

MICROENCAPSULATION AND VIABILITY OF A PROBIOTIC IN A SIMULATED
GASTROINTESTINAL ENVIRONMENT

A Thesis

presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

FEI CHENG

Dr. Fu-hung Hsieh and Dr. Azlin Mustapha, Thesis Supervisors

MAY 2015

The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

MICROENCAPSULATION AND VIABILITY OF A PROBIOTIC IN A SIMULATED
GASTROINTESTINAL ENVIRONMENT

Presented by Fei Cheng,

A candidate for the degree of Master of Science

And hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Fu-hung Hsieh, Bioengineering and Food Science

Dr. Azlin Mustapha, Food Science

Dr. Shramik Sengupta, Bioengineering

ACKNOWLEDGEMENTS

I could never accomplish this thesis without the support and guidance of the following people.

Firstly, I am grateful to my advisors, Dr. Fu-hung Hsieh and Dr. Azlin Mustapha, who have always been supportive, understanding, patient and generously offering assistance to me, so that it was possible for me to work on a topic that was of my interest. It's honored and a pleasure to be their student.

I would like to thank Dr. Shramik Sengupta for serving on my committee in spite of his busy schedule. Without his valuable suggestions, this study would not have been successful.

I would like to thank Dr. Martin Schauflinger, Dr. Tommi White, Dr. Tommas Lam and DeAna Grant in the Electron Microscopy Core, for their abundant help with the scanning electron microscopy training and imaging. I would like to thank Katie Youmans for her assistance with using ChemBioDraw.

I also would like to express my thanks to Prashant Singh for being a peer mentor in my research, to Sha Zhang for the instructions on my data analysis. I would like to give a special appreciation my labmates, Yuan Yuan, Fanding Gao, Lin Li, Yun Wang, Houjie Xu, for helping with my research.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
LIST OF TABLES	v
LIST OF FIGURES	vi
ABSTRACT	vii
Chapters	
1. INTRODUCTION	1
2. LITERATURE REVIEW	4
2.1 Probiotics	4
2.1.1 Strains of probiotics	4
2.1.2 Health benefits from probiotics	6
2.1.3 Dogs as pets and impact on human health	8
2.1.4 Probiotics for dog	10
2.1.5 Viability of probiotics	11
2.2 Microencapsulation techniques	13
2.2.1 Types of microencapsulation materials and methods	14
2.2.2 Application of microencapsulation in food industry	21
2.2.3 Morphology study of microcapsules	21
3. MATERIALS AND METHODS	23
3.1 Bacterial strains and culture conditions	23
3.2 Encapsulation procedures	23
3.3 Morphological study of the microcapsules	24

3.4 Encapsulation yield.....	25
3.5 Simulated gastrointestinal fluids tolerance	25
3.6 Viability during storage up to 6 weeks	26
3.7 Statistical data analysis	26
4. RESULTS AND DISCUSSION.....	27
4.1 Morphology study.....	27
4.2 Encapsulation yield.....	32
4.3 Simulated gastrointestinal fluids tolerance	35
4.3.1 Simulated gastric acid tolerance	35
4.3.2 Simulated intestinal fluid tolerance	39
4.4 Viability during storage up to 6 weeks	44
5. CONCLUSIONS AND FURTHER DIRECTIONS.....	47
APPENDIX A.....	49
APPENDIX B	51
REFERENCES	53

LIST OF TABLES

Table

2.1 Common probiotic strains.....	5
4.1 Four formulations with alginate and xanthan gum and EY	34

LIST OF FIGURES

Figures

2.1 Alginate: α -L-guluronic acid (G), β -D-mannuronic (M) monomers and block types.	15
2.2 Alginate requires G-G blocks to form a gel, which is known as the “egg box” model.	15
4.1a ESEM images of beads.	30
4.1b ESEM images of beads	31
4.2 Effect of SGF on viability of free and entrapped <i>L. rhamnosus</i> GG in four formulations	38
4.3 Effect of SIF on viability of free and entrapped <i>L. rhamnosus</i> GG in four formulations	42
4.4 Effect of SGF+SIF successively on viability of free and entrapped <i>L. rhamnosus</i> GG in four formulations	43
4.5 Viability of encapsulated cells during storage in refrigerator (4°C) up to 6 weeks....	46

MICROENCAPSULATION AND VIABILITY OF A PROBIOTIC IN A SIMULATED
GASTROINTESTINAL ENVIRONMENT

Fei Cheng

Dr. Fu-hung Hsieh and Dr. Azlin Mustapha, Thesis Supervisors

ABSTRACT

The human-origin probiotic *Lactobacillus rhamnosus* strain GG (LGG) proved to exert health benefits in dogs. Like other strains, the viability of this probiotic is reduced while passing through the stomach and upper intestine because of the low pH and presence of bile. In this study, microcapsules loaded with LGG were prepared with the traditional extrusion method. Small drops of alginate (2.0 and 2.5% w/v), with and without xanthan gum (0.15% w/v) solution mixed with cell suspension and formed continuously by a syringe attached to a peristaltic pump, was added to 0.5M CaCl₂ solution and allowed to harden for 30 min, followed by coating with chitosan (1% w/v) for 30 min externally. The capsules of all formulations were able to improve the viability of LGG ($P < 0.05$), maintaining it at a concentration of $>10^7$ CFU/g when separately exposed to simulated gastric fluid (SGF) for 2 h and simulated intestinal fluid (SIF) for 4 h. However, results of sequential incubation in SIF after SGF revealed a dramatic reduction in the viability of encapsulated cells with no difference from control, which cannot be explained with little information in the literature. No difference was detected in the bead appearance by environmental scanning electron microscopy, except that alginate beads incorporated with xanthan gum resulted in less severe wrinkles on the surface. All

the formulations achieved encapsulation yields of around 80% and the cell viability remained at least 10^7 CFU/g after 4 weeks storage at refrigeration temperature ($P>0.05$). The overall situation indicated that the most stable microcapsules were chitosan-coated alginate (2.5% w/v)-xanthan gum (0.15% w/v) beads. These results indicated the potential of industrial application of chitosan-coated alginate-xanthan gum microcapsules.

CHAPTER 1

INTRODUCTION

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO 2001). Statistical data from American Pet Product Association (APPA 2014) revealed that 56.7 million households in the United States own dogs. Moreover, many studies indicated that dogs shared microbiota with co-habiting human owners (Stenske and others 2009; Song and others 2013; Misic and others 2015). Therefore, intake of probiotics not only benefits dogs themselves, but also has positive health impact on human owners. A current research interest is the application of human-origin probiotics in animals. In fact, in some patents, researchers have been using commercial human-origin probiotic strains in companion animals, like *E. faecium* M74 (Knorr and others 2006). In recent studies, one of the strains with the most published human clinical data, *L. rhamnosus* GG, derived from human GI tract, was reported to perform many positive effects in dogs (Weese and Anderson 2002; Rinkinen and others 2000; Rinkinen and others 2003b; Rinkinen and others 2003a; Marsella 2009; Marsella and others 2012). However, to exert health benefits, one was recommended that the minimum concentration of live probiotic bacteria cells should be 10^7 CFU per gram/milliliter of food product/dietary supplement at the time of consumption (Ouweland and Salminen 1998; Capela and others 2006; Mokarram and others 2009; Manojlović and others 2010), while the viability of probiotics is always

challenged passing through the GI tract prior to the final delivery in the host's lower intestine.

Microencapsulation technique has been widely studied for the purpose to enable the probiotic bacteria survive the diverse conditions in the host's digestive tract (Mortazavian and others 2007). Alginate is the most commonly used agent for probiotic encapsulation in food product, owing to its low cost, nontoxicity, biocompatibility, thermo-stability, approved as "generally recognized as safe" (GRAS) food additive and the easiness in its application. Alginate is a linear anionic/acidic diheteroglycan composed of C-5 epimer α -L-guluronate (G) and (1-4)-linked- β -D-mannuronate (M) (Corona-Hernandez and others 2013). From the molecular point of view, in the presence of divalent cations like calcium, a strong molecular framework formed instantaneously, followed by gradual gelation known as the "egg box" (Corona-Hernandez and others 2013). However, alginate gel structure can be weakened by low acidic pH and chelating agents, like phosphate, citrate, before these carriers reach lower small intestine and colon (Zhou and others 2001; Corona-Hernandez and others 2013; Chávarri and others 2010). A controlled release of cells can be realized with a cationic coating material, chitosan, and along with it, a better protective effect can be achieved (Cook and others 2012). Besides, the incorporation of other polymers, such as xanthan gum, proteins and gellan gum, into alginate matrix is another popular direction in exploration of microencapsulation (Fialho and others 2008; Albertini and others 2010; Hébrard and others 2013). So far, there are many publications on encapsulation with alginate-chitosan, alginate-xanthan gum or xanthan gum-chitosan (Iyer and Kailasapathy 2005; Albertini

and others 2010; Chávarri and others 2010; Brinques and Ayub 2011; Cook and others 2011; Sohail and others 2011; Bajracharya and others 2012; Trabelsi and others 2013; Rodklongtan and others 2014; Argin and others 2014; Vodnar and Socaciu 2014); however, beads consisting of these three biocompatible polymers were rarely reported (Fareez and others 2015).

In this study, microencapsulation loaded with *Lactobacillus rhamnosus* strain GG was developed using alginate, chitosan and xanthan gum. Resistance of encapsulated cells to simulated gastrointestinal fluids was evaluated; morphology study was conducted with environmental scanning electron microscopy; encapsulation yield and storage durability were assessed.

CHAPTER 2

LITERATURE REVIEW

2.1 Probiotics

Ever since the concept of using beneficial bacteria as a supplement for health was first introduced by Elie Metchnikoff in 1907, the term “probiotic” appeared in the 1960s and its definition advanced through the years. The latest one, which was internationally endorsed, can be found in The Food and Agricultural Organization of the United Nations and the World Health Organization’s document as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO 2001).

2.1.1 Strains of probiotics

The majority of probiotics comes from the genus *Lactobacillus* and *Bifidobacterium*, and the rest consists of some other bacteria and yeast strains (Table 2.1).

Table 1 Common probiotic strains¹

		Species	Example strains	
Bacterium	<i>Lactobacillus</i>	<i>acidophilus</i>	LA-1/LA-5 NCFM DDS-1 SBT-2062	
		<i>bulgaricus</i>	Lb12	
		<i>casei</i>	Shirota	
		<i>fermentum</i>	KLD RC-14	
		<i>johsonii</i>	La1	
		<i>paracasei</i>	CRL431 F19	
		<i>plantarum</i>	299v	
		<i>reuteri</i>	SD2112	
		<i>rhamnosus</i>	GG GR-1 LB21 271	
		<i>Bifidobacterium</i>	<i>salivarius</i>	UCC118
			<i>adolescentis</i>	ATCC 15703 94-BIM
			<i>animalis</i>	Bb 12
			<i>bifidum</i>	Bb 11
			<i>breve</i>	Yakult
			<i>infantis</i>	Shirota Immunitass 744 01
<i>lactis</i>	Bb 02 B94			
	<i>longum</i>	UCC 35624 SBT-2928 BB536		
	<i>Bacillus</i>	<i>cereus</i> <i>subtilis</i>	toyoi	
	<i>Escherichia</i>	<i>coli</i>	Nissle 1917	
	<i>Enterococcus</i>	<i>faecium</i>	SF68	
	<i>Propionibacterium</i>	<i>freudenreichii</i>	JS	
Yeast	<i>Saccharomyces</i>	<i>boulardii</i>	<i>boulardii</i>	

¹Derived from Ouwehand 2002 and Shah 2007.

2.1.2 Health benefits from probiotics

The consumption of probiotics is proven to bring numerous health benefits for hosts. Some benefits were reported in human studies, some were effective in animal models and many of the rest got positive reports in *in vitro* studies. Those benefits include:

1) **Antimicrobial activity and alleviation of gastrointestinal (GI) infections**

Considered as the most important property to hosts, the antimicrobial activity of probiotics is attributed to the production of organic acids (lactic and acetic acids), hydrogen peroxide, bacteriocins and other metabolites from these bacteria in the gut, which have a bacteriocidal or bacteriostatic effect (Šušković and others 2010). There was sufficient evidence in *in vitro*, *in vivo* and clinical studies that both *Lactobacillus* spp. and *Bifidobacterium* spp. perform antagonistic activities towards GI microbial pathogens including *Escherichia coli*, *Listeria monocytogenes*, *Clostridium perfringens*, *Salmonella* Typhimurium and *Staphylococcus aureus* (Servin 2004).

2) **Improvement in lactose metabolism** Lactose malabsorption results from a deficiency of an enzyme called β -D-galactosidase. Lactic acid bacteria can produce the enzyme, which hydrolyzes lactose, cleaving the carbohydrate into its constituent mono-saccharides in dairy products, thus reducing lactose maldigestors' sensitivity (De Vrese and others 2001).

3) **Treatment against diarrhea** Effectiveness has been found for antibiotic-associated diarrhea caused by *Clostridium difficile* after taking antibiotics with the oral administration of *Saccharomyces boulardii* or *Lactobacillus casei* ssp.

rhamnosus GG (LGG). Also, LGG was reported to be effective in the treatment of gastroenteritis, a main cause of acute diarrhea, by shortening the duration of rotavirus in children (Shah 2007). Though not always promising, some studies found that the intake of probiotics reduced the frequency of traveler's diarrhea (Black and others 1989; Kollaritsch and others 1993; Oksanen and others 1990).

- 4) **Anti-mutagenic activities** Basically, the mechanism lies in the probiotics' ability to bind mutagens in the Ames test (Apás and others 2014; Chalova and others 2008; Darsanaki and others 2012; Korpela and others 2010; Lo and others 2004). Besides, other activities of probiotics were found to account for this property: reducing fecal enzymatic activities which are involved in activation of mutagens (Goldin and Gorbach 1984) and antagonistic role of organic acids produced by probiotic bacteria against several mutagens and promutagens (Lankaputhra and Shah 1998).
- 5) **Anti-carcinogenic properties** This reputed benefit, especially for colorectal health, is ascribed to the induction of apoptosis, immune system stimulation, and suppression of growth of bacteria which produce carcinogens from pro-carcinogens resulting in a reduction of carcinogens in the intestine (Commane and others 2005; Khan and others 2013; Kumar and others 2010; Zhong and others 2014).
- 6) **Reduction in serum cholesterol** Clinical trials have proven the effectiveness of promoting lipid metabolism by probiotics, thus lowering blood cholesterol and preventing hypertension (Ishimwe and others 2015; Khalesi and others 2014).

- 7) **Improvement in inflammatory bowel diseases (IBD)** Beneficial effects on IBD (ulcerative colitis and Crohn's disease) from probiotics have been found in clinical trials owing to their successful competition with pathogens in the intestine, producing bactericidal substances, improving immune system responses and forming a protective barrier on epithelial cell wall (Vanderpool and others 2008).
- 8) **Suppression of *Helicobacter pylori* infection** It was reported that the standard one-week "triple therapy" (consisting of proton pump inhibitors such as omeprazole and the antibiotics clarithromycin and amoxicillin) is considered the priority treatment for this infection but alternative methods are being investigated, including the application of probiotics as a beneficial supplement, which helps reduce the pathogen load in patients' gut. However, it still remains controversial in its effect of eradication rates on *Helicobacter pylori* infection (Hamilton-Miller 2003; Li and others 2014; Lionetti and others 2010).
- 9) **Stimulation of immune system** Oral administration of probiotics is found to be able to modulate the innate immunity or the adaptive immune response or both, which subsequently has a positive effect on prevention or treatment of allergy (Pascal and others 2011).

