Synbiot Encapsulation Employing a Pea Protein-Alginate Matrix

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By

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ABSTRACT

Probiotics and prebiotic are becoming increasingly important to consumers to alleviate issues surrounding gut health, despite the lack of definitive efficacy studies to support health claims. The addition of both probiotics and prebiotics to foods is challenging due to the harsh environmental conditions within the food itself and during transit through the gastrointestinal (GI) tract. To circumvent these challenges encapsulation technology is being explored to protect sensitive ingredients and to control their release within the lower intestines thereby maximizing the health benefiting effects. The overall goal of this research was to design a protein delivery capsule using phase separated pea protein isolate (PPI)-alginate (AL) mixtures for the entrapment of the synbiot which includes the probiotics, *Bifidobacterium adolescentis*, and the prebiotic, fructooligosaccharides (FOS), such that the capsule design provides highly effective protection and release within the GI tract. Research was carried out in three studies.

In study 1, PPI^n (native isolate) and AL interactions were studied in dilute aqueous solutions as a function of pH and biopolymer mixing ratio. Turbidimetric analysis and electrophoretic mobility during an acid titration was used to determine conditions where phase separation occurred. Critical structure forming events associated with the formation of soluble and insoluble complexes in a 1:1 PPI^n-AL mixture were found to occur at pH 5.00 and 2.98, respectively, with optimal interactions occurring at pH 2.10. As the PPI^n-AL ratio increased, critical pH values shifted towards higher pH until a mixing ratio between 4:1 and 8:1was reached, above which structure formation became independent of the ratios through to ratios of 20:1. Electrophoretic mobility measurements showed a similar trend, where the isoelectric point (pI) shifted from pH 4.00 (homogeneous PPI^n) to pH 1.55 (1:1 PPI^n-AL). As the ratio increased towards 8:1 PPI^n-AL, net neutrality values shifted to higher pHs (~3.80) before becoming constant at higher ratios. Maximum coacervate formation occurred at a mixing ratio of 4:1. Based on these findings, capsule design by segregative phase separation was only used in future studies, due to the acidic nature associated with associative phase separation.

In study 2, capsule formation using a native and commercial PPI was studied, and showed no difference between the two formulations during challenge experiments in simulated gastric juice (SGJ). As a result study 3 focused on optimization and characterization of capsules prepared using the commercial PPI. Capsule designs were investigated as a function of protein concentration, prebiotic level, and extrusion conditions (20 vs. 27 G needle) in order to determine protective ability for *B. adolescentis* within SGJ. Capsule designs were also measured in terms of protein and prebiotic retention during the encapsulation process, geometric mean diameter and size distribution, swelling behaviour and release characteristics within simulated intestinal fluids (SIF). All capsules provided adequate protection over the 2 h duration within SGJ. Capsule breakdown and release was similar for all designs within SIF, with a release mechanism believed to be tied to enzymatic degradation of the PPI material within the wall matrix and/or the amount of excessive Na^+ present in the SIF. Capsule size was found to be dependent only on the needle gauge used in the extrusion process. Swelling behaviour of the capsules with SGJ was also found to be dependent only on the protein concentration, where capsules shrank once immersed in SGJ.

A 2.0% PPI-0.5% AL capsule without FOS and extruded through a 20 G needle represents the best and most cost effective design for entrapping, protecting and delivering probiotic bacteria. Future work to establish the role FOS could play post-release as the entrapping probiotics colonize the GI tract, and the protective effect of the capsules wall on FOS structure during transit is recommended.

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LIST OF ABBREVIATIONS

AL	Alginate
ANOVA	Analysis of variance
BSA	Bovine serum albumin
°C	Degrees celsius
CFU	Colony forming units
d	Day
D-value	Thermal death time
DP	Degrees of polymerization
ESP B40	Exocellular polysaccharide B40
F_n	Fructose (n=number of fructose units)
FOS	Fructooligosaccharide
G	Gauge
g	gram
g	Force of gravity
GF_n	Fructose with a terminal glucose unit (n=number of fructose units)
GOS	Galactooligosaccharide
GI	Gastrointestinal
GDL	Glucono-δ-lactone
h	Hour
HPAEC	High performance anion exchange chromatography
M	Molar
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
MRS	De Man, Rogosa, Sharpe
N	Normality

nm	Nanometre
O.D.	Optical density
P95	Orafi® P95 fructooligosaccharide
PAD	Pulsed amperometric detector
PBS	Phosphate buffer saline
PF	Pea flour
pH_c	Critical pH for soluble complex formation
pH_{opt}	pH optimal
$pH_{\phi 1}$	Critical pH for insoluble complex formation
$pH_{\phi 2}$	Citical pH for dissolution of complexes formed during complex formation
pI	Isoelectric point
PPI	Pea protein isolate
PPI^c	Commercial pea protein isolate
PPI^n	Native pea protein isolate
PS	Peptone saline
PSA	Particle size analyzer
RCM	Reinforced clostridial media
RCM-cys	Reinforced clostridial media L-cysteine hydrochloride monohydrate
SGJ	Simulated gastric juice
SIF	Simulated intestinal fluid
WPI	Whey protein isolate
α	Particle radius
ε	Permittivity
$f(\kappa\alpha)$	Smoluchowski approximation
κ	Debye length
ς	Zeta potential
η	Dispersion viscosity
U_E	Electrphoretic mobility
Vb	Volume of HCl used to titrate blank in proximate analysis of protein
Vs	Volume of HCl used to titrate the sample (protein analysis)

W1	Initial weight of capsule (swelling ratio)
W2	Final weight of capsule (swelling ratio)
Wf	Final weight (sample weight + dish weight after drying)
Wi	Initial weight (sample weight + dish weight before drying)
Ws	Sample weight

1. Introduction

1.1 Summary

The overall goal of this research project is to design a novel protein-based delivery capsule using phase separated pea protein isolate-alginate mixtures for the entrapment of the synbiot which includes the probiotic, *Bifidobacterium adolescentis*, and prebiotic fructooligosaccharides (FOS), such that the capsule design provides sufficient protection against simulated gastric juice and release within simulated intestinal fluids.

A probiotic can be described as "a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance" (Fuller, 1989), if administered at therapeutic doses (10^7 live cells per gram of food) (Fooks et al., 1999). Administration of probiotics have postulated health benefits, which include, alleviation of lactose intolerances, reduced risk of cancer, reduction of serum cholesterol, and prevention of intestinal tract infection (Gibson and Roberfroid, 1995b; Collins and Gibson, 1999; Shortt, 1999; Bielecka, 2007). However, once ingested the bacteria are exposed to barriers within the gastrointestinal tract including gastric and bile acids. These barriers cause only a small portion of probiotics to reach the colon intact and in a viable form. Also, once in the colon a bacteria population must become established and active, which may be difficult since the bacteria are most likely within a stressed state due to the exposure to gastric and bile acids, and must compete for nutrients and space against indigenous microflora. Prebiotics are defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid, 1995b). Functionally, prebiotics act to increase the number of beneficial bacteria which already colonize the colon. primarily from the *Lactobacillus* and *Bifidobacterium* genera (Farnworth, 2007). Some of the postulated health benefits prebiotics based on nondigestible oligosaccharides include, improved mineral absorption, resistance to invading pathogens, and regulation of lipid levels (Rastall, 2007; Sarkar, 2007).

To help circumvent challenges faced by probiotics within the food environment and during transit through the harsh conditions of the GI tract, many researchers are now employing encapsulation technology that protect the probiotics through the gastrointestinal tract yet release them in the small intestine at a targeted site (Chen et al., 2005; Crittenden et al., 2006; Muthukumarasamy et al., 2006; Xiao Yan et al., 2009). The encapsulation technology involves encasing the sensitive core, probiotics, ingredient within a biopolymer shell, which can release its contents at controlled rates once triggered by an external sensor (e.g., temperature, pH, enzymes, etc.) (Dziezak, 1988; Desai and Park, 2005; Anal and Singh, 2007). Although a wide variety of biopolymers have been employed as encapsulating agents, alginate-based capsules seem to dominate (Lee and Heo, 2000; Sultana et al., 2000; Chandramouli et al., 2004). Alginate is a linear heteropolysaccharide comprised of D-mannuronic and L-guluronic acids; the latter being highly sensitive to divalent calcium ions, resulting in the formation of strong egg box-like junction zones (Burey et al., 2008). However, alginate-probiotic capsules have not adequately protected probiotic bacteria under SGJ (Lee and Heo, 2000; Sultana et al., 2000). The addition of proteins to the wall material can improve the survival of the probiotic bacteria through SGJ conditions due to a reduced pore size within the capsule, preventing the diffusion of SGJ into the capsules (Wood, 2010).

Within the present research, probiotic capsules will be comprised of PPI-AL mixtures, using controlled phase separation as a means to alter the wall design to improve their survival under SGJ conditions and release within SIF. Phase separation within biopolymer mixtures is governed by biopolymer characteristics (e.g. size, type and distribution of reactive groups and the charge density), mixing ratio and concentration, and solvent conditions (e.g., pH, salt and temperature), and could result in either segregative or associative phase separation (Weinbreck et al., 2003a). The former case typically occurs under conditions where biopolymers carry similar net charges, for instance when an anionic polysaccharide and a protein (within solvent pH > isoelectric point, pI) both carry a net negative charge. Biopolymers tend to phase separate into a protein-rich and a polysaccharide-rich phase due to electrostatic repulsive forces. In the case of associative phase separation (also known as complex coacervation), biopolymers

carry opposing net charges, such as an anionic polysaccharide and a protein (at pH < pI) resulting in the electrostatic attraction between the two biopolymers and phase separation into a biopolymer-rich (protein + polysaccharide) and a solvent-rich phase (Doublier et al., 2000; de Kruif et al., 2004). Encapsulation within biopolymer mixtures involving associative phase behaviour will give a capsule wall stabilized by electrostatic attractive forces, and will not require the need for chemical, enzymatic or salt cross-links. However, in the case of encapsulation within biopolymer mixtures involving segregative phase behaviour, additional cross-linking agents will be required to maintain the wall integrity (e.g., calcium salts).

1.2 Hypotheses

Research in this study focused on the following hypotheses:

- associative phase separation between pea protein isolate and alginate mixtures will occur at solvent pH < pI of the protein and at pH > pK_a of the carboxyl reactive site on the alginate polysaccharide, whereas segregative phase separation within the mixture will occur at solvent pH > pI of the protein;
- biopolymer capsules will act to protect *B. adolescentis* within simulated gastric juice, and release its contents within simulated intestinal fluids;
- the addition of fructooligosaccharides will improve the survival of *B. adolescentis* within the encapsulated wall; and
- changes to the wall material and extrusion conditions will alter the physical properties of the capsule, its ability to protect *B. adolescentis* and to retain fructooligosaccharides in simulated gastric juice and release in simulated intestinal fluid.

1.3 Objectives

To test these hypotheses, the following objectives were established:

- to investigate the effect of pH and biopolymer mixing ratio on the phase behaviour of pea protein isolate-alginate mixtures;

- to develop encapsulation protocols for entrapping *B. adolescentis* within two capsules designs based on associative and segregative phase behaviour, testing their ability to offer protection with in simulated gastric juice;
- to test various wall material ratio (e.g., biopolymer ratio and total concentration, and prebiotic concentration) and extrusion (e.g., needle gauge size) conditions; testing the impact on the physical properties of the capsules and on its ability to protect *B. adolescentis;* and
- to test the release behaviour of *B. adolescentis* within the various capsule designs within simulated gastric juice and simulated intestinal fluid.

2. Literature Review

2.1 Probiotics

Probiotics have been described as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” (Fuller, 1989). The Food and Agriculture Organization/World Health Organization (2001) more recently defined probiotics as “live microorganisms (bacteria or yeasts), which when ingested or locally applied in sufficient numbers confer one or more specified demonstrated health benefits for the host”. Probiotics reach the large intestine in an intact and viable form, therefore helping maintain the microbial balance in the gut (Gibson and Roberfroid, 1995b). For a microorganism to be classified as a probiotic it must: 1) be non-pathogenic; 2) be acid and bile tolerant; 3) withstand technological processes and remain viable during shelf life; and 4) demonstrate health benefits to the host (Gibson and Roberfroid, 1995b; Collins and Gibson, 1999; Shortt, 1999; Bielecka, 2007). Typically, probiotics are associated with two main genera of lactic acid bacteria: *Lactobacillus* and *Bifidobacterium* (Gibson and Roberfroid, 1995b; Shortt, 1999). Other less common probiotic bacteria include *Streptococcus, Leuconostoc, Pediococcus, Propionibacterium, and Bacillus*. Some yeasts have also been classified to have probiotic effects such as *Saccharomyces cerevisiae* and *S. boulardii* (Fuller, 1992).

Lactobacillus spp. are one of the most important microorganisms associated with the human gastrointestinal tract. They are gram positive, non-spore forming rods with anaerobic or microaerophilic respiration (Gomes and Malcata, 1999; Anal and Singh, 2007). Gram positive bacteria have a thick peptidoglycan layer associated within their cell wall, whereas gram negative bacteria have a thin layer making the former more tolerant to environmental stresses (Madigan, 2003). Due to their anaerobic or microaerophilic respiration, growth occurs either in the absence of oxygen (i.e., anaerobic) or in an environment with a reduced concentration of oxygen of air (i.e., microaerophilic) (Madigan, 2003). These bacteria grow best at pH 5.5-6.0 and at temperatures of 35-40°C (Gomes and Malcata, 1999). *Lactobacillus* bacteria are commonly used as starter cultures in yogurt production and are the most widely used

probiotic on the market (Fuller, 1992). Common *Lactobacillus spp.* classified as probiotics include; *Lactobacillus acidophilus*, *L. casei*, *L. delbruekii*, and *L. johnsonii* (Gibson and Roberfroid, 1995b; Shortt, 1999).

Bifidobacterium were first isolated and characterized by Tissier in 1988 in feces of breast fed infants (Fuller, 1992; Gomes and Malcata, 1999). *Bifidobacterium spp.* are Gram positive, non-spore forming anaerobic bacteria (Gomes and Malcata, 1999; Anal and Singh, 2007). These bacteria grow best at 37-41°C and at pHs between 4.5-8.5, with optimal growth occurring between pH 6.0 and 7.0, and virtually no growth at pH <4.5. *Bifidobacterium* make up >95% of the intestinal microflora of infants with the predominant species being *B. infantis* and *B. breve*. *Bifidobacterium* populations are significantly reduced in adults and the predominant *Bifidobacterium spp.* are *B. adolescentis* and *B. longum* (Fuller, 1992). Common *Bifidobacterium spp.* classified as probiotics are *B. adolescentis*, *B. bifidum*, *B. longum*, and *B. animalis* (Gibson and Roberfroid, 1995b; Shortt, 1999).

2.1.1 Health benefits associated with probiotics

Presently, it is generally recognized that an optimum balance in the microbial flora within the digestive tract is associated with good health and nutrition (Lourens-Hattingh and Viljoen, 2001). Increasing evidence suggests that consuming probiotics maintains this balance, leading to several postulated health benefits, such as:

(a) Enhanced lactose absorption: Lactose malabsorption occurs when there is insufficient activity of lactase (β-galactosidase) in the human gut, resulting in a build-up of unabsorbed lactose (Fooks et al., 1999; Farnworth, 2007). Lactase functions to hydrolyze lactose into glucose and galactose units; allowing improved absorption through the intestinal wall. In contrast, a build-up of lactose creates abdominal distension, abdominal pain, excessive flatulence and/or diarrhoea. Some probiotic strains (e.g., *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) found in fermented milk products (e.g., yogurt) are able to produce lactase on their own, which converts the lactose into its monosaccharide end

products (glucose and galactose), allowing the monosaccharides to be more efficiently digested (Fooks et al., 1999; Farnworth, 2007).

(b) Prevention of intestinal tract infections: The addition of probiotics has also been shown to beneficially alter the gastrointestinal flora. By increasing the amount of beneficial bacteria, the resulting competitive exclusion protects the host against gastrointestinal infections caused by pathogenic bacteria (Bielecka, 2007). Gibson and Wang (1994b) found that eight different strains of *Bifidobacteria* (i.e., *B. bifidum, B. adolescentis, B. angulatum, B. catenulatum, B. breve, B. infantis, B. longum* and *B. pseudolongum)* were able to excrete antimicrobials that inhibited the growth of pathogenic bacteria such as *Vibrio cholera, Shigella sonnei, Listeria monocytogenes, E. coli, Campylobacter* spp., *Salmonella* spp., *Clostridium perfringens* and *Bacteroides fragile*.

(c) Reduced serum cholesterol: Fermented dairy products containing probiotic bacteria has been reported to reduce plasma cholesterol levels in humans. Although, there is no conclusive results, studies suggest the potential to use probiotics to lower total or low density lipoprotein cholesterol (Fooks et al., 1999). A high level of serum total cholesterol is generally considered to be a risk factor for coronary heart disease and atherosclerosis (Xiao et al., 2003). A study conducted by Xiao et al. (2003) investigated the ability of a low fat drinking yogurt supplemented with the probiotic, *Bifidobacterium longum* to lower the serum total cholesterol in 32 male volunteers with total serum cholesterol ranging from 220-280 mg/dL. They reported that after 4 wks of ingesting 3 x 100 mL/d there was a significant reduction in serum total cholesterol in subjects with moderate hypercholesterolemia. Schaafsma and co-workers (1998) also investigated the effect of milk products fermented by *L. acidophilus* on blood lipid profiles in adult males; reporting that a daily feeding of 125 mL probiotic milk significantly lower serum low density lipoprotein cholesterol levels.

(d) Reduced risks of cancer: The ability of fermented yogurt products containing probiotics to reduce the risk of cancer is not well established. However, some studies have suggested their ability to reduce the risk of colon cancer. Kulkarni and Reddy

(1994) used rat models to test the ability for *B. longum* to suppress the development of azoxymethane-induced preneoplastic lesions (e.g., aberrant cryptic foci which is associated with colon cancer). The authors found that feeding rats *B. longum* significantly suppressed the formation of aberrant cryptic foci, and could be used as a preventative treatment. A clinical study by Goldin and Gorback (1984) showed *L. acidophilus*, impacted on three bacterial enzymes, which catalyzed the conversion of procarcinogens to carcinogens. Over a 4-wk feeding trial with the milk supplemented with viable *L. acidophilus*, they reported a 2 to 4-fold reduction in bacterial enzyme activities (i.e., for β-glucuronidase, nitroreductase, and azoreductase). Other studies have also reported anti-carcinogenic activity with the use of probiotics in both human and animal models (Benno and Mitsuoka, 1992; Reddy, 1998; Rowland et al., 1998).

The ability for probiotics to improve gastrointestinal health and to act as a preventative treatment remains promising. The Health Canada (2009) suggests that a product must contain 10^9 colony forming units (CFU) of probiotics per serving size to offer any sort of health benefit. As an example, a 100 g serving of yogurt containing 10^7 CFU/g would supply a sufficient amount. Although there are many claims associated with probiotics, there is no conclusive evidence of their benefits and, as such, research efforts are shifting towards efficacy issues.

2.1.2 Challenges associated with the use of probiotics

There are a number of challenges associated with the use of probiotics as preventative treatments, such as:

(a) Exposure to harsh conditions within the gastrointestinal tract: The most common form of probiotic administration is through the direct addition of the probiotic agents to fermented milk products. However, once ingested, the bacteria are exposed to barriers in the gastrointestinal tract (e.g., gastric juice and bile acids) during transit, resulting in only a portion of probiotics reaching the colon intact and viable. Once in the colon, probiotics must colonize and become active which may be difficult if they are in a stressed state. The probiotics must compete for nutrients and

space with indigenous microflora (Gibson and Roberfroid, 1995b; Bezkorovainy, 2001). Furthermore, the low pH of the stomach (pH 1.0-3.0) can also negatively impact the level of viable bacteria reaching the colon. Marteau et al. (1997) investigated the viability of probiotic bacteria (*L. bulgaricus*, *Streptococcus thermophilus, L. acidophilus,* and *B. bifidum*) when exposed to stomach pH and bile using a dynamic model of the stomach and small intestine. The authors indicated that after exposure to the gastric compartment (110 min) two of the bacterial strains, *L. bulgaricus,* and *Streptococcus thermophilus* had viability counts of <1%, while for *L. acidophilus* and *B. bifidum* the viability counts were 60% and 80%, respectively. The authors also exposed the bacteria to bile conditions, reporting viable counts prior to reaching the colon of <10% for *L. bulgaricus* and *Streptococcus thermophilus,* 15% for *L. acidophilus*, and 30% for *B. bifidum*. In another study, *B. adolescentis, B. breve, B. lactis* and *B. longum* cells were exposed to simulated gastric juice at pH 2.0 for 30 min, after which all strains were reduced by 5 log CFU/mL from the initial cell counts of ~8 log CFU/mL (Hansen et al., 2002). The sensitivity of probiotic bacteria to acid and bile salts varies with each individual strain and creates a challenge to deliver sufficient viable cells to the colon. These results suggest that probiotics must be protected in some fashion in order to deliver sufficient numbers to the colon and exert a beneficial effect.

(b) Adhesion to the intestinal wall: The ability for probiotic bacteria to adhere to the intestinal wall is essential in order to exert beneficial effects and survive within the colon. Bouhnik et al. (1992) investigated the fate of orally administered *Bifidobacterium spp.* in the gut. These studies demonstrated that the rate of recovery of *Bifidobacterium spp.* (approximately 30%) in the feces of human volunteers was found to be consistent with the amount able to survive passage to the colon, suggesting that the bacteria was not adhering to the intestinal wall. They reported that once the administration of the probiotic bacteria ended, the presence in the feces also ceased.

(c) Processing and storage conditions: Viability of probiotics agents can also be reduced during product processing and storage (Mattila-Sandholm et al., 2002). Shah and colleagues (1995) investigated the viability of *L. acidophilus* and *B.*

bifidum claimed to be in five commercial yogurt products. They reported three out of the five products contained initial cell counts of $< 10^5$ CFU/g of *L. acidophilus* and two of the products with $< 10^3$ CFU/g of *B. bifidum*. In the yogurt products tested, a 50% decline in *L. acidophilus* viability after 20 d stored at 4°C occurred in two of the products; whereas a rapid decline in *B. bifidum* was observed in all products.

Challenges associated with probiotics can be overcome through: 1) careful strain screening to find a more resilient probiotic; 2) the addition of prebiotic material to enhance growth and viability of the microorganisms; and 3) entrapment technology which involves encasing the probiotics within a protective biopolymer shell.

2.2 Prebiotics

Prebiotics are defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid, 1995b). Prebiotics include food ingredients which pass through the upper part of the digestive system without being absorbed or hydrolyzed by human enzymes. These materials act as nutrients for endogenous colonic bacteria and therefore indirectly provide the host with energy, metabolic substances (short chain fatty acids), and essential micronutrients. For materials to be classified as prebiotic in nature, they must: 1) reach the colon by resisting gastric acidity, hydrolysis by mammalian enzymes, and absorption in the stomach or small intestine; 2) be fermented by intestinal microflora; and 3) selectively stimulate the growth and/or activity of bacteria which promote health (Roberfroid, 2007; Sarkar, 2007). Functionally, prebiotics act to increase the number of beneficial bacteria which already colonize the colon, primarily from the *Lactobacillus* and *Bifidobacterium* genera (Farnworth, 2007).

2.2.1 Sources of prebiotics

The most common prebiotic source are non-digestible carbohydrates because they are naturally occurring, abundant, and meet all the criteria for prebiotic. Non-digestible carbohydrates include, resistant starch (i.e., starch not hydrolyzed in the small

intestine), non-starch polysaccharides (i.e., hemicelluloses, pectin, and gums), and non-digestible oligosaccharides (i.e., inulin, fructo-oligosaccharides).

Oligosaccharides are defined as carbohydrates with low degrees of polymerization (DP) (i.e., 2 to 10-20 monosaccharide units) (Roberfroid and Slavin, 2000; Mussatto and Mancilha, 2007). Oligosaccharides are water soluble and instil a mild sweetness when in solution. Dextrose equivalent values for oligosaccharides range between 0.3-0.6 depending upon the composition and structure (Crittenden and Playne, 1996). Due to the inability of humans to digest many oligosaccharides they are commonly used in low-calorie and diabetic foods. Oligosaccharides are typically produced using three different methods: 1) hot water extraction of roots (i.e., inulin extracted from chicory or Jerusalem artichoke); 2) enzymatic hydrolysis of oligosaccharides or polysaccharides (i.e., oligofructose produced from partial hydrolysis of inulin with endoinulinase); and 3) enzymatic synthesis from one or a mixture of monosaccharides by osyl-transferase (i.e., fructooligosaccharides (FOS) from sucrose) (Roberfroid and Slavin, 2000). A summary of the physicochemical properties of selected prebiotic materials is as follows:

(a) Fructooligosaccharides

Fructans such as inulin represent the most studied prebiotic material. Inulin is a linear chain of fructofuranose residues linked by β-(2→1) linkages (Figure 2.1). A terminal glucopyranosyl residue may be linked in the β-(1→2) configuration, as in sucrose on some of the chains, giving it a non-reducing end. Due to the β-configuration, fructans resist hydrolysis by mammalian enzymes which are specific to α–glycosidic bonds (Mussatto and Mancilha, 2007; Rastall, 2007; Roberfroid, 2007). Commercial inulin is produced from chicory root. Chicory inulin is composed of a mixture of oligosaccharides and polysaccharides, with DP ranging between 2 and ~60 units (DP_{av} = 12). Approximately 10% of the fructans in native chicory inulin have a DP ranging between 2 and 5 (Rastall, 2007; Roberfroid, 2007).

