Microencapsulation of *Bifidobacterium adolescentis* with legume protein isolates crosslinked with genipin

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ABSTRACT

The overall goal of this research was to design a crosslinked legume protein microcapsule capable of increasing viability of *Bifidobacterium adolescentis* when exposed to acidic conditions in order to maintain sufficient probiotic numbers for a host to experience a positive health benefit. Legume protein isolates derived from chickpea (CPI), faba bean (FPI), lentil (LPI) and soy (SPI) were used as wall materials to test the effect of protein-type on the protective nature of the capsule. The research was designed into two phases: first, the characterization of select physicochemical properties of legume proteins and their emulsifying properties; and second, the design of a genipin crosslinked capsule for carrying probiotics.

In study 1, the physicochemical properties of legume protein isolates were investigated for their ability to stabilize an oil-in-water emulsion in the presence and absence of genipin. Solubility, surface (charge and hydrophobicity), and interfacial (interfacial tension) characteristics of all protein isolates were determined along with their crosslinking ability with genipin. Solubility was found to be highest in CPI (~94%), followed by LPI (~90%), FPI (~85%) and SPI (~50%). Surface characteristics of the protein isolates revealed similar zeta potentials (~ -47 mV) for CPI, LPI and FPI, while that of SPI was lower (~ -44 mV). In contrast, surface hydrophobicity was greatest for CPI (~137 arbitrary units, AU), followed by SPI/LPI (~70 AU) and FPI (~24 AU). A significant reduction in interfacial tension (from 16.73 to ~8.42 mN/m) was observed in canola oil-water mixtures in the presence of legume proteins. Genipin crosslinking affinity was found to be similar for each protein isolate as indicated by similar UV spectroscopic values. Overall, emulsion stability as determined by creaming in canola oil-water mixtures increased in the presence of genipin regardless of the legume protein present. Maximum stability in the presence of genipin was highest for SPI (65%), followed by FPI (61%), LPI (56%) and finally, CPI (50%).

Based on this knowledge, all legume proteins were used as wall materials to encapsulate a probiotic core material. Encapsulation was performed using an emulsification technique where canola oil was used to form the continuous phase, and a mixture of legume protein isolate solutions, genipin and *Bifidobacterium adolescentis* was used to form the aqueous discontinuous

phase. Although various capsules formulation (with and without biopolymer coatings/prebiotics) and preparation methods (stir rates, crosslinking times) were tested, the micron sized capsules produced were not adequate for protecting *Bifidobacterium adolescentis* during acid challenge.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
%	Percent
% w/v	Percent by Weight to Volume Ratio
% w/w	Percent by Weight to Weight Ratio
% w.b.	Percent Wet Basis
µL	Microlitre
µm	Micrometre
ANS	8-anilino-1-naphthalenesulfonic acid
APW	Alkaline Peptone Water
AU	Arbitrary Units
BSA	Bovine Serum Albumin
CAP	Cellulose Acetate Phthalate
CFU	Colony Forming Unit
CPI	Chickpea Protein Isolate
CS	Creaming Stability
Da	Dalton
FPI	Faba bean Protein Isolate
FI	Fluorescent Intensity
FOS	Fructooligosaccharides
g	Gram
GIT	Gastrointestinal Tract
GP	Genipin
h	Hour
ha	Hectare
HDL	High-Density Lipoprotein
HPLC	High Performance Liquid Chromatography
IR	Infrared Spectroscopy
kDa	Kilodalton
LDL	Low-Density Lipoprotein

LPI	Lentil Protein Isolate
M	Molar
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
MM	Molecular Mass
MQW	water produced from a Millipore Milli-Q^{TM} water purification system
MRS-cys	Lactobacilli De man, Rogosa, Sharpe media supplemented with L-cysteine hydrochloride monohydrate
n	Number of replicates
NaCas	Sodium Caseinate
NMR	Nuclear Magnetic Resonance
pI	Isoelectric Point
rpm	Rotations Per Minute
s	Second
S	Sevdberg Unit
SGJ	Simulated Gastric Juice
SHIME	Simulation of the Human Intestinal Microbial Ecosystem
SIF	Simulated Intestinal Fluid
SPI	Soy Protein Isolate
WPI	Whey Protein Isolate
g	Gravity

1 INTRODUCTION

1.1 Summary

Probiotics are live microbial organisms that impart a beneficial change to host health, beyond that of general nutrition, through interacting with indigenous, intestinal bacteria and/or by producing a metabolite (Fuller, 1989; O'Riordan et al., 2001; Picot & Lacroix, 2004). The health benefits associated with probiotic consumption are generally preventative in nature and have not been conclusively substantiated in healthy populations. However, these proposed benefits include: decreased lactose malabsorption; reduced risk of coronary heart disease; increased competitive exclusion of detrimental microbes; suppression of some cancer cells; and the production of certain B vitamins (Gibson & Wang, 1994a; Kulkarni & Reddy, 1994; Agerbaek et al., 1995; Gibson & Roberfroid, 1995; Fooks et al., 1999). In addition to having unproven health benefits, there are many challenges associated with probiotic supplementation. The first being that regardless of the probiotic used, it is widely accepted that at least 7.0 log colony forming units (CFU) mL^{-1} must arrive in the colon in a viable state in order for any benefit to be recognized by the host (Bouhnik, 1993; Krasaekoopt et al., 2003). These viable microbial numbers are difficult to achieve because of the natural defenses of the human gastrointestinal tract which have evolved to prevent invasion, colonization and subsequent infection by foreign bacteria.

Encapsulation is the process by which a core material (e.g., probiotics) is entrapped within a wall material to afford protection from harsh environmental conditions and to help facilitate targeted delivery to maximize core material health benefits (Steenson et al., 1987; Champagne et al., 1992; Risch, 1995). Encapsulation technology has the potential to increase survival of probiotics within a food product and during transit through the gastrointestinal tract. However, findings from literature are variable depending on the wall material, the method of encapsulation, and the properties of the capsules themselves (Sultana et al., 2000; Truelstrup-Hansen et al., 2002; Krasaekoopt et al., 2003).

The overall goal in the present study was to design a crosslinked legume protein microcapsule for the delivery of viable *Bifidobacterium adolescentis* to the human colon in

sufficient numbers for the host to experience a positive health benefit. Specifically, microcapsules were prepared using proteins from chickpea, faba bean, lentils and soy, and then crosslinked with the non-toxic chemical fixative, genipin. Plant proteins are becoming increasingly important to the food industry as a replacement for animal-derived proteins (e.g., gelatin, casein and whey) for use as food and encapsulating ingredients. Plant proteins represent an attractive alternative because of their low cost, renewability and functionality, and as replacements for animal proteins based on consumer choices (e.g., vegans) and religious practices. Although soy proteins dominate the plant protein ingredient market, concerns over allergens are driving research activities towards other legume based proteins derived from chickpea, faba bean, lentil and/or pea, due to their similar physicochemical properties (Sánchez-Vioque et al., 1999; Boye et al., 2010b; Joshi et al., 2012). Legume proteins were used for this study to show their applicability for use as encapsulating agents for probiotic bacteria, with the potential of affording improved protection against simulated gastric conditions over alginate wall materials. In literature, micron sized alginate capsules offer little protection to probiotics. It is hypothesised that the globular nature of the legume proteins will reduce pore size within the capsule wall to offer suitable probiotic protection. Legumes are a good source of protein that can be locally and inexpensively produced for use in a variety of food grade products (Comai et al., 2007; Boye et al., 2010b). In addition to their nutritive quality, legume proteins have been proposed to have potential human health benefits including reducing the risk of cancer, cardiovascular disease, diabetes, hypertension and osteoporosis (Hu, 2003; Tharanathan & Mahadevamma, 2003; Boye et al., 2010b).

1.2 Objectives

To address the overall goal of this research, the following objectives were proposed:

- to investigate the relationship between the protein's physicochemical properties and affinity to genipin, with their emulsifying properties within an oil-in-water emulsion;
- to develop genipin crosslinked microcapsules of ≤100 μm in size which are capable of encapsulating *B. adolescentis* using an emulsion-based technology; and
- to study the survival of *B. adolescentis* throughout an acid challenge using various wall formulations (protein-type, prebiotics, and/or coating materials).

1.3 Hypotheses

To address the overall goal and objectives of this research, the following hypotheses were proposed:

- all legume proteins will display properties conducive to stabilizing oil-in-water emulsions, related to their physicochemical properties, and genipin will act to improve their ability to stabilize emulsions;
- legume protein microcapsules crosslinked by genipin can be designed at sizes ≤100 μm that are capable of carrying a sufficient number of cells to give a health benefit to its host; and
- legume protein capsules will afford protection to *B. adolescentis* during an acid challenge study, where survival could be enhanced in the presence of prebiotic material and/or the addition of coatings to the capsule's surface.

2 LITERATURE REVIEW

2.1 Probiotics

2.1.1 Definition

The definition of a probiotic has been modified over time as the relationship between a host and its intestinal microbial flora has become increasingly understood. For the purpose of this study, probiotics are defined as: live microbial organisms that impart a beneficial change to host health, beyond that of general nutrition, through interacting with indigenous bacteria and/or by producing a metabolite (Fuller, 1989; O'Riordan et al., 2001; Picot & Lacroix, 2004). As an extension of this definition a microorganism should exhibit all of the following characteristics to be considered an effective probiotic (Fuller, 1992; Krasaekoopt et al., 2003):

1. retention of viability during processing on an industrial scale and during long periods (ideally greater than 28 days) of storage;
2. retention of viability during passage through the gastrointestinal tract to arrive at the colon with numbers of at least 7.0 log CFU mL^{-1}; and
3. retention of the ability to impart a beneficial outcome upon host health.

2.1.2 Health benefits

The health benefits associated with probiotic consumption are generally preventative in nature and have not been conclusively substantiated in healthy populations. Select benefits are thought to include: decreased lactose malabsorption; reduced risk of coronary heart disease; increased competitive exclusion of detrimental microbes; suppression of some cancer cells; and the production of certain B vitamins (Gibson & Wang, 1994a; Kulkarni & Reddy, 1994; Agerbaek et al., 1995; Gibson & Roberfroid, 1995; Fooks et al., 1999). Lactose malabsorption or intolerance has been reported to affect over half the world's population (Fooks et al., 1999). The condition arises from an inadequate breakdown of ingested lactose in the human gut due to a lack of lactase (β-galactosidase) activity. Symptoms of lactose intolerance may include abdominal distress and discomfort, increased flatulence, and diarrhea. Some probiotic organisms, including certain *Lactobacillus* species, can alleviate these symptoms by producing β-galactosidase in the

colon (Fooks et al., 1999; Montalto et al., 2006). The consumption of probiotics has been linked with low-density lipoprotein (LDL) and total serum cholesterol reduction, as well as LDL: high-density lipoprotein (HDL) ratio reductions, which have been associated with reducing the risk and/or progression of coronary heart disease (Levy et al., 1984; Gordon et al., 1989; Schaafsma et al., 1998). The potential mechanisms proposed for this probiotic action include: interference with cholesterol absorption in the gut, direct uptake of cholesterol by probiotics, and production of metabolites that could impact total blood lipid levels in the host (Fooks et al., 1999). Probiotics may also reduce the number of pathogenic organisms present in the gut through competitive exclusion, which may decrease the frequency of intestinal infections, largely through competition for nutrients. There are approximately 10^{12} live cells per gram of human large intestinal contents, making access to nutrients very competitive, which is the main mechanism by which one type of microbe may reduce the population of another (Gibson & Wang, 1994a). Consumption of probiotics has been identified as having potential as a preventative measure for colon cancer which is one of the leading causes of death in the USA (Benno & Mitsuoka, 1992; Kulkarni & Reddy, 1994). Proposed mechanisms of action include: suppression of carcinogens by blocking, binding or removal; competitive suppression of enzyme producing microbes that have the potential to convert procarcinogens to carcinogens; and a reduction in gastrointestinal transit time which limits exposure to potentially harmful compounds (Fooks et al., 1999). Finally, some *Bifidobacterium* species have the ability to produce water soluble B vitamins including thiamine, nicotinic acid, folic acid, vitamin B12 and biotin, which can impart the host with nutritional benefits beyond those implicit to the organism itself (Deguchi et al., 1985; Noda et al., 1994).

2.1.3 Types and sources

Many bacterial organisms have the potential to be used as probiotics, but traditionally three genera have been utilized with the greatest frequency: *Bifidobacterium*, *Lactobacillus* and *Streptococcus*. *Bifidobacteria* are Gram positive microaerophilic branch-shaped rods that can account for approximately 95% of colonic organisms in infants (Yoshiota et al., 1991; Gibson & Roberfroid, 1995). Although this number generally drops to 25% in adults, they are an important segment of human gut flora and have been associated with many probiotic health benefits (Fuller, 1992; Gibson & Roberfroid, 1995; Gibson et al., 1997). Many *Lactobacillus* species,

which are non-sporulating anaerobic Gram positive rod-shaped bacteria, are used as probiotics as they can survive and grow in a number of acidic fermented foods (e.g., yogurt), are well suited for growth in the human colon, and have β-galactosidase activity (Fuller, 1992; Fooks et al., 1999). Also, some *Streptococcus* species (e.g., *S. thermophilus*) of human oral and nasal origin have been identified as probiotics as they meet the aforementioned definition (Salminen et al., 1998). These bacteria are facultative anaerobic Gram positive coccoids that are usually found in pairs or chains (Fuller, 1992; Fooks et al., 1999). The aforementioned probiotics can be found in many food products and supplements, and may act as starter cultures in food products that employ microbial fermentation.

2.1.4 Challenges of probiotic use

There are many challenges associated with probiotic supplementation. The first being that regardless of the probiotic used, it is widely accepted that at least 7.0 log CFU mL^{-1} must arrive in the colon in a viable state in order for any benefit to be recognized by the host (Bouhnik, 1993; Krasaekoopt et al., 2003). These viable microbial numbers are difficult to achieve because the natural defenses of the human gastrointestinal tract have evolved to prevent invasion, colonization and subsequent infection by foreign bacteria. Primarily, low stomach pH results in a decrease in viable probiotic counts between ingestion and ensuing arrival at the colon, however the presence of bile salts and proteases also play a role (Marteau et al., 1997; Lee & Heo, 2000; Truelstrup-Hansen et al., 2002; Picot & Lacroix, 2004). As an example, Truelstrup-Hansen et al. (2002) reported that *B. breve* 15700 and *B. longum* 15707 populations exposed to pH 2.0 at 37°C for 2 h were reduced by 3.6 and 4.6 log CFU mL^{-1} respectively as compared to controls at pH 6.0. In contrast, after 30 min exposure at pH 1.9, in the presence of pepsin, *B. breve* R070 and *B. longum* R023 populations with initial inoculation levels of approximately 8.0 log CFU mL^{-1}, were reduced by approximately 6.0 and >7.0 log CFU mL^{-1} respectively (Picot & Lacroix, 2004). Fuller (1989) reported that peristalsis resulted in the flushing of invading bacteria out with food to act as another hurdle to colonization of the gut. In another instance, Marteau et al. (1997) demonstrated that viable counts after 110 min in the gastric compartment (pH decrease from 5.0 to 1.8 by 80 min; in the presence of electrolytic salts and pepsinogen (370 U mL^{-1})) of a dynamic GIT model were reduced from initial inoculation levels (7.0 – 8.0 log CFU mL^{-1}) to: less than 1% for *S. thermophiles* and *L. bulgaricus*, 60% for *L. acidophilus* and 80% for *B. bifidum*.

Probiotic numbers and viability in foods may also decrease during processing, which can include freeze and/or spray drying, heating/cooling and during storage (Mattila-Sandhlm et al., 2002).

The inability of a single bacterial strain to exhibit and optimize all of the ideal characteristics of a probiotic is another limitation of these microorganisms (Sanders & Marco, 2010). A single strain probiotic is ideal in simplifying production, proving efficacy, identifying relationships with native host flora and elucidating mechanisms of action. However, with current probiotics, some organisms exhibit better survival characteristics while others have a wider spectrum of proposed probiotic impact. Therefore, because there is no perfect probiotic organism, researchers are obliged to maintain realistic expectations regarding the probiotic strains that are currently available, and develop other means to optimize their efficacy.

A lack of concrete evidence regarding the efficacy of probiotic supplementation in humans with respect to measurable health benefits also impacts this field's acceptance and opportunities. The majority of peer-reviewed probiotic human trials involve subjects with an illness of some kind which has led to a lack of conclusive evidence regarding the effect of probiotics on healthy individuals. Although not fully proven, an intestinal flora shift from unhealthy to healthy is the currently accepted general probiotic mechanism. However, healthy humans carry a wide variety of intestinal flora which displays little similarity between individuals or within one individual through time (Turnbaugh et al., 2009; Lyra et al., 2010; Salonen et al., 2010). Therefore, it has not been conclusively proven that probiotics will have a beneficial impact for an individual with a healthy compliment of microbial flora, or an ability to generate a healthy flora profile (which is not characterized and may vary). This is mainly due to insufficient resolution of human intestinal populations, even when utilizing advanced molecular techniques. It has been shown that even when a shift in intestinal flora population caused by probiotics has been demonstrated, the effect was temporary, with no lasting impact (Lyra et al., 2010; Salonen et al., 2010). Human testing is further complicated as any quantitative or qualitative health increases associated with probiotics may be a factor of a variety of healthy lifestyle choices such as diet and exercise. This becomes a circular problem because ethically there is a requirement for evidence of efficacy in the complex microbial environment of a healthy human prior to widespread trials and testing. The current state of flux in international labeling laws and regulations regarding probiotics may also be doing irreparable damage to the

field, as the current market is flooded with unproven and possibly ineffective products (Sanders & Marco, 2010).

2.2 Prebiotics

2.2.1 Definition

Similar to probiotics, the definition of prebiotics has been refined over time to reflect increased understanding of their role in human gut health. The definition of prebiotics used for this study is: "Indigestible dietary components that pass through the digestive tract to the colon and selectively stimulate the proliferation and/or activity of one, or a limited number, of desirable bacteria (probiotics) in the colon resulting in increased host health" (Gibson & Wang, 1994b; Crittenden, 2001). An important aspect of prebiotics is that they only impact bacteria already present in the colon; they are not microbes themselves with the capacity to change or add to the composition of the native gut flora. As part of the above definition prebiotics must meet the following criteria (Gibson & Roberfroid, 1995):

1. they cannot be hydrolyzed or absorbed in the upper part of the human digestive system;
2. they must cause growth and/or activation of one or a limited number of beneficial colonic bacteria by being a selective substrate; and
3. they must be able to change the microflora of the colon to a healthier composition and as a result induce beneficial luminal or systemic effects to the health of the host.

2.2.2 Health benefits

Prebiotics have the same potential associated health benefits and mechanisms of action as probiotics because these microorganisms metabolize and/or are activated by prebiotics so as to provide beneficial luminal or systemic effects to the host. These effects may include: decreasing the number of detrimental bacteria present in the colon; reducing plasma lipid levels to reduce the risk of coronary heart disease; production of some B vitamins; and providing constipation relief to the host (Delzenne & Roberfroid, 1994; Tomomatsu, 1994; Fiordaliso et al., 1995; Gibson & Roberfroid, 1995; Gibson et al., 1995). In addition to increasing the effect of probiotics, some animal studies have shown that certain prebiotics have increased the intestinal concentration and absorption of ions, including Ca^{2+} and Fe^{2+} (Levrat et al., 1991; Delzenne & Roberfroid, 1994). The mechanism for this effect has not been elucidated but is proposed to

relate to modified osmotic conditions within the large intestine which may impact absorption (Levrat et al., 1991). A significant limitation of prebiotics is that a pathogen may develop a mechanism to utilize a known prebiotic so as to increase its own proliferation and cause harm to the host (Rastall, 2010). This could, in the future, preclude the use of some compounds which otherwise could be prebiotics.

2.2.3 Types and sources

As defined, prebiotics are generally classed as non-digestible carbohydrates (oligosaccharides); however some peptides, proteins and lipids can also be classified as prebiotics (Gibson & Roberfroid, 1995). This review will focus on carbohydrate prebiotics, specifically oligosaccharides, as they are the most thoroughly studied in literature. Oligosaccharides are characterised as molecules of 2-9 covalently linked monosaccharides (Roberfroid, 2000) that are water soluble, have a low caloric impact, and exhibit a low sweetness value (0.3 to 0.6) (Crittenden & Playne, 1996).

A commonly studied group of prebiotics are fructooligosaccharides (FOS), which are characterized by their β-(1→2) glycosidic bonded β-D-fructose moieties which may contain a terminal D-glucose α-(1→2) linkage (Roberfroid, 2000). FOS are usually produced through inulin hydrolysis (acid or enzyme mediated) and these prebiotics have been shown to increase the growth rate of some *Bifidobacterium* species to a greater extent than native inulin (Gibson & Wang, 1994b; Bielecka et al., 2002; Rossi et al., 2005). Other oligosaccharides that have been shown to support the growth of probiotics include: galactooligosaccharides (Vernazza et al., 2006), maltooligosaccharides (Crittenden & Playne, 1996), and xylooligosaccharides (Rastall, 2010).

2.3 Microencapsulation

Microencapsulation is the process of entrapping a core material within a wall matrix so as to form a capsule with a diameter between 0.2 and 5000 μm. The wall matrix provides protection of the core material from harsh environmental conditions and may also facilitate targeted delivery of bioactive ingredients (e.g., probiotics) (Steenson et al., 1987; Champagne et al., 1992; Risch, 1995). Depending of the type of wall matrix used, the encapsulated core material can be a solid, liquid or gas, and can be hydrophilic or hydrophobic. In this research, the core material

used was the acid sensitive probiotic, *B. adolescentis*, which requires protection from the harsh conditions of the stomach. The two major methods of microcapsule formation for bioactive ingredients such as probiotics are based on extrusion and emulsion technologies. These two methods are amenable to a wide variety of wall materials so as to provide diverse physicochemical properties that impact the functionality of the microcapsule.

