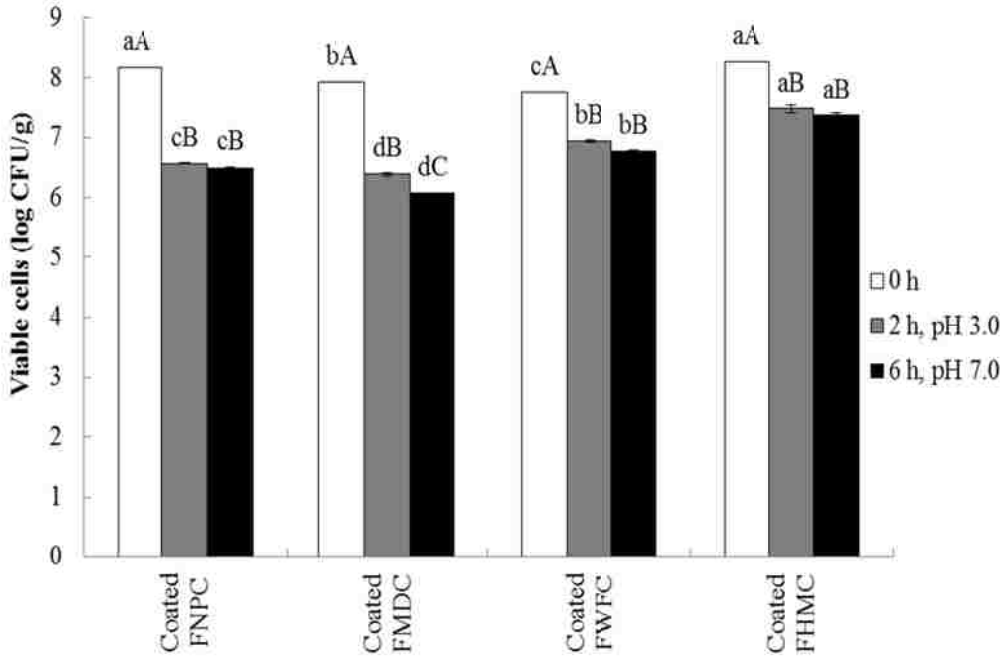

Figure 5.6. Viability of *L. plantarum* loaded in freeze-dried pectin-rice bran extract capsules in a simulated gastrointestinal condition of fed state with a standard meal (□ at 0 h, ▨ at stomach (pH 2.5 and duration time = 2 h), and ■ at intestine (pH 6.5 and duration time =4 h). FNPC, FMDC, FWFC, and FHMC are freeze-dried pectin-rice bran capsules loaded with *L. plantarum* with: no protectants, maltodextrin, wheat dextrin soluble fiber, and hi-maize starch, respectively; $^{a\text{-}c}$Means with different letters within the same color indicate significant differences ($P \leq 0.05$). $^{A\text{-}C}$Means with different letters of the same protective agents before and after incubation indicate significant differences ($P \leq 0.05$).

Incorporation of HM into freeze dried PE-RB capsules coated with WPI contributed to an increase of cell viability during exposure to SGIF (particularly for acidic conditions) in comparison to the other treatments investigated. Wang, Brown, Evans, and Conway (1999) reported that high amylose maize starch had the ability to protect *Bifidobacterium* exposed to *in vitro* low pH conditions and *in vivo* gastrointestinal conditions. It was claimed that the increase of acid resistance was mainly due to the adhesion of the cell to the starch granules. Adhesion of

the bacteria to starch was reported to involve binding of specific cell surface proteins to α-1, 4-linked glucose saccharides (Crittenden et al., 2001). Aggregation of bacteria on starch and its bulking capacity contributed to an increase of cell density, resulting in high cell loading content. Chandramouli, Kailasapathy, Peiris, and Jones (2004) revealed that a high level of initial cells load of probiotic products led to an increase in the number of viable cells in gastrointestinal tract conditions. It is the fact that acid inhibits bacterial growth and their activity by passage of undissociated acid forms through the cell membrane causing acidification of the cytoplasm (Cotter & Hill, 2003). Adhesion to starch by the bacteria possibly blocked or delayed diffusion of the acid to the cell membrane. It was reported that *Lactobacillus casei* mixed with corn starch had more viable cells than free cells after exposure to simulated gastric fluids at pH 3.0, which was likely to be due to entrapment of the cells between the starch granules. Moreover, the cell survival was increased with bacteria fusion protein (starch-binding domain) improving attachment of the cell on the starch and the cells' acid tolerance (Tarahomjoo, Katakura, & Shioya, 2008). And and Kailasapathy (2005) mentioned that addition of hi-maize starch into alginate capsules improved probiotic survival in acid conditions. The starch particles could plug the pores of capsule, preventing diffusion of acidic content into the capsules. The addition of starch was reported to increase the integrated structure and firmness of alginate capsules (Khosravi Zanjani, Ghiassi Tarzi, Sharifan, & Mohammadi, 2014). This also might help enhance the protective effects of FHMC against harsh environmental conditions.

