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Viability of *Lactobacillus acidophilus* DDS 1-10 Encapsulated with an Alginate-Starch Matrix

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L. acidophilus DDS1-10 was encapsulated using an alginate, potato starch and type 4 resistant starch (RS4) matrix. The wall material was optimized by varying levels of alginate (1.6 to 4.4%), while maintaining potato starch and RS4 ratio fixed at 2, and the total solid content at 5%. Particles were prepared using an emulsion and an extrusion method. Particles obtained from the emulsion method were smaller with a non-uniform distribution in size while those obtained by the extrusion method were bigger and uniform in size. The emulsion method did not seem to offer protection to the probiotic cells against pH, bile salt and temperature. However, the particles obtained by the extrusion method provided protection against the effects of pH and bile salts. The particles produced by extrusion were incorporated into two food systems of varying pH levels. Promising results were observed in the milk system; however encapsulation did not provide significant protection when cells were introduced to acai juice during 16 days of storage.

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Chapter 1. Literature review

1.1 Microencapsulation

Microencapsulation is the process by which food ingredients, enzymes, cells or other materials are incorporated in small capsules (Gibbs et al. 1999). The capsule coatings are referred to as wall, shell, or carrier. The entrapped material can be described as the core, fill, actives, or payload (Augustin et al. 2001). Microencapsulation first appeared in the market as part of the manufacturing process of carbonless copy paper (White 1998).

During encapsulation, the core material is usually enclosed by the wall material. Sometimes, there is no obvious boundary between the wall and core material, with both mixed together in what is called the matrix. The shape of the particles can be regular or irregular. Since the particle is usually spherical to elliptical, it is also called a microsphere. If the capsule has a gel-like structure, it is named gel-bead (Amir Mortazavian 2007; Gibbs et al. 1999; Gouin 2004). The surface of the particles can be smooth or rough. There might also be cracks on the particles (Sheu and Marshall 1993). The surface of the particles can be porous too, which usually lower the encapsulation efficiency (Amir Mortazavian 2007). In this case, a second wall or multi-walls can be used to strengthen the inner wall and increase the encapsulation efficiency (Krasaekoopt et al. 2004).

The size of the particles varies with the method applied for encapsulation. Smaller sizes are preferred because they allow for better transportation of nutrients and oxygen, better mechanical strength and better dispersion (Chicheportiche and Reach 1988; Leblond et al. 1999). Singer and Dunn (1990) reported that particles that are smaller than 3 μm are not perceived by the tongue.

Microencapsulation is used for several different reasons. It is used to protect the compound or biological cells from the surrounding environment (heat, oxygen, moisture); disguise unpleasant odor, color or taste of the core material, or convert liquid into powder for ease of handling. It can also be used to better disperse core materials in the product; and control the release of the core material at supposed sites and times (Desai and Park 2005; Dziezak 1988(Desai and Park 2005; Dziezak 1988; Shahidi and Han 1993).

1.2 Microencapsulation techniques

There are various techniques available for microencapsulation. They are normally classified into three categories: (1) Physical techniques (Spray drying, spray chilling, extrusion, centrifugal extrusion, fluid bed coating); (2) Chemical techniques (Interfacial polymerization, polymerization); and (3) Physicochemical techniques (Coacervation). The choice of method is dependent mainly on the wall material, core material, equipment available, cost consideration, application, and scale of production (Gibbs et al. 1999;

Gouin 2004; Krasaekoopt et al. 2004). Table 1.1 shows the advantages and disadvantages of some of the encapsulation techniques, while Table 1.2 shows the major processes involved in some of the encapsulation techniques.

1.2.1 Emulsion method

The emulsion method is achieved by adding the wall material and core material drop-wise into an oil phase to form an emulsion, followed by hardening of the wall material with the addition of a gelling agent or a crosslinking agent (Champagne and Fustier 2007). This method is costly because of the use of vegetable oil. Using this method, Sheu and Marshall (1993) developed a procedure to entrap *L. bulgaricus* by mixing it with 3% sodium alginate and suspending it into oil which contains 0.2% Tween 80. The particles produced protected the bacteria and improved survival in ice milk by 40% (Sheu and Marshall 1993) .

In the emulsion method, the size of the particles is influenced by the speed of agitation and the type of emulsifier used. Smaller sized particles are desired for various reasons: They transport core material more efficiently, disperses better, and carry better mechanical strength (Chicheportiche and Reach 1988; Sugiura et al. 2005). The bead size achieved by the emulsion method varies from 25μm to 2mm (Krasaekoopt et al. 2003).

Capela et al. (2007) indicated that a bead size of 39.2μm could be achieved by using a homogenizing speed of 13,500 rpm for 4 minutes.

1.2.2 Extrusion method

Extrusion is the simplest and most common technique for forming hydrocolloid particles (King 1995). The size of the particles obtained with the extrusion method (2-5mm) is larger than that obtained from the emulsion method (25µm- 2mm) (Sheu and Marshall 1993; Sultana et al. 2000). In this process, the core material and alginate pass through a syringe needle and drop into the hardening solution (containing cations such as calcium). The particles are then formed immediately when the drop comes in contact with the hardening solution. The size of the particles is dependent on the size of the needle, and the distance between the needle and the hardening solution (de Vos et al. 2010; Gouin 2004; Krasaekoopt et al. 2003). The advantage of the extrusion technology is that the wall material could be a shell over the core material, or the wall material and core material could be produced as a matrix (Gibbs et al. 1999).

1.2.3 Other encapsulation methods

Spray-drying is the most widely used and studied technology in the food industry for microencapsulation because of its suitability for economical large-scale industrial

application (Dziezak 1988). The first spray-dryer was constructed in 1878 for pharmaceutical use (Hayashi 1989). The one-step continuous process and readily available equipment allows for easy operation and reproducibility. Encapsulation by this technology involves the atomization of an aqueous suspension into a drying gas, the water evaporates due to high temperature, then the dry particles could be collected (Tonon et al. 2011). The process is controlled by the feed rate, inlet and outlet temperature, and gas flow (Rokka and Rantamaki 2010). Spray drying was once considered an inferior method for encapsulating probiotics since the survival rate of the bacteria was low due to the dehydration and thermal inaction of the microorganism (Fu and Etzel 1995; Saarela et al. 2000). However, a relatively high survival rate of *Bifidobacterium* was reported when utilizing an inlet temperature of 100℃ and an outlet temperature of 45℃ with modified starch as wall material (O'Riordan et al. 2001). But the drawback of this modified method is that it affects the ability of the probiotics to resist the gastrointestinal environment (Del Piano et al. 2008).

Spray chilling is similar to spray drying except that the atomized core material and wall material suspension are chilled by cooled air instead of heated air (Nazzaro et al. 2012). The cool air leads to solidification of the wall material around the core material since the melting point of the wall material is usually higher than the air temperature (Chambi et al. 2008; Champagne and Fustier 2007; Pedroso et al. 2012). The wall material is usually fractionated or hydrogenated vegetable oil which has a melting point ranging from 32-

42℃ (Risch 1995). Therefore, the release of the core material from the microsphere produced by this method can be controlled by the temperature. Frozen liquid, heat-sensitive materials and water-insoluble materials can be used in this method (Gibbs et al. 1999). Spray chilling is considered to be the cheapest encapsulation technique (Gouin 2004).

1.3 Wall material selection

Various substances can be used as wall material to entrap, coat, or encapsulate. However, only a few of them are regarded as "generally recognized as safe" (GRAS) materials. Numerous encapsulation techniques are limited because of the lack of GRAS materials. Food regulations are stricter than the ones applied in the pharmacy industry when regarding wall material choice (Wandrey et al. 2010). Commonly used wall materials are alginate, starch, xanthan gum, cellulose, gelatin, chitosan and ƙ-carrageenan. The choice of the wall material is dependent on the method adopted and the core material used.

Ding and Shah (2009) tested the protection role of alginate, xanthan gum, and carrageenan gum when encapsulating *Lactobacillus rhamnosus* under acidic conditions over 8 hours of incubation. They found that the viability of the probiotics was reduced by 3.63 log CFU/mL, respectively, compared to free cells which showed a reduction of 6 log CFU/mL.

1.3.1 Alginate

Alginates are naturaly occurring polysaccharides either extracted from seaweed or produced by bacteria (Gombotz and Wee 1998). The alginate molecule is a block copolymer composed of sequential M units (1,4-linked ß-D-mannuronic acid) and G unit (α-L-gluluronic acid), with the sequential distribution depending upon the source of the alginate (Martinsen et al. 1989). Divalent cations such as Ca^{2+} tend to bind to the G unit (Krasaekoopt et al. 2003), inducing ionic interchain bridges, which results in an egg-box structure in alginate gel (Rees 1981). Other divalent cations such as Pb^{2+}, Co^{2+} and Ni^{2+} are not used for food applications because of their toxicity. Alginate is the most used biopolymer for encapsulation (Saarela et al. 2000). The unique properties of alginate such as a high gel porosity and ease of processing at room temperature make it an ideal matrix material for encapsulation (Gombotz and Wee 1998).

Various reports have indicated the success of using alginate as wall material alone or in combination with others. Alginate as wall material has been tested with concentrations ranging from 1% to 3%, along with 0.05-1.5M $CaCl_2$ (Chandramouli et al. 2004; Cui et al. 2000; Kearney et al. 1990; Krasaekoopt et al. 2006; Prevost and Divies 1988; Sheu and Marshall 1993). Jankowski et al. (1997) tested 0.6% sodium alginate with 0.05, 0.10, and 0.30 M $CaCl_2$ and found out that the viability and fermentation ability of *L.acidophilus* during yogurt manufacture increased compared to unencapsulated bacteria.

However, alginate is very sensitive to acid. It has been reported to degrade under acidic conditions because the cross-linking network suffers a reduction in molecular weight (Mumper et al. 1994). It is because the hydration links between the M and G units are broken. The network then becomes loose, which fastens the degradation of alginate (Gombotz and Wee 2012).