2.1.3 Dogs as pets and impact on human health

Statistical data from American Pet Product Association (APPA 2014) reported that the top three species as household pets in the United States were freshwater fish, cats and dogs numbering 145, 95.6 and 83.3 in millions, respectively. Another APPA statistic

depicted the trend in the number of dog ownership from 2000 (68 million) to 2014 (83.3 million). The proportion of the United States households that own at least one pet was 68% (82.5 million estimated), 56.7 million households own dogs, 45.3 million of them have cats, while only 16.1 million own fish (APPA 2014). As a very intelligent species, dogs not only are kept as pets, but also can be trained to fulfill specific roles, like in law enforcement, hunting or assistance of people with disabilities.

Like human beings, pets including dogs, can benefit from probiotics with a similar pattern as reviewed above. An *in vivo* study reported that *Lactobacillus acidophilus* DSM 13241 was effective in the aid of digestive processes in dogs (Pascher and others 2008); A patented probiotic strain, *Enterococcus faecium* SF68, was reportedly effective in immunomodulation in young dogs (Benyacoub and others 2003). Supplementation with the canine-derived probiotic, *Bifidobacterium animalis* AHC7, provided a reduced duration time and rate of dogs with acute idiopathic diarrhea (Kelley and others 2009). Oral application of *L. fermentum* CCM 7421 (AD1) and a combination of *Bacillus amyloliquefaciens* CECT5940 and *E. faecium* CECT 4515 resulted in decreased counts of clostridia in canine feces (Gonzalez-Ortiz and others 2013; Stropfová and others 2013).

Beneficial effects on human physiological and psychological health owing to dogs as companion animals have been reviewed (Cutt and others 2007; Knight and Edwards 2008). Besides, many studies indicated that dogs shared microbiota with co-habiting human owners. Comparison of the microbial community between canine and human samples from skin, mouth and feces revealed the transmission of bacteria, which

requested enhances hygiene (Stenske and others 2009; Song and others 2013; Mistic and others 2015). Therefore, intake of probiotics not only benefits dogs themselves, but also has positive health impact on human owners.

2.1.4 Probiotics for dog

Previously, probiotic-enhanced products were mainly designed for human. As more and more domestic animals come into household lives, pet health is attracting more and more attention and currently it has been a growing concern to explore probiotics market for pets and veterinary animals in the food industry. Preferably, a probiotic strain for a specific host should be of host origin. However, due to the complexity of the microbial flora of the animal GI tract, most of the strains isolated from dogs haven't been well profiled yet. Only a few probiotics are commercially available for dogs in the market, and some products that claimed to contain live probiotic bacteria were reported to have lower numbers of live cells than the label description (Weese and Arroyo 2003; Weese and Martin 2011). Thus, a current research interest is the application of human-origin probiotics in animals. In fact, in some patents, researchers have been using commercial human-origin probiotic strains in companion animals, like *E. faecium* M74 (Knorr and others 2006).

In recent studies, one of the strains with the most published human clinical data, *L. rhamnosus* GG, derived from human GI tract, was reported to perform many positive effects in dogs. Essentially, LGG has proved to be safe and survived in dogs (Weese and Anderson 2002). This strain was found to have a significantly better adhesive ability to canine mucus than other both human- and dog-derived LAB strains in *in vitro* tests

(Rinkinen and others 2000; Rinkinen and others 2003b). Another study reported its ability to reduce the number of *C. perfringens* adhesion to canine intestinal mucus (Rinkinen and others 2003a). More positive veterinary clinical effects were found in recent research, such as administration of this strain contributed to reduce immunologic indicators of atopic dermatitis in puppies and a further study proved that early exposure to LGG had long-term clinical and immunological effects, although more studies are still needed (Marsella 2009; Marsella and others 2012). Considering it has been well-documented, LGG can be a probiotic strain for canine use, though reports on its effects for dogs are limited.

2.1.5 Viability of probiotics

In 2001 and 2002, a joint Food Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) Expert Consultation held two meetings successively working on guidelines for selection and evaluation of probiotics in food for humans, and those strains intended to be used in animal feeds were also recognized as they pertain to human health. Of all the restrictions, to exert health benefits, one was recommended that the minimum concentration of live probiotic bacteria cells should be 10^7 CFU per gram/milliliter of food product/dietary supplement at the time of consumption (Ouwehand and Salminen 1998; Capela and others 2006; Mokarram and others 2009; Manojlović and others 2010).

However, the development of food products with adequate doses of live probiotic cells at the time of consumption is challenged by factors like processing procedures, storage conditions and harsh chemicals (especially gastric acid and bile) in the digestive

tract (Tripathi and Giri 2014; Cook and others 2012; Kent and Doherty 2014). Aspects like heating, freezing, thawing, drying and growth media during processing and oxygen content, water activity, storage temperature, packaging, food ingredient and additives during storage, all affect the survivability of probiotics (Tripathi and Giri 2014). The challenges from processing and storage conditions vary depending on the factors but the passage through the GI tract prior to the final delivery in the host's lower small intestine or colon is inevitable for probiotic strains.

After ingestion, probiotics will pass through the esophagus very quickly and reach the stomach, where the greatest viability loss can happen due to the high level of gastric acid. An important quality that a probiotic strain should possess is acid tolerance because the pH in the stomach frequently drops below 2.0 (Kararli 1995), though different subjects under different occasions present different gastric pH (1.7 for human and 1.5 for dogs when being fasted, while 5.0 for human and 2.1 for dogs when being fed) and emptying time (99.8 ± 27.2 min for dogs, 59.7 ± 14.8 min for humans) (Lui and others 1986). Additionally, pepsin, an enzyme, which breaks down proteins, is present in the stomach and functions at low pH. The cells will enter the small intestine after transit through the stomach, where the pH is higher (7.3 ± 0.09 in dog intestine and higher than that in humans by 0.09 - 1.9) (Lui and others 1986) than in the stomach but contains bile salt, which aids in the digestion of lipids and also acts as a bactericide. The small intestine transit time in human is around 3-4 h while dogs showed a shorter transit time (Kararli 1995). Since probiotics delivered in the lower digestive tract exert their health benefits, they should withstand the harsh adverse conditions in the host's upper GI tract.

Probiotics' tolerance against gastric acid and bile has been reported as strain-specific and varies (Ding and Shah 2009b; Succi and others 2005). The request to enable the probiotic bacteria survive the diverse conditions in the human digestive tract has encouraged researchers to find new efficient ways for reducing the loss of probiotic viability (Mortazavian and others 2007), and microencapsulation technique has been widely studied for this purpose.

2.2 Microencapsulation techniques

Encapsulation is determined as a mechanical or physiochemical technique that traps materials that may be sensitive to the external conditions. It provides a protective barrier between the inner material and external conditions. This is different from simple immobilization, in which the core component may be exposed to the outside environment (Mitropoulou and others 2013). In encapsulation, the enclosed materials should be completely sealed inside. Microencapsulation can be concisely defined as a process in which tiny parcels of solids, liquids or gases are enclosed within a coating to give small capsules. Though being termed “microcapsules”, the current devices typically range from tens of microns to around a couple of millimeters in size, and their ability to improve the survival of probiotics in many cases appears to be size-dependent, and there wasn't conclusion drawn from literature on whether micro- or macro- beads provided higher viability (Albertini and others 2010; Sohail and others 2011). The preferred micro-capsulated probiotic product is either in the format of a dry powder, with convenience of storage and long shelf life or a wet gel with better stability in a food product (Cook and others 2012).

2.2.1 Types of microencapsulation materials and methods

Materials used in microencapsulation of probiotic bacteria include polysaccharides extracted from seaweed (alginate, κ -carrageenan), other herbs (starch and its derivatives, gum Arabic), or bacteria (exopolysaccharides like xanthan and gellan) and proteins (soy, gelatin and whey), all of them have been extensively studied (SHEU and Rosenberg 1998; Sun and Griffiths 2000; And and Kailasapathy 2005; Lahtinen and others 2007; Rosas-Flores and others 2013; Tee and others 2014). Among all of the coating materials above, alginate has been the most commonly used agent for probiotic encapsulation in food product. This is owing to its low cost, nontoxicity, biocompatibility, thermo-stability, approved as “generally recognized as safe” (GRAS) food additive and the easiness in its application, which attributes to its widely usage in laboratory-scale microencapsulation.

Alginate is usually commercially available as sodium alginate. Alginate is a linear anionic/acidic diheteroglycan composed of C-5 epimer α -L-guluronate (G) and (1-4)-linked- β -D-mannuronate (M) (Figure 2.1) (Corona-Hernandez and others 2013). From the molecular point of view, in the presence of divalent cations like calcium, a strong molecular framework formed instantaneously, followed by gradual gelation. However, a sufficient amount of G-monomers are required to form gel with calcium, where Ca^{2+} fits into G-blocks known as the “egg box” (Figure 2.2) (Corona-Hernandez and others 2013). The properties of alginate gel are influenced by the concentration and type of polymer and the concentration of the cations in the medium (Ouwere and others 1998).

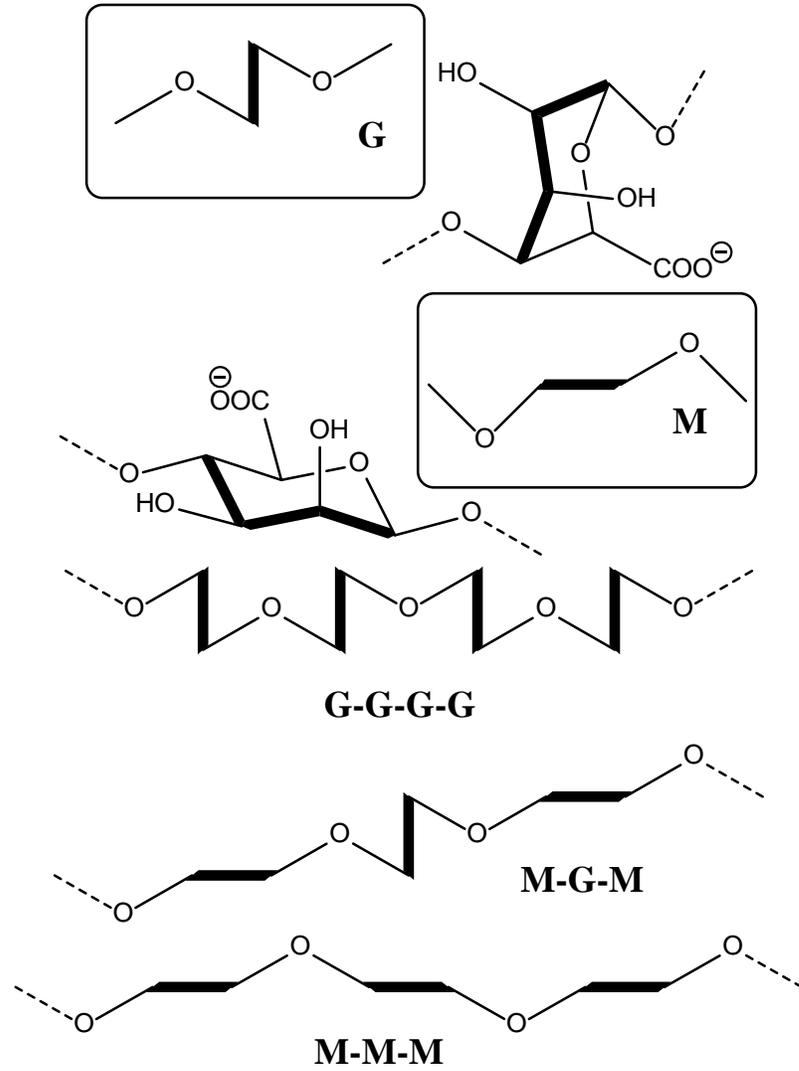


Figure 2.1 Alginate: α -L-guluronic acid (G), β -D-mannuronic (M) monomers and block types.

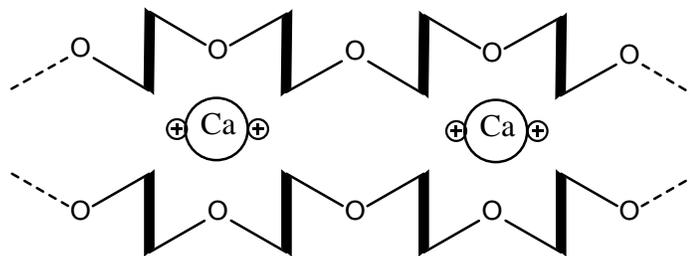


Figure 2.2 Alginate requires G-G blocks to form a gel, which is known as the "egg box" model.

A traditional method to form alginate microcapsules is using an extrusion process. This is accomplished by the dropping of an alginate solution mixture with targeted probiotic bacteria cells, mostly through a syringe needle, into calcium chloride solution, where microcapsules form immediately. The size of microcapsules formed during this process, which could be up to a few millimeters, is determined by the size of droplets formed from the needle. In order to get smaller beads, apart from using a needle with a smaller diameter, in some studies, external forces like coaxial air-flow, an electrostatic field, jet-cutting and spinning-disk atomization were added to form tinier droplets of a few hundreds of microns (Cook and others 2012; Burgain and others 2011; Whelehan and Marison 2011). Due to its mild gelling conditions, no deleterious chemicals are involved and cell viability can be maintained at a high level both aerobically and anaerobically (Burgain and others 2011; Krasaekoopt and others 2003). Though the most important disadvantage of this method is that it's difficult to be implemented in an industrial scale, researchers have been developing advanced techniques like multiple nozzle systems, jet-cutting techniques and spinning-disk atomizers to achieve high-levels of production (Burgain and others 2011; Krasaekoopt and others 2003; Kent and Doherty 2014).

Another popular approach of making microcapsules of alginate matrix is emulsion, a chemical technique based on the interaction between the continuous (oil) and discontinuous (cell-polymer mixture) phases (Krasaekoopt and others 2003). This technique is applied by generating a water-in-oil emulsion through homogenization (e.g. mixture of sodium alginate and cell suspension is suspended in oil, usually with vegetable

oil or Tween 80 as emulsifier), then CaCl_2 is added to break the emulsion and form gel beads, which are subsequently collected by centrifugation or filtration (Burgain and others 2011). Compared to extrusion method, emulsion is easy to scale up and give capsules of a smaller diameter (usually tens of microns), but produces capsule batches which can have large variances in size and shape, and costs more because of the involvement of emulsifier (Burgain and others 2011; Krasaekoopt and others 2003).

The properties of alginate have been well-documented and the encapsulation of all kinds of probiotic cells within alginate matrix has been researched (Martinsen and others 1989; Velings and Mestdagh 1995; Tanaka and others 1984; Haghshenas and others 2015; Corbo and others 2013b; Rosas-Flores and others 2013; Su and others 2011). Microencapsulation with alginate alone has been proved a possible way to improve the viability of probiotic cells when exposed to gastric acid and bile. When the pH value of ambient media is lower than the pKa of M-monomers (3.38) and G-monomers (3.65) of alginate, the G- and M-residues protonate, causing the precipitation of the gel being insoluble alginic acid (Li and others 2009). Many studies have been carried out on different probiotics using both extrusion (Chandramouli and others 2004; Graff and others 2008; Albertini and others 2010; Sabikhi and others 2010; Sohail and others 2011) and emulsion (Mandal and others 2006; Sabikhi and others 2010; Hansen and others 2002; Sultana and others 2000; Ding and Shah 2009b) methods, with ununiformed results. However, alginate gel structure can be weakened by low acidic pH and chelating agents, like phosphate, citrate, which compete for calcium ions and cause “burst release” before these carriers reach lower small intestine and colon (Zhou and others 2001; Corona-

Hernandez and others 2013; Chávarri and others 2010). A controlled release of cells can be realized with a cationic coating material/polymer, also, along with it, a better protective effect can be achieved (Cook and others 2012). Cationic polymers like chitosan, gelatin and poly-L-lysine, have been reported to form ion-exchange interaction with the negatively charged alginate residues and thus provide improved stability of gel structure (Chávarri and others 2010).