Fructooligosaccharides (FOS) are the most extensively studied prebiotic as they meet all the prebiotic criteria. FOS is produced from the partial hydrolysis of inulin through the use of endoinulinase and is mainly constituted of oligosaccharides of D-fructose (F_n) (Bañuelos et al., 2008). The DP of FOS varies from 2 to 7 with a DP_{av} = 4.

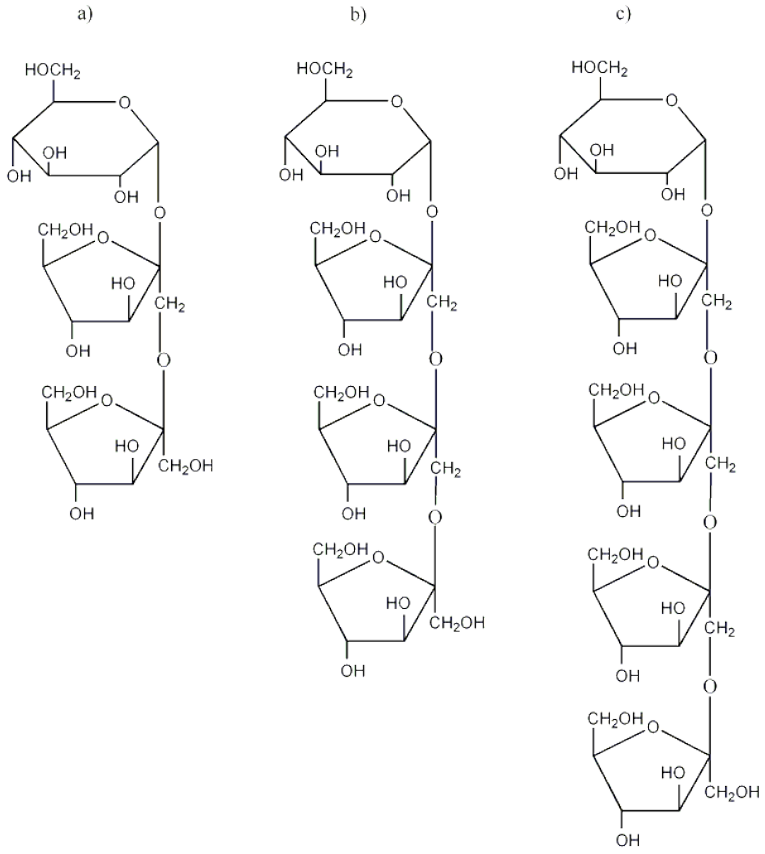

Figure 2.1 Chemical structure of a) 1-kestose (GF_2), b) nystose (GF_3) and c) fructosylnystose (GF_4) fructooligosaccharides (adapted from Bornet, 2001).

FOS can also be produced via enzymatic synthesis (transfructosylation) using β-fructosidase to add fructose to sucrose forming β-(2→1) linkages. The DP ranges from 2 to 4 with a DP_{av} = 3.6 (Rastall, 2007; Roberfroid, 2007). Production of FOS via transfructosylation leads to mixtures of oligosaccharides with terminal D-glucose (GF_n) (Bañuelos et al., 2008). The main FOS are 1-kestose (GF_2), nystose (GF_3), and fructosylnystose (GF_4) (Figure 2.1) (Bornet, 2001). Inulin and FOS are fermented by *Bifidobacterium spp*. and to a lesser extent by *Lactobacillus* spp. The growth of *Bifidobacteria* on these substrates is at the expense of potential pathogenic bacteria (Gibson and Roberfroid, 1995b; Rastall, 2007).

Gibson and Wang (1994a) tested the ability of eight *Bifidobacterium spp., B. infantis, B. catenulatum, B. longum, B. pseudomlongum, B. breve, B. anulatum, and B. adolescentis* to grow on branched FOS (DP_{av}=13, 8% branching), linear FOS (DP_{av} = 4), inulin (DP_{av} = 10), and glucose. They reported that seven of the species grew well with all the fructans tested, but generally the preferred growth substrate was short chain linear oligofructose (DP = 4). Bielecka et al. (2002) revealed subspecies belonging to *B. longum* and *B. animalis* had increased growth rates when grown in media containing 1.0% (w/v) FOS (DP = 2-4) and Rafilose™ (DP = 2-8, glucose + fructose + lactose < 6.8%) compared to inulin sources. Langlands and coworkers (2004) investigated the effects of a diet supplemented with an inulin/fructooligosaccharide mixture (7.5 g/d inulin, DP_{av} = 10; 7.5 g/day fructooligosaccharide, DP = 2-8, glucose + fructose + lactose < 6.8%) for 2 wks on 14 volunteers who were on the waiting list for a colonoscopy. Samples were taken from the caecum, transverse and descending colon and rectum and found that the prebiotic was able to increase the amount of *Bifidobacteria* found within the proximal (5.3 to 6.3 $\log_{10}$ CFU/mL) and distal (5.2 to 6.4 $\log_{10}$ CFU/mL) colon. They also observed an increase in Lactobacilli in the proximal (3.0 to 3.7 $\log_{10}$ CFU/mL) and distal (3.1 to 3.6 $\log_{10}$ CFU/mL) colon. Kaplan and Hutkins (2000) added 2% (w/v) FOS to De man, Rogosa, Sharpe (MRS) agar and tested 28 strains of lactic acid bacteria and *Bifidobacteria*. They reported that 12 out of the 16 *Lactobacillus spp.* and 7 out of the 8 *Bifidobacterium spp.* were able to grow on plates containing the FOS.

(b) Galactooligosaccharides

Galactooligosaccharides (GOS) are produced from lactose by enzymatic synthesis (transgalactosylation) using β-galactosidases. The product consist of oligosaccharides from tri- to pentasaccharides with β-(1→6), β-(1→3), and β-(1→4) linkages, with DP ranging between 2 and 8 (Figure 2.2). The enzyme used during manufacture affects the composition, subsequently determining the DP as well as linkages found within the end product (Mussatto and Mancilha, 2007; Rastall, 2007; Roberfroid, 2007). Galactooligosaccharides have been shown to selectively enhance the growth of *Bifidobacteria* while decreasing pathogenic bacteria (Fooks et al., 1999; Rastall, 2007; Roberfroid, 2007). Depeint et al. (2008) investigated the ability of a novel GOS produced via β-galactosidase originating from a probiotic *Bifidobacterium bifidum* strain against a GOS produced through an industrial β-galactosidase and a placebo on healthy human volunteers. During the first stage, 59 healthy humans (average age 34.4) received 7 g/d commercially available GOS for 7 d, followed by a 7 d washout period after which 30 volunteers received a sequence of treatments of novel GOS at either 0, 3.6, or 7 g/d for 7 d. The stool was evaluated and they reported that the novel GOS significantly increased the *Bifidobacteria* population ratio compared to the placebo. Volunteers receiving 7 g/d of the novel GOS had a significant ($p<0.05$) increase in the *Bifidobacteria* population ratio compared to the commercial GOS. The authors also reported a relation between *Bifidobacteria* proportion and dose of novel GOS received. They conclude that GOS was able to increase the amount of *Bifidobacteria* within the stool and that GOS produced via different sources of β-galactosidases showed different prebiotic properties.

(c) Lactulose

Lactulose is produced by alkaline isomerisation of lactose to produce 4-*O*-β-glactopyranosyl-D-fructose (Figure 2.3) (Mussatto and Mancilha, 2007). Although lactulose has been recognized as a prebiotic for many years, it has also been shown to be a laxative. At sub-laxative levels, lactulose has shown to selectively increase

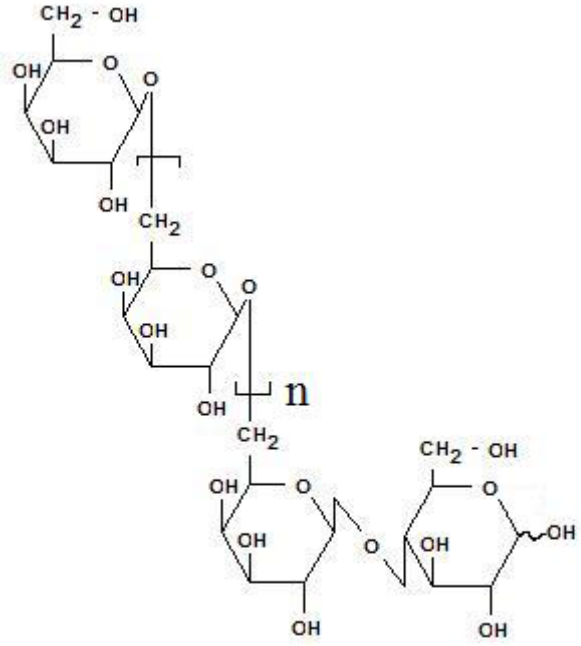

Figure 2.2 Chemical structure of galactooligosaccharides, n = the number of galactose units (adapted from Swennen, 2006).

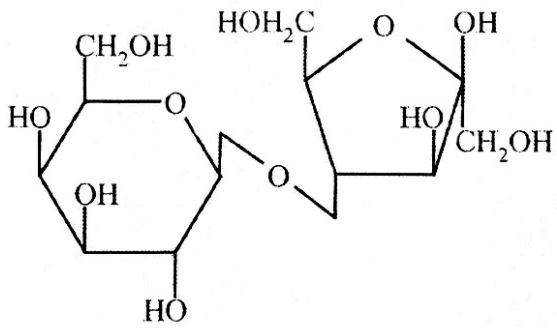

Figure 2.3 Chemical structure of lactulose (adapted from Swennen et al., 2006).

Bifidobacteria population while decreasing less desirable bacterial groups (Casci et al., 2007; Rastall, 2007).A human study by Ballongue (1997) investigated the effect of lactulose on 36 human volunteers in a randomised, double-blind study. When volunteers were fed lactulose (10 g twice a day) for 4 wks an increase in the number of faecal *Bifidobacterium spp.* and *Lactobacilli spp.* along with a decrease in the number of *Bacteroides spp, Clostridium spp.*, and coliforms were reported.

2.2.2 Health benefits associated with prebiotics

There are many health benefits associated with consuming prebiotics, including: significantly modifying the colonic microbial ecosystem, improved mineral/ion absorption, and regulation of lipid levels (Delzenne and Roberfroid, 1994; Mussatto and Mancilha, 2007; Rastall, 2007; Sarkar, 2007).

(a) Improved microbial balance: Clinical trials have shown that the addition of prebiotics to the diet results in an increase in *Bifidobacteria* content within the feces and a decrease in detrimental bacteria (Gibson et al., 1995a; Buddington et al., 1996; Bouhnik et al., 1999). Gibson and co-workers (1995a) measured the affect of FOS and inulin diet addition on colonic bacteria of eight human volunteers over a 45 d period. Individuals were fed controlled diets of 15 g of sucrose for 15 d, then 15 g of FOS (Raftilose, DP = 2-8) or inulin (Raftiline, DP of 10) for 15 d, followed by a second control period of 15 d supplemented with 15 g sucrose. The bowel habit, transit time, stool composition, and breath H_2 and CH_4 were all measured. The authors reported there was an increase in stool frequency by 14% and 34% when FOS and inulin were ingested, respectively. The *Bifidobacteria* content from increased 8.8 to 9.5 $\log_{10}$ g/stool and 9.2 to 10.1 $\log_{10}$ g/stool when FOS and inulin were added to the diets, respectively, whereas pathogenic bacteria, *Bacteriodes, Clostridia,* and *Fusobacteria* decreased. They concluded that the consumption of dietary FOS and inulin may improve intestinal health.

(b) Increased absorption of minerals/ions: The consumption of prebiotics has also been associated with the increased absorption of minerals/ions (Gibson and Roberfroid, 1995b; Gibson et al., 2004; Rastall, 2007). This was demonstrated in

rats fed a diet supplemented with fermentable FOS with high and low degrees of polymerization. The rats were fed 10% Raftilose (DP = 4.8) or Raftiline (DP = 10) and the apparent retention of gross energy, nitrogen, and calcium, magnesium, iron, zinc, and copper contents was measured. The authors reported that there was a decrease in the fecal excretion of minerals suggesting the improved absorption of calcium, magnesium, iron, and zinc (Delzenne et al., 1995). Similar results were observed in a human study with adolescent girls, where a diet supplemented with short chain FOS (DP = 2-4) for 36 d increased magnesium absorption by 18% (van den Heuvel et al., 2009).

(c) Healthier lipid profiles: Prebiotics have also been shown to reduce plasma triglycerides, phospholipids, and cholesterol (Fiordaliso et al., 1995). Rats fed daily 10% FOS in their diet demonstrated a 25% decrease in plasma triglycerides, a 15% decrease in phospholipids, and a 15% reduction in cholesterol. High levels of plasma triglycerides and cholesterol are well known to increase one's risk of coronary heart disease. The ability of prebiotics to reduce the aforementioned can reduce the risk of developing coronary heart disease.

Many studies support the health benefits of consuming prebiotics. However, most of these studies have focused on the ingestion of FOS and inulin as the prebiotics source, with few studies focusing on other prebiotics. More studies are required to clearly establish these health claims and determine the mechanisms and roles prebiotics have in a human diet. There are few problems found to be associated with the use of prebiotics, however a major issue with overconsumption of prebiotics can cause diarrhea or flatulence (Crittenden and Playne, 1996).

2.3 Synbiots

Synbiots are defined by Gibson and Roberfroid (1995b) as "a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare".

Synbiots are a combination of a prebiotic and probiotic bacteria in which the live bacteria is used in conjunction with a specific substrate for growth (Collins and Gibson, 1999; Fooks et al., 1999). Some synbiots suitable for human consumption include, FOS and *Bifidobacterium longum, B. infantis,* and *Lactobacillus acidophilus*; GOS and *B. lactis B. breve,* and *L. casei*; and lactulose and *B. lactis* (Rastall and Maitin, 2002; Hawrelak, 2007; Rouzaud, 2007). The combination of both prebiotics and probiotics could improve survival of the probiotic due to provision of a readily available and specific substrate. The prebiotics provides a carbon source during colonization of the probiotic and may also be used by other beneficial microorganisms already present within the colon (Collins and Gibson, 1999; Fooks et al., 1999; Rouzaud, 2007). Administration of probiotics and prebiotics can be done as separate entities or in combination (synbiot). The combined use of probiotics and prebiotics could produce many nutritional benefits including: improved survival of live bacteria in food products; increased number of ingested bacteria reaching the colon in a viable form; and stimulation of growth and implantation of both exogenous and endogenous bacteria within the colon (Gibson and Roberfroid, 1995b).

2.4 Probiotic and prebiotic utilization

The ability of bacteria within the colon to ferment prebiotic oligosaccharides is influenced by the chemical structure, degree of polymerization, and conformation (linear or branched), as well as water solubility of the prebiotic. In general, fructans which are considered to be the most susceptible to fermentation have short chain lengths, unbranched structure and are soluble in water (Biedrzycka and Bielecka, 2004). Fructans have also been found to show differences in their ability to stimulate growth of *Bifidobacteria* and *Lactobacillus* strains based on DP values (Perrin et al., 2002; Bañuelos et al., 2008). According to Coudray et al. (2003), a DP value of 10 represents a critical physiochemical threshold, where levels < DP 10 are fermented more readily by bacteria than larger chains (> DP 10). The majority of *Bifidobacteria* (except for *B. bifidum*) and some *Lactobacillus spp*. are able to ferment FOS due to the production of relatively high amounts of β-fructosidase, which selectively cleaves β-(2→1) linkages (Bornet, 2001; Hidaka et al., 2001). *Lactobacillus* was shown by Banuelos et al. (2008)

to more efficiently metabolize FOS with a DP ranging between 2 and 3 than FOS with DP >4. The authors studied the ability for two *Lactobacillus* strains; *L. fermentum* CECT5716 and *L. gasseri* CECT5714 to ferment Actilight®, FOS-AES, Raftilose® P95, and Raftline HP®. Actilight® is synthesized by enzymatic transglycosylation and has a DP of 2-4. FOS-AES is syrup containing a mixture of FOS and is produced by enzymatic synthesis using a fungal fructosyltransferase. Raftilose® P95 is produced via enzymatic hydrolysis of chicory inulin and has a DP of 2-8 (DP_{av} = 4). Raftline HP® is chicory inulin with DP_{av} = 23. Banuelos et al. (2008) reported that the *Lactobacillus spp.* had very low growth on Raftilose® P95 and Raftline HP® containing media which were both produced by enzymatic hydrolysis. They also reported that 1-kestose (GF_2) was the only prebiotic which was significantly metabolized by *Lactobacillus spp*. Therefore, fructans produced by enzymatic synthesis with a DP of 2 to 3 were more efficiently metabolized by *Lactobacillus.* The preference for shorter chain oligosaccharides can also be seen in *Bifidobacterium spp.* Biedrzycka and Bielecka (2004) reported results which showed that the majority of *Bifidobacteria* strains tested preferentially utilized short chain FOS (DP = 2-4), oligofructose (DP = 2-8), and low polymerized inulin (DP ≤ 9) compared to highly polymerized inulin and highly purified inulin.

2.5 Encapsulation technology

Encapsulation refers to the entrapment of solids, liquids, or gaseous materials within small capsules/particles that can release their contents at controlled rates under the influences of specific environmental conditions (e.g., pH, temperature, presence of salts, enzymatic degradation, etc.) (Dziezak, 1988; Desai and Park, 2005; Anal and Singh, 2007). Capsules allow for the entrapped ingredient to be separated and protected from its environment until release of the functional ingredient is desired (Ross et al., 2005). Capsules can vary in size, ranging from microns to millimetres (Dziezak, 1988; Desai and Park, 2005) and are grouped into three broad categories based on size: macrocapsules (>5000 μm), microcapsules (5000 μm to 0.2 μm), and nanocapsules (<0.2 μm) (King, 1995). In general, the entrapped ingredient is referred to as the core material or active ingredient, whereas the outer material is referred to as the wall material or coating. The wall materials most commonly used for encapsulation of food

ingredients are hydrocolloids (Roberfroid and Slavin, 2000; Anal and Singh, 2007). Common hydrocolloid wall materials used within the food industry include AL, agar, carrageenan, cellulose, gelatin, gum Arabic, gellan gum, low -methoxy pectin, maltodextrins, starch, and whey protein (King, 1995). In the case of probiotics, capsule materials act to protect the viability of the microbe during food manufacture, storage and transit through the gastrointestinal tract, with the objective of delivering high numbers (10^7 CFU/g of food) to the colon. Literature relating to probiotic encapsulation is dominated by either extrusion (Krasaekoopt et al., 2003; Chandramouli et al., 2004; Chen et al., 2005; Iyer and Kailasapathy, 2005; Muthukumarasamy et al., 2006) or emulsion-based (Sultana et al., 2000; Hansen et al., 2002; Krasaekoopt et al., 2003; Crittenden et al., 2006; Muthukumarasamy et al., 2006) techniques.

2.5.1 Extrusion-based probiotic encapsulation

Extrusion is one of the most commonly used techniques for entrapping probiotics due to its ease, simplicity, and low cost. The gentle formulation conditions during extrusion ensure a high retention of cell viability and produces uniform capsules (King, 1995). The technique involves mixing a hydrocolloid solution with a live probiotic culture, then extruding the mixture through a syringe needle to form droplets which then free fall into a cross-linking or hardening solution (Figure 2.4) (King, 1995; Krasaekoopt et al., 2003). Wall material, hydrocolloid concentration, and size of the capsule all influence survival of the entrapped ingredient. For instance, Muthukumarasamy et al. (2006) looked at various wall materials for the encapsulation of probiotic *Lactobacillus reuteri* and measured the survival of the probiotic during gastric challenges (simulated gastric juice (SGJ), pH 1.5 for 2 h). Alginate (AL) (3.0% w/v), AL (2.0%, w/v) + starch (2.0%, w/v), κ-carrageenan (1.75%, w/v) + locust bean gum (0.75%, w/v), or xanthan (1.0%, w/v) + gellan (0.5%, w/v) were used as the wall material. They reported that AL and AL + starch provided the best protection against SGJ. The concentration of wall material and capsule size can also have an effect on the ability to protect the core ingredients. Lee and Heo (2000) investigated the influence AL concentration (2.0, 4.0, and 6.0% AL) and capsule size (1.03, 1.75, and 2.62 mm) had on the survival of *B. longum* in SGJ (pH 1.55). They reported increased survival of the

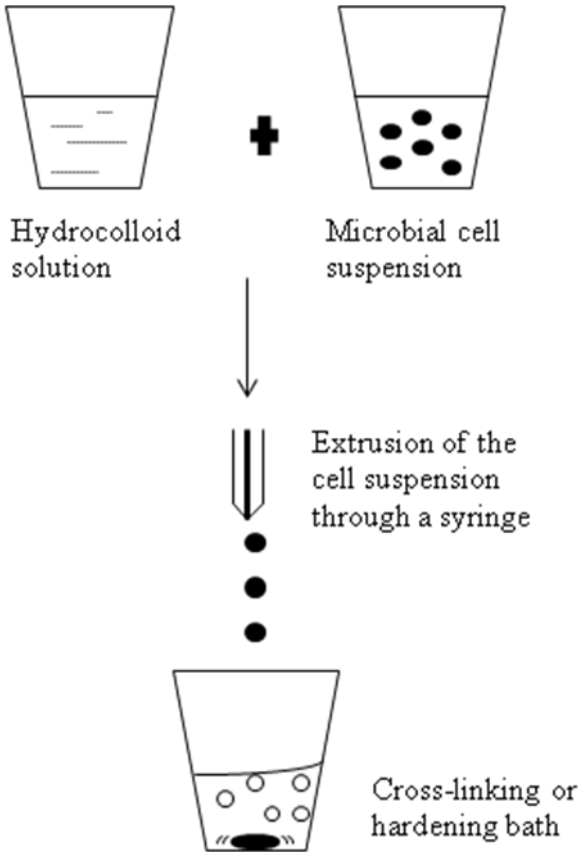

Figure 2.4 Process flow diagram for the encapsulation of probiotic bacteria using an extrusion technique (adapted from Krasaekoopt, 2004).

probiotic with increased AL concentration. Larger capsules were able to protect the probiotic better than small capsules produced. Muthukumarasamy et al. (2006) reported large capsules made by the extrusion technique (2-4 mm) provided better protection compared to smaller capsules produced via the emulsion technique (20-1000 μm).

The size of the capsule produced during extrusion depends on the diameter of the syringe needle, the hydrocolloid concentration, viscosity of the solution, and the distance between the syringe and cross-linking solution. The major factor influencing the capsule size is the diameter of the syringe needle used during extrusion. Muthukumarasamy et al. (2006) formed capsules using a 21gauge (G) needle with various wall materials, AL (2-3 mm), AL-starch (2-3 mm), κ-carrageenan-locust bean gum (3-4 mm), xanthan-gellan (2-3 mm). Lee and Heo (2000) used a 20 G needle to obtain AL capsules between the size of 1-2.6 mm. Chen et al. (2005) encapsulated probiotics in AL capsules with FOS and/or isomaltooliosaccharides and casein using a 31 G needle and produced a capsule size of ~0.5 mm. The shape of the capsule can also be affected by the biopolymers used and biopolymer concentration. Sandoval-Castilla et al. (2010) investigated various biopolymer compositions and their effect on diameter and sphericity of the formed capsules using AL and amidated low-methoxyl pectin blends. They reported that capsule mean sizes ranged from 0.71-0.93 mm using a 30.5 G needle. Additionally, when pectin was added it significantly increased the size of the capsules compared to AL alone. The sphericity of the capsule also was significantly influenced by the presence of pectin. When AL was present the sphericity of the capsules was higher. An important challenge for probiotic encapsulation is the ability to achieve high rate of survival during storage and gastrointestinal transit. Large capsule can achieve high cell loads within the capsule and provide increased survival, however when large capsules are produced the textural and sensorial properties of food products in which they are added can be negatively affected (Muthukumarasamy et al., 2006; Sandoval-Castilla et al., 2010).