An alginate gel is very porous, which usually requires another coating agent to enhance protection of the core material, especially in acidic conditions (Klein et al. 1983; Taqieddin and Amiji 2004). Higher than 5% of alginate is usually avoided because of handling difficulties during processing (Gombotz and Wee 2012). The low viscosity of the alginate solution lacks mechanical and physical stability (Peirone et al. 1998; Smidsrod and Skjakbraek 1990).

1.3.2 Resistant starch type 4

Starches are polysaccharides that are composed of a number of monosaccharides or sugar molecules linked with α-D-(1-4) or α-D-(1-6) linkages. It consists of amylose, which is a linear polymer of glucose with α-D-(1,4) linkages, and amylopectin, which is a branched molecule with α-D-(1-4) and α-D-(1-6) linkages (Sajilata et al. 2006). Resistant starch is defined as a small fraction of the starch, and starch degradation products that are resistant to digestion in the small intestine of healthy humans (Asp 1992). There are four types of

resistant starch. Resistant starch type 1 (RS1) is a physically inaccessible starch trapped within whole grains or seeds. Resistant starch type 2 (RS2) is a high amylose granular starch from certain plants. Resistant starch type 3 (RS3) is retrograded starch after gelatinization, and the linearly/cross-linked starch that is difficult to hydrolyze by α-amylose is called resistant starch type 4 (RS4) (Lee et al. 2007). The ability to resist digestion by pepsin and pancreatin-bile are found in 82% of RS4 (Lee et al. 2007).

Starch has long been used as the wall material for encapsulation since the starch granule is an ideal surface for attachment of probiotic cells. Additionally, resistant starch can also offer the benefit of not being digested, which allows it to reach the colon where it is fermented (Kritchevsky 1995). Sultana et al. (2000) incorporated Hi-Maize starch into alginate and improved the viability of probiotics compared to encapsulation without starch. Resistant starch can also be used as an adherence surface for the probiotics during processing, storage and transit through the upper gastrointestinal tract (Anal and Singh 2007).

1.3.3 Potato starch

Potato Starch consists of starch particles that are oval or spherical in shape with a diameter of 5-100 μm. The pasting temperature is around 60-65°C and it has very high

pasting viscosity. The film strength, flexibility and solubility obtained with potato starch are very high, and provides solutions with high clarity (BeMiller 2009).

The main purpose of using potato starch during encapsulation is to take advantage of its adherence capacity. Crittenden et al. (2001) investigated the adherent capacity of the *Bifidobacterium* strains to adhere to potato starch and a few other starches. They found that the binding capacity was related to the surface area of the granules and was not affected by bile salt. Based on their results, they have proposed the use of potato starch for encapsulation technology.

The VTT Technical Research Center of Finland carried a study to encapsulate the lactic acid bacteria with potato starch. Large potato starch granules (50-100 µm) were enzymatically treated to obtain a porous structure and then used as carrier. During encapsulation, lactic acid bacteria adhere to the pores of the potato starch. With another coating of amylose, the lactic acid bacteria could survive at least 6 months at room temperature (Mattila-Sandholm et al. 2002).

1.3.4 Other wall materials

Chitosan is a linear polysaccharide essentially composed of ß (1-4) glucosamine units together with N-acetylglucosamine units to form a linear structure (Peniche et al. 2003).

The cross-link network is formed in the presence of anions or polyanions (Burgain et al. 2011). During encapsulation, it is preferably used as a second coating due to its low encapsulation efficiency (Mortazavian et al. 2008). Chitosan is commonly used along with alginate. Lee et al. (2004) tested the effect of three different chitosan coatings on alginate beads. They indicated that chitosan with high molecular weight showed higher survival of *L. bulgaricus* after exposure to simulated gastric and intestinal juice, compared to alginate alone as carrier.

Gellan gum and xanthan gum are used in combination to form beads during encapsulation. Gellan gum is a microbial polysaccharide derived from *Pseudomonas elodea*(Jansson et al. 1983). It has a tetrasaccharide repeating unit consisting of two ß-D-glucose, one ß-glururonic acid and one α-L-rhamnose residue (Grasdalen and Smidsrod 1987). Xanthan gum is a heteropolysaccharide consisting of two glucose units, two mannose units, and one glucuronic acid unit (Garcia-Ochoa et al. 2000). Gellan gum and xanthan gum used together as carriers show high resistance to acidic environments compared to alginate. Sun and Griffiths (2000) indicated that the viability of the encapsulated *bifidobacteria* decreased only 0.67 log CFU/mL, while the free cells dropped from 1.23×10^{9} CFU/mL to an undetectable level after 30 minutes of incubation at pH 2.5.

Κ-carrageenan is a natural polymer and is commonly used in the food industry (Burgain et al. 2011). The core material is added to a solution containing this polymer at 40-50°C. After the mixture is cooled down to room temperature, the gelation occurs. Particles are then stabilized by the addition of potassium ions (Krasaekoopt et al. 2003). Dinakar and Mistry (1994) indicated that the encapsulated *Bifidobacterium bifidum* maintained viability for as long as 24 weeks during cheddar cheese ripening.

1.4 Probiotics

Probiotics are defined as "live microbial supplements that beneficially affect the host by improving its intestinal microbial balance" (Fuller 1989). Health claims on probiotics include anti colon cancer properties, reduced risks of irritable bowel syndrome, and prevention of inflammatory bowel disease (Santosa et al. 2006; Wollowski et al. 2001). The probiotic effect mechanisms are attributed to the production of acid, or bacteriocins, reinforcement of body's natural defense, and competition with pathogens (Krasaekoopt et al. 2003). The probiotics obtained from food sources must survive passage through the upper gastrointestinal (GI) tract and function in the gut environment to achieve the defined health benefits (Saarela et al. 2000).

1.4.1 Lactobacillus acidophilus

Lactic acid bacteria (LAB) are a group of bacteria that are gram-positive, non-sporeforming, facultative aerobes, cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates (Sebastian et al. 2011). LAB used as probiotics prevent adherence, establishment, and replication of certain pathogens (Naidu et al. 1999). The main therapeutic and health benefits of *L. acidophilus* are that they enhance the immune system, and prevent intestinal infections, diarrheal disease, colon cancer and upper gastrointestinal tract diseases (Kailasapathy and Chin 2000). It has been reported that certain isolated strains of *Lactobacillus* influence the metabolic activity of the resident microflora in the human gut (Lee and Salminen 1995). Gilliland et al. (1985) reported that *L. acidophilus* had the ability to resist bile salt and decrease cholesterol levels. *L. acidophilus* is one of the most popular bacteria used as probiotics for human consumption (Amir Mortazavian 2007). In the United States, over 80% of the yoghurt in the market contains *L. acidophilus* (Gomes and Malcata 1999). However, Shah et al. (1995) indicated that *L. acidophilus* showed a constant decline in viable cells during storage in commercial yogurt.

The viability of *L. acidophilus* showed a 6-log CFU/mL reduction after 2 hours of incubation at pH 2.0. It also has shown a reduction from 10.2-log CFU/mL to 6.3-log CFU/mL after 4 hours of exposure to bile salt (Ding and Shah 2007). Schillinger (1999) isolated *L. acidophilus* from novel-type probiotic dairy products, and found that four out of eight mild yogurt contained less than 10^4 CFU/g at the end of storage (best before use

day). Therefore, encapsulation may provide the protection needed to retain cell viability of probiotics, such as *L. acidophilus,* when incorporated them into food system, especially acidic foods.

1.5 Encapsulation of probiotics

The ability of beneficial microorganisms to survive GI transit and multiply in the host strongly influences their probiotic benefits. The bacteria should be metabolically stable and active in the product, survive passage through the upper digestive tract in large numbers, and have beneficial effects when in the intestine of the host (Gilliland 1989). Adequate numbers of viable cells are required to be consumed in order to transfer the probiotic effects to consumers. It is suggested that the product should contain at least 10^5 active cells per gram of probiotics to provide the desired benefits (Kebary 1996). However, a low viability of probiotics in dairy products has been indicated by various reports (Gilliland and Speck 1977b; Klaver et al. 1993; Kneifel et al. 1993; Micanel et al. 1997; Phillips et al. 2006; Rybka and Kailasapathy 1995; Schillinger 1999; Shah and Lankaputhra 1997). Vinderola et al. (2000) reported that the viability of *Bifidobacterium*

and *L. acidophilus* in Argentinian yogurt were reduced by 1-4 log after 4 weeks of storage.

Encapsulation, therefore, could provide probiotics with a barrier to resist adverse food environments, since it has been widely used to protect microorganisms from environmental and physiological degradation (Corbo et al. 2011; Lim and Moss 1981). It has been proven that encapsulation increased the viability of microorganisms in both dairy products and the intestinal tract by simulated digestion. Table 1.3 shows successful examples of probiotic encapsulation using different methods with the ultimate goal of applying them in yogurt. Lee and Heo (2000) found that the death rate of *B. longum* decreased proportionally with increased bead size and alginate concentration after exposure to simulated gastric juice and bile salt solution. Chavarri et al. (2010) also found that the encapsulated *B. bifidum* showed significantly decreased death rate after exposure to simulated gastric conditions (pH 2.0, 2h) and bile solution (3%, 2h) when compared with free cells.

1.6 Release mechanism

To determine viability in encapsulated material, bacteria cells need to be released from the beads. When the wall material contains alginate, the cross-linking network can be broken by the removal of cation ions (Ferreira Almeida and Almeida 2004). To achieve

this, chelating agents such as ethylene glycol tetraacetic acid, lactate, citrate, phosphate or a high concentration of ions such as Na^{+} or Mg^{2+} can be used (Gombotz and Wee 2012). Sheu and Marshall (1993) tested the effect of pH of phosphate buffer ranging from 4.8-8.5 on the release of bacterial cells and found no significant differences among the pHs tested. They also found that the cells reached plateau at ten minute with phosphate buffer (0.05M, pH 4.8-8.5). Higher concentrations of phosphate buffer further shortens the release time to 5 minutes.

1.7 Physicochemical characterization of microparticles

1.7.1 Particle size distribution

Laser diffraction is commonly used to determine the particle size of encapsulated materials since it is efficient and repeatable. This method also characterizes volume size distribution (Merkus 2009). Hyndman et al. (1993) tested the mean diameter of the microparticles after encapsulation of *Lactococcus lactis* in gelatin using a particle size analyzer. Brauss et al. (1999) determined the particle size of casein-fat droplets in yogurt using the same method to reveal the connection between fat content and flavor release.