2.3.1 Extrusion technique for encapsulation

The first and oldest method of encapsulation is extrusion, which generally involves the use of hydrocolloids such as alginate as wall materials in conjunction with ionic crosslinking (Tanaka et al., 1984; Risch, 1995). This encapsulation method is simple, inexpensive, and gentle which generally provides high retention of core materials (Risch, 1995). Wall materials including alginate (Risch, 1995; Krasaekoopt et al., 2003), and whey protein isolates (Picot & Lacroix, 2004; Hebrard et al., 2010) can be used for capsule formation. The main limitations of extrusion based technology are that the capsules formed are too large (2-5 mm) to be used in a food product without negatively impacting its textural and mouth-feel characteristics, and the procedure is difficult to efficiently scale up (Truelstrup-Hansen et al., 2002; Krasaekoopt et al., 2003). In this method, the wall and core (bioactive) materials are mixed together in a hydrocolloid solution. As this solution is extruded through a syringe, droplets fall into an ionic solution (e.g., Ca^{2+} in the form of $CaCl_2$) which serves to set and crosslink the hydrocolloid solution into a stable capsule with a three-dimensional lattice structure (Risch, 1995; Krasaekoopt et al., 2003). The size of the resulting capsules is mainly dependant on the gauge of syringe used, although the wall and core material concentration, ionic solution viscosity and distance between the syringe tip and the ionic solution can also have an impact.

Extrusion technology has been successfully used for the encapsulation of probiotics. For instance, Chandramouli et al. (2004) prepared an alginate (1.8%) capsule (~450 μm in diameter), hardened for 30 min in 0.1M $CaCl_2$, containing *L. acidophilus* (initial counts of 9.0 log CFU mL^{-1}) and subjected it to an acid challenge for 3 h at 37^{o}C. The authors reported a protective effect of the wall material where only a ~1.0 and ~2.0 log reduction occurred at pH 3.0 and 2.0, respectively. Lee & Heo (2000) also reported that the encapsulation of *Bifidobacterium* species (initial counts of 7.0 log CFU $capsule^{-1}$) within an alginate (4.0%) capsule (~2.6 mm in diameter) that resulted in only a ~1.0 log reduction during a challenge study at pH ~1.5 (with 0.2% NaCl)

for 3 h at 37^{o}C. In addition, Reid et al. (2005) prepared whey protein (12%) capsules (~2.8 mm in diameter) to entrap *Lactobacillus rhamnosus* at levels of 6.2 log CFU mL^{-1}. When exposed to dynamic simulated gastric conditions (pH decrease from 4.4 to 2.0 over 90 min) encapsulated cells exhibited a 2.4 log CFU mL^{-1} reduction compared to a ~4 log CFU mL^{-1} reduction for free cells.

The extrusion-based method was not considered as a means for encapsulation within the current thesis due to challenges associated with obtaining capsule sizes <100µm.

2.3.2 Emulsification technique for encapsulation

Encapsulation of probiotic bacteria using an emulsification procedure has also been established in literature (Sheu & Marshall, 1993; Sultana et al. 2000; Truelstrup-Hansen et al., 2002; Winder et al., 2003). This process is advantageous relative to extrusion-based capsule production, because micron-sized capsules are produced; the simplicity of the methodology; the reduced amount of wall material required; and the ease of scale up (Winder et al., 2003). However, despite these advantages, the microcapsules produced tend to offer poor protection (reduced survival) to probiotics, have decreased core loading capacity and have a large size distribution (0.025 – 2 mm) (Truelstrup-Hansen et al., 2002). The technique involves the formation of a water-in-oil emulsion upon application of mechanical shear to form micron sized droplets. In this case, the continuous phase typically consists of vegetable oil (e.g., canola or corn), and the discontinuous phase consists of both the wall and core materials (Krasaekoopt et al., 2003). However, the method is flexible and the phases can be reversed as required for a hydrophobic core material. Surfactants may also be added to formulations at various concentrations (e.g., Tween 80: 0.33–1.00 g/g [Devi & Maji, 2010] or 0.02% g/g [Sultana et al., 2000] of wall material polymer) to help increase uniformity in droplet size distribution. The addition of an ionic (e.g., Ca^{2+}) or enzymatic crosslinking agent (e.g., transglutaminase) also acts to stabilize individual droplets by inducing gelation/crosslinking of the wall materials to form capsules, which can then be separated and harvested (Sultana et al., 2000; Truelstrup-Hansen et al., 2002; Krasaekoopt et al., 2003; Winder et al., 2003).

The size of formed microcapsules is primarily related to the level of mechanical shear prior to inducing droplet gelation; however both the wall material concentration and the viscosities of continuous and discontinuous phases play a secondary role in size (Truelstrup-

Hansen et al., 2002; Krasaekoopt et al., 2003). Capsules <100 μm in diameter are typically desired as at this size negative impacts to textural attributes and mouth feel characteristics, upon their addition to foods, do not occur (Krasaekoopt et al., 2003; Picot & Lacroix, 2003). However, challenges associated with the high surface area-to-volume ratios of these small-sized capsules have led to poor probiotic protection under both simulated gastric and acid challenge experiments (Sultana et al., 2000). Surfactant and wall material concentrations within capsule formulations have also been investigated for their effects on capsule size, where overall, increased surfactant load and decreased wall material concentration (lower viscosity) lead to decreases in capsule sizes. Surfactants act to lower the interfacial tension between phases of the emulsion, which enables smaller droplets to form upon shearing, and coating of the surface of the droplets so as to create a physical barrier (Zhuo et al., 2004; Devi & Maji, 2010).

Sultana et al. (2000) utilized a wall material comprised of alginate (2%) and Hi-maze resistant starch (2%), hardened with the addition of 0.1M $CaCl_2$, to encapsulate *Lactobacillus acidophilus* and *Bifidobacterium* species using an emulsification technique in oil. The authors reported capsule size ranges between 500 to 1000 μm, with only a small proportion of sizes <500 μm. Acid challenge studies (pH 2.0 for 3 h) involving the capsule designs indicated a 5 and 3 log CFU mL^{-1} reduction for entrapped *L. acidophilus* and *B. infantis*, respectively. In contrast, Truelstrup-Hansen et al. (2002) produced Ca^{2+}-alginate capsules (20 g L^{-1} alginate, 5 g L^{-1} Tween 80, 62.5 mM $CaCl_2$) containing four strains of *Bifidobacterium* (*adolescentis* 15703, *breve* 15700, *lactis* Bb-12 and *longum* Bb-46) at levels ranging from 6-8 log CFU mL^{-1}. This methodology produced capsule sizes of ~70 μm, however during an acid challenge (pH 2.0, 30 min), a 5 log CFU mL^{-1} reduction for all strains was observed, with the exception of *B. lactis* which remained constant. Annan et al. (2008) reported that an emulsion produced, genipin crosslinked gelatin microcapsule, with an alginate coating, was able to protect encapsulated *B. adolescentis*. When subjected to sequential simulated gastric juice (SGJ) and simulated intestinal fluid (SIF) treatments (to simulate the human gastrointestinal tract) these capsules exhibited only a 1.21 log CFU mL^{-1} reduction in viable numbers in SGJ, and released 7.35 to 7.57 log CFU mL^{-1} of viable probiotics in SIF. In addition, Borza et al (2010) reported that emulsion-produced gelatin (16%)-maltodextrin (3%) composite microcapsules crosslinked with genipin (24 mM), having a size of ~70 μm were able to protect encapsulated *B. adolescentis* from SGJ (pH 2.0;

0.32 mg mL^{-1} pepsin). After 2 h of exposure to SGJ free cells experienced a ~4 log CFU mL^{-1} reduction in numbers while encapsulated cells experienced only a ~2 log CFU mL^{-1} reduction.

2.4 Wall materials

The wall materials used in the formation of microcapsules play an important role in terms of the protection afforded, the type of bioactive ingredient that can be encapsulated and the desired core release profiles in terms of dosage and release rates. Materials should be food grade, and exhibit both emulsifying and gelling properties in the presence of ionic or covalent crosslinkers. Prepared capsules should also be miscible within food products, offer protection to the core ingredient within the food environment and the harsh acidic conditions of the stomach (Hebrard et al., 2010). Typically, microcapsules for carrying probiotics are prepared using either proteins or polysaccharides, alone or in combination. Examples of hydrocolloid wall materials include but are not limited to: alginate, carrageenan, cellulose acetate phthalate, chitosan, gelatin, low methoxy pectins and whey protein isolates (Risch, 1995; Roberfroid, 2000; Krasaekoopt et al., 2004; Anal & Singh, 2007; Hebrard et al., 2010). To afford additional protection to the encapsulated probiotic bacteria, wall materials can be used in combination, and/or coatings can be added using biopolymers with opposing charges to the main capsule wall. For example, the positively charged chitosan (or poly-L-lysine) could be used to coat capsules prepared from negatively charged alginate (Lee & Heo, 2000; Corcoran et al., 2003; Hebrard et al., 2010).

Alginate is a common wall material used for the encapsulation of probiotics because of its low cost and gelling abilities in the presence of calcium ions. Alginate-based capsules have been prepared both by extrusion (Lee & Heo, 2000; Chandramouli et al., 2004) and emulsification (Sultana et al., 2000; Truelstrup-Hansen et al., 2002) based techniques. Alginate is a linear polysaccharide that is extracted from various brown algae species and is comprised of repeating α-L-guluronic acid and β-D-mannuronic acid monomeric units. Alginate may be comprised of homopolymeric blocks of either monomer or heteropolymeric blocks of both monomers randomly arranged within the same molecule (Boguń & Rabiej, 2010). The ratio of these monomers can change the physicochemical properties of this material from a dense but brittle gel if α-L-guluronic acid is the more dominant monomer, to a softer and more elastic gel if β-D-mannuronic is the major monomer unit. Chandramouli et al. (2004) prepared alginate (1.8%) capsules (~450 μm in diameter), hardened for 30 min in 0.1M $CaCl_2$, containing *L.*

acidophilus (initial counts of 9.0 log CFU mL^{-1}) and subjected them to an acid challenge for 3 h at 37°C. The authors reported a protective effect of the wall material where a ~1.0 and ~2.0 log reduction occurred at pH 3.0 and 2.0, respectively. Lee & Heo (2000) also reported that the encapsulation of *Bifidobacterium* species (initial counts of 7.0 log CFU $capsule^{-1}$) within an alginate (4.0%) capsule (~2.6 mm in diameter) resulted in a ~1.0 log reduction during a challenge study at pH ~1.5 for 3 h at 37°C. Truelstrup-Hansen et al. (2002) produced Ca^{2+}-alginate capsules containing four strains of *Bifidobacterium* at levels ranging from 6-8 log CFU mL^{-1}. This methodology produced capsule sizes of ~70 μm, however during an acid challenge (pH 2.0, 30 min), a 5 log CFU mL^{-1} reduction for all strains was observed, with the exception of *B. lactis* which remained constant. This reduction in probiotic viability was proposed to be primarily a factor of capsule size and the resultant high surface area to volume ratio.

Protein isolates have been utilized as capsule wall materials, particularly whey protein isolate (WPI) because of cost, availability, safety and amphiphilic nature (Charteris et al., 1998; Reid et al., 2005; Hebrard et al., 2010). These characteristics of WPI, along with its emulsifier activity, make it a useful wall material in encapsulation studies. WPI consists of β-lactoglobulin (58 % of total protein content; 18.3 kDa), α-lactalbumin (14.1 kDa), serum albumin, immunoglobulins and protease peptones. Gel formation occurs when the protein is heated to induce denaturation and polymerization, followed by cooling and the addition of $CaCl_2$ (Hongsprabhas & Barbut, 1997; Bryant & McClements, 2000). Reid et al. (2005) reported that whey protein capsules (12%) extruded into a $CaCl_2$ crosslinking solution (to produce capsules of ~2.8 mm diameter) were able to encapsulate *Lactobacillus rhamnosus* at levels of 6.2 log CFU mL^{-1}, and afforded better protection during a 90 min SGJ challenge experiment, where viable cell counts were reduced by only 2.4 vs. a ~4.0 log reduction for free cells.

2.4.1 Legume protein wall materials

Chickpea, faba bean, lentil and soy proteins have been shown to have similar physical properties. Iqbal et al. (2006) found that the crude protein levels were 24.0%, 26.1% and 24.9% for chickpea, green lentil and green pea, respectively. Boye et al. (2010b) reported that legume seeds from chickpea, faba bean, soy and lentil present a good source of nutritious proteins. Legumes are dominated by two major classes of proteins: albumins and globulins. The former are water soluble with molecular masses ranging from 5 – 80 kDa. Globulins are salt soluble

proteins consisting of legumin (11S (Sevdberg Unit), hexameric, ~350-400 kDa) and vicilin (7S, trimeric, ~50-60 kDa) (Boye et al., 2010b). Legume proteins display relatively high solubility (>80%) at pH 7.0 (depending on the method of extraction/processing), with isoelectric points ranging between 4.0 and 5.0 (Boye et al., 2010b). The applicability of legume proteins as wall materials for carrying probiotics is limited in the literature. As an example, Klemmer et al. (2011) reported that capsules (~2 mm in diameter) made with pea protein-alginate mixtures via extrusion were able to protect encapsulated *B. adolescentis* from SGJ (pH 2.0) over 2 h at levels of 8.0 log CFU mL^{-1},while free cells were reduced below detectable limits after only 30 min.

2.4.1.1 Chickpea

Although a number of varieties of chickpea (*Cicer arietinum*) exist, the two major ones grown for commercial production are Kabuli and Desi. Chickpea acreage increased dramatically in Saskatchewan between 1996 and 2005 from 6,000-172,000 acres and production peaked in 2001 with 1.1 million acres. In 2011, Saskatchewan produced an estimated 75,200 tonnes of chickpeas with average production of 133,200 tonnes from 2001-2010 (Saskatchewan Ministry of Agriculture, 2011). Over the past several years India has produced and consumed approximately 4-6 million tonnes of the total world chickpea production of 7-9 million tonnes (McVicar et al., 2007). Chickpea protein levels have been reported to range from ~20-25%, with globulins representing the largest total protein fraction (~42%) (Sánchez-Vioque et al., 1999; McVicar et al., 2007; Boye et al., 2010b). Some important amino acid contents of chickpea seeds include lysine (1.29-1.37%), methionine (0.26-0.31%), and threonine (0.66-0.73%) (McVicar et al., 2007). Sánchez-Vioque et al. (1999) produced a chickpea protein isolate by isoelectric precipitation which was found to have a protein content of ~78%, a pI value of 4.3 and protein solubility >80% at pH 6.5. Physicochemical analysis of this isolate revealed water absorption, fat absorption and emulsion capacity values, per 100 g of isolate, of ~343 g, ~409 g and ~48% (w/w) respectively. *In vitro* chickpea protein digestibility was shown to be ~76% due to the largely globular structure of the protein which limits hydrolysis by digestive enzymes (Sánchez-Vioque et al., 1999).

2.4.1.2 Faba bean

Faba beans (*Vicia faba*), belonging to the 'dry/broad bean family', are consumed as both whole foods and as ingredients worldwide, and were first grown in western Canada in 1972 (McVicar et al., 2008). World production and import/export data for faba bean has not been compiled, however dry broad bean production ranged from 4.9-5.1 million tonnes from 2003-2006, where China accounted for almost half of all production (McVicar et al., 2008). Faba bean seeds contain 24-30% protein and have cysteine, lysine and methionine contents of 0.25-0.31%, 1.48-1.61% and 0.17-0.19% respectively (McVicar et al., 2008). Faba bean protein is comprised of 10-30% albumins and 45-78% globulins (El Fiel et al. 2002; Boye et al., 2010b). Faba bean protein solubility in water values of >95% have been reported. Based on literature review there is very little physicochemical data available on faba bean protein.

2.4.1.3 Lentil

Lentils (*Lens culinaris*) are one of the most economically important members of the legume family and Canada is the leading exporting nation of lentils. Production in western Canada began in 1970 with 600 hectares (ha) and increased to 960,000 ha by 2009 (McVicar et al., 2010). In 2011, an estimated 1,455,000 tonnes of lentils were grown in Saskatchewan and a yearly average of 920,200 tonnes were produced from 2001-2010 (Saskatchewan Ministry of Agriculture, 2011). World production of lentils ranged from 2.8-4.0 million tonnes from 2000-2007 with India being the largest producing and consuming nation (McVicar et al., 2010). Lentil is used mainly for human consumption as a protein source, and its seeds have a crude protein content of ~19-26%. Lentils are very high in globulins at approximately 70% of the total protein content (Iqbal et al., 2006; Boye et al., 2010a; McVicar et al., 2010). Lentil protein isolates in particular have been studied as an alternative to soy protein isolates in both food products and encapsulation formulations because of their interfacial and emulsifying properties (Joshi et al., 2012). Joshi et al. (2012) produced a lentil protein isolate by isoelectric precipitation with a final protein content of ~90% and subsequently compared its physicochemical, interfacial and emulsifying properties to a whey protein isolate (WPI) and sodium caseinate (NaCas). The effect of emulsifier concentration, pH and temperature were all evaluated on properties including, but not limited to: interfacial tension, emulsion stability index, zeta potential and surface hydrophobicity. It was reported that a lentil protein isolate can be equally effective as WPI and

NaCas, as an emulsifier, at levels ≥ 20 mg mL^{-1} (Joshi et al., 2012). These results indicate the potential for lentil proteins to be used as a wall material for encapsulation (Reid et al., 2005).

2.4.1.4 Soy

Soybean (*Glycine max*) is an oilseed crop, but because of its high crude protein content (> 35%) it is also used in a variety of food products including soy milk and tofu (Canadian Grain Commission, 2011). From 2006-2010 the average protein content of soybeans grown in Canada was 40.0% and in 2011 4.2 million tonnes of soybeans were harvested (primarily in Ontario, Manitoba and Quebec) (Canadian Grain Commission, 2011). Soy protein is made up primarily of globulin (11S) subunits, but also contains 7S, 15S and 2S subunits in decreasing amounts (Kinsella, 1979; Friedman & Brandon, 2001). Soy protein is one of the most thoroughly studied and economically important plant proteins in terms of its functional properties and its potential to behave as an animal protein alternative (Friedman & Brandon, 2001; Joshi et al., 2012). As a result it is also one of the few plant proteins available for purchase as a protein isolate. As an example, Okezie & Bello (1988) reported that an industrially produced SPI (97% protein) had an emulsion capacity of 8 mL of oil g^{-1} of protein and water and oil adsorption values of 4.10 and 4.88 g/g of protein respectively. Select amino acid contents were also reported as 6.1% lysine, 3.7% threonine and 1.4% tryptophan. Other legume proteins continue to be studied as a soy alternative in part because of the potential for different functional properties and the designation of soybean as one of eight priority allergens in Canada, the USA and the EU (Friedman & Brandon, 2001; Boye et al., 2010b).

2.5 Crosslinking of proteins

The crosslinking of protein wall materials during the encapsulation process provides additional structural integrity, improved bioactive ingredient protection, and may aid in the targeted delivery of bioactives. There are a variety of compounds (chemical reagents and enzymes) and analytical protocols that can be used to induce protein crosslinking, however many of the chemical reagents exhibit significant toxicity and are not approved for food use. For instance, epoxides, formaldehyde and glutaraldehyde are effective crosslinking agents but are cytotoxic (Butler et al., 2003; Nickerson et al., 2006b). Protein crosslinking can also be achieved

by using enzymes such as laccase, transglutaminase and tyrosinase, however only bacterial transglutaminase is considered food grade and commercially available (Buchert et al., 2010).

For this research, a novel chemical crosslinking agent, known as genipin, was used to crosslink the legume proteins during capsule formation. This chemical compound has been approved for pharmaceutical and food-grade use (e.g., as a pigment) in Japan, Korea and Taiwan (Sung et al., 1999; Mi et al., 2000; Paik et al., 2001; Nickerson et al., 2006c), but has not yet received approval in Canada. *Gardenia jasminoides* fruits, which contain the genipin precursor geniposide, have been used in traditional Chinese medicine for their anti-inflammatory, diuretic and haemostatic properties (Mi et al., 2000; Butler et al., 2003; Jin et al., 2004). In addition, a related variety of this plant, *Genipa Americana*, is found in Mexico, the Caribbean and Argentina where its fruits are commonly eaten raw and used to make a sour beverage (Butler et al., 2003).

2.5.1 Genipin

Genipin is a crosslinking agent obtained from the *Gardenia* fruit via enzymatic hydrolysis from its parent compound, geniposide, by β-glucosidase (Butler et al., 2003; Nickerson et al., 2006b). The structure of genipin was elucidated by Djerassi et al. in 1961 (Figure 2.1). Gels crosslinked by genipin exhibit comparable strength to those crosslinked by glutaraldehyde (Nickerson et al., 2006b), but have been reported to be much less cytotoxic (5000-10,000 times) (Sung et al., 1999; Mi et al., 2000; Jin et al., 2004). Genipin has been used to crosslink a variety of materials including but not limited to: bovine serum albumin (BSA), chitosan, gelatin, gelatin-carrageenan mixtures, soy and whey proteins (Butler et al., 2003; Annan et al., 2008; Huang et al., 2009; Devi & Maji, 2010). Genipin is known to form intra- and intermolecular covalent bonds through reactions principally with the primary amines of lysine, but also with those in hydroxylysine and arginine residues (Fujikawa et al., 1988; Butler et al., 2003; Nickerson et al., 2006a,b; Maji & Hussain, 2008). One limitation to the utility of genipin as a crosslinking agent is that the reaction takes 3 to 4 h to complete and is pH dependant (Maji & Hussain, 2008). The proposed mechanism for this reaction involves the following two steps (Figure 2.2): (1) a nucleophilic substitution to the dihydropyran ring of genipin followed by a Schiff's base reaction; and (2) a separate Schiff's base reaction with the ester group of the genipin molecule. For both reactions, the primary amine group, provided by a protein is required for crosslinking with genipin (Butler et al. 2003). The first reaction is initiated by nucleophilic

attack at C3 of genipin by a primary amine group on the protein that results in dihydropyran ring opening and formation of an aldehyde group and a secondary amine. The ring then closes as the secondary amine reacts with the aldehyde group, to form a heterocyclic ring bound to a protein molecule (Butler et al., 2003). The second reaction is a S_N2 nucleophilic attack at the ester group on the genipin molecule by a separate primary amine so as to produce an amide linkage. The evidence for these two reaction mechanisms was based on ^{13}C nuclear magnetic resonance (NMR), infrared spectroscopy (IR) and rheological data collected during genipin crosslinking experiments with chitosan, BSA, gelatin and glucosamine (Butler et al. 2003). In brief, the authors found that the ^{13}C NMR chemical shifts of the genipin ester group did not change during the initial stages of the reaction and the storage modulus did not change until traces of methanol, used to measure this S_N2 reaction, were detected; indicating that amide formation was the second reaction in the crosslinking mechanism. Also, the IR bands for C-N increased upon initial reaction of genipin with the aforementioned polymers at the expense of the IR bands for C-O, indicating the ring opening reaction occurred first. Finally, ^{13}C NMR and spectrophotometric data associated with the immediate formation of the heterocyclic genipin-chitosan compound provided additional evidence for this reaction mechanism (Butler et al., 2003). The authors postulated that the slow rate observed for the second reaction was due to an acid catalysis requirement that is only provided after the first reaction reaches completion.