5.5. Conclusion

The study indicated that pectin-rice bran capsules offered high encapsulation efficiency. Adding of protective agents or coating with whey protein isolate has no effect on the encapsulation efficiency. Whey protein isolate coating significantly improved cell viability of the

encapsulated cells after freeze drying process. Supplementation of uncoated pectin-rice bran capsules with protective agents significantly enhanced the number of viable cells in the freeze dried capsules. Hi-maize starch provided better protection to the encapsulated cells during freeze drying and in simulated gastrointestinal conditions than maltrodextrin and wheat dextrin soluble fiber. Hi-maize starch is classified as a prebiotic. Encapsulation of *L. plantarum* with hi-maize starch in freeze dried pectin-rice bran capsules would become a novel synbiotic supplement that may potentially be incorporated into some food products such as nutrition bars, cereal products or dairy products.

5.6. References

Anal, A. K., & Singh, H. (2007). Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. *Trends in Food Science & Technology, 18*(5), 240-251. doi: http://dx.doi.org/10.1016/j.tifs.2007.01.004

And, C. I., & Kailasapathy, K. (2005). Effect of Co-encapsulation of probiotics with prebiotics on increasing the viability of encapsulated bacteria under in vitro acidic and bile salt conditions and in yogurt. *Journal of Food Science, 70*(1), M18-M23. doi: 10.1111/j.1365-2621.2005.tb09041.x

Caparino, O. A., Tang, J., Nindo, C. I., Sablani, S. S., Powers, J. R., & Fellman, J. K. (2012). Effect of drying methods on the physical properties and microstructures of mango (*Philippine Carabao* var.) powder. *Journal of Food Engineering, 111*(1), 135-148. doi: http://dx.doi.org/10.1016/j.jfoodeng.2012.01.010

Chan, E.-S., Wong, S.-L., Lee, P.-P., Lee, J.-S., Ti, T. B., Zhang, Z., . . . Yim, Z.-H. (2011). Effects of starch filler on the physical properties of lyophilized calcium–alginate beads and the viability of encapsulated cells. *Carbohydrate Polymers, 83*(1), 225-232. doi: http://dx.doi.org/10.1016/j.carbpol.2010.07.044

Chandramouli, V., Kailasapathy, K., Peiris, P., & Jones, M. (2004). An improved method of microencapsulation and its evaluation to protect *Lactobacillus* spp. in simulated gastric conditions. *Journal of Microbiological Methods, 56*(1), 27-35. doi: http://dx.doi.org/10.1016/j.mimet.2003.09.002

Chotiko, A., & Sathivel, S. (2014). Effects of enzymatically-extracted purple rice bran fiber as a protectant of *L. plantarum* NRRL B-4496 during freezing, freeze drying, and storage. *LWT - Food Science and Technology, 59*(1), 59-64. doi: http://dx.doi.org/10.1016/j.lwt.2014.05.056

Chotiko, A., & Sathivel, S. (2016). Development of a combined low-methoxyl-pectin and rice-bran-extract delivery system to improve the viability of *Lactobacillus plantarum* under acid and bile conditions. *LWT - Food Science and Technology, 66*, 420-427. doi: http://dx.doi.org/10.1016/j.lwt.2015.10.058

Corbo, M. R., Bevilacqua, A., Gallo, M., Speranza, B., & Sinigaglia, M. (2013). Immobilization and microencapsulation of *Lactobacillus plantarum*: Performances and in vivo applications. *Innovative Food Science & Emerging Technologies, 18*(0), 196-201. doi: http://dx.doi.org/10.1016/j.ifset.2012.12.004

Cotter, P. D., & Hill, C. (2003). Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiology and Molecular Biology Reviews, 67*(3), 429-453. doi: 10.1128/mmbr.67.3.429-453.2003

Crittenden, R., Laitila, A., Forssell, P., Matto, J., Saarela, M., Mattila-Sandholm, T., & Myllarinen, P. (2001). Adhesion of bifidobacteria to granular starch and its implications in probiotic technologies. *Applied and Environmental Microbiology, 67*(8), 3469-3475. doi: 10.1128/aem.67.8.3469-3475.2001

Dolly, P., Anishaparvin, A., Joseph, G. S., & Anandharamakrishnan, C. (2011). Microencapsulation of *Lactobacillus plantarum* (MTCC 5422) by spray-freeze-drying method and evaluation of survival in simulated gastrointestinal conditions. *Journal of Microencapsulation, 28*(6), 568-574. doi: doi:10.3109/02652048.2011.599435

Ezhilarasi, P. N., Indrani, D., Jena, B. S., & Anandharamakrishnan, C. (2013). Freeze drying technique for microencapsulation of Garcinia fruit extract and its effect on bread quality. *Journal of Food Engineering, 117*(4), 513-520. doi: http://dx.doi.org/10.1016/j.jfoodeng.2013.01.009

Fallingborg, J. (1999). Intraluminal pH of the human gastrointestinal tract. *Danish medical bulletin, 46*(3), 183-196.