Prebiotics can be added into the hydrocolloid solution along with the probiotics allowing them to be entrapped together, or prebiotics can be applied as an additional coating post capsule formation. The addition of prebiotics can enhance the survival of probiotics through the gastrointestinal tract. Iyer and Kailasapathy (2005) studied the

affect of adding inulin (Raftiline), a FOS (Raftilose), and high amylose corn starch (Hi-maize) to the probiotic capsule and reported that bacteria co-encapsulated with prebiotic had increased survival under *in vitro* acid conditions compared to non-encapsulated and encapsulated bacteria with no prebiotic added.

2.5.2 Emulsion-based probiotic encapsulation

Emulsion-based techniques are another common method employed for the encapsulation of probiotic bacteria. This technique consists of a mixture of immiscible liquids (typically oil and water) with the aqueous hydrocolloid phase and cell suspension representing the dispersed phase and the oil representing the continuous phase (Krasaekoopt et al., 2003; McClements et al., 2009). Typically, the continuous phase consists of vegetable oil such as soybean, sunflower, canola or corn. Emulsions are prepared by the addition of a small volume of the discontinuous (aqueous) phase to a larger volume of the continuous phase (oil), followed by homogenization to form a water-in-oil emulsion (Figure 2.5). Typically emulsifiers are also added (such as Tween 80) to the continuous phase to ensure homogeneity (Rokka and Rantamaki, 2010). Once the emulsion is formed a cross-linking agent is added to precipitate the water soluble hydrocolloids to form gel particles (microcapsules) within the oil (Krasaekoopt et al., 2003; Rokka and Rantamaki, 2010). This technique can produce capsules ranging in size between 25 µm - 2 mm. The size of the capsule is controlled by the speed of agitation and concentration of hydrocolloids. This technique can be beneficial due to its ability to produce smaller capsules than can be achieved via extrusion. It is generally accepted that capsules > 100 µm has a negative effect on textural and sensorial properties of the food products (Hansen et al., 2002; Sandoval-Castilla et al., 2010). However, small capsules have limited ability to protect the probiotic bacteria through gastrointestinal transit. Emulsion technology also produces a wide size distribution. Hansen et al. (2002) tested the ability of AL capsules prepared via emulsion technique to protect four *Bifidobacterium spp.* (*B. adolescentis* 15703T, *B. breve* 15700T, *B. lactis* Bb-12, and *B. longum* Bb-46) against SGJ (pH 2.0, 3.0 and 6.0). The capsules produced were 20 µm and 70 µm in size. They reported that the encapsulating the probiotics did not

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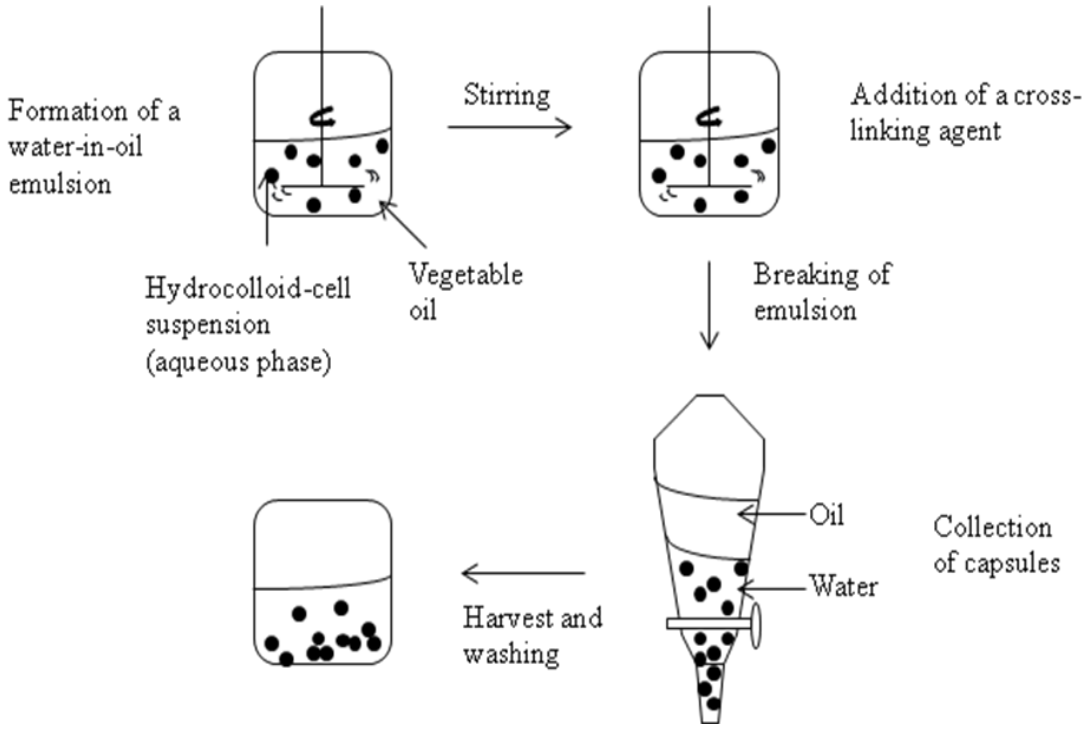

Figure 2.5 Process flow diagram for the encapsulation of probiotic bacteria using an emulsion technique (adapted from Krasaekoopt, 2003).

significantly improve survival within SGJ over the 2 h incubation. Sultana et al. (2000) investigated *L. acidophilus, L. casei,* and *B. infantis* probiotics in 2% AL emulsion-based capsules containing 2% Hi-maize resistant starch. With a capsule size ranging from 0.5 to 1 mm there was no significant increase in survival during SGJ challenge experiments.

Research in the current study will focus only on the use of extrusion processing for the encapsulation of prebiotic oligosaccharides and probiotics, in order to test the effect of wall material properties without having a confounding size effect of variable capsule distribution

2.6 Biopolymer mixtures as a wall material

Typically, probiotic encapsulation matrices are comprised of one or more hydrocolloid material (Chandramouli et al., 2004; Krasaekoopt et al., 2004; Reid et al., 2005), or may have additional coating layers of another material (Krasaekoopt et al., 2004; Iyer and Kailasapathy, 2005; Gbassi et al., 2009). The latter provides additional protection to entrapped viable microorganisms from processing and storage conditions, and during transit through the gastrointestinal tract. The present research investigates the potential of a wall matrix comprised of a protein-polysaccharide mixture (i.e., pea protein isolate and AL polysaccharides) to offer protection to the entrapped bacteria. In mixed systems, biopolymers depending on their electric charge will either undergo: a) segregative phase separation, in which the biopolymers do not interact due to electrostatic repulsive forces between reactive groups of similar charges; or b) associative phase separation, in which biopolymers come together due to electrostatic attractive forces between reactive groups of opposing charges (Dziezak, 1988; de Kruif et al., 2004; Barbosa-Canocas et al., 2005). Depending on the biopolymer conditions (e.g., concentration, ratio, biopolymer-type, reactive group present and molecular weight), solvent (e.g., pH, temperature and salts) and processing (e.g., degree of shear) conditions, the phase behaviour and structure of the resulting matrix can be tailored (Weinbreck et al., 2003a). Although food products containing probiotics have primarily targeted dairy products (e.g., yogurts); transitioning to an entirely plant-based matrix versus animal derived biopolymers may open up new markets and products where the use of animal derived proteins is restricted due to religious, dietary or moral preferences.

2.6.1 Alginate

Alginate is the most commonly used wall material for extrusion-based probiotic encapsulation (Krasaekoopt et al., 2003). Alginate is the salt of alginic acid which naturally occurs in marine brown algae (*Phaeophyceae)* as the main structural component. Alginate is also in the extracellular mucilages secreted by some bacterial species (Draget et al., 2006; Harnsilawat et al., 2006). Alginate is a linear polysaccharide of (1→4)-linked β-D- mannuronic acid (M) and (1→4) -linked α-L-guluronic acid (G) residues which are found in homo- or hetro-polymeric block sequences (Figure 2.6). Alginate polysaccharides are anionic in nature, remaining negatively charged over a wide pH range until their carboxyl reactive sites become fully protonated at pH 1.88 (Liu et al., 2009). The α-L-guluronic acid residues are highly sensitive to calcium salts, forming 'egg-box'-like junction zones which then lead to the formation of polysaccharide-based networks (Sabra and Deckwer, 2005). Alginates rich in G units form strong, dense and brittle gels, whereas ALs which have a high proportion of M units form soft, elastic gels.

Using alginates as an encapsulating agent has given mixed success in terms of offering protection to probiotic bacteria. Chandramouli et al. (2004) encapsulated *L. acidophilus* by extrusion using different AL concentrations (0.75-2.00%, w/v), $CaCl_2$ concentrations (0.1, 0.2, and 1.0 M) and bead size (200, 450, and 1000 µm). The authors found cell viability in SGJ increased with increasing capsule size (200-1000 µm) and AL concentration (0.75–2.0%, w/v), whereas $CaCl_2$ concentration showed no effect on viability of encapsulated cells. At higher capsule sizes (1000 µm vs. 450 µm) and higher AL concentrations (2.00% vs. 1.80% w/v) no significant increases in viability were observed, therefore the optimal conditions for entrapment of *L. acidophilus* (9 log CFU/mL) was determined to be with 1.80% (w/v) AL with a capsule size of 450 µm. Entrapment led to a 3 log reduction in bacterial counts, whereas non-encapsulated cells experienced a 5 log reduction when exposed to simulated gastric conditions at pH 2.0 for 3 h. Muthukumarasamy et al. (2006) investigated different wall materials for entrapping *L. reuteri* in order to protect cell viability during gastric challenges. These materials included: AL, AL plus starch, κ-carrageenan with locust bean gum, or xanthan

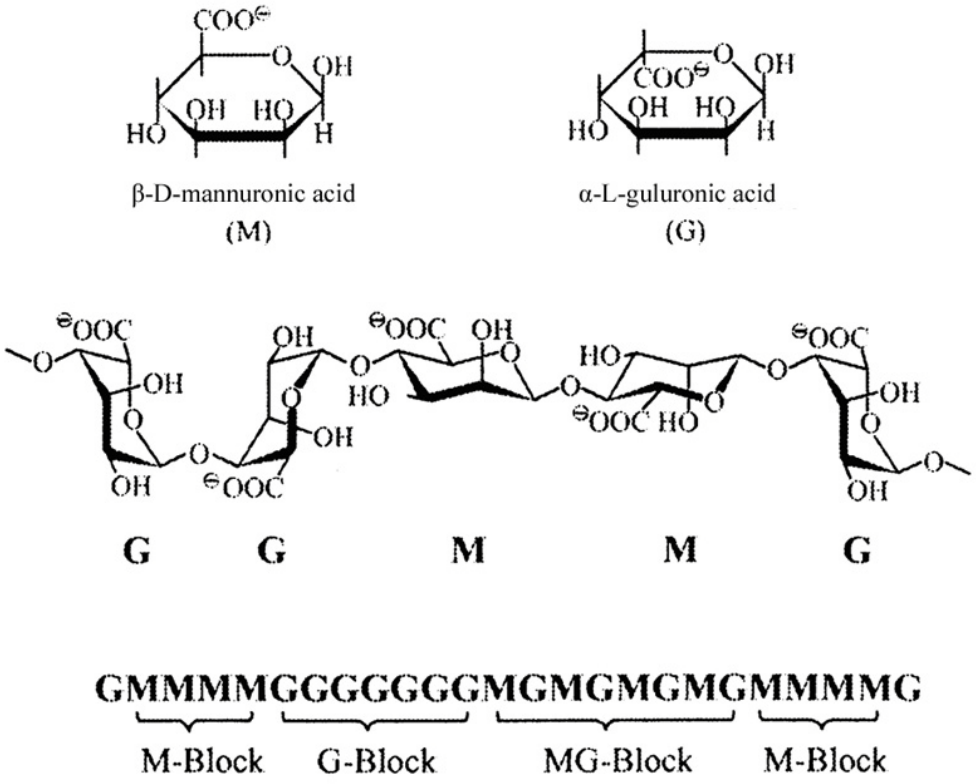

Figure 2.6 Schematic of homo- or hetro-polymeric sequences of (1→4)-linked β-D-mannuronic acid and (1→4)-linked α-L-guluronic acid residues of alginate (Davis et al., 2003).

with gellan. The capsules were prepared by either extrusion or emulsion techniques. They reported the survival of *L. reuteri* was significantly increased when encapsulated compared to non-encapsulated cells and that encapsulation by extrusion with AL or AL/starch provided the greatest protection against gastric juice, with less than 0.5 log reduction from initial cell counts. However, other studies have reported AL capsules do not significantly improve survival of entrapped probiotic bacteria. Hansen et al. (2002) reported AL capsules produced via an emulsion technique did not significantly improve the survival of *B. adolescentis, B. breve, B. lactis,* or *B. longum* compared to the non-encapsulated cells when subjected to SGJ (pH 2.0 and 3.0) for 2 h. Krasaekoopt et al. (2004) investigated the survival of *B. bifidum, L. acidophilus*, and *L. casei* in SGJ (pH 1.55) and sequentially placed into intestinal juice with and without bile salt. They reported no survival of *B. bifidum* either as encapsulated or as free cells in SGJ. They found that the survival rate of *L. acidophilus* in AL beads improved compared to non-encapsulated bacteria after sequential incubation in SGJ and intestinal juices with and without bile salt. However, the highest survival rate was observed when AL beads were coated with chitosan. For *L. casei* they found AL beads only slightly improved survival compared to non-encapsulated cells (1 log CFU/mL difference), however chitosan coated AL beads significantly improved survival. Krasaekoopt et al. (2004) does not report an explanation for the improved survival of chitosan coated beads compared to AL alone and other coating materials used. However, it is assumed chitosan improves the physical barrier separating the probiotic bacteria from the low acid of SGJ. This could be due to reduced pore size within the capsule; however no information was provided on the capsule's structure in this paper.

2.6.2 Pea protein isolates

Field peas (*Pisum sativum)* are comprised of ~20-30% protein, primarily consisting of salt-soluble globulins and water-soluble albumins (Schroeder, 1982). There are two major globulin proteins, legumin and vicilin which represent 65-80% of the extractable pea protein. Legumin is a hexameric 11S protein with a molecular weight between 350-400 kDa, whereas vicilin is a trimeric 7S protein with a molecular mass of 150 kDa (Ducel et al., 2004). The isoelectric point (pI) of a pea protein isolate material

is ~4.5, however depending on the extraction process pIs can range between 4.0 and 6.0 (Koyoro and Powers, 1987). Encapsulation using pea protein materials has been limited, with some research in the entrapment of oil (Ducel et al., 2004), ascorbic acid (Pierucci et al., 2006; Pereira et al., 2009), α-tocopherol (Pierucci et al., 2007), and β-carotene (De Graaf et al., 2001).

2.6.3 Pea protein isolate–alginate mixtures

The use of protein as an encapsulating agent has been previously reported to increase the survival of probiotics against SGJ (Reid et al., 2005; Kotikalapudi et al., 2010; Wood, 2010). Reid et al. (2005) encapsulated *L. rhamnosus* via extrusion using a 70:30 mixture of 12% whey protein isolate (WPI)-bacteria. The authors subjected the capsules to a dynamic gastrointestinal model which varied in pH from 4.4 to 2.0 over 90 min and found that encapsulated cells had a 2.4 log CFU/mL reduction where as free cells experienced a 4 log CFU/mL reduction in viable bacteria. Kotikalapudi et al. (2010) also encapsulated *L. acidophilus* within a PPI-AL. Kotikalapudi et al. (2010) investigated a panel of commercially available probiotics strains (*Lactobacillus acidophilus, Bifidobacterium longum subsp. infantis, B. catenulatum,* and *B. adolescentis*) for their acid resistance, bile resistance, adherence to Caco-2 cells, and carbon source utilization. The authors encapsulated *L. acidophilus* within a 4.0% PPI-0.5% AL capsule and subjected it to simulated gastric juice. They reported non-encapsulated *L. acidophilus* demonstrated the most resistance to SGJ (pH 2.0) compared to the other non-encapsulated probiotics strains, however it could not survive longer that 30 min. They found all probiotics were able to survive for 5 h in MRS media supplemented with 0.3% bile. The authors performed an *in vitro* adherence test of probiotics bacteria to Caco-2 cells as an indicator of which probiotic strain would have the best adherence to the intestinal wall and found that *L. acidophilus* adhered to the Caco-2 cells best. The ability to utilize carbon sources was also investigated and they found that all strains had highest growth on D-xylose; however, no test was performed on prebiotic FOS. Due to their findings, Kotikalapudi et al. encapsulated *L. acidophilus* within a 4.0% PPI-05% AL capsule and subjected it to SGJ (pH 2.0) for 2 h and reported that a capsule size ~3 mm was able to efficiently protect the probiotic bacteria,

with only 1log reduction in cell viability compared to unprotected cells which experienced > 6 log loss.

Wood (2010) investigated the growth of the probiotic, *Bifidobacterium adolescentis*, on various prebiotic sources (i.e., short chain FOS, inulin, FOS/inulin mixtures, glucose and a glucose-free maltooligosaccharide). *B. adolescentis* was better able to utilize short chain FOS as a carbon source. The probiotic was also investigated using various wall materials (i.e., AL, PPI-AL and WPI-AL mixtures) with and without short chain FOS by extrusion, followed by challenge studies within SGJ at pH 2.0 for 2 h. Capsules containing both WPI and PPI protein were able to protect *B. adolescentis*, whereas those with AL only offered minimum protection. The addition of FOS improved the survival of *B. adolescentis* within the PPI-AL capsules relative to without. Wood hypothesized that the protein-based capsules had greater survival than AL alone due to a less porous capsule wall as imaged by cryo-scanning electron microscopy, and that this would likely slow the diffusion of SGJ into the capsule.

Within the current study probiotic capsules will be produced using PPI-AL mixtures to build upon the initial work by Wood (2010) using controlled phase separation as a means to alter the wall design and improve probiotic survival under SGJ conditions and to enhance release within simulated intestinal fluid (SIF). AL and PPI interactions are investigated as a function of pH and biopolymer mixing ratio to determine their phase behaviour based on associative and segregative phase separation. The capsule wall material and processing conditions will be altered to improve the capsule design based on the total biopolymer concentration, addition of a prebiotic source (FOS) and altering needle gauge during extrusion processing. Changes to the capsule wall material and processing conditions will be tested to determine the impact on the physical properties of the capsule and ability to protect the probiotic in SGJ. In addition to work performed by Wood et al. (2010), pea is attractive as a plant protein source since it is commercially available and important to Saskatchewan economy.

2.7 Phase separation within PPI-AL mixtures

Associative phase separation (also known as complex coacervation) occurs when two biopolymers of opposing net charges experience electrostatic attraction, whereas segregative phase separation occurs when biopolymers carry similar net charges and

experience electrostatic repulsive forces (de Kruif et al., 2004). Depending on the biopolymer (e.g., concentration, ratio, biopolymer-type, reactive site present, linear charge density of reactive sites), solvent (e.g., pH, temperature and salt) and processing (e.g., degree and duration of shear) conditions, phase separation can be controlled. When using biopolymers as coating agents, phase separation can be used to tailor the wall properties and ultimately its protective potential for entrapped probiotics. Pea protein isolate assumes an overall positive charge at pHs below the pI (pH ~4.5), creating an environment where associative phase separation could ensue at pH<pI and segregative phase behaviour at pH>pI. A schematic representation describing various phase separating scenarios is given in Figure 2.7 (Goh et al., 2009). Typically in dilute solutions, biopolymers remain suspended and co-soluble, with little interactions regardless of charge (Figure 2.7a). As the total biopolymer concentration rises, biopolymers of similar net charge repel one another, separating into both a protein- and polysaccharide-rich phase (Figure 2.7b). If the biopolymers are of opposing charges, phase separation leads to the formation of both a solvent-rich and biopolymer-rich (protein + polysaccharide) phase. The latter is thought to occur in two structure forming events following nucleation and growth-type kinetics (Weinbreck et al., 2003a; Girard et al., 2004): first, with the formation of soluble complexes (Figure 2.7c); and then with the formation of insoluble complexes (Figure 2.7d) (Ye, 2008; Goh et al., 2009). Depending on the strength and degree of electrostatic attractive forces involved during coacervation, a coacervate structure or precipitate may form. It should be noted that coacervates are not the same as precipitates. Coacervate structure tends to be less compact, entraps more solvent then precipitates, is completely reversible and remains suspended within the biopolymer–rich phase. In contrast, due to their stronger electrostatic interactions precipitates are more compact, entrap less solvent, are less reversible, and tend to fall out of solution rather quickly (Singh et al., 2007).

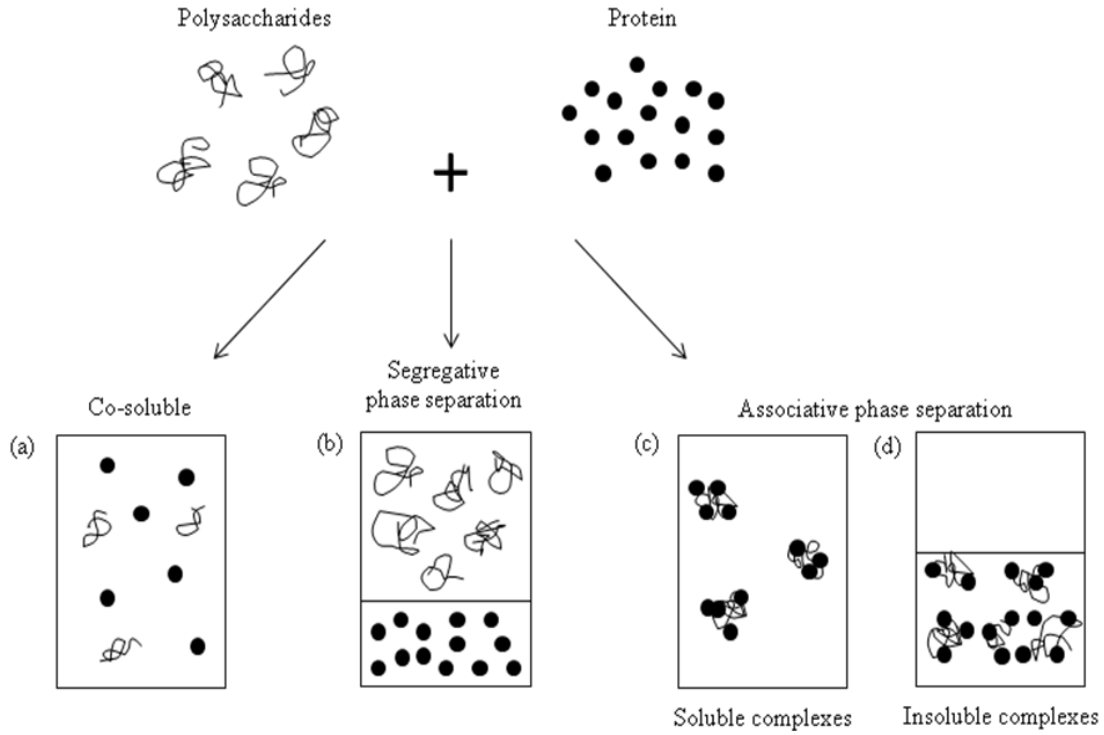

Figure 2.7 Schematic diagram representing various phase separation behaviours in admixtures of proteins and polysaccharides (adapted from Goh et al., 2009).