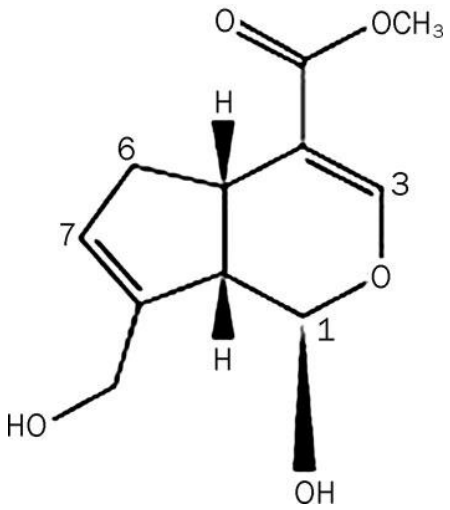

Figure 2.1 Chemical structure of genipin (Feng et al., 2011).

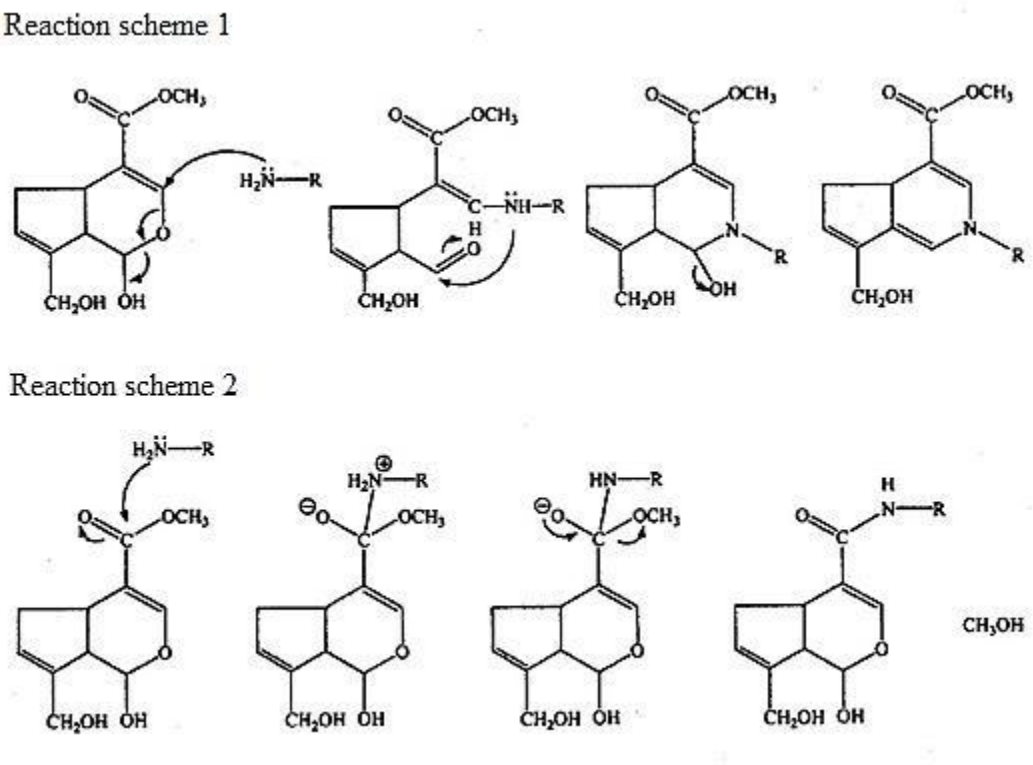

Figure 2.2 Proposed crosslinking reaction mechanism of genipin with primary amines (adapted from Butler et al., 2003)

During genipin crosslinking of proteins/chitosan a blue colour forms. The formation of this colour is associated with oxygen radical induced polymerization of the genipin molecule, which is proposed to only occur following the ring opening step in the first reaction. Polymerization of the genipin molecule also allows it to be sufficiently flexible so as to interact with different protein molecules to form intermolecular crosslinks without high levels of steric hindrance (Butler et al., 2003). A range of environmental conditions, such as temperature and pH, under which the crosslinking reaction takes place, may also impact the degree of intra- and intermolecular crosslinking that is possible due to changes in protein conformation, which may alter the number of available primary amine sites. Protein structure can also impact the degree of crosslinking. For example, globular proteins are generally less available for crosslinking because their compact secondary and tertiary structures limit physical access of the genipin molecule (Buchert et al., 2010). The conformation of the wall materials during capsule formation and

subsequent crosslinking is very important and may be controlled by the reaction conditions utilized. For example, complete or partial denaturation of a reactant protein (via pH, heat treatment, etc.) can modify its crosslinking activity with genipin and impact capsule formation (Meena et al., 2008; Buchert et al., 2010).

2.5.2 Effects of genipin crosslinking

It has been shown that although microcapsules produced via crosslinking have decreased core material concentrations per capsule, they exhibit increased acid resistance to the harsh stomach environment (Annan et al., 2008; Huang et al., 2009; Devi & Maji, 2010). A genipin crosslinked gelatin microcapsule with an alginate coating containing *B. adolescentis* exhibited only a 1.21 log CFU mL^{-1} reduction in viable numbers when exposed to SGJ, and released 7.35 to 7.57 log CFU mL^{-1} of viable probiotics in SIF (Annan et al., 2008). It has been proposed that factors contributing to the protective nature of the wall material included: the increased numbers and strengths of the covalent bonds created by genipin crosslinking, and/or the increase in wall material density created by crosslinking (Berger et al., 2004; Devi & Maji, 2010).

2.6 Capsule coatings

The protection of core materials within a microcapsule can also be improved through the addition of coating materials such as biopolymers, which provide an additional physical barrier to harsh environmental conditions. For example, during transit through the gastrointestinal tract, a significant change in pH occurs from ~6 (mouth) to ~2.0 (stomach) back to ~7.0 (intestines) which can result in swelling and degradation of the capsule wall (Berger et al., 2004; Devi & Maji, 2010). These changes can result in premature release of the core materials, which could lead to issues surrounding dose delivery (Iyer et al., 2005). The addition of coatings can be used to delay this process by providing an additional physical barrier so as to maintain structural integrity of the capsule during transit resulting in a more desirable, delayed release pattern in the colon. Capsule coating can be afforded quite simply through the treatment of a protein-based microcapsule with an oppositely charged biopolymer such as alginate (negatively charged) or chitosan (positively charged) in order to introduce electrostatic binding of the biopolymer coat to the surface of the capsule. In some cases (e.g., alginate), treatment with a hardening agent such as Ca^{2+} is required (Annan et al., 2008; Hebrard et al., 2010). As with wall materials, a wide

variety of biopolymers have been employed as microcapsule coatings including but not limited to: chitosan (Iyer et al., 2005), poly L-lysine (Joki et al., 2001) and alginate (Annan et al., 2008). The ideal microcapsule coating will affect the thickness, permeability, rheology, environmental responsiveness and core release properties of the microcapsule such that it exhibits improved stability and release functionality (Buchert et al., 2010).

For this research, a napin protein isolate, and chitosan polysaccharide were employed as coating materials for the genipin crosslinked legume protein capsules.

2.6.1 Chitosan

Chitosan is produced from chitin by deacylation to yield a polysaccharide comprised of covalently linked β-(1→4)-2-amino-2-deoxy-D-glucopyranose units. Chitosan is both bio-compatible and non-toxic, and has found extensive use in the areas of pharmacology, biomedicine, agriculture, food, and waste treatment (Dutta et al., 2004; Chang et al., 2008). As a coating material, the amino functional groups of chitosan provide an overall positive charge to this biopolymer affording the formation of electrostatic interactions with a negatively charged wall material (e.g., legume protein) (Peng et al., 2012; Wanasundara, 2011). These electrostatic interactions occur without the need of a hardening solution, and result in a microcapsule that may exhibit enhanced protection to the core material (e.g., probiotic). Coating with chitosan leads to an introduction of bioadhesion properties to the microcapsule, resulting from electrostatic interactions at physiological pH (7.3-7.4) between the positively charged groups on chitosan (pKa of 6.3) and the negatively charged mucosal surfaces (due to sialic acid; pKa of 2.6) of the small intestine (He et al., 1998; Woodley, 2001; Bonferoni et al., 2009). The main advantage of microcapsules with bioadhesion properties is an increased residence time in the intestinal system for bioactive core material absorption (Hejazi & Amiji 2003; Bowman & Leong, 2006).

2.6.2 Napin

Napin is a 2S storage protein produced by *Brassicaceae* family embryos during seed development. The *Brassicaceae* family includes many agriculturally important crops such as canola (e.g., *Brassica napus*). Napin is composed of two polypeptide chains with molecular masses of ~4 and ~9 kDa that are covalently linked through disulfide bonds (Ericson et al., 1986). Napin is rich in both glutamic acid and sulfur-containing amino acids and has a very basic

isoelectric point (pI) of ≥ 10 (Wanasundara, 2011). The high overall positive charge of this protein under physiological/capsule formation conditions (e.g., pH 7.0), makes it an ideal candidate as a coating material when used in conjunction with oppositely charged legume proteins. In addition to providing enhanced protection to the core material, napin has been found to exhibit minimal digestibility in the presence of human gastric enzymes and is a plant-based biopolymer (i.e. when coupled with legume proteins the entire microcapsule would be plant-based) (Wanasundara, 2011).

2.7 Acid challenge

The survival of a probiotic can be readily determined by subjecting the organism (free or encapsulated) to an acid (HCl) solution with a pH generally ranging from 1 to 3 for 2-3 h, as an approximation of stomach conditions (Lee & Heo, 2000; Sultana et al., 2000; Truelstrup-Hansen et al., 2002). Because acid exposure is a major detriment to probiotic survival through the stomach (Heatley & Sobala, 1993; Marteau et al., 1997), this method can be used with or without bile salts, for encapsulated probiotics in place of, or prior to the use of more complicated systems (e.g., a Simulation of the Human Intestinal Microbial Ecosystem (SHIME)) as it rapidly provides evidence of the protective properties of the wall material (Lee & Heo, 2000; Sultana et al., 2000; Truelstrup-Hansen et al., 2002). A limitation of this method is that only one factor (i.e. pH) of the body's gastrointestinal environment is examined.

Other gastrointestinal environment testing systems have been employed for determining the protective ability of encapsulation on probiotics. Simulated gastric juice (SGJ) is a solution with a pH of 1.2 to 3.0 that includes bile salts (or NaCl) and pepsin, and is kept at 37˚C to mimic the conditions a capsule would experience in the gastrointestinal tract. Simulated intestinal fluid (SIF) has a pH of 7.5, is kept at 37˚C and contains a variety of digestive enzymes from exocrine cells including amylase, lipase and protease such as trypsin (Hebrard et al., 2010). SGJ and SIF are often used in sequence to simulate the gastrointestinal tract (GIT). A SHIME system is a more complicated model of the human GIT (De Boever et al., 2000) that can also be used to evaluate the functionality of probiotic microcapsules. In this model, five vessels are used in sequence to simulate each aspect of the GIT over ~76 h. Each section has a different pH and digestive enzyme/bile salt composition (which is maintained throughout) and the final three sections are inoculated with bacteria flora commonly found in the GIT, which is also maintained

with nutrient addition. Anaerobic conditions and a temperature of 37°C are maintained in each vessel and the system is generally stabilized (and bacterial populations characterized) for 3 weeks prior to use (De Boever et al., 2000). This is generally a very good model, but one limitation is that it does not account for the selective absorption of nutrients over time and as such is not a perfect representation of the GIT (Hebrard et al., 2010; Sanders & Marco, 2010).

3 MATERIALS AND METHODS

3.1 Materials

The following materials were generously supplied for this research project. Legume seeds were donated by the Crop Development Centre (Saskatoon, SK), and included chickpea (CDC Frontier), faba bean (SSNS) and green lentil (CDC Grandora). Fructooligosaccharide powder (FOS) (Beneo P95: 95% oligofructose) was supplied by BENEO-Orafti (Tienen, Belgium). Napin protein isolate was supplied by Dr. Janitha Wanasundara (Agriculture and Agri-Food Canada, Saskatoon, SK).

The following chemicals and materials were produced by EMD Millipore (Billerica, MA, USA) and purchased from VWR International (Mississauga, ON): *Lactobacilli* De man, Rogosa, Sharpe (MRS) media, potassium chloride (KCl), sodium dihydrogen phosphate (NaH_2PO_4), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium phosphate dibasic (Na_2HPO_4), and Tristar N-Point Indicator.

The following chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON): 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescent probe, chitosan (low molecular weight), L-cysteine hydrochloride monohydrate (L-cys), and polyethylene glycol sorbitan monooleate (Tween® 80).

Glycerol (Acros Organics) and hexane (Certified ACS) were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Ultra high purity (UHP) N_2 and a mixed system containing 80% N_2, 10% CO_2 and 10% H_2 were purchased from Praxair Canada Inc. (Saskatoon, SK).

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

Genipin powder (98% by HPLC) was purchased from Challenge Bioproducts Co., Ltd (Yun-Lin Hsien, Taiwan R.O.C.).

Commercially defatted soy flour (Cargill: Prolia 200/70) was purchased from Cargill Limited (Winnipeg, MB).

Bacto-agar buffered peptone water (alkaline peptone water; APW) from BD (Becton, Dickinson and Co. Difco Laboratories. Sparks, MD) was obtained through VWR International.

Hydrochloric acid (HCl) was purchased from VWR International.

Canola oil was purchased from Loblaw Companies Ltd. (Brampton, ON).

Petri dishes were purchased from Phoenix Biomedical (Mississauga, ON).

Potassium dihydrogen orthophosphate (KH_2PO_4) was purchased from J.T. Baker (Phillipsburg, NJ).

SYTO®-9 nucleic acid stain was purchased from Invitrogen Molecular Probes (Eugene, OR).

Ethanol (95%) was purchased from Commercial Alcohols Inc. (Brampton, ON).

All water used in this research, labelled as MQW, was produced from a Millipore Milli-Q^{TM} water purification system (Millipore Corporation, Milford, MA).

3.2 Study 1: The physicochemical properties of legume protein isolates and their ability to stabilize oil-water emulsions with and without genipin

All protein isolates were prepared in MQW and were adjusted to pH 7.0 using 0.1 M NaOH and/or 0.1 M HCl (Accumet pH meter, Fisher Scientific, Waltham, MA) followed by mechanical stirring at 1000 rpm for 2 h at room temperature (22-23°C) prior to testing, except where noted. All experiments were conducted with adjusted (based on the crude protein results for each isolate) protein concentrations on a weight basis. All results are reported as the mean ± one standard deviation, n = 3.

3.2.1 Legume protein isolate production and proximate analysis

Legume seeds were initially ground employing a bowl grinder (Cuisinart Mini-Prep Plus), followed by a fine grind (IKA A11 basic. IKA Works Inc., Wilmington, NC) to give flour. In the case of soy, commercially defatted flour was used as the starting material. Legume flours from seed were defatted in triplicate with hexane (1:3; w:v; protein:hexane) for 40 min at room temperature (L'Hocine et al., 2006) and then concentrated utilizing a modified isoelectric precipitation procedure (Mondor et al., 2009; Boye et al., 2010; Papalamprou et al., 2010). In brief, the defatted legume flour was dispersed in MQW at a 1 to 10 (w:v; protein:MQW) ratio,

followed by pH adjustment to 9.0 with 1.0 M NaOH so as to facilitate protein solubility. The resulting solution was stirred at 1000 rpm (Ikamag Ret-G, IKA Labortechnik, Germany) for 1 h, and then centrifuged at 5000 x g for 20 min, at 4˚C (Sorvall RC6+; Thermo Fisher Scientific, Waltham, MA). The supernatant was collected for later use, and the process was repeated with a 1 to 5 (w:v) pellet:MQW ratio. Supernatants from both extractions were pooled and adjusted to pH 4.6 with 1.0 M HCl so as to facilitate protein precipitation. The precipitate was collected by centrifugation (5000 x g, 20 min, 4˚C); washed with 25 mL of MQW, frozen (-30°C), and then freeze dried (Labconco FreeZone, Kansas City, MO) to yield a free flowing powder. Protein isolates were stored at 4˚C in sealed tubes for later use.

The crude ash, lipid, moisture and protein (%N x 6.25 for chickpea, faba bean and lentil; x 5.70 for soy) contents for each isolate were determined according to the Association of Official Analytical Chemists (AOAC, 2003) methods: 923.03, 920.85, 925.10, and 920.87, respectively. The carbohydrate content was determined on the basis of percent differential from 100%. All proximate analysis results were performed in triplicate for each protein isolate batch preparation.

3.2.2 Legume protein isolate amino acid composition

The amino acid composition of each protein isolate was determined employing AOAC Official Methods 985.2 and 988.15 (White et al., 1986; AOAC, 2003; Landry & Delhaye, 1993). This work was conducted by POS Bio-Sciences Corp. (Saskatoon, SK). Briefly, to individual 20 x 150 mm screw cap Pyrex tubes was added 20 mg of legume protein isolate. To each tube was added 15.00 mL of 6 N HCl followed by sample flushing with N_2. Tubes were then capped and placed into an oven at 110°C ± 0.5°C for 20 h. Following acid digestion, the individual amino acids were quantified using high pressure liquid chromatography employing the pico-tag amino acid analysis system (Waters Corporation, Milford, MA). Sample amino acid concentration was normalized for each isolate based on its crude protein content (Section 3.2.1).

3.2.3 Physicochemical properties of legume protein isolates

3.2.3.1 Protein solubility

Protein solubility was determined using the following modified (Morr et al., 1985) micro-Kjeldahl analysis protocol. To a protein content weight of 0.20 g for each protein isolate was added 18.00 g of MQW and the resulting suspension was adjusted to pH 7.0 with 0.1 N HCl

and/or 0.1 N NaOH. Sample pH was monitored and maintained throughout a 1 h stirring (1000 rpm) period at room temperature. The total weight of the sample solution was brought to 20.00 g with MQW to give a final protein concentration of 1.00% (w/w). The sample solution was then allowed to remain static for 10 min before being transferred to a 50 mL tube and centrifuged for 10 min at 7200 rpm (Morr et al., 1985). A 5.00 g aliquot of the supernatant was taken for micro-Kjeldahl analysis (Labconco Micro Digester and Labconco Rapid Distillation Apparatus; Labconco Co., Kansas City, MO, USA). Protein solubility was determined by dividing the nitrogen content of the supernatant by the total nitrogen in the sample (x 100%).

3.2.3.2 Zeta potential

Overall surface charge of each protein isolate was determined by measuring electrophoretic mobility (U_E) of prepared protein solutions at pH 7.0 using a Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA). The zeta potential (ζ) was determined from U_E values employing the Henry equation (Eq. 1):

$$U_E = (2\varepsilon \zeta f(\kappa\alpha)) / 3\eta \quad (1)$$

where: ε is the permittivity, $f(\kappa\alpha)$ is a function related to the ratio of particle radius (α) and the Debye length (κ), and η is the dispersion viscosity. For this work, the Smoluchowski approximation, $f(\kappa\alpha)$ of 1.5, was used. Solutions (0.05% w/v) were prepared for each legume protein isolate. A 1 mL syringe was used to inject an aliquot of the sample into the zetasizer sample cell. A refractive index (RI) of 1.450 was used for each sample; water was used as the dispersant (viscosity was 0.8872 cP at 25°C; the RI was 1.330 and the dielectric constant was 78.5. An equilibrium time of 120 s was used for each analysis followed by 10-100 measurements until an acceptable standard deviation was reached, typically 10 measurements were required.

3.2.3.3 Surface hydrophobicity

Surface hydrophobicity for each legume protein isolate was determined using the fluorescent probe, 8-anilino-1-naphthalenesulfonic acid (ANS) (Kato & Nakai, 1980) with modifications developed by Wang et al. (2005). Protein solutions (0.10%, w/v) were prepared by dispersing the powder in 10 mM sodium phosphate buffer (pH 7.0) for 2 h using a magnetic

stirrer (1000 rpm). Each solution was subsequently diluted to obtain protein concentrations of 0.02%, 0.04%, 0.06%, 0.08% and 0.10% (w/v). To 4 mL of each protein solution (0.02%-0.10%; w/v) was added 20 μL of 8 mM ANS solution (in 10 mM sodium phosphate buffer at pH 7.0) and the resulting solution was vortexed (Baxter Diagnostics Inc., Deerfield, IL) at setting 10 for 10 s. Samples were then placed in the dark for 15 min. Fluorescent intensity (FI) was measured using a FluoroMax-4 Spectrofluorometer (HoribaJobin Yvon, Kyoto, Japan) with an excitation wavelength and slit width of 390 nm and 1 nm, respectively, and an emission wavelength and slit width of 470 nm and 1 nm, respectively. FI measurements were also obtained for an ANS blank and protein blanks (without ANS) at each concentration. The FI values of these controls were both subtracted from the FI values of the ANS-protein samples. The initial slope of the plot of FI against % protein concentration was calculated by linear regression analysis and used as an index of average sample surface hydrophobicity.