Floch, M. H., Walker, W. A., Guandalini, S., Hibberd, P., Gorbach, S., Surawicz, C., . . . Dieleman, L. A. (2008). Recommendations for probiotic use—2008. *Journal of Clinical Gastroenterology, 42*, S104-S108 110.1097/MCG.1090b1013e31816b31903f.

Gbassi, G. K., Vandamme, T., Ennahar, S., & Marchioni, E. (2009). Microencapsulation of *Lactobacillus plantarum* spp in an alginate matrix coated with whey proteins. *International Journal of Food Microbiology, 129*(1), 103-105. doi: http://dx.doi.org/10.1016/j.ijfoodmicro.2008.11.012

Gbassi, G. K., Vandamme, T., Yolou, F. S., & Marchioni, E. (2011). *In vitro* effects of pH, bile salts and enzymes on the release and viability of encapsulated *Lactobacillus plantarum* strains in a gastrointestinal tract model. *International Dairy Journal, 21*(2), 97-102. doi: http://dx.doi.org/10.1016/j.idairyj.2010.09.006

Gebara, C., Chaves, K. S., Ribeiro, M. C. E., Souza, F. N., Grosso, C. R. F., & Gigante, M. L. (2013). Viability of *Lactobacillus acidophilus* La5 in pectin–whey protein microparticles

during exposure to simulated gastrointestinal conditions. *Food Research International, 51*(2), 872-878. doi: http://dx.doi.org/10.1016/j.foodres.2013.02.008

Gentès, M.-C., St-Gelais, D., & Turgeon, S. L. (2010). Stabilization of whey protein isolate−pectin complexes by heat. *Journal of Agricultural and Food Chemistry, 58*(11), 7051-7058. doi: 10.1021/jf100957b

Gerez, C. L., Font de Valdez, G., Gigante, M. L., & Grosso, C. R. F. (2012). Whey protein coating bead improves the survival of the probiotic *Lactobacillus rhamnosus* CRL 1505 to low pH. *Letters in Applied Microbiology, 54*(6), 552-556. doi: 10.1111/j.1472-765X.2012.03247.x

Homayouni, A., Azizi, A., Ehsani, M. R., Yarmand, M. S., & Razavi, S. H. (2008). Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream. *Food Chemistry, 111*(1), 50-55. doi: http://dx.doi.org/10.1016/j.foodchem.2008.03.036

Khosravi Zanjani, M. A., Ghiassi Tarzi, B., Sharifan, A., & Mohammadi, N. (2014). Microencapsulation of probiotics by calcium alginate-gelatinized starch with chitosan coating and evaluation of survival in simulated human gastro-intestinal condition. *Iranian Journal of Pharmaceutical Research : IJPR, 13*(3), 843-852.

Kong, F., & Singh, R. P. (2008). Disintegration of solid foods in human stomach. *Journal of Food Science, 73*(5), R67-R80. doi: 10.1111/j.1750-3841.2008.00766.x

Krasaekoopt, W., Bhandari, B., & Deeth, H. (2003). Evaluation of encapsulation techniques of probiotics for yoghurt. *International Dairy Journal, 13*(1), 3-13. doi: http://dx.doi.org/10.1016/S0958-6946(02)00155-3

Krasaekoopt, W., Bhandari, B., & Deeth, H. (2004). The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria. *International Dairy Journal, 14*(8), 737-743. doi: http://dx.doi.org/10.1016/j.idairyj.2004.01.004

Lee, B. B., Ravindra, P., & Chan, E. S. (2013). Size and shape of calcium alginate beads produced by extrusion dripping. *Chemical Engineering & Technology, 36*(10), 1627-1642. doi: 10.1002/ceat.201300230

Lee, J.-S., Kim, E.-J., Chung, D., & Lee, H. G. (2009). Characteristics and antioxidant activity of catechin-loaded calcium pectinate gel beads prepared by internal gelation. *Colloids and Surfaces B: Biointerfaces, 74*(1), 17-22. doi: http://dx.doi.org/10.1016/j.colsurfb.2009.06.018

Martin, M. J., Lara-Villoslada, F., Ruiz, M. A., & Morales, M. E. (2013). Effect of unmodified starch on viability of alginate-encapsulated *Lactobacillus fermentum* CECT5716. *LWT - Food Science and Technology, 53*(2), 480-486. doi: http://dx.doi.org/10.1016/j.lwt.2013.03.019

Meng, X. C., Stanton, C., Fitzgerald, G. F., Daly, C., & Ross, R. P. (2008). Anhydrobiotics: The challenges of drying probiotic cultures. *Food Chemistry, 106*(4), 1406-1416. doi: http://dx.doi.org/10.1016/j.foodchem.2007.04.076

Mokarram, R. R., Mortazavi, S. A., Najafi, M. B. H., & Shahidi, F. (2009). The influence of multi stage alginate coating on survivability of potential probiotic bacteria in simulated gastric and intestinal juice. *Food Research International, 42*(8), 1040-1045. doi: http://dx.doi.org/10.1016/j.foodres.2009.04.023

Nazzaro, F., Fratianni, F., Orlando, P., & Coppola, R. (2012). Biochemical traits, survival and biological properties of the probiotic *Lactobacillus plantarum* grown in the presence of prebiotic inulin and pectin as energy source. *Pharmaceuticals, 5*(5), 481-492.