The most popular coating material is the polysaccharide chitosan. Chitosan (Figure 2.3) is a natural linear cationic homoglycan [β -(1-4)-linked D-glucosamine and *N*-acetyl-D-glucosamine] (Ravi Kumar 2000; Rinaudo 2006). Chitosan is the deacetylated form of chitin, a natural mucopolysaccharide and the supporting material, which can be derived from crustaceans and insects. Many papers reported its efficiency for increasing cell viability as a coat rather than as a capsule matrix (Cook and others 2011; Krasaekoopt and others 2004; Chávarri and others 2010). However, some studies revealed the coat of chitosan did not improve the survival of cells exposed to acid condition, probably due to the strains of microorganism encapsulated had a high acid tolerance (Graff and others 2008; Lin and others 2008). Additional benefits from coating with chitosan were reported as improved survival in drying and freezing (Cook and others 2011; Priya and others 2011).

Other polymers, like proteins, gellan gum, xanthan gum, were less widely used for encapsulation of probiotics, but the incorporation of them into alginate matrix is another popular direction in exploration of microencapsulation (Fialho and others 2008; Albertini and others 2010; Hébrard and others 2013). One notable polymer used is

xanthan gum (Figure 2.4). It is an exopolysaccharide extensively used as a food additive, derived from the plant-pathogenic bacterium *Xanthomonas xampestis* (Katzbauer 1998). Similar to alginate, this polymer is able to form cross-link with calcium cations through a gentle gelling process, which doesn't damage cells. Its efficacy as encapsulating material alone has been investigated (Ding and Shah 2009b). When an alginate-based matrix combined with xanthan gum, improved survival of *L. acidophilus* and *B. lactis* in acidic conditions was reported (Albertini and others 2010); *L. plantarum* LAB12 trapped in chitosan coated alginate-xanthan gum microcapsules yielded highest viability and maintained better controlled releasing behavior in *in vitro* study (Fareez and others 2015).

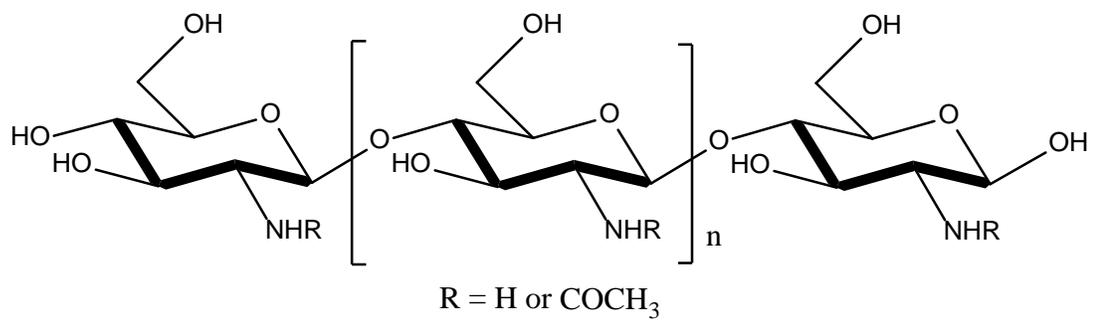


Figure 2.3 The chemical structure of chitosan.

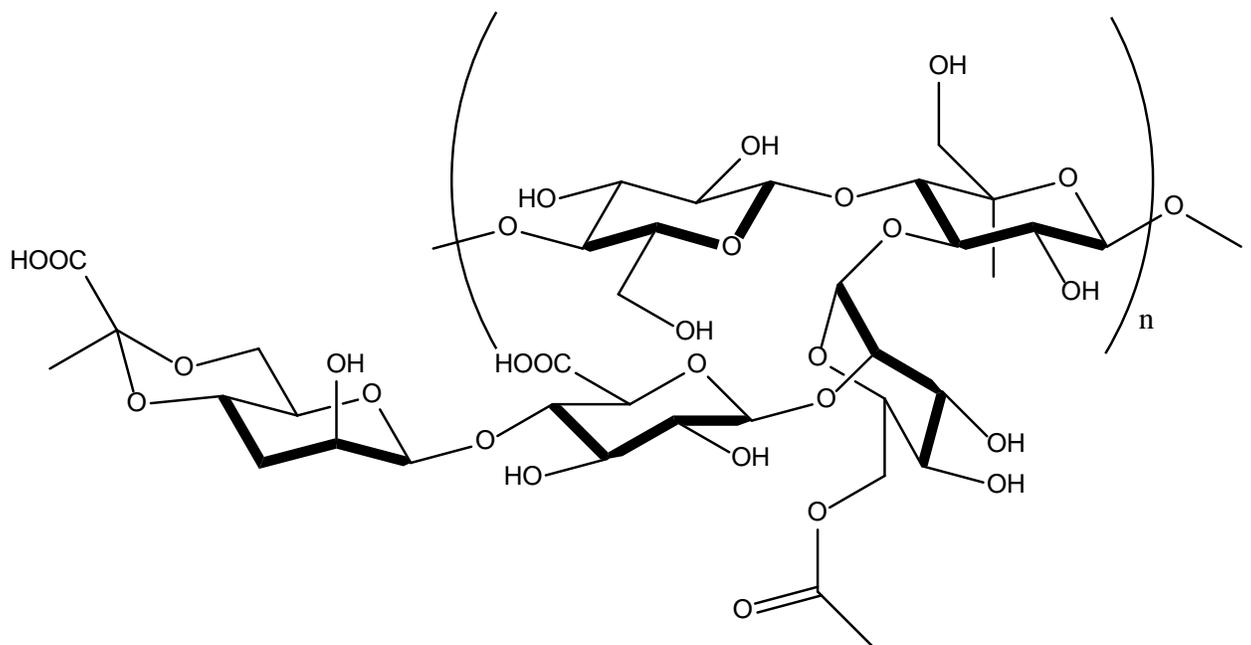


Figure 2.4 The chemical structure of xanthan gum.

2.2.2 Application of microencapsulation in food industry

Microencapsulation is expected to increase probiotics' heat resistance, improve their compression and shear stress resistance, tolerance to drying/freeze-drying (in some occasions), and extend the shelf-life (preferred in room temperature and in various food matrixes) other than enhance their acid and bile tolerance during transiting into the digestive system (Rokka and Rantamäki 2010). Besides, the selection of appropriate techniques, materials and microorganism strains should be nature-friendly, non-toxic and comparatively potent respectively. Also, to commercialize it, the cost added by the application during food product development is another big challenge. A previous report gave the estimate that the price of encapsulated probiotic bacteria may be 2~3 times of that of non-encapsulated ones (Lakkis 2007). Nevertheless, despite those challenges, the application of microencapsulation techniques in food industry has profit-making potential, and until recent years, microencapsulation techniques have been applied in food products such as chocolate, yogurt, juice, nutrient bars and tablets etc (Burgain and others 2011).

2.2.3 Morphology study of microcapsules

Microscopic observations can be used to explicate the surface and walls of microcapsules. To observe cracks, cavities, surface wrinkles, wall thickness, uniformity, and smoothness is very useful in understanding that particle properties and bulk characteristics are related to morphology (Walton and Mumford 1999). Usually, scanning electron microscopy (SEM) is adopted to examine the structural differences of various microcapsules (Jiménez-Pranteda and others 2012; Rosas-Flores and others 2013). SEM technique allows the investigation of surface properties of microcapsules, like cracks,

cavities and smoothness. The presence of these characteristics is generally undesired as they may adversely affect important parameters such as long-term stability, microencapsulation efficiency, volatile retention, or flowability (Rosenberg and others 1985; SHEU and Rosenberg 1998).

A SEM is a type of electron microscope that can produce an image of a sample by scanning it by using a high energy beam of electrons. This technique is widely adopted in studying morphology of microcapsules because it allows for the observation of specimens at a nanometer to micrometer scale by producing three-dimensional-like images of the sample surface. Two common modes – conventional SEM under high-vacuum condition and environmental SEM (ESEM) under low-vacuum conditions - were extensively applied in previous studies (Jiménez-Pranteda and others 2012; Rodklongtan and others 2014; Fareez and others 2015).

For conventional SEM, biological samples go through critical point drying so that they can be imaged in a high vacuumed chamber, which results in high resolution, but it's challenging when it comes to specimens with relatively more water content because the rapid evaporation of moisture from wet samples causes the structure to shrink. Currently, ESEM allows wet samples to be imaged in a gaseous low-vacuumed specimen chamber with relative humidity (up to 100%). Besides, this technique enable the insulators to be imaged without the need for metallic coating after the primary fixation (Thiel and Toth 2005; McGregor and Donald 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strains and culture conditions

For this research, *Lactobacillus rhamnosus* GG (LGG, ATCC 53103), provided by the Food Microbiology Laboratory at the University of Missouri, was sub-cultured twice prior to an anaerobic enrichment in 120 mL MRS broth at 37°C for 48 h. LGG was harvested by centrifugation (Beckman J2-21) at 8000 g for 5 min at 4°C washed twice with sterile peptone water and re-suspended in 15 mL peptone water. The cell suspension was divided into two parts: one was for encapsulation and the other was for control group.

3.2 Encapsulation procedures

Microcapsules were prepared by an adjusted traditional extrusion method (Jiménez-Pranteda and others 2012). The alginate mixture was prepared by adding sodium alginate (2.0, 2.5% w/v, KIMICA ALGIN I-5G-150, Chile) and xanthan gum (0.0, 0.15% w/v, Danisco Cultor, Chile) into deionized water. The solution was autoclaved for 15 min under 121°C prior to the addition of 2 mL cell suspension (around 10^{10} CFU/mL) and stored at 4°C.

The mixture (80 mL) was stirred with a magnetic bar and extruded with the aid of a pump (MASTERFLEX mode 7540-12, Thermo Fisher Scientific, USA) at a flow rate of 2.5 mL/min, through a 26 G syringe needle into a beaker containing 100 mL 0.5 M CaCl₂ solution under gentle stirring with a magnetic bar at room temperature. The

divalent calcium ions cross-linked the droplets of sodium alginate to form spherical alginate beads with a diameter around 2 mm. The beads formed were allowed to harden in CaCl₂ solution for 30 min and filtered through a sterile filter paper (P8, Fisher Scientific).

The filtered alginate beads were rinsed twice with deionized water and transferred to chitosan (Acros Organics, New Jersey, USA) solution (1.0% w/v) in acetic acid solution (0.5% v/v). The solution was autoclaved at 121°C for 15 min and stored in refrigerator before use.

The washed alginate beads were immersed in 100 mL chitosan solution and stirred gently with a magnetic bar for 30 min to coat the surface of the alginate macro particles. The resulting chitosan-coated alginate beads were again separated by filtration and washed twice with sterile deionized water, then transferred to a sterile petri dish, and stored in a refrigerator until further use.

3.3 Morphological study of the microcapsules

The capsules consisting of 2.0% w/v alginate (AL) and 2.0% w/v alginate + 0.15% w/v xanthan gum (AL-XG) loaded with *L. rhamnosus* GG as described in section 3.2 were viewed under a FEI Quata 600 FEG Environmental Scanning Electric Microscope, in order to study the effect result from the addition of xanthan gum. Beads without bacterial cells as control were imaged as well in order to check how the involvement of cells influences the morphology of gel beads. After the primary fixation with 2% glutaraldehyde, 2% paraformaldehyde and 0.1% Ruthenium Red in 0.1 M Na Cacodylate buffer (pH 7.35), samples were placed on a stub with a silicon layer on top and observed

in cold stage under low vacuum and low pressure (107 Pa), with the voltage of 10.00 kV, spot size of 3 d_p and at 4.5°C. The mounting step was not involved because in order to minimize the dehydration, a drop of deionized water was added to keep the sample moisturized, but during the imaging process, specimens still became slightly desiccated.

3.4 Encapsulation yield

To study the efficacy of encapsulation, encapsulation yield (EY) was defined as

$$EY = \frac{N}{N_0} \times 100 \% = \frac{C \times W}{C_0 \times V} \times 100 \%$$

Where N_0 was the number of cells added into alginate mixture and N was the number of cells entrapped in hydrogel beads, C_0 stood for concentration of cell suspension added into alginate mixture (CFU/mL), V was volume of cells suspension added into alginate mixture, C was cell loading in gel beads (CFU/g) and W was mass of beads collected (g).

For enumeration of entrapped cells, 1 g particles were allowed to be liquefied in 99 mL 1% sodium citrate solution (pH 6.8) for 30 min at room temperature, using a stomacher for 2 min afterwards. Cell counts (CFU/mL) were determined by plating on MRS agar plates and anaerobic incubation for 48 h at 37°C. Free cells were enumerated on MRS agar. Peptone water was used to prepare the serial dilutions.

3.5 Simulated gastrointestinal fluids tolerance

Preparation of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) was modified from a previous study (Jiménez-Pranteda and others 2012). SGF was prepared in 0.85% saline solution containing 0.03% pepsin (Sigma, St Louis, MO), and

the pH was adjusted to pH 2.0 with 5M HCl and 1M NaOH. 0.02% (w/v) pancreatin (Merck, Rahway, NJ) and 3% (w/v) bile salt (Oxgall; Sigma Chemical Co., St. Louis, MO) were dissolved in saline solution at pH 8.0 for the preparation of SIF.

Tolerance of probiotic bacteria to simulated gastrointestinal fluids was determined by exposing free (1 mL at a level of around 9 log CFU/mL) and encapsulated (1 g corresponding to around 9 log CFU/g beads) cells at 37°C to 9 mL SGF (0, 30, 60, 90 and 120 min) and SIF (0, 1, 2, 3 and 4 h) individually. Further, the viable count was also examined in both SGF (2 h) and SIF (4 h), successively. At each time point, fluids containing free cells were neutralized by centrifuging at 10,000 g (Beckman GPR) for 5 min and washed twice with peptone water prior to serial dilutions.

3.6 Viability during storage up to 6 weeks

Encapsulated cells were stored in petri dish at 4°C and counted at 0, 2, 4 and 6 weeks. Each treatment was carried out in duplicate. Enumeration was done as described above.

3.7 Statistical data analysis

Logarithmic reductions in bacterial numbers during storage and as a consequence of SGF and SIF incubations were analyzed by one-way ANOVA and Tukey's multiple comparison tests (SPSS Statistics 22, Chicago, IL). Significance was declared at $P < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Morphology study

Preliminary tests with both SEM and ESEM were conducted (images not shown). In the SEM primary test, after critical point drying during preparation, all specimens shrank to around 1 mm. The desiccated samples were mounted on a pin-shaped SEM stub prior to imaging. Two different voltages (5.0 kV and 2.0 kV) were applied for trials. It was observed that the surface of all samples were coarse and aggregated severely after dehydration. Alginate beads with and without cells exhibited very similar shapes, while the addition of xanthan gum resulted in a more spherical shape and comparatively smooth surface. More cavities were observed from alginate beads, but a much more porous structure resulted from the addition of xanthan gum. The randomly distributed rod-shape bumps were microbial cells encapsulated inside. SEM images presented the random distribution of cells in gel beads, but samples shrank significantly after dehydration, not being their original state, which was not desired. ESEM can show the surface morphology with little dehydration, but cannot present the distribution of cells. Additionally, when performing ESEM, the resolution was decreased because the input of water vapor into the chamber scattered the electron beam, which is an inevitable consequence in ESEM (Thiel and Toth 2005). Considering the research objectives (to study the effect on freshly made capsules result from the addition of xanthan gum and

involvement of probiotic cells) and the severe shrinking in SEM, a further morphology study with ESEM was performed.