3.2.3.4 Interfacial tension

The interfacial tensions between prepared legume protein isolate solutions (0.10%; w/w) and canola oil was determined according to the Du Noüy ring method using a semi-automatic tensiometer (Lauda TD2, GmbH & Co., Lauda-Königshofen, Germany). This value was then compared to the interfacial tension between MQW and canola oil (without protein isolates). In this procedure, 40 mL of a prepared protein isolate solution was stirred overnight (16-18 h) at room temperature. To this solution was added 30 mL of canola oil and the interfacial tension between the two discontinuous phases was determined. Interfacial tension was calculated from the maximum force (F_{max}) exerted on the ring as it was pulled through the interface using the following equation (Eq. 2):

$$\gamma = F_{max} / (4\pi R\beta) \qquad (2)$$

where, γ is the interfacial tension, R is the radius of the ring (9.55 mm), and β is a correction factor that is dependent on the dimensions of the ring and the density difference of the liquids used (in these experiments $\beta = 0.1$ g/cm^3).

3.2.4 Crosslinking of legume protein isolates in the presence of genipin

Individual legume protein isolate solutions at a concentration of 0.10% (w/w) were prepared in MQW. After stirring, genipin powder was added to each solution set to achieve final concentrations of: 2.5, 5.0, 7.5, and 10.0 mM. The resulting solutions were stirred (1000 rpm) for 1 h at room temperature and then allowed to crosslink statically for 24 h. An aliquot of each solution was removed and sample absorbance at 288 nm was measured using a UV/Vis Spectrophotometer (Optizen 2120UV. Mecasys Co. Ltd, Korea.). Blanks consisting of each legume protein isolate solution (0.10% w/v) without added genipin were run in conjunction with all sample sets. The initial slope of the plot of absorbance at 288 nm versus genipin concentration was calculated by linear regression analysis and was used as an index of the average genipin induced crosslinking of the legume protein isolates.

3.2.5 Creaming stability of legume protein isolates

Legume protein isolate solutions were prepared with (10.0 mM) and without genipin to determine the impact of crosslinking on creaming stability. Oil in water emulsions (10.0 mL) were prepared by homogenizing 5.0 mL of prepared protein solution (0.50% w/w) with 5.0 mL of canola oil at 13,000 rpm for 5 min using a homogenizer (Polytron® MR PT 2100, Kinematica Inc. Bohemia, NY). Immediately after preparation, emulsions were transferred to a 10 mL sealed graduated glass cylinder and subsequent sample separation into an opaque cream layer (top) and a turbid aqueous layer (bottom) after 24 h of static treatment at room temperature was determined. Percent creaming stability (CS) was calculated using the following equation (Eq. 3):

$$CS\ (\%) = ((V_B - V_A) / V_B) \times 100 \qquad (3)$$

where, V_B is the volume of the aqueous protein solution (5.0 mL) before emulsification and V_A is the volume of the turbid aqueous layer that has ‘fallen out’ of the emulsion after 24 h.

3.3 Study 2: Probiotic encapsulation within genipin crosslinked legume protein microcapsules

Legume protein isolate solutions were prepared in MQW, adjusted to pH 7.0 using 0.5 M NaOH and/or 0.1 M HCl followed by mechanical stirring at 1000 rpm overnight (~16 h) at room temperature prior to encapsulation experiments. All experiments were conducted with adjusted (based on the crude protein results for each isolate) protein concentrations on a weight basis. All experiments were conducted on separately prepared batches of bacteria/capsules and results are reported as the mean ± one standard deviation, n = 3, except where noted. CFU mL^{-1} determination was conducted in duplicate for each replicate at each dilution/sampling time; where the resultant mean accounts for one experimental replicate.

3.3.1 Growth and enumeration of *Bifidobacterium adolescentis*

Bifidobacterium adolescentis (ATCC 15703) was kept at -70°C in a 1:1 (v:v) suspension of glycerol and MRS broth prior to use (Wood, 2010). Viable cell numbers of *B. adolescentis* were obtained by plating of 100 µL aliquots on MRS agar supplemented with 0.05% (w/v) L-cys (MRS-cys), after sequential dilutions were made with sterile 10.0% (w/v) alkaline peptone water (APW), made from buffered peptone water in MQW. Inoculated plates were incubated at 37°C for 48 h under anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2) employing an anaerobic chamber (Forma Scientific Inc., Marietta, GA) and colonies were counted manually (Rodrigues et al., 2012).

3.3.2 Encapsulation of *Bifidobacterium adolescentis*

3.3.2.1 Preparation of *Bifidobacterium adolescentis* for encapsulation

Bifidobacterium adolescentis was inoculated into 10.0 mL of MRS-cys broth and incubated anaerobically at 37°C for 23 h. The bacterial suspension was centrifuged (Sorvall SS-1, Ivan Sorvall Inc. Newtown, CT) for 5 min at 1000 x g and the pellet was re-suspended in 1.00 mL of sterile APW. This protocol was employed to increase initial cell counts.

3.3.2.2 Encapsulation procedure

Genipin crosslinked, legume protein isolate stabilized water in canola oil emulsions were prepared using a technique similar to those reported in literature (Truelstrup-Hansen et al. 2002;

Krasaekoopt et al. 2003; Winder et al. 2003). In this procedure, 9.00 mL of protein isolate solution (10.00% w/w; with respect to the final volume of 10.00 mL) was prepared for each legume protein isolate as previously described. Genipin powder was then added to this mixture so as to reach a final concentration of 15.0 mM. To this mixture was added 1.00 mL of the concentrated bacterial suspension to bring the final volume to 10.00 mL. The resulting mixture was then stirred (1000 rpm) at room temperature for 5 min. This solution (aqueous phase) was added to 100.00 g of canola oil and mixed employing an overhead stirrer (Caframo Real Torque Digital Stirrer, Wiarton, ON) fixed with a compact straight blade impeller (A231: 1.25") (Caframo, Wiarton, ON) for 6 h. Samples were prepared at mixing speeds of 750 and 1000 rpm to form a water-in-oil emulsion. Following the 6 h crosslinking and probiotic encapsulation period, stirring was terminated and 100.00 g of MQW was added in order to break the emulsion into both upper (oil) and lower (aqueous) phases. Due to the amphiphilic nature of the protein wall material, capsules remained associated with both phases and emulsion breakdown was retarded. Harvesting of capsules from this interface and from the aqueous phase was accomplished by centrifugation of ~100 mL of the solution at 1000 x g for 5 min. The upper oil phase was removed by pipette and 10.00 mL of 1.00% Tween 80 in MQW was added to the aqueous capsule solution. This step was used to maintain capsule dispersion and to limit capsule-capsule crosslinking/aggregation. An aliquot of the resulting solution was transferred to a 50 mL separatory funnel to allow the capsules to settle (~5 min) with any remaining oil migrating to the solution surface. Capsules were harvested in solution from the aqueous phase and used immediately.

3.3.2.3 Enumeration of encapsulated *Bifidobacterium adolescentis*

The level of viable *B. adolescentis* (CFU mL^{-1}) was determined by vortexing (30 s at the highest setting of 8) (Fisher Vortex Genie 2^{TM}. Fisher Scientific. Waltham, MA) a 100 µL aliquot of harvested capsule solution in 900 µL of sterile APW, followed by serial dilutions with APW and duplicate spread plating of 100 µL aliquots on MRS-cys agar. Inoculated plates were incubated under anaerobic conditions at 37°C for 48 h and colonies were manually counted.

3.3.3 Effect of stirring speed on the capsule size of genipin induced crosslinked legume protein microcapsules

The effect of stirring speed on capsule size as measured by light scattering was investigated at both 750 and 1000 rpm. Capsules were prepared as described in Section 3.3.2.2 with the exception that 10.00 mL of the aqueous protein isolate solution (10.00% w/w) was employed without the addition of probiotic. Capsule size was determined by light scattering using a Mastersizer 2000 equipped with a Hydro 2000S wet sample cell (Malvern Instruments, Westborough, MA). The measuring parameters of the instrument were set to: 10-20% obscurity; 850 rpm pump speed; a sample absorbance default of 0.1; and software recommended refractive index values of 1.45 and 1.33 for the sample (protein) and dispersant (MQW), respectively. Experiments were conducted on duplicate capsule batches, with size analysis for each batch performed in triplicate. Data was reported as the volume weighted mean capsule diameter, the mode capsule size (of the 100 size bins) and the percentage of capsules with an average size >100 μm.

3.3.4 Acid challenge experiments for free and encapsulated *Bifidobacterium adolescentis*

The survival of free and encapsulated *B. adolescentis* within the four protein isolate wall materials were tested employing a simple acid challenge experiment over a 2 h duration at room temperature. A 10.00 g aliquot of capsule solution (Section 3.3.2.2) was added to 90.00 mL of pH 2.0 MQW (pH adjusted and maintained at this value employing 2.0 M HCl/0.5 M NaOH) with gentle stirring at room temperature. Sample aliquots of 100 μL were removed at times 0, 5, 10, 20, 30, 60, 90 and 120 min, and were immediately diluted (1:10) in sterile APW to partially neutralize the solution without over-dilution. Diluted sample aliquots were vigorously vortexed (highest setting: 8) for 30 s so as to break any intact capsules followed by serial dilution for enumeration studies that were based on acid treatment time. As examples, the CPI-capsules at time 0 and 60 min were serially diluted to 10^{-5} and 10^{-3}, respectively. Serially diluted samples were plated and enumerated as outlined in Section 3.3.2.3.

3.3.5 Effects of biopolymer coatings and/or prebiotics on CPI encapsulated *Bifidobacterium adolescentis* survival in acid challenge experiments

CPI was selected as the encapsulation matrix based on the higher CFU mL^{-1} numbers observed post encapsulation (Section 4.3.2) for this material relative to FPI and LPI. Chitosan (low molecular weight) and napin biopolymers were used as coating materials. Capsule coatings were prepared by the addition of a 10.00 mL aliquot of capsules (Section 3.3.2.2) to 10.00 mL of biopolymer solution (0.50%, w/w in phosphate buffered saline, pH 7.0) followed by gentle stirring (~120 rpm) for 30 min. The resulting mixture was directly added to the acid challenge solution and the same experimental protocol as outlined in Section 3.3.4 was followed with the exception that 100.00 mL of pH 2.0 solution was used. The increased volume (e.g., capsule and coating solutions = 20.00 mL) added to the pH 2.0 solution (total volume = 120.00 mL) was accounted for when determining the final number of survivors (CFU mL^{-1}), as this initial dilution was not 1 in 10, through multiplication by a factor of 1.2.

For the prebiotic experiments, 1.00% (w/w) fructooligosaccharide powder (FOS) was added to the original protein/genipin solution prior to the addition of the concentrated bacterial suspension, as described in Section 3.3.2.2. The acid challenge protocol outlined in Section 3.3.4 was followed for the following experiments, each encapsulating *B. adolescentis*: CPI + FOS, CPI + FOS + chitosan coating, and CPI + FOS + napin coating.

3.3.6 Imaging of chickpea protein microcapsules

A Zeiss Axiostar Plus (240 V) light microscope (Carl Zeiss Canada Ltd. Toronto, ON) was used to examine the size and surface morphology of CPI capsules, containing *B. adolescentis*, with and without a napin coating. Light microscopy digital images, of unmodified harvested capsule solution (Section 3.3.2.2), were made using an AxioCam MRc camera (Carl Zeiss Canada Ltd. Toronto, ON) mounted on a microscope with either 10x or 40x objectives. The software used for light microscopy digital imaging (including scale bar addition) was AxioVision software version 3.1.2.1 (2002).

The resultant digital images were analyzed using ImageJ (Image processing and analysis in JAVA) software version 1.45s (Wayne Rasband. National Institutes of Health, USA) as an alternate size analysis method. To do so, the captured digital light microscopy images (JPEG file format) were adjusted for color threshold (Figure 3.1) to allow the program to distinguish CPI

capsules from the background capsule solution matrix. Limits to particle area (3 – 1000 μm^2) were set in order to exclude air bubbles, free bacteria, protein 'clumps', background noise, etc. from the size analysis. The ImageJ software was then used to run an automated size analysis of the resultant images (e.g., Figure 3.1b) to calculate the area of each particle, which then could be converted to particle diameter (Eq. 4) assuming circular particle shape. The average particle size of the capsules with and without a napin coating was determined over multiple (n = 5) fields of view.

$$Diameter = 2 \times \sqrt{\frac{Area\ of\ circle}{\pi}} \tag{4}$$

A Zeiss Standard 20 (115 V) microscope equipped with a UV lamp (5 Amp; Slo-Blo) (Carl Zeiss Canada Ltd. Toronto, ON) was used to examine the presence and distribution of *B. adolescentis* cells throughout uncoated CPI capsules. In order to visualize cells by fluorescence the SYTO®-9 nucleic acid stain was utilized. SYTO®-9 is a membrane penetrative, green fluorescent nucleic acid dye which is capable of staining live cells (Bunthof et al., 2001; Zotta et al., 2012). To 1.00 mL of harvested capsule solution was added 3.0 μL of SYTO®-9 and the mixture was then kept in the dark for 30 min prior to examination. All fluorescent microscopy digital images of the stained capsule solution were taken with a microscope mounted Photometrics SenSys® camera (Roper Scientific Inc. Tucson, AZ) using either 10x, 16x or 100x objective lenses. The software used for fluorescent microscopy imaging was RS Image software version 1.7.3 (2001) (Roper Scientific Inc. Tucson, AZ). The scale of the fluorescent microscopy digital images was determined using a 0.01-0.1mm stage micrometer (Bausch & Lomb Canada Inc. Vaughan, ON,).

3.4 Statistics

All sample data is reported as the mean ± one standard deviation. A one way analysis of variance (ANOVA) with Holm-Sidak post-hoc test for pairwise comparison procedures was used to detect significant differences ($p<0.05$) between protein levels within the isolate products and between capsule sizes produced based on stir speed. An ANOVA with a Scheffe post-hoc test and T-testing were used to determine statistical differences ($p<0.05$) in physicochemical, crosslinking and encapsulating properties as a function of legume source. Pearson comparison

and a general linear model with backwise stepwise regression were used to determine the ability of legume protein isolate physicochemical properties, without crosslinking, to predict creaming stability in legume protein isolate stabilized emulsions. All statistical analyses were performed using SigmaPlot 12.0 software (Systat Software, Inc. Chicago, IL).

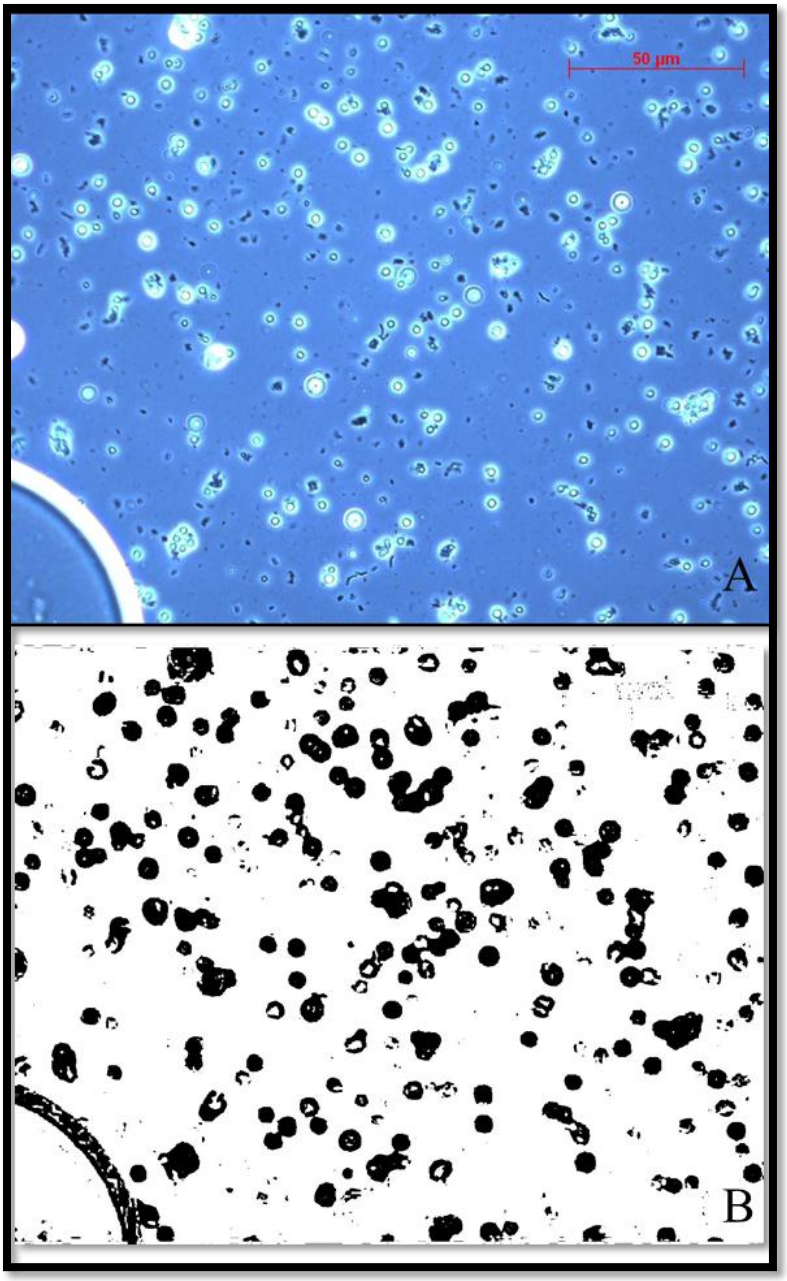

Figure 3.1 Light microscopy digital images of CPI capsules at 400x magnification before (A) and after (B) colour threshold adjustment for ImageJ particle size analysis.

4 RESULTS AND DISCUSSION

4.1 Study 1: The physicochemical properties of legume protein isolates and their ability to stabilize oil-water emulsions with and without genipin

4.1.1 Composition of legume protein isolates

Protein isolates were prepared from raw chickpeas, faba beans and lentils as well as defatted soy flour using isoelectric precipitation. Proximate compositions of the resulting isolates are shown in Table 4.1. Protein content (on a wet weight basis; (w.b.)) was determined to be ~85.8%, ~86.3%, ~83.8% and ~90.9% for CPI, FPI, LPI and SPI, respectively. Differences in protein content among isolates, however, were not significant ($p>0.05$). As there is no universal scheme for classifying legume protein products, all materials were deemed to be an isolate rather than a concentrate in the present study. In the case of soy, Pearson (1983) developed criteria requiring a minimum protein content of 85% on a dry weight basis (using a nitrogen conversion factor of 6.25) to be classified as an isolate. When protein levels were converted from a wet to dry basis in the present study, utilizing the 6.25 conversion factor (5.70 for SPI), the legume protein levels were ~87.6%, ~89.8%, ~91.6%, and ~97.1% (dry weight basis) for CPI, FPI, LPI and SPI, respectively. The isoelectric precipitation method for protein extraction typically involves hydrating defatted flour at alkali pH (9.0) to solubilize the proteins, followed by centrifugation to remove insoluble matter (e.g., fibre, carbohydrates), followed by pH adjustment to near the legume protein's isoelectric point (pI) to induce precipitation. At their pI (~4.5-5.0), legume proteins assume a net neutral charge and tend to aggregate and fall out of solution. Isoelectric precipitation typically yields mainly globulin proteins (Papalamprou et al., 2010), whereas other extraction methods, such as salt extraction, yield isolates comprised of a mixture of globulins and albumins (Liu et al., 2008). Protein levels in the present study were comparable to others found in literature. For example, Can Karaca et al. (2011) using similar legumes and a similar extraction method reported protein levels of ~85.4%, ~84.1%, ~81.9%, and ~87.6% for CPI, FPI, LPI and SPI respectively. In addition, protein levels on a dry weight basis of ~90.2%

and ~78.0% have been reported for LPI (Joshi et al., 2012) and CPI (Sánchez-Vioque et al., 1999) respectively, when prepared using similar isoelectric precipitation extraction procedures.

Table 4.1 Proximate composition of chickpea (CPI), faba bean (FPI), lentil (LPI) and soy (SPI) protein isolates. Data represents the mean ± one standard deviation (n=3).

Sample	Protein (%, w.b.)	Moisture (%)	Lipid (%, w.b.)	Ash (%, w.b.)	Carbohydrate (%, w.b.)
CPI	85.76 ± 0.26	2.39 ± 0.00	0.83 ± 0.04	4.41 ± 3.64	6.89
FPI	86.30 ± 1.26	3.85 ± 0.05	<LOD*	3.89 ± 1.35	5.96
LPI	83.81 ± 1.32	8.48 ± 0.05	0.77 ± 0.01	3.83 ± 1.27	3.11
SPI	90.86 ± 5.20	6.41 ± 0.01	<LOD*	2.19 ± 0.07	0.54

*Below limit of detection (<LOD).

Proximate analysis revealed very low lipid levels (≤ 0.83%) within the isolates due to the defatting procedure. These low lipid levels were not expected to hinder the dispersion of isolates in solution (MQW) during physicochemical testing. Removal of lipids prior to the extraction process helps reduce protein-lipid interactions from occurring, which would inhibit protein solubility and therefore limit isolation (Leyva-Lopez et al., 1995). Moisture levels for isolates were found to be ~2.4%, ~3.9%, ~6.4% and ~8.5% for CPI, FPI, SPI and LPI, respectively, reflecting either the efficiency of the freeze drying process or the relative strength of protein-water interactions (Table 4.1). Ash contents of ~2.2%, ~3.8%, ~3.9% and ~4.4% (w.b.) for SPI, LPI, FPI and CPI respectively and carbohydrate levels of ~0.5%, ~3.1%, ~6.0%, and ~6.9% (w.b.), by differentiation from 100%, for SPI, LPI, FPI and CPI respectively were determined for these materials (Table 4.1). These proximate composition values were similar to those reported in literature for legume protein isolates produced by similar isoelectric precipitation procedures. As examples, Sánchez-Vioque et al.(1999) reported that a CPI produced by isoelectric precipitation had a proximate composition of ~78.0%, ~3.3%, 3.5%, ~2.9% and ~11.8% for protein, moisture, lipid, ash and carbohydrate (by difference) respectively on a dry weight basis. In addition Okezie & Bello (1988) reported protein (%N x 6.25), moisture, crude fat, ash and carbohydrate levels of ~90%, ~4.0%, ~0.0%, ~6.0% and ~4.2% respectively for a winged bean

protein isolate produced by isoelectric precipitation and ~97.0%, ~4.7%, ~0.0%, ~3.4% and ~0.0% respectively for a industrially produced SPI.