2.7.1 Factors affecting phase separation

Phase separation in biopolymer mixtures is primarily influenced by factors that disrupt the electrostatic attractive or repulsive forces occurring between the protein and polysaccharides, such as pH, salt, and biopolymer mixing ratio. The latter refers to the number of reactive sites (positive and negative) available to interact with the neighbouring biopolymer. These factors are discussed below:

pH: Solvent pH plays a significant role in controlling the number of ionizable reactive sites along the biopolymer's backbone: this is particularly important in the case of proteins where the molecule can assume a positive charge at pH < pI and a negative charge at pH > pI. Hence, in the presence of an anionic polysaccharide such as AL associative phase separation with the positively charged protein can occur at pH < pI to form a soluble/insoluble complex. In the case of a protein and polysaccharide mixture complex coacervation occurs over a narrow pH range and generally occurs between the pK_a of the polysaccharide and the pI of the protein (Tolstoguzov, 1997; Ducel et al., 2004). During complex formation there are two main pH-induced structure forming events associated with the formation of soluble and insoluble complexes. The former event (denoted as pH_c) occurs at pH corresponding to the first experimentally detectable increase in turbidity during a pH titration and is the initial site of soluble complex formation. This is followed by a second structure forming event at $pH_{\phi 1}$, associated with the formation of insoluble complexes and a large rise in turbidity. Optimal complex formation is considered to occur at pH where both biopolymers reach their electrical equivalence, donated pH_{opt}, which is followed by dissolution of the complexes at lower pH ($pH_{\phi 2}$), due to protonation of reactive groups on the polysaccharide backbone (Liu et al., 2009). For instance, Liu et al. (2009) investigated the effect of pH on the complex formation between PPI and gum Arabic (biopolymer mixing ratio, 1:1 PPI-gum Arabic; and polymer concentration, 0.05% (w/w)) and reported that structure forming transitions pH_c, $pH_{\phi 1}$, pH_{opt}, and $pH_{\phi 2}$ occurred at pH 4.2, 3.7, 3.5, and 2.5, respectively. Below a pH of 2.5 the carboxyl groups on gum Arabic become protonated (pK_a = 2.2). In both cases, above and below pH_c and $pH_{\phi 2}$, the biopolymers carried similar charges

resulting in electrostatic repulsion to occur. This inhibited complex coacervation. However, in some cases, such as in the presence of a strongly charged polysaccharide (e.g. carrageenan), associative phase separation may occur when the overall net charge on both biopolymers is negative. This phenomenon has been reported for WPI and carrageenan (Weinbreck et al., 2004a), pectin and WPI (Zaleska et al., 2000), and bovine serum albumin and poly(dimethyldiallylammonium chloride (Wen and Dubin, 1997), and is thought to be attributed to the interactions with localized positively charged patches on the protein's surface (Schmitt et al., 1998; Doublier et al., 2000; de Kruif et al., 2004; Weinbreck et al., 2004a).

Salt: As previously stated, the charge on the biopolymers is extremely important and determines the intensity with which the biopolymers interact or repel one another. The addition of salt is another major factor which influences the formation of complexes and affects the screening of charges present on the biopolymers (Schmitt et al., 1999). In general, the addition of salt at low concentrations helps to promote electrostatic interactions between biopolymers, as ions associate with protein structure alter its conformation to expose additional charged groups. However, at high salt concentrations, ions screen charges along the biopolymers, resulting in reduced electrostatic attractive forces which suppresses complex coacervation (Weinbreck et al., 2003a). Depending on the biopolymers used, the amount of salt that must be added to promote complex coacervation varies. Weinbreck et al. (2003a) reported that a concentration of <50 mM NaCl improved complex coacervation between WPI and gum Arabic, whereas at levels >50 mM NaCl complexation was inhibited due to this screening effect. Liu et al. (2010a) reported that 100 mM NaCl suppressed complex formation between pea protein and gum Arabic mixtures.

Biopolymer mixing ratio: Biopolymer mixing ratios also has a significant effect on phase separation. Due to the fixed charge density of proteins and polysaccharides at a given pH, by adjusting the biopolymer mixing ratio, the total charges available for coacervate formation can be modified (Liu et al., 2009). Weinbreck et al. (2003b)

investigated the effect of biopolymer mixing ratios on complex coacervation between WPI and the exocellular polysaccharide B40 (ESP B40). These researchers found that the formation of soluble complexes (pH_c) was independent of mixing ratios between 1:1 and 25:1 (WPI-ESP B40). This stability was thought due to the interaction of a simple polysaccharide chain with given amount to protein. Mattisson et al. (1995) and Weinbreck et al. (2004a) reported a similar pH_c independence of mixing ratios for bovine serum albumin-poly (dimethyl diallyl ammonium chloride) (PDMDAAC) and WPI-carrageenan, respectively. Alternatively, some researchers have found pH_c to be dependent on mixing ratio, for instance in systems of gelatin–agar (Singh et al., 2007) and PPI–gum Arabic (Liu et al., 2009). Liu et al. (2009) reported pH_c to be dependent on biopolymer mixing ratios of <4:1 after which pH_c became constant at higher biopolymer mixing ratios, suggesting that this was due to complexation of gum Arabic with protein-protein aggregates rather than single molecules as reported by Weinbreck et al. (2003b). Weinbreck et al. (2003b) also reported that for WPI-ESP B40 mixtures, the formation of insoluble complexes ($pH_{\phi 1}$) was found to be ratio dependent where $pH_{\phi 1}$ shifted to higher pHs as ratios increased from 1:1 to 9:1 WPI-ESP B40, before becoming constant at ratios >9:1. At the plateau, charged groups on the protein were presumed saturated by the polysaccharide present in solution (Liu et al., 2009).

3. Materials and Methods

3.1 Materials

For this research, pea flour (PF) (Fiesta Flour, lot F147X, 2008) was kindly donated by Parrheim Foods (Saskatoon, SK) and was used to produce a 'native' pea protein isolate (PPI^n). A commercial pea protein isolate (PPI^c) (ProPulse) was kindly donated by Nutri-Pea Ltd. (Portage la Prairie, MB).

Orafti® P95 (FOS, DP 2-8 (6.8% D-glucose + D-fructose + D-sucrose) was kindly supplied by ORAFTI S.A. (Oreye, Belgium).

The following materials and chemicals were purchased from Sigma-Aldrich (Oakville, ON): acetic acid, alginic acid sodium salt from brown algae (viscosity of 2.0%, lot 065K0237), L-cysteine hydrochloride monohydrate, D-fructose, glucono-δ-lactone (GDL), α-D-glucose, pancreatin (amylase, 108 USP units/mg; protease, 100 USP units/mg; lipase and ribonuclease, unknown concentrations; batch 039K1579), phenol, and Tween 80.

Hydrochloric acid (6 N) (HCl) was purchased from Ricca Chemical Company (Arlington, TX).

Calcium chloride dehydrate ($CaCl_2$), N-point indicator, petroleum ether, potassium chloride (KCl), sodium acetic acid ($Na^+CH_3COO^-$), sodium chloride (NaCl), and sodium phosphate dibasic heptahydrate (Na_2HPO_4) were purchased from EMD Chemicals (Darmstadt, Germany).

Sodium hydroxide solution (NaOH, 50% w/w) and sulfuric acid (H_2SO_4) were purchased from Fisher Scientific (Fair Lawn, NJ).

Protein dye reagent (Coomassie brilliant blue G-250 dye) and bovine serum albumin (BSA) (2.0 µg/µL) were purchased from Bio-Rad laboratories, Inc. (Hercules, CA).

Glycerol, dipotassium hydrogen phosphate ($K_2H_2PO_4$), and sodium bicarbonate ($NaHCO_3$) were purchased from BDH Inc. (Toronto, ON).

Boric acid and Kjeldahl catalyst (Kjel-Pak Mixture #200) were purchased from VWR (Edmonton, AB).

Bifidobacterium adolescentis ATCC 15703 was purchased from American Type Culture Collection (Manassas, VA).

Reinforced clostridial media (RCM), and De Man, Rogosa, Sharpe (MRS) media were purchased from Oxoid Ltd. (Basingstoke, England).

Bacto™ Peptone, Difco oxgall dehydrated fresh bile, and granulated Difco agar were purchased from BD (Becton, Dickson and Company, Sparks, MD).

The following anaerobic gases were purchased from Praxair (Saskatoon, SK): ultra high purity nitrogen and a mixed system containing 80% nitrogen, 10% hydrogen and 10% carbon dioxide.

The water used in this research labelled as ddH_2O was produced from a Millipore milli-Q™ water system (Millipore Corporation, Milford, MA) unless otherwise stated.

3.2 Proximate analysis

Proximate analyses on all materials (PF, PPI^n, PPI^c, and AL) were performed according to the Association of Official Analytical Chemists (AOAC, 2003). Methods 925.10, 923.03, 920.87 and 920.85 for moisture, ash, crude protein and lipid (% wet weight basis), respectively. Sample carbohydrate content was determined as percent differential from 100%. All chemical analyses were performed in triplicate. Individual methods are summarized briefly below.

3.2.1 Percent moisture

Percent moisture was determined gravimetrically using a forced air drying oven (Fisher Scientific, Ottawa, ON) and an analytical balance (Metler, Columbus, OH). Samples of ~1 g (± 0.0002 g) were weighed into pre-dried (~16 h at 102 ± 2°C) aluminum dishes (VWR International, Mississauga, ON). Samples were dried overnight (16 h) at 102°C, and then placed within a glass desiccator containing drierite granules (Fisher Scientific, Ottawa, ON) to cool for 30 min, prior to gravimetric analysis. Percent moisture was calculated using the following equation:

$$\%\, Moisture = \frac{(Wi - Wf)}{Ws} \times 100\% \qquad \text{(eq. 1)}$$

where, *Wi* was the initial weight of the sample plus aluminum dish before drying (g), *Wf* was the final weight of the sample plus dish (g) after drying, and *Ws* was the original sample weight (g).

3.2.2 Percent ash

A muffle furnace (Fisher Scientific, Ottawa, ON) was used to determine the ash content of all samples. Crucibles were pre-ashed in a muffle furnace (~550°C) for 3 h and then cooled to room temperature (30 min) within a glass desiccator containing drierite. Samples were weighed ~1 g (± 0.0002 g) using an analytical balance and placed directly into the pre-weighed pre-dried crucibles. Samples were heated within the muffle furnace at ~550°C overnight (~16 h) and then placed in a desiccator to cool to room temperature (30 min) prior to gravimetric analysis. The percent ash content was determined using the follow equation:

$$\%\, Ash = \frac{(Wi - Wf)}{Ws} \times 100\% \qquad \text{(eq. 2)}$$

where, *Wi* was the initial weight of the sample plus the crucible before pyrolysis (g), *Wf* was the final weight of the sample plus the crucible (g) after pyrolysis, and *Ws* was the original sample weight (g).

3.2.3 Percent lipid

A Goldfisch apparatus (Labconco, Kansas City, MO) was used to determine the sample lipid content. Lipid extraction beakers were dried for 16 h in a forced air drying oven at ~102°C, and then placed in a glass desiccator containing dierite to cool to room temperature and weighed using an analytical balance. The materials were weighed (~3 g ± 0.0002 g) on a Whatman #4 filter paper (9.0 cm), folded and placed into individual

20 mm x 80 mm cellulose extraction thimbles (Whatman International Ltd, Maidstone, England). Thimbles were placed in a metal holder and fitted into the Goldfisch apparatus. Approximately 50 mL of petroleum ether was added to the pre-weighed lipid extraction beakers (150 mL) and the extraction process was initiated (5-6 drops/sec). The extraction time employed was ~6 h. Petroleum ether was reclaimed from the samples and the beakers were allowed to cool to room temperature in a fume hood. The lipid extraction beakers were then placed in a forced air drying oven (102°C) overnight (~16 h). After cooling to room temperature (desiccator), sample lipid content was determined gravimetrically. The lipid content was determined using the following equation:

$$\%\,Lipid = \frac{(Wi - Wf)}{Ws} \times 100\% \qquad \text{(eq. 3)}$$

where, *Wi* was the initial weight of the sample plus the lipid extraction flask prior to extraction (g), *Wf* was the final weight of the sample plus lipid extraction flask (g) after extraction, and *Ws* was the original sample weight (g).

3.2.4 Percent protein

The protein content of samples was determined using the Kjeldahl digestion method for nitrogen determination. In brief, 0.5000 ± 0.0002 g of sample was weighed on nitrogen-free weighing paper (9.0 cm; VWR 410), folded and placed within a glass digestion flask. Concentrated H_2SO_4 (20.0 mL) and a catalyst tablet (10.0 g K_2SO_4, 0.3 g $CuSO_4$: Kjel-Pak Mixture #200, VWR International, Mississauga, ON) was added to the flask, which was then placed on a Büchi Digestion Unit (Model K-435; Büchi Analytical Inc.; Potfach, Switzerland) for ~3 h. Individual solutions were cooled to room temperature, followed by dilution with 80.0 mL of ddH_2O. The resulting solutions were steam distillated (Buchi Distillation Unit Model B-324; Büchi Analytical Inc.; Potfach, Switzerland) with the automatic addition of 80.0 mL of 30% (w/v) NaOH and 25.0 mL of 4% (w/v) boric acid. Approximately 100-150 mL of the distillate was collected using a 250 mL Erlenmeyer flask containing 3-4 drops of N-point indicator

(methanol/water/bromocresol green/methyl red). Samples were titrated with 0.2000 N ± 0.0002 HCl until the solution colour turned light pink. The protein content of the sample was calculated as follows:

$$\%Nitrogen = \frac{((Vs - Vb) \times 0.2N\ HCl \times 14.007 g/mol)}{Ws} \times 100\% \qquad \text{(eq. 4)}$$

where, *Vs* and *Vb* was the volume of HCl (mL) used to titrate the sample and the blank, respectively, and *Ws* was the weight of the sample (mg). Percent protein was determined by multiplying the % Nitrogen by 6.25 (conversion factor).

3.3 Prebiotic characterization using high performance liquid chromatography

Carbohydrate analysis of P95 was performed using high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) (Dionex Bio LC 4000, Dionex, Sunnyvale, CA). A CarboPac PA1 (4 x 250 mm) anion exchange column was used in series with a CarboPac Guard PA-1 (4 x 50 mm) column (both from Dionex) for carbohydrate separation. All samples were syringe filtered (13 mm diameter, 0.2 µm pore size, Chromatographic Specialities Inc., Brockville, ON) prior to HPAEC-PAD analysis. The sample injection volume was 50 µL. Oligosaccharides were detected by PAD using the following potentials and durations: E_1 = 50 mV, T_1 = 0.299s; E_2 = 600 mV, T_2 = 0.299s; E_3 = -800 mV, T_3 = 0.499s. Data analysis was afforded using WMSP Chromatography Manager Software (Nanning Weimalong Chromatograph Science Technology Co., China). The mobile phase consisted of three eluents: eluent A, 100 mM NaOH; eluent B, 100 mM NaOH and 250 mM sodium acetate; and eluent C, 300 mM NaOH. The following gradient program was used to achieve carbohydrate separation: initial, 100% eluent A for 8 min, followed by a linear gradient to 100% eluent B at 60 min, followed by a rapid change to 100% eluent C at 61 min, which was held for 29 min. At 91 min a rapid change to 100% eluent A occurred and was held until 120 min. The flow rate was maintained at 1.0 mL/min and the column pressure was approximately 1200 psi.

3.4 pH determination

Sample pH for study 1 was determined using an Accumat pH meter (Fisher Scientific, Ottawa, ON), whereas for studies 2 and 3 an Orion 3-Star pH meter (Thermo Scientific, Waltham, MA) was employed.

3.5 Conductivity determination

Sample conductivity was measured using an Orion 3-Star conductivity meter with a dura probe 4 electrode conductivity cell (Thermo Scientific, Waltham, MA).

3.6 Investigation of the phase behaviour of pea protein isolates–alginate mixtures as a function of pH and biopolymer mixing ratio (Study 1)

3.6.1 Preparation of pea protein isolates

Preparation of PPI^n from PF was performed using methods modified from Bora et al. (1994). Briefly, approximately 400 g of PF was dispersed in 2.4 L of 50 mM phosphate ($K_2H_2PO_4$) buffer (pH 7.20) containing 0.5 M NaCl at a ratio of 6 mL buffer to 1 g PF, with continuous stirring (Ikamag Ret-G, IKA Labortechnik, Germany) for 1 h at room temperature (21-23^{o}C). The dispersion was centrifuged at 18,600 x *g* for 15 min at 4°C (Bechman J2-HC centrifuge; Bechman Coulter Canada Inc., Mississauga, ON). The pellet was discarded and the supernatant was decanted and filtered through glass wool to remove large residual particles. The resulting filtrate was diluted with 5 volumes of cold (4°C) ddH_2O and adjusted to pH 4.50 using 2 N HCl. The solution was left to stand overnight to promote settling of the salt-soluble globulin proteins (e.g., legumin and vicilin). The majority of the solution was decanted to leave a concentrated protein slurry, which was then centrifuged (18,600 x *g*, 15 min, 4°C) to yield a protein-rich pellet. The pellet was washed with approximately 50 mL of ddH_2O, followed by a second centrifugation. The resulting pellet was suspended in 50 mL of ddH_2O and dialyzed (Spectro/Por tubing, 6-8 kDa limit, Spectrum Medical Industries, Inc., Rancho Dominguez, CA) employing 15-20 L of ddH_2O (4^{o}C) with water changing every hour

for the first 5 h and then twice a day until a conductivity of <1.5 µS/cm (~72 h) was reached. The de-salted protein solution was then freeze dried (Labconco Freezone 6 Freeze dryer; Labconco Corporation, Kansas City, MO) for ~72 h. The resulting powder was a light tan colour and was sealed in a plastic screw capped 250 mL container and stored at -20^{o}C.

3.6.2 Turbidimetric acid pH titrations

Turbidimetric acid pH titrations of homogenous and mixed PPI^{n} and AL systems were performed using the method of Liu et al. (2009) to identify critical structure forming events (pH_c, $pH_{\phi1}$, pH_{opt} and $pH_{\phi2}$), and, biopolymer and pH conditions where associative and segregative phase separation occurred. All analyses were made at room temperature and at a total biopolymer concentration of 0.1% (w/v). In brief, a PPI^{n} stock solution was prepared by dissolving the powder in ddH_2O, adjusting the pH to 8.00 using 1 M NaOH, and stirring for 2 h at room temperature using a mechanical stirrer to help facilitate protein solubility. In the case of the AL stock solution, the powder was dissolved in ddH_2O at room temperature for 30 min under constant mechanical stirring. Biopolymer solutions were mixed to obtain the following PPI^{n}-AL (v/v) mixing ratios: 1:1, 2:1, 4:1, 8:1, 12:1 and 20:1. After mixing solution pH was adjusted to pH 8.00 using 0.1 N NaOH. Homogenous PPI^{n} and AL solutions were prepared as controls, at the same protein (or polysaccharide) concentration used in the mixed system. For example, for the aforementioned PPI^{n}-AL mixing ratios, homogenous PPI^{n} concentrations were prepared at 0.050, 0.067, 0.080, 0.089, 0.920, and 0.095% (w/v), respectively. Acidification of the homogenous and mixed systems from pH 8.00 to 1.50 was performed using a combination of 0.06% (w/v) glucono-δ-lactone (GDL) and a gradient of HCl. The internal acidifier (GDL) slowly lowers the pH from 8.00 to ~6.00 followed by the drop wise addition of a gradient of HCl concentrations to minimize sample dilution: 0.05 M HCl for a pH >4.90; 0.5 M HCl for the pH range of 2.80 - 4.90; and 2 M HCl for the pH range of 1.50-2.80. Conductivity was monitored during turbidimetric experiments for mixing ratios of 1:1 and 20:1 PPI-AL. Changes in optical density (O.D.) over the experimental pH range of 7.00 to 1.50 was determined using a ultraviolet-visible (scanning) spectrophotometer (Genesys 10, Thermo Scientific, Fair

Lawn, NJ) at 600 nm employing 1 cm path length plastic cuvettes. Critical pHs corresponding to structure-forming events (pH_c and $pH_{\phi 1}$) were determined as the intersection between two curve tangents as described by Weinbreck et al. (2003a) and Liu et al. (2009). The pH where maximum O.D. occurred was denoted as pH_{opt}. All turbidity profiles were prepared in triplicate, and the associated critical pH values were reported as the mean value ± one standard deviation.

3.6.3 Electrophoretic mobility

Electrophoretic mobility (U_E), that is, the velocity of a particle within an electric field for homogenous and mixed PPI^n-AL solutions were investigated as a function of pH (7.00-1.50) using a Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA). Mixed systems were tested as a function of the biopolymer mixing ratio: 1:1, 2:1, 4:1, 8:1, 12:1 and 20:1 PPI^n-AL. Homogenous PPI^n solutions were tested at concentrations of 0.050, 0.089 and 0.095% (w/v) which corresponded to the 1:1, 8:1 and 20:1 PPI^n-AL mixing ratios. Alginate solutions were tested at a concentration of 0.050% (w/v) which corresponded to the 1:1 PPI^n-AL mixing ratio. All samples were prepared (section 3.6.2) at a total biopolymer concentration of 0.1% (w/v), and were measured at room temperature. Solutions were acidified using an HCl concentration gradient as described in section 3.6.2, and were measured at 0.5 pH increments between pH 7.00 and 1.50. Electrophoretic mobility was calculated from the zeta potential (ς), using the Henry equation:

$$U_E = \frac{2\varepsilon \times \zeta \times f(\kappa\alpha)}{3\eta} \qquad \text{(eq. 5)}$$

where η was the dispersion viscosity, ε was the permittivity, and $f(\kappa\alpha)$ was a function related to the ratio of particle radius (α) and the Debye length (κ). Using the Smoluchowski approximation, $f(\kappa\alpha)$ was 1.5. All measurements were made in triplicate, and were reported as the mean value ± one standard deviation.

3.6.4 Statistics

A one-way analysis of variance (ANOVA) with a Scheffe post-hoc test was used to measure the statistical difference in state diagrams of critical pH values corresponding to structure forming events (pH_c, $pH_{\phi 1}$, and pH_{opt}) as a function of the biopolymer mixing ratio. All statistical analyses were done using SPSS software (SPSS Inc., Ver. 17, 2007, Chicago, IL).

3.7 Entrapment of *Bifidobacterium adolescentis* within pea protein isolate–alginate capsules (Study 2).

3.7.1 Microorganism

Bifidobacterium adolescentis ATCC 15703 (American Type Culture Collection, Manassas, VA) was chosen for this research because of its acid sensitivity and its ability to utilize FOS. The organism was stored at -70°C in a 1:1 (v:v) suspension of glycerol and MRS broth. The cultures were streaked onto RCM agar plates supplemented with 0.05% (v/v) L-cysteine hydrochloride monohydrate (RCM-cys) and incubated at 37°C under anaerobic conditions (80% N_2, 10% CO_2, and 10% H_2) using an anaerobic chamber (Forma Scientific Inc, Marietta, GA).

3.7.2 Growth curve for *Bifidobacterium adolescentis*

Growth experiments were conducted in RCM-cys broth to determine the time required for stationary growth to be reached; along with the corresponding cell concentrations. Side arm flasks containing 100 mL of RCM-cys broth (prepared according to manufacturer's instructions) were autoclaved at 121°C for 15 min, and then placed in the anaerobic chamber to equilibrate overnight. Culture tubes was prepared by inoculating two pure colonies of *B. adolescentis* in 5 mL of sterile RCM-cys broth, followed by incubation at 37^{o}C for 24 h under anaerobic conditions. A 100 µL aliquot of the starter culture was used to inoculate the side arm flask, which was incubated under anaerobic conditions for 40 h at 37^{o}C. The optical density (600 nm) of the suspensions was measured using a Sequoia-Turner Model 340 spectrophotometer (Pegasus

Scientific, Rockville, MD) every 40 min for 8 h (during the lag phase and start of the log phase of growth); every 20 min from 8-15.5 h (during the log phase of growth); and every 40 min from 15.5-21.5 h and then every hour from 21.5-26.5 h (during the stationary phase of growth). A final reading after 40 h of growth was also taken. Experiments were conducted in duplicate.

Aliquots (100 μL) of suspensions were removed from the side arm flasks after 15, 20, and 24 h of incubation and serially diluted with peptone saline (PS: 8.5 g/L NaCl and 1.0 g/L Bacto™ Peptone). Cell concentrations were determined by spread plating onto RCM-cys agar plates. Cell counts were determined after 48 h of incubation at 37°C under anaerobic conditions. Experiments were conducted in duplicate.

3.7.3 Preparation of *Bifidobacterium adolescentis* for encapsulation

Two isolated colonies were inoculated into 5.0 mL of RCM-cys broth and incubated at 37°C under anaerobic conditions. After 20 h of growth (stationary phase), cells were harvested by centrifugation (1, 900 x *g*, 10 min at 4°C), washed with phosphate buffer saline (PBS; 0.01 M Na_2HPO_4, 0.137 M NaCl, 2.68 mM KCl, pH 7.0), centrifuged, and suspended in PS to a final cell concentration of 8-9 log CFU/mL.