Pereira, C. S., & Hünenberger, P. H. (2006). Interaction of the sugars trehalose, maltose and glucose with a phospholipid bilayer: A comparative molecular dynamics study. *The Journal of Physical Chemistry B, 110*(31), 15572-15581. doi: 10.1021/jp060789l

Rajam, R., & Anandharamakrishnan, C. (2015). Spray freeze drying method for microencapsulation of *Lactobacillus plantarum*. *Journal of Food Engineering, 166*(0), 95-103. doi: http://dx.doi.org/10.1016/j.jfoodeng.2015.05.029

Rajam, R., Karthik, P., Parthasarathi, S., Joseph, G. S., & Anandharamakrishnan, C. (2012). Effect of whey protein – alginate wall systems on survival of microencapsulated *Lactobacillus plantarum* in simulated gastrointestinal conditions. *Journal of Functional Foods, 4*(4), 891-898. doi: http://dx.doi.org/10.1016/j.jff.2012.06.006

Ranadheera, R. D. C. S., Baines, S. K., & Adams, M. C. (2010). Importance of food in probiotic efficacy. *Food Research International, 43*(1), 1-7. doi: http://dx.doi.org/10.1016/j.foodres.2009.09.009

Rassis, D. K., Saguy, I. S., & Nussinovitch, A. (2002). Collapse, shrinkage and structural changes in dried alginate gels containing fillers. *Food Hydrocolloids, 16*(2), 139-151. doi: http://dx.doi.org/10.1016/S0268-005X(01)00071-6

Ratna Sudha, M., Chauhan, P., Dixit, K., Babu, S., & Jamil, K. (2009). Probiotics as complementary therapy for hypercholesterolemia. *Biology & Medicine, 1*(4), 1-13.

Reddy, K. B. P. K., Awasthi, S. P., Madhu, A. N., & Prapulla, S. G. (2009). Role of cryoprotectants on the viability and functional properties of probiotic lactic acid bacteria during freeze drying. *Food Biotechnology, 23*(3), 243-265. doi: 10.1080/08905430903106811

Rivera-Espinoza, Y., & Gallardo-Navarro, Y. (2010). Non-dairy probiotic products. *Food Microbiology, 27*(1), 1-11. doi: http://dx.doi.org/10.1016/j.fm.2008.06.008

Saarela, M., Virkajarvi, I., Nohynek, L., Vaari, A., & Matto, J. (2006). Fibres as carriers for *Lactobacillus rhamnosus* during freeze-drying and storage in apple juice and chocolate-

coated breakfast cereals. *International Journal of Food Microbiology, 112*(2), 171-178. doi: http://dx.doi.org/10.1016/j.ijfoodmicro.2006.05.019

Sandoval-Castilla, O., Lobato-Calleros, C., García-Galindo, H. S., Alvarez-Ramírez, J., & Vernon-Carter, E. J. (2010). Textural properties of alginate–pectin beads and survivability of entrapped *Lb. casei* in simulated gastrointestinal conditions and in yoghurt. *Food Research International, 43*(1), 111-117. doi: http://dx.doi.org/10.1016/j.foodres.2009.09.010

Santivarangkna, C., Higl, B., & Foerst, P. (2008). Protection mechanisms of sugars during different stages of preparation process of dried lactic acid starter cultures. *Food Microbiology, 25*(3), 429-441. doi: http://dx.doi.org/10.1016/j.fm.2007.12.004

Santivarangkna, C., Kulozik, U., & Foerst, P. (2008). Inactivation mechanisms of lactic acid starter cultures preserved by drying processes. *Journal of Applied Microbiology, 105*(1), 1-13. doi: 10.1111/j.1365-2672.2008.03744.x

Sathyabama, S., Ranjith kumar, M., Bruntha devi, P., Vijayabharathi, R., & Brindha priyadharisini, V. (2014). Co-encapsulation of probiotics with prebiotics on alginate matrix and its effect on viability in simulated gastric environment. *LWT - Food Science and Technology, 57*(1), 419-425. doi: http://dx.doi.org/10.1016/j.lwt.2013.12.024

Saxelin, M., Tynkkynen, S., Mattila-Sandholm, T., & de Vos, W. M. (2005). Probiotic and other functional microbes: from markets to mechanisms. *Current Opinion in Biotechnology, 16*(2), 204-211. doi: http://dx.doi.org/10.1016/j.copbio.2005.02.003

Semyonov, D., Ramon, O., Kaplun, Z., Levin-Brener, L., Gurevich, N., & Shimoni, E. (2010). Microencapsulation of *Lactobacillus paracasei* by spray freeze drying. *Food Research International, 43*(1), 193-202. doi: http://dx.doi.org/10.1016/j.foodres.2009.09.028