Amino acid profiles for each protein isolate (normalized to 100% based on the protein content of each sample) are given in Table 4.2. Lysine content is of particular importance because of its reactivity with genipin, the crosslinking agent used in this study (Butler et al., 2003; Nickerson et al., 2006a; Maji & Hussain, 2008). Lysine contents for the CPI, FPI, LPI and SPI products were found to be ~6.3%, ~6.0%, ~6.8% and ~5.7% respectively. Similar lysine contents should correspond to similar crosslinking potential with genipin, however, within a MQW in oil emulsion setting, lysine residue exposure to genipin (within the aqueous phase) may be altered as proteins unfold and re-orientate at the oil-water interface (McClements, 2004; Damodaran, 2005). Lysine levels are comparable to those reported for protein isolates in literature. For example, Vioque et al. (2012) reported a ~7.0% lysine level for a FPI prepared by isoelectric precipitation and Okezie & Bello (1988) reported that an industrially produced SPI had a lysine content of 6.1%. In addition, Iqbal et al. (2006) reported lysine contents (adjusted on the basis of protein content) for four legumes with values of: ~7.2% (chickpea), ~7.5% (cowpea), ~7.0% (lentil) and ~8.1% (green pea).

Table 4.2 Normalized amino acid profiles (%) of chickpea (CPI), faba bean (FPI), lentil (LPI) and soy (SPI) protein isolates.

Amino acid	CPI	FPI	LPI	SPI
Phenylalanine	6.40	4.42	5.70	5.02
Isoleucine	4.28	4.38	4.82	4.20
Tryptophan	0.83	0.91	0.83	1.29
Leucine	7.76	7.82	8.19	7.11
Valine	4.22	4.64	4.96	3.93
Methionine	1.50	0.80	0.95	1.31
Tyrosine	2.99	3.88	3.79	3.54
Cysteine	1.17	0.96	0.77	1.31
Alanine	3.85	3.99	4.03	3.23
Threonine	3.34	3.81	3.77	3.57
Histidine	3.04	3.09	2.90	2.95
Glycine	3.63	4.12	3.84	3.76
Serine	6.88	6.77	6.97	6.46
Arginine	9.51	9.72	8.76	7.81
Lysine	6.31	5.95	6.75	5.68
(Glutamic acid + Glutamine)	16.68	17.59	16.45	20.82
Proline	4.27	4.52	4.24	4.98
(Aspartic acid + Aspargine)	13.34	12.63	12.28	13.02
	100	**100**	**100**	**100**

4.1.2 Physicochemical properties of legume protein isolates

4.1.2.1 Surface characteristics

The surface charge or zeta potential values for CPI, FPI, LPI and SPI products at pH 7.0 are shown in Figure 4.1. An analysis of variance showed that all isolates were statistically similar ($p>0.05$), at -47.7, -46.4, -47.2 and -44.3 mV for CPI, FPI, LPI and SPI, respectively. Protein isolates in this study carried a net negative charge at pH 7.0, as all were above their isoelectric points (where zeta potential is 0 mV). The net negative charge at pH 7.0 arises primarily from

the negatively charged R groups found on the aspartate (pK_a = 3.65) and glutamate (pK_a = 4.25) amino acids spatially located on the protein surface (Nelson & Cox, 2005). Can Karaca et al. (2011) and Tang & Sun (2011) reported the isoelectric point of legume globulin proteins to be ~4.5. Surface charge values from this study were similar to those reported in literature. For example, Joshi et al. (2012) reported the surface charge of LPI at pH 7.0 to be -43.3 mV compared to values of ~-55 mV for WPI and BSA. In addition, Tang & Sun (2011) reported zeta potential values at pH 7.0 of ~-40 mV for legume vicilin proteins isolated from kidney, red and mung beans. Having a high protein surface charge is important during the formation of emulsions, as it enhances protein solubility due to electrostatic repulsion between negatively charged proteins, promotes greater hydration of proteins in solution or protein-water interactions resulting in protein migration to the oil-water interface (Schwenke, 2001; McClements, 2004; Damodaran, 2005). High surface charges also play a role in maintaining emulsion stability, as they induce an electric charge (dependant on pH) to the viscoelastic film surrounding the discontinuous droplets. A charged emulsion droplet surface repels others, with a similar charge, to inhibit coalescence and flocculation, which are mechanisms for emulsion instability (McClements, 2004; Damodaran, 2005).

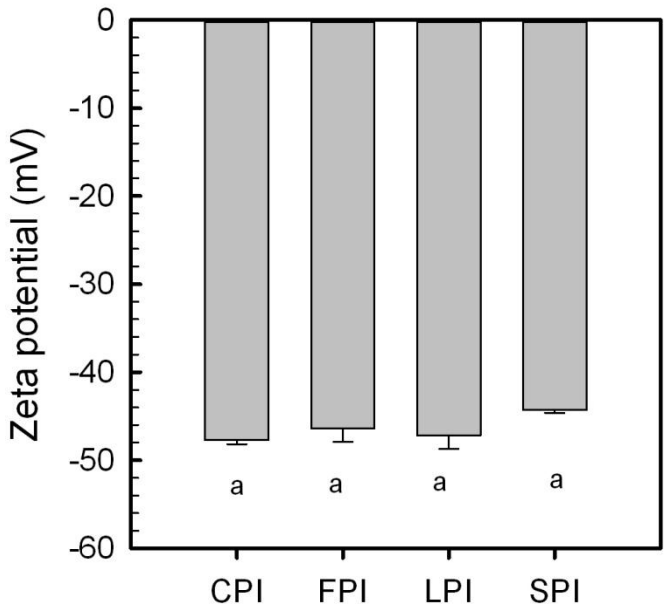

Figure 4.1 Zeta potential (mV) of chickpea (CPI), faba bean (FPI), lentil (LPI) and soy (SPI) protein isolates at pH 7.0. Data represent the mean ± one standard deviation (n=3). Data with same letters signifies no statistical differences ($p>0.05$).

Fluorescence spectroscopy can be a sensitive tool for protein analysis such as, structural changes, folding, aggregation, surface hydrophobicity; based on the intrinsically fluorescent amino acid tryptophan and tyrosine to some degree, and through the use of fluorescent dyes such as ANS. The interaction of dye with protein leads to changes in fluorescence, after excitation, which is the basis of protein characterisation by this method (Hawe et al., 2008). ANS has very low fluorescence in aqueous solution, but becomes highly fluorescent when adsorbed onto hydrophobic binding sites spatially distributed on the protein surface. Ion pairing between the negatively charged sulfonate groups of ANS and positively charged amino acids (histidine, lysine and arginine) also plays a role in dye adsorption to the protein surface. Energy absorption by the dye molecule leads to several spectroscopic events including but not limited to: vibrational, solvent and fluorescence relaxations to ground state. In the case of fluorescence, emission occurs when electrons fall from the lowest vibrational state to ground state (Hawe et al., 2008). Fluorescence magnitude is influenced by solvent polarity, viscosity and temperature,

or processing factors that impact protein conformation and exposure of buried hydrophobic groups.

The average surface hydrophobicity was determined by the ANS fluorescent probe binding method for CPI, FPI, LPI and SPI products at pH 7.0 and is given in Figure 4.2. An analysis of variance indicated that CPI was significantly higher (~137.5 arbitrary units, A.U.) ($p<0.05$) than the other isolates, followed by SPI (~72.8 A.U.) and FPI (~70.4 A.U.) which were similar in magnitude ($p<0.05$), and then FPI (~24.4 A.U.) ($p<0.05$). Tang & Sun (2011) reported a surface hydrophobicity range of ~149 - ~259 A.U. for vicilin isolates produced from kidney, red and mung beans. Can Karaca et al. (2011) reported a similar hydrophobicity pattern for these particular legume protein isolates of, CPI > LPI > SPI = FPI with surface hydrophobicity values (~55 – 80 A.U.), which were generally lower than those reported in this study. The surface hydrophobicity values determined in this study were different than those reported in the aforementioned literature citations, which may be due to the use of different protein concentrations in slope generation.

A protein with high surface hydrophobicity can readily align at the oil-water interface, and re-orientate itself such that its hydrophobic moieties position themselves towards the oil phase and the hydrophilic moieties towards the aqueous phase (Schwenke, 2001; Damodaran, 2005). Depending on its amino acid sequence and the level of folding/unfolding of a protein at the interface, various loops or tails can develop in which sections of the protein extend from the surface of the droplet into the continuous phase creating a steric hindrance that reduces the likelihood of aggregation, flocculation and coalescence between neighbouring droplets. (Schwenke, 2001; McClements, 2004; Damodaran, 2005). The surface characteristics of a protein within an emulsion are reliant on the extent of its interactions with the dispersive solvent/phase, and with the interface. Accessible surface area, coupled with unfolding and re-orientation at the interface, may explain how protein isolates with different physicochemical properties may exhibit similar emulsification properties and *vice versa* (Schwenke, 2001).

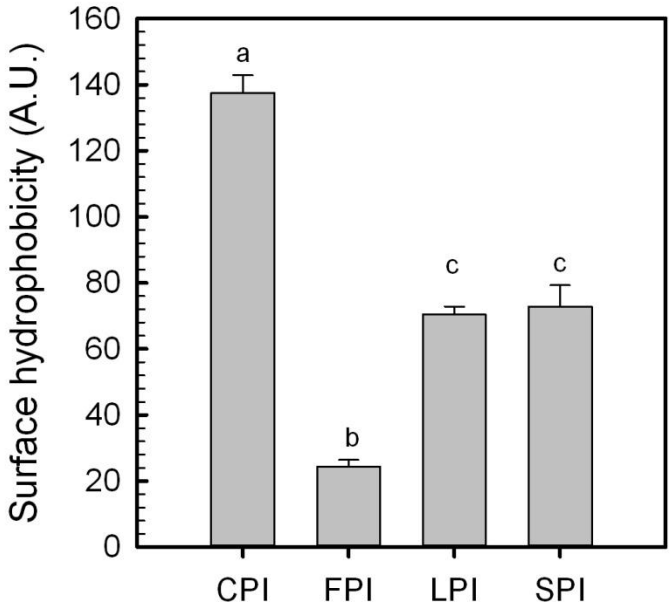

Figure 4.2 Average surface hydrophobicity (arbitrary units, A.U.) of chickpea (CPI), faba bean (FPI), lentil (LPI) and soy (SPI) protein isolates at pH 7.0. Data represent the mean ± one standard deviation (n=3). Data with different letters signifies a statistical difference ($p>0.05$).

4.1.2.2 Protein solubility

Percent protein solubility at pH 7.0 was determined for all legume protein isolates (Figure 4.3). An analysis of variance revealed that all isolates displayed significantly different ($p<0.05$) solubility values, which were found to be the highest for CPI (~94%), followed by LPI (~90%), FPI (~85%) and SPI (~50%). Similar solubility for legume protein isolates prepared by isoelectric precipitation has been reported in literature by Sánchez-Vioque et al. (1999), and Carbonaro et al. (1997) for CPI (>80%), and for FPI, LPI and CPI (all >80%). Solubility is mediated by the balance of protein-protein and protein-solvent (aqueous phase) interactions; the latter promoting solubility, which can further be influenced by environmental factors such as temperature, pH and ionic strength (McClements, 2007; Can Karaca et al., 2011), and by processing (e.g., extraction or post-extraction treatments) (Kinsella, 1979). High surface charge is important for fostering sufficient electrostatic repulsion between proteins, such that they can

overcome electrostatic and van der Waals attractive forces, so as to remain dispersed in solution. In the present study, SPI displayed the lowest surface charge (~-44 mV) relative to the other three isolates (Figure 4.1), which may have contributed to its reduced solubility. However, solubility of proteins is influenced by other factors, such as salts, conformation, pH, level of association/disassociation and hydrophobicity (Carbonaro et al., 1997; McClements, 2004; Damodaran, 2005). In general, proteins that have high surface hydrophobicity tend to be negatively correlated with solubility (Can Karaca et al., 2011), however in the present study, CPI showed both the highest solubility and surface hydrophobicity which reflects the complexity behind fully understanding the structure-function-mechanism related to protein solubility. This could be a factor not of the average surface characteristics (e.g., charge vs hydrophobicity), but rather of the apparent surface characteristics, which are influenced by the frequency and distribution of charges on the folded protein surface (Schwenke, 2001; Tang & Sun, 2011).

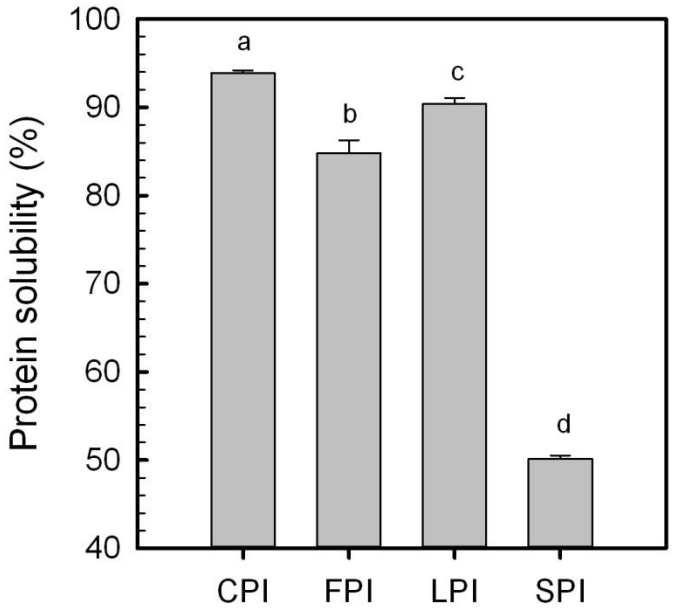

Figure 4.3 Average protein solubility (%) of chickpea (CPI), faba bean (FPI), lentil (LPI) and soy (SPI) protein isolates at pH 7.0. Data represent the mean ± one standard deviation (n=3). Data with different letters signifies a statistical difference ($p>0.05$).

4.1.2.3 Interfacial properties

During emulsion formation, proteins migrate to the oil-water interface and re-align to allow positioning of hydrophobic groups towards the oil phase and hydrophilic groups towards the aqueous phase. This is followed by the formation of a viscoelastic film that resists flocculation or coalescence through electrostatic repulsive forces (depending on the pH) and steric stabilization (Schwenke, 2001; McClements, 2004; Damodaran, 2005; Joshi et al., 2012). The ability of a protein to align at the interface can be described by its ability to reduce the interfacial tension between oil and water phases. Interfacial tension is a measurement of the force (e.g., energy) required to move a probe (e.g., du Nöuy ring) through an interface (Can Karaca et al., 2011). Its ability to reduce this tension will enable smaller emulsion droplets to form, to give a more stable emulsion (Damodaran, 2005). In the present study, interfacial tension was measured through a MQW-canola oil interface for CPI, FPI, LPI and SPI products at pH 7.0, and is shown in Figure 4.4. Each of the four legume protein isolates, when added to the aqueous phase, were shown to reduce the interfacial tension of a MQW-canola oil interface from 16.73 mN/m to values ranging from 8.23-8.62 mN/m (Figure 4.4), however no difference in interfacial reduction was found regardless of protein type ($p>0.05$). Values were similar to those reported in the literature for a protein induced reduction of interfacial tension between water and oil, although materials and/or methods differed. For example, Joshi et al. (2012) reported that the interfacial tension as measured by the pendant drop method for an olive oil-water mixture with LPI added at 10 mg/mL to be ~12 mN/m; reduced from ~22 mN/m without protein. This was reported to be typical for other non-legume globular proteins at an oil-water interface and the reduction of ~10 mN/m is comparable to the ~8.3 mN/m reduction caused by the addition of legume protein isolates in this study. Can Karaca et al. (2011) also reported a similar magnitude in the reduction of interfacial tension (~6.1 mN/m) at a flaxseed oil-water interface with the inclusion of CPI, FPI, LPI and SPI (0.25% w/w), when compared to water-oil alone.

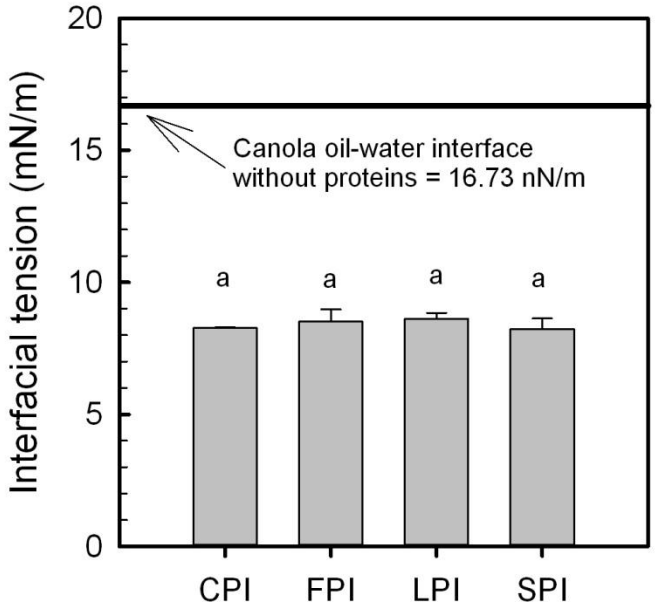

Figure 4.4 Average interfacial tension (mN/m) of chickpea (CPI), faba bean (FPI), lentil (LPI) and soy (SPI) protein isolates at pH 7.0 for a MQW-canola oil interface. Data represent the mean ± one standard deviation (n=3). Data with similar letters signifies no statistical differences ($p>0.05$).

4.1.3 Reactivity of genipin to the legume protein isolates

Genipin is considered to be a novel, non-toxic covalent crosslinking agent produced by *Gardenia* fruit (Sung et al., 1999; Mi et al., 2000; Butler et al., 2003; Nickerson et al., 2006b). Researchers have used genipin to crosslink a variety of materials including, but not limited to: bovine serum albumin (BSA), chitosan, gelatin, gelatin-carrageenan mixtures as well as soy and whey proteins. These materials have been used for a variety of purposes including: wound dressings, hydrogels, micro/nanoparticles and films (Butler et al., 2003; Annan et al., 2008; Huang et al., 2009; Devi & Maji, 2010). To our knowledge, there has been little work on its reactivity with legume proteins and potential for use in stabilizing emulsions. To investigate the crosslinking ability of genipin to our legume protein isolates, sample absorbance was monitored at 288 nm as a function of genipin concentration. A linear increase (R^2 range of 0.870-0.967) was found as genipin concentrations increased from 2.5 to 10 mM when in the presence of the legume protein isolates (Figure 4.5). As such, the slopes were taken as an index of legume

protein isolate-genipin crosslinking reactivity. Slopes for CPI, FPI, LPI and SPI-genipin reactions were found to be similar at 0.0369, 0.0377, 0.0305 and 0.0378, respectively. The differences observed in the spectroscopic results for the four legume protein isolates may be explained by differences in the spatial arrangement and surface exposure of lysine groups, as well as protein flexibility. Spectrophotometric results have been used previously to measure polymer crosslinking with genipin. For example, Butler et al. (2003) found that an absorbance peak developed at 605 nm in a glucosamine:genipin mixture, which increased in intensity as a function of reaction time. The authors also reported the development of peaks at 240 and 280 nm in chitosan:genipin mixtures, which they related to polymer crosslinking.

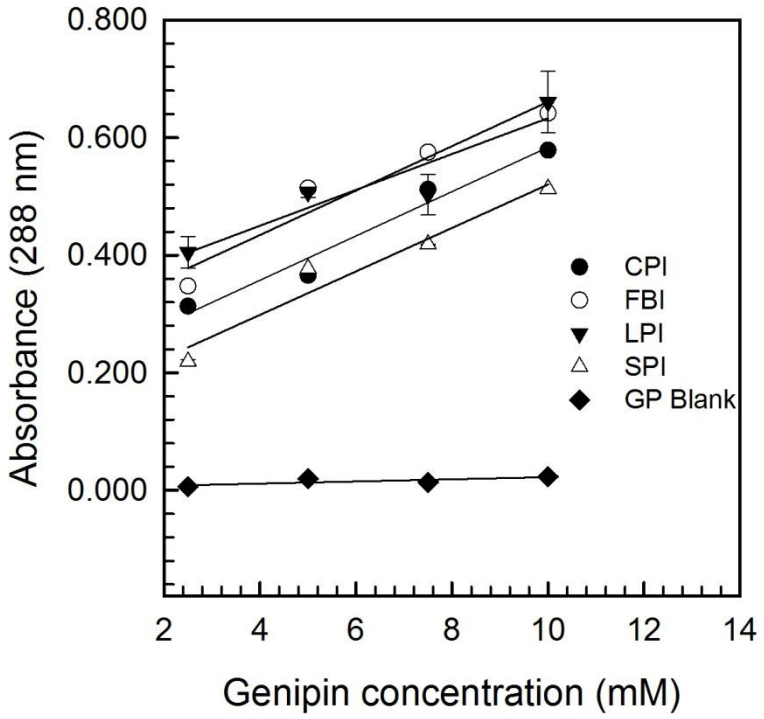

Figure 4.5 Absorbance at 288 nm of mixtures of chickpea (CPI), faba bean (FPI), lentil (LPI) and soy (SPI) protein isolates, as well as a genipin (GP)-MQW blank, as a function of genipin concentration at pH 7.0 after crosslinking for 24 h. Data represent the mean ± one standard deviation (n=3).

The proposed mechanism for protein-genipin crosslinking involves the following two reactions: (1) a nucleophilic substitution to the dihydropyran ring of genipin followed by a Schiff's base reaction; and (2) a separate Schiff's base reaction with the ester group of the genipin molecule. For both reactions, a primary amine group from the protein is required for crosslinking with genipin (Butler et al. 2003). The first reaction is initiated by nucleophilic attack at C3 of genipin by a primary amine group on the protein that results in dihydropyran ring opening and formation of an aldehyde group and secondary amine. The ring then closes as the secondary amine reacts with the aldehyde group, to form a heterocyclic ring bound to a protein molecule (Butler et al., 2003). The second reaction is a S_N2 nucleophilic attack at the ester group on the genipin molecule by a primary amine on the protein so as to produce an amide linkage. The evidence for these two reaction mechanisms was based on ^{13}C nuclear magnetic resonance (NMR), infrared spectroscopy (IR) and rheological data collected during genipin crosslinking experiments with chitosan, BSA, gelatin and glucosamine (Butler et al. 2003).