1.7.2 Morphology of microparticles

It is important to study the morphology of microparticles to check the aggregation and damage on the surfaces of particles. When adopting the emulsion method, the morphology of the microparticles are easily affected by processing changes (speed of adding the calcium chloride, mixing speed, etc.) (Sheu and Marshall 1993). Optical and electron microscopy are widely used to observe the size and shape of particles that are 0.2 μm or larger. Scanning electronic microscopy (SEM) is commonly used to study the surface morphology, such as pores and internal structures by viewing cross-sections of the beads (Zhang et al. 2010).

1.8 Objectives

Microencapsulation technology has been extensively used since its first appearance, with the most well-developed techniques being used by the pharmaceutical industry. In the food industry, research is now focusing on flavor delivery, while the area of encapsulating functional ingredients, which is a huge market, is still waiting for expansion. Probiotics as a functional ingredient has attracted much attention with recent consumer trends that promote consumption of more natural and beneficial diets. Therefore, there is a good opportunity to explore new encapsulation approaches of probiotics. Some fundamental research on encapsulation of *L. acidophilus* encapsulation has been accomplished; however, survival is normally low and inconsistent, and

applications in food systems are not well-studied. Therefore, the goal of this research is to encapsulate *Lactobacillus acidophilus* DDS 1-10 in food grade wall material with both emulsion and extrusion method, to increase the survivability of the probiotics and to incorporate them into food systems.

The specific objectives are to:

1. Select the best alginate-starch mixture/composition to encapsulate *Lactobacillus acidophilus* DDS 1-10.
2. Optimize processing conditions to prepare encapsulated probiotics with maximum encapsulation efficiency with both emulsion and extrusion methods.
3. Evaluate and maximize the viability of the encapsulated cells.
4. Verify the stability of particles in selected conditions (pH, bile salt, and temperature) with particles obtained from both emulsion and extrusion methods.
5. Incorporate the particles obtained from both emulsion and extrusion method and investigate their behavior during storage.

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Table 1.1 Comparison of microencapsulation techniques

Technique	Advantage	Disadvantage	Reference
Emulsion	Easy to scale up Mild process	Non-uniform particle size Expensive	(Krasaekoopt et al. 2003)
Spray drying	Economical Well developed	Limited wall materials High temperature	(Gouin 2004)
Spray chilling	Temperature controlled Inexpensive	Fat as wall material Special storage condition	(Pedroso et al. 2012)
Extrusion	Simple process Uniform particle size	Difficult to scale up Limited wall materials	(Gouin 2004)

Table 1.2 Major steps for encapsulation technique

Technique	Major process
Emulsion	1. Cell is dispersed in the wall material 2. Mixture is added dropwidse into oil to form emulsion 3. Mixture is then solidified to form particles
Spray drying	1. Preparation of the wall material solutions with cells 2. Atomization of the mixture into spray 3. Drying of the spray 4. Separation of the dry particles
Extrusion	1. Preparation of wall material solution with cells 2. Extrusion of the mixture through syringe into the hardening solution 3. Formation of the particles
Spray chilling	1. Preparation of the wall material solutions with cells 2. Contact with cooled air 3. Solidification of the particles 4. Separation of the dry particles

Table 1.3 applications of encapsulating probiotics on yogurt with different techniques

Technique	Wall material	Core material	Reference
Emulsion	Κ-carrageenan	*B. longum*	(Adhikari et al. 2003)
Spray drying	85% milk fat 5-15% whey protein	*B. breve*	(Picot and Lacroix 2004)
Extrusion	2% sodium alginate	*L. acidophilus*	Krasaekoopt et al. (2006)
Spray chilling	Palm oil	*L.acidophilus*	(Pedroso et al. 2012)

Chapter 2 Materials and methods

2.1 *L. acidophilus*

All the glassware and solutions used for the experiments were autoclaved at 121° C for 15 min. The probiotic strain *Lactobacillus acidophilus* DDS 1-10 (*L. acidophilus*) was obtained from a commercial probiotic supplier (Nebraska Cultures, Walnut creek, CA). From a frozen stock culture, an aliquot of 100 μL *L. acidophilus* was streaked on De Man Rogosa Sharp agar (MRS, Acumedia, Neogen Corporation, Lansing, MI). Plates were then incubated anaerobically (Mitsubishi, Tokyo, Japan) for 24 hours at 37°C. Gram stain was then performed on single colonies. Colonies with the expected morphology were transferred and grown overnight at 37 °C in MRS broth (Acumedia, Neogen Corporation, Lansing, MI). Sterile glycerol (7%, v/v) was added to the broth and 1 mL aliquots were stored at -80 °C for future use.

2.2 Preparation of cell suspension

Cultures used for encapsulation were grown for 24 hours by adding 100 μL of a stock culture of *L. acidophilus* stock culture into 9 mL MRS broth and incubating at 37°C anaerobically. They were then transferred to 500 mL MRS broth and incubated for 48 hours at 37°C anaerobically. The optical density of MRS broth (with cells) were measured at 650 nm using a Spectronic 20D+ spectrophotometer (Spectronic

Instruments, Thermo Fisher Scientific, Waltham, MA), with MRS broth (without cells) as a blank. Based on the reading, the volume of the MRS broth (with cells) used were determined by comparison with growth curves previously obtained for *L. acidophilus* (Figure 2.1). Cell pellets were then harvested using a Sorvall Legend XTR centrifuge (Thermo Scientific, Hampton, NH) at 2780×g for 8 minutes. Cell pellets were washed with sterile water twice and decanted under the same centrifugation conditions. The cell pellets were then resuspended in 10 mL phosphate buffer solution (PBS) to obtain a final cell counts of 6×10^8 CFU/mL.

2.3 Preparation of encapsulation matrix

Sodium alginate (Kimica Corp., Chuo-Ku, TY, Japan) of grade IL-1, potato starch (Penford Food Ingredients Company, Centennial, CO), and type 4 resistant starch (RS4) (Fibersym® RW, MGP Ingredients, Atchison, KS) were obtained from commercial sources. Different combinations of matrix materials were tested. The total solid content for all combinations was always maintained at 5% (w/v). Within this 5%, alginate content ranged from 1.6% to 4.4% (w/v), and the ratio of potato starch and RS4 was fixed at 2:1. All the combinations were displayed in Table 2.1. Thermal properties of potato starch and RS4 were measured using differential scanning calorimetry (DSC). Based on the thermal data, the mixture was heated to 85° C in preparation for use.

2.4 Preparation of particles using the emulsion method

The cell suspension was first mixed well with encapsulating material (1:5). The mixture was then pumped through a rubber tube into Hyvee soybean oil (Hyvee Inc., West Des Moines, IA) using a 101U/R pump (Watson-Marlow, Wilmington, MA) at 1.5 mL/min. A gauge-15 needle was connected at the end of the tube to drip the mixture into 250 mL soybean oil containing Tween 80 (0.2%, v/v) (Fisher Scientific, Hampton, NH) which was stirred at 450 rpm by a magnetic stirrer. A water/oil emulsion was formed.

After the dripping was completed, 10 parts of calcium chloride (0.1M) (Aldrich Chemical Company Inc., St. Louis, MO) was added along the side of the beaker to the emulsion fast but gently (>20 mL/s) thus breaking the water/oil emulsion. The mixture was agitated for 30 min to allow the particles to form. After the mixing was finished, the mixture was left to stand for 60 min. During this period, phase separation occurred as the particles with encapsulated *L. acidophilus* started to precipitate and fall to the bottom.

To collect the particles, the oil phase at the top was drained and the clear solution at the bottom was centrifuged (350g×g, 15 min). The particles were washed twice with sterile water and decanted under the same centrifugation conditions and then collected for use. The picture of the particles observed under microscopy is shown in Figure 2.2

2.5 Preparation of particles using extrusion method

The encapsulation matrix material and the cell suspension mixture (1:5) was pumped in the same way as described for the emulsion method using a needle (gauge 25) directly into calcium chloride (0.1M), which was stirred at 200 rpm by a magnetic stirrer. The distance between the needle and the calcium chloride solution was fixed at 3cm. Particles were immediately formed when the mixture came in contact with the calcium chloride solution. The solution was left to mix for 30 to 120 min to allow for the hardening of the particles. The particles were then collected by filtration using cheesecloth which was sterilized in boiling water for 12 min ahead of use. The particles were then washed with sterile water and collected for the following tests. The picture of the particles observed is shown in Figure 2.3.

2.6 Analysis of physical properties of particles

Particle size analysis of the particles from emulsion method was performed using a Malvern Mastersizer 3000 laser diffraction particle size analyzer, equipped with a Hydro MV wet dispersion unit (Malvern instruments Ltd., Malvern, Worcestershire, UK). Analytical parameters were set per the manufacturer's database as follows: refractive index of 1.0, density of 1.0 g/cm^3, mixing speed of 2300 rpm, feed rate of 50%, and absorption index of 0.10. The particles were added into the wet dispersion cell until the obscuration reading became steady (within the limit of 0.1-20%). Data was analyzed using Malvern software (Version 2.20, Malvern instrument Ltd., Malvern, UK).

The size of the particles from extrusion method was measured using a caliper (Mitutoyo, Aurora, IL). A single particle was placed between the jaws of the calipers to measure its diameter. Measurements were performed in triplicate.

2.7 Release and enumeration of the encapsulated cells

Encapsulated cells were released from the particles before enumerating. For the particles obtained with the emulsion method, 1 g of particles were added into 9 mL of potassium phosphate buffer (0.1M, pH 7.0). Then it was macerated using a tissue homogenizer. For the particles obtained with the extrusion method, 1 g of particles were added into a stomacher bag with 9 mL of potassium phosphate buffer (0.1M, pH 7.0). It was then blended in stomacher 400 (Seward, UK) gently for 10 min. An aliquot of 1 mL of the suspension obtained from both methods was serialy diluted for plating. Released cells were enumerated by spreading 100 µL of the diluted suspension into MRS plates and incubating for 48 hours at 37°C anaerobically.