3.7.4 Encapsulation of *Bifidobacterium adolescentis* within PPI^n-AL and PPI^c-AL capsules

This study was designed to test the effect of native versus commercial PPI as it related to both forming the capsule and protecting *B. adolescentis* during pH challenge experiments. Wall material solutions (prepared at 4:1 PPI-AL) for encapsulation of *B. adolescentis* were prepared using 2.0% (w/w) PPI^n-0.5% (w/w) AL and 2.0% (w/w) PPI^c-0.5% (w/w) AL mixtures. Capsules were prepared using slightly different procedures for PPI^n and PPI^c due to inherent differences in protein solubility. Capsule preparation methodologies were as follows:

(a) PPI^n-AL capsules: Capsules were prepared using a modification of the method of (Krasaekoopt et al., 2003) based on an extrusion process. The wall material was prepared by first dissolving PPI^n (weight adjusted for protein content) in 80 g

ddH_2O at pH 8.00 with mechanical stirring for 2 h. The PPI^n solution was then pH adjusted to 7.00 using 1 M HCl, followed by the addition of the required amount of AL. The resulting solution was heated to 80^oC for 30 min, and then cooled to room temperature in a water bath. The final solution was corrected (by weight) for water loss during heating by the addition of ddH_2O. A mixture containing 1.0 g of washed *B. adolescentis* cells in PS (8-9 log CFU/mL) and 18.0 g of the prepared 2.0% (w/w) PPI^n-0.5% (w/w) AL solution was first mixed, then extruded by hand through a 20 gauge (G) needle (25.4 mm in length, 0.584 mm internal diameter; BD, Sparks, MD) and dropped 30 mm into 30 mL of a filter sterilized cross-linking solution (1.0% $CaCl_2$ + 1.0% (w/v) Tween 80). The resulting capsules were mechanically stirred (150 rpm) for 30 min at room temperature to afford hardening, and were then washed twice with PS by decanting. Capsules were used immediately after preparation in survivability studies. Encapsulation experiments were performed in triplicate.

(b) PPI^c-AL capsules: Capsules were prepared by dispersing PPI^c (weight adjusted for protein content) in 80 g of ddH_2O, followed by pH adjustment to 10.00 with 1.0 M NaOH. The resulting mixture was heated to 80^oC with constant mechanical stirring for 30 min. The resulting solution was allowed to cool to room temperature using a water bath and neutralized to pH 7.00 using 1 M HCl. Alginate powder was added, followed by heating at 80^oC for 30 min with constant mechanical stirring. The resulting biopolymer mixture was cooled to room temperature using a water bath, followed by addition of ddH_2O to correct for water loss (by weight) during heating. The mixing of the wall material with *B. adolescentis* cells, and capsule formation was identical to the procedures presented in section (a). Encapsulation experiments were performed in triplicate.

3.7.5 Survival of free and encapsulated *Bifidobacterium adolescentis* in simulated gastric juice at pH 2.0

The ability of free and encapsulated *B. adolescentis* to survive in simulated gastric juice (SGJ; 0.08 M HCl and 0.2% NaCl (w/v)) were tested at pH 2.0 (Rao et al., 1989). Aliquots of 0.5 mL of free or encapsulated cells (8-9 log CFU/mL) in PS were added to 9 glass test tubes (18 mm x 150 mm) containing 9.5 mL of tempered SGJ and

incubated at 37°C for 2 h. Aliquots (0.5 mL) of free or encapsulated cells were removed every 5 min for the first 30 min, and then every 30 min for a total of 2 h. Once removed from the incubator, the samples were neutralized to pH 7.0 using 0.05 M NaOH and transferred into 9.5 mL of PS. For encapsulated systems, samples were homogenized (Omni International Inc., Marietta, GA) at 9,000 rpm for 30 s to break up the capsule wall. To quantify the number of surviving bacteria, aliquots of the culture medium were serially diluted in PS and spread plated onto RCM-cys agar. The plates were incubated at 37°C in an anaerobic chamber for 48 h and enumerated. A D-value was determined which represents the amount of time for 1-log reduction in cell numbers to occur. The D-value was calculated using the equation, $D = t/n$ where $n = (\log N_o - \log N_t) = 1 \log_{10}$ reduction of the cells number, D = Decimal reduction time (min) at pH 2.0, N_o= bacteria at 0 time, N_t = surviving bacteria after an exposure time, t (min) (Kotikalapudi et al., 2010). These experiments were performed in triplicate.

3.8 Encapsulation of *Bifidobacterium adolescentis* within PPI^c-AL capsules: effect of wall characteristics and processing conditions on wall physical properties and their protective nature (Study 3)

3.8.1 Effect of biopolymer concentration and mixing ratio, prebiotic levels and needle gauge size on the PPI^c-AL capsule design

Capsules were prepared using a similar method as described in section 3.7.4, with the following total biopolymer concentration and mixing ratios: 2.5% (w/w) (2.0% PPI^c-0.5% AL – or 4:1), 4.5% (w/w) (4.0% PPI^c-0.5% AL – or 8:1) and 6.5% (w/w) (6.0% PPI^c-0.5% AL – or 12:1). The prebiotic (FOS) concentrations used were; 0, 0.19 (1.0%), 0.38 (2.0%), and 0.57 (3.0%) g, and the needle gauge sizes were 20G (0.584 mm i.d.) and 27G (0.191 mm i.d.). Bacterial cells, biopolymer mixture and prebiotic were mixed together prior to extrusion. All capsule designs were prepared in triplicate batches for statistical purposes.

3.8.2 Capsule size

Wet capsules containing *B. adolescentis* were sized using a laser scattering particle size distribution analyzer (PSA) (Horiban Instruments Inc., Irvine, CA). The PSA measures particle size distributions between 0.01-3000 µm by laser diffraction to determine particle geometric mean and variance. All experiments were done in duplicate.

3.8.3 Capsule protein content

Protein retention within the capsule following cross-linking was determined by measuring protein levels present in the cross-linking and wash solutions post cross-linking using the Bradford protein assay (Bradford, 1976). Capsules were prepared in a similar manner as outlined in section 3.8.1 with the exception of the absence of *B. adolescentis*. Following 30 min of hardening, the cross-linking and wash solutions were vacuum filtered (12.5 cm diameter filter paper; VWR 417, VWR International, Edmonton, AB) to remove particulates. The filtrates were collected, pooled and brought to a final volume of 50 mL using ddH_2O. A 100 µL aliquot of this solution was added to 5.0 mL of Bio-Rad protein assay dye reagent (Coomassie brilliant blue G-250 dye) in a glass test tube (18 mm x 150 mm) and vortexed for 10 s. The sample was incubated at room temperature for 10 min and the absorbance read at 595 nm using a Genesys 20 visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA). A blank was prepared in the same fashion as the sample, without protein (i.e., only AL and prebiotics). Similar to the samples, 18 g of the blank solution was extruded into the cross-linking solution (30 mL) and was allowed to harden for 30 min. This solution (i.e., AL, prebiotics, and cross linking solution) was then homogenized at 9,000 rpm for 1 min. A standard curve was prepared with bovine serum albumin (BSA) at a concentration range of 0.04-0.9 µg/µL with correlation coefficients of 0.9908 or greater. All experiments were conducted in triplicate.

3.8.4 Capsule prebiotic content

Prebiotic (FOS) retention within the capsule following cross-linking was determined by measuring prebiotic levels present in the cross-linking and wash solutions post cross-linking using HPAEC-PAD. Capsules were prepared in a similar manner as outlined in section 3.8.1 with the exception of the absence of *B. adolescentis*. Following 30 min of hardening, the cross-linking and wash solutions were vacuum filtered to remove particulates. The filtrates were collected, pooled and brought to a final volume of 50 mL using ddH_2O. A 3.0 g aliquot of this solution was hydrolyzed by the addition of 1.5 g of 6 N HCl with constant mechanical stirring for 18 h at room temperature. After hydrolysis, the samples were neutralized to pH 7.00 with 1.0 M NaOH and brought to a total volume of 10 mL using ddH_2O. The samples were diluted with ddH_2O for HPAEC analysis, 1/20 (0.19 g FOS), 1/35 (0.38 g FOS), and 1/50 (0.57 g FOS). The amount of FOS entrapped was determined by comparison to a single point standard for each FOS concentration. A standard solution was prepared containing 30 mL of cross-linking agent, 0.19, 0.38 or 0.57 g of FOS, and was brought to volume (50 mL) with ddH_2O. The standard solution was hydrolysed, neutralized, and diluted under the same conditions as the sample. Sample analysis was performed using HPAEC-PAD (section 3.3). In this work an isocratic mobile phase of 100 mM NaOH was used. The flow rate was maintained at 1.0 mL/min and the column pressure was approximately 1200 psi. Experiments were conducted in triplicate.

An estimate of the number of capsules prepared per gram was determined for a 2.0% (w/w) PPI-0.5% AL material extruded through both a 20 G and 27 G needle, through manual counting. Capsules were prepared in a similar manner as outlined in section 3.8.1, except in the absence of *B. adolescentis*. Capsules were first vacuum filtered and dry blotted using Kim wipes (Kimberly-Clark Professional, Huntsville, Ontario), weighed (1 ± 0.0002 g) and counted. Three separated batches were prepared, with data reported as the mean ± one standard deviation.

3.8.5 Capsule weight change in simulated gastric juice at pH 2.0

The swelling properties of freshly prepared capsules within SGJ were investigated using the modified method of Nickerson et al. (2006) over a 2 h period at

37°C to represent gus residence time. Capsules were prepared as outlined in sections 3.7.4 and 3.8.1, except in the absence of *B. adolescentis.* In brief, ~ 1 ± 0.0002 g of capsules that had been dry blotted using Kim wipes (Kimberly-Clark Professional, Huntsville, Ontario) were added to a glass test tube (18 mm x 150 mm) containing 5.0 mL of tempered SGJ at 37°C for 2 h. Samples were vacuum filtered, dry blotted, and weighed to determine weight gain/loss after SGJ treatment. All experiments were performed on triplicate batches of capsules. The swelling ratio was determined using eq. 6:

$$Swelling\ Ratio = \frac{(W2 - W1)}{W1} \qquad \text{(eq. 6)}$$

where, *W1* was the initial weight of the capsules prior to SGJ treatment and *W2* was the final weight after SGJ treatment for 2 h.

3.8.6 Survival of free and encapsulated cells in simulated gastric juice at pH 2.0.

Experiments were performed as outlined in section 3.7.5, and were conducted in triplicate.

3.8.7 Probiotic release in simulated intestinal fluid

The ability of encapsulated *B. adolescentis* to be released from PPI^c-AL capsules (2.0% PPI^c-0.5% AL with 0% and 3.0% FOS and 20G; and 6.0% PPI^c-0.5% AL with 0% and 3.0% FOS and 20G) within simulated intestinal fluid (SIF; 1.25% (w/v) $NaHCO_3$, 0.6% (w/v) oxgall dehydrated fresh bile, and 0.09% (w/v) pancreatin) was tested using methods modified from Laird (2007). Capsule parameters were selected based on extreme conditions for amount of protein and prebiotic within the capsule. A 20 G needle was used due to small difference in size produced during encapsulation and size did not affect survival of probiotics during SGJ experiments. Aliquots of 1.0 mL of free or encapsulated cells (8-9 log CFU/mL) in PS were added to 19.0 mL of tempered SIF (pH 6.50) and incubated (25°C) with mechanical stirring for 3 h within an anaerobic

chamber. Aliquots of 1.0 mL were removed every 30 min for 3 h and serially diluted in PS. Cell concentrations were determined by spread plating onto RCM-cys agar plates and enumerated after 48 h of incubation in an anaerobic chamber (37°C). Samples were performed in triplicate.

3.8.8 Statistics

An analysis of variance (two-way ANOVA) was used to statistically compare differences in capsule size, swelling, and protein/prebiotic retention as a function of protein concentration, prebiotic levels and needle gauge. All statistical analyses were performed using SPSS software (SPSS Inc., ver. 17, 2007, Chicago, IL).

4. Results and Discussion

4.1 Material characterization

Pea flour was found to be comprised of 21.78% protein (%N x 6.25), 7.80% moisture, 1.00% lipid, 4.16% ash and 65.26% carbohydrate; PPI^n was comprised of 93.50% protein (%N x 6.25), 1.55% moisture, 2.40% lipid, 0.79% ash and 1.76% carbohydrate; and PPI^c was comprised of 80.01% protein (%N x 6.25), 2.60% moisture, 1.81% lipid, 4.79% ash and 10.78% carbohydrate. Gwiazda et al. (1980) reported protein levels in their salt extracted pea protein isolate to be 89.10%. Whereas, Sun and Arntfield (2011) reported levels to be 81.90%) for their pea protein isolate extracted from pea flour by salt extraction. The authors reported similar levels, 82.00% in the commercial pea protein isolate. In the case of AL, the powder was found to be comprised of 0% protein, 13.03% moisture, 0.10% lipid, 24.35% ash and 62.52% carbohydrate. The HPAEC-PAD analysis of fructooligosaccharide P95 indicated peaks within the chromatogram corresponding to glucose, fructose, sucrose and oligosaccharides of varying degrees of polymerization (DP) (Figure 4.1). Specifically, inulobiose (DP = 2), inulotriose (DP = 3), inulotetraose (DP =4), inulopentaose (DP = 5), inulohexaose (DP = 6), and inuloheptaose (DP = 7). Peaks present in between the identified peaks (Figure 4.1) contain a terminal glucose end. Wood (2010) reported a similar profile for P95 which contains oligosaccharides ranging between DP 2 to 7 with DPs constituted with a fructose as well as those with a terminal glucose.

4.2 Investigation of the phase behaviour within pea protein isolates (PPI^n)-alginate mixtures as a function of pH and biopolymer mixing ratio (Study 1)

4.2.1 Effect of pH and biopolymer mixing ratio on complex coacervation

The effect of pH (1.50-7.00) and biopolymer mixing ratio (1:1-20:1 PPI^n-AL) on complex formation was investigated in admixtures of PPI^n and AL at a constant total biopolymer concentration (0.1%, w/w) by turbidimetric analysis during an acid titration.

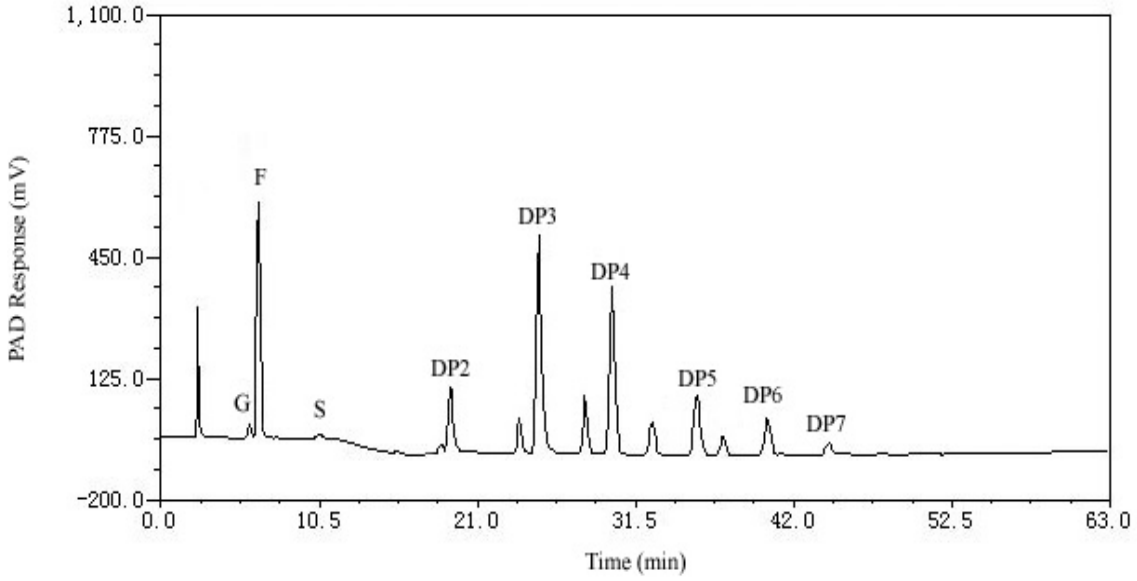

Figure 4.1 HPEAC-PAD carbohydrate profile for fructooligosaccaride P95 (G-glucose, F-fructose, S-sucrose, DP 2-7 with DP 2-6 included FOS structures of Gα 1→2 $[\beta F\ 1\rightarrow 2]_n$ and $[\beta F\ 1\rightarrow 2]_n$ n = 2-7 (i.e., DP 2 includes GF_2 and F_3).

In the case of homogenous PPI^n solutions, O.D. was followed as a function of pH over a concentration range (0.050-0.095%, w/w) (at levels corresponding to those used in the mixed systems). At all concentrations, O.D. were found over a pH range between ~2.00 and 6.00, with a broad peak occurring between ~pH 3.40–4.30 (median pH_{opt} ~3.80) (Table 4.1, Figure 4.2a). However, the peak O.D. was found to increase from 0.500 at 0.05% (w/v) PPI^n, to 0.950 at 0.095% (w/v) PPI^n. The rise in O.D. during the pH titration is thought to be attributed to an increase in protein-protein aggregation associated with reduced charge repulsion between neighbouring protein molecules near the protein's isoelectric point (pI ~3.84-4.23; zeta potential = 0 mV) (Figure 4.2b). At solution pH>pI, proteins assume a net negative charge (zeta potential <0 mV), whereas at pH<pI proteins assume a net positive charge (zeta potential >0 mV) (Figure 4.2b). Harnsilawat et al. (2006) also observed protein-protein aggregation when investigating β-lactoglobulin. The protein had increased turbidity at pH 4.00 and 5.00, which corresponds to the protein's pI (pI ~4.80). In the current study a small concentration effect on pI was seen. At PPI^n levels of 0.050% (w/v) the pI occurred at 3.84, while at PPI^n levels of 0.089% and 0.095% (w/v), pI values were both equivalent to 4.23. Concentration related deviations in pI values may be the result of different levels of exposed charged amino acids on the surface, a consequence of increased protein-protein aggregation at higher concentration levels. Liu et al. (2009) reported that a homogenous PPI^n (prepared using the method of Crevieu et al. 1996) showed optimal O.D. near pH 4.00 which was similar to the present study, however their pI value was slighter higher (pI 5.60) then found in the current study. The differences in pI values between the two studies may reflect slight differences in the methods used during protein extraction from the PF. In the case of homogenous AL solutions, no O.D. was measured over the complete pH range (Figure 4.2a). The surface charge on the AL chain remained negative over the complete pH range measured, however it approached a zeta potential of 0 mV close to pH 1.50. At this pH, carboxyl groups along the AL backbone become protonated (Liu et al., 2009). Harnsilawat et al. (2006) reported similar O.D. and surface charge behavior for AL chains, where no O.D. was observed over a pH range of 3.00 to 7.00 indicating the absence of aggregates, and that AL remained negatively charged over that same pH range. Liu et al. (2009) also reported that for gum Arabic,

Table 4.1 Solution pHs corresponding to maximum O.D. values for homogenous PPI^n solutions as a function of protein concentration, and in mixed PPI^n–AL systems as a function of biopolymer mixing ratio (total concentration of 0.1% (w/v)). Data represent the mean values ± one standard deviation (n = 3).

	pH range of pH_{opt}		Median pH_{opt} value
	Lower	Upper	
Homogeneous PPI^n (%, w/v)[1]			
0.050	3.53 ± 0.21	4.16 ± 0.22	3.84 ± 0.21
0.067	3.43 ± 0.22	4.13 ± 0.21	3.78 ± 0.21
0.080	3.59 ± 0.27	4.30 ± 0.44	3.95 ± 0.33
0.089	3.61 ± 0.19	4.16 ± 0.19	3.88 ± 0.18
0.092	3.47 ± 0.16	4.03 ± 0.21	3.75 ± 0.18
0.095	3.50 ± 0.10	3.99 ± 0.17	3.75 ± 0.13
Mixed PPI^n -AL Ratios			
1:1	1.89 ± 0.11	2.30 ± 0.10	2.10 ± 0.09
2:1	1.97 ± 0.12	2.73 ± 0.06	2.35 ± 0.09
4:1	2.33 ± 0.12	3.17 ± 0.06	2.75 ± 0.05
8:1	2.83 ± 0.15	3.38 ± 0.11	3.11 ± 0.13
12:1	3.00 ± 0.10	3.55 ± 0.28	3.28 ± 0.17
20:1	3.00 ± 0.17	3.67 ± 0.25	3.33 ± 0.21

[1] PPI^n concentrations used in the homogenous solutions correspond to protein levels found in the corresponding mixed systems at different mixing ratios.

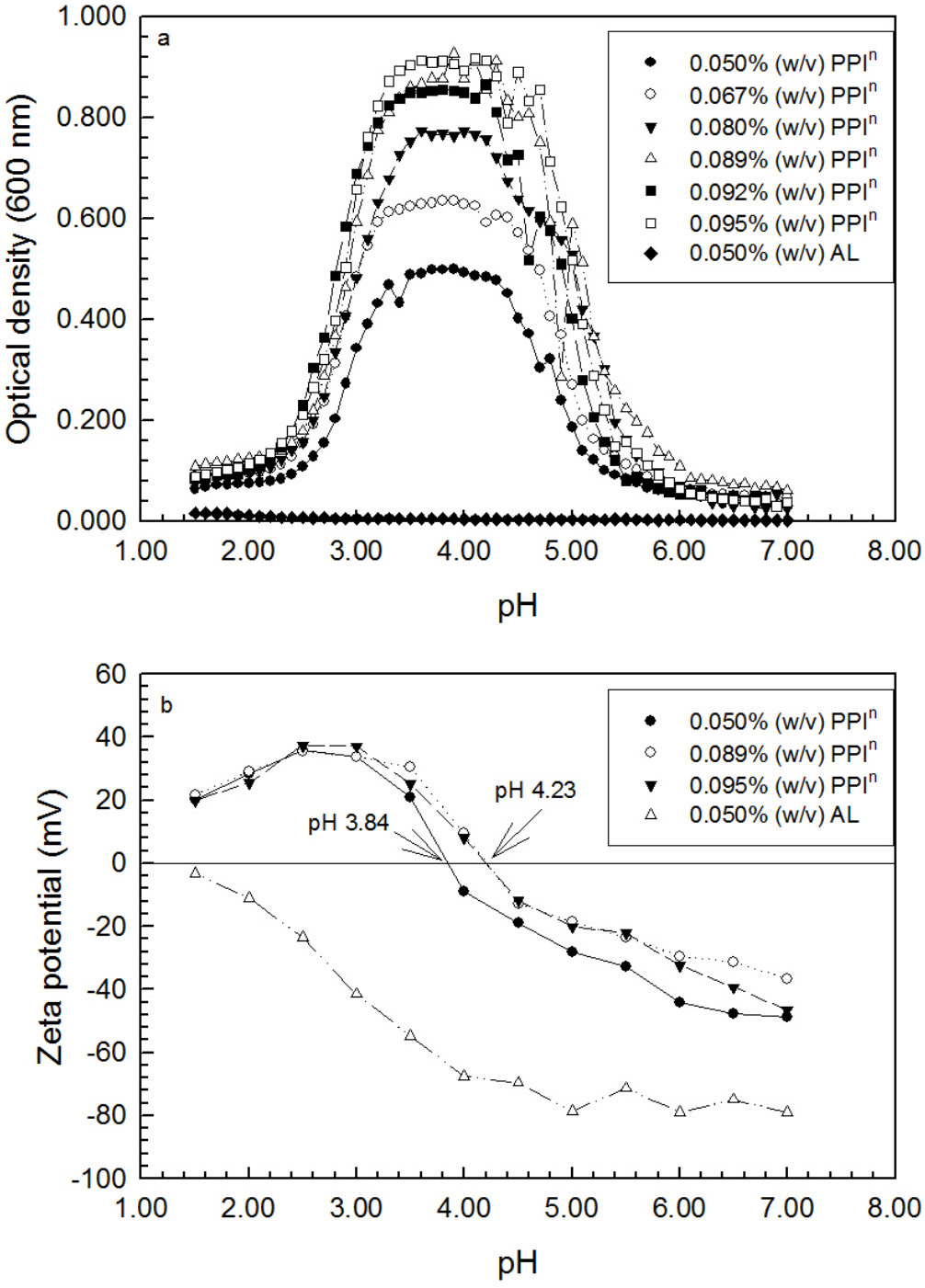

Figure 4.2 Mean turbidity profiles (a) and zeta potential, mV (b) curves for homogenous PPI^n and AL solutions as a function of pH and concentration (n = 3).

another carboxylated polysaccharide, no rise in O.D. was found over the pH range 1.50-7.00, and that net neutrality occurred at pH 1.88.

The addition of AL to a PPI^n solution, at a 1:1 biopolymer mixing ratio caused a significant shift in the initial rise in turbidity from ~pH 6.00 for PPI^n alone to pH 5.00 for the PPI^n-AL mixture (Figures 4.2a, 4.3). This shift was also accompanied by a much slower rise in O.D. during the pH titration, where large increases in O.D. were not seen until pH 3.00 (Figure 4.3) compared with pH 5.00 in the homogenous PPI^n sample (Figures 4.2a). In addition, a slightly lower maximum O.D. was found in the mixed system than the homogenous one, which occurred at a pH near 2.50 instead of 4.00. The pH-dependent turbidity profiles of the PPI^n-AL mixture appeared skewed leftwards (Figure 4.3), whereas the curve for homogeneous PPI^n had a normal distribution (Figure 4.2a). It was hypothesized that the presence of the highly charged polysaccharide AL at a concentration of 0.05% (w/w) caused sufficient electrostatic repulsion to delay protein aggregation and the slight reduction in maximum O.D. may correspond to a small inhibition of structure formation (i.e., protein-protein aggregates).