4.1.4 Creaming stability of legume protein isolates with and without genipin

The creaming stability of MQW–canola oil emulsions, stabilized with CPI, LPI, FPI and SPI were investigated over a 24 h period with and without genipin at pH 7.0, and are shown in Figure 4.6. An analysis of variance revealed that overall, protein stabilized emulsions prepared with genipin showed increased stability (57.75%) relative to those without (51.08%), regardless of protein type ($p<0.05$). Emulsion stability (without genipin) was found to be significantly different ($p<0.001$), where SPI showed the greatest stability (~57.7%), followed by LPI (~52.3%), FPI (~49.3%) and then CPI (~45.0%) (Figure 4.6). A similar trend was found for protein type in the presence of genipin. When relating the physicochemical results to creaming stability (without genipin) using the Pearson correlation, it was found that only solubility was (negatively) correlated ($r = -0.795$, $p<0.01$). A backward stepwise regression model, which was able to explain 63.2% of data variability (Eq. 5), found a similar conclusion where only solubility was a significant factor ($F = 17.142$, $p<0.01$):

$$CS = [-0.229 \times \text{solubility}] + 69.326. \quad (5)$$

Overall, only protein solubility was found to be significantly ($p<0.01$) correlated with creaming stability.

Creaming stability refers to the ability of a protein stabilized emulsion to resist creaming, where oil droplets flocculate and coalescence, then migrate upwards due to the density difference from MQW (Damodaran, 2005; Liu et al., 2010; Can Karaca et al., 2011). The ability of an emulsion to resist creaming is largely dependent on droplet size and density contrast between phases (McClements, 2007). In the present study, it was hypothesized that legume proteins acted to stabilize the emulsions by first migrating to, and then re-aligning at the canola oil-MQW interface to form a viscoelastic film during emulsion formation. This film maintains droplet size by resisting flocculation and coalescence through electrostatic repulsive forces (negative zeta potential) and steric hindrance, and in the presence of genipin, was presumed to become stronger and more resistant to punctures, etc. (Damodaran, 2005; McClements, 2007).

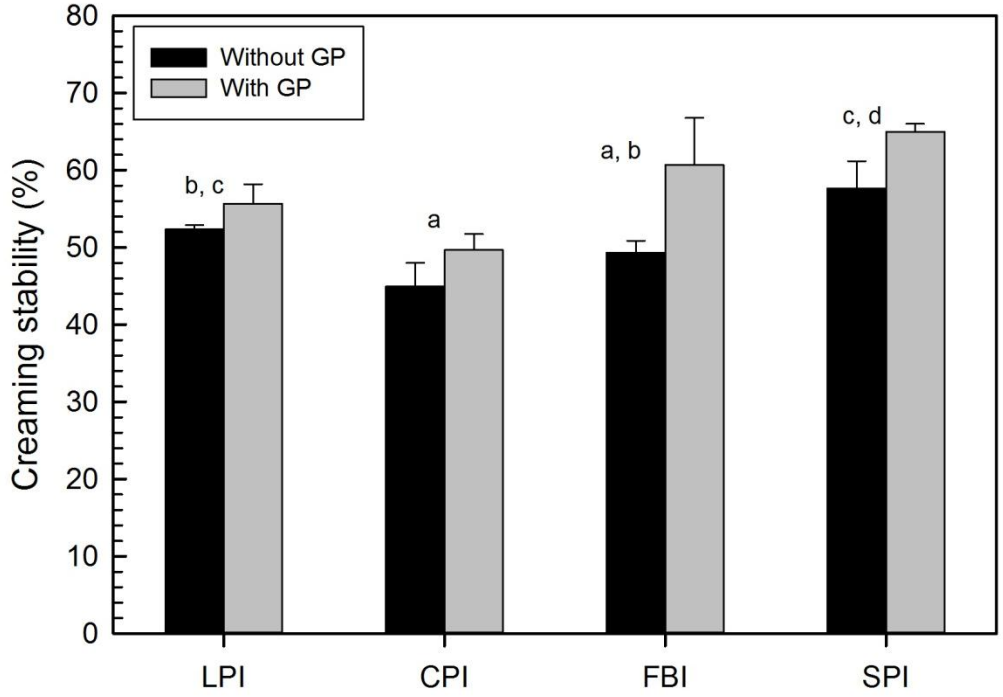

Figure 4.6 Average creaming stability (%) of chickpea (CPI), faba bean (FPI), lentil (LPI) and soy (SPI) protein-stabilized canola oil-MQW emulsions at pH 7.0, with and without 10 mM genipin (GP) after 24 h. Data represent the mean ± one standard deviation (n=3). Data with different letters signifies a statistical difference ($p>0.05$) for legume proteins without GP.

4.1.5 Link to study 2

From Study 1 a great deal of knowledge on the physicochemical properties of the four legume protein isolates was obtained. Of significant importance were their emulsification and genipin crosslinking potentials. These properties were exploited so as to examine their potential as microencapsulating agents for the probiotic *B. adolescentis* in a water-in-oil emulsion system employing genipin as the crosslinking agent. During encapsulation, emulsions are stirred for 6 h to allow sufficient time for the genipin reaction to occur; forming both intra- and inter- molecular covalent crosslinks on proteins surrounding droplets to form a capsule. Study 1 was important, while working with an oil-in-water emulsion, as knowledge gained concerning the physicochemical properties of the legume proteins and their affinity of genipin in the presence of oils will provide a greater understanding of the systems as a whole. The goal of this study was to design an emulsification procedure to produce capsules of <100 µm in diameter that would show increased probiotic protection in an acid challenge system.

4.2 Study 2: Probiotic encapsulation within genipin crosslinked legume protein isolate microcapsules

4.2.1 Growth and enumeration of *Bifidobacterium adolescentis*

Bifidobacterium adolescentis was found to reach 9.26 ± 0.42 log CFU mL^{-1} (n=12) in MRS-cys broth after ~22-24 h of anaerobic growth at 37°C. Similar maximum viable cell counts were reported by Wood (2010) using this same organism under the same growth conditions. These viable cell levels were reported to correspond with the stationary phase of the bacterial growth cycle (Wood, 2010). At this growth stage bacteria may enter a stress resistant state, including acid tolerance, so as to make them more robust, which may be advantageous for their survival through the encapsulation process (Hengge-Aronis, 1993; Hartke et al., 1994; Wood, 2010). *B. adolescentis* was selected for this project because it is generally regarded as an acid sensitive probiotic (Fuller, 1992; Lee & Heo, 2000; Picot & Lacroix, 2004) and builds off earlier research by our group (Wood, 2010; Klemmer et al., 2011). In the present study, it was proposed that encapsulation of *B. adolescentis* within the stationary phase of growth will enhance their survival during the encapsulation process and acid tolerance tests. Based on literature, it is generally accepted that at least 7.0 log CFU mL^{-1} of a probiotic is required to reach the colon in a viable state in order for any benefit to be recognized by the host (Bouhnik, 1993; Krasaekoopt et al., 2003).

4.2.2 Encapsulation of *Bifidobacterium adolescentis*

Conventional practices for encapsulating probiotics within micron-sized capsules involve the use of emulsion-based technology and polysaccharide-based materials, including: alginate (Truelstrup-Hansen et al., 2002), cellulose acetate phthalate (CAP) (Favaro-Trindale & Grosso, 2002), or biopolymer mixtures, such as alginate-starch (Sultana et al., 2000) or κ-carrageenan-locust bean gum (Audet et al., 1988). Polysaccharides are highly advantageous as wall materials due to their low cost and ease of crosslinking with ions (e.g., alginate/pectin with calcium) however, at sizes that are negligible to sensory perception (<100 μm) such capsules regularly fail at protecting probiotics during acid challenge tests. A typical encapsulation process involves dispersing a mixture of polysaccharides and probiotics within a continuous phase of vegetable oil to create a water-in-oil emulsion. Depending on the level and duration of shear, the droplet size within the emulsion can be controlled. After a set stir time, a small amount of calcium ion

solution is added to induce gelation of the polysaccharide rich droplets, followed by a larger volume of the ionic solution to break the emulsion and induce droplet hardening. Following breakage of the emulsion, capsules (i.e. hardened droplets) fall to the bottom of the aqueous phase due to their low affinity with the water-oil interface, and can be harvested.

Literature describes variable success for polysaccharide-based encapsulation of probiotics when challenged by acidic conditions similar to those found in the human GIT. For example, Truelstrup-Hansen et al. (2002) encapsulated *B. adolescentis* within alginate-based capsules of ~20 and ~70 μm in diameter at levels of 6.0-8.0 log CFU mL^{-1}. However, the authors reported a ~5.0 log reduction in CFU mL^{-1} when exposed to a SGJ at pH 2.0 within 30 min. O'Riordan et al. (2001) encapsulated *Bifidobacterium* PL1 within waxy maize starch based capsules ~5 μm in diameter at levels of ~6.6 log CFU mL^{-1}. However, after exposure to an acid challenge at pH 2.8 for both 3 and 6 h, encapsulated probiotic levels were reduced below detectable limits, and the authors reported that this capsule formulation offered no additional protection over free cells. In contrast, Favaro-Trindale & Grosso (2002) encapsulated *B. lactis* and *L. acidophilus* using CAP as a wall material at levels of ~7 log CFU mL^{-1} in capsules with an average size of 22 μm. Probiotic reduction of ~1 log CFU mL^{-1} was reported for both organisms after an acid challenge at pH 1 for 2 h, indicating that this formulation increased core material acid resistance.

Modifying the encapsulation process to better suit protein wall materials faces a number of challenges. First, a higher polymer concentration is generally needed to form a capsule when compared to that of a polysaccharide due to differences in gelation ability (Krasaekoopt et al., 2003). This challenge formed the basis of our hypothesis that the higher biopolymer level used in this study would decrease pore size so as to form a more core protective capsule. Second, genipin induced protein crosslinking requires longer stir times and greater control over environmental conditions (e.g., pH and temperature) than required in an ionic gelation process, which is commonly employed for polysaccharide based wall materials. The longer stir time exposes the probiotics to non-ideal aerobic and mechanical shear conditions, possibly resulting in a loss of cell viability over the extended capsule formation time. Finally, proteins have a greater affinity to the water-oil interface than polysaccharides due to their surface characteristics (e.g., charge and hydrophobicity). A stronger affiliation to this interface negatively impacts capsule sedimentation and their subsequent harvest.

In the present study, *B. adolescentis* was encapsulated within the legume protein isolate wall materials (10% w/w; CPI, LPI, FPI and SPI) characterized in study 1, as discontinuous droplets within a continuous canola oil phase, which were then crosslinked by genipin to create covalent linkages to stabilize droplets into capsules. After 6 h of stirring at low speed (750 or 1000 rpm), stirring ceased and 100 g of MQW was added to break the emulsion. Unfortunately, due to the high surface activity of the proteins (e.g., surface charge and hydrophobicity), separation into two distinct phases (oil and water) failed to occur, although instability of the emulsion was evident by the appearance of large coalescence droplets and the initial separation of layers. Photographs following MQW addition were taken for each legume protein isolate and are shown in Figure 4.7. All materials behaved similarly regardless of their surface charge (Figure 4.1) and hydrophobicity (Figure 4.2), however CPI and SPI based emulsions turned light blue (Figure 4.7A,C), whereas FPI and LPI based emulsions turned dark blue/purple (Figure 4.7B,C). Differences in protein-stabilized emulsion colour upon genipin crosslinking may reflect differences in initial protein isolate colour, the degree of protein crosslinking, capsule porosity (impacting light scattering) or interactions with non-protein materials within the isolate itself (e.g., phenolics). A greater understanding of genipin-induced colour formation in these types of systems represents a future area of research that could be explored.

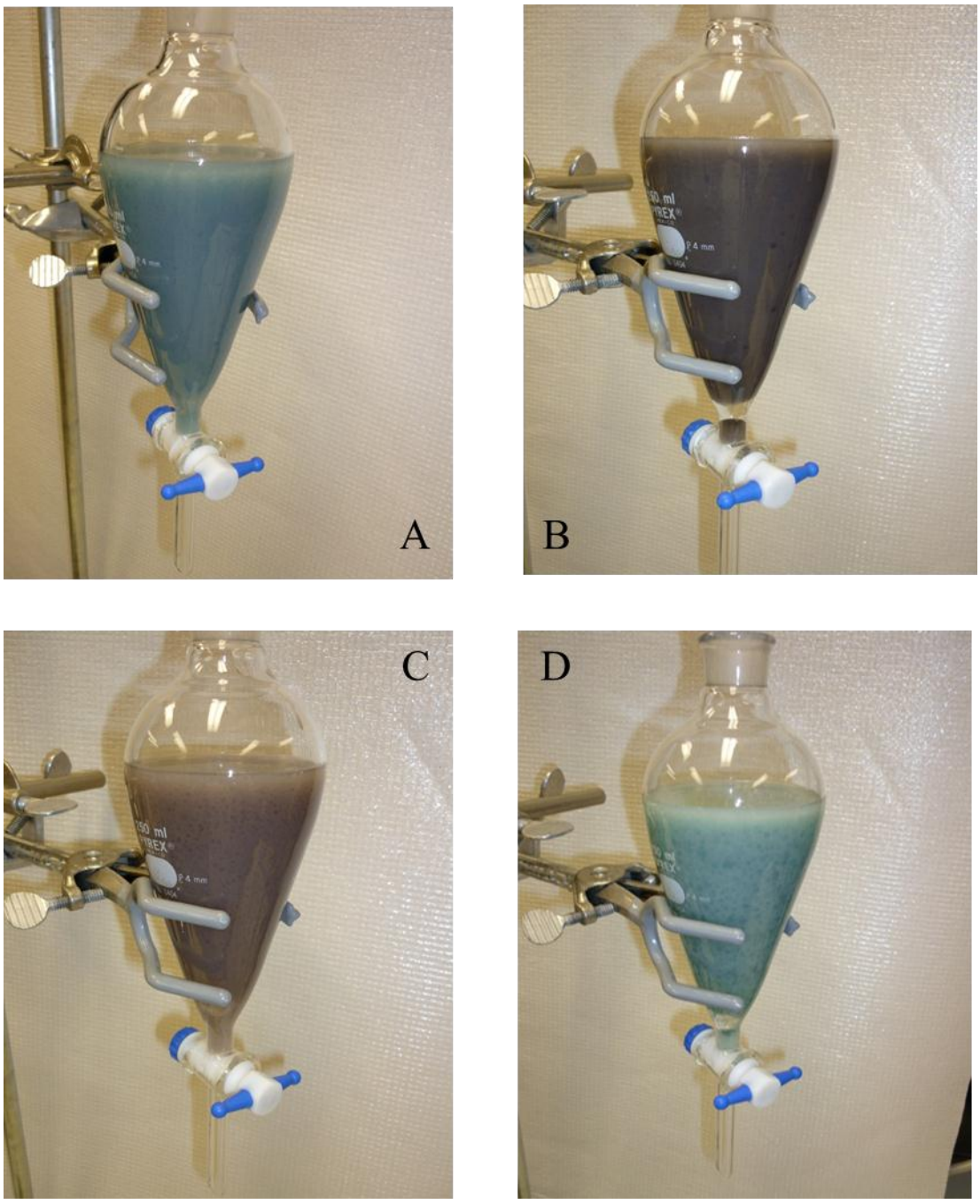

Figure 4.7 Photographs of genipin crosslinked CPI (A), FPI (B), LPI (C) and SPI (D) based emulsions following the addition of MQW to break the emulsion, but prior to centrifugation.

Because the genipin crosslinking reaction continues over time, leading to further aggregation of capsules; immediate harvesting was desired which required emulsion breakdown. To expedite emulsion instability, a centrifugation step was added (1000 x g for 5 min) to help facilitate emulsion breaking and to concentrate the formed capsules in the aqueous phase for harvest; this step was followed by the addition of 1.0% Tween 80 which facilitated capsule dispersion in the aqueous phase. Tween 80 (or polysorbate 80) has a hydrophilic-lipophilic balance (HLB) value of 15, and therefore has a greater affinity to hydrophobic phases (Kloet & Schramm, 2002). It was hypothesized that in an aqueous solution, Tween 80 would coat the surface of the protein capsules by interacting with hydrophobic amino acids, leaving hydrophilic groups accessible to create repulsion between neighbouring capsules so as to preserve them as discrete particles in solution.

4.2.2.1 Enumeration of encapsulated *Bifidobacterium adolescentis*

Once harvested, capsules were broken by vortexing at the highest setting for 30 s in order to determine by spread plating, the number of viable CFU mL^{-1} of encapsulated *B. adolescentis*. Although, homogenization under high shear is commonly used to break open capsule structures for enumeration of encapsulated probiotics at specific time points (Truelstrup-Hansen et al., 2002; Wood, 2010); the vortexing method was more advantageous for these studies due to its ease of use, speed, lack of required cleaning between samples, and low sample volume requirement. These benefits became especially pertinent during acid challenge experiments as capsules were too small to be handled individually and so immediate neutralization of the entire aliquot of capsule solution removed at each time point was required. During a preliminary experiment, CPI capsules containing *B. adolescentis* were broken using both the vortexing and homogenization methods and gave similar ($p>0.05$) viable cell counts of 7.26 and 7.11 log CFU mL^{-1}, respectively.

In the present study, the encapsulation process (with the 1000 rpm stir speed) led to the entrapment of ~7.6, ~7.0, ~7.0 and ~5.6 log CFU mL^{-1} for CPI, FPI, LPI and SPI, respectively (Table 4.3), which represented a ~1-2.5 log CFU mL^{-1} reduction in viable numbers from initial growth. Although there were minor differences between encapsulation numbers, wall material type did not seem to play a significant role. However, the SPI based capsules were found to encapsulate the fewest CFU mL^{-1} relative to the other legume proteins. Based on these results,

and with the exception of SPI, probiotic encapsulation at these levels would only be sufficient to provide a health benefit (7.0 log CFU mL^{-1}) in a 1 mL dose, if full survival and release of the organism was achieved. In study 1, the physicochemical properties of all legume proteins were relatively similar, with the exception of SPI solubility, which was significantly lower ($p<0.05$) (~50%) than the other legume proteins (~90%) (Figure 4.3). This lower solubility may have led to poor crosslinking of the SPI network within the droplet/capsule by genipin. The reduction of viable cell numbers as the result of the encapsulation process was thought to be due to: a) shear induced damage during mixing; b) prolonged exposure to aerobic conditions during the 6 h of crosslinking; and c) exposure to genipin and/or other compounds used in the encapsulation process (e.g., canola oil, Tween 80). Preliminary work (data not shown) indicated that Tween 80 and genipin did not inhibit the growth or viability of *B. adolescentis* individually. However, this would not rule out the possibility of either compound playing a role in the reduction of viable cell numbers during the encapsulation process via a multi-hurdle mechanism of stress. Similar, although lower losses in probiotic viability during encapsulation processes were reported by Annan et al. (2008) with a ~0.8 log CFU mL^{-1} reduction for alginate coated gelatin-based microcapsules containing *B. adolescentis*, and by O'Riordan et al. (2001) with a ~0.78 log CFU mL^{-1} reduction for waxy maize starch-based capsules containing *Bifidobacterium* PL1.

Table 4.3 Mean viable *B. adolescentis* (log CFU mL^{-1}) initially encapsulated within genipin crosslinked legume protein microcapsules. Data represents the mean ± one standard deviation (n = 3). Data with the same superscript were not significantly different ($p > 0.05$).

Sample	**log CFU mL^{-1}**
Chickpea	7.64 ± 0.80^{a}
Faba bean	6.98 ± 0.16^{ab}
Lentil	7.01 ± 0.44^{ab}
Soy	5.63 ± 1.13^{b}

4.2.3 Effect of stirring speed on the size of microcapsules produced by genipin induced crosslinking of legume protein isolates

The effect of stir speed during the encapsulation process on capsule size was tested for each material in the absence of *B. adolescentis*. Capsule size was characterized by light scattering in terms of the volume weighted mean (VWM), the mode, and the size distribution of formed capsules. VWM is the average diameter of all particles analyzed within the scattering field, as determined by the volume they occupy, assuming spherical shape. Using the Mastersizer 2000 instrument, capsule diameters can be separated into 100 size bins from 0.01 to 10,000 μm to give a size distribution curve. The size bin with the greatest percentile of particles is reported as the mode of capsule diameter. The VWM, mode and average percentile <100 μm for CPI, FPI, LPI and SPI based capsules formed with stir speeds of 750 and 1000 rpm are shown in Table 4.4. An analysis of variance was performed on: a) the VWM; b) the mode; and c) the percentage of capsules <100 μm so as to examine the effect of stir speed (750 vs. 1000 rpm), protein type (CPI, FPI, LPI and SPI) and their associated interaction. Statistical analysis of the data found only protein type ($p<0.001$) and the interaction of protein type with stir speed to be significant ($p<0.001$), whereas the effect of stir speed alone was insignificant ($p>0.05$). Based on the strength of the main effect (protein type), it was assumed that it weighted heavily on the degrees of freedom in the interaction term, and as such, the latter was neglected for discussion purposes.