2.8 Optimization of encapsulation matrix material composition

The optimum matrix material composition was determined by comparing the cell survivability of encapsulated cells with free cells after incubation in pH 2.0 MRS broth at 37°C for 3 hours. MRS broth was adjusted to pH 2.0 using 3.0 M hydrochloric acid (Sigma Aldrich, St. Louis, MO). An aliquot of 9 mL of adjusted broths were added into

sterile 15 mL centrifuge tubes. Either 1 g of encapsulated particles or 1 mL of washed cell suspension were added into the prepared tubes tempered at 37°C and vortexed for complete dispersion. The tubes were then incubated at 37°C. One tube was immediately sampled, and then another one after 3 hours for cell enumeration. The samplings were performed in triplicate.

The harvesting of the particles from the emulsion method, or the extrusion method, and free cells used in this experiment were performed as follows. As already mentioned, to harvest the particles from the emulsion method, each tube was centrifuged (350×g, 8 min), the supernatant decanted, and the remaining pellets washed with sterile water twice with water removed under the same centrifugation conditions. For the particles from the extrusion method, they were harvested by filtration using cheesecloth and washed with sterile water. To harvest the 48 hour cultured free cells, each tube was centrifuged (2780×g, 8 min), the supernatant poured out, and the remaining pellet washed twice with sterile water.

At time zero and after incubation time, all enumerations were performed as described under section 2.7, for encapsulated cells. The tubes with free cells were centrifuged (2780×g, 8 min) to recover a pellet that was resuspended in 1 mL of PBS. Aliquots of 0.1 mL were then used for serial dilutions. Viable counts were enumerated by spread plating on MRS agar after anaerobic incubation for 48 hours at 37°C.

2.9 Acid and bile salt resistance of optimized particles

Particles produced by the emulsion and the extrusion method using the optimum composition of the encapsulation matrix material were tested for acid and bile salt resistance and compared to the survivability of free cells. MRS broth without addition of acid or bile salt was used as a control. For the treatments, MRS broth was adjusted to pH of 2.0, 4.0, and 7.0 using 3.0 M hydrochloric acid, and 0.5, 1, and 2% bile salt (Sigma Aldrich, St. Louis, MO). The adjusted broths were added into sterile 15 mL centrifuge tubes in 9-mL aliquots. Either 1 g of encapsulated particles or 1 mL of washed cell suspension were added into the prepared tubes tempered at 37°C and vortexed for complete dispersion. The tubes were then incubated at 37°C. One tube was immediately sampled, and then another at 6, 15, and 24 h for a total of four tubes. During each time point, all tubes, sampled or not sampled, were shaken gently. The samplings were performed in triplicates and enumeration of viable cells was performed using the same procedures described in Sections 2.7 and 2.8.

2.10 Resistance of optimized particles to temperature

Particles produced by the emulsion and the extrusion method using the optimized encapsulation material were tested for their viability at 25, 37, and 50°C and compared to free cells. Either 1 g of encapsulated particles or 1mL of *L. acidophilus* suspension were added into tubes containing 9 mL of MRS broth. The tubes were incubated at 25, 37, and 50°C for 24 hours. Samplings were performed initially and after 24 hours using the same harvest, release and plating procedures described in Sections 2.7 and 2.8.

2.11 Survivability test of optimized particles

Based on results obtained with the pH experiments, the hardening time of the extrusion method was determined to be 120 min. Particles produced with the optimized composition for encapsulation using the extrusion method were tested under different conditions for a week. MRS broth without addition of acid was used as control. Tubes with 9 mL MRS broth adjusted to pH of 4.0, 4.5, and 5.0 using 3.0 M hydrochloric acid were also prepared. Each tube was added with 1 g of encapsulated particles. Enough tubes were prepared to allow the removal of one tube for each pH at each sampling time. Samplings were performed initially and then daily, for 7 days at 7 and 25°C, in triplicate. Encapsulated cells were enumerated as described in sections 2.7 and 2.8 on MRS plates after incubation anaerobically for 48 hours at 37°C. The limit of detection of the method was <100 CFU.

2.12 Shelf life test of optimized particles

Commercial products tested were Hyvee 2% reduced milk (Hyvee, West Des Moines, IA), V8 acai juice (Campbell's Camden, NJ). They were pasteurized in 80°C water bath for 12 minutes to kill all the vegetative cells present in the products. They were then put into ice for cooling down. Particles produced using the optimized matrix composition from the extrusion method were added to the pasteurized products (1 gram/10 mL). Samplings were performed initially and then after 4, 8, 12, 16 days. Encapsulated cells were enumerated as described in sections 2.7 and 2.8 on MRS plates after incubation anaerobically for 48 hours at 37°C.

2.13 Statistical analysis

This study was performed using a completely random design (CRD). Analysis of variance and mean differences were analyzed using SAS 9.4 (SAS Inc., Cary, NC) and JMP 10.0.0 (SAS Institute Inc., Cary, NC). All tests were conducted at a 5% level of significance.

Table 2.1. Selected compositions of encapsulating material used for the extrusion and enumeration methods.

Alginate(%,w/v)	Potato starch: RS4 Ratio
1.6	2:1
3	2:1
4.4	2:1

*Total starch+ alginate= 5% (w/v). All samples were prepared in triplicate.

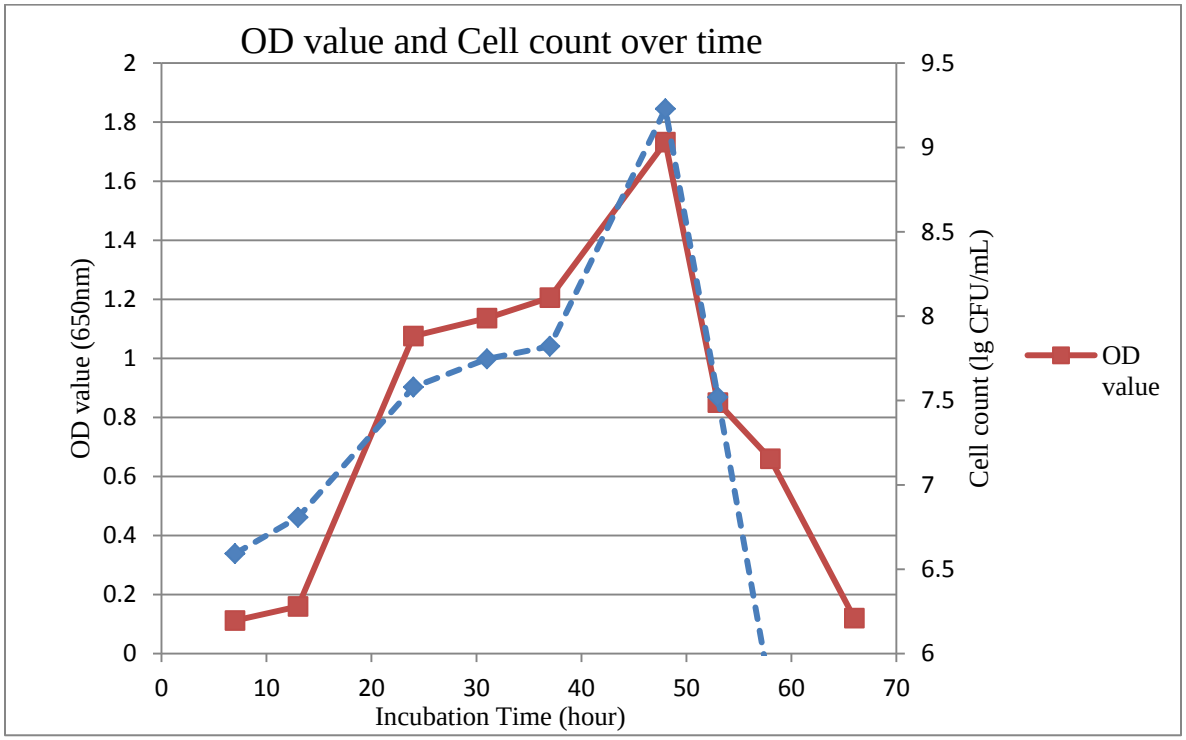

Figure 2.1 Absorbance of MRS broth and *L. acidophilus* growth curve.

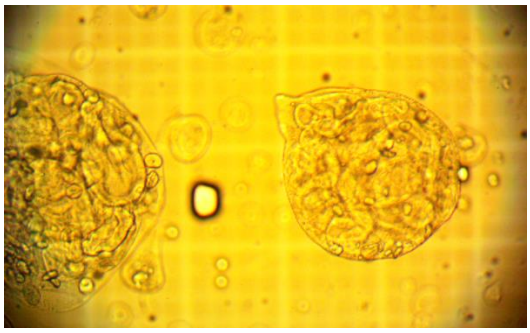

Figure 2.2 Particles obtained using emulsion method under microscopy.

Figure 2.3 Particles obtained using extrusion method.

Chapter 3 Results and discussion

3.1 Emulsion method

3.1.1 Choosing wall material composition

Based on preliminary results (appendix A and B), selected wall materials and compositions were chosen to study which compositions would provide the most protection to the probiotic cells. For the experiments, alginate levels ranged from 1.6 to 4.4%, while the potato starch and RS4 ratio was fixed at 2. Encapsulated particles were obtained with different wall material compositions with an emulsion method. Encapsulated particles were incubated in MRS broth at pH 2 for 3 hours and tested for the viability of the probiotic. The wall material compositions and the results obtained for cell survivability are presented in Table 3.1.

Table 3.1 Bacterial cell reduction in encapsulated *L. acidophilus* when particles were obtained by the emulsion method after 3 hours incubation in MRS broth at pH 2.

Wall material composition		Reduction (log CFU/g)
Alginate (%)	Potato starch: RS4	Mean (SD)
1.6	2	3.08 (0.05)[a]
3	2	2.78 (0.09)[ab]
4.4	2	2.31 (0.38)[b]
Free Cells		2.07 (0.25)[b]

*Different letters within the same column indicate difference based on $p<0.05$.
*Mean and standard deviation was obtained with triplicate samples.