As the PPI^n-AL mixing ratio increased (i.e., PPI^n levels increased; AL levels decreased), the delay in aggregate growth as a function of decreased pH was less substantial. Furthermore, the nature of the turbidity profiles more became normally distributed as the mixing ratio increased (Figure 4.3). Admixtures at ratios between 8:1 and 20:1 had overlapping turbidity profiles suggesting the PPI^n levels were in excess of the carboxyl reactive sites along the AL backbone (Figure 4.3). The shift to maximum O.D. at lower pHs with the addition of AL suggest structures with increased stability at acidic pHs relative to homogeneous PPI^n aggregates. Similar trends were reported by Liu et al. (2009) for admixtures of PPI^n and gum Arabic where at a biopolymer ratio >4:1 turbidity profiles became constant. The authors also reported enhanced aggregate stability at acidic pH (pH<3.10) relative to PPI^n alone. Schmitt et al. (1999) also reported similar findings for β-lactoglobulin-acacia gum when both acacia gum (1:6-1:20) and β-lactoglobulin (6:1-50:1) were in excess.

Critical structure forming events associated with the formation of soluble and insoluble complexes in a 1:1 PPI^n-AL mixture were found to occur at pH 5.00 ± 0.159 (pH_c) and 2.98 ± 0.068 ($pH_{\phi 1}$), respectively, with optimal biopolymer interactions

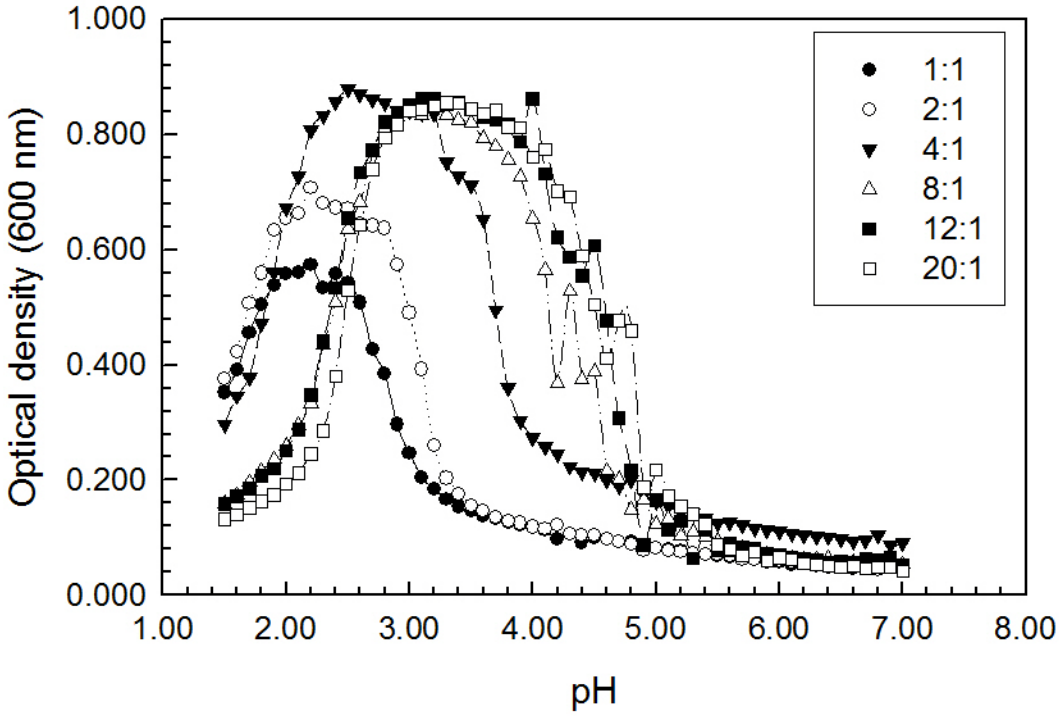

Figure 4.3 Mean turbidity profiles for mixtures of PPI^n-AL as a function of pH and biopolymer mixing ratio (n = 3).

occurring at pH 2.10 ± 0.090 (pH_{opt}). A two-step mechanism for coacervation has been previously reported for WPI-gum Arabic (Weinbreck et al., 2003a), WPI-EPS B40 (Weinbreck et al., 2003b), WPI-carrageenan (Weinbreck et al., 2004a), β-lactoglobulin-pectin (Girard et al., 2004), gelatin-agar (Singh et al., 2007), and PPI-gum Arabic (Liu et al., 2009). This two stage formation is thought to follow nucleation and growth-type kinetics (Turgeon et al., 2003); first with the formation of soluble complexes which act as nucleation sites; and second by an increase in both size and number of complexes in suspension. The increase in size and number of the complexes leads to the formation of insoluble complexes and phase separation into coacervate-rich (or precipitate-rich) and solvent-rich phases.

As biopolymer mixing ratios increased in the current study, critical pH values (pH_c, $pH_{\phi 1}$ and pH_{opt}) shifted towards higher pH until a biopolymer mixing ratio of between 4:1 and 8:1 ($p<0.05$) was reached. Above that ratio a plateau was reached where critical pHs became independent of mixing ratios up to 20:1 ($p>0.05$) (Figure 4.4). Maximum O.D. at pH_{opt} was found to increase from 0.573 at a 1:1 PPI^n-AL ratio to 0.857 at the 4:1 ratio ($p<0.05$), and then became constant at ratios >4:1 ($p>0.05$) (Figure 4.5). Complete dissolution of complexes at $pH_{\phi 2}$ could not be identified in the present study due to enhanced structure stability at acidic pHs in the presence of AL which prevented the O.D. from reaching the baseline. However, a sharp decrease in O.D. at $pH<pH_{opt}$ indicates that partial dissolution of the formed complexes occurred with some dissolution of the protein subunits under acidic conditions (Figure 4.4) (Gueguen et al., 1988). Gueguen et al. (1988) investigated the dissociation and aggregation of pea legumin induced by pH and ionic strength. They reported at both extreme acidic (<3.00) and basic (>11.00) pH most of the molecules were dissociated and at acidic conditions (pH <3.00) the native structure was completely dissociated leading to completely unfolded subunits. Liu et al. (2010b) studied the effect of NaCl, urea, and elevated temperature on the nature of the intramolecular interactions within PPI-gum Arabic mixtures. The authors reported electrostatic interactions were primarily responsible for the complex formed, with secondary stabilization by hydrogen bonding. Complexes formed were thought to involve a single gum Arabic and PPI-PPI aggregate rather than

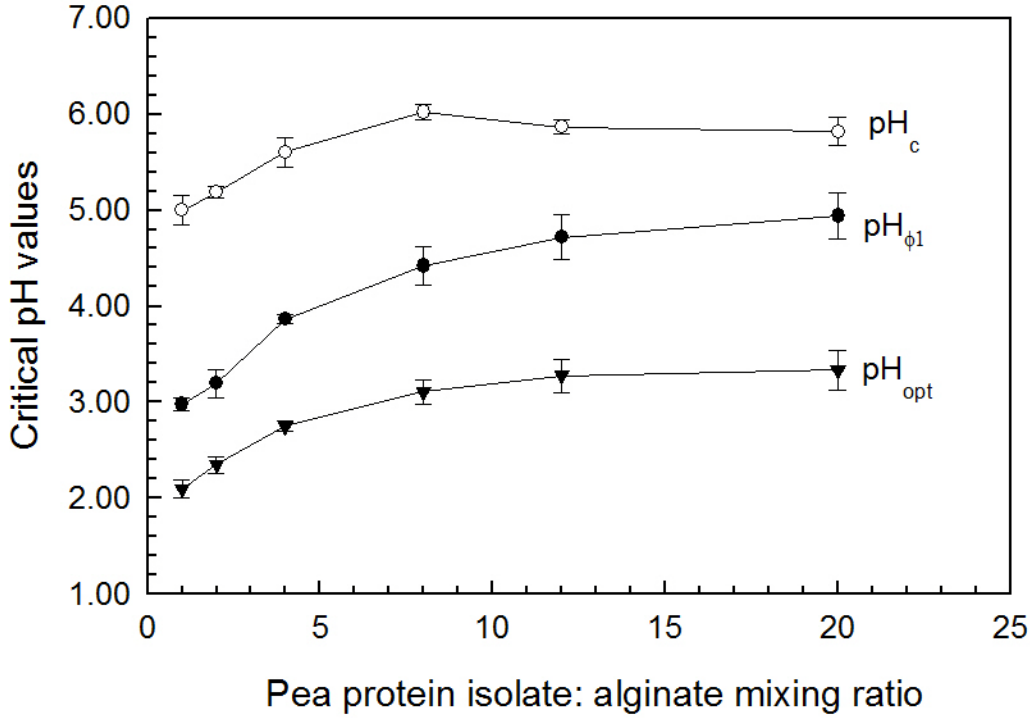

Figure 4.4 Critical pH values associated with the formation of soluble (pH_c) and insoluble ($pH_{\phi 1}$) complexes, and optimal biopolymer interactions (pH_{opt}) as a function of biopolymer mixing ratio for PPI^n-AL mixtures. Data points represent the mean ± one standard deviation (n = 3).

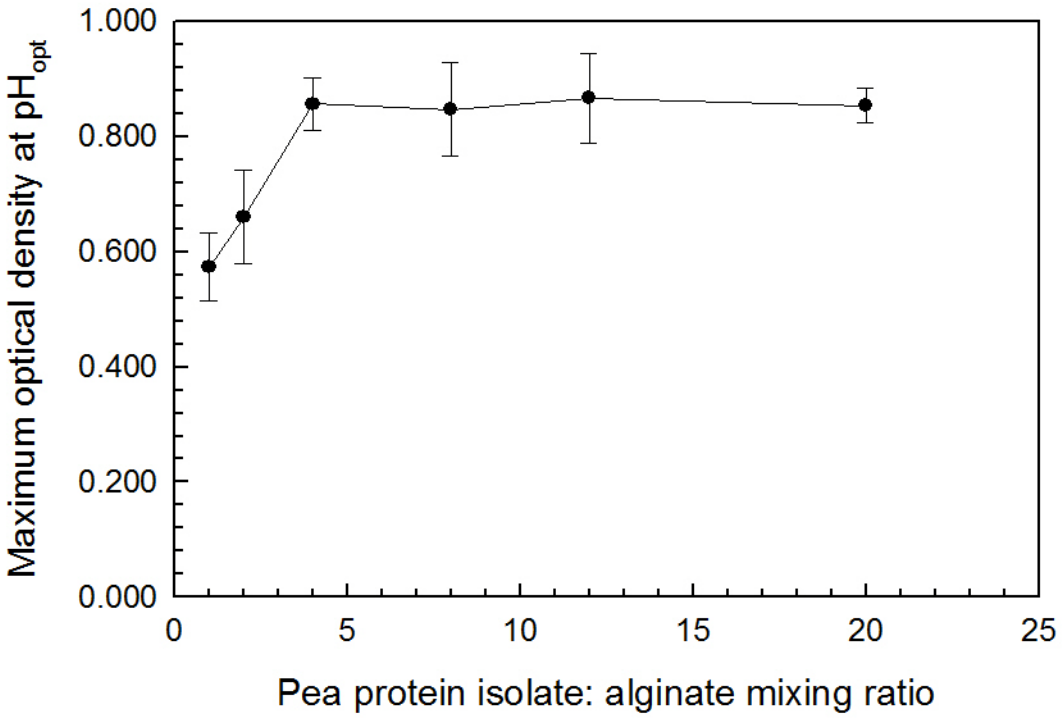

Figure 4.5 Maximum optical density at pH_{opt} in admixtures of PPI^{n}-AL as a function of biopolymer mixing ratio. Data points represent the mean ± one standard deviation (n = 3).

individual PPI molecules. Within the O.D. vs. pH profiles a shoulder was present at acidic pHs (pH <3.00) indicating improved stability at lower pH. As pH was lowered further complexes dissociated. The shoulder and enhanced stability was hypothesized by the authors to be related to hydrophobic interactions that stabilized the PPI-PPI aggregates. At higher temperatures, stability and the shoulder were enhanced which supported their claim. In contrast to Liu et al. (2010b), AL was used in the present study due to its stronger electrostatic interactions with PPI which should lead to a more intact capsule. Similar to Liu et al. (2010b), complexes are thought to be comprised of AL bound to PPI-PPI aggregates, which are further stabilized by hydrogen bonding and hydrophobic interactions.

In the current study, pH_c was found to be somewhat dependent on the biopolymer mixing ratio. As biopolymer mixing ratio increased up to a mixing ratio between 4:1 and 8:1, a shift in pH_c to higher pH values was seen, after which a plateau was reached. The biopolymer mixing ratio dependence of pH_c has been previously reported for PPI-gum Arabic (Liu et al., 2009) and gelatin (Type-A and Type-B)-agar (Singh et al., 2007) systems. Liu et al. (2009) reported similar results for PPI-gum Arabic mixtures, that pH_c was dependent upon biopolymer mixing ratio up to a ratio of 4:1 (PPI-gum Arabic) after which pH_c remained constant. They reported that the shift in pH_c toward higher pHs with increased mixing ratio was caused by the interactions between gum Arabic and protein-protein aggregates rather than a single protein molecule. They also reported that the protein-protein aggregates increased in size as the mixing ratio increased up to the critical limit. Afterwards, pH_c values remained constant. However, these pH_c-mixing ratio dependent trends are contradictory to results found in other literature. For instance, in systems such as bovine serum albumin-polyelectrolyte poly(dimethyl diallyl ammonium chloride) (Mattison et al., 1995), whey protein-exocellular polysaccharide B40 (Weinbreck et al., 2003a,b), whey protein-carrageenan (Weinbreck et al., 2004a), and lactoglobulin (aggregate-free)-pectin (Girard et al., 2004), a constant pH_c as a function of biopolymer mixing ratio was reported. These authors report that the lack of pH_c ratio dependence is caused by complexes formed comprised of a single polysaccharide with only a given amount of proteins. In all cases, large aggregates were removed prior to the complexation studies being performed. In

contrast to the present study where homogeneous protein solutions showed significant O.D. between the pH range 2.00 and 6.00, which overlapped with the mixed turbidity profiles; these authors reported the lack of O.D. within the homogenous samples once the protein aggregates were removed.

In the case of $pH_{\phi 1}$, the mixing ratio dependence is quite prevalent. Weinbreck and co-workers (2003a, b) and Liu et al. (2009) reported a shift in $pH_{\phi 1}$ towards higher pH until a critical biopolymer mixing ratio was reached, beyond which $pH_{\phi 1}$ became stable. The increase in $pH_{\phi 1}$ with increasing biopolymer mixing ratio was thought to be due to a greater amount of protein molecules available per polysaccharide chain after which it becomes stable due to a saturation of the polysaccharide (Liu et al., 2009). Liu et al. (2009) reported after a 4:1 mixing ratio there was saturation of gum Arabic.

Changes to O.D. reported in this study during the pH titration were not believed to be associated with changes to conductivity or dilution effects caused by the addition of HCl during the pH titration. Changes to conductivity were similar regardless of the mixing ratio. For instance, for the 1:1 mixing ratio, conductivity increased from 167.2 µS/cm at pH 7.00 to 19,910 µS/cm at pH 1.50, whereas for the 20:1 mixing ratio, conductivity rose from 160.7 µS/cm at pH 7.00 to 18,050 µS/cm at pH 1.50. Although a large increase in conductivity was observed as pH decreased (1.50), solution conductivity corresponding to the pH range where structure forming events (pH_c, pH_ϕ, and pH_{opt}) occurred remained low (<600 µS/cm). The 1:1 mixing ratio however, due to the shift in critical pH values towards a lower pH had a slightly higher conductivity at $pH_{\phi 1}$ (pH 2.30) at 2478 µS/cm compared with the 20:1 mixing ratio. Dilution effects were also kept to a minimum by using an HCl concentration gradient to acidify the solution only <600µL of HCl was added to the total volume.

Changes to the surface charge were also investigated as a function of pH during a similar acid titration as the turbidity profiles, and as a function of biopolymer mixing ratio (Figure 4.6). The addition of AL to PPI caused a shift of net neutrality from ~4.00 with homogenous PPI^n to ~1.55 at the 1:1 PPI-AL ratio. The 1:1 biopolymer mix ratio displayed similar electrophoretic mobility as the homogenous AL solution (Figures 4.2b, 4.6), where homogenous AL retained a negative charge (zeta potential <0 mV) over nearly the entire pH range examined. Harnsilawat et al. (2006) observed a similar trend

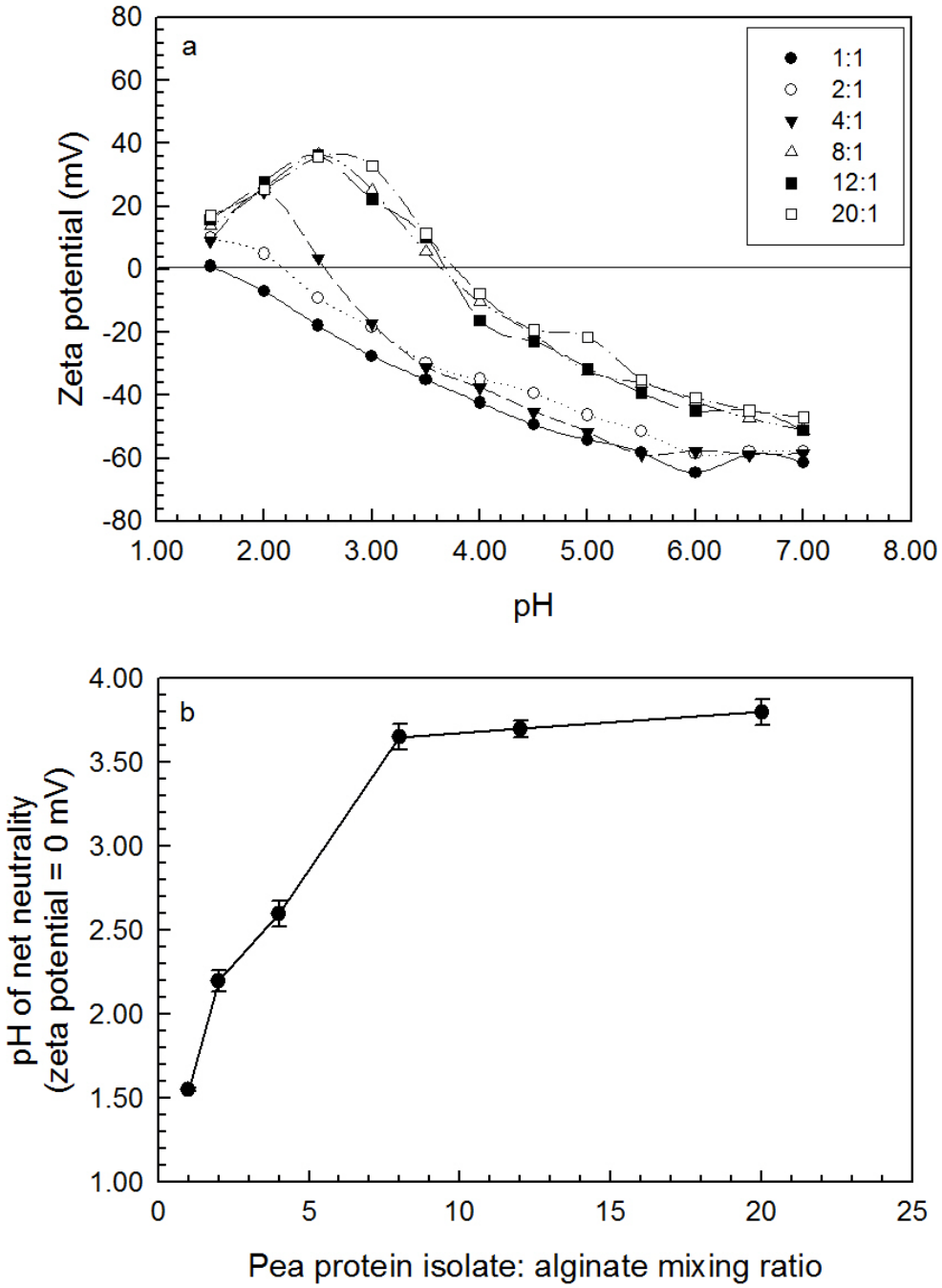

Figure 4.6 Mean zeta potential (mV) profiles for mixtures of PPI^n-AL as a function of pH and biopolymer mixing ratio (PPI^n-AL) (a), and the isoelectric points (pI) of formed complexes as a function of biopolymer mixing ratio (b) (n = 3).

for AL (0.1 wt%), where the zeta potential remained negative over a pH range of 2.00 to 8.00 (-8.7 to -68.4 mV). The authors reported slightly higher zeta values between pHs 2.00 and 4.00, relative to higher pHs since carboxyl groups ($-COO^-$) along the AL backbone were becoming protonated (-COOH). Ducel et al. (2004) also reported a negative surface charge for AL over the pH range of 2.20 to 4.50, with an increase in zeta potential from ~ -60 mV to -20 mV during the pH titration towards pH of 2.20. Similar findings were reported by Liu et al. (2009) and Weinbreck et al. (2004b) for another carboxylated polysaccharide, gum Arabic. Both groups reported pI values of gum Arabic between 1.88 and 2.00. In the current study, as protein levels increased (i.e., as the mixing ratio increased), the pH corresponding to net neutrality shifted towards higher pHs until the ratio reached 8:1 PPI^n-AL ratio after which it then became constant at a pH of 3.65. As in the turbidity profiles shown in figure 4.3, mixing ratios between 8:1 and 20:1 behaved similarly, and resembled that of the individual PPI molecules. Within the O.D. vs. pH profiles a shoulder was present at acidic pHs (pH <3.00) indicating improved stability at lower pH. As pH was lowered further complexes dissociated. The shoulder and enhanced stability was hypothesized by the authors to be related to hydrophobic interactions that stabilized the PPI-PPI aggregates. At higher temperatures, stability and the shoulder were enhanced which supported their claim. In contrast to Liu et al. (2010b), AL was used in the present study due to its stronger electrostatic interactions with PPI which should lead to a more intact capsule. Similar to Liu et al. (2010b), homogenous systems at corresponding concentrations (Figure 4.2a). At or above the 8:1 PPI^n-AL ratio, it was assumed that reactive sites along the AL had become saturated (Liu et al., 2009). In all cases, pH corresponding to net neutrality (zeta potential = 0 mV) was close to pH_{opt} values at the associated biopolymer mixing ratio (within one pH unit difference) (Figures 4.3 and 4.5) suggesting that at pHs near pH_{opt}, charge neutralization was occurring. For instance, pH_{opt} for the 8:1 ratio was reported to occur at pH ~3.00, while net neutrality was found at pH ~3.80. At pHs above net neutrality, PPI-AL complexes carried a net negative charge, whereas at lower pH, complexes carried a positive net charge. Liu et al. (2009) reported net neutrality of mixed PPI-gum Arabic (2:1) systems to occur at pH_{opt}.

4.2.2. Summary

The effect of pH and biopolymer mixing ratio on the formation of soluble and insoluble complexes between PPI^n and AL were investigated. Based on the turbidity profiles (Figure 4.3), critical pHs associated with structure forming events (pH_c, $pH_{\phi 1}$ and pH_{opt}) (Figure 4.4), maximum O.D. at pH_{opt} (Figure 4.5), and electrophoretic mobility (Figure 4.6), optimum conditions for coacervation were thought to occur at a biopolymer mixing ratio of 4:1 and at solution pH near 2.75.

In the next stage of this research the encapsulation of probiotic bacteria (*B. adolescentis*) within a PPI-AL capsule will be investigated. However, the coacervation of PPI-AL mixtures is optimal under acidic conditions (pH 3.00), these pHs are not congruent to support probiotic survival. Consequently mixtures will be developed at suitable pH (7.0) relying on segregative (non-interacting) conditions to prepare the capsule wall, with the addition of Ca^{2+} salts to induce ionic cross-linking between AL chains to support capsule integrity.