Overall, the VWM (independent of stir speed) capsules were found to have the greatest diameter when formed with SPI (~158 μm), followed by CPI (~133 μm), LPI (~127 μm) and FPI (~61 μm) (Table 4.4). Similarly, the mode of capsules followed a similar trend, decreasing in size in the following order: SPI (~145 μm), LPI (~122 μm), CPI (~98 μm) and FPI (~57 μm). In terms of percentages <100 μm, FPI had ~87% of its capsules below 100 μm, whereas CPI, LPI and SPI had ~52%, ~43% and ~32% below 100 μm respectively (Table 4.4). A Holm-Sidak post-hoc pairwise multiple comparison indicated that for VWM only FPI was significantly different from the other three legume protein isolates ($p<0.001$), but considering both the mode of capsule size and percentage of capsules <100 μm, all legume protein types were significantly different from each other ($p<0.05$). The larger size of the SPI capsules relative to the other materials may be explained by the reduced solubility of SPI relative to the other legume proteins (Figure 4.3), which may have resulted in a less compact droplet. During crosslinking by genipin, the droplet/capsule network may not have been homogenous if the protein was not completely

soluble leading to larger average capsule sizes. However, solubility is only one factor impacting capsule size, as the other legume proteins have relatively similar solubility values, but differ widely in size. The size distribution for each material followed a very broad Gaussian-type distribution ranging in size from ~10 to 1000 μm (Figure 4.8). A wide size distribution is common for the emulsification process at relatively low stir speeds and long stirring duration (6 h); but was most likely exacerbated by capsule aggregation caused by the formation of both short and long range (through polymerization of genipin molecules) crosslinking of proteins by genipin within and in-between droplets and by protein-protein interactions between droplets influenced by surface characteristics such as charge and hydrophobicity. Based on these findings, the 1000 rpm stir speed was chosen (arbitrarily) for further encapsulation studies.

Table 4.4 Capsule size parameters (volume weighted mean, average mode, average percentile <100 μm) using chickpea (CPI), faba bean (FPI), lentil (LPI) and soy (SPI) protein isolate wall materials, at two different emulsification stir speeds. Data represents the mean ± one standard deviation (n=2).

Sample	**Stir speed (rpm)**	**Capsule size (μm)**		
		Volume weighted mean	**Average mode**	**Average % <100 μm**
CPI	750	106.70 ± 20.62	61.80 ± 16.91	59.78 ± 9.68
	1000	159.55 ± 62.41	135.29 ± 48.25	44.11 ± 16.88
FPI	750	70.10 ± 21.15	58.02 ± 10.30	82.58 ± 9.52
	1000	51.22 ± 3.18	56.37 ± 4.26	92.36 ± 2.87
LPI	750	124.79 ± 17.03	110.60 ± 16.29	48.75 ± 4.97
	1000	128.43 ± 18.67	133.36 ± 22.14	37.73 ± 8.22
SPI	750	189.98 ± 36.65	178.76 ± 73.47	22.94 ± 10.33
	1000	125.32 ± 6.20	111.38 ± 14.90	41.28 3.89

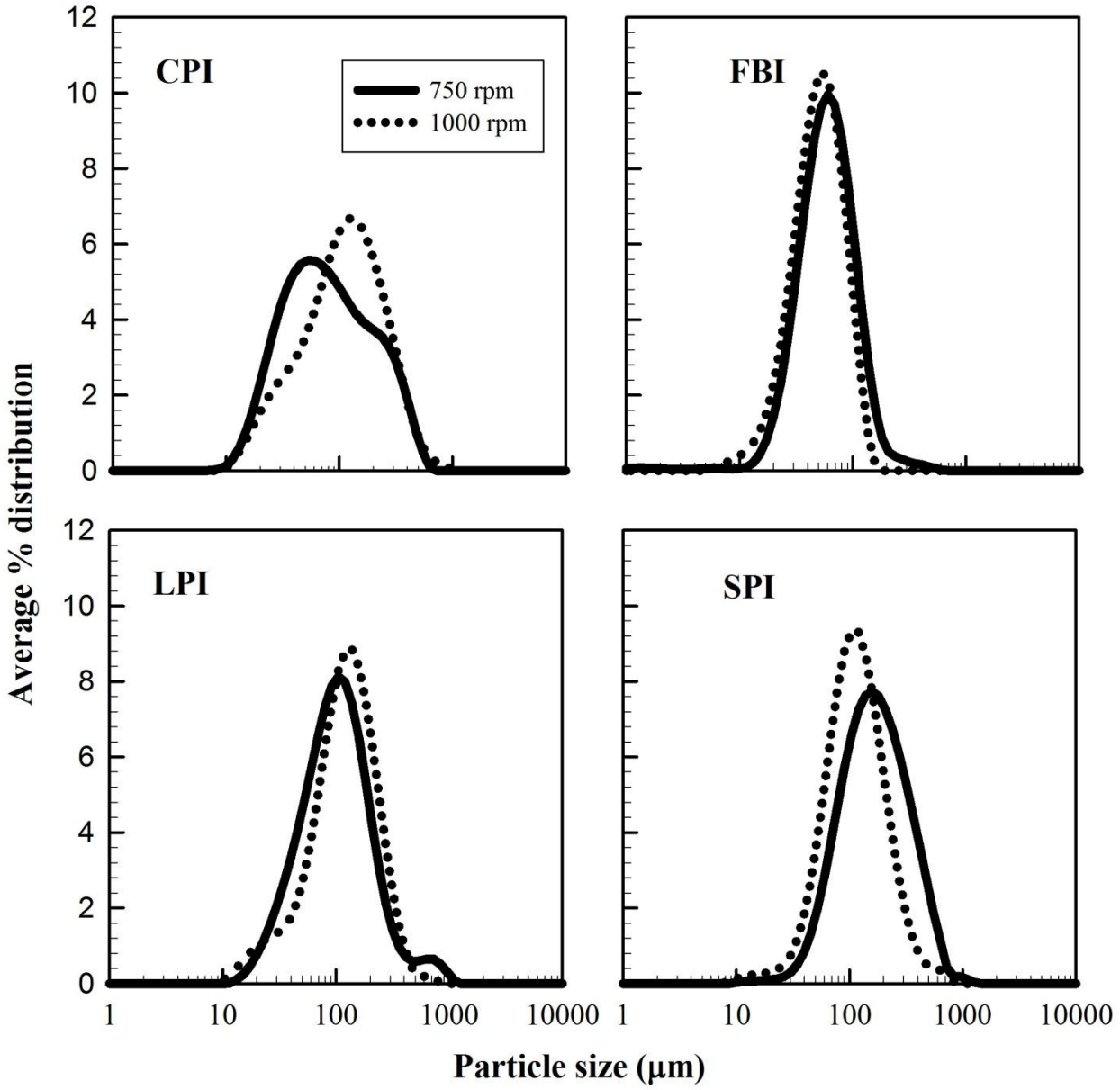

Figure 4.8 Average percent particle size distribution of legume protein capsules produced by emulsions employing stir speeds of 750 and 1000 rpm (n = 2).

Caution should be taken when interpreting the size data (despite being statistically significant) due to the large standard deviations for CPI and SPI (Table 4.4) and challenges associated with light scattering particle size analysis. During light scattering, particles are fitted to an imaginary sphere, and then averaged over all of the particles within the scattering field. If aggregation occurs within the system, the larger structures will skew the averages (due to their

much greater volume) and reduce the accuracy of a spherical particle shape assumption, leading to an overestimation of the true size. Mie theory is used by the Mastersizer software to measure particle size in solution by light scattering. Mie theory was developed in 1908 by the German scientist Gustav Mie, and represents the ideal scattering of electromagnetic radiation (light) by an isotropic spherical particle (Mie, 1908; & Garcia-Rubio, 1992). This theory is now commonly used to determine particle size based on light scattering under the assumption of spherical shape (Garcia-Rubio, 1992; Scholz et al., 1998; Jones, 1999). It also considers that the solvent used to suspend particles refracts light, in addition to suspending the macromolecules, to form an optically heterogeneous system (Garcia-Rubio, 1992). As this method relies on scattering patterns as light interacts with particles in solution, the refractive index of both the solvent and the particle of interest are important in particle size determination (Garcia-Rubio, 1992). The Mastersizer 2000 software has default refractive index values for a variety of compounds and solvents including protein (1.45) and water (1.33). Other authors have used similar refractive index values for water (Michalski et al., 2001) and proteins (Neto et al., 2009). However, in this case, if some canola oil remained in the capsule solution post-harvest it could alter the refractive index of the solvent (water) suspending the capsules to introduce error and high standard deviations to this particle size analysis. The presence of neighbouring particles can also impact the scatter patterns of light as it interacts with a particle (Garcia-Rubio, 1992), making capsule dispersion vs. aggregation an important factor. Furthermore, biopolymers (including proteins) are weak point scatterers, meaning the signal measured in the instrument is relatively low (e.g., compared to a contaminating oil droplet). In the present study, both CPI and SPI produced light blue emulsions/capsules, whereas FPI and LPI produced dark blue/purple emulsions/capsules. Slight differences in capsule hue may also impact scattering intensities measured and in some cases cause high standard deviations. Overall this method of size analysis produced high standard deviations, and did not agree with the direct observation of particle size (Section 4.2.5).

4.2.4 Acid challenge studies for free and encapsulated *Bifidobacterium adolescentis*

Genipin crosslinked CPI, LPI, FPI and SPI capsules with encapsulated *B. adolescentis* were subjected to an acid challenge study at pH 2.0 for 2 h, and then compared to the survival of free cells (Figure 4.9). In all cases (regardless of wall material), significant cell death occurred immediately, and after 30 min no viable cells could be detected (detection limit of 3.0 log CFU

mL^{-1}). Free cells followed a similar profile, except initial viable counts were higher (Figure 4.9) as free cells did not experience the ~1-2.5 log CFU mL^{-1} decrease associated with encapsulation. Since the encapsulation procedure was lengthy, preliminary experiments were performed to test whether the procedure was pre-stressing the bacteria prior to entering the acid challenge, in addition to reducing the total numbers. During these experiments cells were added after 3 and 5 h of stirring to reduce probiotic exposure time to 3 and 1 h respectively. However, cells fared even worse in the acid challenge than those encapsulated over the full 6 h, most likely due to the proteins having already started to form their network, leaving the majority of probiotics to be non-encapsulated or located on, or near the surface of the capsule. Originally, it was hypothesized that capsules prepared using globular proteins may form a more dense wall with smaller pore sizes than those observed in polysaccharide-based designs. This was based on the utilization of a higher wall material concentration (10% w/v), and the use of genipin to induce intra- and inter-molecular protein-protein covalent crosslinking within the capsule (Butler et al., 2003), in order to create a strong and probiotic-protective wall structure. Unfortunately, experimental results from this study did not support this hypothesis. Capsule formulations employed in this study did not offset the high surface area-to-volume ratio of the small capsules produced. Sultana et al. (2000) reported that such ratios enable greater interactions with the surrounding environment (acid) and poor probiotic survival.

Other emulsion-based capsules with polymeric wall materials (e.g., alginate, alginate-starch) have been shown to protect probiotics in a food matrix (milk, yogurt, etc.), but not in simulated gastric juice at sizes <1 mm (Sultana et al., 2000; Truelstrup-Hansen et al., 2002). For example, Sultana et al. (2000) utilized a 2% alginate, 2% Hi-maze resistant starch wall material, hardened with the addition of 0.1 M $CaCl_2$ to encapsulate *Lactobacillus acidophilus* and *Bifidobacterium* species. Capsules were mainly 0.5 – 1.0 mm in size, however a small proportion were found to be < 500 μm. When exposed to pH 2.0 for 3 h, a 5 log CFU mL^{-1} and a 3 log CFU mL^{-1} reduction from initial numbers of ~11.6 log CFU mL^{-1} were found for *L. acidophilus* and *B. infantis*, respectively. Smaller capsules were reported to have even less acid tolerance by Truelstrup-Hansen et al. (2002) who produced Ca^{2+}-alginate capsules (20 g L^{-1} alginate, 5 g L^{-1} Tween 80, 62.5 mM $CaCl_2$) containing four strains of *Bifidobacterium* at levels ranging from 6-8 log CFU mL^{-1}, with an average size of 70 μm, using emulsion technology. When exposed to pH 2.0, a 5 log CFU mL^{-1} reduction in cell numbers occurred after only 30 min for all strains except

B. lactis, which remained constant. In contrast, Borza et al. (2010) reported that an emulsion-based method produced gelatin (16%)-maltodextrin (3%) composite microcapsules crosslinked with genipin (24 mM), having a size of ~70 μm, which were able to protect encapsulated *B. adolescentis* from SGJ (pH 2.0; 0.32 mg mL^{-1} pepsin). After 2 h of exposure to SGJ free cells experienced a ~4 log CFU mL^{-1} reduction in numbers while encapsulated cells experienced only a ~2 log CFU mL^{-1} reduction.

4.2.5 Effect of biopolymer coatings and/or prebiotics on CPI encapsulated *Bifidobacterium adolescentis* survival in acid challenge experiments

The effect of coating materials (chitosan and napin) and/or the addition of the prebiotic, fructooligosaccharides (FOS) were investigated as a means for improving the survival of *B. adolescentis* employing a genipin crosslinked CPI-based capsule during an acid challenge. Only CPI capsules were used in this research as experimental data indicated that all of the legume protein isolates behaved relatively similar in terms of probiotic protection (Figure 4.9). Also, research results showed that CPI displayed the highest initial viable counts after encapsulation (~7.6 log CFU mL^{-1}) than the other materials (Table 4.4). Based on zeta potential data from study 1, CPI carries a negative charge at pH 7.0 (-47.7 mV, Figure 4.1) and therefore is capable of electrostatically coupling to cationic coating materials such as napin and chitosan (Wanasundara, 2011; Peng et al., 2012). Capsules containing an outer napin/chitosan coat are hypothesized to increase the survival of encapsulated *B. adolescentis* during the acid challenge experiment. The prebiotic FOS was also added to capsules in the presence of coatings as it has been demonstrated to be beneficial to the survival of several strains of *B. adolescentis* (Gibson & Wang, 1994; Rossi et al., 2005; Wood, 2010). Prebiotics function to benefit probiotics by selectively stimulating their growth and/or activity (Gibson & Wang, 1994b; Crittenden, 2001), and therefore while FOS may benefit probiotics through the encapsulation process, no increase in acid protection was hypothesized.

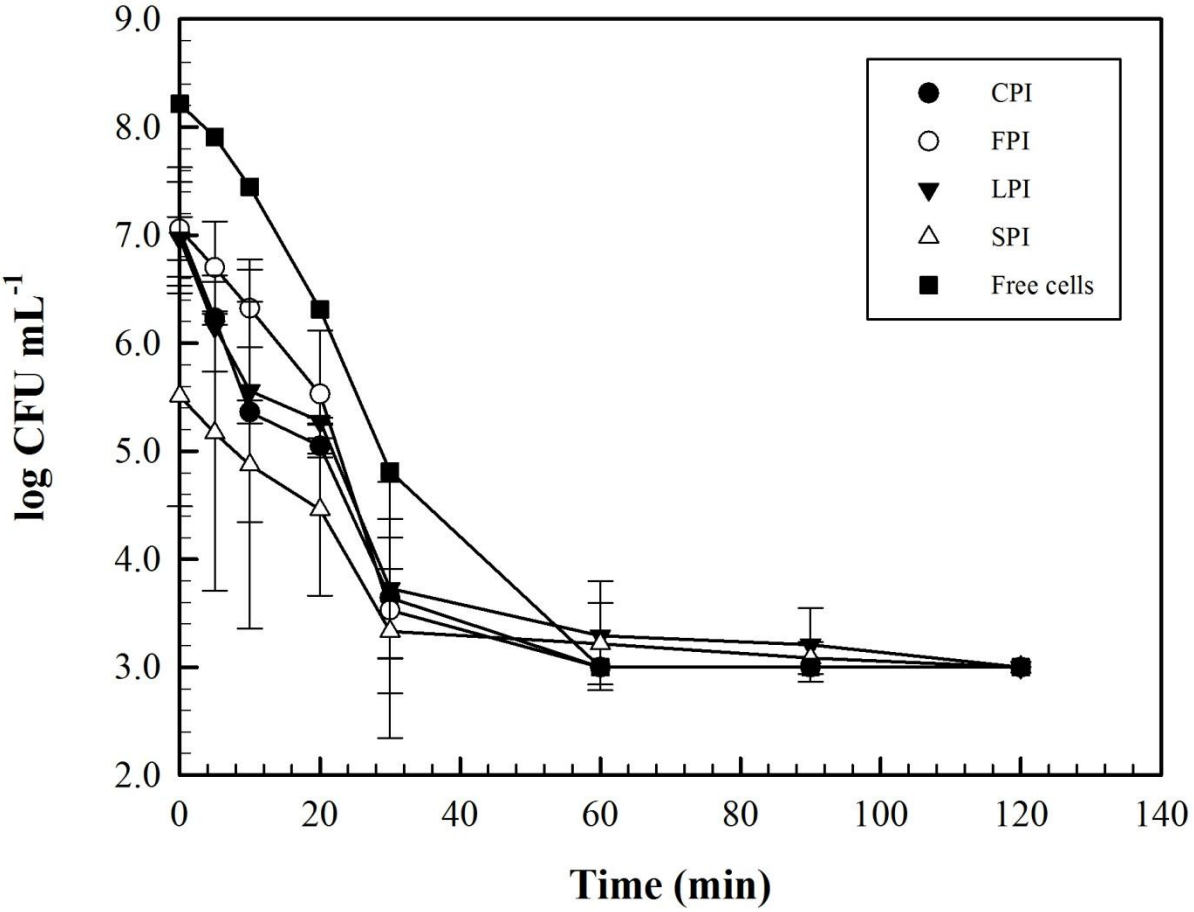

Figure 4.9 Survival of *B. adolescentis* (log CFU mL^{-1}) in genipin crosslinked legume protein isolate microcapsules exposed to an acid challenge (pH 2.0) as compared to free cells. Data represent the mean ± one standard deviation (n=3).

In the present study, the addition of coatings and/or FOS did not significantly reduce cell viability during encapsulation relative to the control (CPI without coatings or FOS) ($p>0.05$), with the exception of the chitosan coating without FOS, where viability was significantly reduced from ~7.6 to ~6.0 log CFU mL^{-1} ($p<0.05$; Table 4.5). Chitosan has known antimicrobial properties and several mechanisms have been proposed for this effect including ionic interaction with cell membranes to alter permeability and chelation of ions leading to cell toxicity. The antimicrobial properties of chitosan also appear to be related to both molecular weight and degree of polymerization (Kim et al., 1997; Chen et al., 1998; Rabea et al., 2003). Formulation of chitosan-coated CPI capsules with FOS seemed to mediate the impact of the antimicrobial

effects of chitosan, as survival was statistically similar to the control, ($p>0.05$). Overall, no additional protective effect was observed with the addition of chitosan coatings with or without FOS (Figure 4.10). During acid challenge, napin-coated CPI capsules with and without FOS seemed to show enhanced survival relative to the control, however the protective effect was not enough to impart a beneficial impact to human health, still showing a ~3 log CFU mL^{-1} reduction within the first 30 min of acid exposure (Figure 4.11). No differences were observed between napin-coated CPI capsules with and without FOS. However, napin coated CPI capsules were able to maintain viable probiotics at 3.97 log CFU mL^{-1} with FOS addition (3.85 CFU mL^{-1} without FOS) after 2 h of acid exposure, which is noteworthy because although the minimum resolution for enumeration by the plate count method in these experiments was 3.00 log CFU mL^{-1}, the trend of rapid cell death (even after only 30 min) in all other cell formulations suggests there was no survival through the acid challenge. Furthermore, this acid challenge may be unnecessarily restrictive to microbial survival as other simulated gastric juice models often utilize variable pH values (~2 – 5) over the course of a 2 h stomach transit time to better reflect *in vivo* stomach conditions as a meal is digested (Marteau et al., 1997; Lee & Heo, 2000). Therefore, there is potential for even greater performance of this capsule formulation, which already shows improvement by protecting nearly 4.0 log CFU mL^{-1} (Figure 4.10) compared to presumed complete death in all other capsule formulations. As such, the use of a more moderate SGJ/SIF model, where capsules are incorporated into a food matrix may merit future investigation.

Polymer coatings have previously been shown to increase encapsulated probiotic survival in literature. For example, Annan et al. (2008) found that gelatin-based (13% w/v) capsules, crosslinked with genipin (1.25 mM) and coated with alginate, having average sizes of ~50 μm were able to protect encapsulated *B. adolescentis* 15703T from both SGJ (pH 2.0; 0.2% NaCl; 0.3g L^{-1} pepsin) and sequential exposure to SGJ and SIF (pH 7.4; 1 g L^{-1} pancreatin; 4.5 g L^{-1} bile salts) better than uncoated capsules and free cells. After 2 h exposure to SGJ a 1.21, 2.55 and 3.45 log CFU mL^{-1} reduction was reported for encapsulated cells with an alginate coating, encapsulated without a coating, and free cells, respectively. After sequential exposure to SGJ (1 h) and SIF (4 h), ~7.6 log CFU mL^{-1} remained within the coated microcapsules compared to ~6.7 and ~6.4 log CFU mL^{-1} for encapsulated without coating, and free cells, respectively. In addition, Iyer et al. (2005) reported that chitosan-coated alginate-starch capsules containing *L.*

casei strain Shirota were able to protect the probiotic through *ex vivo* porcine gastro-intestinal contents for release at levels of ~ 8 log CFU mL^{-1} in ileum and colon fluids after 4 and 8 h incubations respectively.

Table 4.5 Mean viable *B. adolescentis* (log CFU mL^{-1}) initially encapsulated in genipin crosslinked chickpea protein microcapsules with and without chitosan and napin coatings and fructooligosaccharides (FOS) addition. Data represent the mean ± one standard deviation (n = 3). Data with the same superscript is not significantly different ($p>0.05$).