The results showed that the least amount of bacterial reduction was achieved with either free cells, 3.0 or 4.4% alginate, with no significant difference between the three

compositions ($p>0.05$). However, the survival of the bacterial cells encapsulated with 3.0% alginate was not different than the reduction observed in particles made with 1.6% alginate. Therefore, from an economical stand point, the wall material composition of 1.6% alginate and potato starch: RS4 ratio of 2 was chosen for further tests.

3.1.2 Physical properties of encapsulated particles

The particle size distribution of encapsulated particles was determined for different wall material compositions, with alginate varying from 1.6 to 4.4% and the ratio between potato starch and RS4 fixed at 2. Results in Figure 3.1 show that particles prepared with 3.0 and 4.4% alginate showed one peak, while particles with 1.6% alginate showed two peaks. Based on the results, particles with higher alginate level were larger in size. At an alginate level of 1.6%, over 90% of the particles had a mean size of 666µm, while at an alginate level of 3.0%, over 90% of the particles had a mean size of 586µm. Finally, at an alginate level of 4.4%, over 90% of the particles had a mean size of 586µm.

Previous research had reported results regarding levels of alginate and particle size distribution. Sheu and Marshall (1993) used light microscopy to measure the particle size of encapsulated particles. They reported that the particles containing lactobacilli ranged in size from 5-100 µm with means of 25-35µm. When the alginate increased from 0.5 to

4.5%, the mean particle size increased from 15.4 to 22.1µm. Adhikari et al. (2003) measured particles containing *B. longum* B6 with laser diffractometry and found that the particle size was 235.8±25.6 µm, with 91% in the size range of 22 to 350µm.

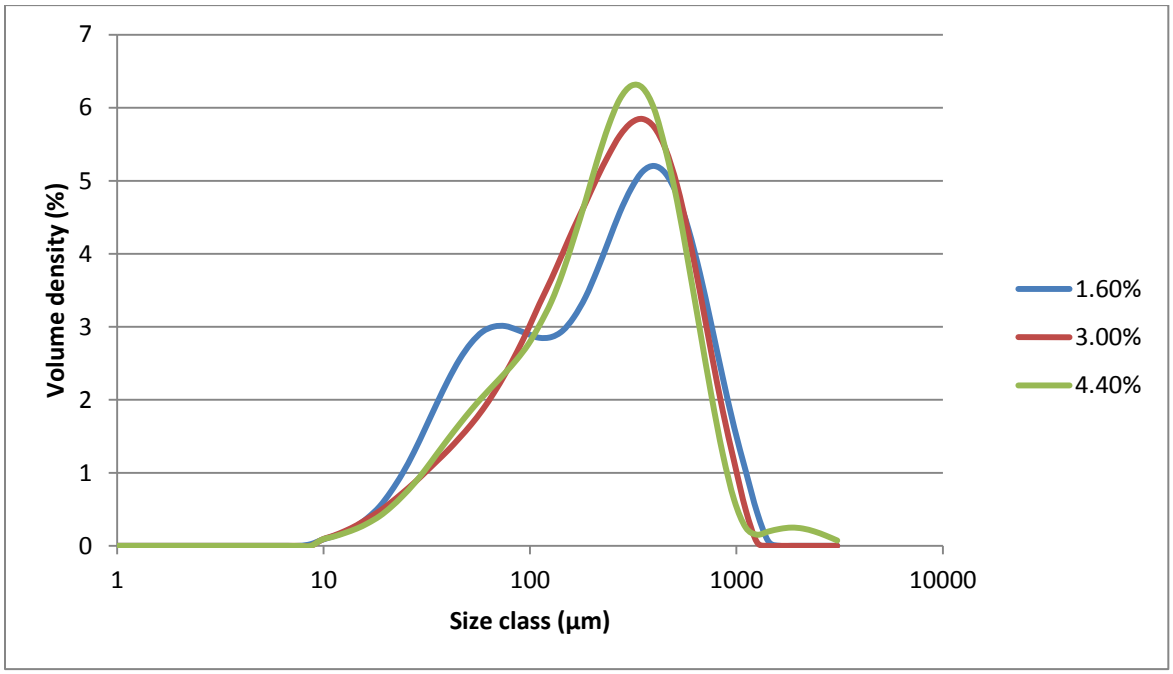

Figure 3.1 Particle size distribution of particles produced by the emulsion method (1.6%, 3.0%, 4.4%- alginate level; potato starch: RS4=2).

3.1.3 Challenging encapsulated L. acidophilus with pH, bile salt and temperature

Encapsulated cells were obtained using the optimized composition for the wall material. The survivability of *L. acidophilus* in the particles after treatment at different pH,

different concentrations of bile salt and temperature was compared to free cells. To test the performance of the encapsulated and free cells at different pH values, they were incubated in MRS broth adjusted to pH 2, 4, and 7. Cell viability was tested after 6, 15, and 24 hours. Viability at pH 2 and 4 appeared to decrease as the incubation period increased (Figure 3.2). However at pH 7, *L. acidophilus* showed growth over the incubation period, with both encapsulated and free cells. Encapsulated cells increased 1.3 log CFU/mL more than free cells. At acidic pH values, encapsulated cells showed a reduction of 1.18 log CFU/g after 24h of incubation at pH 4, while free cells showed a decrease of 2.91 log CFU/mL. Incubation of encapsulated and free cells at pH 2 showed that both types of cells did not survive after 24 hours of incubation.

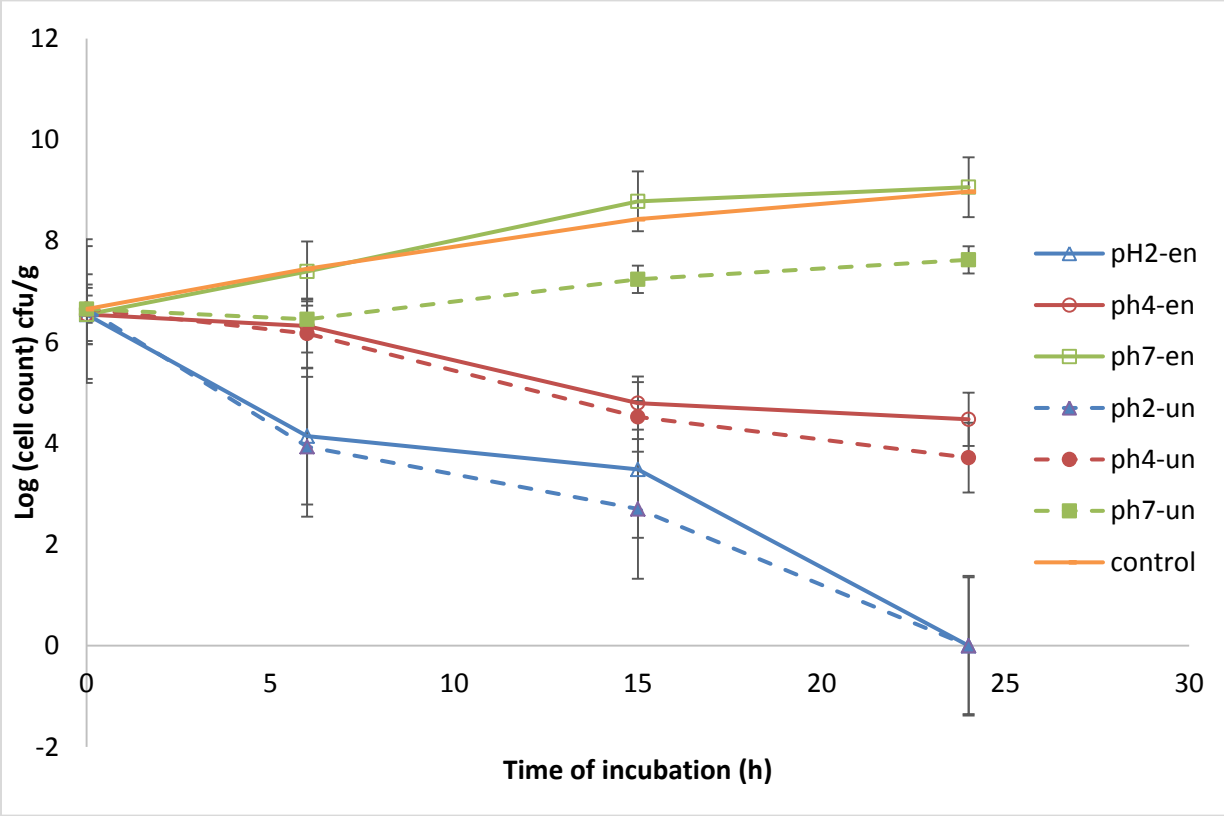

Figure 3.2 Survivability of encapsulated and free *L. acidophilus* over 24 hours of incubation at different pH values.

These results show that encapsulation by emulsion has only a minimal effect in protecting *L. acidophilus* from the effects of low pH. Encapsulation somewhat protected cells at pH 4, but failed to protect the cells at pH 2. These results are in accordance with previous published information. Rao et al. (1989), Hansen et al. (2002), Sultana et al. (2000), Koo et al. (2001) reported that encapsulation did not provide protection against acidic conditions as low as pH 2. However, Sultana et al. (2000) tested the acid tolerance of encapsulated particles at pH 4 after incubation for 3 hours and found 1.71 log CFU/g reduction while the free cells showed almost a 5 log decrease.

Survival of encapsulated cells and free cells was also tested after 6 hours of incubation in MRS broth containing bile salt (0.5, 1.0, and 2.0%). Results shown in Table 3.2 suggest that encapsulation may have provided some protection against bile salt; however statistical analysis showed no difference between reductions obtained with encapsulated and free cells. The encapsulated cells showed a 0.41 log reduction when exposed to 0.5% bile salt broth, while the free cells decreased by 1.36 log. The results from 1.0% and 2.0% bile salt broth showed similar trends, where encapsulated cells were reduced by about 0.70 log and free cells by 1.70 log.

Table 3.2 Reduction in bacterial counts of encapsulated and free *L. acidophilus* over 6 hours of incubation in bile salt at different levels (0.5, 1.0, and 2.0%).