Smilkov, K., Petreska Ivanovska, T., Petrushevska Tozi, L., Petkovska, R., Hadjieva, J., Popovski, E., . . . Mladenovska, K. (2014). Optimization of the formulation for preparing *Lactobacillus casei* loaded whey protein-Ca-alginate microparticles using full-factorial design. *Journal of Microencapsulation, 31*(2), 166-175. doi: doi:10.3109/02652048.2013.824511

Souza, F. N., Gebara, C., Ribeiro, M. C. E., Chaves, K. S., Gigante, M. L., & Grosso, C. R. F. (2012). Production and characterization of microparticles containing pectin and whey proteins. *Food Research International, 49*(1), 560-566. doi: http://dx.doi.org/10.1016/j.foodres.2012.07.041

Tarahomjoo, S., Katakura, Y., & Shioya, S. (2008). New strategy for enhancement of microbial viability in simulated gastric conditions based on display of starch-binding domain on cell surface. *Journal of Bioscience and Bioengineering, 105*(5), 503-507. doi: http://dx.doi.org/10.1263/jbb.105.503

Trabelsi, I., Bejar, W., Ayadi, D., Chouayekh, H., Kammoun, R., Bejar, S., & Ben Salah, R. (2013). Encapsulation in alginate and alginate coated-chitosan improved the survival of

newly probiotic in oxgall and gastric juice. *International Journal of Biological Macromolecules, 61*(0), 36-42. doi: http://dx.doi.org/10.1016/j.ijbiomac.2013.06.035

Vereyken, I. J., van Kuik, J. A., Evers, T. H., Rijken, P. J., & de Kruijff, B. (2003). Structural requirements of the fructan-lipid interaction. *Biophysical Journal, 84*(5), 3147-3154.

Vigsnæs, L. K., Holck, J., Meyer, A. S., & Licht, T. R. (2011). In Vitro fermentation of sugar beet arabino-oligosaccharides by fecal microbiota obtained from patients with ulcerative colitis to selectively stimulate the growth of *Bifidobacterium* spp. and *Lactobacillus* spp. *Applied and Environmental Microbiology, 77*(23), 8336-8344. doi: 10.1128/aem.05895-11

Wang, X., Brown, I. L., Evans, A. J., & Conway, P. L. (1999). The protective effects of high amylose maize (amylomaize) starch granules on the survival of *Bifidobacterium* spp. in the mouse intestinal tract. *Journal of Applied Microbiology, 87*(5), 631-639. doi: 10.1046/j.1365-2672.1999.00836.x

White, R. J., Budarin, V. L., & Clark, J. H. (2010). Pectin-derived porous materials. *Chemistry – A European Journal, 16*(4), 1326-1335. doi: 10.1002/chem.200901879

Zuidam, N., & Shimoni, E. (2010). Overview of microencapsulates for use in food products or processes and methods to make them. In N. J. Zuidam & V. Nedovic (Eds.), *Encapsulation technologies for active food ingredients and food processing* (pp. 3-29): Springer New York.

CHAPTER 6. SUMMARY

This research was focused on developing delivery systems for *L. planrarum* and evaluating the effects of the delivery systems on cell viability at simulated gastrointestinal conditions. Three delivery systems were developed, immobilized *L. plantarum* on purple rice bran fibers (PRF) (delivery system 1), encapsulated *L. plantarum* with combined pectin-rice bran extract (delivery system 2), and double encapsulated *L. plantarum* with protective agents (delivery system 3).

In delivery system 1, *L. planrarum* was immobilized on PRF and freeze-dried to obtain immobilized cell powder. The study indicated that PRF could function as a matrix supporting *L. plantarum* and protecting the viability of *L. plantarum* during freeze drying and storage. PRF may act as a physical barrier which protects the bacterial cells against physicochemical changes caused by freeze drying and unfavorable conditions during storage. Immobilization of *L. plantarum* on PRF also helped to improve cell viability during exposure to bile media. PRF has the ability to bind to bile salts. Cell viability was influenced by freezing rates. Cryogenically frozen cells had lower survival compared to air blast frozen cells, indicating cryogenic freezing may cause cell damages resulting in loss of acid and bile tolerances and loss of cell viability during storage. Immobilized *L. plantarum* on PRF was successfully developed as a new probiotic-fiber supplement that could be incorporated into food products (e.g. nutrition bars, cereal products or dairy products).

In delivery system 2, encapsulated *L. plantarum* in pectin-rice bran extract capsules were prepared by using an ionotropic gelation/extrusion method. The delivery system enhanced *L. plantarum* viability under acid and bile conditions. Rice bran extract possibly had some interaction with pectin, creating a mesh-like network in the delivery system. This contributed to

enhancement of cell viability under acid and bile conditions. Addition of rice bran extract to pectin capsules also improved encapsulation efficiency and capsule sphericity. However, delivery system 2 failed to protect the bacterial cells during freeze drying. Therefore, a third delivery system was developed to enhance survivability of *L. plantarum* during freeze drying.