Figure 4.1 (a and b) shows images of bead structure and surface morphology of the respective chitosan-coated alginate, chitosan-coated alginate-xanthan gum blank beads, and chitosan-coated alginate, chitosan-coated alginate-xanthan gum beads loaded with LGG captured under ESEM. All samples presented the spherical shape at the beginning of imaging, which was also the original state when beads were freshly made. Usually the spheres would make them easier to produce in an industrial scale and package, and be consumed (Wang and others 2013). All samples started out plump and then were dehydrated a little bit over the course of the imaging process. The original size was around 2 mm in diameter (A-1 and D-1), and shrank to near 1.75 mm (A-2 and D-2) at the end of imaging (after around 10 min). With respect to the morphology, the size of beads made through the extrusion method fell into the milliliters range compare to the emulsion method (from tens of microns to hundreds of microns), as reviewed in Chapter 2 (Albertini and others 2010; Sohail and others 2011). A few specimens cracked because of partial collapsing of the polymer cross-link during dehydration, which was observed during examination of surface structure (A-3 and C-2). Also, the ESEM images revealed that cells were completely enclosed in the capsules; no cells were found on the external surface. However, with different levels of dehydration, both SEM (images not shown) and ESEM indicated that the incorporation of cells barely influenced the structure of the polymer network. Further, similar to SEM, the addition of xanthan gum in forming microcapsules resulted in relatively less severe wrinkles (A-4 vs. C-2, B-1 vs. D-3 and B-

2 vs. D-4), probably because the cross-link was changed among polymers with the involvement of xanthan gum. All these observations agreed with previous reports or had a similar pattern (Albertini and others 2010; Pasparakis and Bouropoulos 2006; Sohail and others 2011; Fareez and others 2015).

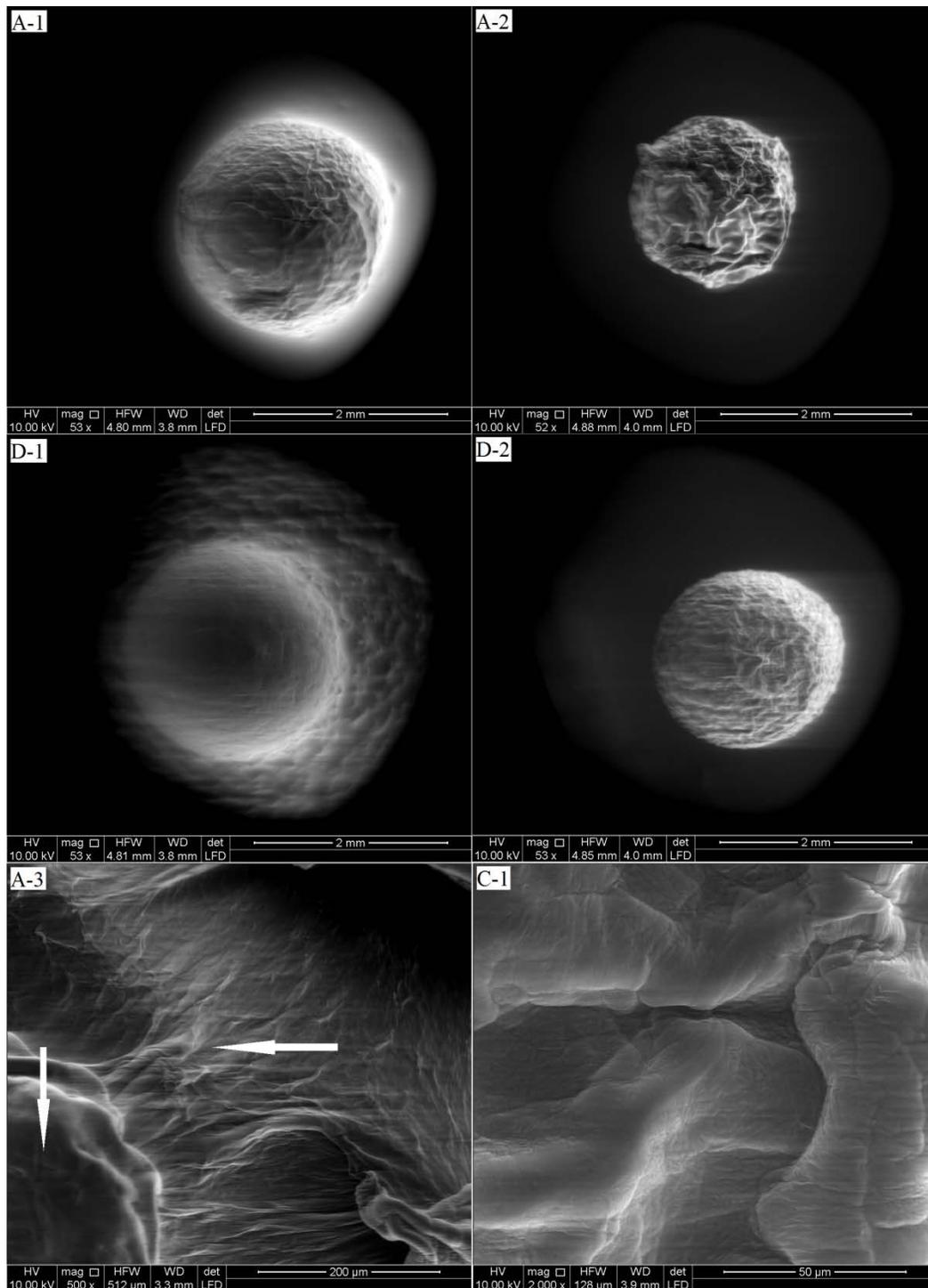


Figure 4.1a ESEM images of beads. A: AL blank beads. C: AL-XG blank beads. D: AL-XG beads loaded with LGG. A-1 and D-1: Beginning of imaging. A-2 and D-2: End of imaging. A-3: The down arrow shows the layer out of crack and the left arrow highlights the inside of a severe crack. C-1: Surface morphology of beads at 2000x.

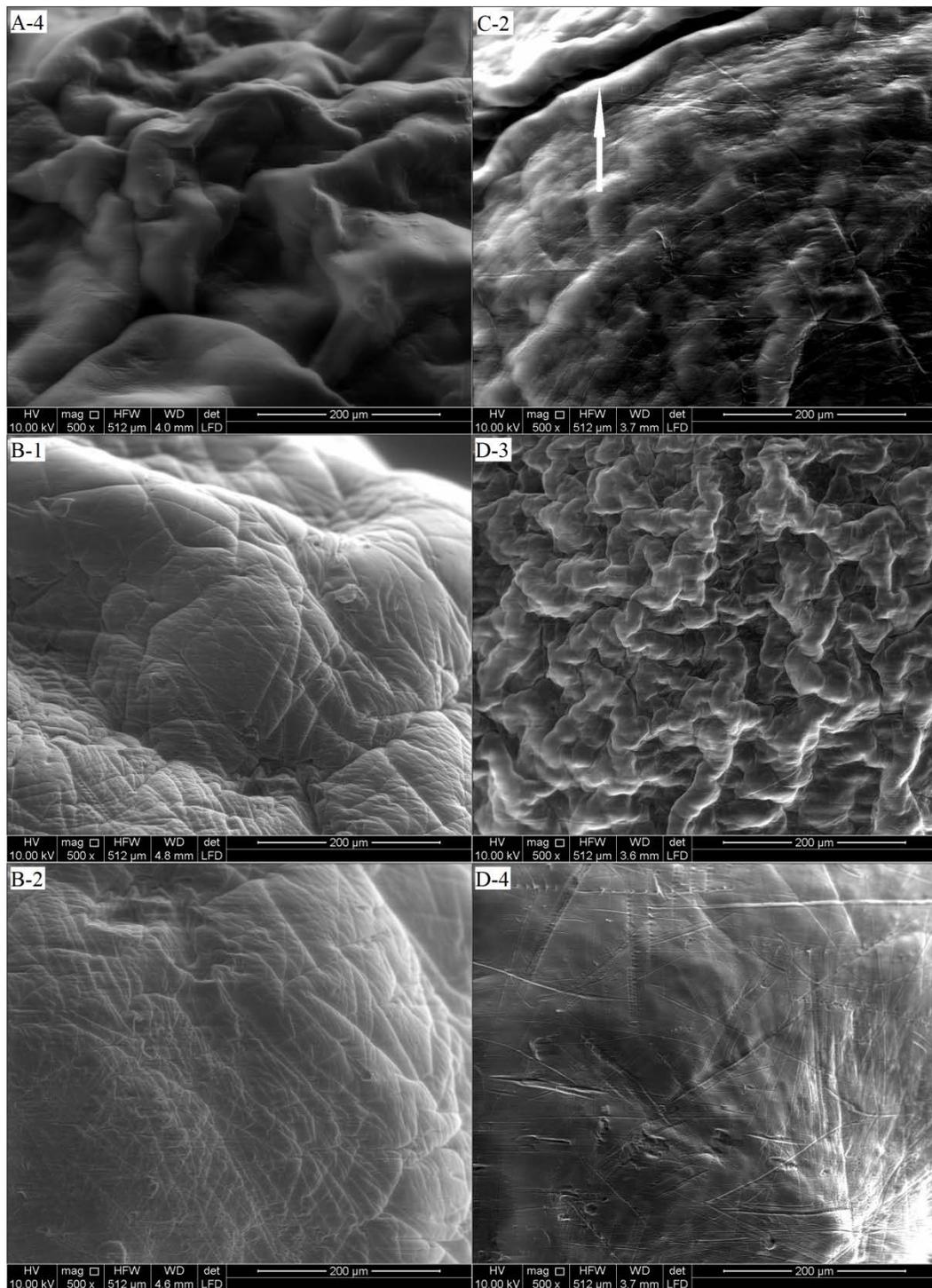


Figure 3.1b ESEM images of beads. A: AL blank beads. B: AL beads loaded with LGG. C: AL-XG blank beads. D: AL-XG beads loaded with LGG. A-4, C-2, B-1 and D-3: Surface morphology of beads at the beginning of imaging. B-2 and D-4: Surface morphology of beads at the end of imaging.

4.2 Encapsulation yield

Encapsulation yield (EY), which is sometimes called encapsulation efficiency (EE), of four formulations was presented in Table 4.1. In most studies using alginate or alginate-chitosan matrix as capsulation materials, EY was reported with the exponential values of the cell counts as in the current research (Chávarri and others 2010; Corbo and others 2013a; Corbo and others 2011; Vodnar and Socaciu 2014; Bajracharya and others 2012). That means EYs of 0.1, 1 and 10% could be derived when the difference of cell counts between cell suspension and beads were 3, 2 and 1 log CFU/mL or log CFU/g. In other cases, some scientists chose logarithmic values when calculating EY (Haghshenas and others 2015; Rodklongtan and others 2014). The latter method of calculation for EY comparatively resulted in a higher rate mathematically. There were no significant differences in EY among these gel formulations. Therefore, the EY in this study was formulation-independent. However, EY varies upon many factors. A previous study reported different EYs with a high concentration of alginate among strains from a minimum 54.8% (*L. reuteri* DSMZ 20016) to a maximum level of 83.33% (*L. rhamnosus* DSMZ 20021) (Corbo and others 2011); the addition of prebiotic substances could possibly reduce the efficiency (Chávarri and others 2010; Vodnar and Socaciu 2014), however, in another recent study, encapsulation with alginate blends, including those added with psyllium and fenugreek gum yielded an efficiency of higher than 98% for all the formulations (Haghshenas and others 2015); a few studies showed that alginate beads presented high EYs of 93% (Corbo and others 2013a) and 99% (Bajracharya and others 2012) compared with data available in the literature; an optimized microencapsulation

condition was obtained with performance of response surface methodology and the model showed that maximum yield of immobilized cells was 80.98% with 0.45M CaCl₂, biomass concentration of 10¹⁰ CFU/mL and 30 min hardening time (Trabelsi and others 2013). Besides, in a study where alginate-based beads were made through an emulsion method instead of extrusion, it was revealed that high pressure by a microfluidizer or high stirring speed by an ultra-turrax homogenizer led to lower EY, though they both efficiently reduced the capsule size (Ding and Shah 2009a); a later report agreed with the lowering in encapsulation efficiency because of high stirring speed, evidencing the sensitivity of the microorganisms to the shear rate (Rosas-Flores and others 2013).

Table 4.1 Four formulations with alginate and xanthan gum and EY

Formulations	Alginate (% w/v)	Xanthan gum (% w/v)	EY (%)
F1	2.0	0.00	88.11 ± 5.53 ^a
F2	2.0	0.15	81.96 ± 0.67 ^a
F3	2.5	0.00	78.38 ± 2.67 ^a
F4	2.5	0.15	83.87 ± 0.02 ^a

^{ab}Means in the same column followed by different lowercase letters were significantly different ($P < 0.05$); Results were expressed as mean ± SEM (standard error of mean).

4.3 Simulated gastrointestinal fluids tolerance

4.3.1 Simulated gastric acid tolerance

One of the major issues that compromise the health benefits of probiotics is its sensitivity to gastric pH. As shown in Figure 4.2, the unencapsulated LGG had poor survivability in SGF with pH 2.0. After 60 min, the viable cell count of free cells was already significantly lower (decreased by 26%) than the initial value ($P < 0.05$), and the count dropped by around 4 log CFU/mL (decreased by 40%) in 120 min. Hydrochloric acid present in the stomach is a strong oxidizing agent, and that can diffuse through the bacterial cell membrane and destroy important cellular components like proteins, fatty acids and nucleic acids (Rodklongtan and others 2014). Considering that the transit time of feed in a dog's stomach can be up to 1.5~2 h (Lui and others 1986), a level of around 5.5 log CFU/mL viable cells is possibly not adequate to exert health benefits.

On the other hand, although the number of cells dropped slightly, the viability of encapsulated cells in the four formulations after 120 min exposure to SGF was not significantly different from their initial values ($P > 0.05$). Thus, the chitosan-coated alginate-based microcapsules in this study showed a protective effect against the strong acid and successfully maintain a recommended bacterial cell count ($\geq 10^7$ CFU/mL) transiting through the simulated gut. The results were in agreement with many previous reports (Ouwehand and Salminen 1998; Capela and others 2006; Mokarram and others 2009; Manojlović and others 2010; Trabelsi and others 2013). The protective effect was likely because of the buffering effect of the hydrogel matrix resulting from its protonation of the acid groups when the pH was lower than its pKa (Li and others 2009), and the

restricted diffusion of the acid medium because of the coating of chitosan (Sohail and others 2011).