4.3 Entrapment of *Bifidobacterium adolescentis* within pea protein isolate–alginate capsules (Study 2)

4.3.1 Growth profile of *B. adolescentis* in RCM-cys media

Growth of *B. adolescentis* within RCM-cys broth was followed over a 40 h duration to determine times associated with various stages of growth (i.e., lag, exponential and stationary phases) (Figure 4.7). Changes in O.D. at 600 nm indicated that the lag phase of growth ranged between 0 and 5 h, followed by exponential growth between 5 and 15 h. The stationary phase of growth for *B. adolescentis* was from 15 to ~20+ h, which corresponded to an O.D. magnitude of 1.500 and cell numbers as determined by pour plating to be 9 log CFU/mL. Work by Wood (2010) on growth profiles of *B. adolescentis* over a similar time period found the stationary phase of growth to range between 15 and 27 h, where cell numbers also remained stable at 9 log CFU/mL. In the past, cells harvested during the stationary phase of growth have shown the greatest survival during the encapsulation process (Hansen et al., 2002; Masco et al., 2007). Masco et al. (2007) investigated 66 *Bifidobacterium* strains in both exponential and stationary phase growth for their tolerance to *in vitro* gastrointestinal transit. The authors reported there was enhanced tolerance when the cells were in the stationary phase compared to the exponential phase. Based on findings from this study, *B. adolescentis* grown for the purposes of entrapment will be harvested during the stationary phase (15 to 20 h) prior to encapsulation.

4.3.2 Survival of free and encapsulated *Bifidobacterium adolescentis* within simulated gastric juice

The survival of *B. adolescentis* within SGJ at pH 2.0 and 37°C was investigated for non-encapsulated cells (denoted as 'free' cells) and those entrapped within PPI^{n}-AL and PPI^{c}-AL based capsules (Figure 4.8). Free *B. adolescentis* cells experienced a large log cycle reduction in cell numbers within the first 5 min of being submerged in SGJ, dropping from 10^{9} to $10^{5.6}$ CFU/mL (D-value 1.44), then again to $10^{3.5}$ CFU/mL after 10

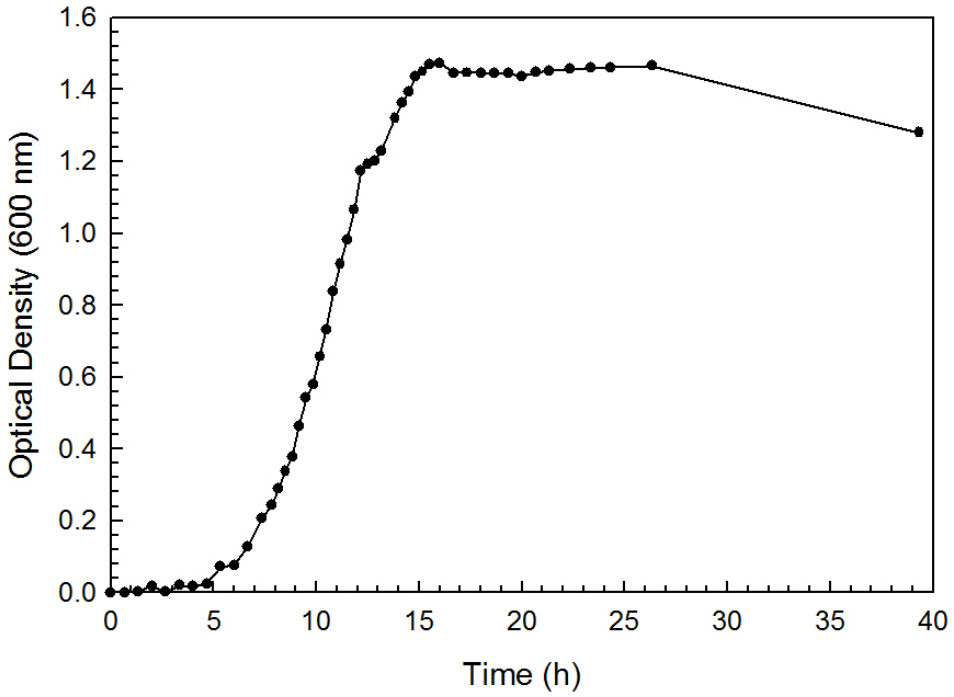

Figure 4.7 Growth curve of *B. adolescentis* grown in RCM-cys broth over time. Data represents the mean value (n = 2).

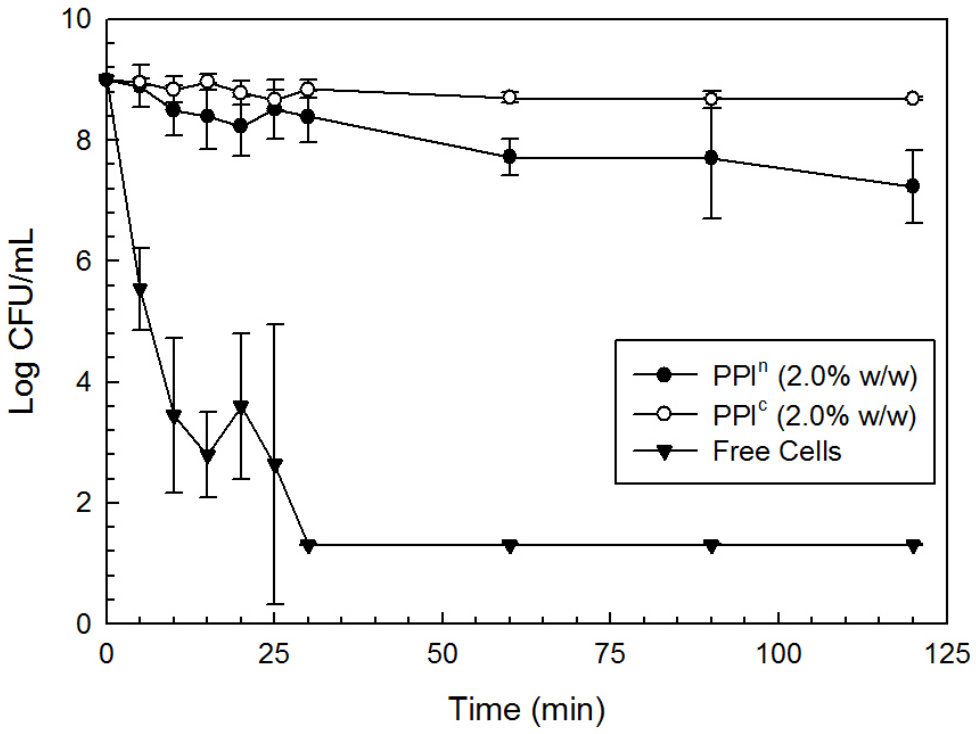

Figure 4.8 Survival of free *B. adolescentis* and entrapped within PPI^{n}-AL and PPI^{c}-AL capsules (2.0% PPI-0.5% AL; or 4:1 mixing ratio) over time within simulated gastric juice at pH 2.0 and 37°C. Data points represent the mean ± one standard deviation (n = 3).

min exposure (Figure 4.8). A D-value was calculated within the first 5 min of exposure to SGJ due to drastic decrease in viable cells numbers. Kotikalapudi et al. (2010) reported a similar D-value for free *B. adolescentis* exposed to SGJ (pH 2.0). They reported that rapid depletion of viable free cells occurred within the first 5 min of exposure, with a D-value 1.12. Free *B. adolescentis* cell counts in the current study reached the lower limit of detection (10^2 CFU/mL) after 25-30 min of exposure to SGJ. In contrast, entrapped *B. adolescentis* cells within the PPI^n-AL and PPI^c-AL capsules provided almost complete protection of the cells from SGJ over a 2 h period. Only a 1-log reduction in cell numbers from 10^9 to 10^8 CFU/mL (D-value 67.99, calculated at 120 min) was observed for PPI^n-AL capsules and less than 1 log reduction for PPI^c-AL capsules (no D-value was calculated since there was less than a 1 log decrease in CFU/mL) (Figure 4.8). Kotikalapudi et al. (2010) showed that encapsulating probiotic *L. acidophilus* within a PPI-AL mixture, improved survival by ~6 times relative to non-encapsulated cells during exposure to SGJ at pH 2.0. Wood (2010) encapsulated *B. adolescentis* within AL, PPI-AL, and WPI-AL capsules. Survival following exposure to SGJ (pH 2.0) was similar to free cells for the AL capsules, however, PPI-AL and WPI-AL capsules improved survival of entrapped cells. Wood reported the PPI-AL and WPI-AL capsules were less porous than the AL capsules which slowed the diffusion of SGJ across the capsule wall. Higher concentrations of wall material also were added in the protein-AL capsules compared to AL alone. More wall material created a denser capsule structure which would also have slowed diffusion of SGJ into the capsule.

4.3.3 Summary

Findings from study 2 suggest that the designed PPI-AL capsules offer highly effective protection against the acidic environment within the gastrointestinal tract, with no difference in protective effect between capsules prepared using PPI^n versus PPI^c. Utilization of PPI^c instead of PPI^n in capsule design could offer tremendous opportunities for product design scale up, due to the greater cost and availability challenges in obtaining a more native (i.e., non-denatured) commercial pea protein ingredient. As such, further research will be based on only the commercial protein product (PPI^c).

4.4 Encapsulation of *Bifidobacterium adolescentis* within PPI^{c}-AL capsules: effect of wall characteristics and processing conditions on the physical properties and the protective nature of the capsules (Study 3)

4.4.1 Capsule size and size distribution

A particle size analyzer was used to investigate the effect of needle gauge (20 and 27 G), PPI protein concentration (2.0, 4.0, and 6.0% w/w plus 0.5% w/w AL), and prebiotic (FOS) concentration (0, 1.0, 2.0, and 3.0%, w/w) on mean capsule size produced in the presence of *B. adolescentis*. Capsules were formed after extrusion of the biopolymer solution through the needle and dropping 30 mm into a cross-linking bath containing 1.0% (w/w) $CaCl_2$ and 1.0% (w/v) Tween 80. For all materials and extrusion conditions, capsule diameter (or size) followed a non-Gaussian distribution and were reported as the geometric mean bead diameter. Figure 4.9 gives a typical capsule size distribution profile for a 2.0% PPI^{c}-0.5% AL system at a 1.0% (w/w) prebiotic level, extruded through both 20 and 27 G needles. For both needle diameters, the size distributions of the capsules are skewed slightly towards the right or towards larger sized capsules. The 20 G needle also gave a larger averaged sized capsule than the 27 G needle. The effect of concentration of prebiotic and the protein concentration used in the wall material, and the needle gauge used in extrusion processing on the geometric mean diameter of the capsules are shown in figure 4.10. Based on an ANOVA analysis the main effect of needle gauge was found to have a significant effect ($p<0.001$) on capsule size, whereas all other main effects and interaction terms were not significant ($p>0.05$). The 20 G needle produced capsules with mean geometric diameters ranging between ~2.00 to 2.30 mm (mean 2.17 ± 0.06) whereas capsules produced using the 27 G needle ranged in size from ~1.70 to 1.90 mm in diameter (mean 1.75 ± 0.04). Wood (2010) using a particle size analyzer and cryo-scanning electron microscopy reported mean diameters for 4.0% PPI-0.5% AL capsules of 2.0 mm and 1.5 mm respectively. The capsule size distribution reported by Wood also followed a non-Gaussian distribution which was similar to the present study.

Typically, encapsulation by extrusion usually produces larger-type beads (0.5-5.0 mm) than those produced by emulsion-based processing (microns)

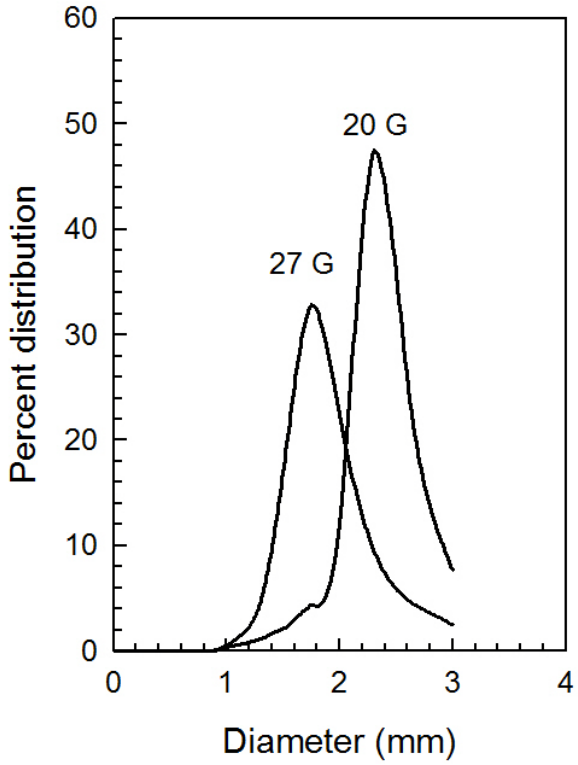

Figure 4.9 Percent distribution of capsule diameter for a 2.0% PPI^{c}-0.5% AL-based capsule with 1.0% prebiotics and containing probiotic bacteria as a function of needle gauge size used in extrusion processing.

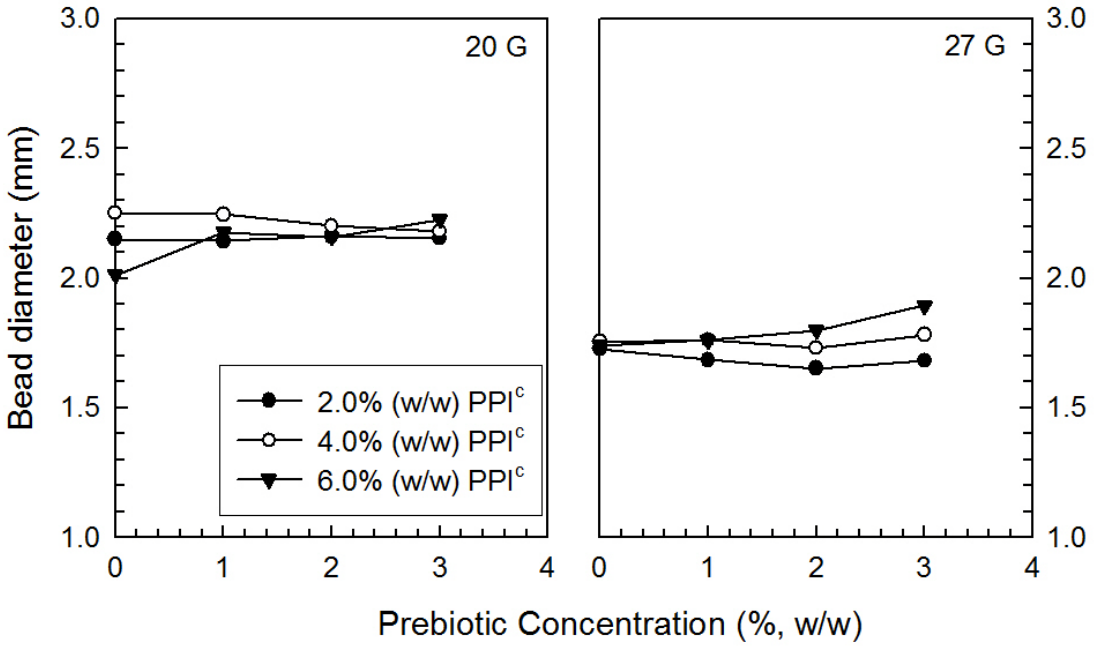

Figure 4.10 Geometric mean diameter (mm) for PPI^{c}-AL based capsules produced in the presence of *B. adolescentis* as a function of prebiotic levels (0, 1.0, 2.0, 3.0%, w/w), protein concentrations (2.0, 4.0, 6.0%, w/w) and needle gauge (20, 27 G) used during extrusion processing. Data represents the mean values for the geometric means of duplicate samples.

(Krasaekoopt et al., 2003). In extrusion processing capsule size is related to; a) needle gauge, where larger gauge needles (i.e., those with smaller inner shaft diameters) produces smaller capsules; b) biopolymer concentration, where higher concentrations (and higher viscosities) lead to the formation of larger capsules; and c) the distance separating the syringe from the cross-linking bath (Krasaekoopt et al., 2003; Anal and Singh, 2007). Sandoval-Castilla et al. (2010) investigated capsule size produced using a variety of AL and low-methoxy pectin mixtures, to find that with increasing total biopolymer concentration, capsules became bigger. Lee and Heo (2000) investigated different concentrations of AL (2.0, 3.0, 4.0%) on capsule size to find that with increased concentrations, capsule size also increased. These results are contradictory to the current study which found no increase in capsule size with increase in biopolymer concentration. In the present study, total biopolymer concentrations were raised by increasing the protein concentration from 2.0-6.0% which would only have a marginal effect on solution viscosity. However, in the case of the previous studies, only small increases in polysaccharide concentrations would lead to dramatic increases in solution viscosity. Another major factor influencing capsule size is the diameter of the syringe needle used during extrusion. For instance, Muthukumarasamy et al. (2006) produced AL capsules with an average size of 2.37 mm using a 21 G needle, whereas Chen et al. (2005) using a smaller inner diameter needle (31 G needle) produced a capsules of 0.5 mm in diameter. This was also observed in the current study, where capsules produced using a 20 G needle produced larger capsules (2.17 mm ± 0.06) and the 27 G needle produced smaller capsules (1.75 mm ± 0.04). In contrast to capsules prepared by emulsion-based techniques, capsules produced by extrusion tend to be more uniform in size. Muthukumarsamy et al. (2006) investigated the size of capsules produced by both emulsion and extrusion techniques using various wall materials (AL, AL + starch, carrageenan + locust bean gum, and xanthan + gellan) and found capsules produced via extrusion were much more uniform in size compared to capsules produced via emulsion.

4.4.2 Protein and prebiotic retention during encapsulation

The effect of prebiotic levels (0, 1.0, 2.0, and 3.0%, w/w), protein concentration (2.0, 4.0, and 6.0%, w/w), and needle gauge (20 vs. 27) on prebiotic and protein

retention post-encapsulation was investigated using HPAEC-PAD and the Bradford assay, respectively. Analyses were performed on residual levels of prebiotic and protein found in the cross-linking bath post-encapsulation, and then compared to original levels added. In the case of protein retention, nearly all of the original wall material (~99%) was retained within the capsule regardless of the protein concentration, prebiotic level or needle gauge. Reid et al. (2005) reported a similar finding for their whey protein isolate capsules, after extrusion through a 23 G needle and into a $CaCl_2$ cross linking bath. The authors reported percent protein as measured by UV absorption at 280 nm of 99.9% within the capsule matrix with only 0.1% remaining within the cross linking bath. Rosenberg and Lee (2004) also reported high levels of protein being entrapped when preparing whey protein isolate capsules coated with AL. When capsules were subjected to either heat treatment or cross-linking, 82.6-87.5% and 78.18-83.4% of the protein was retained, respectively.

The effect of protein concentration, prebiotic levels and needle gauge on capsule design, in terms of its ability to retain prebiotic oligosaccharides within the encapsulation material relative to the original amount added during the batch process (in percent) is given in figure 4.11. Although, slight differences in percentages of FOS retained were evident depending on the main treatment effects and associated interactions, no reliable conclusions could be drawn from the ANOVA analysis. The encapsulation material retained the prebiotic at levels dependent upon the original concentration of FOS added. For instances, the addition of 3.0 % w/w FOS (i.e., 0.57 g total weight added to the biopolymer mixture) led to a capsule retention of 0.081 g ± 0.016 FOS, whereas the addition of 1.0% w/w FOS (i.e., 0.19 g total weight added to the biopolymer mixture) led to a retention of only 0.026 g ± 0.005 FOS. However, when considering equivalent batch weights, the needle gauge influenced the number of capsules produced, and thus the amount of FOS entrapped within an individual capsule. As an example, the number of capsules per gram was counted for a 2.0% (w/w) PPI^n-0.5% (w/w) AL wall material produced with 20 and 27 G needles. In the case of the former, 145.0 ± 6.11 capsules were formed per gram versus 436.67 ± 7.64 capsules per gram produced by the 27 G needle. Findings suggest that higher levels of prebiotic are being entrapped within the larger capsule than the smaller ones. Wood (2010) reported

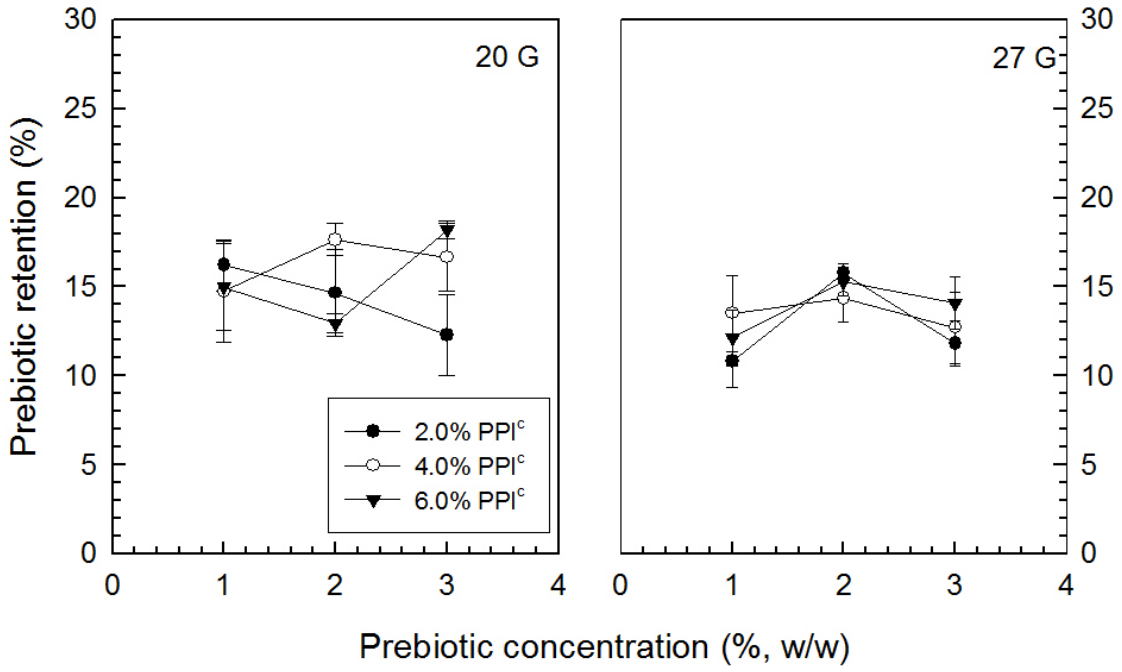

Figure 4.11 The percentage of prebiotic retained within PPI^{C}-AL based capsules post-encapsulation, produced without *B. adolescentis* as a function of prebiotic levels (0, 1.0, 2.0, 3.0%, w/w), protein concentrations (2.0, 4.0, 6.0%, w/w) and needle gauge (20, 27 G) used during extrusion processing. Data represents the mean value ± one standard deviation (n = 3).

similar entrapment efficiencies of FOS at 13.41%, 10.87%, and 16.83% for AL, PPI-AL, and WPI-AL capsules, respectively.

4.4.3 Swelling behaviour of capsules within simulated gastric juice

The effect of protein concentration, prebiotic levels and needle gauge on capsule design was investigated in terms of their swelling behaviour (as described by the swelling ratio) after being immersed for 2 h in SGJ at 37°C (Figure 4.12). The negative swelling ratios suggest that the capsules were shrinking (have reduced volume by weight) or partially degrading (losing wall material into environment) over the 2 h duration. In the case of capsule shrinking, an osmotic gradient might have developed across the capsule wall leading to the migration of water out of the capsule leading to shrinkage.

An ANOVA analysis found that the main effect of protein concentration on the swelling ratio highly significant ($p<0.001$), whereas all other main effects and associated interactions were not significant ($p>0.05$).Figure 4.13 gives swelling ratios for the PPI^c-AL based capsules as a function of protein concentrations used pooled data from Figure 4.14. Overall, swelling ratios increased (less negative) as protein levels were raised from 2.0 to 6.0% (w/w) (or swelling became less negative as protein concentrations increased in the wall material) (Figure 4.13). In the present study, it is hypothesized that a greater amount of intra- and intermolecular interactions are occurring between PPI^c-PPI^c and PPI^c-AL at the 6.0% (w/w) level relative to the 2.0% (w/w) level since the total biopolymer concentration is greater despite having a capsule size that remains relatively constant. It is presumed the higher levels of biopolymers present would lead to more dense capsules that retain greater hydration. As such, this may resist capsule shrinking due to osmotic effects or partial degradation. Gunasekaran et al. (2007) investigated the effect of protein concentration on swelling behaviour of whey protein concentrate hydrogels heated isothermally at 80°C. They observed increased swelling with lower concentrations of whey protein. The authors also reported that at the higher concentration the density of protein network was higher, resulting in less swelling.