Bile salt (%)	Encapsulated (log CFU/g)			Free (log CFU/mL)		
	Initial	6 hours	Reduction	Initial	6 hours	Reduction
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
0.5	6.54 (0.54)	6.13 (0.71)	0.41(0.20)^{b}	6.65 (0.18)	5.29 (0.21)	1.35 (0.26)^{ab}
1.0	6.54 (0.54)	5.79 (0.67)	0.75(0.34)^{ab}	6.65 (0.18)	4.95 (0.31)	1.69 (0.37)^{a}
2.0	6.54 (0.54)	5.85 (0.79)	0.68(0.75)^{ab}	6.65 (0.18)	4.89 (0.34)	1.75 (0.24)^{a}

*Different letters within the same row indicate difference based on $p<0.05$.

* Mean and standard deviation were obtained from triplicate samples.

Survival of encapsulated and free cells at different temperatures was also tested after incubation in MRS broth at 25°C for 24 hours (Table 3.3), at 37°C for 24 hours (Table 3.4), and at 50 °C for 3 hours (Table 3.5). Cell counts were determined either initially and after incubation periods or at set intervals. These results show that none of the temperature was detrimental to either encapsulated or free cells. Actually, bacterial growth was observed in encapsulated and free cells when incubated at 37°C. The cell count of encapsulated and free cells remained stable at 25°C and 50°C. Ding and Shah (2007) found that encapsulated *L. acidophilus* cells obtained from emulsion method died after exposure to 65°C for up to1 hour, indicating that 65°C was the lethal temperature for this organism and encapsulation did not improve heat tolerance.

Table 3.3 Survival of encapsulated and free *L. acidophilus* after incubation over 24 hours incubation in MRS broth at 25°C.

	Bacterial count (log CFU/g)		Bacterial reduction (log CFU/g)
	Initial	3 hours	
	Mean (SD)	Mean (SD)	Mean (SD)
Encapsulated	6.54 (0.54)	6.85 (0.76)	-0.18 (0.27)[a]
Free	6.65 (0.18)	6.49 (0.22)	0.15 (0.12)[a]

*Different letters within the same column indicate difference based on $p<0.05$.
* Mean and standard deviation was obtained with triplicate samples.

Table 3.4 Survival of encapsulated and free *L. acidophilus* over 24 hours incubation in MRS broth at 37°C (log CFU/g).

	Bacterial counts (log CFU/g)				Bacterial reduction (log CFU/g)
	Initial	6 hour	15 hour	24 hour	
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Encapsulated	6.54 (0.54)	7.58 (0.15)	8.14 (0.80)	8.52 (1.08)	-1.97 (1.62)[a]
Free	6.65 (0.18)	7.45 (0.26)	8.42 (0.30)	8.97 (0.53)	-2.29 (0.63)[a]

*Different letters within the same column indicate difference based on $p<0.05$.
* Mean and standard deviation was obtained with triplicate samples.

Table 3.5 Survival of encapsulated and free *L. acidophilus* over 3 hours incubation in MRS broth at 50°C (log CFU/g)

	Initial	3 hours	Bacterial reduction
	Mean (SD)	Mean (SD)	Mean (SD)
Encapsulated	6.54 (0.54)	6.40 (0.69)	0.14 (0.31)[a]
Free	6.65 (0.18)	6.17 (0.14)	0.46 (0.30)[a]

*Different letters within the same column indicate difference based on $p<0.05$.
* Mean and standard deviation was obtained with triplicate samples.

Based on the results obtained and lack of evidence that encapsulation by emulsion was efficient in promoting cell survivability at low pH, and a only a limited effect impacting against bile salts was observed, this method of encapsulation was not further studied.

3.2 *L. acidophilus* encapsulated by the extrusion method

3.2.1 Wall material composition

Based on preliminary experiments, selected wall materials and compositions were chosen that would provide the most protection to the probiotic cells. For these experiments, alginate levels ranged from 1.6 to 4.4%, while the ratio of potato starch and RS4 was fixed at 2. Encapsulated particles of different wall material compositions were obtained using the extrusion method. Viability of bacterial cells was tested initially and after 3 hours of incubation in MRS broth at pH 2. The wall material compositions and the results obtained for cell survivability are presented in Table 3.6.

Table 3.6 Bacterial cell reduction in encapsulated *L. acidophilus* after 3 hours incubation in MRS broth at pH 2 when particles were obtained by the extrusion method.

Encapsulated cells		Bacterial reduction (log CFU/g)
Alginate (%)	Potato starch: RS4	Mean (SD)
1.6	2:1	0.29 (0.06)[b]
3.0	2:1	0.08 (0.09)[bc]
4.4	2:1	0.02 (0.12)[c]
Free cell		2.07 (0.25)[a]

*Different letters within the same column indicate difference based on $p<0.05$.
* Mean and standard deviation was obtained with triplicate samples.

These results showed that there was a significant difference in survivability between encapsulated and free cells ($p<0.05$), indicating that encapsulation by extrusion could be beneficial. Among alginate levels tested there was no difference between 1.6% and 3.0%. However, there was a significant difference between 4.4% and 1.6% level of alginate. Based on these results, to obtain the most protection by encapsulation, 4.4% of alginate and 2:1 ratio of potato starch: RS 4 were chosen as the optimum wall material composition.

3.2.2 Particle size

The size of the particles encapsulated by the extrusion method was measured and the results are shown in Table 3.7. The results indicated that the mean particle diameter ranged from 1.22 to 2.61mm when alginate concentration ranged from 1.6 to 4.4%. It also indicated that the particle mean diameter would increase with increasing alginate concentration. However, no statistical difference was observed among the values measured.

The size of the particles produced by extrusion are influenced by factors such as concentration of the alginate solution, distance between the syringe and hardening solution, and the size of the needle (Smidsrod and Skjakbraek 1990). By comparing results with published research done with same the syringe gauge and alginate solution used here, similar results were found. Lee and Heo (2000) reported obtaining particles with diameters of about 1.03, 1.75, 2.62 mm containing 2, 3, 4% sodium alginate, respectively, which also increased with higher alginate concentration.

Table 3.7 Particle size (mean diameter) of the encapsulated particles from extrusion.

Wall material composition		Particle size (mm)
Alginate (%)	Potato starch: RS4	Mean (SD)
1.6	2	1.22 (0.01)^{ab}
3	2	2.02 (0.01)^{a}
4.4	2	2.61 (0.02)^{a}

*Different letters within the same column indicate difference based on $p<0.05$.
* Mean and standard deviation was obtained with triplicate samples.

3.2.3 Hardening time test

Particles were made with the optimized wall material (4.4% alginate, potato starch: RS4=2) and hardened for different time periods (30, 60, 90, and 120 min). Particles were then tested for the survivability of encapsulated cells by incubating in MRS broth at pH 2 for 3 hours. The results shown in Table 3.8 indicated that as the hardening time increased, the survival of bacterial cells in the particles started to increase as well. The viability of

the encapsulated cells in pH 2 decreased by 1.74 log with a 30 min hardening time which was a significantly higher difference in reduction than the other ones observed with longer hardening times ($p<0.05$). The reduction in all viability at pH 2 was 0.99, 0.55, 0.078 log CFU/g, for a hardening time of 60, 90, 120 min, respectively. Based on the statistical analysis, a hardening time of 120 min was chosen as the optimum processing parameter for the production of the particles.

The results observed here are in accordance with previous research. Chandramouli et al. (2004) tested the hardening time when encapsulating *L. acidophilus* CSCC 2400 with alginate, and found a significant increase in survival of bacteria cells when hardening the particles for 30 min or more in 0.1M calcium chloride solutions. They also indicated that hardening for 8 hours or more had no effect on increasing viability of the encapsulated cells. Also, hardening particles for 12 hours at 4°C did not significantly influence viability of encapsulated cells.

Table 3.8 Effect of hardening time on survival of encapsulated and free *L. acidophilus* after incubation in MRS broth at pH 2 for 3 hours.

Hardening time (min)	Initial (log CFU/g)	3 hours (log CFU/g)	Reduction (log CFU/g)
	Mean (SD)	Mean (SD)	Mean (SD)
30	7.43 (0.35)	5.48 (0.45)	1.74 (0.11)[a]
60	7.91 (0.12)	6.92 (0.31)	0.99 (0.11)[b]
90	8.04 (0.50)	7.49 (0.38)	0.55 (0.11)[bc]
120	8.63 (0.12)	8.55 (0.03)	0.02 (0.11)[c]

*Different letters within the same column indicate difference based on $p<0.05$.
* Mean and standard deviation was obtained with triplicate samples.

3.2.4 Challenging encapsulated L. acidophilus with pH and bile salt test

Survival of encapsulated and free cells after incubation in MRS broth at pH 2.0, 4.0, 4.5, and 5.0 for 24 hours was evaluated and the results are presented in Figure 3.3. Both encapsulated and free cells were stable at pH 4, 4.5, and 5.0 with no significant differences between reductions obtained for encapsulated cells and free cells ($p>0.05$). However, there was a significant difference between the survival of encapsulated and free cells after incubation in pH 2 for 24 hours ($p<0.05$). Encapsulation remarkably improved the acid tolerance of particles at pH 2.