Sample	**log CFU mL^{-1}**
CPI (control: uncoated and without FOS)	7.64 ± 0.80^{a}
CPI capsules with FOS (uncoated)	7.25 ± 0.24^{a}
Chitosan-coated CPI capsules	6.04 ± 0.39^{b}
Chitosan-coated CPI capsules with FOS	7.16 ± 0.12^{a}
Napin-coated CPI capsules	7.80 ± 0.06^{a}
Napin-coated CPI capsules with FOS	7.84 ± 0.03^{a}

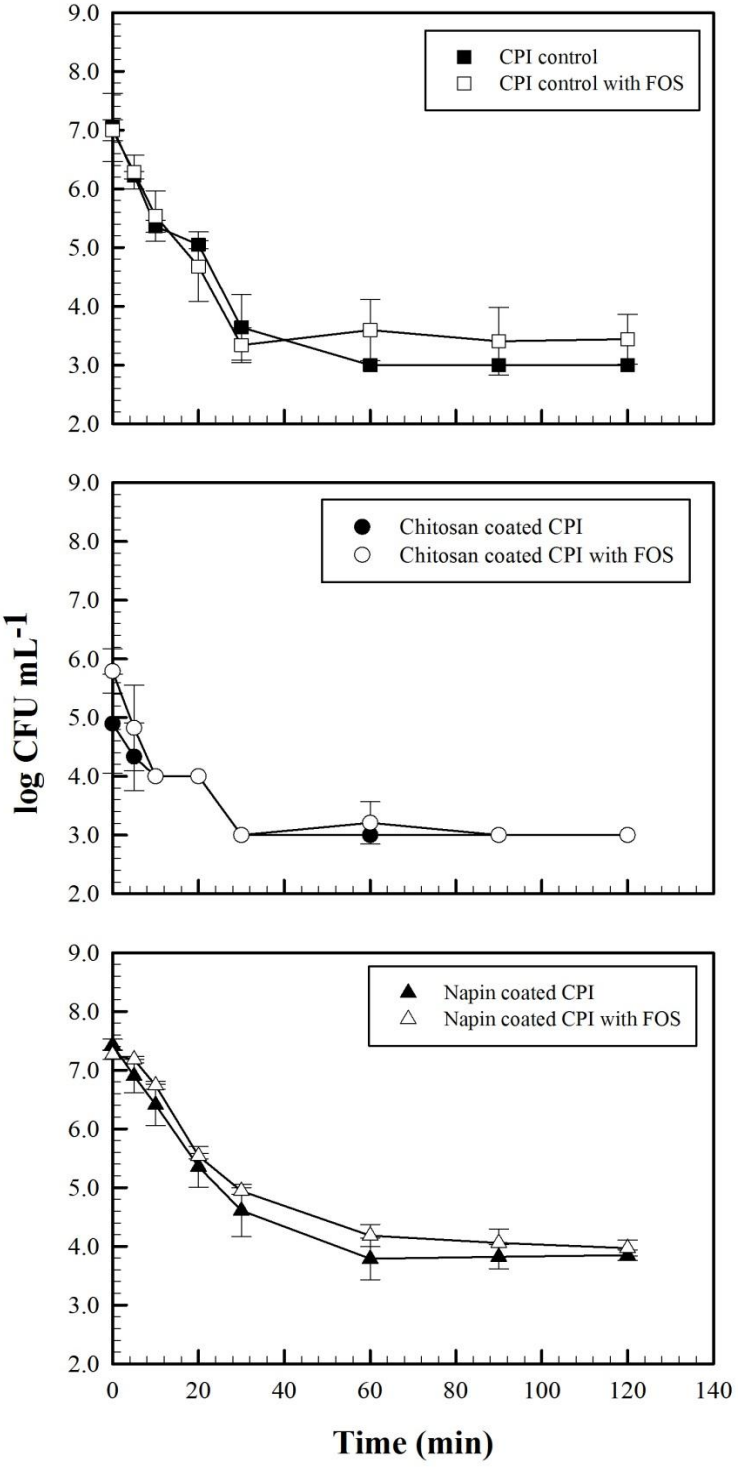

Figure 4.10 The impact of fructooligosaccharides, and chitosan and napin coatings to genipin crosslinked CPI capsules on the survival of encapsulated *B. adolescentis* (log CFU mL^{-1}) during an acid challenge (pH 2.0). Data represent the mean ± one standard deviation (n=3).

4.2.6 Imaging of chickpea protein capsules

Microscopy was utilized to analyze: a) the size and shape of genipin crosslinked CPI capsules with and without a napin coating; and b) the distribution of *B. adolescentis* throughout the capsule matrix (using the SYTO®-9 fluorescent tag) with a napin coating. Representative images taken at 400x magnification for genipin crosslinked CPI capsules in the absence and presence of a napin coating are shown in Figure 4.11. In all cases, capsules appeared spherical in shape and were present as individual and/or flocculated aggregates. Flocculation of capsules may have been the result of genipin crosslinking or associations driven by hydrophobic interactions between proteins on adjacent capsules. ImageJ particle sizing software was used to analyze digitized images from multiple fields of view (n = 5, with 138-254 capsules per field) to determine the average diameters of 5.35 ± 0.30 μm and 4.83 ± 0.99 μm for CPI capsules in the absence and presence of a napin coating, respectively. No statistical differences were found with the addition of the napin coating ($p>0.05$). The size of the formed CPI capsules with and without a napin coating was approximately ~20-25 times smaller (~5 μm in diameter) than the data estimated by light scattering (Table 4.3). The larger sizes estimated by light scattering may reflect the instruments inability to distinguish differences between protein-protein aggregates (non-capsule particles), flocculated capsules and individual capsules as the technique assumes that all particles are point scatters. Furthermore, protein matrices are only weakly scattering particles (e.g., higher noise-to-signal ratio). While light microscopy digital images enabled visualization of the very small CPI capsules produced, undertaking particle size determination using this method may have some bias against large sized particles resulting in lower average size results. Large capsules can be difficult to clearly picture using microscopy and may not appear if they are too thick for sufficient light to penetrate. Regardless, the capsules observed via microscopy were significantly smaller than indicated by light scattering, which may explain their lack of acid protection given their high and detrimental surface area to volume ratios.

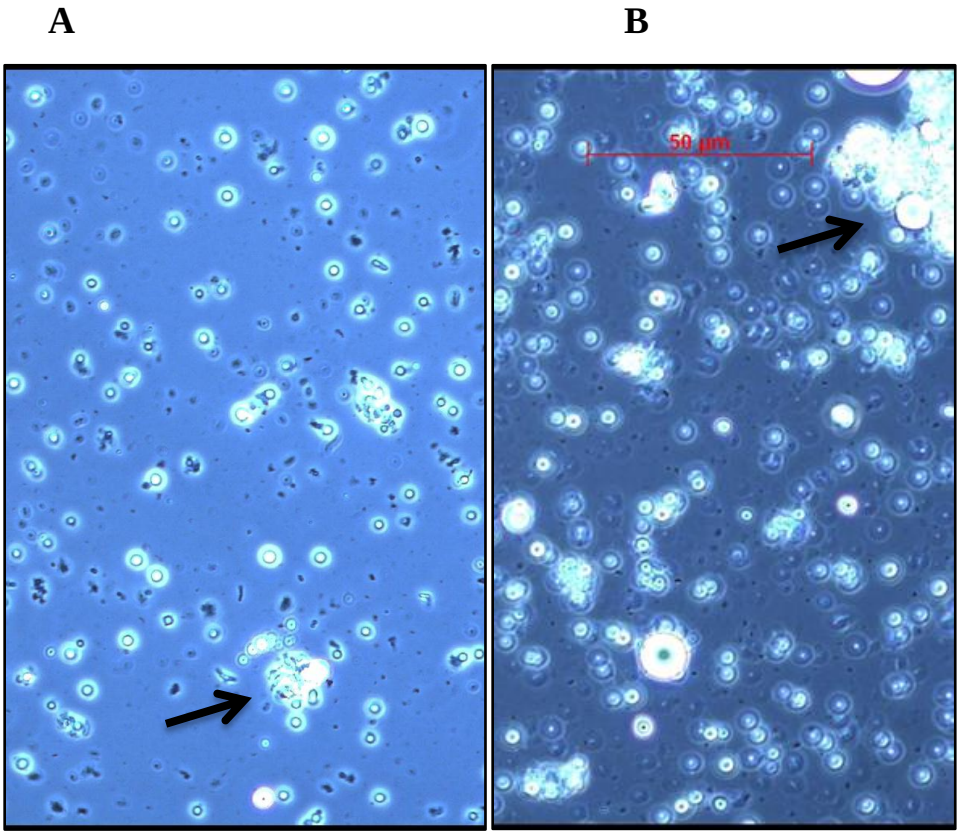

Figure 4.11 Light microscopy digital image of CPI capsules (with encapsulated *B. adolescentis*) in the absence (A) and presence (B) of a napin coating under 400x magnification. Arrows depict areas of capsule aggregation.

Fluorescence microscopy indicated that *B. adolescentis* were present in small as well as larger capsules and in the capsule solution, as indicated by areas of brightened fluorescence (Figures 4.12, 4.13), and that some capsules, or capsule aggregates, were produced near the desired 100 µm size. The SYTO®-9 fluorescent tag is a membrane penetrative green fluorescent nucleic acid dye, capable of staining live, viable cells that results in fluorescence under UV exposure allowing for bacterial visualization. Figure 4.12-A/B show the same very large (~200 µm) capsule at two layers of focus, containing a large number of encapsulated *B. adolescentis*, depicted by bright fluorescent points. Although not the normal arrangement, the thicker capsule wall and smaller surface area to volume ratio of capsules of this type/size may be responsible for a large proportion of exhibited probiotic protection (e.g., 3.97 log CFU mL^{-1}; Figure 4.10) from the previous experiment. The more common, small and dispersed capsule arrangement within the

solution as depicted in Figure 4.12-C likely offered very little acid protection due to the higher exposure to the environment. The apparent probiotic distribution, throughout the capsules, is most likely the result of vigorous stirring during emulsion formation. Figure 4.13 depicts the same large capsule as Figure 4.12, although at a higher magnification and at three levels of focus. Chains of brightened bacteria can be visualized (Figure 4.13-B/C: white arrows), which is the normal growth arrangement for *B. adolescentis* (Gibson & Roberfroid, 1995). The large undefined areas of brightened fluorescence (Figure 4.13-A: black arrow) are presumed to be other *B. adolescentis* on different capsule layers, which are out of focus, as this image depicts a large capsule in 3D. The probiotic protection demonstrated in Section 4.2.4 may have been a result of a small number of capsules of a size near or above 100 μm, which as a result had a thicker capsule wall and a more moderate surface area to volume area ratio allowing them to protect *B. adolescentis* throughout the acid challenge, whereas the individual/dispersed, smaller (e.g., ~5 μm) capsules likely provided little to no acid protection.

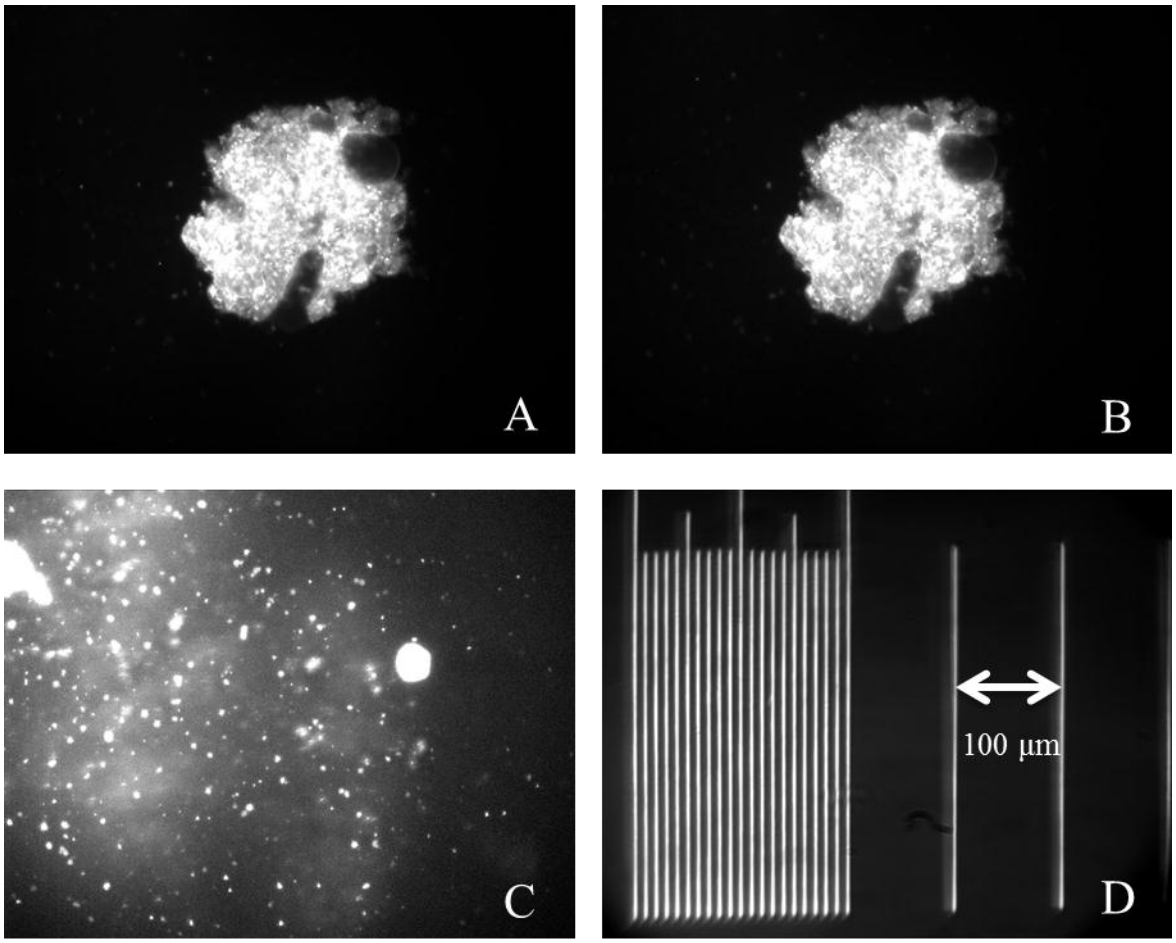

Figure 4.12 Fluorescent microscopy digital images at 160x magnification of a CPI capsule solution demonstrating: large capsule focal plane 1 (A), large capsule focal plane 2 (B), solution background with smaller capsules and free bacteria (C) showing common capsule size and probiotic distribution and stage micrometer (D).

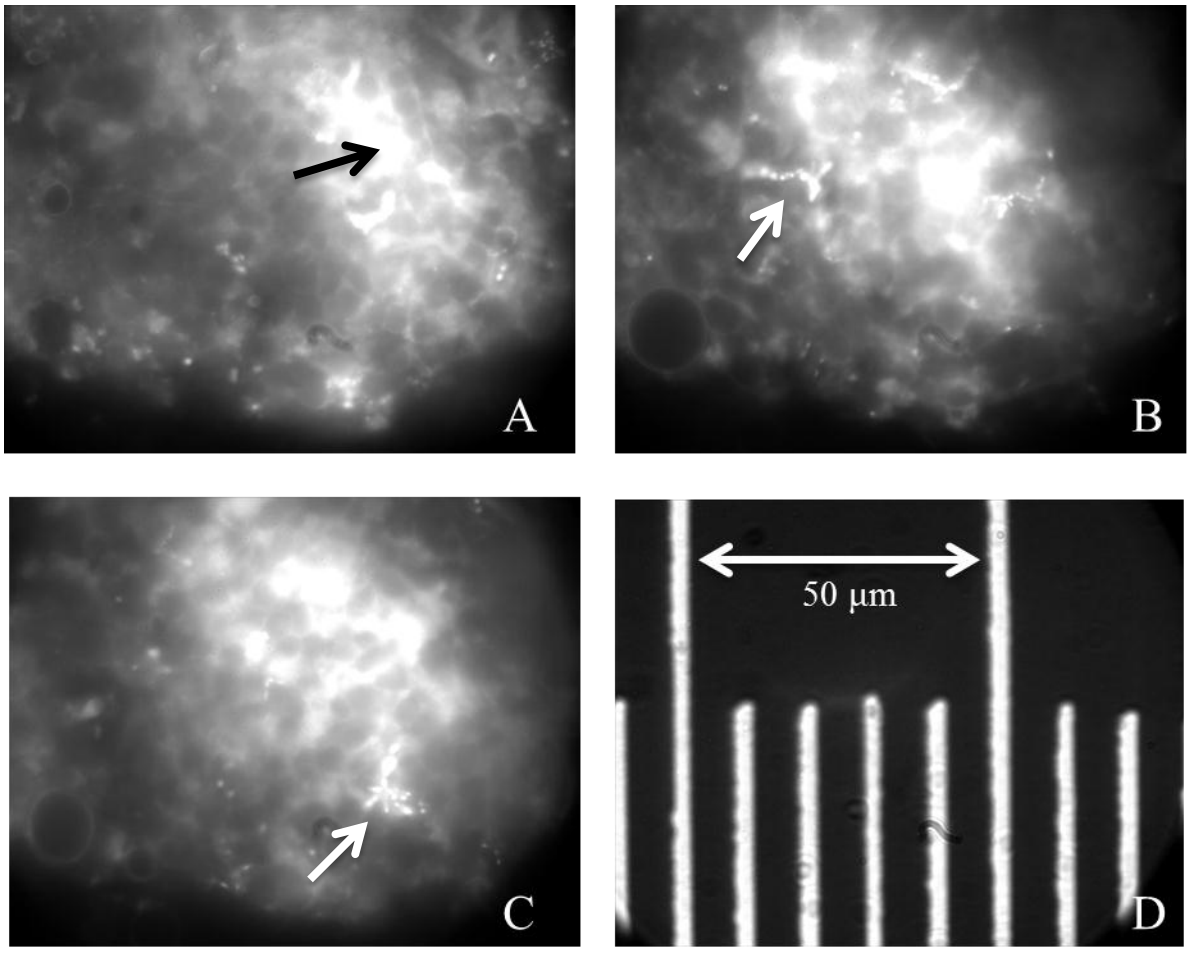

Figure 4.13 Fluorescent microscopy images at 1000x magnification of a CPI capsule solution demonstrating a view of three focal points (A, B and C) of single large capsule with encapsulated bacteria and a stage micrometer view for scale (D). White arrows indicate chains of *B. adolescentis* cells in focus, and the black arrow indicates brightened fluorescence of unfocused cells.

5 CONCLUSIONS

Select physicochemical properties of four legume protein isolates and their crosslinking affinity with genipin were evaluated in terms of their emulsifying properties and potential to act as a wall material for probiotic encapsulation. Overall, legume protein isolates were able to act as emulsifiers due to their amphiphilic nature so as to stabilize Millipore Milli-Q^{TM} produced water (MQW)-canola oil emulsions, which was enhanced in the presence of genipin. However, a full understanding of the impact of the protein isolate's physicochemical properties on emulsion stability was difficult, reflecting the complexity of the system. To be an effective emulsifier, the protein needs to have sufficient surface charge to remain water soluble such that it can migrate to the oil-water interface, and have enough surface hydrophobicity to align and re-orient once there (Schwenke, 2001; Damodaran, 2005). Based on these criteria, it would have been presumed that chickpea protein isolate (CPI) would give the best emulsion stability based on its high solubility and surface hydrophobicity relative to the other protein isolates. However, CPI displayed the lowest stability of all of the four legume protein isolates examined. To better understand the structure-function mechanisms in legume protein isolate stabilized emulsions, further studies on the role of protein characteristics (e.g., conformation, concentration and level of denaturation), solvent effects (e.g., pH, temperature, salts), processing factors (e.g., homogenization rates and duration) and emulsion characteristics (e.g., oil-water ratio, droplet size) are needed.

Encapsulation of *B. adolescentis* within legume protein capsules, crosslinked by genipin was achieved with viable cell numbers of ~7.21 log colony forming units (CFU) mL^{-1}; however this did not translate into a protective effect during an acid challenge. Furthermore, there was disagreement between capsule size as determined by microscopy imaging and light scattering due in part to the presence of aggregates in solution. However, CPI capsules demonstrated an ability to encapsulate the greatest number of *B. adolescentis* and so were utilized to investigate strategies for improving capsule formulation performance. CPI capsules coated with napin protein showed improved survival, however the improvement was only minor; still providing insufficient capabilities as a product. Although microscopy indicated that the capsules produced

by this method were significantly smaller than anticipated, probiotics were found to be distributed throughout capsules. In addition, a small proportion of larger capsules (near or above 100 μm in size) were identified and hypothesized to be responsible for the acid protection that was experienced by the napin coated capsules as a factor of their more moderate surface area to volume ratios. Although this study did not achieve the end goal of encapsulated probiotic survival levels of 7.0 log CFU mL^{-1} novel information of this subject was generated for emulsion-based genipin crosslinked, legume protein encapsulation.

6 FUTURE STUDIES

The current research investigated the relationship between the physicochemical properties of legume protein isolates and their ability to act as emulsifiers with and without genipin-induced crosslinking, and as encapsulating agents for probiotic delivery. However, to better understand the structure-function mechanisms in legume protein-stabilized emulsions, further studies on the role of protein characteristics (e.g., conformation, concentration and level of denaturation), solvent effects (e.g., pH, temperature, salts), processing factors (e.g., homogenization rates and duration) and emulsion characteristics (e.g., oil-water ratio, droplet size) are needed. Furthermore, investigation of crosslinking conditions surrounding protein reactions with genipin is needed in order to optimize crosslinking concentration, temperature and time.

Although some acid protection to the probiotics was afforded by the final napin-coated capsule design, survival levels still remained well below what is needed to offer a beneficial impact to a host. Better control over capsule size, as determined by stir speed, protein concentration, wall-to-core ratio and crosslinking conditions could result in improved/better controlled capsule size and protection to encapsulated probiotics. Manipulating these conditions could also lead to greater control over the pore size of the wall material, where smaller pores may lead to enhanced survival. Differences in pore size among different wall formations/conditions could be assessed using scanning electron microscopy. Possibly, the replacement of genipin with other non-toxic fixatives such as transglutaminase, or using protein-polysaccharide composite materials with ionic fixatives might reduce encapsulation time further, leading to increased control over capsule size.

If a capsule formulation was developed with adequate acid protection capabilities, investigation into performance in a system more closely related to the human gastrointestinal tract (GIT) would be necessary. This research did not investigate capsule release of probiotics, which is an essential function of a probiotic delivery system. Sequential exposure to simulated gastric juice (SGJ) and simulated intestinal fluid (SIF) and/or the use of a simulation of the

human intestinal microbial environment (SHIME) system could provide a better benchmark of capsule performance. The use of an animal model could also provide information on capsule performance in addition to evaluating potential to market capsules for animal feed additives in addition to a human food ingredient. Finally incorporation of capsules into a food matrix or as a food additive, with or without freeze drying, and evaluation of survival, shelf stability and subsequent capsule performance are also necessary investigations prior to marketing probiotic containing capsules of this nature as a human health supplement.

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