No significant difference was detected among the viability of the encapsulated cells under different formulations at 120 min ($P>0.05$). The increase in alginate concentration or the addition of xanthan gum tended to stabilize the structure of capsules. In the literature, the viability of trapped cells in SGF usually was dramatically improved when the alginate concentration increased by at least 1% w/v, and the common used/studied range was 2~4% (w/v). This was because alginate concentration below 2% could not form spherical beads and high concentration ($\geq 5\%$) could not form droplets due to its high viscosity (Lee and Heo 2000; Mandal and others 2006). On the other hand, the addition of xanthan gum did not show significant improved protection for cells when incorporated in 2.0 or 2.5% alginate mixture, but the relatively higher viability of cells after 120 min exposure to SGF was found in chitosan-coated alginate (2.5%)-xanthan gum microcapsules. This agreed with a very recent report (Fareez and others 2015), which was claimed to be the first time that the utilization of chitosan-coated alginate-xanthan gum beads were studied. There are few reports on the interactions among these three polymers, but based on the previous studies, it can be deduced that the enhancement of cell survivability lies in that H^+ ions were captured by negative charged xanthan gum, thus reducing the acidic stress on bacterial metabolism (Jiménez-Pranteda and others 2012).

One thing that can be observed in Figure 4.2 is that cells encapsulated in 2.0% alginate gel beads coated with chitosan were the least stable among all the formulations.

The viable cell count changed from 8.55 ± 0.05 log CFU/g to 7.18 ± 0.50 log CFU/g (decreased by 1.37 log CFU/g, the only group whose reduction was higher than 1 log unit). It might have resulted from the fact that under low pH, chitosan, whose pKa is 6.5, was soluble due to the quaternization of the amino group on D-glucosamine residues (Sogias and others 2010). Thus, the alginate-chitosan interaction was weakened or destroyed, while the junction zone inside the calcium-alginate gel remained. Consequently, at a pH lower than the pKa of alginate, the binding between alginate and chitosan was low, and the swelling behavior of microcapsules might happen and acid might penetrate the gel beads (Dai and others 2008). However, the decrease in cell viability observed was minor, and this indicated the structural stability of capsules passing through stomach.

On the other hand, there were some controversial reports on survivability of probiotics encapsulated in alginate-based polymers, where the entrapped cells did not have a higher viability in SGF (Sultana and others 2000; Brinques and Ayub 2011). These conflicting reports in the literature reaffirmed that it is difficult to compare the results of probiotic survivability experiments published by different authors because of the complicated and differing factors, such as bacterial strain, encapsulation material combination, encapsulation method and simulated media (Sohail and others 2011; Jiménez-Pranteda and others 2012).

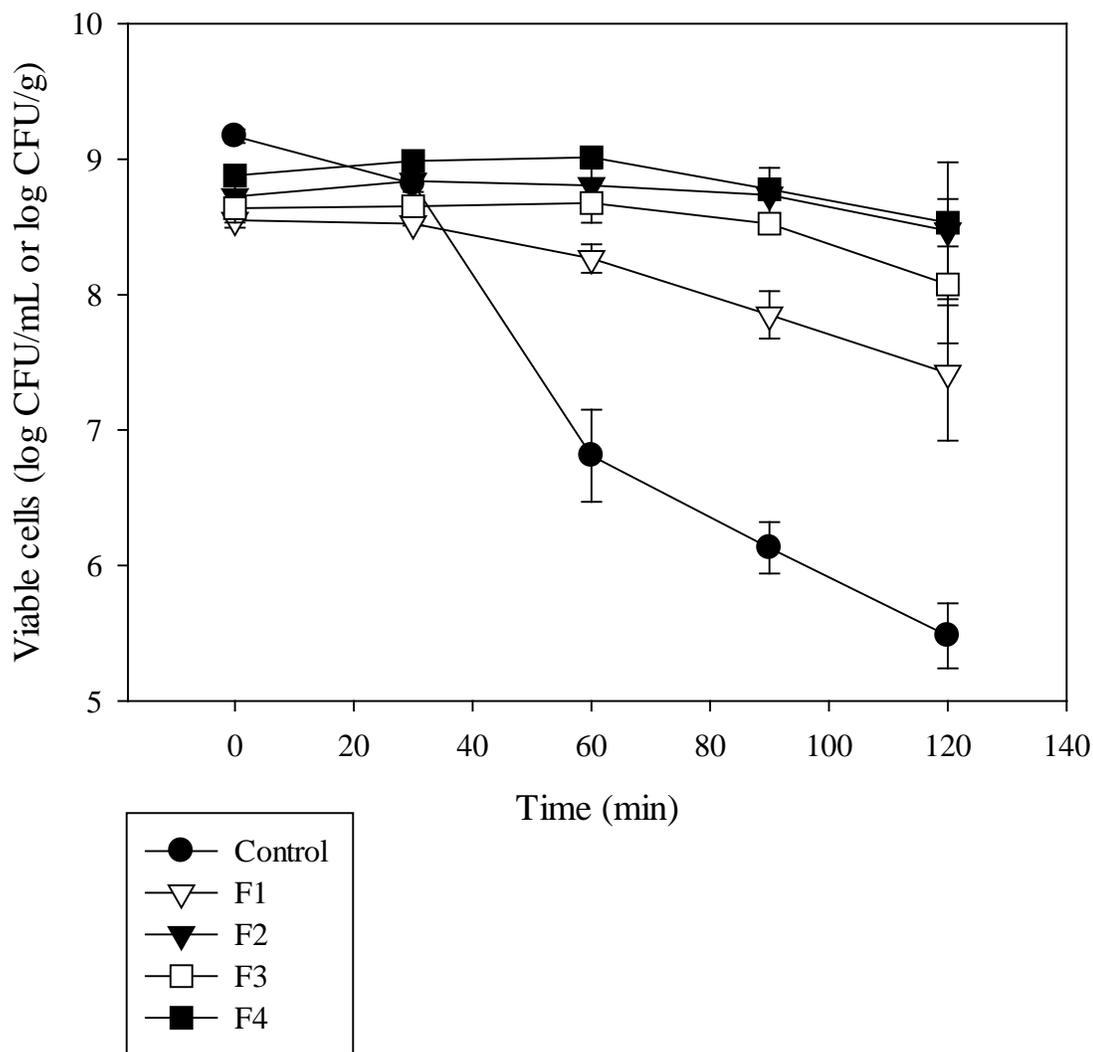


Figure 4.2 Effect of SGF on viability of free and entrapped *L. rhamnosus* GG in four formulations. Control: Free cells. F1: Cells encapsulated in 2.0% alginate. F2: Cells encapsulated in 2.0% alginate+0.15% xanthan gum. F3: Cells encapsulated in 2.5% alginate. F4: Cells encapsulated in 2.5% alginate+0.15% xanthan gum. Error bars present standard error of mean.

4.3.2 Simulated intestinal fluid tolerance

The study of the tolerance of the probiotic to SIF was conducted under two conditions: one treatment was exposing free and encapsulated cells directly to SIF (Figure 4.3), and the other was the exposure of free and encapsulated cells to SIF sequentially after being treated with SGF for 2 h (Figure 4.4). Bile can emulsify lipids, facilitating the formation of micelles, which promote digestion and absorption of fat in a diet. The function of bile at high concentrations is to rapidly dissolve membrane lipids and cause damage of cell membrane, which will result in the leakage of cell content and cell death (Begley and others 2005). The tolerance of bile salts is considered one of the important criteria in the selection of probiotics (Rodklongtan and others 2014). Previous studies revealed that LGG strain could grow in nutritious media with bile up to 3% if being incubated for a sufficient time though it was stressed and damaged at the initial exposure (Gorbach and Goldin 1989; Succi and others 2005; Sohail and others 2011; Jiménez-Pranteda and others 2012). However, it was observed that the SIF treatment alone resulted in significant reduction ($P < 0.05$) in the viability of free LGG while cells exposed to SIF sequentially after incubation in SGF dropped a little in number ($P > 0.05$). Conditions of the ambient environment prior to the entry of a bacterial strain into the small intestine will determine the effects of bile salts on its viability, and exposure to various pHs, growth media and temperatures may select the robust cells from the population and thus decrease the loss of viability in later phase.

The number of encapsulated LGG exposed to only SIF remained stable as observed ($P > 0.05$). At $\text{pH} > 6.5$, chitosan is insoluble and the coating on alginate

microcapsules externally has been extensively proved effective on improving the viability of probiotic bacteria therein (Sogias and others 2010; Trabelsi and others 2013; Fareez and others 2015; Sohail and others 2011; Iyer and Kailasapathy 2005). On the contrary, the viable counts of cells entrapped in all formulations dropped dramatically to a level which had no difference from control. Among the four formulations, cells in 2% (w/v) alginate beads lost maximum viability, and the final survivability was significantly lower than that in the two 2.5% alginate-matrixes. In the literature, most scientists studied the viability of free and encapsulated probiotics in SIF or bile, which was applied directly; the alginate-based capsules were proved effective in enhancing their survival with little decrease in viable cell counts (Brinques and Ayub 2011; Trabelsi and others 2013; Vodnar and Socaciu 2014; Haghshenas and others 2015), which confirmed with what was observed in this study. The viability of free and coated cells during exposure to SIF sequentially after being incubated in SGF or at low pHs was reported in some other explorations (Sathyabama and Vijayabharathi 2014; Mokarram and others 2009; Gbassi and others 2009). Again, comparison is difficult because these authors never used the same strains and simulated media. Cell release and swelling behavior of polymer beads were investigated earlier (Argin and others 2014; Cook and others 2011; Fareez and others 2015). However, beads in this study were integral after the whole process. Also, in preliminary tests (data not shown) the few cells collected from simulated juices were not sufficient to contribute to the viable count of cells remained in capsules. Observations in this study revealed a more complicated performance of encapsulated cells exposed in SIF after sequential incubation in SGF. What possibly happened was, the diffusion of acid

medium weakened the chemical bonds among polymers, continued damaging cells encapsulated and lowering the buffering effect of polysaccharides while these were exposed to SIF. Few studies compared the viability of encapsulated cells in these two conditions and little discussion on the interactions among the three polymers is available. The overall situation, however, indicated that in this study the most and least stable microcapsules in simulated gastrointestinal fluids were chitosan-coated 2.5% alginate-xanthan gum and chitosan-coated 2.0% alginate beads, respectively. These results probably were because that beads of higher density and various polymers offered better buffering effect and thus minimal viability loss occurred (Brinques and Ayub 2011; Su and others 2011; Fareez and others 2015).

It must be noted that the resistance of probiotics in the simulated gastrointestinal systems possibly cannot truly reflect their ability to tolerate the conditions *in vivo*. It is difficult to simulate exactly *in vivo* conditions in laboratory (e.g. Gastric acid and bile acid levels vary in different physiological status). Also, the presence of food in the digestive tract may affect survival as certain microenvironments in food matrix can impair the damages (Begley and others 2005; McConnell and others 2008). Though with these limitations, for reasons of economics or time, simulated buffer systems are consistently necessary in research prior to the application of animal models or clinical study.

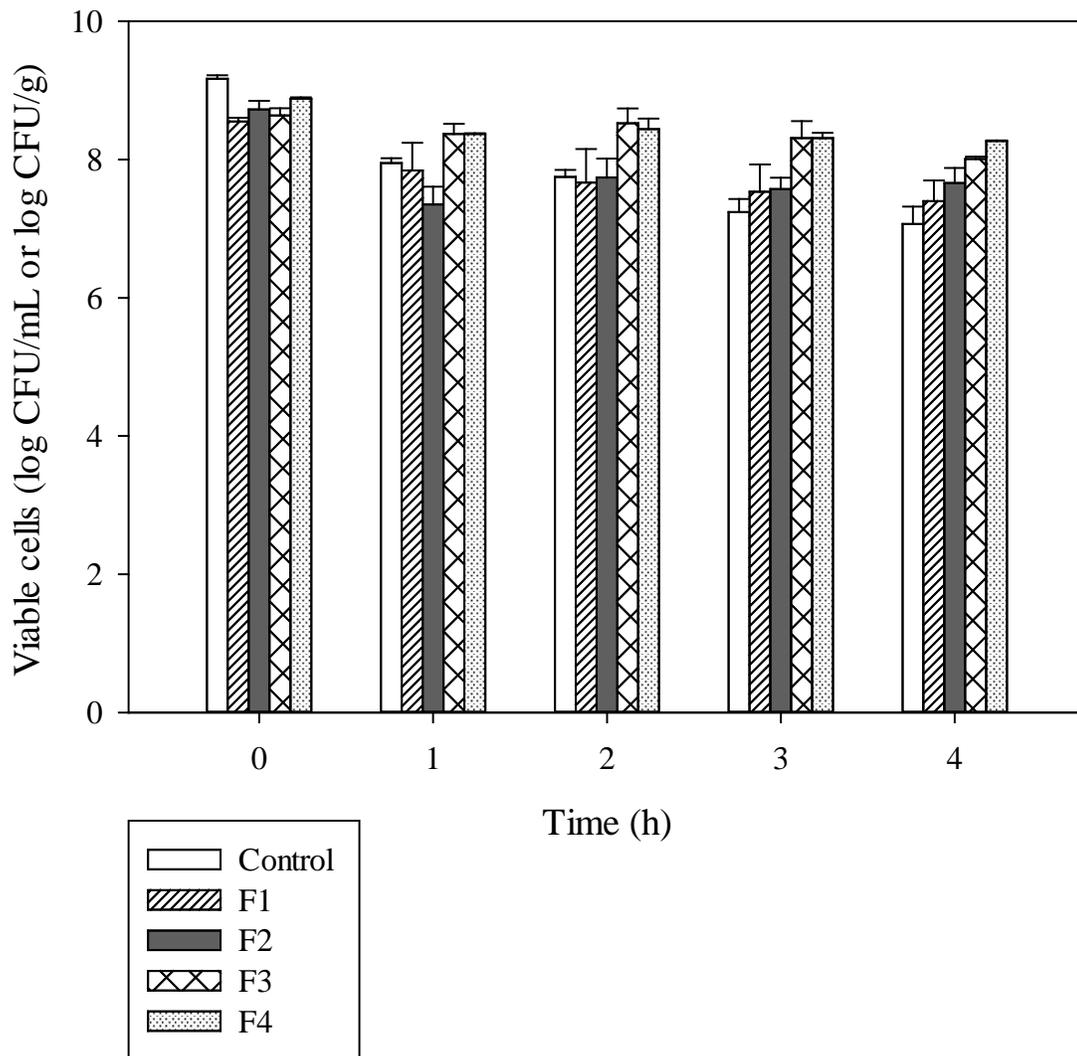


Figure 4.3 Effect of SIF on viability of free and entrapped *L. rhamnosus* GG in four formulations. Control: Free cells. F1: Cells encapsulated in 2.0% alginate. F2: Cells encapsulated in 2.0% alginate+0.15% xanthan gum. F3: Cells encapsulated in 2.5% alginate. F4: Cells encapsulated in 2.5% alginate+0.15% xanthan gum. Error bars present standard error of mean.