Delivery system 3 was developed by mixing *L. plantarum* with a protective agent (maltodextrin, wheat dextrin soluble fiber, or hi-maize starch). Double encapsulation was then performed, by first encapsulating *L. plantarum* with a protective agent in pectin-rice bran extract capsules then coating the capsules with whey protein isolate. Delivery system 3 enhanced cell viability after freeze drying; as evidenced by a greater number of viable cells observed in this delivery system than in delivery system 2. Enhancement of cell viability during freeze drying in delivery system 3 was mainly due to the whey protein isolate (WPI) coating. WPI provided a thin layer covering bacterial cells in the capsule. Hi-maize starch provided better protection to the encapsulated cells during freeze drying and in simulated gastrointestinal conditions than the other protective agents. Hi-maize starch provides surfaces for bacterial cell attachment, and aggregation of starch granules also helps to partially isolate the bacterial cells from harmful environments. This study indicated that double encapsulated *L. plantarum* with hi-maize starch in freeze dried pectin-rice bran capsules could be a novel synbiotic supplement that may be incorporated into food products such as nutrition bars, cereal products or dairy products.

APPENDIX A: FOURIER TRANSFORM INFRARED SPECTROSCOPY (FT-IR) OF PECTIN AND RICE BRAN EXTRACT

Materials and methods

FT-IR spectra of pectin (PE), rice bran extract (RB), and pectin-rice bran extract (PE-2.0RB) powders, as well as PE and PE-2.0RB capsules were analyzed using a TENSOR 27 FT-IR spectrometer (Bruker Opics, Germany). PE-2.0RB powder was prepared by dissolving 2 g/100 mL PE with 2 g/100 mL RB. The solution was stirred for 1 h and freeze dried. To obtain PE-2.0RB capsules, PE-2.0RB solution was prepared by mixing 2 g/100 mL PE and 1 g/ 100 mL RB in distilled water. The solution was stirred for 1 h. It was dropped into a crosslink solution (4 g/100 mL $CaCl_2$) by a syringe pump. PE-2.0RB capsules were immediately formed and continued stirring for additional 30 min to harden the capsules. PE-2.0RB capsules were then harvested. PE capsules were also prepared with the same method as PE-RB capsules, but RB was not added. Samples were placed on the diamond/ZnSe crystal and pressed by a Teflon spacer. Spectra were determined with ATR mode in the range of 4000-650 cm^{-1} using a resolution of 4 cm^{-1}. A total of 256 scans were performed to obtain a high signal-to-noise ratio.

Results and discussion

The bands at about 3300 cm^{-1} of the PE, RB, and PE-2.0RB spectra could represent O–H stretching vibrations of the absorbed water, as displayed in Figure A1. The result was in agreement with that reported by Yu, Wang, Hu, and Wang (2014). Regarding the PE powder spectrum, the peak at 1738 cm^{-1} could be due to C=O stretching vibrations of the methyl esterified carboxylic group. According to Manrique and Lajolo (2002), a band at 1740 cm^{-1} was found in the spectrum of PE from citrus fruit. The peak at 1589 cm^{-1} possibly corresponded to asymmetrical stretching vibrations of the carboxylate group, as reported by Rao (1976). The stretching vibrations of PE's glycosidic linkage (C–O–C) were likely observed at 1012 cm^{-1}. The

high absorbances, between 1200 and 950 cm^{-1}, indicate the fingerprint region of carbohydrates, which is specific for each polysaccharide (Manrique & Lajolo, 2002).

In the spectrum of RB powder, the peak at 3293 cm^{-1} represented N–H stretching vibrations, which could overlap the O–H stretching vibrations. The distinct bands at 2920 and 2852 cm^{-1} indicated asymmetric and symmetric stretching vibrations of aliphatic C–H, respectively, which could result from the fatty acids in RB. The bands at 1740, 1642, and 1544 cm^{-1} were due to the C=O stretching vibrations of ester functional groups, asymmetric stretching vibrations of $C(=O)–O^-$, and N–H bending vibrations of amide (II), respectively. The bands at 1022 and 997 cm^{-1} indicated the C–O–C stretching vibrations of polysaccharides. The results were in accordance with those reported by Chitprasert, Sudsai, and Rodklongtan (2012).