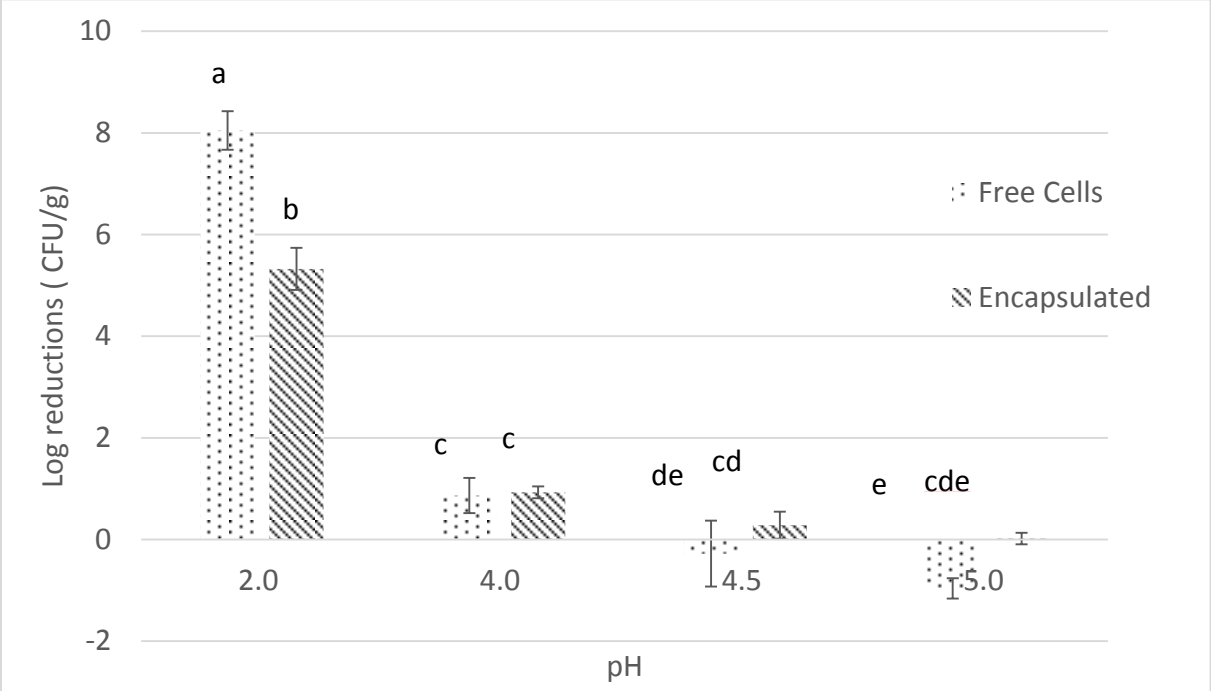

Figure 3.3 Effect of pH on survival of encapsulated cells (cell reduction) after incubation in MRS broth at pH 2.0, 4.0, 4.5, and 5.0 for 24 hours.

Encapsulated and free cells were also exposed to different concentrations of bile salt (0.5, 1.0, and 2.0%) and were tested after 6 hours of incubation. The results in Table 3.9 indicate that encapsulation provided protection for the cells, since the survival of encapsulated cells was significantly better ($p<0.05$) than that of the free cells. Encapsulated *L. acidophilus* with initial cell load of 8.86 log CFU/g, showed only about 0.5 log reduction for all bile salt levels tested. The free cells however showed reduction by 1.36-, 1.70-, and 1.76-log for 0.5, 1.0, 2.0% bile salt, respectively.

Table 3.9 Bacterial counts of encapsulated and free *L. acidophilus* over 6 hours of incubation in bile salt at different levels (0.5, 1.0, and 2.0%).

Bile salt (%)	Encapsulated (log CFU/g)			Free cell (log CFU/Ml)		
	Initial	6 hours	Reduction	Initial	6 hours	Reduction
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
0.5	8.86 (0.14)	8.36 (0.16)	0.50 (0.14)[b]	6.65 (0.18)	5.29 (0.21)	1.36 (0.14)[a]
1.0	8.86 (0.14)	8.27 (0.11)	0.59 (0.14)[b]	6.65 (0.18)	4.95 (0.31)	1.70 (0.14)[a]
2.0	8.86 (0.14)	8.35 (0.02)	0.51 (0.14)[b]	6.65 (0.18)	4.89 (0.34)	1.76 (0.14)[a]

*Different letters within the same column indicate difference based on $p<0.05$.

* Mean and standard deviation was obtained with triplicate samples.

The results show that encapsulation by the extrusion method gives good protection against bile salt. The results related to improved survivability of encapsulated cells treated with bile salt obtained in this study are in accordance with previous published research (Lee and Heo 2000; Sabikhi et al. 2010). Murata et al. (1999) found out that there was a 1- and 0.8-log decrease of free *L. acidophilus* at 1.0% bile salt, while for encapsulated cells there was a 0.5 and 0.3 log decrease, for strains CSCC 2400 and CSCC 2409 respectively, under the same conditions after 6 hours. It was reported that certain stains of *L. acidophilus* had the ability of conjugating bile acid under anaerobic environment (Gilliland and Speck 1977a). Kim et al. (2008) studied strain of *L. cidophilus* ATCC 43121, which was proved by Gilliland and Walker (1990) to be significantly better in bile tolerance than other cultures. Their results showed that encapsulated cells survived better in 0.5% bile salt and the free cells decreased from 6.85-log to 5.96-log, while the encapsulated cells were not affected.

3.2.5 Challenging survival of encapsulated L. acidophilus under different pH and temperature conditions for 7 days

Because the results from the pH experiment were limited to 24 hours, the exposure time to low pH was extended to 7 days to evaluate the long term effect of pH on encapsulated cells. Encapsulated and free cells were incubated in MRS broth at pH 4.0, 4.5, 5.0 at 7 and 25 °C, and were tested every day for 7 days (Figures 3.5 and 3.6). The viability of encapsulated cells remained at 8 logs for the 3 different pHs at 7°C. Meanwhile, the free cells showed a slight decrease of 2.1-, 1.3-, 1.3-log CFU/mL for pH 4.0, 4.5, 5.0, respectively, over time. There was significant difference between encapsulated and free cells at pH 4 when incubated at 7°C ($p<0.05$). When incubation at 25°C was evaluated, the encapsulated cells incubated at pH 4.5 and 5.0 remained stable, showing a slight decrease of 1.7-and 0.7-log respectively, which was not significantly different from the results obtained with free cells ($p>0.05$). Encapsulated cells incubated at pH 4.0 began to die at day 2 and by day 7 a reduction of 5.1 log CFU/g was observed. Despite the 5.0 log reduction, encapsulation was significant in protecting *L. acidophilus* from the effect of pH 4.0 since free cells were completely inactivate by day 6 ($p<0.05$).

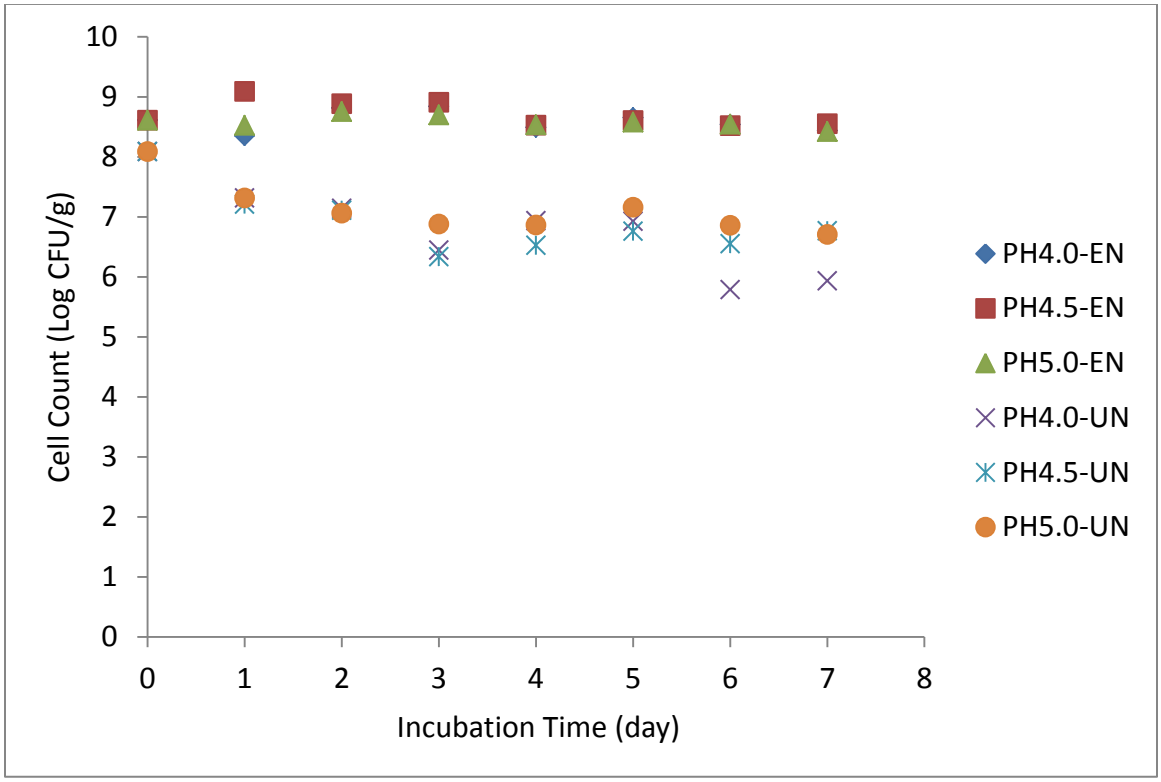

Figure 3.4 Survival of encapsulated and free cells during incubation for 7 days at 7°C.