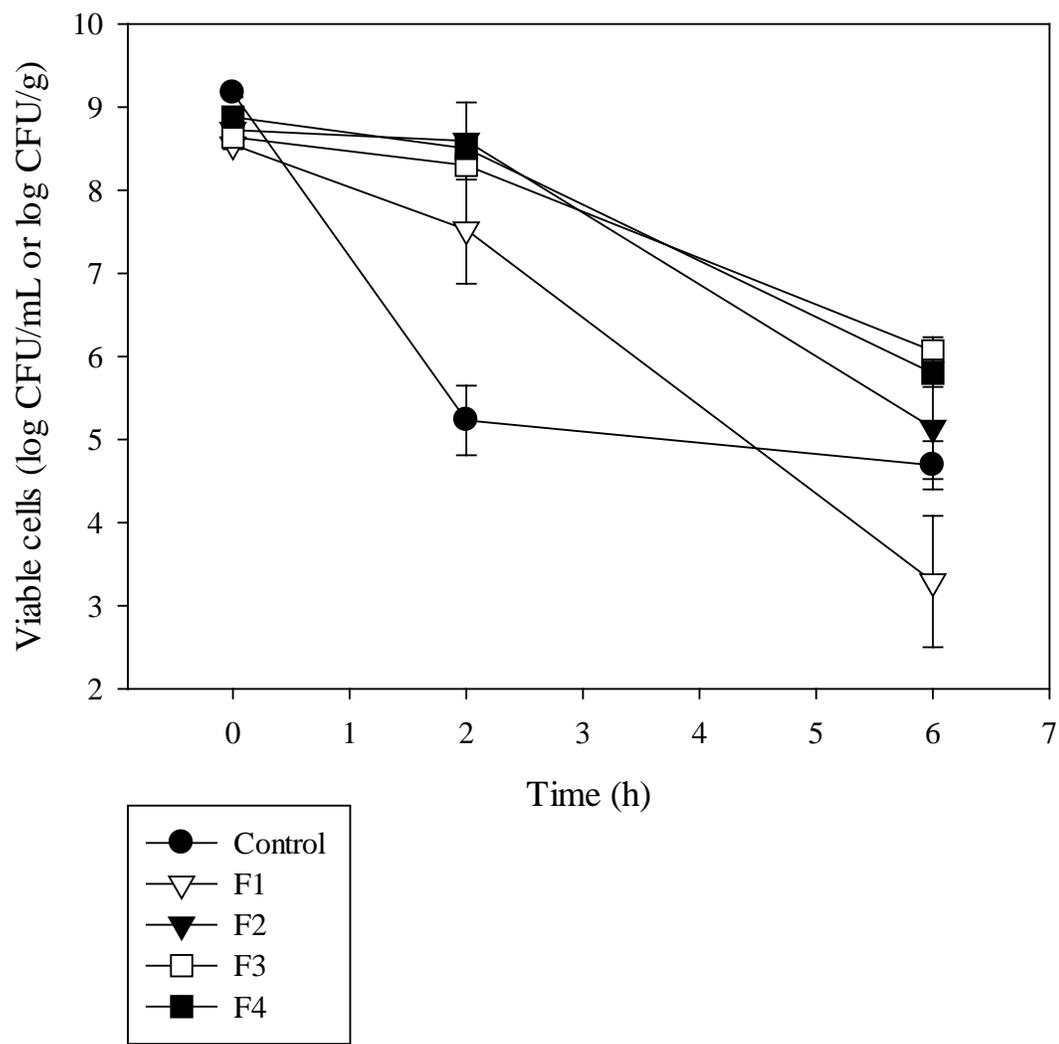


Figure 4.4 Effect of SGF+SIF successively on viability of free and entrapped *L. rhamnosus* GG in four formulations. Control: Free cells. F1: Cells encapsulated in 2.0% alginate. F2: Cells encapsulated in 2.0% alginate+0.15% xanthan gum. F3: Cells encapsulated in 2.5% alginate. F4: Cells encapsulated in 2.5% alginate+0.15% xanthan gum. Error bars present standard error of mean.

4.4 Viability during storage up to 6 weeks

The viability of probiotics during storage is the final concern to evaluate the stability of beads for application in industry. Experiments were performed in order to assess the viability changes of the encapsulated probiotic under refrigeration and the results showing the survival of LGG during 6 weeks of storage at 4°C are presented in Figure 4.5. As the beads were considered the products in this study, the viability of free cells was not tested. The counts of encapsulated cells in the four formulations displayed a gradual reduction of LGG survivability to 4~7 log CFU/g in 6 weeks. No significant difference was detected among viable cell counts in these formulations at each time point ($P>0.05$), although cells in chitosan-coated alginate (2.5%)-xanthan gum remained in high numbers after 6 weeks. Most importantly, all beads showed LGG counts of greater than 10^7 CFU/g for 4 weeks, the minimum value recommended for a product containing probiotics to confer health benefits (Ouweland and Salminen 1998; Capela and others 2006; Mokarram and others 2009; Manojlović and others 2010). The survival of probiotics is significantly influenced by factors like composition of food, types of packaging material and storage environment parameters, such as temperature, moisture content, relative humidity and oxygen content (Tripathi and Giri 2014). With the involvement of microencapsulation, the change in viability lies in how the microcapsule interferes with the reaction of probiotics towards those factors (Tripathi and Giri 2014). Again, due to different strains, materials and methods of microencapsulation, and storage conditions, previous studies reported variously, maintaining a viability of at least 10^6 CFU/g ranging from 2 weeks to 8 months, which are difficult to compare (Corbo and

others 2011; Fareez and others 2015; Iyer and Kailasapathy 2005; Brinques and Ayub 2011; Bajracharya and others 2012; Trabelsi and others 2013; Vodnar and Socaciu 2014; Sohail and others 2013; Albertini and others 2010).

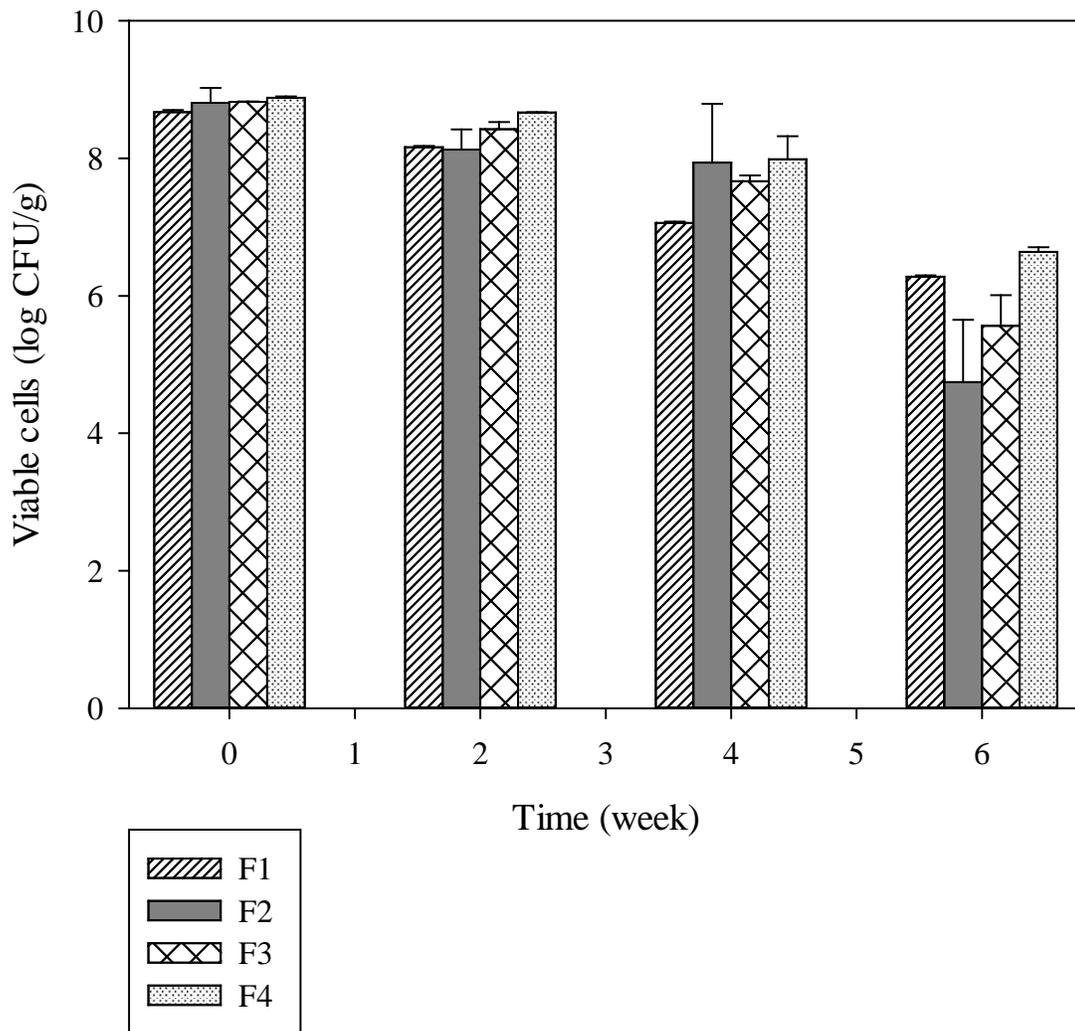


Figure 4.5 Viability of encapsulated cells during storage in refrigerator (4°C) up to 6 weeks. F1: Cells encapsulated in 2.0% alginate. F2: Cells encapsulated in 2.0% alginate+0.15% xanthan gum. F3: Cells encapsulated in 2.5% alginate. F4: Cells encapsulated in 2.5% alginate+0.15% xanthan gum. Error bars present standard error of mean.

CHAPTER 5

CONCLUSIONS AND FURTHER DIRECTIONS

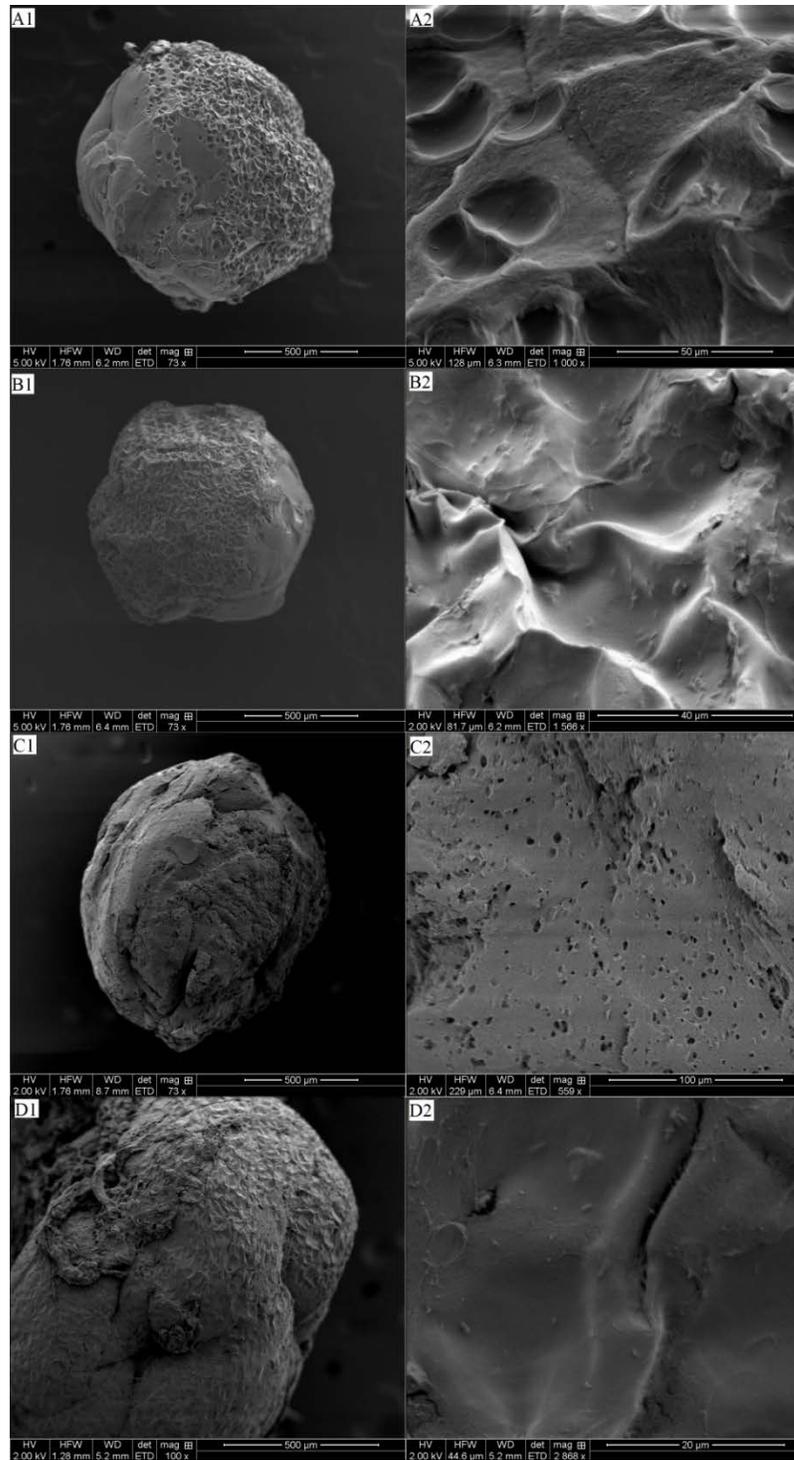
Microencapsulation is critical to ensure viability of probiotics transiting through the digestive system. So far, there are many publications on encapsulation with alginate-chitosan, alginate-xanthan gum or xanthan gum-chitosan (Iyer and Kailasapathy 2005; Albertini and others 2010; Chávarri and others 2010; Brinques and Ayub 2011; Cook and others 2011; Sohail and others 2011; Bajracharya and others 2012; Trabelsi and others 2013; Rodklongtan and others 2014; Argin and others 2014; Vodnar and Socaciu 2014); however, beads consisting of these three biocompatible polymers were rarely reported (Fareez and others 2015). In the present study, microcapsules of LGG were developed using chitosan-coated alginate or alginate-xanthan gum matrix. These capsules were able to improve the viability of LGG ($P < 0.05$), maintaining them at a level of $>10^7$ CFU/g when exposed to SGF and SIF separately. However, results of sequential incubation in SIF after SGF revealed a dramatic reduction in viability of encapsulated cells with no difference from control, which cannot be explained with little information in the literature. No difference was detected in the bead appearance by ESEM, except for the alginate beads incorporated with xanthan gum that resulted in less severe wrinkles on the surface. Other than the above, all the encapsulation formulations achieved EYs of around 80% and the viability was maintained at least 10^7 CFU/g after 4 weeks of storage at refrigeration temperature. The overall situation indicated that the most stable

microcapsules were chitosan-coated alginate (2.5% w/v)-xanthan gum (0.15% w/v) beads. These results indicated the potential of industrial application of chitosan-coated alginate-xanthan gum microcapsules.

To incorporate the microcapsules in this study into pet food, other important properties, such as heat resistance, sensory properties and animal models, should be assessed. Heat treatment is often inevitable in food manufacturing, like spray-drying, cooking or pasteurization (Tripathi and Giri 2014). The particle size and hardness affect the mouthfeel properties of food. In general, soft, rounded and small size ($< 80 \mu\text{m}$) that was not gritty was preferred (Tyle 1993). Encapsulated probiotics may perform differently from *in vitro* studies once being consumed. Therefore, sufficient data from *in vivo* exploration is necessary prior to industrial application (McConnell and others 2008). In addition, shelf-life of the encapsulated probiotics in this study may be improved by developing an advanced microencapsulation method, as it was revealed in many reports that stable viability of encapsulated probiotics were prolonged to months in a dry format stored in refrigeration even though preservation at room temperature is still a challenge (Albertini and others 2010; Bajracharya and others 2012; Sohail and others 2013).

APPENDIX A

Preliminary Test of SEM images for Beads Surface Morphology



A: 2% w/v alginate blank beads. B: 2% w/v alginate beads loaded with cells. C: 2% w/v alginate + 0.15% w/v xanthan gum blank beads. D: 2% w/v alginate + 0.15% w/v xanthan gum beads loaded with cells. The right column shows the respective surface structure to the left.