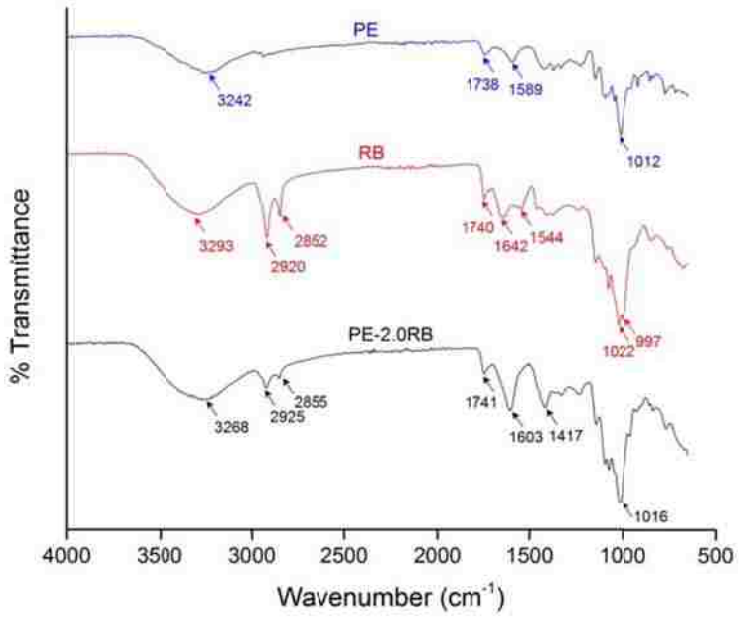

Figure A1. FT-IR spectra of PE, RB, and PE-2.0RB powders. PE, RB, and PE-2.0RB are pectin, rice bran extract, and pectin mixed with 2 g/100 mL rice bran extract, respectively.

The PE-2.0RB powder spectrum showed the bands at 2925 and 2855 cm^{-1}, which could indicate the presence of RB in the powder. The C=O stretching vibrations, which could represent ester functional groups, was shown by the band at 1741 cm^{-1}. The distinct band at 1603 cm^{-1} was

observed in the spectrum of PE-2.0RB powder only. The band at 1642 and 1544 cm^{-1} of the RB powder spectrum, which corresponded to C=O stretching vibrations and N–H bending vibrations, respectively, possibly merged and shifted to the 1603 cm^{-1} band of PE-2.0RB powder. On the other hand, the band at 1589 cm^{-1} from the PE powder spectrum (the vibrations of carboxylate group) was not detected in the spectrum of PE-2.0RB powder. This could suggest that C=O and N-H was likely involved in the interaction.

The spectra of PE and PE-2.0RB capsules had a broad band of O–H stretching vibrations at 3357 and 3550 cm^{-1}, respectively (Figure A2). The O–H stretching vibrations of PE were shifted by +115 cm^{-1}, compared to the spectrum of PE powder. Shifts of the O–H stretching vibrations by +82 cm^{-1} in the PE-2.0RB spectrum were also observed when compared to the spectrum of PE-2.0RB powder. This may confirm the ionic crosslinking of PE and PE-2.0RB solutions to form PE and PE-2.0RB capsules.

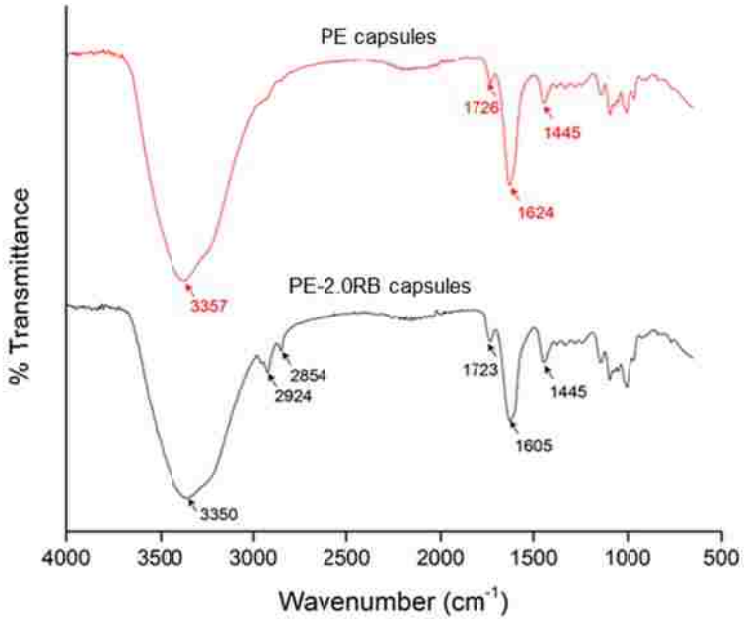

Figure A2. FT-IR spectra of PE and PE-2.0RB capsules. PE and PE-2.0RB are pectin and pectin mixed with 2 g/100 mL rice bran extract, respectively.

The bands at 1726 cm^{-1} of the PE capsule spectrum indicated presence of the methyl esterified carboxylic group, which was similar to the band at 1723 cm^{-1} observed in the spectrum of PE-2.0RB capsules. The peak of methyl ester groups and carboxylic acid groups of pectin were detected in calcium pectinate films at 1735 cm^{-1} (Assifaoui, Loupiac, Chambin, & Cayot, 2010). The researcher mentioned that the shape and wavenumber values of this band depended on the type and amount of cations used in cross-linking solutions. The band at 1445 cm^{-1} was found in both PE and PE-2.0RB capsule spectra, possibly due to deformation of methyl ester ($–OCH_2$) (Wellner, Kacurakova, Malovikova, Wilson, & Belton, 1998).