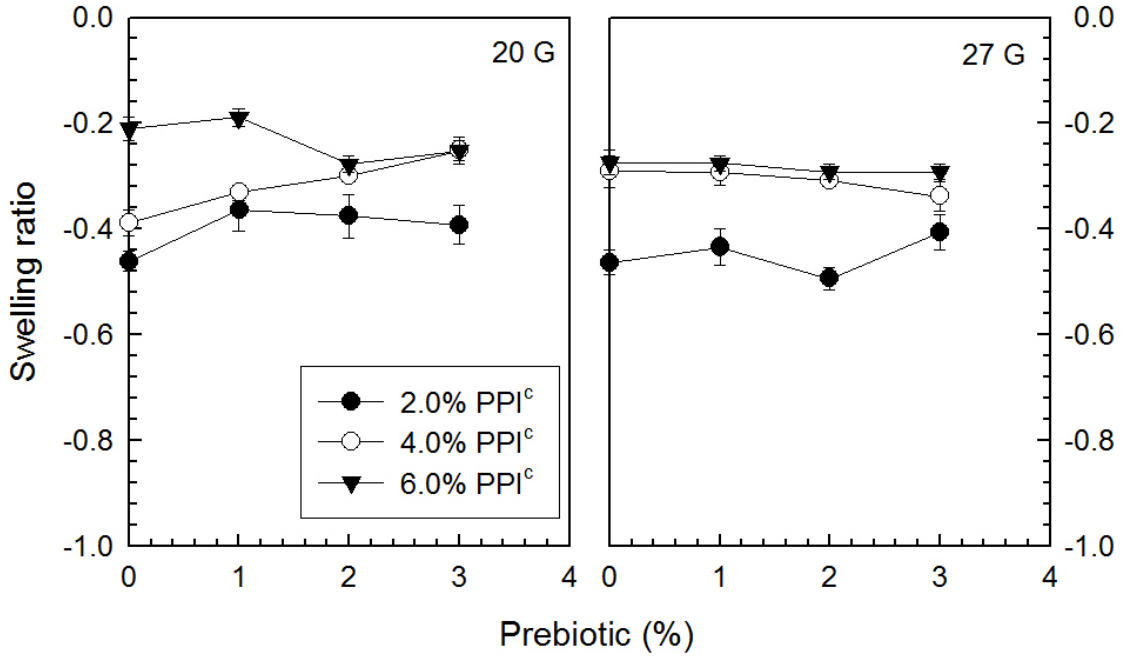

Figure 4.12 The swelling behaviour of PPI^c-AL based capsules immersed in SGJ at 37^{o}C for 2 h. PPI^c-AL based capsules were prepared with differing prebiotic levels (0, 1.0, 2.0, 3.0%, w/w), protein concentrations (2.0, 4.0, 6.0%, w/w) and needle gauges (20, 27 G) used during extrusion processing. Data represents the mean value ± one standard deviation (n = 3).

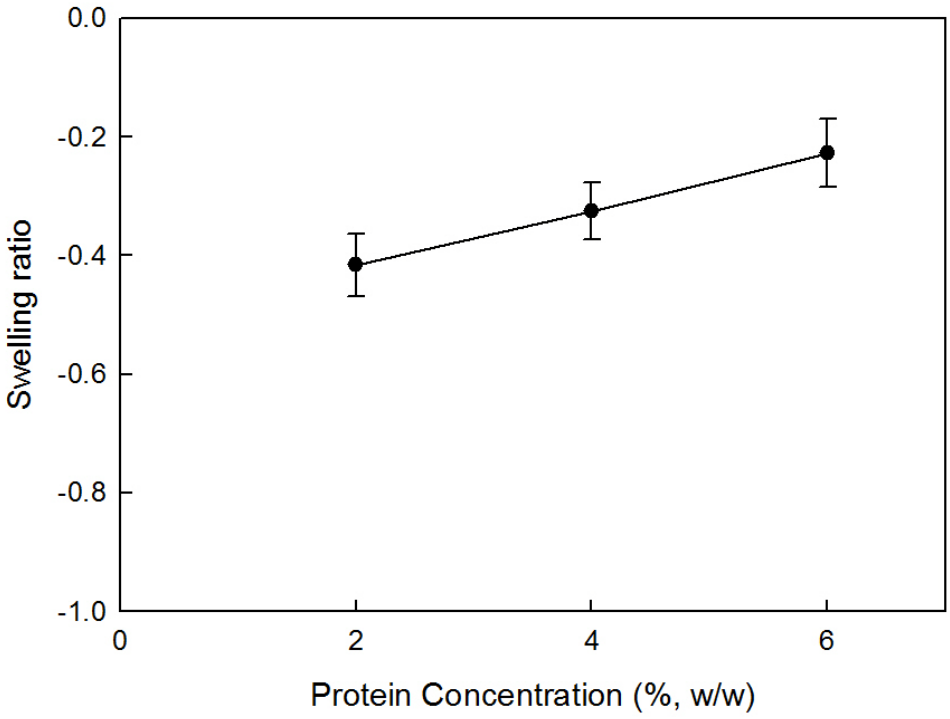

Figure 4.13 The swelling behaviour of PPI^{c}-AL based capsules immersion in SGJ at 37^{o}C for 2 h prepared without *B. adolescentis* and as a function protein concentration (2.0, 4.0, 6.0%, w/w). Data shown in this plot represent pooled data from Figure 4.12 based on an ANOVA analyses. Data represents the mean value ± one standard deviation (n = 24).

4.4.4 Survival of free and entrapped *B. adolescentis* within simulated gastric juice

The effect of protein concentration, prebiotic levels, and needle gauge on the survival of free and entrapped *B. adolescentis* within SGJ, pH adjusted to pH 2.0 was investigated. Overall, all capsule designs, regardless of the protein or prebiotic concentration added to the wall, or the needle gauge used during extrusion provided highly effective protection to the bacteria over 2 h duration of exposure to SGJ (Figure 4.14). As indicated by a less than 1.5 log reduction in viable cell numbers. In contrast, all free cells were lost within the first 25 min of immersion in SGJ (D-value 1.44) (Figure 4.14a). Kotikalapudi et al. (2010) using a PPI-AL capsule to entrap *Lactobacillus acidophilus* also reported a significant protective effect of the capsules versus free cells. Wood (2010) entrapped *B. adolescentis* within AL, PPI-AL, and WPI-AL capsules produced via extrusion and emulsion-based techniques. Survival within SGJ at pH 2.00 was similar to free cells for all materials in capsules produced by emulsion-based processes (size ranging between 10 and 350 µm). However, both PPI-AL and WPI-AL based capsules produced by extrusion (size ranging between 2.0 and 2.3 mm) showed significantly improve protection versus free cells, which died off within the first 10 min. AL capsules produced by extrusion showed no protective effect. Lee and Heo (2000) reported that large AL capsules (2.62 mm) provided better protection to *B. longum* cells within SGJ (pH 1.55) over a 3 h incubation period at 37°C compared to smaller capsules (1.03 mm). Muthukumarasamy et al. (2006) also reported larger AL capsules increased survival of probiotics in SGJ. The authors compared two methods for encapsulation of probiotics, extrusion and emulsion techniques, and found that encapsulation using AL and AL plus starch via extrusion produced significantly larger capsules (2.0-4.0 mm) compared to emulsified capsules (20 µm to 1.0 mm); and reported that the larger capsules were better able to protect *Lactobacillus reuteri* against SGJ. Truelstrup-Hansen et al. (2002) reported capsules of much smaller size (20 and 70 µm) produced via emulsification techniques did not improve survival of *Bifidobacteria* compared to free cells in SGJ.

The addition of prebiotics to the PPI^{c}-AL capsules in the present study did not show any impact on survival through 2 h of immersion in SGJ at 37°C. In the present

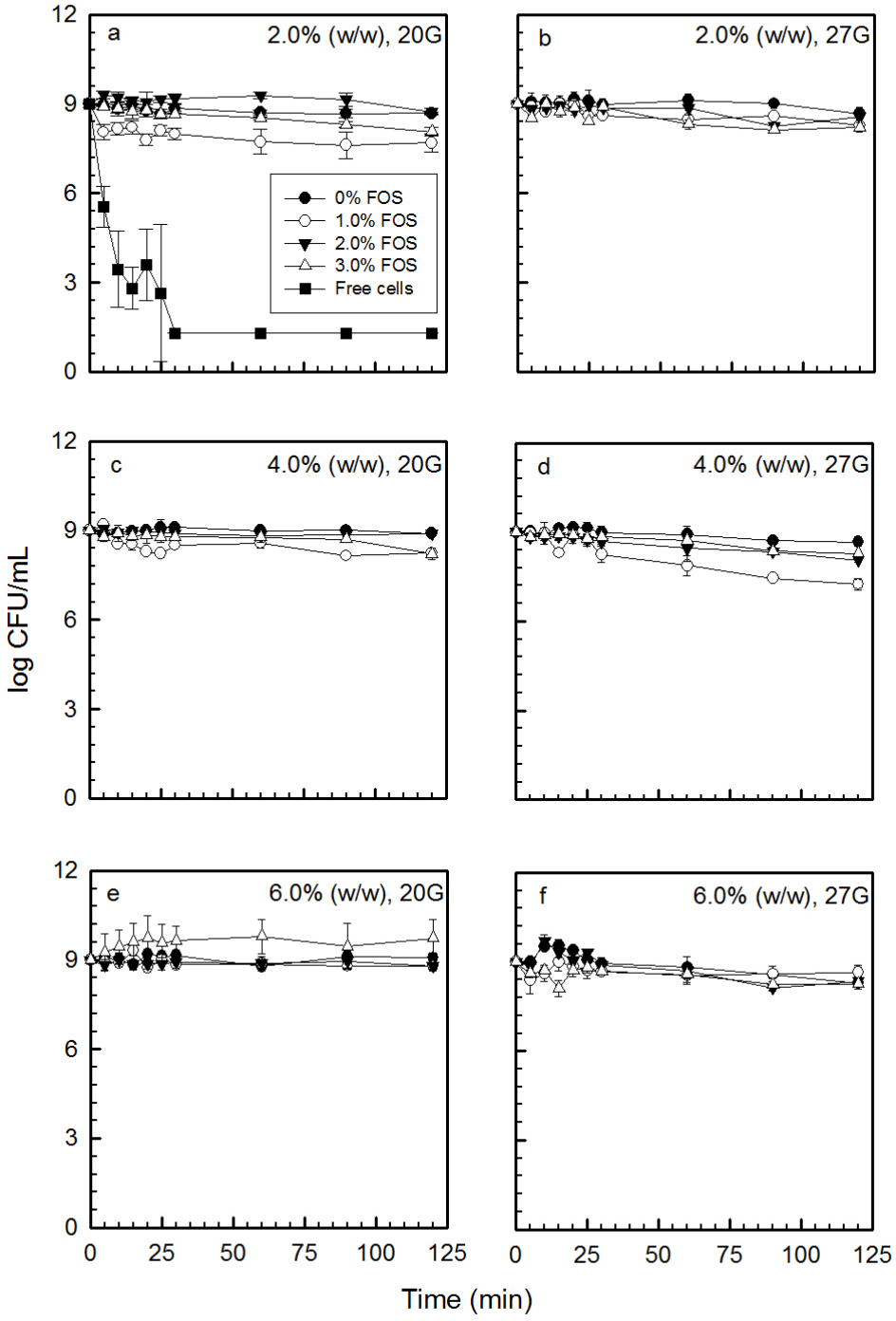

Figure 4.14 Survival of free and entrapped *B. adolescentis* within various PPI^{c}-AL based capsule designs, testing the effect of protein concentration (2.0%- a, b; 4.0%- c, d; 6.0%- e, f), prebiotics levels (0, 1.0, 2.0, 3.0% FOS) and needle gauge (20 G- left; 27 G- right). Data represents the mean value ± one standard deviation (n = 3).

study, designed capsules without FOS provided sufficient protection preventing the effects of FOS addition from being observed. Bielecka et al. (2002) demonstrated that prebiotics and probiotics administered to rats in a synbiot form showed a greater bifidogenic effect compared to probiotics administered alone. These results would indicate the administration of an encapsulated synbiot should also prove to provide better protection of probiotics though gastrointestinal conditions. Wood (2010) reported the addition of 1.0% FOS to PPI-AL capsules containing *B. adolescentis* improved survival within SGJ at pH 2.0 and 37°C. The enhanced protective nature was attributed to the prebiotic increasing the walls ability to limit SGJ diffusion into the capsule. Chen et al. (2005) also reported an increase in survival of probiotic bacteria in SGJ when encapsulated with a prebiotic source. The authors produced capsules via extrusion containing 4.0% (v/v) probiotics (1.0% of each *L. acidophilus, L. casei, B. bifidum,* and *B. longum),* 1.0-3.0% AL, 0-3.0% prebiotics (FOS and isomaltooliosaccharides), and 0-1.0% peptides (pancreatic digested casein). Chen et al. reported that the addition of 3.0% FOS to 1.0% AL and 1.0% peptides provided optimal encapsulation of probiotics and improved survival of probiotics in SGJ (pH 2.0) over a 1 h test period relative to those without FOS. Iyer and Kailasapathy (2005) tested the survival of two *Lactobacillus acidophilus spp.* (CSCC 2400 or CSCC 2409) co-encapsulated with prebiotic (Raftiline®ST, Raftilose®P95, or Hi-maize™) in AL capsule prepared using an Inotech Encapsulator®. They reported that *Lactobacillus acidophilus spp.* co-encapsulated with Hi-maize starch had a heighten survival rate after exposure to acid conditions (pH 2.0) over a 3 h period compared to the free cells, encapsulated cells (without prebiotics), and those co-encapsulated with Raftiline ®ST and Raftilose®P95. The authors report the improved survival of *L. acidophilus* co-encapsulated with Hi-maize is due to the Hi-maize blocking the pores of the capsule, thereby preventing diffusion of SGJ into the capsule.

4.4.5 Release studies of *B. adolescentis* within simulated intestinal fluids

Release within SIF at pH 6.5 at 37°C under anaerobic conditions was investigated over a 3 h duration. Capsule designs containing 2.0% and 6.0% PPI^{c}-AL with 0 and 3.0% FOS were tested. These capsules were selected to represent the most

extreme formulations tested during the capsule design and survival experiments. Overall, all capsule designs had an initial burst release of ~5.5 log CFU/mL at time zero and then a more gradual release, reaching ~7 log CFU/mL within the SIF after ~1.5 h. For all of the capsule designs, release of bacterial cells was shown to have similar behaviour (Figure 4.15). Survival of free cells suggests that once released, *B. adolescentis* will be able to survive the SIF conditions. Release of the bacteria is thought to follow two possible mechanisms. The addition of $NaHCO_3$ in SIF could act as a destabilizing agent. Sodium (Na^+) is an anti-gelling cation, that when present at high concentrations has been shown to destabilize the AL matrix (Smidsrod and Skjakbraek, 1990). Secondly, proteases present within the SIF are also thought help breakdown the capsule by degrading the PPI^c within the wall material to release the cells into the medium.

Many studies have investigated the release of encapsulated probiotics within SIF (Rao et al., 1989; Cui et al., 2000; Krasaekoopt et al., 2004). Cui et al. (2000) found the release of *B. bifidum* from AL-poly-*l*-lysine capsules with SIF to reach 10^8 CFU/g within 8 h, with full release (log 10^9-10^{10} CFU/g) occurring after 12 h of exposure. Rao et al. (1989) reported complete dissolution of capsules prepared with cellulose acetate phthalate by phase separation techniques and release of *B. pseudolongum* (10^9 CFU/g) within 20-40 min of being subjected to SIF.

4.4.6 Summary

In summary, capsule design (i.e., protein concentration, prebiotic levels and needle gauge used in extrusion) showed similar results in terms of their ability to protect *B. adolescentis* under SGJ conditions, and then showed release under simulated intestinal conditions. Release of *B. adolescentis* from the capsule was presumably caused by enzymatic degradation of the PPI^c within the capsule wall from proteases or excess Na^+ in SIF. Capsule size was found to be influenced only by the needle gauge used in extrusion processing, whereby the 27 G needle produced slightly smaller capsules than the 20 G needle. Capsules, depending on the protein concentration used underwent shrinking under SGJ; however this did not translate into reduced survival during challenge studies.

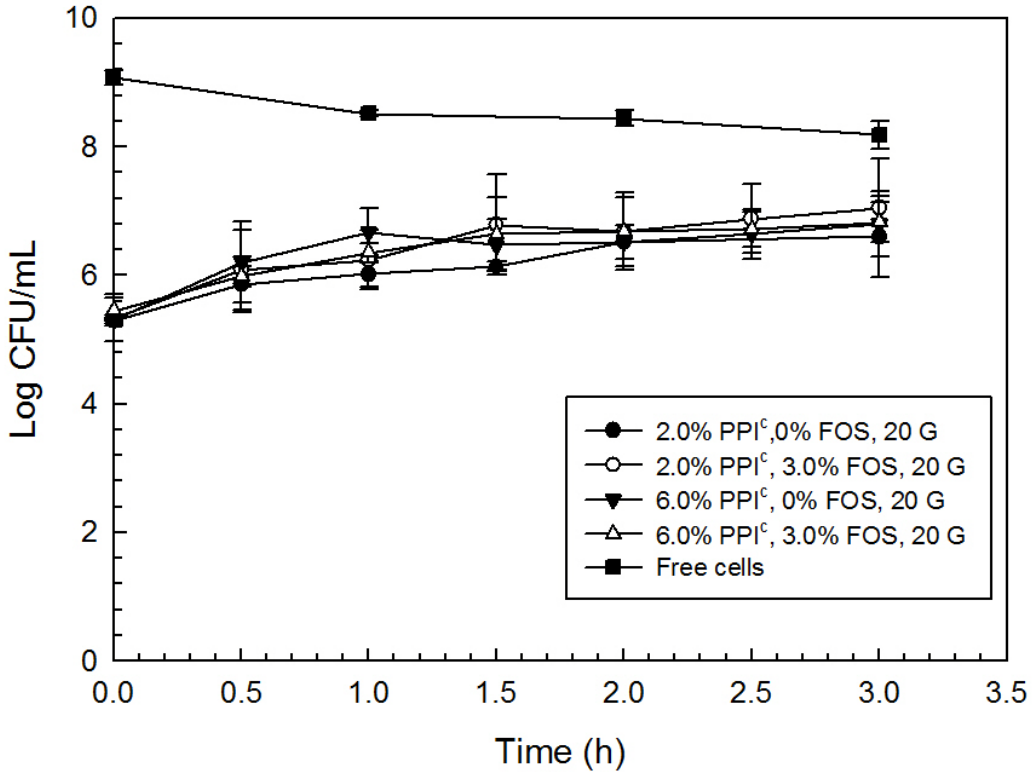

Figure 4.15 Release of entrapped *B. adolescentis* from within PPI^c-AL based capsules over time (h) after being immerged within SIF at 37°C. Capsules designs tested included those with 2.0% and 6.0% PPI^c with (3.0%) and without prebiotics (FOS), and compared to free cells. All capsules germinated using 20 G needle. Data represents the mean value ± one standard deviation (n = 3).

5. Conclusions

The overall goal of this research was to develop a plant protein-based capsule, using phase separation as a means to tailor the wall material, which would offer protection to *B. adolescentis* under simulated gastric conditions while also releasing the probiotic-prebiotic synbiot under simulated intestinal conditions. In the initial study, the effect of biopolymer and solvent conditions on phase separation was investigated for mixtures of pea protein isolate (PPI) and alginate (AL) to identify conditions where associative and segregative phase separation occurred. The resulting biopolymer mixture would constitute the wall material for the capsule design. Both the biopolymer mixing ratio and pH greatly affected associative phase separation. Critical structure forming events (pH_c, $pH_{\phi 1}$, pH_{opt}) were found to shift towards higher pH values as the PPI-AL mixing ratios increased up to mixing ratios between 4:1 and 8:1 after which, critical pH values became independent of biopolymer mixing ratios up to 20:1 and resembled those of homogenous PPI solutions. Associative phase separation of PPI^n-AL was found to be optimal at a biopolymer mixing ratio of 4:1, with maximum interactions occurring at pH 2.75 ± 0.05. Similar trends were also found with electrophoretic mobility measurements where net neutrality shifted towards higher pH as the biopolymer mixing ratio increased to an 8:1, mixing ratio after which the pH corresponding to net neutrality remained relatively constant up to a 20:1 mixing ratio. In contrast segregative phase separation within the biopolymer mixtures was found to occur for these same mixing ratios at solvent pH >~5.00. The acidic pH required for optimal associative interactions prevented associative phase separation from being used for encapsulation of the probiotic bacteria, which have optimal growth at neutral pH (pH 6.00-7.00).

Due to the harsh conditions required for optimal associative phase separation to occur, two capsules designs were developed using segregative phase separation in the presence of calcium for the entrapment of the probiotic bacteria. PPI-AL capsules were designed using both a native PPI (PPI^n) and commercial PPI (PPI^c). PPI^n and PPI^c capsules with entrapped *B. adolescentis* were effective at protecting the probiotic through challenge studies to SGJ (pH 2.00) at 37°C for 2 h. Cell numbers remained high

at 10^8-10^9 CFU/mL through the 2 h period where as the free cells experienced a rapid decline in numbers after the first 10 min within the SGJ.

The use of the commercial PPI in capsule design was further investigated as it will be easier for scale-up within industry compared to using the 'native-form' of the PPI. Capsule performance was investigated as a function of protein concentration (2.0, 4.0, and 6.0%, w/w), prebiotic level (0, 1.0, 2.0, and 3.0%, FOS w/w), and extrusion conditions (20 vs. 27 gauge needle) in order to optimize (for parameters tested) the capsules protective ability for *B. adolescentis* against SGJ and release within SIF. The capsule designs showed similar results in terms of their ability to protect *B. adolescentis* within the SGJ. The capsules also revealed release within SIF, which was presumed to be due to enzymatic degradation of the PPI^c capsule wall and/or due to the excess amount of Na^+ present in the medium (Smidsrod and Skjakbraek, 1990). The capsules retained nearly all of the protein added, where the concentration of prebiotics added was dependent on the amount initially added to the capsule. Larger capsules were able to entrap more FOS per capsule compared to the smaller ones. The capsule size was influenced by the needle gauge, where a 20 G needle produced larger capsules (2.17 ± 0.06) compared to the 27 G needle (1.75 ± 0.04). The capsules were also found to shrink when subjected to SGJ, with high protein rates slowing shrinkage. Although the capsules shrank, this did not affect the ability of the capsule to protect the *B. adolescentis* in SGJ. Based on research performed the overall optimized capsule design to move forward would be 2.0% PPI^c-0.5% AL without FOS and extruded through a 20 G needle. These capsules provided sufficient protection and release of the probiotic bacteria when subjected to challenge experiments. The 2.0% PPI^c would allow for less protein to be used and therefore would reduce the cost of the capsule during production. A 20 G needle during processing would allow for easier production of the capsules and produced only a minimal increase in size compared to the 27 G needle. In the current research prebiotics did not enhance the protective ability of the capsule for *B. adolescentis* during challenge experiments. However, no studies were performed on the release of the prebiotic within the colon. It is hypothesized that the added FOS to the capsules could improve colonization of the bacteria once reaching the colon and beneficially affect the endogenous bacteria within the colon. Further studies would be

warranted to better evaluate the benefit of FOS inclusion and targeted synbiot delivery. Based on capsule size (~2 mm in diameter), developed capsules would be unsuitable for the human food market, however based on their size they may be more appropriate for the feed additive/supplement and/or pet food markets.

6. Future Directions

This study revealed that PPI-AL capsules produced via extrusion can provide 2 h protection for the probiotic bacteria, *B. adolescentis* ATCC 15703 in SGJ at pH 2.0 regardless of capsule parameters and then be released over the course of 3 h within SIF at pH 6.5. Swelling tests also revealed shrinking of the capsules in SGJ at pH 2.0 suggesting either physical degradation of the capsule or passive diffusion of water out of the capsule. Using microscopy to examine the structure during these swelling experiments as well as during degradation of the capsule in SIF (pH 6.5) might give a better understanding of how the capsules release the prebiotics and probiotics.

A synbiot was successfully produced by entrapping *B. adolescentis* and the prebiotic FOS within the PPI-AL capsule. The added FOS did not influence the ability of the capsule to protect the probiotic bacteria when subjected to gastric challenge experiments. Once into the intestine capsule breaks down and releases probiotic. The bacteria are then exposed to acid and bile salt causing the cells to be in a stressed state. The added prebiotic source could provide an accessible carbon source in the colon giving the probiotic bacteria a competitive advantage during adhesion to the intestinal wall. No studies were performed on release of the FOS in SIF. As well as, no studies were done to determine performance of the capsules or entrapped synbiots during sequential transit through the stomach, small intestine and colon conditions. A simulator of the human intestinal microbial ecosystem (SHIME) could provide valuable information on the ability of the capsule to move sequentially through gastrointestinal conditions as well as determine if the added FOS provides a useful carbon source once the probiotic is released in the intestine.

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