APPENDIX B

Viable Cell Counts during Resistance Tests

Effect of SGF on viability of free and entrapped *L. rhamnosus* GG in four formulations

	Viable cell count (log CFU/mL or log CFU/g)				
	0 min	30 min	60 min	90 min	120 min
Control	9.17 ± 0.05	8.82 ± 0.20	6.81 ± 0.34	6.13 ± 0.19	5.48 ± 0.24
F1	8.55 ± 0.05	8.53 ± 0.01	8.26 ± 0.11	7.82 ± 0.18	7.18 ± 0.50
F2	8.71 ± 0.13	8.83 ± 0.08	8.79 ± 0.13	8.69 ± 0.20	8.23 ± 0.51
F3	8.63 ± 0.11	8.65 ± 0.05	8.66 ± 0.15	8.53 ± 0.01	7.89 ± 0.44
F4	8.88 ± 0.02	8.99 ± 0.01	9.01 ± 0.07	8.78 ± 0.05	8.50 ± 0.18

Effect of SIF on viability of free and entrapped *L. rhamnosus* GG in four formulations

	Viable cell count (log CFU/mL or log CFU/g)				
	0 h	1 h	2 h	3 h	4 h
Control	9.17 ± 0.05	7.95 ± 0.07	7.75 ± 0.10	7.24 ± 0.19	7.07 ± 0.25
F1	8.55 ± 0.05	7.68 ± 0.40	7.44 ± 0.49	7.38 ± 0.40	7.30 ± 0.30
F2	8.71 ± 0.13	7.79 ± 0.25	7.66 ± 0.28	7.55 ± 0.17	7.62 ± 0.22
F3	8.63 ± 0.11	8.35 ± 0.15	8.48 ± 0.22	8.25 ± 0.25	8.01 ± 0.03
F4	8.88 ± 0.02	8.37 ± 0.01	8.42 ± 0.16	8.31 ± 0.08	8.27 ± 0.01

Effect of SGF and SIF on viability of free and entrapped *L. rhamnosus* GG in four formulations

	Viable cell count (log CFU/mL or log CFU/g)		
	0 h	2 h	6 h
Control	9.17 ± 0.05	5.23 ± 0.42	4.69 ± 0.29
F1	8.55 ± 0.05	7.16 ± 0.65	2.79 ± 0.79
F2	8.71 ± 0.13	8.39 ± 0.47	4.80 ± 0.62
F3	8.63 ± 0.11	8.29 ± 0.09	6.04 ± 0.17
F4	8.88 ± 0.02	8.50 ± 0.09	5.77 ± 0.17

Viability of entrapped cells during storage in refrigerator up to 6 weeks

	Viable cell count (log CFU/g)			
	0 week	2 week	4 week	6 week
F1	8.67 ± 0.03	8.16 ± 0.02	7.06 ± 0.02	6.28 ± 0.02
F2	8.75 ± 0.22	8.18 ± 0.30	7.38 ± 0.86	4.14 ± 0.91
F3	8.83 ± 0.02	8.42 ± 0.04	7.61 ± 0.25	5.37 ± 0.45
F4	8.88 ± 0.02	8.67 ± 0.00	7.87 ± 0.34	6.64 ± 0.06

REFERENCES

- Albertini B, Vitali B, Passerini N, Cruciani F, Di Sabatino M, Rodriguez L, Brigidi P. 2010. Development of microparticulate systems for intestinal delivery of *Lactobacillus acidophilus* and *Bifidobacterium lactis*. *European Journal of Pharmaceutical Sciences* 40(4):359-66.
- And CI, Kailasapathy K. 2005. Effect of co-encapsulation of probiotics with prebiotics on increasing the viability of encapsulated bacteria under *in vitro* acidic and bile salt conditions and in yogurt. *Journal of Food Science* 70(1):M18-M23.
- Apás AL, González SN, Arena ME. 2014. Potential of goat probiotic to bind mutagens. *Anaerobe* 28:8-12.
- APPA. 2014. APPA National Pet Owners Survey Statistics: Pet ownership and annual expenses. 2013–2014 Survey.
- Argin S, Kofinas P, Lo YM. 2014. The cell release kinetics and the swelling behavior of physically crosslinked xanthan–chitosan hydrogels in simulated gastrointestinal conditions. *Food Hydrocolloids* 40:138-44.
- Bajracharya P, Islam MA, Jiang T, Kang S-K, Choi Y-J, Cho C-S. 2012. Effect of microencapsulation of *Lactobacillus salivarius* 29 into alginate/chitosan/alginate microcapsules on viability and cytokine induction. *Journal of Microencapsulation* 29(5):429-36.
- Begley M, Gahan CG, Hill C. 2005. The interaction between bacteria and bile. *FEMS Microbiology Reviews* 29(4):625-51.
- Benyacoub J, Czarnecki-Maulden GL, Cavadini C, Sauthier T, Anderson RE, Schiffrin EJ, Von der Weid Tv. 2003. Supplementation of food with *Enterococcus faecium* (SF68) stimulates immune functions in young dogs. *Journal of Nutrition* 133(4):1158-62.
- Black FT, Andersen PL, Ørskov J, Ørskov F, Gaarslev K, Laulund S. 1989. Prophylactic efficacy of Lactobacilli on traveler's diarrhea. *Travel Medicine: Springer Berlin Heidelberg*. p. 333-5.
- Brinques GB, Ayub MAZ. 2011. Effect of microencapsulation on survival of *Lactobacillus plantarum* in simulated gastrointestinal conditions, refrigeration, and yogurt. *Journal of Food Engineering* 103(2):123-8.
- Burgain J, Gaiani C, Linder M, Scher J. 2011. Encapsulation of probiotic living cells: From laboratory scale to industrial applications. *Journal of Food Engineering* 104(4):467-83.

- Capela P, Hay TKC, Shah NP. 2006. Effect of cryoprotectants, prebiotics and microencapsulation on survival of probiotic organisms in yoghurt and freeze-dried yoghurt. *Food Research International* 39(2):203-11.
- Chalova VI, Lingbeck JM, Kwon YM, Ricke SC. 2008. Extracellular antimutagenic activities of selected probiotic *Bifidobacterium* and *Lactobacillus* spp. as a function of growth phase. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes* 43(2):193-8.
- Chandramouli V, Kailasapathy K, Peiris P, Jones M. 2004. An improved method of microencapsulation and its evaluation to protect *Lactobacillus* spp. in simulated gastric conditions. *Journal of Microbiological Methods* 56(1):27-35.
- Chávarri M, Marañón I, Ares R, Ibáñez FC, Marzo F, Villarán MdC. 2010. Microencapsulation of a probiotic and prebiotic in alginate-chitosan capsules improves survival in simulated gastro-intestinal conditions. *International Journal of Food Microbiology* 142(1):185-9.
- Commane D, Hughes R, Shortt C, Rowland I. 2005. The potential mechanisms involved in the anti-carcinogenic action of probiotics. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis* 591(1):276-89.
- Cook MT, Tzortzis G, Charalampopoulos D, Khutoryanskiy VV. 2011. Production and evaluation of dry alginate-chitosan microcapsules as an enteric delivery vehicle for probiotic bacteria. *Biomacromolecules* 12(7):2834-40.
- Cook MT, Tzortzis G, Charalampopoulos D, Khutoryanskiy VV. 2012. Microencapsulation of probiotics for gastrointestinal delivery. *Journal of Controlled Release* 162(1):56-67.
- Corbo MR, Bevilacqua A, Gallo M, Speranza B, Sinigaglia M. 2013. Immobilization and microencapsulation of *Lactobacillus plantarum*: Performances and *in vivo* applications. *Innovative Food Science and Emerging Technologies* 18:196-201.
- Corbo MR, Bevilacqua A, Sinigaglia M. 2011. Shelf life of alginate beads containing lactobacilli and bifidobacteria: characterisation of microspheres containing *Lactobacillus delbrueckii* subsp. *bulgaricus*. *International Journal of Food Science & Technology* 46(10):2212-7.
- Corona-Hernandez RI, Álvarez-Parrilla E, Lizardi-Mendoza J, Islas-Rubio AR, de la Rosa LA, Wall-Medrano A. 2013. Structural stability and viability of microencapsulated probiotic bacteria: A review. *Comprehensive Reviews in Food Science and Food Safety* 12(6):614-28.
- Cutt H, Giles-Corti B, Knuiaman M, Burke V. 2007. Dog ownership, health and physical activity: A critical review of the literature. *Health and Place* 13(1):261-72.

- Dai YN, Li P, Zhang JP, Wang AQ, Wei Q. 2008. A novel pH sensitive N - succinyl chitosan/alginate hydrogel bead for nifedipine delivery. *Biopharmaceutics & drug disposition* 29(3):173-84.
- Darsanaki RK, Issazadeh K, Pahlaviani MRMK, Aliabadi MA. 2012. Antimutagenic activity of *Lactobacillus* spp. isolated from fresh vegetables against sodium azide and 2-nitrofluorene. *Journal of Pure and Applied Microbiology* 66(4):1677-82.
- De Vrese M, Stegelmann A, Richter B, Fenselau S, Laue C, Schrezenmeir J. 2001. Probiotics - Compensation for lactase insufficiency. *American Journal of Clinical Nutrition* 73(2):421S.
- Ding WK, Shah NP. 2009a. Effect of homogenization techniques on reducing the size of microcapsules and the survival of probiotic bacteria therein. *Journal of Food Science* 74(6):M231-M6.
- Ding WK, Shah NP. 2009b. Effect of various encapsulating materials on the stability of probiotic bacteria. *Journal of Food Science* 74(2):M100-M7.
- FAO/WHO. 2001. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria.
- Fareez IM, Lim SM, Mishra RK, Ramasamy K. 2015. Chitosan coated alginate-xanthan gum bead enhanced pH and thermotolerance of *Lactobacillus plantarum* LAB12. *International Journal of Biological Macromolecules* 72:1419-28.
- Fialho AM, Moreira LM, Granja AT, Popescu AO, Hoffmann K, Sá-Correia I. 2008. Occurrence, production, and applications of gellan: current state and perspectives. *Applied Microbiology and Biotechnology* 79(6):889-900.
- Gbassi GK, Vandamme T, Ennahar S, Marchioni E. 2009. Microencapsulation of *Lactobacillus plantarum* spp in an alginate matrix coated with whey proteins. *International Journal of Food Microbiology* 129(1):103-5.
- Goldin BR, Gorbach SL. 1984. The effect of milk and lactobacillus feeding on human intestinal bacterial enzyme activity. *The American Journal of Clinical Nutrition* 39(5):756-61.
- Gonzalez-Ortiz G, Castillejos L, Mallo JJ, Calvo-Torras MA, Baucells MD. 2013. Effects of dietary supplementation of *Bacillus amyloliquefaciens* CECT 5940 and *Enterococcus faecium* CECT 4515 in adult healthy dogs. *Archives of Animal Nutrition* 67(5):406-15.
- Gorbach SL, Goldin BR. 1989. *Lactobacillus* strains and methods of selection. U.S. Patent No. 4839281.

- Graff S, Hussain S, Chaumeil J-C, Charrueau C. 2008. Increased intestinal delivery of viable *Saccharomyces boulardii* by encapsulation in microspheres. *Pharmaceutical Research* 25(6):1290-6.
- Haghshenas B, Abdullah N, Nami Y, Radiah D, Rosli R, Yari Khosroushahi A. 2015. Microencapsulation of probiotic bacteria *Lactobacillus plantarum* 15HN using alginate-psyllium-fenugreek polymeric blends. *Journal of Applied Microbiology* 118(4):1048-57.
- Hamilton-Miller JMT. 2003. The role of probiotics in the treatment and prevention of *Helicobacter pylori* infection. Netherlands: Elsevier B.V. p. 360-6.
- Hansen LT, Allan-Wojtas PM, Jin YL, Paulson AT. 2002. Survival of Ca-alginate microencapsulated *Bifidobacterium* spp. in milk and simulated gastrointestinal conditions. *Food Microbiology* 19(1):35-45.
- Hébrard G, Hoffart V, Cardot J-M, Subirade M, Beyssac E. 2013. Development and characterization of coated-microparticles based on whey protein/alginate using the encapsulator device. *Drug Development and Industrial Pharmacy* 39(1):128-37.
- Ishimwe N, Daliri EB, Lee BH, Fang F, Du G. 2015. The perspective on cholesterol-lowering mechanisms of probiotics. *Molecular Nutrition & Food Research* 59(1):94-105.
- Iyer C, Kailasapathy K. 2005. Effect of co-encapsulation of probiotics with prebiotics on increasing the viability of encapsulated bacteria under *in vitro* acidic and bile salt conditions and in yogurt. *Journal of Food Science* 70(1):M18-M23.
- Jiménez-Pranteda ML, Poncelet D, Náder-Macías ME, Arcos A, Aguilera M, Monteoliva-Sánchez M, Ramos-Cormenzana A. 2012. Stability of lactobacilli encapsulated in various microbial polymers. *Journal of Bioscience and Bioengineering* 113(2):179-84.
- Kararli TT. 1995. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharmaceutics and Drug Disposition* 16(5):351-80.
- Katzbauer B. 1998. Properties and applications of xanthan gum. *Polymer Degradation and Stability* 59(1-3):81-4.
- Kelley RL, Minikhiem D, Kiely B, O'Mahony L, O'Sullivan D, Boileau T, Park JS. 2009. Clinical benefits of probiotic canine-derived *Bifidobacterium animalis* strain AHC7 in dogs with acute idiopathic diarrhea. *Veterinary Therapeutics* 10(3):121-30.

- Kent RM, Doherty SB. 2014. Probiotic bacteria in infant formula and follow-up formula: Microencapsulation using milk and pea proteins to improve microbiological quality. *Food Research International* 64:567-76.
- Khalesi S, Sun J, Buys N, Jayasinghe R. 2014. Effect of probiotics on blood pressure: A systematic review and meta-analysis of randomized, controlled trials. *Hypertension* 64(4):897-903.
- Khan AA, Khurshid M, Khan S, Alshamsan A. 2013. Gut microbiota and probiotics: current status and their role in cancer therapeutics. *Drug Development Research* 74(6):365-75.
- Knight S, Edwards V. 2008. In the Company of Wolves: The physical, social, and psychological benefits of dog ownership. *Journal of Aging and Health* 20(4):437-55.
- Knorr R, Cavadini C, Benyacoub J, Satyaraj E. 2006. Probiotic enterococci for improved immunity. U.S. Patent Application 11/544,120.
- Kollaritsch H, Holst H, Grobara P, Wiedermann G. 1993. Prevention of traveler's diarrhea with *Saccharomyces boulardii*. Results of a placebo controlled double-blind study. *Fortschritte der Medizin* 111(9):152.
- Korpela R, Von Wright A, Rätty K, Holma R, Tuure T, Mykkänen H. 2010. The effect of rye bread and *Lactobacillus* GG fermented whey drink on fecal and urinary mutagenicity and fecal enzyme activities. *International Journal of Probiotics and Prebiotics* 5(3):125-32.
- Krasaekoopt W, Bhandari B, Deeth H. 2003. Evaluation of encapsulation techniques of probiotics for yoghurt. *International Dairy Journal* 13(1): 3-13.
- Krasaekoopt W, Bhandari B, Deeth H. 2004. The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria. *International Dairy Journal* 14(8):737-43.
- Kumar M, Henry CJK, Jain S, Yadav H, Kumar A, Nagpal R, Mohania D, Behare P, Verma V, Kumar P, Poddar D, Aggarwal PK. 2010. Cancer-preventing attributes of probiotics: an update. *International Journal of Food Sciences and Nutrition* 61(5):473-96.
- Lahtinen SJ, Ouwehand AC, Salminen SJ, Forssell P, Myllärinen P. 2007. Effect of starch- and lipid-based encapsulation on the culturability of two *Bifidobacterium longum* strains. *Letters in Applied Microbiology* 44(5):500-5.
- Lakkis JM. 2008. Encapsulation and controlled release technologies in food systems. John Wiley & Sons.