Unlike the spectrum of PE capsules, the aliphatic C–H stretching vibrations were detected at 2924 and 2854 cm^{-1} of the PE-2.0RB capsule spectrum due to the fatty acids found in RB. The band at 1624 cm^{-1} of the PE capsule spectrum could be due to the non-esterified carboxyl groups of the pectin molecules, as mentioned by Chatjigakis et al. (1998). This band was likely shifted by -19 cm^{-1} to obtain the band at 1605 cm^{-1} shown in the PE-2.0RB capsule spectrum due to the interaction between PE and RB.

References

Assifaoui, A., Loupiac, C., Chambin, O., & Cayot, P. (2010). Structure of calcium and zinc pectinate films investigated by FTIR spectroscopy. *Carbohydrate Research, 345*(7), 929-933. doi: http://dx.doi.org/10.1016/j.carres.2010.02.015

Chatjigakis, A. K., Pappas, C., N.Proxenia, O.Kalantzi, P.Rodis, & Polissiou, M. (1998). FT-IR spectroscopic determination of the degree of esterification of cell wall pectins from stored peaches and correlation to textural changes. *Carbohydrate Polymers, 37*(4), 395-408. doi: http://dx.doi.org/10.1016/S0144-8617(98)00057-5

Chitprasert, P., Sudsai, P., & Rodklongtan, A. (2012). Aluminum carboxymethyl cellulose–rice bran microcapsules: Enhancing survival of *Lactobacillus reuteri* KUB-AC5. *Carbohydrate Polymers, 90*(1), 78-86. doi: http://dx.doi.org/10.1016/j.carbpol.2012.04.065

Manrique, G. D., & Lajolo, F. M. (2002). FT-IR spectroscopy as a tool for measuring degree of methyl esterification in pectins isolated from ripening papaya fruit. *Postharvest Biology and Technology, 25*(1), 99-107. doi: http://dx.doi.org/10.1016/S0925-5214(01)00160-0

Rao, C. N. R. (1976). *Chemical applications of infrared spectroscopy.* New York: Academic Press.

Wellner, N., Kacurakova, M., Malovikova, A., Wilson, R. H., & Belton, P. S. (1998). FT-IR study of pectate and pectinate gels formed by divalent cations. *Carbohydrate Research, 308*(1–2), 123-131. doi: http://dx.doi.org/10.1016/S0008-6215(98)00065-2

Yu, W.-X., Wang, Z.-W., Hu, C.-Y., & Wang, L. (2014). Properties of low methoxyl pectin-carboxymethyl cellulose based on montmorillonite nanocomposite films. *International Journal of Food Science & Technology, 49*(12), 2592-2601. doi: 10.1111/ijfs.12590

APPENDIX B: LETTERS OF PERMISSION

The Chapter 3 previously appeared as Arranee Chotiko and Subramaniam Sathivel, Effect of enzymatically extracted purple rice bran fiber as a protectant of *L. plantarum* NRRL-B4496 during freezing, freeze drying, and storage, June 9 2014. It is reprinted by permission of Elsevier (see below).

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Estimated size (number of pages)	130
Elsevier VAT number	GB 494 6272 12
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VITA

Arranee Chotiko received her Bachelor's and Master's degrees in Biotechnology from Kasetsart University, Bangkok, Thailand in 2006 and 2009, respectively. In 2010, she was awarded a full scholarship from the Thai Royal Government to pursuit her doctoral degree in the United States. She started her Ph.D. studies under the guidance of Dr. Subramaniam Sathivel in the School of Nutrition and Food Sciences at Louisiana State University in August 2011. During her study, she published five peer reviewed publications. Most notably, she presented her research in the Institute of Food Technologies (IFT) Annual Meeting four times, from 2012 to 2015. Arranee received awards for presenting papers at the local and national meetings. She won first place for presenting her paper "Effects of Enzymatically-Extracted Purple Rice Bran Fiber as a Protectant of *Lactobacillus plantarum* NRRL B-4496 during Freezing, Freeze Drying, and Storage" at 2013 the IFT Biotechnology division's graduate paper competition. The paper resulted from her dissertation research. She published the above mentioned dissertation research in *LWT - Food Science and Technology* in 2014. The article has been downloaded more than 750 times since it was published. In 2013, she was also awarded the Grodner Scholarship from the School of Nutrition and Food Sciences and the Louisiana Gulf Coast Section of IFT Tom Quinn and Associates Scholarship. In 2014, Arranee received a prestigious award, an IFT-Feeding Tomorrow Scholarship for recognizing her outstanding academic activities related to the field of food science. In 2015, she was awarded second place for presenting the paper entitled "Development of A Combined Low-Methoxyl-Pectin and Rice-Bran-Extract Delivery System to Improve the Viability of *Lactobacillus plantarum* at Gastrointestinal Conditions" at the IFT Biotechnology division's graduate paper competition at the IFT Annual Meeting in Chicago, IL.

The paper was also published in *LWT - Food Science and Technology* in the same year. Arranee will receive her doctoral degree in December 2015.