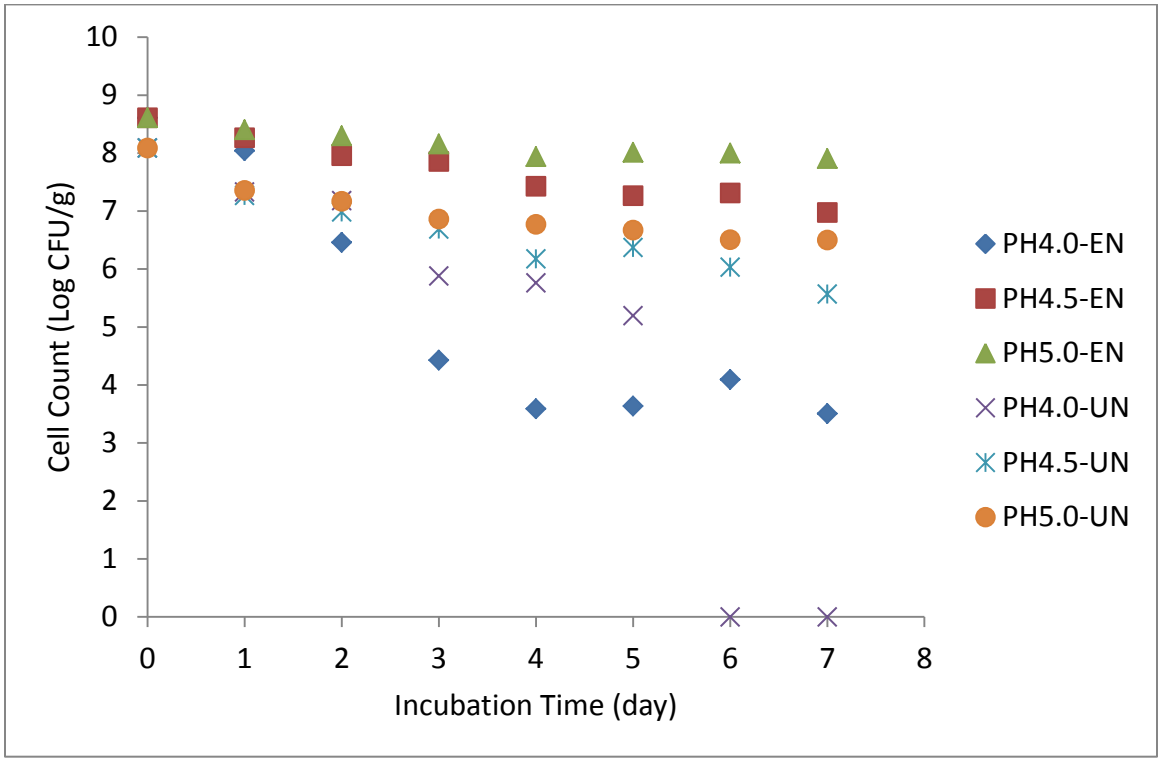

Figure 3.5 Survival of encapsulated and free cells during incubation for 7 days at 25°C.

These results were encouraging because it showed that encapsulation may protect cells long term, especially at pH 4.0 at 7 and 25°C. This may allow for the addition of encapsulated probiotics to acidic beverages with pH around 4.0.

3.2.6 Survival of encapsulated L. acidophilus in low pH beverages

Particles were produced and put into food systems to evaluate their performance over a 16 day shelf life. Based on the results from pH tests over 7 days, encapsulation provided good protection when particles were incubated at low pH conditions at 7 and 25°C. Therefore, two different products within the same pH range were chosen along with these

storage conditions. The two products tested were milk (2% reduced milk, 7°C) and a fruit juice (acai juice, 25°C). The particles were put into the products, stored, and tested for cell viability after 0, 4, 8, 12, 16 days of shelf life. The results are shown in Table 3.10.

Table 3.10 Survival of encapsulated and free cells in milk and juice (log CFU/g).

		Cell count (CFU/g)					Cell reduction (log CFU/g)
		Day0	Day4	Day8	Day 12	Day 16	
		Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Milk	Encapsulated	8.81 (0.10)	8.71 (0.08)	8.30 (0.35)	7.54 (0.29)	7.03 (0.08)	1.74 (0.21)[b]
	Free	8.26 (0.23)	7.73 (0.32)	7.63 (0.17)	7.60 (0.06)	6.97 (0.00)	1.28 (0.33)[b]
Juice	Encapsulated	8.81 (0.10)	7.62 (0.10)	7.39 (0.11)	ND	ND	8.81 (0.12)[a]
	Free	8.26 (0.23)	6.98 (0.13)	5.54 (0.19)	ND	ND	8.26 (0.33)[a]

*Different letters within the same column indicate difference based on $p<0.05$.
*Mean and standard deviation was obtained with duplicated sample.
*ND: None detected; limit of detection of <100 CFU.

The results indicated that both the encapsulated and free cells placed in milk stayed stable. Encapsulated particles showed a decrease of 1.78 log CFU/g in milk after 16 days of shelf life, while the free cells showed a decrease of 1.29 log CFU/mL. Overall, for the particles in milk, the number of probiotics was maintained above the recommended minimum (10^7 CFU/g) during storage, which was feasible for industry applications.

For the particles in acai juice, the results showed that both encapsulated and free cells started to die at day 4. Encapsulation provided protection to some extent at day 4 and 8 with a 1.19 and 1.42 log CFU/g reduction for encapsulated cells, respectively; while free cells suffered a reduction of 1.28 and 2.72 log CFU/mL. However, both encapsulated and free cells completely died at day 12. One reason that may explain why both the encapsulated and free cells died in the juice was that the pH of the beverage was even lower than the pH tested in the MRS broth where protective effects had been observed. The beverage also had a low concentration of calcium cation (2%), which can be beneficial for the stability of the alginate network.

Similar results have been reported for encapsulated probiotics added to yogurt and stored at 4°C. Krasaekoopt et al. (2006) tested the particles in yogurt made from UHT- and conventional milk and found that encapsulated cells were reduced by about 1.2 log while

free cells showed a reduction of 2.5 log after 4 weeks of storage. Ding and Shah (2008) tested encapsulated particles in orange and apple juices over 4 weeks of storage. They found out that there was a rapid decrease of free cells within four weeks and all the free cells died by the fifth week. The encapsulated cells were still detected as high as 10^5 CFU/g after 6 weeks of storage. The reason why the previous results showed a higher survival of the cells might be due to the type of the juice used and strain of the organism. The free cells were still alive by the fifth week while the *L. acidophilus* DDS 1-10 died on day 12 (week 2), which indicates that the organism they used were more resistant to low pH. Therefore, even though previous research also showed a decline in survivability, the rate of cell death was slower than observed in this research.

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Chapter 4 Conclusions and future research

4.1 Conclusions

Alginate-starch matrices were used to encapsulate *L. acidophilus* DDS 1-10 using an emulsion and an extrusion method. For the emulsion method, the optimum wall material composition was 1.6% of alginate and potato starch: RS4 in a ratio of 2:1. Particles obtained with this method showed a particle size distribution that was not uniform. The encapsulation process did not seem to enhance the bacterial resistance to acid conditions. Encapsulation by emulsion provided small but not significant protection against bile salt (0.5, 1.0, and 2.0%) for up to 6 hours. Regarding temperature, none of the different ones tested (25, 37, and 50 °C) was lethal to *L. acidophilus* (encapsulated and free cells), so heat protection by encapsulation was not be observed. Since the encapsulated particles did not seem to offer additional protection against pH and bile salts, the emulsion method was not studied any further.

The extrusion method seemed to overall provide a better protection for *L. acidophilus* compared to the emulsion method. For this process, the optimum wall material was determined to be 4.4% of alginate and potato starch: RS 4 in a ratio of 2:1. The particles produced by extrusion were bigger in size than the ones produced by the emulsion method, but much more uniform, with a mean diameter of 2.61 mm. The hardening time used to produce the particles influenced the survivability of *L. acidophilus* in the

particles. Increasing the hardening time during processing, especially when increased to 2 hours, dramatically increased the stability of the probiotic in the particles. Additionally, the encapsulated particles showed significant resistance to bile salt at 0.5, 1.0, and 2.0% after 6 hours of incubation. When incubation at pH 4.0, 4.5, and 5.0 for 24 hours at 37°C was evaluated, encapsulation did not provide any advantage; since both encapsulated and free cells were stable. However, when the testing time was increased to 7 days and the temperature was changed to 7 and 25°C, encapsulation was protective for the cells when tested at pH 4.0 for both 7 and 25°C. These results indicated the potential to incorporate these particles into a system with a pH around 4.0 followed by storage at 7 and 25°C. When particles were incorporated into food systems, promising results were obtained with milk. When incorporated into this food, the particles maintained counts above or at the levels usually observed in foods added with probiotics during 16 days of storage. However, the cells were not fully protected in acai juice over the storage period most likely due to the low pH of the juice.

4.2 Future work

Areas of interest to continue with this research include:

- Further optimization of the encapsulation processing by adding a second layer to the existing wall material, i.e. chitosan, gelatin.

- Further optimization of wall material compositions by including tests with higher starch composition.
- Evaluation of protection provided to *L. acidophilus* by encapsulation against acid and bile using more complex models.
- Evaluation of protection against temperature provided by encapsulation when higher temperature and longer exposure time are used.
- Freeze drying of the particles to evaluate the survival of *L. acidophilus*, with the potential of adding the dried encapsulated probiotic to a wider variety of food products.
- Incorporation of encapsulated *L. acidophilus* in other dairy products, like cheese and yogurt, as well as in juices with higher pH.
- Shelf life of encapsulated cells.

Appendix A.

Note: The data in Appendix A and B was generated in an attempt to utilize a response-surface design to find the best wall material combinations. The results were inconsistent and did not show any obvious patterns. Therefore this design was abandoned and replaced by a simple model that is described in the thesis under materials and methods.

Bacterial reduction of encapsulated particles produced from emulsion method after incubation in MRS at pH 2 for 3 hours

Alginate (%)	Potato starch : RS4 Ratio	Reduction (log CFU/g) Mean (SD)
1.6	2:1	3.08 (0.05)
3	2:1	2.78 (0.09)
4.4	2:1	2.31 (0.38)
2	1:1	1.36 (0.45)
2	3:1	2.79 (0.07)
4	1:1	2.47 (0.46)
4	3:1	1.58 (0.46)
3	3:5	3.17 (0.28)
3	24:7	3.32 (0.02)

*All samples were done with duplicate, 3% of alginate, ratio of potato starch: RS4 at 2:1 was done with 5 runs.

Appendix B.

Bacterial reduction of encapsulated particles produced from extrusion method after incubation in MRS at pH 2 for 3 hours.

Alginate	Potato: RS4 Ratio	Reduction (Log CFU/g) Mean (SD)
1.6	2:1	2.09 (0.46)
3	2:1	1.97 (0.35)
4.4	2:1	1.74 (0.11)
2	1:1	1.87 (0.01)
2	3:1	1.66 (0.89)
4	1:1	1.90 (0.03)
4	3:1	1.28 (0.30)
3	3:5	2.23 (0.03)
3	24:7	2.10 (0.06)

*All samples were done with duplicate, 3% of alginate, ration of potato starch: RS4 at 2:1 was done with 5 runs.