- Lankaputhra WEV, Shah NP. 1998. Antimutagenic properties of probiotic bacteria and of organic acids. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 397(2):169-82.
- Lee K-Y, Heo T-R. 2000. Survival of *Bifidobacterium longum* immobilized in calcium alginate beads in simulated gastric juices and bile salt solution. *Applied and Environmental Microbiology* 66(2):869-73.
- Li S, He Y, Peng Q-l, Qin X, Zeng Z-y, Huang X-l, Sui J-z, Chen S-y, Xie Y-t, Deng Y, Wang J, Xie L, Li T-j. 2014. Meta-analysis of randomized controlled trials on the efficacy of probiotics in *Helicobacter pylori* eradication therapy in children. *European Journal of Pediatrics* 173(2):153-61.
- Li X, Xie H, Lin J, Xie W, Ma X. 2009. Characterization and biodegradation of chitosan–alginate polyelectrolyte complexes. *Polymer Degradation and Stability* 94(1):1-6.
- Lin J, Yu W, Liu X, Xie H, Wang W, Ma X. 2008. *In vitro* and *in vivo* characterization of alginate-chitosan-alginate artificial microcapsules for therapeutic oral delivery of live bacterial cells. *Journal of Bioscience and Bioengineering* 105(6):660-5.
- Lionetti E, Indrio F, Pavone L, Borrelli G, Cavallo L, Francavilla R. 2010. Role of probiotics in pediatric patients with *Helicobacter pylori* infection: A comprehensive review of the literature. *Helicobacter* 15(2):79-87.
- Lo P-R, Yu R-C, Chou C-C, Huang EC. 2004. Determinations of the antimutagenic activities of several probiotic bifidobacteria under acidic and bile conditions against benzo[a]pyrene by a modified Ames test. *International Journal of Food Microbiology* 93(2):249-57.
- Lui CY, Amidon GL, Berardi RR, Fleisher D, Youngberg C, Dressman JB. 1986. Comparison of gastrointestinal pH in dogs and humans: Implications on the use of the beagle dog as a model for oral absorption in humans. *Journal of Pharmaceutical Sciences* 75(3):271-4.
- Mandal S, Puniya AK, Singh K. 2006. Effect of alginate concentrations on survival of microencapsulated *Lactobacillus casei* NCDC-298. *International Dairy Journal* 16(10):1190-5.
- Manojlović V, Nedović VA, Kailasapathy K, Zuidam NJ. 2010. Encapsulation of probiotics for use in food products. *Encapsulation Technologies for Active Food Ingredients and Food Processing*: 269-302.
- Marsella R. 2009. Evaluation of *Lactobacillus rhamnosus* strain GG for the prevention of atopic dermatitis in dogs. *American Journal of Veterinary Research* 70(6):735-40.

- Marsella R, Santoro D, Ahrens K. 2012. Early exposure to probiotics in a canine model of atopic dermatitis has long-term clinical and immunological effects. *Veterinary Immunology and Immunopathology* 146(2):185-9.
- Martinsen A, Skjåk-Bræk G, Smidsrød O. 1989. Alginate as immobilization material: I. Correlation between chemical and physical properties of alginate gel beads. *Biotechnology and Bioengineering* 33(1):79-89.
- McConnell EL, Fadda HM, Basit AW. 2008. Gut instincts: Explorations in intestinal physiology and drug delivery. *International Journal of Pharmaceutics* 364(2):213-26.
- McGregor J, Donald A. 2010. The application of ESEM to biological samples. *Journal of Physics: Conference Series*: 241(1): 012021.
- Misic AM, Davis MF, Tyldsley AS, Hodkinson BP, Tolomeo P, Hu B, Nachamkin I, Lautenbach E, Morris DO, Grice EA. 2015. The shared microbiota of humans and companion animals as evaluated from *Staphylococcus* carriage sites. *Microbiome* 3(1):2.
- Mitropoulou G, Nedovic V, Goyal A, Kourkoutas Y. 2013. Immobilization technologies in probiotic food production. *Journal of Nutrition and Metabolism* 2013:1-15.
- Mokarram R, Mortazavi S, Najafi MH, Shahidi F. 2009. The influence of multi stage alginate coating on survivability of potential probiotic bacteria in simulated gastric and intestinal juice. *Food Research International* 42(8):1040-5.
- Mortazavian A, Razavi SH, Ehsani MR, Sohrabvandi S. 2007. Principles and methods of microencapsulation of probiotic microorganisms. *Iranian Journal of Biotechnology* 5(1):1-18.
- Oksanen PJ, Salminen E, Siitonen S, Stuckey H, Toppila A, Vapaatalo H, Salminen S, Saxelin M, Hämäläinen P, Ihantola-Vormisto A, Muurasniemi-Isoviita L, Nikkari S, Oksanen T, Pörsti I. 1990. Prevention of travellers diarrhoea by *Lactobacillus* GG. *Annals of Medicine* 22(1):53-6.
- Ouwehand AC, Salminen SJ. 1998. The health effects of cultured milk products with viable and non-viable bacteria. *International Dairy Journal* 8(9):749-58.
- Ouwerx C, Velings N, Mestdagh MM, Axelos MAV. 1998. Physico-chemical properties and rheology of alginate gel beads formed with various divalent cations. *Polymer Gels and Networks* 6(5):393-408.
- Pascal G, Sandra D, Marie B. 2011. Probiotics, prebiotics, and synbiotics: Impact on the gut immune system and allergic reactions. *Journal of Leukocyte Biology* 89(5):685-95.

- Pascher M, Hellweg P, Khol-Parisini A, Zentek J. 2008. Effects of a probiotic *Lactobacillus acidophilus* strain on feed tolerance in dogs with non-specific dietary sensitivity. *Archives of Animal Nutrition* 62(2):107-16.
- Pasparakis G, Bouropoulos N. 2006. Swelling studies and *in vitro* release of verapamil from calcium alginate and calcium alginate–chitosan beads. *International Journal of Pharmaceutics* 323(1):34-42.
- Priya AJ, Vijayalakshmi SP, Raichur AM. 2011. Enhanced survival of probiotic *Lactobacillus acidophilus* by encapsulation with nanostructured polyelectrolyte layers through layer-by-layer approach. *Journal of Agricultural and Food Chemistry* 59(21):11838-45.
- Ravi Kumar MNV. 2000. A review of chitin and chitosan applications. *Reactive and Functional Polymers* 46(1): 1-27.
- Rinaudo M. 2006. Chitin and chitosan: Properties and applications. *Progress in Polymer Science* 31(7):603-32.
- Rinkinen M, Jalava K, Westermarck E, Salminen S, Ouwehand AC. 2003a. Interaction between probiotic lactic acid bacteria and canine enteric pathogens: A risk factor for intestinal *Enterococcus faecium* colonization? *Veterinary Microbiology* 92(1):111-9.
- Rinkinen M, Mättö J, Salminen S, Westermarck E, Ouwehand AC. 2000. *In vitro* adhesion of lactic acid bacteria to canine small intestinal mucus. *Journal of Animal Physiology and Animal Nutrition* 84(1-2):43-7.
- Rinkinen M, Westermarck E, Salminen S, Ouwehand AC. 2003b. Absence of host specificity for *in vitro* adhesion of probiotic lactic acid bacteria to intestinal mucus. *Veterinary Microbiology* 97(1):55-61.
- Rodklongtan A, La-ongkham O, Nitisinprasert S, Chitprasert P. 2014. Enhancement of *Lactobacillus reuteri* KUB-AC5 survival in broiler gastrointestinal tract by microencapsulation with alginate-chitosan semi-interpenetrating polymer networks. *Journal of Applied Microbiology* 117(1):227-38.
- Rokka S, Rantamäki P. 2010. Protecting probiotic bacteria by microencapsulation: Challenges for industrial applications. *European Food Research and Technology* 231(1):1-12.
- Rosas-Flores W, Ramos-Ramírez EG, Salazar-Montoya JA. 2013. Microencapsulation of *Lactobacillus helveticus* and *Lactobacillus delbrueckii* using alginate and gellan gum. *Carbohydrate Polymers* 98(1):1011-7.
- Rosenberg M, Kopelman IJ, TALMON Y. 1985. A scanning electron microscopy study of microencapsulation. *Journal of Food Science* 50(1):139-44.

- Sabikhi L, Babu R, Thompkinson DK, Kapila S. 2010. Resistance of microencapsulated *Lactobacillus acidophilus* LA1 to processing treatments and simulated gut conditions. *Food and Bioprocess Technology* 3(4):586-93.
- Sathyabama S, Vijayabharathi R. 2014. Co-encapsulation of probiotics with prebiotics on alginate matrix and its effect on viability in simulated gastric environment. *LWT-Food Science and Technology* 57(1):419-25.
- Servin AL. 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiology Reviews* 28(4):405-40.
- Shah NP. 2007. Functional cultures and health benefits. *International Dairy Journal* 17(11):1262-77.
- SHEU TY, Rosenberg M. 1998. Microstructure of microcapsules consisting of whey proteins and carbohydrates. *Journal of Food Science* 63(3):491-4.
- Sogias IA, Khutoryanskiy VV, Williams AC. 2010. Exploring the factors affecting the solubility of chitosan in water. *Macromolecular Chemistry and Physics* 211(4):426-33.
- Sohail A, Turner MS, Coombes A, Bhandari B. 2013. The viability of *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* NCFM following double encapsulation in alginate and maltodextrin. *Food and Bioprocess Technology* 6(10):2763-9.
- Sohail A, Turner MS, Coombes A, Bostrom T, Bhandari B. 2011. Survivability of probiotics encapsulated in alginate gel microbeads using a novel impinging aerosols method. *International Journal of Food Microbiology* 145(1):162-8.
- Song SJ, Lauber C, Costello EK, Lozupone CA, Humphrey G, Berg-Lyons D, Caporaso JG, Knights D, Clemente JC, Nakielny S. 2013. Cohabiting family members share microbiota with one another and with their dogs. *Elife* 2(2013): e00458.
- Stenske KA, Bemis DA, Gillespie BE, D'Souza DH, Oliver SP, Draughon FA, Matteson KJ, Bartges JW. 2009. Comparison of clonal relatedness and antimicrobial susceptibility of fecal *Escherichia coli* from healthy dogs and their owners. *American Journal of Veterinary Research* 70(9):1108-16.
- Strompfová V, Lauková A, Cilik D. 2013. Synbiotic administration of canine-derived strain *Lactobacillus fermentum* CCM 7421 and inulin to healthy dogs. *Canadian Journal of Microbiology* 59(5):347-52.
- Su R, Zhu X-L, Fan D-D, Mi Y, Yang C-Y, Jia X. 2011. Encapsulation of probiotic *Bifidobacterium longum* BIOMA 5920 with alginate-human-like collagen and evaluation of survival in simulated gastrointestinal conditions. *International Journal of Biological Macromolecules* 49(5):979-84.

- Succi M, Tremonte P, Reale A, Sorrentino E, Grazia L, Pacifico S, Coppola R. 2005. Bile salt and acid tolerance of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. *FEMS Microbiology Letters* 244(1):129-37.
- Sultana K, Godward G, Reynolds N, Arumugaswamy R, Peiris P, Kailasapathy K. 2000. Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. *International Journal of Food Microbiology* 62(1-2):47-55.
- Sun W, Griffiths MW. 2000. Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellan-xanthan beads. *International Journal of Food Microbiology* 61(1):17-25.
- Šušković J, Kos B, Beganović J, Leboš Pavunc A, Habjanič K, Matošić S. 2010. Antimicrobial activity-the most important property of probiotic and starter lactic acid bacteria. *Food Technology and Biotechnology* 48(3):296-307.
- Tanaka H, Matsumura M, Veliky IA. 1984. Diffusion characteristics of substrates in Ca-alginate gel beads. *Biotechnology and Bioengineering* 26(1):53-8.
- Tee WF, Nazaruddin R, Tan YN, Ayob MK. 2014. Effects of encapsulation on the viability of potential probiotic *Lactobacillus plantarum* exposed to high acidity condition and presence of bile salts. *Food Science and Technology International* 20(6):399-404.
- Thiel BL, Toth M. 2005. Secondary electron contrast in low-vacuum/environmental scanning electron microscopy of dielectrics. *Journal of Applied Physics* 97(5):051101.
- Trabelsi I, Bejar W, Ayadi D, Chouayekh H, Kammoun R, Bejar S, Ben Salah R. 2013. Encapsulation in alginate and alginate coated-chitosan improved the survival of newly probiotic in oxgall and gastric juice. *International Journal of Biological Macromolecules* 61:36-42.
- Tripathi MK, Giri SK. 2014. Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods* 9(0):225-41.
- Tyle P. 1993. Effect of size, shape and hardness of particles in suspension on oral texture and palatability. *Acta Psychologica* 84(1):111-8.
- Vanderpool C, Yan F, Polk DB. 2008. Mechanisms of probiotic action: Implications for therapeutic applications in inflammatory bowel diseases. *Inflammatory Bowel Diseases* 14(11):1585-96.
- Velings NM, Mestdagh MM. 1995. Physico-chemical properties of alginate gel beads. *Polymer Gels and Networks* 3(3):311-30.

- Vodnar DC, Socaciu C. 2014. Selenium enriched green tea increase stability of *Lactobacillus casei* and *Lactobacillus plantarum* in chitosan coated alginate microcapsules during exposure to simulated gastrointestinal and refrigerated conditions. *LWT-Food Science and Technology* 57(1):406-11.
- Walton DE, Mumford CJ. 1999. The morphology of spray-dried particles: The effect of process variables upon the morphology of spray-dried particles. *Chemical Engineering Research and Design* 77(5):442-60.
- Wang W, Waterhouse GI, Sun-Waterhouse D. 2013. Co-extrusion encapsulation of canola oil with alginate: Effect of quercetin addition to oil core and pectin addition to alginate shell on oil stability. *Food Research International* 54(1):837-51.
- Weese JS, Anderson ME. 2002. Preliminary evaluation of *Lactobacillus rhamnosus* strain GG, a potential probiotic in dogs. *Canadian Veterinary Journal* 43(10):771-4.
- Weese JS, Arroyo L. 2003. Bacteriological evaluation of dog and cat diets that claim to contain probiotics. *The Canadian Veterinary Journal* 44(3):212-5.
- Weese JS, Martin H. 2011. Assessment of commercial probiotic bacterial contents and label accuracy. *The Canadian Veterinary Journal* 52(1):43-6.
- Whelehan M, Marison IW. 2011. Microencapsulation using vibrating technology. *Journal of Microencapsulation* 28(8):669-88.
- Zhong L, Zhang X, Covasa M. 2014. Emerging roles of lactic acid bacteria in protection against colorectal cancer. *World Journal of Gastroenterology* 20(24):7878.
- Zhou S, Deng X, Li X. 2001. Investigation on a novel core-coated microspheres protein delivery system. *Journal of Controlled Release* 75(1-2):27-36.